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Research article

The morphological and anatomical variability of the stems of an industrial hemp collection and the properties of its fibres

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ABSTRACT

Industrial hemp (*Cannabis sativa* L.) is identified as a leading fibre crop and there is increasing interest in *C. sativa* fibre due to its new range of industrial applications. However, the complexity of hemp germplasm resulted in insufficient information on the effect of genotypes on fibre quality and quantity. In this study, 16 fibre and non-fibre type hemp genotypes were evaluated to compare the morpho-anatomical differences of stems and physico-mechanical fibre properties under three retting methods and to understand the effect of stem colour on the properties of hemp fibres. Morphological markers were scored and stem anatomy was examined using live and herbarium collections. Stems were retted using chemical, enzymatic, and microbiological methods. The resulting fibres were tested for tensile strength, moisture retention, colour, bast and hurd dry weights. Hemp genotypes showed morphological variations that affect fibre processing and a unique pattern of fibre wedges in cross-sections of the basal internode. Fibre yield, tensile strength, colour, and moisture retention significantly varied among the genotypes. The hemp collection used in this study formed three clusters in principal component analysis and traits such as internodal length, node number, hurd yield, and tensile strength highly contributed to the total variability. Additionally, non-fibre type hemp genotypes that showed important fibre properties were identified. The hemp genotypes that were selected based on our approaches can be tailored towards the specificities of the end-usage of choice. Our methods will enable the exploration of hemp genetic diversity pertaining to fibre properties and contribute to the preliminary identification of genotypes as a supplement to genetic analyses.

1. Introduction

Industrial hemp (*Cannabis sativa* L.) is a multipurpose crop, whose fibre has a wide range of industrial applications (Wang et al., 2013; Crini et al., 2020; Ahmed et al., 2022). The stem of *C. sativa* is used to extract natural fibres. The stem contains two types of fibres, known as bast (used in textile, paper, and automotive industries) and hurd (used for insulation, acoustic absorbers, etc.), which differ in their biological, chemical, and physical properties (van den Broeck, 2008; Taban et al., 2019; Hagnell et al., 2020; Schumacher et al., 2020). Bast fibres are crystalline cellulosic fibre bundles located in the phloem at the periphery of the *C. sativa* stem (Linger et al., 2002; Snegireva et al., 2015). They consist of primary bast fibres, which are generated from the procambium, and secondary bast fibres, which are generated from the vascular cambium (Snegireva et al., 2015). The woody core, which contains xylem vessels, makes up the inner hurd fibres and is rich with lignin (Gorshkova et al., 2012). During development, *C. sativa* stems exhibit basipetal gradient of lignification. Compared to the younger parts, the older parts of the stems show both the primary and secondary bast fibers, and the xylem are more developed (Behr et al., 2016; Guerriero et al., 2017). Retting is a process used to extract bast fibres in the phloem tissues through dissolving certain cells and constituents surrounding the fibre bundles, such as hemicellulose, lignin, and pectin (Ribeiro et al., 2015; Liu et al., 2017). The main retting methods are chemical, mechanical, enzymatic, field retting (expose stems to dew), and microbial approaches, and they influence the quality and quantity of the fibres (Müssig and Martens, 2003; Jankauskienė et al., 2015; Lee et al., 2020; Shuvo, 2020). Other factors that also reportedly contribute to fibre quality and quantity are morpho-anatomical traits and the cellular biochemical composition of *C. sativa* stems (Booth et al., 2004; Marrot et al., 2013). Changes in the *C. sativa* stem from vegetative to flowering stage affect fibre quality and quantity owing to significant chemical and structural changes (Crônier

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et al., 2005; Liu et al., 2015a). However, other variables, such as genotype, environment, management, and their interaction, also affect raw *C. sativa* fibre quality and yield (Struik et al., 2000; Amaducci et al., 2008; Salentijn et al., 2019).

Quantity and quality parameters of *C. sativa* have been extensively studied. For instance, fibre yield was known to be largely influenced by agronomic practices, environment, and genotype (Bennett et al., 2006; Tang et al., 2016; Vandepitte et al., 2020). Similarly, biochemical quality of fibre bundles, their lengths, and stem processability vary between *C. sativa* genotypes (Petit et al., 2019; Vandepitte et al., 2020). Physical properties and mechanical behavior of *C. sativa* fibres such as tensile strength (Sankari, 2000; Rey et al., 2017), compression (Khan et al., 2010), elastic modulus (Rey et al., 2017), thermal properties (Kosiński et al., 2018; Charai et al., 2021; Parcesepe et al., 2021), moisture retention (Pejic et al., 2008; Stevulova et al., 2014), colour (Jankauskienė and Gruzdevienė, 2013; Bleuze et al., 2018), crystallinity (Dai and Fan, 2010), surface properties (Pickering et al., 2007), and bundle architecture (Bourmaud et al., 2017) have been widely evaluated. However, there are limited studies that examine the effect of the genotype on physico-mechanical properties. Therefore, further research on this area will identify suitable genotypes that form different end products.

The *C. sativa* genotype has been shown to be an important factor in determining several quantity and quality parameters of fibres (Jankauskienė et al., 2015; Musio et al., 2018; Petit et al., 2019). However, *C. sativa* germplasm is complex. Genetic reticulation from prolonged domestication and haphazard breeding have made it hard to identify genotypes (Punja et al., 2017; McPartland, 2018; Vergara et al., 2021). Generally, the genotypes are classified according to a wide range of attributes: i) population types–for instance, wild, landraces, and cultivars; ii) gender as they are dioicous or monoecious; iii) stem colours as yellow and green; iv) flowering time, which include early, intermediate, and late flowering genotypes; v) application–fibre, seed, dual (fibre and seed), phytochemical (e.g., cannabidiol-CBD, cannabigerol-CBG), and ornamentals (Salentijn et al., 2015; Schluttenhofer and Yuan, 2017; Musio et al., 2018). Dioecious *C. sativa* genotypes have shown higher fibre yields than monoecious *C. sativa* (Sankari, 2000; Lee et al., 2020). Mechanical properties and the morphology of the fibres of *C. sativa* are also reportedly affected by gender (Faux et al., 2013; Li et al., 2015). Another study has shown that the *C. sativa* genotypes with yellow stems exhibit greater mechanical processability than green-stemmed genotypes and contain more bast fibre yield (Musio et al., 2018). Further, late-flowering genotypes have shown a high fibre yield compared with early flowering genotypes (Höppner and Menge-Hartmann, 2007; Small, 2015). While dual-purpose or multipurpose genotypes have been produced in hemp-breeding programs (Tang et al., 2016; Papastylianou et al., 2021), there is no clear split between fibre, seed, and phytochemical type male plants (Salentijn et al., 2015), and their potential for fibre production has not been investigated. Therefore, more studies are needed to unveil the effect of genotypes on fibre production, linking stem anatomy and morphology with fibre properties. Moreover, the identification of *C. sativa* genotypes belonging to non-fibre categories, which potentially produce quality fibres, is important for the fibre industry.

The objectives of the present study were to; 1) identify the morpho-anatomical differences of the stems of *C. sativa* genotypes, 2) compare the physico-mechanical fibre properties of different *C. sativa* genotypes, 3) understand the effect of the stem colour of genotypes on biological and/or mechanical properties of hemp fibres, and 4) test the fibre quality of non-fibre type *C. sativa* genotypes. To achieve the objectives, we evaluated the physical properties of hurd and bast fibres from various *C. sativa* genotypes extracted using chemical, microbial, and enzymatic retting methods and tested the stem morpho-anatomical differences between the *C. sativa* genotypes.

2. Materials and methods

2.1. Cannabis sativa cultivation and sampling

For this study, we used *C. sativa* genotypes cultivated in the Prairie View A&M (PVAMU) greenhouse (GPS: latitude 30.0901/longitude -95.9824) from September 2020 to May 2021 under a research licence issued by the Texas Department of Agriculture. All genotypes were cultivated under the same conditions, as explained below. Genotypes were grown as one plant per pot in 6 L black plastic pots containing a mixed medium of coconut coir, perlite, and Osmocote (5:3:4, v/v). A soluble fertilizer containing nitrogen, phosphorus, and potassium (20:20:20, v/v) at 750–850 ppm was applied to the plants every two weeks for 30 days. The same fertilizer at 850–1200 ppm was applied to the plants every two weeks from 30 to 90 days. The mean temperature in the greenhouse was 15 ± 5 °C at night and 20 ± 3 °C during the day in winter, and 18 ± 3 °C at night and 25 ± 6°Cduring the day in autumn and spring. The average relative humidity values were 76.6% in winter and 75.2% in autumn and spring. Water was provided using drip irrigation. When the temperature was below 20 °C, the plant watering schedule was 375 ml per day at 8.00 am, and when the temperature was above 20 °C, it was 750 ml twice a day at 8.00 am and 2.00 pm until 30 days. When the temperature was below 20 °C, the irrigation schedule was 650 ml per day

Table 1. Accessions and voucher information of the *Cannabis sativa* male plant collection.

Genotype	Use	Barcode	Col. No.	Seed/plant donor	Collection date
American victory1	Fibre	5746389	8	Patrick Moran, Acquiflow LLC	01/23/2021
Białobrzeskie	Fibre	5746381	1	Canna Farm, Mallory Tate	11/24/2020
Blue genius	CBD	5746386	5	John Bradley, Tetra Hemp Company LLC	02/20/2021
Blue kross	CBD	5746401	25	John Bradley, Tetra Hemp Company LLC	05/12/2021
Cherry blossom	CBD	5746441	39	Eric Johnson, Canabanix	07/05/2021
Cherry wine	CBD	5746404	22	John Bradley, Tetra Hemp Company LLC	05/10/2021
Ditchweed	Wild	5746393	12	Mike Smith, Hempliance Inc.	02/20/2021
Ditchweed-Colorado	Wild	5746397	16	Mike Smith, Hempliance Inc.	04/19/2021
Ditchweed-Nebraska	Wild	5746403	23	Mike Smith, Hempliance Inc.	05/10/2021
Jin Ma	Fibre	5746409	40	Mike Smith, Hempliance Inc.	07/05/2021
KLR2006	CBD	5746407	63	Melissa McDougal, KLR farms	07/05/2021
KLR2014	CBD	5746411	34	Melissa McDougal, KLR farms	07/05/2021
KLR2020	CBD	5746399	18	Melissa McDougal, KLR farms	04/27/2021
Portland	CBD	5746384	3	Shonda Warner, Farmacopia farms LLC	1/24/2020
Tetra	Unknown	5746405	21	John Bradley, Tetra Hemp Company LLC	05/10/2021
US031	Fibre	5746394	13	Chase Simmons	1/23/2021

Note: CBD = cannabinoid genotypes.

at 8.00 am, and when the temperature was above 20 °C, it was 1300 ml twice a day at 8.00 am and 2.00 pm for plants from 30 to 90 days.

Sixteen *C. sativa* accessions of both fibre and non-fibre genotypes (Table 1) were used for this study, and their voucher specimens were deposited at the PVAMU herbarium, and a seed stock was maintained at the Cooperative Agricultural Research Center, PVAMU. Flower heads and leaves were removed, and stems were harvested at the soil level at the full flowering stage (~90 days from planting) from the seeding rate of one plant per pot. All selected genotypes were from dioecious plants, specifically male plants. Samples were harvested in triplicate for each analysis.

2.2. Morphological characters

To score the morphological characters of stems, accessions (triplicates per genotype) were randomly chosen. A total of 10 fibre-relevant discrete and continuous characters were scored for each genotype. Morphological characters, character-state definitions, and methods of scoring are presented in Mendeley Data: Table S1. Heights of live plants were measured using a measuring tape and the number of grooves on the stem, branch number, and leaf number were counted after uprooting at the full flowering stage (~90 days after planting). Stem cross-sections were measured using a digital reticule of the Olympus DP73 stereo microscope (Olympus America Inc., Massachusetts, USA) at 63× on fresh stem sections. The stem curvature and inflorescence position of the herbarium specimens and colours of the freshly peeled stems were visually inspected. The internodal lengths of the digitised herbarium specimens were measured using ImageJ version 2 (Rueden et al., 2017). Only continuous variables were used for statistical analyses.

2.3. Anatomical characters

Only two major positions of the stem were examined because we observed a large variation in the anatomical traits of the hemp stem from the bottom section to the top section during preliminary analyses using one genotype. Immediately after harvest, the stems of each hemp genotype were cut at two stem heights: sections from internodes between nodes 0 (ground level)–1 (position 1) and nodes 3–4 (position 2) positions, starting from the ground level and moving towards the top of the plant. We then determined the cell distribution of each nodal position of each genotype using the manually sectioned transverse sections of stems that were double-stained with methyl green-Congo red (Bonatti et al., 2004). The stained transverse sections were photographed under an Olympus DP73 stereo microscope at 63×. The cortex layer (Liu et al., 2015b), which contains a high percentage of crystalline cellulose, and the xylem, which contains a high percentage of lignified cellular layers, were visually examined. The cortex layer responsible for containing bast fibres (both primary and secondary extra-xylary fibre layers) and the xylem layer, which contains hurd fibres, were measured using the digital reticule of the stereomicroscope, and xylem/cortex values were estimated. The diameter of the pith (on stem position 2) was also measured using the same digital reticule. This procedure was performed in triplicate for each genotype.

2.4. Fibre extraction

Fibres from each genotype (in triplicate) were extracted using chemical, enzymatic, and microbiological retting methods, as described below. To prepare the stems for retting, the top part of the plant, including inflorescences and leaves, was cut away from the stem. All hemp stems were washed with distilled water, wiped, and cut into equal pieces using disinfected secateurs. The stem pieces were disinfected with 70% ethanol. The fresh weight of all stem samples was measured. For the negative control, hemp stems from all genotypes were disinfected with 70% ethanol and immersed in autoclaved distilled water.

Table 2. Morphological and anatomical characters* of the selected hemp genotypes. Average values and standard deviations are provided for the numerical variables. Definitions and delimitations of each character are listed in the Mendeley Data repository: Table S1.

Genotype	Height (cm)	Stem diameter po1* (mm)	Xylem/cortex po2d*	Xylem/cortex po1*	Pith diameter (po2)*(mm)	Stem colour	Stem curvature	Groove no.	Node no.	Internodal length (cm)	Branch no.	Leaf no.	Inflorescence position
American victory1	170.2 ± 66	27.8 ± 6	5.31 ± 1	2.3 ± 1	4.55 ± 1	yellow	curved	6 ± 4	44 ± 6	6 ± 1.5	38 ± 6	55 ± 14	axillary & terminal
Białobrzeskie	79.9 ± 18	22.1 ± 2	1.03 ± 0.3	2.2 ± 1	7.92 ± 2	green	curved	0	22 ± 3	7.1 ± 0.7	26 ± 3	43 ± 8	terminal
Blue genius	210.3 ± 138	28.8 ± 2	3.32 ± 1	7.8 ± 2	11.38 ± 6	yellow	erect	0	14 ± 5	6.2 ± 2	19 ± 4	24 ± 1	axillary & terminal
Blue kross	96.9 ± 11	25.3 ± 8	3.24 ± 1	4.8 ± 1	8.29 ± 4	green	curved	0	22 ± 4	5.2 ± 0.8	16 ± 3	32 ± 4	axillary
Cherry blossom	113.1 ± 43	28.8 ± 9	6.05 ± 2	3.9 ± 1	4.81 ± 2	yellow	curved	4 ± 1	46 ± 9	7.2 ± 1.3	48 ± 7	69 ± 2	axillary
Cherry wine	130.1 ± 31	27.7 ± 3	5.64 ± 2	6.3 ± 2	3.26 ± 1	green	curved	0	59 ± 9	4.9 ± 2.7	41 ± 8	61 ± 20	axillary
Ditchweed	142.2 ± 39	24.4 ± 8	5.24 ± 1	2.2 ± 2	4.24 ± 1	green	erect	4 ± 2	44 ± 9	6.4 ± 0.9	40 ± 7	69 ± 5	axillary
Ditchweed-Colorado	101 ± 33	27.6 ± 3	2.7 ± 1	3.4 ±1	8.35 ± 4	green	erect	4 ± 2	23 ± 10	6.1 ± 2	33 ± 8	60 ± 6	terminal
Ditchweed-Nebraska	97.1 ± 43	25.5 ± 6	6.06 ± 1	2.5 ± 1	7.2 ± 1	green	erect	4 ± 1	25 ± 2	7.1 ± 2.8	20 ± 5	31 ± 12	axillary
Jin Ma	87 ± 17	26.2 ± 12	5.84 ± 1	6.4 ± 1	8.13 ± 2	yellow	erect	0	10 ± 3	9.2 ± 0.7	15 ± 4	19 ± 4	axillary & terminal
KLR2006	110.1 ± 16	25.7 ± 13	2.54 ± 1	4 ± 1	7.05 ± 1	purple	erect	0	46 ± 20	3.6 ± 0.7	48 ± 16	72 ± 22	axillary & terminal
KLR2014	130.2 ± 63	26.2 ± 17	2.96 ± 1	3 ± 0.4	8.06 ± 1	purple	erect	0	51 ± 7	5 ± 1.4	52 ± 6	63 ± 16	axillary & terminal
KLR2020	120.2 ± 72	27.7 ± 16	3.84 ± 0.1	3.8 ± 2	4.83 ± 2	purple	erect	4 ± 1	29 ± 6	3.4 ± 0.6	56 ± 10	41 ± 14	axillary & terminal
Portland	130.1 ± 34	27.6 ± 2	1.57 ± 0.4	4.8 ± 1	11.22 ± 4	yellow	erect	4 ± 3	38 ± 4	6 ± 2.4	41 ± 11	62 ± 10	axillary & terminal
Tetra	215 ± 114	34.3 ± 23	6.21 ± 1	2.8 ± 0.2	9.75 ± 5	yellow	erect	6 ± 2	62 ± 11	6 ± 1.3	64 ± 6	108 ± 51	axillary & terminal
US031	155 ± 26	25.1 ± 8	1.18 ± 0.2	7.7 ± 1	10.01 ± 1	green	erect	0	24 ± 3	7.2 ± 1.4	27 ± 9	44 ± 2	axillary

Note: Avg. = average and no. = number; position 1 = po1, position 2 = po2.

Samples were chemically retted by pre-treating with 0.3% HCl, treated with 6% NaOH at 70 °C for 1 h, and post-treated with 1% acetic acid (Sankari 2000; Mwaikambo and Ansell, 2003). This is a chemical method previously shown to be an optimum yielding approach for bast fibres with minimum loss of mechanical properties (Mwaikambo and Ansell, 2003), and it showed results in the highest yield during our preliminary studies using a series of NaOH concentrations (i.e., 2%, 4%, 6%, 8%, 10%, and 12%). When easily separable by hand, fibres were washed five times with distilled and deionized water at 50 °C to remove chemical residues and cellular debris.

We performed enzymatic retting using a modified version of the method used by George et al. (2014*a* and *b*), as explained below. A mixture of enzymes (pectinase, cellulase, xylanase, and laccase) was used to treat the whole stems. Initially, stems were immersed in 0.1% (w/w) commercial pectinase (Carolina Biological Supply Company, USA) at 30 °C for 30 min at pH 4. The contents were then transferred to 10% (w/w) cellulase at 50 °C for 30 min at pH 6. Finally, they were transferred to 0.05% (w/w) xylanase and 1% (w/w) laccase at 70 °C for 30 min at pH 7. Then the entire cellulase and fibre mixture was heated at 80 °C for 15 min to deactivate enzymes. A pressurized water column was applied to remove the non-fibrous elements, and the separated fibres were collected using tweezers. The fibres were washed five times with distilled and deionized water at 50 °C to remove traces of enzymes and cellular impurities.

We used an uncharacterised microbial solution obtained from okra (*Abelmoschus esculentus* (L.) Moench) (Mendeley Data: Figure S1), which showed soft rot lesions in its fibrous fruit. About 500 g of rotted *A. esculentus* was washed with 5 L of distilled water. The disinfected stems of each hemp genotype were immersed in 100 ml of the microbial solution at room temperature until the fibres separated from the woody core. A pressurized water column was applied to remove the non-fibrous elements. The fibres were washed five times with distilled water, which was heated at 50 °C.

The bast and hurd fibres were tested with toluidine blue staining (Parker et al., 1982) for the purity and evenness of retting. Hurd and bast fibres extracted using all methods were dried naturally at the room temperature. The dry weight of fibres was determined after oven drying them at 80 °C for 5 h (George et al., 2014b), until a constant weight was obtained. As the purpose of this is to demonstrate the efficiency of methods for extraction of raw fibres from each genotype, fibres were not subjected to additional treatments. Samples were stored in polythene bags in a desiccator for subsequent analyses and future studies. The average bast fibre content (%) in the stem was calculated by dividing the fibre dry weight by the dry weight of stems of three replicates. The bast fibre yield was calculated by multiplying the stem yield by the bast fibre content (Sankari, 2000). Dry weight of hurd and bast fibres obtained from different genotypes under three retting methods were compared.

2.5. Tensile strength

The tensile strength of bast fibres was measured using a modified version of the method used by Fan (2010). Tensile strength is an important measure for bast fibres in the industry but not for hurd fibres. Briefly, the extracted fibres were desiccated by oven-drying at 80 °C for 5

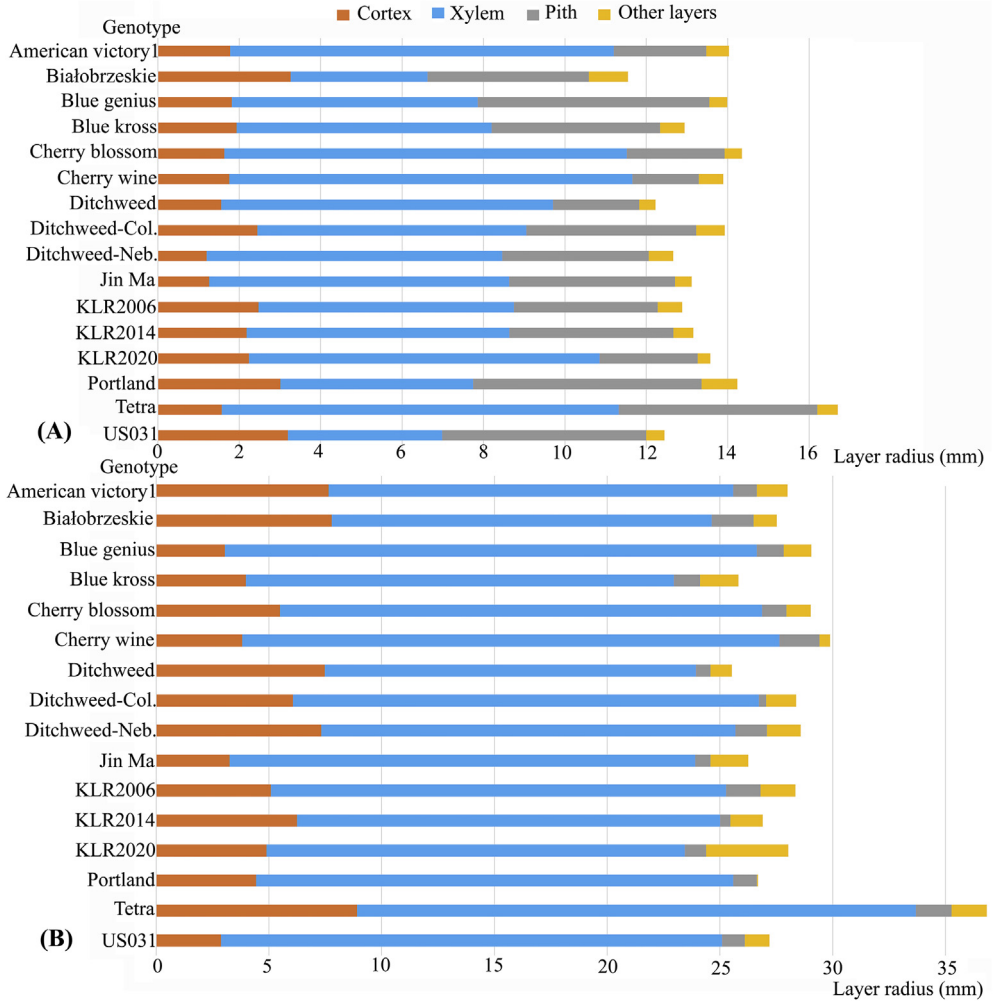

Figure 1. Radii of sections (mm) in stems of different hemp genotypes. (A) position 2 (nodes 0–1) of stems and (B) position 1 (nodes 3–4) sections of stems.

h (George et al., 2014b) and stored at 23 ± 2 °C and 65 ± 2% relative humidity in silica gel. The fibres were mounted on holed (25 mm) paper cards (45 × 20 mm^2) using adhesive tape. We mounted 10 fibre bundles together on the card so that a single bundle would not break in the middle while setting up the experiment. Since fibre bundles do not have even thickness, the average cross-section was measured, as described previously (Shahzad, 2013). The bundle was mounted across the exact centre of the holes. Then both ends of the mounting card were gripped on the TA.XT plus Texture Analyzer (Texture Technologies Corp., New York, USA), using a TA-96B miniature tensile grip attachment. Before recording the reading, both supporting sides of the card were cut using scissors. The test piece was tested at a speed of 0.5 mm/min. The strength of the loaded fibre bundle was analysed by the Texture Exponent Lite software. Five samples per replicate at each treatment level were examined. The tensile strength was calculated by Eq. (1) (Mileiko, 1969):

$$Tensile\ strength\ (MPa) = \frac{Applied\ load\ (N)}{Cross\ section\ area\ (m^2)} \times 10^{-6} \quad (1)$$

2.6. Moisture retention

Moisture retention represents the quantity of water retained in the fibres after being soaked in the water (Pejic et al., 2008). To eliminate initial moisture content, hemp hurd fibres (split into equal halves of equal length) were dried overnight in an oven at 80 °C and then weighed. The water absorption behaviour of hurd fibres was tested by completely immersing the desiccated fibre pieces in 50 ml of sterile deionized water at 23 °C for seven days following the standard centrifuge approach for estimating water retention (ASTM D 2402-78, 1978) at 2200 rpm for 5 min (Bekman Coutler laboratory centrifuge, Indianapolis, Indiana, USA). Additionally, we dried the bast fibres using the same method as for the hurd fibres, weighed them, and transferred them to a Falcon tube. Then the fibres were immersed in 50 ml of sterile deionized water at 23 °C for 1 h. The water absorption of bast fibres was measured using the same centrifuge approach used for hurd fibres. The percentage of water absorption in hemp hurd and bast fibre samples was calculated using Eq. (2) (Hussain et al., 2018):

$$Water\ absorption\ (\%) = \frac{sample\ wet\ weight\ (g) - sample\ dry\ weight\ (g)}{sample\ dry\ weight\ (g)} \times 100 \quad (2)$$

2.7. Colourimetry

The colour of the bast fibres was measured using a HunterLab MiniScan EZ colourimeter (Reston, Virginia, USA), a non-destructive method that has been previously used for fibres (Akin et al., 2000; Jankauskienė and Gruzdevienė, 2013; Bleuze et al., 2018). The colour of the CIELAB system is denoted as L* (lightness), a* [chromaticity from green (-) to red (+)], and b* [chromaticity from blue (-) to yellow (+)] values. Five measurements per replicate were recorded and the average value was calculated. Colour was measured only in raw bast fibres because colour is generally useful for fibres applied in the fabric industry–the colour of hurd fibres is not of interest for main industrial applications.

2.8. Scanning electron microscopy

Following the above fibre examinations, completely desiccated bast and hurd fibres obtained from the microbial retting (genotypes were selected based on the scores in the above tests) were observed under Scanning Electron Microscope (SEM) (JSM-6010LA, JEOL Inc., Massachusetts, USA). The micrographs were taken from the fibre surfaces at both 1000× and 500× using the TouchScope software (JEOL Inc., Massachusetts, USA).

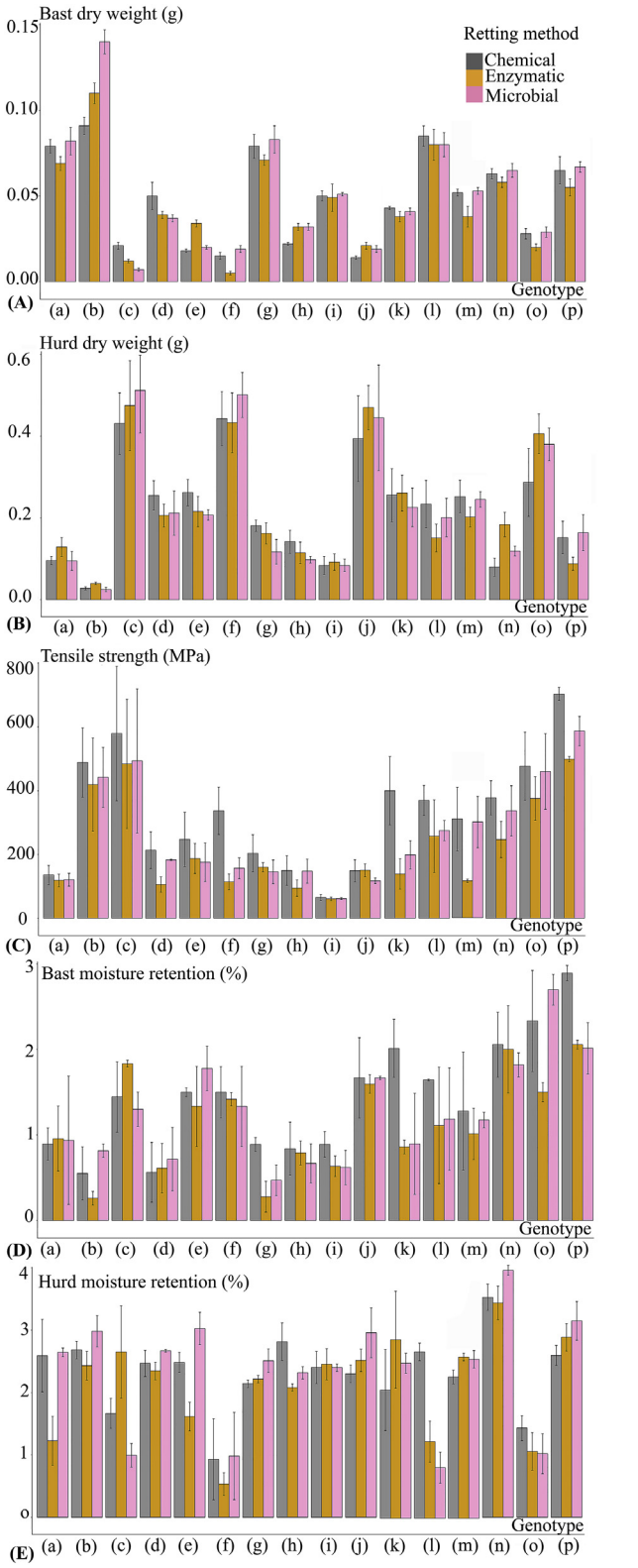

Figure 2. Graphs representing physico-mechanical properties of hemp genotypes. (A) mean dry weights (g) of bast fibres, (B) mean dry weights (g) of hurd fibres, (C) mean tensile strengths (MPa) of bast fibres, and (D) moisture retention (%) of hurd fibres (E) moisture retention (%) of bast fibres under different retting treatments of different hemp genotypes ($p = 0.05$). The hemp genotypes are: (a) American victory1, (b) Białobrzeskie, (c) Blue genius, (d) Blue kross, (e) Cherry blossom, (f) Cherry wine, (g) Ditchweed, (h) Ditchweed-Colorado, (i) Ditchweed-Nebraska, (j) Jin Ma, (k) KLR2006, (l) KLR2014, (m) KLR2020, (n) Portland, (o) Tetra, (p) US031.

2.9. Statistical tests

All experiments were performed in triplicate and results were expressed as mean value ± standard deviation. Atwo-way Analysis of Variance (ANOVA) was performed to determine whether the measurements (bast yield, hurd yield, tensile strength, and moisture retention) are significantly affected by the genotype and retting method, and/or the interaction between them ($p \leq 0.05$). A $p \leq 0.05$ was considered significant. A one-way ANOVA was used to determine the significant differences among the genotypes for all morphological (continuous variables) and anatomical traits. Finally, a principal component analysis (PCA) (Jolliffe and Cadima, 2016) was conducted to determine the variations among the genotypes for morpho-anatomical traits, and mechanical parameters.

As our hemp collection contained green (e.g., Białobrzeskie, Blue kross, and Cherry wine), yellow (e.g., American victory1, Blue genius, and Cherry blossom), and purple (e.g., KLR2007, KLR2014, and KLR2020) stemmed-genotypes, their bast fibre yield data were compared using a two-way ANOVA. The fibres of these genotypes extracted from the chemical, enzymatic, and microbial methods were examined for hurd and bast yield, tensile strength, and moisture retention.

Tukey multiple comparison test ($p \leq 0.05$) was used to calculate the differences between the genotype and retting method. The above statistical analyses were performed using R v3.5 (R Core Team, 2019).

3. Results and discussion

3.1. Morphological diversity of hemp genotypes

The morphological characters of branch number, node number, stem diameter of position 1, and internodal lengths differed significantly ($p \leq 0.05$) (Table 2), reflecting the existence of variability among the tested genotypes. This variability can be further used for hemp genotype improvement programs. The genotype Jin Ma (fibre type) showed the lowest number of branches and nodes, while the genotype Tetra (unknown usage) showed the highest number of branches and nodes. The number of branches and nodes might interfere with fibre-processing methods. Although the curvature of the stems of the different genotypes did not show a significant difference, it is still an important character to be considered as it might affect the fibre-parallelizing process in large-scale fibre processing via machinery. The internodal length was shown to be the highest in the Jin Ma genotype, whereas it was the lowest in the KLR2020 genotype (CBD type). Generally, KLR genotypes (CBD type) showed low internodal lengths. When hemp is retted, it has been observed that tissues in nodes produce more debris, which affects the fibre-refining process. Therefore, genotypes with fewer nodes (i.e., high internodal lengths) can be favourable for fibre production. The stem diameter is an important character of fibre production, and the easily separable thin stems are more suitable in the textile industry (Angelini et al., 2016). The genotype Tetra (unknown usage) showed the highest diameter, and Ditchweed (wild) showed the lowest diameter, on position 1 of the stem. There is a considerable diversity of height in hemp morphology depending on the genotype, soil, and climate conditions (Strzelczyk et al., 2021). Although similar environmental conditions were provided in this research, genotypes did not show any significant differences in height.

3.2. Anatomical diversity of the stems of hemp genotypes

The mean xylem/cortex length of stems of positions 1 and 2 and the mean diameter of the pith layer of position 2 are provided in Table 2. The xylem/cortex value in transverse sections of both positions 1 and 2 hemp stems was not statistically significant ($p = 0.7$). This value may vary depending on the nutrient supply of plants, seeding rate, light conditions, hormonal regulations within plants, etc. (Amaducci et al., 2005; Hall et al., 2014). The microscopic images of transverse sections of positions 1 and 2 hemp stems, respectively, are provided in the Mendeley Data repository: Figures S2 and S3. Although the stem samples were collected at the same plant age (~90 days of planting and at the full flowering stage), their development varied in the secondary bast fibre layer arrangement, lignin deposition, and stem shape (Mendeley Data: Figures S2 and S3). Further, transverse sections were highly variable at position 2 of the

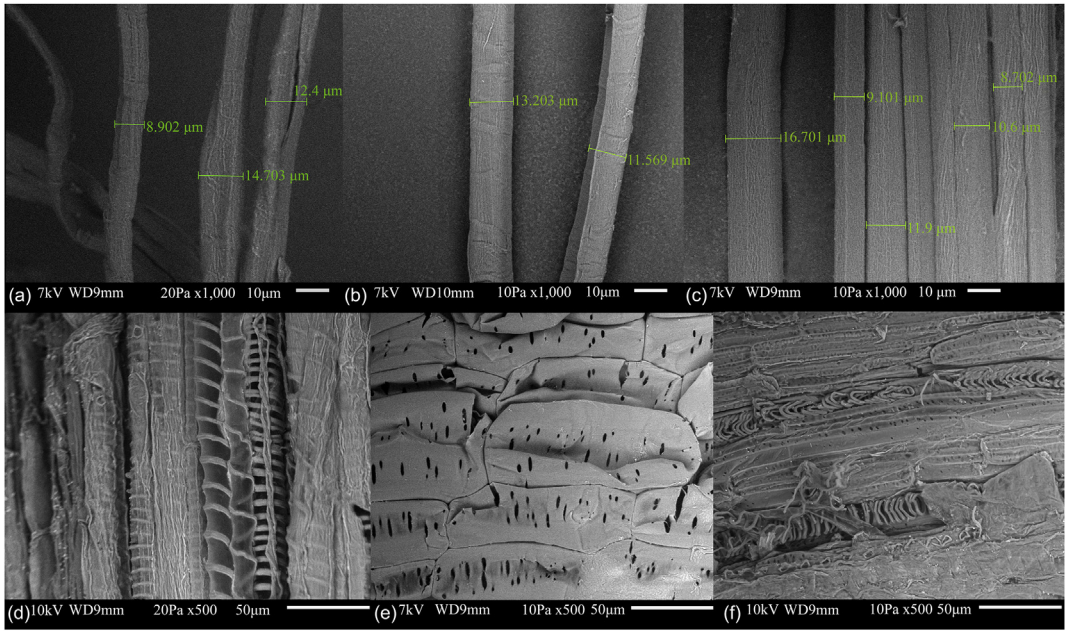

Figure 3. Scanning electron microscope (SEM) micrographs of longitudinal view of hemp fibres. Top panel shows bast fibres of hemp genotypes (a) Białobrzeskie (highest bast yield and lowest bast moisture retention), (b) Ditchweed-Nebraska (lowest tensile strength), and (c) USO31 (highest tensile strength and highest bast moisture retention) and bottom panel shows hurd fibres of hemp genotypes (d) Blue genius (highest hurd yield), (e) Cherry wine (lowest hurd moisture retention), and (f) Portland (highest hurd moisture retention). Magnification and scale bars are provided at the bottom of each image. Diameters of individual bast fibres are marked using green bars.

stems; however, a distinct pattern was not identified (Mendeley Data: Figure S2). Transverse sections of all genotypes were different in overall stem shape and pith shape. The variability in the dimension of each cell layer of stems in positions 1 and 2 is shown in Figure 1. Most of the genotypes (12/16) showed an increase in the dimension of both cortex and xylem layers when they mature. Cortex and xylem characters might not be appropriate to differentiate between hemp genotypes due to their environmental plasticity and variations during different developmental stages. However, the patterns of fibre wedges surrounding the vascular cambium in position 1 were unique in each genotype (Mendeley Data: Figure S3), which we consistently observed in all three replicates of each genotype. Moreover, all transverse sections between the ground level of the stem and the first node showed a similar pattern. However, the section thickness caused by manual sectioning, the used spatial resolution, stains specific to each cellular type are needed to provide deeper insights into the unique patterns of fibre wedges. We provided similar conditions to all accessions used in this study at a greenhouse. However, further studies are warranted to examine whether these patterns vary with environmental conditions during field trials.

3.3. Fibre yield

Genotypes significantly affected both bast ($p < 0.0001$) and hurd ($p < 0.0001$) fibre yields. This finding agrees with a previous study (Sankari, 2000) and is probably due to genotype differences in the formation of stem cellular layers (Mendeley Data: Figures S2 and S3). The Białobrzeskie (fibre type) and Blue genius (CBD type) genotypes resulted in a significantly higher yield of bast and hurd fibres, respectively (Figures 2A and B). Conversely, the Cherry wine genotype (CBD type) showed the lowest bast fibre yield with chemical and enzyme approaches, while the lowest hurd fibre yield was observed with Białobrzeskie (fibre type) in all extraction methods.

Although there was no significant difference between the retting approaches in the overall bast yield ($p = 0.194$), most genotypes (9/16) showed lower weight fibre with the enzymatic method compared with the other two extraction methods (Figure 2A and Mendeley Data: Table S2). The use of enzymes has been suggested as an attractive future area of focus–particularly, for the improvement of fibre thermal and electrical properties (Reddy and Yang, 2005; George et al., 2014a).

Images from SEM indicated that the bast fibres existed as bundles and each fibre was composed of a single cell (Figure 3: top panel). The SEM micrographs showed that microbial treatment resulted in individual bundles to be exposed with a clean surface. This type of surface was obtained in previous studies that used chemical treatments (Bessadok et al., 2008; Islam et al., 2011). Some genotypes showed even thickness throughout the fibres, while others showed uneven thickness and dislocations on their fibre. The diameter of individual bast fibres within one genotype was highly variable. The cell layer that wraps spirally deposited lignin in xylem vessels showed unusual thickness in the genotype Cherry wine (Figure 3: bottom panel), which might link to the lowest moisture retention in this genotype because thick layer might reduce the permeability. The SEM examination was only carried out for selected microbially treated samples due to the cost and time constraints. However, the SEM micrography of all fibres will be helpful to understand the improvement of surface properties by retting methods and the genetic effect on the surface characteristics.

3.3.1. Tensile strength

A comparison of fibre tensile strength results is shown in Figure 2C. Both the retting method and the genotype effect showed a significant difference in tensile strength ($p < 0.0001$ and $p = 0.04$, respectively); however, there was no significant interaction effect between these two factors ($p = 1$). The purpose of this test was not to obtain absolute tensile test values following strict test conditions–such as mounting a certain number of fibre bundles (e.g., ten) in the Texture Analyzer–nor was it to analyze fibre bundle geometry. Naturally, each fibre bundle might differ from another in configuration or number and severity of defects, or both. Therefore, we only examined the tensile strength values to compare each genotype and retting method for fibres stored under the same conditions. Further, we did not examine the effect of the position of fibres in the stem on the tensile strength, because a previous study by Duval et al. (2011) has shown that the stem sampling has less influence on tensile strength.

Table 3. Mean colourimeter values. Colours of the CIELAB system are denoted as L*, a*, and b* values, where L* indicates lightness, a* chromaticity on green (-) to red (+) axis and b* chromaticity on a blue (-) to yellow (+). Standard deviations are provided after mean values.

Genotype	Colour properties	Retting approach		
		Chemical	Enzymatic	Microbial
American victory1	L*	69.80 ± 0.29	69.67 ± 1.09	74.62 ± 1.96
	a*	5.47 ± 0.02	1.77 ± 0.07	2.39 ± 0.07
	b*	21.45 ± 0.22	16.81 ± 0.54	10.55 ± 0.4
Bialobrzeskie	L*	65.6 ± 1.08	69.28 ± 4.92	74.4 ± 0.17
	a*	1.68 ± 0.08	1 ± 0.04	0.97 ± 0.13
	b*	12.9 ± 0.12	13.73 ± 0.94	16.38 ± 0.17
Blue genious	L*	78.38 ± 0.07	73.85 ± 2.77	78.23 ± 1.36
	a*	0.88 ± 0.06	0.3 ± 0.09	0.5 ± 0.05
	b*	11.07 ± 0.34	10.01 ± 0.58	9.7 ± 1.83
Blue kross	L*	82.85 ± 4.58	79.87 ± 1.54	74.27 ± 1.33
	a*	-0.16 ± 0.1	1.1 ± 0.06	0.92 ± 0.14
	b*	8.3 ± 0.89	4.87 ± 0.89	10.24 ± 1.06
Cherry blossom	L*	73.45 ± 5.81	67.58 ± 1.35	71.5 ± 0.9
	a*	1.91 ± 0.29	0.86 ± 0.1	0.49 ± 0.15
	b*	14 ± 1.2	14.22 ± 0.62	13.82 ± 1.56
Cherry wine	L*	67.31 ± 4.85	77.54 ± 1.41	75.64 ± 1.5
	a*	2.28 ± 0.19	1.17 ± 0.05	1.05 ± 0.11
	b*	10.23 ± 2.18	3.29 ± 0.08	2.23 ± 1.18
Ditchweed	L*	55.95 ± 6.83	60.85 ± 3.98	58.84 ± 1.49
	a*	5.1 ± 0.65	0.9 ± 0.12	0.36 ± 0.48
	b*	15.26 ± 1.3	9.91 ± 0.72	6.6 ± 0.71
Ditchweed Colorado	L*	56.34 ± 1.53	70.82 ± 2.41	66.18 ± 5.49
	a*	3.6 ± 0.01	-0.28 ± 4.05	1.9 ± 0.12
	b*	16.93 ± 0.19	13.42 ± 0.42	12.82 ± 2.83
Ditchweed Nebraska	L*	61.55 ± 3.35	62.84 ± 6.75	64.54 ± 2.1
	a*	3.04 ± 0.37	0.25 ± 0.11	1.95 ± 0.21
	b*	12.14 ± 2.48	10.74 ± 4.27	16.92 ± 0.6
Jin Ma	L*	73.64 ± 0.01	75.77 ± 2.02	74.63 ± 0.54
	a*	2.31 ± 0.01	-0.67 ± 0.04	-0.57 ± 0.14
	b*	13.18 ± 0.01	10.88 ± 1.85	11.29 ± 0.23
KLR2006	L*	73.11 ± 1.05	74.29 ± 0.8	73 ± 1.98
	a*	3.28 ± 0.13	2.76 ± 0.1	1.42 ± 0.04
	b*	19.16 ± 0.71	13.79 ± 0.15	7.69 ± 0.39
KLR2014	L*	67.58 ± 0.78	67.09 ± 2.94	62.29 ± 2.42
	a*	5.29 ± 0.13	0.43 ± 0.12	0.61 ± 0.07
	b*	18.27 ± 0.82	7.34 ± 0.37	8.18 ± 0.13
KLR2020	L*	66.73 ± 4.03	70.96 ± 1.49	65.42 ± 0.71
	a*	3.31 ± 0.38	0.76 ± 0.05	1.96 ± 0.07
	b*	14.44 ± 1.46	8.67 ± 0.43	15.45 ± 0.57
Portland	L*	74.1 ± 3.15	66.64 ± 0.79	62.14 ± 4.33
	a*	1.53 ± 0.21	1.47 ± 0.19	0.96 ± 0.05
	b*	13.45 ± 2.49	13.19 ± 0.39	8.21 ± 2.74
Tetra	L*	71.87 ± 1.79	74.8 ± 3.54	73.35 ± 1.58
	a*	3.41 ± 0.17	0.46 ± 0.19	0.61 ± 0.03
	b*	12.08 ± 1.25	6.65 ± 0.51	10.34 ± 1.05
US031	L*	74.88 ± 3.33	73.33 ± 1.97	75.7 ± 0.6
	a*	1.28 ± 0.17	0.97 ± 0.02	-0.9 ± 0.03
	b*	11.54 ± 0.84	11.39 ± 0.54	2.39 ± 0.2

Fibres extracted using the chemical retting method consistently showed high tensile strength in all genotypes (Figure 2C). This is probably because of alkali conditions (NaOH), which can improve adhesion (Mwaikambo and Ansell, 2003; Le Troëdec et al., 2011). Most of the enzymatically treated genotypes (12/16) produced fibres with lower tensile strength compared with the other methods (Figure 2C). Multiple heating steps while transferring enzymes might also weaken fibres (Wong et al., 2001). However, modified enzymatic treatments in previous studies have been shown to enhance the tensile strength of fibres (Liu et al., 2017).

Ditchweed-Nebraska (wild) and US031 (fibre type) genotypes showed the lowest and highest tensile strengths, respectively, with all retting methods (Figure 2C). However, for genotype recommendation, the other factors that affect tensile strength should also be considered, such as fibre-resin interfacial adhesion, growth period of hemp, and fibre length (Pickering et al., 2007; Fan 2010; Duval et al., 2011; Dayo et al., 2018).

3.3.2. Moisture retention

A significant effect of genotype was evident for bast ($p = 0.006$) and hurd fibre ($p < 0.0001$) moisture retention. Bast fibres of USO31 (fibre type) and Białobrzeskie (fibre type) resulted in the highest and lowest moisture retention, respectively (Figure 2D), and hurd fibres of Portland (CBD type) and Cherry wine (CBD type) stains showed the highest and lowest moisture retention, respectively (Figure 2E). The interaction of water molecules with fibre may involve several physical phenomena. For instance, the water penetrating the fibre can be taken up into the capillary space between the fibres (i.e., pores) or absorbed by the fibres via hydrogen bonding (Pejic et al., 2008). Also, amorphous components such as hemicellulose and lignin content play an important role in water storage by fibres (Pejic et al., 2008; Stevulova et al., 2014). Further, water retention is governed by the surface properties such as cavities and the pore structure of the fibres (Pejic et al., 2008). These properties may vary between different genotypes of hemp as they differ in the chemical composition of their stems (Figures S1 and S2). Thus, to further explain the results, additional parameters, such as capillarity, porosity, and water sorption of different genotypes, must be considered.

3.3.3. Colour

We observed a significant effect of the retting method for the values of the three CIELAB colour scale components (i.e., L*, a*, and b*) of bast fibres ($p < 0.0001$). In general, fibres extracted using the chemical method showed darker colours than those extracted using the enzymatic and microbial methods (Table 3). There was also a significant variation in the bast fibre colour of different genotypes ($p < 0.0001$). The highest lightness was significant in Blue kross (CBD type) raw fibres under all retting methods. Furthermore, significant interaction effects between the retting method and the genotype were observed for all three colour components with bast fibres ($p < 0.05$ for each component). The yellowness index was significantly higher in chemically retted fibres compared with the other two methods. Fibres obtained by the enzymatic method exhibited mainly greenish colour shades, which is consistent with a prior study (Bernava et al., 2015). This effect was greater with the microbial retting method, probably due to retaining some pigments

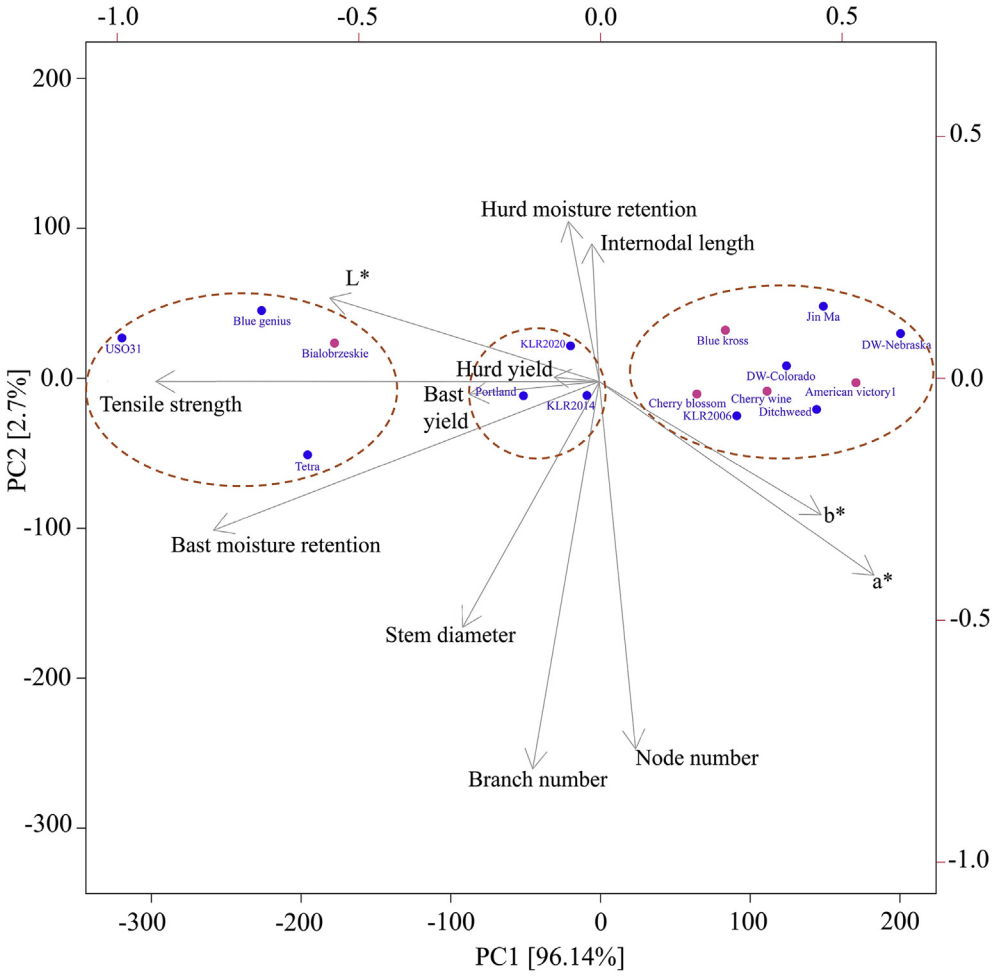

Figure 4. The genotype by trait biplot. This plot illustrates the relationship between the first principal component (PC1) and second principal component (PC2) for 16 genotypes and 10 traits of hemp. The cosine of the angle between the vectors of two traits measures the similarity or the correlation between them relative to their variation among genotypes. Thus, an angle of zero indicates a correlation of +1, an angle of <90° suggests a positive correlation, an angle of 90° indicates no (0) correlation, implying independence, an angle >90° indicates a negative correlation, and an angle of 180° represents a correlation of -1. Axes that represent different characters in multiple dimensions are provided in black fonts. Genotypes are shown in purple fonts. Blue dots represent erect stems and pink dots represent curved stems. Graphically closer genotypes are demarcated with circles.

during the enzymatic and microbial retting compared with the chemical method. Overall, all colour indices were significantly lower in the enzymatic retting method than with the other two methods. If the raw fibre colour is not desirable for downstream applications, bleaching methods can be applied during the processing of the fibres (Wang and Postle, 2004; Gedik and Avinc, 2018).

3.4. Parameter comparison among genotypes with different stem colours

Bast and hurd fibre yield ($p = 0.1$ and $p = 0.5$, respectively), bast and hurd fibre moisture retention ($p = 0.09$ and $p = 0.8$), and tensile strength ($p = 0.9$) did not show significant differences among hemp genotypes with green, yellow, and purple stem colours. The colour variation could be a result of differences in the pigment disposition and phytochemistry within the stems of different genotypes. It was reported that the green stemmed and yellow stemmed genotypes have significant differences in several other fibre characters such as bast fibre yield and processability using machinery (indicated by scutching efficiency, fineness of hackled fibre bundles) (Musio et al., 2018). Further studies on the phytochemical composition of hemp stems are needed to extend our knowledge about the colour differences between hemp fibre genotypes.

3.5. Principal component analysis of traits in different genotypes

A biplot of PCA showing the 2D approximation to the original multi-dimensional space for the trait dataset is presented in Figure 4. The PCA revealed that the five most informative principal components with Eigenvalues of 24738, 695, 209, 48, and 27 were respectively noted which together accounted for 98.84% of the total variance of all the characteristics. Therefore, according to the first principal component (PC1), characters such as internodal length, node number, hurd yield, and tensile strength had relatively higher contribution to the total variability (Figure 4). Of the 98.84% total variation (as indicated by the biplot), 96.14% is explained by PC1. Therefore, mostly the PC1 of the biplot can interpret the differentiation of hemp genotypes based on the characteristics examined in the current study. Three groups of genotypes can be graphically separated along the projected plane to some extent (Figure 4), which infers that these groups are different in terms of traits tested in this study. Each cluster contained a mixture of genotypes used for industrial applications, indicating that non-fibre genotypes contain important fibre/stem characters. To indicate a clear differentiation among the genotypes, more morpho-anatomical and physico-mechanical characters should be included in the PCA. Additionally, DNA sequence and phytochemical data of this hemp collection will provide further insights into the proposed model in future studies. Then it can be used to categorize, differentiate, and select the genetic entities in hemp breeding decisions.

4. Conclusions

This study highlights that industrially useful morpho-anatomical characters of stems and fibre properties are distributed among various genotypes, depicting the complexity of hemp germplasm. Therefore, different genotypes can be recommended based on bast and hurd fibre-specific applications in the industry. Additionally, different genotypes used in this study, which contain important fibre-related characters, can be selected for breeding purposes. Characters such as high bast fibre yields, tensile strength, moisture retention of hurd fibres, and internodal lengths were already present on fibre type hemp. However, some characters such as high moisture retention of bast fibres, natural lightness of bast fibres, and hurd fibre yield were observed on non-fibre type hemp. The properties studied here did not show significant differences among hemp genotypes with different stem colours. Future research avenues should include more genotypes and examine additional fibre characters (such as thermal properties, electrical conductivity, compression, etc.) prior breeding decisions.

Declarations

Author contribution statement

Prabha Amarasinghe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Camille Pierre: Performed the experiments.

Mahta Moussavi: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Addisie Geremew; Selamawit Woldesenbet: Contributed reagents, materials, analysis tools or data.

Aruna Weerasooriya: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at Figshare repository under the accession link: https://figshare.com/s/e92308e6e5de2eb8c267.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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