Studies on the development of callus cultures of *Cannabis sativa* L. regarding plant regeneration

Inês Ferreira – Instituto Superior Técnico, Lisboa, Portugal, October 2018

Abstract

Cannabis sativa L. is known to have a very significant contribution in the medical and pharmaceutical field, mainly due to cannabinoids, the most abundant class of chemical compounds in this plant. *Cannabis* recent legalization in a several number of countries opened a path for the study and expansion of cannabinoids' potential in disease treatment. This study can be performed using genetic transformation techniques in *Cannabis*, requiring the development of an efficient in vitro plant regeneration protocol. In this work indirect organogenesis through formation of a callus, a set of unorganized and proliferating masses of parenchyma cells, was attempted with main focus on establishing the best induction conditions of a healthy and capable of regeneration callus. Young leaves and petioles of *Cannabis sativa* L. drug-type "Euphoria" were used and different carbohydrates, plant growth regulators and additives tested. Even though no shoots could be induced, it could be assessed that the most adequate combination of hormones for that was kinetin and 2,4-Dichlorophenoxyacetic acid (2,4-D) in concentrations of 1 mg/L and 0.5 mg/L, respectively, in MS medium. Regarding carbohydrates, maltose showed to be the most efficient one in MS medium, while in MS modified medium, sucrose was also successful. From all the additives tested, only silver nitrate caused significant changes on calli, stimulating the appearance of roots. The addition of 0.5 mg/L meta-Topolin and of 0.02 mg/L Indole-3-acetic acid + 1.5 mg/L kinetin to calli formed in kinetin and 2,4-D seemed to contribute to the increase of green globular areas in callus surface.

Key words: *Cannabis sativa* L., callus cultures, plant regeneration, indirect organogenesis, shoot induction

Introduction

Cannabis sativa L. (Cannabaceae) is a plant with a worldwide distribution that has applications in different sectors, namely textile, oil and paper production. More importantly, it is very influential in the medicinal and pharmaceutical industries, mainly due to cannabinoids, the most abundant class of chemical compounds in this plant, that have been shown to have a wide range of application in human health. [1][2][3][4]

Despite all the advantages of *Cannabis*, its cultivation has been prohibited in many countries due to its drug potential and past use with focus on psychotropic effects. However, its recent legalization in a several number of countries opened the path for the study and development of cannabinoids' potential in disease treatment. This study can be performed using genetic transformation techniques, such as CRISPR (clustered regularly interspaced short palindromic repeats) CAS9. These techniques offer a way of introducing specific desirable characteristics from a collection of different varieties of the species without changing their quality profiles. However, for that, an effective *in vitro* plant regeneration protocol is required. [5][6][7]

Plant regeneration through tissue culture can occur in two different ways: direct organogenesis or indirect organogenesis.[8] In the last method, the formation of a callus, an unorganized and proliferating mass of parenchyma cells, is the first step. Callus formation can be very heterogeneous. It can vary a lot between explants, species used and conditions it is exposed to - in terms of morphology, color, structure, growth and metabolism.[9][10] *In vitro* propagation of plants can be influenced by a group of factors, categorized as either or hormonal or environmental. In the category of hormonal factors, it can be influenced by the type of media, carbon source, plant growth regulators and additives. Carbohydrates are responsible for the development processes, such as shoot proliferation, root induction, embryogenesis and organogenesis.[11][12] Even though the most commonly used carbon source for the growth of callus is sucrose, other carbohydrates can be used as alternative, like maltose, glucose, fructose or sugar alcohols such as sorbitol and mannitol and some of them have shown to have better outcomes such as minor impact on tissue browning and highest fresh mass and height of plantlets.[13] [14] Plant growth regulators are hormonal factors that are able to influence cell fate in plant tissue culture, being auxins and cytokinins, like naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA) and kinetin (KN), two of the most important types. The combination of these two types of growth hormones usually produces better results than the use of them alone.[15] After callus formation and development, organogenesis occurs. In shoot-forming cultures the developmental steps involve the initiation of the meristematic activity in the callus, followed by cell determination, while meristematic nodules are being formed and, only then, the differentiation of the shoots.[16] The study of the best plant growth regulators that promote the growth of *Cannabis* has been done in several occasions, but the full induction of callus followed by successful shoot and root induction has not been extensively reported. Chaohua Cheng, et al. described a successful callus induction followed by shoot and root induction. Cotyledons of *Cannabis sativa* L. were used and a green, nodular callus and shoot induction of 51.7% were obtained when using 0.4 mg/L thidiazuron (TDZ) and 0.2 mg/L NAA. After that, roots were induced in 80% of the shoots. [3] Lata et. al, 2010, also reported successful plant regeneration through the use of TDZ. Optimal callus growth was obtained in 0.5 µM NAA plus 1.0 µM TDZ and highest shoot production in 0.5 µM TDZ (96.6% induction). [17] However, it was recently shown that the use of TDZ gives rise to high levels of DNA methylation in callus cultures, which were more polymorphic than in the normal leaf tissue. Somaclonal variation in micropropagated plants is a serious concern, since it affects clonal fidelity. Therefore, the use of TDZ in plant cell culture might not be the better option. [5] A later study by Lata et al. found a better alternative to TDZ for shoot induction – meta-topolin (mT). This aromatic natural cytokinin has been shown to enhance shoot proliferation, maintaining histogenic stability and improving rooting efficiency, while at the same time alleviating some physiological disorders in micropropagation. In this one-step micropropagation protocol, 2 µM mT were used and all the explants inoculated were capable of producing shoots. 96 % of regenerated shoots were able to develop roots. [18] In terms of additives added to plant tissue culture amino acids, like proline and glutamine, and casein hydrolysate are often used with the purpose of increasing the nutrition of the media. All of them have been shown to be beneficial in a variety of cases. [19][20] Silver nitrate ($AgNO_3$) is also an often used additive, known to stimulate multiple shoot formation in different plants by inhibiting ethylene production or its function. In other works, the addition of $AgNO_3$ was reported as creating an anti-senescence effect and also it has been stated that it plays a major role in efficient root formation. [8][21] In the present work indirect organogenesis through the formation of callus will be attempted, using *Cannabis* leaves and petioles as explants. The focus will be on the study of the best conditions for the induction of a healthy and capable of regeneration callus. For that, the testing of different carbon sources, plant growth regulators and additives will be performed. After that, shoot regeneration will be attempted in order to access the success of the developed work.

Materials and methods

Plant material

Young leaves and petioles were used as explants. They were collected from plants of the species *Cannabis sativa* L. drug-type "Euphoria" (Royal Queen Seeds, Germany), grown under the controlled conditions of 18h/6h of light/dark at 25/21 °C (day/night) temperature and 70% humidity in the plant growth chamber (CLF Plant Climatics GmbH, Germany), in a semi-hydroponic system. All plant handlings and experimental procedures were carried out under the license No. 4584989 issued by the Federal Institute for Drugs and Medical Devices (BfArM, Germany).

Autoclaving of the media and other compounds

All of the prepared media for plant cell culture were autoclaved before use, with a sterilization period of 20 minutes at 121ºC. In the case of the MS modified medium, the vitamin solution was added only after sterilization.

Collection of leaves and petioles and surface sterilization

Leaves and petioles were collected freshly before use. A sterilization solution was prepared by adding 1.25 ml of NaOCl (0.5% active chlorine) and two drops of Tween 20 to a 50 mL Falcon tube filled with 30 mL of water. The explants stayed in the solution for 10 minutes and then were washed with sterile water. All of the plant cell culture procedures were carried out under aseptic conditions in a laminar flow hood. Leaf discs were cut with the use of a sterile cork borer and about four leaf discs were placed per plate. The petioles were placed three per plate after removing the tissue damaged by sterilization. All the prepared petri dishes were placed in the plant chamber in the Grow Banks under the same controlled conditions mentioned for the *Cannabis* plants.

Callus formation

The formation of the callus was attempted in several conditions: MS medium (4.4 g/L) or MS modified medium (2.7 g/L), both from Sigma-Aldrich, different sugars – sucrose, maltose (both tested in the concentration of 30 g/L), glucose (40 g/L) or sucrose + maltose (15 g/L each) (all from Carl Roth GmbH) different plant growth regulators – BA, NAA, 2,4-D, Kinetin, TDZ, alone or in combinations and different additives – proline, casein hydrolysate, glutamine and silver nitrate. 8 g/L of agar was used in every situation. The medium was renewed usually after 20-30 days.

The plant growth regulators and additives were always added after autoclaving and were used in the following range of concentrations (in mg/L): BA (0.2-6.6), NAA (0.1-3), KIN (1-4), 2,4-D (0.1-5.5), TDZ (0.2-2), $AgNO_3$ (2.5-5), casein hydrolysate (500-1000), proline (50-500), glutamine (50-500).

Shoot formation

The shoot formation was also attempted in the same conditions as for the callus formation. Some pieces of green and healthy callus were, however, also transferred for media known for shoot induction, namely with some concentrations of GA3, TDZ, mT and BA used alone and also some combinations of hormones such as IAA+TDZ, NAA+GA3, BA+GA3, IAA+KIN. Some of the callus was also transferred for media without phytohormones in the presence of 0.5, 0.7 or 1 mg/L activated charcoal with 40 g/L glucose of 30 g/L maltose as a carbon source. Some petri dishes were kept in complete darkness and others under the used light conditions of 16h/8h light/dark. Every medium was renewed usually after 20-30 days.

Suspension cultures

Suspension cultures were prepared. In some of them, callus previously formed in the solid cultures was used (callus from the cultures of 1 mg/L BA+ 1 mg/L NAA and 1 mg/L BA + 0.2 mg/L NAA in maltose). Around 2 g of callus were added per each flask. In others, liquid culture was used from the beginning. For both of them, the protocol that was followed was the one mentioned by Batista et al, 2000, but instead of using 15 g/L sucrose, 15 g/L maltose were used.

Results and discussion

The main aim of this work, as already mentioned goes by the creation of the best induction conditions for the callus. It is known that, when not subjected to the ideal callus induction conditions, morphogenesis might not happen.[7] Therefore the study and selection of the factors that influence callus growth is of major importance, namely the media it is placed on, carbon sources, plant growth regulators and additives. The first studies were performed in MS medium, known for its high salt composition with high levels of nitrogen, potassium and some micronutrients, namely boron and manganese. It is the most standard media used for plant cell cultures. Several carbon sources where tested in this media: maltose, sucrose, maltose + sucrose and glucose.

Maltose versus sucrose

These sugars were tested in a wide range of conditions and both had very different outcomes between each other. For the hormone BA it could clearly be seen that callus growth on leaves was bigger and occurred at a faster rate when sucrose was used as a carbon source. It was also noticed that explants on sucrose didn't last as long as the ones in maltose since, after one month they were already brown and dried. This goes accordingly with the known fact that sucrose's hydrolysis is quicker than maltose's, making the adsorption and sugar metabolization faster. This way, results take less time to appear and callus growth rate is faster in the beginning. At the same time, this quick sugar metabolization is also associated with accumulation of phenolic compounds and cultures becoming hypoxic and accumulating toxic products between their cells, such as ethanol, causing tissue browning and earlier death, like what happened with calli growing on sucrose.[13][24] For the combination of the hormones BA+NAA, the initial great callus growth observed for the usage of BA alone in the case of leaves was not observed - they just ended up completely dried and with no apparent callus. In the case of maltose, calli reached big proportions, growing with a very green and healthy color. In the case of the petioles, when they were placed in sucrose, they lasted longer than in the case of the leaves, however once again, not as long as when they were placed in maltose. Again, a faster development at the beginning was observed for the case of sucrose as it can be seen in figure 1. Overall it can be concluded that maltose was the most adequate sugar between both, even though in the case of the use of BA alone it did not induce callus formation.

Combination of sugars maltose + sucrose

The combination of both sugars was made in an attempt to obtain both the advantages of growing callus in sucrose and in maltose – fast rate of callus growth in case of sucrose and later senescence and healthier callus in the case of maltose. However, this combination was not the most successful one since, even though the callus formed in some cases aged slower than the one solely in sucrose, it was still not as good as the one grown in maltose, as it can be seen in figure 2. That can maybe be explained by the fact that the combination of both sugars does not change the fact that sucrose will be faster hydrolyzed, leading anyway to the accumulation of toxic compounds and to hypoxia in cells. The reason as to why some callus were aging slower could have solely been because a lower concentration of sucrose was used - instead of 30 g/L sucrose, 15 g/L maltose + 15 g/L sucrose were used. Also, callus growth did not always follow this pattern - sometimes callus in sucrose + maltose was worse than in the case of sucrose too.

Glucose

This monosaccharide was additionally tested as a carbon source since in some studies it was found to be better than the previous sugars. It is also said it should decompose faster than sucrose and maltose.[12] The results obtained, however, were less successful than with maltose. That goes accordingly to some studies where it was found that glucose, like sucrose, contributes somehow to cell death also due to hypoxia and accumulation of toxic compounds, even though slightly less. [13][22]

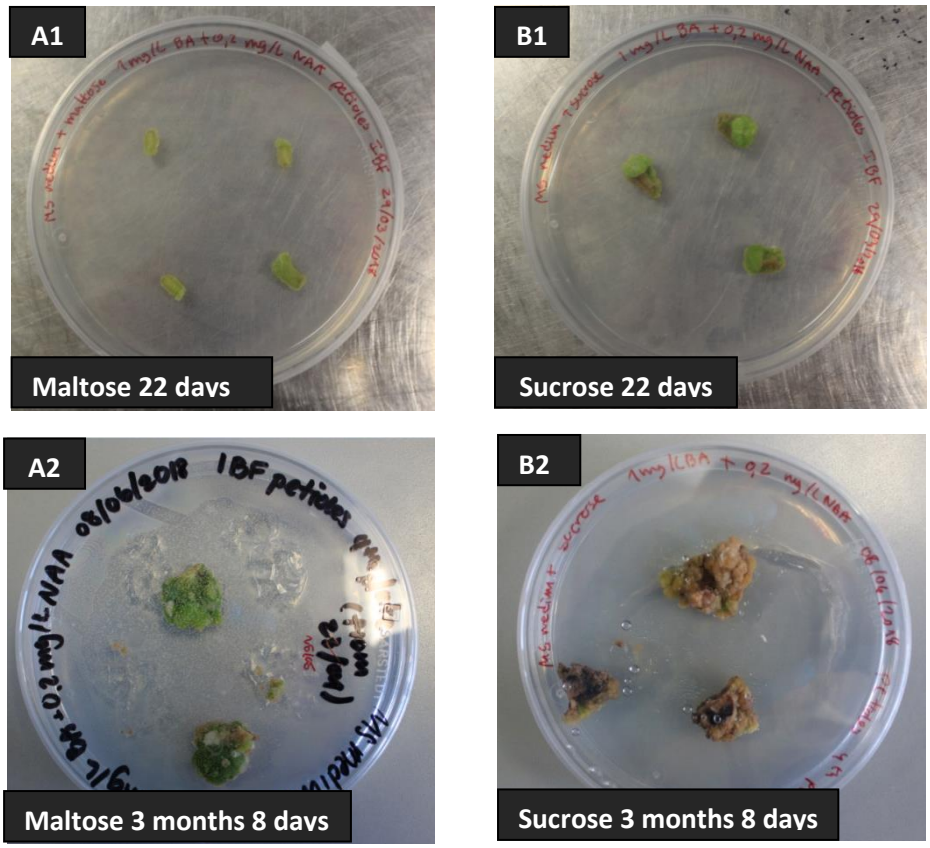

Figure 1 - Development of petiole derived' calli in maltose (A1 and A2) and in sucrose (B1 and B2) over time. It can clearly be seen that calli on sucrose starts developing earlier. However, it dies first than the one in maltose

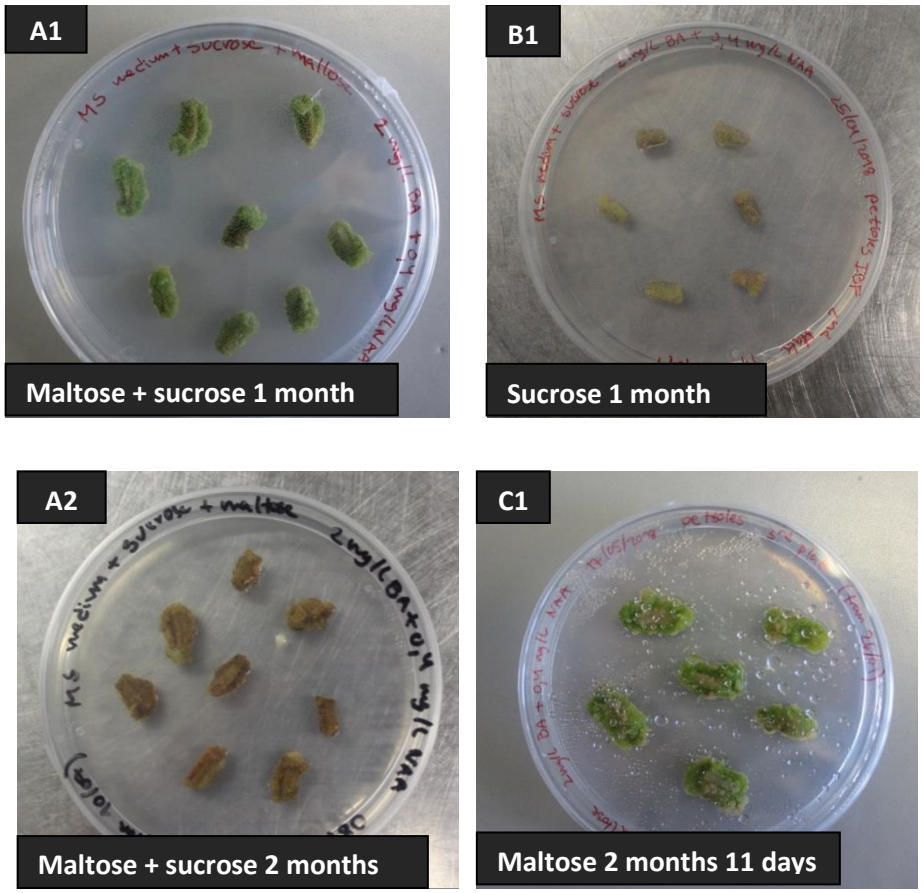

Figure 2 – Petiole' derived calli in maltose + sucrose (A1) and calli in sucrose (B1) both after 1 month and the same calli in maltose+sucrose after 2 months (A2) in comparison with calli in maltose after 2 months and 11 days (C1). It can be seen that in maltose+sucrose callus lasted longer in comparison to sucrose but died earlier than the one in maltose

Plant growth regulators

For the growth of the *Cannabis sativa* callus in MS medium different auxins and cytokinins were tested both for leaves and petioles, some being used alone, as in the case of the auxin 2,4-D and the cytokinins BA and TDZ and some in different combinations and concentrations, such as BA+NAA, KIN+2,4-D and TDZ+NAA. The culture of leaves and petioles was attempted also in basal media without a growth regulator (control), both in the presence of sucrose or maltose, and no growth or callus development was observed. Overall, it could be noticed that, when using a combination of hormones, results were better than when using them alone. For the combination of BA+NAA, in most cases, when using maltose as a sugar, a green and healthy callus developed. The most successful outcome was when using BA and NAA in concentrations of 1 mg/L and 0.2 mg/L, respectively, for callus induction in leaves (figure 3), as well as the concentrations of 2 mg/L BA and 0.2 mg/L NAA. Regarding the combination KIN+2,4-D, the most successful concentration was of 1 mg/L KIN + 0.5 mg/L 2,4-D for leaves on maltose. Even though not developing the same amount of callus as leaves on BA+NAA, it was also green and healthy and presented what looked like bubbles in its surface and also some emerging structures. It could clearly be seen that some parts of the callus were developing into something else as seen in figure 4; therefore it can be assumed that this callus is capable to go through organ regeneration. In some parts of the emerging structures some hairy extremities could be seen, which lead to the assumption that at least some of those were starting to develop into roots. Other parts looked like the beginning of shoots (figure 5). Regarding the combination of TDZ+NAA also some good results were obtained, for the case of petioles on maltose, with some green looking calli that lasted like that for some months. Given the differences between the aspect of calli – different colors and textures, as it can already be seen in figures 3 and 4 – it can also be concluded that plant growth regulators and carbon sources, besides influencing callus development, are able to affect callus morphology.

MS medium versus MS modified medium

MS modified Basal Salt Mixture by Sigma is known for the lack of ammonium nitrate in its composition – being this the main difference between this media and MS medium. The addition of different carbon sources, namely maltose and sucrose, was performed, as well as of different plant growth regulators, like 2,4-D, BA, and the combination of KIN + 2,4-D and BA + NAA. Once again, when adding hormones alone, results were not very successful. For the case of BA+NAA a big callus was obtained for leaves when using the concentrations of 2 mg/L BA + 0.4 mg/L NAA with sucrose. As for petioles, also big callus was achieved in the concentration of 1 mg/L BA+0.1 mg/L NAA in sucrose. For the case of KIN+2,4-D, the callus developed for leaves in maltose was not a very healthy one, however, after a while, just like in the case of MS medium, it could be noticed that some structures also started to develop in the surface of the calli, suggesting again that for this combination of hormones it has organogenic potential. For the petioles, callus on maltose took a bit longer to grow than on sucrose, as expected, but also didn't die as soon. Both sugars had good results.

One striking difference that can be seen between the MS medium and the MS modified is that sucrose, in the case of MS modified has an overall much bigger success rate when it comes to amount of quality callus formed. This difference may be due to the fact that a different ratio of nitrate/sucrose is present between the referred situations. MS modified medium does not have ammonium nitrate in its composition, decreasing a lot the nitrate composition on the media itself. It is known that, in plant growth and development, there is an interactive regulatory mechanism in which the capacity of nitrogen assimilation is coordinated along with the metabolism of carbon, and the outcomes of this interaction vary between species. By these results it could be inferred that such a big ratio of nitrate/sucrose is not beneficial for development of callus in *Cannabis sativa*. [23]

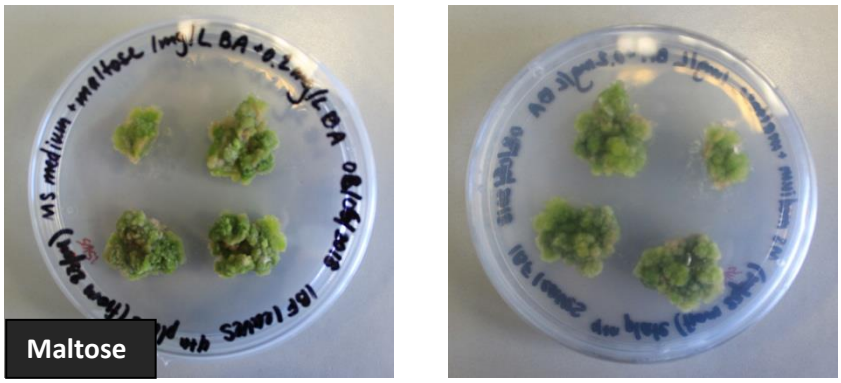

Figure 3 – Callus formation in Cannabis sativa leaves cultured on MS medium supplemented with 1 mg/L BA + 0.2 mg/L NAA in maltose 2 months and 25 days after being placed in the media

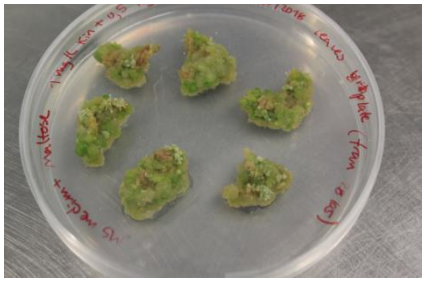

Figure 4 - Callus of *Cannabis sativa* leaves cultured on MS medium supplemented with 1 mg/L KIN + 0.5 mg/L 2,4-D in maltose 3 months and 4 days after being placed in the media

Figure 5 - Structures formed in the surface of *Cannabis sativa* leaves calli cultured on MS medium supplemented with 1 mg/L KIN + 0.5 mg/L 2,4-D in maltose 3 months and 18 days after being placed in the media

Additives

Some additives were tested in order to try to improve callus development or even promote the appearance of shoots, by increasing the nutrition of the media. Some were added in the beginning, when petioles and leaves were first placed in the media and others after a while. Some combinations were also made. The tested additives were glutamine, proline (both amino-acids), casein hydrolysate and silver nitrate. Overall, it could be seen that the addition of glutamine, proline or casein didn't seem to cause major improvements in callus growth. A slight difference could maybe be seen in terms of callus development at the beginning or how green it was but, since the differences were not major it is not possible to address with certainty if the defining factor was the addition of the additives or not. What can be inferred is that the media already had the components needed for efficient callus growth and was not lacking in great amounts any of those that were added.

As for silver nitrate, it was tested in the concentrations of 2.5 mg/L and 5 mg/L. It was either added to the media in the beginning of the culture or after a while (generally around 20 days-1 month after callus being in the media without the additive) and was tested in a whole variety of conditions, namely with the

growth hormones BA+NAA and KIN+2,4-D and also with MS medium and MS modified. Generally speaking, with this concentration it could be noticed that, when not added to the plates in the beginning, in the case of the MS medium with BA+NAA no big difference could be noticed between the plates with this additive and without it, both for the cases of maltose and sucrose. In the case of 4 mg/L BA + 0.4 mg/L NAA in maltose, however, it could be seen that after 2 months and half after being placed in the presence of AgNO3, roots started to develop in the respective callus, while no roots appeared in the one without the additive. With the MS modified a similar but more accentuated and earlier impact was obtained. The addition of $AgNO_3$ to the media with BA+NAA in sucrose, even when not done in the beginning, promoted a huge growth of roots that was not observed in the media without the additive, as it can be seen in figure 6. This huge growth may be can be justified by the already mentioned fact that the ratio of nitrogen/sucrose plays a big role in plant growth and development. As seen already before that, for the case of sucrose, a better outcome was obtained when using it together with MS modified, in this case it can be affirmed again, when such a big difference can be seen between root formation after $AgNO_3$ addition in plates with sucrose in MS medium and with sucrose in MS modified. With maltose + sucrose in MS medium, the addition of $AgNO_3$ was effected in the beginning. The plates had BA+NAA as growth hormones. In the case of the leaves, the addition of this additive made them go darker and dry out, when in comparison to the respective plate without silver nitrate. In the case of the petioles, for the tested conditions it made them grow big roots too, as seen in figure 7. With the addition of 2.5 mg/L $AgNO_3$ to plates with the growth hormones KIN+2,4-D in MS modified, it could be noticed that the callus developed in the presence of $AgNO_3$ seemed slightly more developed. Also it seemed like it was starting to develop more roots than the plate without it. However, again, results were just similar between each other. Overall, it can be concluded that the addition of $AgNO_3$ stimulated the appearance of roots, especially in the case of MS modified with sucrose and the growth hormones BA+NAA and also in the case of maltose + sucrose in MS medium and BA+NAA.

Shoot regeneration

In order to induce the appearance of shoots from callus, different conditions were tested – charcoal in different concentrations was added to the media (0.5 g/L, 0.75 g/L and 1 g/L), where either 40 g/L glucose or 30 g/L maltose were present. These explants were also either placed in complete darkness or under the used light conditions of 16h/8h light/dark. The addition of charcoal is said to be sometimes beneficial to shoot multiplication, development and root formation stages, helping in the elimination of toxic compounds and eventually also releasing growth promoting substances present or absorbed by charcoal. [24] Regarding the light conditions, sometimes exposure to a dark environment can be a promotor of shoot regeneration.

Other than the charcoal addition, the addition of different plant growth regulators was also tested, such as the addition of BA, GA3, mT and TDZ or of several combinations of hormones like IAA+TDZ, NAA+GA3, BA+GA3, IAA+KIN and IAA+BA.

Activated charcoal addition

The addition of charcoal on a media with glucose only seemed to be inducing the appearance of roots and not shoots. Plus, the ones that were placed in the dark on the presence of maltose didn't develop any which can lead to the assumption that not necessarily is the charcoal the only responsible for the formation of the roots in the explants but eventually the use of glucose or the combination of glucose with charcoal. Also, the plates that were placed in the dark caused browning of the tissue.

Plant growth regulators for shoot induction

For shoot induction, mT was added in a concentration of 0.5 mg/L. This was the concentration reported by Lata et al., 2016, to be the most successful in the micropropagation of Cannabis sativa, inducing more and bigger shoots than any concentration of TDZ tested, and not needing the addition of another

hormone for rooting after that. [19] Regarding the combination of 0.02 mg/L IAA + 1.5 mg/L KIN, it had been reported to induce the best response from stem and petiole-derived callus from hop, with successful plant regeneration and high regeneration frequencies. [25] From all the hormones and combination of hormones tested, only these ones that were mentioned showed some type of improvement for the callus. Even if not generating any shoot, they seemed to increase the amount of green globular areas in the callus, as it can be seen in figure 8, which is related to the beginning of shoot regeneration. The lack of success in shoot regeneration with these hormones might be due to genotypic differences between the plants tested.

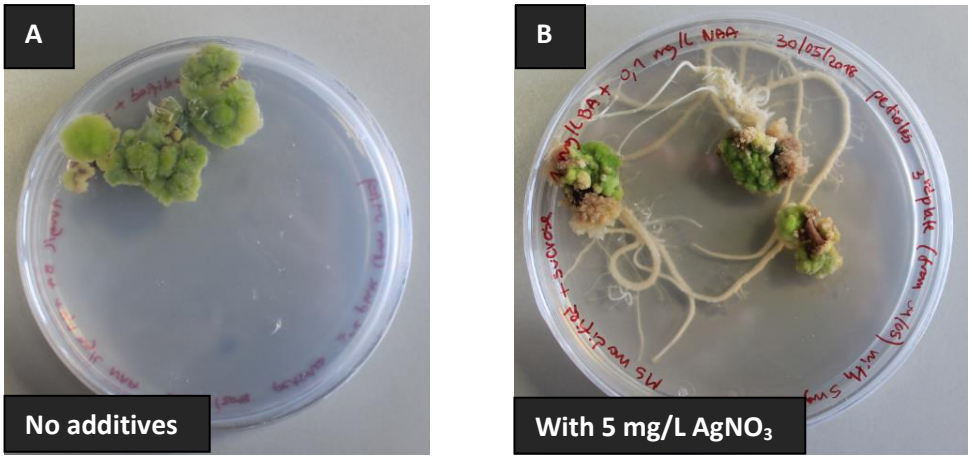

Figure 6 - Callus formation in *Cannabis sativa* petioles cultured on MS modified medium supplemented with 1 mg/L BA + 0.1 mg/L NAA in sucrose without additives (A) and in the presence of 5 mg/L $AgNO_3$ (B) 2 months and 14 days after initial culture

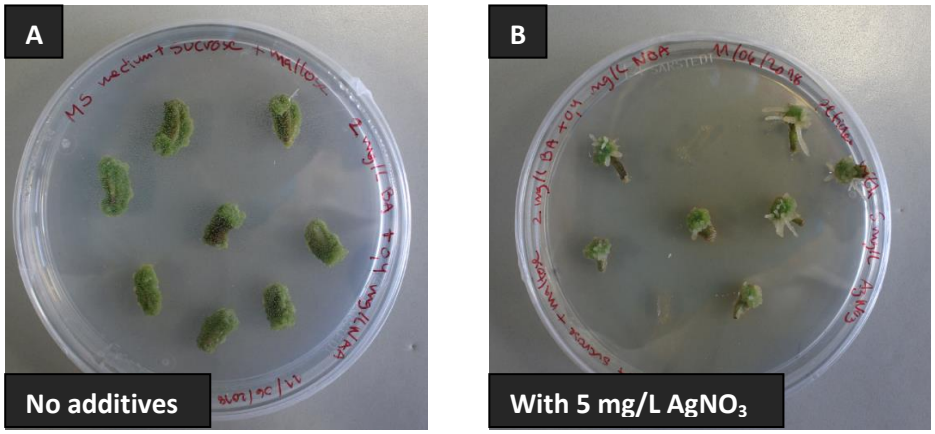

Figure 7 - Callus formation in *Cannabis sativa* petioles cultured on MS medium supplemented with 2 mg/L BA + 0.4 mg/L NAA in sucrose + maltose without additives (A) and in the presence of 5 mg/L $AgNO_3$ (B) 1 month after initial culture

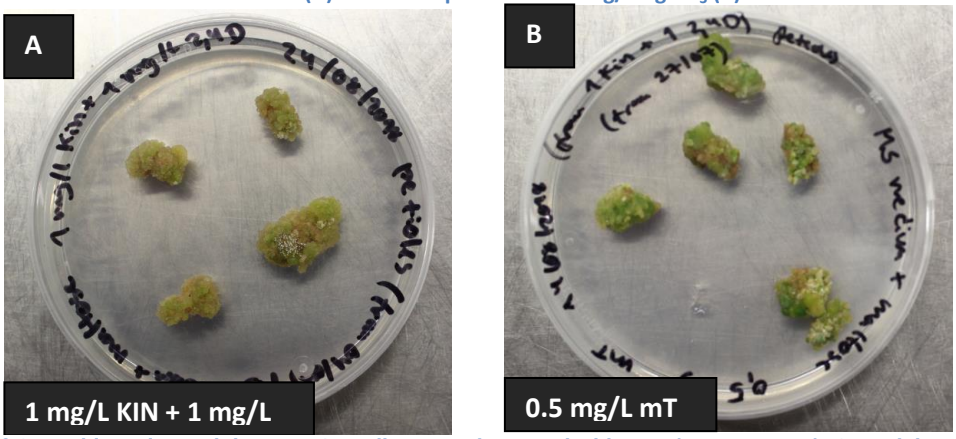

Figure 8 - Callus of *Cannabis sativa* petioles on MS medium supplemented with 1 mg/L KIN + 1 mg/L 2,4-D (A) and with 0.5 mg/L mT (B) in maltose. The petioles in both conditions had the same age and both pictures were taken in the same day. The ones in the right had been transferred to the media with 0.5 mT (after being in the 1 mg/L KIN+ 1 mg/L 2,4-D media) one month before the picture was taken

Conclusion

At the end of this work, even though no shoots could be induced, it could be assessed that the most adequate combination of hormones for that was kinetin and 2,4-D in concentrations of 1 mg/L and 0.5 mg/L, respectively, in MS medium. Regarding carbohydrates, maltose showed to be the most efficient one in MS medium, while in MS modified medium, sucrose was also successful. From all the additives tested, only silver nitrate caused significant changes on calli, stimulating the appearance of roots. The addition of 0.5 mg/L meta-Topolin and of 0.02 mg/L Indole-3-acetic acid + 1.5 mg/L kinetin to calli formed in kinetin and 2,4-D seemed to contribute to the increase of green globular areas in callus surface.

References

[1] Wang, R., He, L. S., Xia, B., Tong, J. F., Li, N., and Peng, F. (2009). A micropropagation system for cloning of hemp (Cannabis sativa L.) by shoot tip culture. *Pak. J. Bot*, 41(2), 603-608.

[2] Grulichova, M., Mendel, P., Lalge, A. B., Slamova, N., Trojan, V., Vyhnanek, T., Winkler, J., Vaverkova, M., Adamcova, D. and Dordevik, B. (2017). Effect of different phytohormones on growth and development of micropropagated *Cannabis sativa* L., 618-623

[3] Chaohua, C., Gonggu, Z., Lining, Z., Chunsheng, G., Qing, T., Jianhua, C., Xinbo G, Dingxiang P and Jianguang, S. (2016). A rapid shoot regeneration protocol from the cotyledons of hemp (*Cannabis sativa* L.). *Industrial Crops and Products*, *83*: 61-65.

[4] Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Frontiers in plant science*, *7*: 19.

[5] Schachtsiek, J., Warzecha, H., Kayser, O., and Stehle, F. (2018). Current perspectives on biotechnological cannabinoid production in plants. *Planta medica*, *84*(04): 214-220.

[6] Salentijn, E. M., Zhang, Q., Amaducci, S., Yang, M. and Trindade, L. M. (2015). New developments in fiber hemp (*Cannabis sativa* L.) breeding. *Industrial crops and products*, *68*: 32-41.

[7] Batista, D., Ascensao, L., Sousa, M. J. and Pais, M. S. (2000). Adventitious shoot mass production of hop (*Humulus lupulus* L.) var. Eroica in liquid medium from organogenic nodule cultures. *Plant Science*, *151*(1): 47-57.

[8] Baghel, S. and Bansal, Y. K. (2015). In vitro Regeneration of Oil Yielding Plants-A Review. *Journal of Essential Oil Bearing Plants*, *18*(5): 1022-1050.

[9] Loyola-Vargas, V. M., De-la-Peña, C., Galaz-Avalos, R. M. and Quiroz-Figueroa, F. R. (2008). Plant tissue culture. *Molecular Biomethods Handbook*, Humana Press, 875-904

[10] Ikeuchi, M., Sugimoto, K. and Iwase, A. (2013). Plant callus: mechanisms of induction and repression. *The Plant Cell*, 25(9): 3159-73.

[11] Muslihatin, W. and Ratnadewi, D. (2012). Effect of carbohydrate source on growth and performance of In Vitro sago palm (*Metroxylon sagu* Rottb.) plantlets. *HAYATI Journal of Biosciences*, *19*(2): 88-92.

[12] Yaseen, M., Ahmad, T., Sablok, G., Standardi, A. and Hafiz, I. A. (2013). Role of carbon sources for in vitro plant growth and development. *Molecular biology reports*, *40*(4): 2837-2849.

[13] Kumar, G. P., Subiramani, S., Govindarajan, S., Sadasivam, V., Manickam, V., Mogilicherla, K., Thiruppathi, S., and Narayanasamy, J. (2015). Evaluation of different carbon sources for high frequency callus culture with reduced phenolic secretion in cotton (*Gossypium hirsutum* L.) cv. SVPR-2. *Biotechnology Reports*, *7*: 72-80.

[14] Rahman, M. H., Islam, R., Hossain, M. and Islam, M. S. (2010). Role of sucrose, glucose and maltose on conventional potato micropropagation. *Journal of Agricultural Technology*, *6*(4): 733-739.

[15] Kumlay, A. M. and Ercisli, S. (2015). Callus induction, shoot proliferation and root regeneration of potato (*Solanum tuberosum* L.) stem node and leaf explants under long-day conditions. *Biotechnology & Biotechnological Equipment*, *29*(6): 1075-1084.

[16] Gurel, S., Ekrem, G. and Kaya, Z. (2000). Callus development and indirect shoot regeneration from seedling explants of sugar beet (*Beta vulgaris* L.) cultured in vitro. *Turkish Journal of Botany*, *25*(1): 25-33.

[17] Lata, H., Chandra, S., Khan, I. A. and ElSohly, M. A. (2010). High frequency plant regeneration from leaf derived callus of high Δ^9-tetrahydrocannabinol yielding *Cannabis sativa* L. *Planta medica*, *76*(14): 1629-1633.

[18] Lata, H., Chandra, S., Techen, N., Khan, I. A. and ElSohly, M. A. (2016). In vitro mass propagation of *Cannabis sativa* L.: A protocol refinement using novel aromatic cytokinin meta-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants. *Journal of Applied Research on Medicinal and Aromatic Plants*, *3*(1): 18-26.

[19] Salehi, M., Moieni, A. and Safaie, N. (2017). A Novel Medium for Enhancing Callus Growth of Hazel (*Corylus avellana* L.). *Scientific reports*, *7*(1): 15598.

[20] Zang, Q., Zhou, L., Zhuge, F., Yang, H., Wang, X. and Lin, X. (2016). Callus induction and regeneration via shoot tips of *Dendrocalamus hamiltonii*. *SpringerPlus*, *5*(1): 1799.

[21] Mohiuddin, A. K. M., Chowdhury, M. K. U., Abdullah, Z. C. and Napis, S. (1997). Influence of silver nitrate (ethylene inhibitor) on cucumber in vitro shoot regeneration. *Plant cell, tissue and organ culture*, *51*(1): 75-78.

[22] Scott, P., Lyne, R. L. and Ap Rees, T. (1995). Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum vulgare* L.). *Planta*, *197*(3): 435-441.

[23] Gabryszewska, E. (2011). Effect of various levels of sucrose, nitrogen salts and temperature on the growth and development of *Syringa vulgaris* L. shoots in vitro. *Journal of Fruit and Ornamental Plant Research*, *19*(2): 133-148.

[24] Pan, M. J. and Van Staden, J. (1998). The use of charcoal in in vitro culture–A review. *Plant growth regulation*, *26*(3): 155-163.

[25] Batista, D., Sousa, M. J. and Pais, M. S. (1996). Plant regeneration from stem and petiole-derived callus of *Humulus lupulus* L.(hop) clone Bragança and var. Brewer's Gold. *In Vitro–Plant*, *32*(1): 37-41.