The bioeconomy potential of hemp (Cannabis sativa L.): challenges of new genotypes and cultivation systems to meet the rising demand for phytocannabinoids

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List of abbreviations and acronyms

%	Percent
°C	Degree centigrade
Δ^9-THC	Δ^9-tetrahydrocannabinol
Δ^9-THCA	Δ^9-tetrahydrocannabinolic acid
Δ^9-THCB	Δ^9-tetrahydrocannabutol
Δ^9-THCH	Δ^9-tetrahydrocannabihexol
Δ^9-THCP	Δ^9-tetrahydrocannabiphorol
BAP	6-benzylaminopurine
BfArM	Bundesinstitut für Arzneimittel und Medizinprodukte
e.g.	exempli gratia (for example)
etc.	et cetera
EU	European Union
CO_2	Carbon dioxide
CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDB	Cannabidibutol
CBDH	Cannabidihexol
CBDP	Cannabidiphorol
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBN	Cannabinol
CC	Coco coir fibre
CK	Cytokinin
DOXP/MEP	Deoxyxylulose phosphate/methylerythritol phosphate
DAB	Deutsches Arzneibuch

DW	Dry weight
EIHA	European Industrial Hemp Association
G30	Peat-mix substituted with 30% of green fibres
GACP	Good Agricultural and Collection Practices
GMP	Good Manufacturing Practices
GOT	Geranyl diphosphate:olivetolate geranyltransferase
GPP	Geranyl diphosphate
h	hours
HPLC	High Pressure Liquid Chromatography
IAA	Auxin
INCB	International Narcotics Control Board
N	Nitrogen
NAA	1-naphthalenaecetic acid
NAA/BAP-mix	1-naphthalenaecetic acid/6-benzylaminopurine solution
OLA	Olivetolic acid
PCR	Phytocannabinoid-rich cannabis
PGR	Plant growth regulators
PKS	Polyketide synthase
PM	Peat-mix
RLD	Root length density
US	United States

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1 Introduction

Recent environmental and health challenges worldwide set the need to act and set ambitious objectives for the transition to a more sustainable society. To address these challenges, in December 2019, the European Union (EU) has set ambitious targets to be climate-neutral by 2050 in order to make Europe the first continent reaching an economy with net-zero greenhouse gas emission (European Commission, 2019). This objective is the central element of the European Green Deal and is in line with the European commitment to global climate action under the Paris Agreement. The implementation of this strategy includes sector-specific action plans, which support the necessary transformation in the areas of transport, agriculture, buildings, research and innovation; and the protection and restoration of biodiversity, air pollution control, the circular economy and raw materials supply. The policy objective in the agricultural sector to improve the sustainability of the agri-food sector includes plans for higher biodiversity and greener food production, as part of the European Green Deal, with a 'farm to fork' strategy at its core (European Commission, 2019). This poses major challenges for farmers to meet the goals and objectives of the European Green Deal while still maintaining a productive, competitive agriculture that produces food with the core objective of food security. Due to its broad fields of application, hemp (*Cannabis sativa* L.) has an enormous potential to actively accompany the economy in an ecologically sustainable way in accordance with the European Green Deal.

1.1 Application fields of *Cannabis sativa* L.

C. sativa L. is an important herbaceous species originating from Central Asia and has been historically used as a source of textile fibre, food, oil and as a medical product (Andre et al., 2016). *C. sativa* is currently experiencing a boom due to its rich phytochemical repertoire, its fibres and its agricultural properties, such as good resistance to drought and pests (Adesina et al., 2020; Amaducci et al., 2015), well-developed root system preventing soil erosion and lower water requirements compared to other textile crops (Andre et al., 2016). Due to its high potential for use in various branches of industry (e.g. textile, paper, building materials, bioenergy, bioplastics, pharmaceutical industry, vegetable oil, animal products, cosmetics, etc.), *C. sativa* is a prime example of a multifunctional crop which is excellently suited for recycling management because of its versatility and the possibility of 100% recycling (grains, leaves, flowers, stem) (Rehder, 1995). Basically, one can differentiate between the **industrial use** of *C. sativa* for paper, clothes or ropes, numerous food products (oils, protein, and flours) or cosmetic products, and its **medical use**. Based on the statements of the hemp

association it is possible to produce more than 50,000 products from *C. sativa* (Carus & Sarmento, 2016). The three product categories with market potential are: (1) oilseed; (2) fibre; and (3) pharmaceuticals (Cherney & Small, 2016) (Figure 1).

Medically used are flowers and extracts of the female plants due to the higher content of active substances. The cannabinoids available in *C. sativa* have a broad range of medically exploitable properties where anti-depressive, anti-oxidative, appetite enhancing, antiemetic, antiphlogistic, analgesic, sedative, muscle relaxing, anxiolytic, antipsychotic and neuroprotective effects belong to. Beside they are also used to treat side effects of chemotherapy, pain, multiple sclerosis, anorexia, epilepsy, anxiety and Parkinson's disease. These broad fields of application show their potential for various indications (Booz, 2011; Consroe, 1998; Englund et al., 2013; Grotenhermen & Müller-Vahl, 2012; Massi et al., 2004; Notcutt et al., 2012; Pertwee, 2008).

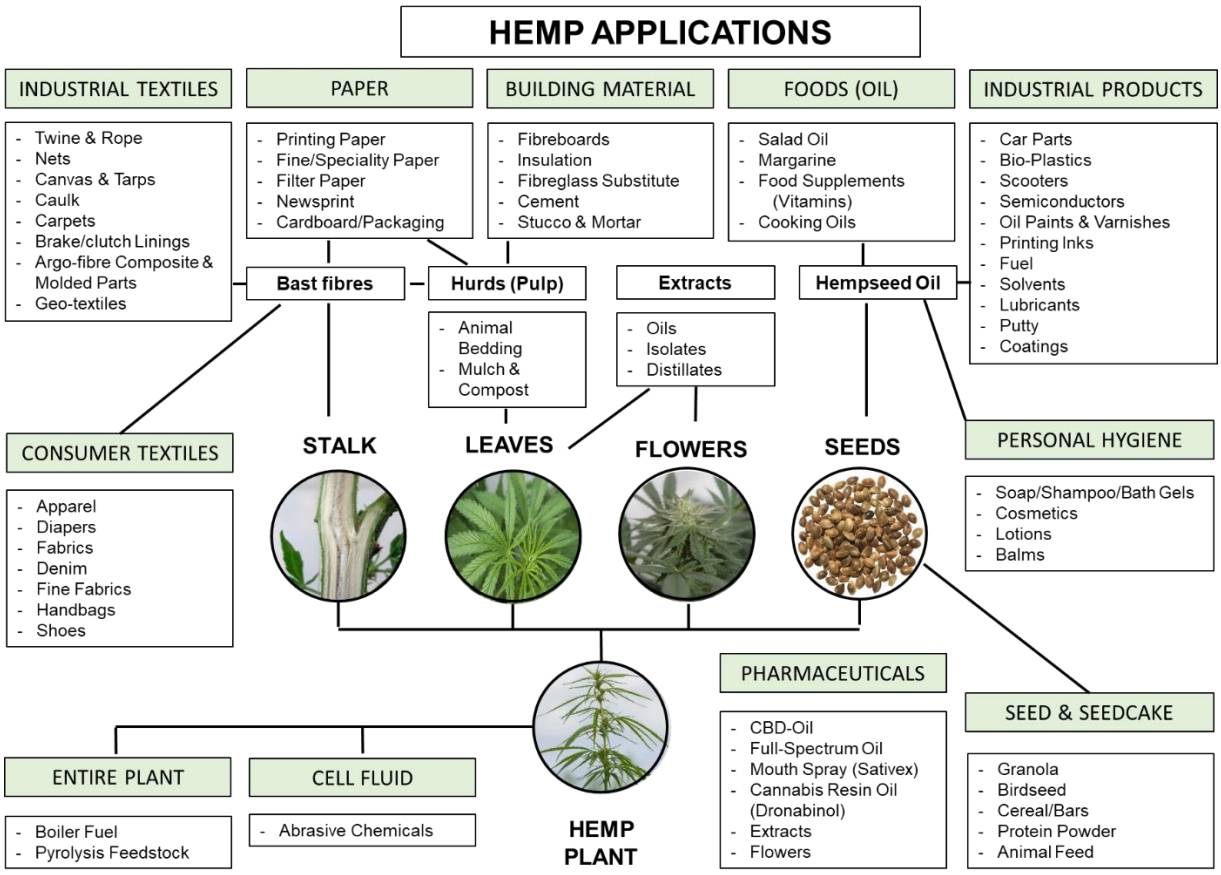

Figure 1 Hemp (*Cannabis sativa* L.) applications (Sumner, 2019 supplemented after L. Burgel).

1.2 Structures and biosynthetic pathway of phytocannabinoids

C. sativa contains 554 identified compounds, among them 113 are classified as phytocannabinoids (Ahmed et al., 2015) and 120 as terpenes (ElSohly & Slade, 2005).

Cannabidiolic acid (CBDA) and over 150 cannabinoids (ElSohly & Gul, 2014) of which 14 are profiled (Jin et al., 2020), are biosynthesized as prenylated aromatic carboxylic acids and stored in the secretory cells of glandular trichomes, which are present in flowers (Russo, 2011; Sirikantaramas et al., 2005; Turner et al., 1978) and to a lesser extent in leaves of *C. sativa* (Gagne et al., 2012). Trichomes, which are present in aerial plant parts in several plant species, are epidermal appendages having certain functions such as protection against predators (de Meijer, 2014; Levin, 1973). The highest cannabinoid concentration is found on perigonal bract trichomes which enwrap pistils and seeds. Glandular trichomes are especially concentrated on the lower surface of younger leaves and on sepals as well as anthers (Cherney & Small, 2016). Commonly known neutral active components Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), and cannabigerol (CBG) do not occur in significant concentrations in fresh plant material of *C. sativa* (Wang et al., 2016). Through the process of decarboxylation of their corresponding acids such as Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), CBDA, and cannabigerolic acid (CBGA), triggered by the influence of light and heat after harvesting, they can convert into their neutral homologues (Aizpurua-Olaizola et al., 2014). The quantity of cannabinoids is subject to fluctuations, depending on the age of the plant (Chandra et al., 2017), genotype (Gorelick & Bernstein, 2017) and the interaction of environmental and physiological processes (Bernstein et al., 2019).

The phenolic fraction of cannabichromenic acid (CBCA), Δ^9-THCA and CBDA is generated via a polyketide-type pathway (PKS) (Eisenreich et al., 1998; Fellermeier et al., 2001; Shoyama et al., 1975). Olivetolic acid (OLA) is formed by the condensation of hexanoyl-CoA with three molecules of malonyl-CoA (Gagne et al., 2012). OLA is further converted by cell extracts and geranyl diphosphate (GPP) with the prenylase geranyl diphosphate:olivetolate geranyltransferase (GOT) into CBGA (Fellermeier & Zenk, 1998), which is the first cannabinoid in the biosynthetic pathway. GPP is delivered as the end product of the deoxyxylulose phosphate/methylerythritol phosphate (DOXP/MEP) pathway (Fellermeier et al., 2001; Rohmer, 1999). In a subsequent step CBDA, Δ^9-THCA and CBCA are formed by their respective syntheses from CBGA (Mechoulam, 1995; Morimoto et al., 1998; Sirikantaramas et al., 2004; Taura et al., 1996) by removing a proton from the methyl group or the hydroxyl group of CBGA (Flores-Sanchez & Verpoorte, 2008). These newly formed acidic cannabinoids can then undergo a non-enzymatic decarboxylation into their respective active forms (Perrotin-Brunel et al., 2011) (Figure 2).

The main cannabinoids best known for their therapeutic potential are CBD and the main psychoactive agent of cannabis Δ^9-THC, with appetite-stimulating, anti-inflammatory, analgesic and antiemetric properties. In contrast, CBD antagonizes the intoxicant euphoric effects of Δ^9-THC (Consroe, 1998; Russo, 2016; Small & Naraine, 2016). Gradually stored Δ^9-THC converts over time into the degradation product cannabinol (CBN), which has 10% of the psychoactive potential of Δ^9-THC (Grotenhermen & Karus, 1998).

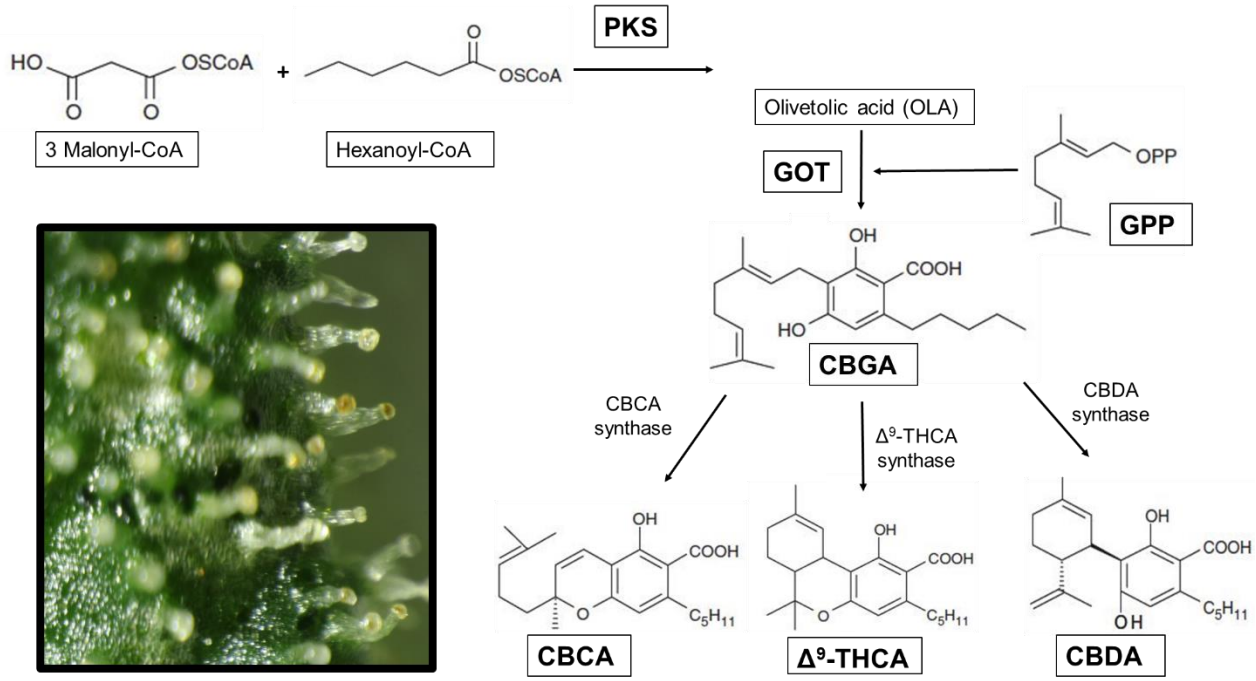

Figure 2 Pathway for biosynthesis of cannabinoids. PKS: polyketide synthase; GPP: geranyl diphosphate; GOT: geranyl diphosphate:olivetolate geranyltransferase; CBGA: cannabigerolic acid; Δ^9-THCA: Δ^9-tetrahydrocannabinolic acid; CBDA: cannabidiolic acid; CBCA: cannabicromenic acid (modified after Flores-Sanchez & Verpoorte, 2008).

All of these cannabinoids are characterized by the presence of an alkyl side chain on the resorcinyl moiety consisting of five carbon atoms (Citti et al., 2019a). However, other phytocannabinoids with a different number of carbon atoms on the side chain, which are called varinoids (with three carbon atoms), e.g. cannabidivarin (CBDV) and Δ^9-tetrahydrocannabivarin (Δ^9-THCV), and orcinoids (with one carbon atom) are known (Hanuš et al., 2016). Still, new phytocannabinoids continue to be discovered, what opens new gaps on their unexplored biological activities. Recently, the presence of a butyl phytocannabinoid series, with a four-term alkyl chain, namely cannabidibutol (CBDB) and Δ^9-tetrahydrocannabutol (Δ^9-THCB) has been reported in CBD samples derived from cannabis

(Citti et al., 2019b; Linciano et al., 2020a). It is proven, that the length of the side alkyl chain has an impact on the biological activity of Δ^9-THC. Citti et al. (2019a) identified a new phytocannabinoid with the same structure than Δ^9-THC, but with a seven-term alkyl side chain, called Δ^9-tetrahydrocannabiphorol (Δ^9-THCP). Additionally, along with Δ^9-THCP the corresponding CBD homologue was isolated, namely cannabidiphorol (CBDP). Moreover, a new series of phytocannabinoids has been reported by Linciano et al. (2020b), which fill the gap between the pentyl and heptyl homologues of CBD and Δ^9-THC, through bearing a *n*-hexyl side chain on the resorcinyl moiety, namely cannabidihexol (CBDH) and Δ^9-tetrahydrocannabihexol (Δ^9-THCH). Further studies currently underway to clarify the mechanism of action of these newly discovered phytocannabinoids are essential to assess their pharmacological potential. However, it can be assumed that the broad spectrum of phytocannabinoids has not yet been fully exploited.

1.3 Medical cannabis market

The medical cannabis market has grown significantly in recent years and further rapid growth is forecasted for the future. The IMARC Group expects the global medical cannabis market to witness strong growth during 2020 – 2025. In the United States (US) as well as Canada, and in countries neighbouring Germany, such as Austria, Italy, Denmark, Portugal and Switzerland, fast growing and innovative markets in the food, cosmetics and pharma industries are developing. CBD products based on phytocannabinoid-rich cannabis genetics, such as drops, capsules, creams, teas, sweets and beverages or flowers are being created (Chandra et al., 2019; Takeaways, 2019). Prognostications indicate that cannabis-infused food and beverage products will account for 60% of the market for cannabis edible products. The rise of CBD as a food ingredient, which is beginning to emerge from the shade of cannabis prohibition, creates a legal challenge for food manufacturers who hope to add it to various products (King, 2019).

In 2018, Germany has imported around 7.6 tonnes of cannabis products such as cannabis flowers, extracts, preparations, and final pharma products compared to 4.5 tonnes in 2017. Particularly, in 2018, German medical cannabis distributors imported 3.13 tonnes of flower material for medical care of patients, while 120 kg were re-exported. Moreover, in Germany, the International Narcotics Control Board (INCB) has estimated a significant increase in demand for medical cannabis from 6.7 – 8.8 tonnes in 2019 to 16 tonnes in 2020, even though the increase was not as high as originally expected (Homberg, 2020).

1.4 Chemotypes of *Cannabis sativa* L.

1.4.1 Classification

C. sativa L. is classified into five chemotypes based on their cannabinoid profiles and concentrations (de Meijer, 2014; ElSohly et al., 2017; Mandolino & Carboni, 2004; Pacifico et al., 2008):

- Chemotype I with a Δ^9-THC/CBD ratio > 1;
- Chemotype II with an intermediate Δ^9-THC/CBD ratio of ≈ 1;
- Chemotype III = fibre-type hemp with a Δ^9-THC/CBD ratio < 1;
- Chemotype IV, containing CBGA as main cannabinoid (Fournier et al., 1987);
- Chemotype V, containing almost no cannabinoids.

Due to the prohibition of cultivation in Germany and many other European countries for decades (Ascrizzi et al., 2019), the medical potential of the cannabis plant has hardly been used and fully researched. In 1996, agricultural cultivation of the plant was re-authorized in Germany only as industrial hemp. Since March 2017, medical products containing substances from the cannabis plant as well as cannabis flowers have been prescribed by doctors. The demand has been increasing since (Bundesvereinigung Deutscher Apothekerverbände e.V., 2018) and is currently covered only by imports from the Netherlands and Canada. However, Germany is aiming to establish cultivation of medical cannabis through the Cannabis Agency set up by the Federal Institute for Drugs and Medical Devices (BfArM). The task of the Cannabis Agency is to 'control and monitor the cultivation of cannabis for medical purposes in Germany' (Bundesinstitut für Arzneimittel und Medizinprodukte, 2018). Medical cannabis from German cultivation will be available in pharmaceutical quality from spring 2021 at the earliest. Until then, cannabis will remain an imported product in Germany and the exporting countries can look forward to a rapidly growing market. In concrete terms, the target market segment of medical cannabis is, therefore, strongly regulated in terms of quantity and price by the Cannabis Agency; and the regulation relates exclusively to cannabis plants with THC contents > 0.2% (average of the currently used genotypes of 5 – 20% THC).

1.4.2 Industrial hemp genotypes

A clear distinction between chemotypes is necessary, as the different regulatory framework for the cultivation of medical cannabis and industrial hemp is expected to result in significant differences in market potential for companies operating in this sector. Industrial hemp

genotypes, that comply with the 0.2% THC limit mandated by the EU legislation, can be cultivated without restrictions by farmers within the EU (Campiglia et al., 2017). In the EU variety list 2020, 102 genotypes are listed, with only 75 having an authorization until at least 2021. The EU catalogue of varieties specifies which industrial hemp genotypes may be grown for commercial purposes in EU countries. Different rules apply to the cultivation of these varieties in different EU countries while the catalogue of varieties is updated annually. In Germany, only farmers who have a licence and a minimum size of cultivated area are allowed to grow industrial hemp. Further, the cultivation must be registered (European Commission, 2020).

Since the versatile use of cannabis in the medical field is not only due to the psychoactive cannabinoid Δ^9-THC, anti-inflammatory phytopharmaceuticals could also be theoretically generated from plant extracts obtained from registered industrial hemp genotypes. In fact, non-psychoactive cannabinoids, such as CBD, CBG and CBC have many more medical uses than Δ^9-THC has (Schultes, 1970). Since CBD is the leading cannabinoid of the cannabis plant, it is arbitrary to consider only genotypes with high Δ^9-THC content as 'medical cannabis'. The extraction of raw materials from industrial hemp genotypes approved for outdoor cultivation could provide a decisive competitive advantage as the quantity harvested could be significantly increased through better land use; also, the safety requirements are significantly lower than for indoor medical cannabis cultivation. In order to be able to guarantee a standardized, reproducible quality of the extracts, existing cultivation system for fibre or oilseed production has to be almost completely redeveloped as the sowing, harvesting time, harvested organ and many other parameters differ greatly from the previous system. In order to achieve this, in a first step, selected approved industrial hemp genotypes have to be tested for the content of cannabinoids in the field, depending on the time of harvest and harvest organ. Screenings from previous studies have shown that medical cannabis genotypes produce a considerably higher concentration of cannabinoids than industrial hemp genotypes do. However, industrial hemp genotypes bred for oilseeds produce more flower biomass than fibre genotypes do. As the principal source of resin is derived from the flowering part of the plant, genotypes bred for oilseeds are a much more promising source for CBD production than fibre genotypes are (Small, 1979). The key factor is whether registered EU hemp genotypes have a sufficiently high cannabinoid content, as it would enable them to achieve a profitable cannabinoid yield through an optimized outdoor cultivation system, which meets the high quality standards for pharmaceuticals. Their potential to be used as raw material for the extraction of several

cannabinoids, with the advantage that they can be cultivated by farmers on a broad scale, has not been investigated yet. Further, the dynamics of cannabinoid accumulation in industrial hemp genotypes during flowering and seed maturity are not well studied (Calzolari et al., 2017). It is hypothesized that EU-registered hemp genotypes vary in respect of biomass yield and cannabinoid content. It needs to be investigated whether the quality and quantity of cannabinoids is high enough to aim for field-scale cannabinoid production.

1.4.3 Breeding efforts for *Cannabis sativa* L.

Due to the increasing relevance of cannabinoids as a further use of the cannabis plant and the growing number of new medical cannabis applications, it is of great importance that seed companies use standard and new breeding techniques to produce new cannabis genotypes (Hein, 2020).

In the past, breeding efforts focused on a high fibre yield and quality as well as increased oilseed yield together with Δ^9-THC reduction (Salentijn et al., 2015). Some cannabis traits show high plasticity, especially the cannabinoid content and phenological development, because of their sensitivity to environmental conditions, e.g. temperature and day length. It is of great interest that the ranking of varieties is stable for most traits in different environments. Furthermore, suitability of cannabis genotypes to a given environment highly depends on the cultivation purpose (Ranalli, 2004; Ranalli & Venturi, 2004). Stable Δ^9-THC reduction as a breeding goal gained importance in 2001, when government regulations limited Δ^9-THC content to only 0.2% within the EU. Within a cannabinoid reduction breeding programme several new monoecious genotypes with very low Δ^9-THC levels (< 0.07%) and without the typical cannabis aroma (e.g. USO 45) were developed (Salentijn et al., 2015). The distinction between drug and fibre accessions can only be made by classification on the basis of the cannabinoid profile (chemotype) (de Meijer et al., 2003).

1.4.4 Phytocannabinoid-rich cannabis genotypes

Due to the reduced concentration of cannabinoids in certified industrial hemp genotypes through breeding, stimulated by a rapidly growing global market for cannabis products, it is of great importance that the focus is placed on breeding new cannabis genetics that contain a high amount of non-psychoactive cannabinoids and a Δ^9-THC content below 0.2%. As a result of this fact and due to the increasing demand for non-psychoactive cannabinoids, an extended chemotype classification has been developed. The term **PCR** hemp (**PCR** hemp = **p**hyto**c**annabinoid-**r**ich cannabis) is used as a synonym for CBD, CBG, CBC-

rich cannabis genetics. In order to comply with the legal framework conditions, it is necessary to differentiate between *C. sativa* containing > 0.2% Δ^9-THC and cannabis rich in PCR (< 0.2% Δ^9-THC) which is classified as an industrial hemp genotype in terms of legal classification (Figure 3). The advantage of such PCR-genetics is that in many EU countries, PCR cannabis genotypes with a high content of non-psychoactive phytocannabinoids (CBD, CBG, and CBC) between 10 – 30% and Δ^9-THC < 0.2% may be legally cultivated as industrial hemp, whereas genotypes with THC content above this limit require a cultivation licence. The higher yield of these active ingredients in PCR cannabis genotypes increases the economic efficiency of the products made from it. These required genetics with < 0.2% Δ^9-THC and CBD contents > 10% hardly exist today. However, as the demand is constantly increasing, such genotypes must be obtained through targeted breeding. In addition, existing genotypes on the market must be tested for stability and potential in order to create the basis for future development of stable varieties.

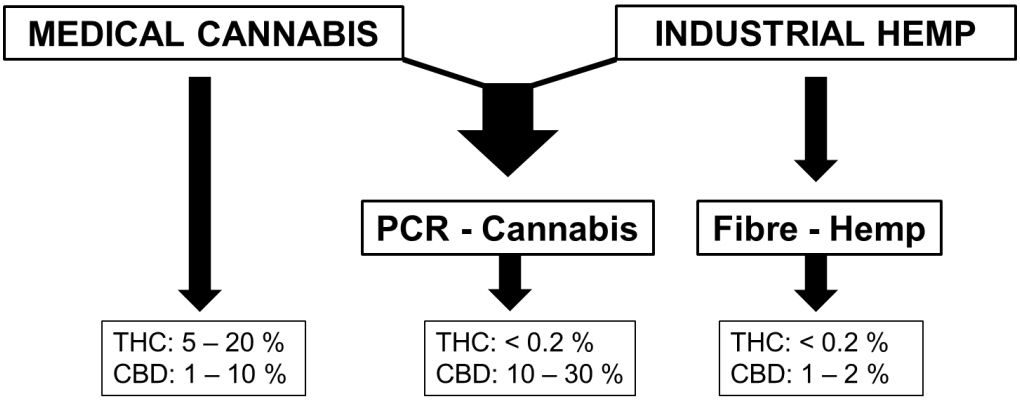

Figure 3 Classification of *Cannabis sativa* L. chemotypes. PCR: Phytocannabinoid-rich cannabis; THC: Tetrahydrocannabinol; CBD: Cannabidiol.

1.5 Cultivation systems for *Cannabis sativa* L.

Basically, two different cannabis cultivation systems can be distinguished: **outdoor cultivation** and **indoor cultivation**. The latter is divided into greenhouse cultivation and cultivation in closed chambers under fully controlled conditions. Depending on the purpose of use, an important aim is to generate uniform plants with appropriate pharmaceutical quality in addition to high yields. The quality characteristics depend on the interaction between the two factors such as genotype and environment. After harvesting, drying, further processing and storage conditions influence the quality. The genotype factor can be influenced by the selection of suitable plant genetic material.

In an **outdoor cultivation system**, the plants are subject to the climatic conditions of the production area, which vary from year to year. The natural life cycle of the cannabis plant allows it to be harvested once a year. Environmental parameters that can be influenced in the field are water supply (irrigation and drainage), soil cultivation measures, and nutrient availability (tillage, crop rotation and fertilization). The photoperiod, light quantity and quality as well as temperature and humidity can hardly be influenced. The suitability of outdoor cultivation of cannabis flowers, which contain phytocannabinoids in a very similar and stable range from batch to batch, has not yet been sufficiently studied. However, if the plant material is used to isolate pure substances such as CBD, or is further processed to prepare extracts, field cultivation is economically an interesting option (Laginha, 2018).

In an **indoor cultivation system**, several environmental factors, such as photoperiod, light intensity and temperature can be specifically controlled, thus standardizing cultivation conditions. Depending on the genotype, this allows an average of 4 – 5 harvests per year and area (Clarke & Watson, 2007). In a greenhouse, all environmental factors such as light quantity as well as intensity, lighting duration, water, nutrients and CO_2 content of the air can be reproducibly adjusted, with precise temperature and humidity control. Greenhouse cultivation is similar to cultivation in closed production facilities (climate chambers) and has the advantage of maintaining very high hygienic standards by means of sluice systems and a corresponding quality assurance system. As a result, the risk of contamination and the spread of plant pests and diseases can be considerably reduced, thus minimizing the use of plant protection products or even completely eliminating them.

1.6 Influence of cultivation methods on plant morphology of *Cannabis sativa* L.

Morphology and cannabinoid profile depend on genetic and environmental factors. Important factors that influence plant growth are: light quantity, lighting duration (photoperiod), water as well as nutrient supply, CO_2 concentration in the air, temperature and humidity. Scientifically, it has yet to be sufficiently tested whether field cultivation is suitable for producing cannabis flowers that form cannabinoids in a very narrow content range from batch to batch. So far, the focus has been on indoor cultivation, as more environmental factors can be controlled and the cultivation conditions can thus be better standardized.

It has been known for long in the industry of crop science and horticulture that plant morphology and metabolism can be manipulated. This can be achieved by influencing various factors such as indoor growing conditions (light quantity, lighting duration (photoperiod), water

as well as nutrient supply, CO_2 concentration in the air, temperature and humidity) and management practices (irrigation, fertilization and pruning techniques) (Jin et al., 2019). Significantly, the composition of the light spectrum has an influence on plant morphology. Whereas it is well established that blue light decreases internode length and subsequently enhances compactness of various plant species (Dong et al., 2014; Khattak et al., 2011), far-red and green light have shown to induce shade avoidance syndrome as well as stem and leaf elongation and premature flowering (Franklin & Whitelam, 2005). Furthermore, pruning techniques can also be used to make morphological adjustments to enhance floral yield by maximizing light interception and creating more air circulation. Another technique uses netting to orientate the flowers optimally towards the light and to give the plant stability in the generative stage (Jin et al., 2019).

Further, the morphology of cannabis genetics can also be adapted by using externally applied plant growth regulators (PGRs) (Burgel et al., 2020b; Lalge et al., 2016; Mendel et al., 2020). By standardizing the genetic plant material and using the same cultivation and processing methods, uniform plants with a uniform phytochemical profile can thus be bred (Fischedick et al., 2010).

For more precise standardization of the cultivation conditions and therefore the plant quality, the use of a quality-assured growing media with uniform water retention and ion exchange capacity is necessary. Since there is the possibility that all environmental factors can be controlled, the growing conditions can be regulated independently of the climate of the location. Through this cultivation technique and processing procedure, uniform plants with a uniform phytochemical profile can be produced (Fischedick et al., 2010).

In order to optimize flower yield per unit area and thus yield of cannabinoids, cultivation systems with minimum space requirements are needed. While outdoor cultivation seems to be more economically feasible, indoor cultivation has advantages, in terms of quality assurance and hygienic standards, to eliminate contaminations and produce homogeneous medical grade cannabis batches under controlled conditions (Laginha, 2018). Even though indoor systems are expensive operations, the interest in efficient use of the available growing space is rising. Additionally, standardization measures at all stages of production are fundamental. For cannabis, qualifying as a pharmaceutical and standardization at the cultivation level are of particular importance. Quality assurance must comply with requirements laid down under the Medicals and Narcotics Law, including **GACP** (Good Agricultural and Collection Practice for

Starting Material of Herbal Origin) and **GMP** (Good Manufacturing Practices). GACP guidelines provide an adequate quality assurance system for cultivation, harvest and primarily processing (WHO, 2003). Further, GMP guidelines, cover all aspects of production, ensuring that cannabis products are steadily made and controlled according to defined quality standards (European Commission, 2017). These quality systems are designed to minimize the risk involved in the manufacturing process, which cannot be eliminated by quality check of the final product. A crucial step to meet these requirements assumes cultivation under controlled environmental conditions and elimination of points of contact between humans and the product in any step of the process chain.

The choice of the appropriate cultivation system, therefore, depends on the intended use of the cannabis raw material and its further processing into the final product.

1.7 Unresolved problems and critical aspects

The commercialization of CBD products opens an important market sector. The production, distribution, and consumption of cannabis are worldwide still traditionally regulated individually by the producing countries, including within the EU. Thus, the legal situation of cannabis products is very fragmented and complex. In Germany, the German Federal Institute for Drugs and Medical Devices ('Bundesinstitut für Arzneimittel und Medizinprodukte, **BfArM**') oversees the cultivation licences, produce, trade, and import as well as export. It also coordinates delivery, sale or purchase of narcotics. Only the cultivation of cannabis for medical purposes is legalized. The cultivation of medical cannabis is subjected to a public tender process. Only companies selected through the process are entitled to medical cannabis cultivation in Germany. CBD products are offered in various products: food and food supplements, cosmetics and others. While, the Δ^9-THC content must always be below 0.2%, the legal status of such products is still not clear because the classification of CBD products in Germany is indistinct. Some difficulties arise from this, as the marketability of CBD products is determined by the Narcotic Act and these products are classified as novel food. At present, the European Authorities consider that CBD irrespective of its source, is a novel food according to Regulation (EU) 2015/2283. Therefore, it is forbidden to market CBD containing foods or food supplements throughout the EU, as long as its sale is not specifically authorized according to the provisions of this Regulation. Opinions differ greatly here (Homberg, 2020).

In Switzerland, cannabis flowers and CBD products may contain up to 1% of Δ^9-THC under the current regulatory setup, thus giving companies based in Switzerland a clear competitive

advantage. Whereas CBD products including extracts, concentrates and flowers are a grey area in EU countries, Swiss producers have the legal clarity which allows them to offer a wide range of CBD products on the market. With the advantage of a higher Δ^9-THC limit of 1%, Swiss producers have access to a wider range of cannabis genetics compared to other European countries that grapple with a Δ^9-THC threshold of 0.2% and cultivation of only those genotypes that are registered within the EU. This limits cannabis research, hampers scientific exchange and restricts breeding progress in Germany compared to its neighbouring countries. It remains to be seen whether there will be an EU-wide regulation in the future (Homberg, 2020).

In summary, this means that presently, the production of pure cannabinoids is an expensive undertaking because of limited availability, production costs, and regulatory limitations due to the various country-specific drug laws (Götz et al., 2019). Therefore, a short, fast and cost-effective production chain together with a reliable supply of the active compound, as well as consistency of quality and safety norms is aimed for (Götz et al., 2019) in order to meet the growing demand for cannabis products.

1.8 General outline and aims

The entire objective of the present thesis is to evaluate industrial hemp genotypes, which are certified by the EU for commercial use, for their cannabinoid content depending on growth stage and harvested organ in an outdoor cultivation system. The ultimate aim is to determine whether the genotypes are suitable for cannabinoid production. Further, the impact of externally applied synthetic phytohormones to modify plant morphology of phytocannabinoid-rich cannabis genotypes in a controlled indoor cultivation system is evaluated. Phytohormones can be used to beneficially influence plant architecture on a genotype-specific level for an optimized flower yield per unit area and thus CBD yield. Investigations should provide basic knowledge about the possible and flexible adaptation of plant morphology to lay the foundation for sustainable indoor cultivation systems with reduced space requirements. Along with indoor production systems of cannabis, soilless cultivation is an important aspect. The evaluation of different substrate compositions, especially substrates substituted with peat alternatives and their impact on plant performance, root growth and cannabinoid content is of crucial importance to further standardize the cultivation conditions aiming for homogeneous cannabis batches in an indoor production system.

These aspects are highly relevant for the establishment of cannabis production and genotype screening as they go along with possible changes in quality and yield due to different cultivation

systems. In view of the diverse potential of the cannabis plant, there is still a lack of information on cultivation and production. Further research in this sector is important in order to aim for a short, fast and cost-effective production chain.

1.9 Objectives

Based on the research gaps identified, the specific objectives of this thesis are:

- to determine the yield potential of different EU-registered hemp genotypes with regard to inflorescence and biomass yield as well as cannabinoid content, depending on genotype, growth stage and biomass fraction in an outdoor cultivation system;
- to investigate whether the tested industrial hemp genotypes are suitable for the production of cannabinoids due to better land use on a field scale;
- to test the impact of exogenously applied plant growth regulators (PGRs), such as 1-naphthalenaecetic acid (NAA), 6-benzylaminopurine (BAP) and a mixture (NAA/BAP-mix) of both PGRs on the plant architecture (total plant height, axillary branch length, and number of internodes) of different phytocannabinoid-rich cannabis genotypes. Furthermore, the biomass yield of inflorescence and leaves together with cannabinoid content should be determined;
- to evaluate a potential beneficial impact of PGRs on plant morphology in order to reach higher cannabinoid yields per plant in an indoor cultivation system;
- to evaluate the growth performance, such as total plant height, biomass yield, root growth and cannabinoid content, of phytocannabinoid-rich cannabis genotypes grown in different substrate compositions substituted with peat alternatives in an indoor pot cultivation system.

In order to achieve the targeted objectives, cultivation of seven industrial hemp genotypes (Finola, Fédora 17, Ferimon, Félina 32, Futura 75, USO 31, Santhica 27) was carried out in a two-year field experiment from 2017 to 2018 at the experimental station ‘Ihinger Hof’ of Hohenheim University (Renningen, Germany) in south-western Germany. Sampling of leaves and inflorescence was performed at four specific growth stages, differentiated into vegetative leaf stage (S1), bud stage (S2), full-flowering stage (S3) and seed maturity stage (S4). At each harvest date, leaves of each plant were divided into upper third and lower two-thirds of leaves and the inflorescence part of the plant. At seed maturity (S4) hemp seeds were manually separated from inflorescence. After the drying process for 75h at 30°C, the biomass of every fraction was determined and dry matter was recorded in gram per square metre. The fractionated

and dried biomass material was ground and stored in a dry and dark place until a quantitative analysis of cannabinoids, namely CBDA, CBD, CBGA, CBG, Δ^9-THCA and Δ^9-THC was performed by high pressure liquid chromatography (HPLC).

Furthermore, greenhouse experiments were conducted at the University of Hohenheim (Stuttgart, Germany), with phytocannabinoid-rich cannabis genotypes, namely KANADA, 0.2x and FED, which were kindly provided by the company AiFame (Wald-Schönengrund, Switzerland). In one experiment, these genotypes were treated with exogenously applied synthetic phytohormones in various concentrations: 10 mg L^{-1} of 1-naphthaleneacetic acid (NAA), 50 mg L^{-1} of 6-benzylaminopurine (BAP) or a 1:5 mixture of 50% NAA and 50% BAP solution (NAA/BAP-mix) to determine the impact of plant growth regulators (PGRs) on the genotype-specific plant morphology, such as total plant height, axillary branch length, and number of internodes. Furthermore, the differences in leaf and flower yields resulting from morphological changes as well as their cannabinoid content were determined.

In a second greenhouse experiment, the impacts of different substrate compositions on growth performance, biomass and flower yields, biomass nitrogen (N) content as well as CBD/A contents on two of the above-mentioned phytocannabinoid-rich cannabis genotypes, KANADA and 0.2x were tested. The substrate compositions were as follows: (a) peat-mix growth media (PM); (b) peat-mix substituted with 30% of green fibres (G30) consisting of coniferous wood and wood chips from pine and spruce wood growth media, and (c) pure coco coir fibres (CC). The harvested aboveground biomass material of each plant was frozen with liquid nitrogen (-196°C) and freeze-dried to determine dry weight, and subsequently, a quantitative analysis of CBD/A was performed by using HPLC. The roots per plant were scanned to calculate root length density (RLD) and root dry weight was measured after a drying process at 110°C for 24h.

The detailed description of all the mentioned experiments is noted in the chapter ‘publications’, which presents the three publications including the findings of this work.

In **publication I**, the results of investigating the biomass and inflorescence dry weight as well as cannabinoid content and yield of CBD, CBG and Δ^9-THC and their acid precursors CBDA, CBGA, and Δ^9-THCA of fractionated leaves and inflorescence, at different growth stages of the seven industrial hemp genotypes, are reported. It is evaluated whether the tested industrial hemp genotypes contain appropriate amounts of cannabinoids and if they are suitable for the production of cannabinoids due to better land use on a field scale.

Publication II presents the results of plant architecture modification through the application of synthetic phytohormones. The study is aimed at a small and compact cannabis plant morphology for various indoor growing systems with the goal of optimizing floral biomass and, therefore, cannabinoid yields per unit area.

Publication III focuses on the evaluation of different substrate compositions on plant performance (total plant height, biomass and flower yields), root growth (root dry weight and RLD) and N content as well as CBD/A content of cannabis leaves and flowers in a pot cultivation. As peat hardly grows back at all, moors are permanently destroyed by peat cutting, which causes massive environmental problems. According to this, a change in societal attitude is taking place with the goal of reducing dependence on peat-based growing media. The substitution of potting substrates with organic alternatives to partially replace fractionated peat can be a viable and sustainable solution to ensure constant plant development, and comparable high floral yield with stable cannabinoid contents, compared to pure peat containing standard substrates.

The presented publications haven been published in peer-reviewed international journals. Thus, the following chapters include the details of each article, which provide the overall frame of the present work. In accordance with the published and already discussed results, a general discussion is included connecting the different parts and aspects of cannabis cultivation for cannabinoid production (chapter 6). A special note is provided with the thesis on the screening of industrial hemp genotypes and their suitability for cultivation with the aim of cannabinoid production. It discusses which further potential for multiple or cascade use of these genotypes exists, as well as the potential of the hemp cultivation in the context of the European Green Deal. Additionally, the cultivation conditions are examined using PGRs to adapt the plant morphology and the different potting substrate compositions for a further standardization of the cannabis cultivation process in the greenhouse. Afterwards, an outlook is provided on existing problems and obstacles in this sector as well as proposed solutions for the future, followed by a summary of the whole thesis (chapter 7).

2 Publications

The present cumulative thesis includes three articles that have been published in peer-reviewed international journals. These three articles constitute the body of the thesis. Please use the references given below for citation of the three articles that are consistent with publications I – III of this thesis.

Publication I (published, Impact Factor 2.603):

Burgel, L., Hartung, J., Graeff-Hönninger, S. (2020). Impact of Growth Stage and Biomass Fractions on Cannabinoid Content and Yield of Different Hemp (*Cannabis sativa* L.) Genotypes. *Agronomy* 2020, 10, 372.

DOI: 10.3390/agronomy10030372

Publication II (published, Impact Factor 2.762):

Burgel, L., Hartung, J., Schibano, D., Graeff-Hönninger, S. (2020). Impact of Different Phytohormones on Morphology, Yield and Cannabinoid Content of *Cannabis sativa* L. *Plants* 2020, 9, 725.

DOI: 10.3390/plants9060725

Publication III (published, Cite Score 2.3 Scopus):

Burgel, L., Hartung, J., Graeff-Hönninger, S. (2020). Impact of Different Growing Substrates on Growth, Yield and Cannabinoid Content of Two *Cannabis sativa* L. Genotypes in a Pot Culture. *Horticulturae* 2020, 6, 62.

DOI: 10.3390/horticulturae6040062

3 Publication I: Impact of Growth Stage and Biomass Fractions on Cannabinoid Content and Yield of Different Hemp (*Cannabis sativa* L.) Genotypes

Burgel, L., Hartung, J., Graeff-Hönninger, S. (2020): Impact of Growth Stage and Biomass Fractions on Cannabinoid Content and Yield of Different Hemp (*Cannabis sativa* L.) Genotypes. Agronomy 2020, 10, 372.

Currently, only industrial hemp genotypes, which comply with the 0.2% THC threshold set by the European Union (EU) legislation, can be grown without restrictions within the EU. As these genotypes are originally bred for fibre and oilseed production, their potential to be used as a raw material for the extraction of various cannabinoids, with the advantage to be grown by farmers on a broad field scale has not been investigated yet. The widely cultivated hemp plant along with its historically used versatility for fibre production, nutrition and medical treatment has led to a renewal of interest with special emphasis on cannabinoids, such as cannabigerol (CBG) and cannabidiol (CBD). Questions regarding the impact of genotype and growth stage on biomass and inflorescence yields as well as cannabinoid contents are still open. Thus, the study, 'Impact of Growth Stage and Biomass Fractions on Cannabinoid Content and Yield of Different Hemp (Cannabis sativa L.) Genotypes', consists of a screening of seven industrial hemp genotypes with regard to the detection of an optimal harvest time and the impact of biomass fraction to optimize CBD yields. Additionally, special attention is paid on a CBG-rich genotype.

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Article

Impact of Growth Stage and Biomass Fractions on Cannabinoid Content and Yield of Different Hemp (*Cannabis sativa* L.) Genotypes

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Abstract: The medicinal use of cannabinoids renewed the interest in industrial hemp (*Cannabis sativa* L.). The aim of this study was to evaluate the impact of growth stage and biomass fractions of seven industrial hemp genotypes. The study focused on biomass yield, content of cannabidiolic acid/cannabidiol (CBDA/CBD), cannabigerolic acid/cannabigerol (CBGA/CBG), and tetrahydrocannabinolic acid (THCA). The experiment was conducted in 2017 and 2018. The biomass samples were taken at the vegetative (S1), bud (S2), full-flowering (S3) and seed maturity stage (S4). Plants were fractionated into inflorescence, upper and lower leaves. The average inflorescence dry yield of genotypes Futura75, Fédora17, Félina32 and Ferimon ranged between 257.28 g m^{-2} to 442.00 g m^{-2}, resulting in a maximum yield of CBDA at S4, with 4568.26 mg m^{-2}, 6011.20 mg m^{-2}, 4975.60 mg m^{-2} and 1929.60 mg m^{-2}, respectively. CBGA was exclusively found in genotype Santhica27, with a maximum CBGA yield of 5721.77 mg m^{-2} in inflorescence at growth stage S4 and a dry weight yield of 408.99 g m^{-2}. Although these industrial hemp genotypes are mainly cultivated for fibre and seed production, however, cannabinoids offer an additional value. For an optimized harvest result, yield of extractable material and overall yield of cannabinoids must be considered.

Keywords: hemp (*Cannabis sativa* L.); genotypes; biomass yield; growth stage; plant fractions; cannabinoids

1. Introduction

Industrial hemp (*Cannabis sativa* L.) is an annual species native to Asia and documented as one of the oldest crops known [1]. Traditionally it is grown in European regions for fibre production [2]. Hemp is considered as a multi-purpose crop. It is widely cultivated and historically used for fibre production, human nutrition and, medicinal purpose [3–6]. Based on the versatility of hemp, high-quality cellulose can be gained from the stems, nutrient-rich oil, and proteins generated out of seeds, as well as valuable essential oils and resins from inflorescence and leaves with abundant glandular trichomes [7,8]. Overall, this has led to a renewed interest in industrial hemp. In addition, cannabinoids extracted from inflorescence and leaf material of *C. sativa* L. have gained interest in recent years [9] with special emphasis on cannabidiol (CBD) and cannabigerol (CBG) [10]. In the years to come the cultivation of hemp is expected to rise based on the favorable regulatory framework, what promotes the constitution of new companies, exploiting products obtainable from hemp [11].

Generally, 113 known phytocannabinoids, which are biosynthesized as phenylated aromatic carboxylic acids are found in *C. sativa* L. [12]. The most abundant ones are cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA). Only small amount of the neutral cannabinoid, in particular CBD and tetrahydrocannabinol (THC) can be found in fresh plant tissue. By spontaneous decarboxylation through heat and light, they convert to their homologues [13,14]. The two main cannabinoids THC and CBD are known for their therapeutic potential. THC, the psychoactive agent, is considered to have anti-inflammatory, appetite-stimulant, analgesic and antiemetic properties [15], while CBD, which modulates the euphoric effects of THC, has antipsychotic, anticancer, antidiabetic antipsychotic and other positive side effects [16–18]. Furthermore, CBG is considered as a promising cannabinoid for various medical applications, although it is not well studied yet [19].

Hemp cultivation was banned in many countries, due to its psychoactive drug component THC [20]. Industrial hemp genotypes, which comply with the 0.2% THC threshold set by the EU legislation, can be cultivated without restrictions by farmers within the EU [21]. However, breeding efforts focused on the increase of seed and fibre yield [22] and in THC reduction. These genotypes have a wide range of industrial applications [20], e.g., bio composites, textiles, construction, paper making, bio-fuel, functional-food, cosmetics and personal care [22]. However, their potential to use as a raw material for the extraction of several cannabinoids, with the advantage to be cultivated by farmers on a broad scale has not been investigated yet. The dynamics of cannabinoid accumulation in industrial hemp genotypes during full-flowering and seed maturity is not known yet [23].

It is hypothesized that EU registered hemp genotypes vary in respect to biomass yield and cannabinoid content. Furthermore, yield and cannabinoid content will depend on growth stage and the plant fraction harvested during the vegetative stage. Hence, the objectives of this study were to test the impact of genotype and harvest time on, biomass yield, fractionated into leaves and inflorescence, as well as cannabinoid yield.

2. Materials and Methods

2.1. Field Trail

The experiment was conducted in two growing seasons (2017, 2018) at the experimental station Ihinger Hof (University of Hohenheim, Upper Neckarland, Lat. N 48°44'40, 70" Lon. E 8°55'26, 36"). During the experimental period in 2017 precipitation amounted to 298.4 mm, the mean temperature was 17.06 °C, and global radiation amounted to 21,814 W h m^{-2} from May to August. In 2018, precipitation amounted to 168.4 mm, mean temperature was 17.95 °C, and global radiation amounted to 32,072 W h m^{-2} from May to September. The weather data was obtained from the weather station located at Ihinger Hof, Germany (Figure 1A–D).

According to the World Reference Base [24], the experimental soils at the trail site can be characterized as vertic Luvisol and vertic Cambisol in 2017 and 2018, respectively. In both years, topsoil of the experimental fields had a similar texture with around 35% clay, 2% sand and 63% silt. The total amount of mineral nitrogen (N_{min}) up to a depth of 90 cm varied only marginally in both years and amounted to 50 kg NO_3 ha^{-1}.

The field trial was set up in both years as a split plot design with six genotypes: Fédora17, Ferimon, Félina32, Futura75, Santhica27, USO31, cut at four growth stages. Genotypes were randomized to main-plots with a plot size of 2 × 10 m according to a randomized complete block design (RCBD) with three replicates. Main plots were further subdivided into four plots, which were used for different sampling dates. In 2018, the genotype Finola was added (Table 1). Around six months before sowing, fields were ploughed to a depth of 0.3 m (winter furrow). The previous crop was winter wheat (*Triticum aestivum* L.) in 2017 and pea (*Pisum sativum* L.) in 2018. Prior to sowing, the seed bed was prepared using a rotary harrow to a depth of 8 cm. Sowing was carried out on May 11, 2017 and on April 25, 2018 with a sowing density of 200 seed m^{-2} and a row distance of 0.15 m in both years. Prior to sowing, the field was fertilized with 50 kg N ha^{-1} (ENTEC 26, EuroChem Agro GmbH, Mannheim,

Germany) in both years. There was no further fertilization of other nutrients. No additional irrigation was implemented over the whole growing season. No pesticides and herbicides were applied during the vegetation period. Also no mechanical weed control took place.

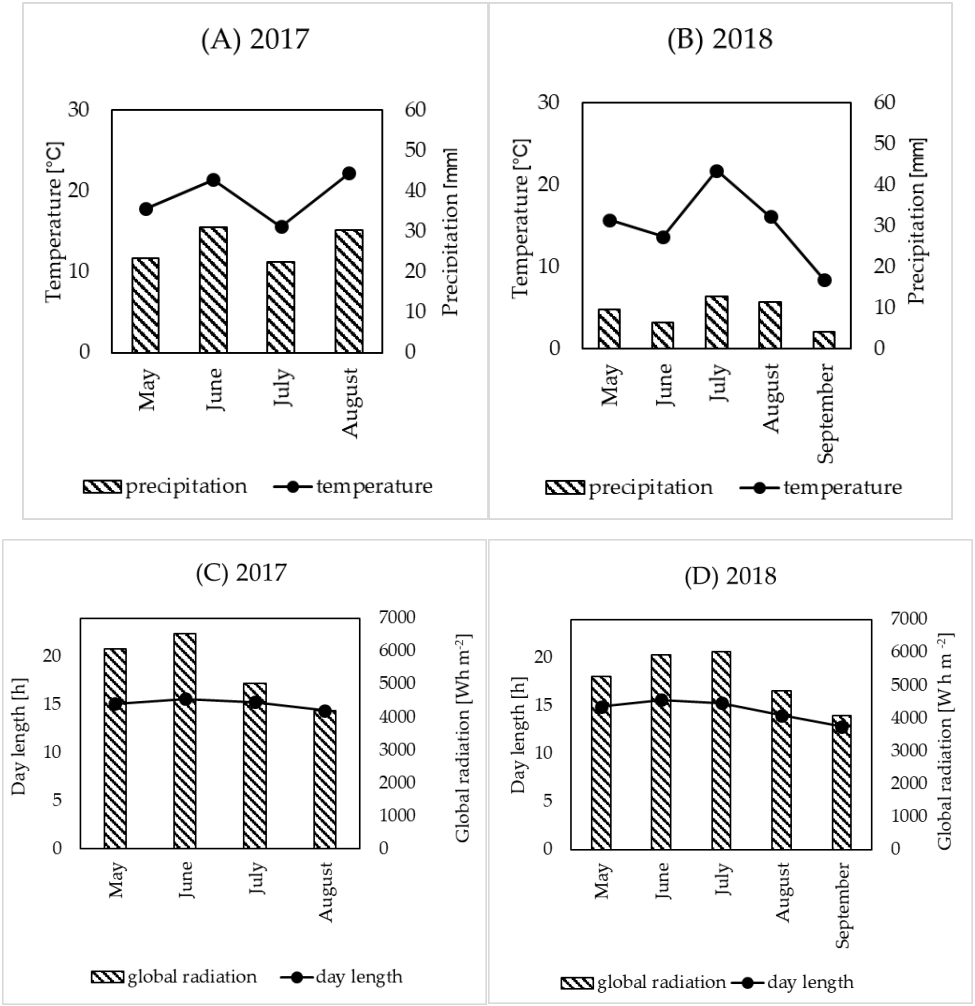

Figure 1. Precipitation in mm (bars) and mean temperature in °C (•) during the cultivation period of hemp at the location Ihinger Hof in 2017 (**A**) and 2018 (**B**). Global radiation in W h m^{-2} (bars) and day length in h (•) during the cultivation period of hemp at the location Ihinger Hof in 2017 (**C**) and 2018 (**D**).

Table 1. Hemp genotypes grown at the location Ihinger Hof, Germany in 2017 and 2018. FNPC*: Fédération Nationale des Producteurs de semences de Chanvre.

Genotype	Year	Origin	Breeder	Sexual Phenotype
Finola	2018	Finland	Ph.D. Jace Callaway	diocious
Fédora17	2017/18	France	FNPC*	monoecious
Santhica27	2017/18	France	FNPC*	monoecious
USO31	2017/18	Ukraine	FNPC*	monoecious
Félina32	2017/18	France	FNPC*	monoecious
Ferimon	2017/18	France	FNPC*	monoecious
Futura75	2017/18	France	FNPC*	monoecious

2.2. Treatments and Sample Preparation

Six monoecious industrial hemp genotypes, and one diocious genotype, were evaluated in this study (Table 1). All genotypes were approved by the European Union for commercial use and certified with a THC content below 0.2%. The seeds were provided by Coopérative Centrale des Producteurs de Semences, France, except for the genotype Finola which was provided by the company BAFA GmbH (Malsch, Germany). Samples of leaves and inflorescence were taken at four different growth stages during the vegetation period. Each of the genotypes had its specific phenological characteristics, such as the length of growing season, until they reached a specific growth stage (Figure 2). For each plot, the growth stage was differentiated into vegetative leaf stage (S1), bud stage (S2), full-flowering stage (S3) and seed maturity stage (S4), according to Mediavilla et al. [25]. If 50% of the plants within a plot had reached the intended growth stage, ten plants were cut and the sampling date was recorded. For the evaluation of the diocious genotype Finola, each replicate was subdivided into male and female plants. If evident, only female plants were harvested. For each harvest date, hemp leaves of each plant were divided into upper third and lower two-thirds of the leaves, as well as hemp inflorescence. Depending on the growth stage inflorescence were, separated manually from stem and seeds. Fractionated hemp samples were dried at a temperature of 30 °C for 75 h. After the drying process, dry matter of every fraction was determined and recorded in gram per square meter to calculate dry weight (DW). Subsequently, the dried plant material was ground with an ultra-centrifugal mill of Retsch, Type ZM 200 (Haan, Germany) to acquire a homogeneous fine powder, with a particle size of maximum 1 mm. The powder was stored in a dark and dry place until used for further chemical analysis. The residual moisture of the samples was measured with a moisture analyser DBS 60-3 of Kern & Sohn GmbH (Balingen, Germany).

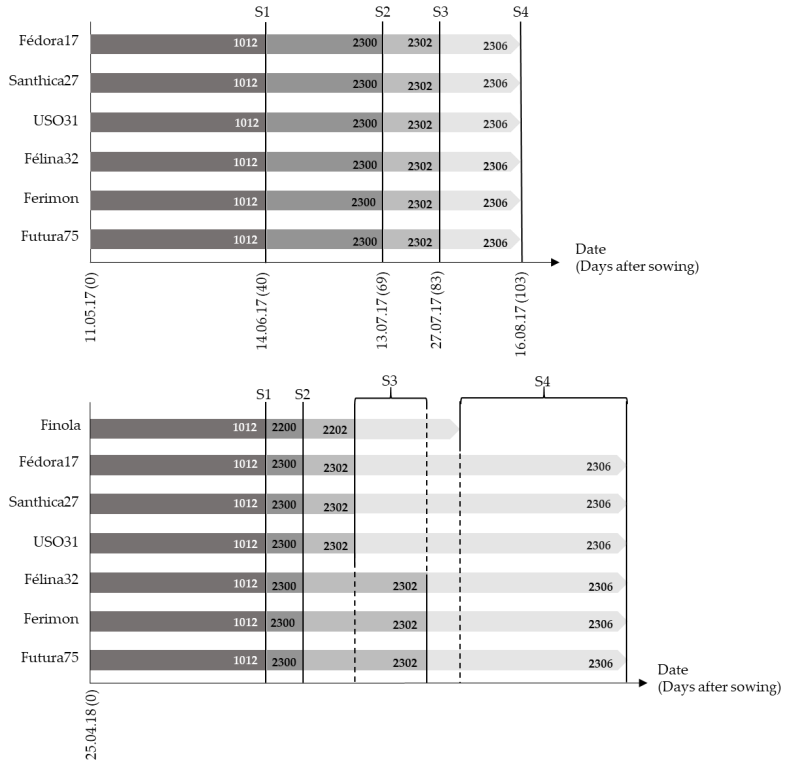

Figure 2. Date and days after sowing of the achievement of growth stages, such as vegetative stage (S1), bud stage (S2), full-flowering stage (S3) and seed maturity stage (S4), during the vegetation period in 2017 and 2018. Code 1012: 6th leaf pair (vegetative stage), 2300/2200: Flower formation, 2302/2202: Flowering, 2306/2204: Seed maturity of monoecious and diocious/female plants, defined according to Mediavilla et al. [25].

2.3. Extraction and Quantification of Cannabinoids by HPLC Analysis

The quantitative analysis of cannabinoids, particularly CBD, CBDA, THC, THCA, CBG, CBGA was performed, accordingly to Lehmann and Brenneisen [26] with slight modifications using liquid chromatography (1290 Infinity II LC System, Agilent, Santa Clara, CA, USA). For the cannabinoid extraction 90 to 110 mg of ground sample was dissolved in 25 mL of a methanol 90%/chlorophorm 10% (*v*/*v*) (9 + 1) composite. The mixture was extracted in an ultrasonic bath for 30 min. After warm down, the supernatant was filtered through syringe filters Polytetrafluorethylen (PTFE), 0.45 µm (Macherey-Nagel GmbH & Co. KG, Germany) into a HPLC vial and injected in the HPLC system. The HPLC instrument was equipped with an autosampler, a quaternary pump, as well as a diode-array spectrophotometer (DAD). Cannabinoids were quantified at a detection wavelength of 230 nm. The chromatographic separation was carried out on a Nucleosil 120-3 C8 column (125 mm x 4 mm i.d., 3.0 µm) with a guard column EC 4/3 Nucleosil 120-3 C8 (Macherey-Nagel, Oensingen, Schwitzerland). The extraction temperature was set to 40 °C. The mobile phase was a mixture of HPLC-grade methanol (solvent A) and 0.1% acetic acid in HPLC-grade distilled H_2O (solvent B; Sigma-Aldrich, Saint Louis, MO, USA) at a constant flow rate of 0.7 mL min^{-1}. The mobile phase composition gradient elution program started with a 50/50 solvent A/solvent B ratio, linearly ramping up to 90% of solvent A over 20 min. This ratio was maintained for 2.0 min. Afterwards the gradient was changed to starting conditions over 5 min. The injection volume was 10 µL and the total run time comprised 27 min.

An external calibration of cannabinoid quantification was performed, using two standards (CAN1 and CAN2), containing the target compounds (CAN1: THC 2%, CBD 2%, THCA 10% (Lipomed, Arlesheim, Switzerland), CBDA 10% and CAN2: CBG 2%, CBGA 2% (Echo Pharmaceuticals BV, Weesp, The Netherlands)).

The data were processed using ChemStation Software for LC Rev. B.04.03-SP2 (Agilent, Santa Clara, CA, USA). The retention time of the respective chromatographic target peak, for example, of the non-psychoactive cannabinoid CBDA was compared with the main chromatographic peak of the reference to carry out a quantitative analysis. In addition, the UV spectra was used to preliminarily allocate the chromatographic peak to the reference spectra visually. The identity of cannabinoid was proven if the deviation of retention time of the chromatographic peak was ≤0.5 min and the optical spectra comparison did not show any difference.

To calculate, the respective cannabinoid content C_{TS} in mass percent [$\%_{m/m}$] of each sample extract, equation [1] was used, where A_{TS} is defined as peak area of the standard analyst, B_{TS} is defined as peak area of the sample analyst in µV × s. V is defined as the volume of the volumetric flask, $EW_{TS_{ijkl}}$ as weight portion of the product in mg, and F_{ijkl} as the residual moisture of the product in $\%_{m/m}$. *Indices* are defined for the *i*-th genotype in the *j*-th replicate, at the *l*-th growth stage and the *k*-th year.

$$C_{TS_{ijkl}}\,[\%_{m/m}] = \frac{(A_{TS}\,[\mu V \times s])}{\left(B_{TS_{ijkl}}\,[\mu V \times s]/100\left[\mu L\,mL^{-1}\right]\right)} \times \frac{V\,[mL]}{EW_{TS_{ijkl}}\,[mg]} \times 100 \times F_{ijkl}\,[\%_{m/m}] \quad (1)$$

2.4. Statistical Analysis

A mixed model approach was used to analyse all traits, which were determined once per plot. For some traits observations of ten single plants were made. In these cases, a mean value across observations was calculated. The traits are dry weight of upper leaf fraction, lower leaf fraction, and inflorescence fraction as well as, the cannabinoids present in the dried material of all fractions. The model [2] is given by:

$$y_{ijkl} = \mu + a_k + r_{kj} + f_{kij} + \tau_i + \varphi_l + (\tau\varphi)_{il} + (a\tau)_{ik} + (a\varphi)_{lk} + (a\tau\varphi)_{ikl} + e_{ijkl} \quad (2)$$

where y_{ijkl} is the observation of the *i*-th genotype in the *j*-th replicate, at the *l*-th growth stage and the *k*-th year, μ is the intercept, τ_i is the fixed effect of the *i*-th genotype, a_k is the fixed effect of the *k*-th year, φ_l is the fixed effect of the *l*-th growth stage, and $(\tau\varphi)_{il}$ is the fixed interaction effect of the *i*-th genotype

and the *l*-th growth stage. $(a\tau)_{ik}$ $(a\varphi)_{lk}$, and $(a\tau\varphi)_{ikl}$ are the random interaction effects of the treatment effects with the year. r_{kj} is the random effect of the *jk*-th replicate, f_{kij} is the *kij*-th random main plot effect and e_{ijkl} is the error associated with y_{ijkl}. For the latter, two year-specific variances were fitted as they increase the model fit measured via Akaike Information Criterion (AIC) [27]. Fitting both, plot and error effects, accounted for the correlation between observations from the same plot assuming a compound symmetry structure. Alternatively, a first order autoregressive variance-covariance structure was used if this increase model fit measured via AIC. Normal distribution and homogeneous variance were checked graphically via residual plots. For the traits dry weight of inflorescence, upper and lower leaves, as well as CBD, CBG, CBGA and THCA, the data were logarithmically transformed to fulfil the requirement concerning homogeneous variance (within one or both years) and normal distribution. In this case, estimates were back transformed for presentation purpose only. Standard errors were back transformed using the delta method. After finding significant differences via global F-test, a *Tukey*-test at a significance level of 5% was used.

Statistical analysis was conducted by using the statistical software SAS version 9.4 (The SAS Institute, NC, USA).

3. Results and Discussion

3.1. Yield Parameters

The fractionated biomass (g m^{-2} DW) of upper leaves, lower leaves and inflorescence was significantly affected by growth stages (S1–S4) across the two growing seasons (2017, 2018). Accordingly, DW means of the different genotypes are described separately for each fraction across both years (Table 2).

Table 2. Mean dry weight (DW) in g m^{-2} of genotypes Fédora17, Félina32, Ferimon, Finola, Futura75, Santhica27 and USO31 over the years 2017 and 2018. Biomass was fractionated into upper leaves, lower leaves and inflorescence. Harvest took place at growth stages S1 to S4 defined after Mediavilla et al. [25]. Results are presented as mean values ± standard error (Mean ± SE). Each mean based on 60 observations. Letters compare the mean dry weight yield, means in one column followed by the same letter are not significantly different as indicated by *Tukey*-test ($\alpha = 0.05$). The *p*-values correspond to global F tests for difference between the levels of the mentioned genotypes, growth stages S1 to S4 or their interactions. S1 = vegetative growth stage, S2 = bud stage, S3 = full-flowering stage and S4 = seed maturity stage.

Genotype	Upper Leaves DW [g m^{-2}]	Lower Leaves DW [g m^{-2}]	Inflorescence DW [g m^{-2}]		
	S1–S4	S1–S4	S2	S3	S4
Fédora17	215.85 ± 43.52^{a}	204.02 ± 44.73^{a}	11.16 ± 5.10^{ab}	60.82 ± 27.79^{a}	442.00 ± 201.98^{a}
Félina32	181.66 ± 36.63^{ab}	168.97 ± 37.26^{ab}	4.90 ± 2.24^{b}	52.35 ± 23.92^{a}	303.39 ± 138.64^{a}
Ferimon	159.10 ± 32.08^{bc}	164.93 ± 36.16^{ab}	6.82 ± 3.12^{b}	56.59 ± 25.86^{a}	257.28 ± 117.57^{a}
Finola	121.65 ± 26.09^{c}	95.75 ± 23.80^{b}	205.48 ± 116.98^{a}	363.92 ± 207.18^{a}	352.54 ± 200.77^{a}
Futura75	185.57 ± 37.42^{ab}	174.85 ± 38.34^{ab}	4.46 ± 2.04^{b}	40.60 ± 18.55^{a}	259.56 ± 118.61^{a}
Santhica27	206.46 ± 41.63^{ab}	229.59 ± 50.34^{a}	9.58 ± 4.38^{ab}	27.17 ± 12.41^{a}	408.99 ± 186.89^{a}
USO31	164.80 ± 33.23^{ac}	184.37 ± 40.42^{ab}	14.45 ± 6.61^{ab}	84.30 ± 38.52^{a}	299.97 ± 140.06^{a}
***p*-values**					
Genotype [G]	0.0015	0.0390		0.1022	
Growth Stage [S]	0.8681	0.5810		0.0343	
G×S Interactions	0.1174	0.1749		0.0135	

DW of upper leaves did not show significant interactions between genotype and growth stage. The average DW (across growth stages) ranged from 121.65 ± 26.09 g m^{-2} (Finola) to 215.85 ± 43.52 g m^{-2} (Fédora17; Table 2). This is also reflected in average DW of lower leaves, which ranged from 95.75 ± 23.80 g m^{-2} (Finola) to 229.59 ± 50.34 g m^{-2} (Santhica27; Table 2). No significant interactions between genotypes and growth stages were obtained. Genotype Finola produced the lowest DW yield of upper and lower leaves across growth stages. In both years, lower leaves were already senescent at

S4. In 2018 the early loss of leaves might have been caused by extremely low precipitation in August and September.

DW yield of inflorescence showed significant interactions between genotype and growth stage. At the beginning of flower formation (S2), when bracts with no pistils where visible, genotype Finola reached the highest inflorescence yield with 205.48 ± 116.98 g m^{-2} while other genotypes, ranged from 4.46 ± 2.04 g m^{-2} (Futura75) to 14.45 ± 6.61 g m^{-2} (USO31; Table 2). At the beginning of full-flowering (S3), where 50% of the bracts are formed, Finola had the highest yields (363.92 ± 207.18 g m^{-2}) compared to the other genotypes, which ranged from 27.17 ± 12.41 g m^{-2} (Santhica27) to 84.30 ± 38.52 g m^{-2} (USO31; Table 2). Campiglia et al. [20] reported for the genotypes Fédora17, Félina32, Ferimon and USO31 ten days after full-flowering, a higher inflorescence yield than for Futura75 and Santhica27 at a fertilization level of 100 kg N ha^{-1} and a plant density of 120 plants m^{-2}.

At growth stage S4, 50% of the seeds were mature. No statistical differences were observed for DW of inflorescence between genotypes. DW of inflorescence ranged between 257.28 ± 117.57 g m^{-2} (Ferimon) and 442.00 ± 201.98 g m^{-2} (Fédora17; Table 2). Tang et al. [28] reported that the threshing residue of inflorescence collected during seed harvest, supplied an estimated biomass yield up to 200 g m^{-2}. For the monecious hemp genotypes (Fédora17, Félina32, Ferimon and Futura75) cultivated in Italy (Lat. 45° N; 10° E), an average inflorescence yield of 250 g m^{-2} and leaf yield of 230 g m^{-2} at full-flowering stage and seed maturity stage, was reported. In contrast to the present study, genotype Fédora17 reached a 7.6% lower inflorescence yield, whereas the other genotypes reached in average a 33% lower inflorescence yield.

Cherney and Small [29] described Finola as a day-length insensitive and early maturing genotype with high inflorescence yield and a short habit. The present study verified the findings of Callaway [30] indicating that Finola produced less dry stem and less leaf biomass than other genotypes, with an average seed yield close to 1.7 t ha^{-1} in Finland. Seed yield surpasses results from other industrial hemp genotypes to date.

3.2. Quality Parameters

3.2.1. CBDA and CBD Content

The highest content of CBDA in inflorescence at S2 was measured in genotype Félina32 (2.941%), followed by Ferimon (2.687%), Futura75 (2.593%) and Fédora17 (2.430%). The lowest content was found at S2 in dried inflorescence of Finola (0.995%), USO31 (0.785%) and Santhica27 (0.185%; Figure 3). At full-flowering stage (S3), average CBDA contents differed significantly between genotypes. The highest contents were measured in Futura75 (2.719%), Félina32 (2.533%), followed by Fédora17 (2.220%) and Ferimon (1.793%; Figure 3). The present results are in line with findings of Sikora et al. [31], indicating a range of 1.444% to 2.039% cannabidiol in the upper third of the plant, including leaves and inflorescence of genotype Fédora19, Ferimon12 and Futura77. Santhica27 and USO31 exhibited the lowest values with 0.647% and 0.589%, respectively (Figure 3). At seed maturity (S4), genotype Futura75 (1.759%), Félina32 (1.639%) and Fédora17 (1.363%), as well as Finola (1.613%) indicated the highest CBDA values in their inflorescence (Figure 3).

Average CBDA contents of dried upper leaves, in S1 ranged from 0.104% (Ferimon) to 0.902% (Futura75; Figure 4). At S2 values ranged from 0.139% (Santhica27) to 1.322% (Futura75; Figure 4). The highest CBDA content of the dried upper leaf fraction was obtained at S3. Genotype Futura75 (2.422%) indicated the highest contents, followed by Félina32 (2.116%), Ferimon (2.014%) and Fédora17 (1.954%; Figure 4). At seed maturity (S4), CBDA contents of dried upper leaves decreased and ranged from 0.203% (Santhica27) to 1.888% (Fédora17; Figure 4).

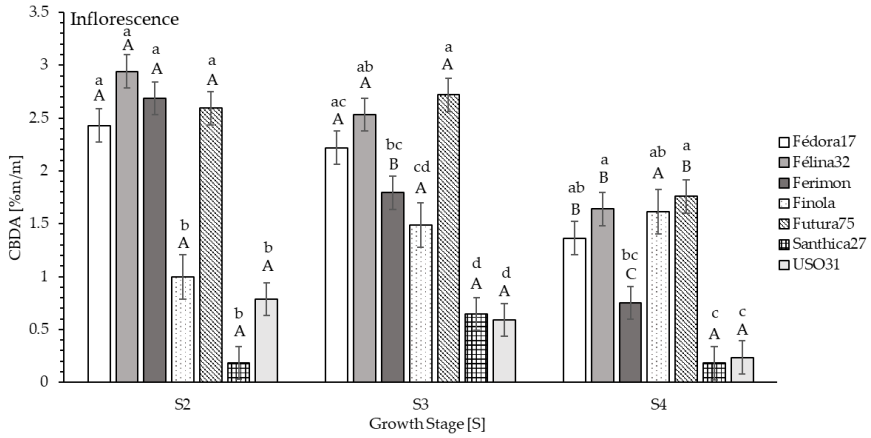

Figure 3. Mean content of CBDA (cannabidiolic acid) in mass percent [$\%_{m/m}$] of genotypes Fédora17, Félina 32, Ferimon, Finola, Futura75, Santhica27 and USO 31, over the years 2017 and 2018. CBDA content was analysed in inflorescence. Harvest took place at growth stages S2 to S4 defined after Mediavilla et al. [25]. Means covered with the same lower-case letter did not differ significantly at $\alpha = 0.05$, within a growth stage. Means covered with the same upper-case letter did not differ significantly at $\alpha = 0.05$, within a genotype as indicated by *Tukey*-test.

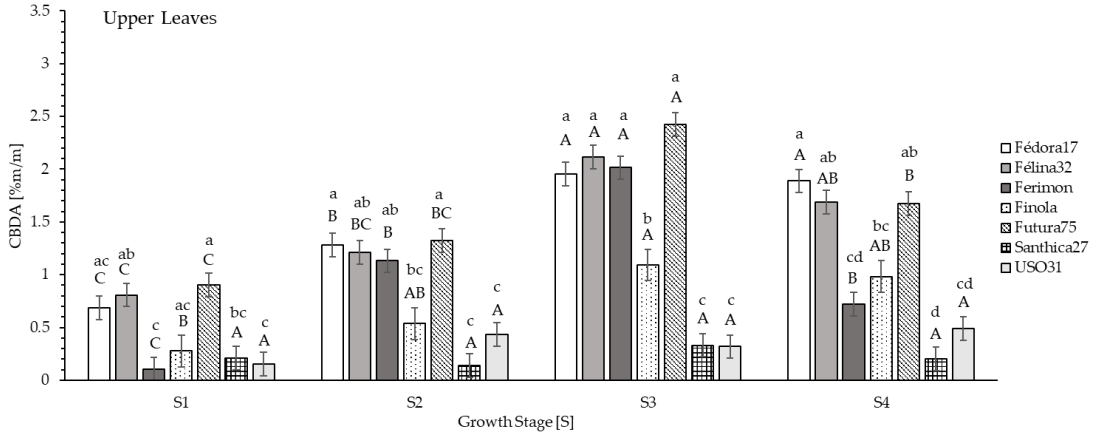

Figure 4. Mean content of CBDA in mass percent [$\%_{m/m}$] of genotypes Fédora17, Félina 32, Ferimon, Finola, Futura75, Santhica27 and USO 31, over the years 2017 and 2018. CBDA content was analysed in upper leaves. Harvest took place at growth stages S1 to S4 defined after Mediavilla et al. [25]. Means covered with the same lower-case letter did not differ significantly at $\alpha = 0.05$, within a growth stage. Means covered with the same upper-case letter did not differ significantly at $\alpha = 0.05$, within a genotype as indicated by *Tukey*-test.

Generally, the CBDA content of the lower leaves was lowest in both growth stages. S2 ranged from 0.041% (Santhica27) to 0.319% (Futura75) and S3 ranged from 0.104% (USO31) to 1.670% (Futura75). The highest content of the lower leaves over all genotypes was measured at S3. Genotype Futura75 indicated the highest CBDA content (1.670%) compared to all other genotypes. The lowest content was obtained in Finola (0.324%), Santhica27 (0.208%), and USO31 (0.104%; Figure 5).

Average CBD contents of inflorescence over the growth stages (S2–S4) ranged from 0.010% (Santhica27) to 0.497% (Ferimon). The highest CBD content was determined in inflorescence of Ferimon (0.497%) at growth stage S2, while e.g., Santhica27 only reached a CBD content of 0.045% at S2. No CBD was determined (n.d.) for the other genotypes at S2. At growth stage S3, CBD content ranged from 0.111% (USO31) to 0.331% (Fédora17) and at S4 from 0.010% (Santhica27) to 0.170% (Finola; Table 3).

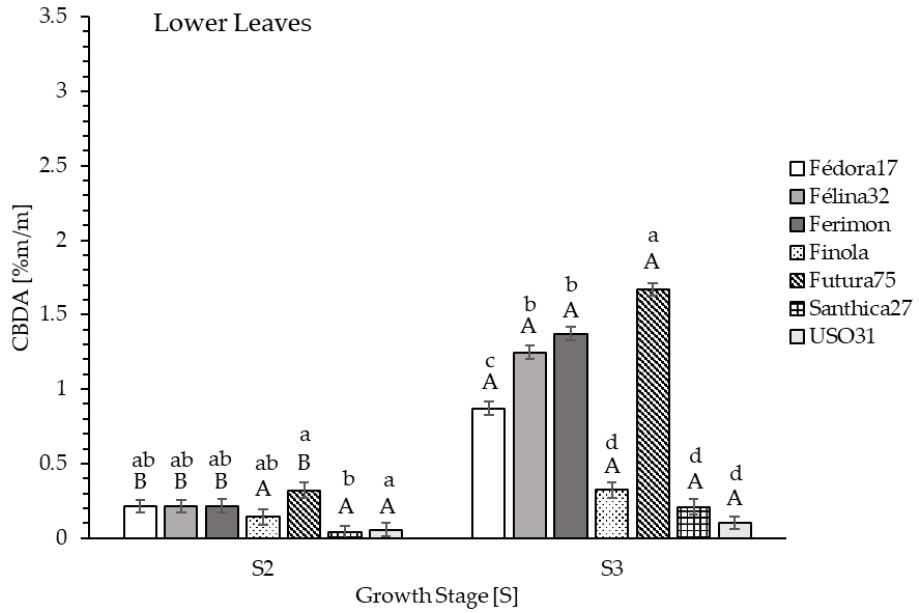

Figure 5. Mean content of CBDA in mass percent [$\%_{m/m}$] of genotypes Fédora17, Félina 32, Ferimon, Finola, Futura75, Santhica27 and USO 31, over the years 2017 and 2018. CBDA content was analysed in lower leaves. Harvest took place at growth stages S2 to S3 defined after Mediavilla et al. [25]. Means covered with the same lower-case letter did not differ significantly at $\alpha = 0.05$, within a growth stage. Means covered with the same upper-case letter did not differ significantly at $\alpha = 0.05$, within a genotype as indicated by *Tukey*-test.

Table 3. Mean content of CBD (cannabidiol) in mass percent [$\%_{m/m}$] of genotypes Fédora17, Félina32, Ferimon, Finola, Futura75, Santhica27 and USO31, over the years 2017 and 2018. CBD content was analysed in inflorescence. Harvest took place at growth stages S1 to S4 defined after Mediavilla et al. [25]. Results are presented as mean values ± standard error (Mean ± SE). Each mean based on 60 observations. Letters compare the mean CBD content, means in one column followed by the same letter are not significantly different as indicated by *Tukey*-test ($\alpha = 0.05$). The *p*-values correspond to global F tests for difference between the levels of the mentioned genotypes, growth stages S1 to S4 or their interactions. S1 = vegetative growth stage, S2 = bud stage, S3 = flowering stage and S4 = seed maturity stage, n.d. = not determined, values were below the detection limit of 0.0015%.

CBD Content [$\%_{m/m}$]			
Genotype	**Inflorescence**		
	S2	**S3**	**S4**
Fédora17	n.d.	0.331 ± 0.107^{a}	0.106 ± 0.034^{ab}
Félina32	n.d.	0.217 ± 0.070^{a}	0.164 ± 0.164^{a}
Ferimon	0.497 ± 0.160^{a}	0.233 ± 0.075^{a}	0.026 ± 0.008^{bc}
Finola	n.d.	0.152 ± 0.070^{a}	0.170 ± 0.078^{ab}
Futura 75	n.d.	0.147 ± 0.047^{a}	0.106 ± 0.034^{ab}
Santhica27	0.045 ± 0.014^{b}	0.114 ± 0.037^{a}	0.010 ± 0.003^{c}
USO31	n.d.	0.111 ± 0.036^{a}	0.041 ± 0.013^{ac}
***p*-values**			
Genotype [G]		0.0087	
Growth Stage [S]		0.0042	
G×S Interactions		<0.0001	

For the upper leaves at S1, the highest value was determined for Félina32 (0.155%), followed by USO31 (0.004%) and Fédora17 (0.003%). No CBD was determined for the other genotypes in upper leaves at the vegetative growth stage. The same applied for upper and lower leaves at growth stage S2, where the highest value was shown for Ferimon (0.060%), followed by Santhica27 (0.030%) and Futura75 (0.007%). At growth stage S3, CBD contents ranged from 0.016% (Santhica27) to 0.162% (Ferimon). The contents decreased at S4, in a range of 0.003% (Santhica27) to 0.090% (Félina32). In general, the mean CBD content of the lower leaf fraction obtained the lowest values. At S2, mostly no CBD was determined while at S3 the contents ranged from 0.080% (Futura75) to 0.006% (USO31; Table 4).

Table 4. Mean content of CBD in mass percent [$\%_{m/m}$] of genotypes Fédora17, Félina32, Ferimon, Finola, Futura75, Santhica27 and USO31, over the years 2017 and 2018. CBD content was analysed in upper leaves and lower leaves. Harvest took place at growth stages S1 to S4 defined after Mediavilla et al. [25]. Results are presented as mean values ± standard error (Mean ± SE). Each mean based on 60 observations. Letters compare the mean CBD content, means in one column followed by the same letter are not significantly different as indicated by *Tukey*-test ($\alpha = 0.05$). The *p*-values correspond to global F tests for difference between the levels of the mentioned genotypes, growth stages S1 to S4 or their interactions. S1 = vegetative growth stage, S2 = bud stage, S3 = flowering stage and S4 = seed maturity stage, n.d. = not determined, values were below the detection limit of 0.0015%.

	CBD Content [$\%_{m/m}$]					
Genotype	**Upper Leaves**				**Lower Leaves**	
	S1	**S2**	**S3**	**S4**	**S2**	**S3**
Fédora17	0.003 ± 0.001^{b}	n.d.	0.106 ± 0.047^{a}	0.074 ± 0.033^{a}	n.d.	0.051 ± 0.017^{ab}
Félina32	0.155 ± 0.070^{a}	n.d.	0.136 ± 0.061^{a}	0.090 ± 0.041^{a}	n.d.	0.061 ± 0.020^{ab}
Ferimon	n.d.	0.060 ± 0.027^{a}	0.162 ± 0.073^{a}	0.014 ± 0.006^{ab}	0.011 ± 0.004^{a}	0.063 ± 0.021^{ab}
Finola	n.d.	n.d.	0.094 ± 0.057^{a}	0.036 ± 0.022^{ab}	n.d.	0.026 ± 0.011^{ac}
Futura75	n.d.	0.007 ± 0.003^{ab}	0.053 ± 0.024^{a}	0.065 ± 0.029^{a}	0.003 ± 0.001^{ab}	0.080 ± 0.026^{a}
Santhica27	n.d.	0.030 ± 0.013^{a}	0.016 ± 0.007^{a}	0.003 ± 0.002^{b}	0.004 ± 0.001^{ab}	0.013 ± 0.004^{bc}
USO31	0.004 ± 0.002^{b}	n.d.	0.036 ± 0.016^{a}	0.026 ± 0.012^{ab}	n.d.	0.006 ± 0.002^{c}
***p*-values**						
Genotype [G]			<0.0001			<0.0001
Growth Stage [S]			<0.0001			<0.0001
G×S Interactions			<0.0001			0.0002

Neutral cannabinoids do not exist at high concentrations in fresh plant material. *C. sativa* L. biosynthesizes mainly the carboxylic acid forms of cannabidiol (CBD), cannabigerol (CBG), tetrahydrocannabinol (THC), namely cannabidiolic acid (CBDA), cannabigerolic acid (CBGA) and tetrahydrocannabinolic acid (THCA) [32]. Cannabinoids are present in all aerial parts of the plant, correlated with the number of grandular trichomes, especially present on leaves and bracts [33].

For CBDA, the acid precursor of CBD as decarboxylated form of the compound in growing plants, genotypes Futura75, Félina32 and Fédora17 indicated the highest values over all growth stages and all fractions of the plant (Figures 3–5). Genotype Ferimon likewise showed the highest CBDA content only at growth stage S2 and S3, whereas genotype Finola obtained the highest CBDA content in inflorescence at S4. In order to optimize CBD/A at field level, the growth stage at harvest and the plant fraction seems to be highly important. The concentration of cannabinoids depends on tissue type, genotype, age, harvest time and growth conditions as reported by Khan et al. [34]. Stout et al. [35] reported the highest CBD/A content in female flowers and a substantially lower content in the other tissues. In particular, dried inflorescence of genotype Futura75 contained the highest content of CBDA at growth stage S2 (2.593%) and S3 (2.719%). According to Hillig and Mahlberg [36] phytocannabinoids are accumulated in inflorescence, which bear most of the trichomes produced by the hemp plant. In the upper leaf fraction, CBDA content increased over time, starting with the lowest value at growth stage S1 (0.902%), 1.322% at S2, while reaching the highest values at growth stage S3 (2.422%). At S4 the levels decreased to 1.674%. Both upper leaves and inflorescence showed the highest increase in CBDA at full-flowering stage (S3; Figures 3 and 4). Results on the increase of CBDA in leaves to a maximum at

S3 followed by a subsequent decrease with plant age and a maximum content in the upper leaves were in line with results of Pacifico et al. [37]. The highest CBDA content was recorded in leaves of a fibre genotype with 2.40% 76 days from sowing. After 76 days, a decrease of the average CBDA content in leaves was observed [37]. Mandolino and Ranalli [38] reported that in proximity of full-flowering, the content of cannabinoids reached a maximum in trichome-rich organs like inflorescence. The same trend was observed for genotypes Fédora17 and Félina32. The content of CBDA in inflorescence showed a maximum at growth stage S2 (2.430% and 2.941%) and S3 (2.220% and 2.533%), respectively (Figure 3).

The higher CBDA content was found in genotypes Futura75, Fédora17, Félina32 and Ferimon. Note that, these industrial hemp genotypes, which comply with the 0.2% THC/A threshold set by the EU legislation restricts the choice of genotypes for European farmers, compared to producers in Switzerland, North America, Asia and Canada (limits from 0.3% up to 1%). However, the CBDA, differs in respect to DW yield between growth stages. Campbell et al. [39] reported, that 83% of variance in CBD/A content resulted from genetic effects, making genotype selection important when seeking high CBD/A contents. Environmental factors such as the positive correlation between CBD/A content and water availability found by Calzolari et al. [23] slightly increase CBD/A content, but the change was small enough that inflorescence yield is far more important to overall yield than a slight change in CBD/A percentage [39].

While genotype Futura75 generated a 84% higher inflorescence yield, genotypes Fédora17, Félina32 and Ferimon reached a 86%, 83% and 78% higher inflorescence yield at S4, compared to S3. No significant differences were recorded for upper leaf DW between growth stage S3 and S4 (Table 2). Taking these results into account, the calculated, total CBDA yield per square meter was higher at S4. In particular, genotype Futura75 reached a 76% higher CBDA yield at S4 (4568.26 mg m^{-2}) compared to S3 (1104.32 mg m^{-2}). The same trend was observed for the other three genotypes Fédora17, Félina32 and Ferimon, with a CBDA yield of 1350.20 mg m^{-2}, 1324.46 mg m^{-2} and 1012.96 mg m^{-2} at S3, in comparison to 6011.20 mg m^{-2}, 4975.60 mg m^{-2} and 1929.60 mg m^{-2} at S4, respectively.

The industrial hemp genotypes are mainly bred for fibre and seed production. Both, Futura75 and Fedora17 are candidate cultivars for a dual-purpose production in the EU, with Futura75 being more suitable for fibre production and Fédora17 for seed production [28]. At full-flowering, stem yield for bast fibres is positively correlated with the duration of vegetative growth, with a tendency to be high in intermediate flowering genotypes, such as Futura75. Inflorescence yield and accordingly seed production of early flowering genotypes, such as Fédora17, Félina32, Fermion and USO31 are proven to be higher [20].

This study found that industrial hemp genotypes are suitable for non-psychotropic cannabinoid production, namely CBD, CBG and their acid precursors. In addition to seed and fibre production, recently, hemp genotypes registered within the EU, have been cultivated for inflorescence to finally extract non-psychotropic cannabinoids [9]. The utilization of harvested inflorescence for cannabinoid extraction, seems to exclude seed production at the same time as plants are harvested at full-flowering stage, thus limiting the full exploitation of the hemp crop [7]. However, threshing residues of inflorescence obtained at seed maturity might offer a unexploited high-value product for the extraction of cannabinoids [21].

Within the tested genotypes, CBD/A was determined in inflorescence at full-flowering stage, as well as in inflorescence resulting from seed threshing. In addition, higher dry matter yields of inflorescence per square meter at S4, supported the assumption, that these genotypes can be cultivated as multi-purpose crop: for seed, oil and fibre production as well as for the additional extraction of cannabinoids out of the remaining material. Cannabinoids can be extracted from the reproductive plant parts and foliage. The inflorescence material has a higher concentration of cannabinoids than foliage material, however foliage part have larger biomass of the hemp plant [40]. The breeding of genotypes with superior characteristics is required to optimize both seed and fibre production, but also the quality and quantity of cannabinoids by residual inflorescence and upper leaves.

Furthermore, the only diocious industrial hemp genotype, Finola, which is specifically bred for oil production [41], recorded a 85% higher DW yield of inflorescence at full-flowering stage (S3) in comparison to the average yield of the other genotypes (Table 2). Subsequently, CBDA yield per square meter amounted to 5422.41 mg m^{-2}, and showed in average a 78% higher yield of CBDA per square meter compared with the other genotypes. No significant differences were shown for Finola in CBDA content between S3 and S4, as well as in DW of inflorescence at S4. If Finola is cultivated for single purpose only, cannabinoid extraction can be carried out from full-flowering to seed maturity. Aiming at dual or multi-purpose, Finola can be harvested after seed maturity. Notable is also the length of the cultivation period: while genotype Finola reached stage S3 after 62 days, together with Fédora17, Santhica27 and USO31, the other genotypes needed 80 days to reach full-flowering (S3) in 2018. Furthermore, after 80 days, Finola reached the seed maturity stage, whereas the other six genotypes reached seed maturity after 130 days. A short vegetation period, combined with a high DW yield and CBDA content, resulted in an optimized land utilization. Moreover, a 10:1 ratio of CBD/THC, is above the recent EU requirements of 2:1 [30], what outlines Finola with a high CBDA/low THCA profile as an interesting genotype for cannabidiol [35].

3.2.2. CBGA and CBG Content of Genotype Santhica27

The first cannabinoid synthetized is cannabigerol (CBG), produced by condensation of a phenol-derived olivetolic acid and a terpene-based geranyl diphosphate. The process is catalysed by geranyldiphosphate:olivetolate geranyltransferase (GOT) [42]. CBG was only determined in considerable amounts in genotype Santhica27 (Figure 6A–D). Fournier et al. [43] reported a new chemotype, initially found in a French fibre hemp population, normally predominant in CBD, having CBG as the major constituent. In these genotypes the pathway CBG to tetrahydrocannabinol (THC) or cannabidiol (CBD) is largely obstructed [44]. In the present study, average CBG contents in the inflorescence over the growth stages S2 to S4 ranged from 0.137% (S4) to 0.520% (S3; Figure 6A). The highest contents were found at growth stage S2 (0.386%) and S3 (0.520%). In the upper leaf fraction the contents ranged from 0.050% (S2) to 0.081% (S1; Figure 6B). The highest contents were found at S1 (0.056%) and S3 (0.081%). The lowest amounts were determined in lower leaves; maximum levels were recorded at growth stage S3 with 0.008%. The content of cannabigerolic acid (CBGA), the acid precursor of CBG, showed the highest content in the inflorescence at growth stage S2, with 3.235%, followed by S3, with 1.534%. The lowest content was determined at growth stage S4, with 1.399%, compared to S2 (Figure 6C). In the upper leaves the content of CBGA did not show any statistical differences among the growth stage S2 to S4, while the values ranged from 0.552% (S1) to 1.040% (S4; Figure 6D). The lowest content was determined again in lower leaves, ranging from 0.085% (S2) to 0.801% (S3). This is in agreement with results on CBG concentrations between 0.4 and 1.2% in leaves and inflorescence of threshing residues reported by Calzolari et al. [23].

Overall, the highest amounts of CBG and the acid precursor CBGA were determined in inflorescence of Santhica27, at growth stage S2 and S3. The CBG content of upper leaves showed a maximum at growth stage S1 and S3, with no statistical differences and a maximum of CBGA at growth stages S2 to S4. Referring to CBG accumulation Pacificio et al. [37], stated that in high-CBG plants, a maximum level of CBG accumulation proceeded before the maximum CBD accumulation in leaves was obtained. With regard to a high exploitation of CBG, over all fractions, a harvest at growth stage S2 or S3 can be recommended. This is in contrast to studies of Calzolari et al. [23], where harvest time did not have an effect on CBG content of genotype Santhica27. Particularly, with regard to DW yield, genotype Santhica27 showed a 93% higher DW yield of inflorescence per square meter at S4, compared to S3. In this respect, a CBGA yield of 416.79 mg m^{-2} was calculated for S3, compared to 5721.77 mg m^{-2} at S4, with a DW yield of 408.99 g m^{-2}. For S3 a CBG yield of 141.28 mg m^{-2} was calculated, while at S4 a yield of 560.32 mg m^{-2} CBG was reached. Whilst CBG/A was found exclusively in genotype Santhica27, it shows the potential of this genotype to be used for CBG/A extraction as well as seed and fibre production.

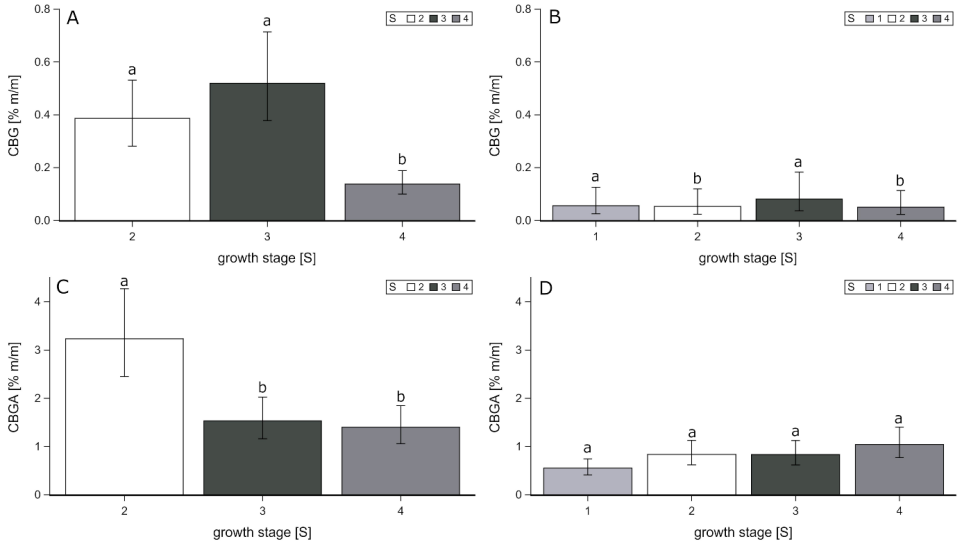

Figure 6. CBG (cannabigerol) content in mass percent [$\%_{m/m}$] of inflorescence (**A**) upper leaf fraction (**B**) of genotype Santhica27, cultivated in 2018. CBGA (cannabigerolic acid) content of extracts in mass percent [$\%_{m/m}$] of flower (**C**) upper leaf fraction (**D**) of genotype Santhica27, cultivated in 2018. Means covered with the same lower-case letter did not differ significantly at $\alpha = 0.05$ as indicated by *Tukey*-test.

3.2.3. THCA and THC Content

Tetrahydrocannabinolic acid (THCA) contents of inflorescence and upper leaves showed significant interactions between genotype and growth stage. Average THCA in the inflorescence, ranged from 0.003% to 0.051% (Table 5) and in upper leaves from 0.004% to 0.051% (Table 6). Average tetrahydrocannabinol (THC) contents in inflorescence ranged from 0.001% to 0.101% with no statistically difference. These values met for all genotypes in all tested growth stages the required EU THC/THCA limit below 0.2% [45], which is a prerequisite for the cultivation and harvest of these genotypes for cannabinoid extraction. No THC/THCA was determined in lower leaf DW.

Table 5. Mean content of THCA (tetrahydrocannabinolic acid) in mass percent [$\%_{m/m}$] of genotypes Fédora17, Félina32, Ferimon, Finola, Futura75, Santhica27 and USO31, over the years 2017 and 2018. THCA content was analysed in inflorescence. Harvest took place at growth stages S1 to S4 defined after Mediavilla et al. [25]. Results are presented as mean values ± standard error (Mean ± SE). Each mean based on 60 observations. Letters compare the mean THCA content, means in one column followed by the same letter are not significantly different as indicated by *Tukey*-test ($\alpha = 0.05$). The *p*-values correspond to global F tests for difference between the levels of the mentioned genotypes, growth stages S1 to S4 or their interactions. S1 = vegetative growth stage, S2 = bud stage, S3 = flowering stage and S4 = seed maturity stage, n.d. = not determined, values were below the detection limit of 0.0015%.

THCA Content [$\%_{m/m}$]			
Genotype	**Inflorescence**		
	S2	**S3**	**S4**
Fédora17	0.003 ± 0.002^{ab}	0.048 ± 0.027^{a}	0.040 ± 0.023^{a}
Félina32	n.d.	0.020 ± 0.011^{a}	0.048 ± 0.027^{a}
Ferimon	0.051 ± 0.029^{a}	0.037 ± 0.021^{a}	0.009 ± 0.005^{a}
Finola	n.d.	0.049 ± 0.037^{a}	0.049 ± 0.037^{a}
Futura75	n.d.	0.014 ± 0.008^{a}	0.046 ± 0.026^{a}
Santhica27	0.004 ± 0.002^{ab}	0.007 ± 0.004^{a}	0.005 ± 0.003^{a}
USO31	n.d.	0.008 ± 0.004^{a}	0.005 ± 0.003^{a}
***p*-values**			
Genotype [G]		0.0991	
Growth Stage [S]		0.0811	
G×S Interactions		0.0029	

Table 6. Mean content of THCA in mass percent [$\%_{m/m}$] of genotypes Fédora17, Félina32, Ferimon, Finola, Futura75, Santhica27 and USO31, over the years 2017 and 2018. THCA content was analysed in upper leaves. Harvest took place at growth stages S1 to S4 defined after Mediavilla et al. [25]. Results are presented as mean values ± standard error (Mean ± SE). Each mean based on 60 observations. Letters compare the mean THCA content, means in one column followed by the same letter are not significantly different as indicated by *Tukey*-test ($\alpha = 0.05$). The *p*-values correspond to global F tests for difference between the levels of the mentioned genotypes, growth stages S1 to S4 or their interactions. S1 = vegetative growth stage, S2 = bud stage, S3 = flowering stage and S4 = seed maturity stage, n.d. = not determined, values were below the detection limit of 0.0015%.

THCA Content [$\%_{m/m}$]				
Genotype	**Upper Leaves**			
	S1	**S2**	**S3**	**S4**
Fédora17	0.004 ± 0.002^{ac}	n.d.	0.051 ± 0.022^{a}	0.048 ± 0.021^{a}
Félina32	0.030 ± 0.013^{a}	n.d.	0.047 ± 0.020^{a}	0.048 ± 0.021^{a}
Ferimon	n.d.	0.039 ± 0.017^{a}	0.040 ± 0.017^{a}	0.009 ± 0.004^{a}
Finola	n.d.	n.d.	0.045 ± 0.025^{a}	0.035 ± 0.020^{a}
Futura75	0.005 ± 0.002^{ac}	0.004 ± 0.002^{ab}	0.011 ± 0.005^{a}	0.049 ± 0.021^{a}
Santhica27	0.024 ± 0.011^{ab}	0.008 ± 0.003^{ab}	0.018 ± 0.008^{a}	0.005 ± 0.002^{a}
USO31	0.031 ± 0.014^{a}	n.d.	0.009 ± 0.004^{a}	0.011 ± 0.005^{a}
***p*-values**				
Genotype [G]	0.1735			
Growth Stage [S]	0.0233			
G×S Interactions	<0.0001			

4. Conclusions

The results of this study showed that the content of terpenophenolic secondary metabolites, namely cannabinoids, highly depend on the genotype and the growth stage of the plant. Biomass yield of leaves and inflorescence must be considered for an optimized harvest result. Industrial hemp genotypes, like Futura75, Fédora17, Félina32 and Ferimon can be cultivated in Europe, as a dual or multipurpose crop for biomass production and CBD/A extraction. Genotype Santhica27 was found to not be appropriate for CBD/A production. But it was found that genotype Santhica27 indicated the highest contents of CBG/A. Further studies should be addressed to the ecological and phytochemical behavior of these industrial hemp genotypes in different environmental conditions. This would be important for the possible end use of the genotypes and support farmers to select the correct variety for their purpose and agronomic environment.

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4 Publication II: Impact of Different Phytohormones on Morphology, Yield and Cannabinoid Content of *Cannabis sativa* L.

Burgel, L., Hartung, J., Schibano, D., Graeff-Hönninger, S. (2020): Impact of Different Phytohormones on Morphology, Yield and Cannabinoid Content of *Cannabis sativa* L. Plants 2020, 9, 725**.**

In 2017, with the legalization of cannabis-based pharmaceuticals in Germany and adaptation of regulatory frameworks throughout the world, studies have focused on a rapidly growing market for cannabidiol (CBD) in pharmaceuticals, nutraceuticals and wellness products. The cannabinoid content of cannabis leaves and flowers depends primarily on the genotype. The amount of reported CBD values may vary due to the method of cultivation. High-quality flower material of the cannabis plant includes a continuous and uniform floral yield and a constant synthesis of a specific cannabinoid compound, produced in an economically feasible framework. Therefore, a better understanding of how the morphology of the cannabis plant affects flower formation to optimize CBD yields per unit area is needed for indoor cultivation with minimum space requirements. Thus, publication II investigates the impact of exogenously applied synthetic plant growth regulators (PGRs), namely 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), and a mixture of both on plant architecture of phytocannabinoid-rich cannabis genotypes, to evaluate a potential benefit of PGR application to adapt plant morphology for various indoor cultivation systems.

plants

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Article

Impact of Different Phytohormones on Morphology, Yield and Cannabinoid Content of *Cannabis sativa* L.

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Abstract: The impact of exogenously applied plant growth regulators (PGR), 1-naphthalenaecetic acid (NAA), 6-benzylaminopurine (BAP), and a mixture of both (NAA/BAP-mix), was investigated in regard to plant height, length of axillary branches, number of internodes, biomass yield and cannabinoid content of three different phytocannabinoid-rich (PCR) *Cannabis* genotypes. The results showed that total plant height was significantly reduced under the application of NAA (28%), BAP (18%), and NAA/BAP-mix treated plants (15%). Axillary branch length was also significantly reduced by 58% (NAA) and 30% (NAA/BAP-mix). BAP did not significantly reduce the length of axillary branches. The number of internodes was reduced by NAA (19%), BAP (10%), and the NAA/BAP-mix (14%) compared to the untreated control. NAA application influenced the plant architecture of the tested cv. KANADA beneficially, resulting in a more compact growth habitus, while inflorescence yield (23.51 g plant^{-1}) remained similar compared to the control (24.31 g plant^{-1}). Inflorescence yield of v. 0.2x and cv. FED was reduced due to PGR application while cannabinoid content remained stable. Overall, the application of PGR could be used on a genotype-specific level to beneficially influence plant architecture and optimize inflorescence yield per unit area and thus cannabinoid yield, especially in the presence of space limitations under indoor cultivation.

Keywords: *Cannabis sativa* L.; growth regulators; indoor growth; plant morphology; length of axillary branches; number of internodes; plant height; biomass yield; cannabinoids

1. Introduction

Cannabis sativa L. has a long history of cultivation for medicinal and food purposes as well as a source of textile fibers [1,2]. Five chemotypes of *Cannabis* were recognized and classified based on their cannabinoid profile and concentration: Chemotype I has a high Δ^9-tetrahydrocannabinol/cannabidiol (THC/CBD) ratio (>1); plants with an intermediate ratio (≈1) are defined as chemotype II; fiber-type plants with a low THC/CBD ratio (<1) are defined as chemotype III; plants containing cannabigerol acid (CBGA) as their main cannabinoid are defined as chemotype IV [3], and chemotype V contains almost no cannabinoids [4–7].

Over the next few years, the cultivation of *C. sativa* is expected to rise based on adaptations to regulatory frameworks throughout the world, which may promote the constitution of new companies and exploit products obtainable from *C. sativa*. Medical cannabis was legalized in Germany in March 2017 [8]. As such, the regulatory framework comprises a policy that provides broad access to medical *Cannabis*. Today, Germany is the leading medical *Cannabis* prescriber in Europe, followed by Italy and the Netherlands [8]. The use of CBD in nutraceuticals, cosmetics, and pharmaceuticals has renewed interest

in the effects of nonpsychotropic cannabinoids in particular [9,10]. Cultivation of *C. sativa* L. was banned in many states due to its psychoactive drug component Δ^9-THC [11]. Industrial hemp genotypes, which comply with the 0.2% THC threshold set by the European Union legislation, can be cultivated without restrictions by farmers within the EU [12]. Breeding efforts for medicinal purposes focused on CBD-enhanced chemotypes, called phytocannabinoid-rich (PCR) *Cannabis*. PCR chemotypes target contents of more than 10% CBD and less than 0.2% THC, with a minimal range of variation. However, to optimize yield and the overall content of nonpsychoactive cannabinoids, a better understanding of the relationship between morphology and flower formation is necessary.

The target for high-quality *Cannabis* production includes a continuous and uniform inflorescence yield and the production of a specific cannabinoid compound [13]. Since the pharmaceutical industry requires the highest quality, it is necessary to ensure consistency in the cannabinoid profile of *Cannabis* plants and the quality of female flowers intended for such use. To optimize inflorescence yield per unit area and thus cannabinoid yield, indoor cultivation systems with minimum space requirements are needed. Outdoor cultivation seems to be economically feasible for the isolation of pure compounds or extract preparations. Indoor cultivation has advantages in terms of quality assurance and hygienic standards, allowing contamination to be eliminated and homogeneous *Cannabis* batches to be produced under controlled conditions [14]. Even though indoor systems are expensive operations, interest regarding the efficient use of available growing space is rising.

Exogenously applied growth regulators, which are chemical analogues to phytohormones, can influence the height and side-branching of plants, resulting in greater biomass and seed production [15,16]. Naturally, they occur in plants, acting as signaling compounds at low concentrations [17]. The first discovered and best understood phytohormones are auxins (IAA) and cytokinins (CK) [18], which are classified as growth promotors according to their main function [19]. IAA, synthesized in the shoot apex in young leaves and transported basipetally to the roots [20], have keys role in the maintenance of apical dominance, as well as responsibility for cell elongation. Their main effects include rooting, stimulation, and inhibition of axillary bud outgrowth [21]. CKs are involved in meristem activity regulations [22], plant shape determination, plant adjustment to side conditions, and responses to the environment [23]. IAA and CK act either antagonistically or synergistically to control developmental processes, such as the formation and maintenance of meristem [24]. *C. sativa* plants grow vertically, focusing on the growth of one dominant main shoot, with smaller side branches surrounding it, producing small buds and resulting in a low inflorescence yield. IAA from intact shoot apexes inhibit axillary branching, whereas CK, induced by removing the shoot apex, stimulates axillar branching [25]. Hence, apical dominance is regulated by IAA and CK [25]. Additionally, shoot branching depends on the genotype, growth stage, and environmental factors, including day-length, light intensity, temperature, and nutrition [26,27]. To generate a large variety of plant forms, shoot branching is a major determinant of the plant architecture, regulated by endogenous and environmental cues [23]. Synthetic compounds with similar activities to endogenous plant hormones are called "plant growth regulators" (PGR) [28]. For example, 1-naphthalenaecetic acid (NAA) and 6-benzylaminopurine (BAP) are synthetic analogues of the endogenous phytohormones IAA and CK, respectively. The effect of phytohormones on the architecture of *C. sativa* L. has not yet been studied in detail [23].

It is hypothesized that both the removal of the shoot apex and the additional exogenous application of PGR can stimulate axillary side-branching and influence the number of short side branches. The targeted plant architecture would be a compact and bushy canopy to promote optimal air circulation and light utilization. Further, shorter side branches give plants more stability during bud development. Nevertheless, the total yield cannot be rated only by the number and weight of inflorescence, therefore the content of cannabinoids is also of great interest [13].

The aim of this study was to evaluate the impact of exogenously applied NAA, BAP, and a mixture of both PGR on plant architecture of three different PCR *Cannabis* genotypes. Total plant height, axillary branch length after removal of the shoot apex, and number of internodes were investigated in

detail. Furthermore, the biomass yield of inflorescence and leaves, as well as cannabinoid content, were determined.

2. Materials and Methods

2.1. Experimental Setup

A greenhouse experiment was set up to test the impact of exogenously applied plant growth regulators (PGR) on morphological characteristics of different phytocannabinoid-rich (PCR) *Cannabis* genotypes, namely cv. KANADA, cv. FED, and cv. 0.2x. The experiment was conducted at the University of Hohenheim, Germany, beginning on 28 November 2018 and finishing on 4 May 2019. Genotypes were kindly provided by the company Ai Fame, Switzerland. Genotypes were treated every fortnight with either 1-naphthaleneacetic acid (NAA) concentrated to 10 mg L^{-1}, or with 6-benzylaminopurine (BAP) concentrated to 50 mg L^{-1}, or a 1:5 mixture of 50% of NAA and 50% of BAP solution (NAA/BAP-mix). The experiment included a control group (control) that was sprayed with deionized water on the respective dates. NAA and BAP were both purchased from Sigma-Aldrich, St. Louis, USA. The first application took place 6 days after planting (DAP) and the last application was at 90 DAP. The applied solution amounted for each application 7.6 mL until 32 DAP and was increased to 11.0 mL for the following applications between day 32 and day 81. On the application days 88 and 90 DAP, the applied solution was increased to 27.0 mL for each application to spray the whole aboveground biomass of the plant.

Genotypes and treatments were randomly allocated to 36 plants according to a row–column design, which was established with four rows and three columns per replicate. Thus, three complete replicates existed.

2.2. Synthetic Growth Regulator Preparation

For the PGR solutions, 30 mg NAA was dissolved in 3 L of distilled water and 150 mg of BAP was dissolved in 3 L distilled water, with 15 mL Tween added to each solution as a surfactant. The NAA/BAP-mixture (NAA/BAP-mix) was prepared using 50% NAA solution and 50% BAP solution. NAA, BAP, and the NAA/BAP-mix were applied by evenly spraying all leaves of the plants. The control group was treated with the same amount of deionized water to simulate the spraying effect on the leaf surface. No Tween was added to the control water.

2.3. Plant Material

The experimental plants were generated by vegetative propagation by cutting only the apical tips of standardized mother plants. The cuttings were dipped into a rooting hormone (0.25% 4-(3-Indolyl)-butyric acid) and gently cultivated in 25 mm × 25 mm slabs, filled up with a growing media mixture of 50% seedling substrate (Klasmann-Deilmann GmbH, Geeste, Germany) and 50% sand. The clones were sprayed with water four times a day to reach a relative humidity above 90%. After 14 days and adequate root growth, the cuttings were transplanted in seedling substrate (Klasmann-Deilmann GmbH, Geeste, Germany) into a pot that was 90 mm in diameter. The shoot apexes were removed to 9 internodes for genotype KANADA and 0.2x-genetic and 11 internodes for the auto-flowering genotype FED. The experimental plants were transferred at 15 DAP into 13 × 13 cm square pots, at 50 DAP into 18 × 18 cm square pots, and finally at 74 DAP into 10 L containers, in a growing media mixture consisting of 15% black peat, 20% fraction 1, 25% milled peat, 20% GF medium, 10% pine bark, 10% leca, 1 kg m^{-3} horn chips, and 1 kg m^{-3} NPK 12-14-24 (Klasmann-Deilmann GmbH, Geeste, Germany). Under an indoor vegetative life cycle of 18 h, sunlight was supplemented with artificial lightning using Gavita high-pressure sodium (HPS) lamps, i.e., E-Series DE FLEX EU Lamp (750 W, 400 V, 1500 µmol s^{-1}), Aalsmeer, Netherlands. The experiment was irrigated by a drip irrigation system and fertilized four days a week with 0.2% of Plantaactiv 18-12-18 Type A during the vegetative growth cycle and with 0.2% of Plantaactive 10-20-30 Type B

during the generative growth cycle, which was purchased from Hauert (Grossaffoltern, Switzerland). The temperature during the vegetative growth stage varied from 23.7 °C to 27.6 °C. Relative humidity varied between 22.4% and 47.5%. At 129 DAP, the experimental plants were moved into a climate chamber to a 12-h photoperiod to initiate floral development. The temperature during the generative growth cycle varied from 17.9 °C to 24.1 °C. Relative humidity varied between 30.8% and 89.2%.

2.4. Measurements

Measurements took place for every plant each seventh day for a total period of 132 days. Plants were measured for their total height and length of axillary branches. Depending on the genotype, 9 (for KANADA and 0.2x-genetic) or 11 (for FED) branches were measured. Nodes of each axillary branch were counted for the KANADA and 0.2x-genetic genotypes. The autoflowering characteristics and dense foliage of genotype FED made it impossible to count axillar branch nodes.

2.5. Plant Samples

Genotypes were harvested when 70% of the pistils had darkened. Gland heads of trichomes are clear or slightly amber at the beginning of the growth cycle. Prior to harvest, when cannabinoid levels reach their maximum, they turn cloudy. The state of trichomes was monitored with binoculars. Genotype FED was harvested at 137 DAP. Harvest of genotype KANADA and 0.2x-genetic took place at 142 and 156 DAP, respectively. Inflorescence and leaves were dried at a temperature of 20 °C for 14 days. After the drying process, dry matter was weighed and recorded in gram per single plant to determine the dry weight (DW). Subsequently, the dried plant material was ground with an ultracentrifugal mill (Retsch, Type ZM 200, Haan, Germany) to acquire a homogeneous powder, with a particle size of 1 mm. The residual moisture of each samples was measured with a moisture analyzer (DBS 60-3 of Kern and Sohn GmbH, Balingen, Germany).

2.6. Extraction and Quantification of Cannabinoids by HPLC Analysis

Quantitative analysis of cannabinoids, particularly cannabidiol (CBD), cannabidiolic acid (CBDA), Δ^9-tetrahydrocannabinol (THC), and Δ^9-tetrahydrocannabinolic acid (THCA), was performed, according to Lehmann and Brenneisen [29] with slight modifications after Burgel et al. [30]. For the cannabinoid extraction, 100 ± 10 mg of the grinded sample was dissolved in 100 mL methanol 90%/chloroform 10% (v/v) (9 + 1) composite.

An external calibration of cannabinoid quantification was performed according to Burgel et al. [30], using one standard (CAN1) containing the target compounds (CAN1: THC 2%, CBD 2%, THCA 10%, CBDA 10%). The reference cannabinoids, CBD, THC, and THCA, were purchased from Lipomed (Arlesheim, Switzerland), and was purchased from CBDA from Echo Pharmaceuticals BV (Weesp, The Netherlands).

Data were processed using ChemStation Software for LC Rev. B.04.03-SP2 (Agilent, Santa Clara, CA, USA). The retention time of the respective chromatographic target peak, was compared with the chromatographic peak of the reference to carry out a quantitative analysis. The UV spectra was used to preliminarily allocate the chromatographic peak to the reference spectra visually. The identity of the target cannabinoid was proven if the deviation of the retention time of the chromatographic peak was ≤0.5 min and the optical spectra comparison did not show any difference.

To calculate the respective cannabinoid content C_{TS} in mass percent [$\%_{m/m}$], Equation (1) was used, where A_{TS} is defined as the peak area of the standard analyst, B_{TS} is defined as the peak area of the sample analyst in µV × s, V is defined as the volume, $EW_{TS_{hijkl}}$ as the weight portion of the product in mg, and F_{ijkl} as the residual moisture of the product in $\%_{m/m}$. Indices are defined for the *i*-th genotype

in the *j*-th row, the *h*-th column of the *k*-th replicate, and the *l*-th treatment; thus, the calculation was performed for each plant.

$$C_{TS_{hijkl}}[\%_{m/m}] = \frac{(A_{TS}[\mu V \times s])}{(B_{TS_{hijkl}}[\mu V \times s]/100[\mu L\ mL^{-1}])} * \frac{V[mL]}{EW_{TS_{hijkl}}[mg]} \times 100 \times F_{hijkl}[\%_{m/m}], \quad (1)$$

2.7. Statistical Analysis

A mixed model approach was used to analyze all traits, which were determined by the measurement of single plants. Thus, the dry weight of the leaves and inflorescence, estimation, and statistical interference of the cannabinoids present in the dried plant material were analyzed by

$$y_{hijkl} = \mu + b_k + r_{jk} + c_{hk} + \delta_i + \tau_l + (\delta\tau)_{il} + e_{hijkl}, \quad (2)$$

where y_{hijkl} is the observation of the *i*-th genotype in the *j*-th row, the *h*-th column of the *k*-th replicate, and the *l*-th treatment, μ is the intercept, δ_i is the fixed effect of the *i*-th genotype,τ_l is the fixed effect of the *l*-th treatment, $(\delta\tau)_{il}$ is the fixed interaction effect of the corresponding main effects, b_k is the fixed effect of the *k*-th replicate, e_{hijkl} is the plant or error effect associated with observation y_{hijkl}, and r_{jk} and c_{hk} are the random row and column effects within the *k*-th replicate, respectively. Normal distribution and homogeneous variance of residuals were checked graphically via residual plots. If needed, the data were logarithmically transformed to fulfill the requirement concerning homogeneous variance and normal distribution. In this case, estimates were back transformed for presentation purposes only. Standard errors were back transformed using the delta method.

Total plant height was measured weekly for 20 weeks. The number of internodes and the length of axillar branches were measured weekly for 14 weeks. Thus, repeated measures were taken and Model (2) was extended by the factor measurement with 20 or 14 levels, as follows:

$$y_{hijklm} = \mu + t_m + b_{km} + r_{jkm} + c_{hkm} + \delta_i + (\delta t)_{im} + \tau_l + (t\tau)_{ml} + (\delta\tau)_{il} + (\delta\tau t)_{ilm} + e_{hijklm}, \quad (3)$$

where t_m is the effect of the *m*-th measurement and all other effects are defined analogous to Model (2) for each measurement *m*. As repeated measures from each row, column, and plant were taken, a first-order autoregressive variance–covariance structure with heterogeneous variance was assumed for these random effects, allowing for a serial correlation between observations taken from the same row, column, or plant. The variance–covariance structure was simplified to a homogeneous variance first-order autoregressive structure if this decreased the AIC [31], thus resulting in a better model fit. After finding significant differences via the global F-test, Fishers LSD test was performed for multiple comparisons. A letter display was used to present the results of the multiple comparisons [32]. All statistical analyses were conducted using the statistical software SAS version 9.4 (SAS Institute, Cary, NC, United States). Figures were also generated using the statistical software SAS version 9.4 and Excel 2013 (Microsoft Corporation, Washington, DC, USA).

3. Results

3.1. Plant Height

In the following section, plant height is described for different plant growth regulator (PGR) treatments (NAA, BAP, and NAA/BAP-mix) separately in comparison to control plants. The height of the treated plants showed significant interactions between treatments and measurements over time. No significant interactions were observed between treatment and genotype. Plant heights of NAA-treated and BAP-treated plants were significantly reduced compared to the control plants, starting 13 and 20 days after planting (DAP), respectively. At 132 DAP, NAA- and BAP-treated plants indicated heights of 69.20 ± 4.43 cm and 79.53 ± 4.43 cm, respectively, compared to the control with 96.47 ± 4.43 cm (Figure 1A,B). Plants treated with the NAA/BAP-mix showed the same trend

(81.74 ± 4.43 cm). A significant reduction in plant height was observed between 20 DAP and 62 DAP compared to the control. No significant growth reduction was determined between 69 and 104 DAP (Figure 1C). Finally, the treatments resulted in 28% (NAA), 18% (BAP), and 15% (NAA/BAP) shorter plants in comparison to the control (Figure 1A–C).

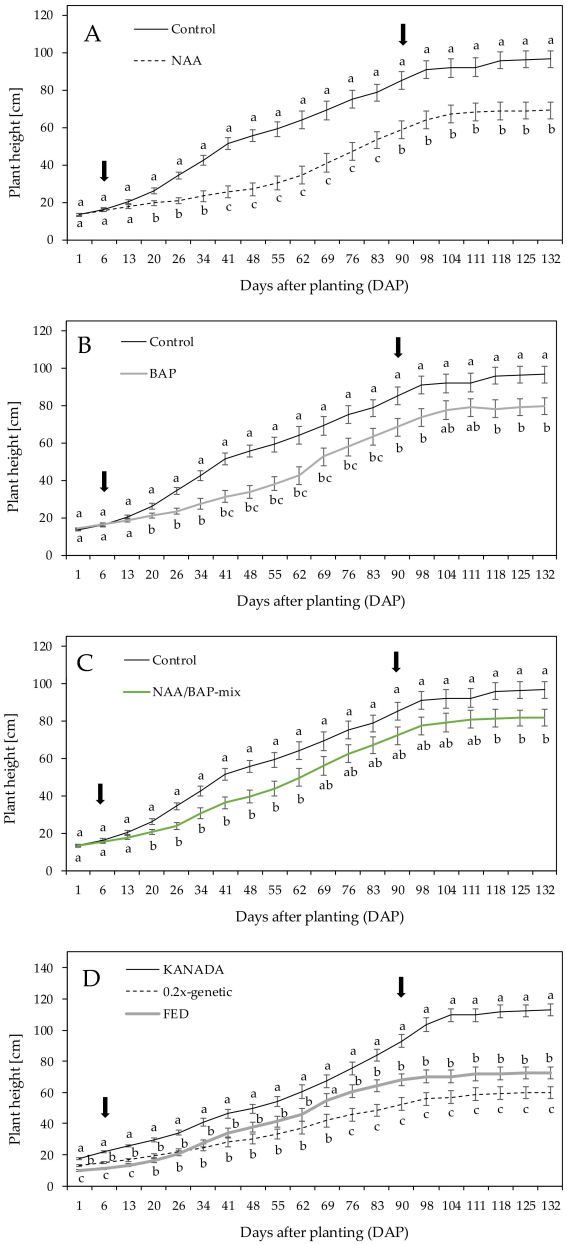

Figure 1. Mean plant height over all three tested genotypes treated with (**A**) 1-naphthalenacetic acid (NAA), (**B**) 6-benzylaminopurine (BAP), and (**C**) NAA/BAP-mix, compared to the nontreated control over a time period of 132 days after planting (DAP). (**D**) Mean plant height of genotype KANADA, 0.2x-genetic, and FED over 132 days. Means covered with at least one identical lowercase letter did not differ significantly at $\alpha = 0.05$. The arrows show the period (6 to 90 DAP) during which application took place.

Across all treatments, the KANADA genotype significantly indicated the highest plants at 132 DAP (113.01 ± 3.83 cm), followed by genotype FED (72.45 ± 3.83 cm), whereas genotype 0.2x-genetic indicated the shortest plants, with a final plant height of 59.88 ± 3.83 cm (Figure 1D).

3.2. Number of Internodes of Axillar Branches

PGR treatments significantly influenced the number of internodes of axillary branches of the genotypes (KANADA and 0.2x-genetic). Internodes of the autoflowering genotype FED could not be measured. Axillary branches of NAA-treated plants showed an inhibited number of internodes after 14 days of application (20 DAP) and resulted in an average number of 17 internodes per axillary branch compared to the control, which showed 21 internodes per branch (Figure 2A). The same trend was observed for the BAP-treated and NAA/BAP-mix-treated plants. BAP and NAA/BAP-mix did not affect the number of internodes per axillary branch during the first 20 days of application (26 DAP). Between 26 and 90 DAP, a reduction of internodes was observed and resulted in 19 and 18 internodes per axillary branch compared to the control (Figure 2B,C). For the final measurement, all PGR were shown to inhibit the average number of internodes during the vegetative period compared to the control. No significant differences were observed between the treatments (Figure 2A–C).

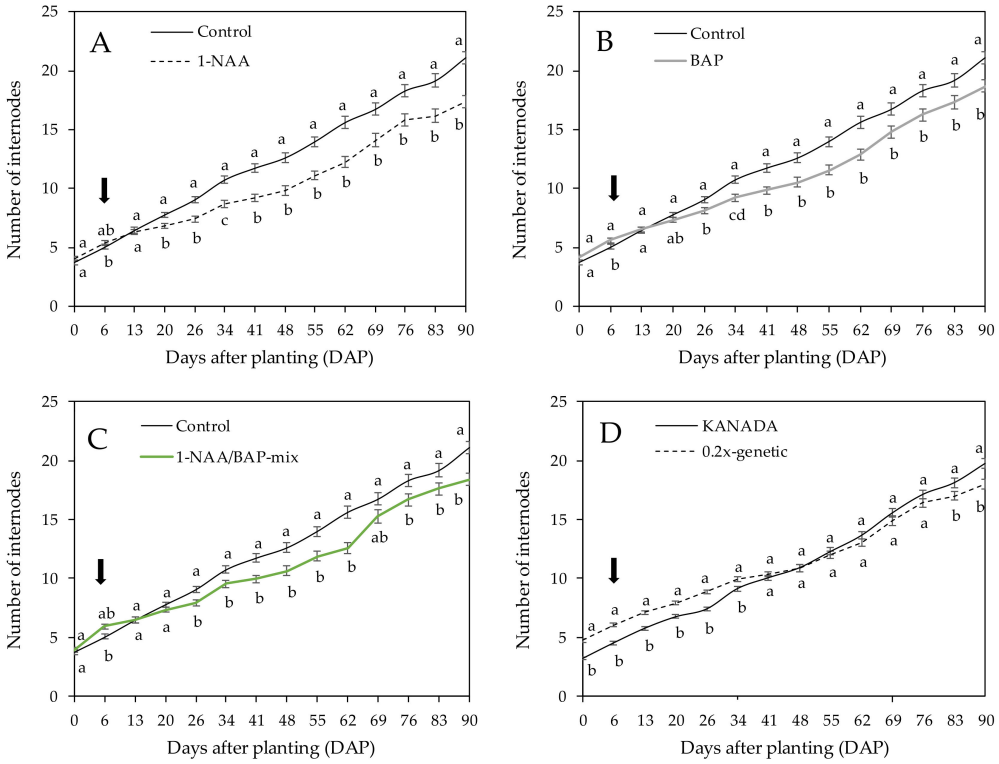

Figure 2. Mean number of internodes per axillary branch over two tested genotypes (KANADA and 0.2x-genetic) treated with (**A**) NAA, (**B**) BAP, and (**C**) NAA/BAP-mix, compared to the nontreated control over a time period of 90 days. (**D**) Mean number of internodes per axillary branch of genotype KANADA and 0.2x-genetic across time. Means covered with at least one identical lowercase letter did not differ significantly at $\alpha = 0.05$. The arrow shows the time point (6 DAP) of the first application.

Across all treatments, genotype KANADA showed an inhibited number of nine internodes until 34 DAP compared to genotype 0.2x-genetic, which showed inhibition of 10 internodes. Finally, at 90 DAP, genotype KANADA demonstrated a significantly higher number of internodes

per axillary branch (20 internodes) compared to genotype 0.2x-genetic, which showed 18 internodes (Figure 2D).

3.3. Length of Axillary Branches

PGR treatments significantly influenced the length of axillary branching. NAA-treated plants started after seven days of application (13 DAP) to inhibit the growth of axillary branches compared to the control. After 84 days of application (90 DAP), the axillar branching growth was reduced and resulted in an average length of 19.23 ± 2.25 cm (Figure 3A). After seven days of application (13 DAP), BAP-treated plants showed a significant reduction in axillar branching over the vegetative period and resulted in 36.46 ± 4.21 cm at 90 DAP compared to the control (45.26 ± 5.41 cm) (Figure 3B). A similar trend was observed for plants treated with the NAA/BAP-mix, with a final length of axillary branches of 31.63 ± 3.77 cm compared to the control (Figure 3C). At 26 DAP and 34 DAP, NAA-treated plants were shown to be influenced to a greater extent compared to the BAP-treated and NAA/BAP-mix-treated plants, respectively. NAA resulted in the highest axillary branch length reduction with 26.03 cm, while BAP and the NAA/BAP-mix showed 8.80 cm and 13.63 cm length reductions compared to the control, which was a significant finding (Figure 3A–C).

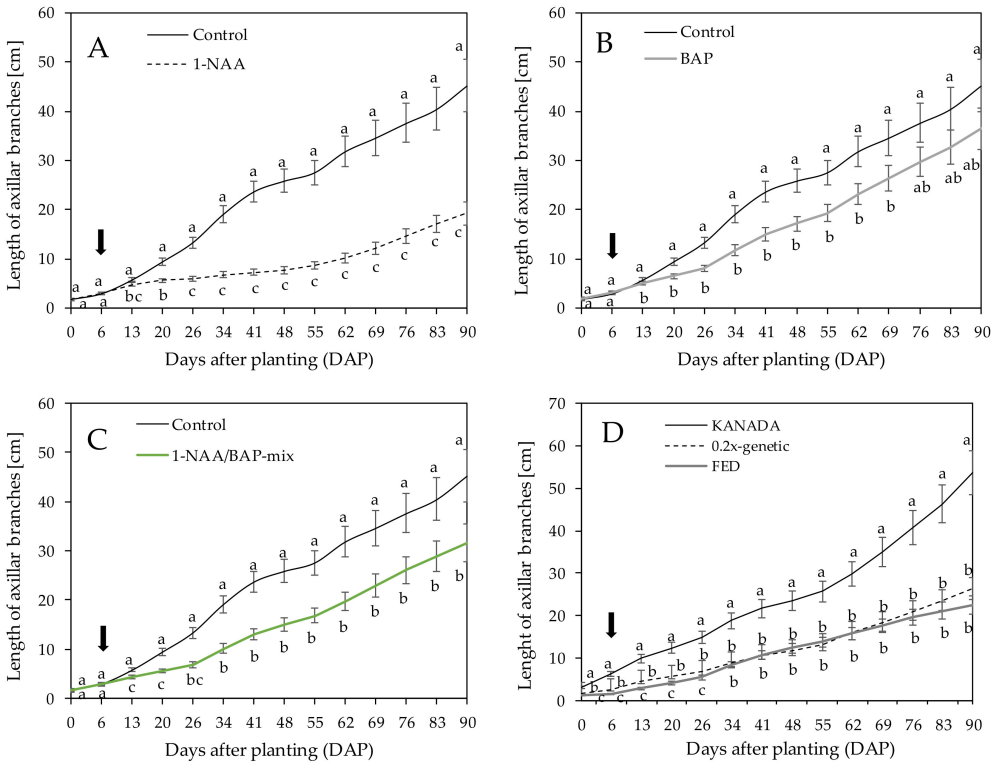

Figure 3. Median length of axillar branches in cm over all three tested genotypes treated with (**A**) NAA, (**B**) BAP, and (**C**) NAA/BAP-mix compared to the nontreated control each over a time period of 90 days. (**D**) Mean length of axillar branches of genotype KANADA and 0.2x-genetic across time. Means covered with at least one identical lowercase letter did not differ significantly at $\alpha = 0.05$. The arrow shows the time point (6 DAP) of the first application.

A genotype-specific difference in axillary branch length across all treatments was observed. Genotype KANADA indicated a final length of axillary branches of 53.64 ± 5.29 cm, which were the longest axillary branches compared to genotype 0.2x-genetic (26.22 ± 2.59 cm) and FED (22.46 ± 2.06 cm) at 90 DAP (Figure 3D).

3.4. Yield Parameters

Dry weight (DW) yield of inflorescence per plant showed significant interactions between genotype and treatment. Accordingly, DW means of the different treatments are described separately for each genotype. Control plants of genotype KANADA reached the highest inflorescence yield with 24.31 g plant^{-1}, followed by plants treated with NAA (23.51 g plant^{-1}) and BAP (22.97 g plant^{-1}). Plants treated with a mixture of NAA and BAP (NAA/BAP-mix) indicated the lowest DW yield of inflorescence, with 14.79 g plant^{-1} compared to the control (Table 1). Control plants of 0.2x-genetic indicated the highest inflorescence yield with 20.83 g plant^{-1}, followed by plants treated with BAP (15.88 g plant^{-1}), which showed no significant difference compared to NAA-treated and NAA/BAP-mix-treated plants, with results of 7.61 g plant^{-1} and 8.57 g plant^{-1}, respectively (Table 1). Control plants of the autoflowering genotype FED showed the highest inflorescence yield with 32.10 g plant^{-1}, which was a significant finding. NAA-treated plants indicated the lowest yield (5.39 g plant^{-1}) compared to the control (Table 1).

Table 1. Mean inflorescence dry weight (DW) in g plant^{-1} of genotypes KANADA, 0.2x-genetic, and FED, treated with NAA, BAP, and NAA/BAP-mix and a nontreated control. Results are presented as mean values ± standard error of mean (mean ± SEM). Means of treatments in one column followed by at least one identical lowercase letter are not significantly different, as indicated by the LSD test ($\alpha = 0.05$). Means of genotypes in one row followed by at least one identical uppercase letter are not significantly different, as indicated by the LSD test ($\alpha = 0.05$). The *p*-values correspond to global F tests for differences between the levels of the mentioned genotypes, treatments, or their interactions.

Trait	Treatment	Genotype		
		KANADA	**0.2x-genetic**	**FED**
Inflorescence DW [g plant^{-1}]	Control	24.31 ± 3.06 ^{aA}	20.83 ± 3.79 ^{aA}	32.10 ± 3.76 ^{aA}
	NAA	23.51 ± 3.10 ^{abA}	7.61 ± 3.10 ^{bB}	5.39 ± 3.10 ^{cB}
	BAP	22.97 ± 3.11 ^{abA}	15.88 ± 3.11 ^{abA}	17.61 ± 3.13 ^{bA}
	NAA/BAP-mix	14.79 ± 3.13 ^{bAB}	8.57 ± 3.11 ^{bB}	19.12 ± 3.80 ^{bA}
***p*-values**				
Genotype [G]			0.0041	
Treatment [T]			0.0003	
G × T Interaction			0.0430	

DW yield of leaves per plant showed significant differences between treatments and genotypes. On average, genotype KANADA showed the highest DW yield of leaves (25.14 g plant^{-1}) over the treatments compared to 0.2x-genetic (18.54 g plant^{-1}) and FED (17.76 g plant^{-1}; Table 2), which was a significant finding.

Plants treated with NAA indicated the lowest DW yield (12.43 g plant^{-1}) of leaves compared to the control and the other treatments over the three genotypes, ranging from 25.60 g plant^{-1} (control) to 20.79 g plant^{-1} (NAA/BAP-mix; Table 2), which was a significant finding.

Table 2. Mean leaves dry weight (DW) in g plant^{-1} of genotypes KANADA, 0.2x-genetic, and FED and mean leaves dry weight (DW) in g plant^{-1} of genotypes KANADA, 0.2x-genetic, and FED, treated with NAA, BAP, and NAA/BAP-mix and a nontreated control. Results are presented as mean values ± standard error of mean (mean ± SEM). Means of genotypes or treatments in one row followed by at least one identical lowercase letter are not significantly different, as indicated by the LSD test ($\alpha = 0.05$). The p-values correspond to global F tests for differences between the levels of the mentioned genotypes, treatments, or their interactions.

Trait	**Genotype**			
Leaves DW [g plant^{-1}]	**KANADA**	**0.2x-genetic**		**FED**
	25.14 ± 1.94 [a]	18.54 ± 2.07 [b]		17.76 ± 2.21 [b]
	Treatment			
	Control	**NAA**	**BAP**	**NAA/BAP-mix**
	25.6 ± 2.60 [a]	12.43 ± 2.24 [b]	22.96 ± 2.24 [a]	20.79 ± 2.43 [a]
***p*-values**				
Genotype [G]	0.0337			
Treatment [T]	0.0045			
G × T Interaction	0.1914			

3.5. Cannabinoid Content

Cannabinoid acids occur in planta and undergo decarboxylation to their neutral forms upon heating or pyrolysis. The following results for cannabidiol (CBD) refer to the sum of cannabidiolic acid (CBDA) and CBD analyzed. Inflorescence and plant leaves treated with NAA, BAP, or NAA/BAP-mix did not show any statistical differences between the plants exposed to growth regulators and the control plants. Significant differences in CBD content were only between genotypes. KANADA indicated the highest content of CBD in inflorescence (10.33%) and leaves (7.03%), followed by the inflorescence (7.91%) and leaves (6.77%) of 0.2x-genetic. The lowest content was measured in the inflorescence and leaves of the autoflowering genotype FED, with 6.34% and 5.59%, respectively (Table 3).

Table 3. Mean content of cannabidiol (CBD) in mass percent [$\%_{m/m}$] of genotypes KANADA, 0.2x-genetic, and FED. CBD was analyzed in inflorescence and leaves. Results are presented as mean values ± standard error of mean (mean ± SEM). Means in one row followed by at least one identical letter are not significantly different as indicated by LSD test ($\alpha = 0.05$). The p-values correspond to global F tests for differences between the levels of the mentioned genotypes, treatments, or their interactions.

Trait	**Genotype**		
CBD [%m/m]	**KANADA**	**0.2x-genetic**	**FED**
Inflorescence	10.33 ± 0.30 [a]	7.91 ± 0.30 [b]	6.34 ± 0.31 [c]
Leaves	7.03 ± 0.18 [a]	6.77 ± 0.20 [a]	5.59 ± 0.28 [b]
***p*-values Inflorescence**			
Genotype [G]	0.0226		
Treatment [T]	0.4411		
G × T Interactions	0.1072		
***p*-values Leaves**			
Genotype [G]	0.0026		
Treatment [T]	0.8755		
G × T Interactions	0.7891		

4. Discussion

Exogenous application of plant growth regulators (PGR; 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), and a mixture of both (NAA/BAP-mix)) was shown to have an impact on modifying the plant architecture of *C. sativa*.

Auxins (IAA) promote stem elongation and maintain apical dominance, including inhibition of axillary bud outgrowth [21]. Hence, an increase in plant height, which leads to taller plants on average, through exogenous NAA treatment was expected. However, the results showed reduced plant height. Between 41 to 83 DAP, a reduction in plant growth in NAA-treated plants was larger compared to NAA/BAP-mix-treated plants. Mendel et al. [23] reported a nonsignificant inhibition of the average plant height of *C. sativa* over a time period of 56 days, in which plants were exposed to different NAA concentrations (5 mg L^{-1}, 10 mg L^{-1}, and 20 mg L^{-1}). In contrast, Lalge et al. [33], documented an increase in total plant height of *C. sativa* plants treated with 10 mg L^{-1} NAA, whereas concentrations of 5 mg L^{-1} and 20 mg L^{-1} showed no significant effect on plant height [33]. Our study indicated that NAA-treated plants showed the highest axillary side-branch reduction, with an average length of 19.23 cm, compared to BAP treated plants (36.46 cm), NAA/BAP-mix treated plants (31.36 cm), and control plants (45.26 cm). However, this was in accordance with the measured number of internodes per axillary side branch, which indicated 19% (four internodes) reduction in the number of internodes per axillary side branch after NAA application compared to the control, whereas no significant differences between treatments were observed. Lalge et al. [33] and Mendel et al. [23] indicated increased lateral branching of plants subjected to NAA treatment at all concentrations that affected the plant. Lalge et al. [33] argued that buds of *C. sativa* demonstrate decreased sensitivity to the inhibitory effects of IAA in apical dominance. Since the apical dominance in our study was broken before application, this may explain the different response of the plant to NAA application, whereas Mendel et al. [23] explained the inhibitory effect in terms of plant height and the significant promoting effect in terms of axillary side-branch length by an over-optimal NAA concentration and an imbalance in the hormonal endogenous balance. Other studies showed that high IAA concentrations induced ethylene synthesis. The release of this gas could be responsible for the decrease in stem elongation [34]. Further, synthetic auxin (NAA) in *C. sativa* could interact with a different set of receptors than the most common and natural auxin indole-3-acetic acid (IAA) [23]. In the case of plant height and axillary side-branch growth, even low concentrations (5 mg L^{-1}) were sufficient to achieve active stem elongation. Higher concentrations of NAA (10 mg L^{-1} and 20 mg L^{-1}) eliminated and restored the hormonal effect. This biphasic behavior of the reaction could depend on adjustment of the ratio, with concentrations of exogenous auxins exceeding the minimum effective dose [23].

PGR, especially growth retardants including CKs, are generally applied in order to obtain short and compact plants [35]. CKs are involved in meristem activity regulation [22], stem elongation inhibition, and plant flowering, among other things [35]. In apical dominance, CK has an antagonistic effect to the IAA effects of. Studies of other species showed increased CK levels (25-fold within 24 h) after decapitation of the shoot apex [36]. Therefore, axillary bud outgrowth is correlated with CK concentration, which is locally biosynthesized in the nodal stem [37]. Further, it is well known that direct application of CKs to axillary buds promotes their outgrowth, even in intact plants [38]. The aim of exogenous CK application is to obtain well-branched plants without removing the apical meristem. The removal of the apical shoot apex is a common pruning technique to control the way plants grow, either to restrict plant height, maximize yield [39], or as a management tool to optimize space utilization for indoor cultivation.

Decapitation of the shoot apex to reach a defined number of side branches, in combination with exogenously applied synthetic analogues of CK (BAP), met expectations and resulted in an 18% reduction in total plant height after 90 days of application in comparison to control plants. The average length of axillary side branches showed a reduction trend of around 20% compared to the control. However, no significant reduction in axillary side branches was shown. The measured number of internodes of the axillary side branches was reduced by 9% on average compared to the control.

In contrast, Mendel et al. [23] and Lalge et al. [33] reported that treatments with BAP did not affect the total plant height of *C. sativa* at concentrations of 10 mg L^{-1}, 25 mg L^{-1}, and 50 mg L^{-1}. A total plant height of 200 to 220 cm was documented by Lalge et al. [33] 56 days after the first application took place for all concentrations, including the control, in accordance with a study by Leite et al. [40], where exogenously applied CK was not effective in modifying evaluated plant growth parameters of other plant species (*Glycine max* L. Merr.). Further, a strongly significant stimulation of axillary side-branch growth was documented at the highest BAP concentration (50 mg L^{-1}). Control plants measured 1 cm in side-branch length on average, whereas BAP-treated plants measured 10 cm in side-branch length on average 56 days after the first treatment took place [23]. Lalge et al. [33] stated a concentration-dependent increase in axillary branch length of BAP-treated plants. The highest dosage (50 mg L^{-1}) showed the most significant results, with around 50% increase in average axillary side-branch length compared to the control, 56 days after first application. These findings were in contrast to the present results, which can be explained by a genotype-specific reaction of PGR. While Mendel et al. [23] and Lalge et al. [33] used an industrial hemp fiber variety called Bialobrzeskie, of chemotype III, a phytocannabinoid-rich (PCR) chemotype was used in the present study. Further, by removing the shoot apex, a change in IAA/CK balance was expected.

A similar trend applied to plants treated with a mixture of both (NAA/BAP-mix). In line with NAA and BAP treatments, a reduction (15%) in total plant height after 84 days of application (90 DAP) was measured compared to the control. The length of axillary side branches was reduced by 31% on average, with the number of internodes reduced by 14% compared to nontreated control plants. IAA and CK interact in a complex manner to control plant growth [41]. Experiments with the model plant *Arabidopsis thaliana* showed a homeostatic regulatory feedback loop model, in which CK functioned as a positive regulator of IAA biosynthesis and IAA repressed CK biosynthesis [41–43]. It remains unclear whether this model can be extrapolated to the functioning of the whole plant, in which IAA and CK concentrations are modified naturally or in response to exogenous application [44]. Nevertheless, the present study showed shorter plants with a reduced length of axillary branches compared to the control, but not as much as plants which were only treated with NAA.

Plant architecture was influenced using PGR. The aim was to modify the plant morphology in order to generate small, compact plants for various indoor growing systems. While NAA-treated plants showed a short habitus, including significant the shortest axillary side branches with a reduced number of internodes. BAP-treated plants and plants treated with the NAA/BAP-mix also showed shorter habitus and demonstrated shorter axillary side branches with reduced numbers of internodes. It is important to note that the use of PGR did not reduce biomass yield and the content of cannabinoids. Results showed that the impact of PGR on yield of inflorescence was dependent on the interaction between the genotype and the treatment. NAA- and BAP-treated plants of genotype KANADA showed an equally high yield of inflorescence DW than the control plants, whereas 0.2x genotype showed an equally high DW yield, but only for BAP-treated plants. However, genotype FED indicated a lower DW yield of inflorescence in PGR treatments. It is important to mention that *Cannabis* genotypes respond differently to production conditions, as reported by Backer et al. [45]. CBD contents in inflorescence and leaves showed no impact on PGR. Considering that a minimal range of variation is aimed for and a THC content of < 0.2% must not be exceeded, the fact that PGR applications have no influence on cannabinoid content may be advantageous.

Above all, the inflorescence yields are decisive, since a higher content of cannabinoids is expected. Stout et al. [46] reported the highest CBD levels in *Cannabis* flowers, with lower amounts in leaves. Cannabinoids can be extracted from the reproductive plant parts and foliage. Inflorescence has higher concentrations of cannabinoids than foliage material, however foliage parts comprise the lager biomass of the *Cannabis* plant [47]. The PCR genetics used in the present study showed lower leaf DW compared to inflorescence DW. Leaf DW yield depended on the genotype and treatment, where genotype KANADA showed the highest DW leaf yields and genotypes 0.2x and FED were not that profitable. The use of PGR did not reduce leaf yield for BAP-treated and NAA/BAP-mix-treated plants.

In summary, the results showed that the used PCR genetics reacted differently to PGR applications. Genotype KANADA was shown to be suitable as a treatment with synthetic auxin (NAA) to adapt plant architecture to corresponding indoor conditions. Short plant height was characterized by stability in the generative phase and avoided breakdown of side branches. A defined number of axillary side branches by apical bud removal and a reduced side-branch length guaranteed homogeneous flower development by uniform exposure. Furthermore, better harvesting conditions were given due to uniform plant height and homogeneous plant development. Despite a more compact habitus, the flower yield was not reduced and the KANADA genotype showed the highest CBD content compared to the other genotypes. Treatment with synthetic CK (BAP) was shown to be disadvantageous regarding the PCR genetics used, because axillary side-branch length could not be significantly shortened. Treatment with a mix of both showed no beneficial effects in any of the three genotypes, as the DW of inflorescence was reduced by the treatment. Only leaf DW was not reduced. If leaf yield is the purpose of use, BAP treatment may be appropriate. In this case, genotype KANADA is also recommended due to the significant finding of the highest leaf DW.

The use of specific PGR is permitted in fruit growing, horticulture, and field-crop cultivation [17,48,49]. In the cultivation of medicinal grade *Cannabis* production, there are currently no approved PGR to modify plant architecture. Since the use of PGR in production under good manufacturing practice (GMP) or good agricultural and collection practice (GACP) guidelines is not clearly defined, the use of PGR in the cultivation of nonmedical *Cannabis* could be of importance for cosmetics or nutraceuticals, for example. Nevertheless, minimum effective chemical inputs, including fertilizers, growth regulators, pesticides, and herbicides, should be achieved and well documented to secure the marketability of the product [50]. When using synthetic PGR, it is very important to know exactly the appropriate application methods and concentrations to avoid possible residues in the final product, as high concentrations of IAA are toxic. Because of these properties, compounds with auxin-like activities were developed and can be applied as herbicides [51]. Synthetic IAAs are more stable than endogenous IAA, because the compounds show reduced metabolic turnover [52]. Thus, natural phytohormones could be an alternative to synthetic ones.

5. Conclusions

The results of this study showed that exogenously applied plant growth regulators (PGR), namely 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), and a mixture of both (NAA/BAP-mix), had significant impacts on the plant architecture of *C. sativa*. Phytocannabinoid-rich (PCR) *Cannabis* genetics reacted in a genotype-specific manner to PGR applications. The use of NAA led to a more compact plant architecture with a consistently high inflorescence yield for the genotype KANADA; cannabidiol (CBD) content was not affected. A beneficial effect on the autoflowering genotype (FED) could not be confirmed. Genotypes 0.2x-genetic and FED showed reduced inflorescence yield due to PGR application. The use of PGR opens up a very interesting field and requires further study to test the use of PGR at different concentrations. Although exogenously applied PGR might be a cultural practice in the future, further studies to screen more PCR-genetics on their specific reactions to PGR applications are required to develop new genotype-specific indoor cultivation systems.

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5 Publication III: Impact of Different Growing Substrates on Growth, Yield and Cannabinoid Content of Two *Cannabis sativa* L. Genotypes in a Pot Culture.

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Several studies focusing on genotype screenings to meet the growing phytocannabinoid needs have been carried out so far. As the content of specific cannabinoids can be influenced by the cultivation methods, the choice of a suitable potting substrate is essential. The most common and standard growing media for greenhouse pot production has a high content of Sphagnum peat, because of its cost-effectiveness, wide availability and suitable physical and chemical properties. However, alternative compounds and their potential to partly replace peat need to be evaluated as peat prices are increasing and there is growing public concern over peat harvesting for commercial horticultural purposes. Furthermore, the physiological aspects, such as water demand and transpiration of the cannabis plant, compared to the water retention of the substrate are not yet fully known and should be considered in order to obtain a suitable standard substrate depending on the genotype. There is a lack of linkage in the literature between the physical properties of peat alternatives with regard to their impact on plant growth and performance. For that reason, publication III examines the impact of a pure peat-mix growth media, a peat-mix growth media substituted with 30% of green fibres, and a pure coco coir growth media on growth performance, root growth, biomass as well as flower yields, and on cannabidiol (CBD/A) contents of two phytocannabinoid-rich cannabis genotypes, namely KANADA and 0.2x, which are grown under greenhouse conditions.

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MDPI

Article

Impact of Different Growing Substrates on Growth, Yield and Cannabinoid Content of Two *Cannabis sativa* L. Genotypes in a Pot Culture

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Abstract: The impacts of different growing substrate compositions, consisting of peat (PM), peat substituted with 30% green fibre (G30) and coco coir fibre (CC) growth media, were investigated in regard to the plant height, biomass and floral yield, biomass nitrogen (N) content, root growth, and cannabidiol content (CBD/A) of two phytocannabinoid-rich cannabis genotypes in an indoor pot cultivation system. Genotypes and substrate treatment combinations were randomly allocated to 36 plants according to a Latin square design. The results showed a higher total plant height for PM (39.96 cm), followed by G30 (35.28 cm), and the lowest in CC (31.54 cm). The N content of leaves indicated the highest values for plants grown in G30 (52.24 g kg DW^{-1}), followed by PM (46.75 g kg DW^{-1}) and a significantly lower content for CC (37.00 g kg DW^{-1}). Root length density (RLD) increased by 40% (PM) and 50% (G30), compared to CC treatments, with no significant differences in root dry weight. Both genotypes, Kanada (KAN) and 0.2x, reacted in a genotype-specific manner. KAN indicated a reduced floral yield of plants grown in G30 (4.94 g $plant^{-1}$) and CC (3.84 g $plant^{-1}$) compared to PM (8.56 g $plant^{-1}$). 0.2x indicated stable high floral yields of 9.19 g $plant^{-1}$ (G30) to 7.90 g $plant^{-1}$ (CC). Leaf DW increased in PM (5.78 g $plant^{-1}$) and G30 (5.66 g $plant^{-1}$) compared to CC (3.30 g $plant^{-1}$), while CBD/A content remained constant. Due to a higher biomass yield, the CBD/A yield of flowers (549.66 mg $plant^{-1}$) and leaves (224.16 mg $plant^{-1}$) revealed 0.2x as an interesting genotype for indoor pot cultivation in a peat-based substrate substituted with 30% green fibres. Overall, the demand for organic green fibres to partly replace fractionated peat showed a genotype-specific option for a homogeneous plant development, with comparable high biomass yields and stable cannabinoid contents compared to a peat containing standard substrate.

Keywords: *Cannabis sativa* L.; indoor cultivation; growing substrates; nitrogen content; biomass yield; root growth; cannabinoids

1. Introduction

Cannabis sativa L. is the golden example of a multifunctional plant with a high potential in various industries (e.g., food, pharma, cosmetics, animal products, etc.). Five chemotypes of cannabis have been classified based on their cannabinoid profile and concentration: Chemotype I with a Δ^9-tetrahydrocannabinol/cannabidiol (THC/CBD) ratio > 1; Chemotype II with an intermediate THC/CBD ratio (≈ 1); fibre-type plants with a THC/CBD ratio < 1 are defined as Chemotype III; Chemotype IV containing cannabigerolic acid (CBGA) as their main cannabinoid [1], and finally Chemotype V containing almost no cannabinoids [2–5]. Cultivation of *C. sativa* L. was banned in

many states due to its psychoactive drug component Δ^9-THC [6]. Industrial hemp genotypes, which meet the 0.2% THC limit mandated by the European Union (EU) legislation, can be cultivated without restrictions by farmers within the EU [7]. Breeding efforts on CBD-amplified chemotypes, called phytocannabinoid-rich cannabis genotypes, are aimed at contents of > 10% CBD and less than 0.2% THC. In many EU countries, phytocannabinoid-rich cannabis with a high content of non-psychoactive phytocannabinoids and THC < 0.2% may be legally grown as industrial hemp, whereas genotypes with a THC content above this limit require a cultivation license for medicinal cannabis in many EU countries. A clear differentiation between chemotypes is necessary, as the different legal framework in the cultivation of medicinal and phytocannabinoid-rich cannabis is expected to result in significant differences in market potential for companies operating in this sector.

For an optimised flower yield with appropriate quality, the choice of genotypes and the cultivation system is crucial, since the content of individual cannabinoids can be influenced by the cultivation method and possible environmental interactions [8]. For the isolation of the pure substance or extract preparation, outdoor cultivation seems to be economically feasible [9]. Indoor cultivation has advantages in terms of quality assurance, as well as hygienic standards as regards avoiding potential contaminations and producing homogeneous cannabis batches under controlled conditions [10]. Soilless growth media have been commonly utilised in greenhouse plant production, and can be formulated by mixing organic components such as peat moss and inorganic components such as vermiculite, perlite and sand [11,12]. Choosing proper substrate components is critical for providing suitable physical and chemical properties required for a specific plant species and its optimum growing conditions [11,13]. Sphagnum peat moss is one of the major components in soilless substrates for the production of greenhouse pot plants because of its wide availability and relative cost-effectiveness, with desirable physical and chemical properties [12,14]. Growing environmental concerns regarding extraction processes and increasing transportation costs have led to a strong need for the exploration of alternative substrates as a partial or total substitution of peat [15,16]. A problem for peat suppliers is the increasing prices of the sods, and together with a growing public concern about peat harvesting for horticultural purposes, experience and knowledge of alternative mixtures and their potential to replace peat are needed [17].

Soilless plant production systems present two challenges for a healthy root growth. First, unlike a normal soil profile, a pot environment provides a shallow layer of substrate, reaching the point of saturation quickly during irrigation. Second, a small pot size provides a limited volume capacity for water storage between irrigation cycles [11]. An effective growing media needs a physical structure with a favourable balance between water storage and air [18]. A balanced mixture of water and oxygen in the medium is required throughout the life cycle at the roots to allow the cannabis plants to grow. This balance at small volumes is a key factor in the development of substrate compositions for soilless plant production systems. These growing media have been a pivotal innovation, allowing the control of water, air and nutrient supplies [19]. In addition, a change in societal attitude is well exemplified by the goal of reducing the reliance on peat-based growing media [20]. In terms of economic considerations as well as performance, including excellent physical, chemical and biological properties [16,21,22], peat is an economically effective component of soilless substrates [23]. It guarantees a high water capacity with simultaneously high air capacity [21], making it challenging to find comparable replacements [23].

By-products have been extensively investigated over the past few decades to replace peat, which include coconut coir dust [24], wood fibre [25] and others. Green fibres, especially different compositions of wood fibres, can improve the physical properties of peat due to their high porosity and water holding capacity [23,26]. There is a lack of linkage between the physical properties of the substrate and their effects on the plant growth and performance of a specific species. Especially in an indoor plant production system, such information is critical as a limited volume of substrate confirms root growth, which in turn makes the plant more vulnerable to suboptimal water and nutrient environments. Further, alternative substrate components should possess similar characteristics to peat in maintaining biomass yield and quality. As the importance of soilless crop production is likely to

increase in the coming years, it is crucial that researchers work with growing media manufacturers to find new materials that are ecologically sustainable and commercially viable, and deliver excellence, as well as those that will replace them [23].

The aim of this study was to evaluate the growth performance, such as the plant height, biomass yield, root growth and cannabinoid content, of cannabis plants grown in different substrates, substituted with peat alternatives in an indoor pot cultivation system.

2. Materials and Methods

2.1. Experimental Setup

A greenhouse experiment was set up to test the impacts of substrate composition on the growth parameters, biomass yield, biomass nitrogen (N) content and cannabidiol (CBD/A) content of two phytocannabinoid-rich *Cannabis sativa* L. genotypes, namely Kanada (KAN) and 0.2x (AI FAME, Wald-Schönengrund, Switzerland). The experiment was conducted at the University of Hohenheim, Germany, from April 3, 2019, until September 4, 2019. Both genotypes were cultivated on the following substrate compositions: (a) peat-mix (PM) growth media, (b) peat-mix substituted with 30% green fibre (G30) growth media, and (c) pure coco coir fibre (CC) growth media (Table 1). The green fibres used consisted of coniferous wood and wood chips from pine and spruce wood. The genotypes and substrate treatment combinations were randomly allocated to 36 plants according to a Latin square design, established with six rows and six columns. Thus, six replicates existed.

Table 1. Substrate composition of peat-mix (PM) growth media, peat-mix substituted with 30% green fibre (G30) growth media, pure coco coir fibre (CC) growth media and pot size during the experimental time from 03.04.2019 until 04.09.2019. Days after planting (DAP).

DAP (Pot Size in cm)	PM	G30	CC
1-35 (9)	70% milled peat 15% sod cut peat fraction 1 15% perlite	70% milled peat 15% sod cut peat fraction 1 15% perlite	100% coco coir fibre
36-70 (12)	45% sod cut peat fraction 2 40% milled peat medium 15% peat fibre	45% sod cut peat fraction 2 25% milled peat medium 30% green fibre	100% coco coir fibre
71-154 (15)	55% sod cut peat fraction 3 15% milled peat 30% green fibre	55% sod cut peat fraction 3 15% milled peat 30% green fibre	100% coco coir fibre

2.2. Plant Material

The experimental plants were produced by vegetative propagation by cutting off only the apical tips of standardised mother plants. Cuttings were cultivated in 25 mm x 25 mm Eazy Plug® seed cubes, EC: 1.0, pH: 5.8 (Goirle, The Netherlands). A rooting hormone (0.25% 4-(3-indolyl)-butyric acid) was used for the fast and homogeneous root development of the cuttings. The clones were covered with a hood and sprayed with water at regular intervals to reach a relative humidity > 90%. After roots emerged, 24 (12 of genotype KAN and 12 of genotype 0.2x) cuttings were transplanted into 70% milled peat, 15% sod cut peat fraction 1 and 15% perlite, in round pots with a 9 cm diameter (Table 1). A group (6 plants of each genotype) was also transplanted into 100% CC fibre for 35 days. A standardised pruning technique was performed. Therefore, the shoot apex was removed to eight internodes for both genotypes.

At 35 days after planting (DAP), both genotypes, six replicates each, were transplanted into PM growth media, G30 growth media and pure CC growth media, all in round pots with a diameter of 12 cm (Table 1). At 70 DAP, genotypes were transplanted into the final substrate composition, a peat-mix substituted with 30% green fibre containing potting substrate (55% sod cut peat fraction 3, 15% milled peat, 30% green fibre), or again into pure CC growth media, all in round pots with a 15 cm diameter

(Table 1). Substrate compositions were kindly provided by the company Klasmann-Deilmann, Geeste, Germany. Under an indoor vegetative life cycle of 18 h, sunlight was supplemented with artificial lightning using Gavita HPS (high-pressure sodium) lamps, E-Series DE FLEX EU Lamp (750 W, 400 V, 1500 µmol s^{-1}), Aalsmeer, Netherlands. During the vegetative growth cycle, plants were irrigated by a drip system and fertilised five days a week with 2 g L^{-1}, 3 DAP to 19 DAP and 3 g L^{-1}, 20 DAP to 61 DAP with Plantaactiv 18-12-18 Type A (Hauert, Grossaffoltern, Switzerland). During the generative growth cycle, 62 DAP to 154 DAP, plants were fertilised with 2 g L^{-1} of Plantaactive 10-20-30 Type B (Hauert, Grossaffoltern, Switzerland) every seventh day. The temperature during vegetative growth varied from 18.8 °C to 30.5 °C. Relative humidity varied between 24.6% and 68.8%. At 83 DAP, experimental plants were moved into a climate chamber with a 12 h photoperiod to initiate floral development. The temperature during the generative growth cycle varied from 22.9 °C to 26.5 °C. Relative humidity varied between 40.8% and 75.4%.

The substrates used had a predefined pH value of 5.5. Substrate pH was determined during the vegetative stage at 25 DAP, using the VDLUFA method (VDLUFA, 1999). Substrate samples (9 g) from each pot were mixed with 22.5 mL of 0.01 M $CaCl_2$ solution and homogenised for 4 h. The pH value was measured using a pH electrode (LE 409, Mettler Toledo®, Columbus, OH, USA).

2.3. Measurements

Measurements took place weekly for each plant for a total period of 136 days. Plants were measured for their total height and SPAD values were measured on the youngest fully developed leaf (average of three measurements $leaf^{-1}$) using SPAD 502 Plus (Konica Minolta, Chiyoda, Japan). SPAD values represent chlorophyll concentrations, which positively correlate with leaf N content [27].

2.4. Plant Samples

The appropriate harvest time of the genotypes was scheduled when 70% of pistils had darkened. Prior to harvest, when cannabinoid levels reach their maximum, the gland head trichome colour changes from clear or slight amber to cloudy. The state of the trichomes was evaluated by a microscope. Harvest took place 154 DAP (4 September 2019). Flowers and leaves from each plant were frozen with liquid nitrogen (−196 °C) to prevent further chemical reactions and finally freeze-dried to determine dry weight (DW) in gram per single plant and cannabinoid content of flowers and leaves. The dried plant material of each sample was ground with an ultra-centrifugal mill (Retsch, Type ZM 200, Haan, Germany) to acquire a homogeneous powder (particle size of 1 mm). The powder was stored in a dark and dry place until use for further analysis. The residual moisture was measured with a moisture analyser (DBS 60-3 of Kern & Sohn GmbH, Balingen, Germany).

The roots of each plant were washed using a 0.5 mm mesh sieve. Every root sample was cleaned of organic debris by hand and put into small laboratory plastic flasks filled with a 50% (v/v) ethanol solution to prevent mould growth. Root development was studied measuring root length density (RLD, cm cm^{-3}) for the total RLD per pot volume (0.0015 m^{-3}) by using images of root samples. The roots were spread over a thin layer of distilled water inside a plexiglas box. Images were acquired using a normal scanner device (Perfection V800 Photo, Epson) with a resolution of 400 DPI. Photo scans were analysed using *WinRhizo Pro V. 2019* (Regent Instruments Inc., Québec, QC, Canada). Root DW was measured after drying root samples at 110 °C for 24 h.

2.5. Extraction and Quantification of Cannabinoids by HPLC Analysis

The cannabinoid extraction was performed according to Burgel et al. [28]. Quantitative analysis of cannabinoids, particularly cannabidiol (CBD), cannabidiolic acid (CBDA), Δ^9-tetrahydrocannabinol (THC) and tetrahydrocannabinolic acid (THCA), was performed, according to the HPLC methods of Lehmann and Brenneisen [29] with slight modifications after Burgel et al. [30].

The data were processed using ChemStation Software for LC Rev. B.04.03-SP2 (Agilent, Santa Clara, CA, USA). The retention time of a respective chromatographic target peak was compared with

the main chromatographic peak of a reference to carry out a quantitative analysis. The UV spectra was used to preliminarily allocate the chromatographic peaks to the reference spectra visually. The identity of a cannabinoid was established if the deviation of the retention time of the chromatographic peak was ≤ 0.5 min from the reference and the optical spectra comparison did not show any difference.

Indices were defined for the *j*-th genotype in the *h*-th row, the *i*-th column and the *k*-th treatment, thus a calculation was done for each plant. For the respective cannabinoid content $C_{TS_{hijk}}$ in mass percent [$\%_{m/m}$] of the extract of each sample *hijk*, equation [1] was used. A_{TS} is defined as peak area of the standard analyst and $B_{TS_{hijk}}$ as the peak area of the sample analyst in µV × s. V is defined as the volume of the volumetric flask, $EW_{TS_{hijk}}$ as the weight portion of the sample in mg, and finally F_{hijk} as the residual moisture of the product in %.

$$C_{TS_{hijk}}\,[\%_{m/m}] = \frac{(A_{TS}\,[\mu V \times s])}{\left(B_{TS_{hijk}}\,[\mu V \times s] \,/\, 100\,[\mu L\, mL^{-1}]\right)} \times \frac{V\,[mL]}{EW_{TS_{hijk}}\,[mg]} \times 100 \times F_{hijk}\,[\%_{m/m}] \quad (1)$$

2.6. Statistical Analysis

A mixed model approach was used to analyse all traits, which were determined by the measurements from single plants. Thus, the DW of leaves, flowers and roots, as well as the biomass N content, RLD and cannabinoids present in dried leaf and flower material, were analysed by:

$$y_{hijk} = \mu + r_h + c_i + \rho_j + \delta_k + (\delta\rho)_{jk} + e_{hijk} \quad (2)$$

where y_{hijk} is the observation of the *j*-th genotype in the *h*-th row and the *i*-th column of the *k*-th treatment, µ is the intercept, ρ_j is the fixed effect of the *j*-th genotype, δ_k is the fixed effect of the *k*-th treatment and $(\delta\rho)_{jk}$ is the fixed interaction effect of the corresponding main effects. e_{hijk} is the error associated with observation y_{hijk}. r_h and c_i are the random row and column effects. Note that a classical Latin square model would fit six treatment effects here. We reparametrized the six treatments as combinations of the two factors genotype and substrate, which also resulted in six combinations. Normal distribution and homogenous variance were checked graphically via residual plots. For the traits RLD and flower DW, a genotype-specific error variance resulted in better model fits measured via AIC [31]. Transformation of the data was not needed.

The total plant height was measured weekly for 19 weeks, and leaf SPAD value was measured weekly for 17 weeks. Thus, repeated measures were taken and a model [2] was extended by the factor measurement with 19 or 17 levels, as follows:

$$y_{hijkm} = \mu + t_m + r_{hm} + c_{im} + \rho_j + (\rho t)_{jm} + \delta_k + (\delta t)_{km} + (\delta\rho)_{jk} + (\delta\rho t)_{jkm} + e_{hijkm} \quad (3)$$

where t_m is the effect of the *m*-th measurement, and all other effects are defined as analogous to the model [2]. Repeated measurements were taken from each row, column and plant. For all three effects, a first order autoregressive variance–covariance structure with heterogeneous variance was assumed. This allows for a serial correlation between observations taken from the same row, column or plant. Additionally, for plant height a logarithmic transformation of the data was not needed to fulfil model pre-requirements. Additionally, a logistic function using days as influencing variable was fitted:

$$f(day) = \frac{max}{1 + e^{-s(day - day_0)}} \quad (4)$$

where day_0 is the value of the sigmoid's midpoint, *max* is the curve's maximum value, and *s* is the logistic growth rate or steepness of the curve. To do so, the parameters of the logistic function were fitted for the data of each plot. Afterwards, the parameter estimates were checked for differences between genotypes and substrates using the model [2]. After finding significant differences via a global F-test, a Tukey test was performed for multiple comparison. The results of the multiple comparison

were presented via letter display [32]. Statistical analysis was conducted by using the statistical software SAS version 9.4 (The SAS Institute, Cary, NC, USA).

3. Results

The height of treated plants showed significant interactions between treatments and measurements over time. Plants treated with PM and G30 grew 35 days in a basic medium mixture + 15% Perlite during the early plant stage till they were potted into PM and G30 growth media, while plants were in 100% CC fibre over the experimental period of 136 days.

Data on plant height indicated that the height of PM- and G30-treated plants was significantly greater compared to plants grown in CC, during vegetative growth at 59 DAP. Between 35 DAP and 70 DAP, when PM and G30 treatments were implemented, the treatments resulted in significantly taller plants compared to CC. At the end of generative growth at 136 DAP, PM-treated plants were significantly taller at 39.96 ± 1.81 cm, whereas G30 (35.28 ± 1.52 cm) and CC (31.54 ± 1.61 cm) showed significant shorter plants (Figure 1; Table 2).

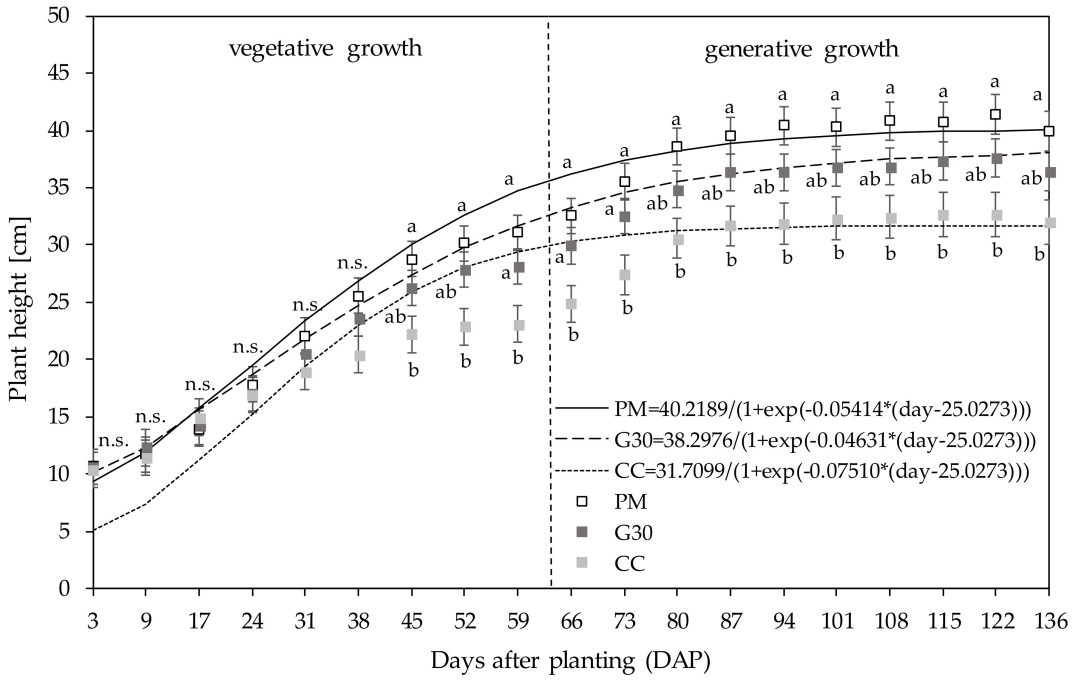

Figure 1. Mean plant height of genotypes treated with peat-mix media (PM), peat-mix + 30% green fibre (G30) compared to those grown in 100% coco coir fibre (CC) through 136 days after planting (DAP). Means with at least one identical lowercase letter did not differ significantly at $\alpha = 0.05$.

Table 2. The p-values correspond to global F tests for differences between the parameters of the logistic function $f(day) = \frac{max}{1+e^{-s(day-day_0)}}$ using days as influencing variables of the measured plant height data.

p-Values	Parameter *s*	Parameter *max*	Parameter day_0
Genotype [G]	0.0280	0.1802	0.0002
Substrate [S]	0.0209	0.0294	0.5794
G × S Interaction	0.1155	0.2382	0.4690

Mean SPAD values measured weekly on the youngest fully developed leaf, representing chlorophyll concentration through 122 DAP, showed a significant three-way interaction between the substrate treatment means, genotype and day of measurement. KAN plants grown in PM (55.30)

and G30 (56.10) had significantly higher SPAD values through 122 DAP, compared to CC plants (43.47) (Figure 2). 0.2x plants grown in G30 (52.77) had significantly higher SPAD values through 122 DAP, followed by plants grown in PM (49.17), whereas the SPAD values of plants grown in CC (41.83) were the lowest (Figure 2).

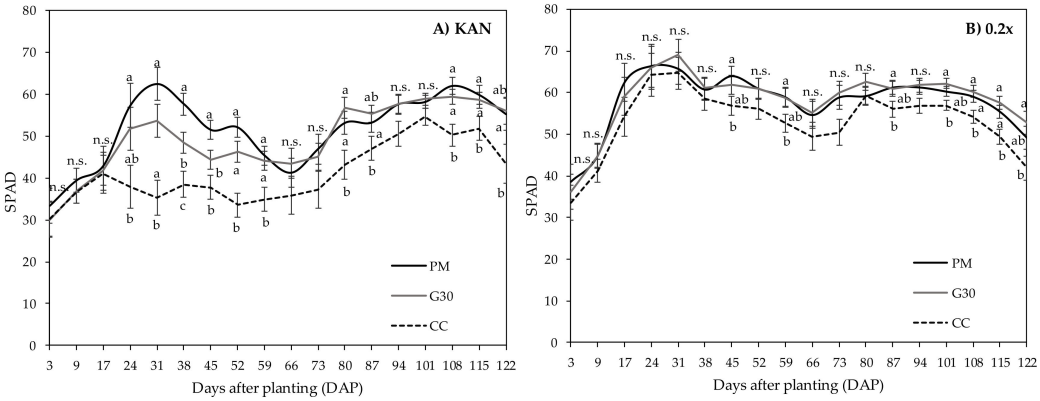

Figure 2. Mean SPAD value of genotype (**A**) KAN and (**B**) 0.2x, grown on peat-mix (PM), peat-mix + 30% green fibre (G30) and 100% coco coir (CC) fibre substrates over a time period of 122 days after planting (DAP). Means with at least one identical lowercase letter did not differ significantly at α = 0.05; n.s. = not significant.

The nitrogen (N) content per plant of leaves and flowers showed a significant difference among substrates. The N content of the leaves of plants grown in G30 was 52.24 g kg DW^{-1}, the highest value, followed by plants grown in PM (46.75 g kg DW^{-1}). Plants grown in CC fibre had 37.00 g kg DW^{-1}, a significantly lower N content compared to G30. No significant differences in the N contents of the flowers between the different substrates were found. The values ranged between 61.87 g kg DW^{-1} (G30) and 53.77 g kg DW^{-1} (CC; Figure 3).

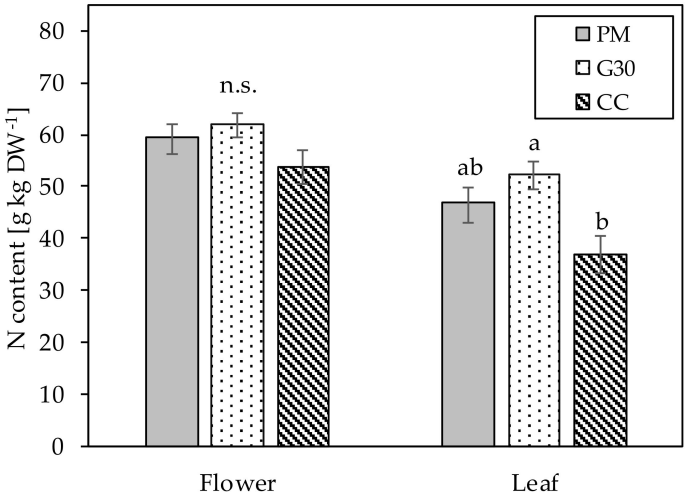

Figure 3. Mean nitrogen (N) content of flower and leaf tissues of genotypes treated with peat-mix media (PM), peat-mix + 30% green fibre (G30) and 100% coco coir fibre (CC). Means with at least one identical lowercase letter did not differ significantly at α = 0.05; n.s. = not significant.

The effects of growth media on flower DW showed genotype-specific differences. The genotype KAN cultivated in PM growth media reached 8.56 g DW plant^{-1}, the significantly highest yield of flowers compared to G30 (4.94 g plant^{-1}) and CC (3.84 g plant^{-1}) (Table 3). The genotype 0.2x ranged between 7.90 g DW plant^{-1} and 9.19 g DW plant^{-1} and showed no significant differences in flower DW between treatments (Table 3). The genotype KAN of the plants grown in G30 and CC fibre had lower DW yields of flowers compared to 0.2x, whereas PM treatments showed no genotype-specific growth differences (Table 3; Figure 4).

Table 3. Mean flower dry weight (DW) in g plant^{-1} of genotypes KAN and 0.2x, grown on peat-mix (PM), peat-mix + 30% green fibre (G30) and 100% coco coir fibre (CC) substrate. Results are presented as mean values ± standard error (Mean ± SE). Letters compare the mean DW yield of flowers. Means in a column followed by at least one identical lower-case letter and means in one row followed by at least one identical upper-case letter are not significantly different as indicated by the Tukey test (α = 0.05). The *p*-values correspond to global F tests for differences between the levels of the mentioned genotypes, substrates or their interactions.

Trait	Substrate	Genotype	
		KAN	0.2x
Flower DW [g plant^{-1}]	PM	8.56 ± 0.74 ^{aA}	8.68 ± 0.94 ^{aA}
	G30	4.94 ± 0.66 ^{bB}	9.19 ± 0.94 ^{aA}
	CC	3.84 ± 0.74 ^{bB}	7.90 ± 0.94 ^{aA}
***p*-values**			
Genotype [G]		0.0002	
Substrate [S]		0.0081	
G × S Interaction		0.0251	

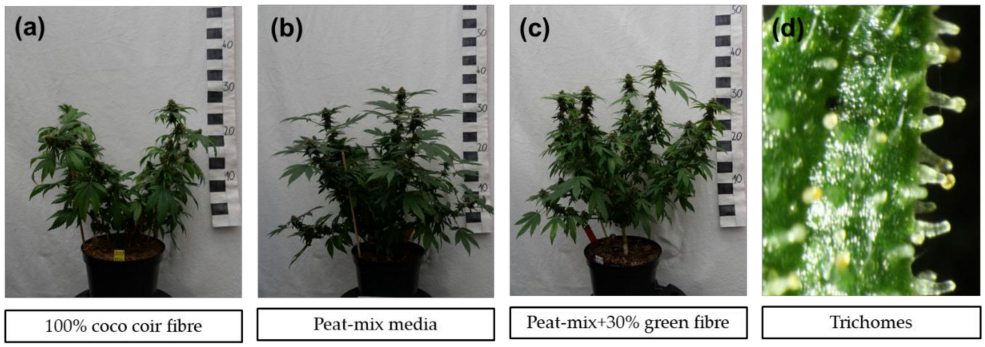

Figure 4. Plants of genotype 0.2x grown on (**a**) 100% coco coir fibre, (**b**) peat-mix media, (**c**) peat-mix + 30% green fibre media and (**d**) binocular imagine of a leaflet top of 0.2x 136 days after planting (DAP).

Significant differences between substrate treatments were found for the DW yield of leaves per plant. Plants grown in PM and G30 reached 5.78 and 5.66 g plant^{-1}, respectively, a higher DW yield than plants grown in CC fibre at 3.30 g plant^{-1} (Table 4).

Root length density (RLD) was significantly affected by the different growth media treatments and genotypes. Plants grown in G30 media had 3087.51 cm cm^{-3}, the significantly highest RLD, followed by PM (2881.65 cm cm^{-3}) and the CC plants with a RLD of 2063.09 cm cm^{-3} (Table 4; Figure 5). Genotype 0.2x (4173.07 cm cm^{-3}) had a significantly higher RLD compared to KAN (1181.76 cm cm^{-3}).

Table 4. Mean leaf dry weight (DW) in g plant^{-1} and root length density (RLD) in cm cm^{-3} of genotypes KAN and 0.2x, grown on peat-mix (PM), peat-mix + 30% green fibre (G30) and 100% coco coir fibre (CC) substrate. Results are presented as mean values ± standard error (Mean ± SE). Letters compare the mean DW yield of leaves and mean RLD. Means in one column followed by at least one identical lower-case letter and means in one row followed by at least one identical upper-case letter are not significantly different as indicated by Tukey's test ($\alpha = 0.05$). The *p*-values correspond to global F tests for differences between the levels of the mentioned genotypes, substrates or their interactions.

Trait	Substrate	Genotype
Leaf DW [g plant^{-1}]	PM	5.78 ± 0.47 ^{a}
	G30	5.66 ± 0.44 ^{a}
	CC	3.30 ± 0.47 ^{b}
RLD [cm cm^{-3}]	PM	2881.65 ± 317.92 ^{ab}
	G30	3087.51 ± 306.93 ^{a}
	CC	2063.09 ± 317.92 ^{b}
***p*-values Leaf**		
Genotype [G]		0.0002
Substrate [S]		0.0010
G × S Interaction		0.2994
***p*-values RLD**		
Genotype [G]		<0.0001
Substrate [S]		0.0279
G × S Interaction		0.4819

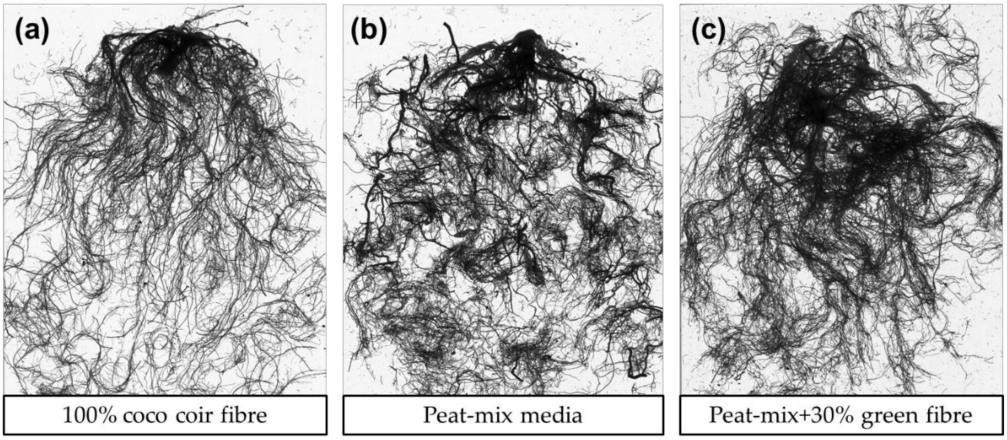

Figure 5. Root scan imagines of plants grown in (**a**) 100% coco coir fibre (CC), (**b**) peat-mix media (PM) and (**c**) peat-mix + 30% green fibre media (G30) of genotype 0.2x.

Genotype-specific differences were found for the DW yield of roots per plant. Plants of genotype 0.2x reached 1.52 g DW plant^{-1}, a higher value than genotype KAN (0.55 g plant^{-1}). Substrate treatments did not affect the root DW of either genotype (Table 5).

The cannabidiol (CBD/A) content of the flowers and leaves of plants grown in PM, G30 and CC did not show any statistical differences. The CBD/A content of flowers ranged between 5.9706%$_{m/m}$ (G30) and 6.2166%$_{m/m}$ (CC) for genotype KAN and between 6.6950%$_{m/m}$ (G30) and 6.7076%$_{m/m}$ (CC) for genotype 0.2x (Figure 6). The CBD/A content of leaves ranged between 3.8393%$_{m/m}$ (G30) and 4.5549%$_{m/m}$ (CC) for genotype KAN and between 3.8586%$_{m/m}$ (G30) and 4.0775%$_{m/m}$ (CC) for genotype 0.2x (Figure 6). The CBD/A yield per plant differed significantly between genotypes. KAN indicated a lower CBD/A yield in flowers (335.28 mg plant^{-1}) and leaves (135.91 mg plant^{-1}) compared to 0.2x, with 549.66 mg plant^{-1} in the flowers and 224.16 mg plant^{-1} in the leaves (Table 5).

Table 5. Mean root DW in g plant^{-1} and mean CBD/A yields in mg plant^{-1} of genotypes KAN and 0.2x, grown on peat-mix (PM), peat-mix + 30% green fibre (G30) and 100% coco coir fibre (CC) substrates. Results are presented as mean values ± standard error (Mean ± SE). Letters compare the mean DW yield of root and CBD/A yields of flower and leaf. Means in one row followed by at least one identical lower-case letter are not significantly different as indicated by Tukey's test ($\alpha = 0.05$). The *p*-values correspond to global F tests for the difference between the levels of the mentioned genotypes, substrates or their interactions.

Trait	Genotype	
	KAN	**0.2x**
Root DW [g plant^{-1}]	0.55 ± 0.10 ^{b}	1.52 ± 0.09 ^{a}
Flower CBD/A [mg plant^{-1}]	335.28 ± 98.67 ^{b}	549.66 ± 79.25 ^{a}
Leaves CBD/A [mg plant^{-1}]	135.91 ± 24.02 ^{b}	224.16 ± 18.63 ^{a}
***p*-values Root**		
Genotype [G]	<0.0001	
Substrate [S]	0.1479	
G × S Interaction	0.2398	
***p*-values Flower**		
Genotype [G]	0.0412	
Substrate [S]	0.7144	
G × S Interaction	0.0937	
***p*-values Leaves**		
Genotype [G]	0.005	
Substrate [S]	0.057	
G × S Interaction	0.3947	

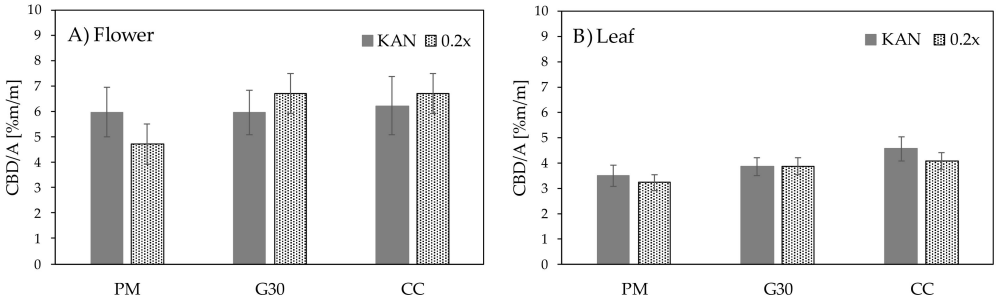

Figure 6. Mean content in (**A**) flower and (**B**) leaf of CBD/A in mass percent [$\%_{m/m}$] of genotypes KAN and 0.2x treated with a peat-mix growth media (PM), peat-mix substituted with 30% green fibre (G30) and 100% coco coir fibre (CC).

4. Discussion

The cultivation of *C. sativa* genotypes in different substrate compositions showed an impact on plant height, biomass yield, root development and biomass N content of the plants in our experiment. Our results indicated that the G30 substrate was suitable for the production of containerised cannabis in comparison to a standard PM-based substrate or pure CC substrate. These results are reflected in the comparable final plant heights of plants grown in PM (40.02 cm) and G30 (36.45 cm). This means an increase of the total plant height of 20% and 12% compared to plants grown in CC fibres (32.03 cm), respectively. Plants grown in PM (54.76) and G30 (53.85) indicated a higher leaf SPAD chlorophyll index compared to plants grown in CC (47.08). Further, these results were substantiated by a higher N content in the leaves of plants cultivated in G30 (52.24 g kg DW^{-1}) and PM (46.75 g kg DW^{-1}) compared to plants grown in CC (37.00 g kg DW^{-1}). No significant differences in flower N content between the substrate treatments were observed. The range between 53.77 g kg DW^{-1} (CC) and 61.87

g kg DW^{-1} (G30) was comparable with the results of Bernstein et al. [33], where flower N contents of 40 to 50 g kg DW^{-1} for cannabis were measured. Bernstein et al. [33] investigated the partitioning of mineral nutrients, e.g., N content, between plant organs, typical uptake and translocation within the plant tissue of *C. sativa*, and showed slightly lower N contents in the leaves of *C. sativa*. The results for N contents ranged between 20 and 30 g kg DW^{-1} and were in line with the results of plants cultivated in CC in the present study, with 37.00 g kg DW^{-1}. The N contents of plants grown in PM and G30 showed higher values, ranging between 46.75 g kg DW^{-1} (PM) and 52.24 g kg DW^{-1} (G30).

The different uptake of N between PM or G30 and CC could have depended on, amongst other things, an adequate air supply and water-holding capacity of the soil. Although CC is a material which rehydrates easily and fast compared to peat, other physical properties of CC, such as water supply and availability, as well as aeration and relative hydraulic conductivity, highly depend on particle size distribution. CC exhibits a higher air content, depending on particle size, but low easily available and total water-holding capacity [34]. Abad et al. [34] reported that CC fibres with a particle size distribution similar to peat showed comparatively higher aeration, and therefore a lower capacity to hold total and easily available water. The described physical differences between CC and peat may have led to this difference in growth performance. Therefore, an irrigation schedule adjusted to the substrate composition, on a crop-by-crop basis, is recommended.

Green fibre is characterised by a high porosity and air-holding capacity [26]. Because of its insufficient plant available water and its tendency to become compressed, it is not recommended as a stand-alone growing media component [35,36]. It can be used to optimise the physical properties of other substrate components, e.g., peat, to increase air space, reduce bulk density and improve re-wetting capacity [23]. Therefore, green fibre may be an ideal component with which peat can be replaced to improve the properties of peat as media.

These positive characteristics of green fibre were also reflected in the present study, in which plants grown in a standard PM media compared to a media where 30% peat was replaced by green fibres (G30) not only provided comparable results in terms of N uptake, but also had comparable positive effects on root growth compared to plants cultivated in CC fibre. The roots of plants grown in PM and G30 had 40% and 50% higher RLDs compared to plants grown in CC, respectively. No significant differences between substrates were observed for root DW. Only a genotype-specific differences were observed. KAN had a lower root DW (0.55 g $plant^{-1}$) compared to 0.2x (1.52 g $plant^{-1}$). Increased root growth in PM and G30 may be attributed to the positive properties of peat and the advantages of green fibres as substitutes. Peat guarantees a high water capacity, whereas air capacity is dependent on the degree of composting [21]. Green fibres are fibrous in structure, elastic, loose and porous, which allows a very high air capacity (good drainability), with a low water capacity in contrast to peat. Due to the low shrinkage properties, green fibres can reduce the shrinkage of peat in pots and improve their value, with very good re-wettability. By contrast, CC fibres have an extremely high air capacity, but a lower water capacity [21].

Above all, the flower yields of genotypes are decisive, since a higher content of cannabinoids in flowers than in leaves is expected [37]. Therefore, a healthy root system is the key factor for high biomass yields, which is essential for optimising cannabinoid yield. The phytocannabinoid-rich cannabis genotypes used in the present study showed a genotype-specific reaction to the different substrate compositions. KAN had the highest floral yield of plants grown in PM (8.56 g $plant^{-1}$), whereas G30 (4.94 g $plant^{-1}$) and CC (3.84 g $plant^{-1}$) were lower by 42% and 55%, respectively. 0.2x indicated no significant difference among the substrates. Floral yield ranged between 7.90 g $plant^{-1}$ (CC) and 9.19 g $plant^{-1}$ (G30). The standard PM-based substrate showed no significant difference between genotypes. KAN had 47% and 51% reduced floral yields when grown in G30 and CC, respectively, compared to PM. In contrast, Caplan et al. [38] documented no significant differences between two coir-based organic substrates and a commercially available peat-based substrate. Floral yield ranged between 34 and 40 g plant $^{-1}$. The differences in flower yield can be explained by the use of a different genotype ('OG Kush × Grizzly') and the different lengths of the vegetative and

generative phases in the experiments of Caplan et al. [38] compared to the current study. Leaves, on the other hand, showed the highest yields for PM and G30 plants, whereas plants grown in CC had in comparison reduced leaf DW yields of 43% and 42%, respectively. No impact on cannabidiol (CBD/A) content between the different substrate treatments was observed. The values of KAN and 0.2x ranged between 5.97%$_{m/m}$ and 5.97%$_{m/m}$ (G30), and 6.22%$_{m/m}$ and 6.70%$_{m/m}$ (CC), in floral dry matter (DM), respectively, whereas the leaves of KAN and 0.2x were between 3.84%$_{m/m}$ and 3.86%$_{m/m}$ (G30), and 4.55%$_{m/m}$ and 4.08%$_{m/m}$ (CC), respectively. The higher CBD/A content in female flowers compared to leaf tissue was in line with Stout et al. [37].

In addition, considering the presence of cannabinoids in the aerial parts of the plant, correlated with their number of glandular trichomes, especially on flowers and upper leaves [24], the biomass yield of flowers and leaves plays a key role in maximising CBD/A yield. In this regard, flower and leaf yield are far more important to overall yield than a slight difference in CBD/A percentage between genotypes, as indicated by Calzolari et al. [39]. This supports 0.2x, with a higher CBD/A yield of flowers (549.66 mg plant^{-1}) and leaves (224.16 mg plant^{-1}), as an interesting genotype for indoor CBD/A production.

Considering that the production of cannabinoids as herbal medicinal products is aimed at, standardisation through compliance with GACP (Good Agricultural and Collection Practice) and GMP (Good Manufacturing Practice), and clear guidelines at the level of cultivation, are essential. Adequate pharmaceutical quality depends not only on the selection of genotypes, but also on external environmental factors that influence plant growth [9]. In addition to lighting, CO_2 concentration, temperature, humidity, water and nutrient supply, the substrate composition of the growing media is also important in order to cultivate cannabis flowers in fully air-conditioned greenhouses, which generate cannabinoids in a narrow range of contents from batch to batch. Since no significant impact of the treatments on CBD/A content, either in the flowers or in the leaves of both genotypes, could be found, it may be beneficial to replace the most frequently used peat substrate with at least 30% green fibre. However, while coco coir fibres could also be considered with respect to CBD/A content, cultivation in CC did not have a beneficial effect on the biomass production of the two genotypes evaluated in this study. A proportionate mixture of coco coir fibres with peat compounds should be tested in the future.

5. Conclusions

The results of this study showed that different substrate compositions, namely coco coir fibres (CC), standard peat-based media (PM) and peat substituted with 30% of green fibres (G30), had significant impacts on the growth, biomass yields, root development and nitrogen (N) tissue content of *C. sativa* after harvest. The use of CC as a growing media indicated a reduction in total plant height, leaf N content, leaf DW yields and root length density (RLD) compared to PM and G30 growing media. Both phytocannabinoid-rich cannabis genotypes reacted in a genotype-specific manner on flower yields. Whereas KAN had the highest floral yield when grown in PM, 0.2x showed no significant differences, with higher yields grown in G30 and CC compared to KAN. A limiting effect on the CBD/A content enacted by the different substrates could not be confirmed. The impact of different substrate compositions on the growth, development and cannabinoid content of *C. sativa* is a major issue when considering cannabis' use as a botanical therapeutic, ideally with a fixed dosage of active compound, with a small range of variation. It can be concluded that the use of organic green fibres to partly replace the fractionated peat showed a genotype-specific option for constant plant development, a comparable high biomass yield and a stable cannabinoid content, compared to a peat containing standard substrate.

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6 General Discussion

Due to the diverse applications and the potential of the bioactive substance groups, above all the cannabinoids, the hemp plant is currently experiencing a revival. The main objectives of the present thesis were to develop a unified cultivation system for the production of cannabinoids from *Cannabis sativa* L. genotypes. In a first step, it was of particular importance to screen the suitability of industrial hemp genotypes, which may be legally cultivated in Germany, for their cannabinoid content in order to be able to make a recommendation about the suitable harvest date and harvest organs of the plant, taking into account other utilization objectives. In a second step, the thesis aimed for the further standardization of phytocannabinoid-rich cannabis genotypes under indoor cultivation conditions, by evaluating different PGRs to adapt the plant morphology for indoor cultivation systems. Further different potting substrate compositions were tested.

Each publication focused on one major topic. **Publication I** focused on the cultivation of seven registered industrial hemp genotypes (THC < 0.2%), namely Fédora 17, Ferimon, Félina 32, Futura 75, USO 31, Santhica 27 and Finola, and their yield potential in terms of biomass and inflorescence as well as cannabinoid content of the different fractions depending on the growth stage. The aim was to identify the most suitable genotypes, their harvest date and plant organ for cannabinoid production on a field scale. In **publication II,** the influence of exogenously applied synthetic PGRs, namely 1-naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP) and a mixture of both, was investigated on the plant architecture of phytocannabinoid-rich cannabis genotypes. The objective was to evaluate the potential of a beneficial PGR application to adapt plant morphology for different indoor culture systems and to meet optimized plant space requirements. Further standardization of the indoor production system for cannabis in pot culture was discussed in **publication III**. It was based on the evaluation of different growth media compositions consisting of (a) peat-based substrate, (b) peat-reduced substrate substituted with 30% of green fibres and (c) pure coco coir fibres. The discussion also took into account the impact of these growth media on quality parameters, such as growth performance, biomass and flower yields, root growth and CBD contents of two different phytocannabinoid-rich cannabis genotypes. The general discussion part will give a deeper insight into the results and findings of publications I – III, draw different perspectives, and evaluate an optimized cannabinoid production chain for outdoor cultivation on a field scale as well as under greenhouse conditions in a pot cultivation system. In addition, the results obtained in this work will be discussed in the context of the current global challenges and the quest for a green

revolution to recover the EU economy, decoupling economic growth from resource consumption from which the environment, society and the economy can permanently benefit.

6.1 Industrial hemp genotypes for CBD/A production

The call within the framework of the European Bioeconomy Strategy to pursue the Green Deal objectives to contribute to the recovery of the EU economy, further increases the farming interest in hemp cultivation.

The agricultural cultivation of hemp has increased since its rebirth in the early 1990s. With the legalization of industrial hemp production, research on health benefits of hemp products was promoted (Rupasinghe et al., 2020). Nevertheless, production in Europe lags far behind demand. Thanks to its superior properties, hemp has already been able to gain a foothold in many sectors of the economy. But the innovation of novel hemp-derived food ingredients and nutraceuticals still requires precise identification as well as quantification of the major bioactive compounds and a standardization of the product (Rupasinghe et al., 2020).

Hemp has a very broad field of application, such food through the incorporation of hemp seed oil and its constituents or the durability of hemp fibres, as its fibre properties gained from the hemp stalk turns it to a valuable product (Andre et al., 2016; Callaway, 2004; Struik et al., 2000; Tang et al., 2006). Nevertheless, in mainstream value-added and supplemented food products, the use of CBD as pharmaceutical or cosmetic additive is currently reported as one of the top trends in the industry.

The newly discovered popularity of the cannabis plant due to its bioactive substances provides growers with new challenges. Generally, these industrial hemp genotypes, which comply with the 0.2% THC limit set by the EU legislation, restricts the choice of hemp genotypes for European farmers, compared to producers of other countries, e.g. Switzerland, North America, Asia and Canada, with their THC limits ranging from 0.3% up to 1%. These tested genotypes were not bred specifically for a high cannabinoid yield but primarily either for fibre extraction or grain production or for dual-purpose production. The question arises whether one of the genotypes tested in the experiment is suitable for a purpose that is new to existing cultivation systems. One major problem is to have an overview of the cannabinoid concentration within plant tissue over the vegetation period.

Generally, some of the tested genotypes, namely Futura 75, Félina 32, Fédora, Finola and Santhica 27 showed potential for non-psychotropic cannabinoid production, such as CBD/A

and CBG/A (Burgel et al., 2020a). Especially, trichome-rich organs at full-flowering showed a maximum of cannabinoids, as also reported by Mandolino and Ranalli, (2002). Besides, the choice of the appropriate genotype, the growth stage at harvest as well as the plant fraction seems to be highly important for optimizing cannabinoid production at field level (Burgel et al., 2020a; Khan et al., 2014). Nevertheless, cannabinoid contents of industrial hemp genotypes (Futura 75, Félina 32, Fédora 17 und Ferimon) ranged from 1.79% to 2.72% at full flowering and contents of the threshing residues of genotypes Futura 75, Félina 32, Fédora 17, and Finola ranged from 1.36% to 1.76% at seed maturity (Burgel et al., 2020a). The approach of optimizing a low cannabinoid content by increasing land use in the open field involves a biomass yield measurement of the plant organs concerned. Fibrous hemp genotypes are certainly characterized by a low flower yield, as the breeding focus is on a high stem biomass. In contrast, genotypes bred for oilseeds produce more flower biomass than fibre genotypes do, according to Small (1979). Here, the inflorescence is the principal source of resin which contains the highest concentration of cannabinoids. Thus, genotypes bred for oilseeds seems to be much more promising for CBD production than fibre genotypes are.

In this context, a common example of a popular oilseed hemp variety is the ‘Finola’ genotype (Smeriglio et al., 2016). Nevertheless, within the present study contents of 1.49% at full-flowering and 1.61% at seed maturity were determined. Higher biomass yields of inflorescence resulted in CBD/A yields of 5422.41 mg m^{-2} and 5686.47 mg m^{-2} respectively, at a row distance of 0.15 m (Burgel et al., 2020a). This does not nearly meet the expectations of high-yield cannabis genetics with a focus on cannabinoid production. For cultivation in Germany, cannabis genotypes with a phytocannabinoid content between 10% and 30% and a THC content of < 0.2% are required to enable companies to cultivate medical cannabis for CBD production, which would help meet the increasing demand on a competitive and economic level. Thus, it is questionable whether the cultivation of these industrial hemp genotypes can cover the high demand for CBD products in Germany or in the rest of Europe. Previous studies have shown that medical genotypes, which have been specially developed with the breeding objective of high cannabinoid concentrations and bigger flower sizes, produce a considerably higher amount of cannabinoids than industrial hemp genotypes do. This is a decisive point as to why industrial hemp should not be reduced to a single production target, and instead, possible multiple and cascading uses should be considered and discussed. Especially, through the newly developed policy framework, which focuses on the European Green Deal, it can be discussed how industrial hemp cultivation can make an important contribution to upscaling innovative

solutions that accelerate the transition to a regenerative growth model and, at the same time, strengthen rural areas by creating attractive jobs there and also employment in the manufacturing industry (Reichenbach, 2020).

6.2 Multipurpose and cascade use of industrial hemp genotypes

Due to the versatility that characterizes the hemp plant, it must not be disregarded as a valuable multi-use crop for further utilization targets. The real added value of industrial hemp lies in the possibility of producing different products with one crop in the annual crop rotation. Industrial hemp cultivation is more efficient and less environmentally degrading than many other crops (Ranalli & Venturi, 2004). The fast growing capacity under various agro-ecological conditions, especially during the first weeks after emergence, makes the hemp plant an ideal crop for carbon sequestration (Adesina et al., 2020; Ranalli & Venturi, 2004; Struik et al., 2000). Therefore, it is an excellent candidate for carbon farming purposes to capture carbon in the soil and in manufactured products. In this context, specific regard has to be given to the planting density, which highly depends on genotypes and the final purpose of utilization. Fibre genotypes are in general planted in high density to promote stalk growth and restrict flowering, reaching an average height of 5 – 6 m. Amaducci et al. (2002) recommended a planting density of 180 plants m^{-2}, when aiming for high yields of long bast fibres. Oilseed or CBD genotypes should do well in farther apart densities to encourage branching and flower yields, reaching an intermediate height of 1 – 1.2 m (Adesina et al., 2020). To achieve high floral yields, a plant density of 15 plants m^{-2} is recommended for hemp grown for CBD production. It is assumed that a high oil production from seed yielding industrial hemp genotypes would require uniform planting densities (Meijer et al., 1995; Williams & Mundell, 2018), whereas a suitable plant spacing to achieve high yields of stem, seed and inflorescence together was determined to be 120 plants m^{-2}, with 0.5 m spacing between rows (Campiglia et al., 2017). Hemp has a 250% higher fibre yield than cotton and 600% higher fibre yield than flax, based on the same acreage (Ranalli & Venturi, 2004). Furthermore, the hemp plant stands out as a fibre plant which is more ecologically neutral than other fibre crops, especially in terms of water usage. The water saving compared to cotton is 75% (Reichenbach, 2020). Hemp fibres were previously used for clothes and for ropes in the sailing ship industry, which over time were replaced by more suitable materials including less extensive cotton or synthetic fibres (nylon, polyester, and acrylic), and finally sailing ships were replaced by steamships (Deitch, 2003). However, the hemp fibre is now becoming more attractive again as a carbon negative raw material in the manufacturing of recyclable, reusable and compostable biomaterials.

Hemp as a construction material has an exceptional thermal quality that reduces power consumption while sequestering carbon. Commonly used are hempcretes, a special hemp-lime composite walling material for isolation; beside hemp wool or fibre-board insulation materials are also used (Reichenbach, 2020). Despite their excellent properties, the use of hemp fibres in the construction industry has not yet been fully established. Hemp as a high-quality novel construction material must be upscaled and mainstreamed.

Furthermore, hemp can be a sustainable alternative to synthetic plastics, which are useful but cause environmental problems. Governments have recognized that there is a growing expectation from the society that some form of intervention and alternatives be shown. Industrial hemp, as a plant-based material, can be used for compostable packaging to reduce the use of plastics; and it can also be utilized in the car industry to make vehicle interiors as it is light weight. In addition, it is as more durable as steel, resulting in a higher energy efficiency and reduced emissions (Reichenbach, 2020).

Further, hemp can act as a natural weed suppressor because of its fast growing and dense canopy, which enables cultivation without herbicides. It can suppress certain nematodes and fungi in the soil, which enables its cultivation without pesticides and fungicides (Adesina et al., 2020; Ranalli & Venturi, 2004). The soil quality can be significantly improved by hemp cultivation through its anchored root system. The rooting system helps to prevent soil erosion as well as nutrient leaching into deeper soil layers. In addition, hemp roots are effective for phytoremediation by absorbing heavy metals from the contaminated soil and storing them in the plant tissue. The organic matter content of the soil increases during the growing season by the continuous soil shading of the hemp leaves (Andre et al., 2016; Struik et al., 2000). Due to the unique properties of increasing soil quality, industrial hemp is a prime candidate for a crop rotation programme to improve yields of the main crop (Adesina et al., 2020).

The growing market of functional and health-promoting food ingredients is due to a steadily increasing public awareness in society for one’s own health. In the case of industrial hemp cultivation for fibre production, harvesting takes place at the flowering stage, and therefore, the seeds cannot mature and be collected. The production of hemp oilseed has recently attracted attention as their macronutrients constitute a balanced health product because of its bioactive components that can support health beyond basic nutrition (Callaway, 2004; Vonapartis et al., 2015). Generally, hemp seeds have been an important nutritional source for thousands of years worldwide, but nowadays especially their huge health promoting properties

are highlighted although most people are still unaware of the valuable nutritional and nutraceutical benefits offered by hemp seeds (Crescente et al., 2018). Indeed, the main constituents of hemp seeds includes 20 – 25% easily digestible proteins, 25 – 30% abundant lipids, polyunsaturated fatty acids, and 20 – 30% carbohydrates (Callaway, 2004; Deferne & Pate, 1996; House et al., 2010; Matthäus & Brühl, 2008; Silversides & Lefrançois, 2005; Tang et al., 2006; Vonapartis et al., 2015). These high-quality and easily digestible proteins, albumin and edestin, present in hemp seeds are rich in essential amino acids; and they are well suited for human consumption as well as for animal feeding (Matthäus & Brühl, 2008; Tang et al., 2006; Vonapartis et al., 2015). Additionally, hemp seed has high levels of the amino acid, 'arginine'. Since it is a rich source of polyunsaturated fatty acid, linoleic acid (omega-6) and alpha-linolenic acid (omega-3), with a ratio of 3:1, hemp seed oil is regarded as balanced for human nutrition (Ditrói et al., 2013; Schultz et al., 2020; Tang et al., 2006; Vonapartis et al., 2015). These fatty acids, which cannot be synthesized endogenously, but are required for a proper nutrition, must be acquired from the diet (Deferne & Pate, 1996; Dimić et al., 2009; Kriese et al., 2004; Tang et al., 2006; Vonapartis et al., 2015). The seeds can be consumed as raw or peeled seeds, seed oil and seed meal or flour. Further, the pressing of hemp seed generates hemp seedcake as a co-product, also rich in protein and dietary fibre. According to nutritional recommendations, 15 – 20% of the daily caloric intake should come from fats. In this context, one-third of it should be essential fatty acids with an omega-3 to omega-6 ratio of 3:1. Due to its favourable composition, hemp seed oil is an excellent choice to meet this need. The European Industrial Hemp Association (EIHA) expects the plant-based food market to grow to €2.4 billion by 2025 from €1.5 billion in 2018, as hemp represents a perfect sustainable protein source to be grown locally and organically (Reichenbach, 2020).

The above-mentioned fields of application and positive agronomic properties of industrial hemp cultivation provide an overview of the diverse uses and potential of the hemp plant. Publication I indicated that the growth stage at harvest and the plant fraction seemed to be highly relevant. Genotypes, such as Futura 75, Fédora 17, Félina 32 and Ferimon, which were highlighted for a higher CBD/A content, reached between 78% and 86% higher dry weight yields of threshing residues after seed maturity, compared to floral yields at full flowering. This consequently leads to a higher total CBD/A yield per square metre, with values up to 6011.20 mg m^{-2} (Fédora 17) (Burgel et al., 2020a). Focusing on these results, harvesting after seed maturity seems to be more economical in terms of CBD extraction due to higher biomass

yields. This makes selected genotypes such as Finola, Futura 75, Fédora 17, Félina 32 and Ferimon excellent candidates for dual- or multi-purpose cropping.

In publication I, special attention was given to the industrial hemp genotype Santhica 27, which had a significantly lower CBD/A content in inflorescence at full flowering, compared to the other genotypes. This genotype became noticeable due to a higher CBG/A content in its inflorescence material, with the CBGA content ranging from 1.40% to 3.24% and CBG content, from 0.14% to 0.52% over the harvest dates (Burgel et al., 2020a). Considering that in many studies CBG/A is described as having a promising medical potential for the treatment of various diseases, it has not been extensively studied so far (Aizpurua-Olaizola et al., 2016; Deiana, 2017). The availability of CBG/A in the cannabis plant is very low because it is rapidly converted into THC (Mechoulam, 1995), CBD (Taura et al., 1996), and CBC. A recessive allele that reduces the efficacy of the synthases into THC, CBD and CBC is responsible for higher CBG/A concentrations in this plants, which allow the generation of CBG-dominant cannabis genotypes (de Meijer & Hammond, 2005). In this context, a new chemotype was reported by Fournier et al. (1987). Consequently, it is important not only to focus on the CBD/A content, when screening suitable industrial hemp genotypes for the extraction of non-psychoactive cannabinoids, but also to consider other promising cannabinoids such as CBG/A and CBC/A.

Overall, industrial hemp indeed shows potential for the extraction of non-psychoactive cannabinoids, but due to low contents of these cannabinoids, the exploitation possibilities of individual genotypes should be evaluated separately in order to fully use the economic value of the plant.

Still, there are many obstacles preventing hemp from getting the place it deserves in our economy, both in the fibre as well as food industry and in the pharmaceutical sector. Due to the drastic reduction in the cultivation of hemp during prohibition, hardly any investments are being made in the field of hemp fibre use, despite the high potential of this environment- and climate-friendly recyclable raw material; beside, there is no planning security. Intensive and transparent support is needed from governments, interest groups and citizens, in order to revolutionize hemp cultivation on a large scale, from which the environment, society and the economy will benefit in a sustainable way.

6.3 Cannabinoid production under indoor conditions

The European Monitoring Centre for Drugs and Drug Addiction, (2012) stated that a driving force behind the change in the diversity of cannabis products in the EU was the dramatic increase in domestic production in parts of Europe.

The cannabis grown in the EU comes from both indoor production and outdoor cultivation. While outdoor cultivation allows only one harvest a year in most countries, indoor production techniques can yield several harvests per year and area (Clarke & Watson, 2007). The possibility of year-round cultivation under stable conditions, which can result in a maximum of six harvests per year, makes the indoor exercise 15 – 30 times more productive than outdoor cultivation (Magagnini et al., 2018). The multiple harvests can be made possible if the production facilities are set up in separate rooms for each of the three cannabis growth phases (propagation, vegetative growth and flowering phase) due to the different photoperiods as well as other environmental requirements (Magagnini et al., 2018).

The type of production and cultivation methods used affect yields. Factors, such as plant density, water supply (irrigation or rain-fed crops), soil pH and climate conditions can have a significant effect on outdoor produced cannabis, and these factors can differ from year to year depending on the growing region and genotype (Clarke & Watson, 2007).

Furthermore, it should be noted that the overall yield cannot be assessed by the weight of flowers alone; it is mainly the chemical composition of the bioactive ingredients that is of interest to producers and end users. For a medical producer, a uniform and continuous yield as well as production of a certain cannabinoid compound or a specific cannabinoid ratio between growth cycles is important. Due to this fact, indoor cultivation is moving strongly into focus. Within these indoor systems light intensity, light spectrum, temperature, humidity, and air CO_2 concentration can be adjusted (Magagnini et al., 2018).

The development of suitable cannabis cultivation techniques for the production of high-quality raw material to feed the pharmaceutical sector must be discussed and facilitated. In a first step, it should be defined, what ‘medical grade cannabis’ means for the production of flowers and the whole production chain. Generally, herbal drugs and extracts, as with all pharmaceutical raw materials used in the production of medical products, must be of suitable pharmaceutical quality. Corresponding quality standards are described in the individual monographs of the pharmacopoeia, according to § 55 of the German Medicines Law. They contain, among other things, information on ingredients and required active substance content (Bundesinstitut für

Arzneimittel und Medizinprodukte, 2020). One of the problems is that quality standards are currently not harmonized between EU member-states or between the EU and/or Canada, the USA, etc. In Europe, these products must meet the relevant requirements laid down in the Medicines Act (including pharmacopoeias, EU GMP/GDP and GACP) and the Narcotics Act. In Germany, for example, the BfArM has taken on new tasks by establishing the Cannabis Agency. This agency ensures the supply of cannabis in of medical grade. The agency will manage and supervise the cultivation, harvesting, processing, quality assurance, storage, packaging and distribution of cannabis to wholesalers, pharmacists or manufacturers. To meet the pharmaceutical quality and specifications originally required by the Cannabis Agency for cannabis flowers as herbal medical products, only cultivation in closed production facilities or high-tech greenhouses is suitable (Laginha, 2018). Due to this fact, it is essential to discuss the indoor cultivation of cannabis for the pharmaceutical sector.

Fundamental to the production of medical products of uniform pharmaceutical quality are standardization measures at all stages of manufacture to achieve a defined standard prescribed by pharmacopoeial monographs or other official collections and in-house specifications (Teuscher et al., 2012). In the case of cannabis flowers, the German Pharmacopoeia Monograph (‘Deutsches Arzneibuch, DAB’) ‘Cannabis flowers’ and the general monograph ‘Herbal drugs’ of the European Pharmacopoeia are particularly authoritative. According to the definition of the DAB monograph, cannabis flowers should contain at least 90% and at most 110% of the cannabinoids, such as THC and CBD, and it should be indicated in the labelling (Bundesinstitut für Arzneimittel und Medizinprodukte, 2017). This is due to the fact that the individual genotypes sometimes differ considerably in terms of their THC and CBD content. Consequently, a fluctuation range of ± 10% is tolerated within a batch in order to enable correct dosing. The fact that there can also be considerable differences within a variety from harvest to harvest is not taken into account. This regulation would be problematic for genetics with low cannabinoid content, as a relative variation of ± 10% around the mean cannabinoid content would mean a much too narrow content range. This is another aspect of not only focusing on low-content industrial hemp genotypes, but also placing new phytocannabinoid-rich genotypes on the market. In principle, the monograph for ‘herbal drugs’ contains the general requirements that a drug must fulfil, with a particular focus on purity (foreign components, moisture, pesticide residues, heavy metals, mycotoxins and microbial impurities) (Europäische Arzneibuch-Kommission, 2017). Therefore, standardization at the level of cultivation is of particular importance, although the **GACP** guidelines of the EU provide that there is a quality

assurance system for cultivation, harvesting and primary processing (European Medicines Agency - Committee on herbal products, 2006). For further processing steps, the **GMP** guidelines also apply. The primary goal in medical plant cultivation is to generate uniform plants of appropriate pharmaceutical quality. The characteristic expression (phenotype) and, therefore, the quality properties of medical plants depend on the interaction of genotype and environment.

Taking this into account and since it is known that plant morphology and metabolism can be manipulated by influencing various factors such as indoor growing conditions and management methods such as pruning techniques and netting for morphological adjustments, publication II focused on adaptation of plant morphology through PGRs. The adaptation of plant morphology through pruning and training techniques is very costly and has an increased risk of infections. Therefore, the influence of different elicitors, such as synthetic phytohormone-based PGRs, can offer an alternative to morphological adaptation, without injuring the plant by cutting or pruning.

6.4 Plant growth regulator application for morphology adaptation

Since the morphology of phytocannabinoid-rich cannabis genotypes differs considerably, the aim is to adapt the plant architecture for various indoor cultivation system in order to achieve the best possible area use for an optimized space requirement and an increased floral yield (Burgel et al., 2020b). The results of publication II have shown that exogenously applied synthetic phytohormones, such as PGRs, can beneficially influence the plant architecture of the tested genotypes. The applied PGRs, NAA and BAP, are synthetic analogues of the endogenous phytohormones auxin (IAA) and cytokinins (CK), which naturally occur in plants, acting as signalling compounds (Sauer et al., 2013). By applying NAA during the vegetative phase, the genotypes showed significantly reduced height. A BAP treatment also reduced height, while a mixture of both did not differ from the height of the non-treated control and the BAP treatment. After the last application and entry into the generative phase, all treatments resulted in significantly shorter plants compared to the untreated plants. This was accompanied by a significantly greater reduction in axillary side branches caused by the application of NAA, whereas BAP did not reduce the length of axillary side branches, but a mixture of both showed a reduction compared to the untreated cannabis plants. Additionally, the average number of internodes per side branch was reduced by all application treatments. Overall, PGRs have the potential to modify the architecture of the cannabis plant (Burgel et al., 2020b).

Results from Lalge et al. (2016) and Mendel et al. (2020) reinforce the assumption of a genotype-specific reaction of phytohormon-based PGRs. Lalge et al. (2016) noted in their study either an increase in total plant height through NAA applications or no impact, depending on the concentration that was applied to the plants. Mendel et al. (2020) reported that there was no impact of NAA treatments on plant height. The results of BAP applications by Lalge et al. (2016) and Mendel et al. (2020) are also in contrast to the present study, presenting no impact on the total plant height and a rising stimulation on side branching accompanied by an increase in BAP concentration. This is despite the fact that growth retardants including CKs are used to obtain short and compact plants (Pobudkiewicz, 2008). In this regard, it is important to mention that in both studies an industrial fibre hemp variety ('Bialobrzeskie') was used, which again clearly supported the assumption that the impact of PGRs might show genotype-specific differences.

The reproductive inflorescence yield potential in cannabis largely depends on the morphology of the plants at the vegetative phase, as reported by Shiponi and Bernstein (2021). One of the genotypes (KANADA) tested in publication II showed a positive response to NAA treatment during the vegetative phase, expressed by a short habitus, shortened axillary side branches and a reduced number of internodes per branch. This finally led to high floral yields and a stable high CBD content (Burgel et al., 2020b). It can be suggested that the morphological modification of this genotype results in a higher floral yield potential per area.

The results of this work have provided information about the different genotype-specific reactions of pohytocannabinoid-rich cannabis genotypes to PGR applications. Generally, each cultivar should be treated as an individual case to balance potential positive as well as negative aspects of different growth regulating chemicals (Cutting & Wolstenholme, 1993). Cannabis genotypes bring with them the complexity that existing genotypes can be very different morphologically even though they belong to the same genus. Therefore, it might be recommended that the influence of PGR be individually screened on all used cannabis genotypes existing for indoor cultivation. Since the morphology of the genotypes can differ greatly from one another, the first step is to assess which morphological features should be adapted, depending on the cultivation system and purpose. The exogenous application of a synthetic phytohormone, such as NAA, can have a positive effect on genotypes characterized by increased height growth and can achieve more compact plants, whereas a genotype with naturally bushier growth can be negatively affected by the same phytohormone.

The application of specific PGRs has already found a permanent place in horticulture, fruit growing and field cultivation for a wide range of crops to improve quality (Correia et al., 2019; Cutting & Wolstenholme, 1993; Sauer et al., 2013). Nevertheless, to establish the use of PGRs sustainably in the long-term indoor cannabis cultivation, in order to adapt the plant architecture in a space-saving way depending on the cultivation system, it must be clarified in a first step whether the use of PGRs for medical cannabis cultivation is permitted so that the high quality standard is achieved as per the DAB monograph guidelines. It must also be clarified whether the application is only meant for the extraction of CBD as an ingredient for food supplements or for the wellness products sector.

Since it is known that synthetic phytohormones are more stable than endogenous phytohormones, it is crucial to develop a better understanding of how to regulate and concentrate them into artificial growing systems (Dunlap et al., 1986). Further, endogenous phytohormones, which occur naturally in plant tissue, could alternatively be used for their positive properties especially in medical grade cannabis cultivation which is gaining greater acceptance.

6.5 Growing media evaluation for further standardization of indoor cannabis production

Soilless growing substrates for pot plants used in greenhouse, container, and other indoor production systems consist of pure organic materials or are substituted with inorganic materials. Suitable attributes of these materials for horticulture are low costs and physical properties that support adequate aeration and water retention for an optimal growth performance (Londra et al., 2018). Sphagnum peat is probably the most commonly used substrate for ornamental plant cultivation (Bunt, 1988; Heiskanen, 1993; Raviv et al., 2002). As reported in numerous studies, economics, peat environmental awareness and the availability of new recyclable materials increase the interest in new substrate compositions (Samadi, 2011). Since it is known that the most promising alternatives are coco coir, wood fibres, and composts (Carlile & Coules, 2013), with comparable growth results to peat (Evans & Stamps, 1996; Londra et al., 2012; Mak & Yeh, 2001; Noguera et al., 2000), it is of great importance to evaluate the potential of these alternative substrate compositions and integrate them into the rapidly growing indoor cannabis sector.

The findings of the present work, which are reported in publication III, are in agreement with results of previous studies that investigated growth performance of different plant species in

various substrate compositions. The substrate composition consisting of a pure coco coir growth media (CC), a standard peat-based growth media (PM) and a peat substituted with 30% of green fibre growth media (G30), indicated an impact on plant growth and performance of two phytocannabinoid-rich cannabis genotypes (KANADA and 0.2x). This was reflected in a final plant height of cannabis plants grown in PM-based media (40.02 cm) and in G30-based (36.45 cm) growth media, which meant a growth increase of 20% and 12%, respectively, compared to the growth in pure coco coir fibres. Higher SPAD values together with an increased leaf N content ranging from 46.75 g kg DW^{-1} (PM) to 52.24 g kg DW^{-1} (G30) indicated a better N uptake, compared to CC cultivation (Burgel et al., 2020c). This points to a better nutrient supply for plants grown in PM and G30 and is consistent with results of Londra et al. (2018), where increased N supply to plants led to higher leaf N contents and an increased canopy size.

These results highlight that cannabis plants cultivated in a substrate where 30% peat has been replaced by green fibres, provide comparable growth rates in terms of N uptake and further comparable positive effects on root growth, which is represented by a 40% (PM) and 50% (G30) increase in root length density (RLD) compared to CC growth media (Burgel et al., 2020c). This promotes a healthy root system crucial to achieving a high biomass production that is essential for an increased cannabinoid yield.

Considering that *C. sativa* is a fast growing plant species with a high and valuable biomass production, and also a plant that uses resources efficiently, the final biomass yields and N-use efficiency may be dependent on water availability (Tang et al., 2018). Although it is reported that cannabis is tolerant to water limitations due to improved water-use efficiency (Cosentino et al., 2013), the final biomass production and N-use efficiency are restricted by a reduced water availability during growth. Therefore, it is important to evaluate the water retention capacity as well as other physical properties of the individual substrate materials in order to create an optimal root growth with sufficient water and nutrient supply, made through a suitable medium, for the cannabis plant to provide high yields. Of course, the selection of the appropriate genotype, which is characterized by a suitable cannabinoid profile, is very important, but biomass and floral yields play a key role in maximizing CBD/A yields, which is far more important than slight differences in CBD/A percentages (Calzolari et al., 2017).

The differences in growth achieved by the different substrate treatments revealed by the study can be attributed to the different chemical and physical properties of the substrate compositions used. Although, peat and coco coir offer many advantages in terms of low bulk density, high

water retention capacity and easy root penetration, disadvantages such as a poor aeration and low pressure heads, due to a low percentage of large particles present, create a problem in water-air balance and gas-exchange, depending on the watering regime (de Boodt & Verdonck, 1972; Heiskanen, 1993; Londra et al., 2012). In particular, coco coir is characterized by its dependence on particle sizes for air content and water retention capacity (Abad et al., 2005). The results are in line with studies on other species and indicate the lowest growth increase of plants cultivated in pure coco coir (Londra et al., 2018). This was also observed in the present study and resulted in higher leaf yields for plants grown in PM and G30 (Burgel et al., 2020c).

Overall, the study reveals that different genotypes react differently to the physical properties of the soil, such as water-storage of the substrate, etc., and the substrate compositions should be selected in accordance with the genotype. Results of several comprehensive studies, which have investigated the complexity of different cannabis genotypes and the acclimatization strategies to cope with soil and water conditions (Herppich et al., 2020), on the physiological properties of the cannabis plant, such as photosynthetic performance as well as gas exchange, response to N supply variations (Chandra et al., 2011; Maļceva et al., 2011; Tang et al., 2017, 2018) and soil water availability (Chandra et al., 2011; Tang et al., 2017, 2018), illustrate the genotype-specific properties of different substrate compositions.

In contrast, green fibres, which are elastic, loose and porous, thus exhibiting good drainability due to their high porosity and air-holding capacity (Maher et al., 2008; Schmilewski, 2008), can be ideal as an additive for peat-based substrates, as they can upgrade the substrates by reducing the shrinkage of peat in pots and providing very good re-wettability. Green fibres are, however, unsuitable as a stand-alone growth media due to its low water capacity in contrast to peat, and its tendency to become compressed (Domeño et al., 2010; Gruda & Schnitzler, 2004).

The present study points out that organic green fibres can be a perfect genotype-specific option to partly replace peat for a constant plant growth together with comparable high floral yields and a stable cannabinoid content. The study showes a high potential for green fibres as a substitute for commercial cannabis potting substrates and lays the foundation for further research to explore and evaluate mixing ratios of peat and green fibres in indoor cannabis cultivation. As evaluated in the present study, pure coco coir fibres did not show any beneficial effects on growth performance and biomass productions of the genotypes in this cropping system; however, they have been shown in various studies to be a stand-alone growth media or an ingredient for use in horticultural substrate mixtures to replace peat (Evans & Stamps, 1996;

Meerow, 1994). Because of their positive physical properties (Abad et al., 2005; Evans & Stamps, 1996; Londra & Valiantzas, 2011; Londra, 2010; Londra et al., 2012; Meerow, 1995; Schindler et al., 2015; Valiantzas et al., 2007; Valiantzas & Londra, 2012), it is recommended that coco coir fibres are not disregarded completely as a potting substrate in cannabis cultivation and that their potential in different mixing ratios is evaluated as a substitute for peat. Coco coir fibres are less hydrophobic than peat with a naturally higher cation exchange capacity, so flushing is recommended as part of the irrigation routine. In addition, the potential of coco coir fibres should be compared to water-based growing operations with soilless media mixtures, such as rock wool, perlite and clay pebbles in hydroponic systems. It has to be considered that hydroponic systems are very cost-intensive and demanding in terms of installation and requirement for water as well as nutrients. Also, they have a lower buffer capacity than that of substrates. However, hydroponic cropping systems allow most of the growing process to automate and also allow a comprehensive control, but there is no evidence that floral yields and potency are improved (Jin et al., 2019; European Monitoring Centre for Drugs and Drug Addiction, 2012).

Nevertheless, the substitution and mixture of different compositions raise questions about the ability of the components in their mixing ratios to produce a high-quality substrate for optimum genotype-specific cannabis plant growth, so that it is commercially used as standard growing media on a large scale.

Finally, it must be mentioned that due to the stringent quality requirements for the flowering raw material, indoor cultivation of cannabis will continue to be a focus of research. The present study has shown that optimization through morphological modifications and the choice of a suitable potting substrate have potential for further standardization of cultivation, but the cannabis plant has such a high degree of complexity that both the use of synthetic phytohormones in the form of PGRs and the choice of a suitable substrate composition must always be assessed on a genotype-specific basis what underlines the diversity of existing cannabis genotypes.

6.6 Outlook

The present thesis fundamentally discussed relevant parts of cannabis cultivation, firstly through the cultivation of approved industrial hemp genotypes in the field for cannabinoid extraction and, secondly, the production of cannabinoids indoors through phytocannabinoid-rich cannabis genotypes.

The thesis found that selected industrial hemp genotypes had potential to integrate cannabinoid extraction as an additional use into hemp cultivation, taking into account growth stage and harvested organ. Further research should be addressed to the ecological and phytochemical behaviour of approved industrial hemp genotypes in different environmental conditions, in order to provide farmers with scientifically based genotype information and assist in the selection of appropriate genotypes. A major challenge is that many EU countries still prohibit the use and marketing of flowers as they are considered narcotics, even if the THC content is below the established limit. For industrial hemp to be integrated into the agricultural sector as a profitable crop, farmers must be able to maximize the income from the whole plant, especially the flowers and leaves. The additional income from the CBD market can help finance the construction of processing plants for fibres and shives, which could be sold at low costs for industrial purposes. Further, the maximum THC content allowed in the field should be increased to 0.3% to allow breeding of new genotypes and to bring the EU in line with international standards, thus making farmers more competitive. Hemp-derived food, CBD and cosmetic markets have already proved how they can deliver added value in terms of quality and sustainability. However, a common and science-based regulatory framework is still lacking. This uncertainty limits investment and hampers the development of a value chain for cannabis products.

The marketability of CBD products under the Narcotics Act and their classification as novel foods is still a highly controversial issue. Furthermore, the marketability of CBD as an ingredient for cosmetics is another problem. It has to be seen if cannabis-derived raw materials will be permitted as natural ingredients for cosmetic products in the future. It also remains to be seen whether cannabis and cannabis preparations containing naturally occurring cannabinoid contents will be considered as novel food.

Specifically, the production of medical cannabis poses great challenges to potential producers. Although the demand for medical cannabis is growing strongly, it is predicted that medical cannabis will not be available in Germany until spring 2021 at the earliest. As a result, Germany is currently dependent on imports and cannot participate in a rapidly growing market. Further research is needed to efficiently standardize the cost-intensive indoor cultivation and to meet the high demand and quality requirements in this sector. In short, to revive the unique potential of *C. sativa*, an EU-wide approach has to be created and harmonized across the different member-countries.

7 Summary

Cannabis sativa L. as a prime example of a multifunctional crop is excellently suited for recycling management due to its versatility and the usability of the whole plant (grains, leaves, flowers, stems). The cannabis plant currently experiences a boom due to its rich phytochemical repertoire, its fibres and valuable oil required in numerous products, and its unique agricultural properties. Hence, the real added value of *C. sativa* lies in its ability to produce different products with one crop and, at the same time, achieving positive environmental effects with one rotational crop. In addition, the demand for cannabis products is constantly increasing and a further rapid growth is forecasted. The medical benefits of *C. sativa*, based on the phytocannabinoids available in flowers and leaves, are the main focus of attention worldwide. A broad field of application has been highlighted, including the therapeutic potential of non-psychoactive phytocannabinoids for various indications. Above all, innovative markets in the food, cosmetics and pharma industry are growing fast, with a focus on cannabidiol (CBD), which is the leading cannabinoid of the cannabis plant, as an ingredient for cannabis products.

Basically, it is important to differentiate between industrial hemp genotypes, medical phytocannabinoid-rich (PCR) cannabis genotypes and Δ^9-tetrahydrocannabinol (Δ^9-THC)-rich genotypes. Industrial hemp genotypes meet the 0.2% Δ^9-THC limit mandated by the European Union (EU) legislation, and therefore, can be legally cultivated by farmers on a field scale. Medical PCR cannabis genotypes contain high amounts of non-psychoactive cannabinoids such as CBD, cannabigerol (CBG), and cannabichromene (CBC), in the range of 10 – 30% while their Δ^9-THC content is also below 0.2%. These genotypes are currently being bred but are still barely available on the market.

Cannabinoid extraction from industrial hemp genotypes cultivated on a field scale could provide a decisive advantage as the harvested biomass quantities could be significantly increased through better land use and cost management, compared to an indoor production system. In addition, the multi-functionality of the industrial hemp plant can provide added economic value. Therefore, existing cultivation systems for fibre and oilseed production have to be modified as the harvesting time, harvested organ and other parameters are expected to differ greatly from those of the present systems. In order to achieve this, **publication I** dealt with the following specific objectives (**1**) to determine the yield potential of different EU-registered hemp genotypes with regard to inflorescence and biomass yield as well as cannabinoid content, depending on genotype, growth stage and biomass fraction in an outdoor

cultivation system, and (**2**) to investigate whether the tested genotypes are suitable for the production of cannabinoids due to better land use on a field scale.

The cultivation of seven industrial hemp genotypes (Finola, Fédora 17, Ferimon, Félina 32, Futura 75, USO 31 and Santhica 27) was carried out in a two-year field experiment. Sampling of leaves and inflorescence, took place at four specific growth stages: vegetative leaf stage (S1), bud stage (S2), full-flowering stage (S3), and seed maturity stage (S4). Dry matter of the sampled biomass fractions was recorded, and cannabinoids, such as cannabidiolic acid (CBDA), CBD, cannabigerolic acid (CBGA), CBG, and Δ^9-tetrahydrocannabinolic acid (Δ^9- THCA) were analysed. The results indicated that the content of cannabinoids highly depended on the genotype and the growth stage. Thus, biomass and inflorescence yields must be considered for an optimized harvest result. It was found that genotype Santhica 27 indicated the highest contents of CBG/A. Further, it was found that genotypes such as Futura 75, Fédora 17, Félina 32, Ferimon, Finola and Santhica 27, which were highlighted to have a higher CBD/A or CBG/A content compared to other evaluated genotypes, reached the highest yields of threshing residues after seed maturity, and thus a higher CBD/A and CBG/A yield per area. In conclusion, harvesting after seed maturity seems to be economically beneficial. These findings make selected industrial hemp genotypes excellent candidates for multipurpose cropping with respect to biomass production and CBD/A or CBG/A extraction, thus realizing the full potential of the plant.

Additionally, the thesis aimed at further standardization of PCR cannabis genotypes in indoor cultivation systems. Due to the prescribed requirement of high-quality medical cannabis material by various institutions, indoor cultivation is in focus as under the system all production parameters can be standardized. The production of cannabinoids under indoor conditions is an expensive undertaking due to processing costs and regulatory limitations, thus there is an increasing interest in using the available space requirements efficiently. Therefore, a cost effective production chain together with a high floral yield of consistent quality is aimed at. **Publication II** evaluated the adaptation of the plant architecture, through the targeted use of synthetic phytohormones, aiming for a small and compact plant morphology for various indoor cultivation systems to reach high floral yields, and thus, high yields of CBD/A per unit area. The specific objectives were (**3**) to test the impact of exogenously applied plant growth regulators (PGRs), such as 1-naphthalenaecetic acid (NAA), 6-benzylaminopurine (BAP) and a mixture (NAA/BAP-mix) of both PGRs on the plant architecture (total plant height, axillary branch length, and number of internodes) of different PCR cannabis genotypes (KANADA,

0.2x and FED). Furthermore, the biomass yield of flowers and leaves together with CBD/A content were determined, and (**4**) the potential beneficial impact of PGRs on plant morphology in order to reach higher CBD/A yields per plant was evaluated. For this objective, genotypes were treated exogenously with synthetic phytohormones in various concentrations in a greenhouse experiment to determine the impact of PGRs on the genotype-specific plant morphology. Furthermore, the differences in leaf and flower yields resulting from morphological changes in these genotypes and their CBD/A content was investigated. As a result of publication II, a genotype-specific impact of applied PGRs on the plant architecture was determined. NAA led to more compact plant morphology with a consistently high floral yield for genotype KANADA, whereas CBD/A content was not affected. Genotypes 0.2x and FED showed reduced floral yields due to the applications of PGRs.

Publication III dealt with the objective on (**5**), the evaluation of the growth performance, such as total plant height, biomass yield, root growth and cannabinoid content, of PCR cannabis genotypes (KANADA and 0.2x) grown in different substrate compositions substituted with peat alternatives in an indoor cultivation system. In a pot experiment, the impacts of the following substrate compositions: (a) peat-mix growth media (PM); (b) peat-mix substituted with 30% of green fibres (G30) consisting of coniferous wood and wood chips from pine and spruce wood growth media, and (c) pure coco coir fibres (CC), on growth performance, biomass and flower yields, biomass nitrogen (N) content as well as CBD/A contents were tested. The results of publication III showed that the different substrates had significant impacts on the growth, biomass and floral yields, root development and N tissue content of the tested genotypes. Pure CC as a growing media indicated a reduction in total plant height, leaf N content, leaf dry weight and RLD compared to PM and G30 growth media. Further, a genotype-specific reaction on floral yield was investigated. While genotype KANADA had the highest floral yields when grown in PM, 0.2x showed no significant differences, with higher floral yields grown in G30 and CC. For both genotypes, no limiting effect on CBD/A content was enacted by the different growing media. Based on the results, it can be concluded, that organic peat alternatives such as green fibres, partly replacing peat in standard growing media, offers a genotype-specific option for constant plant growth, a comparable high floral yield and a stable CBD/A content.

Overall, the preceding publications highlight that selected industrial hemp genotypes can be used for cannabinoid production in combination with further applications. Thus, their multi-functional properties make them ideal for integration into agriculturally sustainable cropping systems for an economically efficient circular economy. Further, the optimization through

morphological modifications and the choice of a suitable potting substrate have the potential to improve the standardization of cannabis indoor cultivation systems. This indicates that the high complexity of PCR cannabis genotypes needs to be evaluated for their response to new indoor growing systems developed at the genotype-specific level, with the overall aim of expanding high-quality cannabis production to meet the growing demand.

8 Zusammenfassung

Cannabis sativa L. eignet sich aufgrund der Vielseitigkeit und der Möglichkeit, die ganze Pflanze (Körner, Blätter, Blüten, Stängel) zu nutzen, hervorragend für die Kreislaufwirtschaft und ist daher ein Paradebeispiel für eine multifunktionale Nutzpflanze. Die Cannabispflanze erlebt derzeit einen Boom aufgrund ihres reichhaltigen Repertoires an sekundären Pflanzeninhaltsstoffen, ihrer Fasern und ihres wertvollen Öls in zahlreichen Industriezweigen sowie ihrer einzigartigen landwirtschaftlichen Eigenschaften. Der Mehrwert von *C. sativa* liegt in der Nutzung für verschiedene Produkte und gleichzeitig der Erzielung positiver Umwelteffekte innerhalb einer Fruchtfolge. Zudem steigt die Nachfrage nach Cannabisprodukten stetig an. Das Hauptaugenmerk liegt dabei im medizinischen Nutzen von *C. sativa*, basierend auf den in Blüten und Blättern vorhandenen Phytocannabinoiden. Cannabidiol (CBD), das führende Cannabinoid der Cannabispflanze, steht dabei als Inhaltsstoff diverser Cannabisprodukte im Fokus wodurch sich schnell wachsende und innovative Märkte in der Lebensmittel-, Kosmetik- und Pharmaindustrie entwickeln.

Dabei ist es wichtig, zwischen Nutzhanf Genotypen, medizinischen phytocannabinoid-reichen (PCR) Cannabis Genotypen und Δ^9-Tetrahydrocannabinol (Δ^9-THC) -reichen Genotypen zu unterscheiden. Nutzhanf erfüllt den von der EU-Gesetzgebung vorgeschriebenen Δ^9-THC-Grenzwert von 0,2% und kann daher von Landwirten im Feldmaßstab legal angebaut werden. Medizinische PCR Cannabis Genotypen, enthalten hohe Mengen an nicht-psychoaktiven Cannabinoiden, wie CBD, Cannabigerol (CBG) und Cannabichromen (CBC), in einem Bereich von 10 – 30%, während ihr Δ^9-THC-Gehalt ebenfalls unter 0,2% liegt. Diese Genotypen werden derzeit gezüchtet und sind noch kaum auf dem Markt erhältlich.

Die Cannabinoid-Extraktion von aus Nutzhanf gewonnenen Rohstoffen, die im Feldmaßstab angebaut werden, könnte einen entscheidenden Wettbewerbsvorteil bieten, da die geerntete Biomasse durch eine bessere Flächennutzung und mehr Kosteneffizienz im Vergleich zu einem Indoor-Produktionssystem deutlich erhöht werden könnte. Darüber hinaus kann die Multifunktionalität der Nutzhanfpflanze einen wirtschaftlichen Mehrwert bieten. Bestehende Anbausysteme für die Faser- und Ölsaatenproduktion müssen neu entwickelt werden, da sich der Erntezeitpunkt, das Ernteorgan und andere Parameter stark von den bisherigen Systemen unterscheiden dürften. Um dies zu erreichen, befasst sich **Publikation I** mit folgenden Zielen:

(**1**) Ermittlung des Ertragspotenzials verschiedener in der EU zugelassener Nutzhanf Genotypen hinsichtlich Blütenstand- und Biomasseertrag sowie Cannabinoidgehalt in

Abhängigkeit von Genotyp, Wachstumsstadium und Biomassefraktion in einem Freilandanbausystem und (**2**) Untersuchung, ob die getesteten Genotypen aufgrund einer besseren Flächennutzung im Feldmaßstab für die Cannabinoid Produktion geeignet sind.

In einem zweijährigen Feldversuch wurden sieben Nutzhanf Genotypen (Finola, Fédora 17, Ferimon, Félina 32, Futura 75, USO 31 und Santhica 27) angebaut. Die Beprobung von Blättern und Blütenständen erfolgte zu vier spezifischen Wachstumsstadien: vegetatives Blattstadium (S1), Knospenstadium (S2), Vollblütestadium (S3) und zur Samenreife (S4). Die Trockensubstanz der beprobten Biomassefraktionen wurde erfasst sowie der Cannabinoidgehalt, im Speziellen die Cannabidiolsäure (CBDA), CBD, Cannabigerolsäure (CBGA), CBG und Δ^9-Tetrahydrocannabinolsäure (Δ^9-THCA), analysiert. Die Ergebnisse zeigten, dass der Gehalt an Cannabinoiden stark vom Genotyp und dem Wachstumsstadium abhängt. Daher müssen für ein optimales Ernteergebnis die Biomasse und der Ertrag der Blütenstände berücksichtigt werden. Es wurde festgestellt, dass der Genotyp Santhica 27 den höchsten Gehalt an CBG/A aufwies. Die Genotypen Futura 75, Fédora 17, Félina 32, Ferimon, Finola und Santhica 27, welche im Vergleich zu den anderen Genotypen die höchsten CBD/A- bzw. CBG/A Gehalte aufwiesen hatten zur Samenreife die höchsten Biomasseerträge an Druschrückständen und somit einen höheren CBD/A- und CBG/A-Ertrag pro Fläche. Zusammenfassend lässt sich sagen, dass die Ernte nach der Samenreife wirtschaftlich vorteilhaft ist. Diese Ergebnisse machen ausgewählte Nutzhanf Genotypen zu idealen Kandidaten für den Mehrzweckanbau in Bezug auf Biomasseproduktion und CBD/A- bzw. CBG/A-Gewinnung, um das volle Potenzial der Hanfpflanze auszuschöpfen.

Zusätzlich befasste sich die Arbeit mit einer weiteren Standardisierung von PCR Cannabis Genotypen in Indoor-Anbausystemen. Aufgrund der vorgeschriebenen hohen Qualitätsanforderungen für medizinisches Cannabismaterial rückt der Indoor-Anbau immer mehr in den Fokus, da alle Produktionsparameter standardisiert werden können. Die Produktion von Cannabinoiden unter Indoor-Bedingungen ist aufgrund von Verarbeitungskosten und regulatorischen Einschränkungen kostenintensiv. Daher wird vor allem eine kosteneffektive Produktionskette zusammen mit einem hohen Blütenertrag bei gleichbleibend hoher Qualität angestrebt. In **Publikation II** wurde die Anpassung der Pflanzenarchitektur durch den gezielten Einsatz von synthetischen Phytohormonen evaluiert. Mit dem Ziel eine kleine und kompakte Pflanzenmorphologie zu generieren sowie hohe Blütenerträge um folglich hohe CBD/A Erträge pro Flächeneinheit zu erreichen. Dies umfasste folgende Zielsetzungen: (**3**) den Einfluss exogen applizierter Pflanzenwachstumsregulatoren (PGRs), wie 1-Naphthalinessigsäure (NAA),

6- Benzylaminopurin (BAP) und einer Mischung (NAA/BAP-Mix) aus beiden PGRs auf die Pflanzenarchitektur (Gesamtpflanzenhöhe, Länge der axillaren Seitentriebe und deren Anzahl an Internodien) verschiedener PCR Cannabis Genotypen (KANADA, 0.2x und FED) zu prüfen. Darüber hinaus den Biomasseertrag von Blüten und Blättern sowie den CBD/A Gehalt zu bestimmen und (**4**) einen möglichen positiven Einfluss von PGRs auf die Pflanzenmorphologie zu evaluieren, um folglich höhere CBD/A-Erträge pro Pflanze zu erzielen. In einem Gewächshausexperiment wurden die Genotypen mit exogen applizierten synthetischen Phytohormonen in verschiedenen Konzentrationen behandelt, um den Einfluss von PGRs auf die genotyp-spezifische Pflanzenmorphologie zu bestimmen. Weiterhin wurden die aus den morphologischen Veränderungen resultierenden Unterschiede in den Blatt- und Blütenerträgen sowie deren CBD/A-Gehalt untersucht. Als Ergebnis der Publikation II wurde ein genotyp-spezifischer Einfluss der applizierten PGRs auf die Pflanzenarchitektur festgestellt. NAA führte beim Genotyp KANADA zu einer kompakteren Pflanzenmorphologie mit einem konstant hohen Blütenertrag, während der CBD/A-Gehalt nicht beeinflusst wurde. Die Genotypen 0.2x und FED zeigten durch die Anwendungen reduzierte Blütenerträge.

Publikation III befasste sich mit der Bewertung von (**5**) Ertragsparametern und Cannabinoidgehalt von PCR Cannabis Genotypen (KANADA und 0.2x), welche in verschiedenen Substratzusammensetzungen in einem Indoor-Topfanbausystem kultiviert wurden. In einem Gewächshausexperiment wurde der Einfluss folgender Substratzusammensetzungen: (a) Torf-Mix (PM); (b) Torf-Mix, substituiert mit 30% Grünfasern (G30), bestehend aus Holzspänen von Kiefern- und Fichtenholz, und (c) reine Kokosfaser (CC), auf Wachstumsleistung, Blatt- und Blütenerträge, Stickstoff (N)-Gehalt, Wurzelwachstum sowie CBD/A-Gehalt untersucht. Die Ergebnisse der Publikation III zeigten, dass die verschiedenen Substrate signifikante Auswirkungen auf die Wachstumsleistung und die Wurzelentwicklung der getesteten Genotypen aufwiesen. Reines CC als Wachstumsmedium zeigte eine Reduktion der Gesamtpflanzenhöhe, des Blatt-N-Gehaltes, des Blattertrags und der Wurzellängendichte (RLD) im Vergleich zu den Wachstumsmedien PM und G30. Weiterhin wurde eine genotyp-spezifische Reaktion auf den Blütenertrag untersucht. Während Genotyp KANADA, kultiviert in PM Medium, die höchsten Blütenerträge aufwies, zeigte Genotyp 0.2x keine signifikanten Unterschiede auf, wobei die Blütenerträge bei einer Kultivierung in G30 und CC Medium signifikant höher waren. Für beide Genotypen wurde kein limitierender Effekt auf den CBD/A-Gehalt festgestellt. Basierend auf den Ergebnissen lässt sich schlussfolgern, dass organische Torfalternativen wie Grünfasern, die Torf in

Standardtopfsubstraten teilweise ersetzen, eine genotyp-spezifische Option für konstantes Pflanzenwachstum, einen vergleichbar hohen Blütenertrag und einen stabilen CBD/A-Gehalt bieten.

In Summe zeigte sich, dass ausgewählte Nutzhanf Genotypen für die Cannabinoidproduktion in Kombination mit weiteren Verwendungsmöglichkeiten genutzt werden können. Somit sind sie aufgrund ihrer multifunktionalen Eigenschaften ideal für die Integration in landwirtschaftlich nachhaltige Anbausysteme für eine ökonomisch effiziente Kreislaufwirtschaft. Weiterhin hat die Optimierung durch morphologische Modifikationen und die Wahl eines geeigneten Topfsubstrats das Potenzial, die Standardisierung von Cannabis Indoor-Anbausystemen zu verbessern. Dies deutet darauf hin, dass die hohe Komplexität der PCR Cannabis Genotypen hinsichtlich ihrer spezifischen Reaktion bewertet werden muss, um neue Indoor-Anbausysteme auf genotyp-spezifischer Ebene zu entwickeln.

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Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic
The bioeconomy potential of hemp (Cannabis sativa L.):
challenges of new genotypes and cultivation systems to meet the rising demand for phytocannabinoids

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

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I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

_____ _____

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