



universität  
wien

# DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Rooting and Acclimatization of Populus Hybrids under  
Photoautotrophic Conditions“

verfasst von / submitted by

Edith Trautenberger

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of  
Magistra der Pharmazie (Mag.pharm.)

Wien, 2019 / Vienna, 2019

Studienkennzahl lt. Studienblatt /  
degree programme code as it appears on  
the student record sheet:

A 449

Studienrichtung lt. Studienblatt /  
degree programme as it appears on  
the student record sheet:

Diplomstudium Pharmazie

Betreut von / Supervisor:

Ass. Prof. Mag. Dr. Christoph Wawrosch



# Acknowledgements

I would like to thank my supervisor Ass. Prof. Mag. Dr. Christoph Wawrosch for his support in every way. I am highly thankful for the possibility to get to know working in the field of biotechnology, specifically in plant tissue culture. It was a pleasure to learn about a completely new topic in a highly supportive work group. My hands-on experiments in the laboratory were a lot of fun and I am grateful for the knowledge and skills I have gathered over time through my everyday practice. Dr. Wawrosch is a great academic advisor, was always highly supportive and took plenty of time to get my research going. Many thanks to Dr. Andrea Kodym, for all the scientific and non-scientific chats we had over time and the advice she offered when I asked for help. I highly appreciate having had the opportunity to become her project coworker right after my thesis experiment ended. While working for her I could broaden my mind enormously by being confronted with new tasks. After all, a special thanks to Mag. Florian Gössnitzer who showed me around and introduced me to all the basic working steps in tissue culture. He would listen to any upcoming problems and helped by offering solutions and his insights. I will always remember the laughs we shared, and the amusing talks we had. Further my dearest regards to my family for supporting me financially as well as mentally in rough times on my academic pathway in general. Thank you, Mom for always being there for me when I needed someone. Thank you, Dad for always listening to my insights. Regards to my friends who supported me all the way. My deepest thanks to my partner Taylor Peer for believing in me and for convincing me to trust in myself, and for cheering me on when I was stuck.



## Abbreviations

PPF        Photosynthetic Photon Flux

½LS        nutrient media after Linsmaier and Skoog (1965), half concentrated



# Contents

|            |                                                                               |           |
|------------|-------------------------------------------------------------------------------|-----------|
| <b>1</b>   | <b>Introduction and aims of the work.....</b>                                 | <b>1</b>  |
| 1.1        | <i>Populus</i> species .....                                                  | 1         |
| 1.2        | Photoautotrophic micropropagation .....                                       | 4         |
| <b>2</b>   | <b>Material and methods .....</b>                                             | <b>9</b>  |
| 2.1        | Plant material .....                                                          | 9         |
| 2.2        | Culture medium .....                                                          | 10        |
| 2.3        | Culture condition .....                                                       | 12        |
| 2.4        | Culture evaluation .....                                                      | 14        |
| <b>3</b>   | <b>Results .....</b>                                                          | <b>18</b> |
| <b>3.1</b> | <b>Formation of roots .....</b>                                               | <b>18</b> |
| 3.1.1      | Root length affected by the culture conditions .....                          | 18        |
| 3.1.2      | Number of roots affected by the culture conditions.....                       | 24        |
| 3.1.3      | Percentage of root formation by the culture conditions.....                   | 30        |
| <b>3.2</b> | <b>Formation of shoots .....</b>                                              | <b>34</b> |
| 3.2.1.1    | Length of shoots affected by the culture conditions .....                     | 34        |
| 3.2.1.2    | Initially formed length of shoots affected by the<br>culture conditions ..... | 34        |
| 3.2.1.3    | Average length of shoots affected by the<br>culture conditions .....          | 41        |
| 3.2.1.4    | Percentage of shoot formation affected by the culture conditions .....        | 48        |
| 3.2.2      | Plant development.....                                                        | 51        |
| <b>4</b>   | <b>Discussion .....</b>                                                       | <b>61</b> |
| <b>5</b>   | <b>Abstract .....</b>                                                         | <b>73</b> |

**Zusammenfassung ..... 76**

**6 References..... 79**

## 1. Introduction and aims of the work

Due to recent, and increasingly severe, deforestation and the growing world population, with its demand for more food, wooden plants are becoming of more and more interest than ever before. With the destruction of forests comes a decrease in biomass leading to a shortage of food (Hazarika, 2003; Stettler et al., 1996). Billions of plants are needed to overcome this global problem, and therefore the usage of in vitro micropropagation is the most efficient method in order to cultivate such a high amount of plants (Hazarika, 2003; Kozai, 2005). Poplars are gaining importance in contributing to a favorable carbon balance and providing wood, high in demand, for production (Stettler et al., 1996).

### 1.1. *Populus* species

Woody plants are one of the oldest, largest living organisms, and therefore important objects for biological studies (Kozłowski, 1971).

The genus *Populus* has been the second most studied tree in biotechnology and in genetic modification after the genus *Pinus* (Khattab, 2011).

*Populus* species belong to the willow family Salicaceae, the genus consisting of a broad spectrum of more than hundred species (Hanak, 2014). Further, poplars are widely distributed over the northern hemisphere, with North America as the native habitat, and have been planted in other parts of the world (Stettler et al., 1996). Within the *Populus* species the black poplar (*Populus nigra*) is widely distributed in Europe (Dudonné et al., 2011). Besides the importance for fish and wildlife, poplars are commonly used as energy crops in energy production, as well as in the field of paper manufacturing and the timber industry, mainly because of its fast growing characteristics (Stettler et al., 1996).

**Biomass for bioenergy**

Poplar is an important energy crop and is used worldwide (Hanak, 2014). Its wood can serve as a source of lignocellulose to produce bioethanol for biofuel usage (Sims et al., 2006). Since hybrid poplars are one of the fastest growing trees of North America, they are chosen for plantation (Felix et al., 2008) and provide a significant sustainable energy resource to displace fossil fuel resources (Sims et al., 2006).

## **Endophytes**

Poplar represents a model system for tree-endophyte-interaction studies, with the purpose to understand bacterial plant-growth-promotion mechanisms (Hanak, 2014). Endophytic bacteria provide several advantages to their host plant, like enhanced plant growth (Alistair et al., 2012). They colonize inside the host and have the ability to control pathogens. Moreover, they increase stress resistance in addition to inducing plant growth, but without showing signs of pathogen infection. Hanak's inoculation of hybrid poplar, with a poplar-specific endophyte *Paenibacillus* sp. P22, led to stimulated shoot and root growth, among other effects (Hanak, 2014).

Inoculating hybrid poplar with endophytic bacteria is therefore an attractive option to increase the total biomass compared to un-inoculated control plants (Alistair et al., 2012).

## **Ethnopharmacological relevance**

Poplar species have been used traditionally in medicine in Asia, mainly because of their anti-inflammatory properties. Propolis, as a hive product collected by honey bees, is well known for its antioxidant properties, and consequently has long been used in traditional medicine (Castaldo & Capasso, 2002). Especially for the treatment of various inflammatory diseases as well as diarrhea, Poplar flower (Flos populi) are in use in Asia (Xu et al., 2014). A high total phenolic content with phenolic acids and flavonoid aglycons as the main compounds seems to be responsible for antioxidant properties of Poplar buds and bud extracts. Poplar bud extract significantly regulated genes involved in antioxidant defenses, inflammatory response and cell renewal in in-vitro tests. Additionally, poplar bud extract seems to have potential antiaging properties due to interfering with the transcriptional effect on skin aging. This could be potentially utilized in cosmetic and functional food formulation (Dudonné et al., 2011).

## 1.2. Photoautotrophic in vitro cultivation

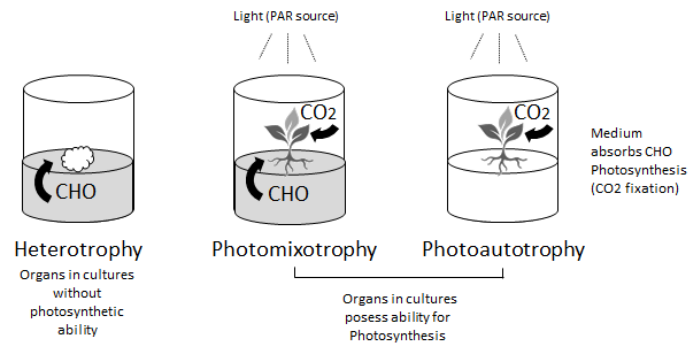


**Figure 1. Nutritional types of plant in vitro cultures (adapted from (Kozai & Kubota, 2005a).**

There are different forms of plant nutrition metabolisms, which are shown in figures 1 and 2: heterotrophy, photomixotrophy and photoautotrophy. Heterotrophy is a nutritional type where an exogenous carbohydrate source represents the only energy source. Compared to the two other nutritional types the plant lacks photosynthetically active organs and consequently the capability to perform photosynthesis without the addition of an exogenous sugar source. Photomixotrophy refers to the plant's ability to perform photosynthesis due to possessing photosynthetically active organs and a carbon source in the medium. The energy source comes from a combination of CO<sub>2</sub> fixation (photosynthesis) and a carbon source. This form is common in tissue culture and referred to as the "conventional" type of cultivation. This type of cultivation form consists of four different stages: isolation of aseptic cultures from ex vitro plants,

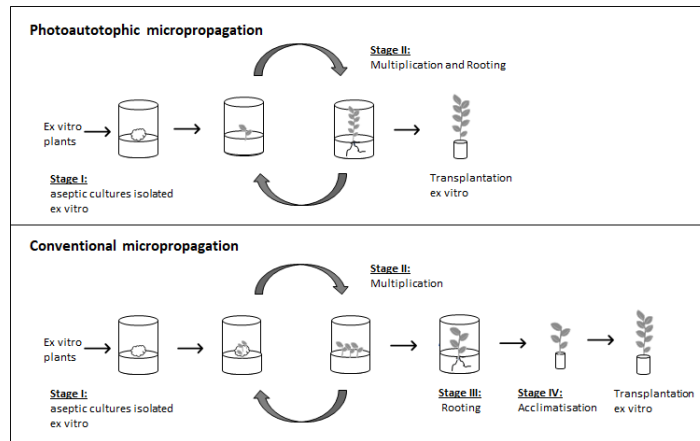


**Figure 2. Photoautotrophic and conventional micropropagation (adapted from (Kozai & Kubota, 2005a).**

multiplication of plant tissue (for example nodes), rooting, and acclimatization. Afterwards, the plant can be transplanted ex vitro.

Unfortunately, this strategy has some downsides. One of the disadvantages is a high relative humidity (95%) and ethylene accumulation in the culture jar because the gas exchange and air movement are restricted. This might lead to aberrations of morphology and physiology (Zobayed et al., 2000). Further disadvantages are a low light intensity of about  $50\text{-}100\ \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, a low  $\text{CO}_2$  concentration during photoperiod and a sugar-containing medium ( $20\text{-}30\ \text{g}\cdot\text{L}^{-1}$  sucrose), which increases the risk of contamination (Kozai, 2005; Zobayed et al., 2000).

Photoautotrophy is a newer approach (Kozai, 2005) to overcome problems caused in conventional in vitro environment and offers several advantages to micropropagation (Hazarika, 2003).

This strategy is more simplified by consisting of fewer work stages than the conventional micropropagation. Rooting does not need to be an additional stage (like it is in the conventional approach) but takes place during the stage of multiplication, as shown in figure 2. Acclimatization is made easier and rooting seems to be improved (Hazarika, 2003). In general, in vitro micropropagation assumes genetically identical plants, but the photoautotrophic method is supposed to even bring outcomes with a high photosynthesis capability and growth (Kubota, 2001). Persistent leaves seem to live longer and obtain more photosynthetic ability ex vitro (Grout & Millam, 1985). The characteristics of this method are the absence of the

carbon source ( $0\text{ g}\cdot\text{L}^{-1}$  sucrose) in the medium, higher  $\text{CO}_2$  levels, and higher light intensities ( $100\text{--}150\text{ }\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF) (Afreen, 2005; Zobayed et al., 2000). Due to the combination of higher  $\text{CO}_2$  and light intensities, the plant's mechanism of  $\text{CO}_2$  fixation (photosynthesis) and net photosynthetic rate (Kozai & Kubota, 2005a), which is otherwise mainly restricted by low  $\text{CO}_2$  concentration (Kozai & Kubota, 2005b), seems to be enhanced along with plant growth. Net photosynthetic rate is the total rate of  $\text{CO}_2$  fixation minus the rate of loss during respiration (Shin et al., 2013). Regarding relative humidity, high levels, as is the case in conventional micropropagation due to lack of gas exchange and ventilation, tend to lead to physiological and morphological issues (Kozai et al., 1995) for example incorrect development of the cuticle, a hydrophobic and waxy covering produced by the epidermal cells of leaves. Normally it serves as a protection against desiccation, especially during acclimatization (Zobayed et al., 2001a). Hyperhydricity (vitrification) is a morphological disorder that occurs as a plant's response to stress generated in unsuitable in vitro environment such as high relative humidity or accumulation of ethylene gas, among others (Afreen, 2005). The chance for occurrence is reportedly diminished under photoautotrophic conditions because of reduced relative humidity and ethylene gas (Kozai & Kubota, 2005a). Only low levels of the phytohormone ethylene produced by plants seem to promote plant development (Kozai & Kubota, 2001).

Higher light penetration to the container contents, which can be acquired by using a plastic film as a jar closure (also increasing gas exchange) (Kozai et al., 1995) and forced ventilation seem to reduce relative humidity contributing to normal functioning of stomata (Zobayed et al., 2001b). Consequently, this results in greater survival after transfer ex vitro with normal anatomical structure and stomata function, due to improved net photosynthetic rate and enhanced transpiration and therefore increase in water and nutrient uptake (Zobayed et al., 1999). Also, yearly gain appears to be increased (Xiao et al., 2011) and compared to the conventional method plant aberrations are more likely to be reduced (Hazarika, 2003). The absence of sucrose in the medium decreases chances of microbial contamination (Kozai & Kubota, 2005a). Moreover, sugar seems to reduce photosynthesis performance and increase production costs (Hazarika, 2003). The plant

uses the carbon for CO<sub>2</sub> fixation from the increased CO<sub>2</sub> in the gas phase inside the jar, as shown in fig.1 (Kozai & Kubota, 2005a). Though, an increased airflow for gas exchange is essential for high CO<sub>2</sub> levels inside the jars (Kozai & Kubota, 2001). Airflow can either be improved by natural ventilation or by a forced ventilation system. Natural ventilation can be promoted through usage of gas permeable filters whereas forced ventilation is achieved by using air pumps (Zobayed, 2005). However, enhanced CO<sub>2</sub> levels are not enough for the plants to perform photosynthesis photoautotrophically. Therefore, light intensity needs to be increased as well (Kozai & Kubota, 2005a).

The purpose of our study was to improve rooting, plant growth, and therefore plant survival. In previous experiments with poplar hybrids by Schuhleitner (2014), Gindler (2014), and Schnur (2013) the main focus was placed on photoautotrophic micropropagation and improvement of plant development. Our experiment was based on Pachala-Ghoreishi (2018) work. She might have been the first observing the development of a root system in poplar hybrids under photoautotrophic micropropagation and showed a beneficial effect on root length. Additionally, she observed that the auxins IBA and IAA led to good rooting in heterotrophic cultures. Therefore, in this experiment IBA was used and could also have given insights into how IBA promotes plant and root development of poplar hybrids under photoautotrophic conditions. However, the control was missing for further investigations.

The objective was to implement photoautotrophic conditions in the plant's surrounding in addition to an added growth regulator, to promote plant growth, overcome plant dropouts, and promote plant survival. The goal was to identify correlations between the changes in plant development and the variations of parameters. The impact of IBA was not accounted as a parameter. The parameters were set as different light intensity (40, 100 und 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF) and CO<sub>2</sub> concentration (0%, 5%) in the plant's surrounding and a variation of sucrose concentration (0, 10, 20, 30  $\text{g}\cdot\text{L}^{-1}$ ) in the media. The impact on length of shoots, shoots formation, number of roots, length of roots and roots formation was measured

after 21 days. Plant development (length of shoots *ex vitro*) was further measured until (and including) the 10th week.

## 2. Material and methods

### 2.1. Plant material

The plant material used for the experiments originally came from the Institute of Forest Genetics, located in Waldsieversdorf, Germany. It was then provided to the Department of Pharmacognosy by the Department of Ecogenomics and System Biology, both at the University of Vienna. The material was labeled as *Populus* hybrid 741 [*Populus alba* x (*P. davidiana* + *P. simonii*) x *P. tomentosa*] and originated from China. This poplar hybrid was genetically modified to harbor two insect-resistance genes. These features are considered to improve the overall plant growth by protecting the plant from damage and subsequently death in the growth process (Tian et al., 2000).

The poplar clone was multiplied via conventional micropropagation through nodal explants to obtain enough material to get started, and once again during the experiment. To this purpose, leafless nodes were used, whereas for the experiment leaves were not removed from the nodal explants to allow for photosynthesis. The preparation was always carried out in a laminar airflow bench.

## **2.2. Culture medium**

The medium consisted of half strength LS (Linsmaier and Skoog, Linsmaier E.M. (1965) basal medium (a powder formulation of micro and macro elements), sucrose, a gelling agent, distilled water, and the growth regulator indole-3-butyric acid (IBA). Sucrose concentration was varied for each treatment group, altogether four different concentrations of sucrose (0, 10, 20 or 30 g·L<sup>-1</sup>) were used separately for each treatment group. Media were solidified with 4 g·L<sup>-1</sup> of the gelling agent Gelrite® (Carl Roth GmbH + Co. KG). The growth regulator IBA, which was shown to be very efficient for an improved root development in poplar (Wei et al., 2017), was added at a concentration of 0.5 µM. IBA, like IAA (indole-3-acetic acid), belongs to the group of auxins, which promote plant cell expansion and elongation beside acting as a gene expression regulator (Osborne & McManus, 2005). In her experiment, Pachala-Ghoreishi (2018) found that both auxins IBA and IAA (0.5 µM IBA and 1 µM IAA) were favorable for rooting.

### **Preparation of the culture medium**

A specific amount of sucrose (0, 10, 20 or 30 g·L<sup>-1</sup>) was dissolved in distilled H<sub>2</sub>O in an Erlenmeyer flask. After addition of the proper amount of LS powder (L0230.0025, Duchefa Biochemie) 4 g·L<sup>-1</sup> Gelrite® was set by in small portions to avoid clumping. After filling up with distilled H<sub>2</sub>O to the final volume the growth regulator IBA was added (0.5 µM). After adjustment of the pH to 5.7±0.1 the medium was then portioned (40 ml) into 250 ml baby food jars, which were then closed with Magenta® B-caps. The gap between Magenta® B-cap and jar was sealed with Parafilm® to keep the area inside the jar free from contamination. To encourage the airflow and therefore higher CO<sub>2</sub> levels inside the glass jar (photoautotrophic conditions), a hole was drilled in the middle of the Magenta® B-caps and a membrane (pore widening 0.04 µm; Sun Biofilter, Mitomi Industrial Corporation, Japan) was used as a closure (figure 3). Enhanced growth of poplar hybrid 741 was observed by Gindler (2014) when the Magenta® B-caps were made more gas permeable. The media were finally autoclaved for 20 minutes at 121°C and 1

bar. After a cooldown phase, the sterilized media were then used either for micropropagation or stored at  $5\pm1^{\circ}\text{C}$  in the dark.



**Figure 3. Magenta<sup>®</sup> B-caps, regular and with an integrated membrane.**

### 2.3. Culture conditions

The whole micropropagation procedure was implemented in the laminar air flow bench to maintain sterile standards. Four equally-sized nodes retaining the leaves were inoculated per jar, as already mentioned the jars were closed with Magenta® B-caps with an attached membrane. Through the membrane the air flow rate and the gas exchange could be enhanced, and the relative humidity decreased. With an increase in air exchange, the ethylene concentration is reduced, which leads to better plant growth. Ethylene is a phytohormone originally produced by the plant itself, especially in times of stress (Wang et al., 2007). Poplar stock cultures were micropropagated conventionally to maintain material for further investigations and closed with regular Magenta® caps.

The test series were set as five jars per medium with four nodes per jar. One test series contained 20 nodes as a sample. Four different media (0, 10, 20 or 30 g·L<sup>-1</sup> sucrose) were prepared. Altogether 20 glass jars were placed into one aquarium. Two inversed aquaria differentiated in CO<sub>2</sub> concentration (0%, 5%) were placed on a wooden plate in a Heraeus® growth cabinet with a specific light radiation inside. The purpose of the aquaria was to keep the different CO<sub>2</sub> concentrations steady in the Heraeus® cabinet. Two test series were put into one aquarium each, into one of three Heraeus® cabinets with a light intensity of 40, 100 and 200 μM·m<sup>-2</sup>·s<sup>-1</sup> PPF, respectively. The duration of the experiment was limited to 21 days under a 16-hour photoperiod at 25±1°C. Concerning the different CO<sub>2</sub> concentrations, 0% CO<sub>2</sub> represents the pressure of an atmospheric CO<sub>2</sub> concentration of 0.04%, which was naturally present in the aquarium, and 5% CO<sub>2</sub> was created with the help of a puffer solution adapted from the method by Warburg O. (1962). The puffer solution was mixed out of 3 M solutions of KHCO<sub>3</sub> and K<sub>2</sub>CO<sub>3</sub> (ratio 170:30 ml) in a crystallizing dish (230 ml), which is supposed to generate a constant CO<sub>2</sub> concentration in the gas phase above the puffer solution. A magnetic stir bar was placed into the crystallizing dish keep the puffer solution in motion. The stir bar was connected magnetically with a stirrer placed underneath the wooden plate. As mentioned above, the CO<sub>2</sub> escapes into the gas phase above the puffer mixture. The result is a constant CO<sub>2</sub> concentration until the H<sub>2</sub>CO<sub>3</sub> in

the puffer solution is used up. The plants absorb  $\text{CO}_2$  during photosynthesis ( $\text{CO}_2$  fixation) to convert it to organic sugar compounds to fulfill the nutritional need.

The requirement to obtain the equilibrium, and therefore 5%  $\text{CO}_2$  in the gas phase, is to ensure that the puffer solution is replaced regularly. In this experiment the puffer solution was changed every 3 to 4 days.



**Figure 4. Aquaria located in Heraeus® growth cabinet.**

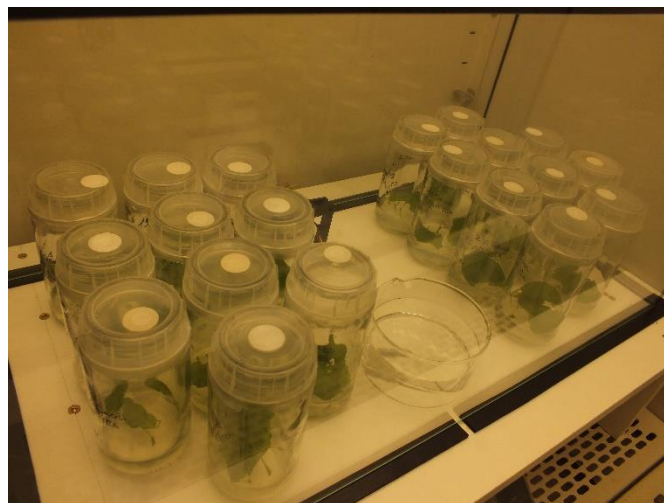


**Figure 5. Jars placed in the aquarium next to a puffer solution to enhance  $\text{CO}_2$  (5%) in the gas phase.**

## **2.4. Culture evaluation**

After a 21 days culture period the plant growth and root formation were evaluated visually and documented via photography. In case of microbial infection, the culture jar was taken out of the test series. Shoot and root formation, length and number of roots, along with the length shoots were measured with graph paper and/or a tweezer.

### **Length of shoots:**

Two types of shoot length were measured. Initially formed shoot length is the length that was measured right after the 21-day cycle in the aquarium. Average shoot length is the mean of length over 10 months of observation.

### **Shoots formation:**

The formation of shoots was calculated in the data analysis process.

### **Number of roots:**

The root's count formed during the 21-day period was analyzed. Some transplants showed beginnings of roots formations with length less than 1 mm and others did not show any formation.

### **Length of roots:**

The length was measured equally to the length of shoots.

### **Roots formation:**

The formation of roots was calculated in the data analysis process.



**Figure 4. Evaluation of plantlet's root number, length and shoot length.**



**Figure 5. Root and shoot development from poplar nodal explants after 21 days of culture.**

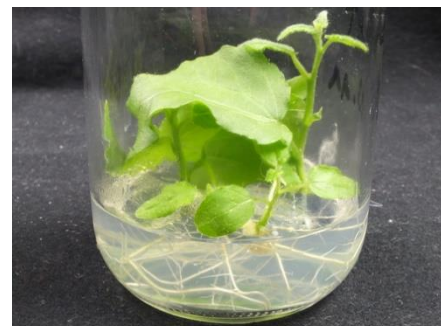


**Figure 6. Newly inoculated poplar explants (left) and poplar culture after 21 days (right).**

Shortly after the examination plantlets were transplanted into small pots filled with a mixture of peat and Perlit® (4.5:1), watered and sprayed with a 0.1% solution of Chinosol® (8-hydroxyquinoline sulfate), an antimicrobial agent, to avoid microbial infections and to keep the survival rate high and the explant dropout rate low. For the acclimatization phase of three weeks, plantlets were placed into an acclimatization chamber with controlled relative humidity that was lowered every week starting from 80%. Assuming the plantlets were exposed to a relative humidity of about 80-90% in the culture jar during the experiment (Kozai et al., 1995), in order to survive in the greenhouse (*ex vitro*) later on, the plant's system had to acclimate to a relative humidity of 60% first (acclimatization phase). During the acclimatization phase, the plantlets were observed visually, measured and sprayed with Chinosol® once a week. The observation, documentation via photography and the measurements continued for seven weeks, once a week, after which the plants were transferred to the greenhouse. In the fourth week of growing in the greenhouse, plants cultivated at  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF showed an infestation of an aphid species and spider mites. Therefore, affected plants were treated with a 0.15% solution of Samba®K, an insecticide and acaricide, to prevent further infestation or damages caused by present infestation. Explants that were micropropagated at  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF one month later, were treated equally to prevent infestation. One explant group cultivated at  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF was left without being treated against aphid species, because the greenhouse period of eight weeks ended for this group, before the first infestations occurred.



**Figure 7. Acclimatization of poplar plantlets.**

**Statistical analysis**

Descriptive statistics and significance testing (one-way and two-way ANOVA) was conducted with the program Statistica (version 6.1). For significance testing the effect of each treatment on the parameters was quantified by mean and standard error. These were obtained from analysis of variance (ANOVA).

The XLSTAT add-on for Excel was used for the significance testing on binary outcomes in root formation and shoot formation. Logistic regression was the applied model in this case. Further, a Pivot table in Excel served for the analysis of plant development.

### 3. Results

#### 3.1. Formation of roots

##### 3.1.1. Root length as affected by the culture conditions

###### Analysis of one parameter

First, an analysis of descriptive statistics and a one-factor analysis of variance (ANOVA) was conducted to evaluate the effect of one specific parameter (light intensity, sucrose concentration, CO<sub>2</sub> concentration) on the length of roots. Regarding the results, the light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF revealed significantly longer roots (5.1 cm) than other light intensities (40, 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF; 3.75 cm). Concerning the parameter sucrose, groups treated with 1-3% showed again significantly longer roots (4.4 cm) than without any sucrose (3.4 cm) in the media. Observing the impact of CO<sub>2</sub> (0%, 5%), both groups had plantlets similar in their root length (4 cm, 4.2 cm).

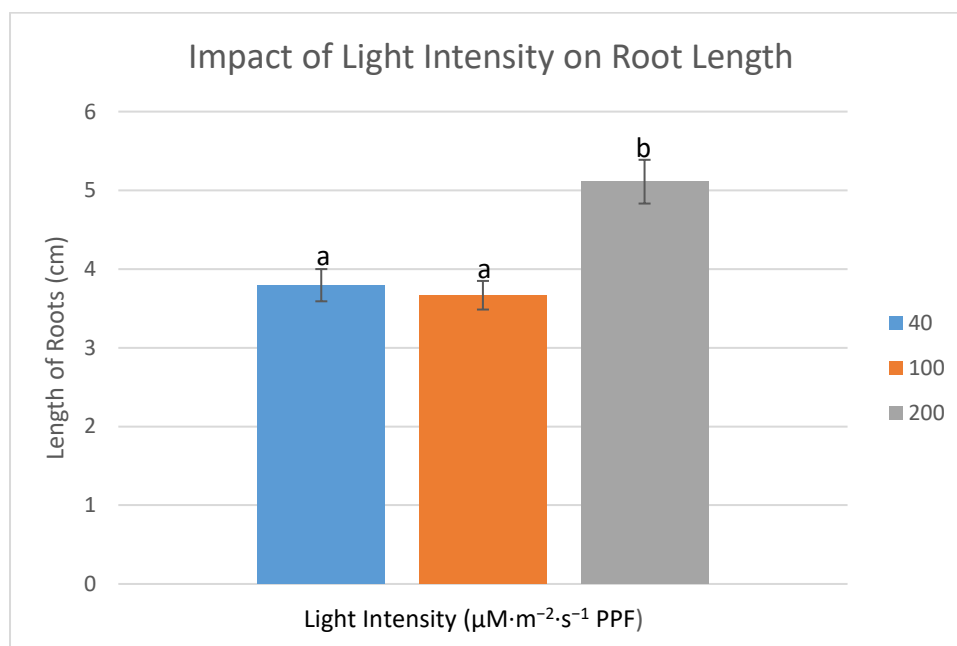


**Figure 8.** Root length (cm) affected by light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

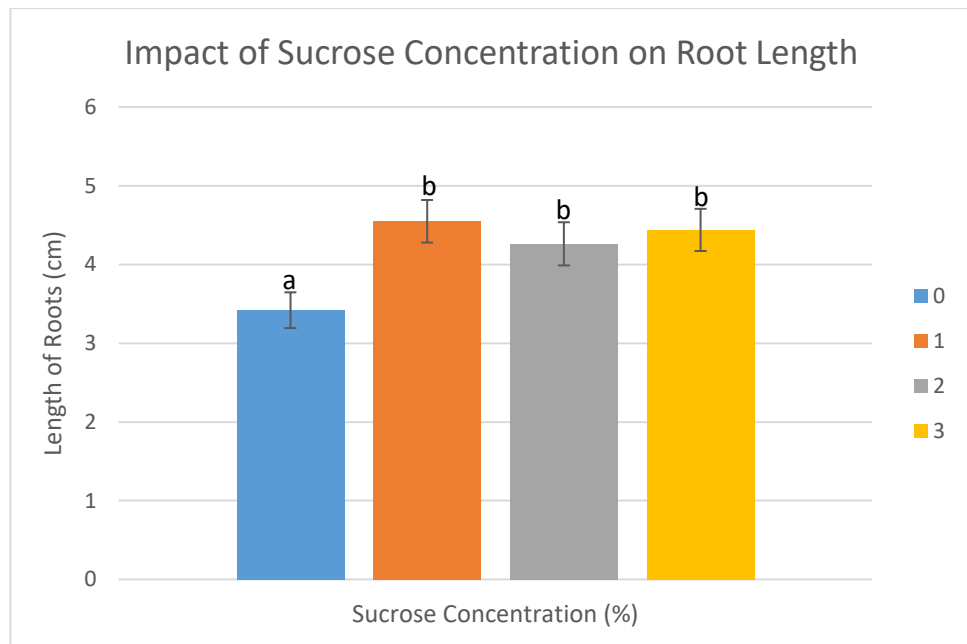


**Figure 9.** Root length (cm) affected by 0%-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

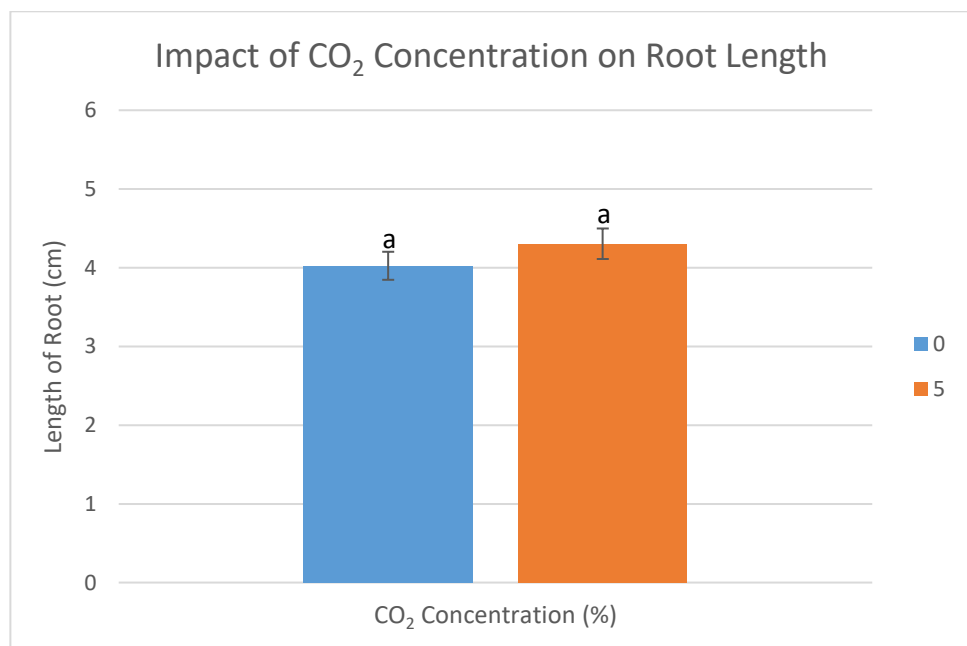


**Figure 10.** Root length (cm) affected by 0% or 5% CO<sub>2</sub> concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

**Interpretation:** The explants root's length was significantly affected by light 200, leading to longer roots than when grown at lower light intensities (40, 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF). With the absence of sugar as a carbon source for the plant, the root length was significantly lower.

### Analysis of multiple parameters

The single factor analysis (ANOVA) revealed a significant, direct impact of light intensity and sucrose on the root length. Then an analysis of two parameters (ANOVA) was conducted. The data set was therefore filtered by light and the effect of both factors, sucrose and CO<sub>2</sub> on the root length was examined.

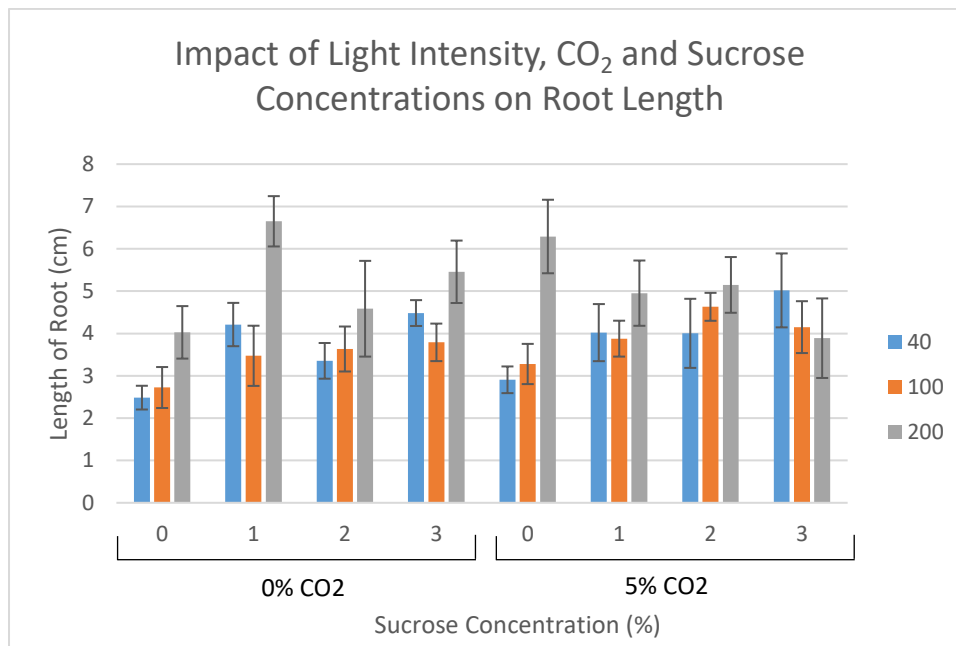


**Figure 11. Descriptive Statistics of Root length (cm) affected by light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, sucrose concentration 0-3% and CO<sub>2</sub> of 0% or 5%.**

#### **40 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The explants root length was significantly lowest (2.5 cm) (when grown at 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF without sucrose (0%) compared to the highest amount of sucrose with 3% (4.9 cm) independent of CO<sub>2</sub>. At 0% or 5% CO<sub>2</sub> the exact trend could be detected.

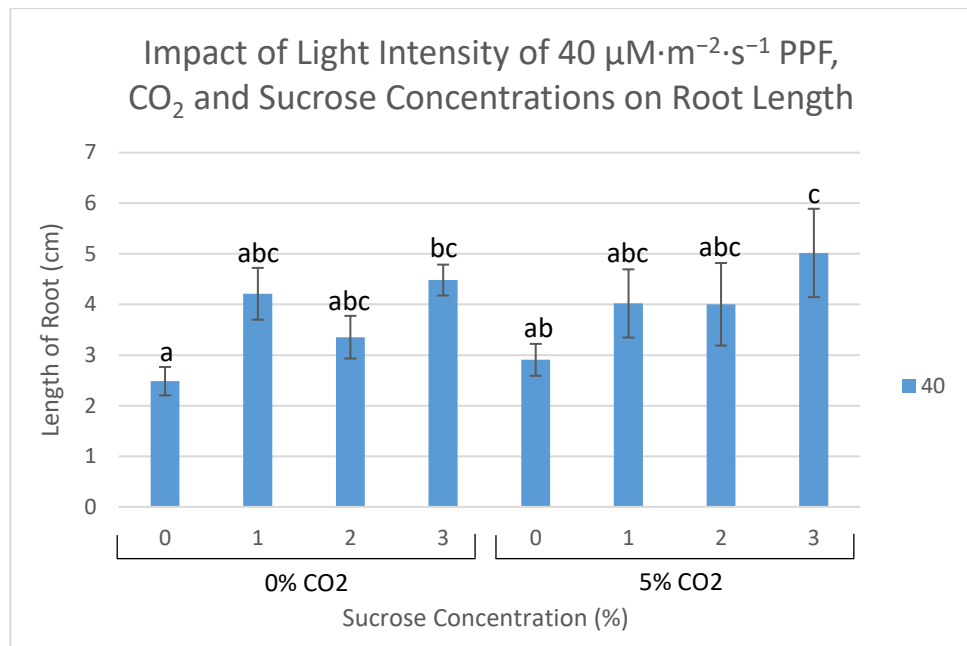


**Figure 12.** Root length (cm) affected by  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

#### **$100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The explants developed at the light setting of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0% sucrose, and 0% CO<sub>2</sub> had significantly shorter roots (2.7 cm) than explants grown under the influence of 5% CO<sub>2</sub> and 2% sucrose (4.6 cm).

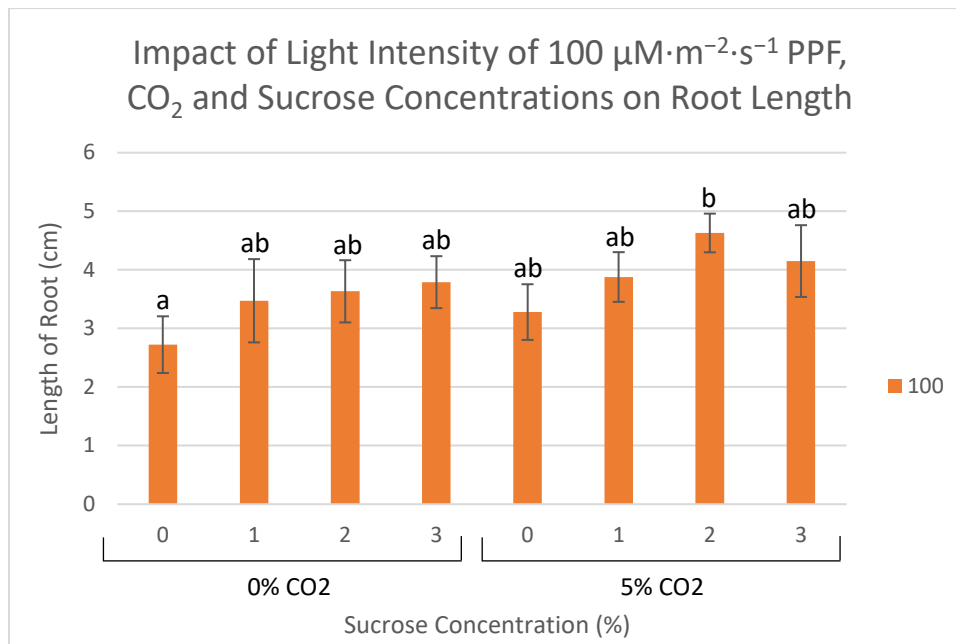


**Figure 13. Root length (cm) affected by 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).**

### **200 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

Grown at 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub> explants showed significantly shorter roots (4 cm) in absence of sucrose (0%) than with the usage of a low amount of sucrose (1%) (6.7 cm). At a high level of sucrose (3%) and a 5% CO<sub>2</sub> concentration explant's roots were significantly shorter too (3.9 cm), compared to roots formed at a 0% CO<sub>2</sub> concentration and 1% sucrose in the media (6.7 cm).

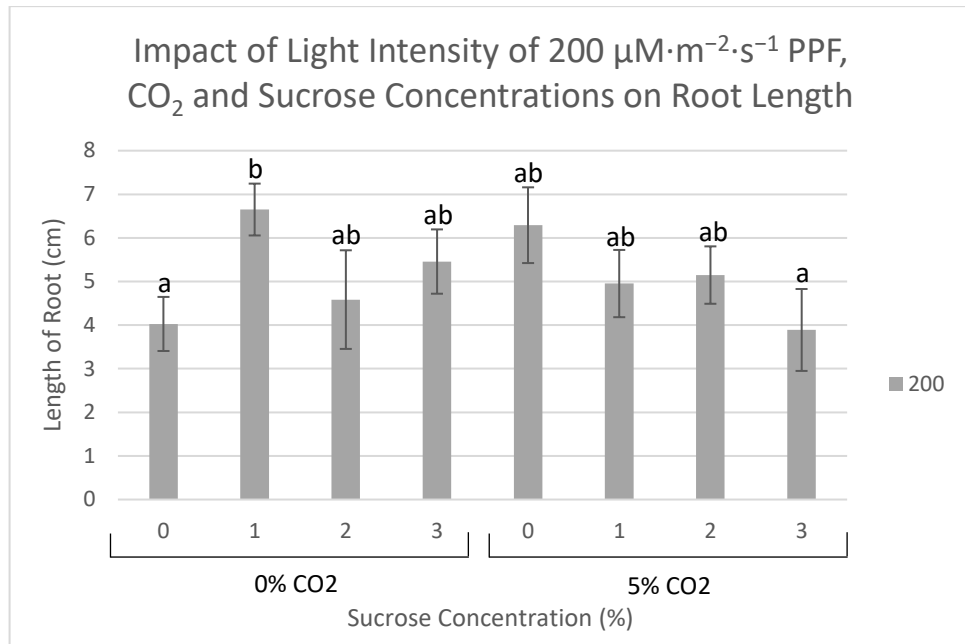


**Figure 14.** Root length (cm) affected by 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

Interpretation: Grown at 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, roots were significantly shorter when sucrose concentration was kept at 0% compared to the highest amount of sucrose (3%) independent of CO<sub>2</sub> concentration. At 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF explants affected by 5% CO<sub>2</sub> concentration and 2% sucrose had longer roots than 0% sucrose and 0% CO<sub>2</sub> concentration. At 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub> concentration and 1% sucrose roots were longer than 0% sucrose or 3% sucrose and 5% CO<sub>2</sub> concentration.

### 3.1.2. Number of roots as affected by the culture conditions

#### Analysis of one parameter

Equivalent to the analysis used to evaluate the roots length, again an analysis of descriptive statistics and a one-factor analysis of variance (ANOVA) was performed to examine the effect of one specific parameter (light intensity, sucrose concentration, CO<sub>2</sub> concentration) on the number of roots. The analysis has shown a significantly lower number of roots at light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Whereas different sucrose concentrations did not seem not to have any significant effect on the root number. Concurrent to the observations concerning sucrose, neither 0% CO<sub>2</sub> nor 5% CO<sub>2</sub> had a significant impact on the number of roots.

Interpretation: Only light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF showed a significant impact with fewer roots (2.4) than the other light intensities (3.5).

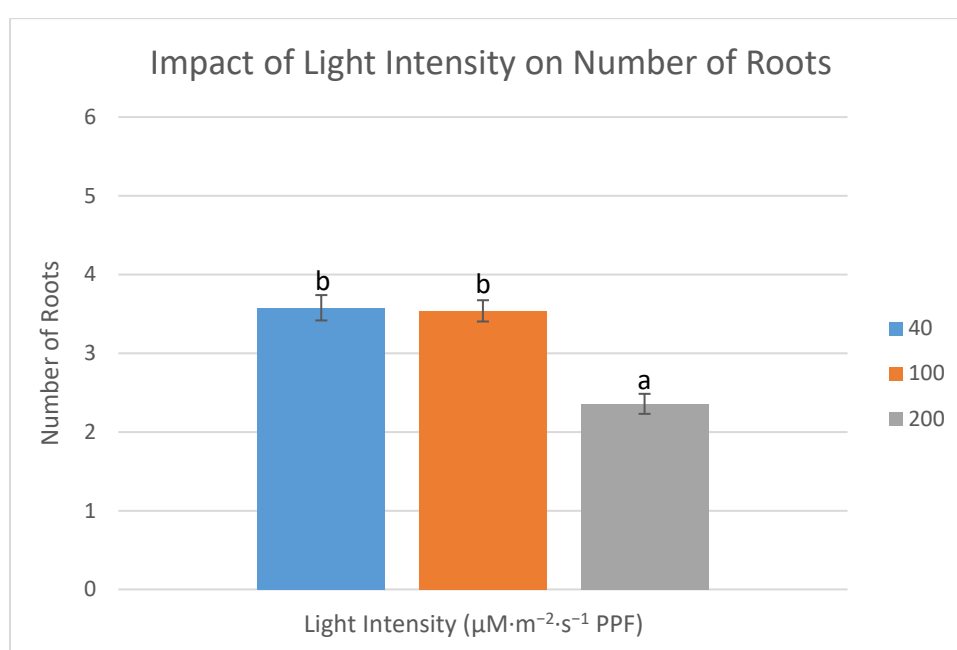


**Figure 15.** Number of roots affected by light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

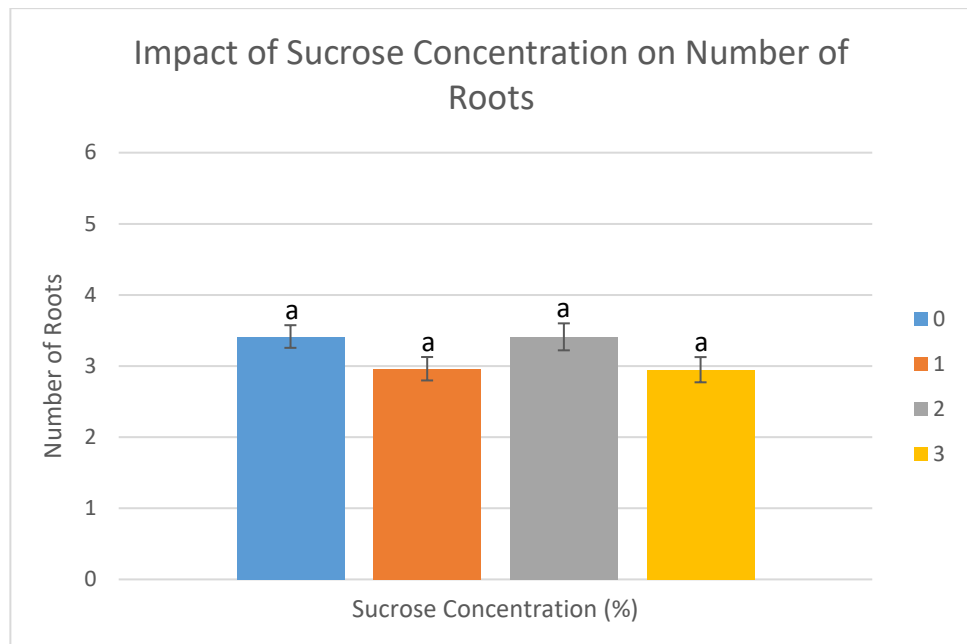


**Figure 16.** Number of roots affected by 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

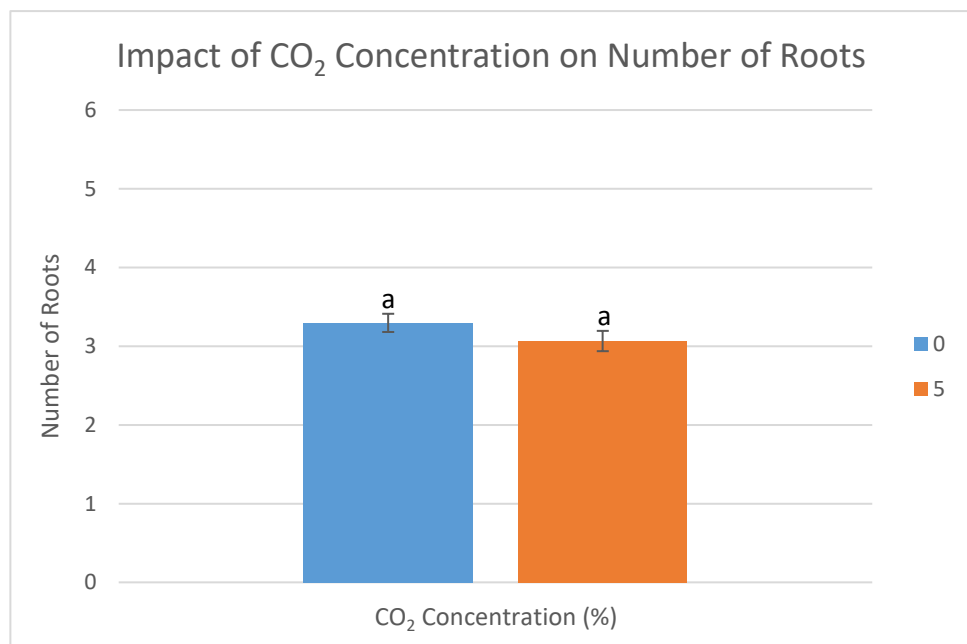


**Figure 17.** Number of roots affected by 0% or 5% CO<sub>2</sub> concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### Analysis of multiple parameters

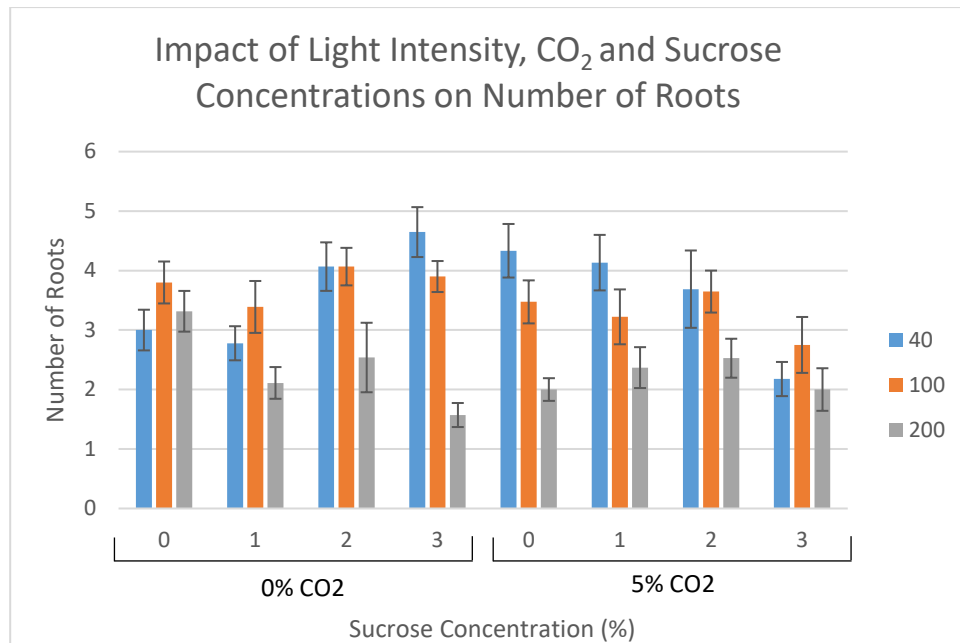


**Figure 18. Descriptive Statistics of Number of Roots affected by light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, sucrose concentration 0-3% and CO<sub>2</sub> of 0% or 5%.**

#### **40 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

At light 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub> a two-factor analysis of variance (ANOVA) demonstrated an upward trend of number of roots with the increase of sucrose concentration in the media. Therefore, 3% sucrose led to more roots (4.6) than 0% or 1% in comparison (3,2.7). Whereas a downward trend of the number of roots could be observed with the usage of a CO<sub>2</sub> buffer solution (5%). Thus, at 5% CO<sub>2</sub> the explants grown under the influence of the highest sucrose concentration of 3% had significantly fewer roots (2.2) compared to other sucrose concentrations (0-2%) (3.7-4.3).

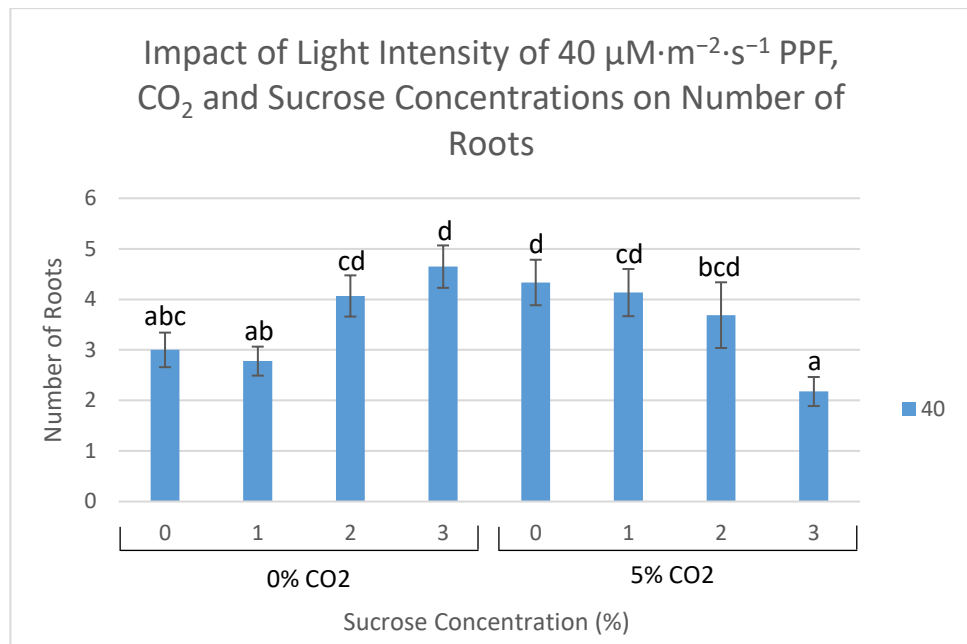


**Figure 19.** Number of roots affected by  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### **$100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

Explants lighted with  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF had all a similar number of roots with just a slightly insignificant variance in their number. In terms of significance the explants treated without CO<sub>2</sub> (0%) and with 2% sucrose formed more roots (4.1) than explants affected by 5% CO<sub>2</sub> and 3% sucrose (2.8).

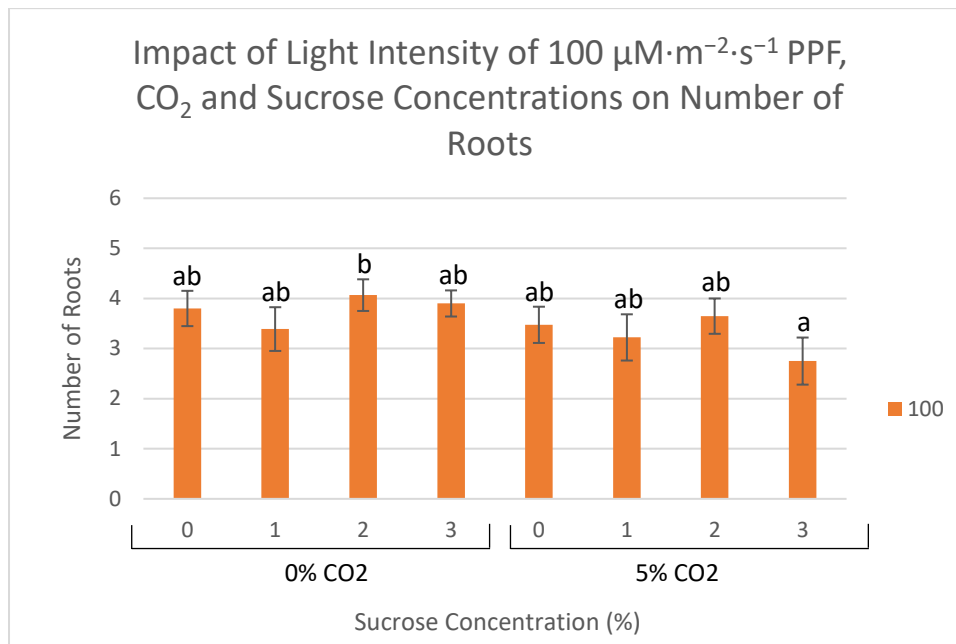


**Figure 20.** Number of roots affected by  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### **$200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The influence of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF on the explants resulted in a much higher number of roots (3.3) in absence of a CO<sub>2</sub> buffer solution (0%) and without sucrose in the media compared to explants treated with a small amount or the highest amount of sucrose (1.6-2.5). Also, in comparison to explants (2) grown under 5% CO<sub>2</sub> either without sucrose or with the highest amount of sucrose in the media, number of roots seemed to be higher.

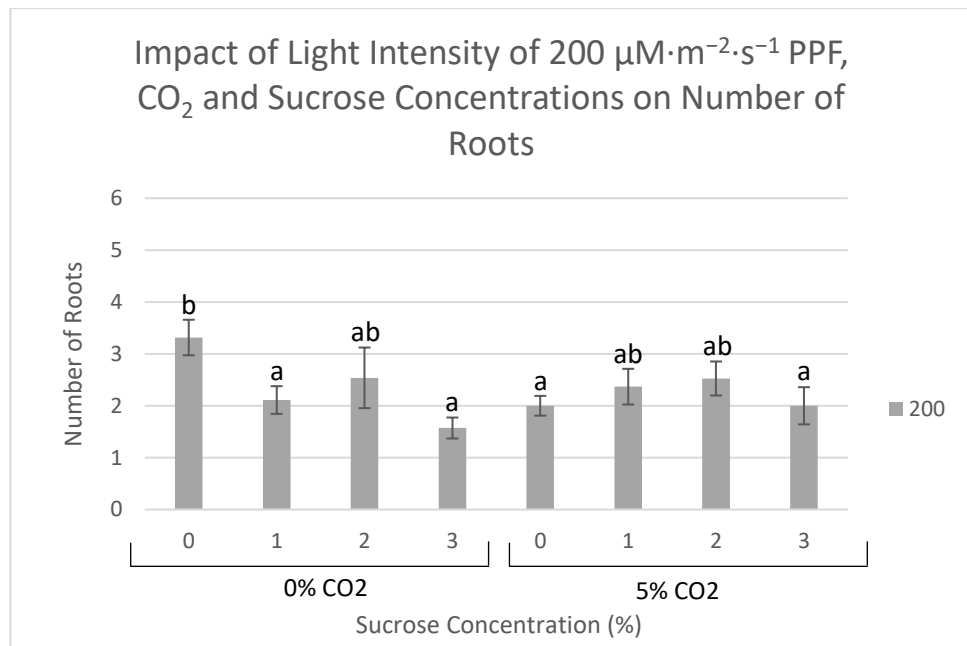


**Figure 21. Number of roots affected by 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).**

Interpretation: The parameter CO<sub>2</sub> seemed to interfere with sucrose at light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. 0% CO<sub>2</sub> combined with the highest sucrose concentration (3%) brought more roots than without sucrose or a low sucrose concentration (1%). Whereas at 5% CO<sub>2</sub> an inverse relationship was detectable, with the fewest roots observed with 3% sucrose. With higher light intensities, significance was only observed among specific combinations, but not as clear a trend as with light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Rather, single specific combinations with CO<sub>2</sub> and sucrose concentration had a more significant impact than others.

### 3.1.3. Percentage of root formation as affected by the culture conditions

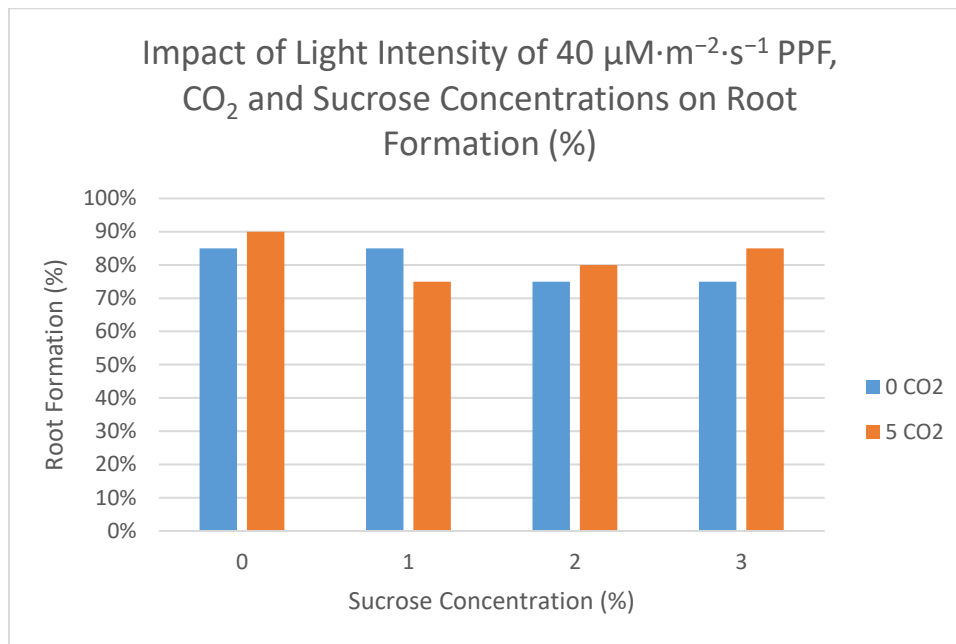


**Figure 22. Root formation (%) affected by 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0-3% sucrose concentration and 0% or 5% CO<sub>2</sub> concentration.**

For information about root formation, data was analyzed through descriptive statistics and for the test of significance logistic regression was chosen. Results at light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF demonstrated that the variability among explants that formed roots (%) seemed low, and fortunately 70-90% of explants formed roots. At light intensity of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF the percentage of root formation was similar to the outcomes from treatment of light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Only explants that grew under conditions of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0% CO<sub>2</sub> with the highest amount of sucrose or without sucrose even seemed to all form roots (100%).

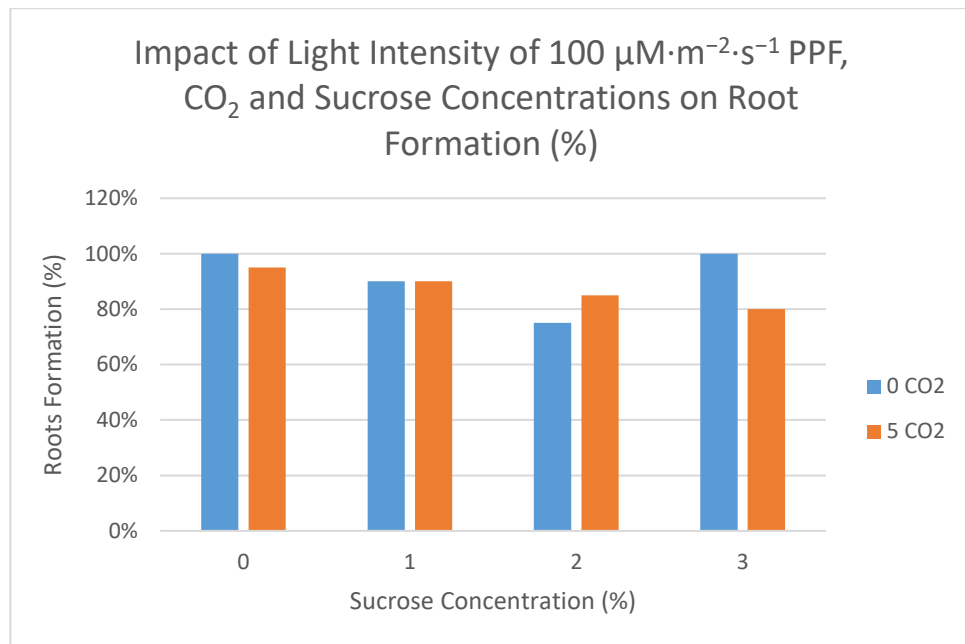


**Figure 23. Root formation (%) affected by 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF 0-3% sucrose concentration and 0% or 5% CO<sub>2</sub> concentration.**

When the light concentration was set to 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, descriptive analysis showed more variability of root formation among explants though the impact of the different treatments did not differ significantly. Without sucrose and at 0% CO<sub>2</sub>, the percentage of explants that formed was almost double than that of the 5% CO<sub>2</sub> treatment group. Explants treated with 3% sucrose and either CO<sub>2</sub> concentration (0%, 5%) formed less roots overall than at other light intensities. An inverse relationship could be detected. This meant, with the increase of sucrose level to 2%, a trend downwards among the 0% CO<sub>2</sub> groups was observed, but not at a significant level. Opposing this, an increasing trend resulted between the 5% CO<sub>2</sub> groups, when the sucrose concentration was enhanced (up to the 2% line). However, both CO<sub>2</sub> groups showed a lower rate of root formation with about 60-70% with the addition of 3% sucrose. These descriptive statistics outcomes described above were not of significance.

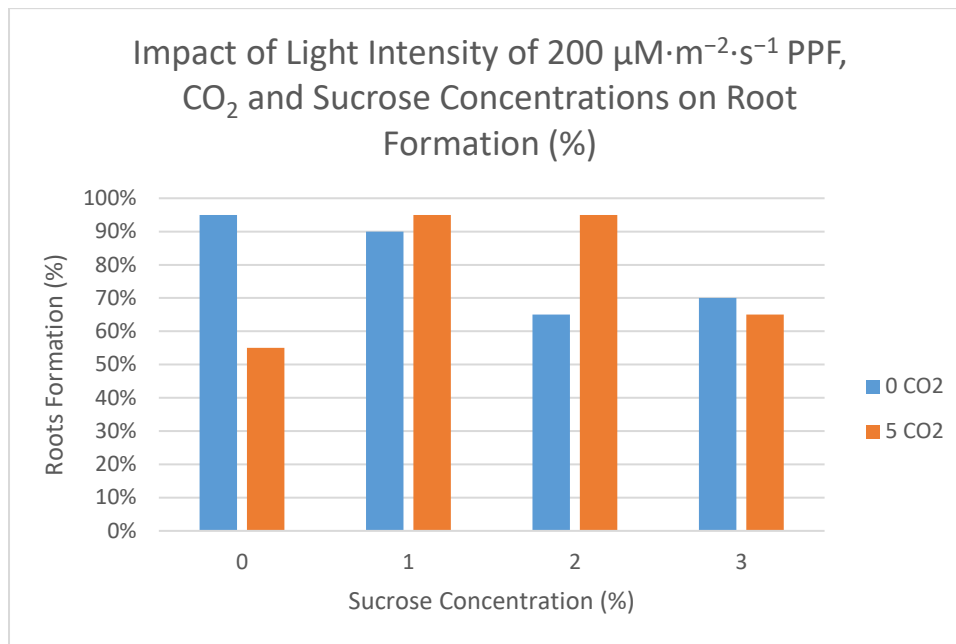


**Figure 24. Root formation (%) affected by 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF 0-3% sucrose concentration and 0% or 5% CO<sub>2</sub> concentration.**

The analysis of a logistic regression with a binary response type (root formation = yes/no) was chosen to detect any significant difference in root formation among the groups. According to the results of the analysis, the data did not reveal any significant impact by any of the parameters: light intensity (40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF), CO<sub>2</sub> concentration (0%, 5%) or sucrose concentration (0%, 1%, 2%, 3%) ( $P$  Value= > 0.05; Table 1.). The Wald Chi-Square value of the observed parameters did not predict that any parameter has a significant impact on the formation of roots. The same can be interpreted from the  $P$ -value as well, shown in the Table 1.

**Table 1. Root formation (%) test of significance: logistic regression table**

| <b>Predictor variables</b> | <b>Logistic Regression Coefficient (A)</b> | <b>Standard Error (B)</b> | <b>Wald Chi-Square* (A/B)<sup>2</sup></b> | <b>P-Value</b> | <b>Odds ratio</b> |
|----------------------------|--------------------------------------------|---------------------------|-------------------------------------------|----------------|-------------------|
| Light-40                   | 0.000                                      | 0.000                     | -                                         | -              | -                 |
| Light-100                  | 0.449                                      | 0.339                     | 1.759                                     | 0.185          | 1.567             |
| Light-200                  | -0.383                                     | 0.294                     | 1.703                                     | 0.192          | 0.682             |
| CO <sub>2</sub> -0         | 0.000                                      | 0.000                     | -                                         | -              | -                 |
| CO <sub>2</sub> -5         | -0.258                                     | 0.255                     | 1.026                                     | 0.311          | 0.772             |
| Sucrose-0                  | 0.000                                      | 0.000                     | -                                         | -              | -                 |
| Sucrose-1                  | 0.000                                      | 0.405                     | 0.000                                     | 1.000          | 1.000             |
| Sucrose-2                  | -0.701                                     | 0.366                     | 3.680                                     | 0.055          | 0.496             |
| Sucrose-3                  | -0.595                                     | 0.370                     | 2.585                                     | 0.108          | 0.552             |

\* Logistic Regression Statistic

Interpretation: explant's roots percentage was not impacted by any parameter.

### 3.2. Formation of shoots

#### 3.2.1. Length of shoots as affected by the culture conditions

##### 3.2.1.1. Length of initially formed shoots

The shoots were evaluated after 21-day exposure to the culture conditions in the aquaria. The measured shoot length was documented as initially formed shoot length whereas the average shoot refers to the mean of measurements of every week. Analysis was performed equivalently to the one which was used to evaluate the explant's root system.

#### Analysis of one parameter

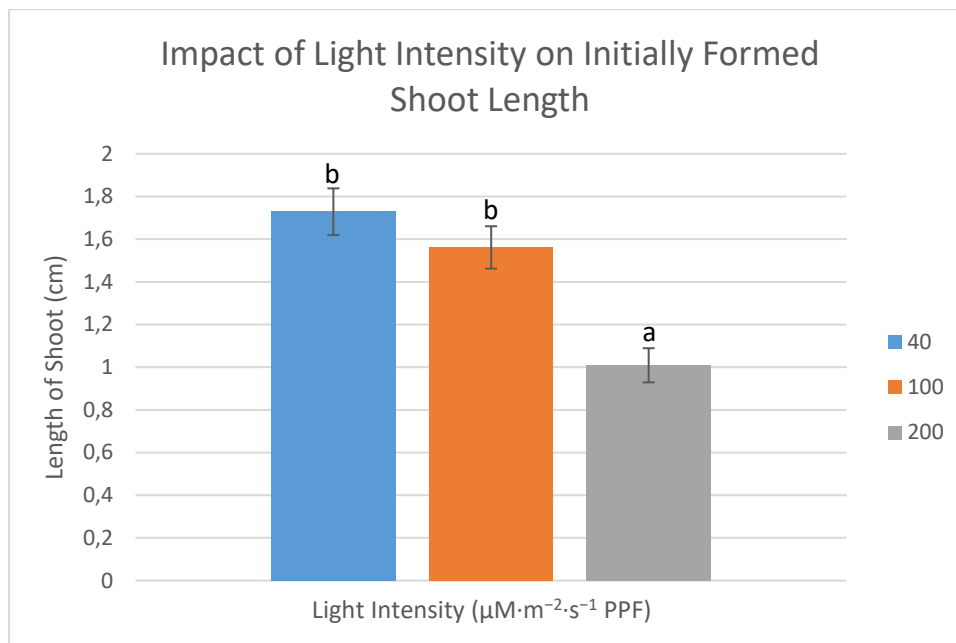


**Figure 25.** Initially formed shoot length (cm) affected by light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

First, every parameter was examined via one-factor analysis of variance (ANOVA), as it was performed in the analysis of the roots. The aim was to observe a possible predominant effect on initially formed shoots length. The outcome revealed a significant impact between light 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF with the shortest shoots (1 cm) compared to those treated with other light intensities (1.5-1.7 cm). In terms of sucrose, lower concentrations resulted in significantly longer shoots (1.2-2 cm).

The most impact was seen with a 1% concentration leading to longest shoots (2 cm), while the absence of sucrose led to the second longest shoots (1.6 cm). The difference in CO<sub>2</sub> concentration appeared important as well. At 0% CO<sub>2</sub> concentration explants grew longer shoots (1.6 cm) than those treated with 5% CO<sub>2</sub> (1.3 cm) during the experiment.

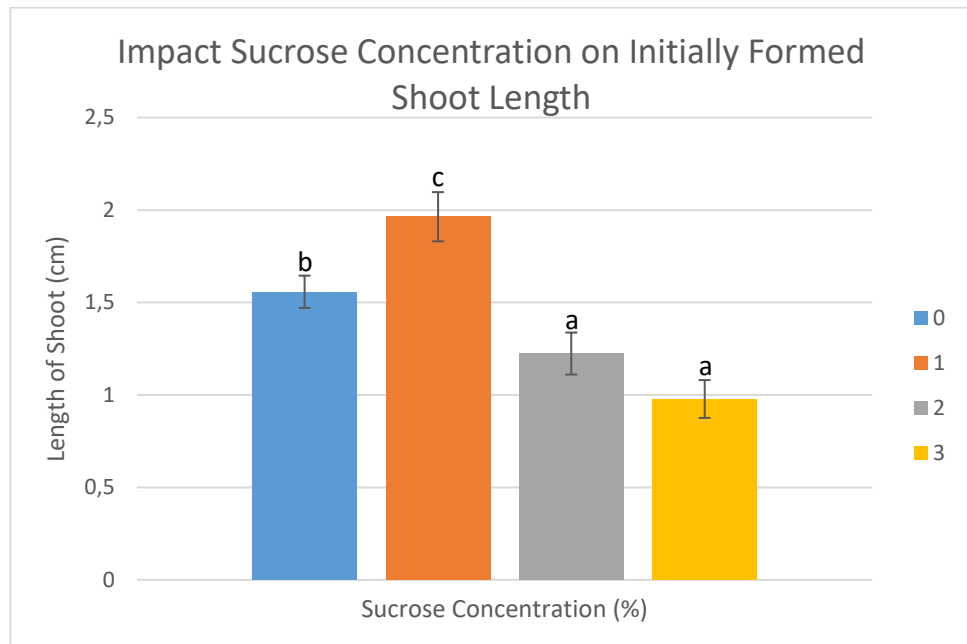


**Figure 26.** Initially formed shoot length (cm) treated with 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

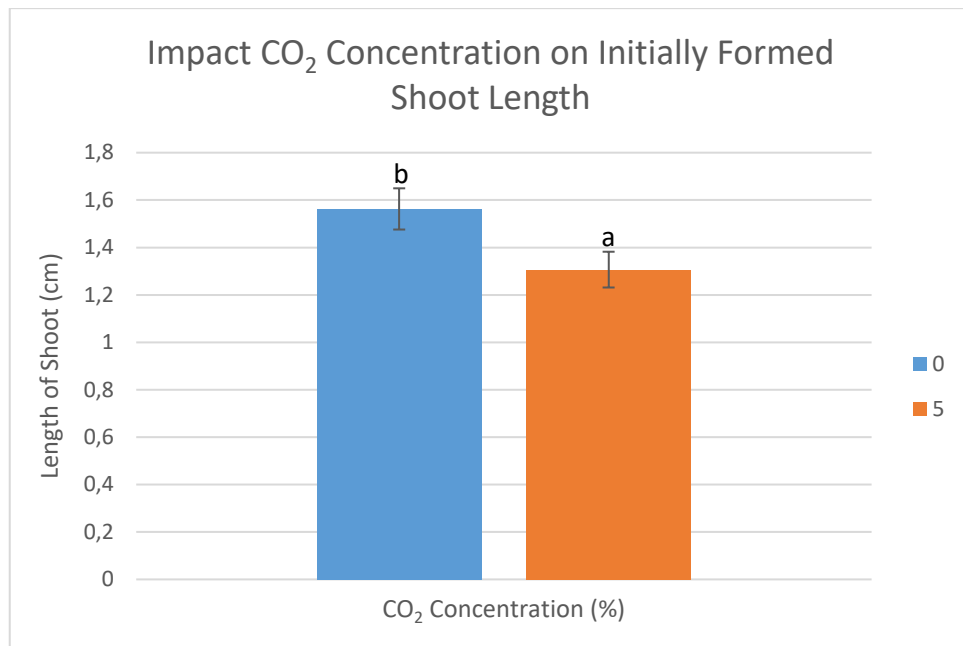


**Figure 27. Initially formed shoot length (cm) treated with 0% or 5% CO<sub>2</sub> concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).**

#### Interpretation:

Each of the three parameters analyzed separately, seemed to have a significant impact on the initial formation of shoots. Low to medium light intensities (40, 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF) promoted the growth of longer shoots. The same was valid for 1% sucrose concentration and 0% CO<sub>2</sub>.

### Analysis of multiple parameters

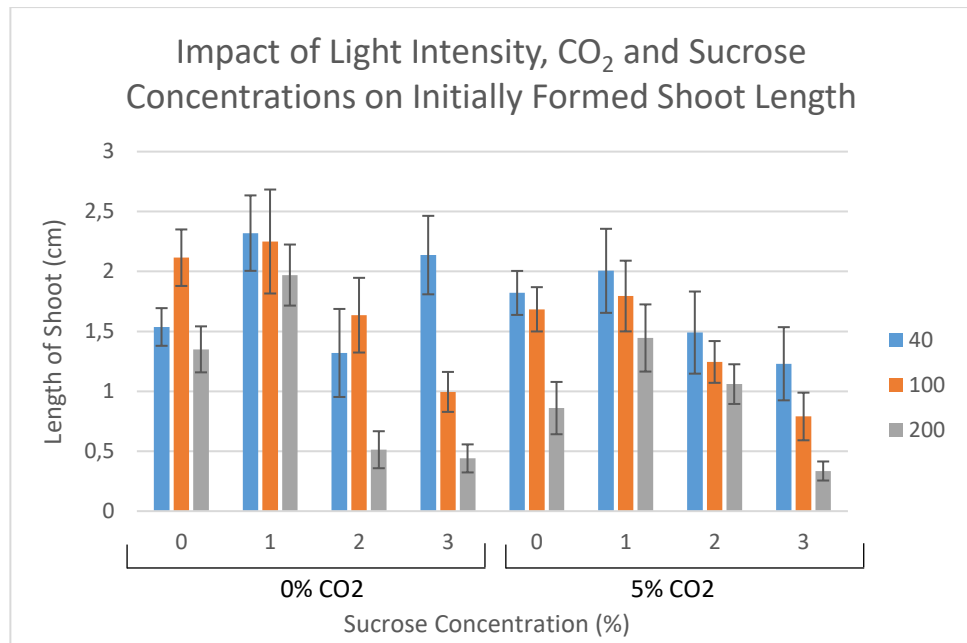


**Figure 28.** Descriptive statistics of initial shoot length affected by light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, sucrose concentration 0-3% and CO<sub>2</sub> of 0% or 5%.

#### **40 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF in combination with 0% CO<sub>2</sub> and 1% sucrose concentration promoted longer initially formed shoots (2.3 cm) compared to explants grown in 2% sucrose concentrated media (1.3 cm). This outcome was limited to 0% CO<sub>2</sub> treatments. Observations with 5% CO<sub>2</sub> showed the impact on the development of shoot length was not statistically significant.

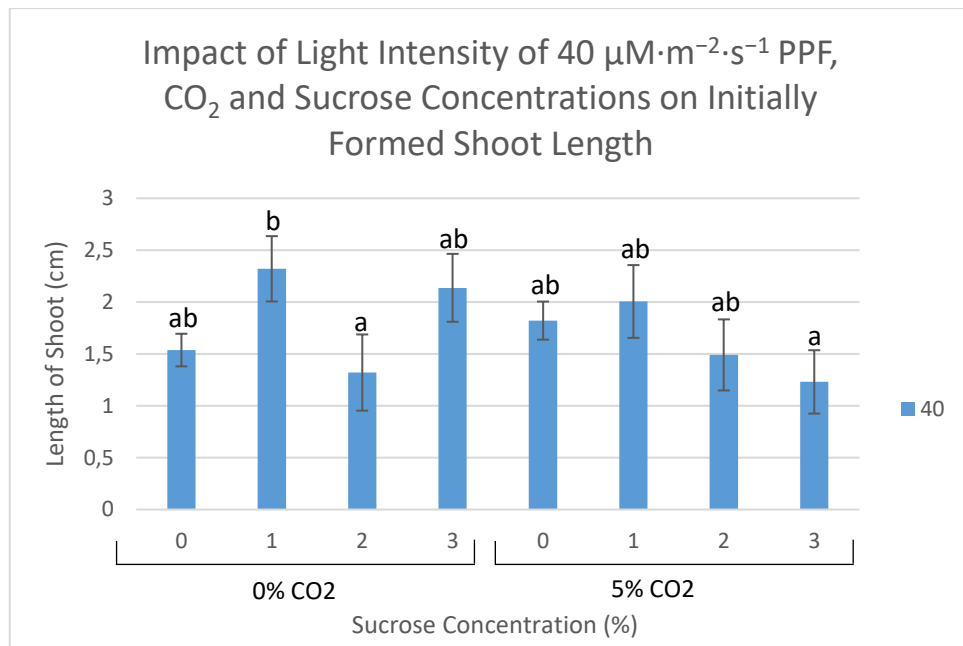


**Figure 29. Initially formed shoot length (cm) treated with 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).**

### **100 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

Explants treated with 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub> developed significantly shorter initially formed shoots (1 cm) when the highest sucrose concentration (3%) was used compared with the absence of sucrose (2.1 cm), or the usage of 1% concentrated media (2.3 cm). The same trend of decreasing shoot length with increased sucrose concentration was seen with the implementation of a CO<sub>2</sub> buffer solution (5%) next to the jars containing the explants.

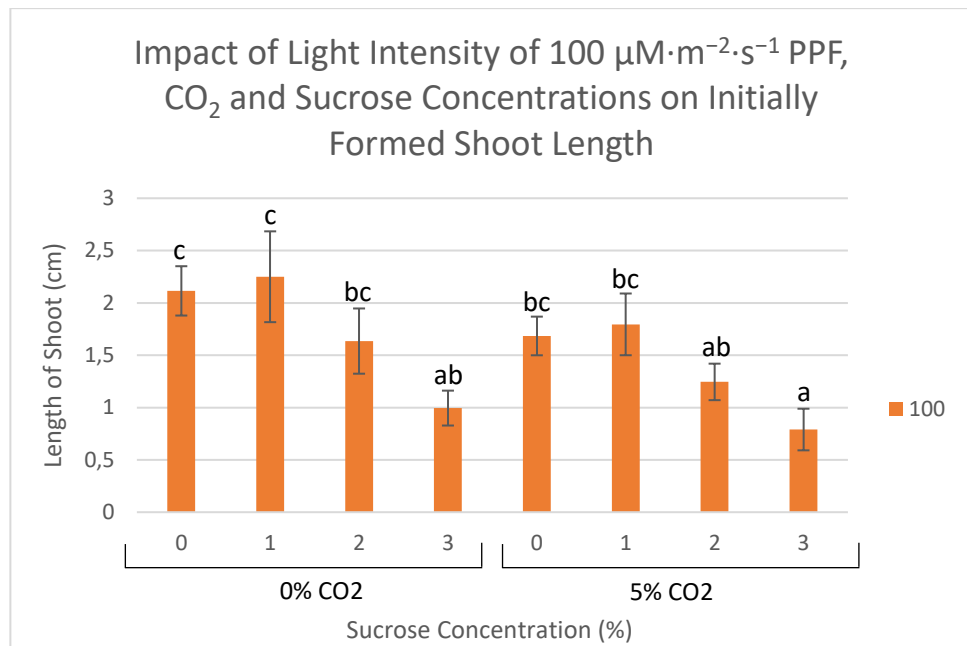


**Figure 30.** Initially formed shoot length (cm) treated with  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### **$200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The combination of light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub> led to the significantly longest initially formed shoots (2 cm) with the use of 1% sucrose concentration. Without any sucrose the explants had the second longest shoots (1.4 cm). Treatments of 5% CO<sub>2</sub> in addition to 1% or 2% sucrose concentration resulted in a significant increase of length of shoots (1-1.5 cm) compared to the outcomes of the group treated with 3% sucrose concentration (0.4 cm).

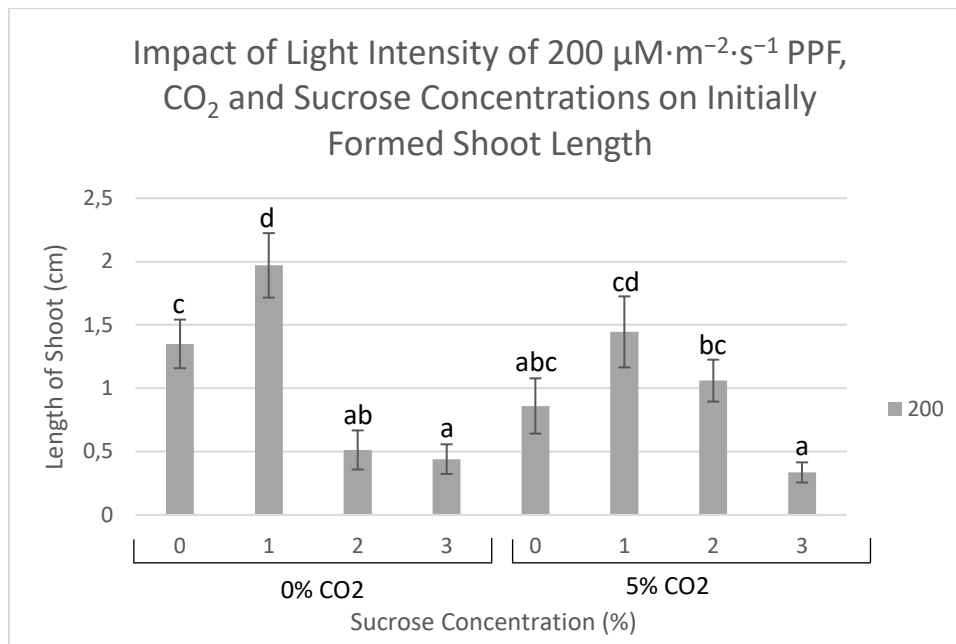


**Figure 31.** Initially formed shoot length (cm) treated with  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

#### Interpretation:

A low light intensity ( $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF) and 0% CO<sub>2</sub> with low sucrose concentration (1%) seemed to bring longer initially formed shoots compared to a higher sucrose concentration (2%). The same trend could be observed at light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF with a low amount or without sucrose led to longer shoots. Likewise, light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF combined with 5% CO<sub>2</sub> concentration brought the same results whereas for light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF was best combined with a minimum to medium sucrose concentration (1-2%) for longer shoots.

#### **3.2.1.2. Average length of shoots**

The length of shoot was measured every week over ten weeks. In the end, the average shoot length was calculated as the mean of measurements of every week. The impact of parameters was then analyzed for significance.

##### Analysis of one parameter

The analysis of the average length of shoots was the same as the analysis of the previously discussed plant objects. The impact of each of the three parameters on the average length of shoot was analyzed, first separately (one-factor analysis of variance (ANOVA)) and later, various combinations of these parameters and their impact was tested for significance (multiple factor analysis of variance (ANOVA)). According to the outcomes of the one-factor analysis the longest average length of shoot (6.8 cm) was observed on explants treated with light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, followed by those explants grown under the influence of light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF (6.2 cm). Lighting of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF resulted in explants with shortest shoots (4.3 cm). In terms of sucrose, the significantly longest average shoots (6 cm) came either from the media of 1 % sucrose concentration or without sucrose at all. Concentration of 2-3% sucrose led to outcomes of significant shorter average shoots (5.5 cm). The observations of  $\text{CO}_2$  as a parameter revealed that 0%  $\text{CO}_2$  led to significant longer average shoots (6.2 cm) than 5%  $\text{CO}_2$  (5.4 cm).

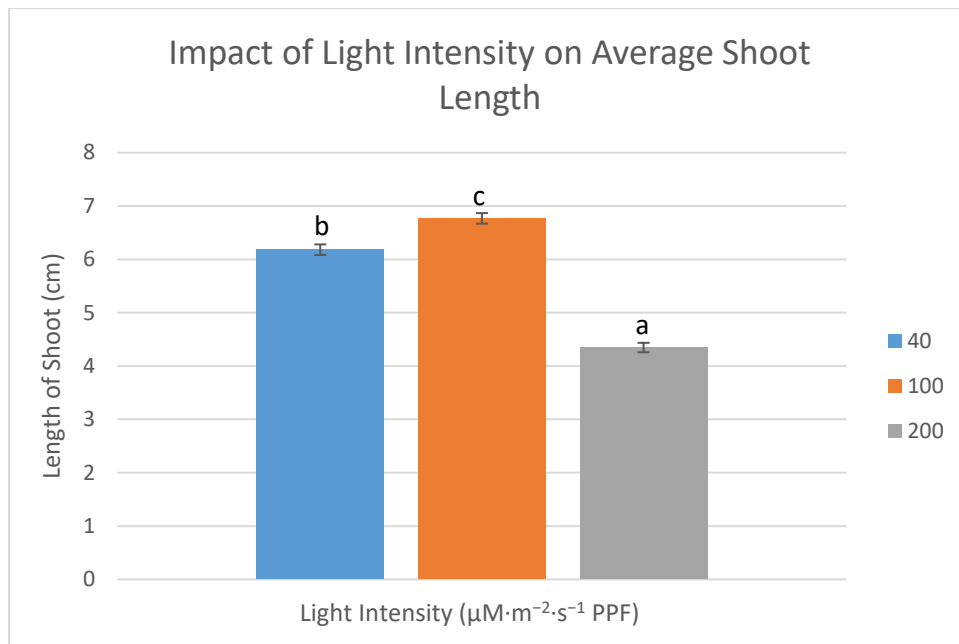


**Figure 32.** Average shoot length (cm) treated with light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

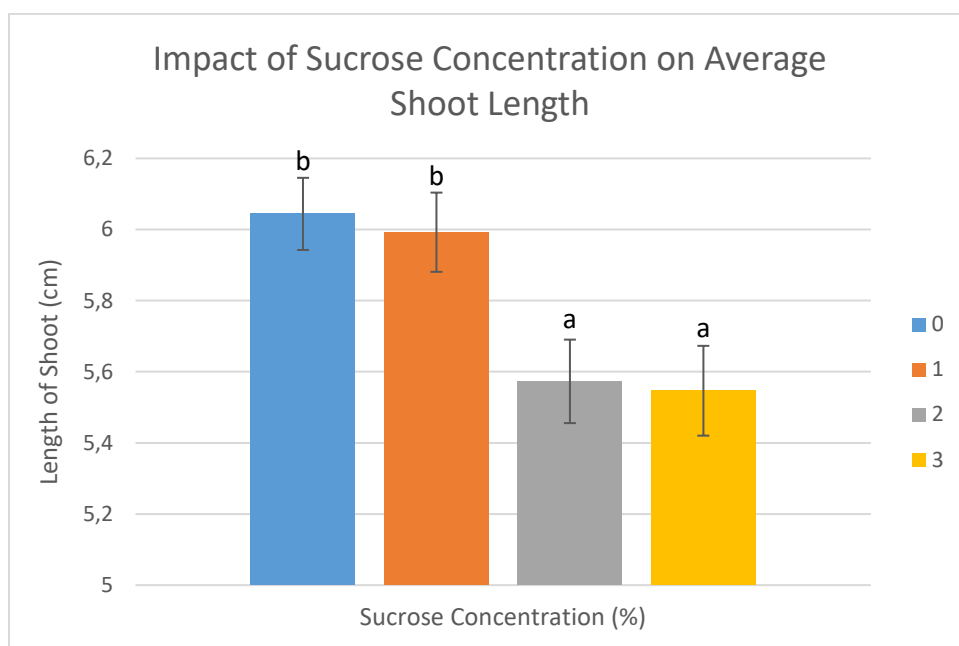


**Figure 33.** Average shoot length (cm) treated with 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

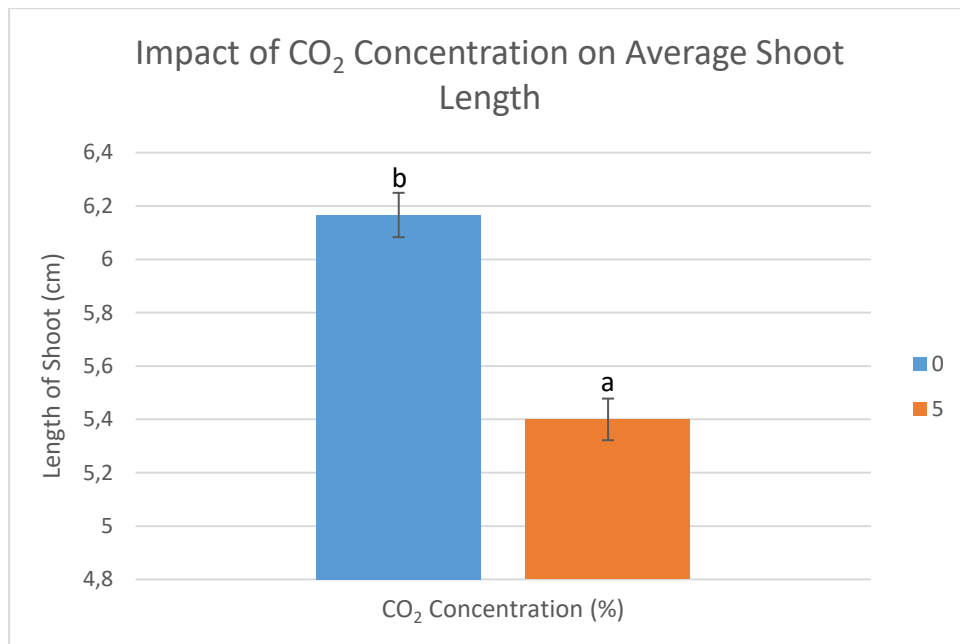


**Figure 34.** Average shoot length (cm) treated with 0% or 5% CO<sub>2</sub> concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

#### Interpretation:

The one-factor analysis offered insights of strong significant impact on average shoot length. Each parameter had a significant impact on shoot length. Light intensities of 40 and 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0-1% sucrose concentration and 0% CO<sub>2</sub> led to longer shoots on average. 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF brought the longest shoots on average.

### Analysis of multiple parameters

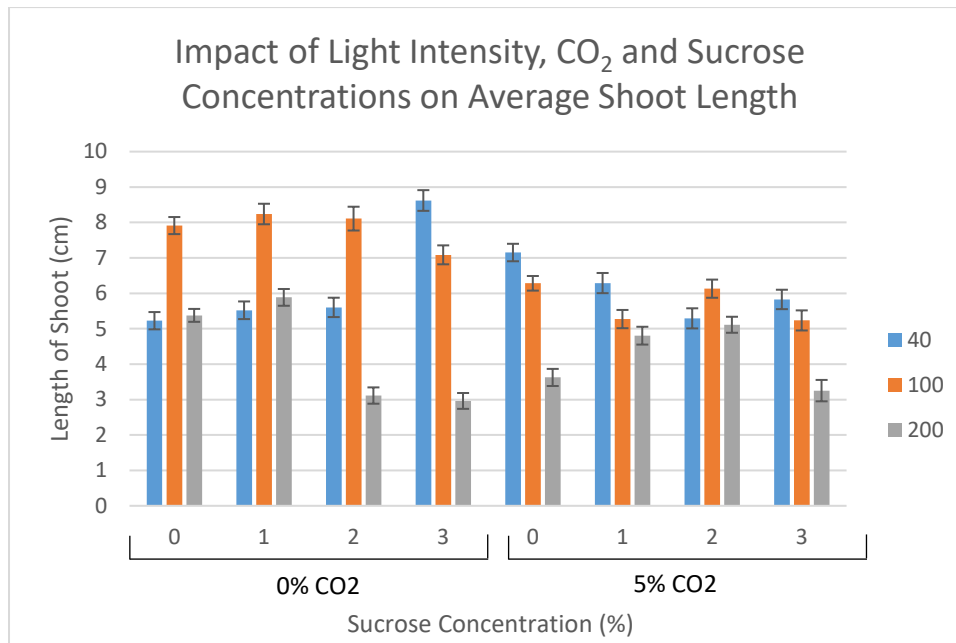


**Figure 35. Descriptive statistics of average shoot length treated at light intensity 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, sucrose concentration 0-3% and CO<sub>2</sub> of 0% or 5%.**

#### **40 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The effect of CO<sub>2</sub> and sucrose concentration at light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF on the average shoot length was investigated. The explant groups treated with 0% CO<sub>2</sub> and 3% sucrose concentration had significant longer shoots (8.6 cm) than at any other sucrose concentration (5.2-5.6 cm). All explants affected by 5% CO<sub>2</sub> in the absence of sucrose developed the longest shoots (7.2 cm). Regarding sucrose concentration as a parameter, explants grown in 1% concentrated media had significant longer shoots (6.3 cm) than those grown in 2% concentrated media (5.3 cm).

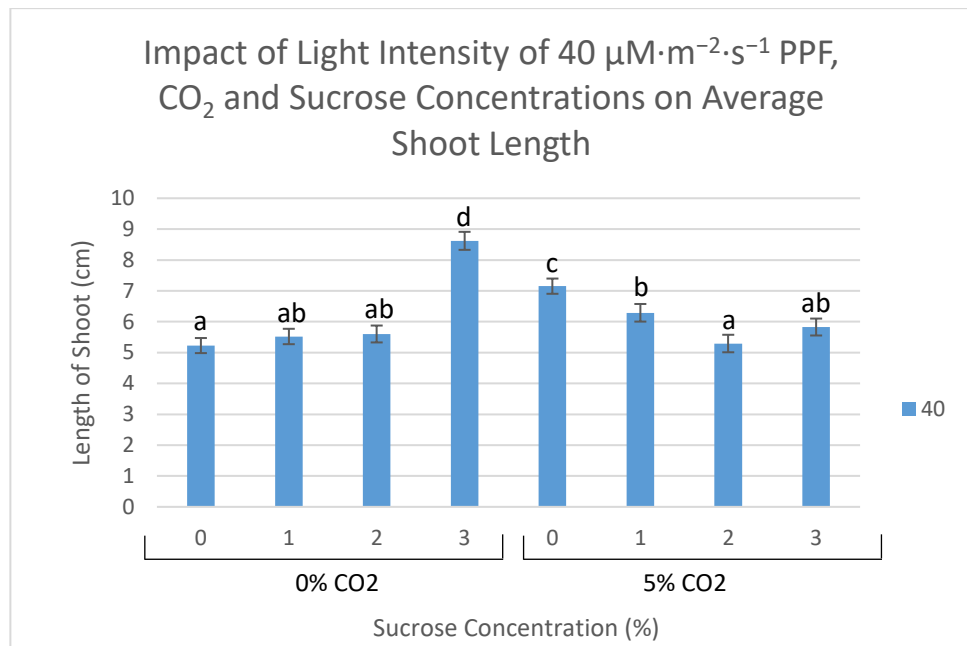


**Figure 36.** Average shoot length (cm) treated with  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

#### **$100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

Light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF combined with either of the various sucrose concentrations and 0% CO<sub>2</sub> led to significant longer shoots (7.1-8.2 cm). The highest sucrose concentration remained as an exception with the shortest shoots (7.1 cm). Explants exposed to 5% CO<sub>2</sub> showed the longest shoots when combined with 2% sucrose (6.1 cm) or without sucrose (6.3 cm).

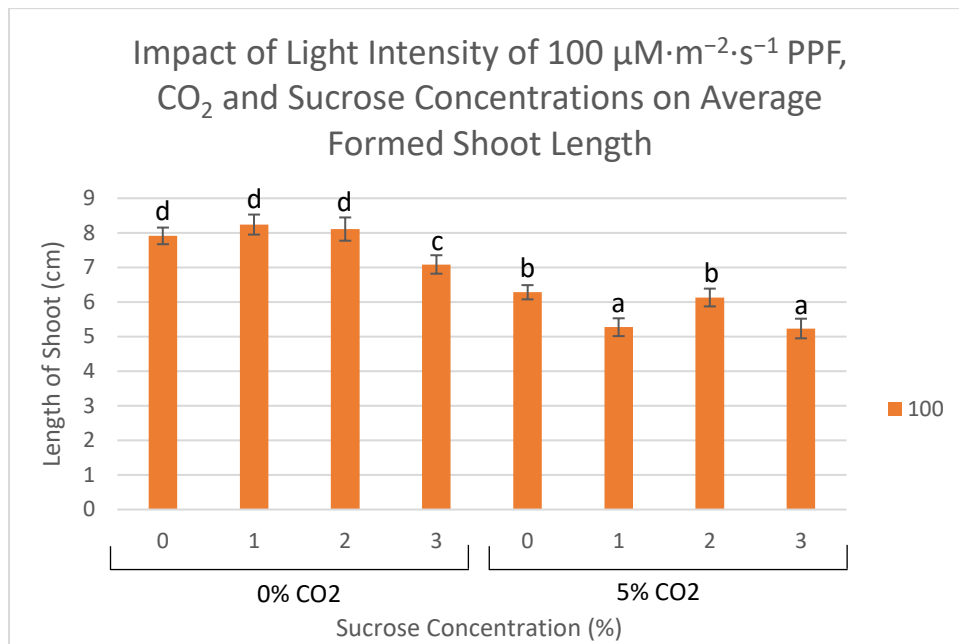


**Figure 37.** Average shoot length (cm) treated with  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### **$200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The exposition to light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF led to results of significantly shorter shoots (3-3.1 cm) in the 0%  $\text{CO}_2$  group with the usage of 2-3% sucrose. In contrast, in the 5%  $\text{CO}_2$  group the absent of sucrose and the highest sucrose concentration (3%) were associated with shorter shoots (3.3-3.6).

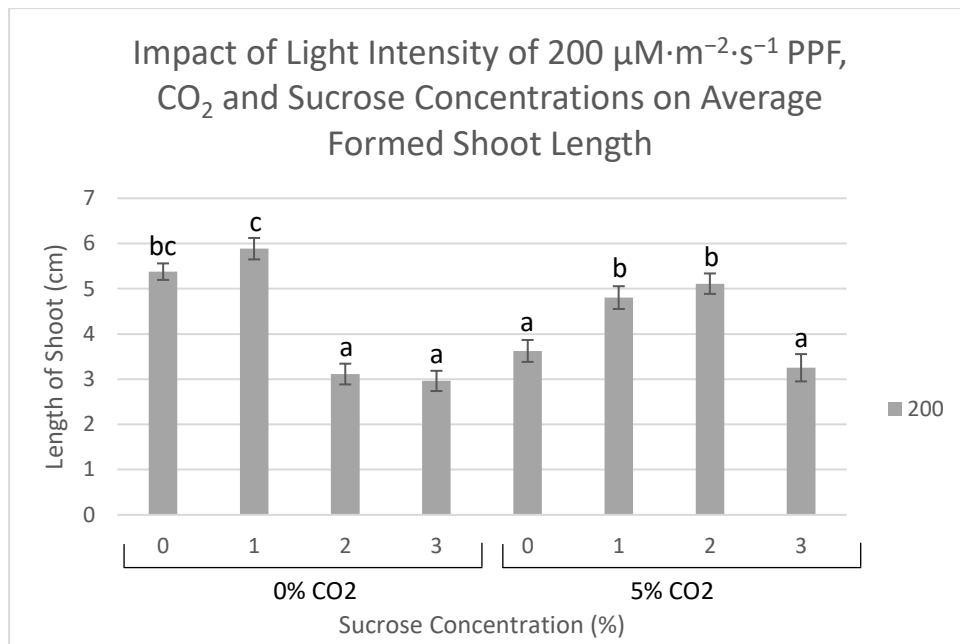


**Figure 38.** Average shoot length (cm) treated with  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

#### Interpretation:

Explants exposed to a light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF without CO<sub>2</sub> enrichment and combined with the highest amount of sucrose (3%) had the longest shoots. All treatments with  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub> led to longer shoots. 3% sucrose concentration brought shorter shoots within the 0% CO<sub>2</sub> group. Treated with  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0% CO<sub>2</sub>, and low amount of sucrose (1%) or without explants developed longer shoots. However, medium amount of sucrose (1-2%) in the media, led to the longest shoots in the 5% CO<sub>2</sub> group.

### 3.2.2. Percentage of shoot formation as affected by the culture conditions

Descriptive statistics analysis of shoots formation was conducted. Test for significance was later determined through logistic regression. Descriptive statistics separately revealed a trend of shoot formation increase at light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. However, a slight difference among treatment groups of 0% and 5%  $\text{CO}_2$  with the highest sucrose concentration or without any sucrose could be found. Explants of the 0%  $\text{CO}_2$  group seemed to form less shoots overall. Plant groups grown at light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF reached a relatively high formation level of 95-100%. Only the group treated at 0 %  $\text{CO}_2$  combined with 2% sucrose concentration stopped forming shoots at 85%. Plants exposed to light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF led to 100% of shoot formation, meaning every explant regenerated a shoot. The 0%  $\text{CO}_2$  treatment group with 2% sucrose concentration was the only group with a lower formation rate, namely of 80%. This would mean a greater plant development for the  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF light group overall compared to other light concentrations. However, this result was not found to be statistically significant. Contrary, the significance test (Table 2.) did not reveal any significant difference among these parameters.

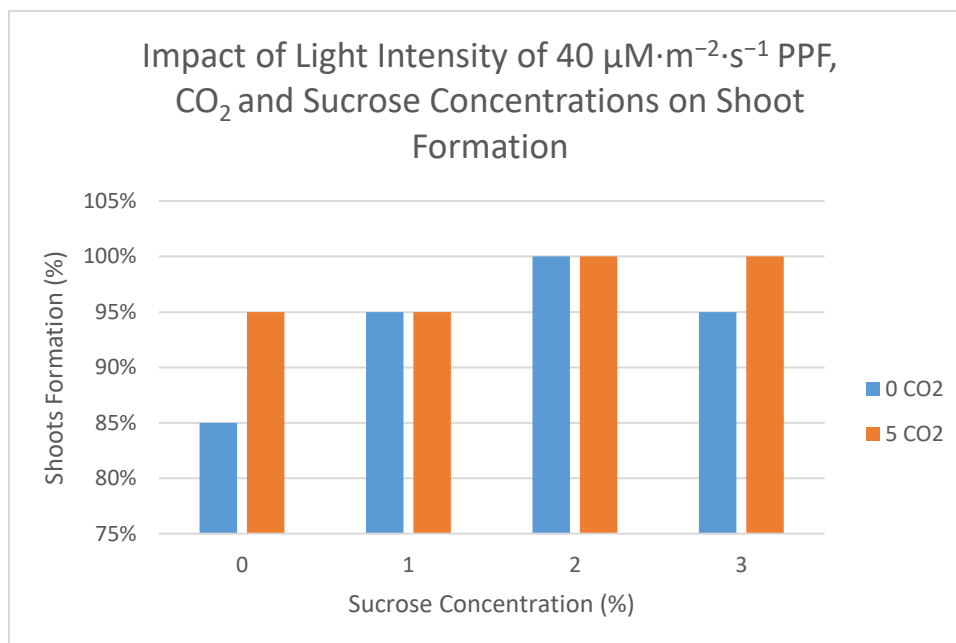


**Figure 39. Shoot formation (%) treated with  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0-3% sucrose concentration and 0% or 5%  $\text{CO}_2$  concentration.**

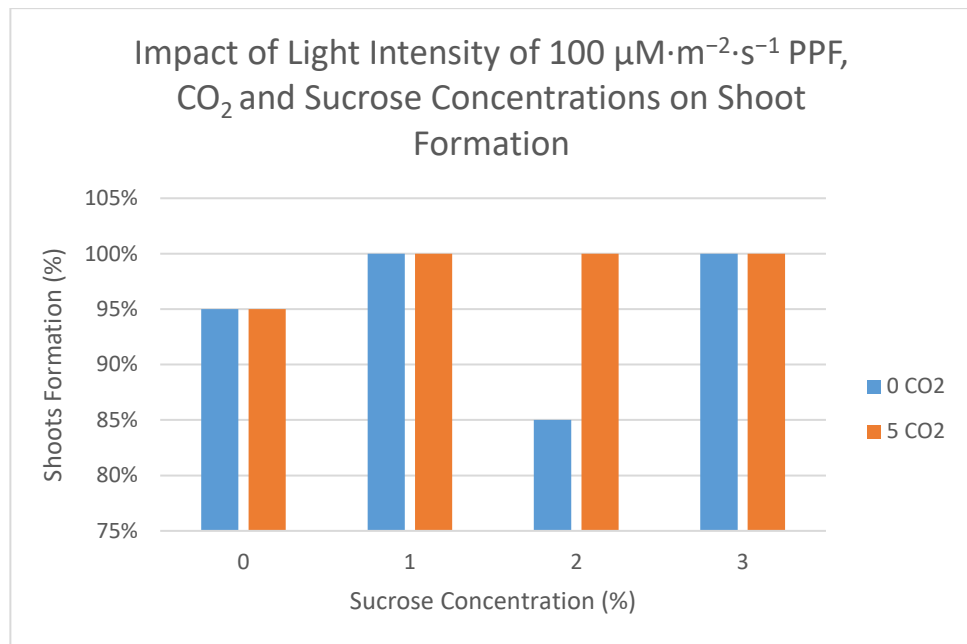


**Figure 40.** Shoot formation (%) treated with  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0-3% sucrose concentration and 0% or 5%  $\text{CO}_2$  concentration.

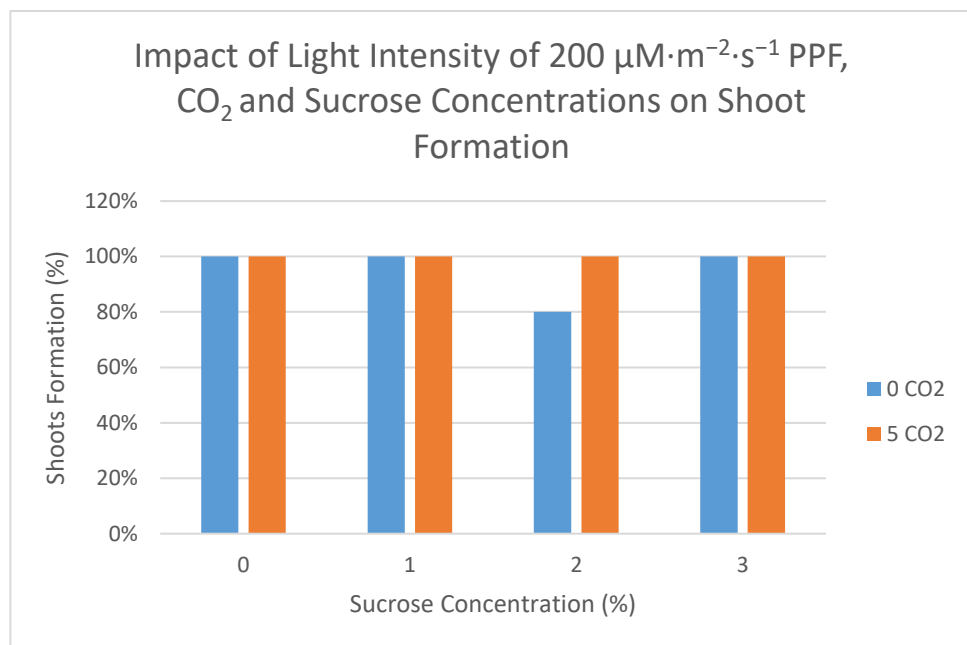


**Figure 41.** Shoot formation (%) treated with  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0-3% sucrose concentration and 0% or 5%  $\text{CO}_2$  concentration.

As mentioned above, a logistic regression with a binary response type (root formation: yes/no) represented the test for significance equivalent to root formation

analysis. Logistic regression was used to detect a possible significant difference of the impact on the formation percentage. Since a response data record was defined for the data analysis, logistic regression was necessary. Logistic regression outcomes revealed parameters that did not have any influence on explant's shoot formation. This was valid for each parameter: light intensity ( $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF,  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF,  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF),  $\text{CO}_2$  concentration (0%, 5%) and sucrose concentration (0%, 1%, 2%, 3%) ( $P=0.05$ ; Table 2) shown in Table 2. Wald Chi-Square Value of the observed parameters did not predict that any parameter has a significant impact on the formation of roots. The same can be interpreted from the  $P$ -Value.

**Table 2. Shoot formation (%) test of significance: logistic regression table**

| Predictor variables | Logistic Regression Co-efficient (A) | Standard Error (B) | Wald Chi-Square*<br>(A/B) <sup>2</sup> | P-Value | Odds ratio |
|---------------------|--------------------------------------|--------------------|----------------------------------------|---------|------------|
| Light-40            | 0.000                                | 0.000              |                                        |         |            |
| Light-100           | 0.000                                | 0.726              | 0.000                                  | 1.000   | 1.000      |
| Light-200           | 0.000                                | 0.726              | 0.000                                  | 1.000   | 1.000      |
| CO <sub>2</sub> -0  | 0.000                                | 0.000              |                                        |         |            |
| CO <sub>2</sub> -5  | 1.142                                | 0.678              | 2.842                                  | 0.092   | 3.134      |
| Sucrose-0           | 0.000                                | 0.000              |                                        |         |            |
| Sucrose-1           | 1.120                                | 1.164              | 0.926                                  | 0.336   | 3.065      |
| Sucrose-2           | -0.891                               | 0.706              | 1.594                                  | 0.207   | 0.410      |
| Sucrose-3           | 1.120                                | 1.164              | 0.926                                  | 0.336   | 3.065      |

\* Logistic Regression Statistic

### 3.2.3. Plant development

Plants were observed over a period of ten weeks after they were transferred out of the aquaria. Plant development was interpreted as the average measured length of shoot per week, assuming they would increase over time. Over the first three weeks the plant growth enhanced to a great amount. This change was visually detectable and verified via measurements. Statistical analysis was conducted equivalently to those of previous objectives with descriptive statistics and significance testing afterwards. Measurements at week ten were used to determine significance. Each parameter was first tested for significant impact on the plant growth separately and subsequently, combinations of parameters were tested to identify the impact caused by the interaction of those parameters.

#### Analysis of one parameter

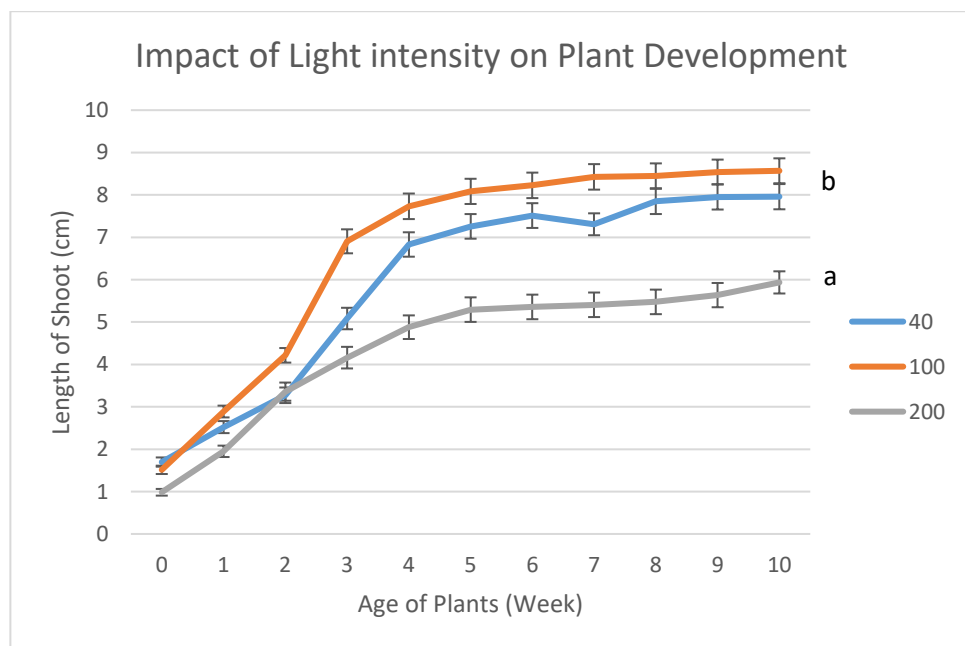


**Figure 42.** The impact of light concentrations 40, 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF on the length of shoots over time. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

The impact of each individual parameter on the explant's growth was analyzed. Interest was placed on how these explants, impacted by parameters during the growth cycle in the aquarium, developed over ten weeks. Parameters were defined as light intensity (40, 100, 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF),  $\text{CO}_2$  concentration (0%, 5%)

and sucrose concentration (0-3%). Observations of light as a parameter showed plants exposed to light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF had significantly shorter shoots at week 10. On the other hand, explants placed in a Heraeus® heating cabinet with a light intensity of 40 or  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF reached a greater height but did not differ significantly from each other in terms of  $\text{CO}_2$  and sucrose concentration. The different concentrations did not seem to influence plant development significantly. Standardized coefficients compare the strength of the effect of each individual independent variable (light intensity,  $\text{CO}_2$  concentration, sucrose concentration) to the dependent variable (plant development). If the absolute value of the coefficient is higher, the effect is stronger. The regression analysis showed the greatest standardized coefficient of light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF (shoot length of 8.7 cm). The standardized coefficient of light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF was the second greatest (shoot length of 8 cm) followed by the standardized coefficient of 0%  $\text{CO}_2$  (shoot length of 7.8 cm). This implies the impact of light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF might have been the strongest. Even though, parameters of  $\text{CO}_2$  and sucrose concentration did not differ significantly in their impact, standardized coefficients of 0% sucrose concentration (shoot length of 7.7 cm) and 0%  $\text{CO}_2$  were slightly higher. This means that the effects might still have been stronger.

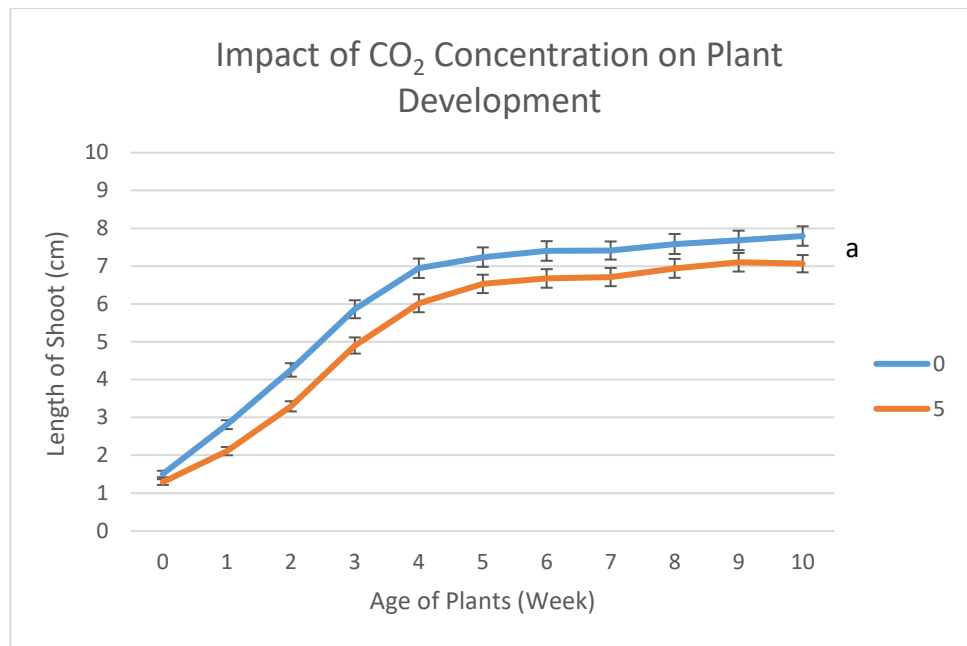


**Figure 43.** The impact of 0% or 5% concentration of CO<sub>2</sub> on the length of shoots over time. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

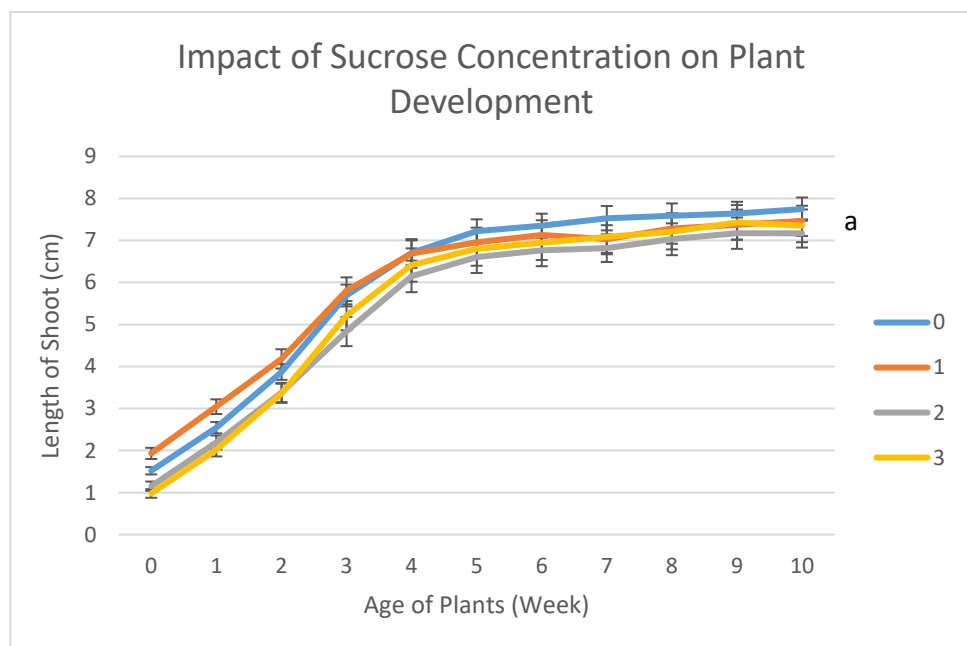


**Figure 44.** The impact of 0-3% sucrose concentration on the length of shoots over time. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

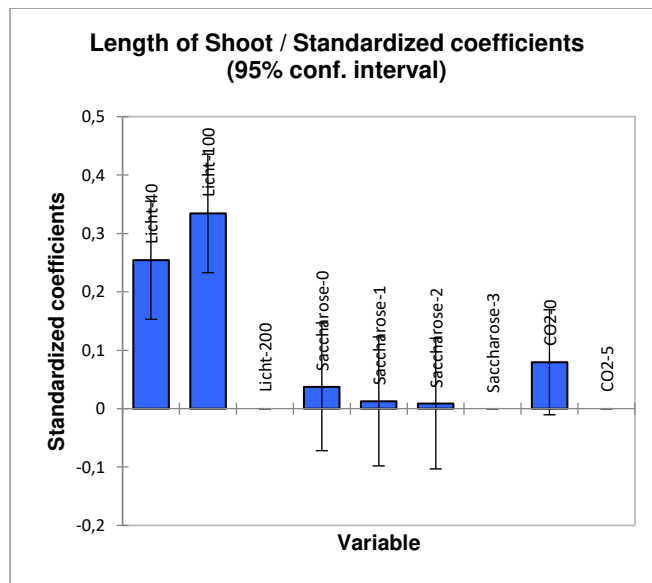


**Figure 45. Standardized coefficients of parameters.**

### Analysis of multiple parameters

The one factor analysis showed that light intensity as a parameter influenced plant growth over time. Lower light intensities seem to correlate with greater plant growth. A two factor ANOVA was conducted to detect the impact on plant development due to interactions of the parameters.

#### **40 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

Out of the plants developed from the explants treated with light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub>, those exposed to the highest sucrose concentration (3%) were significantly taller, with an average length of shoot of 10.6 cm. Among plant groups that were exposed to 0-2% sucrose concentration, height did not significantly differ (7-7.1 cm). Light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF but with 5% CO<sub>2</sub> seemed not to have any significant impact on plant growth. In this group the tallest plants, had an average length of shoot of 9 cm (0% sucrose concentration group), did not differ significantly from the average shortest length of shoot of 7.6 cm (3% sucrose concentration group).

Interpretation: Out of all light intensity 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF groups, plants from the group of 0% CO<sub>2</sub> with 3% sucrose concentration and plants from the group of 5% CO<sub>2</sub> with 0% sucrose concentration were slightly (but insignificant) taller in week 10.

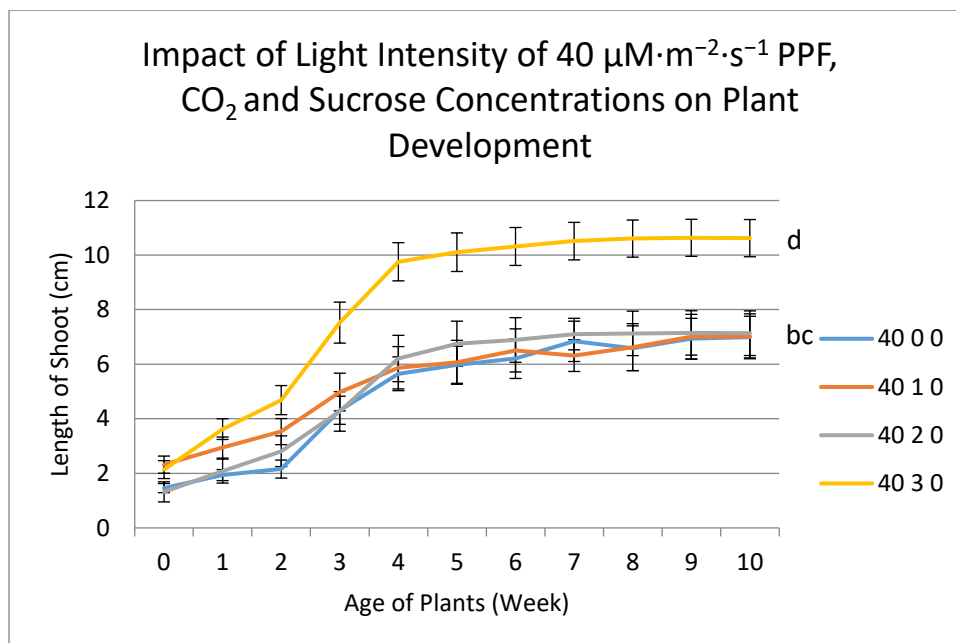


Figure 46. Plant development after treatment with light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPf, 0%  $\text{CO}_2$  and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

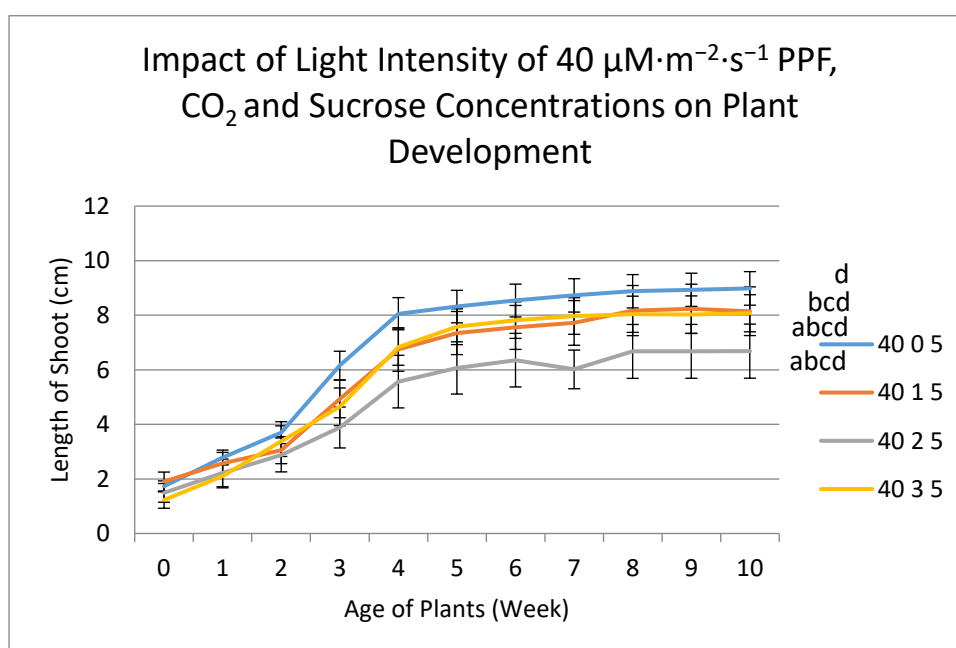


Figure 47. Plant development after treatment with light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPf, 5%  $\text{CO}_2$  and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### **100 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF**

The treatment combination of light intensity of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0%  $\text{CO}_2$  led to similar outcomes in terms of increase of shoot length. In addition, 3% sucrose concentration explants seemed to be insignificant shorter (9 cm) than those grown at 0-2% sucrose concentration (9.7-10.2 cm). Explants exposed to light intensity of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and treated with 5%  $\text{CO}_2$  did not vary significantly in their growth when compared among each other (6.3-8.5 cm). Though, descriptive statistics outcomes showed 2% sucrose concentration to bring the tallest (8.5 cm) in week 10 whereas 1% sucrose concentration to correlate with shorter plants (6.3 cm). Observing all treatments of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF together, the combination of 1% sucrose concentration and 0%  $\text{CO}_2$  led to significantly longer shoots with an average of 10.0 cm compared to 6.3 cm in the 5%  $\text{CO}_2$  group.

Interpretation: Both treatment combinations of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF with 0% or with 5%  $\text{CO}_2$  did not have any significant impact on plant development. The only exception was the combination of 1% sucrose concentration and 0%  $\text{CO}_2$  correlated with significant longer shoots compared to the 5%  $\text{CO}_2$  group.

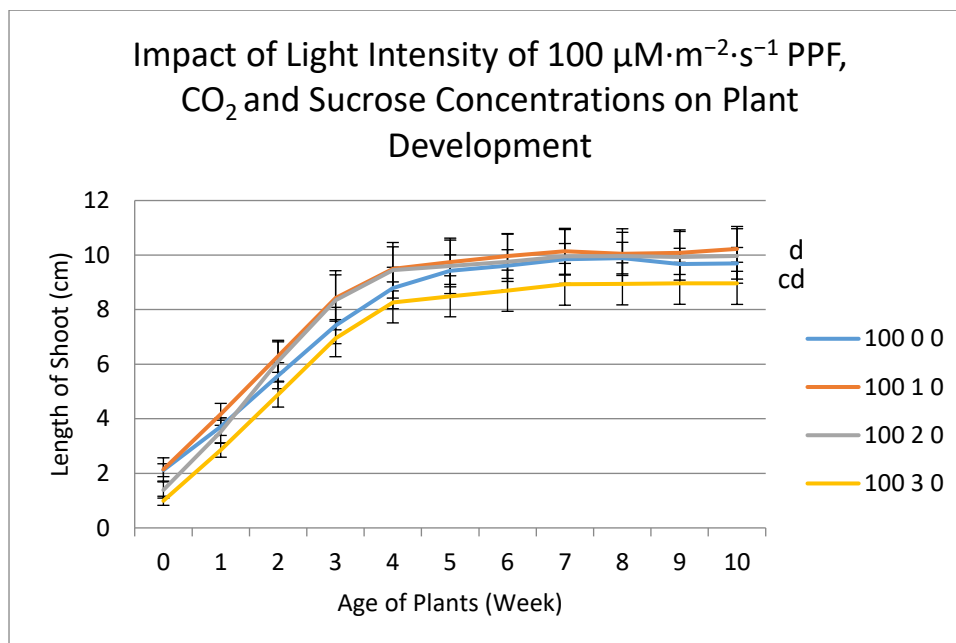


Figure 48. Plant development after treatment with light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0%  $\text{CO}_2$  and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

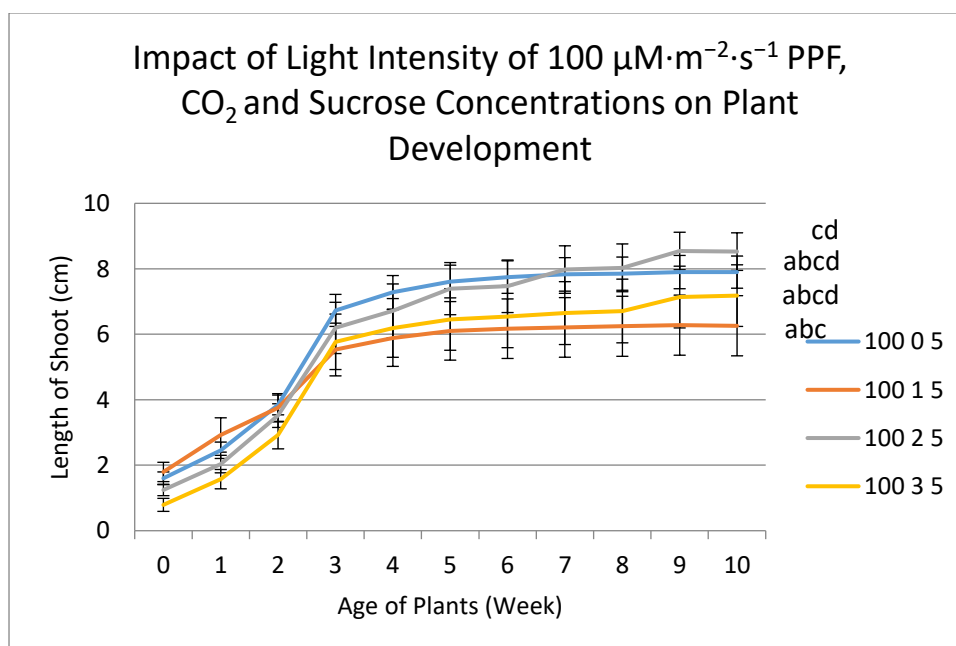


Figure 49. Plant development after treatment with light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 5%  $\text{CO}_2$  and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### 200 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF

The shoots of explants exposed to light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF PP combined with 0%  $\text{CO}_2$  and 0-1% sucrose concentrations turned out to be significantly longer (6.8-7.3 cm) at week ten than those from the groups treated with either 2-3% sucrose concentration (3.8-4.4 cm). In addition to a  $\text{CO}_2$  buffer solution (5%), all plants were similar in terms of their height (5.4-6.7 cm). Comparing all 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF groups with each other, plants of the 0%  $\text{CO}_2$  group without sucrose (0%) in the media, showed significantly longer shoots (6.8 cm) at week ten than those of the 5%  $\text{CO}_2$  group (5.4 cm). Likewise, plants treated with 1% sucrose concentration and 0%  $\text{CO}_2$  had longer shoots (7.3 cm) than those grown at 1% sucrose concentration and 5%  $\text{CO}_2$  (6.1 cm). Regarding descriptive statistics, plants grown in some amount of sucrose seemed to do better in their growth than those exposed to 0% or 3% sucrose concentration.

Interpretation: 0-1% sucrose concentration combined with light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PP seemed to improve plant growth significantly within the 0%  $\text{CO}_2$  group and compared to 0% sucrose concentration with 5%  $\text{CO}_2$ .

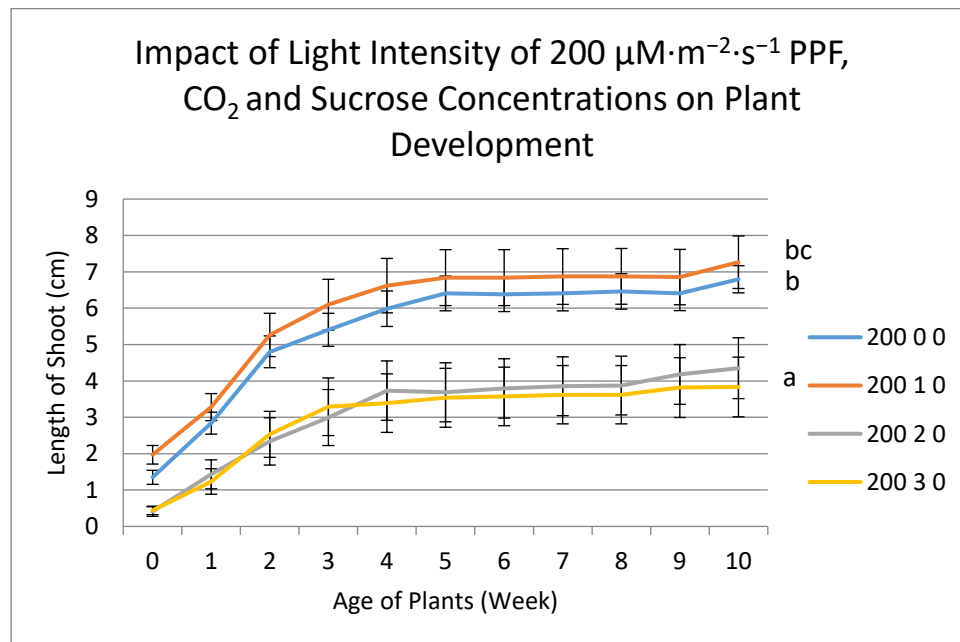


**Figure 50.** Plant development after treatment with light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0%  $\text{CO}_2$  and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

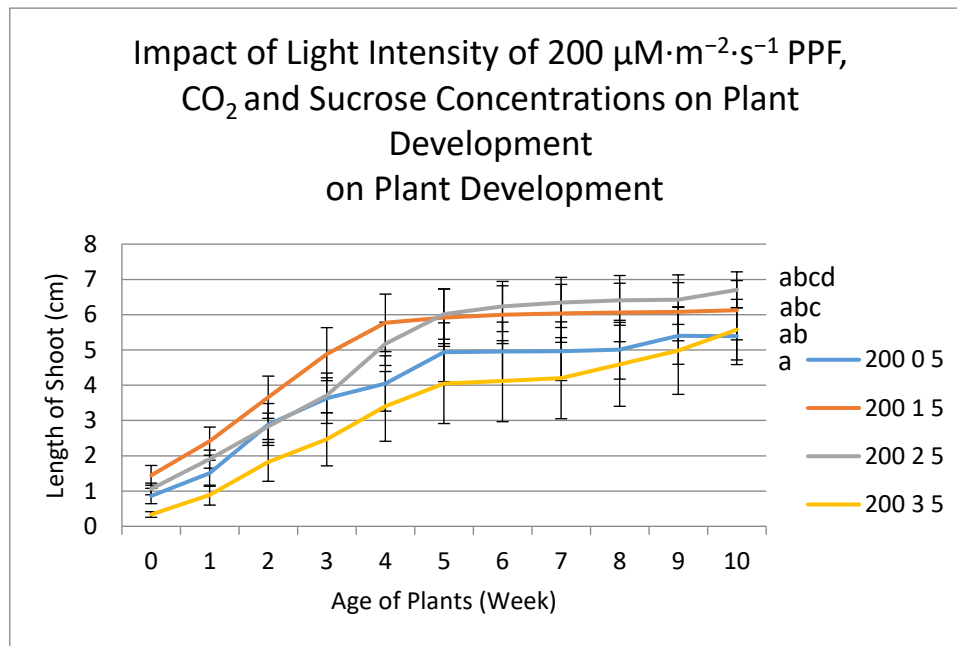


**Figure 51.** Plant development after treatment with light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 5%  $\text{CO}_2$  and 0-3% sucrose concentration. Illustration of median and standard error, columns with different letters differ significantly from each other ( $p=0.05$ ).

### Interpretation of Plant Development

Overall most of the plants survived up to week ten. Analysis results have brought insight into possible treatment combination to enrich plant growth. Light intensity as a parameter seems to impact plant development for the better (towards a higher growth). Out of all light intensity  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF groups, plants from the group of 0%  $\text{CO}_2$  and 3% sucrose concentration and plants from the group of 5%  $\text{CO}_2$  and 0% sucrose concentration were slightly (but insignificantly) taller in week ten. Both treatment combinations of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF with 0% or 5%  $\text{CO}_2$  did not have any significant impact on plant development. The only exception was the combination of 1% sucrose concentration and 0%  $\text{CO}_2$  correlated with significant longer shoots compared to the 5%  $\text{CO}_2$  group. 0-1% sucrose concentration combined with light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF seemed to improve plant growth significantly within the 0%  $\text{CO}_2$  group compared to 0% sucrose concentration in the 5%  $\text{CO}_2$  group.

Otherwise callus formations on some explants and vitrified shoots were observed. A possible connection could have been the limitation of applied sugar concentration. At least a minimum amount of sugar is necessary to carry on normal growth and keep symptoms of hyperhydricity at bay (Langford & Wainwright, 1987) or the relative humidity and/or ethylene gas concentration might have been too high (Kozai & Kubota, 2005a). This issue has not been investigated further in this experiment.

## 4. Discussion

In this observational study the poplar hybrid 741 [*Populus alba* x (*P. davidiana* + *P. simonii*) x *P. tomentosa*] was photoautotrophically micropropagated in vitro. The goal was to investigate how the plant adapts to these culture conditions and to find optimal treatment combinations for successful rooting and acclimatization.

*Populus sp.* belongs to the family of Salicaceae and represents an important crop for the paper and wood industry due to its rapid and enormous growth (Lubrano, 1992) as well as of interest for biomass production (Tuskan, 1998). The high demand for this plant could be satisfied with the technique of in vitro micropropagation. Since conventional micropropagation brings several downsides with it, the alteration to a photoautotrophic in vitro micropropagation is conducive to better outcomes in terms of plant growth and development (Kozai & Kubota, 2005a).

Photoautotrophic conditions are defined as the combination of higher light intensity, higher CO<sub>2</sub> concentration in the plant surrounding in addition of media free from a carbon source like sucrose or other kinds of sugar components. Under these circumstances various studies have shown improvement in how plant perform photosynthesis, meaning how the plant generates their own energy.

Under “normal” conditions energy would be generated from the carbon source in the media and from the light in the surroundings. Since the photosynthetic rate of conventionally in vitro grown plant is low, they require the addition of sugar to the culture medium (Shin et al., 2013).

With the implementation of photoautotrophic conditions, the plant switches to utilize the higher light intensity and CO<sub>2</sub> gas from the surrounding to generate energy instead.

The poplar hybrid 741 used in this study was genetically modified with two insect-resistant genes. These features are considered to improve the overall plant growth by protecting the plant from damage and dying in the growth process (Tian et al., 2000). Likewise, an endophyte interaction on poplar hybrid 741 seems to be another promotor for growth as well (Hanak et al., 2014).

This observational study is based on prior observations of photoautotrophic effect on poplar hybrid 741 plantlets. Those showed a positive impact on plant objects when photoautotrophic conditions were implemented in micropropagation. Gindler (2014) observed that an enhanced airflow in the jar due to usage of Magenta® caps, with a membrane with a hole, seemed to improve plant growth. However, enhanced CO<sub>2</sub> (2%) combined with higher sucrose concentration seemed to reduce shoot elongation. Likewise, Schnur's objectives (fresh weight, number of leaves, leaf area, number of nodes and concentration of chlorophyll in leaves) seemed to improve under photoautotrophic conditions.

In Gindler's and Schuhleitner's work Magenta® caps with a implemented membrane, higher light intensity and a higher CO<sub>2</sub> concentration came out as promotive parameters regarding plant growth. Pachala-Ghoreishi (2018) was the first to investigate how photoautotrophic conditions impact the root system, among other observations. She found a lower light intensity to already have a positive influence on rooting and acclimatization.

This work represents the continuation of these previous observations with the focus placed on rooting and plant development.

The impact on shoot and root formation, length and number of roots, along with the length shoots were measured after 21 days of culture condition exposure. Plant development (length of shoots *ex vitro*) was further measured until (and including) the 10th week to detect the adaptation to prior the stimuli in the jar.

Regarding the materials, a half strength LS basal medium (a powder formulation of micro and macro elements) by Linsmaier and Skoog (1965), was used in this study. This formulation was shown by Hanak (2014) to improve development of poplar hybrid Type 741. As a gelling agent Gelrite® was chosen. Gelrite® might be superior to Agar in terms of shoot growth, plant survival and performance (Kang et al., 2009). As a growth regulator Indole-3-butyric acid (IBA), an Auxin, was added to initiate root formation. IBA was shown to be the one growth regulator very necessary for an improved root development (Wei et al., 2017). Similarly Auxins IBA and IAA seem to bring good rooting results (Pachala-Ghoreishi, 2018).

Magenta® B-caps with a drilled hole and an attached membrane served as a lid to enhance ventilation without the risk of microbial infection. This is meant to improve aerial microenvironment inside the jar.

Conventional micropropagation techniques are typically performed using small culture jars without ventilation and under lower light intensities. Contrary, a larger jar size, for example baby food jars (200 ml), instead of small culture jars like culture tubes, was shown to have a positive impact on the root length, root system visual density and on the shoot height and fresh weight (McClelland & Smith, 1990). However, a larger jar aerial environment seems to be better controlled with a forced ventilation system (Kozai & Kubota, 2005a).

For this reason, in this experiment 250 ml baby food “Hipp” jars were combined with the membrane-lid system to encourage airflow, and consequently enhance CO<sub>2</sub> levels inside the jar.

The benefits of implementing a photoautotrophic system over the conventional micropropagation are various.

It was shown that under properly controlled environmental conditions, the photosynthesis, and consequently plant growth, of plants that were micropropagated photoautotrophically was improved due to enhanced net photosynthetic rate. Net photosynthetic rate is the total rate of CO<sub>2</sub> fixation minus the rate of loss during respiration (Afreen, 2005; Shin et al., 2013). Even though photoautotrophic conditions were shown to bring the highest net photosynthetic rate, a photomixotrophic system seems to surpass conventional tissue culture in terms of growth promotion (Kozai & Kubota, 2005a).

Further, better survival rates seem to result from improved stomata function and transpiration rates.

Even hyperhydricity, a morphological disorder generated as a plant’s response to stress, seems to be reduced. Stress is likely to occur in unsuitable in vitro environments. Unsuitable environmental conditions like high relative humidity or accumulation of ethylene, among others. This phenomenon might be less likely to occur with the reduction of relative humidity and ethylene.

Further, the chance for microbial contamination is minimized due to the absence of sugar in the media.

Advantages of engineering aspects exist as well (Kozai & Kubota, 2005a) but are not discussed here any further.

The purpose of this observational study was to investigate the impact of different light intensities, sucrose concentrations in the media, and CO<sub>2</sub> concentration inside the jar on rooting and plant growth. Since Auxins (IBA, IAA) showed good rooting results in heterotrophic cultures in Pachala-Ghoreishi (2018) experiment, IBA was used in this experiment. Observing the impact of IBA on rooting, growth and development of poplar hybrids under photoautotrophic conditions was another aspect of this work.

Light intensities of 40, 100, and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF were selected for the experiment. Likewise, light intensities of 25, 50, 100, or 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  were used previously in an experiment on *Momordica grosvenori* plantlets by Zhang et al. (2009). In another experiment, light intensities of 70, 150, 230, or 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  were chosen in combination with a Florialite-based medium to observe the effect on neem plants (Nguyen & Kozai, 2001).

The assumption behind the usage of the highest light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF in this experiment was to stimulate the plant's capability to perform photosynthesis and consequently support plant growth and rooting. All of that takes place without the requirement of an exogenous carbohydrate source but rather on its own. Such results were observed in many findings present in the literature. Net photosynthetic rates and growth of *Doritaenopsis* hybrid plantlets were promoted significantly by increasing the light intensity and CO<sub>2</sub> concentration in the culture jar. The greatest amount of growth was measured either under photoautotrophic or photomixotrophic conditions (Shin et al., 2013). Lower light intensities are commonly used in conventional in vitro micropropagation. In our study, 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF was chosen as a representation of the conventional micropropagation technique when combined with 3% sucrose and atmospheric CO<sub>2</sub> concentration.

Regarding sucrose, media concentrations were set between 0-3%. The highest percentage of 3% sucrose is commonly used in the conventional micropropagation whereas in photoautotrophic micropropagation, any kind of exogenous

carbohydrate source is left out (Shin et al., 2013; Wilson et al., 2000). Mingozi et al. observed effects with similar sucrose concentration layout of 0%, 1% and 3% sucrose on *Populus deltoids* (Mingozi et al., 2009). Findings in the literature revealed 0% sucrose to often be compared to 3% sucrose. This makes sense, since one corresponds with a photoautotrophic approach (0% sucrose) and the other with a conventional approach (3% sucrose). The idea behind could be understood as a comparison of the effects of both strategies on plant objects (Shin et al., 2013; Valero-Aracama et al., 2001).

However, exogenous sugar seems to be a burden by attracting microorganisms with further plant infections and damage (Long, 1997) and in an experiment with rose cultures an inverse correlation of sucrose concentration and photosynthetic activity was investigated. With a low concentration of sucrose (10 g l<sup>-1</sup>) photosynthetic activity was higher than with a high concentration of sucrose (40 g l<sup>-1</sup>). A minimum amount of sugar (2.5 g l<sup>-1</sup>) was therefore required, otherwise plantlets displayed symptoms of hyperhydricity and eventually died (Langford & Wainwright, 1987). Besides infections caused by microorganisms attracted by sugar, other findings in the literature even stated that exogenous sugars supplied in excessive amount could have a negative effect on photosynthesis (Desjardins et al., 1995).

In terms of CO<sub>2</sub> as a controlled parameter, concentration in the gas phase inside the culture jar was set to be either atmospheric with 0.04% CO<sub>2</sub> or elevated to 5% CO<sub>2</sub> through usage of a buffer solution inside the aquaria. According to Desjardins et al. (1995) low CO<sub>2</sub> concentrations might lead to low photosynthesis rate whereas CO<sub>2</sub> enrichment seems to do the opposite, increasing photosynthesis and therefore having a beneficial effect on growth because of it. Higher levels of CO<sub>2</sub> are therefore necessary to stimulate photoautotrophic capability of plants (CO<sub>2</sub> fixation). Having higher light intensities and a medium free of sugar alone is not enough for the plant to perform photosynthesis (Kozai & Kubota, 2005b). The combination of high CO<sub>2</sub> concentrations with high light intensity make oxygen exchange rates to go up (Cournac et al., 1991).

For healthy plant growth, internal gases of all parts of a plant need to be exchanged with those in surrounding air (Kozai et al., 1991). Plants release substances and when those accumulate this can have a significant effect on growth and development (Heyser & Mott, 1980).

For example, a rapid accumulation of ethylene gas inside the culture jar was shown to reduce rooting time and improved rooting percentage in an experiment on a hybrid Peach rootstock. However, different concentration of the gas did not seem to have any impact on root number and length.

Plant growth and plant development were defined as objectives. Length of root, number of roots and formation of root were picked as parameters to measure the root system. Length of shoot represented the parameter to analyze plant growth and plant development.

Regarding the parameter length of root, results of the single parameter analysis (ANOVA) showed the highest light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF to bring longer roots as an outcome. In an experiment on *Momordica grosvenori* plantlets higher light intensities also offered a well-developed rooting system, better shoot growth, and the ex vitro survival percentage (Zhang et al., 2009).

Sucrose concentration of 0% led to shorter roots. Since root formation is an energy demanding process the additional sugar could be essential. Contrary, literature shows sugar added to the media seems to decrease the root length of macadamia plantlets under CO<sub>2</sub>-enriched conditions. Whereas CO<sub>2</sub>-enriched photoautotrophic conditions increased the root length (Cha-um et al., 2011).

With the multiple parameter analysis (ANOVA) interference of the parameter could be investigated. Explants grown at  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% sucrose had significant shortest roots compared to the highest amount of sucrose with 3% independently of the CO<sub>2</sub> concentration in the surrounding. Low light intensities therefore be rather combined with a higher sugar concentration as is common in conventional micropropagation to enhance root length.

In order for the plant to perform photoautotrophy, a high light intensity is needed when exogenous carbon source is left out (Afreen, 2005). At light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF explants affected by 5% CO<sub>2</sub> and 2% sucrose had longer roots than

0% sucrose and 0% CO<sub>2</sub>. In the analysis 0% sucrose was the single factor impacting root length negatively. Maybe this parameter led to overall shorter root length in the aforementioned combination or there might be other subtle explanations. Roots regenerated at a light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0% CO<sub>2</sub> and 1% sucrose were longer than those influenced by 0% sucrose. The negative impact of 0% sucrose detected through single parameter analysis, might surpass the positive one of the light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF after all. 0% CO<sub>2</sub> and 1% sucrose combined led to an increase in roots length compared to 3% sucrose and 5% CO<sub>2</sub>.

To summarize: explants might be best treated with the highest light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF since it correlated with the greatest positive impact on root length. The same was shown by Naguyen and Kozai on neem plants. Root elongation was promoted the most strongly with 230  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, the highest light intensity besides light intensities of 70  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 150  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF (Nguyen & Kozai, 2001).

A supporting material like Vermiculite instead of the gelling agent used might improve root development further (Ermayanti et al., 1999). However, the advantages of alternative types of supporting material will be discussed later in more detail.

For the parameter number of roots, light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF showed a significant impact with less roots than with any of the other light intensities. Pachala-Ghoreishi (2018) stated in her work that light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF significantly increased number of roots compared to 100 and 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Even though in this study 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF was not found to be a parameter of significant impact on number of roots, her outcome indirectly coincides with the result of this experiment regarding 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF being less effective. The effects of all parameter interactions were examined as their combined effect could have differed from the effect of one parameter alone.

Treatment group of light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF with 0% CO<sub>2</sub> and 3% sucrose, which represented a conventional approach, developed more roots than when treated with 0% or 1% sucrose. This might be because light intensity of 40

$\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF is probably too low for the plant to generate energy solely from solar energy and  $\text{CO}_2$  fixation (Afreen, 2005).

Obviously, through the addition of exogenous sugar to the media, plants will have more energy reserves to form roots. As mentioned in the section above, some amount of sugar seems to be necessary for the root formation (George, 1996). 5%  $\text{CO}_2$  led to more roots when combined with 0%-2% sucrose than when the highest sugar concentration of 3% sucrose was applied. This is in accordance with Cha-um's work (2011), where the number of roots of macadamia plantlets increased without sugar in the medium but with enhanced  $\text{CO}_2$  conditions at  $120 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Why 2% sucrose led to similar number of roots is not clear.

Combining light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF with 0%  $\text{CO}_2$  and 0% sucrose led to more roots than 5%  $\text{CO}_2$  with 0% sucrose or 3% sucrose. Even though  $\text{CO}_2$  as a parameter alone did not have any significant effect, 0%  $\text{CO}_2$  seems to be one of the parameters that repeatedly occurred in treatment combinations with higher number of roots. It seems  $\text{CO}_2$  does interfere with the other parameters when combined. In addition,  $\text{CO}_2$  gas might prevent root induction and root initiation in combination with sugar during stage of root formation.

In another observational study *Vitis vinifera* produced fewer roots with enhanced  $\text{CO}_2$  during root initiation stage on a sugar-containing medium. However, a rooting pre-culture on medium with sucrose before the  $\text{CO}_2$ -enriched culture ( $1200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF) on sucrose-free medium led to improved root and even shoot growth (Fournioux & Bessis, 1993).

Yet alone,  $\text{CO}_2$  might not impact rooting overall (Marino & Ventura, 1997). The same statement is true for this study.

Light intensity of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF showed better results in terms of root number when combined with 0%  $\text{CO}_2$  and 2% sucrose than when explants were affected by 5%  $\text{CO}_2$  and 3% sucrose. Pachala-Ghoreishi (2018) as well found the combination of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF with higher sugar concentrations to bring more roots. Highest light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF stimulated the explants to grow more roots in combination with 0%  $\text{CO}_2$  and 0% sucrose than with 1% or 3% sucrose. This shows that photoautotrophy might have a positive influence on

the number of roots.

Interestingly, a high light intensity ( $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF) seems to counteract the process of root formation, but a combination of all parameters to create a photoautotrophic environment seems to be essential for a higher number of roots and therefore water and nutrient supply for the plant.

Other parameters were formation of roots and shoots. Single parameter ANOVA outcomes showed formation of roots and shoots to not be affected by any parameters: light intensity,  $\text{CO}_2$  or sucrose concentration. Even though descriptive statistics results raised suspicion whether there was a difference between the treatment groups but applied logistic regression outcomes did not reveal any significant difference between the groups, in terms of the percentage of rooting or shooting per explant.

Ermayanti (1999) demonstrated in her Mangosteen experiments that a high number of air exchanges, vermiculite as a supporting material, sucrose ( $30 \text{ g}\cdot\text{L}^{-1}$ ) and growth regulators (2-IP/IBA) brought the same percentage of rooting as when sugar and growth regulators were absent.

Shoot length measurements were divided into two parameters. Shoot length measured right after the experiment is defined here as initially formed shoot length, and average shoot length as the other parameter. Regarding initially formed shoot length, analysis of single parameters showed light intensity of 40 and  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF to lead to longer shoots. Consequently, a higher light intensity of  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF as the only parameter might not be optimal. In the previously mentioned study of neem plants, elongation of shoots was also impaired at a higher light intensity ( $230 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF) (Nguyen & Kozai, 2001). However, 0% and 1% sucrose increased both shoot length parameters (initial, average). The effect of some amount of sucrose (1%) on initial shooting was even stronger than without sucrose. Like sucrose, 0%  $\text{CO}_2$  had a positive impact on both shoot length parameters (initial, average).

Combining all three parameters, light intensity of  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0%  $\text{CO}_2$  and 1% sucrose resulted in longer initially formed shoots compared to those of explants treated with 2% sucrose. Explants affected by light intensity of 100

$\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% or 1% sucrose 0%  $\text{CO}_2$  evolved longer primary shoots compared to 3% sucrose. Light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0%  $\text{CO}_2$  and 1% sucrose led to the longest initially formed shoots, followed by the treatment with 0% sucrose, which led to the formation of the second longest initially formed shoots.

Treatments of 5%  $\text{CO}_2$  combined with 1% or 2% sucrose resulted in longer initially formed shoots when compared 3% sucrose. It seems the combination with lower sugar concentration, 0%  $\text{CO}_2$  and low to medium light intensities to be most promotive for longer initial shoot growth.

The second parameter concerning shoot length observations is, as mentioned above, average shoot length. In a single parameter analysis (ANOVA) light intensity of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF correlated with plants of longer average shoot length. Results based on analysis of all three parameters and their interconnections showed the longest shoots on average in the combination of light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0%  $\text{CO}_2$  and 3% sucrose (conventional approach).

Whereas with elevated  $\text{CO}_2$  of 5%, 0% sucrose led to the longest average shoots opposing observation of the 0%  $\text{CO}_2$  group. An increase in length of roots of macadamia plantlets under photoautotrophic  $\text{CO}_2$ -enriched conditions was also observed by Cha-um (2011). With the addition of 1% sucrose explants had longer average shoots than those treated with 2% sucrose.

Elevated  $\text{CO}_2$  levels combined with no or low sugar concentrations brought on the best results. The combination of light intensity of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0%  $\text{CO}_2$  and 3% sucrose brought shorter average shoots. All explants grown at atmospheric  $\text{CO}_2$  levels (0%) formed longer shoots on average than those affected by 5%  $\text{CO}_2$ . However, in the 5%  $\text{CO}_2$  group explants had the longest shoots on average grown in a 0% or 2% sucrose containing medium. In contrast, 1% and 3% sucrose led to the lowest average shoot length.

Regarding light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, a combination with 0%  $\text{CO}_2$  and 0% or 1% sucrose resulted in longer average shoots. Combined with 5%  $\text{CO}_2$ , the longest shoots were found with 1% or 2% sucrose. Basically, only the combination with 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF seems to be independent of  $\text{CO}_2$  concentrations in regard to 0-1% sucrose leading to longer shoots on average.

For all other light intensities 5% CO<sub>2</sub> seems to be best combined with 0-1% sucrose for longer average shoots. Schuhleitner (2014) as well found an increase in shoot length with higher light intensities (100, 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF), whereas CO<sub>2</sub>-enhancement did not impact the shoot's development.

Contrary, Adelberg (1999) showed that enhanced light intensity together with increased CO<sub>2</sub> to have a positive interaction on shoot proliferation on two triploid genotypes of melon (*Cucumis melo* L.).

Plant development was set as another parameter in this study to investigate how plantlets might adapt and grow as a response to prior experienced environmental conditions. Of further interest was of course the survival rate.

According to Zobayed et al. (1999) high net photosynthetic rate, normal anatomical structure and functional stomata are factors that contribute to enhance plant survival percentage in ex vitro environment. These are all factors that were shown to be improved under photomixotrophic or photoautotrophic conditions (Kozai & Kubota, 2005a). According to the literature (George, 1996), besides high photosynthetic rate contributing to enhanced survival percentage, by reducing relative humidity with an open system (as is the case under photoautotrophic conditions) leads to more vigorous plants during acclimatization and higher survival rates due to improved stomata closure, thicker cuticula and therefore less water loss and wilting.

Analysis was conducted equivalently to all other parameters: first single parameter analysis and then multiple parameter analysis of light intensity, sucrose concentration and CO<sub>2</sub> concentration.

Single parameter analysis revealed plants treated with light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF or 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF reached a significantly higher size in week 10. However, investigating the other two parameters separately in a single parameter analysis led to the conclusion that those did not have any significant effect on the explant's development. A combination of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0% CO<sub>2</sub> and 3% sucrose brought the highest grown plants in week 10 within the group treated with light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF (conventional approach). Since there is an essential need for sugar as a carbon source for the plant's energy

supply at lower light intensities, the results are less surprising but high amounts of sugar might suppress photosynthesis again (Kozai & Kubota, 2005c).

Within the group of light intensity  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0%  $\text{CO}_2$ , explants treated with 0% or 1% sucrose were the tallest at week 10. This leads to the conclusion that a high light concentration alone might not be effective, but photoautotrophic conditions seem to be an option to improve plant development and acclimatization. The same positive impact was observed on growth of coconut seedlings and a better survival rate upon transfer to ex vitro (Samosir & Adkins, 2014). This combination of parameters also led to significantly longer shoots than when 5%  $\text{CO}_2$  was used. Likewise, plants treated with 1% sucrose and 0%  $\text{CO}_2$  had longer shoots than those grown at 1% sucrose and 5%  $\text{CO}_2$ . Combinations of  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, whether combined with 0% or 5%  $\text{CO}_2$ , did not have any significant impact on plant development in week 10 except for plants treated with  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 1% sucrose and 0%  $\text{CO}_2$  which resulted in greater growth only in comparison to the 5%  $\text{CO}_2$  group.

For further research about the impact of each treatment, ethylene concentrations might be of interest. Therefore, measurements of the exact concentration of ethylene gas in the headspace inside the jar would be recommended. Higher ethylene concentrations were shown to have a negative impact on plant development and rooting and might lead to aberration of morphology and physiology (Kozai & Kubota, 2005a; Zobayed et al., 2000), therefore it might be of interest to measure the concentration of ethylene gas in the headspace. Under photoautotrophic conditions ethylene concentrations are supposed to be lower than under conventional conditions.

Another potential approach would be to focus on the right kind of substrate. The substrate represents an important part of in vitro microclimate and subsequently for root development. The implementation of fibrous supporting materials with high air porosity like Vermiculite or Floralite seems promising since relative humidity inside the jar could be decreased (Kozai & Kubota, 2001). Floralite, a mixture of Vermiculite and paper pulp (7:3), might be a better replacement for a gelling agent like Gelrite® or Agar (Afreen-Zobayed et al., 2000). Oxygen and nutrient

availability seem to be enhanced and improves rooting and plant growth. Acclimatization seems to be improved as well due to an enhanced root system (Afreen-Zobayed et al., 1999). It also has been shown to enhance root and shoot development under photoautotrophic conditions (Afreen-Zobayed et al., 2000). Vermiculite was shown to be beneficial under CO<sub>2</sub>-enriched conditions (Kirdmanee et al., 1995). Other alternatives might be Rockwool (Nagae et al., 1996) and cellulose plugs (Afreen-Zobayed et al., 1999). Activated charcoal might be another possible innovation to try since improvement in plant growth in poplar species has been observed (Kang et al., 2009) and in other plant species (Pan & Staden, 1998).

In this observational study natural ventilation was used to increase the air flow. However, forced ventilation would be an asset to reach the desired CO<sub>2</sub> concentrations. In that case it ought to be combined with large culture jars, so concentrations inside the jar would be more predictable and controllable (Kozai & Nguyen, 2003).

## 5. Abstract

The present thesis dealt with the photoautotrophic in vitro micropropagation of the poplar hybrid 741 [*Populus alba* x (*P. davidiana* + *P. simonii*) x *P. tomentosa*]. The purpose of the study was to investigate how poplar in vitro cultures adapt to certain environmental conditions. These were created by factorial combination of three parameters: light intensity (40,100, or 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF), sucrose concentration (0-3%) and CO<sub>2</sub> concentration (0% or 5%). The conventional (40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 3% sucrose, 0% CO<sub>2</sub>) and photoautotrophic approach (200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0% sucrose, 5% CO<sub>2</sub>) were tested, among others. The impact of each of these combinations (treatments) on root and shoot formation as well as their development was investigated. To that end, length, number and formation (%) of roots as well as initially formed length, average length and formation (%) of shoots were evaluated. Subsequently, plant development was monitored over the next ten weeks following the experiment.

Regarding the results, all light intensities had a significant impact on the observed growth parameters. The highest light intensity of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF led to the formation of longer roots but with a lower number of roots per explant overall. However, the light intensities of 40 and 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF led to longer initially formed shoots and average shoots. With a light intensity of 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF the effect on average shoot length seemed to be even stronger.

Lack of sucrose (0%) led to a shorter root length without an impact on the number of roots. 0% and 1% sucrose increased the initially formed shoot length and average shoot length. The impact of 1% sucrose on initially formed shoot length was even stronger than 0%.

0% CO<sub>2</sub> also had a positive impact on both shoot length types (initially formed and average shoot length), whereas no effect on the root system could be observed. In general, the formation of roots and shoots has not been affected by any parameter.

In terms of plant development, a single factor analysis showed significant higher plant growth in week 10 when the cultures were kept at a light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF or 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF. Each of the other parameters, sucrose and

CO<sub>2</sub> concentrations, observed on their own, did not seem to have any significant effect on the plant development. A combination of 3% sucrose led to the highest grown plants in week 10 within the group that was treated with a light intensity of 40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF and 0% CO<sub>2</sub>. Within the group of 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF light intensity and 0% CO<sub>2</sub> explants treated with 0% or 1% sucrose were the tallest in their growth. No significant effect could be detected between the other treatment combinations. It can therefore be concluded that lower sugar concentrations, as used in the photoautotrophic method, seemed to have an impact on root and shoot development. However, lower CO<sub>2</sub> levels only affected shoot lengths positively. Photoautotrophic conditions seemed to promote plant development (shoot length elongation over time). Therefore, the photoautotrophic approach could be a reliable method for improved plant development of poplar hybrid 741. Depending on which part of the plant is of interest, certain combinations of parameters brought better results than others. For this experiment in general the photoautotrophic approach was a solution to improve outcomes in certain cases but did not lead to better outcomes overall. Further research could be conducted to investigate the impact of each treatment on ethylene concentrations. Since higher ethylene concentrations affect plant development and rooting, and might lead to aberration of morphology and physiology, it might be of interest to measure the concentration of ethylene gas in the headspace. The substrate represents an important part of in vitro microclimate and subsequently for root development. Therefore, the use of supporting material with high air porosity might be of further research interest. By using such a supporting material instead of a gelling agent, relative humidity inside the jar could be decreased. Natural ventilation was used in this experiment to increase the air flow. However, forced ventilation might be even more beneficial to reach the desired conditions. In that case it ought to be combined with large culture jars. In this way concentrations of CO<sub>2</sub> and/or ethylene inside the jar would be more predictable and controllable. In conclusion, using a photoautotrophic micropropagation system seems to be an appropriate method to propagate poplar hybrid 741.

## Zusammenfassung

Die vorliegende Arbeit beschäftigte sich mit der photoautotrophen in vitro-Kultivierung der Pappel-Hybride Typ 741 [*Populus alba* x (*P. davidiana* + *P. simonii*) x *P. tomentosa*].

Das Ziel der Studie war zu ermitteln, wie sich in vitro Kulturen der Pappel an bestimmte Kulturbedingungen adaptieren. Diese wurden durch eine Kombination der drei Parameter Lichtintensität (40, 100, 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF), Saccharose-Konzentration (0-3 %) und  $\text{CO}_2$ -Konzentration (0 % oder 5 %) geschaffen. Unter anderem wurden der konventionelle (40  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 3% Saccharose, 0%  $\text{CO}_2$ ) und der photoautotrophe Ansatz (200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF, 0% Saccharose, 5%  $\text{CO}_2$ ) untersucht. Der Einfluss jeder dieser Kombinationen (Behandlungen) auf Wurzel- und Sprossbildung und -Entwicklung wurden eruiert. Dazu wurden die Länge, die Anzahl und die Bildungsrate (%) der Wurzeln als auch die anfängliche sowie die durchschnittliche Sprosslänge und die Sprossbildungsrate (%) evaluiert. Anschließend wurde die Pflanzenentwicklung über die auf das Experiment folgenden zehn Wochen beobachtet.

Alle Lichtintensitäten zeigten einen signifikanten Einfluss auf die erhobenen Parameter. Die höchste Lichtintensität von 200  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF führte zu längeren Wurzeln, jedoch insgesamt mit einer verminderten Anzahl an Wurzeln pro Explantat. Die Lichtintensitäten von 40 and 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF wiederum resultierten in längeren anfänglich gebildeten Sprossen und durchschnittlicher Sprosslänge. Bei einer Lichtintensität von 100  $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF scheint der Effekt auf die durchschnittliche Sprosslänge stärker gewesen zu sein.

Die Konzentration von 0% Saccharose führte zu einer kürzeren Wurzellänge ohne Einfluss auf die Anzahl der Wurzeln. 0% und 1% Saccharose erhöhten die anfänglich gebildete sowie die durchschnittliche Sprosslänge. Der Einfluss auf die anfänglich gebildete Sprosslänge durch 1% Saccharose war stärker als jener durch 0%. 0%  $\text{CO}_2$  hatte auch einen positiven Einfluss auf beide Typen von Sprosslängen (die anfänglich gebildete und die durchschnittliche Sprosslänge), wohingegen keine Auswirkungen auf das Wurzelsystem zu beobachten waren. Generell wurde

die Wurzel- und Sprossbildung von keinem der Kulturparameter beeinflusst. Die Pflanzenentwicklung betreffend zeigte eine einfaktorielle Analyse ein signifikant höheres Pflanzenwachstum in der Woche 10, wenn die Pflanzen bei einer Lichtintensität von  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF als auch  $100 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF kultiviert worden waren. Keiner der anderen Parameter (Saccharose und  $\text{CO}_2$  Konzentration) zeigte einzeln betrachtet einen signifikanten Einfluss auf die Entwicklung der Explantate. Eine Kombination mit 3% Saccharose führte zu den längsten Sprossen in der 10. Woche innerhalb der Gruppe, welche bei der Lichtintensität von  $40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF und 0%  $\text{CO}_2$  kultiviert worden war. In der Gruppe, die der Lichtintensität von  $200 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PPF und 0%  $\text{CO}_2$  ausgesetzt wurde, regenerierten die Explantate, die auf Medien mit 0% oder 1% Saccharose kultiviert worden, die längsten Sprosse. Zwischen den anderen Behandlungskombinationen war kein signifikanter Effekt festzustellen. Daraus kann geschlossen werden, dass niedrige Zuckerkonzentrationen, die bei der photoautotrophen Methode verwendet werden, auf die Wurzel und Sprossentwicklung einen Einfluss haben. Jedoch beeinflussten niedrige  $\text{CO}_2$ -Konzentrationen nur die Sprosslänge positiv. Photoautotrophe Bedingungen scheinen die Pflanzenentwicklung (Sprosslängenelongation im Zeitverlauf) zu fördern. Deshalb könnte der photoautotrophe Ansatz eine zuverlässige Methode für eine verbesserte Pflanzenentwicklung der Pappelhybride 741 sein. Abhängig davon, welcher Teil der Pflanze von Interesse ist, brachten bestimmte Kombinationen von Parametern bessere Ergebnisse als andere. In weiterführenden Untersuchungen könnte der Einfluss der Kulturparameter auf die Ethylenkonzentration eruiert werden. Nachdem höhere Ethylenkonzentrationen die Pflanzenentwicklung und die Bewurzelung beeinflussen und zu morphologischen und physiologischen Anomalien führen könnten, wäre es von Interesse, die Konzentration des Ethylen im Gasraum zu messen. Das Substrat stellt für das in vitro-Mikroklima und folgend für die Entwicklung der Wurzel einen wichtigen Parameter dar. Deshalb wären Untersuchungen zu einem Trägermaterial mit einer hohen Luftdurchlässigkeit von Interesse. Durch die Verwendung eines solchen Trägermaterials statt eines Gelbildners könnte die relative Luftfeuchtigkeit innerhalb des Kulturgefäßes gesenkt werden. In unseren Versuchen wurde eine natürliche Belüftung verwendet, um die Luftzirkulation zu erhöhen. Jedoch könnte eine

forcierte Belüftung sogar noch vorteilhafter sein, um die gewünschten Konzentrationen an CO<sub>2</sub> und/oder Ethylen zu erreichen. In diesem Fall müsste diese jedoch mit größeren Kulturgefäßen kombiniert werden. Dadurch wären die Gaskonzentrationen im Gefäß besser berechenbar und steuerbar. Abschließend lässt sich sagen, dass die photoautotrophe in vitro-Kultivierung eine geeignete Methode zur Vermehrung der Pappelhybride 741 zu sein scheint.

## 6. References

- Adelberg, J., Fujiwara, K., Kirdmanee, C., & Kozai, T. (1999). Photoautotrophic shoot and root development for triploid melon. *Plant Cell, Tissue and Organ Culture*, 57(2), 95-104.
- Afreen-Zobayed, F., Zobayed, S. M., Kubota, C., Kozai, T., & Hasegawa, O. (2000). A combination of vermiculite and paper pulp supporting material for the photoautotrophic micropropagation of sweet potato. *Plant Sci*, 157(2), 225-231.
- Afreen-Zobayed, F., Zobayed, S. M. A., Kubota, C., Kozai, T., & Hasegawa, O. (1999). Supporting material affects the growth and development of in vitro sweet potato plantlets cultured photoautotrophically. *In Vitro Cellular & Developmental Biology - Plant*, 35(6), 470-474.
- Afreen, F. (2005). Physiological and Anatomical Characteristics of in Vitro Photoautotrophic Plants. In T. Kozai, F. Afreen, & S. M. A. Zobayed (Eds.), *Photoautotrophic (sugar-free medium) Micropropagation as a New Micropropagation and Transplant Production System* (pp. 61-90). Dordrecht: Springer Netherlands.
- Alistair, R., Kelly, M., F., M. M., Adam, H., Kaitlyn, K., Lee, N., . . . Daniel, L. (2012). Inoculation of hybrid poplar with the endophytic bacterium *Enterobacter* sp. 638 increases biomass but does not impact leaf level physiology. *GCB Bioenergy*, 4(3), 364-370.
- Castaldo, S., & Capasso, F. (2002). Propolis, an old remedy used in modern medicine. *Fitoterapia*, 73 Suppl 1, S1-6.
- Cha-um, S., Chanseetis, C., Chintakovid, W., Pichakum, A., & Supaibulwatana, K. (2011). Promoting root induction and growth of in vitro macadamia (*Macadamia tetraphylla* L. 'Keaau') plantlets using CO<sub>2</sub>-enriched photoautotrophic conditions. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 106(3), 435.
- Cournac, L., Dimon, B., Carrier, P., Lohou, A., & Chagvardieff, P. (1991). Growth and Photosynthetic Characteristics of *Solanum tuberosum* Plantlets Cultivated in Vitro in Different Conditions of Aeration, Sucrose Supply, and CO<sub>2</sub> Enrichment. *Plant Physiol*, 97(1), 112-117.
- Desjardins, Y., Hdider, C., & de Riek, J. (1995). Carbon nutrition in vitro — regulation and manipulation of carbon assimilation in micropropagated systems. In J. Aitken-Christie, T. Kozai, & M. A. L. Smith (Eds.), *Automation and environmental control in plant tissue culture* (pp. 441-471). Dordrecht: Springer Netherlands.
- Dudonné, S., Poupard, P., Coutière, P., Woillez, M., Richard, T., Mérillon, J.-M., & Vitrac, X. (2011). Phenolic Composition and Antioxidant Properties of Poplar Bud (*Populus nigra*) Extract: Individual Antioxidant Contribution of Phenolics and Transcriptional Effect on Skin Aging. *Journal of Agricultural and Food Chemistry*, 59(9), 4527-4536.
- Ermayanti, T., Imelda, M., Tajuddin, T., Kubota, C., & Kozai, T. (1999). Growth Promotion by Controlling the In Vitro Environment in the Micropropagation of Tropical Plant Species. *Intl. Workshop on Conservation and Sustainable Use of Tropical Bioresources.*, 10-25.
- Felix, E., Tilley, D. R., Felton, G., & Flamino, E. (2008). Biomass production of hybrid poplar (*Populus* sp.) grown on deep-trenched municipal biosolids. *Ecological Engineering*, 33(1), 8-14.
- Fournioux, J. C., & Bessis, R. (1993). Use of carbon dioxide enrichment to obtain adult morphology of grapevine in vitro. *Plant Cell, Tissue and Organ Culture*, 33(1), 51-57.
- George, E. F. (1996). Plant propagation by tissue culture. Part 2: in practice. In (pp. xii + pp. 575-1361). Westbury: Exegetics Limited.
- Gindler, N. (2014). *Untersuchung zur photoautotrophen Mikrovermehrung der Pappel (Populus sp.)*. Universität Wien,

- Grout, B. W. W., & Millam, S. (1985). Photosynthetic Development of Micropropagated Strawberry Plantlets following Transplanting. *Annals of Botany*, 55(1), 129-131.
- Hanak, A. M. (2014). *Studies on poplar-endophyte-interactions with plant-growth-promotin effects*. (PhD), University of Vienna,
- Hanak, A. M., Nagler, M., Weinmaier, T., Sun, X., Fragner, L., Schwab, C., . . . Weckwerth, W. (2014). Draft Genome Sequence of the Growth-Promoting Endophyte *Paenibacillus* sp. P22, Isolated from *Populus*. *Genome Announc*, 2(2), e00276-00214.
- Hazarika, B. N. (2003). Photoautotrophic Micropropagation - A Review. *Agric.Rev.*, 24(2), 152-156.
- Heyser, J., & Mott, R. (1980). The relationship between the production of phenolic compounds and the decline in growth of loblolly pine cultures. *Plant Physiol*, 65(Suppl), 90.
- Kang, B.-g., Osburn, L., Kopsell, D., Tuskan, G. A., & Cheng, Z.-M. (2009). Micropropagation of *Populus trichocarpa* 'Nisqually-1': the genotype deriving the *Populus* reference genome. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 99(3), 251.
- Khattab, S. (2011). Effect of Different Media and Growth Regulators on the in vitro Shoot Proliferation of Aspen, Hybrid Aspen and White Poplar Male Tree and Molecular Analysis of Variants in Micropropagated Plants. *Life Sci J*, 8(1), 177-184.
- Kirdmanee, C., Kitaya, Y., & Kozai, T. (1995). Effects of CO<sub>2</sub> enrichment and supporting material in vitro on photoautotrophic growth of *Eucalyptus* plantlets in vitro and ex vitro. *In Vitro Cellular & Developmental Biology - Plant*, 31(3), 144-149.
- Kozai, T. (2005). Introduction. In T. Kozai, F. Afreen, & S. M. A. Zobayed (Eds.), *Photoautotrophic (sugar-free medium) Micropropagation as a New Micropropagation and Transplant Production System* (pp. 1-5). Dordrecht: Springer Netherlands.
- Kozai, T., Iwabuchi, K., Watanabe, K., & Watanabe, I. (1991). Photoautotrophic and photomixotrophic growth of strawberry plantlets in vitro and changes in nutrient composition of the medium. *Plant Cell, Tissue and Organ Culture*, 25(2), 107-115.
- Kozai, T., Kitaya, Y., Fujiwara, K., Smith, M. A. L., & Aitken-Christie, J. (1995). Environmental measurement and control systems. In J. Aitken-Christie, T. Kozai, & M. A. L. Smith (Eds.), *Automation and environmental control in plant tissue culture* (pp. 539-574). Dordrecht: Springer Netherlands.
- Kozai, T., & Kubota, C. (2001). Developing a Photoautotrophic Micropropagation System for Woody Plants. *Journal of Plant Research*, 114(4), 525-537.
- Kozai, T., & Kubota, C. (2005a). Concepts, Definitions, Ventilation Methods, Advantages and Disadvantages. In T. Kozai, F. Afreen, & S. M. A. Zobayed (Eds.), *Photoautotrophic (sugar-free medium) Micropropagation as a New Micropropagation and Transplant Production System* (pp. 19-30). Dordrecht: Springer Netherlands.
- Kozai, T., & Kubota, C. (2005b). In Vitro Aerial Environments and Their Effects on Growth and Development of Plants. In *Photoautotrophic (sugar-free medium) Micropropagation as a New Micropropagation and Transplant Production System* (pp. 31-52). Dordrecht: Springer Netherlands.
- Kozai, T., & Kubota, C. (2005c). In Vitro Root Zone Environments and Their Effects on Growth and Development of Plants. In T. Kozai, F. Afreen, & S. M. A. Zobayed (Eds.), *Photoautotrophic (sugar-free medium) Micropropagation as a New Micropropagation and Transplant Production System* (pp. 53-60). Dordrecht: Springer Netherlands.
- Kozai, T., & Nguyen, Q. T. (2003). Photoautotrophic Micropropagation of Woody and Tropical Plants. In S. M. Jain & K. Ishii (Eds.), *Micropropagation of Woody Trees and Fruits* (pp. 757-781). Dordrecht: Springer Netherlands.
- Kozlowksi, T. T. (1971). *Growth and Development of Trees* (Vol. 1): Academic Press Wisconsin.

- Langford, P. J., & Wainwright, H. (1987). Effects of Sucrose Concentration on the Photosynthetic Ability of Rose Shoots In Vitro. *Annals of Botany*, 60(6), 633-640.
- Linsmaier E.M., S. F. (1965). Organic Growth Factor Requirements of Tobacco Tissue Cultures. *Physiologia Plantarum*, 18(1), 100-127.
- Long, R. D. (1997). Photoautotrophic Micropropagation — A Strategy for Contamination Control? In A. C. Cassells (Ed.), *Pathogen and Microbial Contamination Management in Micropropagation* (pp. 267-278). Dordrecht: Springer Netherlands.
- Lubrano, L. (1992). Micropropagation of Poplars (*Populus* spp.). In Y. P. S. Bajaj (Ed.), *High-Tech and Micropropagation II* (pp. 151-178). Berlin, Heidelberg: Springer Berlin Heidelberg.
- Marino, G., & Ventura, M. (1997). The influence of ethylene on in vitro rooting of GF 677 (*Prunus persica* × *Prunus amygdalus*) hybrid peach rootstock. *In Vitro Cellular & Developmental Biology - Plant*, 33(1), 26-29.
- McClelland, M., & Smith, M. (1990). Vessel type, closure, and explant orientation influence in vitro performance of five woody species. *HortScience*, 25(7), 797-800.
- Mingozzi, M., Montello, P., & Merkle, S. (2009). Adventitious shoot regeneration from leaf explants of eastern cottonwood (*Populus deltoides*) cultured under photoautotrophic conditions. *Tree Physiol*, 29(3), 333-343.
- Nagae, S., Takamura, T., Tanabe, T., Murakami, A., Murakami, K., & Tanaka, M. (1996). In vitro shoot development of *Eucalyptus citriodora* on Rockwool in the film culture vessel under CO<sub>2</sub> enrichment. *Journal of Forest Research*, 1(4), 227-230.
- Nguyen, Q. T., & Kozai, T. (2001). Photoautotrophic Micropropagation of Tropical and Subtropical Woody Plants. In N. Morohoshi & A. Komamine (Eds.), *Progress in Biotechnology* (Vol. 18, pp. 335-344): Elsevier.
- Osborne, D. J., & McManus, M. T. (2005). Hormones and Signals: Identification and Description of Signalling Molecules. In *Hormones, Signals and Target Cells in Plant Development* (pp. 6-41). New York, USA: Cambridge University Press.
- Pachala-Ghoreishi, N. (2018). *Photoautotrophe in vitro-Bewurzelung der Pappel*. (diploma thesis), University of Vienna,
- Pan, M. J., & Staden, J. v. (1998). The use of charcoal in in vitro culture – A review. *Plant Growth Regulation*, 26(3), 155-163.
- Samosir, Y. M. S., & Adkins, S. (2014). Improving acclimatization through the photoautotrophic culture of coconut (*Cocos nucifera*) seedlings: an in vitro system for the efficient exchange of germplasm. *In Vitro Cellular & Developmental Biology - Plant*, 50(4), 493-501.
- Schnur, P. (2013). *Untersuchungen zur Etablierung von photoautotrophen in vitro Kulturen der Pappel (Populus sp.)*. (diploma thesis), University of Vienna,
- Schuhleitner, S. (2014). *Klonale Vermehrung einer Pappel-Hybride unter photoautotrophen Bedingungen*. (diploma thesis), University of Vienna,
- Shin, K.-S., Park, S.-Y., & Paek, K.-Y. (2013). Sugar metabolism, photosynthesis, and growth of in vitro plantlets of *Doritaenopsis* under controlled microenvironmental conditions. *In Vitro Cellular & Developmental Biology - Plant*, 49(4), 445-454.
- Sims, R. E. H., Hastings, A., Schlamadinger, B., Taylor, G., & Smith, P. (2006). Energy crops: current status and future prospects. *Global Change Biology*, 12(11), 2054-2076.
- Stettler, R. F., Bradshaw, H. D., Jr., Heilman, P. E., & Hinckley, T. M. (1996). Preface. In *Biology of Populus and its Implications for Management and Conservation* (pp. 539). Ottawa, Ontario, Canada: NRC Research Press.
- Tian, Y., Zheng, J., Yu, H., Liang, H., Li, C., & Wang, J. (2000). Studies of transgenic hybrid poplar 741 carrying two insect-resistant genes. *Acta Botanica Sinica*, 42(3), 263-268.

- Tuskan, G. A. (1998). Short-rotation woody crop supply systems in the United States: what do we know and what do we need to know? *Biomass and Bioenergy*, 14(4), 307-315.
- Valero-Aracama, C., Zobayed, S. M. A., Roy, S. K., Kubota, C., & Kozai, T. (2001). Photoautotrophic Micropropagation of Rhododendron. In N. Morohoshi & A. Komamine (Eds.), *Progress in Biotechnology* (Vol. 18, pp. 385-390): Elsevier.
- Wang, Y., Liu, C., Li, K., Sun, F., Hu, H., Li, X., . . . Li, X. (2007). Arabidopsis EIN2 modulates stress response through abscisic acid response pathway. *Plant Mol Biol*, 64(6), 633-644.
- Warburg O., G. A.-W., Lorenz S. (1962). Neue Methode zur Bestimmung der Kohlensäuredrucke über Bicarbonat-Carbonatgemischen. In *Weiterentwicklung der zellphysiologischen Methoden, angewandt auf Krebs, Photosynthese und Wirkungsweise der Röntgenstrahlen* (pp. 578-581). Stuttgart: Thieme Verlag.
- Wei, F., Zhao, F.-f., & Tian, B.-m. (2017). In vitro regeneration of Populus tomentosa from petioles. *Journal of Forestry Research*, 28(3), 465-471.
- Wilson, S. B., Kubota, C., & Kozai, T. (2000). Effects of Medium Sugar on Growth and Carbohydrate Status of Sweetpotato and Tomato Plantlets in Vitro. In C. Kubota & C. Chun (Eds.), *Transplant Production in the 21st Century: Proceedings of the International Symposium on Transplant Production in Closed System for Solving the Global Issues on Environmental Conservation, Food, Resources and Energy* (pp. 258-265). Dordrecht: Springer Netherlands.
- Xiao, Y., Niu, G., & Kozai, T. (2011). Development and application of photoautotrophic micropropagation plant system. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 105(2), 149-158.
- Xu, Q., Wang, Y., Guo, S., Shen, Z., Wang, Y., & Yang, L. (2014). Anti-inflammatory and analgesic activity of aqueous extract of Flos populi. *J Ethnopharmacol*, 152(3), 540-545.
- Zhang, M., Zhao, D., Ma, Z., Li, X., & Xiao, Y. (2009). Growth and Photosynthetic Capability of Momordica grosvenori Plantlets Grown Photoautotrophically in Response to Light Intensity. *HortScience*, 44(3), 757-763.
- Zobayed, S. M. A. (2005). Ventilation in Micropropagation. In T. Kozai, F. Afreen, & S. M. A. Zobayed (Eds.), *Photoautotrophic (sugar-free medium) Micropropagation as a New Micropropagation and Transplant Production System* (pp. 147-186). Dordrecht: Springer Netherlands.
- Zobayed, S. M. A., Afreen-Zobayed, F., Kubota, C., & Kozai, T. (1999). Stomatal characteristics and leaf anatomy of potato plantlets cultured in vitro under photoautotrophic and photomixotrophic conditions. *In Vitro Cellular & Developmental Biology - Plant*, 35(3), 183-188.
- Zobayed, S. M. A., Afreen, F., & Kozai, T. (2000). Quality Biomass Production via Photoautotrophic Micropropagation. *Acta Hortic.*, 377-386.
- Zobayed, S. M. A., Afreen, F., & Kozai, T. (2001a). Physiology of Eucalyptus plantlets grown photoautotrophically in a scaled-up vessel. *In Vitro Cellular & Developmental Biology - Plant*, 37(6), 807-813.
- Zobayed, S. M. A., Armstrong, J., & Armstrong, W. (2001b). Leaf anatomy of in vitro tobacco and cauliflower plantlets as affected by different types of ventilation. *Plant Science*, 161(3), 537-548.