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Comparative Study of Cannabis Sativa Ecotypes Found in Three Villages at Lusikisiki, Eastern Cape, South Africa

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ABSTRACT

This study comparatively assesses variation in growth, development, and yield of *Cannabis sativa* ecotypes from three villages (V1, V2, and V3) in Lusikisiki, Eastern Cape, South Africa. The study was conducted on physiologically mature plants. Six plants per village were selected, and the morphological features, namely, plant height, number of branches, nodes, flowers, plant fresh and dry weights, and flower fresh and dry weights, were determined. One thousand seeds per village were collected, and their weights and moisture levels were determined using a digital scale and a moisture meter. Seeds, leaves, and flowers from each village were fixed and prepared for the determination of anatomical traits, including sizes of the seed, micropyle, and hilum. Trichome types and density on leaves and flowers were determined using a Scanning Electron Microscope (SEM). It was observed that morpho-anatomical traits enable

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differentiation among ecotypes, influenced by the locally specific environment. Thus, revealing significant variations in vegetative traits (plant height, number of branches, plant fresh and dry weights). V1 recorded the highest vegetative traits, whilst V3 had the highest reproductive and anatomical traits (number of flowers, flower fresh and dry weights, cannabinoid content, trichome density, and seed germinability, aiding features such as the sizes of seed and micropyle). Flowers recorded a higher density of glandular type trichomes than leaves across the villages, although the highest were recorded in V2, followed by V3. In situ conservation of these naturalized cannabis populations through area zoning to ensure genetic preservation for sustainable product supply and economic growth is recommended.

Keywords: Cannabis; Ecotype; Variation; Morphological Traits; Anatomy; Preservation; Ecology

1. Introduction

The continual growth in global population, variation in climatic patterns, and the current economic pressure require a high level of human intervention for sustainable utilization of natural resources [1,2]. Plants are a valuable, precious natural resource with diverse applications in human life, such as the supply of phytochemicals, food, timber, and fibre, hence the need to understand and conserve [3]. *Cannabis sativa*, commonly known as Marijuana, is an erect annual herb in the *Cannabaceae* family [4]. Cannabis was first domesticated for human use more than 12,000 years ago in East Asia [5]. Its domestication resulted from the multiple benefits acquired from different parts of this plant [6]. The versatility of the Cannabis plant for medicinal and recreational uses propelled its spread to several other countries across the globe [7]. The therapeutic effect of this plant is derived from a diverse array of chemical compounds it contains [8]. These are secondary metabolites including terpenoids, flavonoids, sterols, and phyto-cannabinoids [4]. The phyto-cannabinoids produced by this plant have long been used as medicine [9]. Whilst its psychoactive effect has subjected the plant to strict regulatory restrictions worldwide [10,11]. Recently, the medicinal uses of cannabis have been widely recognized, which has led to an increase in the relaxation of Cannabis laws in many parts of the world [12]. The multi-usage of this plant, such as in textile, industrial, and medicinal applications, has intensified the interest and the effort to further understand the plant. This would result in the ability to explore the full potential of the plant as well as to optimize its production with efficient methods to obtain desirable characteristics [13]. *Cannabis sativa* growth, morphology, and secondary metabolite composition are strongly influenced by environmental factors through complex physiological and biochemical mechanisms [14]. Environmental variables, including temperature, light intensity, photoperiod, soil moisture, nutrient availability, and humidity, interact with the plant's genetic framework to regulate key metabolic pathways and the plant's developmental processes [15,16]. Numerous studies that highlighted the interactive effect of these environmental factors on growth and development traits of *Cannabis sativa* are listed in **Table 1** below.

Mechanistically, environmental factors outline *Cannabis sativa* traits by modulating gene expression, enzymatic activity, and hormonal balance, which in turn affect growth, morphology, and secondary metabolite production [29]. Understanding these physiological pathways is crucial for optimizing cultivation practices and achieving consistent cannabinoid yields, especially under variable field conditions such as those in Lusikisiki. Furthermore, to optimize production, the morphological characteristics play a fundamental role in growth, yield potential, and quality of cannabis plants [30]. Cannabis is divided into two morphological growth stages, namely, vegetative and reproductive stages [31]. The vegetative stage comprises stems and leaves, while the reproductive stage is characterized by flowers and seeds [32]. In general, the morphological features of plants can evolve as influenced by the environmental conditions of their growing locality [33]. Furthermore, due to the environmental differences of local habitat, a native single species of cannabis may exhibit differences in both the phytochemical and morphological characteristics [1,34,35].

Table 1. Summary of previous studies on the mechanistic effect of environmental factors on *Cannabis sativa* growth and development traits.

Environmental Factor	Mechanistic Effects on Plant Growth & Development	References
Light Intensity and Photoperiod	Light intensity and quality directly regulate photosynthesis and photomorphogenesis in *Cannabis sativa*. The plant, through phytochrome and cryptochrome receptors, senses the changes in the light spectrum and duration, activating hormonal and genetic responses that influence vegetative growth and flowering. Thus, short light duration (photoperiods) triggers THE FLOWERING LOCUS T (FT) genes, which promote floral initiation. While high light intensity increases photosynthetic rates and carbohydrate accumulation, it provides more energy for biosynthesis of secondary metabolites such as cannabinoids and terpenes.	Ahsan *et al.* [17]; Razzaq *et al.* [18]; Li *et al.* .
Temperature	In the Cannabis plant, temperature influences the enzyme kinetics, membrane fluidity, and hormonal signalling. The optimal plant growth occurs at 25–30 °C, where photosynthesis, respiration, and transpiration processes are balanced. High temperatures can induce stress responses mediated by heat shock proteins (HSPs), altering metabolic flux toward secondary metabolite production. On the other hand, low temperatures reduce metabolic activity and nutrient uptake, often resulting in stunted growth or delayed flowering. Thermal stress may also modify the ratio of Δ^9-tetrahydrocannabinol (THC) to cannabidiol (CBD) by influencing enzyme specificity and gene expression in the cannabinoid biosynthetic pathway.	Langa *et al.* [20]; Rehman *et al.* [21]; Archer
Environmental humidity (EH)	Indirectly modulates phytochemical profiles through its influence on plant water relations and defence mechanisms. It affects transpiration rates and stomatal conductance. Thus, low humidity increases transpiration and can cause water stress, whereas high humidity reduces transpiration and may favour pathogen development. These changes influence nutrient uptake, leaf anatomy, and trichome density. Notably, trichomes are the main site of cannabinoid and terpene biosynthesis.	Ogwu *et al.* [23]; Da Cunha Leme Filho *et al*.
Water availability	Water availability regulates turgor pressure, nutrient transport, and stomatal conductance. While water stress activates abscisic acid, leading to stomatal closure to preserve water. These stress responses usually regulate secondary metabolite pathways, improving cannabinoid and terpene synthesis as part of the plant's defence mechanism. Prolonged water stress reduces biomass accumulation and yields due to impaired photosynthetic capacity.	Scharwies *et al.* [25]; Zhao *et al*.
Nutrients	Macronutrients such as nitrogen (N), phosphorus (P), and potassium (K) are vital for structural and metabolic functions. Nitrogen influences chlorophyll synthesis and vegetative vigour, while phosphorus supports energy metabolism (ATP synthesis) and flowering. Nutrient deficiency or excessiveness disturbs carbon-nitrogen balance and alters hormonal signalling (cytokinin and auxin), affecting morphology and cannabinoid concentration. As such, limited nitrogen supply may reduce growth but increase cannabinoid concentration, due to carbon redirection from primary to secondary metabolism.	Cao *et al.* [27]; Song *et al.*

In its morphological structure, *Cannabis sativa* possesses two types of hair-like outgrowth called glandular trichomes and non-glandular trichomes that are a powerhouse of secondary metabolites [36]. These glands or cells are found on the surface of plant organs such as leaves, stems, and flowers [37]. Glandular trichomes are specialized plant hairs that have a secretory function, producing and storing secondary metabolites such as essential oils, resins, terpenes, and cannabinoids [38]. Whereas non-glandular trichomes are simple hair-like structures that do not produce or secrete any chemical compounds, but their main functions are physical protection and environmental adaptation [39]. The presence of these glands makes Cannabis an economically significant plant as it contains complex herbal medicine properties [2]. As described by Lawrence [40], the plant is usually referred to as medicinal cannabis. Several varieties and hybrids of *Cannabis sativa* Lam have been established, which are suitable for both industrial and medicinal processing [41]. These varieties have different chemical composition, growth habit and agronomic requirements [42]. Cannabis encompasses hundreds of identified organic chemical constituents, collectively known as cannabinoids [43,44]. These are mostly concentrated in the essential oils of unfertilized female flowers [45]. However, two major and well-characterized cannabinoids have economic significance, namely, D-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which have historical use as recreational and medicinal agents [43]. The THC is a psychoactive compound that is abundantly found in the inflorescences of the cannabis plant [46]. Whilst CBD is found in all plant tissues and is non-psychoactive but is known to have important medicinal and pharmaceutical properties [47].

These two alkaloids are structurally different, as the CBD is a bicyclic compound containing a hydroxyl group, while the THC is tricyclic and is characterized by a cyclic ring [48].

The concentration and content of these two cannabinoids determine whether the plant belongs to the category of industrial and or medicinal type [46]. The medicinal cannabis contains concentrations of THC exceeding 0.3% [42]. Although the medicinal value of this species is attributed to the cannabinoids, which are concentrated in the essential oils of female flowers [45], the medicinal properties are highly influenced by the chemo-type, which is the ratio between CBD and THC [49]. The quality of these cannabinoids (i.e., THC and CBD) is strongly influenced by environmental factors, such as temperatures, elevation, and humidity [5]. Hence, the variation in morphology and phytochemical composition should be traced in the species background and the agro-ecological conditions where they have grown [43]. Thus, the best ecotype must be understood to ensure efficiency, improved yields, and quality produce for sustainable product development.

2. Materials and Methods

2.1. Study Location

The study was conducted in Lusikisiki, Ingquza Hill Local Municipality, OR Tambo District Municipality in the Eastern Cape Province, South Africa (**Figure 1**). The plant material for this study was obtained from three villages located around the wilderness of Lusikisiki, where the cannabis grows in abundance. Due to current legal implications regarding cannabis in South Africa, the names of the villages where samples were collected are discreetly referred to as Village 1 (V1), Village 2 (V2), and Village 3 (V3) to protect their identities. These villages are situated in the Indigenous valley forests alongside the riverbanks and are characterized by different geographic altitudes as well as different slope orientations. Thus, Villages 1 and 3 are the North-facing slopes, while Village 2 is a South-facing slope. They are situated at the geographic altitudes of 200, 300, and 800 m above sea level (ASL), respectively.

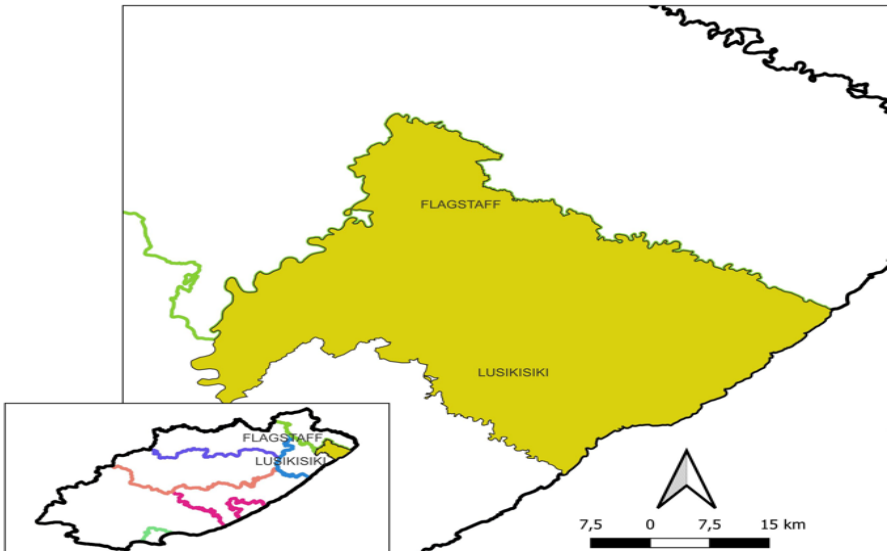


Figure 1. A schematic map of Eastern Cape Province showing OR Tambo district and Lusikisiki.
Source: Early warning—Döhne ADI.

In the Eastern Cape, O.R. Tambo district municipality is situated in the Eastern part of the province. The district lies along the Indian Ocean coastline, stretching up to 250 km and at an altitude of 1000 m above sea level, rising to the southern Drakensberg mountains [50]. The north-eastern parts of OR Tambo (15,946.84 km^2) is boarded by the Alfred Nzo district while others are boarded by the parts of Amathole and Chris Hani districts of the Eastern Cape [51]. As described by Mucina and Rutherford [52] Eastern region vegetation consists of 4 bioregions namely, Zonal and Intra-zonal forest, Indian coastal belt, Sub-escarpment Savanna and sub-escarpment grassland bioregion. The OR Tambo district receives summer season rainfall with mean annual rainfall ranging between 800 and 1000 mm [51].

Hence, the area is well suited for rain-fed arable agriculture where slopes and soils permit for tillage practices. The area is characterized by a range of farming activities from crop production to livestock farming [53]. OR Tambo has the richest natural resources and the most fertile soils and favourable climatic conditions for diversified plant production [54]. Prior collection, soils in each of the three villages where cannabis is found growing abundantly in the wild were classified according to the South African Soil Classification System (Soil Classification Working Group, 2018). The soil samples were collected with the aid of hand-held auger for determination of chemical properties at Döhne Analytical Laboratory. Rainfall, mean maximum and minimum monthly temperatures for the past 3 years was sourced from ARC-Institute for soil, water and climate (**Table 2**).

Table 2. Monthly mean maximum and minimum temperature, maximum and minimum relative humidity (RH) and rainfall for the past 3 years.

		Period/ Month											
Season/ Year	**Climatic Factors**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2021	**Mean Max T (°C)**			25.4	23.8	23.9	21.7	20.8	21	21.7	22.3	22.6	24.2
	Mean Min T (°C)			15.4	12.1	8.2	7.5	4.3	6.8	10.4	11.1	13.1	15.1
	Mean Max RH (%)			99.7	99.9	98.2	98.2	96.1	98.6	98.7	99.2	99.6	99.4
	Mean Min RH (%)			63.4	60.1	40.3	45.3	30.8	43.7	56.4	55.4	64.4	66.3
	Total Rain (mm)			55.8	148	22.6	231	0	55.2	173.8	97	183	213.4
2022	**Mean Max T (°C)**	26.4	25.9	26.1	21.6	22.7	21.6	21.4	20.9	22.2	23.8	22.9	24.7
	Mean Min T (°C)	17.5	17	15.5	11.9	9.3	5.8	7.1	7.5	11.2	13.7	13.6	15.4
	Mean Max RH (%)	100	99.9	99.8	99.7	99.4	97.1	98.2	99.2	99.3	99.6	99.4	99.7
	Mean Min RH (%)	69.2	71.7	65	68.1	52.2	40	42.2	45.7	59.8	62.7	69.3	67.9
	Total Rain (mm)	65	150.8	147	434.2	50.8	7.8	28.2	28.6	126.4	71.2	81.8	131
2023	**Mean Max T (°C)**	26.9	26.3	25.6	24.9	21.9	20.4	20.5	22.6	22.3	21.6	24.8	22.8
	Mean Min T (°C)	16.5	16.8	16.1	11.8	9.4	6.5	5.3	7.4	8.9	11.3	13.7	15
	Mean Max RH (%)	99.8	99.8	99.8	99.1	99.4	99.2	97.7	98.1	98.7	99.3	99.7	99.9
	Mean Min RH (%)	64.8	69.4	66.4	53.2	57.6	45.9	39.4	40.9	50.8	65.8	62.9	76.2
	Total Rain (mm)	224.6	177.2	97	33.8	255.2	106	28.4	4.2	75.8	355.8	86.6	184.8

2.2. Plant Material Collection for Morphological Analysis of *Cannabis sativa* Ecotypes

The study was conducted during the late winter season of 2023, when the *Cannabis sativa* plants were at physiological maturity, with their floral resin at 50% amber coloration. Six (6) plants per village, totaling eighteen (18) plants for the entire study, were selected, tagged, and the following parameters were recorded:

i. Morphological:

a. Plant height was measured from the ground level to the apex of the plant using a flexible 5 m measuring tape.

b. The number of nodes, branches and flowers per plant was obtained by physical counting.

ii. Biological yield:

After the assessment of morphological parameters, the tagged plants were cut at the 1st node above-ground level, kept in airtight plastic bags and taken to Döhne, where their biological yield parameters were assessed.

a. Plant fresh weight was obtained using a digital weighing scale.

b. Flowers were removed from stems with the aid of secateurs, and their fresh weight was also determined.

c. Samples were dried at room temperature for fourteen (14) days following Tipple *et al*. [55], and the plant's dry weight was determined. The flowers were then cured in dark-cool room conditions as per the United Nations Office on Drugs and Crime (2009) standards, and the flower dry weight was determined.

iii. Oil yield:

a. The oil yield and chemical composition were determined through GC-MS at Döhne Analytical Laboratory.

Oil yield was determined by use of an analytical balance, which determined their mass with an accuracy of 0.001 g using the following formula by Demirel *et al*. [56]:

$$Oil\ yield(\%) = \frac{Mass\ of\ oil}{Mass\ of\ a\ sample} \times 100$$

b. Determination of oil chemical comp osition following the protocol by Olascuaga-Castillo *et al.* [57]; Alzarieni *et al.* [58]; Aljamali *et al*. [59].

Oil was extracted from 310 grammes (g) of dried flower using Clevenger equipment and the hydro-distillation process. The vapour oil combination was passed through the condenser, where it condensed, collected into a flask, and the oil was separated from the water through a process known as decantation. The distillation time was three hours, and the temperature of the heating mantle was 100 degrees Celsius (°C). 10 µL of the sample was pipetted and diluted with 1mL of ethanol. The sample was injected using the injector port. The essential oil was analyzed using gas chromatography-mass spectrometry (GC-MS) on a fused silica polar capillary column (30 m 0.25 mm 0.25 m film thickness) on a Thermo-Fischer ISQ TM7610 single quadrupole. The oven temperature was programmed from 50 to 250 °C and maintained for 10 minutes at a rate of 2 °C/min. The injector temperature of 250 °C and the transfer line temperature of 280 °C were utilized. The mass spectrometer's ion source and analyzer were kept at 280 and 100 °C, respectively. The mass spectrometer was run in full scan mode with electron impact ionisation (EI) positive mode, and data were collected from 40 to 600 m/z. The carrier gas was hydrogen, with a flow rate of 1.2 mL/min. The chemical components were identified by comparing their respective retention periods and mass spectra with data from the NIST library.

iv. Seed collection:

One thousand (1000) seeds of cannabis per village were collected in the wild in August 2023. After collection the seeds were kept in airtight containers for moisture retention until use, and the container was labelled following Turner *et al.* [60] collection protocols, such as date of collection, name of district and locality details and the altitude. The average weight of a thousand (1000) cannabis seeds was determined using an Adam ACBplus-600g scale. Seed moisture content was recorded using a moisture meter.

2.3. Anatomical Analysis of *Cannabis sativa* Ecotypes under Scanning Electron Microscope (SEM)

The expanded young leaves were collected from the eighth node of a plant from each village as per Asikin *et al.* [61]. At collection, both the morphology and the density of trichomes were observed using the scanning electro-microscope (SEM) in the Department of Botany, University of Fort Hare, as per Lemus-Barrios [62]. The morphology of trichomes was classified following Talip *et al*. [63]. Trichome density was calculated on five replicate plants per village, and each replicate represented an average number of trichomes per leaflet. The total number of trichomes on the adaxial and abaxial leaf surface, as well as in the flower, was recorded. The density of trichomes was calculated as per Asikin *et al*. [61], by dividing the total number of trichomes by leaf area and was expressed in cm². Thus,

$$\text{Trichome density} = \frac{\text{Total number of trichomes}}{\text{Leaf area}}$$

Seed macro and micromorphological features, such as the total seed size (length and width), micropyle and hilum size, were investigated using SEM following the consideration by Abdelhameed *et al*. [64].

2.4. Preparation of Samples for Anatomical Observation in the SEM

The leaves and flower samples collected from three villages were placed into a closed container, and the 6% Glutaraldehyde solution was applied to preserve the specimens from the field until the laboratory, where the specimens were prepared for SEM analysis in the department of Botany at the University of Fort Hare. During specimen preparation, the samples were dissected into small pieces of 0.5–1cm with the aid of a sharp razor blade. The dissects were cleaned using a sodium cacodylate (NaCo) solution for 24 hours without collapsing the cells and tissues. The specimens were then subjected to a series of alcohol from 10–100% ethanol at 2-hourly intervals for 24 hours for cell cleaning. The specimens were hardened at a critical point by drying using helium gas for approximate-

ly 45 minutes. Hardened specimens were gold-coated to maintain the electrons, and the specimens were subjected to SEM (JEOLJSM-IT100InTouchScopeTM, Tokyo, Japan) for viewing and analysis.

2.5. Data Analysis

Before running the analysis, the assumptions of ANOVA were verified. The normality of residuals for each variable was tested using the Shapiro-Wilk W-test and confirmed by visual inspection of the Normal Probability Plots (Q-Q plots). The homogeneity of variances across the three villages (V1, V2, and V3) was confirmed using Levene's test. The Analysis of Variance (ANOVA) was performed for each parameter and treatment. The Least Significant Difference (LSD post-hoc test was used to separate the means at a significance level of $p = 0.05$. Data was subjected to statistical analysis using Statistica Ver. 13.2 (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Classification and Chemical Composition of Soils in Three Villages in Lusikisiki

This study has shown that the is variation amongst soils of the three villages where V1 is predominantly a Glenrosa (Gs) soil (greyish brown, apedal, loamy sand to sandy loam topsoil on weathering rock), 350 mm depth, with a low agricultural potential. However, V2 is mostly Oakleaf (Oa) soils (dark greyish brown, apedal, fine sandy loam topsoil grading into deep brown, weakly to moderately structured, clay loam subsoil with weakly developed darker clay cutans in the upper parts to depth), 1000+ mm depth with a high agricultural potential. Whilst V3 was found to have the Tubatse (Tb) soils (dark greyish brown, apedal, fine sandy loam topsoil grading into brown, weakly to moderately structured, clay loam subsoil with weakly developed darker clay cutans in the upper parts. Underlying material is hard to weathering rock, 600 mm and is moderately suitable for agriculture.

The results of the chemical composition of the soil collected from V1, 2 and 3, respectively, are presented in **Table 3**. The highest pH level was recorded on soils collected from V1 (7.3), followed by V2 (6.4) and the lowest was recorded in soils collected in V3 (6.3). The results of the soil mineral analysis for major nutrients such as nitrogen (N), phosphorus (P), potassium (K), organic carbon (OC) and calcium (Ca) as well as the exchangeable zinc (Zn), were found to be higher in the soils of V1 followed by V3 and the soils of V2 had low nutrient concentration.

Table 3. Chemical composition of soils collected in three villages in Lusikisiki.

Treatment	Properties						
						Exchangeable cations (cmol+kg)	
	pH	OC	N	P	K	Ca	Zn
	(kcl)	____ (%) ____			_____ Mg/L _____		
V1	7.3	3.21	1.00	48	552	21,890	13.9
V2	6.4	3	0.11	8	227	8584	2.9
V3	6.3	2.21	0.92	18	426	9683	4.6

Note: V1: village one, V2: village two, V3: village three, pH : potential of hydrogen, OC: organic carbon, N: nitrogen, P: phosphorus, K: potassium, Ca: calcium, Zn: Zinc.

3.2. Local Environmental Effect on Vegetative Growth and Development of *Cannabis sativa* Ecotypes

The results showed that geographic location had a significant influence on vegetative growth parameters, namely, plant height, number of nodes, and number of flowers per *Cannabis sativa* plant found in three different villages in Lusikisiki (**Table 4**). The tallest plants were attained in V1 with an average height of 128.3 cm, followed by V3 (75.2 cm), and the shortest plants were found in V2 (63.9 cm) and were significantly different from each other. Plants growing in V3 had the highest average number of nodes (14.2), followed by V1 (12.3), and the lowest number of nodes was found in Plants growing in V2. Although not significant, plants of V1had the highest average number (6) of branches compared to others. Plants of V3 (51.5) yielded the highest number of flowers, followed by V1(47.6), and the lowest was recorded in V2 (15.6), which

were significantly different from each other.

3.3. Differences in Biomass of *Cannabis sativa* Ecotypes Found in Three Villages in Lusikisiki

Results (**Table 5**) showed significant differences in all the measured biomass parameters between the three villages in the Lusikisiki region, except for the plant dry weight, which was not significantly different. The highest average plant fresh weight was obtained from V1 (154 g), followed by V3 (138.5 g), and then V2 (46.9 g) had the lowest plant fresh weight. However, the highest average flower fresh weight was found in V3 (46.3 g) followed by V1 (35.1 g), and the lowest was found in V2 (9.2 g). Similarly, plants of V1(42 g) recorded the highest average plant dry weight, followed by V3 (40.3 g), and V2 (12.2 g) recorded the lowest plant dry weight. The highest flower dry weight was attained in plants of V3 (12.4 g), followed by V1 (11.6 g), and were not significantly different from each other. While V2 (2.7) yielded the lowest flower dry-weight and was significantly different from the others, V3 and V1.

3.4. Differences in Moisture, Oil Content, and Weight of *Cannabis sativa* Seeds from Three Localities in Lusikisiki

Results showed that seeds collected from V2 (26.15 g) had the highest seed weight, followed by V3 (25.5 g), and the lowest seed weight per 1000 seeds was recorded in V1 (24.11g) (**Figure 2**). A similar trend was observed concerning seed moisture with V2 (14.4%) followed by V3 (11.6%), and the lowest was found in V1(10.3%). Seeds collected from V2 (5.87 g) had the highest oil content, followed by V3 (4.95 g), and V1 (4.77 g) had the lowest oil content compared to the others.

3.5. Differences in Phytochemical Composition of *Cannabis sativa* Ecotypes Collected in Lusikisiki

The results of the major cannabinoid concentrations in *Cannabis sativa* ecotypes collected from three villages in Lusikisiki are shown in **Table 6**. Six types of cannabinoids, namely: cannabidiol (CBD), Cannabicyclol (CBL), Cannabichromene (CBC), delta-8-tetrahydrocannabinol (Δ8-THC), delta-9-tetrahydrocannabinol (Δ9-THC), and the rare isomer of Δ9-THC called delta-11-tetrahydrocannabinolic (Δ11-THC), were detected in all samples, with their concentrations varying by location. The analysis indicated that the highest cannabinoid concentration levels were found in V3 samples, followed by V1, while V2 samples contained lower levels compared to the others.

Table 4. Effect of locality on vegetative plant growth and development of *Cannabis sativa* ecotypes.

Locality	Plant Height (cm)	No of Nodes	No of Branches	No of Flowers
V1	128.3^{a}	12.3^{b}	6^{a}	47.6^{b}
V2	63.9^{c}	9.5^{c}	5^{a}	15.5^{c}
V3	75.2^{b}	14.2^{a}	5.3^{a}	51.5^{a}
Mean	89.9	12	5.4	38.2
Cv	17	22.9	35.9	44.4
p-Value	0.001	0.03	0.67	0.004

Note: V1: village one, V2: village two, V3: village three, Values in a column followed by a different letter (a,b,c) are significantly different at $p \leq 0.05$. *p*-Value: probability value, Cv (%): coefficient of variance.

Table 5. Differences in plant biomass of *Cannabis sativa* ecotypes collected from three localities/villages in Lusikisiki.

Locality	Plant Fresh Weight (g)	Flower Fresh Weight (g)	Plant Dry Weight (g)	Flower Dry Weight (g)
V1	154^{a}	35.1^{b}	42^{a}	1.6^{a}
V2	46.9^{c}	9.2^{c}	12.2^{b}	2.7^{b}
V3	138.5^{b}	46.3^{a}	40.3^{a}	12.4^{a}
Mean	107	28.9	30.3	8.5
Cv	57.3	58.2	75.3	53.2
p-Value	0.02	0.00	0.08	0.00

Note: V1: village one, V2: village two, V3: village three, Values in a column followed by a different letter (a,b,c) are significantly different at $p \leq 0.05$. *p-Value*: probability value, Cv (%): coefficient of variance.

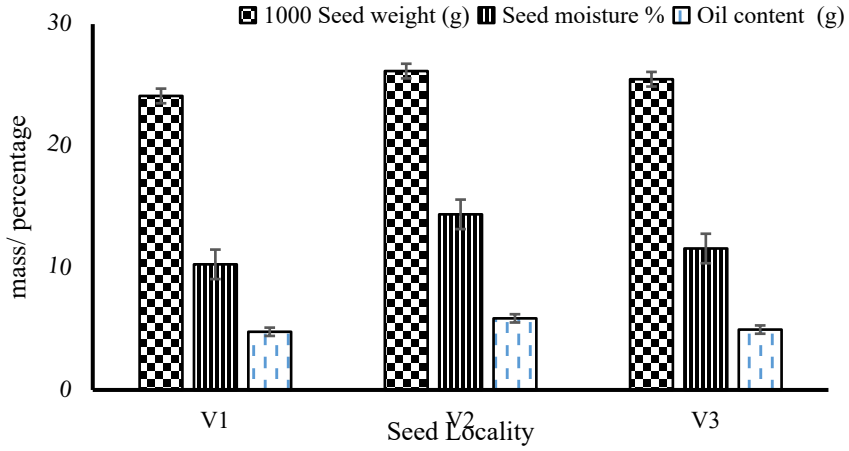
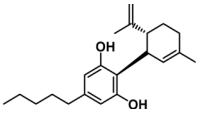
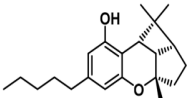
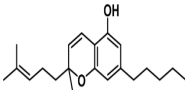
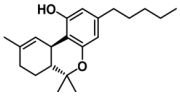
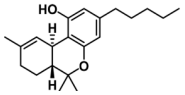
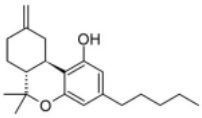


Figure 2. Locality effect on oil yield attributes and oil content of *Cannabis sativa* ecotypes.

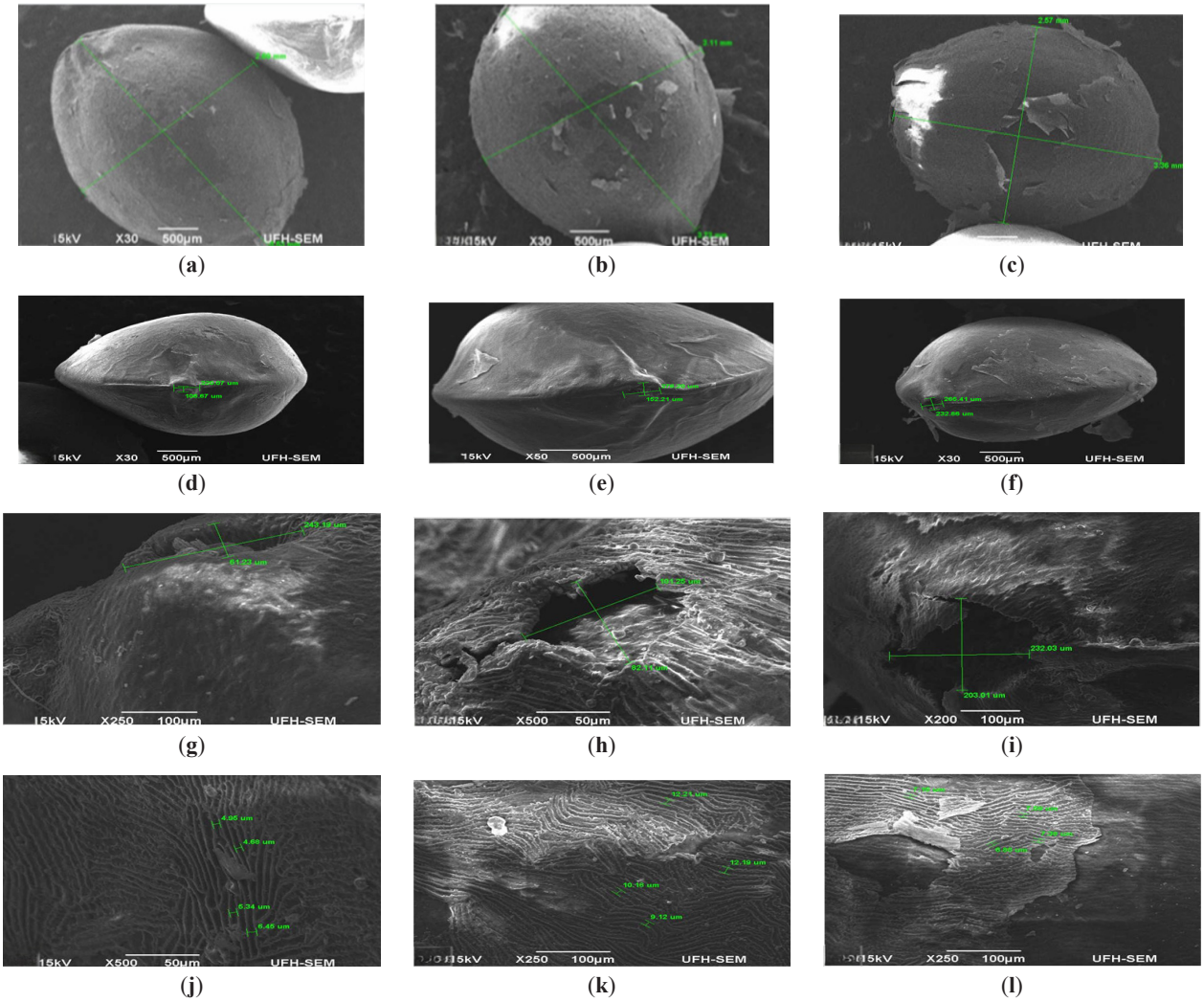
Table 6. Effect of locality on the concentration % of major cannabinoids of *Cannabis sativa* ecotypes collected in the three villages of Lusikisiki.

Compound	Concentration %			
	Molecular structure	Village 1	Village 2	Village 3
Cannabidiol (CBD)		13.9	12.2	14.8
Cannabicyclol (CBL)		0.44	0.40	0.75
Cannabichromene (CBC)		0.05	0.04	4.31
Delta 8-tetrahydrocannabinol (d8-THC)		51	51.1	50.6
Delta 9-tetrahydrocannabinol (d9-THC)		16.5	14.5	19.6
Delta 11-Tetrahydrocannabinol (exo THC)		9.25	8.01	9.36

3.6. Comparative Analysis of the Seed Traits of *Cannabis sativa* Ecotypes Found in Three Villages in Lusikisiki

This study found that all the seeds of *Cannabis sativa* ecotypes collected in three (3) villages in Lusikisiki had a globose shape, smooth, glossy, grey-green, and showed fine puzzle-like patterns in khaki-coloured lines on the surface when examined under a light microscope. The results of the seed dimensions as presented through the SEM micrographs (**Figure 3**). The results of the SEM (**Figures 3a–c**) showed that the seeds collected in three villages were small seeds with an average seed length and diameter of 3.53 × 2 mm, 3.73 × 3.11 mm, and 3.36 × 2.57 mm, respectively. Thus, seeds from V2 were bigger, followed by V3 and V1. Seeds of V3 (62 um^2) had a wide open micropyle, followed by V2 (42 um^2), and a small opening was observed in seeds of V1 (34.4 um^2). A wide-open hilum

(seed base) was observed in seeds of V3 (47.3 um^2), followed by V1 (14.9 um^2), and the least was in seeds of V2 (8.3 um^2). With regards to seeds testa sculpturing, seeds of V2 were more sculptured (coarse textured), followed by V1 and V3 were less sculptured (smoother) than the others (**Figures 3d–l**).



(**a**) (**b**) (**c**)
(**d**) (**e**) (**f**)
(**g**) (**h**) (**i**)
(**j**) (**k**) (**l**)

Figure 3. (**a**) determination of seed size (mm) in V1; (**b**) determination of seed size (mm) in V2; (**c**) determination of seed size (mm) in V3; (**d**) determination of micropyle size (mm) in V1; (**e**) determination of micropyle size (mm) in V2; (**f**) determination of micropyle size (mm) in V3; (**g**) determination of hilum size (µm) in V1; (**h**) determination of hilum size (µm) in V2; (**i**) determination of hilum size (µm) in V3; (**j**) determination of seed Testa sculpturing (µm) in V1; (**k**) determination of seed Testa sculpturing (µm) in V2; (**l**) determination of seed Testa sculpturing (µm) in V3.

3.7. Comparative Analysis of Trichomes of *Cannabis sativa* Ecotypes

The results of trichome analysis are presented through the SEM micrographs (**Figure 4**). The results of the SEM showed that the plants in V1 had the highest density of swollen trichomes on the upper (abaxial) leaf surface, followed by V3 and the plants in V2 had lowest number of non-glandular trichomes (**Figures 4a–c**). However, V3 plants had the highest density of non-glandular trichomes on the lower (adaxial) leaf surface, followed by V1 and the lowest number was recorded in V2. Cannabis flowers had glandular types of trichomes irrespective of the villages. The flower collected from V2 had the highest density of glandular trichomes in the floral part (flower), followed by V3 and the lowest density was observed in V1 flower (**Figures 4d–i**).

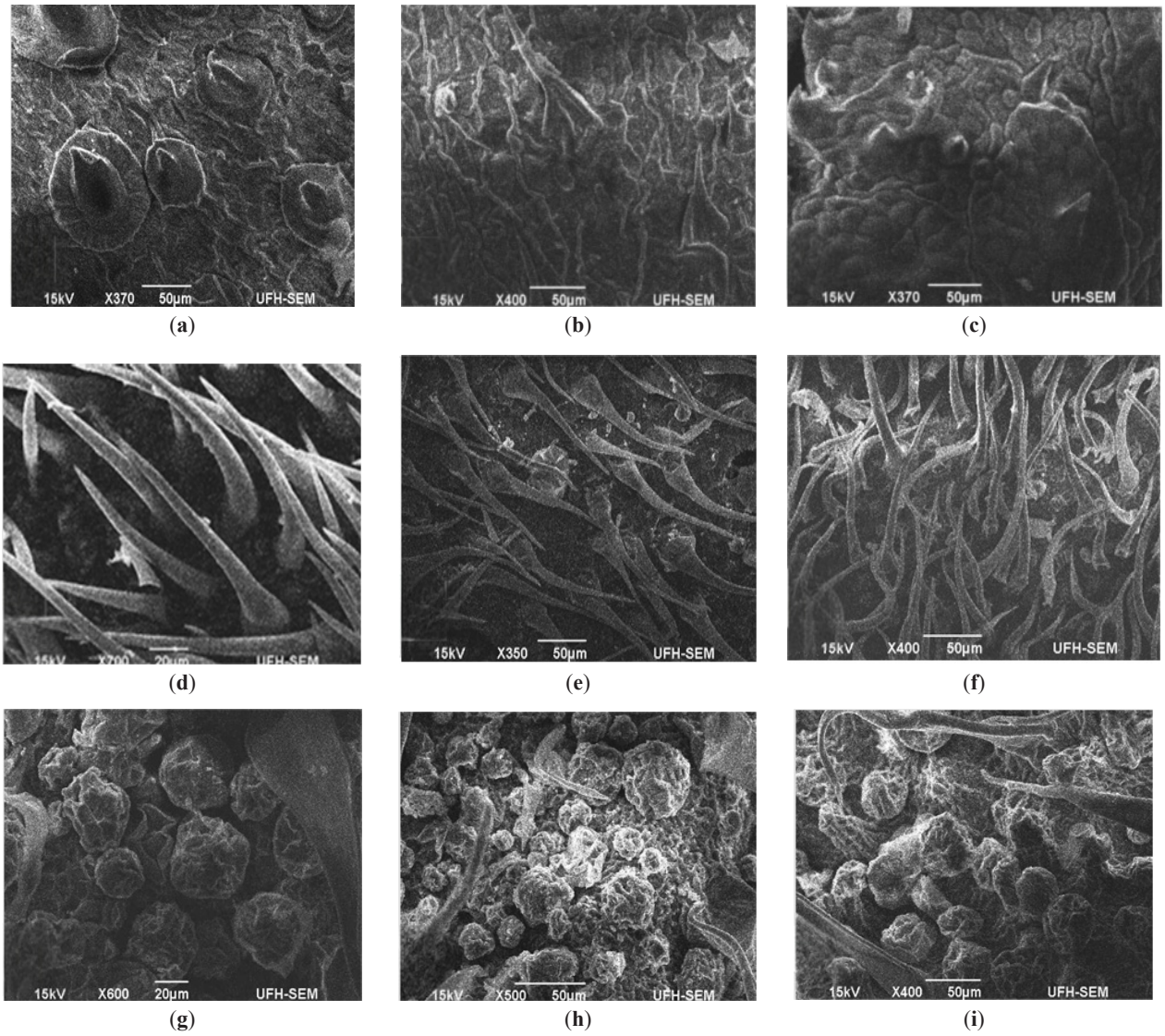


Figure 4. (**a**) abaxial leaf side trichomes (upper leaf side) in V1; (**b**) abaxial leaf side trichomes (upper leaf side) in V2; (**c**) abaxial leaf side trichomes (upper leaf side) in V3; (**d**) adaxial leaf side trichomes (lower leaf side) in V1; (**e**) adaxial leaf side trichomes (lower leaf side) in V2; (**f**) adaxial leaf side trichomes (lower leaf side) in V3; (**g**) flower trichomes in V1; (**h**) flower trichomes in V2; (**i**) flower trichomes in V3;

4. Discussion

This study revealed that although soils of Village 1 (V1) are characterized as Glenrosa, they are rich in soil nutrient content, followed by the Tubatse soils in Village 3 (V3), and the Oakleaf soils of V2 had the lowest nutrient content. This may be attributed to the low altitude geographic situation of V1 (200 m asl) with a slope aspect facing north-east, supported by a dense diversity of indigenous forest flora in which the plant debris and nutrients are deposited during the rainy seasons. Whereas village 2 (V2) is situated at the south shady facing slope aspects at the medium (300 m asl) elevation. Ecological conditions such as temperature and precipitation along altitudinal gradients can influence the soil nutrient status in the mountainous ecosystems [65,66]. Soil nutrient status in forest ecosystems can provide valuable insights into the nutrient cycling under different geographic conditions [67]. North-facing slopes are supported with thick and dense vegetation with nutrient-rich soil compared to soils of the south-facing slopes [68]. Furthermore, Filimonenko *et al*. [69] reported that higher radiation results in higher soil temperature, leading to rapid decomposition of soil organic matter.

In the current study, it has been observed that plants

growing at V1 were vegetatively taller with more branches compared to Cannabis plants found in V2 and V3. While V1 had plants with higher vegetative traits, plants from V3 yielded the highest reproductive traits (i.e., number of flowers). It is assumed that the available high soil nutrient content, slope exposure (sunlight) and temperature favoured the robust vegetative growth of cannabis plants in V1 and 3. Slope aspect is a vital ecological factor that affects the daily cycle of solar radiation and has a strong influence on aspects of the microclimate, particularly the air and soil temperature, moisture and nutrition [70]. Plant height and number of branches are indicators of plant interactions with their environment [71]. Irrespective of elevation, the plants growing in the north-facing slope aspect had increased crop productivity by 15%–25% compared to the south slope aspect and low elevation [72]. According to Köner [73], plants growing in areas of limited exposure to sunlight and nutrients have limited meristem activity, resulting in shorter growth. Plant height plays a significant role in circumventing the risk of shady slope with less light radiation, low temperature and strong wind [74,70]. According to He *et al*. [75]; Li *et al*. [76], plants tend to increase branch number when solar radiation is strong to enhance photosynthetic efficiency.

Flowers are the key factor affecting the evolution of plants and the guarantee of sexual reproduction [77]. Flower yield in cannabis plants increased with increasing exposure to light and adequate nutrients [78]. The greater the number, the better the possibility to produce offspring and an assured, sustainable population [70]. In this study, the highest plant biomass (fresh and dry plant weight) was obtained from plants of V1, followed by V3. It is assumed that many ecological factors, such as slope exposure to sunlight, temperature, and available soil nutrients in V1 and V3, may be the contributing factors. Plants adjusted their functional traits and biomass allocation to adapt to a harsh habitat when subjected to environmental stress or resource constraints to improve competitiveness and survival fitness [79]. Xue *et al*. [70] reported a significantly greater plant biomass on the north sunny slope than in the south shady slope due to increased photosynthetic period for plants. Plants growing at V3 exhibited the highest fresh and dry flower weight. This might be because plants growing in V3 have a longer exposure to sunlight (photoperiod) with adequate soil nutrients for cannabis plant growth and development. Photoperiod influences photosynthesis and photomorphogenesis through plant photoreceptors that sense light and control plant growth [80]. Moreover, light properties play a vital role in plant vegetative growth and reproductive (flowering) developmental stages, as well as in biomass, secondary metabolite synthesis, and accumulation [81]. Dang *et al*. [82]; Cheng *et al*. [83] reported the maximised floral biomass when the day length was approximately 14 hours long. Based on the results, the highest seed yield, 1000-seed weight, moisture, and oil yield were found on seeds collected from V2, followed by V3 collection. The seed weight, moisture content, and seed oil yield, together with the number of fatty acids in the oil, are dependent on the plant's origin and the climatic conditions of the cultivation region [84]. Industrial cannabis (Hemp), when grown in areas of shorter day length and south-facing shady slopes, produced both the highest seed weight and oil yield [85,86].

The current study revealed that *Cannabis sativa* ecotypes collected in three different villages in Lusikisiki had similar types of common cannabinoids, namely: cannabidiol, Cannabicyclol, Cannabichromene, delta-8-tetrahydrocannabinol, delta-9-tetrahydrocannabinol, and the rare positional isomer of d9-THC called delta-11-tetrahydrocannabinolic. However, their concentrations vary by location, with V3 recording the highest concentrations, followed by V1. This might be attributed to the interactions between the slope aspect and the elevation. Thus, V3 is situated at higher elevations with a north aspect, allowing sufficient plant exposure to sunlight, whereas V2 is situated in a south aspect at a lower elevation compared to V3. Solar-exposed slopes (e.g., north-facing in South Africa) may promote higher THC levels due to increased light intensity and UV exposure [87]. The highest altitude (2652 m) exhibited a unique profile characterized by higher CBD: THC ratios, suggesting an adaptive response to environmental conditions [88]. Environmental factors play a pivotal role in the cultivation of Cannabis, influencing not only the growth and health of the plants but also the potency and profile of the produced cannabinoids [89]. Elevation and slope orientation influence cannabinoid concentration in *Cannabis sativa* through changes in environmental stressors and light conditions [15]. Several studies have reported that the Cannabinoid variation in *Cannabis sativa* occurs

from the intricate interaction between genetic makeup and environmental conditions [90,15,91]. For instance, higher light intensity and UV-B exposure are associated with increased THC accumulation due to the plant's protective response against radiation stress [18]. Similarly, temperature and nutrient regimes can influence enzymatic activity within the cannabinoid biosynthetic pathway, altering both total cannabinoid concentration and ratios among specific compounds [21,28]. Stress conditions such as drought, nutrient deficiency, or pathogen attack can trigger secondary metabolite production, including cannabinoids and terpenes, as part of the plant's adaptive defense mechanism [92]. Moreover, environmental micro-conditions such as humidity, soil microbiota, and altitude or slope orientation contribute to the observed variability even among genetically identical plants [93]. This suggests that phenotypic plasticity plays a pivotal role in the formation of cannabinoid profiles under different cultivation environments.

The macro and microstructure of seeds are important, particularly for the characterization and classification of the Angiosperm taxa [94]. Seed morphology enables plants to inhabit and adapt to new ecological positions, resulting in plants growing in different environments and climatic conditions [95]. Within species, diversity occurs in seed shape, size, colour, texture, micropyle, and hilum size that assist in the observation of systematic relationships amongst the variety of families and, eventually, differentiate taxa [96]. In plant biology, seed shape and size are key morphological traits that play a critical role in the distribution, development, and survival of seedlings into an individual plant [97]. However, it is greatly influenced by a variety of ecological and biological factors [98]. Moreover, the morphology of the seed coat is considered the most important part of the seed for taxonomic and evolutionary observations due to its fixed structures that are not easily affected by external environmental factors during seed development and ripening [99]. The seed coat patterns provide information that solves classification problems, establishes evolutionary relationships, and serves as genetic markers for discovering genotypes in segregating hybrid progenies [100,20]. This study established that all the seeds of *Cannabis sativa* ecotypes collected in three (3) villages in Lusikisiki were ellipsoid in shape, slightly flattened, smooth, glossy grey-green, and showed fine puzzle-like patterns in khaki-coloured lines on the surface when examined under a light microscope. These observations are supported by Stoilkovska Gjorgievska *et al.* [101], who reported that *Cannabis sativa,* the marijuana type, has an ellipsoid seed shape, slightly flattened with a smooth seed coat. EiSohly *et al*. [102] also observed a brownish cannabis seed colour with mottled patterns in the perianth. These results indicate that seed shape might be a useful taxonomic marker for medicinal cannabis species. However, similar seed shapes exist in other species of the genus, such as the industrial hemp [102]. Therefore, they should be considered in combination with other macro-morphological characteristics when applied to species identification within the genus Cannabis. Similar findings were reported by Zhao *et al*. [103] in their studies on seed morphology of the genus Oxytropis in the Fabaceae family. The current study has established that the seeds collected in three villages had an average seed length and diameter of 3.53 × 2 mm, 3.73 × 3.11 mm, and 3.36 × 2.57 mm, respectively. The cannabis seed size doesn't exceed 5mm [101]. Boesewinkel *et al*. [104] describe the micropyle as the small opening in the seeds through which water and pollen grains enter seeds during fertilization. Seeds of V3 (62 um^2) had a wider micropyle opening compared to the other sites (V2 and V1). The size of the apertures in the micropyle sections varied depending on the temperature of the seed origin [105]. Studies showed that seeds with a wide micropyle size exhibited a high quantity of water uptake as well as rapid seed germination [105,106]. According to Doyle [107], the hilum is the scar on the seed coat that marks the place at which seeds get fastened to the tissues of the ovaries. It regulates the dynamics between the embryo and the external surroundings, thus controlling water content in the seeds during the last phase of the formation of seeds [108]. Zhao *et al*. [109] reported that hilum size could be considered as a proxy trait for soybean yield and quality. Zhao *et al*. [109] further stated that micropyle and hilum are complex seed morphological traits that can be affected by genetic and environmental factors. *Thus,* the current study observed the wide-open hilum on seeds of V2.

Essential oil-bearing plants possess cells or glands called trichomes that have a significant economic value [110]. Trichomes are a powerhouse of secondary metabolites such as cannabinoids and terpenes, which are the crucial

compounds for the biological and therapeutic properties of cannabis [111]. Investigation of trichome density on both the abaxial, adaxial sides of the leaf and in the flower revealed a notable increase in non-glandular trichome density on the adaxial side of the leaf, with V3 recording the highest. Trichome population was higher on cannabis plants grown in locations of 12 hours or more day length compared to short photoperiod areas, irrespective of cultivar [112]. The results of this study contradict the findings of Punja *et al*. [113], who reported the increased trichome density on the abaxial surface of the leaf. However, remarkably higher glandular trichome densities emerged on the flowers across the villages, with the highest recorded in the V2 flower. This trend suggests that, as the plants progressed through growth stages, there is a consistent generation of glandular trichomes, which are fundamental spots for resin synthesis and cannabinoid production, particularly in the flower part. This distribution of trichomes and their types in different developmental stages of the plant might be attributed to the varied environmental conditions affecting the two leaf surfaces differently or could be due to genetic predisposition influencing the trichome placement in the plant [113]. Moreover, trichomes represent the main cells for secondary metabolite manufacturing and sequestration. This increase in trichome density and distribution aligns with the common understanding that secondary metabolite levels peak around the time that plants are considered mature [7].

5. Conclusion

Differences in ecotypes are inherently attributed to the selective local ecological factors such as climate, altitude, slopes, etc. Consequently, different ecotypes of the same species may adopt different survival strategies to adapt to the environmental stress. The results of this study reflect a notable and distinct diversity amongst the natural populations of *Cannabis sativa* concerning morpho-anatomical features, which indicates the genetic value that necessitates the conservation of these locally naturalized species. Thus, plant interactions with their environment, particularly the north-facing slope orientation, were evident when plants from (V1) exhibited vegetative growth with taller, more branches, and higher biomass (both fresh and dry). Whereas plants from (V3) yielded the highest reproductive traits (i.e., number of flowers, fresh and dry flower weights). However, the highest seed yield (1000-seed weight), moisture, and oil yield were found on seeds collected from V2, followed by V3. Major cannabinoid content was high in V3 and V1. This study established no differences in the seeds' morphological traits (size and colour) of *Cannabis sativa* ecotypes. Ecological differences such as altitude and slope orientation, as well as plant interactions with the environment, affected the morpho-anatomic traits of seeds, leaves, and flower trichomes, with a gradual increase in locality altitude and different slope orientations. Remarkably, higher glandular trichome densities emerged on the flowers across the villages, with the highest recorded in the V2 flower. This trend suggests that, as the plants progress through the growth stages, there is a consistent generation of glandular trichomes, which are fundamental spots for resin synthesis and cannabinoid production, particularly in flowers. Environmental factors play pivotal roles in cannabinoid production. Moreover, to ensure the long-term preservation and sustainability of these *Cannabis sativa* genetic materials, the integration of in-situ and ex-situ conservation strategies is essential. In-situ conservation fosters the maintenance of these ecotype populations within their natural habitats, allowing for the continuous adaptation of plant species to local environmental conditions and promoting the preservation of ecological interactions and genetic diversity. However, due to the growing threats from habitat degradation, overharvesting, and environmental change, ex-situ preservation methods such as seed banking and vegetative propagation can provide complementary protection of these cannabis ecotypes. These techniques enable the protection of genetic material outside its natural habitat, ensuring that valuable traits are not lost and can be reintroduced into natural populations and breeding programs when required. Therefore, an integrated conservation approach that combines in-situ management of wild populations with ex-situ propagation and storage strategies represents the most effective pathway toward maintaining the genetic integrity, adaptability, and medicinal potential of *Cannabis sativa* ecotypes found in Lusikisiki, Eastern Cape. These findings provide actionable insights for optimizing cultivation practices and advancing cannabinoid research, production, and species conservation.

6. Recommendations

The results of this study have shown that each local environment has a specific influence on the morpho-anatomical traits of the studied cannabis ecotypes. However, the plants from Village 3 exhibited superior reproductive traits in terms of flower biomass, flower yield, cannabinoid concentration, and the number of trichomes, making them a desirable ecotype for domestication to support sustainable medicinal cannabis industry development. Furthermore, special emphasis on genetic improvement programmes for ex-situ preservation of these genetic characteristics is recommended. Future research should focus on studying the seed germinability of these ecotypes under environmental stresses, such as varying minimal temperature levels, as well as their response to different asexual propagation techniques, like cuttings, to improve yields and quality.

Author Contributions

A.D., Conceptualization, Writing—Initial Draft, Writing, Revisions, Data Curation, Investigation, Visualization, I.E., Conceptualization, Writing, Review and Editing, Supervision; T.T.S., Conceptualization, Writing Review and Editing; A.O.O., Data Curation and Reviewing, B.M. (Babalwa Mpambani), B.M.(Buyisile Mayekiso), laboratory investigation and reviewing; Conceptualization, Review and Editing, H.A.S., Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted following the permit granted in terms of Section 22A(9)(a)(i) of the Medicines and Related Substances Act, 1965, to acquire, possess, and use Schedule 6 AND 7 substances for analytical and research purposes. Permit No: POS289/2025/2026. Granted by the South African Health Products Regulatory Authority (SAHPRA). The study was conducted according to ethical clearance as approved by the Ethics Committee of Walter Sisulu University (protocol number WSU/FNS-GREC/2024/03/11/G10 and date of approval 09 January 2024–16 January 2027).

Informed Consent Statement

Not applicable.

Data Availability Statement

All data generated in this study are included in this article for publication.

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Conflicts of Interest

The authors declare no conflict of interest.

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