Aquaporins-mediated water availability in substrates for cannabis cultivation in relation to CBD yield.

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Abstract

Aims

The objective of this study was to identify the most suitable substrate for *Cannabis sativa L*. cultivation based on its effects on water relations and CBD production.

Methods

Biomass production, physiological parameters, mineral contents, changes in the expression levels of the PIP aquaporins and the concentration of CBD was measured in *Cannabis sativa L. (var. Tiborszallasi)* plants cultivated on 5 substrates with different physical-chemical characteristic under controlled conditions.

Results

The substrates available water (AW) was the main factor affecting growth and production. The efficiency of the water use was governed fundamentally by transpiration. S1 and S3 were those in which the plants grew optimally and allows plants to invest energy in secondary metabolites production acquiring high levels of CBD. The plants grown in S2 and S5, composed by coconut fiber and perlite, showed the lowest growth in agreement with low transpiration rates which reduce the water uptake. S5 substrate, with some AW still available, is forcing plants to invest energy in improve water and nutrients transport as observed by the high levels of nutrients *in planta* and high PIPs expression levels. S4 plants presented the highest inflorescences production and CBD content which can be attributed to plant stress due to the low levels of AW and high pH and electrical conductivity (EC).

Conclusion

The absorption of water and minerals by plants has been affected by PIP-mediated water transport, playing key roles for an optimal use of the water present in the substrates with specific isoforms involved in this responses.

Introduction

Cannabis sativa L., is a plant with great interest in several industries as pharmaceutical, animal products, cosmetics and food (Burgel et al. 2020). Belonging to the Cannabaceae family, with annual flowering, it has been cultivated and used for thousands of years for its fiber and psychotropic and medicinal properties (Holmes et al. 2021). During the last decade, cannabis research related to the genetic and photochemical characteristics of the different varieties has increase (Rull 2022). More than 100 unique cannabinoids have been identified (Ahmed et al. 2015; Caplan et al. 2017;), predominantly Δ 9-

tetrahydrocanbinolic acid (THCA) and cannabidiolic acid (CBDA). These acids undergo a decarboxylation process during storage and when heated to become neutral cannabinoids such as tetrahidrocannabinol (THC) and cannabidiol (CBD) (Strzelczyk et al. 2021). Years ago, CBD received less attention as a potential drug candidate than Δ9-THC, although it has been frequently used in cannabis-based formulations. Nowadays, due to several medicinal beneficial properties, the interest around the use of CBD in different products is increasing and therefore crops requires much more investigation (Navarrete et al. 2021).

Cannabis presents a life cycle that is divided into two growth stages, a vegetative stage and a flowering stage. The vegetative development stage of plants is characterized by accelerated growth, increasing biomass and, frequently, a higher nutrient requirement (Raviv & Lieth 2008). During the growth stage, hemp also had high water requirements (Adesina et al. 2020). The accumulation of cannabinoids occurs mainly in the flowering stage, but, as secondary metabolites, their concentration may vary depending on the physiological conditions of the plant. Moreover, cannabis mineral nutrition can influence floral dry weight and secondary metabolites concentrations (Caplan et al. 2019; Nemati et al. 2021). However, the water needs and their relation with water uptake and transport through plant have not been studied.

Soilless greenhouse agriculture focuses on the development of new substrates that are profitable, renewable and sustainable in order to facilitate sustainable agricultural production. Thus, it is necessary to identify organic substrates capable of sustaining efficient crop production (Mejía et al. 2022). An adequate substrate is essential to provide the necessary physical and chemical properties for the specific plant or crop and its optimal growth conditions (Burgel et al. 2020; Ortiz-Delvasto et al. 2023). An appropriate growing medium should have good drainage capacity and ample air-filled pore (Zheng et al. 2007), but also it would have good water availability (Nemati et al. 2021).

Apart from the water availability in soils, the ability of plants to uptake water need to be addressed. Therefore, aquaporins (AQPs) that are the membrane proteins directly involved in water transport, need to be studied. Up to date, five subfamilies have been found in plants: intrinsic plasma membrane proteins (PIPs), intrinsic tonoplast proteins (TIPs), intrinsic nodulin26-like proteins (NIPs), small basic intrinsic proteins (SIPs), and intrinsic X proteins (XIPs) (Verdoucq and Maurel 2018). Of the different types of aquaporins, PIPs1 and PIPs2 clusters play an important role in maintaining the transport of water in cells and because of that, they are involved in many physiological processes essential for plant survival and plants response to stress (Yepes-Molina et al. 2020). Generally, overexpression of these PIP1 and PIP2 aquaporins enhances root, stem and leaves growth and development (Chen et al. 2022). Moreover, PIP aquaporins are also involved in reproductive organ development such as flowering, fruit and seed formation (Chen et al. 2022; Yang et al. 2022). Different PIP2 are up-regulated or down-regulated according to fruit initiation or ripening (Zhu and Ming 2019). In this way, the PIP aquaporins of *C. sativa* should play a key role in both, the vegetative growth stage, which requires a large amount of water and nutrients, and the flowering stage. Recently, 30 Cannabis AQPs has been identified (Guerriero et al. 2019). However, the involvement of aquaporins in *C. sativa* physiology has not been analyzed to date.

For all this reasons, the objective of this study was to determine the changes caused by different substrates on cannabis physiology, growth and CBD production. The investigation was done with 5 experimental substrates (S1-S5) showing different physico-chemical characteristics. For this, the analysis of the biomass production, photosynthesis, transpiration, mineral nutrition and changes at the expression level of PIP aquaporins in the cannabis plant grown on the different substrates were analyzed. Also, the concentration of CBD in the bud of the plant was associated with the composition of the substrates and the physiological response of the plant.

Materials and Methods

Plant material and growth conditions

Seeds of cannabis plants (*Cannabis sativa L. var. Tiborszallasi*), provided by Cultivo Manuel y Rafa 3000 S.L, were germinated in vermiculite, in the dark at 28°C, for 5 d. Subsequently, they were transferred to round pots of 9 cm diameter with 50% vermiculite and 50% perlite, one plant per pot. Plants were placed in a controlled environment growth chamber with 70–85% relative humidity (RH), 25–30°C, 485 ppm CO_2 and a photosynthetically active radiation (PAR) of 400 ± 50 µmol m^{-2} s^{-1} with a photoperiod of 18h/6h (day/night). The plants were irrigated with a modified Hoagland nutrient solution consisting of NO_3^- (15mM), $H_2PO_4^-$ (1mM), K^+ (6 mM), Ca^{2+} (5 mM), Mg^{2+} (2 mM), SO_4^{2-} (2 mM), Fe^{2+} (72 µM), Mn^{2+} (18 µM), Cu^{2+} (3 µM), Zn^{2+} (3 µM), BO_3^{3-} (45 µM) y MoO_4^{2-} (0,1 µM) (Cockson et al. 2019). The plants were irrigated twice a week, until saturation. After 30 days, plants were transplanted into 15 cm diameter pots with 5 different experimental substrates (provided by Projar, Valencia, Spain) composed by: (S1) 70% coconut fiber and 30% peat, (S2) 70% coconut fiber and 30% perlite, (S3) 60% coconut fiber, 30% peat and 10% perlite, (S4) 100% coconut fiber (S5) 90% coconut fiber and 10% of perlite. The experimental design was completely randomized with 6 plants for each of the 5 types of substrate. On day 60 of plant development, conditions were changed in order to stimulate the flowering: photoperiod 12/12 h, PAR 400 ± 50 µmol·m^{-2} ·s^{-1}, 60–75% RH and 22–25°C. Plants were irrigated with same modified Hoagland solution but with NO_3^- reduced to 9mM. The harvest was carried out when 70% of the pistils had changed their color to a dark amber (100–103 days). The whole experiment was done in three different assays.

Characterization of the substrates

For the physical analysis of the substrates, a volume of 20 liters of each substrate was used. The granulometry was carried out on a dried sample at 40°C. Total porosity, air space, bulk density, available water (AW), reserve water (RW) and unavailable water (UAW) were determined according to De Boodt et al. (1974). For mineral content analysis, previously lyophilized and finely ground substrate from 5 samples were digested with HNO_3:$HClO_4$ (2:1). Elements were detected by inductively coupled plasma (ICP) assay (Optima 3000, PerkinElmer). pH and electrical conductivity (EC) in the drained solution was

measured 4 times throughout the experiment. They were measured in the percolate of the discharge solutions by means of a portable pH/EC meter CM 35 (Hach, Düsseldorf, Germany).

Physiological parameters

Plant height of 6 plants grown on each type of substrate were measured once a week during the experiment. This measurement was made with a tape measure from the base of the stem to the apex of the plant. The floral, leaves and total dry weight were measured in 3 plants per substrate. For this, plants were placed in an oven at 22°C for 12 days.

Chlorophyll content was determined in fully developed leaves, using a SPAD-502Plus chlorophyll meter (Konica Minolta, Langenhagen, Germany). Photosystem II fluorescence was determined with a miniaturized pulse-width-modulated photosynthesis performance analyzer (mini-PAM; Walz, GmbH, Germany). Measurements were made on 6 plants of each substrate, using 5 leaves per plant. 12 measurements were made throughout the experiment.

The transpiration rate was calculated using the gravimetric method (Aroca et al. 2006). For this, the surface of the pots was covered with aluminum foil and the pot-plant system was weighed obtaining the initial weight (W_0). After 24 h, all the pots were weighed again, obtaining the final weight (W_f). The leaf transpiration rate is calculated as $(W_0-W_f)/t*FW$, where t = time in hours and FW = fresh weight in grams. Measurements were made on 4 plants of each substrate.

Mineral contents have been analysed in leaves and inflorescences. All leaves and inflorescences were collected from the dried plant material. The material was ground with an IKA model A10 grinder (Staufen, Germany) to obtain granulometries between 0.5 and 0.7 mm. Samples were digested with HNO_3:$HClO_4$ (2:1). Elements were detected by inductively coupled plasma (ICP) assay (Optima 3000, PerkinElmer).

Concentration of CBD

For CBD extraction, 100 mg of flowers, previously dried and ground, were mixed with 10 mL of absolute ethanol extraction solvent and stirred in a vortex. Subsequently, the sample was sonicated for 30 minutes and then stirred at 25ºC for 15 minutes, according to Berman et al. (2018), then allowed to settle at room temperature and subsequently filtered through a filter 0.22 µm PVDF (Milipore, Beford, MA, USA). CBD was quantified by UPLC-QtoF-MS, using a Waters Acquity I-Class UPLC coupled to a quadrupole flight-type mass spectrometry detector (Bruker maXis impact model) and equipped with an electrospray source type ESI (Bruker Daltonics, Bremen Germany). Quantification by UPLC-MS was performed using the external standard method, measuring the peak areas of the cannabinoids. To do this, the mass of the pure standard (m/z = 313.2162) was measured in negative mode [M-H] and subsequently said ion was identified in the samples of ethanolic extracts of flowers. Cannabidiol Solution – 1.0 mg/mL in methanol, ampule of 1 mL (Sigma, Darmstadt, Germany) was used as a pure standard to perform the quantification.

PIP genes expression

RNA isolation was performed using the NZYtech total RNA extraction kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. 3 plants per group were used. The quantity and purity of RNA were analysed with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to synthesise cDNA from 2 µg of total RNA, according to the manufacturer's protocol.

For Primers design all the sequences (8) of plasma membrane intrinsic proteins (PIPs) available for *C. sativa* were obtained from NCBI database and the primer sets were specifically designed in the 3' or 5' non-coding region of each gene, in order to avoid the non-specific amplification of other aquaporin genes (Online Resource 1). The efficiency of the primer sets was evaluated with the software QuantStudio 5 (QuantStudio Design and Analysis Software version 1.4.0.0), by analysing the threshold cycle (Ct)/fluorescence ratio at six independent points of PCR curves (Ramakers et al. 2003), giving values between 95 and 100% (Online Resource 1). Five housekeeping primers— 18S ribosomal RNA (id: XM_030651156.1), Elongation factor 1-gamma (id: XM_030649893.1), Protein phosphatase 2A subunit (id: XM_030625838.1), E3 ubiquitin-protein ligase (id: XM_030633681.1) and tubulin alpha-3 chain (id: XM_030654744.1)— for *C. sativa* were selected according to Deguchi et al. (2021), checked in each cDNA using the quantitative PCR quantifcation (qPCR) and analysed with Visual basic application for Excel (GeNorm) that automatically calculates the gene stability (Vandesompele et al. 2002). 18S ribosomal RNA (18S) was then selected as the reference gene for the standardisation.

Real-time PCR analysis was performed on 3 independent samples for each treatment (biological replicates) and each sample reaction was carried out in triplicate (technical replicates) in 96-well plates in a QuantStudio 5 Flex, a Real-Time qPCR system (Applied Biosystems by Thermo Fisher Scientific), following the manufacturer's instructions. The qPCR program consisted of 10 min initial denaturation at 95°C, and then amplification in a two-step procedure: 15 s of denaturation at 95°C and 60 s of annealing and extension at a primer-specific temperature for 40 cycles, followed by a dissociation stage. Data collection was carried out at the end of each round in step 2. These conditions were used for both target and reference genes, and the absence of primer-dimers was checked in controls lacking templates. The transcript levels were calculated using the $2^{-\Delta Ct}$ method (Schmittgen and Livak 2008) and presented as relative units.

Data analysis

Statistical analysis was performed using the SPSS 25.0.0.1 software package. Data were analyzed by one-way ANOVA and later Duncan's multiple comparison test. Significant differences were determined between the values of each determination at $p \leq 0.05$, according to Duncan's test. Values presented are means ± SE.

Results

Physical and chemical characteristics of the substrates

Physical properties of substrates are shown in Table 1. Substrates S4 and S5 presented higher total porosity, airspace and less bulk density than substrates S1, S2 and S3, presenting S2 the lowest airspace and maximum bulk density. In case of water parameters, S3 showed the most quantity of available water (AW), followed by S1 and S5, whereas S4 accumulated the less values of water availability. Reserve water (RW) was highest in S2 followed by S3, been S4 the substrate with less RW. The analysis of unavailable water (UAW) showed that S3 had the highest value of UAW. On the opposite, S1 and S2, in that order, were the substrates which had less UAW.

Table 1
Physical properties of substrates: Total porosity, air space, bulk density, available water (AW), reserve water (RW), unavailable water (UAW). Values are the mean of 3 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$).

Substrate	Total porosity (%)	Air Space (%)	Bulk density (g cm-3)	AW (%)	RW (%)	UAW (%)
S1	91 ± 0.15b	23 ± 1.43b	0.131 ± 0.003b	29.8 ± 3.42b	4.6 ± 0.05d	25.5 ± 0.97d
S2	91.6 ± 1.16b	16 ± 1.72c	0.155 ± 0.007a	19.7 ± 1.58c	11.5 ± 0.01a	29.3 ± 0.88c
S3	90.8 ± 0.65b	17 ± 0.84c	0.119 ± 0.004c	38.9 ± 1.92a	8.2 ± 0.03b	34.3 ± 0.76a
S4	96.6 ± 2.97a	35 ± 0.62a	0.089 ± 0.009d	7 ± 0.53d	3.9 ± 0.02e	32.5 ± 0.83b
S5	94.4 ± 1.38a	36 ± 0.83a	0.09 ± 0.003d	25.6 ± 1.64b	6.1 ± 0.08c	32.4 ± 0.84b

The content of minerals (Table 2), macronutrients and sodium (Na) (Table 2a) and micronutrients (Table 2b), was determined in substrate samples. Na showed differences among substrates being higher in S4 and S5, followed by S1 and S3, while S2 had the lowest concentration. Ca and Fe were higher in S4 and S5 substrates and minimum in S3. By contrary, Mg and Zn were highest in S3 and lowest in S4 and S5. S2 showed the second higher concentration of Mg and Zn (followed by S1), and the higher P, B, Cu and Mn values, which were minimum in S4 and S5. Also S2 showed the highest concentration of K, followed by S5 whereas, while S4 had the lowest K content.

Table 2
Analysis of mineral content in substrates: Macronutrients and sodium (a) and micronutrients (b) of each substrate. Values are the mean of 3 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$).

a. Macronurients and sodium (mmol/Kg DW)					
Substrate	**Ca**	**K**	**Mg**	**P**	**Na**
S1	40,968 ± 0,083c	530,719 ± 0,623c	53,396 ± 0.004b	130,579 ± 0.004b	5.224 ± 0.008c
S2	41,051 ± 0,002c	614,218 ± 0,004a	54,777 ± 0,004b	143,955 ± 0,003a	3.795 ± 0.005d
S3	38,476 ± 0,517d	534,752 ± 4,266c	73,581 ± 2,966a	112,468 ± 2,090c	4.959 ± 0.144c
S4	169,957 ± 0,201a	521,393 ± 2,971d	43,442 ± 0,063c	16,615 ± 1,208d	49.228 ± 0.176a
S5	153,324 ± 0,682b	570,852 ± 1,540b	42,208 ± 0,063c	10,615 ± 0,151e	46.435 ± 0.144b
b. Micronutrients (mmol/Kg DW)					
Substrate	**B**	**Cu**	**Fe**	**Mn**	**Zn**
S1	2.164 ± 0.002b	0.067 ± 0.003b	0.712 ± 0.004d	0.765 ± 0.006c	0.348 ± 0.005c
S2	2.390 ± 0.004a	0.077 ± 0.001a	0.821 ± 0.003c	0.961 ± 0.002a	0.394 ± 0.002b
S3	2.057 ± 0.387c	0.075 ± 0.001a	0.581 ± 0.005e	0.907 ± 0.001b	0.406 ± 0.002a
S4	1.195 ± 0.009d	0.048 ± 0.003c	5.800 ± 0.006a	0.679 ± 0.002d	0.109 ± 0.004d
S5	1.005 ± 0.003e	0.032 ± 0.003d	4.010 ± 0.006b	0.461 ± 0.019e	0.090 ± 0.009e

The pH and the EC of the percolated watering solution (Fig. 1) were analyzed. Both, pH and EC, decreased over time in all substrates. In both measurements substrate S4 presented the highest average pH and EC values followed by S5 with significant differences. Both presented significant higher values than S1, S2 and S3, which not present significant differences among them at the end of the experiments.

Physiological measurements

Plant height (Fig. 2) grown on the different substrates was measured over time. Plants grown on the S3 substrate were significantly taller compared to all other plants. The plants cultivated in S1 and S4 were the seconds in plant height and did not present significant differences between them. Plants grown in S5 substrate had the lowest height.

The dry weight of leaves and inflorescences were measured at the end of the experiments (Table 3). Plants grown on S1 and S3 reached a significantly higher leaf dry biomass compared to S2, S4 and S5. The plants cultivated in the substrates S3 and S4 presented the highest yield in the dry weight of the inflorescence, followed by plants cultivated in S1 without significant differences. The S5 substrate presented the lowest quantity of dry leaf and inflorescences biomass.

Table 3
Average dry weight of the leaves and the inflorescence of plants grown in the different substrates. Values are the mean of 3 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$).

Substrate	Leaves DW (g Plant^{-1})	Inflorescence DW (g Plant^{-1})
S1	14.18 ± 1.22a	12.22 ± 0.90a
S2	9.53 ± 1.18b	11.48 ± 0.84a
S3	15.69 ± 1.77a	13.16 ± 1.51a
S4	9.49 ± 1.28b	13.38 ± 1.37a
S5	8.78 ± 1.82b	8.83 ± 0.95b

SPAD values (Fig. 3) measured weekly in fully developed young leaves, indicate the concentration of chlorophyll both in the vegetative stage and in the reproductive stage. The results showed a lot of variations during the experiment with significant differences among treatments. Plants grown on substrates S1, S2, S3 and S5 presented strong variations during time course of the vegetative stage. S3 generally presented significantly the highest values in reproductive stage, followed by S1 and S2. Plants grown on the S4 substrate presented the lowest values with significant differences during all the experiment compared with the rest of the plants.

The efficiency of photosystem II (Fig. 4) showed that plants grown on the substrate S1 and S3 presented the highest efficiency and non-significant differences between them, followed by plants grown on S2 and S5, also without significant differences. The plants cultivated on S4 substrate presented the lowest efficiency.

Figure 5 shows the transpiration rate of plants grown on different substrates at the end of the experiment. The plants cultivated on substrate S1, followed by plants grown on S3, presented the highest transpiration rate. Plants cultivated on S4 and S5 showed low levels of transpiration with no significant differences between them. Plants cultivated on S2 showed the lowest transpiration rate.

Nutrients content

Table 4 shows the concentration of macronutrients (Table 4a) and micronutrients (Table 4b) of leaves of plants grown on different substrates. Plants grown in S3 presented the highest content of N followed by S4 and S5 plants. Ca content did not present changes in the leaves of the plants of any of the substrates.

K was higher in the leaves of plants grown in S4 and S5, while these plants presented the lowest Fe and Mn contents. By his side, plants grown in S1 presented the highest contents of P, Cu, Fe and Mn in leaves, followed by S3 plants, which presented high values in all nutrients measured with K exception. In general, S2 plants presented the lowest content in almost all the nutrient in leaves (N, Ca, Mg, S, B and Cu), being significant in case of B and Cu respect to all other substrates. B presented its highest values in S5 plants followed by S3 and Zn was similar in all leaves.

Table 4
Analysis of mineral content in cannabis leaves: Leaf macronutrients (a) and leaf micronutrients (b) content. Values are the mean of 3 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$)

a. Leaf macronutrients (mmol/Kg DW)

Substrate	Ca	K	Mg	P	S	N
S1	277.54 ± 33.58a	1082.24 ± 29.75c	94.50 ± 4.06b	207.29 ± 5.03a	101.21 ± 0.06b	2867.26 ± 42.26b
S2	230.61 ± 33.53a	1236.99 ± 57.43bc	90.14 ± 7.13b	171.67 ± 2.93b	98.73 ± 5.52b	3080.64 ± 40.39ab
S3	327.91 ± 46.73a	1243.31 ± 40.24bc	121.05 ± 11.57a	172.79 ± 7.51b	127.84 ± 5.70a	3282.73 ± 117.92a
S4	300.64 ± 13.95a	1498.51 ± 67.90a	120.64 ± 2.27a	148.20 ± 16.27b	106.40 ± 3.96ab	3187.28 ± 75.61a
S5	351.85 ± 45.07a	1375.74 ± 94.28ab	137.51 ± 8.87a	147.58 ± 2.89b	121.65 ± 13.98ab	3181.16 ± 89.02a

b. Leaf micronutrients (mmol/Kg DW)

Substrate	B	Cu	Fe	Mn	Zn
S1	4.097 ± 0.379abc	0.123 ± 0.009a	1.524 ± 0.101a	1.002 ± 0.094a	0.599 ± 0.028a
S2	2.925 ± 0.227c	0.065 ± 0.003b	1.166 ± 0.015b	0.927 ± 0.037ab	0.534 ± 0.035a
S3	4.512 ± 0.257ab	0.118 ± 0.013a	1.519 ± 0.101a	0.875 ± 0.108ab	0.589 ± 0.061a
S4	3.314 ± 0.185bc	0.097 ± 0.010a	0.816 ± 0.115c	0.719 ± 0.048b	0.491 ± 0.031a
S5	5.245 ± 0.865a	0.104 ± 0.008a	1.157 ± 0.060b	0.866 ± 0.083ab	0.584 ± 0.093a

The concentration of all mineral nutrients was determined in samples of dry inflorescences (Table 5), divided in macronutrients (Table 5a) and micronutrients (Table 5b). In general, plants grown on S1 and

S3 showed the highest nutrients accumulation in the inflorescences, with the highest values in almost all nutrients (Mg, P, Cu, Fe, Mn and Zn). By contrary, Plants cultivated in S2 showed the lowest content in all nutrients, with significant lower values in Ca, Mg and Cu contents. The N, K and B content in the inflorescence of the plants grown on the different substrates did not show significant differences.

Table 5
Analysis of mineral nutrients in cannabis inflorescences: Inflorescences macronutrients (a) and inflorescences micronutrients (b). Values are the mean of 3 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$).

a. Inflorescences macronutrients (mmol/Kg DW)						
Substrate	**Ca**	**K**	**Mg**	**P**	**S**	**N**
S1	155.76 ± 14.76a	1054.36 ± 7.61a	115.97 ± 1.89a	327.76 ± 11.05a	104.23 ± 9.34a	3388.30 ± 56.09a
S2	60.98 ± 21.21b	1132.25 ± 34.39a	60.15 ± 8.72c	227.10 ± 16.65b	91.81 ± 10.35a	3335.57 ± 79.51a
S3	147.78 ± 19,29a	977.84 ± 40.95a	122.45 ± 8.21a	239.52 ± 5.78b	105.80 ± 5.41a	3326.97 ± 170.41a
S4	110.44 ± 2.54ab	1060.77 ± 69.35a	89.51 ± 2.71b	231.10 ± 8.35b	105.16 ± 2.81a	3441.40 ± 195.92a
S5	158.21 ± 19.26a	1011.26 ± 62.99a	94.29 ± 3.03b	230.13 ± 11.07b	101.61 ± 0,20a	3632.94 ± 238.22a
b. Inflorescences micronutrients (mmol/Kg DW)						
Substrate	**B**	**Cu**	**Fe**	**Mn**	**Zn**	
S1	2.086 ± 0.105a	0.235 ± 0.023a	1.886 ± 0.009a	1.212 ± 0.098a	1.189 ± 0.030a	
S2	1.819 ± 0.104a	0.066 ± 0.007c	0.883 ± 0.098b	0.517 ± 0.118b	0.879 ± 0.069b	
S3	2.216 ± 0.045a	0.153 ± 0.012b	1.403 ± 0.095ab	1.072 ± 0.114a	0.994 ± 0.004ab	
S4	2.018 ± 0.136a	0.123 ± 0.009b	0.854 ± 0.256b	0.655 ± 0.044b	0.953 ± 0.147ab	
S5	2.430 ± 0.352a	0.128 ± 0.017b	1.191 ± 0.218b	0.739 ± 0.022b	0.883 ± 0.071b	

Concentration of CBD

The content of cannabidiol (CBD) in the inflorescences of plants grown in the different substrates showed significant differences (Fig. 6). The inflorescences of the plants grown on S4 substrate, presented the highest CBD content followed by plants from S3 substrate, then S1 plants and finally S2 and S5

plants, being the flowers of plants grown in S5 substrate the ones that presented the lowest content of CBD.

Expression of the aquaporins

The analysis of the expression of the PIP aquaporins in leaves (Fig. 7) revealed that the plants cultivated on S5 showed the highest expression of the analyzed PIPs1 aquaporins (Fig. 7a, 7b, 7c and 7d), and these plants had significant differences compared with the plants grown on the other substrates. Plants grown on the rest of substrates showed no differences in their *PIP1.1*, *PIP1.2* and *PIP1.3* expressions, while *PIP1.4* of plants grown on S4 had the second highest expression of this aquaporin, followed with no significant differences by plants grown on S2. Plants cultivated on S1 showed the lowest *PIP1.4* expression. In relation to the expression of PIPs2 (Fig. 7e, 7f, 7g and 7h), significant differences were also observed between the plants grown in the different substrates. *PIP2.1* showed a significant higher expression in plants grown on S4 substrate (Fig. 7e), followed by S2 and S3 plants. *PIP2.2* and *PIP2.7* had a similar expression pattern, S5 showed the highest expression, whereas plants cultivated on the others substrates showed no significant difference among them. In case of *PIP2.3* expression, plants grown on S5 showed the highest expression of this aquaporin followed, without significant differences, by plants grown in S3. Plants cultivated on S1, S2 and S4 did not show any significant differences among them in *PIP2.3* expression.

Discussion

It is known that substrates present physical-chemical characteristics which will influence plants growth and development. Cannabis plants require high amount of water, especially during their vegetative stage (Adesina et al. 2020). In that sense, S3 is the substrate with more water (AW, UAW and RW), showing a great capacity to support water requirements of cannabis. Also S1 with high AW presented good characteristics for cannabis growth. Both substrates have peat in their composition and share similar physical properties, such as low porosity and air space and high amount of AW. S2 by his side, with 70% coconut fiber but without peat in its composition, has similar air space and porosity to S3 and S1, but its AW is strongly reduced, while RW is the highest, which point to perlite content to be related with this parameter. By his side, coconut fiber substrates are known to have high aeration capacity but little water retention. Therefore, S4 and S5 substrates, which are made mainly with coconut fiber, presented higher total porosity and air space, but lower bulk density and lower total available water (AW and RW).

It has been reported that pH influences greatly the nutrient absorption of plants depending soil composition (Sarkar and Wynjones 1982). During the assays, the pH gradually decreased in all the substrates percolates. These results coincide with those reported by Caplan et al (2017), who report a gradual decrease in pH over time. Some authors reported that cannabis plants yield was the highest when soil pH was 6.6–6.8 (Coffman and Gentner 1975) due to nutrient availability. In this sense, S1, S2 and S3 has optimal values, while S4 and S5 showed the highest values of pH in the filtered solution (from 7.9 to 7.7 in S4 and from 7.6 to 7.3 in S5) which is above the optimal pH for cannabis growth. EC of

watering solution also exerts an influence in plant growth, and it has reported that there is a negative correlation between plant growth and EC on hemp (Anderson et al. 2021). In our results, S4 showed the highest EC (2.5 mS cm^{-1}) compared with the others, followed by S5 (1.5 mS cm^{-1}), and because we irrigated all plants with the same growth solution, we assume that this difference in EC must be due to the different substrate composition. Both, S4 and S5, are made with > 90% coconut fibre but with different water retention due to the small amount of perlite in S5, and analysing the mineral composition, both had the highest levels of Na, Ca and Fe compared to all the others substrates, being higher in S4 than S5. Thus, we can assume that EC differences in our substrates are mainly due to Na, Ca and Fe contents.

To analyse the effect on plant growth and development induced by the substrates, plant biomass and physiological parameters were measured. Plants grown on S1 and S3 had the highest amount of leaves per plant, high height and inflorescence production, being those parameters greater in S3 plants. In agreement, these S1 and S3 plants showed high amount of chlorophylls and were the ones which showed the highest efficiency in the photosystem II (85%). It has been reported that the increase of AW in soils enhances transpiration, stomatal conductance and maximum net assimilation rate, resulting in higher photosynthetic capacity in species such as *Robinia pseudoacacia* (Liu et al. 2013) or *Jatropha curcas* (Pérez-Vázquez et al. 2013). Also in that sense, S1 and S3 plants showed the highest levels of transpiration, as a direct consequence of the high amount of AW, pointing to a great importance of apoplastic pathway in cannabis when high level of water is available. As said before, both substrates have peat in their composition and share similar physical properties. Besides, their mineral content is slight different, with S3 having the greatest amounts of Mg, Cu, Mn and Zn, and the highest amount of water in all stages (AW, UAW and RW), so it can be assumed that these characteristics enhanced greatly plant growth in S3. It seems that perlite presence in the composition increase reserve water and nutrients retention in these substrate.

Coconut fiber has been commonly considered as a good substrate for cannabis because it presents high aeration capacity, even when it has little total and easily available water retention (Abad et al. 2005; Vargas-Tapia et al. 2008), but in a study carried out by Burguel et al (2020), they found that cannabis plants grown in a substrate composed of 100% coconut fiber had lower height in relation to plants grown in peat or peat + green fiber, which is concordant with our results. Indeed, plants grown on S2 and S5 showed less plant height, leaves and inflorescences compared with plants grown on substrates with peat in its composition. Nevertheless, between them, S2 plants grew better, been higher and with more inflorescences than S5 plants. These differences can be partially explained because substrates are made with coconut fibre, but differs in their quantity (70% in S2 and 90% in S5), which affect directly to porosity, air space and available water, being less in S2, while bulk density and reserve water are greater in S2 substrate. Again, higher perlite content seems to have an effect in increased RW and nutrients retention. Also, the contain of perlite alters the percolated solution, which has less pH and EC in S2 than S5. So, the higher % of perlite in substrates mixed with coconut fibres favours plant growth and development, probably by reducing drainage and lowering pH and EC of watering solution to more adequate levels for hemp growth.

It should be noted that the lower availability of water in substrates based on coconut fiber (S2, S4 and S5) generates a reduction in the transpiration rates of the plants that grow on them, which could be a symptom of water stress (Aroca et al. 2006) that could be leading to the reduction of vegetative growth and leaf development. In that sense, is interesting that plants cultivated on S4 (100% coconut fibre) were the second tallest (equally with S1) and had similar amount of inflorescences to S3, but they had much less leaf biomass with the lowest levels of chlorophylls (SPAD) and photosynthetic efficiency, as well as low transpiration rate, indicating that the stress situation is strongly affecting these plants. This stress situation could be attribute to substrate physical properties, as it is the substrate with lowest AW and RW contents and highest pH and EC values, not optimal for cannabis vegetative growth. However, the stress situation leads to greater flowering and, as we will discuss later, greater accumulation of CBD.

The analysis of mineral content of leaves showed that there is a positive correlation between growth and the content of Fe. Fe content in leaves is necessary to photosystems structure and function (Higuchi and Saito 2022) and in our experiments is directly correlated to this parameter, being efficiency in the photosystem II maximum in S1 and S3 plants and with lowest values in S4 plants. The nutrient uptake by plants and their growth is modulated by physical properties of soils (Raviv et al. 2004; Soong et al. 2020) and in this sense, we can observed that S1 and S3 somehow enhances mechanisms to boost Ca and Fe uptake when these cations are available. The availability of Fe is strongly dependent on pH (Kim and Guerinot 2007) and the correlation between pH in the substrate percolate and Fe content in leaves is clear in our results. Also, the high transpiration levels, which improve transport of Fe and Ca by apoplastic pathway to the leaves, (Kim and Guerinot 2007; Pathak et al. 2021) could be the driven way in the nutrients transport in S1 and S3 plants. Interestingly, S2 substrate present the highest values of K, P, B, Cu and Mn and high amount of RW that could, at a given moment, be available for the plants. However, this water and nutrients are being retained in the substrate by the high perlite content, but not being absorbed by the plants, resulting in lower levels of nutrients in the leaves and inflorescences of those plants compared to all other plants, driving to reduced growth, leaves and inflorescences DW compared to S1 and S3 plants. Again, the level of transpiration, extremely low in this case, seems to be key in the absorption of water and nutrients in cannabis, which points to a great importance of the apoplastic pathway in the growth and develop of cannabis plants in relation to water and nutrients transport.

By other side, S4 and S5 showed lower levels of Fe and Mn in leaves. Between them, Fe is abundant in these substrates, but the uptake of Fe was repressed, especially in S4, probably due to the high pH and EC that percolated solutions showed. Interestingly, S5 presented the highest accumulation of B in leaves, been this substrate the one with less B content, pointing to a boosted uptake and transport of B in those plants. Although is commonly accepted that boron transport is mainly by passive diffusion through the lipid bilayers (Dannel et al. 2002), some proteins belonging to the major intrinsic protein family (MIP) such as zmPIP1 or atPIP1.1b aquaporins have been reported to transport B (Dordas et al. 2000). Taking into account the previous fact, and that plants cultivated on S5 showed the highest expression of PIPs, it suggests that S5 plants enhance B uptake at least partially by increasing their PIPs expressions.

In plant inflorescences, the mineral contents are in agreement with previous data. In general, inflorescences accumulated more levels of nutrients in S1 and S3 plants, followed by S5 plants, and plants cultivated in S2 and S4 showed lower content in almost all nutrients, been Ca, Mg and Cu lower in S2 plants. All this data strongly points to water availability as main factor in nutrient uptake in both, vegetative and reproductive stages of cannabis.

Levels of CBD in inflorescences were also measured. It has been reported a positive correlation among plant growth and CBD content in hemp cultivars (Kakabouki et al. 2021), which is in correlation with the founded results, where S3 and S1 (in this order) showed high levels of CBD, high levels of nutrients accumulated in leaves and inflorescences and better growth parameters, while S5 plants has the minimum inflorescence production and CBD content, and the lowest growth. This results are expected, as the plants that have optimal growth conditions can invest energy in the production of secondary metabolites while plants with slower development will tend to use their resources in growth. However, S4 plants showed the highest amount of CBD. Cannabinoids such as CBD are also synthetized by plants in response to some stresses such as drought (Caplan et al. 2019). The main difference between S4 and S5 is due to the presence of small amounts of perlite (10%) in S5, but both substrates are made mainly by coconut fiber. This different composition is affecting exclusively the AW and RW, which are higher in S5, while S4 substrate had the lowest amount of AW from all the analysed substrates. This, together with all physiological parameters, suggest that S4 plants suffered from high stress caused by the lack of water. Thus, it seems that S4 and S5 plants are dealing with water stress, S4 plants investing energy in growing their production of inflorescences and accumulating protectant molecules such as CBD, while S5 plants invest in improving nutrients and water absorbance and mobilization, being the AW in the substrate the main responsible of the different behavior.

Results indicate that water relations are crucial in growth and development of cannabis. Cannabis growth have been shown to be strongly dependent from substrates available water. As discussed before, it exists a clear relationship between transpiration and growth in cannabis plants, pointing to great importance of apoplastic pathway for water and nutrients transport in cannabis when water is available in high amounts, promoting the high growth in S1 and S3 plants. However, when the water available for plants is lower (S2, S4 and S5), transpiration of the plant is reduced and water transmembrane transport acquire great significant roll. In our results, almost all the PIPs (all PIP1s, *PIP2.2*, *PIP2.3* and *PIP2.7*) were overexpressed in leaves of plants grown on S5 compare with plants cultivated in all other substrates. These plants showed the lowest height, leaves and inflorescences dry weight, but interestingly, although S5 substrate showed the lowest levels of nutrients, S5 plants accumulated higher levels of macro and micronutrients in leaves, especially striking in the case of B, Mn and Zn, and S5 substrate presented high total water amounts (AW + RW) that could be still accessible for plants. These facts, together with higher expression of the PIPs, suggest that plants in S5 are induced to transport higher levels of water and nutrients to the leaves in order to stimulate vegetative growth under unfavorable conditions, and that PIPs are directly involved in this adaptation, usually by overexpression of PIPs (Zhang et al. 2020; Yang et al. 2022). By contrary, plants grown on S2 and S4 which had no access to water available, tends to water preservation mechanisms, with low transpiration levels and no changes in PIPs expression in contrast to

the upregulation of PIPs in S5 plants. Plants have mechanisms to cope with dehydration, and one of them is the repression of PIP genes (Šurbanovski et al. 2013). Curiously, the only PIP isoform that is higher in those S2 and S4 plants in comparison to S5 plants was *PIP2.1*, which is the most expressed aquaporin in our experiments. This aquaporin presented its higher gene expression values in S4 plants, followed without significant differences by S2 and S3 plants. In that sense, it seems that the expression of *PIP2.1* has some relationship with the mobilization of water or nutrients towards the inflorescences, improving their production and somehow related to CBD accumulation, which are stimulated in S2, S3 and specially in S4 plants in comparison to plants grown in S5. Since PIP2 family has been reported to be involved in flowering stage in plants such as *Gentiana scabra* (Nemoto et al. 2022) or rose (Ma et al. 2008), the high expression of *PIP2.1* could be the key to promote flowering in cannabis.

Another interesting pattern is showed by *PIP1.4*. This aquaporin isoform has general low levels of expression but is higher in S2, S4 and maximum in S5. It has been usually described that PIPs aquaporins are regulated in plants under water stress, with specific isoforms upregulated in leaves (Barzana et al. 2021), specifically many PIPs1 has been associated to water and gas exchange functions and stomatal behavior and ROS detoxification (Rodrigues et al. 2017). It seems that PIP1.4 could be playing a role in response to low water availability, counteracting the reduced transpiration or playing a role in mitigating the water stress situation.

Also in optimal conditions, aquaporins mediated water transport could make the difference in behavior between plants growth and development. It has been said that S1 had highest transpiration rates but, however, S3 plants grew better than S1 plants. This can be partially ascribed to physical properties of the substrates, because S3 accumulates more water (AW + RW), presenting high water availability. In this sense, some aquaporin isoforms were high expressed in leaves of S3 plants which are not in S1 plants, pointing to the significance of the cell to cell pathway to cooperate with the apoplastic pathway to improve growth when high amount of water is still available in substrates. These isoforms were *PIP2.1* and *PIP2.3*, which has been described as important in leave tissues (Guerriero et al. 2019). *PIP2.3* is higher in S5 and S3 and seems to have a role in elongating stem and leaf tissues, been related to vegetative growth more than inflorescence production. All these data suggest that the aquaporins-mediated water transport in cannabis play key roles in both, optimal and water stress conditions.

Concluding remarks

Data obtained showed that AW in substrates is the main factor affecting cannabis growth. Substrates with peat in its composition accumulate more AW and apoplastic pathway promoted by transpiration seems to be clue to water and nutrients uptake in the plants grown in this substrates. By his side, substrates composed mainly by coconut fiber have lower AW giving rise to water stress. The plants grown on these substrates reduce the transpiration rate with consequences in water and nutrients uptake affecting vegetative growth and leaves development. However, symplastic pathway related to aquaporins have key roles when sufficient water is still available in substrates in both cases: in plants grown in

optimal water conditions, were improvement expression of some PIP isoforms makes the difference in growth, production and higher CBD concentration; And in stressed plants grown in coconut fiber, were PIPs promotes growth and nutrients accumulation if there is still some water available in the substrate. In concrete, *PIP2.3* seems related to vegetative growth while *PIP2.1* is somehow involved in better inflorescence production, and *PIP1.4* could be involved in the response to water stress. All the data suggest that the aquaporins-mediated water availability is crucial for an optimal use of the water present in the substrates.

Declarations

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Conception and design: Gloria Bárzana and Micaela Carvajal. Material preparation, data collection, analysis and interpretation of data were performed by Nidia Ortiz-Delvasto, Pablo Garcia-Gomez and Gloria Bárzana. The first draft of the manuscript was written by Nidia Ortiz-Delvasto and Pablo Garcia-Gomez and all authors commented on previous versions of the manuscript. Final Draft: Gloria Bárzana. Supervision and validation: Gloria Bárzana and Micaela Carvajal.Funding acquisition: Micaela Carvajal. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

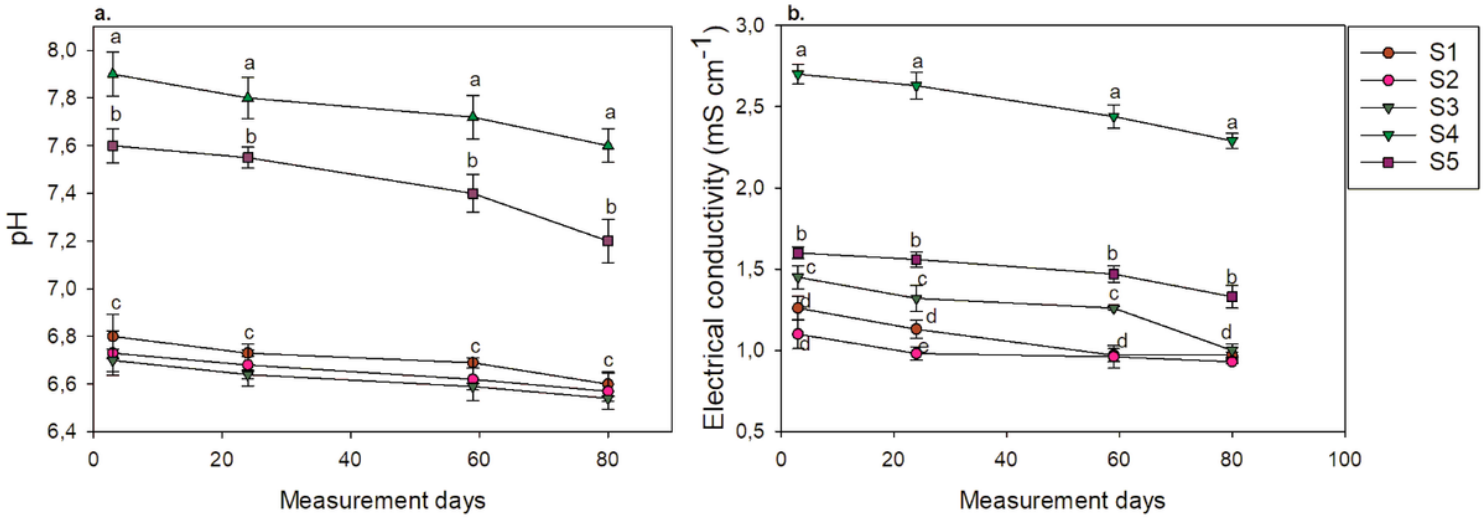

Figure 1

pH (a) and electrical conductivity (b) of the substrates measured during the development of the experiment. Each substrate is represented with a different color: S1 brown, S2 pink, S3 grey, S4 green and S5 purple. The points show the mean of 6 plants ± SE. Means of each day followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$). The graph was made with SigmaPlot 14.5.

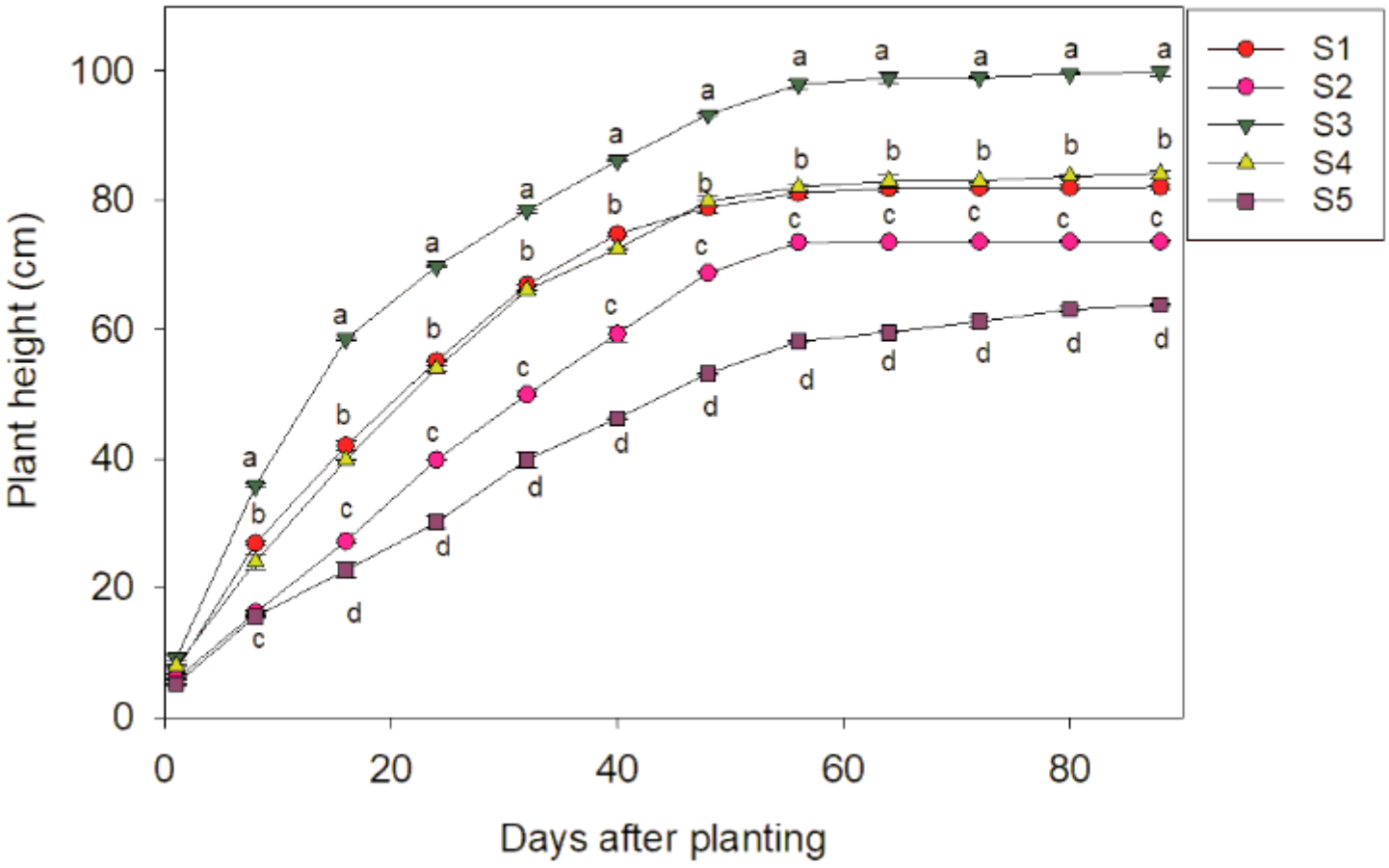

Figure 2

Average height of plants grown in different substrates measured during the development of the experiment. Each substrate is represented with a different color: S1 brown, S2 pink, S3 grey, S4 green and S5 purple. The points show the mean of 6 plants ± SE. Means of each day followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$). The graph was made with SigmaPlot 14.5.

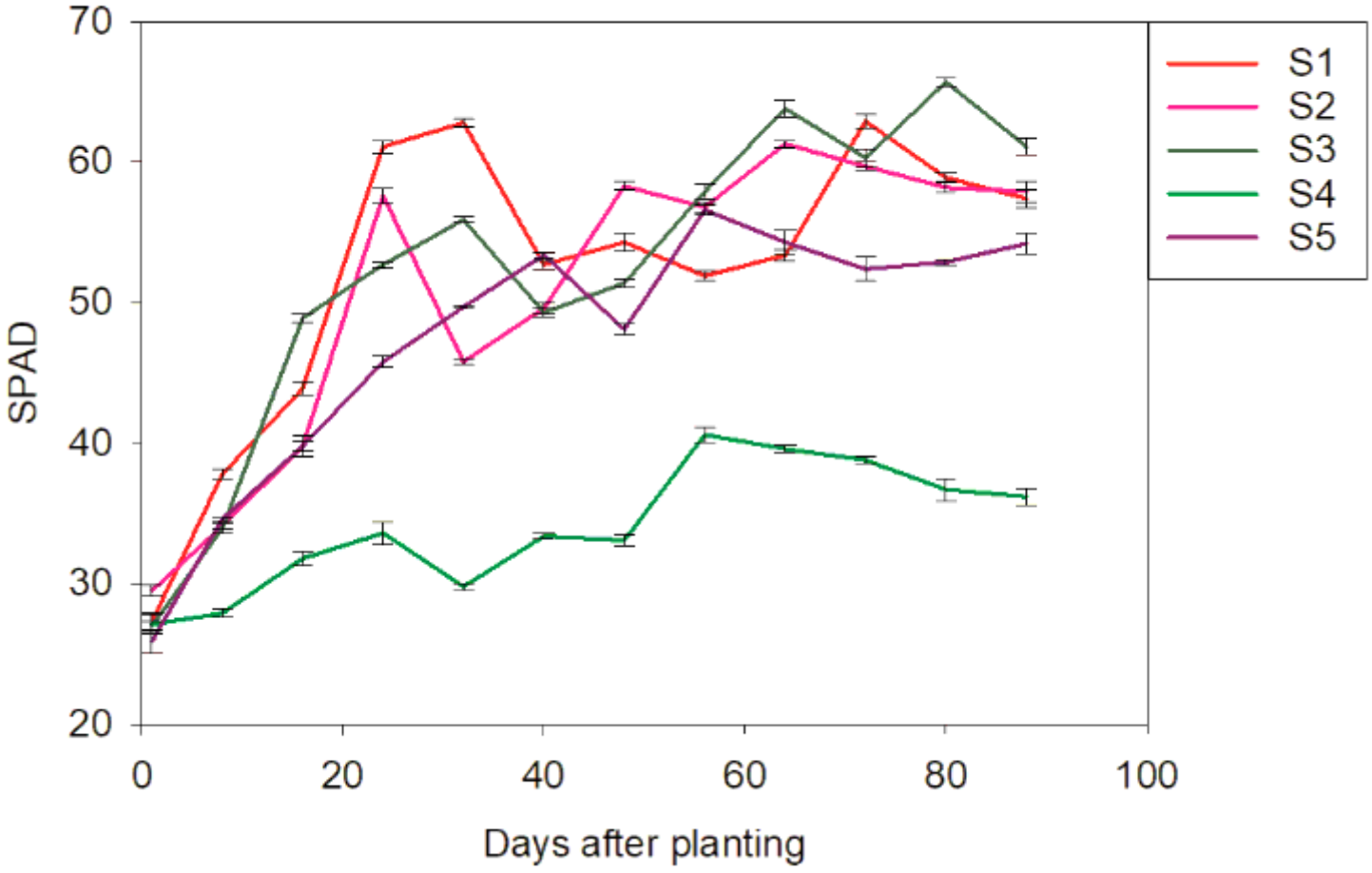

Figure 3

SPAD values of plants grown in the different substrates measured during the development of the experiment. Each substrate is represented with a different color: S1 brown, S2 pink, S3 grey, S4 green and S5 purple. The points show the mean of 6 plants ± SE. The graph was made with SigmaPlot 14.5.

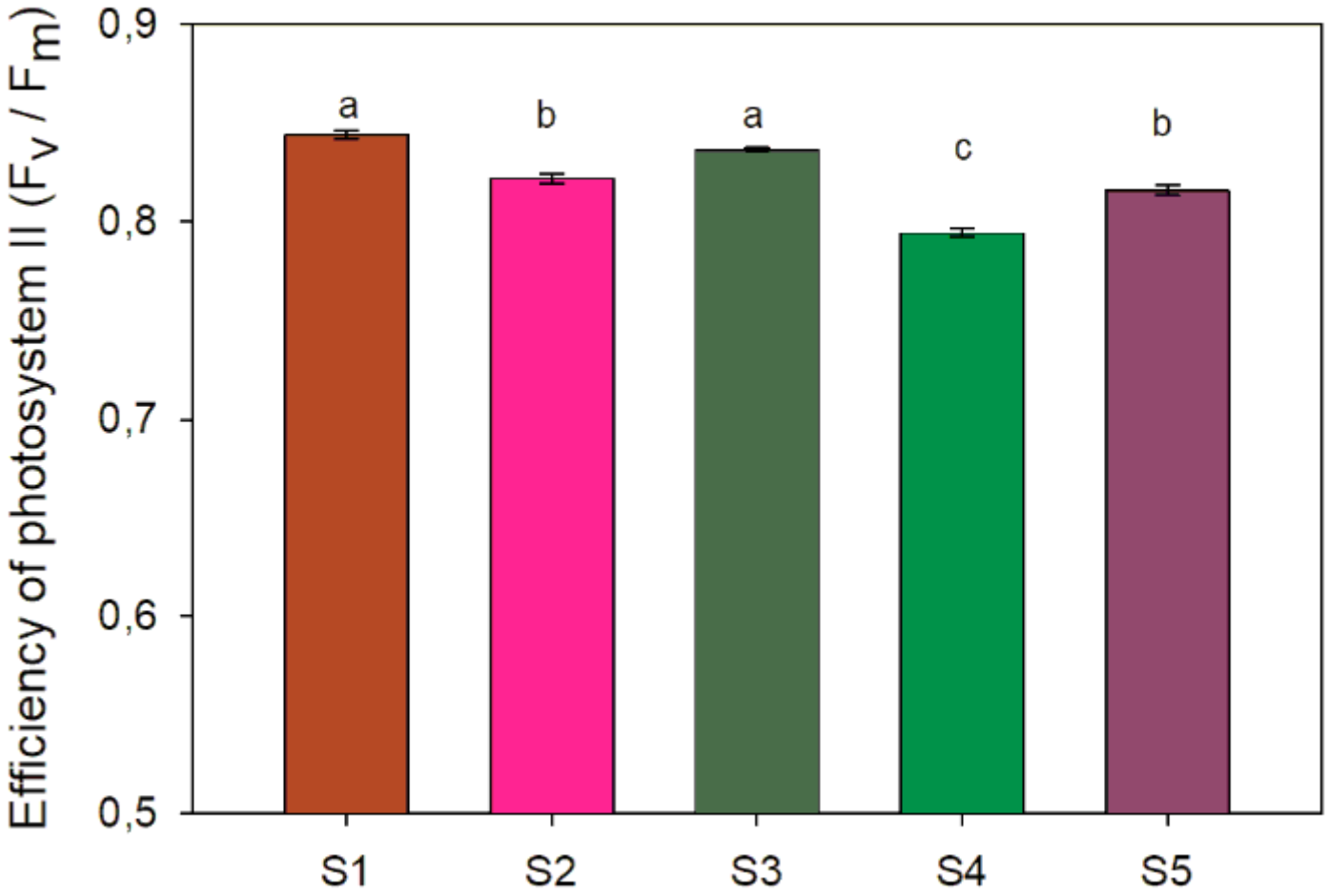

Figure 4

Efficiency of photosystem II of plants grown in the different substrates. Each substrate is represented with a different color: S1 brown, S2 pink, S3 grey, S4 green and S5 purple. Values are the mean of 6 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$). The graph was made with SigmaPlot 14.5.

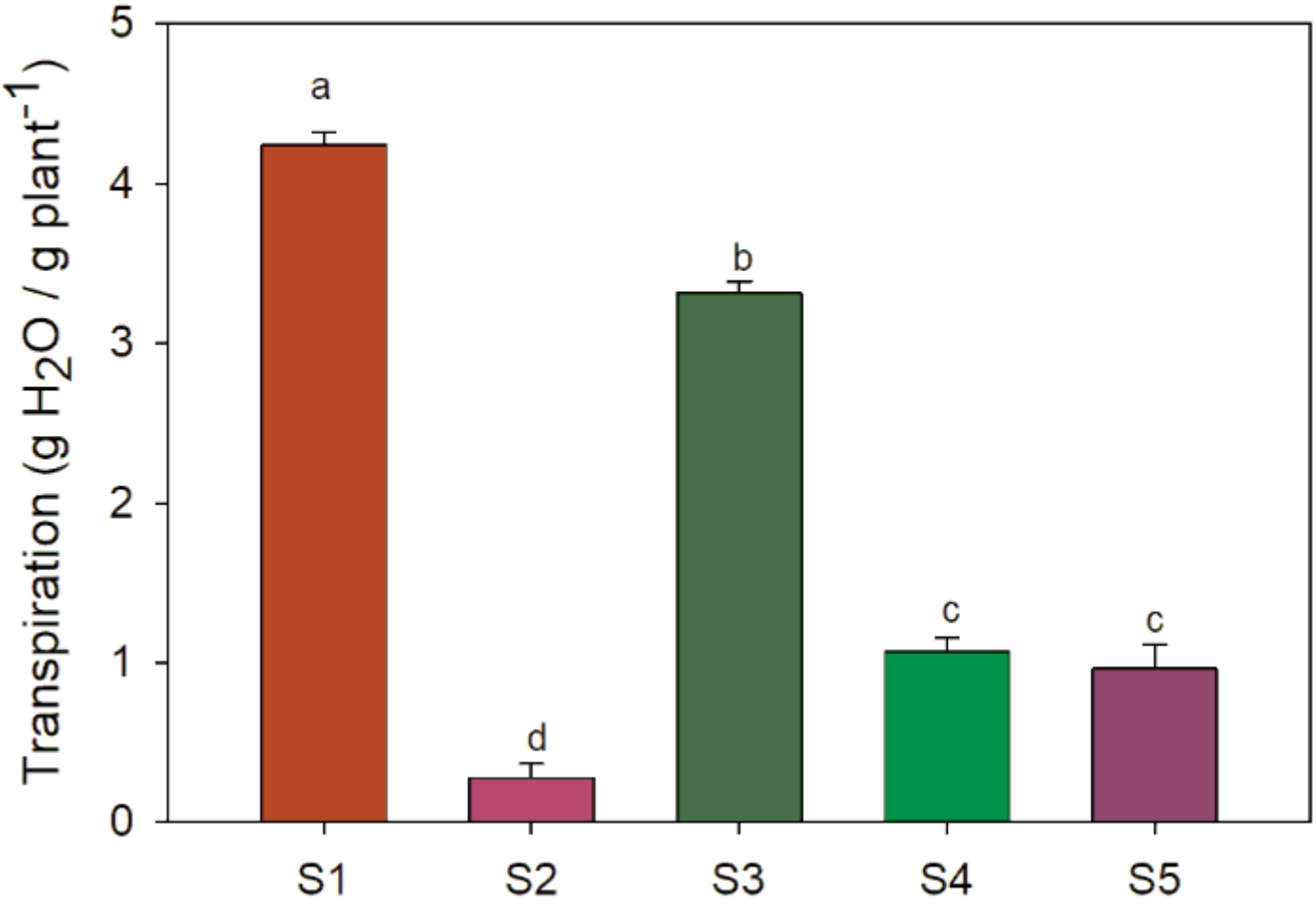

Figure 5

Transpiration of the cannabis plants grown in the different substrates. Each substrate is represented with a different color: S1 brown, S2 pink, S3 grey, S4 green and S5 purple. Values are the mean of 6 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$). The graph was made with SigmaPlot 14.5.

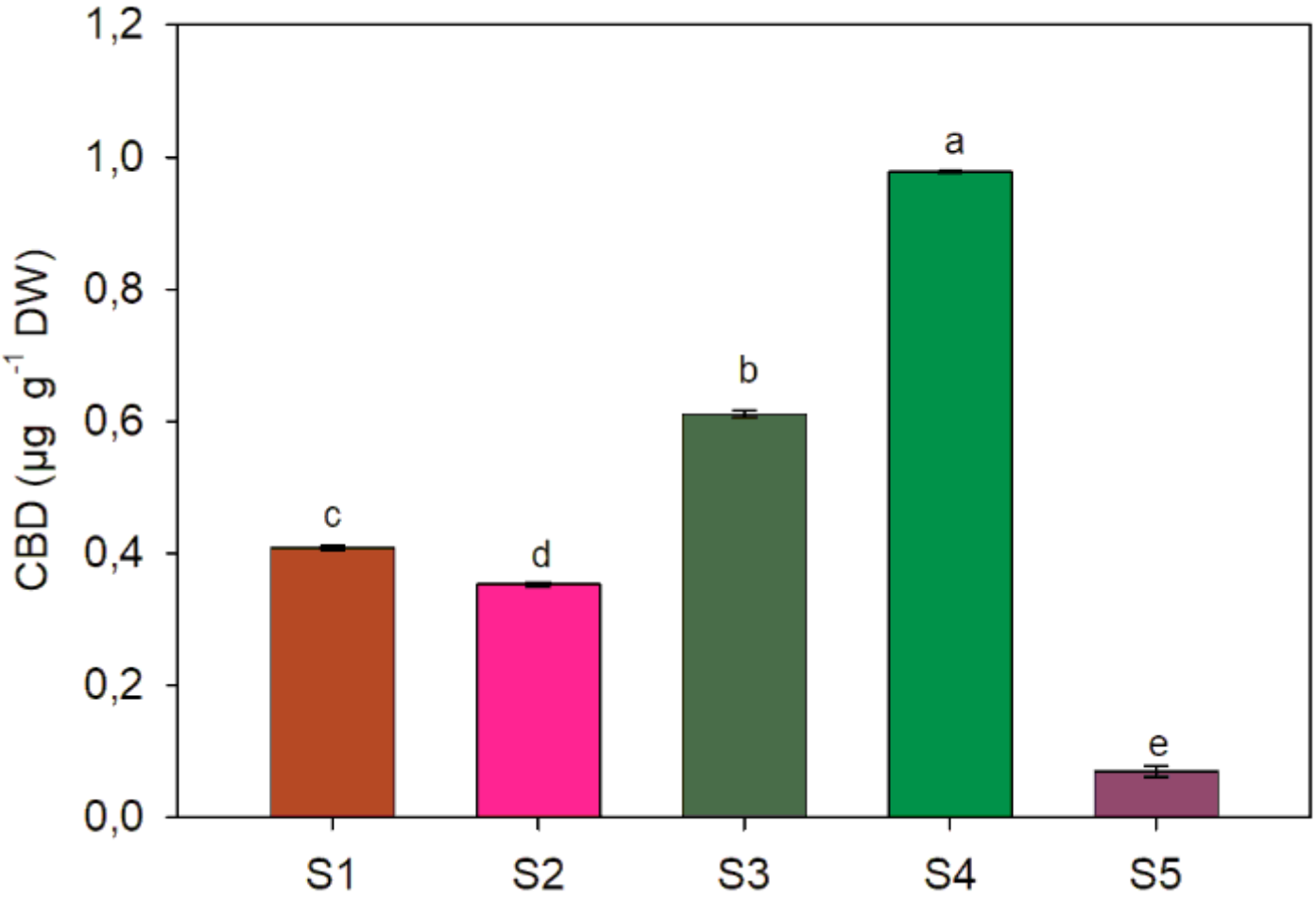

Figure 6

Cannabidiol (CBD) content present in the inflorescences of cannabis plants grown in the different substrates. Each substrate is represented with a different color: S1 brown, S2 pink, S3 grey, S4 green and S5 purple. Values are the mean of 3 samples ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$). The graph was made with SigmaPlot 14.5.

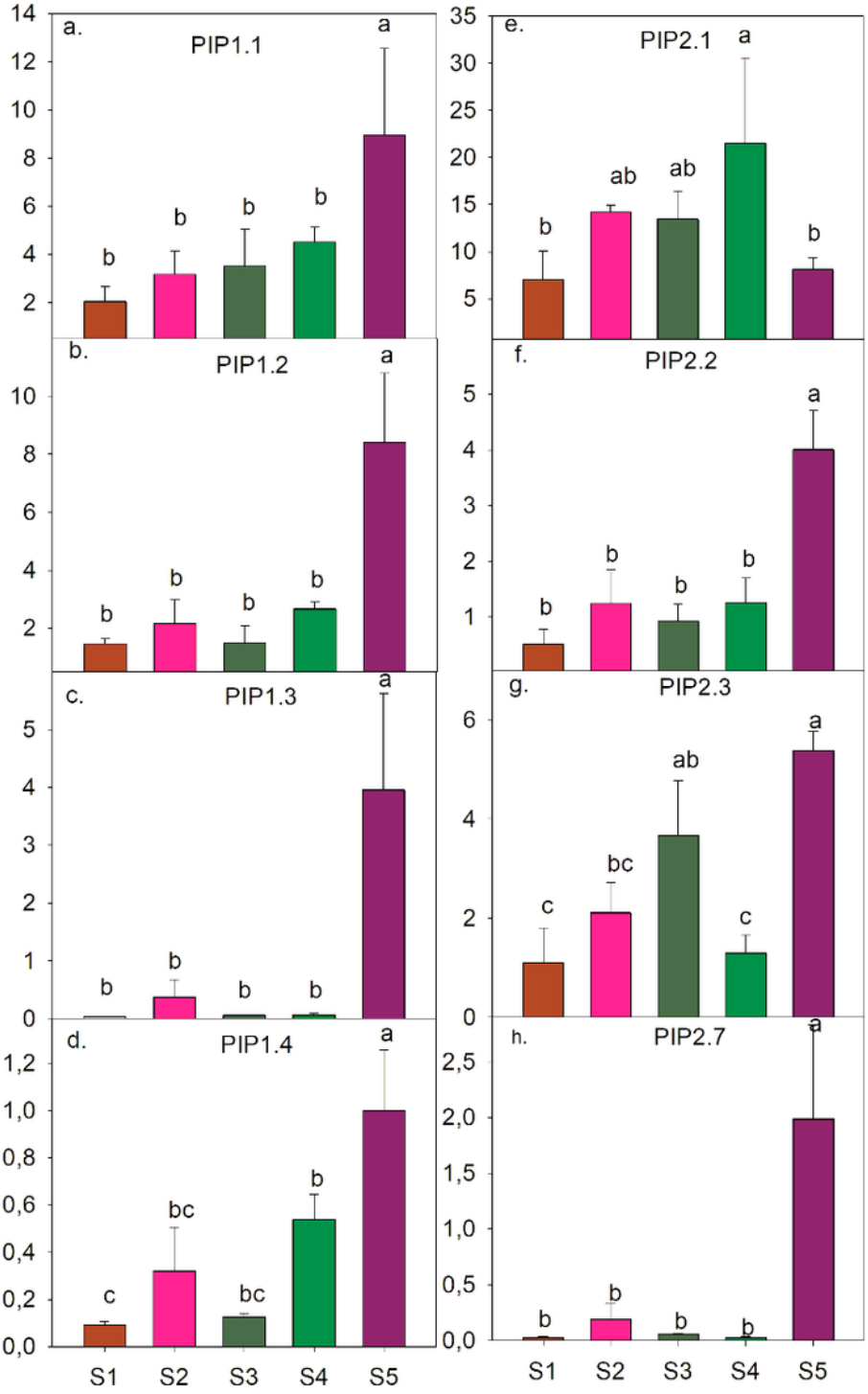

Figure 7

Relative expression of PIPs genes (a) PIP1.1, (b) PIP1.2, (c) PIP1.3 (d) PIP1.4 (e) PIP2.1 (f) PIP2.2 (g) PIP2.3 and (h) PIP2.7 in cannabis leaves of plants grown in the different substrates. Each substrate is represented with a different color: S1 brown, S2 pink, S3 grey, S4 green and S5 purple. Each value represents the mean of 3 young leaves mix from 3 different plants with 3 technical replicates of each

measurement ± SE. Means followed by different letters were significantly different according to Duncan's test ($p \leq 0.05$). The graph was made with SigmaPlot 14.5.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OnlineResource1.pdf