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Research paper

High added-value compounds from *Cannabis* threshing residues

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ABSTRACT

Industrial hemp cultivation in Europe is dual-purpose, with stalks providing fibers and hurds, and seeds being used for food, feed and pharmaceutical applications. Economic sustainability of hemp cultivation should encompass the possibility of recovering non-narcotic secondary metabolites from hemp by-products (leaves, leaflets and bracts) originating from seed harvest and seed cleaning procedures. Surprisingly, no information is currently available on the contents of high added value bioactive compounds (CBD, CBG, cannflavin A, Δ^9-THC) in industrial hemp inflorescence and threshing residues. This observation provided a rationale for investigating the issue on three monoecious varieties grown in Northern Italy. The concentration of target compounds was monitored from full-flowering until plant senescence by LC–MS/MS analysis of methanolic extracts of the plant biomass. The anti-inflammatory prenylated flavonoid cannflavin A was present in all varieties at levels mainly affected by genotype and air temperature. Conversely, the concentration of CBD, currently the clinically most promising non-narcotic cannabinoid, correlated to the overall extent and distribution of precipitation during growing cycle. Our findings suggest that postponing harvest after seed maturity increases the CBD contents and increase the CBD/Δ^9-THC ratio in harvest threshing.

1. Introduction

Hemp has been traditionally cultivated as a source of fiber, but a growing interest in the nutritional properties of the seeds has fostered its further development as a dual-purpose crop (Tang et al., 2016). Hemp is also a prolific producer of bioactive secondary metabolites, and their recovery from by-products of the harvest of fiber and seeds could further qualify this plant as a multipurpose crop (Amaducci and Gusovius, 2010, http://multihemp.eu). In this context, the recovery of non-narcotic phytocannabinoids like CBD and CBG and of lipophilic flavonoids (cannflavins) from hemp side-products has the potential to complement the economy of hemp growing, but, while there is no shortage of studies on the effect of environment and agronomical techniques on fiber hemp (see Amaducci et al., 2015 for a review), the dynamics of cannabinoids accumulation in industrial hemp during flowering and seed ripening is still poorly known, and nothing is known on the one of cannflavins. The accumulation and profile of phytocannabinoids is affected by Ca/Mg and N-P-K fertilization (Coffman and Gentner, 1975; Coffman and Gentner, 1975; Hanuš 1994), with harvest time being a major factor affecting their concentration in the flowerheads (Fournier et al., 2004; Pacifico et al., 2008; Turner et al., 1981). As regards breeding, efforts have focused on the increase in fiber and seed yield (Salentijn et al., 2015) and in the reduction of the Δ^9-THC content in order to comply with the 0.2% threshold set by the EU legislation [(EC) No 2860/2000] to dissect narcotic *Cannabis* (marijuana) from fiber hemp. This legal constraint, while substantially draining resources that could have been otherwise used to improve traits linked to the yield and quality of fiber and seeds, has nevertheless resulted in the development of a number of varieties low in THC but with a relatively high content in other phytocannabinoids. These meroterpenoids, most of which are exclusive of the genus *Cannabis* (Hanuš et al., 2016) are accumulated at the level of the inflorescence on the pistillate flowers that bear most of the glandular trichomes produced by the plant (Hillig and Mahlberg, 2004). This plant biomass, rich in cannabinoids and also containing the lipophilic flavonoids typical of hemp, makes up most of the threshing residue recovered during seed harvest and seed cleaning procedures, and in a recent study it was estimated that up to 2 t ha^{-1} of this material can be potentially collected (Tang et al., 2016). To qualify these residues as a source of valuable phytochemicals for added value applications in the realm of medicine, nutrition and cosmetic, the factors affecting their accumulation should be clarified, and suitable cultivation practices developed.

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Table 1
Life cycle phases and climatic conditions experienced in the year 2014 in two different locations in Italy (PC: 45° 0′ N, 9°42′ E, 72 m asl and RO: 45° 4′.N, 11° 45′ E, 3 m asl).

	End date	Rainfall (mm)	Mean solar radiation (MJ m^{-2} d^{-1})	Mean average air hum. (%)	Mean max temp. (°C)	Mean average temp. (°C)
Locality PC 1						
Sowing date	28 Apr					
Emergence	07 May	77	17.4	79.6	19.9	14.8
Vegetative	23 Jun	86	22.9	63.9	26.8	20.4
Ripening	26 Jul	101	19.6	76.2	27.8	22.1
Senescence	26 Ago	36	16.7	77.1	27.2	22.1
Total		299	20.0	72	26.6	20.8
Locality RO 2						
Sowing date	12 May					
Emergence	20 May	20	23.1	64.6	23.7	16.8
Vegetative	07 Ago	241	23.8	69.6	28.3	21.6
Ripening	04 Sep	45	20.2	75.7	28.7	22.4
Senescence	02 Oct	66	14.6	77.2	25.1	19.2
Total		373	20.0	73.3	27.2	20.8
Locality PC 3						
Sowing date	28 May					
Emergence	04 Jun	35	22.1	67.4	25.1	18.9
Vegetative	26 Ago	169	19.3	74.2	28.0	22.4
Ripening	22 Sep	47	14.8	78.5	26.1	20.7
Senescence	06 Oct	8	12.7	78.6	23.2	17.2
Total		259	17.9	75	26.9	21.3

In the framework of the EC project Multihemp, we have monitored the content of added value secondary metabolites in industrial hemp leaves and bracts, the major “threshing residues” from the production of fiber and seeds. To evaluate the role of environmental conditions on the accumulation of secondary metabolites during the flowering phase, three sequential sowings were planned. Three monoecious cultivars (Ermes, Santhica 27 and Ermo) exemplifying distinct chemotypes of hemp were compared at two sites in Northern Italy, investigating the dynamics of accumulation of the three major phytocannabinoids (CBG, CBD and THC) and of the major cannflavin (cannflavin A) from flowering until seed ripening. To the best of our knowledge, this is the first study where the kinetics of accumulation of cannabinoids was compared to the one of cannflavins.

2. Materials and methods

2.1. Field management and sampling operations

Two experimental fields were established in 2014 in the Po valley, the first one in Piacenza at CERZOO, Research Centre for Zootechnics and Environment, (45°0′15.98″ N, 9°42′20.44″ E, 72 m asl) and the second one in Rovigo at CREA-CIN, Research Centre for Industrial Crops (45°4′40.1″ N, 11°45′57.1″ E, 3 m asl). Field layout was a completely randomized block design to compare three sowing times and three cultivars. Single plot dimension was 1 m^2 (1 × 1 m). The three sowings were scheduled between end of April and the end of May, at fourteen days distance between each other. The date of sowings were planned in order to cover the period with high and still increasing day length to avoid pre-flowering stimulated by short days (Amaducci et al., 2012, 2008) (Table 1). The selected varieties for this study were Ermes, Santhica 27 and Ermo: three monoecious cultivars characterized by three different chemotypes. Ermes has a CBD prevailing chemotype, or chemotype III according to Hillig and Mahlberg (Hillig and Mahlberg, 2004) and Santhica 27 is a CBG chemotype, totally lacking CBD and THC (Fournier et al., 2004). The variety Ermo, is derived from parental selection of Ermes and both are Italian breed by Dr. Grassi G. in Rovigo and registered at CPVO with n° 20100208 and n° 20020483 respectively. The Ermo variety almost totally lacking cannabinoids production with a total content of cannabinoids lower than 0.05% (Onofri et al., 2015). Sowing was performed by hand using 100 viable seeds per m^{-2}. At the end of the emergence 38,7 g m^{-2} of Calcium nitrate (YaraLiva Tropicote) were applied to provide 60 kg ha^{-1} of N. Plants were monitored periodically until the beginning of flowering, when 10 plants per plot were labeled and subsequently used for samples collection. At each sampling time and for each plant one axillary bud, randomly chosen along the top of the inflorescence, was harvested. The axillary buds, harvested on the 10 plants per each plots were pooled to obtain a single sample, that was oven dried at 40 °C for 48 h. Meteorological data were obtained from an on-site meteostation (WatchDog 2900ET) for the field located in Piacenza, while data from the field of Rovigo were acquired from ARPA network (Regional Environmental Protection Agency, www.arpa.veneto.it).

2.2. Secondary metabolites extraction

After drying, samples were crushed carefully and seed and secondary stems were removed using tweezers. The remaining bracts and leaves, representing the threshing residue fraction, were milled using an electric spice grinder. Two sub samples of 150 mg were prepared using a precision scale, one was placed in oven at 105 °C until constant weight to asses dry matter content of the sample, the other was extracted using 15 mL of methanol (CHROMASOLV, Sigma-Aldrich), into an ultrasonic bath at 50 °C for 60 min. Tubes were spin at 6000 rcf for 5 min and the solvent was separated from plant residues. For the quantification of cannflavin A the extract was analyzed without further treatment. To quantify the main cannabinoids (i.e., CBD, CBG and THC), an aliquot of the extract was evaporated until dryness at 50 °C and subsequently maintained for 120 min at 120 °C in order to achieve the total decarboxylation of cannabinoids (Appendino et al., 2008), after the thermal treatment the samples were re-dissolved in the initial volume of methanol. In this study the organic solvent chosen for extraction was anhydrous methanol instead of *n*-hexane or solvent mixture (Raharjo and Verpoorte, 2004) in order to achieve high recovery of both the lipophilic cannabinoids and the more polar cannaflavins in one single step. The recovery for target compounds was not investigated in this paper, but methanol is, in general, a very good solvent for cannabinoids and flavonoids. The Cannabis monograph of the American Herbal Pharmacopoeia makes references to this solvent for preparation