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Yield of new hemp varieties for medical purposes under semi-arid Mediterranean environment conditions

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Abstract

Under the effects of climate change new drought tolerant crops are imperative to introduce in irrigated agricultural areas of Mediterranean countries. In this sense, hemp (*Cannabis sativa* L.) represents an alternative in many semi-arid agricultural areas of Mediterranean basin because of its low water requirements and cost effectiveness when it is developed under non controlled conditions. The aim of this work was to evaluate the potential yield of five new hemp varieties (Sara, Pilar, Aida, Theresa, and Juani) cultivated under high tunnel conditions in a semi-arid Mediterranean area, and also to study the effect of plant density on active biomass production and cannabinoids biosynthesis (cannabidiol, CBD and cannabigerol, CBG) at different plant positions. The trial was conducted under plastic macro-tunnels during two seasons (2014 and 2015), from May to October. The agronomic response and the chemical profiles of the studied varieties were evaluated at the end of each season. Moreover, it was monitored the differentiation in terms of active biomass production and cannabinoids biosynthesis in different plant organ positions (at upper, medium, and lower). Additionally, during the second season, three different plant densities (PD_1, 9,777; PD_2, 7,333; and PD_3, 5,866 plants · ha^{-1}) were tested in order to define the the best of them for maximizing CBD and CBG productions. The findings highlighted significant differences in yield between cultivars within the CBD and CBG. Moreover, plant density was a determinant factor related to active biomass production and cannabinoids contents, PD_3 representing a suitable strategy to maximize the cannabinoids production minimizing the requirements of rooted apical cuttings. These results allowed concluding that these new hemp cultivars together with the adopted agronomic practices in this experience would be very appropriate for CBD and CBG productions, being determinant to consider the plant density and the cultivar for both studied chemotypes.

Keywords: cannabidiol, cannabigerol, *Cannabis sativa* L., plant density

Introduction

Hemp (*Cannabis sativa* L.) is one of the most attractive multi-purpose crops because of the demand of non-food crops in agriculture and other derivatives as food sources (Carus et al., 2013; Andre et al., 2016). Traditionally, the most known uses and applications attributed to this crop were related to the textile industry, and in some cases, to obtain oil and seeds for animal feed or human food (Struik et al., 2000; Cappelletto et al., 2001; Bennet et al., 2006). However, one of the most promising hemp applications is related to the production of cannabinoids and other secondary metabolites (Maule, 2015; Cooper et al., 2018; Fiorini et al., 2019). In response to this situation, in recent times hemp cultivation for biomedical applications is increasing because of the booming demand for minor not psychotropic cannabinoids, particularly for cannabidiol (CBD) and cannabigerol (CBG) (Maxmen, 2018).

Within the most known cannabinoids, delta-9-tetrahydrocannabínol (Δ9-THC), CBD and CBG are the most important compounds (Cascio et al., 2010), although there are several other secondary metabolites, such as terpenoids and flavonoids, whose effects and interactions are being investigated (Potter, 2014; Lewis et al., 2018). Δ9-THC and CBD are biosynthesized from the same precursor, CBG. The ratio between Δ9-THC and CBD depend on the inheritance of two codominant genes (B_T and B_D) (Meijer et al., 2003). Thus, it can be found homozygous genotypes which have inherited the B_T genes from each parent; and these genotypes will mainly produce Δ9-THC. On the contrary, other homozygous genotypes which have inherited the B_D genes from each parent can be found and will mainly produce CBD. Nevertheless, large variations exist in the ratio between Δ9-THC and CBD in varieties characterized by Δ9-THC and CBD chemotypes. Another chemotype is

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represented by those genotypes characterized by CBG as the dominant cannabinoid, obtained by the almost complete blockage of the metabolic pathways involved in the biosynthesis of CBD and Δ9-THC (Pacifico et al., 2008).

Biosynthesis and accumulation of cannabinoids is not exclusively governed by the plant genetic profile, but also by the growth conditions. In this regard, to obtain high concentrations of secondary metabolites together with a high yield on plant biomass, abundant light irradiation is required (Amaducci et al., 2008), and for this reason the hemp cultivation for biomedical applications in semi-arid Mediterranean areas seems to be promising. In this regard, recently, García-Tejero et al. (2019) developed a novelty experience in order to define the best agronomical practices (plant densities, irrigation dose, fertilization and sowing time) to maximize the cannabinoids production by using two monoecious industrial hemp cultivars (cv. Ermes, with a CBD-chemotype; and cv. Carma, with a CBG-chemotype). Moreover, these authors reported significant improvements when these plants were grown under polyethylene-covered tunnel structures, in comparison to the obtained results under open-field conditions.

Within each chemotype, the most determinant factor to obtain a homogeneous chemical profile is related to the plant propagation material, this being very heterogeneous when plants are seed propagated (Huestis, 2007). Potter (2009) reported that it will be essential to achieve the maximum plant homogeneity for maximizing the yield, being the origin of plant propagation material a key factor to ensure plant homogeneity at harvest. In this context, the use of seeds would not be recommendable because a wide heterogeneity normally derives in progenies of traditional industrial hemp varieties; whereas the use of rooted apical cuttings would be the most advisable technique to obtain "clones" of a specific variety. Thus, asexually propagated new hemp varieties would be an efficient alternative for CBD and CBG production under open field conditions in comparison to traditional industrial hemp varieties. However, it is necessary to improve the knowledge about the effects of different agronomic practices, in terms of CBD and CBG synthesis, especially if hemp is going to be developed under non-controlled conditions.

Studies with experimental data related to field performance and potential yield of new hemp varieties for biomedical uses are scarce, and much less if these experiments are developed under field conditions, having a broad range of questions related to the hemp varieties adaptation and response, cannabinoids synthesis or agronomic practices to optimize the yields on specific cannabinoids.

Considering the previous experiences; the aim of this work was to evaluate the potential yield of five new hemp varieties (Sara, Pilar, Aida, Theresa, and Juani) cultivated under high tunnel conditions in a semi-arid Mediterranean area, and also to study the effect of plant density on active biomass production and cannabinoids biosynthesis (cannabidiol, CBD and cannabigerol, CBG) at different plant positions.

Material and Methods

Experimental site

The trial was carried out during two consecutive years (2014 and 2015) in an experimental farm belonging to the Andalusian Institute of Training and Agricultural Research (IFAPA, in Spanish); located in the Guadalquivir river basin, SW Spain (37° 301 47" N; 05° 581 0211 W) as the result of the established collaboration between IFAPA and Phytoplant Research S.L., this being one of the few Spanish companies authorized to carry out R&D on hemp for biomedical applications.

The local climate of the experimental area is typically Mediterranean-dry, with an annual reference evapotranspiration (ET_0) of 1,200 mm and an annual rainfall of 550 mm, with a seasonal pattern, mainly distributed from October to April, which generates a potential deficit of -700 mm.

Regarding to the soil type, it is a Typical Fluvisol (USDA, 2016), with a useful depth up to 2.5 m, low content of organic matter (<15 $g \cdot kg^{-1}$), and available water holding capacity of 170 $mm \cdot m^{-1}$. More details about the experimental site characteristics can be found at García-Tejero et al. (2019).

Plant material and field experimental conditions

Three new hemp varieties with CBD chemotype (Sara, Pilar, Theresa) and two with CBG chemotype (Aida and Juani) were tested. All of them were obtained by Phytoplant Research S.L. in indoor facilities by means of conventional plant breeding techniques, and then registered in the European Union Community Plant Variety Office.

Plants were grown under polyethylene-covered tunnel structures (plastic macro-tunnels), whose dimensions were 50 x 6 m, with a maximum height of 3.5 m with inside average temperature between 23 and 35 ºC (with maximum temperatures > 40 ºC), relative humidity roughly 65%, and vapor pressure deficit (VPD) about 3.5

kPa (Figure 1A). The total surface for each high tunnel was 300 m^2 but considering that it was necessary to keep a distance of 1 m from each side, and 3 m from each extreme, the useful surface was 176 m^2.

Table 1 summarizes the experimental conditions and the properties for each hemp variety used during the two-monitoring seasons.

During the studied seasons, irrigation doses were estimated according to the methodology proposed by Allen et al. (1998), and following the recommendations described by Cosentino et al. (2012).

For the first season (2014), three varieties were tested: Sara, Pilar, and Aida. Rooted cuttings were transplanted on 28 May; these ones were disposed in ridges of 0.3 m of height, with 1 m between ridges, and 0.6 m between plants within each row; and a real plant density for each high-tunnel of 9,777 plants · ha^{-1}. Previously to the transplanting operations, the soil was completely covered with a black plastic mesh, with the aim of controlling weeds. Irrigation and fertilization were independently managed. At the end of season, the total irrigation volume applied was close to 400 mm. Fertilization started on 13 June (16 days after transplanting), applying exclusively Terra Vega (CANNA España SL) fertilizer till 7 July. After this, from 8 July to 31 July, two types of fertilizers (Terra Vega + Terra Flores, by CANNA España SL) were incorporated. Finally, from 31 July to seven days before harvesting, it was exclusively applied Terra Flores fertilizer. As Terra Vega as Terra Canna, both are liquid fertilizers for fertigation with a NPK composition of 3-1-1 and 2-2-4, respectively. Both compositions contain other relevant micronutrients as Mg, B, S, Na, Cu, Fe, Mn, Mo and Zn in different composition and chelates as EDDHA, EDDTA, and DTPA to ensure the nutrient absorption by roots. At the end of the season, plants had received 115-75-160 kg · ha^{-1} of NPK.

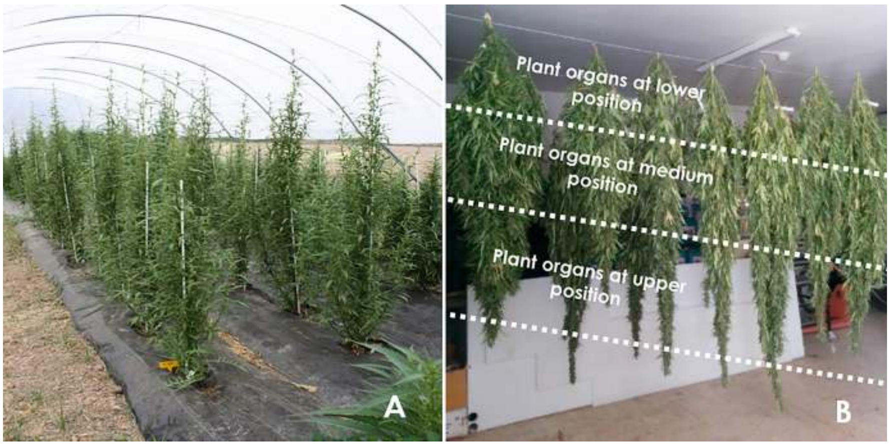

Figure 1. Hemp cultivation inside of polyethylene-covered tunnel structures (plastic macro-tunnels) (A) and plant organ positions studied at harvest (B).

Throughout 2015, Pilar was suppressed and two new varieties (Theresa and Juani with CBD and CBG chemotypes, respectively), were tested. All varieties were cultivated within the same high tunnels used in the previous season, and the rooted cuttings were

Table 1. Experimental conditions and chemotype characteristic for hemp varieties used.

Hemp variety	2014			2015		
	PD	TD	HD	PD	TD	HD
Sara (CBD)	PD_1	28 May	29 Sept	PD_1 PD_2 PD_3	27 May	21 Oct
Pilar (CBD)	PD_1	28 May	29 Sept	---	---	
Aida (CBG)	PD_1	28 May	13 Oct	PD_1 PD_2 PD_3	27 May	21 Oct
Theresa (CBD + CBD_V)	---	---	---	PD_1 PD_2 PD_3	27 May	14 Sept
Juani (CBG + CBG_V)	---	---	---	PD_1	3 June	2 Oct

PD, plant density; PD_1, PD_2 and PD_3 are 9,777, 7,333, and 5,866 plants ha^{-1}, respectively; TD, transplanting date; HD, harvesting date; CBD, cannabidiol; CBG, cannabigerol; CBD_V and CBG_V are propyl variants of CBD and CBG, respectively.

transplanted at the end of May - beginning of June (Table 1). During this second season, and considering the scarce development of plant lateral growing in 2014, three different plant densities (PD) were tested: PD_1, in which plants were disposed in rows 1 m spaced, and 0.6 m between plants within each row; PD_2, plants in rows 1 m spaced between them and 0.8 m between plants within each row; and PD_3, with plants in rows 1 m spaced, and 1 m between plants wthin each row. As it was pointed out before, according to the useful surface of the high-tunnel structure, the real plant density for PD_1, PD_2 and PD_3 was 9,777, 7,333 and 5,866 plants · ha^{-1}, respectively. In this regard, Sara, Aida and Theresa were grown under PD_1, PD_2 and PD_3; whereas Juani, considering its morphology and reduced plant development (previously described by the company), was exclusively grown under PD_1. As in the previous season, plants were grown above ridges of 0.3 m of height and completely covering the soil with a black plastic mesh to control the weeds.

Related to the irrigation and fertilization scheduling, nutrients started to be applied one week after transplanting. As in the previous season, the first applications were done with Terra Vega, until 10 August. From 11 August till harvesting was combined the application of Terra Vega with Terra Flores. At the end of the season, 100-50-150 kg · ha^{-1} of NPK was applied, these doses being equal for all varieties and PD. Regarding to the total irrigation water for each variety, it was 310 mm for Sara, Aida and Theresa, whereas Juani received 220 mm, this difference was related to height and the minor foliage development, but also to a shorter cultivation cycle. To ensure the harvesting date, stigma observations on weekly basis were done; stablishing the optimum maturity point by tracking the evolution of stigma's color from white/yellow to orange/brown. Once approximately 80% of buds had reached this point; plants were harvested (this point differed depending on the variety and the growing conditions experimented during the seasons (Table 1).

Plant measurements

At the end of the studied seasons, fresh weight (FW) of each plant was measured, using a precision balance (0.001 kg), and these were air dried in darkness for 7-10 days. Once the active biomass was dry enough (relative humidity below 12%), dry weight (DW) was recorded, leaves and flowers were pulled apart from the stems, obtaining dry weight of leaves and flowers [$DW_{(F+L)}$], and the dry weight of stems and branches (DW_{stems}) separately. Additionally, the active biomass content in plant organs distributed in three equal positions was measured: upper, medium, and lower (Figure 1B).

Cannabinoids contents [g per 100 g $DW_{(F+L)}$] were measured by using the official method, (EC) No 1177/2000 of 31 May 2000 Annex C, named "Community method for the quantitative determination of THC content of hemp varieties". This method is based on the quantitative determination of Δ9-THC by gas chromatography although slight modifications were made in the temperature ramp of the chromatographic oven for the simultaneous determination of cannabinoids (CBD_V, Δ9-THC_V, CBD, CBC, Δ8-THC, Δ9-THC, CBG, and CBN).

More details about the proposed methodology to quantify the cannabinoids content can be found at García-Tejero et al. (2019).

Experimental design and statistical analysis

In 2014, the experimental design was of randomized complete blocks, considering four replications per variety with an experimental plot unit of 4 m x 6 m; with 4 rows per unit and 40 plants. Data obtained were subjected to a one-way variance analysis (ANOVA, SPSS 15 .0 statistical package; SPSS, Chicago, IL, USA), using a Tukey's test for mean separations ($p < 0.05$), and comparing all varieties. In this regard, the differences in terms of yield and biochemical compounds were studied, in order to define the best varieties for the production purposes.

In 2015, the experimental design was similar to the previous season, in this case with two factors; the variety and the PD; considering four replications per variety and PD, respectively. Each experimental plot (replication) had a surface of 24 m^2, with 40, 32, and 24 plants, depending on the plant density (PD) considered. The studied parameters were subjected to a two-way ANOVA, studying the effects in terms of cultivar, PD, and the interactions between them. Previously, a Levene's test was applied to verify the variances homogeneity.

All statistical analysis was developed by using the SPSS 15.0 statistical package (SPSS, Chicago, IL, USA).

Results

Biomass production of the studied varieties

During 2014, Sara, Pilar and Aida varieties were transplanted on the same date and plant density with equal irrigation and fertilizer amounts. Related to the CBD varieties, Sara evidenced the most promising results in terms of fresh weight (FW), and total dry weight (DW), although referring to the active biomass ($DW_{(F+L)}$), the differences with Pilar were not significant (Table 2).

However, it was observed a significant improvement in terms of the ratio of active biomass; Pilar being a variety much smaller with a lower lateral development and producing much less stalks than Sara. In relation to the active biomass distribution, both Sara and Pilar accumulated the higher amounts of $DW_{(F+L)}$ in the lower third of the plant, although in percentage terms, Pilar had a higher amount of active biomass in the upper third of the plant than Sara (12.5 vs 8.3%), especially if we consider the total biomass produced for these cultivars. On the contrary, although Aida corresponded to a different chemotype, this variety was included within the statistical analysis with the remaining varieties, and no differences were found with Sara in terms of FW, DW, $DW_{(F+L)}$, being observed a similar pattern with the remaining cultivars, in terms of $DW_{(F+L)}$ distribution along the plant.

During 2015, two varieties with CBG chemotype (Aida and Juani) and two varieties with CBD chemotype (Sara and Theresa) were tested, and all of them (except for Juani) were grown at three different plant densities, analyzing the differences in terms of variety, plant density, and the interaction between these parameters.

In the case of CBD chemotype, significant differences in yield were found between cultivars and between the different PD considered. In this agreement, in terms of cultivar, these effects were observed in the FW, DW, $LDW_{(F+L)}$, DW_{Stems} and the ratio of active biomass (Table 3). It is noticeable that Theresa registered a significant improvement in terms of the ratio of active biomass (0.62), although on overall, Sara was much more productive than Theresa, obtaining almost 83% more active biomass.

Relating to the PD effects, both PD_1 and PD_3 showed the best results, especially for the different parameters of DW studied in this work (Table 3). When there were studied the effects of PD within each cultivar; the most relevant findings were fixed for Sara PD_1, followed by Sara PD_3 and Theresa PD_1 and PD_3, focusing the differences in these cases in terms of the ratio of active biomass (Table 3). Finally, as it was observed in the previous season the highest amounts of active biomass were produced in the lower plant section, this fact being observed in both CBD cultivars.

In the case of CBG chemotype, regarding to the variety effect, significant effects were observed exclusively for $MDW_{(F+L)}$, DW_{Stems} and the ratio between DW vs. $DW_{(F+L)}$. In this sense, Juani evidenced a significant improvement in terms of active biomass production regarding to total dry weight in the medium position of the plant (Table 4), this fact being related with the specific plant morphology and development of Juani, which was able to produce on overall better results of active biomass than Aida. Moreover, although no significant differences between cultivars were observed in terms of DW, Juani offered improvements in terms of the ratio of active biomass in comparison to Aida, when both varieties were grown under the same PD. In this agreement, regarding to the effects promoted by PD, this was exclusively studied for Aida, because Juani was only grown under PD_1. Nevertheless, no effects were detected in terms of PD for Aida (Tables 3 and 4), being these results different from those obtained for the CBD varieties.

Cannabinoids production of the studied varieties

Table 2. Yield parameters for studied hemp varieties during 2014.

Hemp variety	FW	DW	$DW_{(F+L)}$	$UDW_{(F+L)}$	$MDW_{(F+L)}$	$LDW_{(F+L)}$	DW_{Stems}	Ratio
				(kg · ha^{-1})				
Sara (CBD)	25,938a	7,836a	3,478ab	290.2b	671.6ab	2,516a	4,358a	0.44b
Pilar (CBD)	17,069b	4,075b	2,769b	344.9ab	558.6b	1,865b	1,306b	0.68a
Aida (CBG)	32,414a	8,241a	4,190a	532.8a	904.9a	2,752a	4,051a	0.51ab

CBD, cannabidiol; CBG, cannabigerol; FW, fresh weight; DW, total dry weight; $DW_{(F+L)}$, dry weight of flowers and leaves; $UDW_{(F+L)}$, dry weight of flowers and leaves at upper position; $MDW_{(F+L)}$, dry weight of flowers and leaves at medium position; $LDW_{(F+L)}$, dry weight of flowers and leaves at lower position; Ratio, DW_{Stems}, dry weight of Stem and branches; $DW_{(F+L)}$ vs. DW. Different letters evidence significant differences among varieties at $p < 0.05$.

During 2014, the main composition in terms of cannabinoids in the three varieties was studied by selecting different sections of the plant position (upper, medium or lower). Significant differences in the chemical profile were observed among varieties, these contents not being affected by the plant position (Table 5).

Pertaining to the variety, Sara was the most effective in terms of CBD and CBD_V cannabinoids, even for the remaining cannabinoids analyzed; except for the case of CBG, in agreement with the chemotype represented for this variety (Table 5). In this line, the total amount of cannabinoids synthetized by this variety was 77% higher than for the case of Pilar and Aida, this being an important characteristic of this variety (Table 5). These results would reinforce those previously discussed related to the active biomass production, being Sara the best variety as it has been previously described. On the other hand, in relation to the distribution of cannabinoids biosynthesis in different plant organs position, it was noted a certain trend to produce higher contents in the upper and medium parts, in accordance to the maximum concentration of flowers in these parts. This fact was detected for CBG (Aida being the only variety corresponding to this chemotype) and total cannabinoids, with significant differences among the upper and medium positions with respect to the lower position.

Table 3. Impact of hemp variety and plant density on yield parameters for CBD chemotype during 2015.

Yield parameters	Variety		Plant Density			Variety x Plant Density					
	Sara	Theresa	PD_1	PD_2	PD_3	Sara PD_1	Sara PD_2	Sara PD_3	Theresa PD_1	Theresa PD_2	Theresa PD_3
	(kg · ha^{-1})										
FW	17,408a	12,332b	18,835a	12,570b	13,204ab	23,806a	12,392b	16,026ab	13,864b	12,748b	10,382b
DW	6,214a	3,390b	6,330a	3,613b	4,462a	8,494a	4,368bc	5,781b	4,167bc	2,859c	3,144c
$DW_{(F+L)}$	2,369a	2,067a	2,901a	1,636b	2,117ab	3,307a	1,631b	2,170ab	2,495ab	1,641b	2,064ab
$UDW_{(F+L)}$	354.5a	284.6a	388.8a	207.6b	362.1a	425.1a	196.9b	441.5a	352.7ab	218.3b	282.7ab
$MDW_{(F+L)}$	873.1a	859.9a	890.1ab	598.3b	1,111a	582.2bc	488.7c	1,548a	1,198ab	707.9c	673.7bc
$LDW_{(F+L)}$	1,863a	922.5b	1,622a	917.8b	1,639a	2,299a	1,120b	2,170a	944.8b	714.7b	1,107b
DW_{Stems}	3,776a	1,318b	3,385a	1,912b	2,345a	5,099a	2,673bc	3,556ab	1,671c	1,151c	1,134c
Ratio	0.40b	0.62a	0.50a	0.51a	0.52a	0.38c	0.43bc	0.39c	0.62a	0.59ab	0.65a

FW, fresh weight; DW, total dry weight; $DW_{(F+L)}$, dry weight of flowers and leaves; $UDW_{(F+L)}$, dry weight of flowers and leaves at upper position; $MDW_{(F+L)}$, dry weight of flowers and leaves at medium position; $LDW_{(F+L)}$, dry weight of flowers and leaves at lower position; DW_{Stems}, dry weight of Stem and branches; Ratio, $DW_{(F+L)}$ vs. DW; PD_1, PD_2 and PD_3 are 9,778, 7,333, and 5,866 plants per hectare. Different letters evidence significant differences among varieties at $p < 0.05$.

Table 4. Impact of hemp variety and plant density on yield parameters for CBG chemotype during 2015.

Yield parameters	Variety		All cases			
	Aida	Juani	Aida PD_1	Aida PD_2	Aida PD_3	Juani PD_1
	(kg · ha^{-1})					
FW	18,752a	23,318a	21,085a	15,605a	19,567a	23,318a
DW	6,501a	7,249a	7,598a	5,353a	6,553a	7,249a
$DW_{(F+L)}$	2,965a	4,777a	3,284a	2,588a	3,024a	4,777a
$UDW_{(F+L)}$	359.9a	460.7a	446.3a	365a	268.5a	460.7a
$MDW_{(F+L)}$	695.5b	1,664a	866.1ab	480.9b	739.5ab	1,664a
$LDW_{(F+L)}$	1,910a	2,652a	1,972a	1,742a	216.4a	2,652a
DW_{Stems}	3,464a	2,370b	4,225a	2,693ab	3,475ab	2,370b
Ratio	0.46b	0.66a	0.43b	0.49b	0.46b	0.66a

FW, fresh weight; DW, total dry weight; $DW_{(F+L)}$, dry weight of flowers and leaves; $UDW_{(F+L)}$, dry weight of flowers and leaves at upper position; $MDW_{(F+L)}$, dry weight of flowers and leaves at medium position; $LDW_{(F+L)}$, dry weight of flowers and leaves at lower position; DW_{Stems}, dry weight of Stem and branches; Ratio, $DW_{(F+L)}$ vs. DW; PD_1, PD_2 and PD_3 are 9,777, 7,333, and 5,866 plants per hectare. Different letters evidence significant differences among varieties at $p < 0.05$.

Table 5. Impact of hemp variety and plant organ positions on cannabinoids content during 2014.

Cannabinoids	Varieties			Plant organ positions		
	Sara	Pilar	Aida	Upper	Medium	Lower
	(g per 100 g DM)					
CBD_V	0.114a	0.041b	0.020c	0.040a	0.039a	0.039a
CBC_V	0.024a	0.008b	0.007b	0.009a	0.009a	0.009a
CBD	8.090a	4.781b	1.051c	3.538a	3.511a	3.473a
CBC	0.370a	0.194b	0.120b	0.183a	0.178a	0.167a
Δ9-THC	0.547a	0.303b	0.065c	0.232a	0.223a	0.216a
CBG	0.499b	0.170b	4.484a	2.264a	2.105a	1.383b
CBN	0.201a	0.073b	0.002c	0.057a	0.055a	0.048a
Total	9.900a	5.600b	5.600b	6.349a	6.144a	5.356b

CBD, cannabidiol; CBG, cannabigerol; CBC, cannabichromene; Δ9-THC, Delta-9-tetrahydrocannabinol; CBN, cannabinol. CBDV and CBCV are propyl variants of CBD and CBC, respectively. Different letters evidence significant differences among varieties at $p < 0.05$

In the second experimental year (2015), three different densities (9,777; 7,333, and 5,866 plants · ha^{-1}) were studied for Sara, Theresa, and Aida; whereas for the case of Juani, this variety was grown exclusively at 9,777 plants · ha^{-1}. Additionally, as it was done during the previous season, it was considered the differentiation of cannabinoids biosynthesis taking into account the plant aerial position (upper, medium and lower).

Assuming the effects of the variety, the results showed significant differences in all the cannabinoids analyzed, something similar to the observations in the previous season (Table 6). In this sense, Sara and Theresa produced higher amounts of CBD, in comparison to Juani and Aida, which were the varieties that produced higher amounts of CBG as shown in Table 6. Among those cultivars belonging to the same chemotype, some interesting differences between Sara and Theresa were observed. Thus, Theresa reached higher values of CBD_V, whereas Sara reached higher levels of CBC and CBN. Something similar was observed in the CBG-varieties.

Thus, Aida produced the highest content of CBG and total cannabinoids on overall, highlighting the levels of CBC, in comparison to Juani.

More relevant were the results related to the effects of PD in the cannabinoids production. In this sense, significant effects were observed in all of them except of CBG, something interesting to be considered in order to achieve equilibrium between cannabinoids and plants production. In this regard, it is remarkable that PD_3 was the most productive density in terms of total cannabinoids, followed by PD_2 and PD_1 (Table 6). Moreover, between the most affected cannabinoids because of the PD were CBD, Δ9-THC or CBC. A significant effect derived from the plant organs position was found. On overall, upper and medium positions were the most productive, with slight variations depending on the considered cannabinoid (Table 6). It is noticeable the improvements obtained for CBD_V, Δ9-THC_V, CBD, CBC or CBN. By the contrary, only CBG was the cannabinoid that reached the highest contents in the lower position.

In order to normalize the obtained results in terms of total cannabinoids production ($kg·ha^{-1}$) these were calculated taking into account the active biomass ($DW_{(F+L)}$) obtained under the different growing conditions and the ratio of cannabinoids (g cannabinoid per 100 g $DM_{(F+L)}$) in the different cultivars, PD and plant organs positions (g cannabinoid per 100 g $DM_{(F+L)}$). According to our findings, the highest productions of CBD were obtained in Sara under PD_1 and PD_3, and the worst in Theresa (Table 7). Related to the CBG varieties, no substantial differences were found between Aida and Juani, although the highest CBG productions in these cultivars and under PD_1 were obtained by Juani (Table 7).

Table 6. Impact of variety, plant density, and plant organ positions on cannabinoid contents during 2015.

Cannabinoids	Variety				Plant Density			Plant organ position		
	Sara	Aida	Theresa	Juani	PD_1	PD_2	PD_3	Upper	Medium	Lower
	(g cannabinoid per100 g $DM_{(F+L)}$)									
CBD_V	0.24b	0.13c	1.55a	0.15c	0.63b	0.89a	0.65b	0.77a	0.71a	0.62c
Δ9-THC_V	0.05b	0.01c	0.21a	0.01c	0.08c	0.13a	0.10b	0.10a	0.10a	0.08c
CBD	10.1a	0.85c	4.17b	0.36d	3.64b	3.67b	4.96a	4.05a	3.97a	3.72b
CBC	0.55a	0.22c	0.30b	0.19d	0.30b	0.30b	0.36a	0.37a	0.29b	0.28b
Δ9-THC	0.92a	0.11c	0.53b	0.05d	0.38c	0.45b	0.53a	0.41b	0.45a	0.42ab
CBG	0.78c	4.84a	0.45c	3.98b	2.29a	2.15a	2.00a	1.65b	2.49a	2.41a
CBN	0.12a	0.04b	0.05b	0.02c	0.05b	0.06b	0.07a	0.10a	0.04b	0.03b
Total	12.8a	6.20c	7.29b	4.78d	7.38b	7.52b	8.83a	7.53b	8.05a	7.55b

CBD, cannabidiol; CBG, cannabigerol; CBC, cannabichromene; Δ9-THC, Delta9-tetrahydrocannabinol; CBN, cannabinol. CBD_V and Δ9-THC_V correspond to the propyl variants of CBD and Δ9-THC, respectively; PD_1, PD_2 and PD_3 are 9,777, 7,333, and 5,866 plants per hectare. Different letters evidence significant differences among varieties at $p < 0.05$.

Table 7. Average cannabidiol, cannabigerol and total cannabinoids production for the studied varieties under different plant densities during the two-monitoring seasons.

Hemp variety	CBD contents				CBG contents				Total cannabinoids			
	2014	2015			2014	2015			2014	2015		
	PD_1	PD_1	PD_2	PD_3	PD_1	PD_1	PD_2	PD_3	PD_1	PD_1	PD_2	PD_3
	($kg·ha^{-1}$)											
Sara	281.5a	354.5a	155a	206.7a	17.4b	26.5c	12.4b	16.7b	344.4a	448.8a	197.4a	218.6a
Pilar	132.4b				4.7c				155.1c			
Theresa		104.8b	66.0b	91.5b		11.0bc	7.22b	10.1b		180.2b	115.7b	165.6b
Aida	44.0c	27.3c	22.8c	25.4c	187.9a	144.9b	134.6a	150a	234.6b	188.9b	171.4a	190.5a
Juani		17.2c				190.2a				228.4b		

CBD, cannabidiol; CBG, cannabigerol; Total, total cannabinoids (whole cannabinoids studied in this work). PD_1, PD_2, and PD_3 are 9,777, 7,333, and 5,866 plants per hectare. Different letters differ significantly for each cannabinoid, and between the cases of a same chemotype (Sara, Pilar, and Theresa are CBD chemotype; Aida and Juani are CBG chemotype). Different letters within each column evidence significant differences among varieties at $p < 0.05$.

Discussion

This work examines two novelties approaches for cannabis production for biomedical purposes: i) the usage of new hemp varieties with a negligible synthesis of psychotropic cannabinoids, and higher concentrations of other therapeutic cannabinoids (mainly CBD and CBG); ii) and stablishing the most suitable PD in order to maximize the cannabinoids production.

The first experience focused on studying the cultivation of traditional industrial hemp varieties for biomedical applications under non-controlled conditions were recently developed by García-Tejero et al. (2019). These authors concluded that several improvements in terms of final yield could be obtained when this crop was grown under high tunnels, in comparison to open-field conditions.

In relation to active biomass production, comparing our findings with those reported when fibre varieties were used, and using the similar PD, irrigation doses, sowing time and cropping system, García-Tejero et al. (2019) reported $DW_{(F+L)}$ values close to 2,400 kg · ha^{-1} for Ermes (CBD Chemotype), whereas, for the new CBD varieties tested in this work, the results oscillated between 3,478 and 3,307 kg · ha^{-1} in case of Sara (Tables 2 and 3); 2,495 and 1,641 kg · ha^{-1} for Theresa (Table 3); and 2,769 kg · ha^{-1} for Pilar (Table 2). According to this, on overall, a significant improvement in $DW_{(F+L)}$ production was reached with in the varieties tested in this experience.

In addition, considering the CBG varieties, and the previous results provided by García-Tejero et al. (2019), these authors reported maximum values of $DW_{(F+L)}$ for Carma variety around 4,550 kg · ha^{-1}. These results were slightly higher than those harvested for Aida, with maximum productions of 4,190 kg · ha^{-1} in 2014 and between 3,284 to 3,024 kg · ha^{-1} in 2015, depending on the PD (Tables 2 and 4). For the case of Juani, (the other CBG variety tested in this work) the $DW_{(F+L)}$ values were close to 4,800 kg · ha^{-1} (Table 4), even higher than those reported for Carma by García-Tejero et al. (2019).

In order to define the most appropriate cultivar and plant PD to maximize the total cannabinoids production (cannabinoids harvesting; kg·ha^{-1}), it will be determined by two factors: i) the total active biomass produced (DW_{F+L}); ii) and the plant capability to synthesize cannabinoids in its tissues (very high in flowers and leaves, low or very low in stems and branches; and almost non-existent in seeds and roots), (Khan et al. 2014; Andre et al. 2016).

Focusing the attention in the total active biomass, on overall, PD_1 and PD_3 reported the best results in terms of DW_{F+L}. This controversy would be explained according to these reasonings: the number of plants per hectare and the plant capability for an optimum lateral spread. In this sense, PD_1 relied on the highest number of plants per hectare; and thus, the potential production would be very relevant. However, taking into consideration that the distance between plants in the same row for PD_1 (0.6 m) was the lowest; the lateral development is limited, unlike PD_3, with the lowest number of plants but the highest distance between plants in the same row, and hence the highest lateral development. These conditions would explain the similar results in terms of DW_{F+L} for PD_1 and PD_3, as it was observed for the case of Aida (Table 4) with a lateral development more pronounced than the observed in Juani. Likewise, and focusing exclusively the improvements in terms of active biomass production, no significant differences were observed between Aida and Juani for $PD_{1.}$ Relating to the CBD cultivars, the best results were obtained for Sara cultivar under PD_1 and PD_3, much higher than the productions values of $DW_{(F+L)}$ obtained in Pilar (Table 2) and Theresa (Table 3) in 2014 and 2015, respectively. These differences are specially related with the plant morphology and size. In accordance to this, Sara highlights for a very relevant volume and a lateral growing very pronounced. Although its inflorescences are not very compact, this variety counts on with a high capability of developing them, and hence, it is considered as one of the most relevant CBD chemotype currently obtained by the company Phytoplant Research S.L.

In relation to the ratio of cannabinoids, in 2014 (Table 5) Sara reported higher CBD concentrations than Pilar and other related cannabinoids, whereas in 2015 (Table 6) this variety was again the most relevant within the CBD chemotype. These results would reinforce the advantage of Sara as a promising CBD variety, comparing to other cultivars that at present, are being studied. For the case of CBG chemotype, in 2014 Aida reported a ratio of CBG of 4.48 g·100 g^{-1} DW_{F+L} (Table 5), whereas in 2015 (Table 6) this same cultivar .reported similar concentrations of CBG (4.84 g 100^{-1} $DW_{F+L})_{L}$; in comparison to Juani, with CBG values around 3.98 g·100 g^{-1} DW_{F+L}.

Linking the active biomass production and the synthesis of cannabinoids (Table 7), the cultivar would be the most determinant factor within the CBD chemotype, Sara reporting the best results, whereas for the case of CBG chemotype, Juani would be the most relevant.

Comparing these results with those reported by García-Tejero et al. (2019) when studying the cannabinoids production by using varieties for industrial uses; it was fixed in the best of the cases (under high-tunnels) a total amount of CBD close to 48.67 kg · ha^{-1} for Ermes (14% of total CBD produced by Sara under PD_1 in 2015).

Moreover, considering the CBG amount recorded for Carma (118.0 kg · ha^{-1}) in the best of the studied cases, this represents 62% of the total CBG produced by Juani under PD_1.

According to Barrat et al. (2012), in recent years, this crop is witnessing a revival and many researchers have studied the potential usage and applications of different non psychotropic cannabinoids (such as CBD and CBG) for biomedical purposes because of its interaction capability to contribute to the pharmacological power of medicinal hemp extracts (Russo & Taming, 2011). One example of these new applications was the approval of Sativex in June 2011 for treating spasticity caused by multiple sclerosis, or the development of different experiments for synthetic production of Δ9-THC and cannabidiolic acid by means of genetically modified microorganisms (Zirpel et al., 2015).

Conversely, several authors have reported the significant improvements in terms of cannabinoids concentration in different plant tissues when comparing fiber versus drug cultivars. In this agreement, THC concentrations were between 4 to 30 times higher in leaves of drug cultivars than fiber type cultivars (Potter, 2004; Pacifico et al., 2008; Bruci et al., 2012). Something similar was found in case of CBD and CBG concentrations in flowers and leaves, these levels are among 2 and 20 times higher in drug-type cultivars than in fiber-types (Cappelletto et al., 2001; Stout et al., 2012).

In particular, the CBD content was higher than those reported by Janatová et al. (2018) when they used similar cultivars belonging to this same chemotype. These authors obtained between 0.49 and 0.60 g CBD per 100 g of dry matter, developing six production cycles under indoor conditions. Therefore, according to the findings of the present study, the CBD productions were up to 350 $kg \cdot ha^{-1}$, whereas, under indoor controlled conditions, Janatová et al. (2018) would be able of producing, and taking into account their best results, around 84 $kg \cdot ha^{-1}$ CBD, with the difference that these authors developed six production cycles during a year and under indoor facilities, with high energy costs. Thus, the use of these new varieties under the growing conditions defined in previous experiments would ensure a high cost-effective production of non-psychotropic cannabinoids with significant energy savings and positioning the hemp as a good alternative to other irrigated crops in semi-arid Mediterranean areas such as South Spain.

Conclusions

This work presents novelty findings in terms of active biomass and cannabinoids production when using new hemp varieties for biomedical purposes, obtained from traditional plant breeding techniques. Plant density was identified as a determinant factor to maximize production, being PD_1 (9,777plants ha^{-1}) the most advisable in terms of total yield of cannabinoids, although higher costs must be considered for the nursery stage. However, some improvements were recorded for PD_3 (5,866 plants ha^{-1}), especially because of the higher plants' capability for a lateral development.

Regarding the studied varieties, Sara resulted the CBD variety with the highest yield, followed by Pilar and Theresa, whereas with the CBG varieties no significant differences were found among them. Nevertheless, it is worth to develop and breed new hemp varieties with biomedical applications in order to increase the final production of its main cannabinoid under Mediterranean semiarid environment.

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