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1 Aim of the Work

The aim of this work was to develop a reliable regeneration protocol for medicinal cannabis from callus culture as part of a collaboration with Prof. Oliver Kayser and Dr. Felix Stehle from the Technical University of Dortmund, Germany with the common goal to produce transgenic cannabis plants with an optimized cannabinoid spectrum.

Since a lack of regeneration into plantlets occurred, callus was assessed for changes in ploidy level as endopolyploidy might inhibit regeneration.

2 Abstract

For the development of a reliable indirect *in vitro* plant regeneration protocol for *Cannabis sativa* L., the effects of different concentrations and combinations of eight different plant growth regulators, different carbon sources (maltose, sucrose), different basal media (full MS, half MS, two component cannabis fertilizer), activated charcoal and the influence of light/dark culture environment on callus growth were investigated in five chemotypes. Callus was successfully induced (100 %) from ovule and leaf explants of all chemotypes with two growth regulators in light conditions on a cannabis fertilizer-containing culture medium. For plant regeneration, 29 already published and 9 self-designed media were tested. However, no callus showed signs of regeneration into plantlets. Since somaclonal variation is known to occur in callus cultures and can be manifested in regeneration problems due to the loss of culture health, the ploidy level of callus cells nuclei was assessed via flow cytometry analysis. The results showed at least 73 % of analyzed calli being endopolyploid. For a further evaluation of the data, the endopolyploidy index (EI) was assessed. The resulting high EI-values of even young callus cultures showed the rather low influence of the age on somaclonal variation in cannabis callus cultures. Maltose as carbon source seemed to have the greatest influence on the ploidy level of cannabis callus cultures, since all, except for one analyzed maltose media derived callus, showed endopolyploidy. Maltose in combination with two growth regulators had the highest occurrence of endopolyploidy.

3 Zusammenfassung

Für die Entwicklung eines verlässlichen Protokolls für die indirekte *in vitro* Regeneration von *Cannabis sativa* L., wurden die Auswirkungen von verschiedenen Konzentrationen und Kombinationen von acht Pflanzenwuchsstoffen, verschiedene Kohlenstoffquellen (Saccharose, Maltose), verschiedene Basalmedien (MS voll, MS halb, zwei-Komponenten Cannabis Dünger), Aktivkohle und der Einfluss von hellen oder dunklen Kulturbedingungen bezüglich Kalluswachstum mit fünf verschiedenen Chemotypen getestet. Es konnte mit allen Chemotypen erfolgreich (100 %) Kallus von Blättern und den weiblichen Samenanlagen mit zwei Wuchsstoffen auf einem Kulturmedium mit Cannabis Dünger in hellen Bedingungen initiiert werden. Für die Regeneration in Pflanzen wurden 29 bereits publizierte und 9 selbsterstellte Medien getestet. Jedoch zeigte kein Kallus Zeichen von einer Regeneration in Pflanzen. Da bekannt ist, dass in Kalluskulturen häufig somaklonale Variation vorkommt, und sich diese in Form von Regenerationsproblemen aufgrund ungesunder Kulturen zeigt, wurde der Ploidie-Status der Zellkerne via Durchflusszytometrie untersucht. Die Ergebnisse zeigten, dass mindestens 73 % der analysierten Zellkerne endopolyploid waren. Um die Daten weiter zu evaluieren, wurde der Endopolyploidie Index (EI) berechnet. Es konnte durch die hohen EI-Werte der sogar jungen Kalluskulturen gezeigt werden, dass das Alter der Cannabis Kalluskulturen eine eher geringe Rolle für somaklonale Variation spielt. Maltose als Kohlenstoffquelle schien den größten Einfluss auf den Ploidie Status von Cannabis Kalluskulturen zu haben, da alle, bis auf einen analysierten Maltose-Medium abstammenden Kallus, Endopolyploidie zeigten. Maltose in Kombination zwei Wuchsstoffen hatte das größte Auftreten von Endopolyploidie.

4 Introduction

4.1 Cannabis

Cannabis (Figure 1) is one of the oldest by mankind cultivated crops, it was used for its pain soothing qualities in China, India and Egypt already 5000 years ago (Blaschek 2016). Because of its many qualities it became widely spread (Pertwee 2014).

Figure 1: Cannabis as illustrated in Köhler's Book of Medicinal Plants, 1897

The genus *Cannabis* belongs to the family Cannabaceae, with controversial opinions on the number of species. In literature, the most found perception is that there is only one

species, *C. sativa,* with the subspecies *C. sativa ssp. sativa* and *C. sativa ssp. indica.* Others assume the genus *Cannabis* comprises three species: *C. sativa*, *C. indica* and *C. ruderalis.* (McPartland and Guy 2017)

Amongst many attempts to establish a reasonable taxonomic system for cannabis plants, so far no such effort has been successful. For example, the differentiation between “fiber-type” and “drug-type” cannabis plants (Kojoma et al. 2006) using THC or CBD content as distinguishing characteristic, and also many other systems using THC/CBD ratio (Small and Cronquist 1976; Hillig 2004) are problematic, as the cannabinoid spectrum, which determines the chemotype, is extremely variant, also within morphologically similar cannabis plants not least because of human selection for hundreds of years (de Meijer 2014).

Cannabis sativa L. (cannabis) grows up to 6 m high, however, the most common height of cannabis is up to 2 m. It is an annual, naturally dioecious plant, well known for its characteristic palmately, 100 - 250 mm long leaves with mostly 5 – 7 serrate leaflets. The dense inflorescences are often sticky from the resin produced by glandular trichomes. The fruits are 3 – 5 mm long, grey achenes. (Small 2008; Pertwee 2014; Blaschek 2016)

4.1.1 Cannabinoids and other Phytochemicals

In cannabis plants at least 120 phytocannabinoids, 200 different terpenoids and about 20 different flavonoids have been identified. The main therapeutic properties of cannabis are attributed to the phytocannabinoids. Several studies showed benefits of THC with either smoked or vaporized cannabis (Corey-Bloom et al. 2012; Wilsey et al. 2013; Eisenberg et al. 2014; Wallace et al. 2015; Ware et al. 2015), or of both THC and CBD with the oral-mucosal spray Sativex (Johnson et al. 2010; Collin et al. 2010; Langford et al. 2013).

Despite the knowledge on the synergistic and antagonistic effects of phytochemicals, no study has so far considered phytochemicals other than THC and CBD. Phytochemicals

can influence bioavailability, interfere with cellular transport processes, activate pro-drugs or deactivate active compounds.

For example, cannabis extracts have stronger muscle-anti-spastic effects compared to pure THC, or non-THC cannabinoids can attenuate side-effects induced by THC. CBD has influence on the pharmacokinetics of THC by inhibiting the P450-mediated hepatic drug metabolism, consequently the elimination of THC is slowed down. CBD also can reduce cognitive and memory deficits induced by smoking cannabis.

Terpenes can increase the blood-brain barrier permeability, thus can even be used as permeation agents for cannabinoids in transdermal patches. Synergistically, they may modulate the affinity of THC to CB1 receptors and also interact with neurotransmitter receptors.

Flavonoids can also influence the pharmacokinetics of THC by inhibiting hepatic P450 enzymes. (Andre et al. 2016; Baron 2018)

Phytocannabinoids

At least 120 phytocannabinoids have been identified in cannabis (Elsohly et al. 2017). Marked by their C21-terpenophenolic skeleton, all phytocannabinoids arise from two precursors: olivetolic acid and geranyl diphosphate, which originate from two distinct biosynthetic pathways, the polyketide and the plastidal 2-C-methyl-D-erythritol 4-phosphate pathway (Figure 2). Three oxidocyclases then convert the precursors to the diverse phytocannabinoids (Andre et al. 2016), which accumulate in the secretory cavity of the glandular trichomes primarily in female flowers. Therefore seed-based “CBD oil” or “hemp oil” products are subtherapeutic as only traces of phytocannabinoids can be found in cannabis seeds. (Baron 2018)

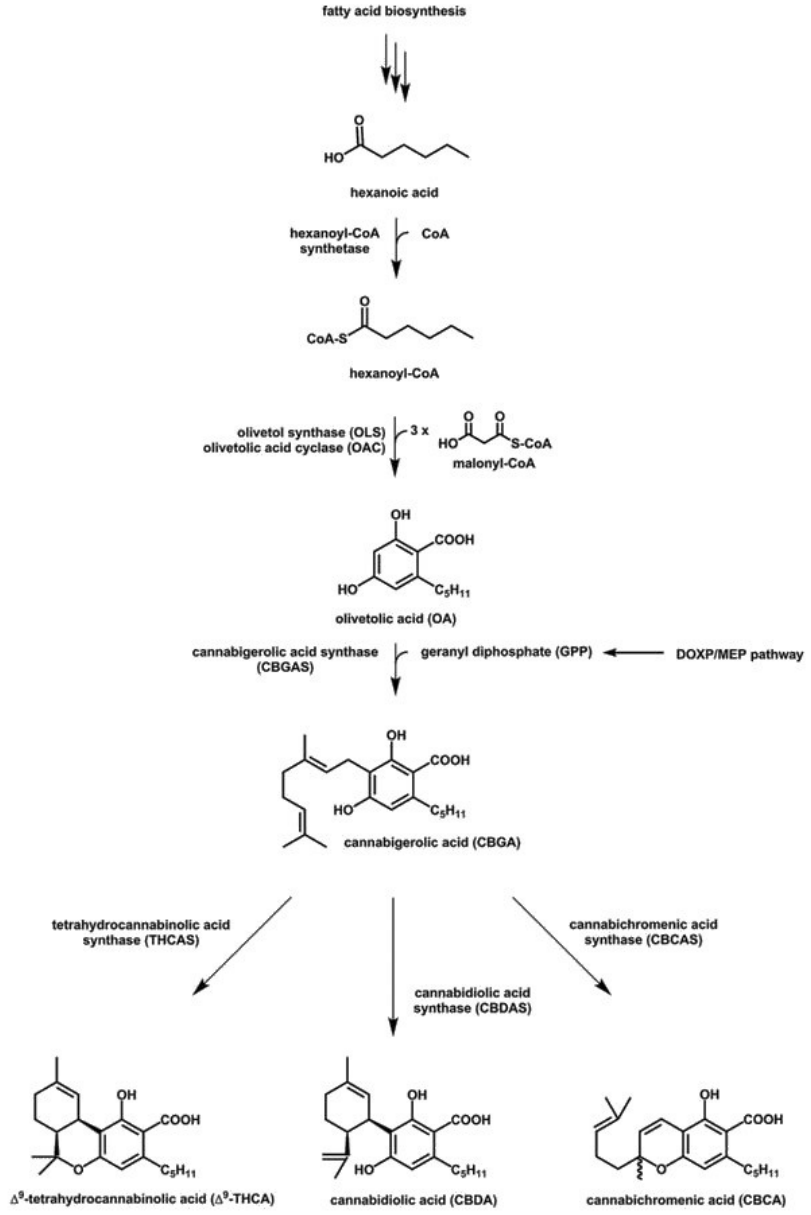

Figure 2: Biosynthesis of cannabinoids in cannabis (Schachtsiek et al. 2018)

Psychoactive effects are shown by only three naturally occurring phytocannabinoids: Δ9-tetrahydrocannabinol, Δ8-tetrahydrocannabinol and cannabinol. 11-OH-tetrahydrocannabinol, a product of the human metabolism of THC, triggers the greatest effects. (Colom and Gual 2018)

Most of the pharmacological effects of phytocannabinoids are based on the ability to activate two G-protein coupled cannabinoid receptors, CB_1 and/or CB_2. CB_1 and CB_2 receptors belong to the human endocannabinoid system which is thought to be involved

in many physiological functions, such as appetite, pain sensation, mood, memory, inflammation, insulin level and fat and energy metabolism (Andre et al. 2016).

CB_1 receptors are located primarily in the central nervous system and throughout the brain, but also in the immune cells, the gastrointestinal, reproductive, adrenal, heart, lung and bladder tissues. CB_2 receptors can be found on immune cells where the expression is induced when there is active inflammation (Bie et al. 2018). Moreover, PPAR-δ, TRPA1, 5HT3A, glycine receptor, CMR1 and other nuclear receptors are modulated by cannabinoids (Baron 2018).

This great amount of receptors affected by phytocannabinoids show a high therapeutic potential, but also means a higher number of possible side effects requiring more rigorous research.

The seven predominant phytocannabinoid acids - tetrahydrocannabinolic acid, cannabidiolic acid, cannabinolic acid, cannabigerolic acid, cannabichromenic acid, tetrahydrocannabivarin acid and cannabidivarinic acid- are found as the primary metabolite precursors to the cannabinoids in cannabis plants. Heat, UV exposure and prolonged storage convert these acidic phytocannabinoids into the active cannabinoids Δ^9-tetrahydrocannabinol, cannabidiol, cannabinol, cannabigerol, cannabichromene, tetrahydrocannabivarin and cannabidivarin. (Baron 2018)

In medicinal cannabis plants, Δ^9-tetrahydrocannabinol (THC) (Figure 3) is the most prevalent phytocannabinoid. It is a partial agonist of both CB_1 and CB_2 receptors, but has higher affinity for the CB_1 receptor, which seems to be linked with the psychotropic effects potentially mediated by the modulation of both glutamate and gamma-aminobutyric acid (GABA) systems. Its actions at the N-methyl-D-aspartate (NMDA) receptors are associated with the analgesic and anti-inflammatory benefits, but also antioxidant neuroprotective effects, which play a significant role in chronic pain syndromes such as fibromyalgia and chronic migraine.

THC is 20 times more anti-inflammatory than acetylsalicylic acid, twice as anti-inflammatory as hydrocortisone and enhances analgesia from kappa opioid receptor agonist medications. It also has potent anti-emetic benefits in adults and children, which

led to approval for dronabinol (isolated THC from cannabis plants for pharmaceutical preparations) and nabilone (Canemes, synthetic drug) for the second line treatment of chemotherapy related nausea and vomiting. Nabiximols (Sativex, tincture of cannabis) is approved for spasticity as symptom of multiple sclerosis. (Schnattinger 2020)

Due to the wide range of actions there are reported benefits for many diseases: Alzheimer´s disease, amyotrophic lateral sclerosis (ALS), Multiple sclerosis (MS), autism, Parkinson´s, Tourette´s syndrome, Huntington´s disease/chorea, depression, posttraumatic stress disorder, traumatic brain injury, hypothermia, duodenal ulcers, anorexia and cachexia, inflammatory bowel disease, spinal cord injury, diabetes, obesity, glaucoma, and as an antipruritic in cholestatic jaundice. (Baron 2018)

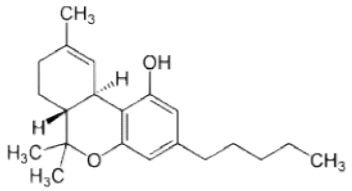

Figure 3: Δ^9-tetrahydrocannabinol (THC)

Cannabidiol (CBD) (Figure 4), the second major cannabinoid, has according to the World Health Organization no evidence for abuse or dependence potential. Due to its lack of psychoactivity, it has gained increased attention during the past years. Amongst interacting with many ion channels, enzymes and other receptors, it is a low-potent CB_1 and CB_2 receptor antagonist, thus attenuating negative side effects of THC such as anxiety, tachycardia and sedation. CBD has strong analgesic and anti-inflammatory effects due to its ability to inhibit cyclooxygenase and lipoxygenase. The anti-inflammatory effect is several hundred times more potent than aspirin. Several studies show the possible treatment of epilepsy, Alzheimer´s disease, Parkinson´s disease, Multiple sclerosis, Huntington´s disease, Amyotrophic lateral sclerosis, anxiety disorders including posttraumatic stress disorder, depression, dystonia, schizophrenia and psychosis, stroke and hypoxic-ischemic injury, traumatic brain injury, spinal cord injury, inflammatory disorders, psoriasis, rheumatoid arthritis, a wide range of cancers across multiple organ systems including brain, blood, breast, lung, prostate, colon, inflammatory bowel diseases, nausea, osteoporosis, hepatic encephalopathy and cirrhosis, cardiovascular diseases

including hypertension, cardiomyopathy and myocardial ischemia, and diabetic complications, including diabetes-induced peripheral neuropathy. (Baron 2018)

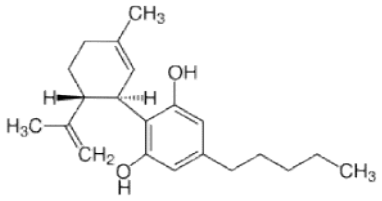

Figure 4: Cannabidiol (CBD)

Terpenes

The approximately 200 different terpenes found in cannabis are typically simple mono- and sesqui-terpenes which have very low toxicity and are already widely used in the food and cosmetic industry. Consequently, they have been proven safe and are well-accepted. Myrcene, β-caryophyllene, α-pinene, humulene, linalool, limonene, terpinolene, terpineol and geraniol are the most common terpenes found in cannabis with varying concentrations depending on the chemotype. They work individually and synergistically with the cannabinoids. Terpenes show, among others, anti-inflammatory, antioxidant, analgesic, anxiolytic, antidepressant, anti-insomniac, skin penetration enhancing, anticancer, antitumor, antiviral, antibacterial, antifungal, antiparasitic, anticonvulsive, neuroprotective, anti-allergic and even antidiabetic effects.

Myrcene (Figure 5) is a common monoterpene found in high amounts in cannabis, which due to different mechanisms protects the brain, heart and skin tissues from inflammation and oxidative damage.

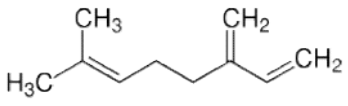

Figure 5: Myrcene

Also ß-caryophyllene (Figure 6) can be found in high concentrations in most cannabis strains. It is a selective, full agonist of CB_2 thus participates in cell protection, neuroprotection, nociception, feeding behavior, and in preventing alcohol-induced damage. Together with its anti-inflammatory and anti-convulsive characteristics it shows multi-

target potential for the treatment of neuroinflammatory diseases like MS and PD. (Nuutinen 2018)

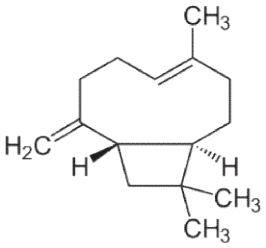

Figure 6: ß-caryophyllene

Flavonoids

Apigenin, luteolin, quercetin, ß-sitosterol, vitexin, cannflavin A and cannflavin B (Figure 7), the latter being unique to cannabis, are some of the 20 cannabis-produced flavonoids. Through their phenolic character flavonoids act as antioxidants and protect against oxidative stress. Many of them also have anti-inflammatory, neuroprotective and anti-cancer effects. Cannflavin A shows even a 30 times more potent inhibitor of prostaglandine E-2 than aspirin, and ß-sitosterol was shown to reduce topical inflammation by 65% in skin models.(Baron 2018)

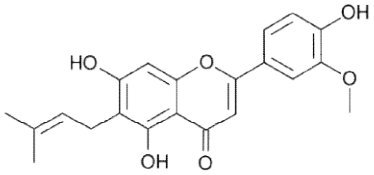

Figure 7: Cannflavin B

Due to the legal restrictions in the cultivation and application of cannabis, research is lagging behind and more data are needed to show the therapeutic actions and mechanisms for a safe medicinal application.

4.1.2 Cannabis as medicinal Plant

Today, cannabis-derived medicinal products are used for many indications due to positive feedback from patients and doctors, although there is reliable evidence based on large-scaled random controlled trials available only for a few indications:

Nabiximols (Sativex) has been approved for the treatment of spasticity in multiple sclerosis in the UK since 2010, followed by a few European countries.

Dronabinol (Marinol) is licensed for the treatment of nausea and vomiting caused by cytostatic therapy and for loss of appetite in HIV/AIDS- related cachexia in the United States and Belgium.

Nabilone (Cesamet) has been approved for the treatment of the side effects caused by chemotherapy in patients with cancer in Great Britain, the United States and Canada.

CBD (Epidiolex) was approved in 2018 for the treatment of patients suffering from rare pediatric epilepsy syndromes (Dravet and Lennox-Gastaut syndromes) in the United States. (EMCDDA 2019)

Further, due to the reported positive effects, medicinal cannabis and products are used for many more indications off label: For chronic pain of different types such as neuropathic pain or migraine, for chronic inflammatory diseases such as Crohn's disease and rheumatism, for psychiatric conditions such as depression, obsessive compulsive disorders, posttraumatic stress disorder, neurological diseases such as multiple sclerosis, epilepsy, Tourette syndrome, appetite loss and nausea and also for irritable bowel syndrome, asthma and glaucoma. (Grotenhermen and Müller-Vahl 2016)

In Austria, preparations of dronabinol and cannabidiol are not registered for specific indications, but approved and clinically used as add-ons, when Dronabinol is classified as narcotic drug and its use requires special prescription conditions. Sativex is registered for the treatment of spasticity in multiple sclerosis if other medical therapy failed. (Lampl et al. 2017)

4.1.3 Legal Framework

Because of its psychotropic effects, the cultivation, distribution, sale, possession and consumption of cannabis is illegal in many countries. Only a few countries allow the recreational use of cannabis, with varying legality and restrictions: Canada (Government of Canada 2020), Georgia (Wayne 2018), South Africa (Child 2018), Uruguay (Malena and Felipe 2013), the Australian Capital Territory in Australia (Lowrey 2019) and 14 states and territories in the USA (Contributors 2020).

However, the medical use is legal in many more countries: Argentina, Australia, Barbados, Bermuda, Brazil, Canada, Chile, Colombia, Croatia, Cyprus, Czech republic, Denmark, Ecuador, Finland, Georgia, Germany, Greece, Ireland, Israel, Italy, Jamaica, Lebanon, Lithuania, Luxembourg, Malawi, Malta, Netherlands, New Zealand, North Macedonia, Norway, Peru, Poland, Portugal, Saint Vincent and the Grenadines, San Marino, South Africa, Sri Lanka, Switzerland, Thailand, United Kingdom, 33 states in the USA, Uruguay, Vanuatu, Zambia and Zimbabwe. (Figure 8)

In Australia, Antigua and Barbuda, Argentina, Austria, Bangladesh, Barbados, Belgium, Belize, Bermuda, Bolivia, Brazil, Cambodia, Chile, Colombia, Costa Rica, Croatia, Czech Republic, Ecuador, Egypt, Estonia, Finland, Germany, India, Iran, Israel, Italy, Jamaica, Laos, Lesotho, Luxembourg, Malta, Mexico, Moldova, Morocco, Myanmar, Nepal, Netherlands Pakistan, Paraguay, Peru, Poland, Portugal, Saint Kitts and Nevis, Saint Vincent and the Grenadines, Slovenia, Spain, Switzerland, Thailand, Trinidad and Tobago and 16 states in the USA cannabis is illegal, but decriminalized.

In addition, each country has its own regulations regarding amount or sale of cannabis.

Also there are different restrictions about cannabis-derived pharmaceuticals. Sativex, for example, has recently been approved in 21 European Union countries for the treatment of spasticity in multiple sclerosis and is therefore the most widely approved cannabis-based product in Europe. (EMCDDA 2019)

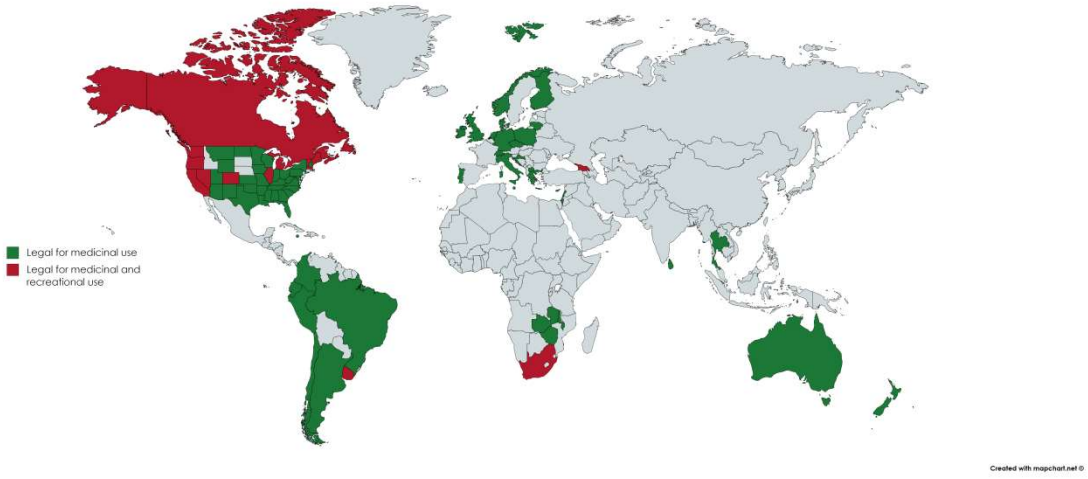

Figure 8: Legal status of cannabis (status 2020) (created with mapchart.net)

In Austria the cultivation of cannabis plants containing not more than 0.3 % THC is generally allowed. Since stems and leaves of high THC chemotypes contain an amount less than 0.3 % THC, the possession and sale of such plants is allowed, as long as they are not flowering. Thus, seeds and plants in a vegetative state of high THC producing chemotypes are available to customers above 18 years as “ornamental plants” in grow shops all over Austria.

Agricultural fiber hemp production is regulated and farmers are authorized to only use seeds listed in a catalog published by the EU for the production of fiber and seeds. Fiber derived from hemp stems is used for the production of insulation material, paper and textiles. Seeds are used for cooking and baking, as bird food, but also for the production of hemp oil. In 2018, Austria was the third largest fiber hemp producer in Europe with a planting area of 1.6 ha. Lower Austria was the main producer, followed by Upper Austria, Burgenland and Styria. Across Europe, France is the largest hemp producer with 16.6 ha of hemp growing area. (Statista 2019; Statistik Austria 2019)

The sale of CBD products such as CBD oils or CBD extracts, referred to as nutritional supplements, foods or medicines, is prohibited, since they are classified as novel food and have not been authorized yet. These include foods like cakes or sweets but also cosmet-

ics which are produced with CBD oils or extracts. This is a legally diffused area and many CBD shops get away with selling these products, by not labeling nutritional or medicinal purposes. (Mahmood 2018)

Pharmacies produce CBD or THC containing preparations only for patients with a doctor's prescription (Lampl et al. 2017).

4.2 Biotechnological Cannabinoid Production

Large-scale field cultivation of hemp for meeting the increasing demand of medicinal cannabis and cannabis products is difficult to control and cannabinoid content in plants is variable.

4.2.1 Metabolic Engineering

Desired plants with an optimized cannabinoid profile can be generated by metabolic engineering, when genes and metabolic pathways within a living cell are altered to achieve and increase the production of specific substances (Khosla and Keasling 2003).

One important tool for genome engineering is CRISPR (clustered regularly interspaced short palindromic repeats) CAS9 (CRISPR associated protein 9), which our partner Oliver Kayser and his team in the Faculty of Chemistry and Chemical Biology of TU Dortmund University are using to generate transgenic cannabis plants. Their aim is to establish a reliable transformation protocol for cannabis.

For the generation of transgenic cannabis plants, a culture of cannabis leaf discs is inoculated with Cas9- and sgRNA-carrying *Agrobacterium tumefaciens*. The resulting transformed leaf discs are stimulated to produce callus from which primary transgenic plantlets are regenerated. Plantlets with the desired chemotype can then be propagated via vegetative cuttings (micropropagation). (Schachtsiek et al. 2018)

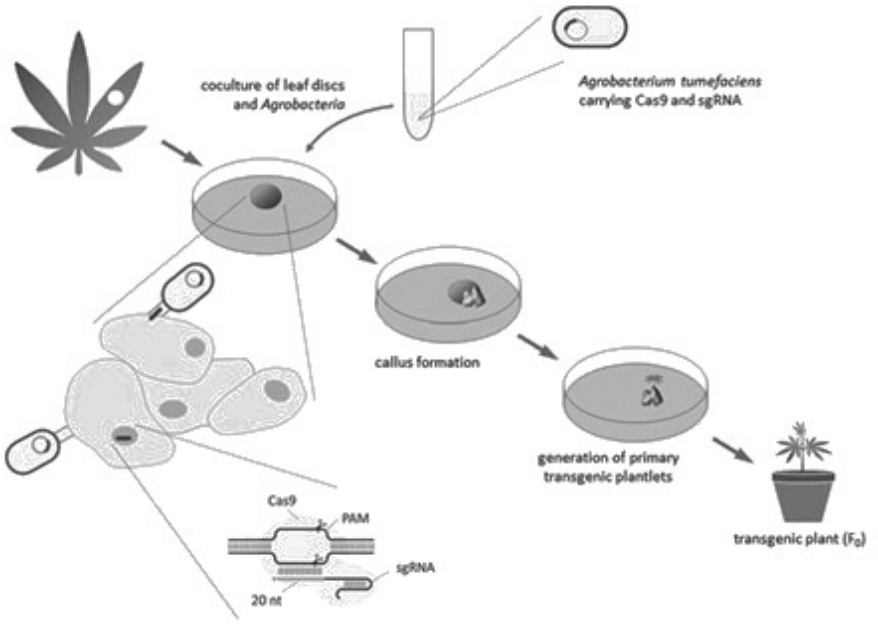

Figure 9: Generation of transgenic cannabis plants (Schachtsiek et al. 2018)

Successful genetic transformation was reported by several laboratories (Mackinnon et al. 2001; Feeney and Punja 2003, 2015, 2017; Sirkowski 2012; Wahby et al. 2013, 2017).

However, successful plantlet regeneration of transformed cells was only reported by two authors (Mackinnon et al. 2001; Sirkowski 2012), although details on regeneration rate and conditions of plantlets are missing in the publication. (Table 1)

4.2.2 *In vitro* Techniques

Establishing an effective regeneration protocol is an essential prerequisite for genetic transformation. In recent years, several plant regeneration and transformation studies have been carried out. Table 1 shows an overview of the current research on cannabis cell culture, regeneration and transformation. Recently, progress has been made, but an efficient and reliable regeneration system that works for various chemotypes is still needed.

In cannabis reasonable rates of plant regeneration from nodal segments have been reported (Lata et al. 2009, 2016b, a; Chaohua et al. 2016; Smýkalová et al. 2019; Galán-Ávila et al. 2020).

However, indirect organogenesis studies with callus obtained from tissues such as young leaves, petioles, internodes and axillary buds (Ślusarkiewicz-Jarzina et al. 2005), cotyledons, stems and roots (Wielgus et al. 2008), leaves (Pacifico et al. 2008), leaves and hypocotyls (Movahedi and Torabi 2015; Movahedi et al. 2016) showed no or low rates of plant regeneration. The same applies to successfully transformed callus (Mackinnon et al. 2001; Sirkowski 2012), successfully transformed hairy roots (Wahby et al. 2013, 2017; Feeney and Punja 2017), and suspension cultures (Feeney and Punja 2003, 2015; Sirikantaramas et al. 2005; Flores-Sanchez et al. 2009). (Lata et al. 2010) seem to be successful in indirect cannabis plant regeneration. (Table 1)

There is significant influence of the chemotype on the rate of plant regeneration, but no difference could be seen between the tested varieties in callus induction, which works generally well. Further, the age of the donor explants was an important factor, as younger cotyledons produced a higher number of explants forming shoots than older ones. (Galán-Ávila et al. 2020)

Hairy root cultures were successful in cannabinoid production, but the efficiency was very low and upscaling these cultures would not be possible.

Callus and cell suspension cultures are not useful for cannabinoid production, because undifferentiated callus tissues, even those derived from flowers, are not able to synthesize cannabinoids. (Wróbel et al. 2018)

In addition, the heterologous production of cannabinoids in tobacco is a promising technique. However, the high nicotine and alkaloid content are major drawbacks and alternative model plants possessing glandular trichomes, like tomato, may provide a good alternative. This area still needs further research. (Schachtsiek et al. 2018)

Table 1: Current research on cannabis cell culture (Schachtsiek et al. 2018 adjusted).

Cell culture system	Plant regeneration	Successful transformation	Reference
Callus	yes (not specified)	yes	(Mackinnon et al. 2001)
Callus, suspension cultures	no	yes	(Feeney and Punja 2003)
Suspension cultures	no	no	(Sirikantaramas et al. 2005)
Callus	1.35 %	no	(Ślusarkiewicz-Jarzina et al. 2005)
Callus	1.40 %	no	(Wielgus et al. 2008)
Callus	no	no	(Pacifico et al. 2008)
Direct organogenesis	yes (100.00)	no	(Lata et al. 2009)
Suspension cultures	no	no	(Flores-Sanchez et al. 2009)
Callus	yes (96.60)	no	(Lata et al. 2010)
Callus	yes (not specified)	yes	(Sirkowski 2012)
Hairy roots	no	yes	(Wahby et al. 2013)
Suspension cultures	no	yes	(Feeney and Punja 2015)
Callus	yes (not specified)	no	(Movahedi and Torabi 2015)
Direct organogenesis	yes (54.80)	no	(Chaohua et al. 2016)
Direct organogenesis	yes(100.00)	no	(Lata et al. 2016b)
Direct organogenesis	yes (not specified)	no	(Lata et al. 2016a)
Hairy root culture	no	yes	(Feeney and Punja 2017)
Hairy root culture	no	yes	(Wahby et al. 2017)
Direct organogenesis	yes (49.00)	no	(Smýkalová et al. 2019)
Direct organogenesis	yes (49.45)	no	(Galán-Ávila et al. 2020)

4.3 Somaclonal Variation

Somaclonal variation is defined as genetic and phenotypic variation among clonally propagated plants of a single donor clone (Kaeppler et al. 2000). Some years ago, oil palm tree farmers experienced a huge financial loss because of somaclonal variation: High-yielding varieties of oil palm were propagated through tissue culture techniques and supplied to plantations. However, some of these clonal, genetically identical trees developed abnormal flowers and yielded much less oil. Since young palms need several years of intensive care before they start to fruit, this ended in a serious economic problem. (Paszkowski 2015)

Explant preparation like wounding and sterilization, different media components and *in vitro* culture environment exposes explants to oxidative stress. This oxidative stress results in the production of free radicals like hydrogen peroxide, which cause:

- Hyper/hypo-methylation of DNA
- Changes in chromosome number
- Chromosomal rearrangements
- DNA base deletion/substitution

However, these mechanisms not only cause problems like in the oil palm production or other micropropagation programs, where it is highly desirable to produce true-to-type plant material. They have also provided an alternative tool to breeders for obtaining genetic variability rapidly in horticultural crops, which are difficult to breed. For example, carrots' resistance to drought, early flowering chili peppers, or bananas with larger bunch size were developed through somaclonal variation. (Krishna et al. 2016)

Somaclonal variation can be either of somatic or meiotic nature, and while meiotic variation is heritable, somatic variation is often not. Somatic variation is of most impact in situations where the primary regenerant is the end product. For example, when ornamental plants or trees are multiplied *in vitro*. Meiotic variation is important in situations where the end product of the tissue culture process is further propagated in the field or nursery and sold as seed. Mechanisms producing both somatically and meiotically herit-

able variation contribute to the loss of culture health and regenerability of cultures over time. (Kaeppler et al. 2000)

Further, it can be distinguished between genetic and epigenetic variation: not only mutations in DNA sequences can lead to a phenotypic variant, but also a different epigenetic regulation can play a major role and is frequently observed in micropropagation (Marum 2011) (Figure 10).

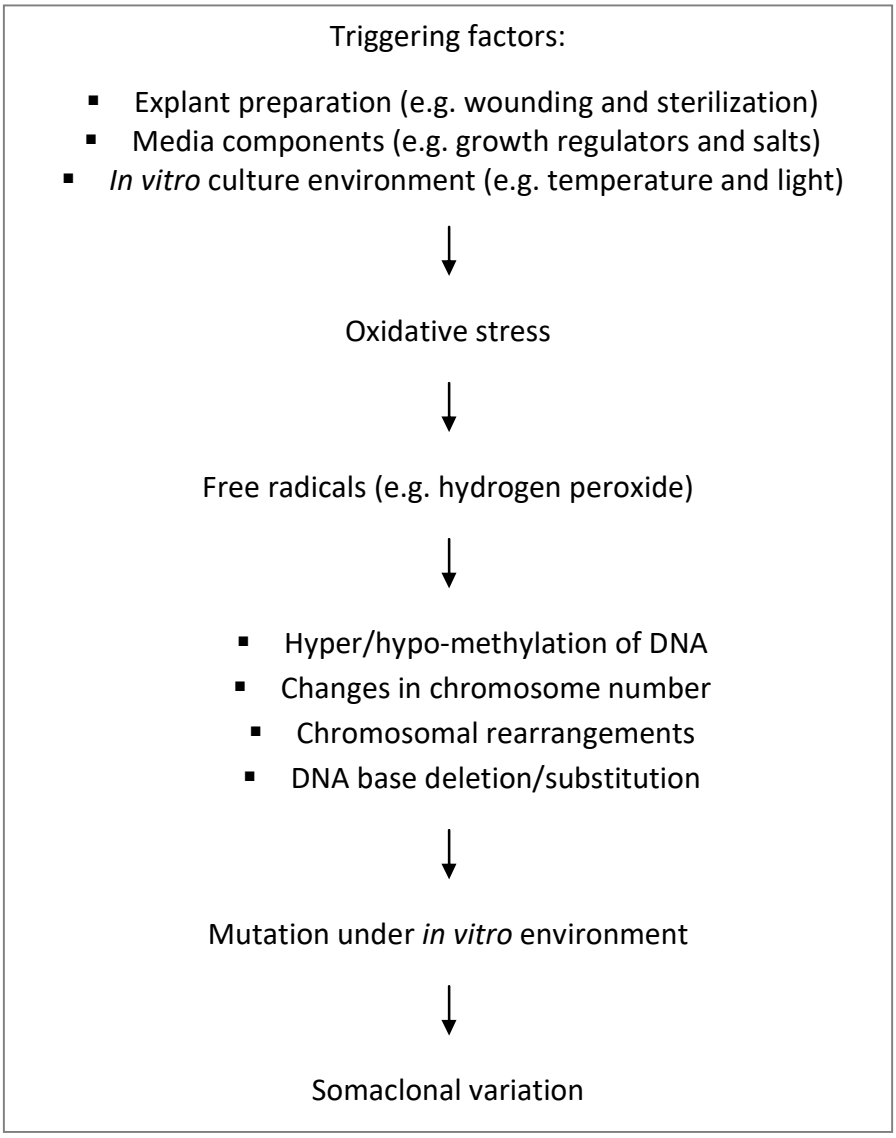

Figure 10: Mechanism of somaclonal variation in *in vitro* culture (Krishna 2016 adjusted)

4.3.1 Epigenetic Variation

Besides mutations in DNA sequence, the chromatin structure and subsequently the phenotype is highly affected by epigenetic mechanisms such as

- DNA methylation,
- histone modification and
- RNA interference.

In vitro plant regeneration systems are based on cell de-differentiation and re-differentiation processes. During these processes, highly dynamic mechanisms of chromatin remodeling take place (Marum 2011). This may occur due to the activation of transposable genetic elements, since insertions of transposable genetic elements in the plant genome can result in chromosome rearrangement. This in turn can lead to genetic misregulation, aneuploidy and new transposon insertions. (Samarina et al. 2019)

4.3.2 Triggering Factors

The main factor affecting genetic stability in *in vitro* propagation seems to be the chemotype itself (Shen et al. 2007; Tican et al. 2008). Triggers to induce variation can be multiple stress factors such as tissue damage, sterilizing agents, imbalance of nutrient media components, excessive concentrations of auxins and cytokinins and their imbalances (Samarina et al. 2019).

Moreover, the chemical nature of the growth regulators and the culture time play an important role. For example, the widely used synthetic cytokinin TDZ was shown to cause high levels of DNA methylation in callus cultures (Ghosh et al. 2017).

(Sales and Butardo 2014) showed somaclonal variation in tissue culture derived bananas due to prolonged subculture and high 2,4-D concentration. Also (Mamedes-Rodrigues et al. 2018) and (Samarina et al. 2019) proved genetic instability of some species during long term *in vitro* conservation. However, many aspects of the mechanisms leading to somaclonal variation remain unclear.

4.4 Genome Size Measurements with Flow Cytometry

DNA flow cytometry is a popular method for the indirect determination of DNA content in cell nuclei by measuring fluorescence emission. To estimate nuclear DNA content, aqueous suspensions of intact nuclei whose DNA is stained using a DNA-specific fluorochrome are prepared. The amount of light emitted by each nucleus is quantified and the result is usually displayed in form of a histogram. The relative fluorescence intensity represents the relative DNA content. (Doležel and Bartoš 2005)

The diploid genome of cannabis consisting of 18 autosomes and a pair of sex chromosomes has an estimated size of 1636 Mb for female plants and 1686 Mb for male plants. The female plants are homogametic (XX), the male plants heterogametic (XY), owning the larger Y chromosome (van Bakel et al. 2011). With 978 Mb of DNA equaling one picogram (Dolezel et al. 2003), the haploid genome of female cannabis is around 0,84 pg (Sakamoto et al. 1998).

5 Materials and Methods

5.1 Callus Initiation

5.1.1 Plant Material

Explants for callus induction were obtained from female cannabis plants purchased from a cannabis nursery (Flowery Field, 1070 Vienna, Austria) (Figure 11). Mother plants of five different chemotypes (Table 2) were cultivated in the controlled environment of a greenhouse with a minimum temperature of 16 °C (Figure 12). A photoperiod of 18 hours, using fluorescent tubes (OsramBioLux 58w) and LEDs (Aequator 150w / 300w) was provided.

Every four weeks Guanokalong (Femeg, the Netherlands) was added and in order to prevent pests, vaporized sulphur was applied once a week during the night period.

Table 2: Plant material

Name	Genetics	Origin of Material
Austrian Power Kush (APK)	Indica	-
Black Domina (BD)	Indica	Sensi Seeds
Cannalope (CNH)	Hybrid	DNA Genetics
Orange Bud (OB)	Hybrid	Dutch Passion
Wappa (W)	Hybrid	Paradise Seeds

Figure 11: Young cannabis plants from the cannabis nursery

Figure 12: Mother plants in the controlled environment of a greenhouse

Healthy, newly developed leaves were cut with scissors from the mother plant. Inflorescences were harvested, when seeds were still green and the ovules about 1 mm in size.

5.1.2 Surface Sterilization

For surface sterilization an aqueous sodium hypochlorite solution (NaOCl) of 0.5 % (w/v) (Kodym and Leeb 2019) was prepared: 37 ml of the NaOCl stock solution with 14 % active chloride (Sigma Aldrich) were mixed with 963 ml sterile water and several drops of Polysorbat 20 (Tween 20) as wetting agent in a ventilated hood.

Newly harvested leaves and inflorescences were rinsed in tap water and put into a sterile beaker. After sufficient sterilization solution was added, the beaker was covered with sterile aluminum foil and put on a magnetic stirrer for 20 minutes (Figure 13). After exactly 20 minutes, the beaker was transferred to the sterile hood and the sterilization solution was strained quickly. The plant material was rinsed with sterile water shortly for the first time, followed by three rinses for 10 minutes.

Figure 13: Surface sterilization of cannabis leaves and inflorescences

5.1.3 Callus Induction

The callus initiation assay was carried out in four independent experiments:

All preparation work was carried out in the sterile environment of a laminar air flow bench, until the culture vessels were closed and sealed with cling film or Parafilm (Figure 14).

Experiment 1:

Multi well plates with 12 wells were used for media screening, each plate containing one type of medium. Each well was filled with 2 ml of the media 1 – 24 with the growth regulators KIN or BAP in combination with IAA in various concentrations (Table 5).

Leaf explants of two different chemotypes (BD, CNH) were cut with scalpels and tweezers on sterile paper. The resulting explants with the size of 5 – 10 mm had two cut edges with a middle rib. For each treatment 24 explants were used, with two explants per well. Half the explants were placed on the medium with the leaf top facing up, the other half with the top facing down (Figure 15). The experiment was evaluated after nine weeks of culture in a dark environment.

Experiment 2:

For the second experiment, two media and multi well plates were used. Two plates were used per medium, one plate for dark and the other for light culture conditions. Each well was filled with 2 ml of medium 25 with the growth regulators TDZ (2 µM) and NAA (1 µM) or medium 26 with the growth regulator 2,4-D (4.5 µM) (Table 5).

Leaf explants of four different chemotypes (BD, CNH, OB, W) with the size of 5 – 10 mm and two cut edges with a middle rib were prepared. For each treatment 24 explants were cultured, with two top facing down explants per well. The experiment was evaluated after nine weeks of culture in a dark and light environment.

Experiment 3:

For experiment 3, ovules from the chemotype APK were cultured on media 25 and 26 in multi well plates under light and dark conditions (Table 5). In each well, one ovule was placed. 12 ovules per treatment were analyzed. The experiment was evaluated after nine weeks of culture in a dark and light environment.

Experiment 4:

Ovules from the chemotype APK were cultured in eprouvettes filled with 7.5 µM KIN and 7.5 µM IAA medium (No. 27) (Table 5). In each eprouvette one isolated ovule was placed. A total number of 20 eprouvettes was used, ten were kept under light and the other ten under dark conditions. The experiment was evaluated after nine weeks.

Figure 14: Laminar air flow bench for explant preparation in sterile environment

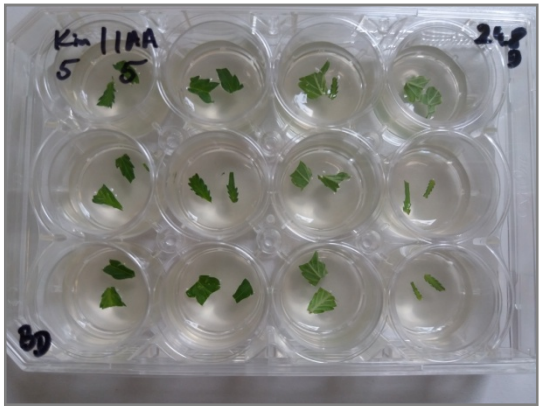

Figure 15: Example for callus induction well plate

For experiments 1 – 3 a basal medium (BM) containing a two component fertilizer (Bio-nova Nutri Forte A+B) (Table 3) prepared according to the manufacturer's instructions (2 mL/L) with MS vitamins (Murashige-Skoog, 1962) was prepared (Table 4). As carbon source 3 % w/v maltose was added. The gelling agent Gelrite (Duchefa, The Netherlands) was used in a concentration of 0.2 % w/v. (Leeb 2018)

For the fourth experiment a different BM was used: a BM with MS half concentration of macronutrients, full micronutrients (Duchefa) and vitamins with 3 % w/v sucrose and 0.8 % w/v agar (Merck) was prepared. (Murashige and Skoog 1962) (Table 6, Table 4)

Table 3: Nutrient content of Bionova Nutri Forte A+B

Nutrients	Content (mmol/L)
Nitrogen (N)	13.450
Phosphor (P)	0.910
Potassium (K)	2.525
Sulfur (S)	1.211
Calcium (Ca)	4.100
Magnesium (Mg)	2.600
Iron (Fe)	0.028
Manganese (Mn)	0.0081
Copper (Cu)	0.00132
Zinc (Zn)	0.003
Boron (B)	0.018
Chlorine (Cl)	2.676
Silicon (Si)	0.019

Table 4: Ingredients of MS – Vitamins (Murashige-Skoog, 1962)

Ingredient	Content (mg/L)
Myo-inositol	100.0
Nicotinic acid	0.5
Pyridoxine	0.5
Thiamine	0.1
Glycine	2.0

Various types and concentrations of growth regulators were added to the BM (Table 5):

For media 1 –24, 6-Benzylaminopurine (BAP), Kinetin (KIN) and Indole-3-acetic acid (IAA) in various concentrations were added to the BM (Kodym et al. 2017). For medium 25, the BM was supplemented with 2 µM Thidiazuron (TDZ) and 1 µM 1-Naphthaleneacetic acid (NAA) (Chaohua et al. 2016). Medium 26 contained 4.5 µM 2,4-Dichlorophenoxyacetic acid (2,4-D) (Flores-Sanchez et al. 2009) and medium 27 7.5 µM KIN and 7.5 µM IAA.

The pH was adjusted to 5.7–5.8 with NaOH or HCl stock solutions before autoclaving at 121^{o}C for 15 min.

Cultures were kept in the dark or light for callus induction. For the light treatment a photoperiod of 16 hours was provided by three Sylvania Grolux tubes (58W T8) and one Philips LED tube (24W865 T8) per shelf. The temperature in the growth chamber was

25±1°C with 50 % relative humidity. The shelf cooling was on, but cardboard pads were placed on top of the shelves to prevent the cultures from becoming too cold.

The cultures for the dark treatment were kept in the same growth chamber, but placed in light tight boxes.

After 1 – 3 months the obtained callus was separated from the original tissue and transferred to fresh medium of the same composition. The callus was then subcultured every 4 – 6 weeks and kept under light conditions for fast proliferation. The propagated callus was used for plant regeneration assays and flow cytometry analysis.

Table 5: Growth regulators for callus induction

Medium	Growth regulators (µM)					
ID	BAP	KIN	IAA	2,4-D	NAA	TDZ
1	1		0			
2	1		1			
3	1		5			
4	1		10			
5	5		0			
6	5		1			
7	5		5			
8	5		10			
9	10		0			
10	10		1			
11	10		5			
12	10		10			
13		1	0			
14		1	1			
15		1	5			
16		1	10			
17		5	0			
18		5	1			
19		5	5			
20		5	10			
21		10	0			
22		10	1			
23		10	5			
24		10	10			
25					1	2
26				4.5		
27		7.5	7.5			

5.2 Plant Regeneration

The plant regeneration screening was performed with 38 different media (28 – 65) (Table 7).

Three BM were used for plant regeneration essays:

1. MS full (Murashige and Skoog 1962) (Table 6, Table 4)
2. MS half concentration of macronutrients, full micronutrients and vitamins (Murashige and Skoog 1962) (Table 6, Table 4)
3. Plant fertilizer Bio Nova Nutri Forte A+B (Bio Nova B.V., The Netherlands) with MS vitamins (Table 3, Table 4)

Table 6: Nutrient content of MS full and MS half (Murashige and Skoog 1962)

Nutrients	Content	
	MS full	MS half
Micro Elements		
$CoCl_2.6H_2O$ (Cobalt(II) chloride hexahydrate)	0.11 µM	0.11 µM
$CuSO_4.5H_2O$ (Copper(II) sulphate pentahydrate)	0.10 µM	0.10 µM
FeNaEDTA (Ethylenediaminetetraacetic acid, ferric-sodium salt)	100.00 µM	100.00 µM
H_3BO_3 (Boric acid)	100.27 µM	100.27 µM
KI(Potassium iodide)	5.00 µM	5.00 µM
$MnSO_4.H_2O$ (Manganese(II) sulfatemonohydrate)	100.00 µM	100.00 µM
$Na_2MoO_4.2H_2O$ (Sodium molybdite dihydrate)	1.03 µM	1.03 µM
$ZnSO_4.7H_2O$ (Zinc sulphate heptahydrate)	29.91 µM	29.91 µM
Macro Elements		
$CaCl_2$ (Calcium chloride)	2.99 mM	1.50 mM
KH_2PO_4 (Potassium dihydrogenphosphate)	1.25 mM	0.63 mM
KNO_3 (Potassium nitrate)	18.79 mM	9.40 mM
$MgSO_4$ (Magnesium sulphate)	1.50 mM	0.75 mM
NH_4NO_3 (Ammonium nitrate)	20.61 mM	10.31 mM

Media were prepared with two types of sugar: maltose or sucrose at a concentration of 3 %. As gelling agents, 0.2 % Gelrite (Duchefa, The Netherlands) or 0.8 % agar (Merck) were used. Seven different growth regulators in various combinations and concentrations were analyzed. (Table 7)

For this assay, small pieces of calli were used from the various callus induction experiments. There was no original plant material attached to the calli. For callus propagation, all calli were subcultured in light culture conditions because of better growth. After propagation, some calli went through two different regeneration treatments: calli on regeneration media 37, 63 and 64 were first placed on regeneration media 28, 30, 32, 35. (Table 8)

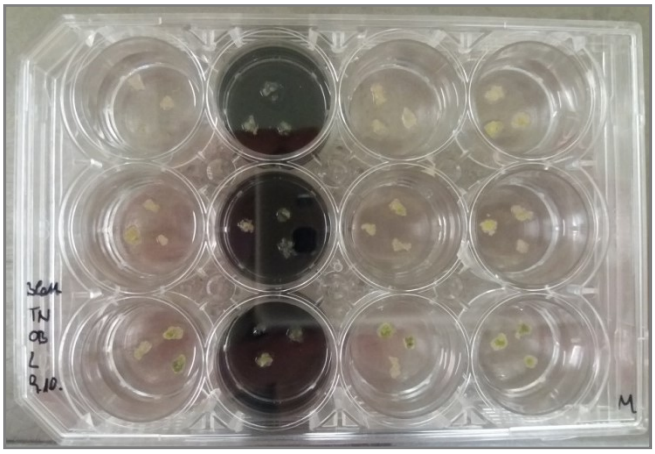

Figure 16: Well plate with 3 pieces of calli in each well for plant regeneration assays

Plant regeneration studies with media 28 – 33 and 35 – 37 were carried out with multi well plates with 3 ml per well, each row containing a different medium. Studies with medium 34 and media 38 – 67 were carried out with Petri dishes (92 mm x 16 mm). In each well, 3 pieces of callus and in each Petri dish about 15 pieces of callus were placed (Figure 16). The cultures were incubated under light conditions.

Final evaluation took place after four weeks of cultivation on regeneration media.

Callus can be stored for prolonged time without subculturing for at least 9 months at 15°C.

Table 7: Growth regulators and additives for plant regeneration

Medium ID	Growth regulators (µM)							Activated charcoal (%)	Carbon source	Gelling agent	Basal medium
	TDZ	IAA	BAP	IBA	GA3	KIN	Z				
28									Maltose	Gelrite	Bionova
29									Sucrose	Gelrite	Bionova
30								0.15	Maltose	Gelrite	Bionova
31								0.15	Sucrose	Gelrite	Bionova
32	0.25								Maltose	Gelrite	Bionova
33	0.25								Sucrose	Gelrite	Bionova
34	0.25								Sucrose	Gelrite	MS full
35	0.50								Maltose	Gelrite	Bionova
36	0.50								Sucrose	Gelrite	Bionova
37	0.50								Sucrose	Gelrite	MS full
38	0.50								Sucrose	Agar	MS full
39	1.00								Sucrose	Gelrite	MS full
40	5.00								Sucrose	Gelrite	MS full
41	10.00								Sucrose	Gelrite	MS full
42	0.25	1.00							Sucrose	Gelrite	MS full
43	0.25	5.00							Sucrose	Gelrite	MS full
44	0.50	1.00							Sucrose	Gelrite	MS full
45	0.50	5.00							Sucrose	Gelrite	MS full
46	1.00	1.00							Sucrose	Gelrite	MS full
47	1.00	5.00							Sucrose	Gelrite	MS full
48	5.00	1.00							Sucrose	Gelrite	MS full
49	5.00	5.00							Sucrose	Gelrite	MS full
50	10.00	1.00							Sucrose	Gelrite	MS full
51	10.00	5.00							Sucrose	Gelrite	MS full
52	10.00	10.00							Sucrose	Gelrite	MS full

53	0.50				7.00				Sucrose	Gelrite	MS full
54	2.50				7.00				Sucrose	Gelrite	MS full
55	5.00				7.00				Sucrose	Gelrite	MS full
56		7.50				7.50			Sucrose	Gelrite	MS half
57		0.057					6.84		Sucrose	Gelrite	MS full
58		0.057					13.69		Sucrose	Gelrite	MS full
59			0.50						Sucrose	Gelrite	MS full
60			1.00						Sucrose	Gelrite	MS full
61			5.00						Sucrose	Gelrite	MS full
62			10.00						Sucrose	Gelrite	MS full
63			8.88	2.46					Sucrose	Agar	MS full
64			17.78				9.12		Sucrose	Agar	MS full
65				5.00	1.00			0.10	Sucrose	Agar	MS full

Table 8: Source of callus and culture vessels used for plant regeneration assays

Regeneration medium ID	Explant (callus source)	Chemo-type	Callus induction medium ID and culture condition (dark/light)	Callus propagation medium ID and culture condition (dark/light)	Intermediate callus regeneration medium ID and culture condition (dark/light)	Culture vessel
28	Leaves	BD	26 (L)	26 (L)		Multi well plate
	Leaves	BD	26 (D)	26 (L)		Multi well plate
	Leaves	BD	25 (L)	25 (L)		Multi well plate
	Leaves	CNH	25 (L)	25 (L)		Multi well plate
	Leaves	CNH	25 (D)	25 (L)		Multi well plate
	Leaves	OB	25 (L)	25 (L)		Multi well plate
	Leaves	OB	25 (D)	25 (L)		Multi well plate
	Leaves	W	25 (L)	25 (L)		Multi well plate
	Leaves	W	25 (D)	25 (L)		Multi well plate
	Ovules	APK	27 (L)	27 (L)		Multi well plate
29	Leaves	BD	26 (L)	26 (L)		Multi well plate
	Leaves	BD	26 (D)	26 (L)		Multi well plate
	Leaves	BD	25 (L)	25 (L)		Multi well plate
	Leaves	CNH	25 (L)	25 (L)		Multi well plate
	Leaves	CNH	25 (D)	25 (L)		Multi well plate
	Leaves	OB	25 (L)	25 (L)		Multi well plate
	Leaves	OB	25 (D)	25 (L)		Multi well plate
	Leaves	W	25 (L)	25 (L)		Multi well plate
	Leaves	W	25 (D)	25 (L)		Multi well plate
	Ovules	APK	27 (L)	27 (L)		Multi well plate
30	Leaves	BD	26 (L)	26 (L)		Multi well plate
	Leaves	BD	26 (D)	26 (L)		Multi well plate
	Leaves	BD	25 (L)	25 (L)		Multi well plate

	Leaves	CNH	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (D)	25 (L)	Multi well plate
	Leaves	OB	25 (L)	25 (L)	Multi well plate
	Leaves	OB	25 (D)	25 (L)	Multi well plate
	Leaves	W	25 (L)	25 (L)	Multi well plate
	Leaves	W	25 (D)	25 (L)	Multi well plate
	Ovules	APK	27 (L)	27 (L)	Multi well plate
31	Leaves	BD	26 (L)	26 (L)	Multi well plate
	Leaves	BD	26 (D)	26 (L)	Multi well plate
	Leaves	BD	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (D)	25 (L)	Multi well plate
	Leaves	OB	25 (L)	25 (L)	Multi well plate
	Leaves	OB	25 (D)	25 (L)	Multi well plate
	Leaves	W	25 (L)	25 (L)	Multi well plate
	Leaves	W	25 (D)	25 (L)	Multi well plate
	Ovules	APK	27 (L)	27 (L)	Multi well plate
32	Leaves	BD	26 (L)	26 (L)	Multi well plate
	Leaves	BD	26 (D)	26 (L)	Multi well plate
	Leaves	BD	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (D)	25 (L)	Multi well plate
	Leaves	OB	25 (L)	25 (L)	Multi well plate
	Leaves	OB	25 (D)	25 (L)	Multi well plate
	Leaves	W	25 (L)	25 (L)	Multi well plate
	Leaves	W	25 (D)	25 (L)	Multi well plate
	Ovules	APK	27 (L)	27 (L)	Multi well plate
33	Leaves	BD	26 (L)	26 (L)	Multi well plate
	Leaves	BD	26 (D)	26 (L)	Multi well plate

	Leaves	BD	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (D)	25 (L)	Multi well plate
	Leaves	OB	25 (L)	25 (L)	Multi well plate
	Leaves	OB	25 (D)	25 (L)	Multi well plate
	Leaves	W	25 (L)	25 (L)	Multi well plate
	Leaves	W	25 (D)	25 (L)	Multi well plate
	Ovules	APK	27 (L)	27 (L)	Multi well plate
34	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
35	Leaves	BD	26 (L)	26 (L)	Multi well plate
	Leaves	BD	26 (D)	26 (L)	Multi well plate
	Leaves	BD	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (L)	25 (L)	Multi well plate
	Leaves	CNH	25 (D)	25 (L)	Multi well plate
	Leaves	OB	25 (L)	25 (L)	Multi well plate
	Leaves	OB	25 (D)	25 (L)	Multi well plate
	Leaves	W	25 (L)	25 (L)	Multi well plate
	Leaves	W	25 (D)	25 (L)	Multi well plate
	Ovules	APK	27 (L)	27 (L)	Multi well plate
36	Leaves	BD	26 (L)	26 (L)	Multi well plate

	Leaves	BD	26 (D)	26 (L)		Multi well plate
	Leaves	BD	25 (L)	25 (L)		Multi well plate
	Leaves	CNH	25 (L)	25 (L)		Multi well plate
	Leaves	CNH	25 (D)	25 (L)		Multi well plate
	Leaves	OB	25 (L)	25 (L)		Multi well plate
	Leaves	OB	25 (D)	25 (L)		Multi well plate
	Leaves	W	25 (L)	25 (L)		Multi well plate
	Leaves	W	25 (D)	25 (L)		Multi well plate
	Ovules	APK	27 (L)	27 (L)		Multi well plate
37	Leaves	BD	26 (L)	26 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	29, 31, 33, 36 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	W	25 (D)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Ovules	APK	27 (L)	27 (L)	29, 31, 33, 36 (L)	Petri dishes
	Ovules	APK	27 (L)	27 (L)	28, 30, 32, 35 (L)	Petri dishes
38	Leaves	BD	25 (L)	25 (L)		Petri dishes
	Leaves	W	25 (L)	25 (L)		Petri dishes
	Leaves	CNH	25 (L)	25 (L)		Petri dishes
	Leaves	OB	25 (D)	25 (L)		Petri dishes
	Leaves	OB	25 (L)	25 (L)		Petri dishes
	Leaves	W	26 (D)	26 (L)		Petri dishes
	Leaves	CNH	26 (L)	26 (L)		Petri dishes
	Leaves	BD	26 (D)	26 (L)		Petri dishes
	Leaves	BD	26 (L)	26 (L)		Petri dishes
	Ovules	APK	25 (L)	25 (L)		Petri dishes

39	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
40	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
41	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes

	Ovules	APK	25 (L)	25 (L)	Petri dishes
42	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
43	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
44	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes

	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
45	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
46	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
47	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes

	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
48	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
49	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
50	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes

	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
51	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
52	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (D)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
53	Leaves	W	26 (L)	26 (L)	Petri dishes
	Leaves	OB	26 (L)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes

	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (L)	26 (L)	Petri dishes
	Leaves	OB	26 (L)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
54	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (L)	26 (L)	Petri dishes
	Leaves	OB	26 (L)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
55	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
	Leaves	OB	26 (L)	26 (L)	Petri dishes
	Leaves	W	26 (L)	26 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	Petri dishes
56	Leaves	BD	26 (L)	26 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes

	Ovules	APK	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (L)	26 (L)	Petri dishes
	Leaves	OB	26 (L)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
57	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (L)	26 (L)	Petri dishes
	Leaves	OB	26 (L)	26 (L)	Petri dishes
	Leaves	CNH	26 (L)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
58	Leaves	W	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
	Leaves	W	26 (L)	26 (L)	Petri dishes
	Leaves	OB	26 (D)	26 (L)	Petri dishes
	Leaves	CNH	26 (D)	26 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	Petri dishes
59	Leaves	OB	25 (L)	25 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	Petri dishes
	Ovules	APK	25 (L)	25 (L)	Petri dishes
60	Leaves	W	26 (L)	26 (L)	Petri dishes

	Leaves	OB	26 (D)	26 (L)		Petri dishes
	Leaves	CNH	26 (D)	26 (L)		Petri dishes
	Leaves	BD	26 (D)	26 (L)		Petri dishes
	Leaves	OB	25 (L)	25 (L)		Petri dishes
	Leaves	CNH	25 (L)	25 (L)		Petri dishes
	Leaves	BD	25 (L)	25 (L)		Petri dishes
	Leaves	W	25 (L)	25 (L)		Petri dishes
	Ovules	APK	25 (L)	25 (L)		Petri dishes
	Leaves	W	26 (L)	26 (L)		Petri dishes
	Leaves	OB	26 (D)	26 (L)		Petri dishes
	Leaves	CNH	26 (D)	26 (L)		Petri dishes
	Leaves	BD	26 (D)	26 (L)		Petri dishes
61	Leaves	OB	25 (L)	25 (L)		Petri dishes
	Leaves	CNH	25 (L)	25 (L)		Petri dishes
	Leaves	BD	25 (L)	25 (L)		Petri dishes
	Leaves	W	25 (L)	25 (L)		Petri dishes
	Ovules	APK	25 (L)	25 (L)		Petri dishes
	Leaves	W	26 (L)	26 (L)		Petri dishes
	Leaves	OB	26 (D)	26 (L)		Petri dishes
	Leaves	CNH	26 (D)	26 (L)		Petri dishes
	Leaves	BD	26 (D)	26 (L)		Petri dishes
62	Leaves	OB	25 (L)	25 (L)		Petri dishes
	Leaves	CNH	25 (L)	25 (L)		Petri dishes
	Leaves	BD	25 (L)	25 (L)		Petri dishes
	Leaves	W	25 (L)	25 (L)		Petri dishes
	Ovules	APK	25 (L)	25 (L)		Petri dishes
	Leaves	BD	26 (L)	26 (L)	28, 30, 32, 35 (L)	Petri dishes
63	Leaves	BD	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	28, 30, 32, 35 (L)	Petri dishes

	Leaves	BD	25 (L)	25 (L)	29, 31, 33, 36 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	W	25 (D)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Ovules	APK	27 (L)	27 (L)	29, 31, 33, 36 (L)	Petri dishes
	Ovules	APK	27 (L)	27 (L)	28, 30, 32, 35 (L)	Petri dishes
64	Leaves	BD	26 (L)	26 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	BD	26 (D)	26 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	BD	25 (L)	25 (L)	29, 31, 33, 36 (L)	Petri dishes
	Leaves	CNH	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	OB	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	W	25 (D)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Leaves	W	25 (L)	25 (L)	28, 30, 32, 35 (L)	Petri dishes
	Ovules	APK	27 (L)	27 (L)	29, 31, 33, 36 (L)	Petri dishes
	Ovules	APK	27 (L)	27 (L)	28, 30, 32, 35 (L)	Petri dishes
65	Leaves	BD	25 (L)	25 (L)		Petri dishes
	Leaves	BD	26 (D)	26 (L)		Petri dishes
	Leaves	CNH	25 (L)	25 (L)		Petri dishes
	Leaves	CNH	26 (D)	26 (L)		Petri dishes
	Leaves	OB	25 (L)	25 (L)		Petri dishes
	Leaves	OB	26 (D)	26 (L)		Petri dishes
	Leaves	W	25 (L)	25 (L)		Petri dishes
	Leaves	W	26 (L)	26 (L)		Petri dishes
	Ovules	APK	25 (L)	25 (L)		Petri dishes
	Ovules	APK	27 (L)	27 (L)		Petri dishes

5.3 Flow Cytometry Analysis

Leaves from the mother plants in the greenhouse and calli were analyzed together with Dr. Eva Temsch at the Department of Botany and Biodiversity Research, University of Vienna.

Various calli from different chemotypes (APK, BD, CNH, OB, W), explant sources (leaves or ovules), media and carbon sources (maltose, sucrose) were analyzed (Table 9). All calli came from light treatments.

Table 9: Sample of various chemotypes, callus sources, growth regulators and carbon sources subjected to flow cytometry.

Chemotype	Source	Growth regulators (µM)			Carbon source
		TDZ	NAA	2,4-D	
APK	Motherplant				
	Leaves	2	1		Sucrose
	Leaves	2	1		Maltose
	Leaves			4.5	Maltose
	Ovules	2	1		Maltose
BD	Motherplant				
	Leaves	2	1		Sucrose
	Leaves	2	1		Maltose
	Leaves	2	1		Maltose
	Leaves			4.5	Maltose
	Leaves			4.5	Maltose
	Ovules	2	1		Maltose
CNH	Motherplant				
	Leaves	2	1		Sucrose
	Leaves	2	1		Maltose
	Leaves	2	1		Maltose
	Leaves			4.5	Maltose
	Leaves			4.5	Maltose
	Ovules	2	1		Maltose
W	Motherplant				
	Leaves	2	1		Sucrose
	Leaves	2	1		Maltose
	Leaves	2	1		Maltose
	Leaves			4.5	Maltose
	Leaves			4.5	Maltose
	Ovules	2	1		Maltose

	Motherplant				
	Leaves	2	1		Sucrose
OB	Leaves	2	1		Maltose
	Leaves	2	1		Maltose
	Leaves			4.5	Maltose

For sample preparation, approximately 25 mg callus was co-chopped (Galbraith et al. 1983) along with an appropriate fresh weight of the internal standard organism in Otto´s buffer I (Otto et al. 1981). As internal standard organism *Solanum pseudocapsicum* was selected since its genome (1C=1.295 pg (Temsch et al. 2010)) is within an appropriate size range compared to the cannabis genome size (~0,836 pg) for flow cytometry.

The obtained suspension was filtered using a 45 µm nylon mesh to remove large cell debris before adding RNAse and incubating at 37°C. The removal of RNA is important as propidium iodide (PI) also intercalates with double stranded RNA. After 30 minutes, PI containing Otto buffer II was added (Otto et al. 1981) and the sample was incubated for another 60 minutes at the refrigerator.

The instrument PartecCyFlow ML flow cytometer equipped with a 100 mW and 532 nm diode pumped laser (Cobolt Samba, Cobolt AB, Stockholm, Sweden) was used for the analysis. For the *in vivo* leaves, 3 runs with 3,333 particles each were carried out. For measurements in calli, 3,333 particles in 3 runs or 10,000 particles within one run were measured. The PI fluorescence intensity was recorded and analyzed with the instrument analysis software (FloMaxsoftware, Partec, Münster, Germany).

The 1C values were calculated according to the formula:

$$1C\ (C.s.) = \frac{Mean\ G1\ nuclei\ peak\ position\ (C.s.)}{Mean\ G1\ nuclei\ peak\ position\ (S.p.)} \times 1C(S.p.)$$

In addition, the endopolyploidy index (EI, (Barow and Meister 2003)) was calculated according to the formula:

$$EI = \frac{0 * n(2C) + 1 * n(4C) + 2 * n(8C) + 3 * n(16C) + 4 * n(32C) \ldots}{n(2C) + n(4C) + n(8C) + n(16C) + n(32C) \ldots}$$

6 Results

6.1 Callus Initiation

The callus initiation assay was carried out in four independent experiments using the following types of explants (Table 10):

1. Leaf explants of chemotypes BD and CNH on media 1 – 24 in the dark.
2. Leaf explants of four different chemotypes (BD, CNH, OB, W) on media 25 and 26 under dark and light conditions.
3. Ovule explants of chemotype APK on media 25 and 26 under dark and light conditions.
4. Ovule explants of chemotype APK on medium 27 under dark and light conditions.

Table 10: For callus induction experiments 1 - 4 used explant types, chemotypes, media and culture conditions.

Experiment	Explant type	Chemotype	Media	Dark/light
1	Leaves	BD, CNH	1 - 24	D
2	Leaves	BD, CNH, OB, W	25, 26	D, L
3	Ovules	BD, CNH, OB, W	25, 26	D, L
4	Ovules	APK	27	D, L

Evaluation after nine weeks was based on two criteria:

1. Presence of callus - yes/no.
2. Amount of callus produced. 0 meaning no callus produced, 1 meaning little callus, 2 meaning sufficient callus produced and 3 meaning a large amount of callus was produced. (Figure 17)

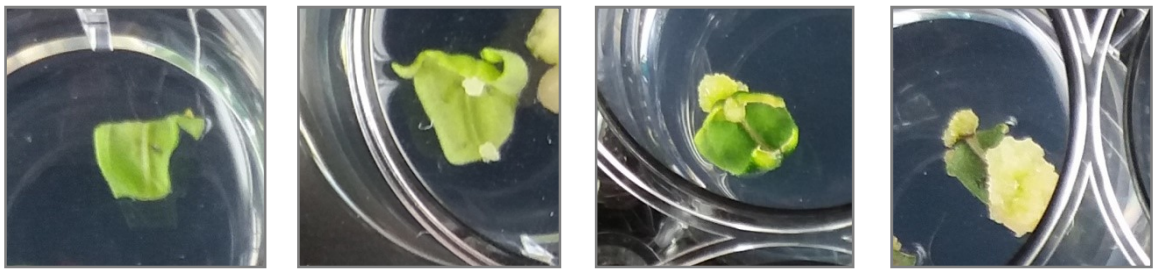

Figure 17: Amount of callus produced: no callus formation (0), little (1), sufficient (2) and large (3) callus formation in leaf explants (left to right).

Experiment 1:

Leaf explants of both chemotypes (BD, CNH) produced callus with all tested concentrations of KIN/IAA and BAP/IAA (Table 11).

Table 11: Results experiment 1 for leaf explants

Medium ID	Growth regulators	BD Explants producing callus (%)	CNH Explants producing callus (%)
1	1 µM BAP	29	13
2	1 µM BAP/1 µM IAA	13	46
3	1 µM BAP/5 µM IAA	71	49
4	1 µM BAP/10 µM IAA	42	52
5	5 µM BAP	29	68
6	5 µM BAP/1 µM IAA	21	68
7	5 µM BAP/5 µM IAA	63	28
8	5 µM BAP/10 µM IAA	36	68
9	10 µM BAP	**9**	13
10	10 µM BAP/1 µM IAA	38	35
11	10 µM BAP/5 µM IAA	42	48
12	10 µM BAP/10 µM IAA	21	47
13	1 µM KIN	35	**2**
14	1 µM KIN/1 µM IAA	25	43
15	1 µM KIN/5 µM IAA	63	57
16	1 µM KIN/10 µM IAA	**88**	45
17	5 µM KIN	32	14
18	5 µM KIN/1 µM IAA	50	52
19	5 µM KIN/5 µM IAA	60	53
20	5 µM KIN/10 µM IAA	58	**82**
21	10 µM KIN	42	31
22	10 µM KIN/1 µM IAA	42	35
23	10 µM KIN/5 µM IAA	58	62
24	10 µM KIN/10 µM IAA	46	73

Chemotype BD responded best to medium 16 with 88 % of explants producing callus and the worst to medium 9 with 9 % of the explants producing callus. For chemotype CNH, medium 20 was the most effective with 82 % callus producing explants and medium 13 with 2 % the least effective. Medium 16 and 20 both contained 10 µM IAA, while media 9 and 13 were without IAA.

A higher number of chemotype BD explants produced callus on media with the growth regulator combination KIN/IAA (50 %) than BAP/IAA (34 %). In CNH, the number of callus producing explants was similar with KIN/IAA (46 %) and BAP/IAA (44 %). (Figure 18, Figure 19)

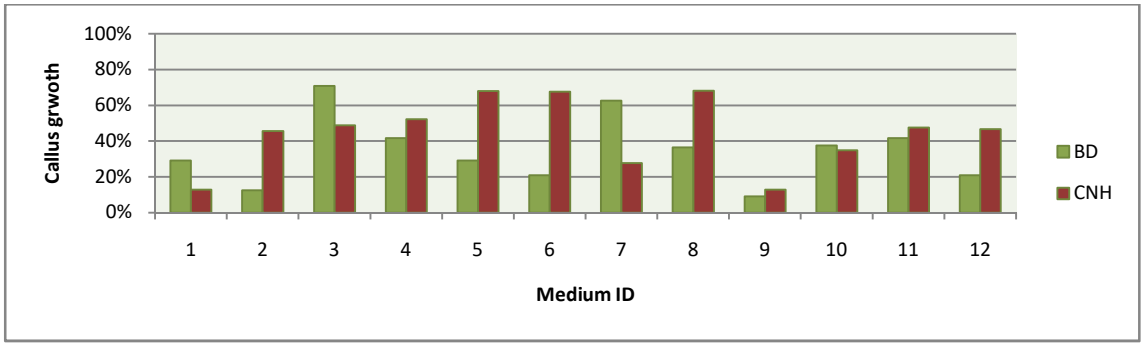

Figure 18: Percentage of average callus induction of BD and CNH leaf explants with various concentrations of BAP and IAA.

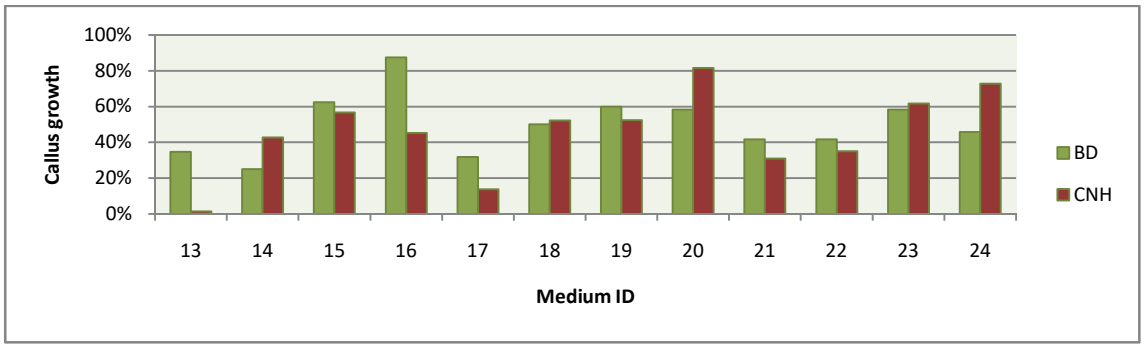

Figure 19: Percentage of average callus induction of BD and CNH leaf explants with various concentrations of KIN and IAA.

The largest amount of callus produced by explants of chemotype BD was on medium 16 and the smallest on medium 9. Chemotype CNH explants produced most callus on medium 20 and least on medium 13. Explants of chemotype CNH produced more callus in general, especially with BAP/IAA containing media. (Figure 20, Figure 21)

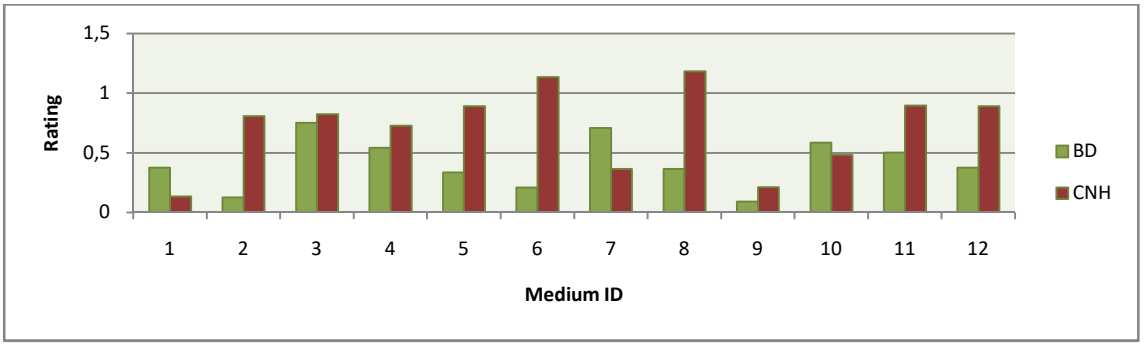

Figure 20: Average rating of callus induction of BD and CNH leaf explants with various concentrations of BAP and IAA.

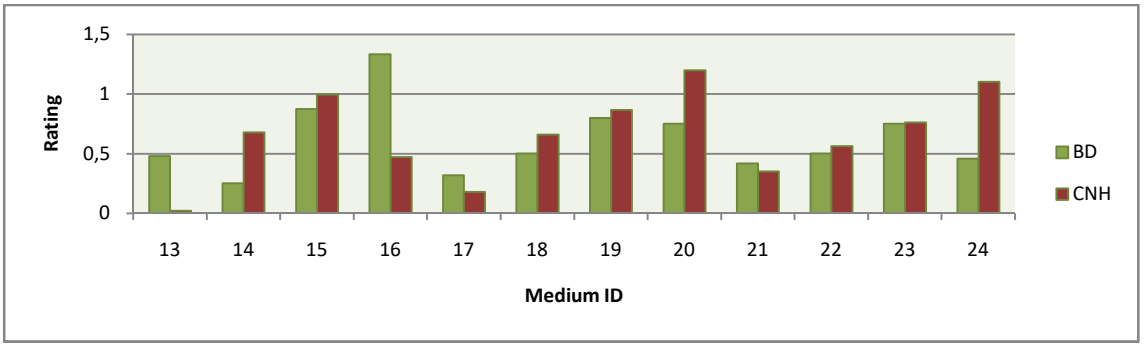

Figure 21: Average rating of callus induction of BD and CNH leaf explants with various concentrations of KIN and IAA.

Experiment 2:

After 9 weeks of culturing leaf explants in light or dark culture environment, all tested chemotypes (W, OB, BD, CNH) produced callus on the two induction media (Table 12). Chemotype W on medium 25 and light culture conditions dropped out after five weeks of culture because of bacterial contamination. However, after five weeks each explant had already produced a sufficient amount of callus.

Table 12: Results experiment 2 for leaf explants

Medium ID	Growth regulators	Culture conditions (D/L)	Explants producing callus (%)			
			W	OB	BD	CNH
25	2 µM TDZ/1 µM NAA	L	100	100	100	100
25	2 µM TDZ/1 µM NAA	D	100	100	100	100
26	4.5 µM 2,4-D	L	88	83	96	73
26	4.5 µM 2,4-D	D	86	100	100	91

Medium 25 was the optimal callus initiation medium for all tested chemotypes, independent of the light conditions. Medium 26 also gave good results with over 83 – 100 % of the explants responding positively, depending on the genotype. (Figure 22)

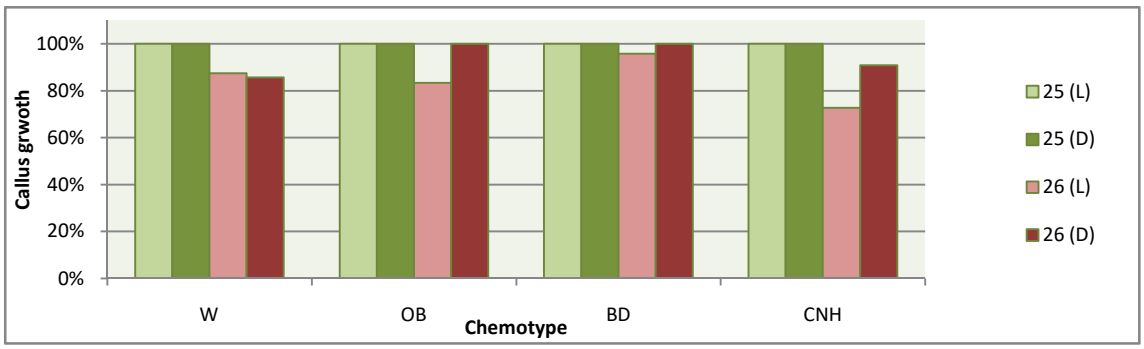

Figure 22: Percentage of average callus induction in the leaf explants of the chemotypes W, OB, BD and CNH on medium 25 (2 µM TDZ/1 µM NAA) and 26 (4.5 µM 2,4-D) and in dark and light culture environment.

Considering the amount of callus produced, medium 25 was the most effective medium. Even after five weeks, the contaminated culture with chemotype W had already produced a sufficient amount of callus. (Figure 23)

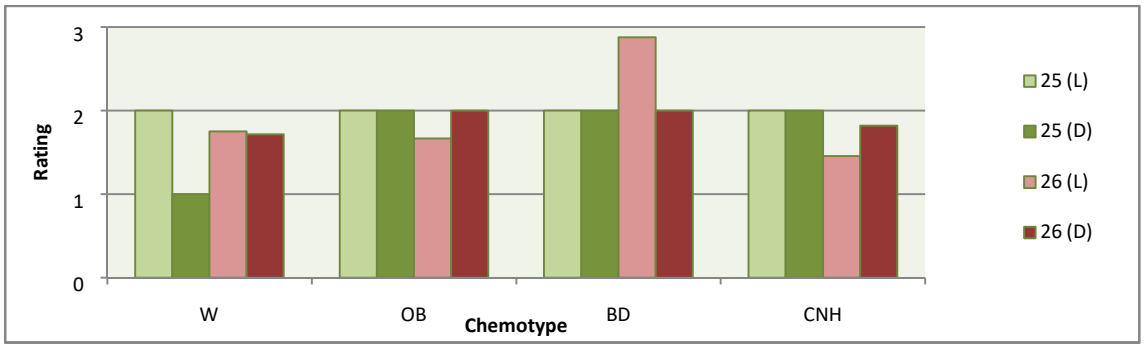

Figure 23: Average rating of callus induction of W, OB, BD and CNH leaf explants with 4.5 µM 2,4-D and 2 µM TDZ/1 µM NAA each in dark and light culture environment.

Experiment 3:

Callus initiation of isolated ovules from chemotype APK on two different media showed clearly that medium 25 containing 2 µM TDZ and 1 µM NAA was more effective than medium 26 with 4.5 µM 2,4-D (Table 13). Callus formed both under dark and light conditions. Regarding the amount of callus, explants under light conditions produced twice as much callus as those in the dark. On medium 26, callus production was very low with 8 % of explants producing callus in the dark, but none in the light. (Figure 24)

Table 13: Results experiment 3 for ovule explants

Medium ID	Growth regulators	Culture conditions (D/L)	Explants producing callus (%)
25	2 µM TDZ/1 µM NAA	L	100
25	2 µM TDZ/1 µM NAA	D	100
26	4.5 µM 2,4-D	L	0
26	4.5 µM 2,4-D	D	8

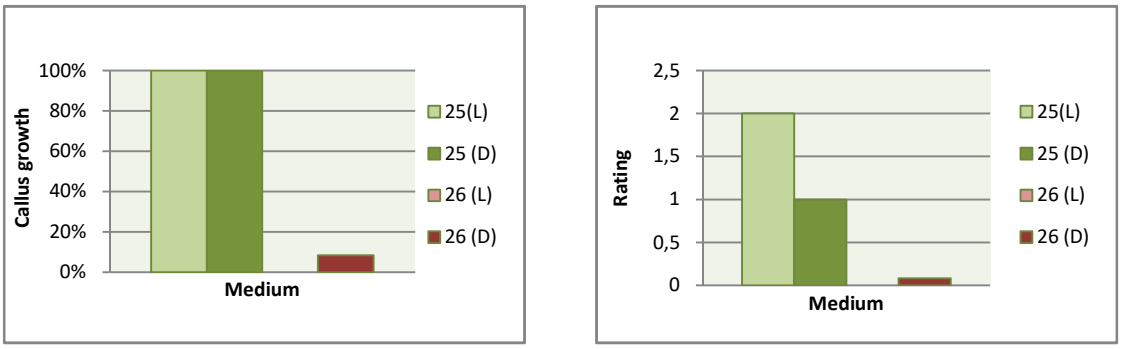

Figure 24: Percentage of average callus induction of ovule explants with 4.5 µM 2,4-D and 2 µM TDZ/1 µM NAA in each dark and light culture environment. The right graph shows the average rating of callus induction.

Experiment 4:

Callus induction of ovule explants in dark and light culture conditions on medium 27 resulted in 100 % callus induction in light, but no callus induction in dark conditions. Explants in the light had produced a large amount of callus after nine weeks (rating 3). (Table 14)

Table 14: Results experiment 4 for ovule explants

Medium ID	Growth regulators	Culture conditions (D/L)	Explants producing Callus (%)
27	7.5 µM IAA/7.5 µM KIN	L	100
27	7.5 µM IAA/7.5 µM KIN	D	0

Callus initiation from leaves could be achieved on all 27 media used. Success was chemotype-dependent and the quantity of callus varied amongst culture conditions and chemotypes. The most effective callus induction treatment was medium 25 in light culture conditions, where all explants of the four tested chemotypes produced a sufficient amount of callus within nine weeks.

For callus initiation from ovules, medium 27 in light culture conditions was the most effective treatment with 100 % of explants producing a large amount of callus.

Characteristics of induced calli

The calli differed from each other regarding shape, color, size and surface, with its color ranging from white, pale yellow to green and brown (Figure 25). It was mostly soft, especially the ovule-induced calli, but also compact calli were produced. A cross-section of an ovule-induced soft callus shows its large, watery cells (Figure 26). Its characteristics did not depend on the chemotype, explant, growth regulator or culture conditions.

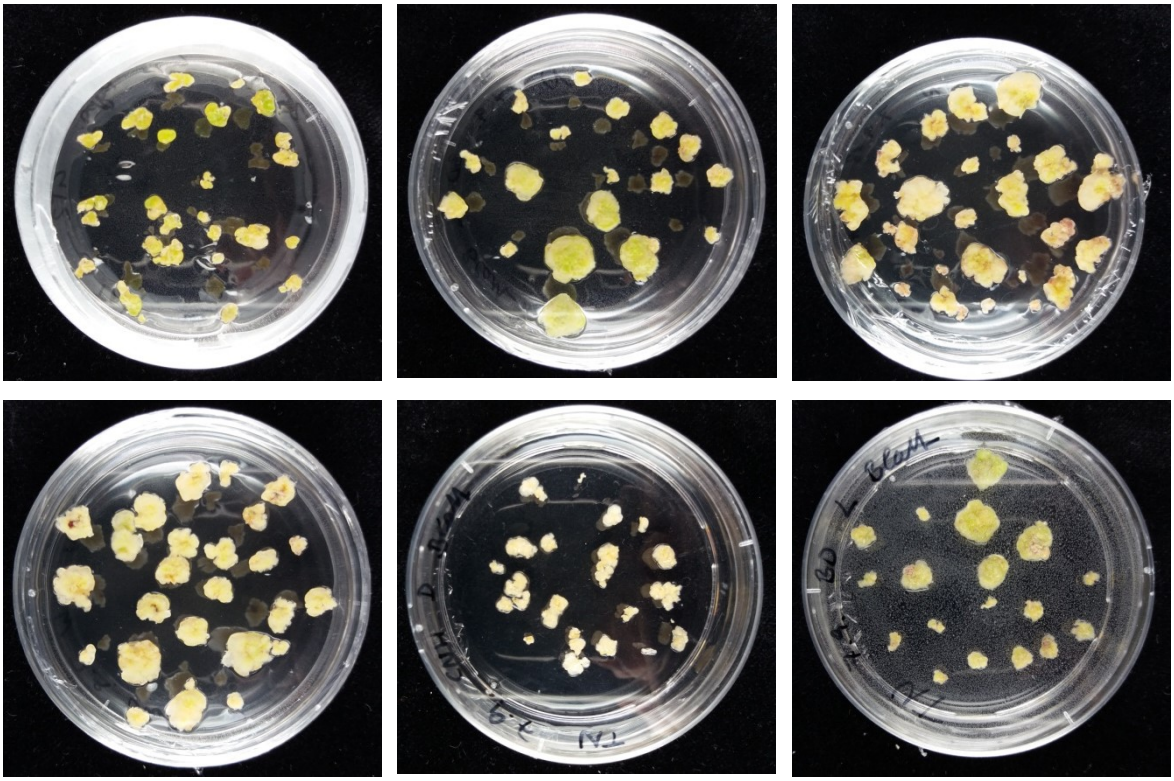

Figure 25: Differently characterized calli after propagation

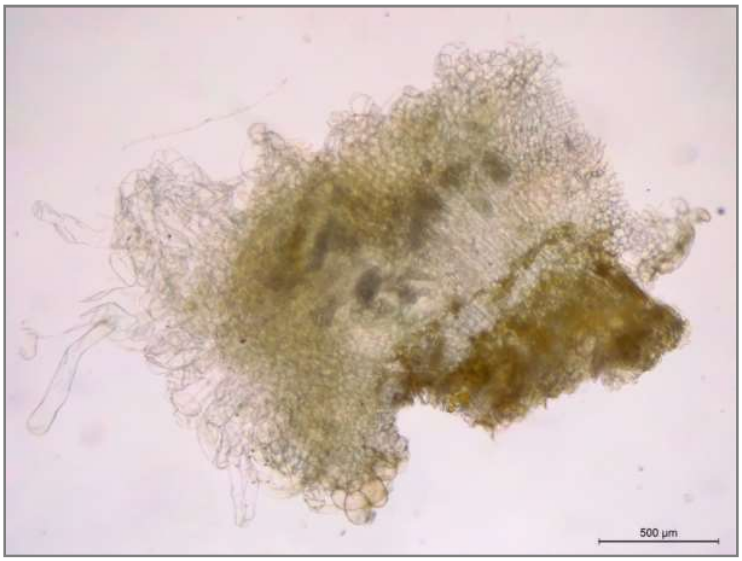

Figure 26: Cross-section of an ovule explant induced soft callus

6.2 Plant Regeneration

Some calli produced roots, especially on media with high concentrations of growth regulators (e.g. media 40, 41, 52), but there were no signs of shoots in any of the 38 tested media (media 28 – 65), despite including published media that had shown successful plant regeneration in other chemotypes (Table 15).

Table 15: Source and their results of the plant regeneration assay media

Medium ID	Reference	Plant species used for	Results in literature/ further information
28	-		
29	-		
30	-		
31	-		
32	-		since lower TDZ concentrations seem to be more effective (Chandra et al. 2010; Lata et al. 2010)
33	-		
34	-		
35	(Lata et al. 2010)	Cannabis	96.6%
36	(Lata et al. 2010)	Cannabis	96.6 %
37	(Lata et al. 2010)	Cannabis	96.6 %
38	(Lata et al. 2010)	Cannabis	96.6 %
39	(Lata et al. 2010)	Cannabis	93.3%
40	(Lata et al. 2010)	Cannabis	86.6%
41	(Lata et al. 2010)	Cannabis	83.3%
42	(Kodym et al. 2017)	Tobacco	
43	(Kodym et al. 2017)	Tobacco	
44	(Kodym et al. 2017)	Tobacco	
45	(Kodym et al. 2017)	Tobacco	
46	(Kodym et al. 2017)	Tobacco	
47	(Kodym et al. 2017)	Tobacco	
48	(Kodym et al. 2017)	Tobacco	
49	(Kodym et al. 2017)	Tobacco	
50	(Kodym et al. 2017)	Tobacco	
51	(Kodym et al. 2017)	Tobacco	
52	(Kodym et al. 2017)	Tobacco	

53	(Chandra et al. 2010)	Cannabis	84.84 %
54	(Chandra et al. 2010)	Cannabis	95.62 %
55	(Chandra et al. 2010)	Cannabis	79.81 %
56	-		
57	(Batista et al. 1996)	Hops	
58	(Batista et al. 1996)	Hops	
59	(Chandra et al. 2010)	Cannabis	71.42 %
60	(Chandra et al. 2010) adjusted	Cannabis	
61	(Chandra et al. 2010)	Cannabis	65.52 %
62	(Chandra et al. 2010)adjusted	Cannabis	
63	(Movahedi and Torabi 2015)	Cannabis	Highest length of shoots: 12.3 mm
64	(Sirkowski 2012)	Cannabis	
65	-		

6.3 Flow Cytometry Analysis

6.3.1 Endopolyploidy

C-values of mother plant material and leaf and ovule calli of the five different chemotypes were statistically analyzed. The results were also grouped regarding carbon source and age, and accordingly analyzed. Calli younger than 100 days were the youngest calli examined. Calli older than 100 days were understood as older calli. (Table 16)

Table 16: Variable parameters of calli induced for flow cytometry analysis: Chemotypes, explants, growth regulators, carbon source and callus age.

Chemotypes	Explants	Growth regulators	Carbon source	Callus age
▪ APK ▪ BD ▪ CNH ▪ OB ▪ W	▪ Ovules ▪ Leaves	▪ 2,4-D (4.5 µM) ▪ TDZ (2 µM) + NAA (1 µM)	▪ Maltose ▪ Sucrose	▪ Below 100 days ▪ Over 100 days

The results of flow cytometry measurements are presented in histograms, which show the relative DNA content (based on the fluorescence intensity) on the x-axis and the number of particles counted as the peak area (Figure 27).

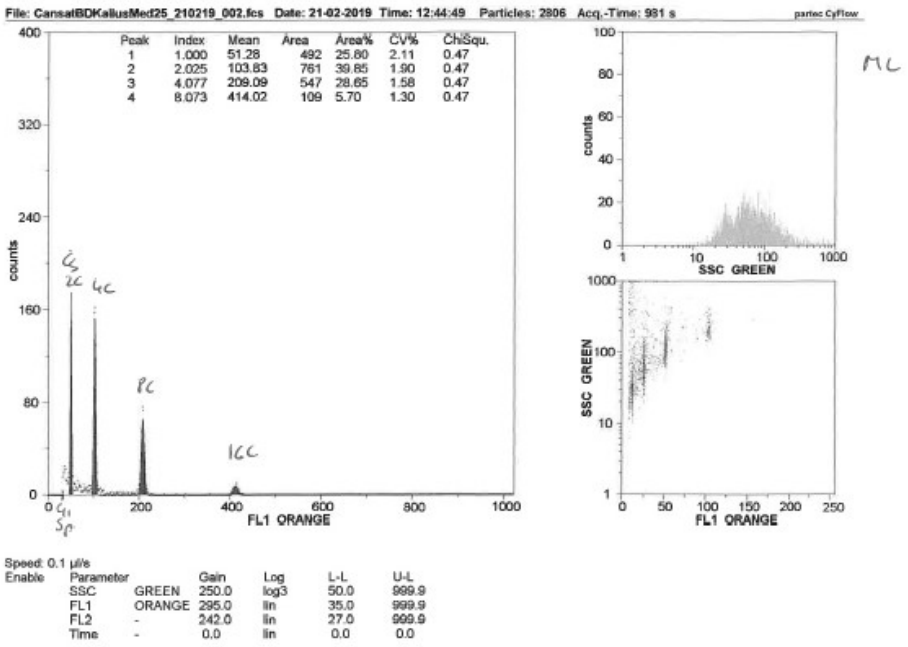

Figure 27: Flow cytometry histogram of cannabis callus nuclei with up to 16C.

Since cannabis is a diploid plant, the 2C-value corresponds to the genome size of cannabis nuclei in its G_1 or G_0 phase of the cell cycle. The 4C-value corresponds to the G_2 phase right before nuclear division. Nuclei in their S phase can be found between the 2C and 4C-values. Cannabis nuclei with a C-value higher than 4C are considered endopolyploid. A certain amount of 4C nuclei also originates from endopolyploidy.

The endopolyploidy index (EI) is an important value to show the extent of endopolyploidy and was determined for a comparable evaluation of endopolyploidy in the individual samples. It indicates the mean number of endoreplication cycles for the nuclei. For this the number of nuclei at each replication stage (C-value) is multiplied by the number of endocycles necessary to reach the corresponding replication stage. Hence EI-values over 1 mean many of the measured nuclei have high DNA amounts.

All mother plants were, as expected, generally diploid. Out of 26 analyzed calli, 19 calli showed endopolyploidy, seven had a maximum of 4C. In this experiment they are interpreted as diploid nuclei. However, some of these 4C calli had higher EI-values than 8C calli. This indicates a stagnation of the cell cycle after DNA replication, thus cell division is missing and these diploid nuclei seem to have been affected by somaclonal variation.

The highest C-value of 32C was found in callus of leaf explants from chemotype BD on medium with 2 µM TDZ/1µM NAA with maltose, which was analyzed after 100 days. The highest EI-value of 2 was found in callus from leaf explants of chemotype CNH with 2 µM TDZ/1µM NAA and maltose that was younger than 100 days. This callus' maximum C-value was only 16C, but a high number of nuclei were found in the high C values. Interestingly, in callus obtained from leaf explants of chemotype W with 4.5 µM 2,4-D and maltose medium over 100 days of age, no nuclei with 2C could be found. Its high EI of 1.58 and C-values of 4C and 8C indicate a high number of nuclei with 8C. (Table 17)

Table 17: Flow cytometry analysis results arranged by chemotype.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
APK	Mother-plant						2, 4	
	Leaves	2	1		Sucrose	Over	2, 4	0.78
	Leaves	2	1		Maltose	Below	2, 4, 8	0.57
	Leaves			4.5	Maltose	Below	2, 4, 16	0.81
	Ovules	2	1		Maltose	Over	2, 4, 8	0.80
BD	Mother-plant						2, 4	
	Leaves	2	1		Sucrose	Over	2, 4	0.75
	Leaves	2	1		Maltose	Over	2, 4, 8, 16, 32	**1.82**
	Leaves	2	1		Maltose	Below	2 ,4, 8, 16	0.92
	Leaves			4.5	Maltose	Below	2, 4, 8	0.52
	Leaves			4.5	Maltose	Over	2, 4	0.35
	Ovules	2	1		Maltose	Below	2, 4, 8	**1.14**
CNH	Mother-plant						2, 4	
	Leaves	2	1		Sucrose	Over	2, 4	0.63
	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.30**
	Leaves	2	1		Maltose	Below	2, 4, 8, 16	**2.00**
	Leaves			4.5	Maltose	Below	2, 4, 8	0.51
	Leaves			4.5	Maltose	Over	2, 4, 8	0.37
	Ovules	2	1		Maltose	Below	2, 4, 8	0.66
W	Mother-plant						2, 4	
	Leaves	2	1		Sucrose	Over	2, 4, 8	0.48
	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.00**
	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
	Leaves			4.5	Maltose	Below	2, 4	0.58
	Leaves			4.5	Maltose	Over	4, 8	**1.58**
	Ovules	2	1		Maltose	Below	2, 4	0.73
OB	Mother-plant						2, 4	
	Leaves	2	1		Sucrose	Over	2, 4	0.50
	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.41**
	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
	Leaves			4.5	Maltose	Over	2, 4, 8	0.61

In leaf calli, high EI-values and C-values were commonly found in calli induced with 2 µM TDZ/1 µM NAA and maltose which were over 100 days old. However, a callus younger than 100 days had the highest EI. (Table 18)

Table 18: Leaf explant calli flow cytometry results sorted by descending EI.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
CNH	Leaves	2	1		Maltose	Below	2, 4, 8, 16	**2.00**
BD	Leaves	2	1		Maltose	Over	2, 4, 8, 16, 32	**1.82**
W	Leaves			4.5	Maltose	Over	4, 8	**1.58**
OB	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.41**
CNH	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.30**
W	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.00**
BD	Leaves	2	1		Maltose	Below	2, 4, 8, 16	0.92
APK	Leaves			4.5	Maltose	Below	2, 4, 16	0.81
APK	Leaves	2	1		Sucrose	Over	2, 4	0.78
BD	Leaves	2	1		Sucrose	Over	2, 4	0.75
CNH	Leaves	2	1		Sucrose	Over	2, 4	0.63
OB	Leaves			4.5	Maltose	Over	2, 4, 8	0.61
W	Leaves			4.5	Maltose	Below	2, 4	0.58
APK	Leaves	2	1		Maltose	Below	2, 4, 8	0.57
W	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
OB	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
BD	Leaves			4.5	Maltose	Below	2, 4, 8	0.52
CNH	Leaves			4.5	Maltose	Below	2, 4, 8	0.51
OB	Leaves	2	1		Sucrose	Over	2, 4	0.50
W	Leaves	2	1		Sucrose	Over	2, 4, 8	0.48
CNH	Leaves			4.5	Maltose	Over	2, 4, 8	0.37
BD	Leaves			4.5	Maltose	Over	2, 4	0.35

Calli derived from ovules didn't show as high C-values as leaf derived calli, but still three out of the four tested calli were endopolyploid. (Table 19)

Table 19: Ovule explant calli flow cytometry results sorted by descending EI.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
BD	Ovules	2	1		Maltose	Below	2, 4, 8	**1.14**
APK	Ovules	2	1		Maltose	Over	2, 4, 8	0.80
W	Ovules	2	1		Maltose	Below	2, 4	0.73
CNH	Ovules	2	1		Maltose	Below	2, 4, 8	0.66

When sucrose rather than maltose was used in the medium, the highest EI was 0.78. In general, all EI-values were rather low. Only one out of five calli had a C-value over 4. (Table 20)

Table 20: Sucrose media calli flow cytometry results sorted by descending EI.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
APK	Leaves	2	1		Sucrose	Over	2, 4	0.78
BD	Leaves	2	1		Sucrose	Over	2, 4	0.75
CNH	Leaves	2	1		Sucrose	Over	2, 4	0.63
OB	Leaves	2	1		Sucrose	Over	2, 4	0.50
W	Leaves	2	1		Sucrose	Over	2, 4, 8*	0.48

*8C only in one run

On media with maltose on the other hand, there was a wide range of EI-values, starting from 0.35 to a maximum of 2. Across all flow cytometric measurements, all 8C, 16C and 32C were found on maltose media, except for one. (Table 21, Table 20)

Table 21: Maltose media calli flow cytometry results sorted by descending C-value.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
BD	Leaves	2	1		Maltose	Over	2, 4, 8, 16, 32	**1.82**
CNH	Leaves	2	1		Maltose	Below	2, 4, 8, 16	**2.00**
OB	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.41**
CNH	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.30**
W	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.00**
BD	Leaves	2	1		Maltose	Below	2, 4, 8, 16	0.92
APK	Leaves			4.5	Maltose	Below	2, 4, 16	0.81
W	Leaves			4.5	Maltose	Over	4, 8	**1.58**
BD	Ovules	2	1		Maltose	Below	2, 4, 8	**1.14**
APK	Ovules	2	1		Maltose	Over	2, 4, 8	0.80
CNH	Ovules	2	1		Maltose	Below	2, 4, 8	0.66
OB	Leaves			4.5	Maltose	Over	2, 4, 8	0.61
APK	Leaves	2	1		Maltose	Below	2, 4, 8	0.57
W	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
OB	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
BD	Leaves			4.5	Maltose	Below	2, 4, 8	0.52
CNH	Leaves			4.5	Maltose	Below	2, 4, 8	0.51
CNH	Leaves			4.5	Maltose	Over	2, 4, 8	0.37

W	Ovules	2	1		Maltose	Below	2, 4	0.73
W	Leaves			4.5	Maltose	Below	2, 4	0.58
BD	Leaves			4.5	Maltose	Over	2, 4	0.35

All calli except for one initiated with TDZ/NAA on maltose media were endopolyploid, independent from the age of calli (Table 23).

Table 22: Maltose media calli initiated with TDZ/NAA flow cytometry results sorted by descending C-value.

Chemo-type	Source	Growth regulators (µM)		Carbon source	100 Days	C-value	EI
		TDZ	NAA				
BD	Leaves	2	1	Maltose	Over	2, 4, 8, 16, 32	**1.82**
CNH	Leaves	2	1	Maltose	Below	2, 4, 8, 16	**2.00**
OB	Leaves	2	1	Maltose	Over	2, 4, 8, 16	**1.41**
CNH	Leaves	2	1	Maltose	Over	2, 4, 8, 16	**1.30**
W	Leaves	2	1	Maltose	Over	2, 4, 8, 16	**1.00**
BD	Leaves	2	1	Maltose	Below	2, 4, 8, 16	0.92
BD	Ovules	2	1	Maltose	Below	2, 4, 8	**1.14**
APK	Ovules	2	1	Maltose	Over	2, 4, 8	0.80
CNH	Ovules	2	1	Maltose	Below	2, 4, 8	0.66
APK	Leaves	2	1	Maltose	Below	2, 4, 8	0.57
W	Leaves	2	1	Maltose	Below	2, 4, 8	0.54
OB	Leaves	2	1	Maltose	Below	2, 4, 8	0.54
W	Ovules	2	1	Maltose	Below	2, 4	0.73

Concerning the age of callus cultures, more diploid calli were found in older cultures (36 %) than in younger ones (17 %). (Table 23, Table 25)

Table 23: Over 100 days old calli flow cytometry results sorted by descending C-value.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
BD	Laves	2	1		Maltose	Over	2, 4, 8, 16, 32	**1.82**
OB	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.41**
CNH	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.30**
W	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.00**
W	Leaves			4.5	Maltose	Over	4, 8	**1.58**
APK	Ovules	2	1		Maltose	Over	2, 4, 8	0.80
OB	Leaves			4.5	Maltose	Over	2, 4, 8	0.61
W	Leaves	2	1		Sucrose	Over	2, 4, 8	0.48
CNH	Leaves			4.5	Maltose	Over	2, 4, 8	0.37
APK	Leaves	2	1		Sucrose	Over	2, 4	0.78

BD	Leaves	2	1		Sucrose	Over	2, 4	0.75
CNH	Leaves	2	1		Sucrose	Over	2, 4	0.63
OB	Leaves	2	1		Sucrose	Over	2, 4	0.50
BD	Leaves			4.5	Maltose	Over	2, 4	0.35

On maltose media, all older calli than 100 days initiated with TDZ/NAA and also 3 out of 4 calli initiated with 2,4-D were endopolyploid. Initiated with sucrose media, only one callus older than 100 days out of five showed endopolyploidy. (Table 24, Table 25)

Table 24: Over 100 days old calli on maltose media flow cytometry results sorted by descending C-value.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
BD	Leaves	2	1		Maltose	Over	2, 4, 8, 16, 32	**1.82**
OB	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.41**
CNH	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.30**
W	Leaves	2	1		Maltose	Over	2, 4, 8, 16	**1.00**
W	Leaves			4.5	Maltose	Over	4, 8	**1.58**
APK	Ovules	2	1		Maltose	Over	2, 4, 8	0.80
OB	Leaves			4.5	Maltose	Over	2, 4, 8	0.61
CNH	Leaves			4.5	Maltose	Over	2, 4, 8	0.37
BD	Leaves			4.5	Maltose	Over	2, 4	0.35

In the younger callus cultures only 2 out of 12 tested calli (17 %) were diploid. (Table 25)

Table 25: Below 100 days old calli flow cytometry results sorted by descending C-value.

Chemo-type	Source	Growth regulators (µM)			Carbon source	100 Days	C-value	EI
		TDZ	NAA	2,4-D				
CNH	Leaves	2	1		Maltose	Below	2, 4, 8, 16	**2.00**
BD	Leaves	2	1		Maltose	Below	2, 4, 8, 16	0.92
APK	Leaves			4.5	Maltose	Below	2, 4, 16	0.81
BD	Ovules	2	1		Maltose	Below	2, 4, 8	**1.14**
CNH	Ovules	2	1		Maltose	Below	2, 4, 8	0.66
APK	Leaves	2	1		Maltose	Below	2, 4, 8	0.57
W	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
OB	Leaves	2	1		Maltose	Below	2, 4, 8	0.54
BD	Leaves			4.5	Maltose	Below	2, 4, 8	0.52
CNH	Leaves			4.5	Maltose	Below	2, 4, 8	0.51
W	Ovules	2	1		Maltose	Below	2, 4	0.73
W	Leaves			4.5	Maltose	Below	2, 4	0.58

Regarding growth regulators, 75 % of calli initiated from leaves over maltose on 2,4-D medium were endopolyploid, but only one callus had over 8C. Lower EI-values were found with this growth regulator than with TDZ/NAA. (Table 26)

Table 26: 2,4-D initiated calli flow cytometry results sorted by descending C-value.

Chemo-type	Source	Growth regulators (µM)	Carbon source	100 Days	C-value	EI
		2,4-D				
APK	Leaves	4.5	Maltose	Below	2, 4, 16	0.81
W	Leaves	4.5	Maltose	Over	4, 8	**1.58**
OB	Leaves	4.5	Maltose	Over	2, 4, 8	0.61
BD	Leaves	4.5	Maltose	Below	2, 4, 8	0.52
CNH	Leaves	4.5	Maltose	Below	2, 4, 8	0.51
CNH	Leaves	4.5	Maltose	Over	2, 4, 8	0.37
W	Leaves	4.5	Maltose	Below	2, 4	0.58
BD	Leaves	4.5	Maltose	Over	2, 4	0.35

Leaf explant calli initiated by TDZ/NAA on maltose media showed the highest C-values and EI-values. (Table 27)

Table 27: TDZ/NAA initiated, maltose media leaf explant calli flow cytometry results sorted by descending C-value.

Chemo-type	Source	Growth regulators (µM)		Carbon source	100 Days	C-value	EI
		TDZ	NAA				
BD	Leaves	2	1	Maltose	Over	2, 4, 8, 16, 32	**1.82**
CNH	Leaves	2	1	Maltose	Below	2, 4, 8, 16	**2.00**
OB	Leaves	2	1	Maltose	Over	2, 4, 8, 16	**1.41**
CNH	Leaves	2	1	Maltose	Over	2, 4, 8, 16	**1.30**
W	Leaves	2	1	Maltose	Over	2, 4, 8, 16	**1.00**
BD	Leaves	2	1	Maltose	Below	2, 4, 8, 16	0.92
APK	Leaves	2	1	Maltose	Below	2, 4, 8	0.57
W	Leaves	2	1	Maltose	Below	2, 4, 8	0.54
OB	Leaves	2	1	Maltose	Below	2, 4, 8	0.54

Of all the factors, carbon source and growth regulators had the greatest influence on the endopolyploidy level and EI-value. 86 % of calli initiated with maltose media were endo-polyploid. Especially leaf derived calli initiated with TDZ/NAA on maltose media showed a high rate of endopolyploidy (100 %).

6.3.2 Genome Size in Cannabis

The relative DNA content of the analyzed samples was calculated using *Solanum pseudocapsicum* as internal standard (Figure 28).

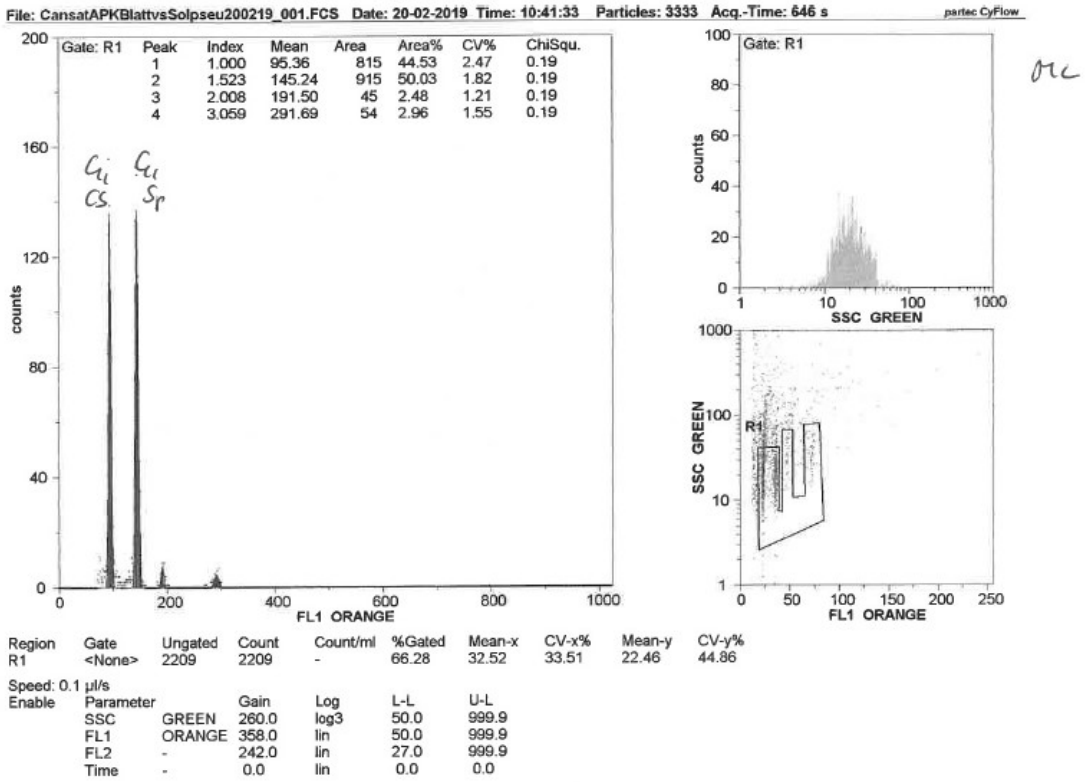

Figure 28: Flow cytometry histogram of the relative DNA content of mother plant (APK) leaf sample vs. standard *Solanum pseudocapsicum.*

The 1C-values of the analyzed samples ranged from 0.8227 – 0.8946 pg DNA, when the CV% values of the peaks in the histogram ranged from 0.0471 – 1.2609. A CV% of less than 3 % is desirable, which means a good quality of the sample. (Table 28)

Table 28: Results of flow cytometry measurements of the different samples including mean 1C-values, SD and CV% 1C-values.

Chemotype	Source	Growth regulators (µM)			Carbon source	100 Days	Mean 1C (pg)	SD	CV% 1C-values
		TDZ	NAA	2,4-D					
APK	Motherplant						0.8524	0.0023	0.2747
	Leaves	2	1		Sucrose	Over	0.8375	0.0036	0.4328
	Leaves	2	1		Maltose	Below	0.8357	0.0016	0.1902
	Leaves			4.5	Maltose	Below	0.8750	0.0110	1.2609
	Ovules	2	1		Maltose	Over	0.8664	-	-
BD	Motherplant						0.8464	0.0014	0.1620
	Leaves	2	1		Sucrose	Over	0.8408	0.0013	0.1529
	Leaves	2	1		Maltose	Over	0.8379	-	-
	Leaves	2	1		Maltose	Below	0.8446	0.0033	0.3924
	Leaves			4.5	Maltose	Below	0.8901	0.0081	0.9077
	Leaves			4.5	Maltose	Over	0.8724	-	-
	Ovules	2	1		Maltose	Below	0.8946	0.0029	0.3284
CNH	Motherplant						0.8697	0.0004	0.0471
	Leaves	2	1		Sucrose	Over	0.8635	0.0066	0.7629
	Leaves	2	1		Maltose	Over	0.8583	-	-
	Leaves	2	1		Maltose	Below	0.8411	0.0067	0.7913
	Leaves			4.5	Maltose	Below	0.8758	-	-
	Leaves			4.5	Maltose	Over	0.8563	-	-
	Ovules	2	1		Maltose	Below	0.8679	0.0059	0.6838
W	Motherplant						0.8604	0.0033	0.3837
	Leaves	2	1		Sucrose	Over	0.8467	0.0009	0.1098
	Leaves	2	1		Maltose	Over	0.8772	-	-

	Leaves	2	1		Maltose	Below	0.8509	0.0013	0.1567
	Leaves			4.5	Maltose	Below	0.8462	0.0018	0.2115
	Leaves			4.5	Maltose	Over	0.8476	-	-
	Ovules	2	1		Maltose	Below	0.8553	0.0072	0.8399
OB	Motherplant						0.8426	0.0027	0.3176
	Leaves	2	1		Sucrose	Over	0.8710	0.0055	0.6361
	Leaves	2	1		Maltose	Over	0.8227	0.0016	0.1951
	Leaves	2	1		Maltose	Below	0.8474	0.0021	0.2426
	Leaves			4.5	Maltose	Over	0.8535	-	-

7 Discussion

Recently, considerable effort in cannabis *in vitro* cultivation techniques has been made since the medicinal use of cannabis plants is rising and further cannabis research is dependent on reliable *in vitro* protocols for callus initiation, plant regeneration and callus/plant propagation. In this study, already published and newly designed media were used for callus initiation and regeneration.

Callus initiation as first step appears to work well: callus could be easily obtained in our study as well as by other authors reported (Mackinnon et al. 2001; Feeney and Punja 2003; Ślusarkiewicz-Jarzina et al. 2005; Pacifico et al. 2008; Wielgus et al. 2008; Lata et al. 2010; Sirkowski 2012; Movahedi and Torabi 2015).

Best results in callus initiation for leaf and ovule explants from all five tested chemotypes (APK, CNH, BD, OB, W) showed the medium with the growth regulators TDZ/NAA, which (Chaohua et al. 2016) have used successfully for plant regeneration from cannabis cotyledons. In our study, we used this medium for callus initiation and it worked equally well for all tested chemotypes with 100 % induction rate in light and dark culture environments.

Medium with 2,4-D suggested for drug-type cannabis callus induction from leaves by (Flores-Sanchez et al. 2009), showed good results for leaf explants but a very low to none callus induction rate for ovule explants. Therefore, this medium seems to be rather chemotype independent, but may have different effects on the explants. Different callus initiation results depending on the cannabis explants were also reported by (Movahedi and Torabi 2015).

Also the 27 different media (growth regulators Kin, BAP and IAA in various combinations and concentrations) originally used for callus initiation in tobacco (Kodym et al. 2017) worked for callus initiation in cannabis leaf explants, although with a rather high chemotype dependency. Indeed, the callus induction rate of both chemotypes (BD and CNH) was highest with media containing 10 µM IAA and lowest with media without auxin.

Most calli also partly had large, watery cells, especially ovule derived calli. According to (Betekhtin et al. 2017), these cells function as nurse tissue in morphogenic callus. They do not divide, but are metabolically active and support the growth of pro-embryogenic masses by providing sugars, proteins and other conditioning factors. Thus, calli with watery cells have morphogenetic potential and are suitable for plant regeneration assays.

Some of the plant regeneration protocols found in literature promised a good chance to successfully regenerate plantlets, especially one publication has to be mentioned: (Lata et al. 2010). According to the authors, plant regeneration was very successful, they report even plant regeneration rates up to 96.6 %. Since cannabis is very heterogeneous, published protocols work for some chemotypes but are not universally applicable. Unfortunately, our calli did not respond to any of the treatments reported by these authors. When trying to repeat also other published protocols for indirect organogenesis of cannabis usually unsatisfactory results in terms of regeneration are obtained.

Thus, alternative approaches were examined which included using: salts, in form of fertilizers, especially formulated for cannabis (Kodym and Leeb 2019) and maltose instead of sucrose, since (Chutipaijit and Sutjaritvorakul 2018a) recently showed enhanced callus induction and plant regeneration for aromatic rice by using maltose as carbon source. The same authors also showed better results in callus induction and plant regeneration in aromatic rice when activated charcoal was added to the media. Activated charcoal adsorbs, amongst other substances, growth regulators and implies the regeneration on media without growth regulators. This is what (Galán-Ávila et al. 2020) recently showed in their direct *in vitro* regeneration studies with cannabis plants: hypocotyls cultured in medium without growth regulators showed an excellent response (61.54 %) and even spontaneous rooting of the regenerants, which were acclimatized just 6 weeks after culture initiation. Our indirect regeneration studies included media with activated charcoal, media without any growth regulators and some calli even went through charcoal treatment before they were placed on various other media. Nevertheless, no callus on any of the 38 tested media showed organogenesis.

Since callus cultures are known for a high occurrence of somaclonal variation, we assumed this could be the reason for our regeneration problems. As a marker of somac-

lonal variation, we assessed the endopolyploidy level of our calli with flow cytometry analysis. As hypothesized, we found endopolyploid calli, and the endopolyploidy levels were high. Endopolyploidy is common in plant cells that undergo specialized differentiation like trichomes on roots, leaves, stems and anthers. For example, in cotton fiber cells can endoreduplicate up to 32 – 64C. Endoreduplication also occurs in metabolically highly active cells like in those with secretory or nutritive functions, where the extreme case of 2,4567C (13 endocycles) in *Arum maculatum* has been measured. In terms of cannabis, endopolyploidy in roots has been known for a long time (Litadière 1925) and recently (Galán-Ávila et al. 2020) described endopolyploidy in cotyledons and hypocotyls of cannabis plantlets. According to our results and (Galán-Ávila et al. 2020), leaves of cannabis plants seem to preserve the diploid pattern.

However, endopolyploidy seems to not only play a role in forming specialized cells and tissues, it might also enable plants to cope with various stresses they are exposed to. For example, the formation of large endopolyploid trichomes on leaves can protect plants from drought (by reflecting light), herbivores (irritable trichomes) or frost (by protecting underlying cells). Since plants in tissue culture are exposed to diverse stress factors, such as wounding and sterilization in explant preparation, growth regulators and salts in media or the *in vitro* culture environment like temperature or light, endopolyploidy can be the protective response of the cells. This general stress response might be a reason for the high occurrence of somaclonal variation in plant *in vitro* cultivation. (Dodsworth et al. 2017)

In our studies, we tested sucrose and maltose as carbon source and detected its significant influence on the C-value and EI. When maltose was used, nuclei with 8C, 16C and 32C were found in the callus cultures. On the other hand, on sucrose media only one callus culture showed 8C nuclei. Considering only TDZ/NAA induced calli on maltose media, all calli except for one were endopolyploid, thus in this composition triggering factors seem to add up. To our knowledge, in literature rather positive effects of maltose on *in vitro* plant cultivation can be found. As already mentioned (Chutipaijit and Sutjaritvorakul 2018b) the positive effect of maltose on callus induction and plant regeneration in aromatic rice, with no signs of somaclonal variation issues like regeneration problems or unhealthy regenerated plantlets was recently reported. Also (Smýkalová et

al. 2001) used maltose media for a successful micropropagation of hops. However, in our study maltose shows a high potential for inducing somaclonal variation in form of endopolyploidy in cannabis.

The growth regulators TDZ/NAA and 2,4-D also had different influence on the endoploidy level: endopolyploid calli initiated with 2,4-D had C-values of 2C, 4C, 8C and only 1 callus over 8C (in only 1 run), whereas the endopolyploid calli initiated with TDZ/NAA had very high C-values with many 16C and even up to 32C. The somaclonal variation-inducing potential of the growth regulators TDZ and 2,4-D has already been reported several times (Sales and Butardo 2014; Ghosh et al. 2017).

Contrary to the many in literature reported findings about the age of callus cultures representing a triggering factor for somaclonal variation (Bairu et al. 2011; Mamedes-Rodrigues et al. 2018; Samarina et al. 2019), we could not directly verify this with our cannabis studies. Since calli from cultures under 100 days old were mostly endopolyploid, only 17 % were diploid (Table 25) and even the callus with the highest EI (2.0, Table 17) was under 100 days old, culture age does not seem to play a major role in somaclonal variation in cannabis *in vitro* cultivation. Studies with pea (Smýkal et al. 2007) and fennel plants (Bennici et al. 2004) in long term *in vitro* cultivation showed the absence of somaclonal variation after even 17 months and 24 years respectively. According to these aspects, long term *in vitro* cultivation not necessarily triggers somaclonal variation.

Contrary to reported studies with *Dieffenbachia* plants (Shen et al. 2007) and potato plants (Tican et al. 2008), our flow cytometry results showed barely differences in C-values and EI generated by the cannabis chemotype.

Also the explant type showed low differences in the C-values and EI. In our studies, leaf and ovule explant derived calli, i.e. calli initiated from highly differentiated plant tissue were analyzed. Hence, the chance for somaclonal variation in these cultures was higher than in cultures originating from undifferentiated starting material such as pericycle, procambium and cambium (Bairu et al. 2011). Recently, (Galán-Ávila et al. 2020) reported the high regenerative capacity of cannabis hypocotyls explants, potentially originating from the xylem cells. Therefore, further research concerning hypocotyl induced

callus cultures and their regenerative potential would be desired in the next step of investigation.

In conclusion, the bottleneck of indirect plant regeneration of cannabis plants seems to be the plant regeneration, since in our study we could achieve callus initiation rates of even 100 % with TDZ/NAA and also various successful callus initiation protocols have already been published. However, reliable protocols for indirect plant regeneration in cannabis plants are still missing, although recently progress in direct plant regeneration could be achieved. With the new findings, in the next step indirect regeneration studies with hypocotyl derived callus on media without any growth regulators should be analyzed. In terms of somaclonal variation of cannabis callus cultures, we could show that maltose as carbon source seems to play an important role in the occurrence of somaclonal variation. Especially the combination of maltose media with TDZ/NAA showed very high C-values up to 32C and an endopolyploidy rate of 100 % in leaf derived calli.

8 References

Andre CM, Hausman JF, Guerriero G (2016) Cannabis sativa: The plant of the thousand and one molecules. Front Plant Sci. https://doi.org/10.3389/fpls.2016.00019

Bairu MW, Aremu AO, van Staden J (2011) Somaclonal variation in plants: Causes and detection methods. Plant Growth Regul 63:147–173. https://doi.org/10.1007/s10725-010-9554-x

Baron EP (2018) Medicinal Properties of Cannabinoids, Terpenes, and Flavonoids in Cannabis, and Benefits in Migraine, Headache, and Pain: An Update on Current Evidence and Cannabis Science. Headache 58:1139–1186

Barow M, Meister A (2003) Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. Plant, Cell Environ 26:571–584. https://doi.org/10.1046/j.1365-3040.2003.00988.x

Batista D, Sousa MJ, Pais MS (1996) Plant regeneration from stem and petiole-derived callus of Humulus lupulus L. (hop) clone Bragança and var. Brewer's Gold. Vitr - Plant 32:37–41. https://doi.org/10.1007/BF02823011

Bennici A, Anzidei M, Vendramin GG (2004) Genetic stability and uniformity of Foeniculum vulgare Mill. regenerated plants through organogenesis and somatic embryogenesis. Plant Sci 166:221–227. https://doi.org/10.1016/j.plantsci.2003.09.010

Betekhtin A, Rojek M, Jaskowiak J, et al (2017) Nuclear genome stability in long-term cultivated callus lines of Fagopyrum tataricum (L.) Gaertn. PLoS One 12:e0173537. https://doi.org/10.1371/journal.pone.0173537

Bie B, Wu J, Foss JF, Naguib M (2018) An overview of the cannabinoid type 2 receptor system and its therapeutic potential. Curr. Opin. Anaesthesiol. 31:407–414

Blaschek W (2016) Ein Handbuch für die Praxis Wichtl-Teedrogen und Phytopharmaka

Chandra S, Lata H, Mehmedic Z, et al (2010) Assessment of cannabinoids content in micropropagated plants of cannabis sativa and their comparison with conventionally propagated plants and mother plant during developmental stages of growth. Planta Med 76:743–750. https://doi.org/10.1055/s-0029-1240628

Chaohua C, Gonggu Z, Lining Z, et al (2016) A rapid shoot regeneration protocol from the

cotyledons of hemp (Cannabis sativa L.). Ind Crops Prod 83:61–65. https://doi.org/10.1016/j.indcrop.2015.12.035

Child K (2018) The highest court has spoken: You are allowed to smoke - and grow - dagga at home. Timeslive

Chutipaijit S, Sutjaritvorakul T (2018a) Improvement of Plant Regeneration Frequency from Carbon Sources in Aromatic Rice (Oryza sativa L.). Iran J Sci Technol Trans A Sci 42:1131–1137. https://doi.org/10.1007/s40995-017-0169-1

Chutipaijit S, Sutjaritvorakul T (2018b) Application of activated charcoal and nanocarbon to callus induction and plant regeneration in aromatic rice (Oryza sativa L.). Chem Speciat Bioavailab 30:1–8. https://doi.org/10.1080/09542299.2017.1418184

Collin C, Ehler E, Waberzinek G, et al (2010) A double-blind, randomized, placebo-controlled, parallel-group study of Sativex, in subjects with symptoms of spasticity due to multiple sclerosis. Neurol Res 32:451–459. https://doi.org/10.1179/016164109X12590518685660

Colom J, Gual A (2018) Psychoactive constituents of cannabis and their clinical implications: a systematic review Constituyentes psicoactivos del cannabis y sus implicaciones clínicas: una revisión sistemática. 30:140–151

Contributors W (2020) Legality of cannabis. In: Wikipedia, Free Encycl. https://en.wikipedia.org/w/index.php?title=Legality_of_cannabis&oldid=978101031. Accessed 22 Jun 2020

Corey-Bloom J, Wolfson T, Gamst A, et al (2012) Smoked cannabis for spasticity in multiple sclerosis: A randomized, placebo-controlled trial. CMAJ 184:1143–1150. https://doi.org/10.1503/cmaj.110837

de Meijer E (2014) The Chemical Phenotypes (Chemotypes) of Cannabis. Handb Cannabis

Dodsworth S, Leitch IJ, Breuer C, Sugimoto-Shirasu K (2017) Endopolyploidy in Plants Endopolyploidy in Plants The Occurrence of Endopolyploidy in Plants • The Phylogenetic Distribution of Endopolyploidy across Plants • During the Endoreduplication Cycle, Cells Replicate their Nuclear DNA without Cell Division • A Role for Endopolyploidy in a Plant's Response to Stress. https://doi.org/10.1002/9780470015902.a0020097.pub2

Doležel J, Bartoš J (2005) Plant DNA flow cytometry and estimation of nuclear genome

size. In: Annals of Botany. Oxford Academic, pp 99–110

Dolezel J, Bartoš J, Voglmayr H, Greilhuber J (2003) Nuclear DNA content and genome size of trout and human. Cytom Part A 51 51A:127–128. https://doi.org/10.1002/cyto.a.10013

Eisenberg E, Ogintz M, Almog S (2014) The pharmacokinetics, efficacy, safety, and ease of use of a novel portable metered-dose cannabis inhaler in patients with chronic neuropathic pain: A phase 1a study. J Pain Palliat Care Pharmacother 28:216–225. https://doi.org/10.3109/15360288.2014.941130

Elsohly MA, Radwan MM, Gul W, et al (2017) Phytochemistry of Cannabis sativa L .

EMCDDA (2019) Developments in the European cannabis market. 1–19

Feeney M, Punja Z (2017) The role of agrobacterium-mediated and other gene-transfer technologies in cannabis research and product development. In: Cannabis sativa L. - Botany and Biotechnology. Springer International Publishing, pp 343–363

Feeney M, Punja ZK (2003) Tissue culture and Agrobacterium-mediated transformation of hemp (Cannabis sativa L.). Vitr Cell Dev Biol - Plant 39:578–585. https://doi.org/10.1079/IVP2003454

Feeney M, Punja ZK (2015) Hemp (Cannabis Sativa L.). Methods Mol Biol 1224:319–329. https://doi.org/10.1007/978-1-4939-1658-0_25

Flores-Sanchez IJ, Peč J, Fei J, et al (2009) In Vitro cell culture of cannabis sativa for the production of cannabinoids. J Biotechnol 143:157–168. https://doi.org/10.1016/j.jbiotec.2009.05.006

Galán-Ávila A, García-Fortea E, Prohens J, Herraiz FJ (2020) Development of a Direct in vitro Plant Regeneration Protocol From Cannabis sativa L. Seedling Explants: Developmental Morphology of Shoot Regeneration and Ploidy Level of Regenerated Plants. Front Plant Sci 11:645. https://doi.org/10.3389/fpls.2020.00645

Galbraith DW, Harkins KR, Maddox JM, et al (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science (80-) 220:1049–1051. https://doi.org/10.1126/science.220.4601.1049

Ghosh A, Igamberdiev AU, Debnath SC (2017) Detection of DNA methylation pattern in thidiazuron-induced blueberry callus using methylation-sensitive amplification polymorphism. Biol Plant 61:511–519. https://doi.org/10.1007/s10535-016-0678-3

Government of Canada (2020) What you need to know about cannabis.

https://www.canada.ca/en/services/health/campaigns/cannabis/canadians.html. Accessed 22 Jun 2020

Grotenhermen F, Müller-Vahl K (2016) Medicinal Uses of Marijuana and Cannabinoids. CRC Crit Rev Plant Sci 35:378–405. https://doi.org/10.1080/07352689.2016.1265360

Hillig KW (2004) A chemotaxonomic analysis of terpenoid variation in Cannabis. Biochem Syst Ecol 32:875–891. https://doi.org/10.1016/j.bse.2004.04.004

Johnson JR, Burnell-Nugent M, Lossignol D, et al (2010) Multicenter, Double-Blind, Randomized, Placebo-Controlled, Parallel-Group Study of the Efficacy, Safety, and Tolerability of THC:CBD Extract and THC Extract in Patients with Intractable Cancer-Related Pain. J Pain Symptom Manage 39:167–179. https://doi.org/10.1016/j.jpainsymman.2009.06.008

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43:179–188. https://doi.org/10.1007/978-94-011-4183-3_4

Khosla C, Keasling JD (2003) Metabolic engineering for drug discovery and development. Nat Rev Drug Discov 2:1019–1025. https://doi.org/10.1038/nrd1256

Kodym A, Leeb CJ (2019) Back to the roots: protocol for the photoautotrophic micropropagation of medicinal Cannabis. Plant Cell Tissue Organ Cult 138:399–402. https://doi.org/10.1007/s11240-019-01635-1

Kodym A, Wawrosch C, Gössnitzer F (2017) In vitro-Kultivierung von Arzneipflanzen (Einführung in die Arbeitstechniken der pflanzlichen Gewebekultur)

Kojoma M, Seki H, Yoshida S, Muranaka T (2006) DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in “drug-type” and “fiber-type” Cannabis sativa L. Forensic Sci Int 159:132–140. https://doi.org/10.1016/j.forsciint.2005.07.005

Krishna H, Alizadeh M, Singh D, et al (2016) Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech 6:1–18

Lampl C, Donnerer J, Herbert M, et al (2017) Positionspapier der Österreichischen Schmerzgesellschaft zum klinischen Einsatz von Cannabinoiden in der Schmerzmedizin. Schmerz Nachrichten 2:

Langford RM, Mares J, Novotna A, et al (2013) A double-blind, randomized, placebo-controlled, parallel-group study of THC/CBD oromucosal spray in combination with

the existing treatment regimen, in the relief of central neuropathic pain in patients with multiple sclerosis. J Neurol 260:984–997. https://doi.org/10.1007/s00415-012-6739-4

Lata H, Chandra S, Khan I, Elsohly M (2016a) In vitro propagation of Cannabis sativa L. And evaluation of regenerated plants for genetic fidelity and cannabinoids content for quality assurance. In: Methods in Molecular Biology. pp 275–288

Lata H, Chandra S, Khan I, ElSohly MA (2009) Thidiazuron-induced high-frequency direct shoot organogenesis of Cannabis sativa L. Vitr Cell Dev Biol - Plant 45:12–19. https://doi.org/10.1007/s11627-008-9167-5

Lata H, Chandra S, Khan IA, Elsohly MA (2010) High frequency plant regeneration from leaf derived callus of high Δ9-tetrahydrocannabinol yielding cannabis sativa L. Planta Med 76:1629–1633. https://doi.org/10.1055/s-0030-1249773

Lata H, Chandra S, Techen N, et al (2016b) 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. J Appl Res Med Aromat Plants 3:18–26. https://doi.org/10.1016/j.jarmap.2015.12.001

Leeb CJ (2018) Cannabis sativa L.: In vitro Micropropaggation

Litadière RD (1925) Sur l'existence de figures didiploides dans le meristeme radiculaire du Cannabis sativa L. Cellule 35:24–25

Lowrey T (2019) ACT legalises personal cannabis use, becoming first Australian jurisdiction to do so. ABC News

Mackinnon L, Mcdougall G, Aziz N, Millam S (2001) Progress towards transformation of fibre hemp. Annu Rep Scottish Crop Res Inst 2000/2001 84–86

Mahmood A (2018) Erlass. Bundesministerium für Arbeit, Soziales, Gesundh und Konsum

Malena C, Felipe L (2013) Uruguay becomes first country to legalize marijuana trade. In: Reuters. https://www.reuters.com/article/us-uruguay-marijuana-vote/uruguay-becomes-first-country-to-legalize-marijuana-trade-idUSBRE9BA01520131211. Accessed 22 Jun 2020

Mamedes-Rodrigues TC, Batista DS, Vieira NM, et al (2018) Regenerative potential, metabolic profile, and genetic stability of Brachypodium distachyon embryogenic calli as affected by successive subcultures. Protoplasma 255:655–667.

https://doi.org/10.1007/s00709-017-1177-x

Marum L (2011) An epigenetic view of plant cells cultured in vitro : somaclonal variation and beyond. J Exp Bot 62:3713–3725. https://doi.org/10.1093/jxb/err155

McPartland JM, Guy GW (2017) Models of Cannabis Taxonomy, Cultural Bias, and Conflicts between Scientific and Vernacular Names. Bot Rev 83:327–381. https://doi.org/10.1007/s12229-017-9187-0

Movahedi, Ghasemiomran, Torabi (2016) Effect of explants type and plant growth regulators on in vitro callus induction and shoot. Iran J Med Aromat Plants 32:83–96

Movahedi, Torabi (2015) The effect of different concentrations of TDZ and BA on in vitro regeneration of Iranian cannabis (Cannabis sativa) using cotyledon and epicotyl explants. J Plant Mol Breed 3:20–27

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant 15:473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nuutinen T (2018) Medicinal properties of terpenes found in Cannabis sativa and Humulus lupulus. Eur. J. Med. Chem. 157:198–228

Otto FJ, Oldiges H, Göhde W, Jain VK (1981) Flow cytometric measurement of nuclear DNA content variations as a potential in vivo mutagenicity test. Cytometry 2:189–191. https://doi.org/10.1002/cyto.990020311

Pacifico D, Miselli F, Carboni A, et al (2008) Time course of cannabinoid accumulation and chemotype development during the growth of Cannabis sativa L. Euphytica 160:231–240. https://doi.org/10.1007/s10681-007-9543-y

Paszkowski J (2015) Epigenetics: The karma of oil palms. Nature 525:466–467

Pertwee R (ed) (2014) Handbook of Cannabis. Oxford University Press

Sakamoto K, Akiyama Y, Fukui K, et al (1998) Characterization; Genome sizes and morphology of sex chromosomes in hemp (Cannabis sativa L.). Cytologia (Tokyo) 63:459–464. https://doi.org/10.1508/cytologia.63.459

Sales EK, Butardo NG (2014) Molecular Analysis of Somaclonal Variation in Tissue Culture Derived Bananas Using MSAP and SSR Markers. Int J Biol Biomol Agric Food Biotechnol Eng 8:603–610

Samarina L, Gvasaliya M, Koninskaya N, et al (2019) A comparison of genetic stability in

tea [Camellia sinensis (L.) Kuntze] plantlets derived from callus with plantlets from long-term in vitro propagation. Plant Cell Tissue Organ Cult 138:467–474. https://doi.org/10.1007/s11240-019-01642-2

Schachtsiek J, Warzecha H, Kayser O, Stehle F (2018) Current Perspectives on Biotechnological Cannabinoid Production in Plants. Planta Med. 84:214–220

Schnattinger H (2020) https://www.gesundheitskasse.at/cdscontent/?contentid=10007.848815&portal=oegksportal

Shen X, Chen J, Kane ME, Henny RJ (2007) Assessment of somaclonal variation in Dieffenbachia plants regenerated through indirect shoot organogenesis. Plant Cell Tissue Organ Cult 91:21–27. https://doi.org/10.1007/s11240-007-9273-9

Sirikantaramas S, Taura F, Tanaka Y, et al (2005) Tetrahydrocannabinolic Acid Synthase, the Enzyme Controlling Marijuana Psychoactivity, is Secreted into the Storage Cavity of the Glandular Trichomes. Plant Cell Physiol 46:1578–1582. https://doi.org/10.1093/pcp/pci166

Sirkowski EE (2012) Marked Cannabs for Indicating Medical Marijuana. 1:

Ślusarkiewicz-Jarzina A, Ponitka A, Kaczmarek Z (2005) Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of Cannabis sativa L. Acta Biol Cracoviensia Ser Bot 47:145–151

Small E (2008) Morphological variation of achenes of Cannabis. Can J Bot 53:978–987. https://doi.org/10.1139/b75-117

Small E, Cronquist A (1976) A PRACTICAL AND NATURAL TAXONOMY FOR CANNABIS. Taxon 25:405–435. https://doi.org/10.2307/1220524

Smýkal P, Valledor L, Rodríguez R, Griga M (2007) Assessment of genetic and epigenetic stability in long-term in vitro shoot culture of pea (Pisum sativum L.). Plant Cell Rep 26:1985–1998. https://doi.org/10.1007/s00299-007-0413-9

Smýkalová I, Ortová M, Lipavská H, Patzak J (2001) Efficient in vitro micropropagation and regeneration of Humulus lupulus on low sugar, starch-Gelrite media. Biol Plant 44:7–12. https://doi.org/10.1023/A:1017901817063

Smýkalová I, Vrbová M, Cvečková M, et al (2019) The effects of novel synthetic cytokinin derivatives and endogenous cytokinins on the in vitro growth responses of hemp (Cannabis sativa L.) explants. Plant Cell Tissue Organ Cult 139:381–394.

https://doi.org/10.1007/s11240-019-01693-5

Statista (2019) Europische Länder mit der größten Anbaufläche von Hanf im Jahr 2018

Statistik Austria (2019) Anbau auf dem Ackerland 2012

Temsch EM, Temsch W, Ehrendorfer-Schratt L, Greilhuber J (2010) Heavy Metal Pollution, Selection, and Genome Size: The Species of the Žerjav Study Revisited with Flow Cytometry. J Bot 2010:1–11. https://doi.org/10.1155/2010/596542

Tican A, Chiru N, Ivanovici D (2008) Vasile Goldiș

van Bakel H, Stout JM, Cote AG, et al (2011) The draft genome and transcriptome of Cannabis sativa. Genome Biol 12:R102. https://doi.org/10.1186/gb-2011-12-10-r102

Wahby I, Caba JM, Ligero F (2013) *Agrobacterium* infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures. J Plant Interact 8:312–320. https://doi.org/10.1080/17429145.2012.746399

Wahby I, Caba JM, Ligero F (2017) Hairy root culture as a biotechnological tool in C. sativa. In: Cannabis sativa L. - Botany and Biotechnology. Springer International Publishing, pp 299–317

Wallace MS, Marcotte TD, Umlauf A, et al (2015) Efficacy of Inhaled Cannabis on Painful Diabetic Neuropathy. J Pain 16:616–627. https://doi.org/10.1016/j.jpain.2015.03.008

Ware MA, Wang T, Shapiro S, Collet JP (2015) Cannabis for the Management of Pain: Assessment of Safety Study (COMPASS). J Pain 16:1233–1242. https://doi.org/10.1016/j.jpain.2015.07.014

Wayne S (2018) Smoking Marijuana Legalized in Georgia. In: Georg. Today. http://georgiatoday.ge/news/11592/Smoking-Marijuana-Legalized-in-Georgia. Accessed 22 Jun 2020

Wielgus K, Luwanska A, Lassocinski W, Kaczmarek Z (2008) Estimation of cannabis sativa L. tissue culture conditions essential for callus induction and plant regeneration. J Nat Fibers 5:199–207. https://doi.org/10.1080/15440470801976045

Wilsey B, Marcotte T, Deutsch R, et al (2013) Low-dose vaporized cannabis significantly improves neuropathic pain. J Pain 14:136–148. https://doi.org/10.1016/j.jpain.2012.10.009

Wróbel T, Dreger M, Wielgus K, Słomski R (2018) The application of plant in vitro

cultures in cannabinoid production. Biotechnol Lett 40:445–454. https://doi.org/10.1007/s10529-017-2492-1

9 Appendix

Abbreviations

APK	Chemotype Austrian Power Kush
BAP	6-Benzylaminopurine
BD	Chemotype Black Domina
BM	Basal medium
CBD	Cannabidiol
CNH	Chemotype Cannalope
CRISPR	Clustered regularly interspaced short palindromic repeats
D	Dark culture conditions
2,4-D	2,4-Dichlorophenoxyacetic acid
EI	Endopolyploidy index
GA3	Gibberellic acid
GABA	gamma-Aminobutyric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
L	Light culture conditions
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
NMDA	N-Methyl-d-aspartic acid
OB	Chemotype Orange bud
TDZ	Thidiazuron
THC	(−)-trans-Δ^9-tetrahydrocannabinol
W	Chemotype Wappa
Z	Zeatin

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