# CHARACTERIZATION AND GERMINATION DYNAMICS OF CANNABIS LANDRACES ACROSS DIVERSE ENVIRONMENTAL CONDITIONS

**By**

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## ABSTRACT

*Cannabis sativa* L. is globally esteemed for its diverse applications, yet historical legal barriers impeded research and industrial advancement. The pharmaceutical relevance of its cannabinoids drives recent regulatory relaxations. Nevertheless, delineating cultivars remains pivotal for optimizing cannabis products. Understanding the physiological intricacies of cannabis germination and seedling establishment, particularly for South African genotypes, remains limited. Therefore, this study aims to differentiate cannabis genotypes and explore seed germination and seedling development to enhance sustainable farming practices across diverse environmental conditions. Eleven cannabis genotypes were used, including landraces Ladysmith Ugwayi wesiZulu ('L1') and Iswazi ('L2'), Durban Poison ('H1'), Bergville Ugwayi wesiZulu ('B1'), Natal ('B2') and Iswazi ('B3'), and Msinga Ugwayi wesiZulu ('M1') and Iswazi ('M2'), and the commercially available 'Hemp', 'Cherry bubble gum' ('High-cannabidiol (CBD)') and 'White Rhino' ('High-$\Delta^9$-tetrahydrocannabinol (THC)') genotypes. Characterizing cannabis landraces involved assessing physical traits, mineral element composition and Short Tandem Repeats (STR) markers of the seeds. Pre-treatment effects on seed dormancy and germination were also evaluated, alongside the effect of temperature regimes, polyethylene glycol (PEG)-induced drought stress, accelerated aging, and sodium chloride (NaCl) salinity stress. The study revealed a strong positive correlation ($p$<0.001) among cannabis accessions based on STR markers, physical traits, and mineral profiling. However, clustering based on mineral composition varied from clustering based on physical traits, and STR markers produced different groupings, highlighting a complex relationship among these characteristics. As a result, the genotypes, especially the landraces, could not be classified as 'High-CBD', 'High-THC', or 'Hemp' based on the measured parameters. On the other hand, landraces and environment interactions generally exhibited significant differences

($p$<0.05) in germination characteristics, with sodium chloride (NaCl), and polyethylene glycol (PEG) levels generally impeding germination; however, tolerance varied among genotypes. ‘H1’, ‘L2’, and ‘B2’ are ideal for saline conditions, while ‘L2’, ‘B1’, ‘H1’, and ‘B3’ are recommended for farmers with limited water resources. Accelerated aging also significantly reduced germination performance in cannabis landraces, highlighting the importance of optimal storage for seed viability. Gibberellic acid ($GA_3$), potassium nitrate ($KNO_3$), and prechilling proved effective in enhancing germination and breaking physiological dormancy. Temperature also played a crucial role in cannabis germination percentage and germination rate, as well as biochemical composition. Genotypes ‘L1’, ‘B1’, ‘H1’, ‘B2’, and ‘M1’ demonstrated higher germination at cooler temperatures of 20/15 °C, while ‘B3’, ‘M2’, and ‘L2’ performed better under warmer conditions of 30/25 °C. However, higher temperatures were generally detrimental to the germination of cannabis landraces. Our findings reveal that genotype selection and optimal planting dates are sensitive to temperature variations and depend on genotype-specific traits and biochemical factors. These results highlight the importance of carefully considering these variables in cannabis cultivation practices. In summary, the study provides crucial information for genotype classification, helping licensed producers and plant breeders optimize genotype selection for medicinal cannabis and hemp, ensure regulatory compliance, and maximize yields. It details the physiological and biochemical processes in cannabis germination and seedling development, laying a foundation for future research, clinical studies, and production strategy optimization.

## ABBREVIATIONS

| | |
|---|---|
| φ | water potential |
| $\phi$ | sphericity |
| $\Delta^9$-THC or THC | (-)-trans-$\Delta^9$-tetrahydrocannabinol |
| $^1$H NMR | proton nuclear magnetic resonance |
| AA | accelerated aging |
| ABA | abscisic acid |
| Al | aluminium |
| ANOVA | analysis of variance |
| AR | after-ripening |
| AUC | Area Under the Curve |
| $a_w$ | Water activity |
| C | carbon |
| Ca and $Ca^{2+}$ | calcium and calcium ions |
| $CB_1$ and $CB_2$ | cannabinoid receptors 1 and 2 |
| CBD | cannabidiol |
| CBDAS | cannabidiolic acid synthase |
| CBG | cannabigerol |
| CBN | cannabinol |
| Cl and $Cl^-$ | chlorine and chlorine anion |
| $ClO_2$ | chlorine dioxide |
| CRD | completely randomized design |
| Cu | copper |
| CVG | coefficient of velocity of germination |
| $D_g$ | geometric mean diameter |

| | |
|---|---|
| EC | electrolyte conductivity |
| EDX | energy-dispersive X-ray detector |
| FAMEs | fatty acids methyl esters |
| Fe | iron |
| FTIR | Fourier transform infrared |
| FT-NIR | Fourier transform near-infrared |
| GA and $GA_3$ | gibberellins and gibberellic acid |
| GC | gas chromatography |
| GC-FID | gas chromatography coupled with flame ionization detection |
| GP | germination percentage |
| GRI | germination rate index |
| GPR | germination percentage recovery |
| GSI | Germination Stress Tolerance Index |
| $H_2SO_4$ | sulfuric acid |
| HCL | hydrochloric acid |
| $HNO_3$ | nitric acid |
| HPLC | high-performance liquid chromatography |
| HPLC-DAD | high-performance liquid chromatography coupled with diode-array detector |
| HPS | high-pressure sodium |
| HW | hot water |
| IR | infrared |
| K and $K^+$ | potassium and potassium ion |
| $KNO_3$ | potassium nitrate |

| | |
|---|---|
| L | length |
| LC | liquid chromatography |
| LEDs | light-emitting diodes |
| LSD | least significance difference |
| Mg | magnesium |
| Mn | manganese |
| MS | mass spectrometry |
| N | nitrogen |
| Na and $Na^+$ | sodium and sodium ion |
| $Na_2CO_3$ | sodium carbonate |
| $Na_2SO_4$ | sodium sulfate |
| NaCl | sodium chloride |
| $NaHCO_3$ | sodium bicarbonate |
| NIR | near-infrared |
| NIR-HSI | near-infrared hyperspectral imaging |
| NMR | nuclear magnetic resonance |
| NO | nitrite/nitric oxide |
| O | oxygen |
| P | phosphorus |
| PC | principal component |
| PCA | principal component analysis |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PGRs | plant growth regulators |
| pH | potent of hydrogen |

| | |
|---|---|
| PI | promptness Index |
| PINS | promptness index under control. |
| $P_N$ | net photosynthesis |
| Ra | aspect ratio |
| RH | relative humidity |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| S | sulfur |
| SEM | scanning electron microscopy |
| Si | silicon |
| SL | seedling length |
| SPE-PEDOT | screen-printed electrode modified with poly-(3,4-ethylenedioxythiphene) |
| SPEs-CB | screen-printed electrodes modified with carbon black |
| SPME-GC-MS | Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry |
| STR markers | Short Tandem Repeats |
| STS | silver thiosulfate |
| SVI | seed vigour index |
| T | thickness |
| TAG | triacylglycerol |
| TCA | tricarboxylic acid |
| TCC | 2,3,5-triphenyltetrazolium chloride |
| THCAS | tetrahydrocannabinolic acid synthase |
| TLC | Thin-layer chromatography |

| | |
|---|---|
| UPLC-MS | Ultra-Performance Liquid Chromatography-Mass Spectrometry |
| UV-B | ultraviolet blue |
| W | width |

## DECLARATIONS

### DECLARATION 1: PLAGIARISM

I, **Sabeliwe Langa,** declare that:

1. The research reported in this thesis is my original research, except where otherwise indicated.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. their words have been re-written, but the general information attributed to them have been referenced, and
   b. where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References section/s.

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## DECLARATION 2: PUBLICATIONS

1. **Langa, S**., Magwaza, L.S., Mditshwa, A and Tesfay, S.Z, 2024. Characterization of cannabis varieties and the intrinsic and extrinsic factors affecting cannabis germination and seedling establishment: A descriptive review. *Industrial Crops and Products,* 208*:* 117861. https://doi.org/10.1016/j.indcrop.2023.117861.

2. **Langa, S**., Magwaza, L.S., Mditshwa, A and Tesfay, S.Z, 2024. Seed dormancy and germination responses of cannabis landraces to various pre-treatments. *South African Journal of Botany*, 165: 91-100. https://doi.org/10.1016/j.sajb.2023.12.021.

3. **Langa, S**., Mditshwa, A., Tesfay, S.Z., and Magwaza, L.S, 2024. Temperature effects on seed germination and seedling biochemical profile of cannabis landraces. *International Journal of Plant Biology*, 15, 1032-1053. https://doi.org/10.3390/ijpb15040073.

## DEDICATION

This thesis is dedicated to the queen mother in heaven, **Ms. Zanele T. Mchunu**, who passed away in the first year of my PhD studies. Robala ka khotso Ntombi yeChunu, and continue to shine brightly, just like the star that you are!!!

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# PREFACE

This thesis is a compilation of manuscripts collected into chapters, each of which is introduced disjointedly. Hence, some repetition throughout the various chapters has been unavoidable.

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# 1. CHAPTER 1: INTRODUCTION

## 1.1. Background

*Cannabis sativa* L., commonly known as marijuana, dagga, or hemp, is an annual dioecious and dicotyledonous plant belonging to the *Cannabaceae* family (Flores-Sanchez *et al*., 2009; Chaohua *et al*., 2016; Lata *et al*., 2016). Cannabis is one of the most consumed illicit drugs worldwide, and it also provides informal income to rural communities in South Africa (Citti *et al*., 2018; Duvall, 2019). Its classification as an illicit drug is linked to the intoxicating effects of the psychotropic activity of (-)-trans-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), which resulted in the ban of cannabis worldwide for centuries (Citti *et al*., 2018).

Until recently, many countries have uplifted the regulations after the discovery of cannabis as a plant of both medicinal and agricultural value (Decorte *et al*., 2011; Sawler *et al*., 2015; Gouws, 2017; Citti *et al*., 2018; Adebisi and Olaoye, 2022). The growing interest in the medical industry follows the recent discovery of the relevance of cannabinoids in the pharmaceutical industry. On the other hand, there has been a renewed interest in hemp, which is grown globally as a multifunctional agricultural crop for its oily seeds and long, durable fibers (Salentijn *et al*., 2015; Smith, 2019).

Being an annual summer crop, cannabis grows well under temperatures ranging from 15 to 27 °C (Humphries and Florentine, 2019). It is particularly sensitive to low soil moisture levels and necessitates adequate moisture throughout its growing season (Cole and Zurbo, 2008; Williams and Mundell, 2018; Adesina *et al*., 2020). Previous studies showed that water stress reduced the germination rate and potential, delayed radicle and hypocotyl growth in 'Yunma 1' YM and 'Bamahuoma' BM hemp genotypes (Du *et al*., 2022). Water stress also reduced biomass, seed yield, and water potential of the 'Black Label' hemp cultivar, mostly grown for industrial purposes (Gill *et al*., 2022). Hemp has a widespread distribution and is characterized by deep

root system, rapid growth, and high biomass production which contributes to its salinity stress resistance (Cheng *et al*., 2016). However, the tolerance to salt stress in hemp varies depending on the salt type, concentration, genotypic differences, and growth stages (Cheng *et al*., 2016; Hu *et al*., 2018; Di Mola *et al*., 2021).

Cannabis is characterized by physiological dormancy, and wild genotypes, referred to as feral or ditch weed, can remain dormant in the soil for up to 10 years while remaining viable (Jovičić *et al*., 2019; Önol and Yildirim, 2021). On the other hand, cannabis seeds are orthodox, allowing storage in seed banks at 5 °C with 5-7% relative humidity for up to 5 years or at 15 °C for up to 12 months, provided the seeds maintain a moisture content of 6% (Parihar *et al*., 2014; Suriyong *et al*., 2015; Oseyko *et al*., 2020; Oseyko *et al*., 2021). However, uncontrolled storage conditions previously reduced seed viability by 70 – 80% (Small and Brookes, 2012).

Generally, cannabis genotypes are categorized based on non-chemical characteristics and cannabinoid composition, known as chemotaxonomy (de Meijer, 1994; Cerrato *et al*., 2021). Scientists have identified five distinct cannabis chemotypes, namely, chemotypes I, II, III, IV, and V (Broseus *et al*., 2010; Piluzza *et al*., 2013; Khan *et al*., 2014; Schluttenhofer and Yuan, 2017; Soorni *et al*., 2017; Cerrato *et al*., 2021). Chemotype I represents high THC genotypes with less than 0.5% (cannabidiol) CBD and 1% to over 20% $\Delta^9$-THC (Broseus *et al*., 2010; Piluzza *et al*., 2013; Khan *et al*., 2014; Soorni *et al*., 2017; Cerrato *et al*., 2021). Chemotype III includes high CBD genotypes with below 0.3% $\Delta^9$-THC, and chemotype II has equal amounts of $\Delta^9$-THC and CBD (Broseus *et al*., 2010; Piluzza *et al*., 2013; Khan *et al*., 2014; Schluttenhofer and Yuan, 2017). Chemotype IV is predominant in cannabigerol (CBG), and Chemotype V has no detectable phytocannabinoids (Cerrato *et al*., 2021).

Additionally, classification using non-chemical attributes, including morphological and physiological features, has resulted in three distinct groups, namely, drug hemp, oilseed hemp,

and fiber hemp (de Meijer, 1994; Salentijn *et al*., 2015). Despite recent regulatory relaxations, the historical illegality of cannabis cultivation, breeding, and distribution has hindered research advancement on the crop and consequently delayed the development of industrial products in many countries, including South Africa. Consequently, the taxonomic classification of cannabis is still under evaluation.

### 1.2. Problem statement

Currently, there are hundreds of cannabis genotypes around the world as a result of a long history of hybridization (Jin *et al*., 2017). These genotypes have varying chemical components, highlighting the importance of characterizing cannabis as a foundational requirement for standardizing and regulating the quality of cannabis for industrial and pharmaceutical purposes (Farag and Kayser, 2015; Jin *et al*., 2017). Variation in chemical composition among cannabis genotypes indicates that prolonged hybridization can lead to the degradation of seed purity and a reduction in crop yield (Huang *et al*., 2016; Jin *et al*., 2017; Zhang *et al*., 2020). Seed purity is amongst many seed quality components, including seed viability, health, mechanical damage, physiology, germinability, and physical characteristics (Finch-Savage and Bassel, 2016; Rahman and Cho, 2016).

Therefore, seed quality parameters are essential for high-quality seeds that exhibit vigorous growth, consistent germination, and rapid establishment, which ultimately results in high yields (Kandpal *et al*., 2016; Rahman and Cho, 2016; Feng *et al*., 2019). Additionally, high-quality seeds use radiation and nutrients more effectively and exhibit resilience to biotic and abiotic stresses (Bianchini *et al*., 2021). Therefore, assessing seed quality is critical where cannabis cultivation relies heavily on seed propagation, particularly in South Africa (Kodym and Leeb, 2019).

Seed germination is a biochemical and physiological process that starts with water imbibition and relies on stored proteins, lipids, and carbohydrates to sustain good seedling development (Alencar *et al*., 2012; Erbas *et al*., 2016; Ali and Elozeiri, 2017; Zhao *et al*., 2018; Reis *et al*., 2020). The mobilization of seed reserves during germination and early stages of seedling growth have been previously studied on various crops such as cereals, oil crops (*Helianthus annuus* L.), ornamental and medicinal plants (*Cereus jamacaru* D.C., *Morinda citrifolia* L.) (Alencar *et al*., 2012; Erbas *et al*., 2016; Paula *et al*., 2016). The findings of the previous studies emphasize that seed reserve content is correlated with germination percentages and rates (Zhao *et al*., 2018).

Environmental factors, such as temperature, light, oxygen, water availability, and salinity, however, significantly influence seed germination, seedling development and reserve mobilization (Marques *et al*., 2013; Mohammad *et al*., 2014; Tabatabaei and Naghibalghora, 2014; Zucareli *et al*., 2015; da Silva *et al*., 2019; Reis *et al*., 2020; Bano *et al*., 2021). For instance, salt stress results in both ion toxicity and water deficit (da Silva *et al*., 2019). Ion toxicity prevents reserve mobilization during seedling establishment, whereas water deficit often results in a reduced water influx, which limits germination percentage and rate, water potential, and plant establishment (Macar *et al*., 2009; Mohammad *et al*., 2014; Tabatabaei and Naghibalghora, 2014; da Silva *et al*., 2019). On the other hand, temperature influences imbibition, reserve mobilization, and seed aging (Sikder *et al*., 2009; Gajanayake *et al*., 2011; Zucareli *et al*., 2015; Reis *et al*., 2020).

Dormancy mechanisms, including physical, morphological and physiological, may also affect seed germination success and plant adaptation (Golmohammadzadeh *et al*., 2015; Chahtane *et al*., 2017; Soltani *et al*., 2018). Despite favourable conditions, many seeds may fail to germinate, indicating varying dormancy types and their impact on seed viability and plant

survival (Önol and Yildirim, 2021). Understanding these factors is crucial for optimizing cannabis seed germination and seedling establishment.

### 1.3. Justification

Differentiating cannabis genotypes before propagation is essential for targeting and recommending appropriate genotypes that meet purity standards, ensuring the expected quality and yield for specific markets (Zhang *et al*., 2012; Bai *et al*., 2020). Characterizing cannabis genotypes is particularly important in South Africa, where research on the crop has been limited due to its legal status in the past decades. Thus, classifying South African genotypes will provide the groundwork for production for industrial uses, genotype improvement interventions, and clinical research. Additionally, it will provide guidance for individual growers regarding genotype selection in light of recent legislative relaxations regarding hemp and medicinal cannabis.

Cannabis, being an oil crop, is prone to seed deterioration due to the lower chemical stability of lipids compared to starch in crops with high oil content (Suriyong *et al*., 2015; Prudente and Paiva, 2018). However, there is little information about the storage potential of South African genotypes. Understanding this information is critical for selecting genotypes with higher performance potential in regions with high temperatures and relative humidity during seed maturation (de Lima *et al*., 2019).

Additionally, the germination and seedling growth responses of South African cannabis genotypes to environmental factors such as temperature, salinity, and drought stress are not well-documented. This information is vital as environmental factors can adversely affect seed germination and seedling development, ultimately impacting yield and chemical composition. This information will provide valuable insights for cannabis growers and plant breeders to

implement good agricultural practices, product development, and genotype selection and improvement.

Moreover, cannabis seeds may not germinate under favourable growing conditions due to physiological dormancy linked to the indeterminate growth pattern of the female inflorescence and the physical barrier imposed by their hard-outer shell (Elias *et al*., 2020; Kildisheva *et al*., 2020; Schultz *et al*., 2020; Önol and Yildirim, 2021). Therefore, studying seed dormancy and dormancy-breaking techniques is critical to enhancing germination and improving seedling development.

### 1.4. Aim of the study

This study aims to distinguish the physical properties, genetic traits and mineral element composition of seeds used for producing cannabis material for different industrial applications. The study also aims to investigate the effect of seed quality and environmental conditions on the germination performance of landraces.

### 1.5. Research questions

- How effective are physical properties, genetic traits, and mineral element composition in characterizing cannabis genotypes for categorizing landraces as ‘High-CBD’, ‘High-THC’ and ‘Hemp’?
- How do dormancy mechanisms impact the germination and seedling development of cannabis landraces, and what are effective dormancy-breaking techniques to alleviate these effects?
- What is the impact of accelerated aging on the germination rates of cannabis landrace seeds?
- How does drought stress influence the seed germination and seedling development of cannabis landrace seeds?

- How does salt stress influence the seed germination and seedling development of cannabis landrace seeds?
- How do alternating temperature regimes influence the seed germination and biochemical properties of cannabis landraces?

### 1.6. Specific objectives

This research study has the following specific objectives:

- To characterize cannabis genotypes based on physical properties, Short Tandem Repeats (STR) markers, and mineral element composition.
- To assess the effect of various pre-treatments on seed dormancy and germination of cannabis landraces.
- To examine the effect of accelerated aging on seed germination performance of cannabis landraces.
- To investigate the effect of polyethylene glycol (PEG)-induced drought stress on seed germination and seedling development of cannabis landraces.
- To assess the effect of soil salinity on seed germination and seedling development of cannabis landraces.
- To assess the effect of different temperature regimes on seed germination of cannabis landraces and seedlings carbohydrates, fatty acids methyl esters, and amino profile.

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# 2. CHAPTER 2: CHARACTERIZATION OF CANNABIS GENOTYPES AND THE INTRINSIC AND EXTRINSIC FACTORS AFFECTING CANNABIS GERMINATION AND SEEDLING ESTABLISHMENT: A DESCRIPTIVE REVIEW

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**Abstract**

*Cannabis sativa* L. is the utmost consumed, grown, and produced illicit drug worldwide. The psychotropic activity of the component (-)-*trans*-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), however, led to the banning of cannabis worldwide for many centuries. Besides being the most popular crop for recreational purposes, cannabis is grown globally as a multifunctional agricultural crop for its oily seeds and long, durable fibers. These properties have led to cannabis application in various industries, including agriculture, textile, bio-composite, papermaking, automotive, construction, and biofuel. Furthermore, medicinal cannabis constitutes of high concentration of cannabinoids, which have been discovered relevant in the pharmaceutical industry. As a result, various countries worldwide have relaxed regulations on cannabis. However, the cannabis products' final use depends on the cultivar. Yet, the crop has hundreds of cultivars owing to constant breeding and human selection, which has altered the original plant with the spread of the crop worldwide. Generally, these cultivars possess chemical composition that

varies genetically, and with the growing environmental conditions. Therefore, the current descriptive review of literature highlights the techniques available for classifying cannabis genotypes, given the ongoing debate over classifying cultivars into distinct taxonomic groups. The effect of intrinsic and extrinsic factors on cannabis germination and seedling establishment are also highlighted.

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**Keywords**: cannabis, environmental factors, seed quality, taxonomy, reserve mobilization

### 2.1. Introduction

*Cannabis sativa* L., commonly known as marijuana, dagga, or hemp, is an annual dioecious and dicotyledonous plant belonging to the *Cannabaceae* family (Flores-Sanchez *et al*., 2009; Chaohua *et al*., 2016; Lata *et al*., 2016). Cannabis which originated from Central Asia, is one of the earliest plants domesticated in eastern Asia since 3500 BC (Decorte *et al*., 2011). Furthermore, it is one of the most consumed, grown, and produced illicit drug worldwide, with about 151 countries cultivating cannabis plants from 2010 to 2018 (Citti *et al*., 2018; Duvall, 2019; Shao *et al*., 2023). South Africa being among the world's largest producers of *Cannabis*, with its cultivation occurring mainly in remote rural areas of the Eastern Cape and KwaZulu-Natal provinces (Gastrow, 2003; Peltzer and Ramlaan, 2007).

Cannabis classification as an illicit drug owes to the intoxicating effects of the psychotropic activity of the component (-)-*trans*-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) (Citti *et al*., 2018). This compound then led to the ban of cannabis worldwide for centuries (Citti *et al*., 2018). Until recently when many countries across the world considered uplifting the regulations controlling the cultivation of cannabis, particularly for hemp and drug type for medicinal purposes (Gouws, 2017; Citti *et al*., 2018; Allaf *et al*., 2023). Since regulatory changes have been made in various countries worldwide, the global cannabis market has seen significant growth, making it a lucrative industry (Qobo and Sihlobo, 2019). In 2023, the legal cannabis market was estimated

to be worth $57.18 billion, with a projected value of $147 billion by the end of 2027 (Stefkov *et al*., 2022).

The growing interest, particularly in medicinal cannabis, follows the recent discovery of the relevance of cannabinoids in the pharmaceutical industry (Citti *et al*., 2018). It is suggested that cannabinoids usually interact with cannabinoid (CB) receptors ($CB_1$ and $CB_2$) and other receptor systems to show their pharmacological effects in humans (Lewis-Bakker *et al*., 2019). As a result, various medicines known to activate cannabinoid $CB_1$ and $CB_2$ receptors have been manufactured from cannabis derivatives (Pertwee, 2009). Amongst these medicinal products include Cesamet® (nabilone), Marinol® (dronabinol; $\Delta^9$-tetrahydrocannabinol), and Sativex® ($\Delta^9$-tetrahydrocannabinol with cannabidiol) (Pertwee, 2009; Turri *et al*., 2018; Gaisey and Narouze, 2021; Harrison and Simpson, 2021).

On the other hand, hemp has been grown globally as a multifunctional agricultural crop for its oily seeds and long, durable fibers (Salentijn *et al*., 2015; Smith *et al*., 2019). It has been used in many industries, including agriculture, papermaking, textile, automotive, construction, bio-composite, and biofuel (Salentijn *et al*., 2015; Chaohua *et al*., 2016; Schluttenhofer and Yuan, 2017). Hemp seed oil has desirable quantities of omega-6 and omega-3 lipids for human and animal diets, cooking, or processing cosmetics and fuels (Salentijn *et al*., 2015; Chaohua *et al*., 2016; Schluttenhofer and Yuan, 2017). The rediscovery of the multifunctional properties of cannabis has, thus, sparked scientific interest in this plant (Gouws, 2017; Citti *et al*., 2018).

Scientific research studies in this crop are critical even for developing medicinal products in many countries, including South Africa. Particularly, considering that variation in chemical composition amongst cannabis cultivars or species is generally prevalent (Farag and Kayser, 2015; Jin *et al*., 2017; Procaccia *et al*., 2022). This is largely owed to the existence of hundreds of different cannabis cultivars as a result of the long history of hybridization by humankind

(Jin *et al*., 2017; Birenboim *et al*., 2022; Procaccia *et al*., 2022). These cultivars are known to have varying chemical composition resulting not only from genetic factors, environmental conditions, morpho-spatial position in the plant and crop management practices (Piluzza *et al*., 2013; Toth *et al*., 2021; Danziger and Bernstein, 2021a; De Prato *et al*., 2022; Song *et al*., 2023;). Therefore, the growing cannabis industry and the accessibility of cannabis products for medical and other industrial purposes demand improved product analysis, which is essential for improving product standardization and quality assurance (Jin *et al*., 2017; Monti *et al*., 2022).

Furthermore, despite the increased interest in hemp and medicinal cannabis, the physiology of cannabis germination and seedling establishment is not very well understood. Seed germination and seedling establishment are crucial phases for crop production and plant density and are also the most sensitive stages of plant growth (Jorenush and Rajabi, 2015; Lamichhane *et al*., 2018). Generally, the germination of seeds can occur in the field if certain conditions triggering the germination process exist, including sufficient temperature, moisture, and light (Čanak *et al*., 2020). Hence, seed germination is considered a quick and preliminary screening as germination patterns provide insight into plant establishment and favourable conditions (Hu *et al*., 2018). Therefore, this descriptive review seeks to provide insights into the chemical composition of cannabis to determine optimal analytical procedures for the quantitative determination of compounds in samples. This is so that the discovered compounds provide useful information for differentiating cannabis genotypes (i.e., medicinal and drug-type genotypes) for choosing planting material based on the desired end-use product. It also seeks to give an overview of the physiology and biochemistry of cannabis seed germination and plant development and the influence of internal and external factors in these growth processes.

## 2.2. Classification of cannabis genotypes

Plant physiological features determine crop yield and quality; hence, they are critical in breeding and crop management (Sarsenbaev *et al*., 2013). Those of cannabis are even more important as it is known for its significant degree of heterogeneity, which is believed to be partly caused by its sexual dimorphism (Sarsenbaev *et al*., 2013). Sexual dimorphism of cannabis is witnessed through the rate of growth and development as they differ significantly between female and male plants (Sarsenbaev *et al*., 2013). Male plants are known for flowering and senescing earlier than female plants (Sarsenbaev *et al*., 2013). Besides, cannabis has spread globally, and its dispersion from its natural habitat has altered the original plant through constant human selection (Thomas, 2012).

Human selection has resulted in cannabis having enormous phenotypic diversity observed in various physiological features, including leaf shape, seed size, fiber quality, branching, height, phytochemistry, phenology, and ecology (Vergara *et al*., 2016). Consequently, it has been difficult to classify cultivars taxonomically, and it is still unclear if the existing cultivars belong to two or more species (Fischedick *et al*., 2010). Even though taxonomic classification is still under evaluation, scientists have identified three subspecies under the species *Cannabis sativa*, namely *C. sativa* subsp. *sativa* and *C. sativa* subsp. *indica*, and *C.* subsp. *ruderalis* (Thomas, 2012; Procaccia *et al*., 2022; Simiyu *et al*., 2022). Otherwise, the cultivars classified thus far were categorized through cannabinoid composition (chemical composition) and non-chemical characteristics (de Meijer, 1994), as briefly described in subsections 2.1 and 2.2.

### 2.2.1. Morphological and physiological (non-chemical) classification

Non-chemical attributes generally involve classifying cannabis genotypes through morphological and physiological features (de Meijer, 1994; Salentijn *et al*., 2015). These characteristics are mostly resultants of domestication and are based on the climatic adaptation

of the plant (de Meijer, 1994). Under this classification, plants are distinguished based on: (a) population types, which are wild and naturalized plants, landraces, and cultivars; (b) end-use of the plant, such as fiber cultivars, seed cultivars, drug strains, and ornamentals; (c) time of flowering, namely, short- and long-day flowering, intermediate flowering genotypes (d) gender of the plant which are monoecious and dioecious cultivars; and (e) geographic origin.

De Meijer (1994) discovered three demarcated groups using the morphological and physiological traits, namely, drug hemp, oilseed hemp, and fibre hemp. The drug hemp genotypes were categorized as short, highly branched plants, and their leaves' distinct features were small in size and dark green colour (de Meijer, 1994). The oilseed hemp genotypes were also categorized as short but were early maturing plants and rich in seed production (de Meijer, 1994). Whereas fibre hemp genotypes were categorized as tall unbranched plants with poor seed production (de Meijer, 1994). On the other hand, tall plants with slender morphology and short life cycles were categorized into male plants (Small *et al*., 1975; Lubell and Brand, 2018).

However, this review identified that categorizing *C. sativa* plants using sex morphology is flawed. This is considering that sex morphology can be modified or reversed by exogenously applying plant growth regulators (PGRs) and chemicals, such as ethylene and cytokinin, gibberellins, silver nitrate, and silver thiosulfate (STS) (Lubell and Brand, 2018). For instance, female flower formation can be promoted in male plants by applying exogenous ethylene and cytokinin (Lubell and Brand, 2018). Whereas hormones and chemicals such as gibberellins, silver nitrate, and STS promote male flowers to be formed on female plants (Lubell and Brand, 2018).

However, reversing the sex morphology from male to female cannabis plants is prevalent, following the great importance of female cannabis plants in various industrial applications. This is associated with the fact that unfertilized female plants achieve pure chemical

compounds and produce high metabolite content compared to male, as well as fertilized female and monoecious plants (Lata *et al*., 2016). As a result, there has been an increasing interest in feminized seeds and vegetative female clones (Kurtz *et al*., 2020). However, feminized seeds and vegetative production are mostly not easily accessible to growers and are costly compared to normal seeds (DiMatteo *et al*., 2020). Therefore, the subsubsections 2.1.1 and 2.1.2 discusses the options available for classification of genotypes as a seed level, which should provide useful information that will minimize the costs of cannabis production.

#### 2.2.1.1. Seed morphology for genotype classification

The macro- and microstructure of seeds are very significant for classifying Angiosperm taxa, particularly (Kaya *et al*., 2011; Rashid *et al*., 2018). Among species, diversity exists in seed shape, size, texture, hilum size, and colour, which can help study systemic relationships in a large genotype of plant families and, subsequently, distinguish taxa (Rashid *et al*., 2018; Rashid *et al*., 2021). However, the morphology of the seed coat, in particular, is categorized as the most important part of the seed for studies related to taxonomy and evolution as it generally has stable parameters and is unlikely affected by external environmental factors during seed development and ripening within the fruit (Kaya *et al*., 2011; Rashid *et al*., 2018; Karaismailoğlu, 2019; Waheed *et al*., 2021;). The seed coat patterns provide information that solves classification problems, establishes evolutionary relationships, elucidates the seed coat's adaptive significance, and serve as genetic markers for discovering genotypes in segregating hybrid progenies (Kaya *et al*., 2011).

Usually, stereomicroscopes are used to examine plant species to identify features that characterize them into taxa (Candan *et al*., 2016). In cannabis, such studies are very limited, with only Naraine *et al*. (2020), who studied the morphological divergence of seeds among ruderal, fibre, oilseed, dioecious/monoecious, and marijuana variants using a stereoscope.

Naraine *et al*. (2020) found that the seeds of hemp cultivars are larger and lighter in colour compared to marijuana seeds because they have been subjected to more selection for these features. Fibre cultivars have larger seeds compared to oilseed cultivars, associated with the former having poor seed production, and thus, more energy is channelled into the seeds (de Meijer, 1994; Naraine *et al*., 2020). The seeds of monoecious oilseed cultivars were characterized as slimmer with a more pronounced elongated base compared to those of dioecious cultivars. As mentioned previously, oilseed genotypes are rich in seed production, and thus less energy is channelled into them; hence, they are slimmer (de Meijer, 1994; Naraine *et al*., 2020).

However, optical microscopy often fails to reveal qualitative differences in the research objects, of which scanning electron microscopy (SEM) has been useful in identifying these differences (Candan *et al*., 2016; Majeed *et al*., 2023). According to Candan *et al*. (2016), SEM has been useful in identifying differences in micromorphological characteristics of leaves, pollens, fruit, and seeds when there are disagreements on the identity of taxa. This is because SEM detects ultrastructural characteristics by combining extreme magnification to reveal qualitative differences that optical microscopy cannot detect (Majeed *et al*., 2023). These ultrastructural characteristics include exo- and endomorphic, macro- and micromorphological, seed coat morphology, and microsculpturing features (Kaya *et al*., 2011). As a result, SEM has been widely adopted for reviewing phenetic linkages and species classification (Kaya *et al*., 2011; Rashid *et al*., 2018), but no such information is available on cannabis.

#### 2.2.1.2. Near-infrared and Raman spectroscopy for classification of genotypes

Differentiating the sex of the cannabis seed before sowing is a key factor in minimizing the yield losses, required labour, and high costs (DiMatteo *et al*., 2020; Kurtz *et al*., 2020). This is

associated with the fact that the sex of the cannabis plant is often indistinguishable until the onset of flowering (DiMatteo *et al*., 2020; Toth *et al*., 2020). Cannabis cultivation may always be associated with yield losses, following that male and monoecious plants are removed at a later stage of development so that unfertilized female plants can be produced (Lata *et al*., 2016; DiMatteo *et al*., 2020; Toth *et al*., 2020). This ensures the production of an end product with a highly consistent chemical profile from unfertilized female plants (Lata *et al*., 2016; Toth *et al*., 2020).

Generally, traditional methods such as manual inspection, protein electrophoresis, DNA molecular marker technique are used for discrimination of different seed genotypes (Bai *et al*., 2020; Liu *et al*., 2022). In cannabis, Cascini *et al*. (2019) revealed that genetic markers can differentiate between cannabis drug-type and fiber-type genotypes, both in seeds and during the early stages of plant development, well before cannabinoids accumulate. In their study they conducted sequencing of tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) genes from 21 *C. sativa* genotypes (Cascini *et al*., 2019). From this study, Cascini *et al*. (2019) were able to successfully distinguish between drug-type and fiber-type cannabis samples, which was achieved with a perfect discrimination level of Area Under the Curve (AUC) 100%. This distinction was subsequently validated through chemical analyses to confirm the actual cannabinoid concentrations (Cascini *et al*., 2019). However, studies similar to the one conducted by Cascini *et al*. (2019), requires that a standard DNA fingerprint of seeds must be constructed first and the establishing of a complete DNA molecular marker identification system requires professional technicians and is expensive.

As a result, technology devices such as near-infrared hyperspectral imaging (NIR-HSI) have been identified as a prospective approach for genotype characterization at the seed level as it provides detailed spatial information and spectral information inside the research object (Zhang *et al*., 2018; He *et al*., 2019; Cui *et al*., 2020; Jin *et al*., 2022). The spatial and spectral

information is then used for physicochemical and external morphological characterization of the sample (Zhang *et al*., 2018; He *et al*., 2019; Cui *et al*., 2020; Jin *et al*., 2022). NIR-HSI analysis allows to obtain results within a short period without any variations and destructing the sample, unlike protein electrophoresis and DNA molecular marker technologies where variations are prevalent (He *et al*., 2019; Zhang *et al*., 2019; Bai *et al*., 2020). Additionally, protein electrophoresis and DNA molecular marker approach are found only suitable for application in detection on a small scale as they involve reagent-dependent treatment processes and are destructive and time-consuming (Wu *et al*., 2019). However, these technologies must be coupled with machine learning methods for deep mining the information in hyperspectral images (Jin *et al*., 2022). Deep learning has been the most preferred method because of its self-learning feature (Jin *et al*., 2022).

### 2.2.2. Chemical classification of cannabis

It is well known that *C. sativa* L. comprises of about 441 non-cannabinoid compounds and 125 phytocannabinoids (Thomas and ElSohly, 2016; Radwan *et al*., 2021; Kopustinskiene *et al*., 2022). However, the current literature review focuses on cannabinoids because they have been and potentially are still to be used to identify different cannabis genotypes, particularly $\Delta^9$-THC and cannabidiol (CBD). CBD and $\Delta^9$-THC remain the most prevalent cannabinoids found in cannabis plants (Citti *et al*., 2018; Burnier *et al*., 2019). $\Delta^9$-THC is known to possess about 90% of the psychoactive effects of cannabis and was isolated and characterized by Raphael Mechoulam in 1964 (Citti *et al*., 2018; Burnier *et al*., 2019). On the other hand, CBD, an isomer of THC, is characterized as a non-psychoactive compound (Lata *et al*., 2016). Cannabinoids are the most active secondary metabolism compounds uniquely found in cannabis (Radwan *et al*., 2008; Kill *et al*., 2016; Agarwal *et al*., 2018; Bonini *et al*., 2018). The isolation and characterization of these compounds date back to the 1940s, and over about 125 cannabinoids

have been discovered since that year (Citti *et al*., 2018; Radwan *et al*., 2021; Kopustinskiene *et al*., 2022).

However, generally, cannabis chemical composition tends to vary with genotypes; hence, chemotaxonomic classification involves distinguishing different cannabis genotypes through cannabinoids and their concentration into different chemotypes (de Meijer, 1994; Broseus *et al*., 2010; Piluzza *et al*., 2013). Apart genotypes, the production and accumulation of cannabinoids are influenced by environmental conditions, morpho-spatial position in the plant and crop management practices, such as light, fertilizer regimes, water availability, temperature, plant architecture, plant density and biotic stresses (Danziger and Bernstein, 2021a; Toth *et al*., 2021; De Prato *et al*., 2022; Song *et al*., 2023). For instance, Wei *et al*. (2021) found that a higher proportion of blue light promoted the synthesis and accumulation of THC and CBD cannabinoids in the 'Xinma' hemp genotype. UV-B irradiation within a wavelength range of 280- 320 nm has also been shown to increase THC content in certain cannabis genotypes (Lydon *et al*., 1987; Potter and Duncombe, 2012; Wei *et al*., 2021).

The application of nitrogen (N), potassium (K) and phosphorus (P) fertilizers have also been found to alter the accumulation of secondary metabolites (Saloner and Bernstein, 2021; Shiponi and Bernstein, 2021; Saloner and Bernstein, 2022; Song *et al*., 2023). Generally, higher N and K availability tends to decrease the concentration of cannabinoids and terpenoids (Saloner and Bernstein, 2022; Song *et al*., 2023). To maintain a high secondary metabolite profile in medical cannabis cultivars, it is recommended to use an N level of 160 mg/L, a K level of 60 mg/L, and a P level ranging between 5 and 15 mg/L (Saloner and Bernstein, 2020; Saloner and Bernstein, 2021; Saloner and Bernstein, 2022; Song *et al*., 2023).

Pruning techniques have also been shown to impact the chemical composition of medical cannabis plants, particularly in 'Topaz' cultivar, as noted by Danziger and Bernstein (2021b).

However, the increased floral yield biomass appears to be the primary factor in cannabinoid production per plant, not the specific chemical changes induced by the pruning methods (Danziger and Bernstein, 2021b). In addition, salinity has also shown impact on the accumulation of secondary metabolites in cannabis as Yep *et al*. (2020) reported that the yield and potency of cannabis cultivar 'Nordle' decreased as cannabis roots were exposed to increasing NaCl concentrations.

Amongst the cannabinoids, $\Delta^9$-THC and CBD are the most important chemotaxonomic markers. This is because the chemotaxonomic markers involve categorizing genotypes using the relative proportions of $\Delta^9$-THC and CBD (Broseus *et al*., 2010; Piluzza *et al*., 2013). The use of the relative proportions of $\Delta^9$-THC and CBD is associated with the fact that these compounds are hardly affected by external factors, including environmental conditions. Therefore, it is often relatively constant throughout the plant's life, which makes it advantageous distinguishing cannabis genotypes using chemotaxonomic markers (Staginnus *et al*., 2014). Over and above, the THC: CBD ratio of the cannabis raw material is critical for industrial applications and thus assists in selecting genotypes suitable for developing plant material for industrial applications or medicinal purposes (Fischedick *et al*., 2010).

Chemotaxonomy was used to identify five distinct chemotypes that currently exist in cannabis, which are chemotypes I, II, III, IV and V (Cerrato *et al*., 2021). Chemotype I represents high THC genotypes, possessing CBD of less than 0.5% and $\Delta^9$-THC content ranging between 1 to 20% or more (Broseus *et al*., 2010; Piluzza *et al*., 2013; Khan *et al*., 2014; Soorni *et al*., 2017; Cerrato *et al*., 2021). Chemotype III represents high CBD genotypes, possessing $\Delta^9$-THC content below 0.3% (Broseus *et al*., 2010; Khan *et al*., 2014; Schluttenhofer and Yuan, 2017). Chemotype II represents the intermediate type, which possesses an equal amount of $\Delta^9$-THC and CBD (Piluzza *et al*., 2013; Khan *et al*., 2014). Chemotype IV represents cannabigerol

(CBG) predominant genotypes, and chemotype V represents genotypes with no detectable amount of phytocannabinoids (Cerrato *et al*., 2021).

Therefore, concerning chemotaxonomy, determining the chemical composition of cannabis plays a crucial part in selecting genotypes suitable for developing plant material for industrial applications or medicinal purposes. Various effective techniques have been used to analyze cannabis plant metabolites, including cannabinoids and terpenes (Birenboim *et al*., 2022; Koo *et al*., 2023). Nonetheless, these techniques come with certain challenges during sample analyses. Therefore, subsubsection 2.2.1 briefly discusses the existing techniques and highlight the challenges that need to be overcome to ensure that chemical classification yields the desired results.

#### 2.2.2.1. Techniques for analyzing cannabis composition for the classification of genotypes.

Direct analyses of the cannabis plant metabolites have been critical for chemotaxonomy, and this has been achieved through the application of various analytical techniques (Leghissa *et al*., 2018; Gaafar *et al*., 2018; Birenboim *et al*., 2022; Cicaloni *et al*., 2022; Ramos-Guerrero *et al*., 2022; Koo *et al*., 2023). These techniques include gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS or MS/MS), GC coupled with flame ionization detection (FID), high-performance liquid chromatography (HPLC), HPLC coupled with diode-array detector (DAD), Thin-layer chromatography (TLC), Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry and Ultra-Performance Liquid Chromatography-Mass Spectrometry (SPME-GC-MS and UPLC-MS). (Gaafar *et al*., 2018; Birenboim *et al*., 2022; Cicaloni *et al*., 2022; Ramos-Guerrero *et al*., 2022; Stefkov *et al*., 2022; Koo *et al*., 2023). Among these techniques, vibrational spectroscopy methods, including nuclear magnetic resonance (NMR), infrared (IR), near-infrared (NIR), Fourier transform

infrared (FTIR), Raman, and Fourier transform near infrared (FT-NIR), are also used for analysis of cannabis secondary metabolites (Birenboim *et al*., 2022; Ramos-Guerrero *et al*., 2022; Stefkov *et al*., 2022).

Nonetheless, GC-FID and HPLC with UV detection remain the most commonly used methods for analysing the phytocannabinoids in cannabis extracts (Happyana and Kayser, 2016; Berman *et al*., 2018; Procaccia *et al*., 2022; Koo *et al*., 2023). The GC analytical method, together with principal component analysis (PCA), has been successfully used to categorize the chemotaxonomic of the cannabis genotypes (Happyana and Kayser, 2016). Moreover, the GC analytical method has been used to control the quality of plant materials (Happyana and Kayser, 2016). Cannabis terpenoids, on the other hand, are volatile in nature, and GC coupled with either FID or MS is suggested as the most suitable technique for analysing such compounds (Shapira *et al*., 2019). Terpenoid volatility also requires a careful selection of extraction methods, and liquid extraction is commonly used in cannabis (Shapira *et al*., 2019). The liquid extraction is usually conducted using a genotype of solvents, such as ethanol, *n*-pentane, petroleum ether, and chloroform (Shapira *et al*., 2019; Birenboim *et al*., 2022).

However, the analytical methods mentioned above pose some challenges during sample analyses. One of the challenges associated with GC coupled with FID and HPLC coupled with UV detection is the required reference compounds to prepare a calibration curve, which is generally unavailable for many cannabinoids (Hazekamp *et al*., 2004). Both analytical methods, GC with FID and HPLC with UV detection, can also be affected by chlorophyll or lipids impurities in the sample (Hazekamp *et al*., 2004). As a result, these methods require a sample clean-up step before analysis (Hazekamp *et al*., 2004). Moreover, GC with FID requires prior derivatization to distinguish between cannabinoids and their carboxylic counterparts (Hazekamp *et al*., 2004; Procaccia *et al*., 2022). Using the GC analysis method, measuring both acid and neutral cannabinoids is challenging because decarboxylation may occur in the

injection ports (Berman *et al*., 2018). As a result, only decarboxylated phytocannabinoids are measured (Berman *et al*., 2018). It has also been reported that cannabinoids may degrade (oxidize, isomerize) in the injector port and column (Berman *et al*., 2018).

Berman *et al*. (2018) suggest that metabolite extracts can be derived through silylation for measuring both acid and neutral phytocannabinoids; however, obtaining a complete derivatization yield is still challenging. Hence, LC is mostly preferred over GC as it allows for determining the cannabinoid composition of both acid and neutral derivatives without a derivatization step (Citti *et al*., 2018). HPLC has been identified as the only most suitable technique for the simultaneous analysis of acid and neutral compounds; however, it must still be coupled with UV detection to obtain the desired results (Hazekamp *et al*., 2004). However, it is also flawed as it is usually challenging to separate the entire components and thus requires multiple chromatographic runs (Hazekamp *et al*., 2004). It also becomes difficult to detect contaminations with UV detection (Hazekamp *et al*., 2004). Proton nuclear magnetic resonance ($^1$H NMR) has been identified as the solution to this challenge as it allows analysing cannabinoids without conducting any chromatographic purification, chromatographic separation or use of certified reference standards (Hazekamp *et al*., 2004; Stefkov *et al*., 2022).

A proton nuclear magnetic resonance ($^1$H NMR) analytical method has been identified as a novel approach for characterizing cannabis cultivars and is suitable for metabolic profiling as it can describe the observable chemical profile (Happyana and Kayser, 2006; Lesma *et al*., 2014). Over and above, $^1$H NMR is suggested to have high accuracy, highly reproducible within a short analysis time, and effective in analyzing large samples and requires simple steps for sample preparation (Hazekamp *et al*., 2004; Lesma *et al*., 2014; Gaafar *et al*., 2018; Stefkov *et al*., 2022).

Recently, there has been a growing importance in characterizing the chemical profile of cannabis inflorescence throughout its development, before harvesting, as it plays a crucial role in determining the time of chemical maturation of the plants (Harpaz *et al*., 2022). This is associated with the fact that the cannabinoid concentration tends to increase as the inflorescence and overall plant development progress (Harpaz *et al*., 2022). Consequently, this information is critical for determining the optimal stage with the aim of achieving the highest possible cannabinoid levels (Harpaz *et al*., 2022). However, conventional and commonly used techniques such as GC-FID and HPLC with UV detection use lab-based non-portable equipment and require sample preparation and a complex, time-consuming approach that demands specialized expertise and thus, do not allow its use for on-site analysis (Harpaz *et al*., 2022; Monari *et al*., 2023). This has resulted to numerous methods, such as electrochemical detection, being proposed as novel approach over conventional techniques for rapid screening of cannabinoids in cannabis plants (Pholsiri *et al*., 2022; Monari *et al*., 2023).

Electrochemical detection, which includes portable biosensor essays, provide on-site analysis capabilities with high sensitivity, rapid and low-cost analysis, and has proven to yield the desired results (Harpaz *et al*., 2022; Pholsiri *et al*., 2022). The study by Monari *et al*. (2023) compared two electrochemical sensors, namely screen-printed electrodes (SPEs) modified with carbon black (CB) (SPEs-CB) and SPE modified with poly-(3,4-ethylenedioxythiphene) (PEDOT) (SPE-PEDOT) electrodes, with the HPLC for detection and quantification of cannabinoids in different types of cannabis inflorescences. Their study revealed eminent correlation between the results obtained by the electrochemical method and those obtained by HPLC (Monari *et al*., 2023). Furthermore, electrochemical detection allows monitoring the THC levels to ensure that the plant material consist of the permitted levels (Pholsiri *et al*., 2022).

However, the electrochemical detection has also faced some challenges, primarily stemming from the closed oxidation potential of THC and CBD due to their similar chemical structures, which resulted to overlapping peaks (Pholsiri *et al*., 2022). Additionally, other cannabinoids such as cannabigerol (CBG) and cannabinol (CBN) were interfering with the quantification of THC and CBD (Pholsiri *et al*., 2022). However, these issues have been effectively resolved through the combination of large-pore silica gel coated on paper chromatography, which enables the high-efficiency separation of THC and CBD while minimizing interference from other cannabinoids (Pholsiri *et al*., 2022). As a result, the paper chromatography procedure has been integrated with electrochemical detection for detection and quantification of THC and CBD in cannabis oil (Pholsiri *et al*., 2022). Despite these challenges, the advantages of electrochemical detection have established it as a highly suitable on-site tool for detection and quantification of cannabinoids (Harpaz *et al*., 2022).

## 2.3. Factors critical for cannabis seed germination and seedling development

### 2.3.1. Internal factors

#### 2.3.1.1. Seed quality

Seed quality generally comprises viability, seed lot purity, health, mechanical damage, physiology, germinability, and physical characteristics (Finch-Savage and Bassel, 2016; Rahman and Cho, 2016). In the field production, a high-quality seed has strong vigour and steady germination and establishes quickly in the field (Kandpal *et al*., 2016). Furthermore, a high-quality seed is more tolerant of biotic and abiotic stresses and more efficiently utilizes available radiation and nutrients (Bianchini *et al*., 2021). As a result, these seeds produce seedlings that provide rapid soil coverage, which is advantageous for minimizing the potential negative effects of weed-crop competition (Bianchini *et al*., 2021).

However, seeds get exposed to various threats during production, including freezing and heat damage, mechanical damage, fungal infection, mildew, and insect damage (Zhang *et al*., 2020). These factors normally result in a decline in seed vitality; consequently, their germination and emergence get affected, directly impacting yield (Medeiros *et al*., 2020; Zhang *et al*., 2020). Seed viability is also influenced by various features, including plant species, seed development and maturation environmental conditions, the physiological status of the matured seed, and techniques for seed storage (discussed in depth in 3.2.1 of subsection 3.2) (Parihar *et al*., 2014).

Furthermore, seeds are prone to damage during drying, processing, and storage after harvest for later sale, which may decrease seed viability and vigour (Feng *et al*., 2018). For instance, it is reported that quality indicators of cannabis seeds are influenced by various drying factors, such as layer thickness, air flow rate, flow temperature above 60 °C, and layer mixing (Oseyko *et al*., 2021). Developing hybrids from different seed genotypes also leads to severe seed purity degradation, consequently decreasing crop yield (Huang *et al*., 2016; Zhang *et al*., 2020). The development of hybrids is a serious issue in cannabis, as there are currently hundreds of cultivars due to a long history of hybridization (Jin *et al*., 2017).

Hence, successful plant breeding and production requires high-quality seeds (Goodarzian Ghahfarokhi *et al*., 2014; Feng *et al*., 2019). Using high-quality seeds is advantageous in plant breeding as it minimizes the costs of conducting field trials and increases the likelihood of discovering a superior crop genotype (Medeiros *et al*., 2020). High-quality seed is a good start for plant growth in plant production as it can increase yield significantly, indicating an abundant harvest (Rahman and Cho, 2016; Feng *et al*., 2019).

Certification of seed quality attributes is usually accomplished by measuring seed germinability or physicochemical attributes through widely used methods, germination and vigour tests (Feng *et al*., 2018; Medeiros *et al*., 2020). However, it is difficult to screen and

categorize viable and non-viable seeds in large quantities using these conventional methods or naked eye as they are time-consuming, subjective, and destructive (Ambrose *et al*., 2016; Medeiros *et al*., 2020). Over and above, they are inadequate in detecting any changes in the chemical composition and internal anatomical features of seeds, which are characteristics that relate to seed viability and vigour (Medeiros *et al*., 2020). Following the shortcomings linked to conventional methods, there has been a desire for a method that can provide detection of seed quality in a quick, reliable, non-destructive, and objective manner (Feng *et al*., 2018; Feng *et al*., 2019; Medeiros *et al*., 2020).

Rahman and Cho (2016) suggest machine vision, spectroscopy, hyperspectral imaging, and X-ray imaging for seed quality testing. These techniques are highly recommended for collecting information on complex traits related to seed quality (Medeiros *et al*., 2020). Fourier Transform Near-Infrared (FT-NIR) spectroscopy has been previously used to determine the viability of seeds, and it detects seed compounds by acquiring a large number of spectral data details (Medeiros *et al*., 2020). Near-infrared (NIR) spectroscopy is a rapid technology previously used to examine the chemical properties of complex food matrices and intact seeds or grains (Caporaso *et al*., 2018).

On the other hand, the hyperspectral imaging technique has recently been recognized as a great analytical tool for assessing seed quality and safety in a non-destructive manner (Feng *et al*., 2019; Mukasa *et al*., 2020). It has been widely used to assess seed viability, vigour, defect, disease, and cleanness and determine seed composition (Feng *et al*., 2019). Moreover, NIR-HSI is being explored for its benefits in the seed industry to evaluate seed viability against traditional methods (He *et al*., 2019). NIR-HSI technology is also perceived to have high detection speed and is inexpensive for seed viability detection (Cui *et al*., 2020).

However, such studies on cannabis seed quality are still limited, and this has been associated with its ban for many centuries. Chumchu and Patil (2023) recently used computer vision and machine learning to classify cannabis seeds into 17 categories according to their external features, such as colour, shape, and size.

#### 2.3.1.2. Seed dormancy

Seed dormancy and germination are interrelated physiological traits significantly impacting seed plant adaptation and survival (Yan and Chen, 2020). When orthodox seeds are produced, they develop desiccation tolerance and remain dormant until the ambient environment favours germination (Yan and Chen, 2020). However, a dormant seed may not germinate even under favourable conditions (Chahtane *et al*., 2017; Yan and Chen, 2020; Longo *et al*., 2021). Seed dormancy is categorized based on the embryo's developmental state during seed dispersal, seed physical features, and/or physiological responses to environmental factors (McGinty *et al*., 2021).

According to Longo *et al*. (2020), dormancy may start during seed maturation and then be finely regulated by many transcription factors interacting in a complex molecular network that regulates hormonal levels and signaling. Plant hormones known as abscisic acid (ABA) and gibberellins (GA) are critical for inducing, maintaining, and releasing seed dormancy (Longo *et al*., 2021). These hormones act antagonistically; while ABA promotes dormancy during seed maturation and inhibits seed germination, GA promotes seed germination (Yan and Chen, 2020; Longo *et al*., 2021). Thus, when the level of ABA is reduced, and GA is increased, the seed dormancy gets released, and subsequently, germination commences (Yang *et al*., 2020). Increased GA levels after imbibition are required for the rupture of the testa and endosperm, as well as the emergence of the radicle (Yang *et al*., 2020). Thus, dormancy breaking encompasses

a net shift to increased GA biosynthesis and ABA degradation, resulting in a low ABA/GA ratio (Xu *et al*., 2020).

Independent of how seeds are released from dormancy, there have been various studies on the release of seed dormancy using different methods, and some assessing and quantifying the dormancy level of seed lots (Buijs *et al*., 2018; Pausas and Lamont, 2022). Among these methods are cold stratification, in which seeds imbibe cold for a period of time, and dry after-ripening (AR), in which the seeds are stored in a dry environment (Buijs *et al*., 2018; Chen *et al*., 2020; Elias *et al*., 2020). However, environmental factors such as temperature and soil moisture are critical in dormancy release (Pausas and Lamont, 2022). Previously, it has been demonstrated that cold stratification releasing seed dormancy varies with soil moisture content and temperature and the duration of the cold stratification exposure (Chen *et al*., 2020). It is reported that a temperature of about 0- 10 °C of cold stratification is effective for seed dormancy release (Elias *et al*., 2020). However, it is highly dependent on the species as there are also differences between species and genotypes in relation to the degree of dormancy (Elias *et al*., 2020).

Studies on cannabis seed dormancy and dormancy release, though very limited owing to illegality issues in the past centuries, have revealed that cannabis also experiences a certain level of dormancy (Jovičić *et al*., 2019). Wild cannabis, also known as feral or ditch weed, was reported to stay dormant in soils for up to 10 years and still be viable (Jovičić *et al*., 2019). The study by Elias *et al*. (2020) showed dormancy was at its peak in newly harvested seeds of 'Merlot' and 'Berry Blossom' hemp genotypes, which they could break with wet prechilling treatment at 10 °C within 5 days. Prechilling is considered hydropriming because it activates germination enzymes to initiate the breakdown and movement of stored seed reserves into embryonic axes during the treatment (Elias *et al*., 2020).

In another study, Islam *et al*. (2021a) found that wet prechilling at 4 °C for 5 days decreased the germination of 'Ferimon', 'Han NW', and 'Morpeth' hemp genotypes compared to control but enhanced shoot and root growth. Thus, even though the study by Elias *et al*. (2020) recommended prechilling seeds at 10 °C for breaking dormancy and attaining maximum germination, assessing its effect on early seedling growth parameters, such as shoot and root growth, is still critical. Besides prechilling, Islam *et al*. (2021a) found that other pre-treatment methods, such as gibberellic acid ($GA_3$) at 500 and 1000 $mg \cdot L^{-1}$ and chlorine dioxide ($ClO_2$) at 500 and 1000 $mg \cdot L^{-1}$ also decreased the germination of 'Ferimon', 'Han NW' and 'Morpeth' hemp genotypes. Similar to prechilling at 4 °C, $GA_3$ and $ClO_2$ enhanced the shoot and root growth of these genotypes compared to the control. Over and above, it was revealed that research studies assessing the germination performance of different cannabis genotypes under various pre-treatments, including phytohormones and scarification are still essential (Önol and Yildirim, 2021; Utami *et al*., 2021).

### 2.3.2. External factors

#### 2.3.2.1. Seed storage conditions

Seeds are not always intended for immediate use after collection and are often stored for varying amounts of time before being used in propagation programs (De Vitis *et al*., 2020). During prolonged storage, the seed quality, viability, and vigour decrease gradually with a change in lipid peroxidation, which is known as seed deterioration (Zheng and Ma, 2014; Kandpal *et al*., 2016; Feng *et al*., 2018). Like any other seed, the compounds, including lipids and proteins in cannabis seed, as well as other quality characteristics deteriorate during storage, entering an aging process after natural maturity and consequently affecting their further processing (Kandpal *et al*., 2016; Feng *et al*., 2018; Oseyko *et al*., 2021). Seed degradation is largely caused by free radicals-mediated lipid peroxidation, enzyme inactivation or protein degradation, cellular membrane disruption, and damage to genetic (nucleic acid) integrity

(Zheng and Ma, 2014). Seed deterioration is mainly influenced by genetics and environmental interactions during seed maturation and harvests, in which temperature, seed moisture, and relative humidity are the leading influential environmental factors (Mohammadi *et al*., 2011; Zheng and Ma, 2014; Suriyong *et a*l., 2015; Feng *et al*., 2018).

In addition, crops with high oil content have been found prone to seed deterioration due to the lower chemical stability of lipids in relation to starch (Suriyong *et al*., 2015; Prudente and Paiva, 2018). The oil content of the oil crops, such as soybean, peanut, and sunflower, is a major contributor to the reduction in seed storage potential (Suriyong *et al*., 2015). The high protein content also contributes to the reduction in seed storage potential due to the high affinity of this substance to water (Prudente and Paiva, 2018). Thus, cannabis seed is no exception as it contains an oil content of 25-35%; in comparison, sunflower possesses an oil content of 35-42%, peanut has 45-56%, and soybean has 18-22% (Suriyong *et al*., 2015). Cannabis also contains a protein content of 20-25%, whereas sunflower has a protein content of 20-40%, peanut has 22-33%, and soybean has 38-42% (Suriyong *et al*., 2015).

Cannabis seed is orthodox; thus, it can be stored in seed banks for an extended time, and its viability may not be impacted (Parihar *et al*., 2014; Suriyong *et al*., 2015). However, seeds require storage with lower temperature and humidity control as it often delays physiological and physiochemical changes and, ultimately, seed aging, thereby leading to an extended viability period (Mohammadi *et al*., 2011; Kandpal *et al*., 2016; Feng *et al*., 2018). Previous studies suggest that 5 °C and 5-7% relative humidity are the ideal conditions for the preservation of cannabis seed viability under long-term storage of 5 years and 15 °C for a storage period of up to 12 months; however, seeds must have a moisture content of 6% (Oseyko *et al*., 2020; Oseyko *et al*., 2021). Seed moisture content reportedly plays a significant role in cannabis seed storage as an 11% moisture content at 20 °C significantly reduced the

germination capacity within 18 months compared to when seeds had a moisture content of 6% (Oseyko *et al*., 2021).

Some farmers may afford to maintain such favourable conditions, with the right seed moisture content, humidity, and temperature level for storing seeds for further processing. However, such conditions are not always available for small resource-poor farmers in developing countries; hence, issues of seed deterioration have become a serious problem (Mohammadi *et al*., 2011). Resource-poor farmers from developing countries often save seeds from harvest under normal conditions for use in the following planting season (Hlatshwayo *et al*., 2021). This form of seed saving is disadvantageous as it is reported that normal storage conditions resulted in a sharp decline in seed germination energy and capacity of Ukrainian cannabis genotypes, which was completely lost after four years of storage (Oseyko *et al*., 2021). Small and Brookes (2012) also reported that the longevity of cannabis seeds decreases rapidly to about 70-80% after 2 years under uncontrolled storage conditions.

#### 2.3.2.2. Water/soil moisture

Water availability is a paramount limiting factor in crop development as seed germination begins with water uptake (Wolny *et al*., 2018; López *et al*., 2021). Therefore, an optimum moisture level is essential for imbibition, enzyme activation, embryo stimulation, reserve mobilization, and plumule emergence and elongation (Wijewardana *et al*., 2018). Reduced soil moisture leads to a reduced percentage and rate of germination and, subsequently, low seed germination (Wijewardana *et al*., 2018; Rahmah *et al*., 2020). Reduced water availability also results in high mortality rates and low crop performance during the initial stages of crop development (Tounekti *et al*., 2020). Cannabis is sensitive to soil moisture conditions and requires high moisture during its growing season, especially throughout the first six weeks of plant establishment (Cole and Zurbo, 2008; Williams and Mundell, 2018; Adesina *et al*., 2020).

Williams and Mundell (2018) suggested that a lack of soil moisture at planting hinders germination and affects the overall cannabis yield. Therefore, adequate moisture must be ensured at the germination stage to achieve maximum seed germination and early canopy closure, which is beneficial for the plant as weeds can be suppressed effectively (Cole and Zurbo, 2008).

Reduced soil moisture or water deficit, also known as drought, is largely determined by various factors, including rainfall patterns, the moisture-holding capacity of the soil, and water losses through evapotranspiration (Fahad *et al*., 2017). It threatens the success of plant establishment in areas with low rainfall, xeric soil, and high temperatures during warm seasons such as spring and summer (López *et al*., 2021). Poor germination and impaired seedling establishment are the initial manifestations of drought (Fahad *et al*., 2017). Thereafter, the extreme water deficit disrupts growth, nutrient and water relations, photosynthesis, and assimilate partitioning in plants (Babaei and Ajdanian, 2020). It eventually leads to the death of the plant and, ultimately, a significant reduction in crop yields through the negative impacts of water stress on plant growth, physiology, and reproduction (Fahad *et al*., 2017; Babaei and Ajdanian, 2020).

In cannabis, Du *et al*. (2022) reported that water stress induced through 20% PEG reduced the germination rate and germination potential and delayed radicle and hypocotyl growth in hemp seedlings of 'Yunma 1' YM and 'Bamahuoma' BM. In 'Black Label', an industrial hemp cultivar, water deficit reduced biomass, seed yield, and water potential (Gill *et al*., 2022). Notably, 'Black Label' did not increase the root production relative to shoot under water stress like other plants (Gill *et al*., 2022). Thus, hemp may have yet undiscovered alternative mechanisms for optimizing water use within the plant without allocating a higher proportion of biomass to the roots (Gill *et al*., 2022). Water stress, together with continuous lighting, has been found beneficial for seed ripening in cannabis (Schilling *et al*., 2023).

#### 2.3.2.3. Salinity

Salinity is a serious soil degradation arising from natural causes and human-mediated activity, including irrigation in arid and semi-arid regions (Mohammad *et al*., 2014). Salinity severely affects plant growth and productivity through water deficit and ionic toxicity (Voigt *et al*., 2009; Al-Musa *et al*., 2012; da Silva *et al*., 2019). The first effect of high salinity is a decrease in soil water potential and thus restricting water uptake by the seeds, which delays seed imbibition and radicle protrusion (Voigt *et al*., 2009; da Silva *et al*., 2019). As seeds germinate, they absorb saline ions, which trigger toxic effects on several biochemical and physiological processes, delaying the emergence of seedlings and increasing mortality (Voigt *et al*., 2009). Salinity stress also leads to overproduction of reactive oxygen species (ROS) and eventually to toxicity which is linked to their reactions with numerous cell components (Wang *et al*., 2012; Sharma *et al*., 2013). This toxicity then causes a cascade of oxidative reactions and the consequent inactivation of enzymes, lipid peroxidation, protein degradation, and DNA damage (Wang *et al*., 2012). Together, these effects restrict growth and development and thus reduce crop yields (Krasensky and Jonak, 2012; Sarabi *et al*., 2017).

Hemp is known for its high tolerance to saline environment stress due to its wide distribution, deep root system, fast growth, high biomass, strong resistance to stress, and ease of cultivation (Cheng *et al*., 2016). However, salt stress tolerance in various species, including hemp, varies with the salt type and salt concentration and among genotypes and with plant growth and development stages, affecting alterations in plant morphology, anatomy, and physiology (Cheng *et al*., 2016; Hu *et al*., 2018; Di Mola *et al*., 2021). It also varies with plant growth and development stages, affecting alterations in plant morphology, anatomy, and physiology (Cheng *et al*., 2016; Di Mola *et al*., 2021). Furthermore, salt tolerance is associated with seed reserve mobilization, and the tolerant seeds reportedly tend to accumulate more reserves (Hu *et al*., 2018). For instance, salt stress negatively affected seed germination and seedling

development of the ‘Yunma 5’ and ‘Bamahuoma’ hemp genotypes through inhibition of germination rate and the length of the radical and hypocotyl in the study conducted by Hu *et al*. (2018). However, ‘Yunma 5’ was found to be more salt tolerant as the germination rate of this genotype was not significantly reduced by the salinity levels up to 150 mM NaCl and $Na_2SO_4$, whereas that of ‘Bamahuoma’ was significantly reduced at salinity level as low as 100 mM NaCl and 50 mM $Na_2SO_4$ (Hu *et al*., 2018).

Furthermore, radicles and hypocotyls at each salt treatment of $Na_2CO_3$, $NaHCO_3$, $Na_2SO_4$, and NaCl were longer in ‘Yunma 5’ compared to in ‘Bamahuoma’. However, the radicle and hypocotyl growth were more salt-sensitive compared to the germination rate as the salt concentration increased to 300 mM (Hu *et al*., 2018). The salt stress tolerance also varied with salt type among the genotypes, such as saline and alkaline; however, both genotypes exhibited alkali sensitivity (Hu *et al*., 2018). This is because sodium ions in salt stress cause ion toxicity and reduce the osmotic potential, leading to osmotic stress and, eventually, plant metabolic disorders (Fang *et al*., 2021). On the other hand, alkali stress induced by $NaHCO_3$ and $Na_2CO_3$, the most commonly used compounds, results in similar effects as saline stress. However, it further increases the pH, which typically disturbs the cell pH stability, destroys cell membrane integrity, and reduces root vitality and photosynthetic function (Fang *et al*., 2021). Moreover, Yep *et al*. (2020) found that the yield and potency of the drug-type cultivar ‘Nordle’ decreased with continuous root exposure to increasing NaCl concentrations.

#### 2.3.2.4. Temperature

The environmental conditions to which the plants are exposed often influence the ecophysiological responses, and among the key factors for seed germination is the temperature (Zucareli *et al*., 2015). Temperature influences seed germination rate and maximum seed germination in three ways: seed aging, dormancy loss, and germination (Gajanayake *et al*.,

2011). Temperature is also a modifying factor as it influences the germination percentage and rate by affecting the imbibition rate and mobilization of storage reserves (Sikder *et al*., 2009; Zucareli *et al*., 2015; Reis *et al*., 2020). Furthermore, it influences the supply of substrates necessary for seed reserve mobilization, including water, and consequently affects plant growth and development (Sikder *et al*., 2009). The mobilization of seed reserves and seed germination also vary in different temperature regimes; hence, the temperature is a modifying factor in germination (Sikder *et al*., 2009).

Cannabis is an annual summer crop; thus, it grows well in warm temperatures averaging between 15 and 27 °C (Humphries and Florentine, 2019). The study by Kumar *et al*. (2020) reports that the optimum germination temperature for the *C. sativa* of 'Kausani' accession was 25 °C. On the other hand, a study by Byrd (2019) found that the tested hemp cultivars showed a difference in temperature and origin as germination percentage decline at temperatures above 25 °C was observed for Canadians and at about 30 °C for Northern European cultivars. Byrd (2019) also found that the germination percentage declined rapidly for all cultivars at temperatures near 40 °C and displayed limited germination capacity at temperatures as high as 45 °C. Generally, extreme and fluctuating temperatures are critical in the sense that they restrict plants' distribution, adaptability, and yield potential (Gajanayake *et al*., 2011). High temperature can reduce the germination potential of the seeds as a result of damage to the proteins, interrupted protein synthesis, inactivation of major enzymes, and damage in membranes, resulting in poor germination and stand establishment (Fahad *et al*., 2017). High temperature can also majorly affect the process of cell division and favour oxidative damage (Fahad *et al*., 2017). Furthermore, it is reported that high temperatures may accelerate maturity by inducing flowering, resulting in stunted cannabis growth (Humphries and Florentine, 2019). Low soil temperatures at sowing also tend to influence plant populations and may result in poor

standing, and notably, cannabis is intolerant to frost (Cole and Zurbo, 2008; Gajanayake *et al*., 2011; Humphries and Florentine, 2019).

Over and above, temperature is also a limiting factor in cannabis growth as it affects net photosynthesis ($P_N$) (Jin *et al*., 2019). Low temperatures slow $P_N$, and the $P_N$ stops when there is excessive heat, resulting in plants using up energy in cooling under high temperatures by acquiring water and transpiring it through the stomata (Jin *et al*., 2019).

#### 2.3.2.5. Lighting

Light influences germination and other aspects of plant development and physiology and the content of its pigments and other metabolites (Chandra *et al*., 2015; Jin *et al*., 2019; Namdar *et al*., 2019). Light features, such as light quantity (intensity), light quality (spectrum), and photoperiod (the duration of exposure to light), affect the functions of the plant through the regulation of photosynthesis, photoperiodism, and photomorphogenesis (Magagnini *et al*., 2018; Wei *et al*., 2021; Cheng *et al*., 2023). Cannabis is a photophilic plant, and thus, its growth and development are directly influenced by light intensity and quality variations (Cheng *et al*., 2022; Li *et al*., 2023). For instance, the study conducted by Rodriguez-Morrison *et al*. (2021) found that the yield in relation to harvest index and the size and density of the apical inflorescence in cannabis increased with the increasing light intensity. Light intensity also greatly influences the synthesis of plant metabolites. Notably, Rodriguez-Morrison *et al*. (2021) also found that light intensity had no effects on the potency of cannabinoids and had minor effects on the potency of terpenes (Rodriguez-Morrison *et al*., 2021).

The cannabis plant is sensitive to photoperiod and is a short-day plant, which strongly influences the productivity of different crop genotypes (Adesina *et al*., 2020; Ahrens *et al*., 2023). Cannabis requires 2 days of darkness for radicle protrusion, whereas seedlings require continuous light for 2 weeks to promote maximum biomass accumulation (Bilodeau *et al*.,

2019; Pierroz, 2023; Schilling *et al*., 2023). The cannabis plant also measures the length of daylight falling onto its leaves to regulate its development and flower production (Thomas, 2012). It is reported that cannabis requires 12 h of uninterrupted darkness to transition from the vegetative to the flowering stage (Thomas, 2012; Chandra *et al*., 2017; Bilodeau *et al*., 2019; Schilling *et al*., 2023). The light must then be reduced when the seedlings have fully grown into a flowering stage. After that, continuous lighting is required for seed ripening (Schilling *et al*., 2023).

However, the effect of light generally varies with several factors, including the photoperiod sensitivity of a genotype. Schilling *et al*. (2023) found that photoperiod-insensitive cannabis cultivars of 'Finola' flowered under different light regimes of ultra-short days (8:16), short days (12:12), long days (16:8), and continuous light (24:0), While on the other hand, the photoperiod-sensitive cannabis plants of 'Fedora 17' and 'Felina 32' cultivars flowered shortly after exposure to 12 h of uninterrupted darkness, with reduced or no flowering under continuous light and long days. Furthermore, light impacted the physiology of the photoperiod-sensitive cannabis plants, as plants produced under ultra-short days were shorter and feebler than those grown under short days, long days, and continuous light (Schilling *et al*., 2023). Moreover, 'Fedora 17' reportedly yielded the highest number of seeds under short-day conditions with no seed setting under continuous light. On the contrary, 'Finola' yielded the highest number of seeds under continuous light compared to the other light regimes.

Notably, specific light wavelengths enable plants to adapt and modify their biological cycle, and plants use different types of photoreceptors, including phytochromes, to perceive changes in light quality (Demotes-Mainard *et al*., 2016). Blue light has been recognized as the best quality light for hemp as it has been found to promote growth parameters, such as fresh and dry biomass of the shoot, number of leaves per plant, stem diameter, root length, and chlorophyll content (Cheng *et al*., 2022; Cheng *et al*., 2023). It also promotes net

photosynthesis, stomatal conductance, and transpiration (Cheng *et al*., 2022; Cheng *et al*., 2023). Supplemental blue light has also been found to increase cannabinoid concentration (% *w/w*), and green light leads to the accumulation of both cannabinoids and terpenes (Morello *et al*., 2022). Wei *et al*. (2021) also found that the higher proportion of blue light promoted the synthesis and accumulation of cannabinoids in hemp.

As a result, most commercial production of cannabis occurs under greenhouses with supplemental electric lighting or growth chambers with sole source electric lighting to ensure sufficient light is provided for the plants (Hawley *et al.*, 2018; Trancoso *et al*., 2022). Light wavelengths/spectrum is one of the important factors in controlled cultivation as it regulates growth, development, and the accumulation of metabolites (Sng *et al*., 2021). In the cultivation of cannabis under a controlled environment, various lamps, such as high-pressure sodium (HPS) lamps and light-emitting diodes (LEDs), have been used to provide the required light spectrum (Trancoso *et al*., 2022). However, LEDs have been widely used for their versatile mobility, narrow spectrum range, energy efficiency, and longevity compared to HPS lamps (Wei *et al*., 2021; Morello *et al*., 2022).

Artificial lighting and light spectrum influence plant development and the profile of terpenes and cannabinoids (Potter and Duncombe, 2012; Danziger and Bernstein, 2021a; Morello *et al*., 2022). Terpenes represent the largest group of cannabis phytochemicals found abundantly on the surface of the female inflorescence (Calvi *et al*., 2018). They are responsible for the unique aromatic properties and influence the flavour quality of cannabis inflorescence and its therapeutic effect (Calvi *et al*., 2018). Lighting has been recognized for producing cloned female cannabis yielding a drug product of consistently high potency (Potter and Duncombe, 2012; Morello *et al*., 2022). Higher cannabinoid concentration has been found under LED light compared to HPS treatment (Magagnini *et al*., 2018). As previously mentioned in subsection 2.2, an irradiation with UV-B at a wavelength of 280- 320 nm has also been found to increase

the THC content of drug-type cannabis genotypes (Lydon *et al*., 1987; Potter and Duncombe, 2012). This is associated with the fact that plants use the wavelength of blue light and UV to synthesize carotenoids and anthocyanins (Islam *et al*., 2021b). Generally, UV-B radiation penetrates plant tissues, inducing DNA damage, photoinhibition, and lipid peroxidation, and resulting in growth, physiology, and productivity retardation (Lydon *et al*., 1987; Chen *et al*., 2018; Islam *et al*., 2021b). The plants then produce secondary metabolites in higher concentrations, in this case, cannabinoids, which absorb and prevent actinic UV-B radiation (Lydon *et al*., 1987; Ning *et al*., 2012; Chen *et al*., 2018; Takshak and Agrawal, 2019). However, the response of cannabis to spectral changes varies with cultivars (Danziger and Bernstein, 2021a; Li *et al*., 2023).

Various studies have investigated the influence of light intensity, spectrum, and photoperiodism on cannabis growth. This includes the study conducted by Livadariu *et al*. (2019), where they reported that LED treatments were most effective in promoting fresh weight and sprouting in hemp compared with the control (sunlight). However, such information is lacking, or little is known with respect to the effect of light on cannabis germination parameters. Yet, plants generally respond to light variations for the completion of their life cycle, including seed germination, while different colours of light influence differently on plant growth stages (Cheng *et al*., 2022). Furthermore, the light requirement may act as a mechanism that indicates conditions suitable for seed germination (Motsa *et al*., 2015). This is why some seeds germinate better under only light or darkness, and some seeds can germinate either under light or darkness (Motsa *et al*., 2015; Flores *et al*., 2016). Meeting the light requirement is also important as it prevents the depletion of stored seed reserves and, thus, aids the plant species to survive (Motsa *et al*., 2015).

## 2.4. Conclusion and future prospects

The physiology of cannabis germination and seedling establishment is not very well known due to limited research studies on the crop resulting from being banned for centuries owing to its psychoactive compound THC. The review provides useful information for choosing genotypes for application in different industries to meet the growing demand of the pharmaceutical industry and assist growers in meeting the purity standards of the target market. It also highlights the effect of intrinsic and extrinsic factors on seed germination and seedling development and their impact on the final product in terms of yield and chemical composition. Furthermore, it highlights the progressive initiatives in detection and quantification of cannabis chemical profile from inflorescence and plant development to harvested material. Through this overview, it is clear that research on the classification of different genotypes is still much needed, not only to determine the end-use product but to determine the response of genotypes to intrinsic and extrinsic factors during germination and seedling development on the morphological and biochemical level.

Notably, few studies have quantified the chemical composition of cannabis using highly sought-after advanced technologies such as $^{1}$H NMR. Thus, this overview suggests that these technologies should also be used for classifying cannabis genotypes, as it is known that chemical profiling has been useful in classifying cannabis genotypes. The use of technology devices such as NIR-HSI for cannabis classification is also recommended, as they have the potential to differentiate genotypes and determine sex at a seed level effectively. This is critical in cannabis to minimize the yield losses, labour required, and high costs of cannabis cultivation. Furthermore, this overview suggests that SEM is incorporated into the investigations on cannabis classification. This follows that SEM reportedly has a high potential for reviewing phenetic linkages and classification of various crop species. This is because cannabinoid composition and non-chemical characteristics have been previously used to categorize

cannabis species, and there is still ongoing evaluation. Therefore, seed morphology seems prospective to solve taxonomy-related issues, hence the suggestion of SEM research.

Determining seed quality before planting is also an important factor, just as genotype classification, for determining seed vitality, germination, and emergence and achieving high yields. Thus, the use of advanced non-destructive techniques is recommended to collect information on complex traits related to seed quality and to reduce the turnaround time. Therefore, this overview recommends investigating the efficiency and effectiveness of machine learning, spectrometry, X-ray imaging, FT-NIR, NIR spectroscopy, and hyperspectral imaging in detecting cannabis seed quality. The investigations can further incorporate different storage conditions to study their influence on seed quality, germination, and plant development. However, these investigations should be mindful of inequalities among cannabis growers in relation to access to controlled storage conditions and the rising climate change, and therefore, must consider a wide spectrum of storage environmental variations.

Besides seed quality, cannabis germination is influenced by the embryo's developmental state, nutrients stored in the endosperm or embryo, and environmental factors. However, there are very limited studies on these factors in cannabis. Therefore, this overview recommends investigations on cannabis dormancy and different techniques for releasing seed dormancy and their interaction with environmental conditions. It also recommends studies on hydrolysis and mobilization of seed reserves during germination and how these processes are influenced by intrinsic factors as well as extrinsic factors, especially considering the ever-increasing climate change.

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**Sabeliwe Langa, Lembe S. Magwaza, Asanda Mditshwa and Samson Z. Tesfay**.: Supervision, **Lembe S. Magwaza, Asanda Mditshwa and Samson Z. Tesfay**.: Project administration, **Sabeliwe Langa**.: Funding acquisition, **Sabeliwe Langa**. All authors have read and agreed to the published version of the manuscript.

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# 3. CHAPTER 3: GENETIC AND PHENOTYPIC DIVERSITY IN CANNABIS GENOTYPES: INSIGHTS FROM SEED DIMENSIONS, MINERAL PROFILES, AND SHORT TANDEM REPEATS (STR) MARKERS

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**Abstract**

This study aimed to characterize cannabis genotypes based on seed physical properties, mineral element composition and Short Tandem Repeats (STR) markers. Eleven cannabis genotypes were analyzed, including landraces Ladysmith Ugwayi wesiZulu ('L1') and Iswazi ('L2'), Durban Poison ('H1'), Bergville Ugwayi wesiZulu ('B1'), Natal ('B2') and Iswazi ('B3'), and Msinga Ugwayi wesiZulu ('M1') and Iswazi ('M2'), and the commercially available 'Hemp', 'Cherry bubble gum' ('High-CBD') and 'White Rhino' ('High-THC') genotypes. To characterise these genotypes, measurements were taken of physical traits including seed length, width, thickness, geometric mean diameter, surface area, aspect ratio, and sphericity. Sixty-eight genetic markers were selected for genetic analysis, and the mineral element composition was examined using a Scanning Electron Microscope (SEM) coupled with an energy-dispersive X-ray (EDX) detector. Significant differences ($p$<0.001) were found among genotypes for seed dimensions and surface area, while sphericity and aspect ratio showed no significant differences ($p$>0.05). Variations in mineral composition were also observed, with

significant differences ($p$<0.05) for elements such as carbon, iron, potassium, oxygen, phosphorus, sulfur, and silicon. All genotypes primarily contained carbon and oxygen, with 'High-THC' having the highest carbon (75.10%), while 'H1' had the lowest (55.58%). Conversely, 'H1' had the highest oxygen level (42.82%) whereas 'High-THC' had the lowest level (23.79%). Positive correlations ($p$<0.001) between different genotypes indicated similarities in physical traits, mineral composition, and STR markers. However, hierarchical clustering revealed distinct groupings among genotypes, suggesting a complex diversity pattern beyond the observations made in physical traits, STR markers, and mineral composition. The genotypes, particularly the landraces, could not be categorized as 'High-CBD', 'High-THC', or 'Hemp' based on the measured parameters. Observed variations in seed dimensions and mineral profiles highlight the diverse genetic and phenotypic landscape of the tested samples. These findings could potentially influence breeding programs by guiding decisions aimed at optimising desirable traits.

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**Keywords**: Cannabis, mineral element, seed genotype, sphericity, short tandem repeats (STR)

### 3.1. Introduction

Cannabis has enormous phenotypic diversity due to human selection, resulting in over 700 genotypes around the world (Vergara *et al*., 2016; Woodbridge, 2018; Monti *et al*., 2022). However, research on this crop was limited in the past centuries due to legal restrictions owing to cannabis's intoxicating compound delta-9-tetrahydrocannabinol ($\Delta^9$-THC), preventing researchers from investigating cannabis and making use of its full potential (Adhikary *et al*., 2021). Consequently, the taxonomic classification of cannabis is still under evaluation. Cannabis genotype characterization has become even more crucial for clinical research and agricultural improvement programs as public perception and laws in many nations have begun acknowledging cannabis as a plant of both medical and agricultural value (Sawler *et al*., 2015).

Generally, cannabis cultivars are categorized through non-chemical characteristics, such as plant morphological and physiological features, as well as widely accepted chemotaxonomy based on cannabinoid composition (de Meijer, 1994; Cerrato *et al*., 2021). To date, scientists have identified five distinct chemotypes that currently exist in cannabis, which are chemotypes I, II, III, IV, and V (Broseus *et al*., 2010; Piluzza *et al*., 2013; Khan *et al*., 2014; Schluttenhofer and Yuan, 2017; Soorni *et al*., 2017; Cerrato *et al*., 2021). Chemotype I represents high THC genotypes with less than 0.5% cannabidiol (CBD) and 1% to over 20% $\Delta^9$-THC (Broseus *et al*., 2010; Piluzza *et al*., 2013; Khan *et al*., 2014; Soorni *et al*., 2017; Cerrato *et al*., 2021). Chemotype III includes high CBD genotypes with below 0.3% $\Delta^9$-THC, and chemotype II has equal amounts of $\Delta^9$-THC and CBD (Broseus *et al*., 2010; Piluzza *et al*., 2013; Khan *et al*., 2014; Schluttenhofer and Yuan, 2017). Chemotype IV is predominant in cannabigerol (CBG), and Chemotype V has no detectable phytocannabinoids (Cerrato *et al*., 2021; Langa *et al*., 2024).

On the other hand, the non-chemical attributes involved morphological and physiological features, resulting in three main groups: drug hemp, oilseed hemp, and fibre hemp (de Meijer, 1994; Salentijn *et al*., 2015). However, these characteristics are largely influenced by domestication and climatic adaptation (de Meijer, 1994). The number of cannabinoids in cannabis plants can also vary greatly depending on harvest time, storage conditions, genetic factors, as well as morpho-spatial position in the plant and crop management practices (Tipparat *et al.*, 2014; Danziger and Bernstein, 2021; Toth *et al*., 2021; De Prato *et al*., 2022; Song *et al*., 2023).

Furthermore, most cannabis genotypes commonly found today are hybrids of *C. sativa* and *C. indica*, making it difficult to differentiate them based solely on their morphological characteristics (Jin *et al*., 2015; Lapierre *et al*., 2023). Therefore, seed genotype identification has the potential to address these challenges since seeds of different genotypes under the same

plant species possess distinct internal chemical compositions, external morphological characteristics, and commercial value (Feng *et al*., 2018; Rashid *et al*., 2018; Zhang *et al*., 2020; Rashid *et al*., 2021). In cannabis, Naraine *et al*. (2020) studied the morphological divergence of seeds among ruderal, fibre, oilseed, dioecious/monoecious, and marijuana variants using a stereoscope. These researchers found that the seeds of hemp or fibre cultivars are larger compared to marijuana seeds and those of oilseed cultivars, which was associated with the fibre cultivars having poor seed production, leading to more energy being channelled into the seeds (Naraine *et al*., 2020; de Meijer, 1994).

Information about the physical properties of seeds plays a critical role in designing and modelling equipment for seed handling, transporting, planting, processing, marketing, and storage (Gharibzahedi *et al.*, 2009; Munder *et al*., 2017; Sastry *et al*., 2019; Gojiya *et al*., 2020; Saini *et al.*, 2022). Besides seed physical properties, there are other various techniques for seed genotype identification, including high-performance liquid chromatography (HPLC), protein electrophoresis, near-infrared hyperspectral imaging (NIR-HSI), and DNA molecular marker technology (Zhang *et al*., 2018; He *et al*., 2019; Cui *et al*., 2020; Jin *et al*., 2022; Xu *et al*., 2022; Zhang *et al*., 2024). Every cultivar has a unique DNA fingerprint according to DNA-based analysis, and molecular marker-based fingerprinting is non-tissue specific and is not influenced by environmental factors (Dalusong *et al*., 2019). Therefore, molecular markers are one of the essential seed genotype identification techniques in crop development as they provide a new degree of accuracy, precision, and perfection in germplasm screening (Kachare *et al*., 2020).

Genotype identification can be achieved by using carefully selected DNA-based markers such as microsatellites, also known as short tandem repeats (STR) or simple sequence repeats (SSRs) (Dalusong *et al*., 2019). Microsatellites are more dependable than other marker systems in establishing high-fidelity profiles not only because of their co-dominant nature but also their

chromosomal selectivity (Dalusong *et al*., 2019). Cascini *et al*. (2019) sequenced the tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) genes in 21 cannabis genotypes to distinguish drug-type from fiber-type cannabis. Cascini *et al*. (2019) successfully differentiated between the two types in seeds and early plant development stages, achieving a perfect Area Under the Curve (AUC) of 100%.

Cannabis seeds, particularly hemp have been traditionally used for food and feed owing to their remarkable nutritional content, and their consumption is increasing nowadays (Alonso-Esteban *et al*., 2022; Saini *et al.*, 2022). These seeds are composed of an oil content of 25 – 35%, protein content of 20 – 25%, and carbohydrates ranging between 20 – 30% (Suriyong *et al*., 2015; Kaliniewicz *et al.*, 2021; Teleszko *et al*., 2022). Cannabis seeds are also composed of phytic acid, choline, trigonelline, lecithin, chlorophyll, vitamins B, C, E, and K, tocopherols, and are a valuable source of mineral components such as iron, calcium, zinc, phosphorus, potassium, and magnesium (Mihoc *et al*., 2012; Kaliniewicz *et al.*, 2021; Teleszko *et al*., 2022). Considering their remarkably high nutritional value, cannabis seeds may also be used for biofortification, which is the process of enhancing nutrition with certain micronutrients (Garg *et al*., 2018; Stonehouse *et al*., 2020).

Biofortification of crops holds significant global significance in providing micronutrients to communities with limited access to diversified diets and alternative micronutrient interventions (Garg *et al*., 2018; Stonehouse *et al*., 2020). However, the mineral composition of the hempseeds is influenced by both the genotypes and environmental factors (Rehman *et al*., 2019; Farinon *et al*., 2020). Therefore, it is fundamental to search for genotypes with the desirable mineral content. Even though studies have been conducted on cannabis in relation to the characterization of genotypes based on physical traits, molecular markers, and mineral composition, it is worth noting that such research work is still very limited in the South African context.

Characterization of genotypes based on physical traits, molecular markers, and mineral composition is very critical, considering that the uplifting of the regulations controlling the cultivation of cannabis is specifically for hemp and drug type for medicinal purposes (Gouws, 2017; Citti *et al*., 2018; Allaf *et al*., 2023). Furthermore, seed genotype identification is very critical for achieving optimum yield potential and securing a uniform product (Bai *et al*., 2020; Taheri-Garavand *et al*., 2021; Tu *et al*., 2021; Xu *et al*., 2022). It is also the foundation of post-harvest processes, including drying, hulling, transportation, or mechanical oil extraction (Munder *et al*., 2017; Pérez-Barcena *et al*., 2021). Additionally, genotype identification is crucial for crop development and registration processes, seed production, trade, and inspection (Korir *et al.*, 2013). Therefore, this study seeks to distinguish cannabis genotypes based on their physical properties, STR markers, and mineral element composition.

## 3.2. Materials and methods

### 3.2.1. Genotype collection and experimental design

Eleven cannabis genotypes were used for genotype characterization, of which nine of the genotypes were landraces collected from local growers in four regions of KwaZulu-Natal Province in South Africa, namely: Bergville, Hammersdale, Ladysmith, and Msinga. The other three genotypes used as reference are ‘Hemp’, ‘Cherry bubble gum’ (‘High-CBD’), and ‘White Rhino’ (‘High-THC’), obtained from a seed supplier in Johannesburg, Durbanseedco under Grohydro (Pty) Ltd and Nirvana Shop South Africa, respectively. The collected cannabis landraces were assigned pseudonyms to ensure uniform naming of landraces for this experiment, as detailed in Table 3.1.

**Table 3.1:** The pseudonyms of the cannabis landraces and their geographic location information

| Location | Geographic information | | | | | Common local name | Name used for the experiment |
|---|---|---|---|---|---|---|---|
| | Latitude: | Longitude: | Elevation | Average rainfall | Average temperature | | |
| Ladysmith | -28.57533 | 29.85948 | 1009.53 m | 108.7 mm | 23.3 °C | 'Ugwayi wesiZulu' | 'L1' |
| | | | | | | 'Iswazi' | 'L2' |
| Msinga | -28.700960 | 30.303320 | 559 m | 340.0 mm | 23.0 °C | 'Ugwayi wesiZulu' | 'M1' |
| | | | | | | 'Iswazi' | 'M2' |
| Bergville | -28.677544 | 29.125553 | 1137.86 m | 109.4 mm | 23.5 °C | 'Ugwayi wesiZulu' | 'B1' |
| | | | | | | 'Iswazi' | 'B3' |
| | -28.667679 | 29.034111 | | | | 'Natal' | 'B2' |
| Hammersdale | -29.870903 | 30.633519 | 597 m | 110.3 mm | 23.7 °C | 'Durban Poison' | 'H1' |

The collected seed samples were stored at room temperature in the Horticultural Sciences Laboratory of the University of KwaZulu-Natal, Pietermaritzburg (latitude: -29.636311 and longitude: 30.409060). Seeds showing defects were removed from the seed lot, and then the remaining seeds were sorted based on colour. Dark-coloured seeds were associated with maturity, whereas greenish-coloured seeds were associated with immaturity (Miraji *et al.*, 2021). Therefore, dark-coloured seeds were used for the experiment laid out in a completely randomized design (CRD).

### 3.2.2. Physical parameters of seeds

The evaluation of the physical traits of the seeds of different cannabis genotypes was carried out at the Seed Technology Laboratory, University of KwaZulu-Natal, South Africa, following the procedures by Perea-Flores *et al*. (2011) and Gojiya *et al*. (2020), with some modifications. Briefly, the length (L), width (W), and thickness (T) of the seeds, all in mm, were measured using digital calliper (150 mm (6"), China). Twenty randomly selected seeds of each genotype were measured for all dimensions, and the average values of L, W, and T were recorded.

The results of the three axial dimensions, namely, L, W, and T, were then used for further calculation of other physical properties like geometric mean diameter, surface area, aspect ratio, and sphericity, following the equations by Perea-Flores *et al*. (2011) and Gojiya *et al*. (2020).

The geometric mean diameter ($D_g$) was calculated by using Eq. 1 as follows:

$$D_g(mm) = (LWT)^{1/3} \quad (1)$$

where L is the length, W is the width, and T is the thickness in mm.

The results obtained from the geometric mean diameter were used to calculate the surface area (S) by using Eq. 2 as follows:

$$S\ (mm^2) = \pi {D_g}^2 \quad (2)$$

The aspect ratio (Ra) was calculated as the ratio of the width to its respective length using Eq. 3 as follows:

$$Ra = \frac{W\ X\ 100}{L} \quad (3)$$

where, W = Width and L = Length in mm.

The sphericity ($\phi$) was calculated as the ratio of the geometric mean diameter to the length of the seed by using Eq. 4 as follows:

$$\phi = \frac{D_g}{L} \quad (4)$$

where $D_g$ = geometric mean diameter and L is the length in mm.

### 3.2.3. Quantification of mineral elements using scanning electron microscopy

The mineral element concentration and composition of the endosperm of seeds from different cannabis genotypes were evaluated. The elements analyzed included aluminium (Al), carbon (C), calcium (Ca), chlorine (Cl), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), nitrogen (N), sodium (Na), oxygen (O), phosphorus (P), sulfur (S), and silicon (Si). The mineral element profiling was carried out following the procedure by Perea-Flores *et al*. (2011), with some modifications. Briefly, five seeds of each genotype were cryo-fractured in liquid nitrogen and divided into longitudinal and transversal sections. The halves of each seed were mounted on stubs and secured using carbon double-sided insulating tape. The seeds were gold-coated using a Quorum Q150R ES Gold Sputter Coater (Quantum Design GmbH, Breitwieserweg 9, 64319 Pfungstadt, Germany). The microanalysis of the minerals on the endosperm of the seeds was then conducted using a Zeiss Ultra Scanning Electron Microscope (SEM) coupled with an energy-dispersive X-ray (EDX) detector (Zeiss, Oberkochen, Germany). The EDX detector was set at an active area ranging from 8 to 10 $mm^2$. It was also used to perform the X-ray mapping technique, which constituted using pseudo-colours to represent the bi-dimensional spatial distribution of the energy emission of the chemical elements of the sample. Five fields of view, 100.0 $\mu m^2$ each, were analyzed for 60 s at a 20 kV accelerating voltage and at a count rate of 1000-3000 cps. The results obtained from the EDX analysis were processed using Oxford INCA & Aztec EDX Analysis Software. The elemental distribution from the X-ray mapping technique is presented in Figure 3.1, with each

element represented by a specific colour. This mapping provides visual insight into the spatial distribution of elements such as C, Fe, K, O, P, S, Si, Al, Ca, Cl and Mg, which is essential for understanding the mineral element composition in cannabis seeds. Mineral elements such as Cu, Mn, N and Na were detected in only one or two genotypes, with each element found in one field of view per genotype out of a total of 11 genotypes and 5 fields of view. Due to their irregular occurrence and insufficient data, these elements were excluded from further analysis. The proportion of each element was then quantified in percentage of the total mineral concentration in each sample (wt%).

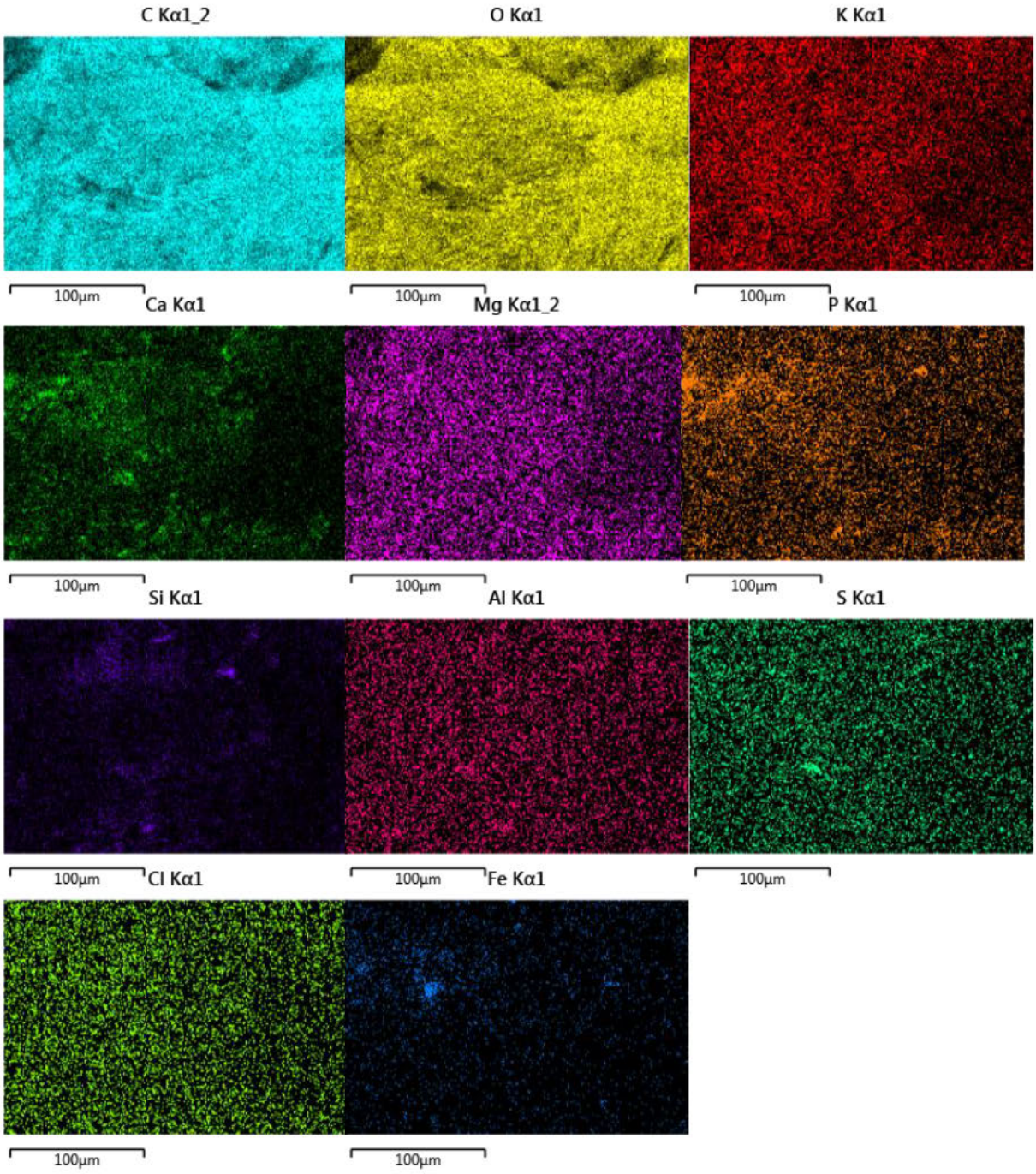



**Figure 3.1**: Scanning electron microscopy micrographs of the endosperm cell elemental distribution by X-ray mapping technique from EDX analysis spectrum. **C** (carbon), **O** (oxygen), **Cl** (chlorine), S (sulfur), K (potassium), P (phosphorus), **Ca** (calcium), **Al** (aluminum), **Si** (silicon), **Fe** (iron), **Mg** (magnesium).

### 3.2.4. STR markers analysis

#### 3.2.4.1. DNA extraction

The DNA extraction was performed using the Quick-DNA™ Plant/Seed Miniprep Kit (Catalog No. D6020, Zymo Research Corp, 17062 Murphy Ave., Irvine, CA 92614, U.S.A.), following the manufacturer's instructions. Briefly, 150 mg of finely cut seed was added to a ZR BashingBead™ Lysis Tube (2.0 mm) along with 750 µL of BashingBead™ Buffer. The tube was tightly capped, secured in a bead beater with a 2 mL tube holder assembly, and processed at maximum speed for at least 5 min. The lysis tube was centrifuged at ≥10000 x *g* for 1 min. The supernatant was transferred to a Zymo-Spin™ III-F Filter in a collection tube and centrifuged at 8000 x *g* for 1 min. Thereafter, 1200 µL of Genomic Lysis Buffer was added to the filtrate in the collection tube and mixed well. Then, 800 µL of the mixture was transferred to a Zymo-Spin™ IICR Column in a collection tube and centrifuged at 10000 x *g* for 1 min. This step was repeated twice, discarding the flow-through each time. Following this, 200 µL of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IICR Column in a new collection tube and centrifuged at 10000 x *g* for 1 min. Subsequently, 500 µL of g-DNA Wash Buffer was added to the column and centrifuged at 10000 x *g* for 1 min. The Zymo-Spin™ IICR Column was then transferred to a clean 1.5 mL microcentrifuge tube, and 100 µL of DNA Elution Buffer was added directly to the column matrix. The sample was centrifuged at 10000 x *g* for 30 sec to elute the DNA. A Zymo-Spin™ III-HRC Filter was placed in a clean collection tube, and 600 µL of Prep Solution was added before centrifuging at 8000 x *g* for 3 min. Finally, the eluted DNA was transferred to the prepared Zymo-Spin™ III-HRC Spin Filter in a clean 1.5 mL microcentrifuge tube and centrifuged at 16000 x *g* for 3 min. The samples were then immediately analyzed.

### 3.2.4.2. Polymerase chain reaction (PCR) primers and multiplex conditions

Based on information from the literature, sixty-eight genetic markers of the STR type, representative of cannabis, were selected for the genetic analysis of cannabis genotypes. PCR amplification was conducted in an Eppendorf® Mastercycler® Nexus gradient thermocycler (Eppendorf® 6331000025, Eppendorf AG, Hamburg, Germany). For the PCR protocol, the study used Q5 High-Fidelity 2X Master Mix Catalogue No. M0292 (New England Biolabs (NEB), Ipswich, Massachusetts, United States), 10mM of each primer pair in a primer mix, nuclease-free water Catalogue No. E476 (AMRESCO LLC, OH 44139, USA). The forward primers were fluorescently labelled with M13-FAM, PGEX3-ATTO532, PGEX5-ATTO550, and T7-ATTO565. The multiplex was carried out in two rounds; in the first round, all the primers were added to each corresponding panel. The DNA concentration used ranged from 1 to 2 µL. The second round only consisted of the markers that were not performing well in the multiplex. Each panel folder for the second round had the marker name labelled on the folder that was present for those runs. The three markers, namely, CS1, D02-CANN1, and ANUCS204, were not performing well, resulting in very messy data that could be called inaccurate. Therefore, these markers were excluded from the analysis.

For fragment analysis, fragment preparation was carried out using two microliters of the fluorescently labelled PCR amplicons and 15 µL LIZ500 sizing standard and Hi-Di™ Formamide Catalogue No. 4311320 (Thermo Fisher Scientific, Carlsbad, USA). The fragments were run on the ABI PRISM™ 3730xl Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA), 50 cm capillary array, POP-7TM. The data was analyzed and interpreted using GeneMarker V2.9.5 (SoftGenetics, State College, Pennsylvania, USA).

### 3.2.5. Data analysis

The data was analyzed using GenStat®, 20.1 Edition (VSN International, Hamel Hampstead, UK, 2020) at the 5% significance level. The means of significantly different variables were separated using Duncan's multiple-range test. The statistical data analysis for correlation, Principal Component Analysis (PCA), and Agglomerative Hierarchical Clustering (AHC) was conducted using Statistical Computing Environment, R software, version 4.4.0 using the RStudio platform (Posit Software, PBC, 250 Northern Ave Suite 420 Boston, MA 02210).

## 3.3. Results

### 3.3.1. Physical parameters

There were statistically significant differences ($p<0.001$) among the cannabis genotypes concerning seed width, thickness, length, geometric mean diameter, and surface area (Table 3.2). In contrast, no statistically significant differences ($p>0.05$) were observed in seed sphericity and aspect ratio among the cannabis genotypes. The seeds of 'B1' and 'M2' had relatively high mean values of width (3.74 and 3.72 mm), thickness (3.01 and 3.07 mm), length (4.80 and 4.92 mm), geometric mean diameter (3.78 and 3.82 mm) and surface area (44.92 and 46.01 mm) compared to the other genotypes. On the other hand, the seeds of 'M1' and 'B2' had relatively low mean values of seed width (3.00 and 3.05 mm), thickness (2.48 and 2.46 mm), geometric mean diameter (3.15 and 3.09 mm) and surface area (31.24 and 29.92 mm) compared to the other genotypes. Furthermore, 'B2' seeds had the lowest mean length of 3.91 mm amongst the genotypes.

**Table 3.2:** Physical properties of eleven cannabis genotypes.

| Genotype | Width (mm) | Thickness (mm) | Length (mm) | Geometric mean diameter (mm) | Surface area (mm) | Sphericity | Aspect ratio (%) |
|---|---|---|---|---|---|---|---|
| B1 | 3.74a | 3.01ab | 4.80a | 3.78a | 44.92a | 0.79a | 77.90ab |
| M2 | 3.72a | 3.07a | 4.92a | 3.82a | 46.01a | 0.78a | 75.70ab |
| L2 | 3.64ab | 2.85abc | 4.53ab | 3.61ab | 40.91ab | 0.80a | 80.39ab |
| Hemp | 3.59ab | 2.71abc | 4.79a | 3.60ab | 40.76ab | 0.75a | 75.01ab |
| H1 | 3.49abc | 2.89abc | 4.63ab | 3.60ab | 40.79ab | 0.78a | 75.62ab |
| High-THC | 3.49abc | 2.88abc | 4.12bc | 3.46bc | 37.61bcd | 0.84a | 84.83a |
| High-CBD | 3.47abc | 2.74abc | 4.59ab | 3.52ab | 39.00abc | 0.77a | 75.60ab |
| B3 | 3.27bcd | 2.53c | 4.42abc | 3.32bcd | 34.60bcde | 0.75a | 74.15ab |
| L1 | 3.06cd | 2.59bc | 4.13bc | 3.20cd | 32.10cde | 0.78a | 74.40ab |
| B2 | 3.05cd | 2.46c | 3.91c | 3.09d | 29.92e | 0.79a | 78.10ab |
| M1 | 3.00d | 2.48c | 4.22bc | 3.15cd | 31.24de | 0.75a | 70.96ab |
| *p*-value | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.051 | 0.060 |
| LSD | 0.24 | 0.25 | 0.29 | 0.17 | 3.77 | 0.05 | 7.22 |

*The means with different letters are significantly different according to Duncan's multiple range test ($p$<0.05; number of replicates = 3). LSD = least significance difference.

### 3.3.2. Mineral element composition

The EDX spectrum revealed that endosperm cells contained various mineral elements, including C, Fe, K, O, P, S, Si, Al, Ca, Cl, Mn, Cu, N, Na, and Mg (Figure 3.2). Among the genotypes analyzed, 'M1' was identified as the only genotype containing Na, however, in small amounts. 'B3' was also the only genotype containing N. 'High-THC' and 'High-CBD' were the only genotypes found to have Mn. Furthermore, 'High-CBD' exhibited trace amounts of Cu. A detailed elemental distribution obtained from the X-ray mapping technique is presented in Table 3.3.

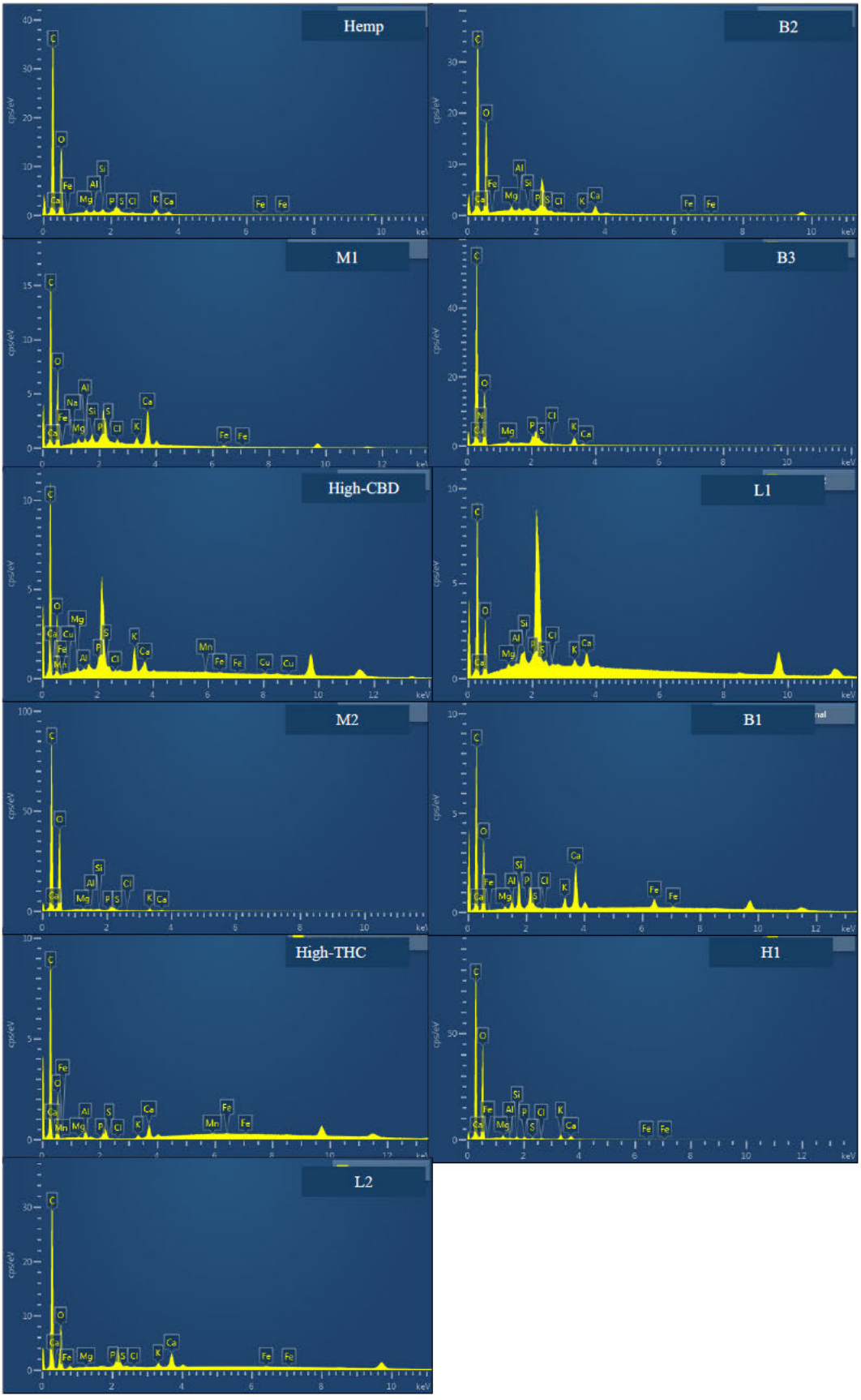



**Figure 3.2**: Scanning electron microscopy micrographs of the EDX spectrum of endosperm cells on the mineral elements of different cannabis landraces.

The cannabis genotypes predominantly comprised essential elements such as S, C, and O, along with ultra-trace and toxic elements like Al, as well as micro-mineral elements like Fe, and macro-mineral elements such as K, O, P, Si, Ca, Cl, and Mg. Notably, there were statistically significant differences ($p<0.05$) among the cannabis genotypes in relation to seed C, Fe, K, O, P, S, and Si. In contrast, no statistically significant differences ($p>0.05$) were observed among the genotypes in relation to Al, Ca, Cl, and Mg (Table 3.3). Among the essential elements, carbon was the most abundant, ranging from 55.58% to 75.10%, followed by oxygen, ranging from 23.79% to 42.82%.

Among the macro-mineral elements, chlorine levels were generally the lowest in all the genotypes at 0.064, 0.104, 0.036, 0.048, 0.054, 0.028, 0.036, 0.112, 0.036, 0.068 and 0.01% for 'High-CBD', 'H1', 'Hemp', 'B3', 'B1', 'B2', 'M2', 'M1', 'L2', 'L1' and 'High-THC', respectively. In contrast, potassium was the highest amongst the macro-mineral elements in 'High-CBD', 'H1', 'Hemp', 'B3', and 'M2' at 1.31, 0.536, 0.182, 0.694, and 0.392%, respectively. Calcium was the highest amongst the macro-mineral elements in 'B1', 'M1', and 'L2' at 1.04, 1.16 and 0.478%, respectively. Silicon was the highest amongst the macro-mineral elements in 'B2', 'L1', and 'High-THC' at 0.3255, 2.7727, and 0.5677%, respectively.

Among the genotypes, 'High-THC' genotype exhibited the highest carbon concentration, while 'H1' had the lowest. On the other hand, 'H1' showed the highest oxygen concentration, with 'High-THC' having the lowest. Certain genotypes exhibited very low levels of specific elements. For instance, 'B2', 'M2', and 'L1' had aluminium, known as an ultra-trace and toxic element, in the lowest quantity compared to other elements at 0.014, 0.024, and 0.029%, respectively. Chlorine was also found in low quantities in 'High-CBD', 'B3', 'B1', and 'High-THC' compared to the other elements at 0.064, 0.048, 0.054, and 0.010%, respectively. Iron was lowest in 'H1' and 'Hemp' compared to the other elements at 0.057 and 0.030%,

respectively. Lastly, sulfur was found in lowest quantities in 'Hemp' and 'L2' compared to the other elements at 0.032 and 0.030%, respectively.

**Table 3.3**: Analysis of variance (ANOVA) for mineral element composition of eleven cannabis genotypes.

| | Concentration (wt%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Genotype** | **Al** | **Ca** | **C** | **Cl** | **Fe** | **K** | **Mg** | **O** | **P** | **S** | **Si** |
| High-CBD | 0.193b | 0.570a | 65.97bc | 0.064abc | 0.131efg | 1.310a | 0.214a | 30.85bc | 0.354ab | 0.244a | 0.568b |
| H1 | 0.063b | 0.388a | 55.58d | 0.104ab | 0.057fg | 0.536b | 0.208a | 42.82a | 0.124bc | 0.078b | 0.263b |
| Hemp | 0.057b | 0.076a | 68.97abc | 0.036bc | 0.030g | 0.182b | 0.070a | 30.44bc | 0.036c | 0.032b | 0.053b |
| B3 | 0.197b | 0.199a | 66.74abc | 0.048abc | 0.462b | 0.694b | 0.118a | 29.82bc | 0.424a | 0.150ab | 0.568b |
| B1 | 0.379ab | 1.040a | 68.01abc | 0.054abc | 2.588a | 0.432b | 0.154a | 28.87bc | 0.278abc | 0.144ab | 0.718b |
| B2 | 0.014b | 0.248a | 68.58abc | 0.028bc | 0.138ef | 0.170b | 0.224a | 30.43bc | 0.172bc | 0.072b | 0.326b |
| M2 | 0.024b | 0.092a | 64.18c | 0.036abc | 0.462b | 0.392b | 0.050a | 35.03b | 0.068c | 0.072b | 0.065b |
| M1 | 0.153b | 1.160a | 68.68abc | 0.112a | 0.181de | 0.314b | 0.154a | 28.94bc | 0.136bc | 0.092b | 0.268b |
| L2 | 0.197b | 0.478a | 74.54ab | 0.036abc | 0.242cd | 0.246b | 0.048a | 24.42c | 0.134bc | 0.030b | 0.079b |
| L1 | 0.029b | 0.608a | 67.80abc | 0.068abc | 0.462b | 0.408b | 0.180a | 29.41bc | 0.194abc | 0.132ab | 2.773a |
| High-THC | 0.854a | 0.408a | 75.10a | 0.010c | 0.332c | 0.170b | 0.058a | 23.79c | 0.124bc | 0.046b | 0.568b |
| p-value | 0.112 | 0.561 | 0.002 | 0.090 | <0.001 | 0.009 | 0.146 | <0.001 | 0.025 | 0.023 | 0.022 |
| LSD | 0.561 | 1.084 | 7.744 | 0.066 | 0.104 | 0.562 | 0.156 | 6.213 | 0.221 | 0.117 | 1.405 |

*The means with different letters are significantly different according to Duncan's multiple range test ($p$<0.05; number of replicates = 3). LSD = least significance difference. **C** (carbon), **Fe** (iron), **K** (potassium), **O** (oxygen), **P** (phosphorus), **S** (sulfur) and **Si** (Silicon), **Al** (aluminum), **Ca** (calcium), **Cl** (chlorine), and **Mg** (magnesium).

### 3.3.3. Short Tandem Repeat markers

Thirty-four markers were identified in the seeds of the different cannabis genotypes, and their ranges are presented in Table 3.4.

**Table 3.4**: Detailed list of markers in cannabis genotypes and their ranges.

| MARKER | RANGE | MARKER | RANGE |
|---|---|---|---|
| **Plex 1** | | **Plex 3** | |
| B02-CANN2 | 163–172 | D02-CANN1 | 93–113 |
| H09-CANN2 | 204–224 | ANUCS205 | 172–242 |
| 5159 | 312–350 | nH09 | 385–430 |
| 9269 | 114–140 | ANUCS302 | 125–173 |
| ANUCS308 | 177–203 | 9043 | 320–355 |
| H11-CANN1 | 285–297 | ANUCS204 | 128–184 |
| ANUCS303 | 141–156 | ANUCS203 | 169–267 |
| B05-CANN1 | 217–244 | **Plex 4** | |
| B01-CANN1 | 314–460 | 1528 | 175–200 |
| ANUCS202 | 147–185 | H06-CANN2 | 255–273 |
| 4910 | 240–300 | DM016_Y | 230 |
| **Plex 2** | | ANUCS307 | 105–108 |
| 3735 | 70–115 | ANUCS201 | 161–223 |
| ANUCS206 | 159–167 | DM029 | 219 |
| ANUCS301 | 209–261 | E07-CANN1 | 85–113 |
| C11-CANN1 | 138–180 | | |
| ANUCS305_1 | 410–450 | | |
| ANUCS501_X | 72–105 | | |
| ANUCS304 | 167–230 | | |
| ANUCS306 | 92–95 | | |
| CS1 | 134–370 | | |

### 3.3.4. Multivariate analysis

#### 3.3.4.1. Multivariate analysis for physical characteristics

##### 3.3.4.1.1. Correlation matrix

A Pearson's correlation was conducted on the physical parameters and amongst the genotypes (Figure 3.3A and B). Under physical parameters, there was a very strong positive correlation between seed sphericity and aspect ratio ($r = 0.93$, $p<0.001$) (Figure 3.3A). Thickness had a positive correlation with length ($r = 0.71$, $p<0.05$) while width had a strong positive correlation with length ($r = 0.83$, $p<0.01$) and thickness ($r = 0.91$, $p<0.001$). Geometric mean diameter had a strong positive correlation with the surface area ($r = 1.00$, $p<0.001$), width ($r = 0.97$, $p<0.001$),

thickness (r = 0.93, $p$< 0.001) and length (r = 0.90, $p$<0.001). On the other hand, there was a very strong positive correlation ($p$<0.001) between the seeds of different genotypes (Figure 3.3B), indicating that genotypes had similarities in their physical characteristics.

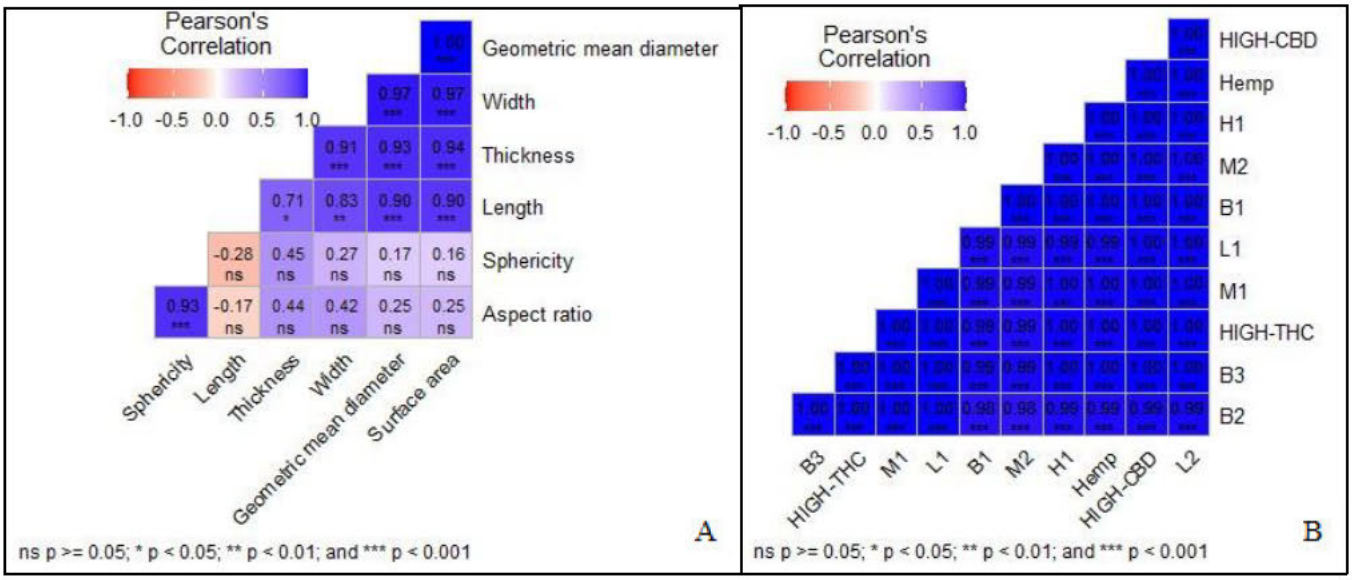



**Figure 3.3**: The Pearson's correlation between (A) physical properties and (B) among genotypes based on their physical traits.

#### 3.3.4.1.2. Principal component analysis for physical characteristics

The principal component analysis (PCA) was conducted on physical features (Table 3.5). PCA identified two PCs with an eigenvalue of ≥ 1, which accounted for 80.11% of the total variation. PC1 accounted for 55.51% of the total variation and positively correlated to seed width, thickness, surface area, and geometric mean diameter. PC2 accounted for 24.60% of the total variation and positively correlated to seed sphericity and aspect ratio.

**Table 3.5**: Summary of factor loadings, eigenvalue, percent, and cumulative variation for eleven cannabis genotypes based on their physical traits.

| **Traits** | **Dim 1** | **Dim 2** |
|---|---|---|
| Width | 0,974 | -0,068 |
| Thickness | 0,969 | 0,048 |
| Length | -0,497 | 0,482 |
| Water activity | 0,236 | -0,380 |
| Surface Area | 0,963 | -0,213 |
| Sphericity | 0,410 | 0,884 |
| **Eigenvalue** | 444,073 | 196,815 |
| **Variability (%)** | 55,509 | 24,602 |
| **Cumulative (%)** | 55,509 | 80,111 |

#### 3.3.4.1.3. Hierarchal Cluster Analysis

Agglomerative hierarchical clustering for means of physical traits classified the 11 landraces into four groups (Figure 3.4). Cluster 1 comprised of ‘B2’, ‘B3’, ‘M1’ and ‘L1’. Cluster 2 comprised of ‘B1’, ‘M2’, ‘Hemp’, ‘High-CBD’, and ‘H1’. Cluster 3 and 4 were comprised of ‘L2’ and ‘High-THC’, respectively.

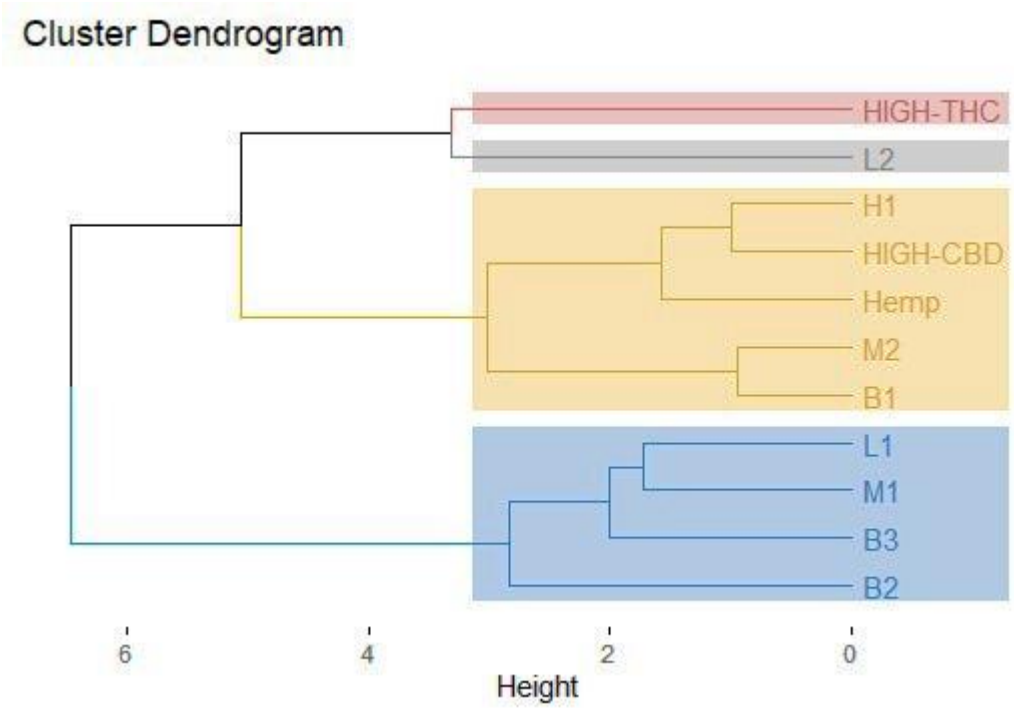



**Figure 3.4**: Agglomerative hierarchical cluster of eleven cannabis genotypes based on their physical traits.

#### 3.3.4.2. Multivariate analysis for mineral elements composition

##### 3.3.4.2.1. Correlation matrix

Generally, there was no significant correlation among the mineral elements. Except for S, which had a strong positive correlation with K (r = 0.90, $p<0.001$) and P (r = 0.83, $p<0.01$) (Figure 3.5A). There was also a positive correlation between P and K (r = 0.71, $p<0.05$). On the other hand, C had a significant negative correlation with O (r = -0.98, $p<0.001$) and Cl (r = -0.61, $p<0.05$). Notably, a very strong positive correlation ($p<0.001$) was evident among the seeds of various genotypes (Figure 3.5B), suggesting a similarity in their mineral element composition.

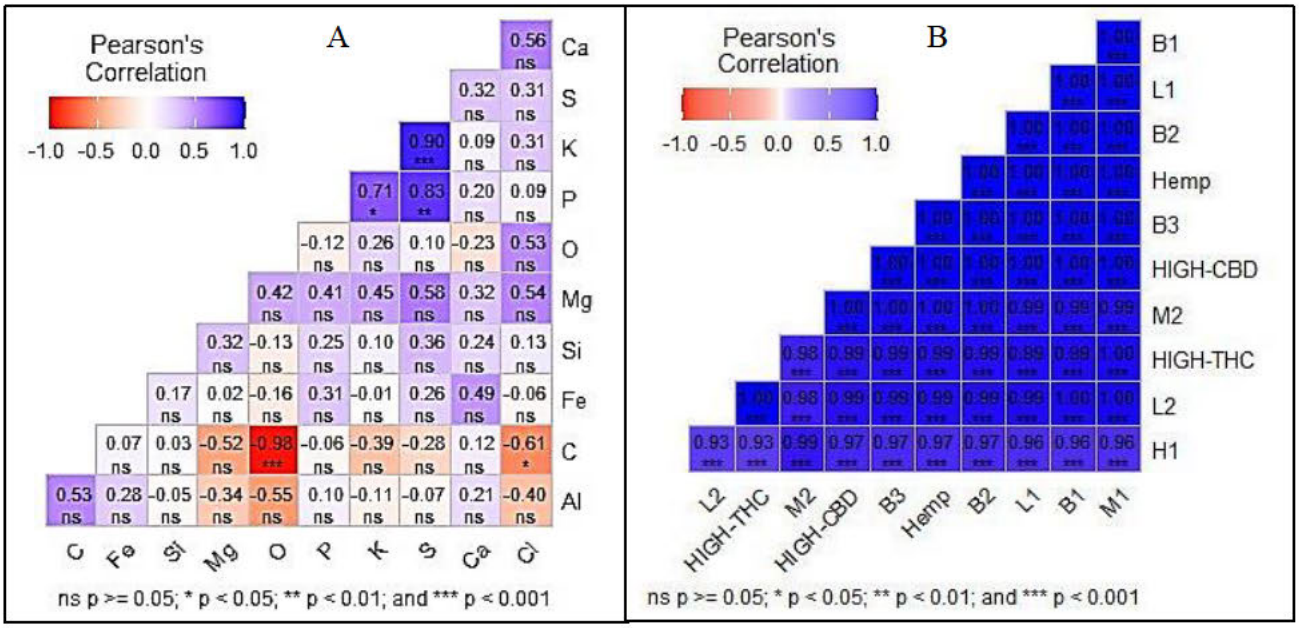



**Figure 3.5**: The Pearson's correlation between (A) mineral element composition and (B) among genotypes based on their mineral element composition. **C** (carbon), **Fe** (iron), **K** (potassium), **O** (oxygen), **P** (phosphorus), **S** (sulfur) and **Si** (Silicon), **Al** (aluminium), **Ca** (calcium), **Cl** (chlorine), and **Mg** (magnesium).

#### 3.3.4.2.2. Principal component analysis

PCA identified three PCs with an eigenvalue of ≥ 1, accounting for 73.92% of the total variation (Table 3.6). PC1 accounted for 35.99% of the total variation, which negatively correlated to carbon (C) and positively correlated to Cl, K, S, and Mg. PC2 accounted for 24.71% of the total variation, which positively correlated to Al and P and negatively correlated to O. PC3 accounted for 13.22% of the total variation and positively correlated to Ca.

**Table 3.6**: Summary of factor loadings, eigenvalue, percent, and cumulative variation for eleven cannabis genotypes based on their mineral elements. **C** (carbon), **Fe** (iron), **K** (potassium), **O** (oxygen), **P** (phosphorus), **S** (sulfur) and **Si** (Silicon), **Al** (aluminium), **Ca** (calcium), **Cl** (chlorine), and **Mg** (magnesium).

| **Mineral element** | **Dim 1** | **Dim 2** | **Dim 3** |
|---|---|---|---|
| Al | -0.434 | 0.607 | -0.014 |
| C | -0.732 | 0.582 | -0.021 |
| Ca | 0.306 | 0.539 | 0.720 |
| Cl | 0.710 | -0.216 | 0.528 |
| Fe | 0.093 | 0.571 | 0.345 |
| K | 0.759 | 0.243 | -0.488 |
| Mg | 0.809 | 0.013 | 0.134 |
| O | 0.592 | -0.723 | 0.022 |
| P | 0.570 | 0.617 | -0.401 |
| S | 0.797 | 0.496 | -0.287 |
| Si | 0.294 | 0.375 | 0.193 |
| **Eigenvalue** | **3.958** | **2.718** | **1.455** |
| **Variability (%)** | **35.986** | **24.711** | **13.225** |
| **Cumulative (%)** | **35.986** | **60.697** | **73.922** |

#### 3.3.4.2.3. Hierarchal cluster analysis

Agglomerative hierarchical clustering for means of mineral element composition classified the 11 landraces into four groups (Figure 3.6). Cluster 1 comprised of ‘L2’, ‘High-THC’, ‘B2’, ‘Hemp’, and ‘M2’. Cluster 2 comprised of ‘H1’ and Cluster 3 comprised of ‘B3’ and ‘High-CBD’. Cluster 4 comprised of ‘B1’, ‘M1’ and ‘L1’.

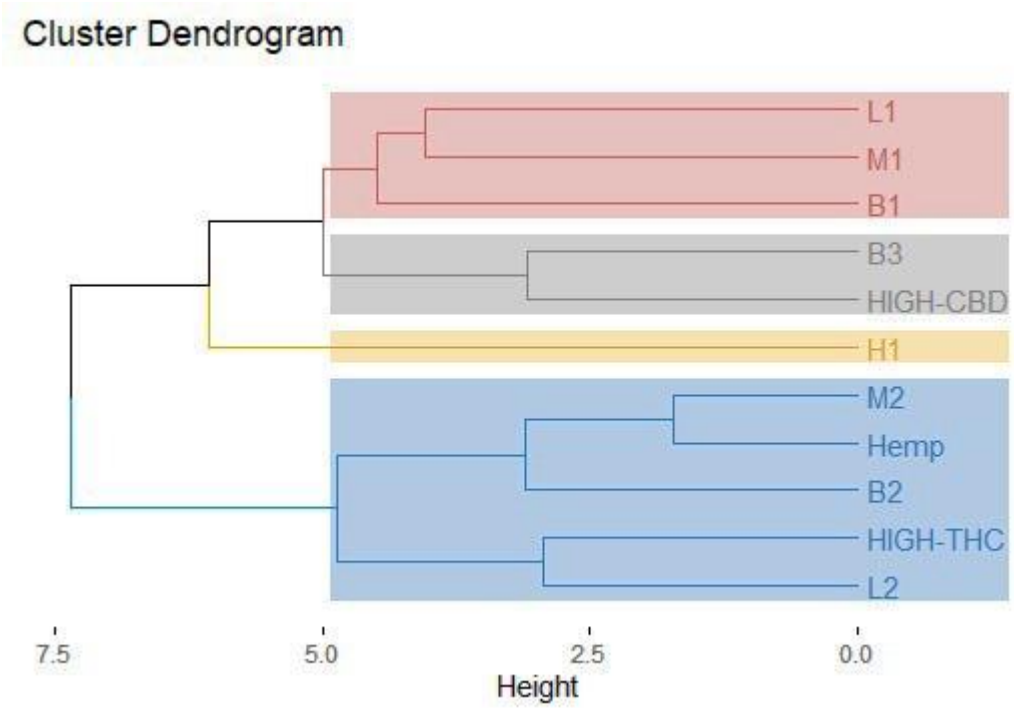



**Figure 3.6**: Agglomerative hierarchical cluster for eleven cannabis genotypes based on their mineral element composition.

#### 3.3.4.3. Multivariate analysis for short tandem repeats (STR) markers

In addition to CS1, D02-CANN1, and ANUCS204 loci which were eliminated due to displayed inaccuracy of data, B02-CANN2 1, B02-CANN2 2, ANUCS308 1, ANUCS308 2, H11-CANN1 1, H11-CANN1 2, ANUCS202 1, ANUCS202 2, ANUCS206 1, ANUCS501_X 1, ANUCS501_X 2, ANUCS306 1, ANUCS306 2, H06-CANN2 1, DM016_Y 1, DM016_Y 2, ANUCS307 1, ANUCS201 1, ANUCS201 2, DM029 1, DM029 2 and E07-CANN1 1 were excluded from multivariate analysis because they had zero variation. The remaining STR markers were then subjected to further analysis as presented in the sub-sections 3.3.4.3.1, 3.3.4.3.2 and 3.3.4.3.3 of 3.3.4.3.

##### 3.3.4.3.1. Correlation matrix

The Pearson correlation analysis showed variations among the STR markers, revealing significant correlations among the alleles, with some showing strong positive or negative

relationships (Figure 3.7). Notably, alleles 5159 1 and 5159 2 had a perfect positive correlation (r = 1.00; $p$<0.001). Similarly, 9269 2 had a perfect positive correlation with ANUCS305_1 2 and ANUCS302 1 (r = 1.00; $p$<0.001). ANUCS303 2 also showed a perfect positive correlation with ANUCS304 1 (r = 1.00; $p$<0.001). B05-CANN1 1 and B05-CANN1 2 exhibited a perfect positive correlation (r = 1.00; $p$<0.001). Additionally, allele 4910 1 had a perfect negative correlation with ANUCS304 2, NH09 1, and NH09 2 (r = -1.00; $p$<0.001). ANUCS305_1 1 showed a perfect negative correlation with H06-CANN2 2 (r = -1.00; $p$<0.001), while ANUCS305_1 2 had a perfect positive correlation with ANUCS302 1 (r = 1.00; $p$<0.001). ANUCS304 2 displayed a perfect positive correlation with NH09 1 and NH09 2 (r = 1.00; $p$<0.001), and NH09 1 and NH09 2 also had a perfect positive correlation with each other (r = 1.00; $p$<0.001).

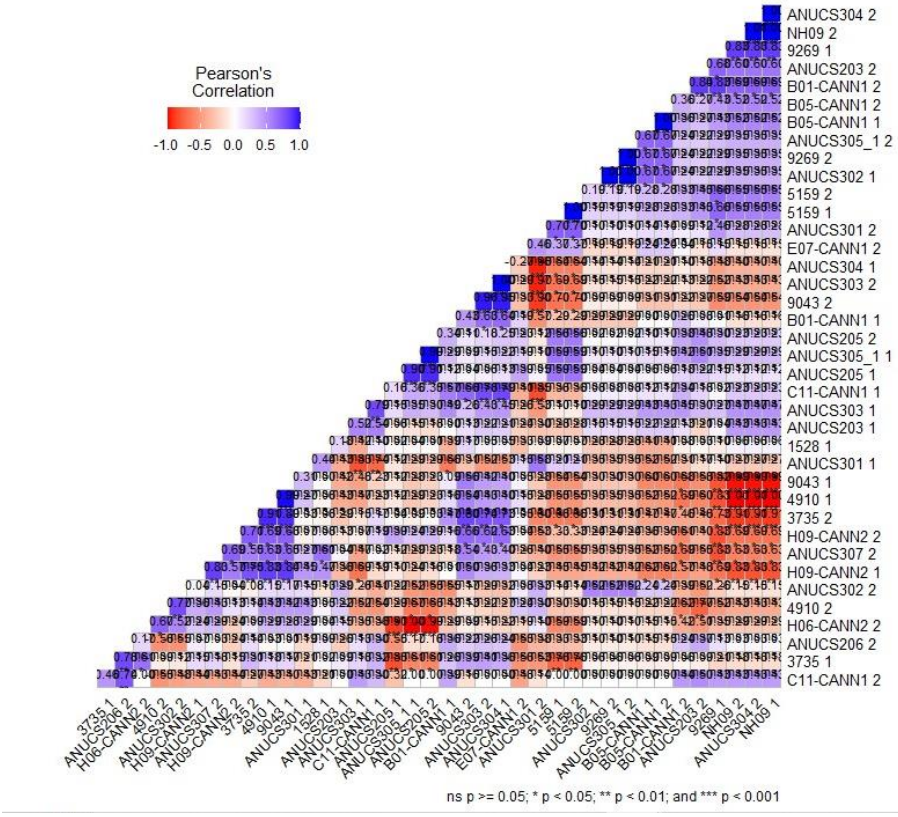



**Figure 3.7**: The Pearson's correlation for Short Tandem Repeats (STR) markers assessed in eleven cannabis genotypes.

A very strong positive correlation ($p$<0.001) was evident among the seeds of various genotypes (Figure 3.8), suggesting a similarity in their STR markers.

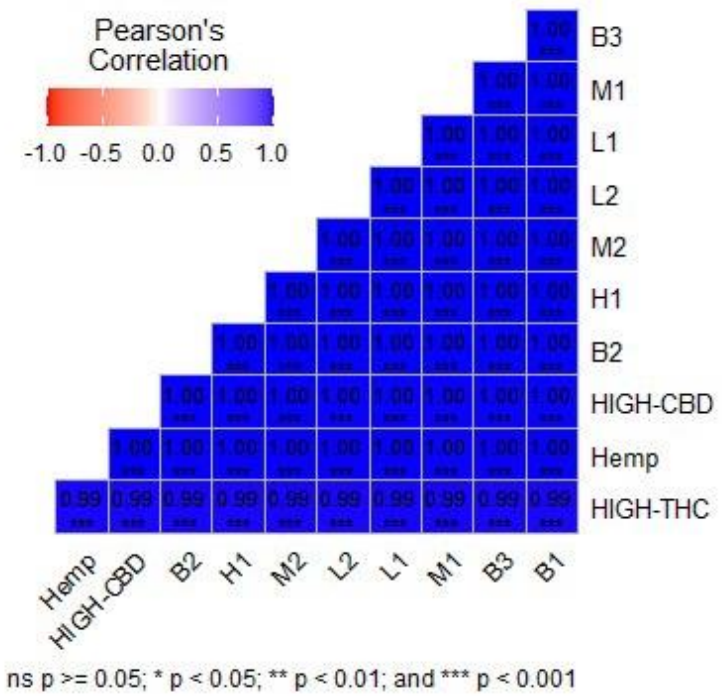



**Figure 3.8**: The Pearson's correlation for eleven cannabis genotypes based on their STR markers.

#### 3.3.4.3.2. Principal component analysis

PCA identified six PCs with an eigenvalue of ≥ 1, which accounted for 93.53% of the total variation. However, only five PCs, accounting for 88.03%, were used for representation of the PCA (Table 3.7). PC1 accounted for 35.30% of the total variation and positively correlated to 5159 1, 5159 2, 9269 1, B05-CANN1 1, B05-CANNA1 2, B01-CANNA1 2, ANUCS304 2, NH09 1, NH09 2, ANUCS203 2, and negatively correlated to H09-CANN2 1, H09-CANN2 2, 4910 1, 3735 2, 9043 1, 9043 2 and ANUCS307 2. PC2 accounted for 20.30% of the total variation and positively ANUCS303 1, ANUCS303 2, B01-CANN1 1, C11-CANN1 1, ANUCS304 1 and 9043 1; and negatively correlated to 4910 2, ANUCS301 1 and ANUCS301 2. PC3 accounted for 14.67% of the total variation and positively correlated to ANUCS305_1

1, ANUCS205 1, and ANUCS205 2, and negatively correlated to 3735 1 and H06-CANN2 2. PC4 accounted for 11.01% of the total variation. which positively correlated to 9269 2, ANUCS305_1 2, ANUCS302 1 and ANUCS302 2, and negatively correlated to ANUCS206 2. PC5 accounted for 6.75% of the total variation and positively correlated to ANUCS203 1 and 1528.1 allele calls.

**Table 3.7**: Summary of factor loadings, eigenvalue, percent, and cumulative variation for eleven cannabis genotypes based on their physical traits.

| Traits | Dim 1 | Dim 2 | Dim 3 | Dim 4 | Dim 5 |
|---|---|---|---|---|---|
| H09-CANN2 1 | -0.860 | -0.146 | 0.216 | -0.092 | 0.056 |
| H09-CANN2 2 | -0.682 | 0.253 | 0.455 | 0.345 | 0.142 |
| 5159 1 | 0.732 | -0.346 | 0.543 | -0.027 | -0.025 |
| 5159 2 | 0.732 | -0.346 | 0.543 | -0.027 | -0.025 |
| 9269 1 | 0.901 | 0.004 | 0.009 | -0.260 | -0.109 |
| 9269 2 | 0.469 | 0.034 | -0.321 | 0.658 | -0.119 |
| ANUCS303 1 | 0.403 | 0.767 | -0.196 | 0.213 | 0.012 |
| ANUCS303 2 | -0.552 | 0.808 | 0.016 | 0.160 | 0.085 |
| B05-CANN1 1 | 0.636 | 0.082 | -0.240 | 0.530 | -0.215 |
| B05-CANN1 2 | 0.636 | 0.082 | -0.240 | 0.530 | -0.215 |
| B01-CANN1 1 | -0.131 | 0.730 | 0.150 | -0.120 | -0.416 |
| B01-CANN1 2 | 0.724 | 0.352 | 0.007 | -0.232 | -0.097 |
| 4910 1 | -0.925 | -0.097 | 0.170 | 0.132 | -0.292 |
| 4910 2 | -0.474 | -0.679 | -0.237 | 0.277 | 0.196 |
| 3735 1 | -0.352 | 0.364 | -0.753 | -0.318 | -0.186 |
| 3735 2 | -0.872 | 0.309 | 0.174 | 0.122 | -0.272 |
| ANUCS206 2 | -0.064 | 0.456 | -0.381 | -0.617 | -0.305 |
| ANUCS301 1 | -0.246 | -0.774 | 0.202 | -0.350 | 0.139 |
| ANUCS301 2 | 0.452 | -0.859 | 0.135 | -0.092 | -0.152 |
| C11-CANN1 1 | 0.049 | 0.938 | -0.101 | 0.081 | 0.295 |
| C11-CANN1 2 | 0.357 | 0.423 | -0.343 | -0.552 | -0.255 |
| ANUCS305_1 1 | 0.419 | 0.430 | 0.777 | 0.127 | 0.049 |
| ANUCS305_1 2 | 0.469 | 0.034 | -0.321 | 0.658 | -0.119 |
| ANUCS304 1 | -0.514 | 0.829 | 0.073 | 0.167 | 0.088 |
| ANUCS304 2 | 0.925 | 0.097 | -0.170 | -0.132 | 0.292 |
| ANUCS205 1 | 0.267 | 0.164 | 0.866 | 0.323 | 0.176 |
| ANUCS205 2 | 0.353 | 0.433 | 0.815 | 0.088 | 0.036 |
| NH09 1 | 0.925 | 0.097 | -0.170 | -0.132 | 0.292 |
| NH09 2 | 0.925 | 0.097 | -0.170 | -0.132 | 0.292 |
| ANUCS302 1 | 0.469 | 0.034 | -0.321 | 0.658 | -0.119 |
| ANUCS302 2 | -0.107 | -0.566 | -0.412 | 0.665 | 0.093 |

| Traits | Dim 1 | Dim 2 | Dim 3 | Dim 4 | Dim 5 |
|---|---|---|---|---|---|
| 9043 1 | -0.923 | -0.103 | 0.157 | 0.112 | -0.240 |
| 9043 2 | -0.646 | 0.671 | 0.026 | 0.202 | 0.128 |
| ANUCS203 1 | 0.168 | 0.254 | -0.440 | 0.267 | 0.658 |
| ANUCS203 2 | 0.676 | 0.293 | 0.152 | -0.328 | -0.157 |
| 1528 1 | -0.191 | -0.088 | 0.070 | -0.344 | 0.761 |
| H06-CANN2 2 | -0.419 | -0.430 | -0.777 | -0.127 | -0.049 |
| ANUCS307 2 | -0.807 | -0.049 | 0.030 | 0.040 | 0.454 |
| E07-CANN1 2 | 0.082 | -0.364 | 0.496 | 0.212 | -0.357 |
| **Eigenvalue** | **13.768** | **7.916** | **5.721** | **4.293** | **2.633** |
| **Variability (%)** | **35.302** | **20.297** | **14.669** | **11.007** | **6.752** |
| **Cumulative (%)** | **35.302** | **55.600** | **70.269** | **81.276** | **88.029** |

#### 3.3.4.3.3. Hierarchal cluster analysis

Agglomerative hierarchical clustering for means of STR markers classified the 11 landraces into four groups (Figure 3.9). Cluster 1 comprised of ‘M1’, ‘L2’, ‘B3’, ‘B1’ and ‘L1’. Cluster 2 comprised of ‘Hemp’ and ‘High-CBD’, and cluster 3 comprised of ‘High-THC’. Cluster 4 comprised of ‘B2’, ‘H1’ and ‘M2’.

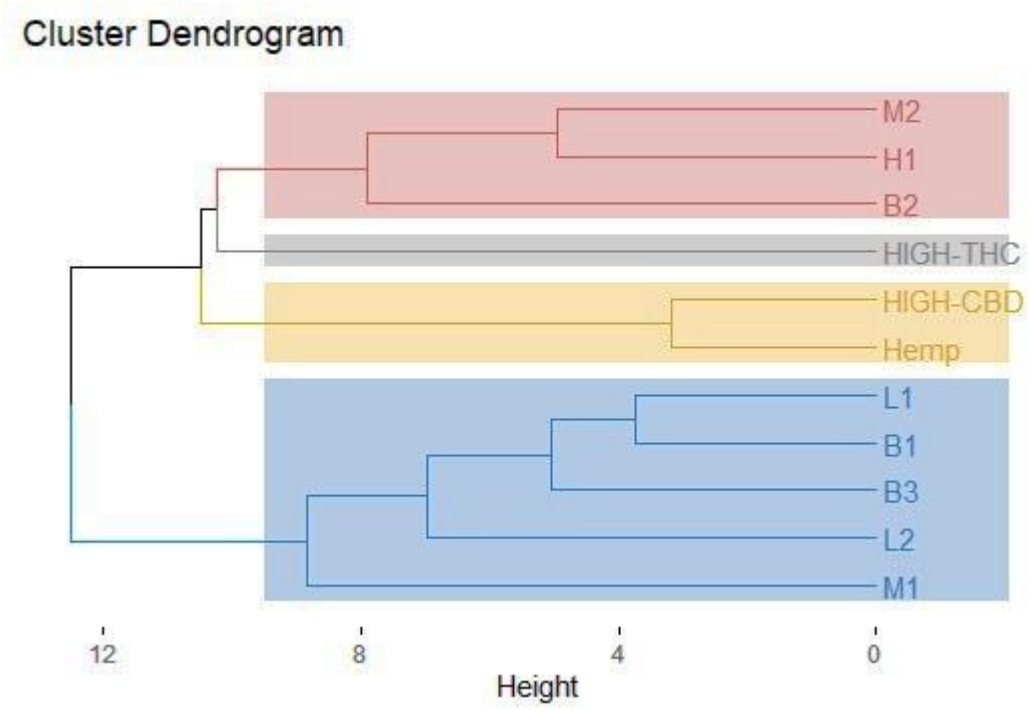



**Figure 3.9**: Agglomerative hierarchical cluster for eleven cannabis genotypes based on their STR markers.

## 3.4. Discussion

### 3.4.1. Physical traits

The findings of the current study showed diverse physical traits of cannabis seeds, with significant variations observed among different genotypes. Notably, the seeds of 'B1' and 'M2' exhibited relatively high mean values for width, thickness, length, geometric mean diameter, and surface area compared to other genotypes. In contrast, 'M1' and 'B2' seeds displayed relatively lower mean values for width, thickness, geometric mean diameter, and surface area compared to other genotypes. Thus, when designing seed-handling equipment, the dimensions of the seeds' length, width, and thickness must be taken into consideration to determine the appropriate aperture sizes (Taheri-Garavand *et al*., 2012). On the other hand, the geometric mean diameter is useful for characterizing the seeds with dimensions of irregular shapes (Taheri-Garavand *et al*., 2012). Additionally, the mean geometric mean diameter is useful for

estimating the projected area of a particle moving in a turbulent or near-turbulent air stream (Taheri-Garavand *et al*., 2012).

Pearson's correlation analysis revealed significant relationships among physical parameters and different cannabis genotypes. Notably, seeds with higher sphericity tended to have higher aspect ratios. Moreover, greater thickness correlated with longer lengths, while wider seeds tended to be longer and thicker. Additionally, seeds with larger geometric mean diameters exhibited larger surface areas, widths, thicknesses, and lengths. The principal component analysis (PCA) revealed that PC1 variation was contributed by seed width, thickness, surface area, and geometric mean diameter, which was key in significant differences among the genotypes. In contrast, PC2 variation was contributed by seed sphericity and aspect ratio. The Agglomerative hierarchical clustering of physical traits grouped the 11 landraces into four distinct clusters. Cluster 1 included 'B2', 'B3', 'M1', and 'L1', while Cluster 2 comprised 'B1', 'M2', 'Hemp', 'High-CBD', and 'H1'. Cluster 3 consisted of only 'L2', and Cluster 4 was represented by 'High-THC'. However, Clusters 3 and 4 were closely related, and this relationship was associated with their seeds being more spherical compared to the other genotypes. Notably, seeds from 'B1' and 'M2' within Cluster 2 displayed relatively higher mean values for width, thickness, length, geometric mean diameter, and surface area compared to other landraces. Contrarily, Cluster 1 consisted of seeds with relatively low surface area, width, and thickness.

### 3.4.2. Mineral element composition

The cannabis genotypes consisted of essential (i.e., S, C, and O), ultra-trace and toxic elements, such as Al, and micro minerals (Fe), as well as macro minerals such as K, O, P, Si, Ca, Cl, and Mg. However, the composition of these elements varied among the different cannabis genotypes. The major macro-elements, such as P, K, Mg, and Ca, as well as trace elements,

such as Fe, were previously reported in hempseeds (Farinon *et al*., 2020). Additionally, the findings of the current study are consistent with Farinon *et al*. (2020), who reported that the mineral profile of seeds can vary significantly depending on a number of factors, including the type of plant genotype, the type and amount of fertilizer applied, and the mineral composition of the soil (Farinon *et al*., 2020).

Notably, the essential elements were found in abundance compared to the other ones, with carbon being the dominant element, followed by oxygen. Particularly, the 'High-THC' genotype exhibited the highest carbon content, while 'H1' displayed the lowest. Conversely, 'H1' had the highest oxygen levels, with 'High-THC' genotypes showing the lowest. These findings are in inconsistent with the findings by Farinon *et al*. (2020) and Alonso-Esteban *et al*. (2022), who reported that phosphorus was the most abundant mineral found in both whole and hulled hemp seeds. Additionally, potassium was the highest amongst the macro-mineral elements in genotypes such as 'High-CBD', 'H1', 'Hemp', 'B3', and 'M2'; similarly, calcium was the highest in 'B1', 'M1' and 'L2', and silicon was the highest in 'B2', 'L1' and 'High-THC'. The findings on potassium are consistent with the reports by Alonso-Esteban *et al*. (2022), who revealed that potassium content was also high in hemp seeds, especially in hulled seeds. Barčauskaitė *et al.* (2022) also found that hemp seeds were rich in K.

Furthermore, specific genotypes exhibited exceptionally low levels of certain elements, such as 'B2', 'M2', and 'L1' having the lowest aluminium concentration, and 'High-CBD' genotypes, along with 'B3', 'B1', and 'High-THC' having lowest chlorine levels. Moreover, 'H1' and 'Hemp' demonstrated the lowest iron concentrations. Iron, however, was the only microelement in the cannabis genotypes that was present in significant amounts. Notably, iron was found to be the primary microelement in whole hemp seeds by Alonso-Esteban *et al*. (2022) and Barčauskaitė *et al.* (2022). Whereas 'Hemp' and 'L2' showed the lowest sulfur levels. However, it is important to highlight that several anti-nutrient elements in food, such as

phytates and oxalates, which are included in hemp seeds, may reduce the bioavailability of some mineral elements (Alonso-Esteban *et al*., 2022).

In general, there were minimal correlations observed among the mineral elements, except for S, which exhibited a strong positive correlation with K and P. Additionally, a positive correlation was found between P and K. Conversely, C demonstrated a significant negative correlation with O and Cl. The correlation between these elements is important for the development and selection of a genotype of the desired minerals, which could improve the level of another mineral (Yeken *et al*., 2018). However, the interactions between the mineral elements may be influenced by genetic and environmental factors (Yeken *et al*., 2018; Nadeem, 2021). Nonetheless, a very strong positive correlation was observed among the seeds of various genotypes, indicating a similarity in their mineral element composition.

The findings of the current study showed distinct patterns of variation in mineral element composition among the cannabis genotypes, with PC1 primarily negatively associated with C and positively correlated with Cl, K, S, and Mg. PC2 was linked to Al, P, and O, and PC3 was related to Ca. On the other hand, the Agglomerative hierarchical clustering approach showed similarities and differences in mineral element composition among the cannabis landraces, providing insights into potential relationships and distinctions in their genetic backgrounds. For instance, Cluster 1 included ‘L2’, ‘High-THC’, ‘B2’, ‘Hemp’, and ‘M2’, indicating similarities in their mineral element composition. Cluster 2 consisted of ‘H1’, while cluster 3 comprised ‘B3’ and ‘High-CBD’. Finally, cluster 4 encompassed ‘B1’, ‘M1’, and ‘L1’.

### 3.4.3. Short Tandem Repeat markers

The findings of Pearson's correlation analysis indicated a strong network of interactions, both positive and negative, between the different alleles, with multiple significant correlations among them. The correlations observed suggest complex interactions that may provide insight

into the underlying biological processes and genetic linkages. The perfect correlations observed, such as those between 5159 1 and 5159 2 and ANUCS304 2 with NH09 1 and NH09 2, highlighted a very close genetic linkage. Similarly, the correlation analysis revealed a strong relationship among the different genotypes, suggesting genetic linkage. The STR markers chosen for the current study are known to possess superior discriminative ability for the identification of species, gender determination, and individual identification of *C. sativa* (Xia *et al.,* 2022). Therefore, the inconsistencies between the current study and findings by Köhnemann*et al.* (2012) and Marcińska *et al.* (2022) may be associated with plant material used as they analyzed leaves or inflorescence. Thus, this could imply that there is still a need for the development and validation of a unique identification technique for cannabis seed genotype using the STR system. Otherwise, other genes may be critical for the characterization of the genotypes used in the current study at a seed level. Tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) gene sequencing from 21 *C. sativa L*. variations, for example, has previously been used to discriminate cannabis seeds of drug-type and fiber-type genotypes, with a perfect discriminating score of AUC 100% (Cascini *et al*., 2019; Langa *et al*., 2024).

Principal Component Analysis (PCA) further explained the genetic structure, identifying six principal components, with the first five explaining a substantial portion of the total variance. The PCA identified six principal components, explaining 93.53% of the total variation. PC1, the most influential component, positively correlated with several markers while negatively correlated with others. PC2, PC3, PC4, PC5, and PC6 each contributed to explaining variance in the dataset with distinct marker associations. Agglomerative hierarchical clustering grouped the 11 landraces into four clusters, revealing patterns of similarity and dissimilarity in their STR marker profiles. Cluster 1 included ‘M1’, ‘L2’, ‘B3’, ‘B1’, and ‘L1’, while Cluster 2

comprised ‘Hemp’ and ‘High-CBD’. ‘High-THC’ formed Cluster 3, and Cluster 4 consisted of ‘B2’, ‘H1’, and ‘M2’.

In summary, the correlation revealed similarities among the cannabis genotypes. However, further analysis, particularly agglomerative hierarchical clustering, showed differences amongst the genotypes. This study identified four distinct clusters among the landraces by evaluating their physical traits, mineral element composition, and STR markers separately using agglomerative hierarchical clustering. However, the clustering based on mineral composition grouped the landraces differently compared to the clustering based on physical traits. Similarly, clustering based on STR markers resulted in distinct groupings that differed from those based on physical traits and mineral composition. These differences across clustering analyses highlighted the complex nature of the diversity of the genotypes and the need for consideration of multiple characteristics for comprehensive landrace characterization and classification.

### 3.5. Conclusion

Physical parameters, particularly of ‘M1’ and ‘B2’, are fundamental in determining suitable aperture sizes for efficient processing and aiding in characterizing seeds with irregular shapes. These findings emphasize the need for genotype-specific considerations in equipment design and offer valuable insights for breeding programs targeting seed trait optimization, ultimately enhancing processing techniques and equipment efficiency. Potassium, calcium, and silicon were the major macro-elements, and their distribution differed among genotypes. Carbon and oxygen were abundant elements in cannabis genotypes, with carbon being dominant. Understanding these variations is vital for optimizing agricultural practices and processing techniques for facilitating the development of high-quality hemp-based products. Strong correlations between certain alleles suggested close genetic linkage, emphasizing the

importance of understanding genetic relationships among cannabis genotypes. Generally, the correlation highlighted similarities among cannabis genotypes. However, clustering based on mineral composition differed from that based on physical traits, and STR markers also resulted in distinct groupings, indicating a complex relationship among these characteristics. Therefore, the genotypes, particularly the landraces, could not be categorized as 'High-CBD', 'High-THC', or 'Hemp' based on the measured parameters. Further studies are recommended to develop and validate a unique identification technique for cannabis seed genotypes using the STR system. Given that Cascini *et al*. (2019) successfully discriminated between drug-type and fiber-type cannabis seeds using Tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) gene sequencing, the current study recommends further exploration of these gene sequencing methods for the characterization of cannabis genotypes.

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# 4. CHAPTER 4: SEED DORMANCY AND GERMINATION RESPONSES OF CANNABIS LANDRACES TO VARIOUS PRE-TREATMENTS

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**Abstract**

The current study evaluated the effectiveness of different pre-treatments for dormancy breaking and seed germination of five cannabis landraces. These landraces were collected from local growers in three regions of KwaZulu-Natal Province in South Africa, namely, Bergville (B), Hammersdale (H), and Ladysmith (L). Each genotype was assigned an alphabet and number based on where the seed material was collected. The five landraces were as follows, 'B2', 'B1', 'H1', 'L1', and 'L2'. The study was conducted using various pre-treatments, including potassium nitrate ($KNO_3$), hydrochloric acid (HCl), hot water (HW) at 70 °C, nitric acid ($HNO_3$), dry prechilling at 10 °C, gibberellic acid ($GA_3$), sulfuric acid ($H_2SO_4$), and water, which served as the control. Before the germination test, the seed viability of cannabis landraces was assessed using the tetrazolium chloride (TCC) test. Afterward, the seeds were germinated in Petri dishes with cotton pads moistened with distilled water for five (5) days. The study was later validated under greenhouse conditions. The parameters such as germination percentage, germination rate, seedling length, and seed vigour index of the

germinated seeds were then measured. The landraces, dormancy breaking treatments, and their interactions showed significant differences ($p$<0.001) in germination percentage, germination rate index, seedling length, and seed vigour index under both laboratory and greenhouse conditions. In summary, $GA_3$, $KNO_3$ and prechilling were the most effective pre-treatments to improve the germination and seedling growth of cannabis genotypes and breaking seed dormancy. Therefore, it was concluded that cannabis genotypes had physiological dormancy.

**Keywords**: cannabis, dormancy breaking, germination enhancement, landrace

## 4.1. Introduction

"*Cannabis sativa* L., commonly known as hemp or marijuana (or dagga), is an annual dioecious and dicotyledonous plant belonging to the *Cannabaceae* family" (Flores-Sanchez *et al*., 2009; Chaohua *et al*., 2016; Lata *et al*., 2016). Cannabis is useful in "several agro-industrial fields, namely agriculture, textile, bio-composite, papermaking, automotive, construction, biofuel, functional food, oil, cosmetics, and personal care, as well as in the pharmaceutical industry" (Salentijn *et al*., 2015; Chaohua *et al*., 2016; Grulichova *et al*., 2017; Zuk-Golaszewska and Golaszewska, 2018). The multi-industrial application of cannabis owes to its oily seeds, long and durable fibers, and high content of cannabinoids, terpenes, and phenolic compounds (Grulichova *et al*., 2017; Zuk-Golaszewska and Golaszewska, 2018; Smith, 2019). With the recent discovery of medicinal cannabis, there have been re-regulations on the cultivation of cannabis since it was banned worldwide due to the intoxicating effects of its compound, delta-9-tetrahydrocannabinol (Thomas, 2012).

Traditionally, cannabis usually grows in disturbed areas such as the "borders of fields, on rubbish heaps near or habitations in farmyards, vacant lots, and in disturbed areas of pastures" (Small, 2015). If cultivated, it requires a low level of irrigation and fertilization, particularly after its establishment (Forapani *et al*., 2001; Ascrizzi *et al*., 2019). However, seed germination

involves complex physiological processes that respond to environmental signals such as water potential, light, and other factors (Bano *et al*., 2021). Many seeds still fail to germinate after being processed and placed in favourable growing conditions (Önol and Yildirim, 2021). Seed germination and performance also vary from plant to plant, resulting in different kinds of dormancy (Önol and Yildirim, 2021).

The cause of dormancy varies among plant species, but it is categorized into physical, morphological, physiological, morpho-physiological or a combination of physical and physiological dormancy, and thus, the seed must undergo the necessary changes for the germination to start (Golmohammadzadeh *et al*., 2015; Chahtane *et al*., 2017; Soltani *et al*., 2018). "Under natural conditions, necessary changes occur gradually as a result of varying combinations of aeration, moisture, temperature, and light". Dormancy could also be released using different methods, including phytohormones or chemical treatments (Önol and Yildirim, 2021). The standard procedures for breaking dormancy include prechilling, scarification, and treatments with gibberellic acid ($GA_3$) or nitric acid ($HNO_3$), as well as soaking seeds in hot water, or heat shock (Majd *et al*., 2013; Golmohammadzadeh *et al*., 2015; Labbafi *et al*., 2018). Scarification can be done mechanically or chemically (Utami *et al*., 2021). Mechanical scarification includes seeds shaken with an abrasive material such as sand or scratched with a knife (Utami *et al*., 2021). Under chemical scarification, seeds are soaked in chemical materials such as sulphuric acid ($H_2SO_4$), potassium nitrate ($KNO_3$), and hydrochloric acid (HCl) (Utami *et al*., 2021).

Pre-soaking the seeds in $GA_3$ or $KNO_3$ is known to alleviate both the physical and physiological dormancy (Tapfumaneyi *et al*., 2023). Prechilling simulates cold winter conditions for seeds exhibiting physiological dormancy, which leads to increased synthesis of $GA_3$ in the embryo and thereby aiding seed germination (Golmohammadzadeh *et al*., 2015). On the other hand, HCl, $H_2SO_4$, $HNO_3$ and hot water (HW) are known to break physical

dormancy by thinning the seed coat, which may harm the seed and damage the embryo (Gunes *et al*., 2013; Jaganathan *et al*., 2019; Jin *et al*., 2023). However, the results often vary as "induction of germination in dormant seeds may depend on the plant species, environmental conditions, growth form, seed origin and type of dormancy" (Golmohammadzadeh *et al*., 2015; Kildisheva *et al*., 2020; Islam *et al*., 2021; Flores-Sánchez *et al*., 2022).

Like any other crop, cannabis also has certain residual dormancy even though it is known to grow easily in any environment (Jovičić *et al*., 2019; Elias *et al*., 2020). In fact, it is suggested that wild cannabis can stay dormant but viable in soils for up to 10 years (Jovičić *et al*., 2019). Physiological dormancy is prevalent in cannabis because of the indeterminate growth habit of the female inflorescence, resulting in varied maturation times for seeds on the inflorescence (Önol and Yildirim, 2021). Elias *et al*. (2020) studied dormancy breaking through wet prechilling at 10 °C in 'Merlot' and 'Berry Blossom' hemp genotypes. They were able to break the seed dormancy within 5 days and achieved higher germination compared to the control treatment. In another study, the germination of 'Ferimon', 'Han NW', and 'Morpeth' hemp genotypes exposed to wet prechilling at 4 °C for 5 days, $GA_3$ at 500 and 1000 $mg \cdot L^{-1}$ and chlorine dioxide ($ClO_2$) at 500 and 1000 $mg \cdot L^{-1}$ was reduced compared to control (Islam *et al*., 2021). However, wet prechilling at 4 °C enhanced shoot and root growth of 'Ferimon', 'Han NW', and 'Morpeth' hemp genotypes compared to control (Islam *et al*., 2021).

However, studies on seed dormancy and dormancy breaking techniques are still lacking in South African cannabis genotypes. It is also evident from the previous studies that there is limited information of the existence of dormancy breaking technique that promote both seed germination and seedling growth in cannabis. Furthermore, hemp seeds are characterized by hard outer shell (pericarp, also known as hull) (Schultz *et al*., 2020). It is known that a hard seed coat (i.e., shell) may act as a physical barrier to the emergence of radicles and leaves, leading to physical dormancy (Elias *et al*., 2020; Kildisheva *et al*., 2020). Understanding that

the seed coat exerts such germination-restrictive action, therefore, there is a need for determining the influence of cannabis seed hardness on seed germination (Chaves *et al*., 2017). Therefore, there is a need to assess different methods for breaking dormancy and determine how different cannabis genotypes respond to these techniques in order to enhance germination and seedling development and reduce the duration of seed dormancy. Thus, the aim of the study is to evaluate the effect of various pre-treatments in breaking dormancy of cannabis seeds and determine the most effective pre-treatment for germination and seedling growth.

## 4.2. Materials and methods

### 4.2.1. Genotype collection

Seeds of five (5) cannabis landraces were used in the current study. The landraces were collected from cannabis growers in different regions of KwaZulu-Natal, namely, Ladysmith (Ugwayi wesiZulu and Iswazi), Bergville (Bergville Natal and Ugwayi wesiZulu), and Hammersdale in Durban (Durban poison). These seeds were kept at room temperature until use at the Horticultural laboratory in Agricultural Science Campus (with latitude: -29.636311 and longitude: 30.409060) at the University of KwaZulu-Natal Pietermaritzburg, South Africa. The collected cannabis landraces were assigned pseudonyms to ensure uniform naming of landraces for this experiment, as detailed in Table 4.1. The cannabis seeds were sorted according to colour. Dark-coloured seeds were associated with maturity whereas greenish coloured seeds were associated with immaturity (Miraji *et al*., 2021). Therefore, dark-coloured seeds were used for the experiment. Seeds showing defects were removed from the seed lot.

**Table 4.1:** The geographic location information and pseudonyms of the cannabis landraces collected from different regions of KwaZulu-Natal.

| Location | Geographic information | | | | | Landrace name | Pseudonym: |
|---|---|---|---|---|---|---|---|
| | Latitude: | Longitude: | Elevation | Average rainfall | Average temperature | | |
| Ladysmith | -28.57533 | 29.85948 | 1009.53 m | 108.7 mm | 23.3 °C | Ugwayi wesiZulu | 'L1' |
| | | | | | | Iswazi | 'L2' |
| Bergville | -28.667679 | 29.034111 | 1137.86 m | 109.4 mm | 23.5 °C | Natal | 'B2' |
| | -28.677544 | 29.125553 | | | | Ugwayi wesiZulu | 'B1' |
| Hammersdale | -29.870903 | 30.633519 | 597 m | 110.3 mm | 23.7 °C | Durban Poison | 'H1' |

### 4.2.2. Experimental design

The experiment was set under laboratory conditions and was later validated under greenhouse conditions. Each experiment was laid out in a factorial completely randomized design, consisting of 5 genotypes x 8 different seed dormancy breaking treatments. Each experiment was replicated three times, resulting in a total of 120 experimental units (90 mm Petri dishes for the laboratory and Polystyrene seedling trays for the greenhouse experiment). The seed dormancy breaking treatments consisted of control, sulphuric acid, potassium nitrate, hydrochloric acid, gibberellic acid, nitric acid, hot water, and prechilling.

The techniques for seed dormancy breaking were adopted from Tiwari *et al*. (2018) with minor modifications. The selected seed dormancy-breaking treatments were applied as described below.

*Hydrochloric acid:* Cannabis seeds were immersed in HCl 32% AR (Minema Chemicals (Pty) Ltd, 34 Ridge Rd, Laser Park; Johannesburg, South Africa) for 1 h under a fume hood. After soaking in HCl, the seeds were rinsed three times with distilled water. This procedure was followed by soaking seeds in distilled water for 24 h at room temperature, which were air-dried thereafter. The study followed this procedure for all the applied treatments.

*Sulphuric acid:* Cannabis seeds were immersed in $H_2SO_4$ 98% AR (Minema Chemicals (Pty) Ltd, 34 Ridge Rd, Laser Park; Johannesburg, South Africa) for 1 h under a fume hood.

*Hot water treatment:* Seeds in the dry state were placed in test tubes sealed with aluminum foil and placed in a water bath set at 70 °C for 1 h. Thereafter, seeds were soaked in distilled water for 24 h at room temperature and then air dried.

*Nitric acid:* Seeds were immersed in $HNO_3$ 60% AR (Minema Chemicals (Pty) Ltd, 34 Ridge Rd, Laser Park; Johannesburg, South Africa) for 1 h under a fume hood.

*Gibberellic acid:* Seeds were immersed in a gibberellic acid ($GA_3$) (Merck KGaA, 64271 Darmstadt, Germany) solution for 24 h at room temperature. The gibberellic acid solution was prepared by adding 10 mg of $GA_3$ into 1 litre of distilled water.

*Potassium nitrate:* Seeds were immersed in a 0.2% potassium nitrate ($KNO_3$) (Merck KGaA, 64271 Darmstadt, Germany) solution for 1 h under a fume hood.

*Prechilling:* Prechilling in the current study is referred to as dry prechilling. The current study adopted the prechilling technique by Elias *et al*. (2020) with some modifications. The seeds in the dry state, instead of wet, were stored in a sealed zipper bag and kept in a cold room set at

10 ± 0.5 °C for 5 days. Thereafter, seeds were soaked in distilled water for 24 h at room temperature and then air dried.

*Water:* The untreated seeds, which served as control, were soaked in distilled water for 24 h before germination, and then air-dried.

### 4.2.3. Seed viability test

Seeds were subjected to the viability test using 2,3,5-triphenyltetrazolium chloride (TCC) (Sigma Aldrich, Merck KGaA, 64271 Darmstadt, Germany) and the procedure was adopted from Mandizvo and Odindo (2019). TCC is a rapid and effective method for evaluating viability or physiological quality in seeds (Paiva *et al*., 2017). It is a water-soluble compound which promotes changes in colour when in contact with living seed tissues due to the activity of dehydrogenase enzymes (Virgens *et al*., 2019). Seed samples were preconditioned by soaking in distilled water for 18 h, at 25 °C in an incubator, and then excised with a scalpel blade through the embryo. The excised seeds were immersed in a 1% tetrazolium salt solution (TCC) for 12 h at room temperature. The experiment was replicated three times, and each replicate consisted of 30 seeds. Seed viability evaluation was done by placing the seeds in categories as viable and non-viable according to the coloration of the embryonic axis. Viable seeds were characterized by embryo showing uniform shiny pink colour, and non-viable seeds were characterized by embryo showing totally white colour (Paiva *et al*., 2017). Only the percentage of viable seeds was recorded.

### 4.2.4. Germination test

Thirty (30) seeds of each cannabis genotype were placed on cotton pads moistened with distilled water in 90 mm Petri dishes or in a seedling tray containing lawn dressing compost obtained from Duzi Turf, New England Rd, Pietermaritzburg, South Africa. Lawn dressing compost is characterized as a finely sifted black organic weed free medium. It is manufactured

of a mixture of organic waste and sewage sludge, with a composition of 85% humus and 15% 2 mm - 8 mm larger particles. Each seed was planted in a seedling tray hole and the germinating seeds were irrigated with tap water regularly to ensure adequate moisture. The experiment was replicated three times for consistency. The seeded Petri dishes were then transferred into a germination chamber (Micro-Clima Arabidopsis Chamber, ECD01E, Snijders, Netherlands) maintained at a temperature of 30/25 °C and light cycle of 16/8 h. The germination progress was monitored daily, and the number of germinated seeds was recorded for each replicate of 30 seeds for a period of 5 days under laboratory conditions and 14 days under greenhouse conditions. A seed was considered to have germinated when the emerging radical elongated to a length of 2 mm or more. The germination percentage (GP) was calculated according to Khalaki *et al*. (2019) using Eq. 1 and was expressed in percentages (%).

$$\mathrm{GP} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100 \quad (1)$$

The germination rate index (GRI) was calculated according to Awasthi *et al*. (2016) using Equation 2 and was expressed as percentage per day (%/day).

$$\mathrm{GRI} = \frac{\text{Number of germinated seeds}}{\text{Day of first count}} + \cdots + \frac{\text{Number of germinated seeds}}{\text{Day of final count}} \quad (2)$$

#### 4.2.5. Seedling growth test

After the germination test, the seedling length of germinated seeds was measured on the final day of germination in millimetres using a ruler. The seedling length was calculated as an average for all the germinated seeds for each landrace and treatment. The seedling vigour index (SVI) was calculated from the final germination percentage and seedling length according to Awasthi *et al*. (2016) using Equation 3.

$$\mathrm{SVI} = \frac{\text{Final germinated percentage} \times \text{Seedling length}}{100} \quad (3)$$

### 4.2.6. Data analysis

The data was analyzed using GenStat®, 20.1 Edition (VSN International, Hamel Hampstead, UK, 2020) at the 5% significance level. Tukey's test with GenStat® was used to separate the means of significantly different variables.

## 4.3. Results

### 4.3.1. Seed viability

The results showed no significant differences (*p*=0.102) amongst landraces for seed viability as assessed using tetrazolium chloride (TCC). The results showed that all seeds of landrace 'B1' were stained red (completely or partially stained), indicating 100% seed viability, as shown in Figure 4.1. Landrace 'B2' recorded a seed viability mean of 98.89%, while 'L2' and 'L1' recorded a seed viability mean of 92.22%. Landrace 'H1' recorded the lowest seed viability mean of 88.89%.

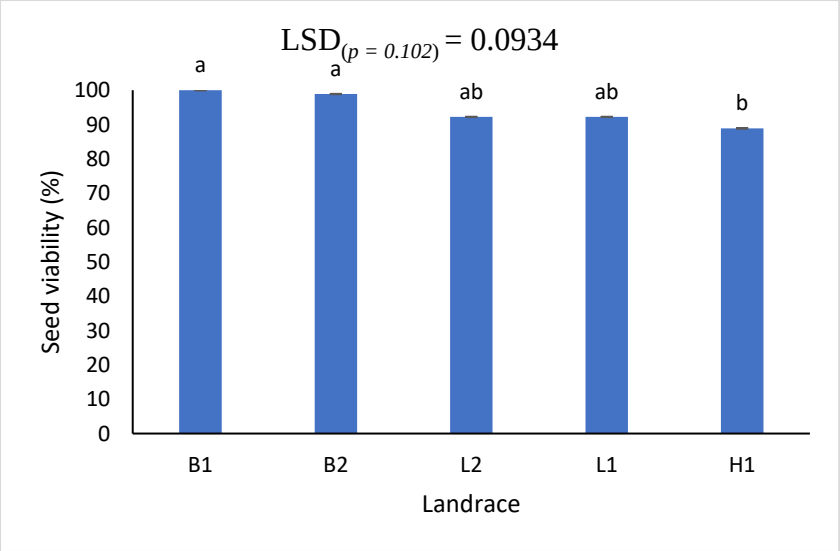



**Figure 4.1:** Seed viability of cannabis landraces before dormancy-breaking treatments (p=0.102; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 4.3.2. Germination and seedling growth under laboratory conditions

The landraces, pre-treatments, and their interactions showed significant differences ($p$<0.001) in germination percentage, germination rate index, seedling length and seed vigour index under laboratory conditions, as shown in Table 4.2.

**Table 4.2:** Analysis of variance (ANOVA) for seed germination percentage (GP), germination rate index (GRI), seedling length (SL), and seed vigour index (SVI) of cannabis landraces subjected to various dormancy breaking pre-treatments under laboratory conditions.

| Source | df | Mean square | | | |
|---|---|---|---|---|---|
| | | GP | GRI | SL | SVI |
| Landrace | 4 | 0.1864** | 1.2587** | 220.06** | 0.0269** |
| Treatment | 7 | 1.7199** | 4.6427** | 5479.68** | 0.3432** |
| Landrace*Treatment | 28 | 0.0341** | 0.0178** | 39.78** | 0.0043** |

ns - not significant ($p$>0.05); *significant ($p$<0.05); **highly significant ($p$<0.001**)**

#### 4.3.2.1. Seed germination

The results revealed that there were no significant differences between control and treatments such $GA_3$, $KNO_3$, $H_2SO_4$ and prechilling in all the cannabis genotypes. In landrace 'B2', the germination recorded under $H_2SO_4$, $KNO_3$, and $GA_3$ was higher compared to the control, as shown in Figure 4.2. $H_2SO_4$, $KNO_3$, and $GA_3$ recorded germination percentages of 92.22, 91.11, and 88.89%, respectively, and control recorded 82.22%. Similarly, $H_2SO_4$, $KNO_3$, and $GA_3$ recorded higher germination percentages in landrace 'L2' and 'L1' compared to control. In landrace 'L2', $H_2SO_4$, $GA_3$, and $KNO_3$ recorded germination of 86.67, 83.33, and 82.22%, respectively. In landrace 'L1', $KNO_3$, $H_2SO_4$, and $GA_3$ recorded a germination of 85.56, 83.33, and 74.44%, respectively. In landrace 'B1', none of the applied pre-treatments recorded germination higher than 73.33% recorded under control. Instead, 'B1' germination was completely inhibited under HCl. In landrace 'H1', $KNO_3$ and $GA_3$ recorded higher germination

of 91.11 and 85.56% compared to control. HCl and HW completely inhibited the germination of landrace 'L1'.

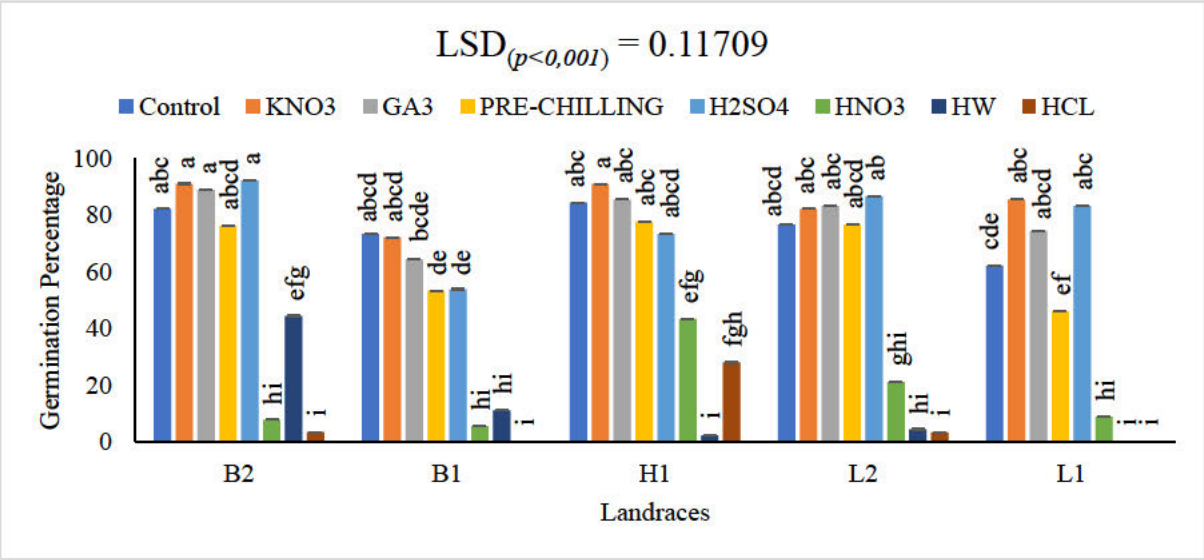



**Figure 4.2**: Effect of eight different seed dormancy-breaking treatments on germination percentage among five (5) cannabis landraces under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 4.3.2.2. Germination rate index

The results revealed that $H_2SO_4$ recorded the highest GRI in cannabis landraces, except 'H1' as the control recorded the highest, as shown in Figure 4.3.

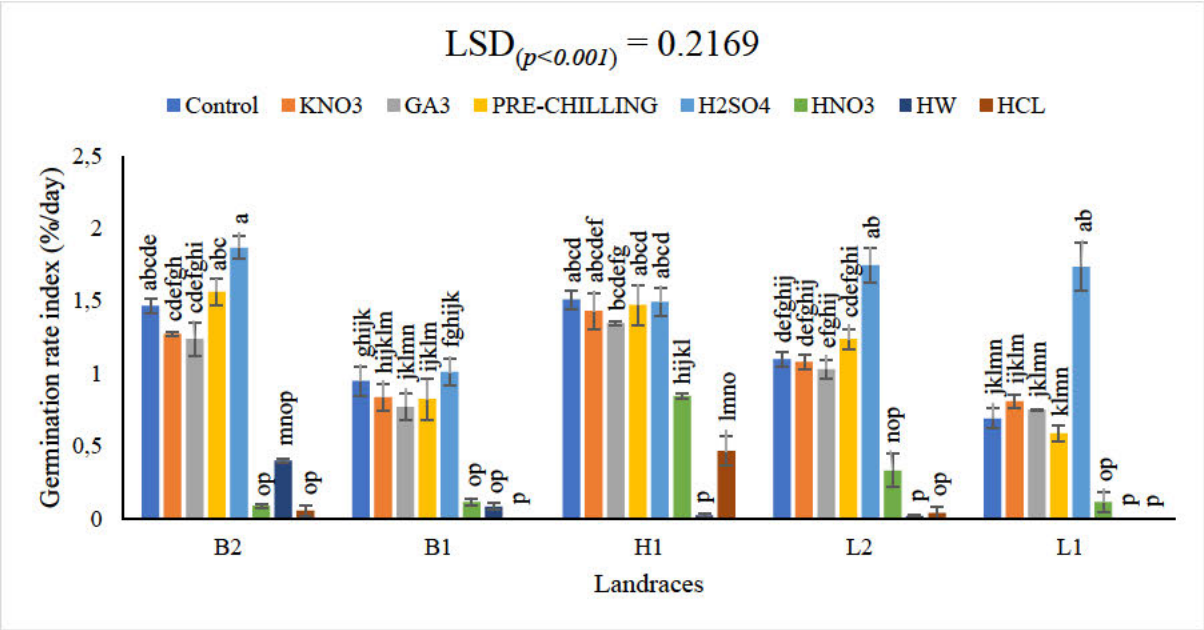



**Figure 4.3:** Effect of eight different seed dormancy-breaking treatments on germination rate index among five (5) cannabis landraces under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 4.3.2.3. Seedling length

The results revealed that the control recorded higher seedling length means compared with the applied pre-treatments. Control recorded higher seedling length means of 51.74, 48.18, 40.50, and 33.88 mm for landrace 'B2', 'B1', 'L2', and 'L1', respectively. The lowest seedling length means of 1.56, 1.00, and 1.50 mm for landrace 'B2', 'B1', and 'L1' were recorded under $HNO_3$ treatment, respectively. For landrace 'L2', the lowest seedling length mean of 1.00 mm was recorded under HCl treatment, as shown in Figure 4.4. Compared to the control, prechilling and $KNO_3$ treatment recorded higher seedling length means of 42.46 and 40.42 mm for landrace 'H1', respectively. The lowest seedling length mean of 1.33 mm for landrace 'H1' was recorded under HW treatment.

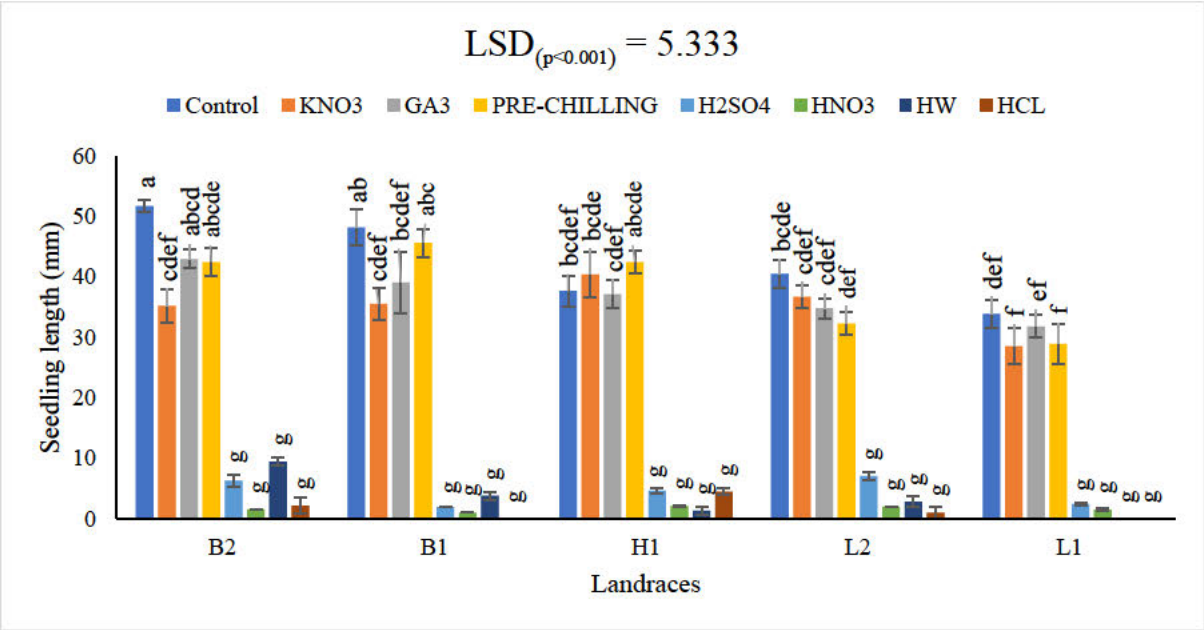



**Figure 4.4:** Effect of eight different seed dormancy-breaking treatments on the radicle length among five (5) cannabis landraces under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 4.3.2.4. Seed vigour index

The results revealed that the control recorded higher SVI compared with the applied dormancy breaking treatments. Control recorded higher SVI of 42.53, 35.21, and 31.20 for landrace 'B2', 'B1', and 'L2', respectively. The lowest SVI means of 0.12 and 0.06 were recorded under $HNO_3$ treatment in landrace 'B2' and 'B1', respectively. The lowest SVI mean for 'L2' of 0.10 was recorded under HCl treatment, as shown in Figure 4.5. Compared to the control, $KNO_3$ and prechilling treatment recorded higher SVI means of 36.89 and 32.73 for landrace 'H1', respectively. For landrace 'L1', $KNO_3$ and $GA_3$ treatment recorded higher SVI means compared to the control of 24.47 and 23.64, respectively. The lowest SVI means of 0.04 and 0.00 were recorded for landrace 'H1' and 'L1', respectively, under HW treatment.

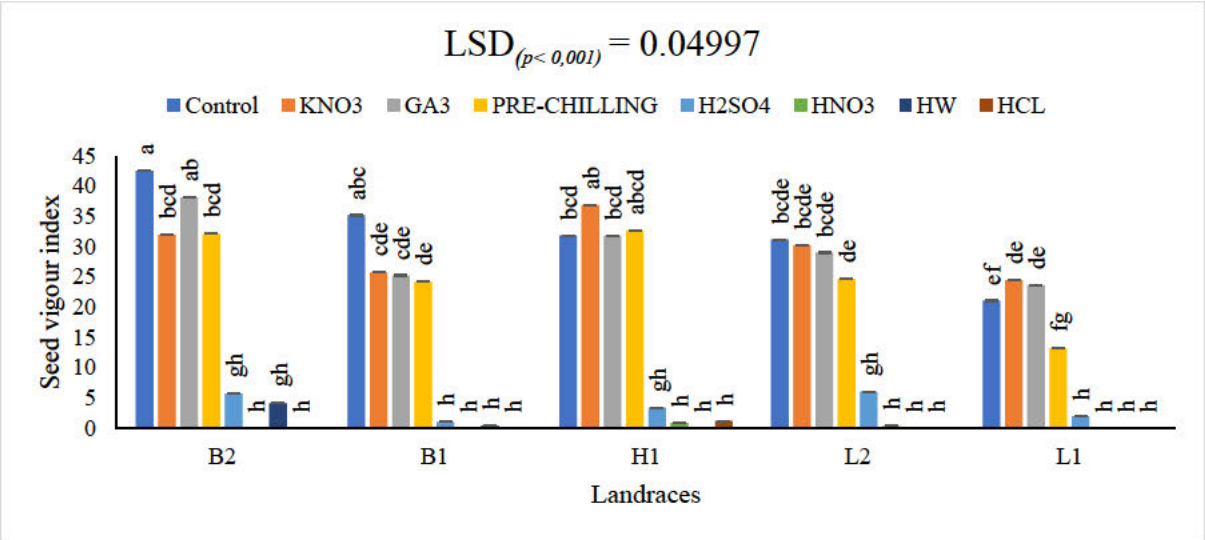



**Figure 4.5:** Effect of eight different seed dormancy-breaking treatments on seed vigour index among five (5) cannabis landraces under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 4.3.3. Germination and seedling growth under greenhouse conditions

The landraces, pre-treatments, and their interactions showed significant differences ($p$<0.001) in germination percentage, germination rate index, seedling length, and seed vigour index under greenhouse conditions, as shown in Table 4.3.

**Table 4.3:** Analysis of variance (ANOVA) for seed germination percentage (GP), germination rate index (GRI), seedling length (SL), and seed vigour index (SVI) of cannabis landraces subjected to various dormancy breaking pre-treatments under greenhouse conditions.

| Source | df | Mean square for GP | df | Mean square | | |
|---|---|---|---|---|---|---|
| | | | | GRI | SL | SVI |
| Landrace | 4 | 3435.74** | 4 | 1687.08** | 3387.3** | 11019.6** |
| Treatment | 7 | 21623.90** | 4 | 3943.95** | 39655.0** | 32001.3** |
| Landrace*Treatment | 28 | 16667.41** | 16 | 214.97** | 1133.1** | 1218.4** |

**highly significant ($p$<0.001)

#### 4.3.3.1. Seed germination

The results revealed that the applied seed dormancy breaking treatments such as HCl, $HNO_3$ and $H_2SO_4$ completely inhibited the germination of all the cannabis genotypes and, therefore, their analyzed data was excluded. HW also hindered the germination of all the cannabis genotypes compared to the control, with complete inhibition in 'H1' and 'L2', as shown in Figure 4.6. Compared to the control, prechilling treatment recorded significantly higher germination of 65.56% in 'H1', The results also reveal that there was no significant difference between control and treatments such $GA_3$, $KNO_3$ and prechilling in 'B2', 'B1', 'L2', and 'L1'. Similarly, there was no significant difference between control and treatments such $GA_3$ and $KNO_3$. However, $KNO_3$ recorded higher germination of 40% in 'H1' compared to 36.67% of control. $GA_3$, $KNO_3$ and prechilling recorded higher germination of 91.11, 87.78 and 90.00% in 'B2' compared to 86.67% of control. $GA_3$ and $KNO_3$ recorded higher germination of 80.00 and 95.56% in 'B1' compared to 78.89% of control. $GA_3$ and $KNO_3$ also recorded higher germination of 81.11 and 71.11% in 'L2' compared to 66.67% of control. $GA_3$ also recorded higher germination of 95.56% in 'L1' compared to 93.33% of control.

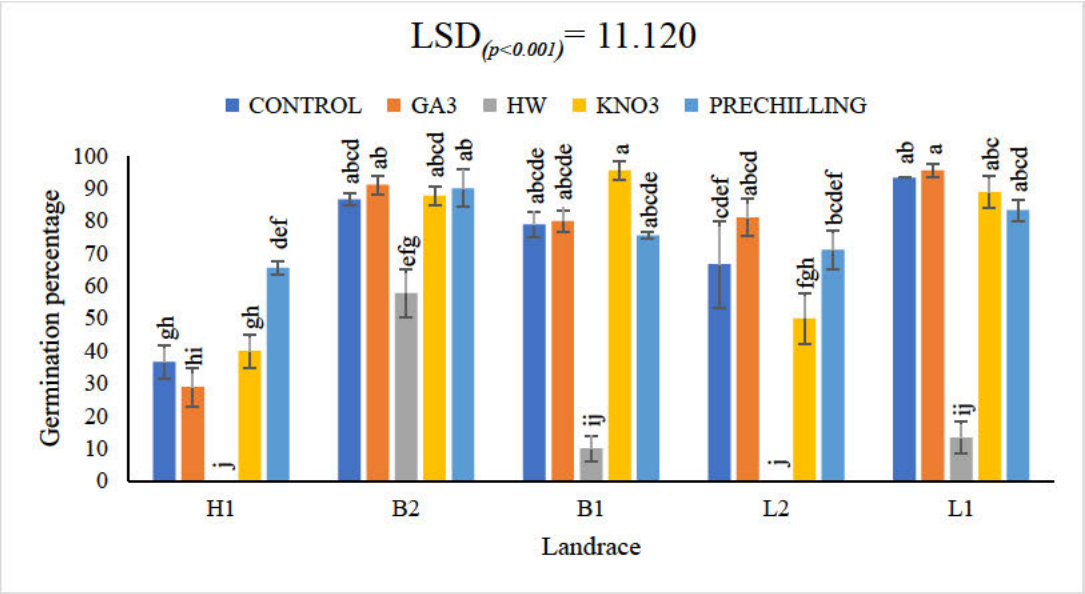



**Figure 4.6:** Effect of five different seed dormancy-breaking treatments on germination percentage among five (5) cannabis landrace under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 4.3.3.2. Germination rate index

The results revealed that the germination rate index varied in genotypes with the applied seed dormancy breaking treatments. However, the germination rate index recorded for all cannabis genotypes was significantly lower under HW compared to the control, as shown in Figure 4.7. $GA_3$, $KNO_3$ and prechilling recorded a higher germination rate index in 'H1' of 15.8, 24.35 and 21.93%/day, respectively, compared to 13.49%/day recorded for the control. $GA_3$ and $KNO_3$ recorded a higher germination rate index of 57.22 and 47.97%/day in 'B2', respectively, compared to 40.03%/day recorded for control. $GA_3$ and $KNO_3$ also recorded a higher germination rate index of 58.16 and 64.49%/day in 'B1', respectively, compared to 44.59%/day recorded for control. Furthermore, $GA_3$ and $KNO_3$ also recorded a higher germination rate index of 45.55 and 43.10%/day in 'L1', respectively, compared to 39.70%/day recorded for control. $GA_3$ and prechilling recorded a higher germination rate index of 42.63 and 27.74%/day in 'L2', respectively, compared to 26.53%/day recorded for control.

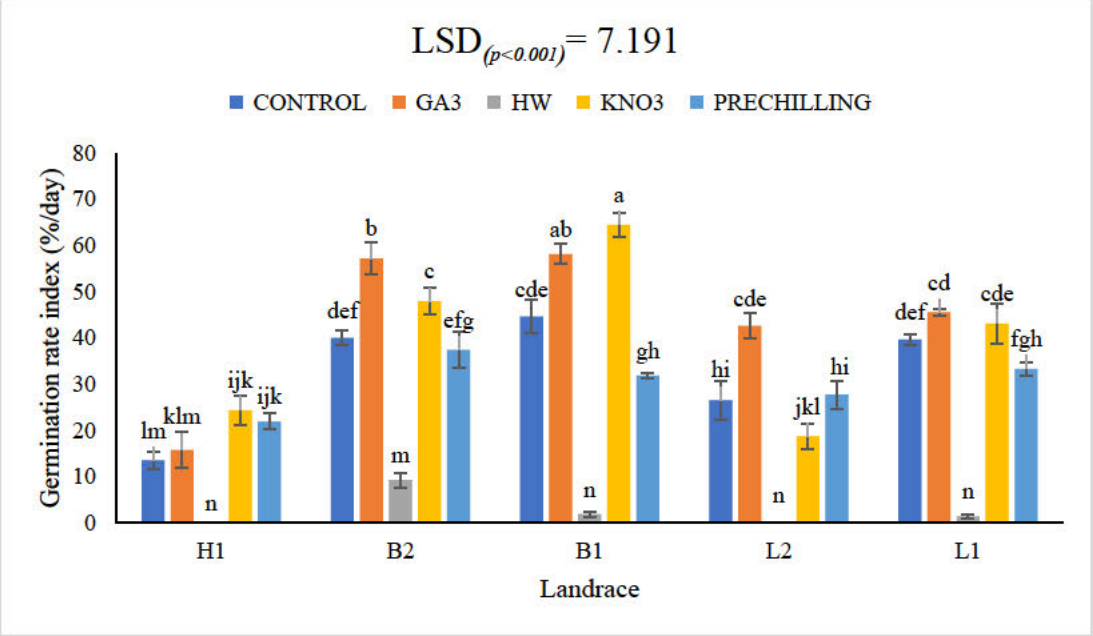



**Figure 4.7:** Effect of five different seed dormancy-breaking treatments on germination rate index among five (5) cannabis landraces under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 4.3.3.3. Seedling length

The results revealed that seedling under $GA_3$, $KNO_3$ and prechilling recorded longer length in 'H1', 'B2', 'B1' and 'L2' compared to control, as shown in Figure 4.8. $GA_3$, $KNO_3$ and prechilling recorded a seedling length of 138.0, 160.9 and 176.6 mm in 'H1', respectively, and control recorded 111.2 mm. $GA_3$, $KNO_3$ and prechilling recorded a seedling length of 148.4, 157.3 and 208.7 mm in 'B2', respectively, and control recorded 138.5 mm. $GA_3$, $KNO_3$ and prechilling recorded a seedling length of 154.6, 150.7 and 161.7 mm in 'B1', respectively, and control recorded 144.0 mm. $GA_3$, $KNO_3$ and prechilling recorded a seedling length of 138.3, 140.2 and 142.2 mm in 'L2', respectively, and control recorded 130.5 mm. The results also revealed that seedlings under $GA_3$ and prechilling had longer length of 133.2 and 151.7 mm in 'L1', respectively, compared to 110.8 mm recorded for control.

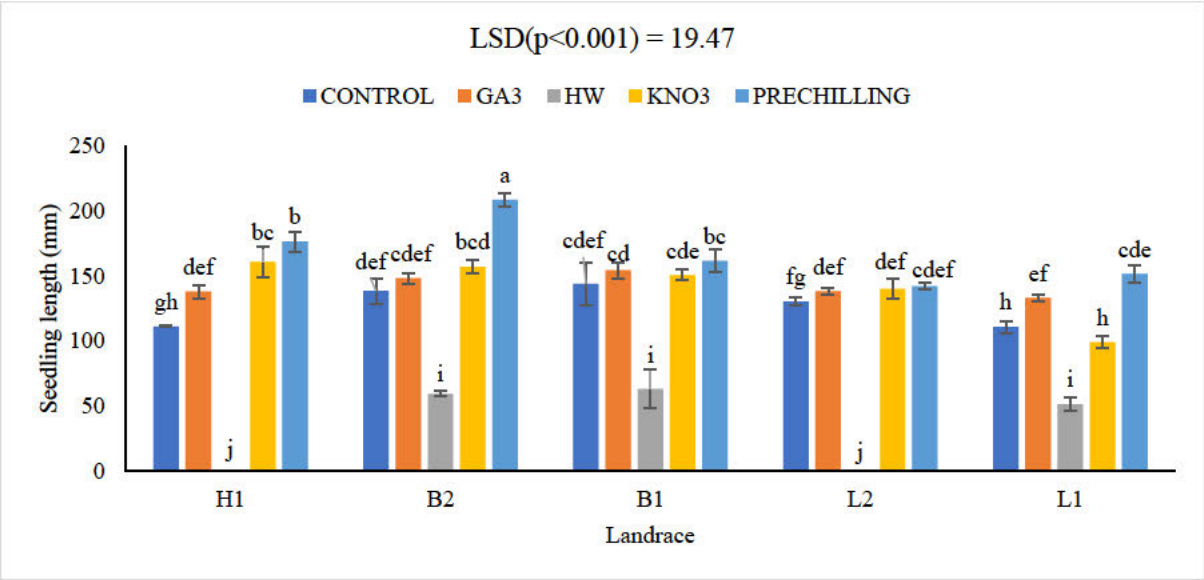



**Figure 4.8:** Effect of five different seed dormancy-breaking treatments on the radicle length among five (5) cannabis landraces under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 4.3.3.4. Seed vigour index

The results revealed that seed vigour index was higher in 'B2' and 'B1' under $GA_3$, $KNO_3$ and prechilling compared to control, as shown in Figure 4.9. $GA_3$, $KNO_3$ and prechilling recorded a higher seed vigour index of 135.23, 138.28 and 188.18 in 'B2', respectively, and control recorded 120.17. $GA_3$, $KNO_3$ and prechilling recorded a higher seed vigour index of 124.11, 144.21 and 121.96 in 'B1', respectively, and control recorded 113.53. The results also revealed that seed vigour index was higher in 'L2' and 'L1' under $GA_3$ and prechilling compared to control. $GA_3$ and prechilling recorded a seed vigour index of 112.41 and 100.81 in 'L2', respectively, and control recorded 87.63. $GA_3$ and prechilling recorded a seed vigour index of 127.33 and 126.19 in 'L1', respectively, and control recorded 103.44. Furthermore, the results revealed that $KNO_3$ and prechilling had a higher seed vigour index of 63.24 and 116.13 in 'H1', respectively, compared to 40.79 recorded for control.

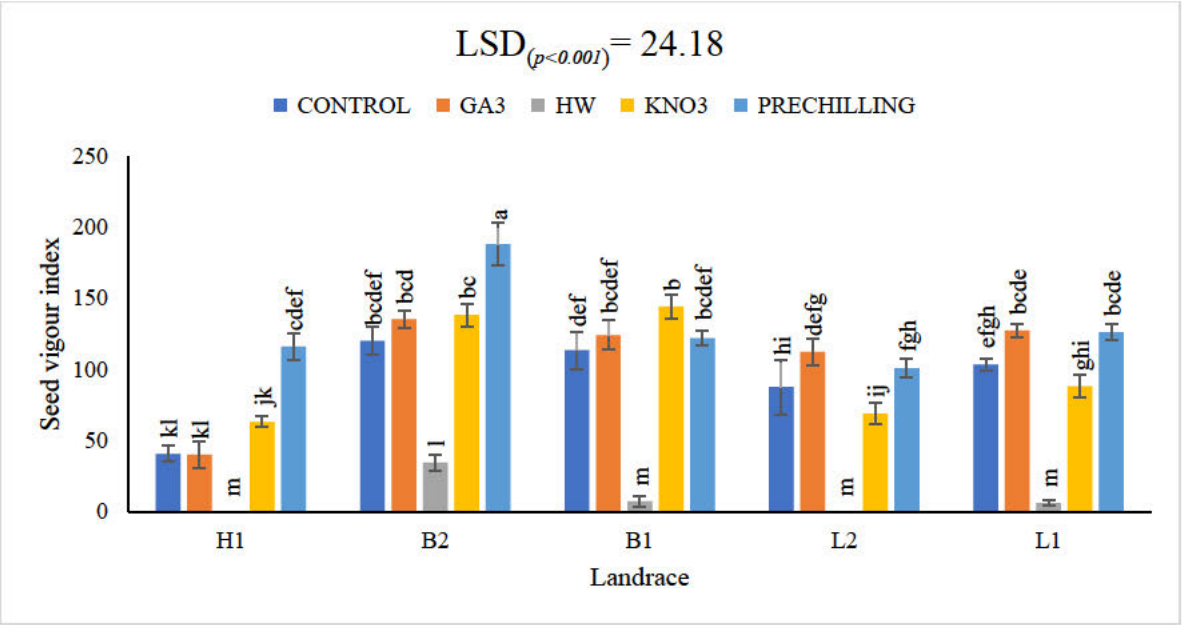



**Figure 4.9:** Effect of five different seed dormancy-breaking treatments on seed vigour index among five (5) cannabis landraces under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 4.4. Discussion

Generally, germination percentage, germination rate index, seedling length and seed vigour index varied among cannabis genotypes with the applied treatments under both laboratory and greenhouse conditions. Amongst the cannabis genotypes, 'B2' generally had higher germination percentage, seedling length and seed vigour under both greenhouse and laboratory conditions. On the other hand, 'H1' and 'L1' generally had poor germination performance compared to other genotypes under greenhouse and laboratory conditions, respectively. The results revealed a relationship between germination and seedling growth performance and the viability of the seeds of certain cannabis genotypes. This follows that 'B2' had second highest seed viability percentage, while 'H1' had the lowest and 'L1' had the second lowest seed viability percentage. These findings were expected as it is known that viable seed indicates its capability to germinate and produce a normal seedling when it is not dormant (Quintana *et al*., 2023).

Under the laboratory conditions, $H_2SO_4$, $KNO_3$, and $GA_3$ generally proved highly effective in breaking the seed dormancy of cannabis landraces, while prechilling, $HNO_3$, HCl, and HW proved ineffective. These results were then validated under greenhouse conditions which simulated field conditions. Under the greenhouse conditions, $KNO_3$, $GA_3$ and prechilling proved highly effective in breaking the seed dormancy of cannabis genotypes, while $H_2SO_4$, $HNO_3$, HCl, and HW proved ineffective. Notably, prechilling performance proved effective in breaking the seed dormancy of cannabis landraces under greenhouse conditions but was ineffective under laboratory conditions. Specifically, prechilling was generally the most effective treatment in enhancing seed germination and seedling length under greenhouse conditions and ultimately, seed vigour index. Such findings about the cannabis genotypes and applied treatments were expected as previous studies suggests that these germination and seedling growth parameters varies with environmental conditions, in response to pre-treatments (Golmohammadzadeh *et al*., 2015; Kildisheva *et al*., 2020; Islam *et al*., 2021; Flores-Sánchez *et al*., 2022). The greenhouse findings corroborate with Elias *et al*. (2020), who achieved dormancy breaking through wet prechilling at 10 °C in 'Merlot' and 'Berry Blossom' hemp genotypes, although the greenhouse study used dry prechilling. In contrast to the findings of both Elias *et al*. (2020) and the current study under greenhouse conditions, prechilling proved ineffective in breaking the seed dormancy of cannabis landraces under laboratory conditions. It is worth highlighting that dry prechilling is associated with not providing enough moisture like wet prechilling does to activate the hydrolytic enzymes in preparation of seeds for germination once they are moved to favourable conditions (Amini *et al*., 2015).

Furthermore, the seedling length and seed vigour index was generally higher under control compared to the applied pre-treatments under laboratory conditions. The findings of the laboratory study are in contrast with the findings of Aliloo and Darabinejad (2013), who reported that prechilling, $KNO_3$ and $GA_3$ pre-treatments recorded higher seedling length and

seed vigour in *Heliotropium europaeum* compared to the control. However, Aliloo and Darabinejad (2013) used 1.5% $KNO_3$ for 1 h and $GA_3$ at a concentration of 250 mg per litre for 12 h. Thus, besides pre-treatment concentration and duration of seed contact, the findings of the laboratory study on the seedling length and seed vigour lower than control were associated with the fact that species response to pre-treatments may vary with the growth stage (Golmohammadzadeh *et al*., 2015; Kildisheva *et al*., 2020; Islam *et al*., 2021). However, the greenhouse results revealed that prechilling, $KNO_3$ and $GA_3$ generally improved the seedling length and seed vigour index of the cannabis landraces compared to control. Thus, the findings of the current study under greenhouse conditions agree with the findings by Aliloo and Darabinejad (2013), however, in contradiction with the laboratory findings as prechilling, $GA_3$ and $KNO_3$ improved seedling length and seed vigour index in cannabis genotypes. The differing findings between the laboratory and greenhouse studies may indicate that the cannabis thrived under greenhouse conditions because it simulated the actual growth environment of the cannabis genotypes.

The complete germination inhibition of $H_2SO_4$, $HNO_3$, HCl in all cannabis genotypes under greenhouse conditions was associated with high concentrations which could have been corrosive and extended seed soaking duration resulting into seed and embryo damage; and ultimately inhibiting germination (Maesaroh and Demirbağ, 2020). $H_2SO_4$ having been effective in breaking seed dormancy through germination and germination rate index under laboratory conditions revealed that it oxidized the seed coats, resulting in softening and rupture of the seed coats (Zare *et al*., 2011; Mensah and Ekeke, 2016). Thereafter, the germination process in the seed was initiated, and eventually, protrusion of the radical and subsequent germination (Zare *et al*., 2011; Rasebeka *et al*., 2014; Mensah and Ekeke, 2016). However, the results under similar conditions proved that $H_2SO_4$ was not effective in producing healthy and vigorous quality seedlings. This is evident in the poor seedling length and seed vigour index

revealed under laboratory conditions. Poor seed vigour index was associated with seeds unable to germinate under the greenhouse conditions.

However, HCl, HW, and $HNO_3$ results were expected because they have been previously reported as ineffective in breaking seed dormancy (Kambizi *et al*., 2006; Bhardwaj *et al*., 2016). For $H_2SO_4$, the findings of the current study are in contradiction with Ansari *et al*. (2016), who reported that scarification of the seed coat with 95% $H_2SO_4$ stimulated the seed germination of *Malva sylvestris* L. As a result, germination of 88% was recorded under acid scarification, and no germination was recorded under control (Ansari *et al*., 2016). Mensah and Ekeke (2016) also reported that $H_2SO_4$ enhanced the germination of *Senna obtusifolia* L. to 100% against intact seeds that did not germinate.

On the other hand, $KNO_3$ is well-known as a compound that promotes the germination of photo-dormant seeds (Rouhi *et al*., 2013). "$KNO_3$ increases ambient oxygen levels by reducing the amount of oxygen available for the tricarboxylic acid (TCA) cycle" (Rouhi *et al*., 2013), which is a critical metabolic pathway for germination as it unifies carbohydrate, fat, and protein metabolism (Zhang *et al*., 2017). "$KNO_3$ is related to the activity of the enzyme nitrate reductase in the production of nitrite/nitric oxide (NO)" (Lara *et al*., 2014). "NO breaks a seed dormancy through the interaction with phytochrome signaling pathways, ethylene biosynthesis and interplays with reactive oxygen species" (Lara *et al*., 2014). Hence, it was effective in breaking the seed dormancy of cannabis landraces. However, the results of the current study contrast the findings by Mensah and Ekeke (2016), who reported that $KNO_3$ was ineffective in breaking seed dormancy and promoting seed germination of *Senna obtusifolia* species (Mensah and Ekeke, 2016).

For $GA_3$, the results of the current study were associated with the fact that "$GA_3$ acts as a natural plant growth regulator, releasing the seeds from dormancy by promoting protein

synthesis, elongation of coleoptiles, and production of ethylene" (Camara *et al*., 2018; Polaiah *et al*., 2020). Similar results have been previously reported on plants such as *Papaver rhoeas* L. and *Papaver dubium* L. where $GA_3$ promoted germination when exogenously applied in these plants (Golmohammadzadeh *et al*., 2015). $GA_3$ being effective in breaking seed dormancy may also be associated with that both with abscisic acid (ABA) are key for inducing, maintaining, and releasing seed dormancy (Longo *et al*., 2021). These hormones are known to act antagonistically; ABA promotes dormancy during seed maturation and inhibits seed germination, whereas GA promotes seed germination (Longo *et al*., 2021; Yan and Chen, 2020). Thus, the seed dormancy gets released when the ABA/GA ratio is low, and eventually, germination commences (Yang *et al*., 2020).

Therefore, taking into consideration that greenhouse conditions simulate the actual cannabis growing conditions, the summary of findings is that $GA_3$, $KNO_3$ and prechilling were the most effective pre-treatments to improve the germination and seedling growth of cannabis genotypes. Also taking into consideration of the pre-treatments that improved the germination, it was found that the seed coat of the cannabis genotypes had no effect on germination as the pre-treatments that were effective are associated with breaking physiological dormancy. Notably, prechilling is known to induce cold environmental conditions that leads to increased synthesis of $GA_3$ in the embryo and thereby aiding seed germination. Thus, the physiological dormancy in cannabis genotypes was largely associated with ABA/GA ratio.

## 4.5. Conclusions

The current study revealed the significancy of adequate growing conditions in cannabis genotypes for assessing their germination and seedling growth studies. The current study concluded that using prechilling, $GA_3$, and $KNO_3$ pre-treatments enhanced the germination capacity and seedling growth of cannabis landraces, and alleviated seed dormancy. However,

the effect on the germination of cannabis varied with landrace, treatment, and seed viability. It was concluded that the physiological state of the embryo caused dormancy in cannabis landraces, ABA/$GA_3$ levels, and possibly a decreased gas permeability of the seed coat was the major barrier to cannabis seed germination. This is because the pre-treatments prechilling and $GA_3$ are associated with the increased synthesis of $GA_3$ which "promotes protein synthesis, elongation of coleoptiles, and production of ethylene". On the other hand, $KNO_3$ is associated with the increased ambient oxygen levels; oxygen is associated with metabolism reactivation during seed imbibition. Prechilling is known to induce cold environmental conditions that leads to increased synthesis of $GA_3$ in the embryo and thereby aiding seed germination. The current study, therefore, recommends further investigation of the effect of growth hormones or chemicals at different concentrations and duration of seed exposure on seed reserve mobilization during germination of cannabis seeds of known ABA/$GA_3$ ratio.

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# 5. CHAPTER 5: SEED GERMINATION PERFORMANCE OF CANNABIS LANDRACES UNDER ACCELERATED AGING

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**Abstract**

The aim of the study was to investigate the effect of accelerated aging on seed germination of five cannabis landraces. The seeds of five cannabis landraces, namely, Ladysmith Ugwayi wesiZulu ('L1') and Iswazi ('L2'), Bergville Natal ('B1'), Msinga Ugwayi wesiZulu ('M1') and Natal ('B2') were subjected to accelerated aging at 42 °C consisting of six (6) levels, i.e., control (unaged), 24, 48, 72, 96 and 120 h under laboratory conditions. The seed viability was assessed using a Tetrazolium chloride solution (TCC) test. Electrolyte conductivity, pH, and water activity were taken for five days before germination. The germination test was conducted under laboratory conditions under 30/25 °C in Petri dishes with cotton pads moistened with distilled water. The study was later validated under greenhouse conditions. Thereafter, the germination was monitored daily, and the collected data was used to assess the germination percentage, germination rate, and coefficient of velocity of germination were measured. The results showed that accelerated aging significantly affected seed germination characteristics, seed viability, electrolyte leakage, pH, and water activity ($p$<0.05). Accelerated aging increased seed electrolyte leakage, causing progressive deterioration and reduced germination

performance of cannabis landraces. Thus, optimal storage conditions are key for maintaining seed viability and germinability of cannabis seeds.

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**Keywords**: Seed viability, accelerated aging, cannabis, electrolyte leakage, landraces, germination

### 5.1. Introduction

Seeds undergo various cellular, physical, physiological, and biochemical changes under natural conditions, which leads to decreased viability and, eventually, seed death, and this event is termed seed aging (Lakshmi *et al*., 2021). The unfavourable environmental factors during production and storage aggravate the potential loss of vigour, viability, and quality of seeds with a change in lipid peroxidation, which is known as seed deterioration (Zheng and Ma, 2014; Kapilan and Thiagarajah, 2015; Kandpal *et al*., 2016; Feng *et al*., 2018; Lakshmi *et al*., 2021). The rate of the aging process depends on several factors, including heritable genetics, mechanical damages, seed oil and moisture content, relative humidity and temperature, pests and diseases, oxygen pressure, and other factors such as direct sunlight, fumigation procedures, seed treatments (Kapilan and Thiagarajah, 2015; Bijanzadeh *et al*., 2017; Thirusendura and Saraswathy, 2017). The main factors influencing the rate of deterioration are temperature and relative humidity (RH), which controls the moisture content of the seed (Lakshmi *et al*., 2021).

Deterioration changes in aged seeds are attributed to oxidative damage (Kapilan and Thiagarajah, 2015). Free radical-mediated lipid peroxidation, enzyme inactivation or protein degradation, disruption of cellular membranes, and damage to genetic (nucleic acids) integrity are identified as the major causal agents of seed deterioration (Zheng and Ma, 2014). As a series of biochemical reactions, seed aging is relatively progressive and irreversible, and it can significantly reduce seedling establishment (Bijanzadeh *et al*., 2017; Nazari *et al*., 2020). Therefore, effective seed storage is critical to preserving seed viability after harvesting or

purchasing seed (De Vitis *et al*., 2020). Storing seeds in a place with proper temperature and humidity control often delays physiological and physiochemical changes and, ultimately, seed aging (Mohammadi *et al*., 2011; Kandpal *et al*., 2016; Feng *et al*., 2018). Generally, the natural aging of seeds is a long-duration procedure; as a result, many research studies have applied artificial accelerated aging tests to simulate the seed aging procedure in a short time compared with natural aging (Feng *et al*., 2018).

Accelerated aging damages DNA and mRNA, ultimately causing biochemical deterioration of the stored material and reducing seedling vigour and early plantlet development shortly after germination (Hussein *et al*., 2012). Thus, accelerated aging is critical for understanding the events that lead to the loss of seed viability (Hussein *et al*., 2012). Furthermore, accelerated aging is very effective in determining the relative storage potential of seed lots (Feng *et al*., 2018). Additionally, it enables the selection of cultivars with higher performance potential in breeding programs for regions with a high probability of high temperature and relative humidity during seed maturation (de Lima *et al*., 2019). In developing countries, small resource-poor farmers often lack access to storage with proper temperature and humidity control, leading to prevalent seed deterioration (Mohammadi *et al*., 2011). Therefore, the accelerated aging results can also assist in recommending cultivars for producers with inadequate infrastructure for seed storage (de Lima *et al*., 2019).

Crops with high oil content have been found prone to seed deterioration due to the lower chemical stability of lipids in relation to starch (Suriyong *et al*., 2015; Prudente and Paiva, 2018). The oil content of oil crops, such as *Glycine max* (L.) Merr., *Arachis hypogaea* L. and *Helianthus annuus* L., is a major contributor to the reduction in seed storage potential (Suriyong *et al*., 2015). Moreover, the high protein content also contributes to the reduction in seed storage potential due to the high affinity of this substance to water (Prudente and Paiva, 2018). Thus, cannabis seed is no exception as it contains an oil content of 25 – 35%; sunflower

has an oil content of 35 – 42%; peanut has 45 – 56%; and soybean has 18 – 22% (Suriyong *et al*., 2015). Cannabis also contains a protein content of 20 – 25%, whereas sunflower has a protein content of 20 – 40%, peanut has 22 – 33%, and soybean has 38– 42% (Suriyong *et al*., 2015). According to Small and Brookes (2012), the longevity of hemp seeds drops to 70-80% after two years of storage in a sheltered but otherwise uncontrolled environment. However, there is little information on the physiology of cannabis seed aging, particularly in South African landraces, and its effect on germination due to the illegality issues on the crop over the past centuries. Such information is critical in order to produce a sufficient supply of high-quality seeds needed to perpetuate production. Therefore, the current study aims to investigate the response of cannabis landraces to different durations of accelerated aging.

## 5.2. Materials and methods

### 5.2.1. Genotype collection

Seeds of five (5) cannabis landraces were used in the current study. The landraces were collected from cannabis growers in different regions of KwaZulu-Natal, namely, Ladysmith (Ugwayi wesiZulu and Iswazi), Bergville (Bergville Natal and Ugwayi wesiZulu), and Msinga (Ugwayi wesiZulu). The collected cannabis landraces were assigned pseudonyms to ensure uniform naming of landraces for this experiment, as detailed in Table 5.1.

**Table 5.1:** The pseudonyms of the cannabis landraces and the description of their geographic location.

| Location | Geographic information | | | | | Landrace name | Pseudonym: |
|---|---|---|---|---|---|---|---|
| | Latitude: | Longitude: | Elevation | Average rainfall | Average temperature | | |
| Ladysmith | -28.57533 | 29.85948 | 1009.53 m | 108.7 mm | 23.3 °C | Ugwayi wesiZulu | 'L1' |
| | | | | | | Iswazi | 'L2' |
| Bergville | -28.667679 | 29.034111 | 1137.86 m | 109.4 mm | 23.5 °C | Natal | 'B2' |
| | -28.677544 | 29.125553 | | | | Ugwayi wesiZulu | 'B1' |
| Msinga | -28.700960 | 30.303320 | 559 m | 340.0 mm | 23.0 °C | Ugwayi wesiZulu | 'M1' |

The collected seed samples were stored at room temperature in the Horticultural Sciences Laboratory of the University of KwaZulu-Natal, Pietermaritzburg in South Africa (latitude: -29.636311 and longitude: 30.409060). Prior to germination studies, the cannabis seeds were sorted based on colour. Dark-coloured seeds were associated with maturity whereas greenish coloured seeds were associated with immaturity (Miraji *et al*., 2021). Therefore, dark-coloured seeds were used for the experiment. Seeds showing defects were removed from the seed lot.

### 5.2.2. Experimental design

The experiment was set under laboratory conditions and was later validated under greenhouse conditions. Each simulated seed aging experiment was structured using a factorial completely randomized design consisting of 5 genotypes and 6 accelerated seed aging periods. Each

experiment was replicated three times, resulting in a total of 90 experimental units (90 mm Petri dishes for the laboratory and polystyrene seedling trays for the greenhouse experiment). Seeds of various cannabis landraces were subjected to accelerated aging following the procedures by Mandizvo and Odindo (2019). Accelerated seed aging was done for 24, 48, 72, 96, and 120 h using an incubator consisting of 11 x 11 x 3.5 cm inverted plastic sandwich boxes containing a 10 x 10 x 3 cm copper wire mesh tray. One hundred millilitres of saturated NaCl solution were poured into each plastic box, and a dry screen tray was inserted. Seeds of each landrace were placed on the surface of the screen trays. After leveling the seed sample on the surface of the screen tray, the lid was secured on each plastic AA box. The AA boxes were placed in a germination chamber (Labcon L.T.I.E, South Africa) set at 42 ± 0.3 °C.

### 5.2.3. Seed viability test

Seeds were subjected to the viability test using 2,3,5-triphenyltetrazolium chloride (TCC) (Sigma Aldrich, Merck KGaA, 64271 Darmstadt, Germany) and the procedure was adopted from Mandizvo and Odindo (2019). TCC is a rapid and effective method for evaluating viability or physiological quality in seeds (Paiva *et al*., 2017). It is a water-soluble compound which promotes changes in colour when in contact with living seed tissues due to the activity of dehydrogenase enzymes (Virgens *et al*., 2019). Seed samples were preconditioned by soaking in distilled water for 18 h at 25 °C in an incubator, and then excised with a scalpel blade through the embryo. The excised seeds were immersed in a 1% tetrazolium salt solution (TCC) for 12 h at room temperature. The experiment was conducted in seeds that had not been subjected to accelerated aging (control) and to those that were subjected to accelerated aging for 120 h. The experiment was replicated three times for consistency, and each replicate consisted of 30 seeds. Seed viability evaluation was done by placing the seeds in categories as viable and non-viable according to the coloration of the embryonic axis. Viable seeds were characterized by embryo showing uniform shiny pink colour, and non-viable seeds were

characterized by embryo showing totally white colour (Paiva *et al*., 2017). Only the percentage of viable seeds was recorded.

### 5.2.4. Electrical conductivity and pH test

One gram of aged seed for different periods (24, 48, 72, 96 and 120 h) of each landrace were placed into beakers filled with 10 ml of distilled water, unaged seed was used as control. Initial and final electrolyte conductivity and pH of distilled water were recorded using H198129 pH/EC waterproof tester (HANNA Instruments, Romania). Readings on electrolyte conductivity and pH were taken after 2, 12, and 24 h of soaking. The readings were replicated three times. The pH/EC waterproof tester probe was rinsed in distilled water after each successive measurement to eliminate cross-contamination of measurements. The electrical conductivity was calculated by dividing the obtained value by seed mass and expressed as μS $cm^{-1}$ $g^{-1}$.

### 5.2.5. Water activity test

Seeds from different accelerated aging periods (0, 24, 48, 72, 96 and 120 h) were subjected to a water activity test. Water activity ($a_w$) is a non-destructive, portable, rapid technique and reliable moisture indicator of hygroscopic materials (e.g., agro-food, pharmaceuticals) and particularly of intermediate products such as orthodox seeds (Baldet *et al*., 2009). This is because it is directly related to the water potential (φ), which indicates the energy status of water in hygroscopic matrices (Baldet *et al*., 2009). This involves the determination of the relative humidity at which the seed moisture content remains constant; this is termed the equilibrium relative humidity (ERH) (Kavak and Eser, 2009). The water activity of the seed is ERH/100. The water activity of aged and unaged seeds was recorded using an AquaLAB CX-2 water activity meter (Decagon Devices Inc., Pullman, Wash., USA). The water activity test readings were replicated three times for consistency.

### 5.2.6. Germination test

Thirty (30) seeds of each cannabis genotype were placed on cotton pads moistened with distilled water in 90 mm Petri dishes or in a seedling tray containing lawn dressing compost obtained from Duzi Turf, New England Rd, Pietermaritzburg, South Africa. Lawn dressing compost is characterized as a finely sifted black organic weed-free medium. It is a mixture of organic waste and sewage sludge, with a composition of 85% humus and 15% 2mm-8mm larger particles. Each seed was planted in a seedling tray hole and the germinating seeds were irrigated with tap water regularly to ensure adequate moisture. The experiment was replicated three times for consistency. The seeded Petri dishes were then transferred into a germination chamber (Micro-Clima Arabidopsis Chamber, ECD01E, Snijders, Netherlands) maintained at a temperature of 30/25 °C and light cycle of 16/8 h. The germination progress was monitored daily, and the number of germinated seeds was recorded for each replicate of 30 seeds for 5 days under laboratory conditions and 14 days under greenhouse conditions. A seed was considered to have germinated when the emerging radical elongated to a length of 2 mm or more. The germination percentage (GP) was calculated according to Khalaki *et al*. (2019) using Eq. 1 and was expressed in percentages (%):

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100 \quad (1)$$

The germination rate (GRI) was calculated according to Awasthi *et al*. (2016) using the following equation and was expressed as seeds day$^{-1}$:

$$\text{GRI} = \frac{\text{Number of germinated seeds}}{\text{Day of first count}} + \cdots + \frac{\text{Number of germinated seeds}}{\text{Day of final count}} \quad (2)$$

The coefficient of velocity of germination (CVG) was calculated according to Sobarzo-Bernal *et al*. (2021) using the following equation and was expressed in percentage:

$$\text{CVG} = 100 \text{ X } \frac{\text{Total of germinated seeds}}{\Sigma(\text{N1D1+N2D2...+NnDn})} \quad (3)$$

Where Nn represents seeds germinated on a specific day, and Dn represents the day corresponding with the number of seeds germinated.

### 5.2.7. Data analysis

The data was analyzed using GenStat®, 20.1 Edition (VSN International, Hamel Hampstead, UK, 2020) at the 5% significance level. The means of significantly different variables were separated using Tukey's test.

## 5.3. Results

The landraces and treatments showed significant differences ($p$<0.05) in the seed viability of cannabis seeds, and their interaction showed no significant differences ($p$>0.05). On the other hand, the landraces, treatments, and their interaction showed significant differences ($p$<0.001) in pH, electrolyte conductivity (EC), and water activity ($a_w$) in cannabis (Table 5.2).

**Table 5.2**: Analysis of variance (ANOVA) for seed viability, pH, electrolyte conductivity (EC), and water activity ($a_w$) of cannabis landraces subjected to different periods of accelerated aging (number of replicates = 3).

| **Source of variation** | **df** | **F Value** | **df** | **F Values** | | |
|---|---|---|---|---|---|---|
| | | **Seed viability** | | **pH** | **EC** | **$a_w$** |
| Landrace | 4 | 3.23* | 4 | 684.65** | 125.23** | 70.27** |
| Treatment | 1 | 20.32** | 5 | 80.65** | 305.00** | 262.64** |
| Landrace*Treatment | 4 | 2.01ns | 20 | 2.90** | 55.64** | 7.50** |

ns - not significant ($p$>0.05); *significant ($p$<0.05); **highly significant ($p$<0.001**)**

### 5.3.1. Seed viability

The results revealed that seed viability decreased with accelerated aging (Figure 5.1). Seeds of 'B1', aged and unaged, showed the highest seed viability mean of 100% and 94.41%, respectively, compared to the other landraces. Meanwhile, the unaged seeds of 'L1' and 'L2'

exhibited the lowest seed viability mean of 92.22%, respectively. The aged seeds of 'M1' also showed the least seed viability mean of 70.63%.

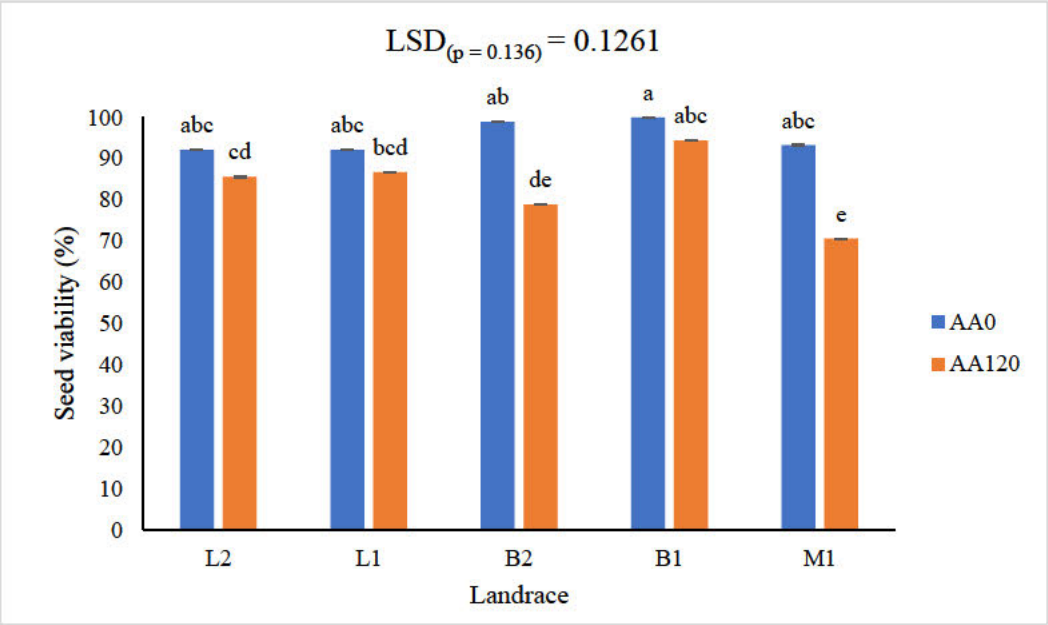



**Figure 5.1:** Seed viability among different cannabis landraces in response to zero and 120 h of accelerated aging treatments ($p$=0.136; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 5.3.2. Electrical conductivity of seed leachate and pH

The results revealed that the EC showed increased electrolyte leakage with seed aging in all landraces (Figure 5.2). 'M1' had the lowest EC amongst the landraces of 172.8 and 201.4 µS $cm^{-1}$ $g^{-1}$ under control and AA24, respectively. Landrace 'B2' had the lowest EC of 216.6, 226.2, 237.7, and 258.3 µS $cm^{-1}$ $g^{-1}$ under AA48, AA72, AA96, and AA120, respectively. Meanwhile, 'B1' had the highest EC of 252.4, 253.6, and 256.1 µS $cm^{-1}$ $g^{-1}$ under control, AA24, and AA48, respectively. 'M1' also showed the highest EC of 309.5, 358.0, and 393.2 µS $cm^{-1}$ $g^{-1}$ under AA72, AA96, and AA120, respectively.

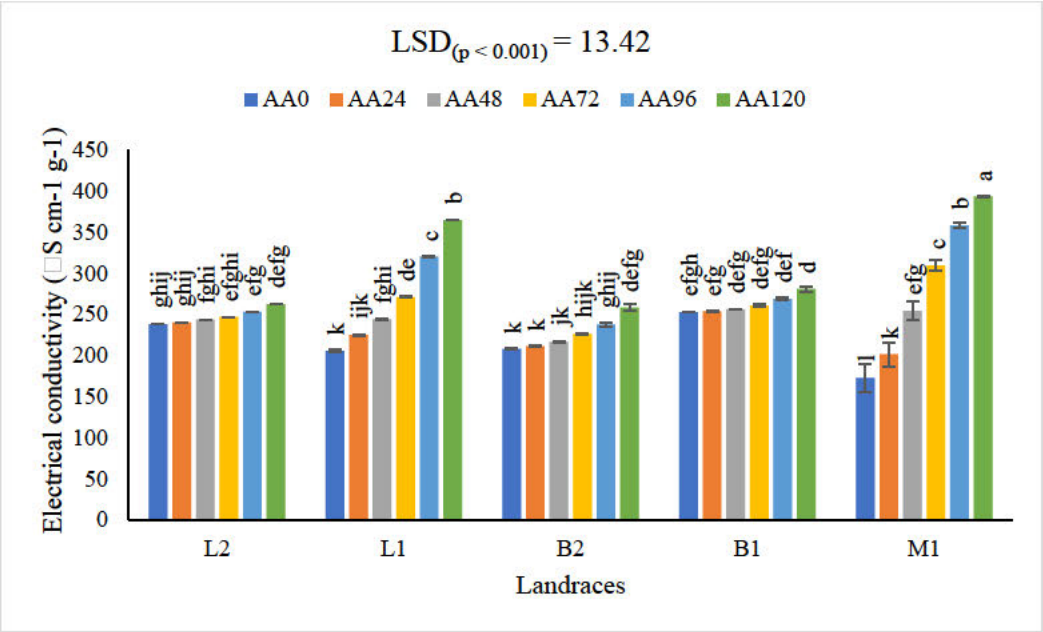



**Figure 5.2**: Electrical conductivity among different cannabis landraces in response to various accelerated aging treatments ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

The results revealed that pH decreased with seed aging in all landraces (Figure 5.3). Generally, 'M1' recorded the lowest pH irrespective of the accelerated aging period compared to other landraces, whereas 'B1' recorded the highest pH.

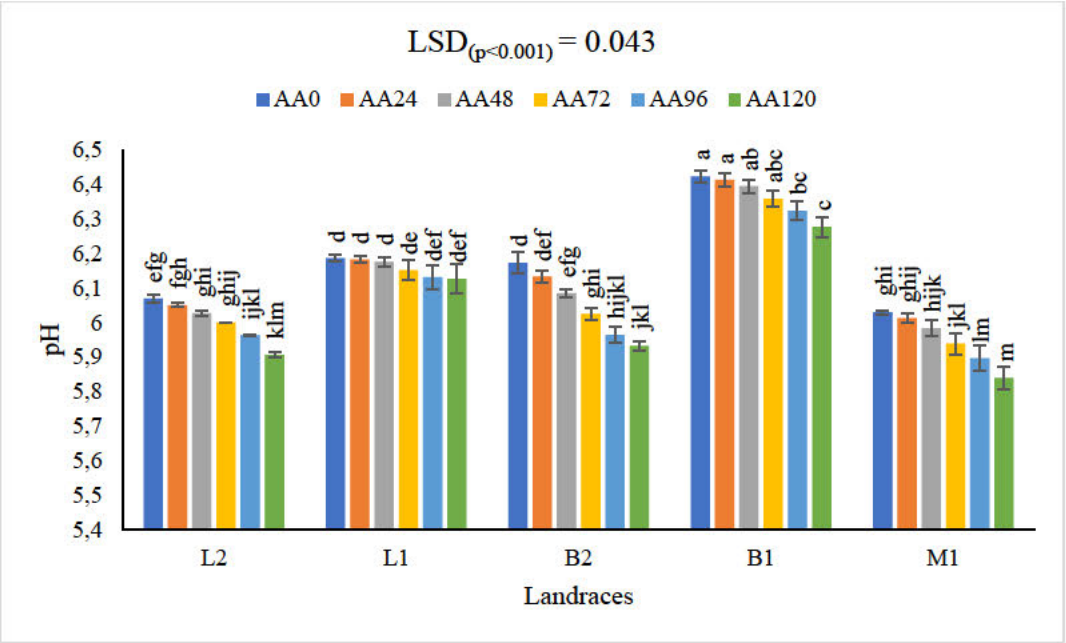



**Figure 5.3**: The pH of different cannabis landraces in response to various accelerated aging treatments ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 5.3.3. Seed water activity

The results revealed an increasing trend in water activity of cannabis landraces with the increasing accelerated aging period (Figure 5.4). 'L2' exhibited the lowest water activity of 0.561 under control compared to the other landraces, and 'B1' had the highest mean of 0.638. 'L1' showed the lowest water activity of 0.590, 0.615, 0.646, 0.680, and 0.692 under AA24, AA48, AA72, AA96, and AA120, respectively. Meanwhile, 'B1' recorded the highest water activity of 0.684, 0.693, and 0.699 under AA24, AA48, and AA72, respectively. Landrace 'L2' also exhibited the highest water activity of 0.720 and 0.724 under AA96 and AA120, respectively.

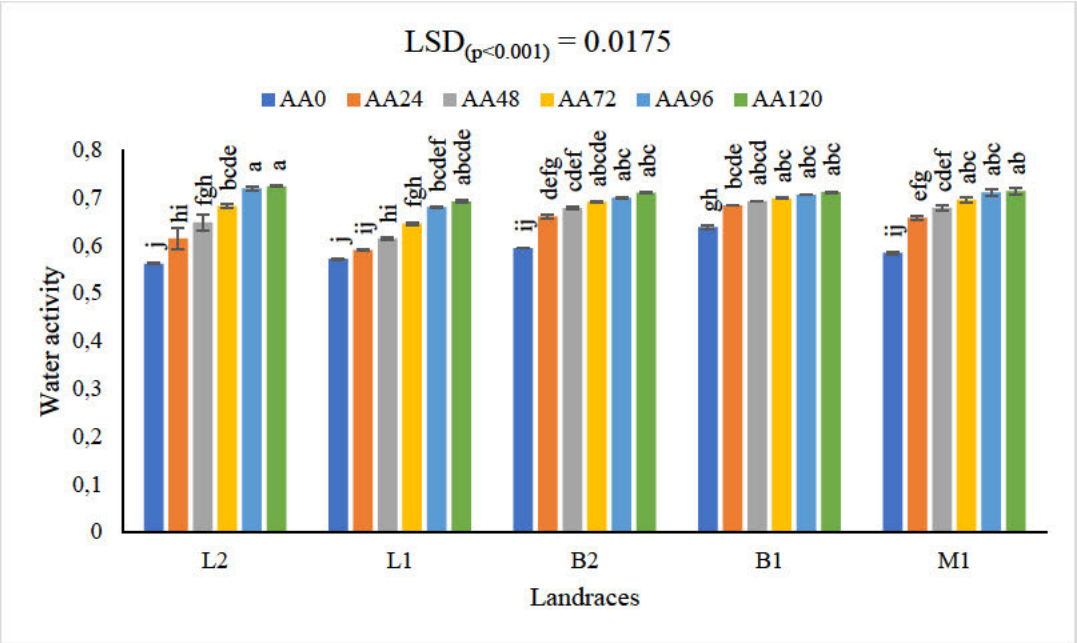



**Figure 5.4**: The water activity of various cannabis landraces under different accelerated aging treatments ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 5.3.4. Germination indexes

#### 5.3.4.1. Germination indexes under laboratory conditions

The landraces and treatments showed significant differences ($p$<0.001) in germination percentage (GP), germination rate index (GRI), and coefficient of velocity of germination (CVG) under laboratory conditions (Table 5.3). The landraces and treatment interactions also showed significant differences ($p$<0.05) in GRI and CVG under laboratory and showed no significant differences ($p$>0.05) in GP.

**Table 5.3**: Analysis of variance (ANOVA) for seed germination percentage (GP), germination rate index (GRI), and coefficient of velocity of germination (CVG) of cannabis landraces subjected to different periods of accelerated aging under laboratory conditions (number of replicates = 3).

| **Source of variation** | **df** | **F Values** | | |
|---|---|---|---|---|
| | | **GP** | **GRI** | **CVG** |
| Landrace | 4 | 34.87** | 43.39** | 11.17** |
| Treatment | 5 | 60.02** | 83.95** | 20.19** |
| Landrace*Temperature | 20 | 0.71ns | 3.78** | 0.011* |

ns - not significant ($p$>0.05); *significant ($p$<0.05); **highly significant ($p$<0.001**)**

#### 5.3.4.1.1. Germination

The results revealed a significant reduction in seed germination of cannabis landraces with the increasing accelerated aging duration (Figure 5.5). 'L2' demonstrated the highest germination percentage amongst the landraces irrespective of the accelerated aging duration. Similarly, 'M1' showed the lowest germination percentage irrespective of the accelerated aging period. About 50% germination reduction was observed in 'L2' and 'B2' after AA120 as 'L2' germination was reduced to 32.22% from 82.22% recorded under control, and the germination of 'B1' was reduced from 75.56 to 24.83%.

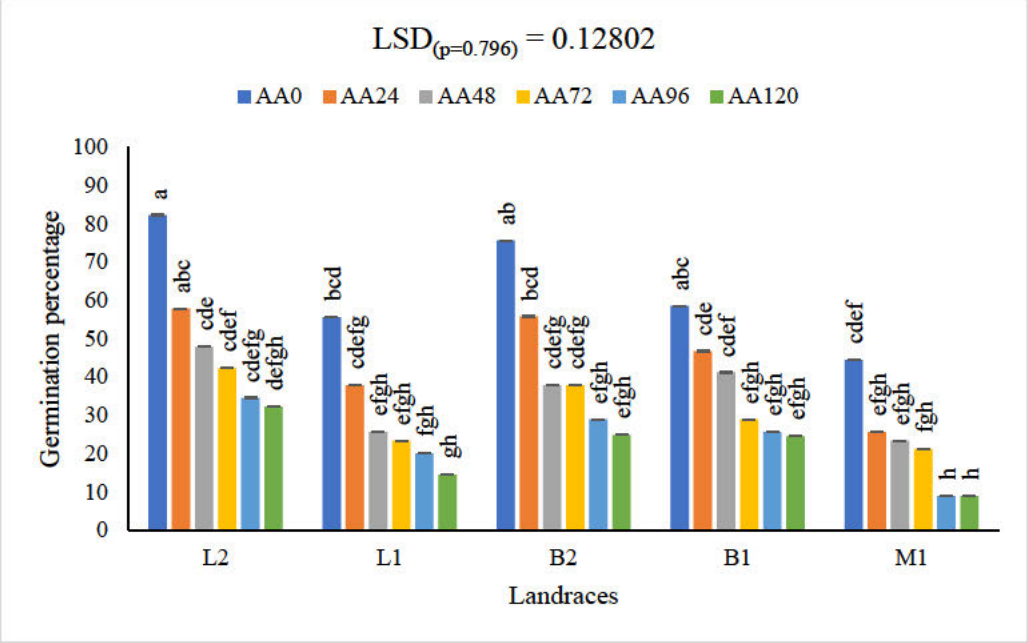



**Figure 5.5:** The germination percentage of various cannabis landraces under laboratory conditions in response to different accelerated aging treatments ($p$=0.796; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 5.3.4.1.2. Germination rate

The results demonstrated that the germination rate index generally decreased with the increasing accelerated aging duration (Figure 5.6). Notably, 'L2' showed the highest germination rate index amongst the landraces irrespective of the accelerated aging period, which decreased with the increasing duration of accelerated aging. 'M1' also generally exhibited the lowest germination rate index under the accelerated aging treatments except under AA48, where 'L1' showed the lowest germination rate index of 2.872 seeds day$^{-1}$. Notably, 'B2' showed a higher germination rate index of 3.717 seeds day$^{-1}$ under AA120 compared to 3.467 seeds day$^{-1}$ under AA96. Similarly, 'M1' exhibited a lower germination rate index of 0.972 seeds day$^{-1}$ under AA96 compared to 0.983 seeds day$^{-1}$ recorded under AA120. 'B1' showed the lowest germination rate index of 3.161 seeds day$^{-1}$ under AA72 compared to 3.644 and 3.239 seeds day$^{-1}$ under AA96 and AA120, respectively.

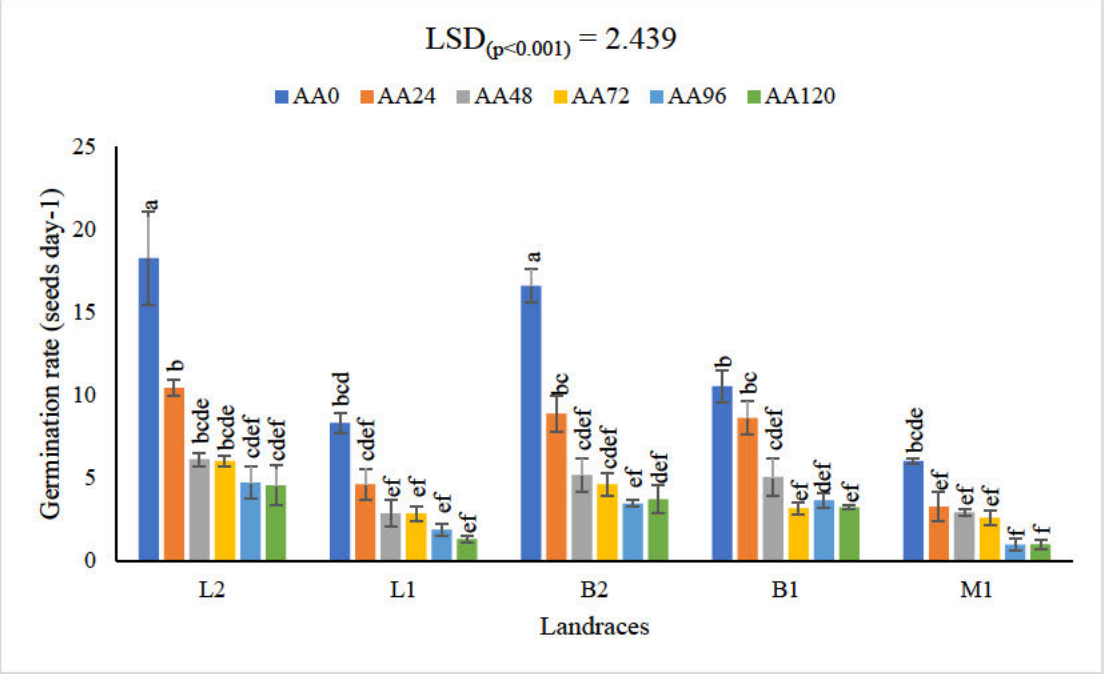



**Figure 5.6**: The germination rate of various cannabis landraces under laboratory conditions in response to different accelerated aging treatments ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 5.3.4.1.3. Coefficient of velocity of germination

The results revealed that accelerated aging reduced the coefficient of velocity of germination (CVG), showing variations with the duration of accelerated aging and landrace (Figure 5.7). 'B2' exhibited the highest CVG of 61.70 and 40.85% under control and AA48, respectively, compared to the other landraces. 'L2' showed the highest CVG of 48.45, 39.99, and 42.35% under AA24, AA72, and AA120, respectively, compared with the other landraces. 'B1' also showed the highest CVG of 41.8% under AA96. On the other hand, 'L1' exhibited the lowest CVG of 34.27, 27.88, and 29.06% under AA48, AA96, and AA120, respectively, compared to the other landraces. 'M1' also exhibited the lowest CVG of 40.22 and 37.73% under control and AA24, respectively. Additionally, 'B1' showed the lowest CVG of 31.88% under AA72.

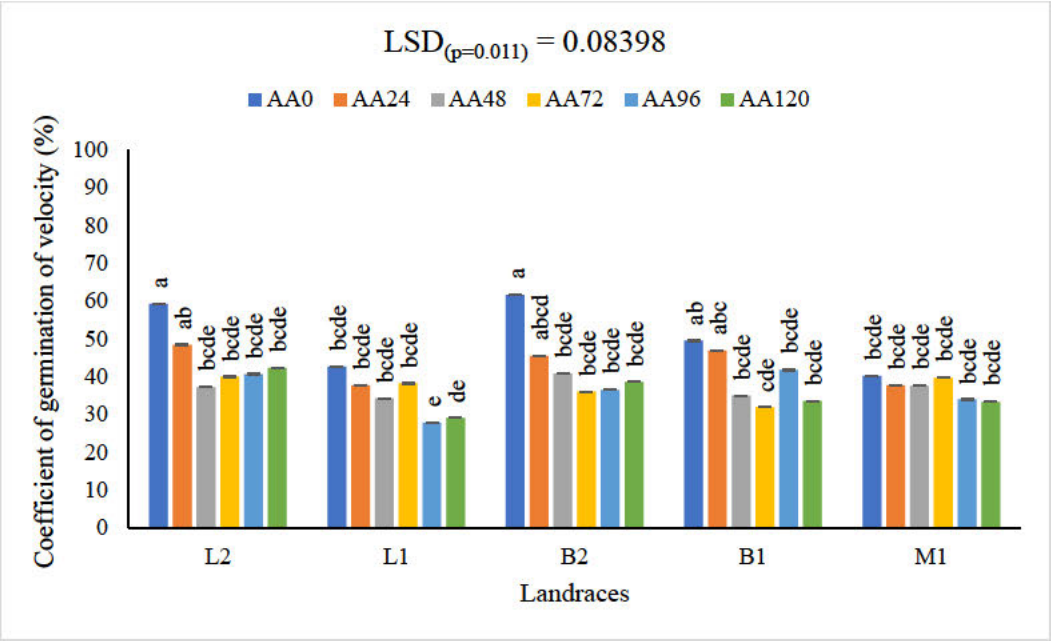



**Figure 5.7**: The coefficient of velocity of germination of various cannabis landraces under laboratory conditions in response to different accelerated aging treatments ($p$=0.011; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 5.3.4.2. Germination indexes under the greenhouse conditions

The landraces and treatments showed significant differences ($p$<0.001) in germination percentage (GP), germination rate index (GRI), and coefficient of velocity of germination (CVG) under greenhouse conditions (Table 5.4). The landraces and treatment interactions also showed significant differences ($p$<0.05) in GP and GRI under greenhouse conditions and showed no significant differences ($p$>0.05) in CVG.

**Table 5.4**: Analysis of variance (ANOVA) for seed germination percentage (GP), germination rate index (GRI), and coefficient of velocity of germination (CVG) of cannabis landraces subjected to different periods of accelerated aging under greenhouse conditions (number of replicates = 3).

| Source of variation | df | F Values | | |
|---|---|---|---|---|
| | | GP | GRI | CVG |
| Landrace | 4 | 57.84** | 76.01** | 58.62** |
| Treatment | 5 | 15.83** | 26.94** | 64.50** |
| Landrace*Temperature | 20 | 4.26** | 2.13* | 0.070ns |

ns - not significant ($p$>0.05); *significant ($p$<0.05); **highly significant ($p$<0.001**)**

#### 5.3.4.2.1. Germination

The results revealed that seed germination varied greatly with landrace and accelerated aging treatment (Figure 5.8). The germination percentage of 'B2' decreased with the duration of accelerated aging. The germination percentage of 'L2' also generally decreased with the increasing duration of accelerated aging, except that it showed increased germination of 67.78% under AA48 compared to 64.44% exhibited under AA24. Similarly, the germination percentage of 'L1' tended to decrease with the increasing duration of accelerated aging. However, it had higher germination of 90.00 and 87.78% under AA48 and AA72, respectively, compared to 84.44% under control and 83.33% under AA24. In 'B1', control, AA48, and AA72 showed an increased germination percentage of 93.33%, respectively, compared to 84.44% exhibited under AA24. Fluctuations were observed in 'M1', with AA24 exhibiting the highest germination percentage of 90.00%, followed by AA96 recording 87.78%, which both had higher germination compared to 82.22% recorded under control.

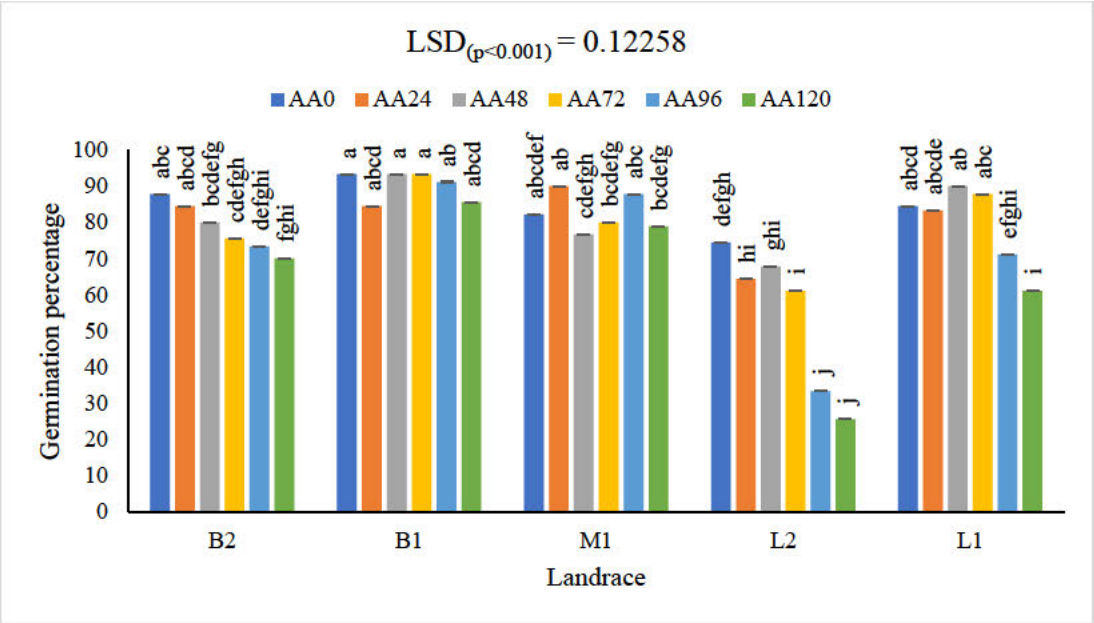



**Figure 5.8**: The germination percentage of various cannabis landraces under greenhouse conditions in response to different accelerated aging treatments ($p$<0.001, number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 5.3.4.2.2. Germination rate index

The findings demonstrated that the germination rate index varied with landrace and duration of accelerated aging treatment. However, there was generally a decrease with the increasing duration of accelerated aging treatment (Figure 5.9). For instance, the germination rate index decreased with the increasing duration of accelerated aging in 'B2', 'M1' and 'L2'; however, it showed a higher germination rate under AA120 compared to AA96. 'B2', 'M1', and 'L2' showed a germination rate index of 7.266, 6.278, and 1.625 seeds day$^{-1}$ under AA120, respectively, with the corresponding 6.192, 4.723, and 1.376 seeds day$^{-1}$ under AA96. The germination rate index also decreased with the increasing duration of accelerated aging in 'L1'; however, it showed a lower germination rate of 5.121 seeds day$^{-1}$ under AA24 compared to 7.515 seeds day$^{-1}$ showed under AA48. Additionally, 'B1' exhibited a lower germination rate index of 12.466 seeds day$^{-1}$ under AA24 compared to 12.815 and 14.819 seeds day$^{-1}$ under AA48 and AA72, respectively.

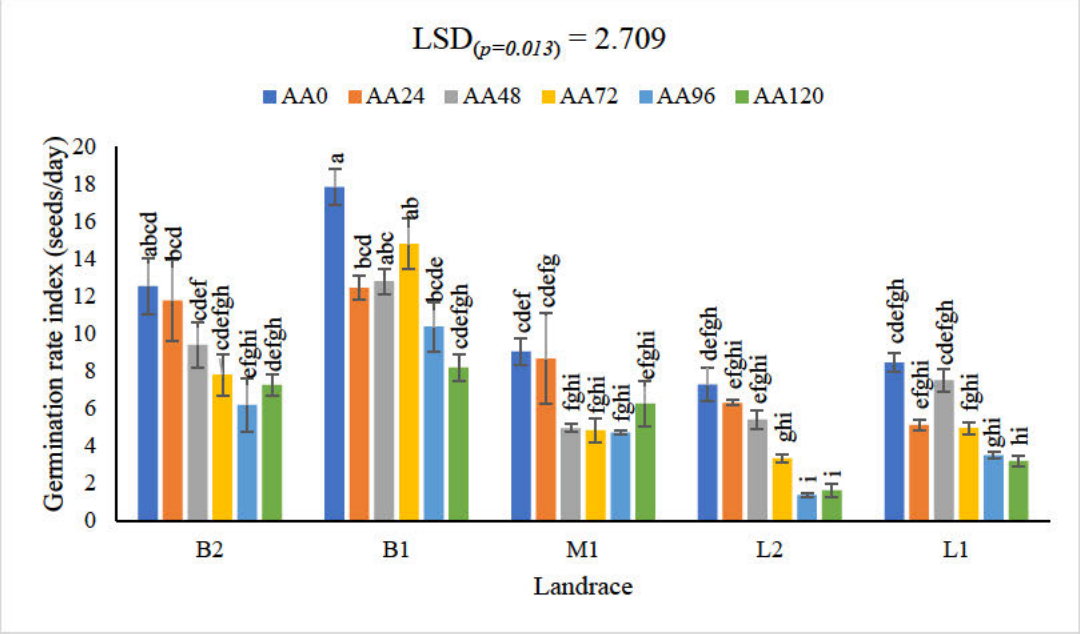



**Figure 5.9**: The germination rate index of various cannabis landraces under greenhouse conditions in response to different accelerated aging treatments ($p$=0.013; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 5.3.4.2.3. Coefficient of velocity of germination

The findings showed that the coefficient of velocity of germination (CVG) varied with landrace and duration of accelerated aging treatment. However, similar to the germination rate index, CVG generally decreased with the increasing duration of accelerated aging treatment (Figure 5.10). For instance, the CVG decreased with the increasing duration of accelerated aging in 'B2', 'M1', and 'L2'; however, they showed increased CVG under AA120 compared to AA96. 'B2', 'M1', and 'L2' exhibited a CVG of 22.50, 17.57, and 17.59% under AA120, respectively, with the corresponding 20.20, 16.65, and 13.50% under AA96. The CVG also decreased with the increasing duration of accelerated aging in 'L1'; however, it showed an increased CVG of 21.29% under AA48 compared to 18.66% under AA24. Additionally, 'B1' showed a higher CVG of 35.86% under AA72 compared to 33.71 and 31.92% recorded under AA24 and AA48, respectively.

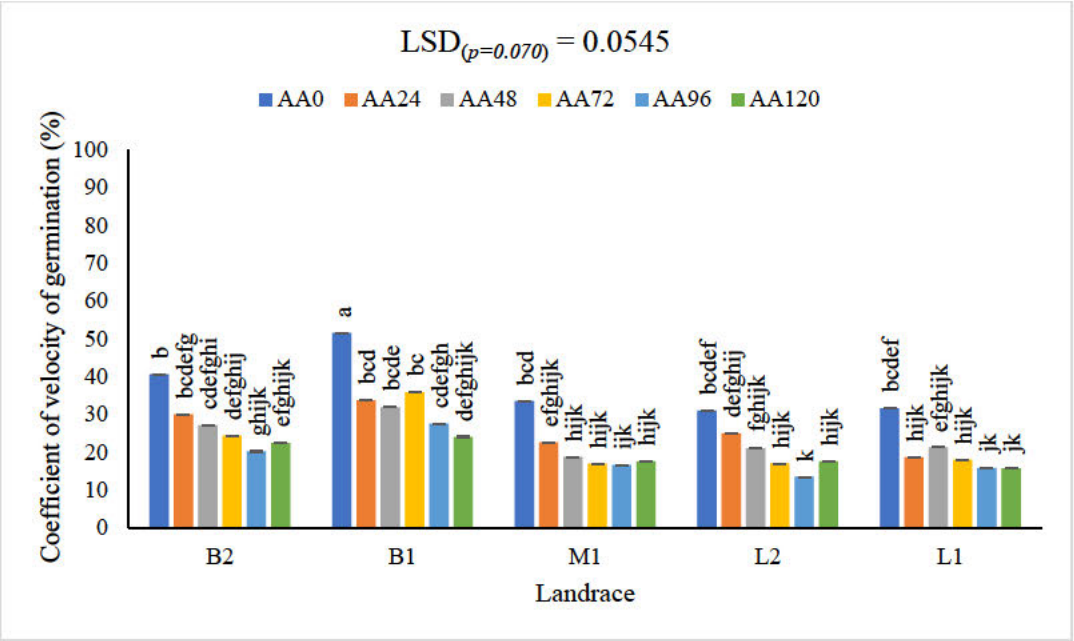



**Figure 5.10**: The coefficient of velocity of germination of various cannabis landraces under greenhouse conditions in response to different accelerated aging treatments ($p$=0.070; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 5.4. Discussion

The current study investigated the response of various cannabis landraces to different accelerated aging (AA) treatments, a simulation of seed aging procedure in a short time. The AA indicator is recognized as an accurate measure of seed vigour and storability since it correlates with field emergence (Kumar *et al.*, 2019). The aging process induced the deterioration of seed viability, increased electrical conductivity of seed leachate and water activity, and decreased the potent of hydrogen of seed exudates. It also appears that accelerated aging treatments caused a significant decrease in germination characteristics in the current study under laboratory and greenhouse conditions, as further discussed in the succeeding subsections.

### 5.4.1. Seed viability

The aging process induced the deterioration of seed viability, which was associated with loss of seed vigour due to ultracellular changes. A decrease in seed viability was associated with increased solute leakages due to seed aging, leading to a decrease in germination capacity. Increased solute leakage during imbibition indicates an inability to reform coherent membranes during seed rehydration, leading to a loss of viability and vigor (Barreto and Garcia, 2017; Lerin *et al*., 2021). This was evident in landrace 'M1' on the final day of aging, which, compared to other landraces, had reduced seed viability, germination percentage, and germination rate under laboratory conditions, as well as increased electrical conductivity. Additionally, the control treatment generally demonstrated higher germination, germination rate, and coefficient of velocity of germination under both germination conditions compared to the accelerated aging treatments. The results of the current study correlate with the findings by Al-Maskri *et al*. (2003), who reported that the decline in seed viability and relative growth rate was accompanied by an increase in solute leakage and lipid peroxidation product in *Daucus carota* L. seeds. According to Hussen *et al*. (2011), the reduction in seed viability might be associated with declining biochemical activities in seeds as aging damages enzymes necessary for converting reserve food in the embryo to a usable form.

### 5.4.2. Electrolyte conductivity of seed leachate and pH

The principle of positive correlations between the amount of leaching from the seed and loss of seed viability has been used to assess seed viability using seed leaching (Min and Kang, 2011). Therefore, seed solute leakage can be used to assess seed deterioration. The findings of the current study show that the electrical conductivity of seed leachate increased with aging, irrespective of the landrace, which indicates that all seeds became inferior. Solute leaking from seeds occurs during the membrane reorganization stage after rehydration. As a result, seed

leakage rates correspond with the degree of cell membrane damage and repair in response to aging, and leakage can be used to measure membrane damage (Bijanzadeh *et al*., 2017).

As seed deterioration progresses, the cell membrane becomes less rigid and more water permeable, allowing the cell contents such as sugars, organic acids, and H+ ions to escape, which acidify the solutions with water, resulting in lower pH (Singh *et al*., 2017; Junior *et al*., 2019). The results of the current study conform with the statement by Singh *et al*. (2017) and Junior *et al*. (2019) as seeds aged, their hydrogen potency reduced. Therefore, the decrease in pH of seed exudates was associated with cell membrane damage, as demonstrated by the increase in electrical conductivity of seed leachate. It was also attributed to acidity not promoting the synthesis of enzymes needed to germinate cannabis landraces. The results of the current study correlate with the findings by Mandizvo and Odindo (2019) who also reported that the pH of seed exudates in *Vigna subterranea* (L.) Verdc. decreased with aging. Additionally, the findings on electrical conductivity correlate with the previous study by Hussein *et al*. (2012), who reported that the electrical conductivity of *Zea mays* L. seeds leachate increased linearly with accelerated aging. Govindaraj *et al*. (2014) also reported that the lowest electrical conductivity was observed in unaged seeds and increased gradually with an increase in the duration of accelerated aging, irrespective of the treatment and *Oryza sativa* L. genotypes.

### 5.4.3. Water activity

Water activity can be used to determine if the moisture in the seed is at an acceptable level for storage. Water activity below 0.60 is known to have no microbial proliferation; this level is recommended for oilseeds due to the risk of heating during storage and the susceptibility of the oil to quality loss (Caddick, 2001). In the current study, the landraces recorded a water activity of below 0.60 under control, except for ‘B2’. According to Kelly *et al*. (2011), storage oil

(triacylglycerol [TAG]) breakdown plays an important role in the life cycle of many plants by providing carbon skeletons that support seedling growth immediately following seed germination and enable seedling establishment. Therefore, it explains the increased water activity while germination decreased with accelerated aging. The observed increase in seed water activity with seed aging correlates with the previous studies. For instance, Kumar *et al*. (2015) reported that the water activity of soybeans increased with accelerated aging. Similarly, Krishnan *et al*. (2004) reported that the water activity of wheat seeds increased with long-term storage under ambient conditions. Therefore, the increased water activity in cannabis landrace seeds during accelerated aging highlighted the critical role of proper storage conditions to maintain seed viability.

### 5.4.4. Germination indexes

A decrease in germination indexes such as germination rate, and coefficient of velocity of germination was observed with seed aging under both laboratory and greenhouse conditions. While germination also decreased with the increasing accelerated aging under laboratory conditions, fluctuations were observed under the greenhouse conditions. The decrease in germination characteristics was associated with the EC of seed leachate, which was used to measure membrane damage. It has been previously reported that the reduction in germination is associated with the degradation of the mitochondrial membrane, leading to a reduction in the energy supply necessary for germination (Hussein *et al*., 2012). Whereas the fluctuation in seed germination under greenhouse conditions demonstrated that the seeds might have had some dormancy, particularly as ‘M1’ and ‘L1’ showed reduced germination compared to other artificial aging treatments. Thus, the moist heat used for accelerated aging helped release physiological seed coat-imposed dormancy, often associated with increased hydrogen peroxide levels (Visscher *et al*., 2018; Smychniuk *et al*., 2020). Cannabis seeds showed some level of

dormancy only under greenhouse conditions and this was linked to the greenhouse simulating the actual cannabis growing conditions.

Nonetheless, the observed decrease in germination indexes in the current study correlates with the findings of Rastegar *et al*. (2011) who found that accelerated aging caused a remarkable decrease in germination characteristics of soybean. Our findings corroborate those of Tabatabaei (2015) who reported that the germination percentage, germination index, and mean time to germination of *Hordeum vulgare* L. seeds were reduced with the duration of aging. In their study on various *Triticum aestivum* L. cultivars, Ghahfarokhi *et al*. (2014) reported that seed aging significantly affected germination percentage, germination index, normal seedling percentage and mean germination time. Moreover, these researchers also found that seed aging greatly influenced malondialdehyde content, seedling dry weight, the weight of utilized (mobilized) seed reserve, and electrical conductivity. Thus, similar to previous studies on other crops, our findings demonstrate that accelerating aging affects seed germination in various ways, including seed reserve mobilization and seed biochemistry of cannabis landraces.

### 5.5. Conclusions

Accelerated aging negatively affected the seed germination performance of cannabis landraces as there was progressive seed deterioration during aging. Reduced seed viability, pH, and germination indexes were associated with increased seed electrolyte leakage. The accelerated aging treatments were found to have an impact on the germination potential of cannabis seeds, which was also associated with reduced seed viability. Thus, it demonstrated that cannabis seed germination was sensitive to elevated temperature and relative humidity. Therefore, maintaining high seed viability under prolonged uncontrolled storage conditions is essential for cannabis landraces, as effective storage delays physiological and physiochemical changes, ultimately preventing seed aging. However, the current study did not provide information on

the biochemical alterations that occur during the germination of aged seeds. This information is crucial since it has been discovered that crops with high oil content, such as cannabis, are more susceptible to seed deterioration due to the lower chemical stability of lipids in relation to starch. The current study, therefore, recommends investigations on the effect of accelerated aging in seed germination, seed reserve mobilization, and the biochemistry of different cannabis genotypes during germination.

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# 6. CHAPTER 6: SEED GERMINATION RESPONSE OF VARIOUS CANNABIS LANDRACES TO POLYETHYLENE GLYCOL (PEG)-INDUCED DROUGHT STRESS

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**Abstract**

The current study investigated the effect of PEG-induced drought stress on the seed germination of cannabis landraces. The genotypes were collected from local growers in four regions of KwaZulu-Natal Province in South Africa, namely, Bergville (B), Hammersdale (H), Ladysmith (L), and Msinga (M). These were assigned an alphabet and number designation corresponding to their respective collection locations, resulting in eight genotypes, namely, 'B1', 'B2', 'B3', 'H1', 'L1', 'L2', 'M1', and 'M2'. Drought stress was induced in a laboratory setting using PEG 8000 at 0, -0.2, -0.4, -0.6, -0.8, and -1.0 MPa, and the findings were subsequently validated under greenhouse conditions. The parameters measured included germination percentage, germination rate, germination stress tolerance index, seedling length, and seed vigour index. In general, there were statistically significant differences ($p$<0.05) among landraces, PEG treatments, and their interactions for germination characteristics in both greenhouse and laboratory environments. However, the interaction between landraces and PEG treatments did not show statistical differences ($p$>0.05) in germination percentage under greenhouse conditions. The germination characteristics were reduced under PEG treatments

compared to the control, and higher concentrations completely inhibited the germination of ‘M1’ and ‘L1’. In laboratory conditions, ‘L2’ generally showed the highest germination and germination rate, with ‘B1’ displaying the highest germination stress tolerance index. In greenhouse conditions, ‘H1’ exhibited the highest germination, while ‘B3’ showed the highest germination rate, germination stress tolerance index, seedling length, and seed vigor index. Thus, it indicates that ‘L2’, ‘B1’, ‘H1’, and ‘B3’ may be suitable choices for farmers facing limited access to climate adaptation resources like irrigation technologies.

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**Keywords**: Cannabis, landrace, polyethylene glycol, germination recovery, drought

### 6.1. Introduction

Climate change directly and significantly impacts agricultural production as it influences various climate variables, including temperature, rainfall patterns, growing seasons, humidity, water availability, crop genotype selection, and soil health (Maponya and Mpandeli, 2012). Changes in these factors can disrupt traditional agricultural practices and have far-reaching consequences for crop yields, thus threatening sustainability, food security, and livelihoods of the farmers who primarily depend on rain for crop production (Schreiner *et al*., 2018; Myeni *et al*., 2019). Of these changes, drought and water stress have the most significant impact on crop production. Water stress interferes with plant cellular functions, including protein synthesis, nitrogen assimilation, and cell membrane activity (Zia *et al*., 2021). Water-stressed plants typically exhibit poor growth, reduced leaf water content, low turgor pressure, and low transpiration rate (Zia *et al*., 2021). As a result, growers largely rely on irrigation to supply water to their crops (Materechera, 2011; Jordán and Speelman, 2020).

*Cannabis sativa* is a multi-purpose crop and has been involved in a wide range of products and has been integrated into several agro-industrial fields, namely agriculture, textile, bio-composite, papermaking, automotive, construction, biofuel, functional food, oil, cosmetics,

and personal care (Salentijn *et al*., 2015; Chaohua *et al*., 2016). Cannabis exhibits therapeutic and pharmaceutical properties owing to its high concentrations of cannabinoids, terpenes, and phenolic compounds (Grulichova *et al*., 2017; Żuk-Gołaszewska and Gołaszewski, 2018). The cannabis crop is highly adaptable; it grows in various climatic and agronomical conditions (Decorte *et al*., 2011). It grows well on loose, well-aerated loam soil with high fertility, abundant organic matter, and adequate sunlight, warmth, and moisture (Clarke and Merlin, 2016; Sera *et al*., 2017). Good soil moisture and ample rainfall are required in cannabis for good growth, particularly during the first 6 weeks of planting (Sera *et al*., 2017). However, with reduced precipitation due to climate change, the required water during seed germination and seedling development may not be met, ultimately negatively impacting seed emergence (Zia *et al*., 2021).

It is well understood that seed germination is the first and most important and sensitive stage in the life cycle of plants, and the successful establishment of seedlings largely depends on germination (Gorai and Neffati, 2007; Petrovic *et al*., 2016). Water availability is crucial for seed germination for various processes, such as imbibition, enzyme activation, embryo stimulation, reserve mobilization, and plumule emergence and elongation (Wijewardana *et al*., 2018; Wolny *et al*., 2018; López *et al*., 2021; Langa *et al*., 2024a). Insufficient water availability reduces both the percentage and rate of germination, ultimately leading to poor seed germination (Wijewardana *et al*., 2018; Rahmah *et al*., 2020; Langa *et al*., 2024a). Additionally, it causes higher mortality rates and reduces crop performance during the early stages of development (Tounekti *et al*., 2020; Langa *et al*., 2024a).

Research studies on drought response and tolerance during germination are very limited in cannabis crops, particularly in South African landraces, due to the ban of the crop from usage for research and industrial applications for the past centuries. Such research is now critical following the recent legalization of cannabis cultivation and the rapidly increasing demand for

hemp products (Schluttenhofer and Yuan, 2017; Citti *et al*., 2018). Information on drought tolerance in the early stages of plant development is vital because seeds with more rapid germination water deficit conditions may be expected to achieve a rapid seedling establishment, resulting in higher yields (Petrovic *et al*., 2016). PEG is a natural polymer that is water-soluble and nonionic; it is mainly used to simulate water-stress conditions as it lowers plants' water potential due to osmotic stress (Petrovic *et al*., 2016; Ahmad *et al*., 2020). It is an inert osmotic agent whose molecules are too large to penetrate the seed, thus preventing any toxic effects on plants (Petrovic *et al*., 2016; Shivakrishna *et al*., 2018).

PEG has been used to study the effect of drought stress on cannabis, including studies by Li *et al*. (2018), Blandinières *et al*. (2021), and Du *et al*. (2022). However, Li *et al*. (2018) and Du *et al*. (2022) studied the effect of combining pre-treatments and drought stress under a single concentration of 20% PEG. Blandinières *et al*. (2021) also used a similar concentration, and these studies were conducted in industrial hemp, whereas different genotypes might have a different response to different PEG concentrations. Thus, the response of different cannabis genotypes to different PEG concentrations is still limited. Certain seeds can retain their viability even after prolonged exposure to drought stress and germinate once the abiotic stress is alleviated (Ahmadloo *et al.*, 2011; Vicente *et al*., 2020). However, such information is limited in cannabis. Therefore, the objective of the current study was to investigate the response of different cannabis landraces to PEG-induced drought stress under laboratory and greenhouse conditions.

## 6.2. Materials and methods

### 6.2.1. Genotype collection

Seeds of eight cannabis genotypes (landraces) were collected from local growers in four regions of KwaZulu-Natal Province in South Africa: Bergville, Hammersdale, Ladysmith, and

Msinga. The collected cannabis landraces were assigned pseudonyms to ensure uniform naming of landraces for this experiment, as detailed in Table 6.1.

**Table 6.1:** The pseudonyms of the cannabis landraces and their geographic location information.

| Location | Geographic information | | | | | Common local name | Name used for the experiment |
|---|---|---|---|---|---|---|---|
| | Latitude: | Longitude: | Elevation | Average rainfall | Average temperature | | |
| Ladysmith | -28.57533 | 29.85948 | 1009.53 m | 108.69 mm | 23.31 °C | 'Ugwayi wesiZulu' | 'L1' |
| | | | | | | 'Iswazi' | 'L2' |
| Msinga | -28.700960 | 30.303320 | 559 m | 340.00 mm | 23.00 °C | 'Ugwayi wesiZulu' | 'M1' |
| | | | | | | 'Iswazi' | 'M2' |
| Bergville | -28.677544 | 29.125553 | 1137.86 m | 109.36 mm | 23.45 °C | 'Ugwayi wesiZulu' | 'B1' |
| | | | | | | 'Iswazi' | 'B3' |
| | -28.667679 | 29.034111 | | | | 'Natal' | 'B2' |
| Hammersdale | -29.870903 | 30.633519 | 597 m | 110.34 mm | 23.66 °C | 'Durban Poison' | 'H1' |

The collected seed samples were stored at room temperature at the Horticultural Sciences Laboratory of the University of KwaZulu-Natal, Pietermaritzburg (latitude: -29.636311 and longitude: 30.409060). Prior to germination studies, the cannabis seeds were sorted based on colour. Dark-coloured seeds were associated with maturity, whereas greenish-coloured seeds were associated with immaturity (Miraji *et al*., 2021). Therefore, dark-coloured seeds were used for the experiment. Seeds showing defects were removed from the seed lot.

### 6.2.2. Experimental design

The simulated drought stress experiment was structured using factorial completely randomized design consisting of 8 genotypes and 6 PEG treatments. The seeds were exposed to polyethylene glycol (PEG) BioUltra 8000 (Sigma Aldrich, Merck KGaA, 64271 Darmstadt, Germany) at different concentration levels, following the procedure by Midaoui *et al*. (2003), with some modifications. Midaoui *et al*. (2003) used PEG 6000 at concentrations of 0, -0.6, and -1.0 MPa. In the current study, the PEG solution was prepared by dissolving PEG 8000 in distilled water at 25 ± 2 °C. The concentrations were set at intervals of -0.2 MPa: -0.2, -0.4, -0.6, -0.8, and -1.0 MPa. Distilled water was used as the control treatment. The amount of solute for different concentrations was determined using Eq. 1 previously described by Michel (1983).

$$\text{PEG}= \frac{4-(5.16\varphi T-560\varphi +16)\text{^}0.6}{2.58T-280} \quad (1)$$

Where φ represents water potential and T represents Celsius temperature.

### 6.2.3. Seed viability test

Seeds were subjected to the viability test using 2,3,5-triphenyltetrazolium chloride (TCC) (Sigma Aldrich, Merck KGaA, 64271 Darmstadt, Germany), and the procedure was adopted from Mandizvo and Odindo (2019). TCC is a rapid and effective method for evaluating viability or physiological quality in seeds (Paiva *et al*., 2017). It is a water-soluble compound that promotes changes in colour when in contact with living seed tissues due to the activity of dehydrogenase enzymes (Virgens *et al*., 2019). Seed samples were preconditioned by soaking in distilled water for 18 h, at 25 °C in an incubator, and then excised with a scalpel blade through the embryo. The excised seeds were immersed in a 1% tetrazolium salt solution (TCC) for 12 h at 25 °C at room temperature. The experiment was replicated three times, and each replicate consisted of 30 seeds. Seed viability evaluation was done by placing the seeds in

categories as viable and non-viable according to the coloration of the embryonic axis. Viable seeds were characterized by embryos showing a uniform shiny pink colour, and non-viable seeds were characterized by embryos showing a totally white colour (Paiva *et al*., 2017). Only the percentage of viable seeds was recorded.

### 6.2.4. Germination test

The cannabis seeds were initially soaked in distilled water for 24 h to promote germination. Twenty-five (25) seeds of each cannabis genotype were placed on cotton pads moistened with distilled water in 90 mm Petri dishes, and 25 seeds were placed in a seedling tray containing lawn dressing compost obtained from Duzi Turf, New England Rd, Pietermaritzburg, South Africa. Lawn dressing compost is characterized as a finely sifted black organic weed-free medium. It is manufactured of organic waste and sewage sludge, comprising 85% humus and 15% 2mm-8mm larger particles. Each seed was planted in a seedling tray hole, and the germinating seeds were irrigated with tap water regularly to ensure adequate moisture. The experiment was replicated three times for consistency. The seeded Petri dishes were then transferred into a germination chamber (Micro-Clima Arabidopsis Chamber, ECD01E, Snijders, Netherlands) maintained at 30/25 °C and a light cycle of 16/8 h. The germination progress was monitored daily, and the number of germinated seeds was recorded for each replicate for 7 days under laboratory conditions and 14 days under greenhouse conditions. A seed was considered to have germinated when the emerging radical elongated to a length of 2 mm or more. The germination percentage (GP) was calculated according to Khalaki *et al*. (2019) using Eq. 2 and was expressed in percentages (%).

$$\text{Germination percentage (\%GP)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100 \qquad (2)$$

### 6.2.5. Germination recovery

Seeds that had not germinated during the germination test under different PEG treatments in laboratory conditions were rinsed and put in Petri dishes with distilled water for an additional 3 days. Germination recovery aimed to determine the number of seeds that successfully germinated after the drought stress was alleviated. The number of recovered seeds was then added to the number of seeds germinated under osmotic treatments of solutes (PEG). The percentage of germination recovery was calculated using Eq. 3 by Chen *et al*. (2022).

$$\text{Germination recovery percentage (\%GP)} = \frac{a+b}{c} \times 100 \quad (3)$$

Where *a* is the number of germinated seeds after being transferred to distilled water, *b* is the number of seeds germinated from osmotic treatments of solutes (PEG), and *c* is the total number of seeds.

### 6.2.6. Germination rate index

After the germination recovery, the total number of germinated seeds was recorded, and the germination rate index (GRI) was calculated using Eq. 4 by Awasthi *et al*. (2016).

$$\text{GRI} = \frac{\text{Number of germinated seeds}}{\text{Day of first count}} + \cdots + \frac{\text{Number of germinated seeds}}{\text{Day of final count}} \quad (4)$$

### 6.2.7. Germination stress tolerance index

The total number of germinated seeds was recorded and subsequently used to calculate the germination stress tolerance in cannabis landraces under salt and water stress conditions. The germination stress index was calculated using the Promptness Index (PI), which was calculated using Eq. 5 (Giancarla *et al*., 2012).

$$\text{PI} = \text{nd2}\ (1.0) + \text{nd4}\ (0.8) + \text{nd6}\ (0.6) + \text{nd8}\ (0.4) + \text{nd10}\ (0.2) \quad (5)$$

Where nd2, nd4, nd6, nd8, and nd10 represent the number of germinated seeds in 2$^{nd}$, 4$^{th}$,6$^{th}$, 8$^{th}$, and 10$^{th}$ day, respectively.

After that, the Germination Stress Index (GSI) was calculated using Eq. 6 by Giancarla *et al*. (2012).

$$\text{GIS (\%)} = \frac{PIS}{PINS}\ X\ 100 \quad (6)$$

Where PIS is the promptness index under water and salt stress conditions, and PINS is PI under control.

#### 6.2.8. Seedling growth test

After the germination test, the seedling length of germinated seeds was measured on the final day using a ruler and expressed in millimetres. The seedling length was calculated as an average for all the germinated seeds for each landrace and treatment. The seedling vigour index (SVI) was calculated from the final germination percentage and seedling length according to Awasthi *et al*. (2016) using Equation 7.

$$\text{SVI} = \frac{\text{Final germinated percentage} \times \text{Seedling length}}{100} \quad (7)$$

#### 6.2.9. Data analyses

The data was analyzed using GenStat®, 20.1 Edition (VSN International, Hamel Hampstead, UK, 2020) at the 5% significance level. The means of significantly different variables were separated using Tukey's test.

### 6.3. Results

#### 6.3.1. Seed viability

The results showed significant differences ($p$<0.001) amongst landraces for seed viability as assessed using TCC. Landrace ‘B1’ exhibited the highest seed viability of 95.67% amongst the

landraces, while ‘L1’ showed the lowest seed viability of 70.00% (Figure 6.1). Notably, both ‘H1’ and ‘M1’ exhibited the seed viability of 76.67%; however, there were no significant differences between these genotypes and ‘L1’ and ‘M2’.

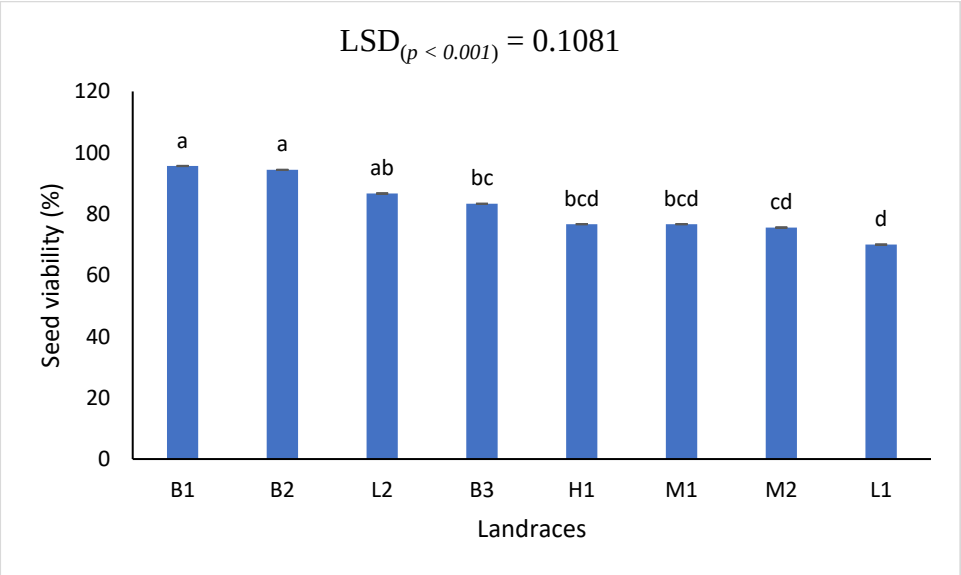



**Figure 6.1:** Seed viability of cannabis landraces before dormancy-breaking treatments ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 6.3.2. Germination of cannabis landraces under laboratory conditions

The landraces, PEG treatment, and their interaction showed significant differences ($p$<0.001) in germination percentage, germination recovery percentage, germination rate index, and germination stress tolerance index under laboratory conditions (Table 6.2).

**Table 6.2**: Analysis of variance (ANOVA) for seed germination percentage (GP), germination recovery percentage (GPR), germination rate index (GRI), and germination stress tolerance index (GSI) of cannabis landraces subjected to different PEG concentrations under laboratory conditions (number of replicates = 3).

| Source of variation | df | F Values | | | df | F Value |
|---|---|---|---|---|---|---|
| | | GP | GPR | GRI | | GSI |
| Landrace | 7 | 57.57** | 69.43** | 56.73** | 7 | 50.40** |
| Treatment | 5 | 446.84** | 114.87** | 381.05** | 4 | 31.33** |
| Landrace*treatment | 35 | 11.76** | 5.08** | 13.29** | 28 | 7.61** |

*Significant ($p$<0.05); **highly significant ($p$<0.001**)**

#### 6.3.2.1. Germination

The results revealed that the germination of all cannabis landraces was reduced with the increasing PEG concentration (Figure 6.2). Generally, 'L2' exhibited the highest germination among the landraces. Meanwhile, 'L1' generally exhibited the lowest germination, with complete inhibition under PEG -0.6, -0.8, and -1.0 MPa. However, except under control as 'H1' demonstrated the lowest germination, with complete inhibition under PEG -1.0 MPa. Similar to 'H1', the germination of 'B3' and 'M2' was completely inhibited under PEG -1.0 MPa. Similar to 'L1', the germination of 'M1' was completely inhibited under PEG -0.6, -0.8 and -1.0 MPa. Furthermore, 'B2' exhibited complete germination inhibition under PEG -0.8 and -1.0 MPa.

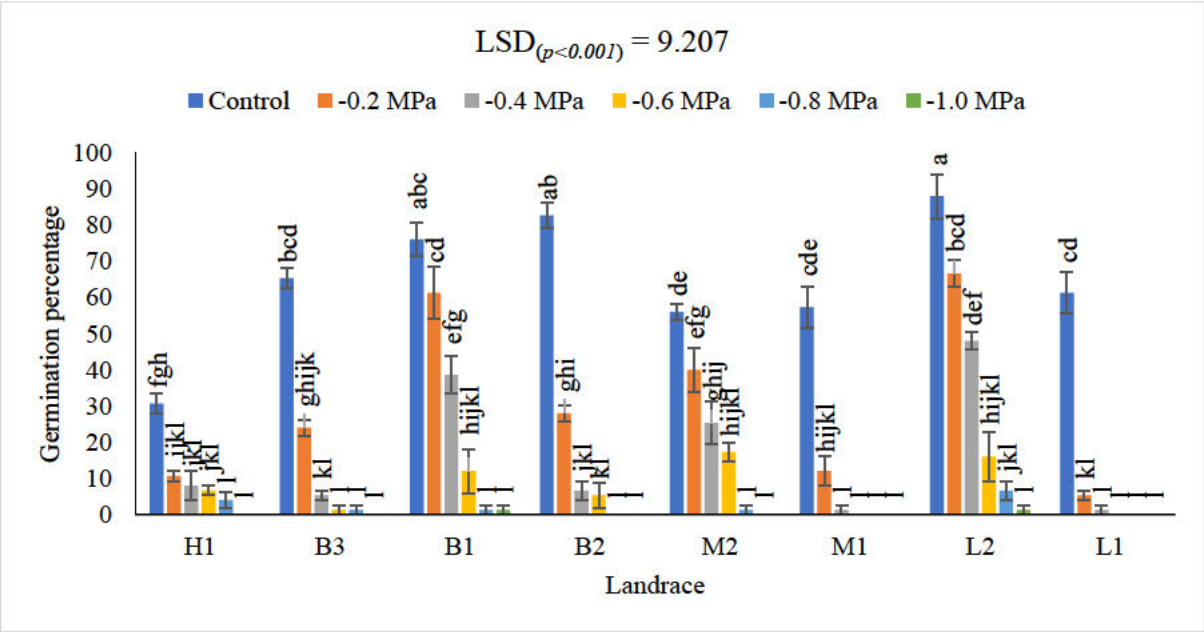



**Figure 6.2**: Germination of various cannabis landraces in response to different PEG concentrations under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 6.3.2.2. Germination recovery

The results revealed that the increasing PEG concentration generally reduced germination after recovery (Figure 6.3). However, some variations in the interaction between landrace and PEG concentration were observed in 'H1', 'M2', and 'L2'. 'H1' exhibited increased germination of 26.67, 34.67 and 18.67% under PEG -0.6, -0.8 and -1.0 MPa, respectively, compared to 16.00% under PEG -0.4 MPa. 'M2' demonstrated a higher germination rate of 41.33% under PEG -0.8 MPa compared to 36.00% recorded under PEG -1.0 MPa. 'L2' also exhibited increased germination of 42.67 and 49.33% under PEG -0.8 and -1.0 MPa, respectively, compared to 25.33% recorded under PEG -0.6 MPa. Notably, the germination of 'M1' and 'L1' was permanently inhibited. Meanwhile, 'L2' generally demonstrated the highest germination amongst the landraces, while 'L1' showed the lowest.

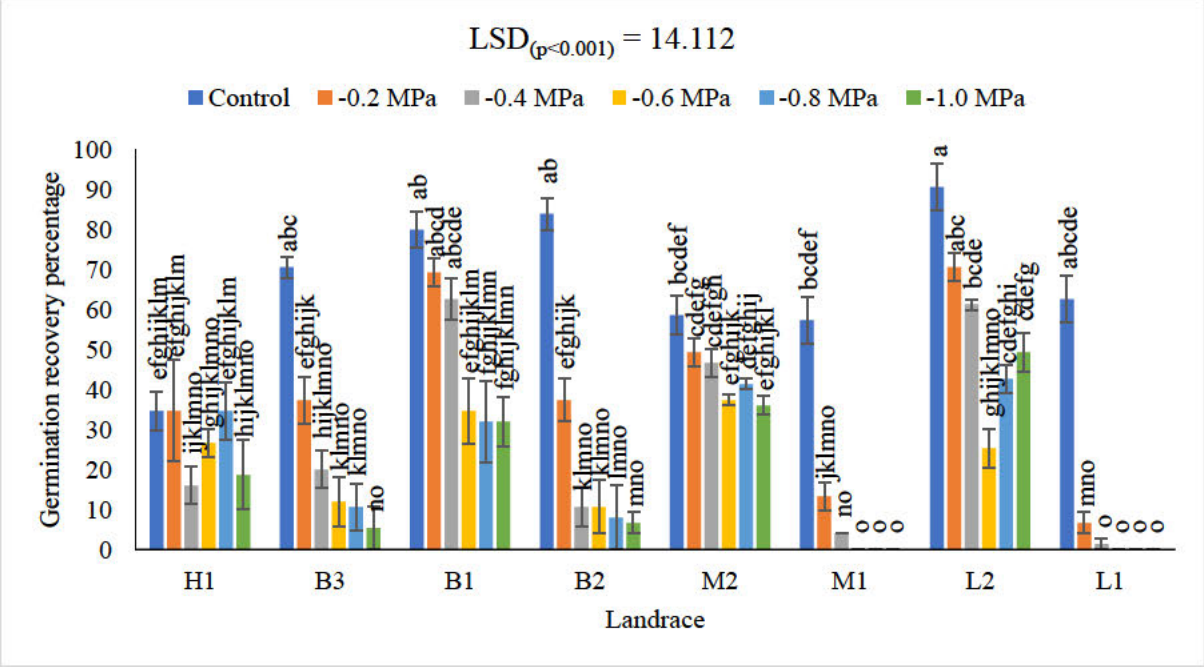



**Figure 6.3**: Germination recovery of various cannabis landraces subjected to different PEG concentrations under laboratory conditions ($p$<0.001). LSD 5% = least significance difference at 5 percent.

#### 6.3.2.3. Germination rate index

The results revealed that the germination rate index decreased with the increasing PEG concentration (Figure 6.4). However, some variations in interaction between landraces and PEG treatments were observed, particularly in 'H1' and 'M2'. 'H1' showed an increased germination rate index of 1.083 and 1.442 seeds/day under PEG -0.6 and -0.8 MPa compared to 0.868 seeds/day under PEG -0.4 MPa. 'M2' also exhibited an increased germination rate index of 1.396 and 1.479 seeds/day under PEG -0.8 and -1.0 MPa compared to 1.339 seeds/day under PEG -0.6 MPa. Furthermore, 'B1' exhibited a higher germination rate index of 0.971 seeds/day under PEG -1.0 MPa compared to 0.922 seeds/day under PEG -0.8 MPa. The results also revealed that 'L2' generally demonstrated the highest germination rate index amongst the landrace while 'L1' exhibited the lowest.

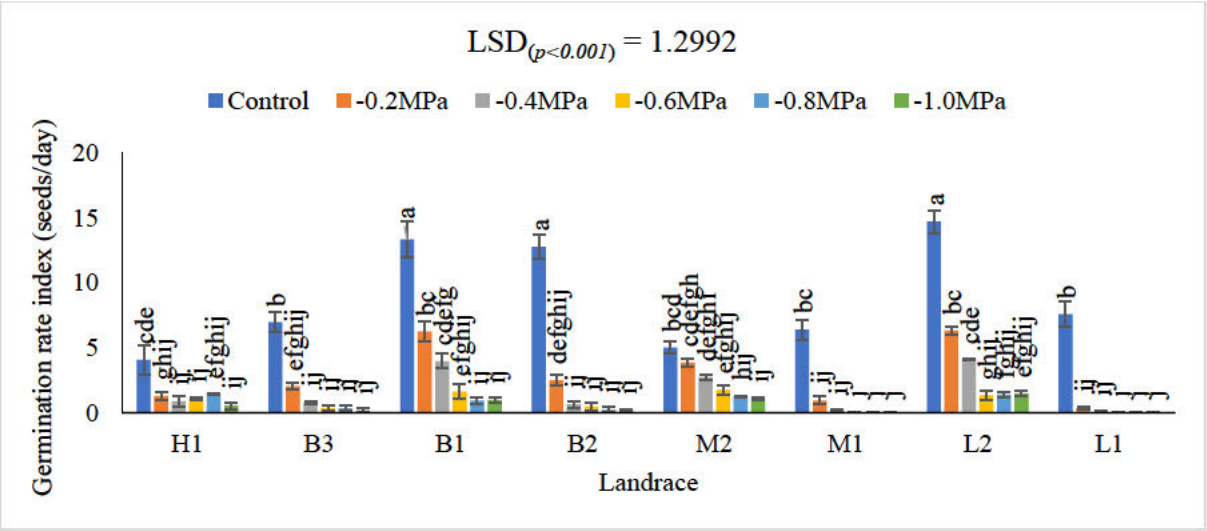



**Figure 6.4**: Germination rate index of various cannabis landraces subjected to different PEG concentrations under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 6.3.2.4. Germination stress tolerance index

The results showed a decreasing germination stress tolerance index with the increasing PEG concentration under greenhouse conditions, particularly in landraces 'B3', 'B2', 'M1', and 'L1' (Figure 6.5). However, there were variations in some landraces with the PEG treatments, with different landraces established to have the highest germination stress tolerance index across the PEG concentrations. These landraces included 'H1', 'B1', 'M2', and 'L2'. 'H1' showed an increased germination stress tolerance index of 45.75 and 102.09% under PEG -0.6 and -0.8 MPa compared to 17.60% under PEG -0.4 MPa. It also demonstrated the highest germination stress tolerance index amongst the landraces at 102.09% under PEG -0.8 MPa.

'B1' also exhibited a higher germination stress tolerance index of 18.41% under PEG -1.0 MPa compared to 14.86% under PEG -0.8 MPa. 'L2' also showed an increased germination stress tolerance index of 28.83 and 21.52% under PEG -0.8 and -1.0 MPa compared to 18.43% recorded under PEG -0.6 MPa. Additionally, 'M2' demonstrated a slightly higher germination stress tolerance index of 85.98% under PEG -0.4 MPa compared to 85.02% recorded under

PEG -0.2 MPa. 'M2' also exhibited a higher germination stress tolerance index of 45.44% under PEG -0.8 MPa compared to 41.11% recorded under PEG -0.6 MPa. Notably, 'L1', followed by 'M1', generally demonstrated the lowest germination stress tolerance index amongst the landraces under all the treatment groups. Meanwhile, 'M2', followed by 'B1', generally exhibited the highest germination stress tolerance index amongst the landraces in response to PEG treatments.

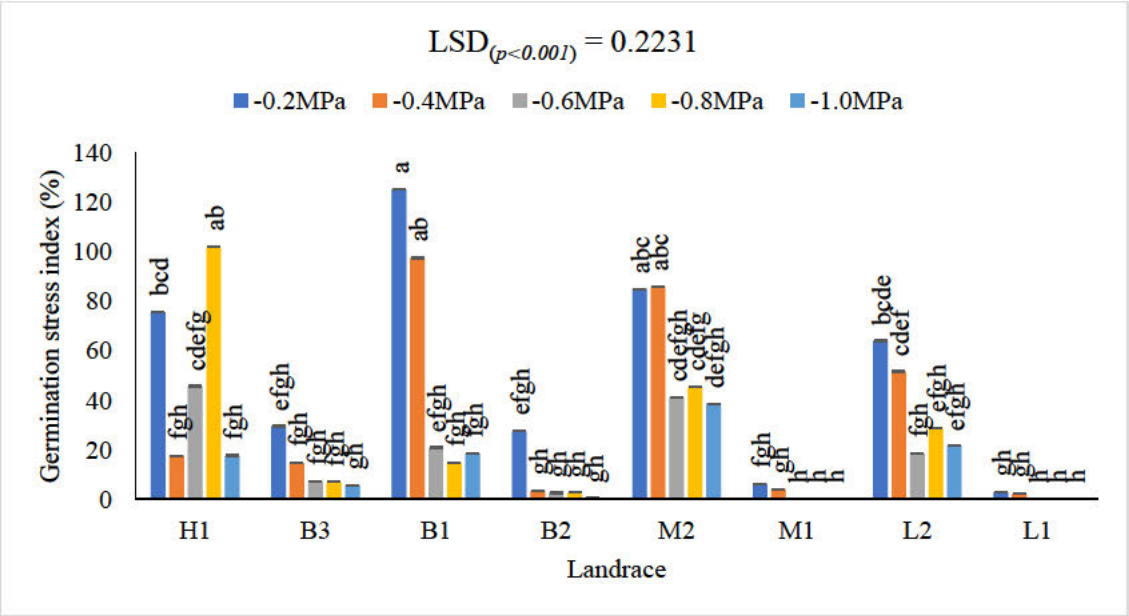



**Figure 6.5**: Germination stress index of various cannabis landraces subjected to different PEG concentrations under laboratory conditions ($p$<0.001). LSD 5% = least significance difference at 5 percent.

### 6.3.3. Germination and seedling growth of cannabis landraces under greenhouse conditions

The landraces, PEG treatment, and their interaction showed significant differences ($p$<0.05) in germination rate index, seedling length, seed vigour, and germination stress index (Table 6.3). The landraces and PEG treatment also showed significant differences ($p$<0.05) in germination percentage. Meanwhile, the interaction between landrace and PEG treatments revealed no significant difference ($p$>0.05) in germination percentage.

**Table 6.3**: Analysis of variance (ANOVA) for seed germination percentage (GP), germination rate index (GRI), seedling length (SL), seed vigour index (SVI), and germination stress tolerance index (GSI) of cannabis landraces subjected to different PEG concentrations (number of replicates = 3).

| Source of variation | df | F Values | | | | df | F Value |
|---|---|---|---|---|---|---|---|
| | | GP | GRI | SL | SVI | | GIS |
| Landrace | 7 | 2.65* | 19.60** | 10.52** | 5.06** | 7 | 9.64** |
| Treatment | 5 | 12.66** | 32.58** | 21.15** | 31.05** | 4 | 6.30** |
| Landrace*Treatment | 35 | 0.41ns | 3.23** | 4.48** | 1.84* | 28 | 1.93* |

ns - not significant ($p$>0.05); *significant ($p$<0.05); **highly significant ($p$<0.001**)**

#### 6.3.3.1. Germination

The results showed that the germination generally decreased with the increasing PEG concentration in all the cannabis landraces (Figure 6.6). However, 'B1' exhibited slightly higher germination of 78.67% under PEG -0.6MPa compared to 77.33% under PEG -0.4MPa. Notably, 'M1' and 'L2' exhibited no changes in germination under PEG -0.2MPa compared to control. 'M1' exhibited a germination of 86.67% under control and PEG -0.2MPa, respectively. 'L2' exhibited a germination of 77.33% under control and PEG -0.2MPa, respectively. 'H1' generally exhibited higher germination, followed by 'B3', compared to the other landraces. Meanwhile, 'B2', followed by 'L2', generally showed the lowest germination compared to the other landraces.

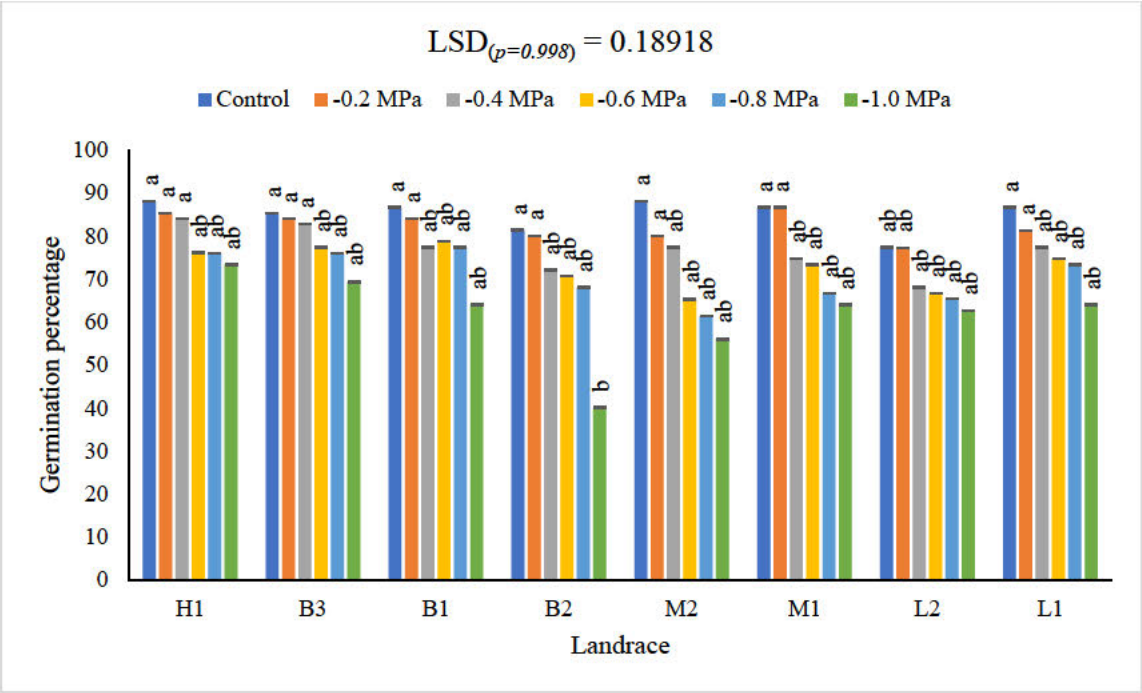



**Figure 6.6**: Germination of various cannabis landraces subjected to different PEG concentrations under greenhouse conditions ($p$=0.998; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 6.3.3.2. Germination rate index

The results revealed that the germination rate varied with the cannabis genotype and PEG concentration (Figure 6.7). However, the landraces generally demonstrated the highest germination rate under the control treatment. Nonetheless, 'H1', 'M2', and 'M1' exhibited the highest germination rate under PEG treatments compared to control. Specifically, 'H1' demonstrated the highest germination rate of 3.365 and 3.621 seeds/day under PEG -0.2 and -0.4 MPa, respectively, while the control recorded 3.285 seeds/day. 'M1' exhibited the highest germination rate of 5.129 seeds/day under PEG -0.2 MPa, while control showed 4.660 seeds/day. 'M2' showed the highest germination rate of 6.443 seeds/day under PEG -0.2 MPa, while control exhibited 4.423 seeds/day. The results also revealed that 'B3', followed by 'B1', generally exhibited the highest germination rate amongst the landraces under PEG treatments.

Meanwhile, 'H1' and 'M2' showed the lowest germination rate amongst the landraces under PEG treatments.

Notably, 'B3' generally exhibited a higher germination rate amongst the landraces under the PEG treatments. Meanwhile, 'H1' generally showed the lowest germination rate compared to the other landraces.

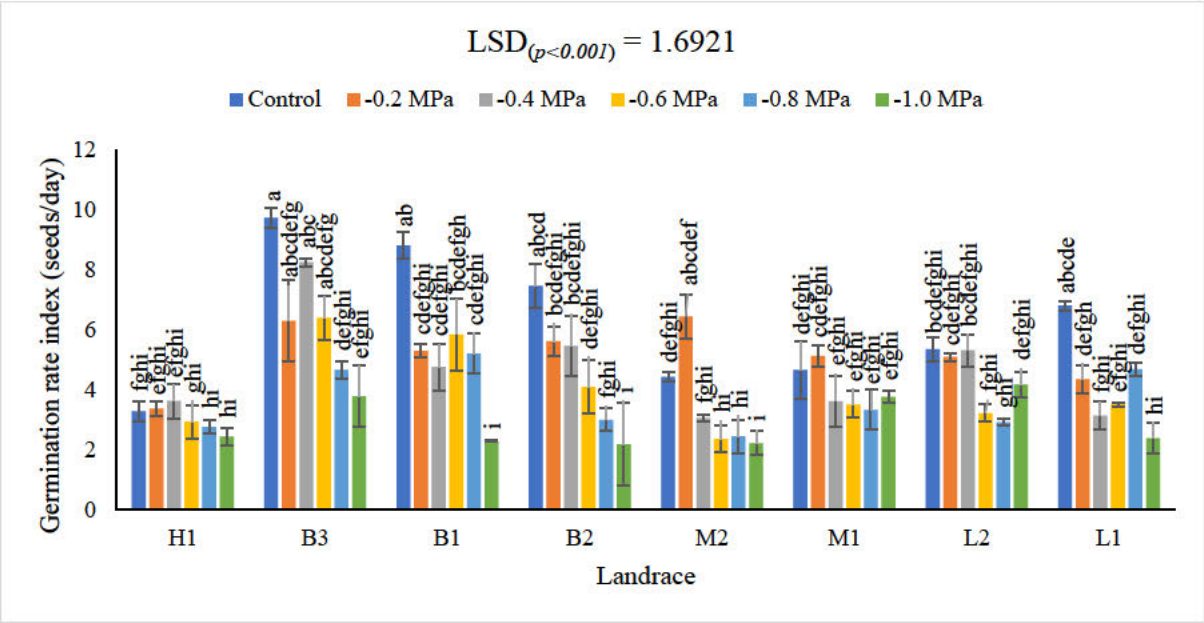



**Figure 6.7**: Germination rate index of various cannabis landraces subjected to different PEG concentrations under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 6.3.3.3. Germination stress tolerance index

The results demonstrated that the germination stress tolerance varied with the cannabis genotype and PEG treatment under greenhouse conditions (Figure 6.8). However, the germination stress tolerance was generally reduced with the increasing PEG concentration. The germination stress tolerance was generally the lowest under PEG -1.0 MPa, except for 'H1' and 'M2'. 'H1' showed the highest germination stress tolerance of 100.99% under PEG -1.0MPa, while the lowest was 90.36% under PEG -0.4MPa. 'M2' exhibited a higher

germination stress tolerance of 83.09% under PEG -1.0MPa compared to 79.96% recorded under PEG -0.8MPa. Notably, 'B3', 'B1', and 'B2' exhibited the highest germination stress tolerance of 222.17, 89.16, and 64.21% under PEG -0.4 MPa, respectively. The results also revealed that 'B3' generally showed higher germination stress tolerance among the landraces under PEG treatments. Meanwhile, 'B2' and 'L1' generally demonstrated lower germination stress tolerance among the landraces.

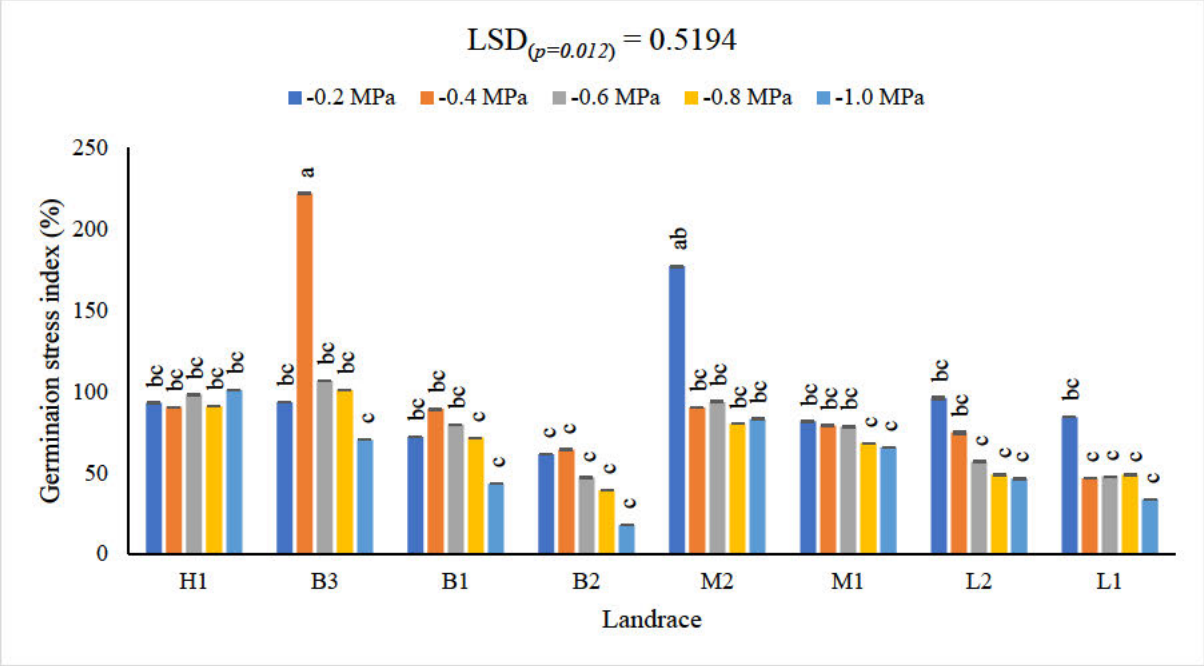



**Figure 6.8:** Germination stress index of various cannabis landraces subjected to different PEG concentrations under greenhouse conditions ($p$=0.012; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 6.3.3.4. Seedling length

The results revealed that the seedling length varied with the cannabis genotypes and PEG concentration (Figure 6.9). However, the seedling length of the landraces was generally higher under control and lower under PEG -1.0MPa compared to the other treatment groups. However, 'M2' and 'M1' showed the highest seedling length of 167.7 mm under PEG -1.0 MPa and

155.3 mm under PEG -0.6 MPa, respectively. 'M2' and 'M1' also showed the lowest seedling length of 107.0 and 115.0 mm under PEG -0.2 and -0.4 MPa, respectively. 'H1' and 'B3' also showed the lowest seedling length of 101.1 and 136.1 mm under PEG -0.8 MPa, respectively. Whereas 'B1', 'B2', 'L2', and 'L1' showed the lowest seedling length under PEG -1.0 MPa of 113.5, 82.8, 129.1, and 103.5 mm, respectively. The results also revealed that the landraces tended to have higher seedling length under PEG -0.6MPa compared to PEG -0.2 and -0.4MPa. Among the landraces, 'B3' and 'H1' generally had the highest and lowest seedling lengths under PEG treatments, respectively.

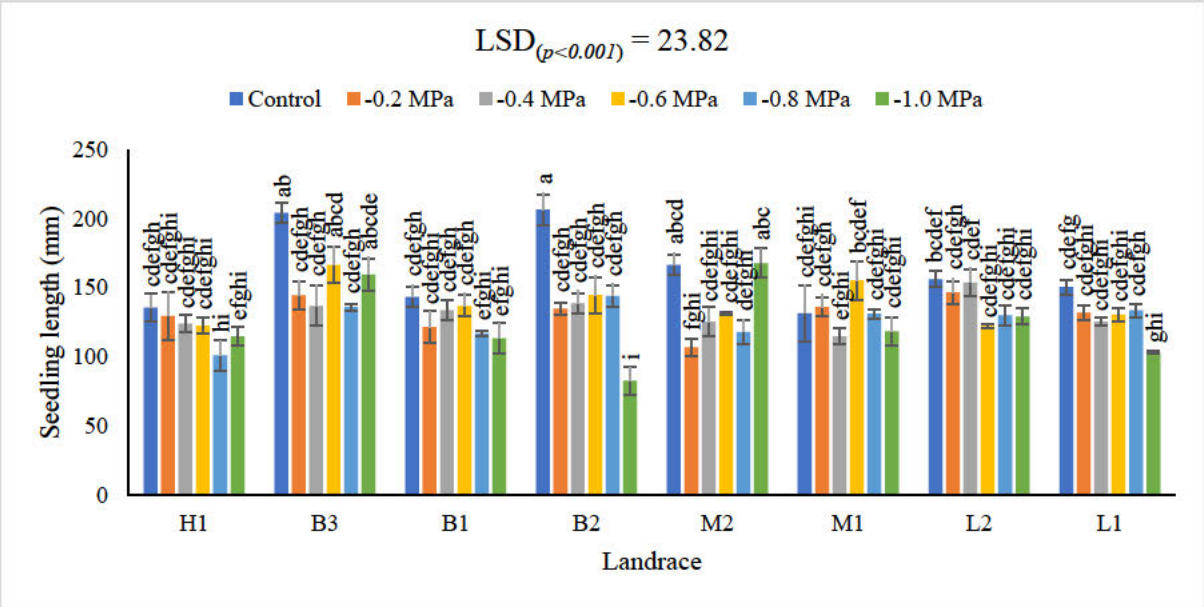



**Figure 6.9:** Seedling length of various cannabis landraces subjected to different PEG concentrations under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 6.3.3.5. Seed vigour index

The results showed that the seed vigour index varied with the cannabis genotypes and PEG concentration (Figure 6.10). However, the landraces generally demonstrated a higher seed vigour index under control compared to PEG treatments, except for 'M1', which showed the

highest index of 1.184 under PEG -0.2 MPa. Notably, 'H1', 'B3', and 'M2' exhibited the lowest seed vigour index under PEG -0.8 MPa of 0.759, 1.035, and 0.738, respectively. The seed vigour was also lower compared to 0.841, 1.114, and 0.929 exhibited by 'H1', 'B3', and 'M2' under PEG -1.0 MPa, respectively. Meanwhile, the other landraces had the lowest seed vigour under PEG -1.0 MPa. The results also revealed that 'B3', 'B1', 'M1', and 'L1' exhibited a higher seed vigour index of 1.297, 1.067, 1.124, and 0.974 under PEG -0.6 MPa, respectively, compared to corresponding 1.127, 1.026, 0.863 and 0.967 under PEG -0.4 MPa. In 'B3'and 'B1', the seed vigour index demonstrated under PEG -0.6 MPa was also higher compared to 1.214 and 1.018 exhibited under PEG -0.2 MPa, respectively. Notably, 'B3' and 'M2' generally exhibited the highest and lowest seed vigour index amongst the landraces under PEG treatments, respectively.

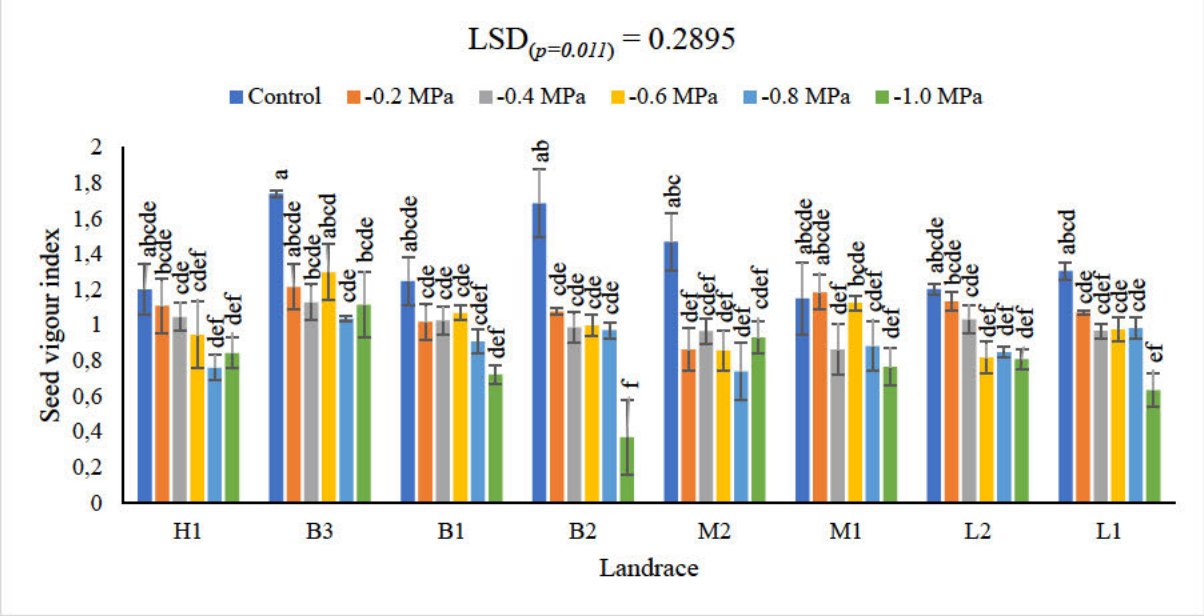



**Figure 6.10**: Seed vigour index of various cannabis landraces subjected to different PEG concentrations under greenhouse conditions ($p$=0.011; number of replicates = 3. LSD 5% = least significance difference at 5 percent.

## 6.4. Discussion

### 6.4.1. Germination

The germination percentage in landraces generally decreased with the increasing PEG 8000 concentration under laboratory and greenhouse conditions as the water potential decreased. Laboratory conditions resulted in the most significant decrease in germination, reaching a minimum of 0.00%, compared to the 40.00% observed under greenhouse conditions. The lower germination in laboratory settings compared to greenhouse conditions can be attributed to the precisely controlled conditions, ensuring a constant temperature and relative humidity level. The findings on the decreased germination with the increasing PEG concentrations were associated with the low water potential of the germinating seeds due to the osmotic stress imposed by PEG 8000. Such leads to germination water requirements not being met, as it is known that seeds generally do not imbibe enough water under scarce water conditions (Zia *et al*., 2021). Eventually, the metabolic processes of the seeds get disrupted and may cease (Violita and Azhari, 2021). This disruption includes degradation and inactivation of the essential hydrolytic and other groups of enzymes that convert endosperm starch into simple sugars that play a key role in germination, ultimately reducing the germination percentage (Vijay *et al*., 2018; Bukhari *et al*., 2021; Violita and Azhari, 2021). The results of the current study corroborate with the findings by Queiroz *et al*. (2019), who reported that maize showed reductions in germination percentage, germination rate index, and mean germination time with the increasing PEG concentration. It has also been previously reported that water deficit stress reduced the dry matter production of hemp (Babaei and Ajdanian, 2020). Thus, limited water availability causes a reduction in plant growth attributes from germination to harvest.

The germination was reduced even after the germination recovery steps were taken; the control treatment still demonstrated the highest germination percentage compared to the landraces under different PEG concentrations. However, the germination recovery was successful in all

the landraces, and it varied with landraces and PEG concentrations. Notably, higher PEG concentration completely inhibited the germination of ‘M1’ and ‘L1’ under the laboratory conditions, which was associated with the metabolic processes of their seeds being ceased by the higher PEG concentrations as from PEG -0.6MPa. According to Balouch *et al*. (2023), drought stress may cause significant gene damages and may also inhibit the synthesis of gibberellin hormone, which plays a role in the activation of α-amylase. All these genetical and enzymatical disruptions may lead to the reduction of seed germination under moderate stresses and non-germination in severe pressures. Additionally, polyethylene glycol disrupts water imbibition, thus preventing the hydrolysis of stored materials and, consequently, reducing the percentage and rate of germination (Balouch *et al*., 2023).

The recovery in germination in other landraces under PEG treatments was associated with that the seeds maintain viability when the abiotic stress exceeds their tolerance limit and thus can still germinate when the stress is reduced (Vicente *et al*., 2020). Hence, the seeds subjected to PEG-induced drought stress still germinated when transferred to reduced water stress, which was distilled water. When the landraces exhibited reduced germination with the increasing PEG concentration, some landraces, such as ‘M1’ and ‘L2’, showed adaptability under PEG -0.2 MPa as they exhibited a similar germination percentage as control. Thus, low PEG concentrations might have had no disruptive impact on the metabolic processes in the seeds of these landraces. These results corroborate the findings by Queiroz *et al*. (2019), who reported that the treatment PEG -0.2MPa was not a limiting factor for sorghum (*Sorghum bicolor* (L.) Moench.), as the germination results obtained under this concentration were similar to the control.

### 6.4.2. Germination rate index

The germination rate of cannabis landraces generally decreased with increasing PEG concentrations. The delayed germination observed in landraces was attributed to cannabis seeds not absorbing sufficient water under PEG treatments. Consequently, more time was required for the seeds to adjust their internal osmotic potential to align with the surrounding environment, which reduced the germination rate (Queiroz *et al*., 2019; Zia *et al*., 2021). According to Balouch *et al*. (2023), seeds require time to recover from damage to the membrane and other cellular components caused by polyethylene glycol. This recovery involves restarting the activity of the antioxidant system to prevent oxidative stress (Balouch *et al*., 2023). The results of the current study corroborate with the findings of Tounekti *et al*. (2020), who reported that the germination rate index of sorghum seeds was considerably reduced with the increase in PEG 8000 concentration. The results of the current study also agree with the findings of Naim and Qadarif (2015), who reported that the final germination and germination rate index in 'Pirooz' and 'Arman' chickpea (*Cicer arietinum* L). decreased significantly with the decreasing water potential due to PEG 8000 solutions. Landraces 'M2' and 'M1', however, showed the highest germination rate under PEG -0.2 MPa compared to control, which was associated with these species having tolerance to moderate stress.

### 6.4.3. Germination stress index

Generally, the germination stress index in cannabis landraces decreased with the increasing PEG concentration. This showed a similar trend as germination percentage and germination rate index, which generally decreased with the increasing PEG concentration. The reduced germination stress tolerance was associated with cannabis being known to be sensitive to water stress and requires high moisture throughout its growing season, particularly during the first six weeks of plant establishment (Cole and Zurbo, 2008; Williams and Mundell, 2018; Adesina

*et al*., 2020). The results of the current study corroborate with the findings by Mirza *et al*. (2015), who reported that the germination stress tolerance index was reduced under water-stressed wheat seeds. However, it was also notable that the germination stress index in specific cannabis landraces varied with PEG treatments, such as 'H1', 'M2'and 'L2' under laboratory conditions and 'H1', 'B3', 'B1', 'B2' and 'M2' under greenhouse conditions. The germination stress index in these landraces was sometimes improved under increased PEG concentrations, and this was associated with the different genotypes and environmental conditions (Razzaq *et al*., 2017). In 'H1' and 'M2', it was more closely linked with the species. In other landraces under greenhouse conditions, it was more associated with the environmental effects since this trend was exclusively observed under these conditions.

Among the landraces, M2 showed the highest germination stress tolerance towards PEG treatments under laboratory conditions, while L1 and M1 showed the lowest water stress tolerance. 'B3' generally showed higher germination stress tolerance among the landraces under PEG treatments under greenhouse conditions. Meanwhile, 'B2' generally demonstrated lower germination stress tolerance. Thus, the water stress tolerance varied with species. However, some level of consistency in 'M2' and 'L1' under laboratory and greenhouse conditions exhibited the second highest and lowest germination stress tolerance index, respectively. According to Giancarla *et al*. (2012), most plants show some drought stress tolerance; however, it varies from species to species and even within species. Even though the germination stress tolerance index is being suggested as a screening criterion for stress tolerance in different plants, the findings of the current study contradict this suggestion and were associated with it reflecting seed quality differences (Ahmad *et al*., 2015). This follows that it did not reflect the stability in seedling length and seed vigour index in cannabis landraces, and it has previously failed to reflect stress tolerance in various plants and the yield stability of genotypes (Ahmad *et al*., 2015).

### 6.4.4. Seedling length

The seedling length generally decreased under PEG treatments relative to control. The reduced seedling length was associated with PEG creating a low water potential resulting in protoplasm being dehydrated, reducing the turgidity of the cell, which ultimately reduced the cell elongation and cell division and eventually reduced the seedling length (Vijay *et al*., 2018; Bukhari *et al*., 2021). These changes mentioned above in cell wall extensibility further result in an imbalance in hormones such as cytokinin and abscisic acid, which then affects growth through reduced seedling height (Bukhari *et al*., 2021). Among the plant hormones, ABA and auxin have been found to be critical in the response of hemp to drought stress (Gao *et al*., 2018). Additionally, ABA's rapid accumulation and mediation in plant survival when subjected to various stresses has led to this hormone being categorized as a stress hormone (Gao *et al*., 2018). In cases where seedling length was higher compared to control, it was associated with tolerance to moderate stress and adaptation to drought stress, possibly through increased root length, as seedling length comprises both root and shoot length.

### 6.4.5. Seed vigour index

The seed vigor index, comprising both seed germination and shoot growth, reflects the combined seed properties influencing its potential activity and performance in the early stages of germination and seedling growth (Bouchyoua *et al*., 2024). It is known that this index is particularly sensitive to drought conditions and can be regarded as a reliable indicator of the tolerance of a crop to drought stress (Bouchyoua *et al*., 2024). In the current study, the seed vigour index generally decreased under PEG treatments relative to control and varied in landraces with the PEG concentrations. The reduced seed vigour index was associated with osmotic or ionic effects of PEG, which possessed inhibiting effects on cell division and cell elongation processes linked with the growth of seedlings and the decrease in plastic

extensibility of the growing cell walls (Vijay *et al*., 2018). Thus, exposure to low water potential during the seedling stage has detrimental effects on seed emergence, impeding seedling growth, establishment, and vigour (Bukhari *et al*., 2021).

In general, the impact of drought on cannabis landraces showed variability depending on the growing conditions and specific landrace. Among the landraces tested under laboratory conditions, 'L2' generally exhibited the highest germination percentage and rate, and 'B1' demonstrated the highest germination stress tolerance index. Meanwhile, 'L1' and 'M1' exhibited the lowest germination percentage, germination rate, and germination stress tolerance index. Under the greenhouse conditions, ‘H1’ generally exhibited the highest germination, and ‘B3’ had the highest germination rate, germination stress tolerance index, seedling length, and seed vigour index. These variations were associated with the growing media and attributed to compost which provided well-aerated conditions compared to the cotton pad in a Petri dish used under laboratory conditions (Tiawoun *et al*., 2017). These differences in water-holding capacity manifested in the significant reduction of germination under laboratory environments compared to the greenhouse conditions. Furthermore, the germination differences were attributed to the environmental conditions.

### 6.5. Conclusion

The current study investigated the effect of PEG-induced drought stress on the germination of different cannabis landraces. PEG treatments reduced germination characteristics, including germination percentage, germination rate, germination stress tolerance index, seedling length, and seed vigour index. However, the findings varied with the PEG concentration, landrace, and environment. Landraces ‘L2’, ‘B1’ and ‘B3’ showed drought tolerance amongst the landraces. However, their tolerance depended on the growing environment, with ‘L2’ and ‘B1’ indicating drought tolerance under controlled growing conditions while ‘B3’ exhibited drought tolerance

under greenhouse conditions. Thus, these findings indicate that 'L2', ‘B1’, ‘H1’, and ‘B3’ may be a suitable choice for farmers facing limited access to climate adaptation resources like irrigation technologies. Furthermore, these landraces may be suitable for further study for breeding programs targeted at the development of drought-resistant genotypes. Based on the current findings, it is recommended that further studies investigate the effect of drought stress on cannabis landraces in different growth stages, taking into consideration the morphological, biochemical, and genetic traits.

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# 7. CHAPTER 7: SALT TOLERANCE IN CANNABIS LANDRACES UNDER NACL-INDUCED SALT STRESS DURING SEED GERMINATION AND EARLY SEEDLING GROWTH

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**Abstract**

Overreliance on irrigated agriculture to intensify production and meet growing food demands threatens productivity and sustainability through soil salinization. Hence, understanding plant salt tolerance during early development is vital, given the high sensitivity of seed germination to such environmental stresses. There is currently a lack of information regarding seed germination of drug-type cannabis genotypes in saline conditions. This study, therefore, investigated the seed germination and seedling growth responses of cannabis landraces under sodium chloride (NaCl)-induced salt stress. The genotypes were collected from local growers in four regions of KwaZulu-Natal Province in South Africa, namely, Bergville (B), Hammersdale (H), Ladysmith (L), and Msinga (M). Each genotype was assigned an alphabet and number based on where the seed material was collected. The eight genotypes were as follows, 'B1', 'B2', 'B3', 'H1', 'L1', 'L2', 'M1', and 'M2'. Salt stress was induced in a laboratory setting using NaCl at levels 0, 50, 100, 150, 200, and 250 mM, and the findings were subsequently validated under the greenhouse conditions. The parameters measured included germination percentage and rate, stress tolerance index, seedling length, and seed vigour index.

Generally, there were significant differences ($p$<0.001) in landrace and environment interactions in the measured parameters under both laboratory and greenhouse settings. Except that, there were no significant differences ($p$>0.05) in germination under greenhouse conditions as well as in germination stress tolerance index in both laboratory and greenhouse settings. Additionally, the elevated levels of NaCl generally reduced the germination and seedling growth characteristics of all tested cannabis genotypes. However, the results varied with landrace, seed viability, salt concentration, and environmental conditions. 'L2' generally indicated tolerance to saline conditions under the laboratory setting, while 'H1' and 'B2' showed resilience to saline conditions under the greenhouse conditions. These findings have practical implications, indicating that 'H1', 'L2', and 'B2' could be a better selection for planting under saline conditions imposed by poor irrigation management.

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**Keywords**: cannabis, landrace, germination, osmotic stress, germination stress tolerance

### 7.1. Introduction

The increasing global demand for food requires agricultural intensification, placing unprecedented pressure on land and water resources (Mettetal, 2019; Rosa *et al*., 2020). Amongst various food production influencers, water and nutrients play crucial roles as key biophysical factors (Rosa *et al*., 2020). Over the years, advanced technologies have enabled the production of fertilizers in massive quantities, while water remains a critical input limiting global food production (Rosa *et al*., 2020). This is largely attributed to changing climate, resulting in heightened warming conditions, alterations in rainfall patterns and distribution, and an increase in the intensity and frequency of droughts and floods (Maponya and Mpandeli, 2012; Nhemachena *et al*., 2020). Climate change worsens water stress and hydrologic variability, particularly in semi-arid and arid regions (Materechera, 2011; Van Rensburg *et al*., 2011; Schreiner *et al*., 2018; Nhemachena *et al*., 2020). This phenomenon adversely affects

agricultural food supply and demand, as well as local food systems, posing a threat to the progress toward achieving food security (Materechera, 2011; Myeni *et al*., 2019; Nhemachena *et al*., 2020).

Therefore, irrigation has emerged as a crucial element of agricultural intensification, and its expansion is essential to enhance agricultural production and meet the rising demand for food commodities (Mettetal, 2019; Rosa *et al*., 2020). The overreliance on irrigated agriculture has led to the categorization of this field as the most water-demanding, accounting for over 85% of human water consumption (D'Odorico *et al*., 2020). Although irrigation is remedial for climate change effects, it also threatens the productivity and sustainability of irrigated agriculture through soil salinization (Materechera, 2011). Across the globe, approximately 19.5% of irrigated lands and 2.1% of drylands are affected by salinity issues, which are continuously increasing mainly due to mismanaged irrigation practices (Dehnavi *et al*., 2022). Thus, irrigation may also require access to technologies to desalinate irrigation water. This is because using saline water or excessive groundwater pumping can increase the concentration of salts in the soil, which may increase with climate (Di Mola *et al*., 2021).

Salt accumulation in the soil root environment limits water and nutrient uptake, leading to osmotic stress, ion toxicity, nutrient imbalances, and water deficit (Zörb *et al*., 2019; Dehnavi *et al*., 2022). The increased osmotic potential of the soil solution results in suppressed plant growth and decreased yield (Materechera, 2011; Dehnavi *et al*., 2022). Hence, salinity is one of the major abiotic stresses limiting plant productivity, resulting in about a 50 – 80% decrease in crop productivity depending on the crop and geographical location (Koyro and Eisa, 2008; Derese *et al*., 2018). Therefore, understanding salt tolerance in the early stages of plant development is vital as seeds with more rapid germination under saline conditions may be expected to achieve a rapid seedling establishment, resulting in higher yields (Petrović *et al*., 2016). It is well understood that seed germination is the first and most important and sensitive

stage in the life cycle of plants, and the successful establishment of seedlings largely depends on their successful germination (Gorai and Neffati, 2007; Petrović *et al*., 2016). However, little or no information exists concerning the seed germination of cannabis under saline environmental conditions in South Africa, where reliance on irrigation has been significant, with irrigated agriculture consuming the most water compared to other economic sectors (Materechera, 2011). Previous studies (Hu *et al*., 2018; Hu *et al*., 2019; Dixit, 2022; Varga *et al*., 2022; Rabbani *et al*., 2023; Zhang *et al*., 2023) have revealed that salt stress induced by different salt types such as NaCl, $Na_2SO_4$, $Na_2CO_3$ and $NaHCO_3$ ranging between 0 –300 mM inhibited the germination and plant growth of different fiber and seed-type hemp genotypes. Generally, the crops' response depends on several factors, including the type of salt and its concentration, as well as plant species or cultivars, which have varying tolerance to salt stress (Di Mola *et al*., 2021).

Cannabis is known to be comprised of drug-, oilseed-, and fibre-type hemp (De Meijer, 1994). Therefore, it is critical to investigate the response of drug-type hemp to salinity stress, considering that genotypes have varying tolerance. South Africa comprises well-known and popular strains, such as Swazi Gold, Malawi Gold, and Durban Poison, which are mainly produced for recreational drug purposes (Peltzer and Ramlagan, 2007; Gevaaalik, 2018). Thus, the response of South African genotypes to predominantly used salt for simulating salinity stress, i.e., ssodium chloride (NaCl), could be investigated to understand their salinity tolerance during seed germination (Vicente *et al*., 2020). Therefore, the objective of the current study was to investigate the germination and seedling growth response of cannabis landraces to NaCl-induced salt stress under laboratory and greenhouse conditions.

## 7.2. Materials and methods

### 7.2.1. Genotype collection

Seeds of nine cannabis genotypes (landraces) were collected from local growers in four regions of KwaZulu-Natal Province in South Africa, namely, Bergville, Hammersdale, Ladysmith, and Msinga. The collected cannabis landraces were assigned pseudonyms to ensure uniform naming of landraces for this experiment, as detailed in Table 7.1.

**Table 7.1:** The pseudonyms of the cannabis landraces and their geographic location information

| Location | Geographic information | | | | | Common local name | Name used for the experiment |
|---|---|---|---|---|---|---|---|
| | Latitude: | Longitude: | Elevation | Average rainfall | Average temperature | | |
| Ladysmith | -28.57533 | 29.85948 | 1009.53 m | 108.7 mm | 23.3 °C | 'Ugwayi wesiZulu' | 'L1' |
| | | | | | | 'Iswazi' | 'L2' |
| Msinga | -28.700960 | 30.303320 | 559 m | 340.0 mm | 23.0 °C | 'Ugwayi wesiZulu' | 'M1' |
| | | | | | | 'Iswazi' | 'M2' |
| Bergville | -28.677544 | 29.125553 | 1137.86 m | 109.4 mm | 23.5 °C | 'Ugwayi wesiZulu' | 'B1' |
| | | | | | | 'Iswazi' | 'B3' |
| | -28.667679 | 29.034111 | | | | 'Natal' | 'B2' |
| Hammersdale | -29.870903 | 30.633519 | 597 m | 110.3 mm | 23.7 °C | 'Durban Poison' | 'H1' |

The collected seed samples were stored at room temperature at the Horticultural Sciences Laboratory of the University of KwaZulu-Natal, Pietermaritzburg (latitude: -29.636311 and longitude: 30.409060). Prior to germination studies, the cannabis seeds were sorted based on

colour. Dark-coloured seeds were associated with maturity, whereas greenish-coloured seeds were associated with immaturity (Miraji *et al*., 2021). Therefore, dark-coloured seeds were used for the experiment. Seeds showing defects were removed from the seed lot.

### 7.2.2. Experimental design

The simulated salt stress experiment was structured using a factorial completely randomized design consisting of 8 genotypes and 6 sodium chloride (NaCl) treatments. Thus, seeds of various cannabis landraces were subjected to artificial salt stress by incubating them in sodium chloride (NaCl) (ACS reagent, ≥ 99.0%; Sigma Aldrich, Merck KGaA, 64271 Darmstadt, Germany) solutions. The prepared concentrations adopted from Zhang *et al*. (2012) involved six levels, which were 0, 50, 100, 150, 200, and 250 mM.

### 7.2.3. Seed viability test

Seeds were subjected to the viability test using 2,3,5-triphenyltetrazolium chloride (TCC) (Sigma Aldrich, Merck KGaA, 64271 Darmstadt, Germany), and the procedure was adopted from Mandizvo and Odindo (2019). TCC is a rapid and effective method for evaluating viability or physiological quality in seeds (Paiva *et al*., 2017). It is a water-soluble compound that promotes changes in colour when in contact with living seed tissues due to the activity of dehydrogenase enzymes (Virgens *et al*., 2019). Seed samples were preconditioned by soaking in distilled water for 18 h, at 25 °C in an incubator, and then excised with a scalpel blade through the embryo. The excised seeds were immersed in a 1% tetrazolium salt solution (TCC) for 12 h at 25 °C at room temperature. The experiment was replicated three times, and each replicate consisted of 30 seeds. Seed viability evaluation was done by placing the seeds in categories as viable and non-viable according to the coloration of the embryonic axis. Viable seeds were characterized by embryos showing a uniform shiny pink colour, and non-viable

seeds were characterized by embryos showing a totally white colour (Paiva *et al*., 2017). Only the percentage of viable seeds was recorded.

### 7.2.4. Germination test

The cannabis seeds were initially soaked in distilled water for 24 h to promote germination. Thirty (30) seeds of each cannabis genotype were placed on cotton pads moistened with distilled water in 90 mm Petri dishes, and 25 seeds were placed in a seedling tray containing lawn dressing compost obtained from Duzi Turf, New England Rd, Pietermaritzburg, South Africa. Lawn dressing compost is characterized as a finely sifted black organic weed-free medium. It is manufactured of a mixture of organic waste and sewage sludge, with a composition of 85% humus and 15% 2 mm – 8 mm larger particles. Each seed was planted in a seedling tray hole, and the germinating seeds were irrigated with tap water regularly to ensure adequate moisture. The experiment was replicated three times for consistency. The seeded Petri dishes were then transferred into a germination chamber (Micro-Clima Arabidopsis Chamber, ECD01E, Snijders, Netherlands) maintained at a temperature of 30/25 °C and a light cycle of 16/8 h. The germination progress was monitored daily, and the number of germinated seeds was recorded for each replicate for 7 days in a laboratory setting and 14 days under greenhouse conditions. A seed was considered to have germinated when the emerging radical elongated to a length of 2 mm or more. The germination percentage (GP) was calculated according to Khalaki *et al*. (2019) using Eq. 1 and was expressed in percentages (%).

$$\text{Germination percentage (\%GP)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100 \quad (1)$$

### 7.2.5. Germination recovery

The seeds that did not germinate during the germination test under different NaCl treatments in laboratory conditions were rinsed and put in Petri dishes with distilled water for an additional 3 days. Germination recovery aimed to determine the number of seeds that successfully

germinated after the salt stress was alleviated. The number of recovered seeds was then added to the number of seeds germinated under osmotic treatments of NaCl solute. The percentage of germination recovery was calculated using Eq. 3 by Chen *et al*. (2022).

$$\text{Germination recovery percentage (\%GP)} = \frac{a+b}{c} \times 100 \quad (2)$$

Where *a* is the number of germinated seeds after being transferred to distilled water, *b* is the number of seeds germinated from osmotic treatments of solutes (NaCl), and *c* is the total number of seeds.

### 7.2.6. Germination rate index

After the germination recovery, the total number of germinated seeds was recorded, and the germination rate index (GRI) was calculated using Eq. 3 by Awasthi *et al*. (2016).

$$\text{GRI} = \frac{\text{Number of germinated seeds}}{\text{Day of first count}} + \cdots + \frac{\text{Number of germinated seeds}}{\text{Day of final count}} \quad (3)$$

### 7.2.7. Germination stress index

The total number of germinated seeds was recorded and subsequently used for calculating the germination stress tolerance in cannabis landraces under salt stress conditions. The germination stress index was calculated by using the Promptness Index (PI), which was calculated using Eq. 4 (Giancarla *et al*., 2012).

$$\text{PI} = \text{nd2}(1.0) + \text{nd4}(0.8) + \text{nd6}(0.6) + \text{nd8}(0.4) + \text{nd10}(0.2) \quad (4)$$

Where nd2, nd4, nd6, nd8, and nd10 represent the percentage of germinated seeds in $2^{nd}$, $4^{th}$,$6^{th}$, $8^{th}$, and $10^{th}$ day, respectively.

After that, the Germination Stress Tolerance Index (GSI) was calculated using Eq. 5 by Giancarla *et al*. (2012).

$$\text{GIS (\%)} = \frac{PIS}{PINS}\ X\ 100 \quad (5)$$

Where PIS is the promptness index under salt stress conditions, and PINS is PI under control.

#### 7.2.8. Seedling growth test

After the germination test, the seedling length of germinated seeds was measured on the final day using a ruler and expressed in millimetres. The seedling length was calculated as an average for all the germinated seeds for each landrace and treatment. The seedling vigour index (SVI) was calculated from the final germination percentage and seedling length according to Awasthi *et al*. (2016) using Equation 6.

$$\text{SVI} = \frac{\text{Final germinated percentage} \times \text{Seedling length}}{100} \quad (6)$$

#### 7.2.9. Data analyses

The data was analyzed using GenStat®, 20.1 Edition (VSN International, Hamel Hampstead, UK, 2020) at the 5% significance level. The means of significantly different variables were separated using Tukey's test.

### 7.3. Results

#### 7.3.1. Seed viability

The results showed significant differences ($p$<0.001) amongst landraces for seed viability as assessed using TCC. Landrace ‘B1’ exhibited the highest seed viability of 95.67% amongst the landraces, while ‘L1’ showed the lowest seed viability of 70.00% (Figure 7.1). Notably, both ‘H1’ and ‘M1’ exhibited the seed viability of 76.67%; however, there were no significant differences between these genotypes and ‘L1’ and ‘M2’.

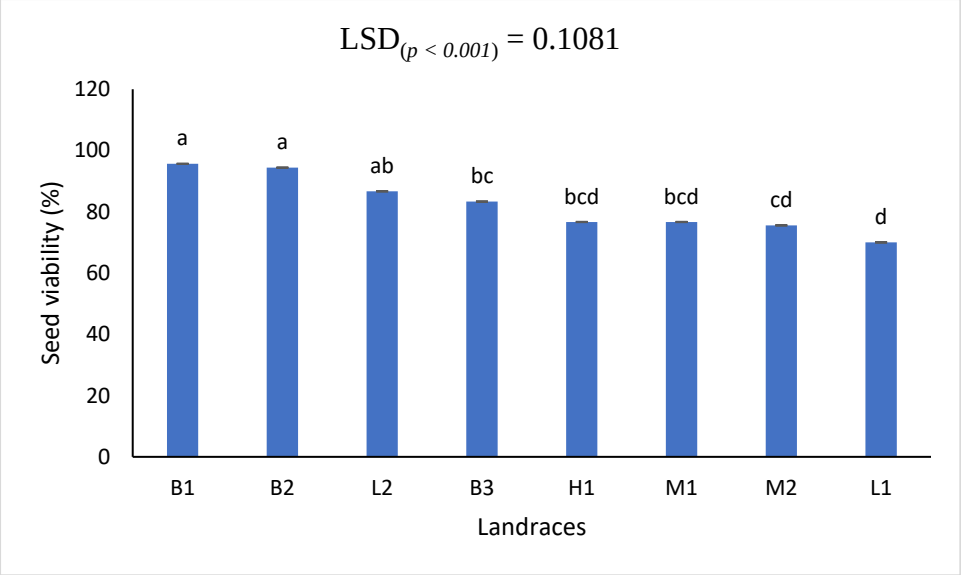



**Figure 7.1:** Seed viability of cannabis landraces before dormancy-breaking treatments ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 7.3.2. Germination and seedling growth under laboratory conditions

The landraces, NaCl treatments, and their interactions showed significant differences ($p$<0.001) in germination percentage, germination percentage recovery, and germination rate index under laboratory conditions (Table 7.2). The landraces and NaCl treatments showed significant differences ($p$<0.001) in germination stress tolerance index, while their interactions showed no significant differences ($p$=0.153).

**Table 7.2:** Analysis of variance (ANOVA) for seed germination percentage (GP), germination percentage recovery (GPR), germination rate index (GRI), and germination stress tolerance index (GSI) of cannabis landraces subjected to NaCl-induced salt stress under laboratory conditions (F values; number of replicates = 3)

| Source of variation | df | GP | GPR | GRI | df | GSI |
|---|---|---|---|---|---|---|
| Landrace | 7 | 81.75** | 72.71** | 63.63** | 7 | 12.66** |
| Treatment | 5 | 195.14** | 115.86** | 158.77** | 4 | 52.19** |
| Landrace*treatment | 35 | 3.62** | 3.78** | 3.46** | 28 | 1.35ns |

ns - not significant ($p$>0.05); **highly significant ($p$<0.001**)**

### 7.3.2.1. Germination

Generally, the results revealed a trend of decreasing germination percentage with increasing NaCl concentration (Figure 7.2). However, landrace 'H1' under NaCl 100 mM exhibited germination of 6.67%, which was lower compared to the 9.33% recorded under NaCl 150 mM. Similarly, 'M2' showed germination of 48.00% under NaCl 100mM, which was also lower compared to 56.00% recorded under NaCl 150mM. Additionally, both 'B3' and 'L1' exhibited the lowest germination of 4.00% under 150 mM, respectively. Furthermore, the germination of 'M1' was inhibited under NaCl 200 mM. NaCl 250 mM also inhibited the germination of 'L1', 'B3' and 'M1'. Notably, 'L2' exhibited the highest germination percentage amongst the landraces under NaCl treatments, while 'H1' showed the lowest.

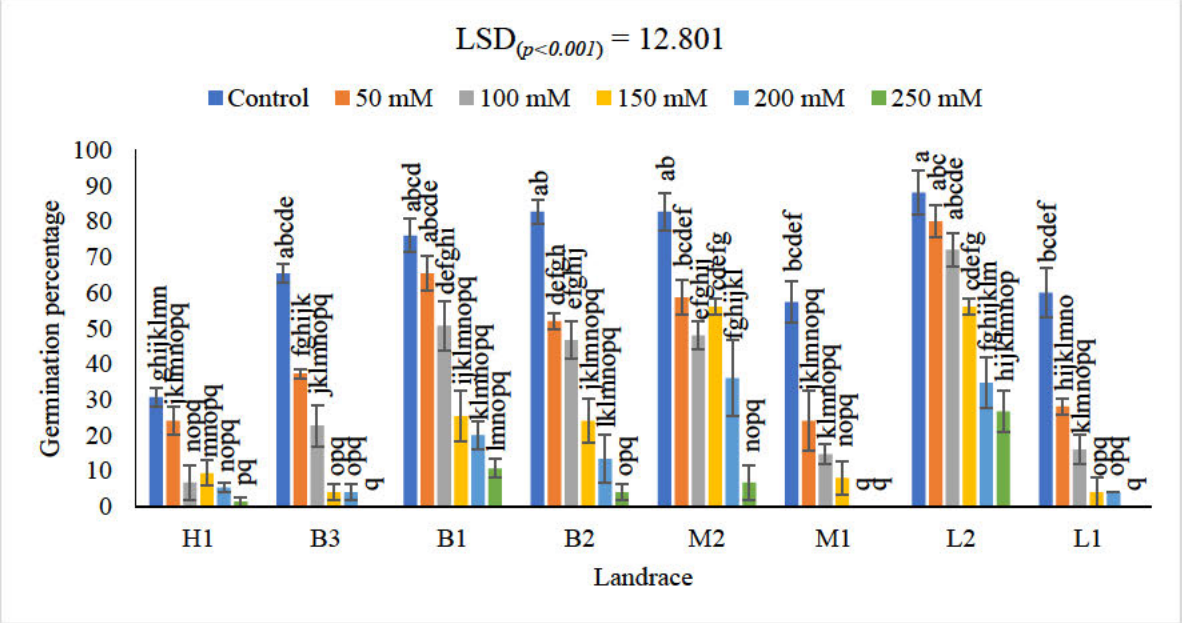



**Figure 7.2**: Germination percentage of various cannabis landraces subjected to different concentrations of NaCl under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 7.3.2.2. Germination recovery

The results revealed that the germination after recovery decreased with the increasing NaCl concentration (Figure 7.3). Generally, 'L2' showed the highest germination amongst the landraces under all the treatment groups. While 'M1' exhibited the lowest germination after recovery compared to the other landraces under NaCl treatments. The results also showed that the germination of 'M1' was permanently inhibited under NaCl 200 and 250mM as no germination recovery was observed. The germination of 'L1' was also permanently inhibited under NaCl 250mM. Furthermore, no germination recovery was observed for 'M1' under NaCl 50 and 150mM, 'L1' under NaCl 150mM, and 'B3' under NaCl 100mM.

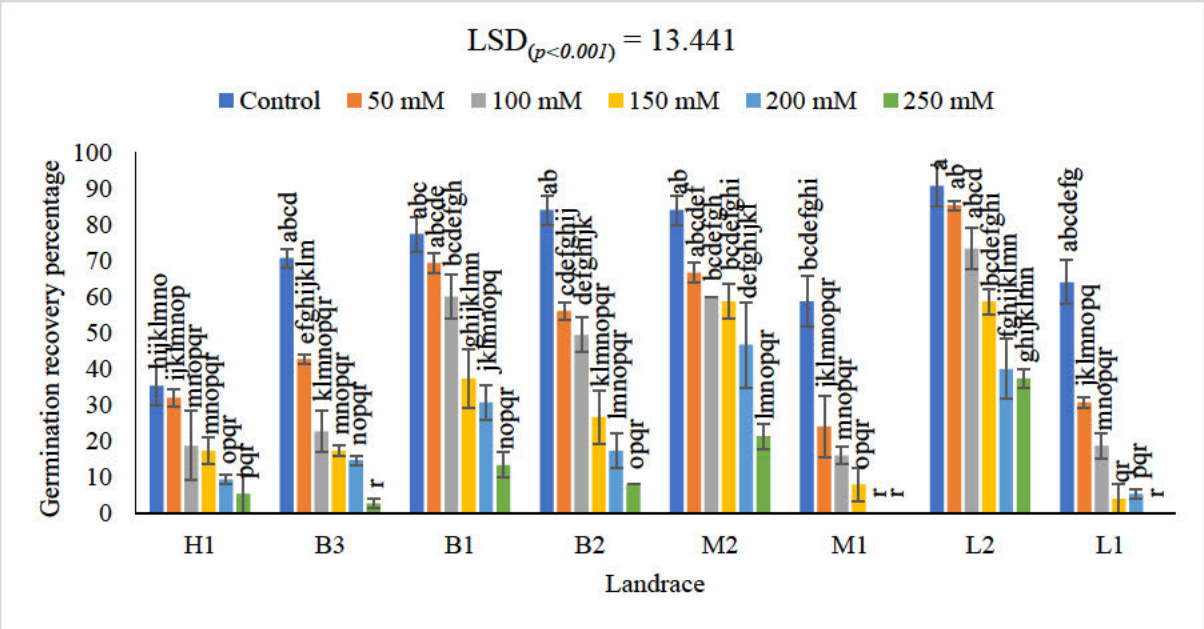



**Figure 7.3**: Germination recovery percentage of various cannabis landraces subjected to different concentrations of NaCl under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 7.3.2.3. Germination rate index

The results revealed that the germination rate index decreased with the increasing NaCl concentration under laboratory conditions (Figure 7.4). Notably, 'L2' exhibited the highest

germination rate index among the landraces under all the treatment groups, whereas 'M1' showed the lowest germination rate index.

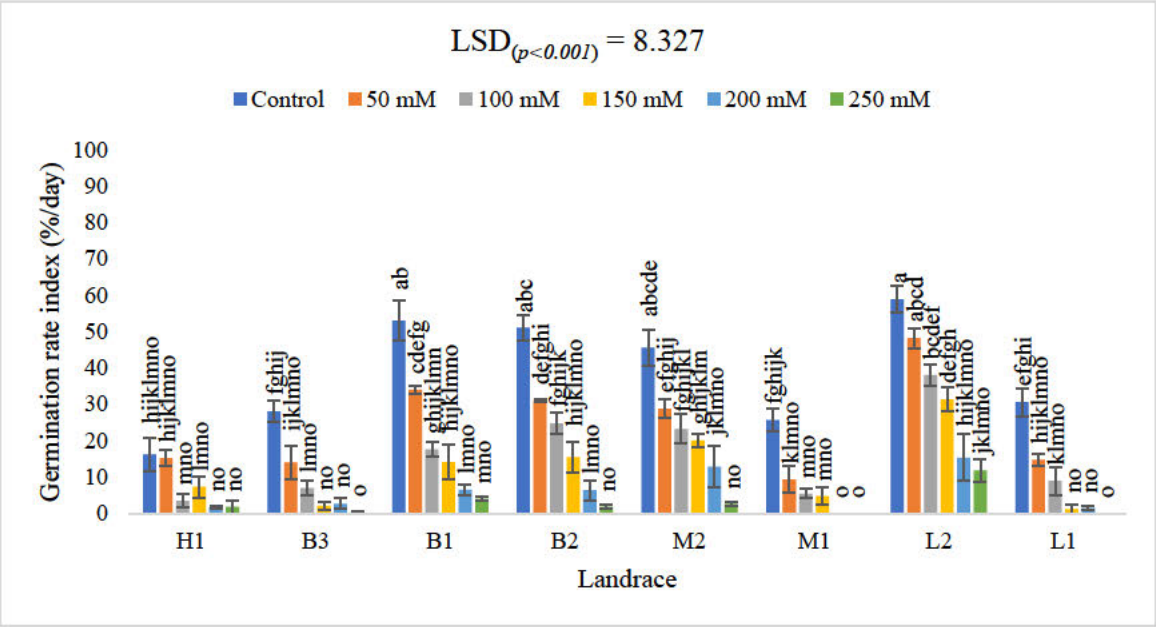



**Figure 7.4**: Germination rate index of various cannabis landraces subjected to different concentrations of NaCl under laboratory conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 7.3.2.4. Germination stress tolerance index

The results revealed a decrease in germination stress tolerance index with the increasing NaCl concentration (Figure 7.5). However, 'H1' exhibited a higher germination stress tolerance index of 44.23% under NaCl 150 mM compared to 26.06% under NaCl 100 mM. 'M2' also showed a higher germination stress tolerance index of 58.56% under NaCl 150 mM compared to 54.81% under NaCl 100 mM. Furthermore, 'L1' demonstrated a higher germination stress tolerance index of 5.79% under NaCl 200 mM compared to 5.43% under NaCl 150 mM. 'H1' had the highest germination stress tolerance index of 97.47% amongst the landraces under NaCl 50mM. The results also demonstrated that 'L2' generally had the highest germination stress tolerance index amongst the landraces under NaCl treatments, while 'M1' generally exhibited the lowest germination stress tolerance index.

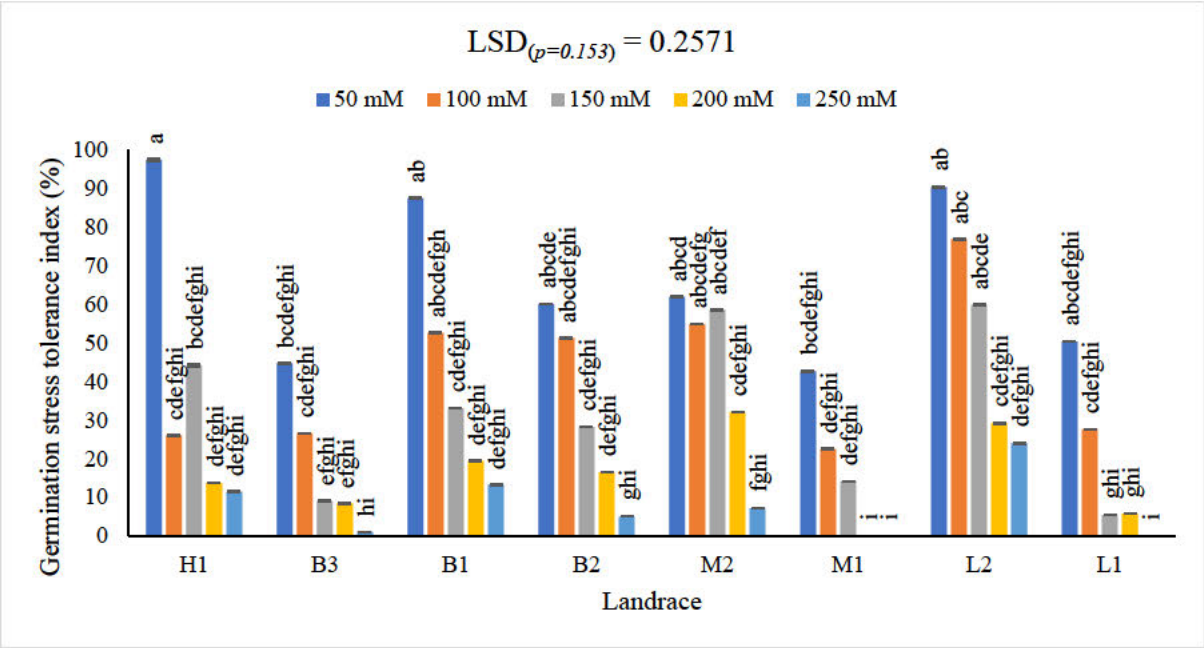



**Figure 7.5**: Germination stress index of various cannabis landraces subjected to different concentrations of NaCl under laboratory conditions ($p$=0.153; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 7.3.3. Germination and seedling growth under greenhouse conditions

The landraces, NaCl treatments, and their interactions showed significant differences ($p$<0.001) in germination rate index, seedling length, and seed vigour index under the greenhouse conditions (Table 7.3). The landraces and NaCl treatments showed significant differences ($p$<0.001) in germination percentage, while their interactions showed no significant differences ($p$=0.346). The landraces showed significant differences ($p$<0.05) in germination stress tolerance index, while NaCl treatments showed no significant differences ($p$=0.111). Landraces and NaCl treatment interactions also showed no significant differences ($p$=0.843) in the germination stress tolerance index.

**Table 7.3**: Analysis of variance (ANOVA) for seed germination percentage (GP), germination rate index (GRI), seedling length (SL), seed vigour index (SVI), and germination stress tolerance index (GSI) of cannabis landraces subjected to NaCl-induced salt stress under greenhouse conditions (F values; number of replicates = 3).

| Source of variation | df | GP | GRI | SL | SVI | df | GSI |
|---|---|---|---|---|---|---|---|
| Landrace | 7 | 4.08** | 11.68** | 21.19** | 17.62** | 7 | 3.37* |
| Treatment | 5 | 25.17** | 79.12** | 95.84** | 117.79** | 4 | 1.94ns |
| Landrace*Treatment | 35 | 1.10ns | 4.21** | 3.92** | 3.64** | 28 | 0.71ns |

ns - not significant ($p$>0.05); *significant ($p$<0.05); **highly significant ($p$<0.001**)**

#### 7.3.3.1. Germination

Generally, the germination percentage decreased with the increasing NaCl concentration (Figure 7.6). Notably, 'H1' followed by 'M2' exhibited the highest germination percentage amongst the landraces. On the other hand, 'L1' followed by 'L2' generally exhibited the lowest germination percentage amongst the landraces. 'L2' exhibited the lowest germination percentage of 81.33, 76.00, and 73.33% under control and NaCl 50 and 100 mM, respectively. Whereas 'L1' showed the lowest germination percentage of 69.33 and 44.00% under NaCl 150 and 250 mM, respectively. Furthermore, 'B3' exhibited the lowest germination percentage of 46.67% under NaCl 200 mM.

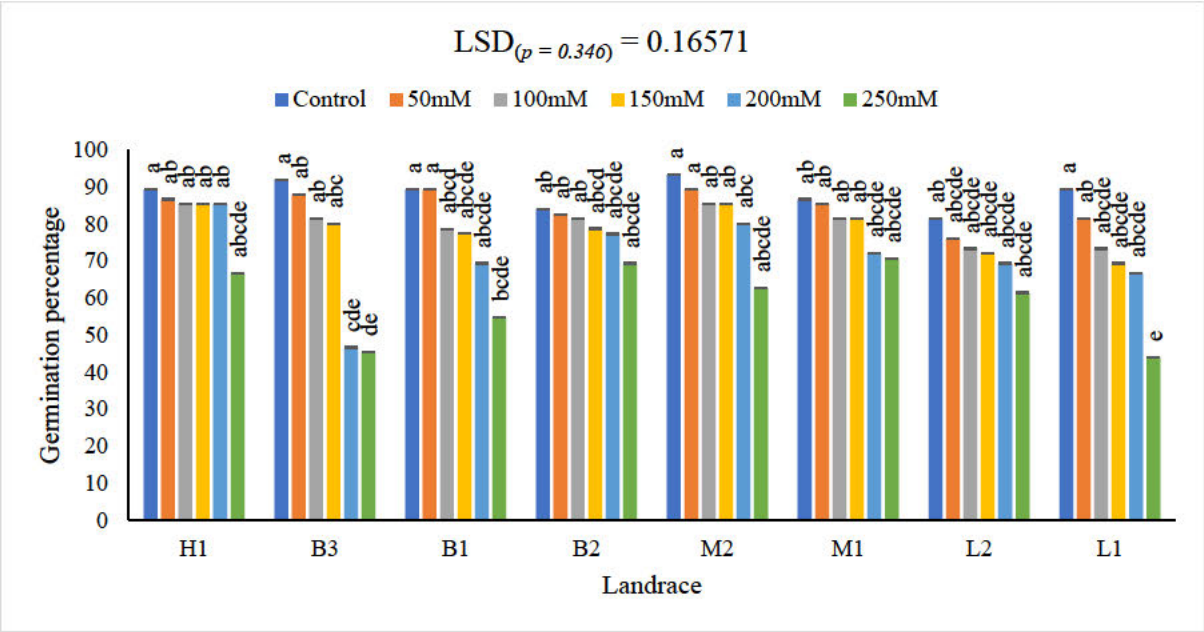



**Figure 7.6**: Germination percentage of various cannabis landraces subjected to different concentrations of NaCl under greenhouse conditions ($p$=0.346). LSD 5% = least significance difference at 5 percent.

#### 7.3.3.2. Germination rate index

The results revealed that the germination rate decreased with the increasing NaCl concentration (Figure 7.7). However, 'M1' exhibited a higher germination rate of 5.4 seeds/day under NaCl 50 mM compared to 4.3 seeds/day recorded under control. 'B1' also exhibited a lower germination rate of 3.2 seeds/day under NaCl 150 mM compared to 3.5 seeds/day under NaCl 200 mM. Additionally, 'M2' showed a lower germination rate of 4.0 seeds/day under NaCl 100 mM compared to 5.3 seeds/day under NaCl 150 mM. Furthermore, 'L2' exhibited a lower germination rate of 3.5 seeds/day under NaCl 50 mM, followed by 3.7 seeds/day, compared to 4.4 and 4.1 seeds/day recorded under NaCl 100 and 200 mM, respectively. Generally, 'B2' demonstrated the highest germination rate amongst the landraces under NaCl treatment, while 'L1' exhibited the lowest.

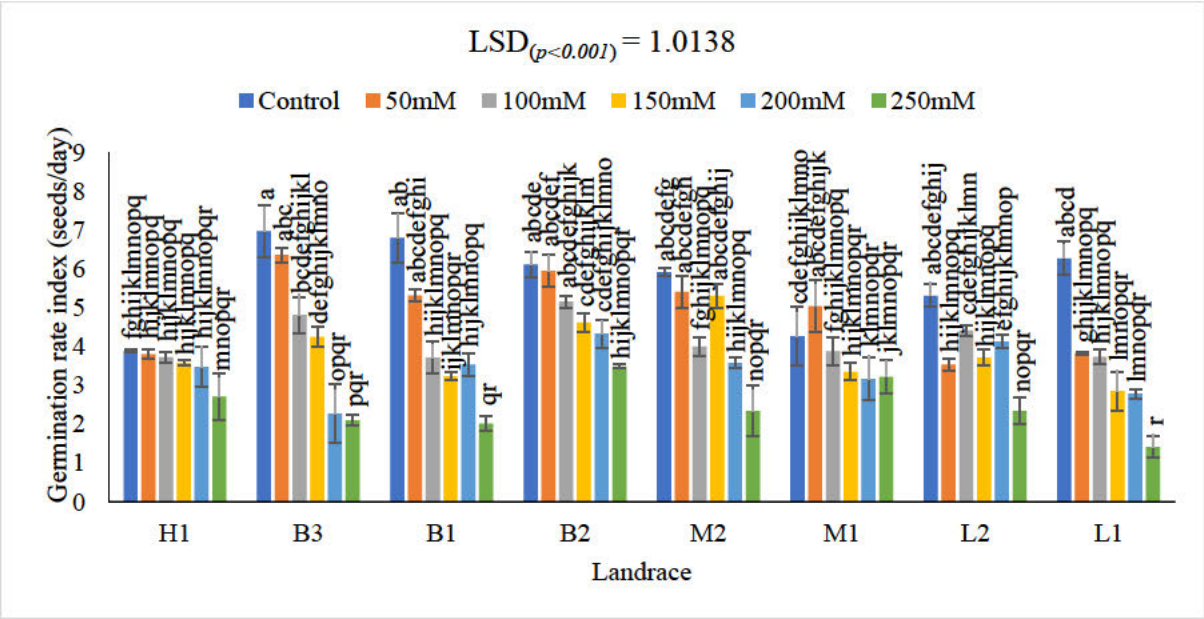



**Figure 7.7:** Germination rate index of various cannabis landraces subjected to different concentrations of NaCl under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 7.3.3.3. Germination stress tolerance index

The results revealed that the germination stress tolerance index varied with landrace and NaCl but generally decreased with increasing NaCl concentration (Figure 7.8). The variations were notable in 'H1', which exhibited a higher germination stress tolerance index of 200.00% under NaCl 200 mM compared to 100% under NaCl 100 mM. Similarly, 'B1' exhibited a higher germination stress tolerance index of 24.08% under NaCl 200 mM compared to 19.85 and 6.49% under NaCl 100 and 150 mM, respectively. 'M2' also exhibited a higher germination stress tolerance index of 91.36% under NaCl 150 mM compared to 25.48% under NaCl 100 mM. Additionally, 'M1', 'L2', and 'L1' exhibited a higher germination stress tolerance index of 385.93, 70.60 and 28.68% under NaCl 100 mM compared to 285.50, 30.01 and 22.35% under NaCl 50 mM, respectively. 'L2' also exhibited a higher germination stress tolerance index of 59.47% under NaCl 200 mM compared to 30.01 and 32.60% under NaCl 50 and 150

mM, respectively. Furthermore, 'M1' exhibited a higher germination stress tolerance index of 51.95% under NaCl 250 mM compared to 44.81 and 25.97% under NaCl 150 and 200 mM, respectively. Notably, 'H1' generally demonstrated the highest germination stress tolerance index amongst the landraces, while 'L1' exhibited the lowest.

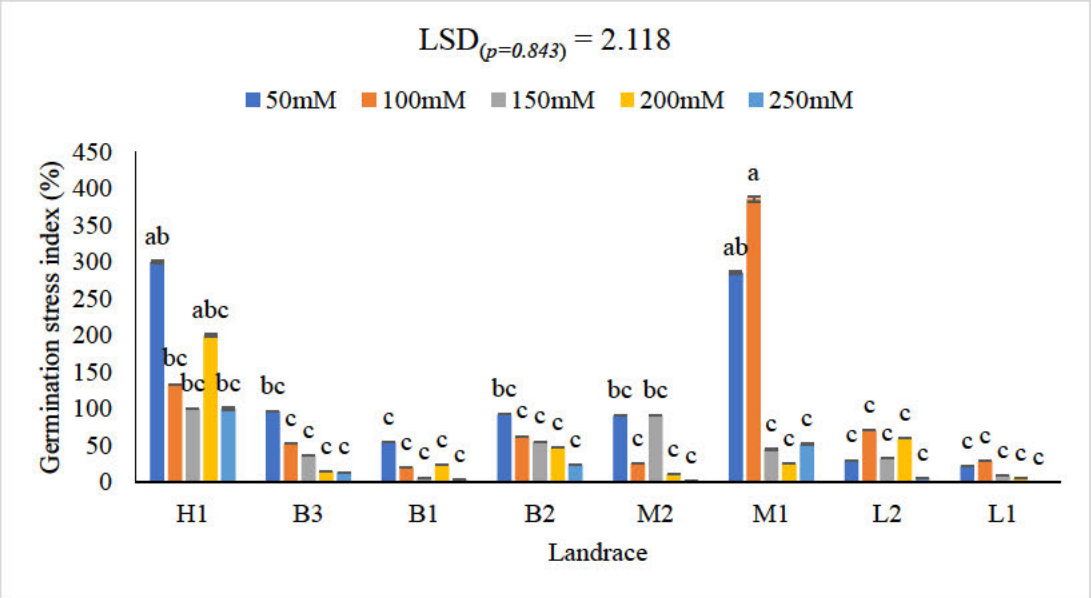



**Figure 7.8**: Germination stress tolerance index of various cannabis landraces subjected to different concentrations of NaCl under greenhouse conditions ($p$=0.843; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 7.3.3.4. Seedling growth

##### 7.3.3.4.1. Seedling length

The results revealed that the seedling length of cannabis genotypes generally decreased with the increasing NaCl concentration (Figure 7.9). However, 'M2' and 'M1' exhibited a higher seedling length of 167.1 and 153.6 mm under NaCl 50 mM, respectively, compared to the corresponding 155.3 and 131.0 mm under control. Additionally, 'M2' and 'L2' exhibited a higher seedling length of 129.6 and 120.7 mm under NaCl 150 mM, respectively, compared to the corresponding 120.6 and 111.6 mm under NaCl 100 mM. Notably, 'B2' generally

demonstrated the highest seedling length amongst the landraces under NaCl treatments, while 'L1' showed the lowest.

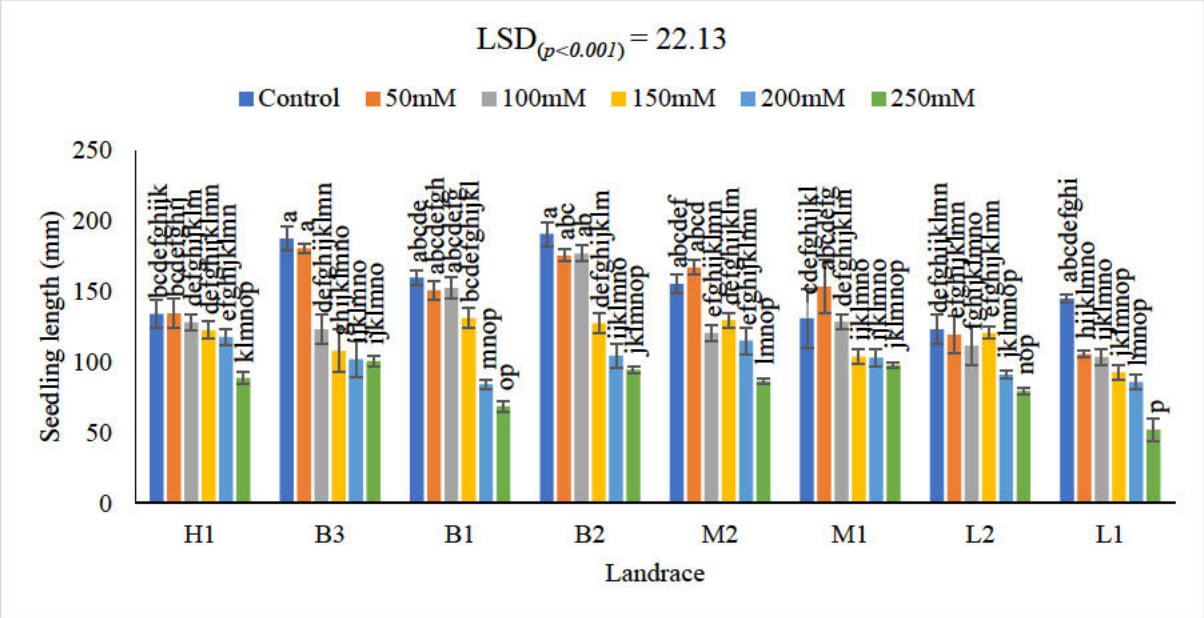



**Figure 7.9:** Seedling length of various cannabis landraces subjected to different concentrations of NaCl under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 7.3.3.4.2. Seed vigour index

The results revealed that the seed vigour index of the cannabis genotypes decreased with the increasing NaCl concentration (Figure 7.10). However, 'M2' and 'M1' exhibited a higher seed vigour index of 1.5 and 1.3 under NaCl 50 mM, respectively, compared to the corresponding 1.4 and 1.1 under control. Additionally, 'M2' and 'L2' exhibited a higher seed vigour index of 1.1 and 0.9 under NaCl 150 mM, respectively, compared to the corresponding 1.0 and 0.8 under NaCl 100 mM. Notably, 'B2' generally exhibited the highest seed vigour index amongst the landraces under NaCl treatments, while 'L1' showed the lowest.

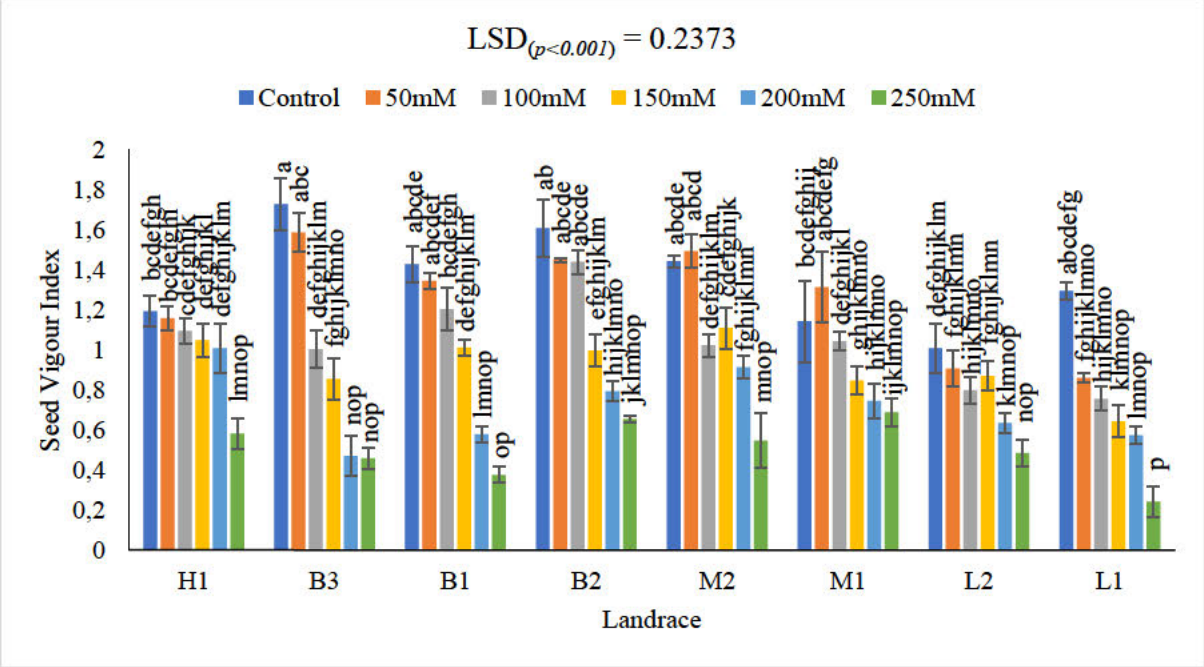



**Figure 7.10:** Seed vigour index of various cannabis landraces subjected to different concentrations of NaCl under greenhouse conditions ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

## 7.4. Discussion

### 7.4.1. Germination

The results highlighted a consistent trend of decreasing germination percentage with the increase in NaCl concentration observed in both laboratory and greenhouse conditions. Notably, there was a complete germination inhibition in 'M1', 'L1', and 'B3' under higher NaCl concentrations of NaCl 200 and 250 mM. The findings of the current study were associated with osmotic stress inhibiting water uptake essential for seed imbibition and the accumulation of $Na^+$ and $Cl^-$ in the embryo, which affects the breakdown and translocation of seed reserves, structural organization of protein and radicle protrusion, thereby inhibiting seed germination (Voigt *et al*., 2009; da Silva *et al*., 2019; Saberali and Moradi, 2019; Desheva *et al*., 2020; Podder *et al*., 2020). These findings corroborate those of Saberali and Moradi (2019), who reported that high salinity reduced the germination of the seeds of *Trigonella foenum-*

*graecum* L., *Dracocephalum moldavica* L., *Satureja hortensis* L., and *Anethum graveolens* L. Furthermore, NaCl might have affected germination through ionic stress, inhibiting critical metabolic steps in dividing and expanding cells, and became lethal at high concentrations.

The results further highlighted that a certain level of variability in the response of landraces to NaCl concentrations existed. These variations were attributed to the differences in environmental conditions, with controlled laboratory conditions and field-simulating greenhouse conditions. This follows that 'L2' consistently demonstrated the highest germination percentage amongst the landraces subjected to NaCl treatments under laboratory conditions, demonstrating its high saline-stress tolerance. Conversely, 'H1' consistently exhibited the lowest germination percentage under laboratory. 'H1' also consistently exhibited the highest germination percentage amongst the landraces when subjected to NaCl treatments under greenhouse conditions, followed by 'M2'. On the other hand, 'L1', followed by 'L2', consistently exhibited the lowest germination percentages amongst the landraces. Reduced germination in 'L1' was also associated with the viability of the planted seeds as it had the lowest amongst the landraces.

Besides the environmental factors and seed viability, the effect of salt stress and plant responses depends on many parameters, including the concentration and time of exposure to salt, and plant genotypes play a key role in the effect of salt stress and plant responses. Thus, the variability in landraces response to NaCl-induced salt stress was also associated with the variations in salt tolerance amongst the landraces. The results of the current study agree with the findings of Hu *et al*. (2019), who reported variation in saline-alkaline tolerance in different industrial hemp genotypes of fiber and seed type. Typically, tolerant genotypes develop cellular defense strategies to mitigate the adverse effects of salt stress, including stress adaptation effectors that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport, and long-distance response co-ordination (Tlahig *et al*., 2021).

Furthermore, enzymatic (catalase, glutathione reductase, and several peroxidases) and non-enzymatic (ascorbate, carotenoids, flavonoids, and other phenolics) antioxidant systems are also amongst defense strategies in tolerant genotypes (Tlahig *et al*., 2021). Cao *et al*. (2021) reported a significant increase in glutamate with saline-alkaline stress in industrial hemp which was associated with response and adaptation to stress. Glutamate is a novel signalling molecule that plays a role in the response and adaptation of plants to various abiotic stressors, including salinity, by mediating calcium ($Ca^{2+}$) signaling through glutamate receptor-like proteins (GLRs) (Qui *et al*., 2020; Cao *et al*., 2021). This signaling activates the salt overly sensitive (SOS) pathway, enhancing salt tolerance in plants (Qui *et al*., 2020). Therefore, 'L2' and 'H1' having had the highest germination under different conditions might suggest that the environmental conditions also influenced the activation of cellular defense mechanisms.

### 7.4.2. Germination recovery

The results also highlighted improved germination after the NaCl-induced salt stress was alleviated, except in 'M1' and 'L1', where its effects were lethal, indicating a lack of adaptation capacity under salt stress conditions. However, the seeds of 'M1' and 'L1' had already shown reduced viability even before planting. Seeds resuming germination once the salt stress was alleviated suggested that the inhibition of germination was temporary, and the seeds possessed the capability to maintain viability under NaCl treatments (Nisar *et al*., 2019). The temporary germination inhibition in the other landraces under saline conditions was associated with an 'enforced dormancy', which was linked to osmotic constraint rather than ionic toxicity of NaCl-induced salt stress. Bhatt and Santo (2016) also suggested that salt stress can alter the balance of germination regulators, leading to the onset of secondary physiological dormancy.

Notably, the germination recovery tended to increase with the increasing NaCl concentration. However, there was still a consistent trend of decreasing germination percentage with the

increasing NaCl concentration. The results of the current study corroborate with the findings of Hu *et al*. (2018), who reported that the percentage germination recovery in hemp cultivars increased with the increasing salt concentration. The results of the current study also corroborate with the findings of Duan *et al*. (2007), who reported that the inhibition of *Suaeda salsa* by salt stress was temporary, and the germination recovery was better in seeds with a stronger germination inhibition.

### 7.4.3. Germination rate index

The findings from the study indicated a consistent decrease in the germination rate index as the NaCl concentration increased in both laboratory and greenhouse conditions. This was associated with the increasing concentration of NaCl reducing the water potential in the growth media due to an imbalance of anion and cation, which creates ion toxicity and, consequently, limits water absorption in seeds (Akram *et al.*, 2020). Resulting in delayed germination and slowed down germination speed of cannabis landraces under saline conditions compared to normal conditions (Thiam *et al*., 2013; Akram *et al.*, 2020). The results of the current study agree with the findings of Hu *et al*. (2018), who reported that the germination rate of 'Yunma' and 'Bamahuoma' hemp cultivars was reduced with the increasing salt concentration. As salinity results in limited water absorption due to high osmotic potential, seeds require time to implement mechanisms for adjusting their internal osmotic pressure; hence, the reduced germination rate under NaCl treatments (Thiam *et al*., 2013).

The results further revealed variations in the response of different landraces to salt stress in different environmental conditions, with controlled laboratory conditions and more realistic greenhouse environments. For instance, 'L2' consistently exhibited the highest germination rate index amongst the landraces under laboratory conditions, indicating a notable resilience to the effects of NaCl-induced salt stress. In contrast, 'M1' consistently exhibited the lowest

germination rate index among the landraces under NaCl treatments in the same setting. Additionally, ‘B2’ consistently exhibited the highest germination rate amongst the landraces under NaCl treatment, while ‘L1’ consistently demonstrated the lowest germination rate. The lowest germination rate index in ‘M1’ and ‘L1’ was associated with reduced seed viability before planting. The variations in the response of different landraces to salt stress indicated differences in NaCl-induced salt tolerance and adaptability under various environmental conditions.

### 7.4.4. Germination stress tolerance index

There was a significant decrease in the germination stress tolerance index with the increasing NaCl concentrations in laboratory conditions. In greenhouse conditions, the germination stress tolerance index also generally showed a decline with increasing NaCl concentrations; however, the responses varied amongst the landraces. The results of the current study corroborate with the findings by Tanveer *et al*. (2020), who reported that the NaCl-induced salt stress significantly decreased the germination stress index in tomato plants.

Furthermore, there were variations in the response of different landraces to NaCl-induced salt stress in different environmental conditions. In laboratory conditions, ‘L2’ consistently exhibited the highest germination stress tolerance index, indicating greater adaptability to NaCl-induced salt stress. On the other hand, ‘M1’ consistently showed the lowest germination stress tolerance index, indicating a relatively lower tolerance to such stress. In greenhouse conditions, ‘H1’ showed the highest germination stress tolerance index amongst the landraces subjected to NaCl-induced salt stress, while ‘L1’ exhibited the lowest. These findings were associated with ‘L2’ and ‘H1’ adjusting their osmotic potential to counter soil water potential, control intracellular ROS levels, and avoid ion toxicity to be saline tolerant Jacob *et al*. (2020). The findings were also associated with these landraces activating various physiological and

biochemical responses to salt stress, including ion compartmentalization, protein and metabolite biosynthesis, accumulation of organic solutes and antioxidants, and heightened activity of antioxidant enzymes (Jacob *et al*., 2020). The germination stress tolerance response in 'M1' and 'L1' was associated with reduced seed viability.

### 7.4.5. Seedling growth

#### 7.4.5.1. Seedling length

The findings of the study indicated a consistent trend of decreasing seedling length in cannabis genotypes with increasing NaCl concentrations. These findings were associated with NaCl limiting water uptake and signaled the adverse impact of excessive salts on photosynthetic rate (Kamran *et al*., 2019; Desheva *et al*., 2020). NaCl treatments were also associated with a decrease in the level of carbohydrates and growth hormones (resulting in growth inhibition) and reduced protein synthesis by altering antioxidant enzyme activities, thereby limiting the supply of metabolites to young growing tissues (Kamran *et al*., 2019; Desheva *et al*., 2020; Shiade and Boelt, 2020; Navyashree *et al*., 2023). Additionally, the reduced seedling length was associated with a higher accumulation of salts in the cell walls, resulting in a more rigid cell wall (Shiade and Boelt, 2020). A rigid cell wall is linked with diminishing turgor pressure efficiency, resulting in slower and shorter cell enlargement and shoot elongation (Shiade and Boelt, 2020). Furthermore, excess accumulation of $Na^+$ induces efflux of cytosolic $K^+$ and $Ca^{2+}$, resulting in an imbalance in plants intracellular potassium homeostasis, nutrient deficiency, oxidative stress, retarded growth, and cell death (Singh *et al*., 2017; Kamran *et al*., 2019).

Notably, 'B2' consistently exhibited the longest seedling length amongst the landraces under NaCl treatments, suggesting a relatively greater tolerance to NaCl-induced salt stress. On the other hand, 'L1' consistently showed the shortest seedling length, indicating a comparatively lower ability to withstand the adverse effects of NaCl-induced salt stress, which was also

attributed to reduced seed viability. These findings demonstrated the differences in salt stress tolerance amongst the landraces. The tolerance of cannabis landraces to NaCl-induced stress was associated with metabolic adjustments, altering various enzymes (Nasri *et al*., 2015). For instance, phosphatases, crucial for plant adaptation to salinity, regulate physiological functions, including soluble phosphorus levels (Pi) (Nasri *et al*., 2015; Banerjee and Roychoudhury, 2020). Acid phosphatases are vital in providing inorganic phosphate during germination from seeds and seedlings (Nasri *et al*., 2015). Hydrolysis of stored phosphate esters in seeds aids germination and growth, enhancing plant tolerance to salt stress (Nasri *et al*., 2015).

#### 7.4.5.2. Seed vigour index

There was a consistent decrease in the seed vigour index of cannabis genotypes as the NaCl concentration increased, suggesting that the ability to germinate and establish healthy seedlings in cannabis landraces was reduced under saline conditions. This was attributed to a decrease in water absorption and an increase in ion uptake, inhibiting imbibition, and seedling establishment under saline conditions (Khan *et al*., 2022). The absorption of vital nutrients like P and K also gets inhibited, limiting seedling growth and vigor index (Khan *et al*., 2022). Notably, ‘B2’ consistently demonstrated the highest seed vigour index amongst the landraces under NaCl treatments, suggesting a greater capacity for maintaining a better seed performance under saline conditions. This better performance was also attributed to increased seed viability. Conversely, ‘L1’ consistently displayed the lowest seed vigour index, indicating a reduced ability to preserve seed vitality under NaCl-induced salt stress. Nevertheless, ‘L1’ had the lowest seed viability, and thus, the results on the seed vigour index were expected. The overall findings of the current study suggested that the germination and seedling growth responses were significantly dependent on seed viability, landrace, NaCl concentration, and environmental conditions. However, the germination and seedling growth characteristics were inhibited by the NaCl treatments. These findings corroborate the findings by Hu *et al*. (2018),

Hu *et al*. (2019), Dixit (2022), Varga *et al*. (2022), Rabbani *et al*. (2023), and Zhang *et al*. (2023), who reported that salt stress inhibited the germination and plant growth of different fiber and seed-type hemp genotypes. The findings of the current study further indicated that 'L2' had better tolerance to NaCl-induced salt stress among the landraces under the laboratory conditions, attributed to it having demonstrated the highest germination percentage and rate and germination stress tolerance. 'H1' and 'B2' had a better tolerance to salt stress among the landraces under the greenhouse conditions. These findings were attributed to 'H1' having demonstrated the highest germination percentage and stress tolerance index among the landraces. 'B2' had faster germination, longer seedling length, and the highest seed vigour index. On the other hand, 'M1' and 'L1' were shown to be less tolerant to salt stress, attributed to the poor performance in terms of germination and seedling growth characteristics under laboratory and greenhouse conditions, respectively.

### 7.5. Conclusion

The current study investigated the effect of NaCl-induced salt stress on the germination and seedling growth of different cannabis landraces. It was found that the NaCl treatments reduced the germination and seedling growth characteristics, including germination percentage and rate, germination stress tolerance, seedling length, and seed vigour index of cannabis landraces. There were notable variations in germination and seedling growth responses with landraces, NaCl treatments, and the growing environment, as well as with the viability of the seed before germination. The growing environmental conditions also had a significant impact on the germination and seedling growth responses, with 'L2' indicating tolerance to saline conditions under the laboratory conditions and 'H1' and 'B2' showing resilience to saline conditions under the greenhouse conditions. Thus, 'L2', 'H1', and 'B2' could be a better selection for planting under saline conditions imposed by poor irrigation management. Furthermore, these landraces may be suitable for further study for breeding programs targeted at the development of saline-

resistant genotypes. Based on the current findings, it is recommended that future research investigate the effect of salt stress on drug-type cannabis landraces at various growth stages, considering morphological, biochemical, and genetic traits.

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# 8. CHAPTER 8: TEMPERATURE EFFECTS ON SEED GERMINATION AND SEEDLING BIOCHEMICAL PROFILE OF CANNABIS LANDRACES

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**Abstract**

This current study investigated the effect of temperature on the germination and seedling biochemical profile of cannabis landraces, namely Ladysmith Ugwayi wesiZulu (L1) and Iswazi (L2), Durban Poison (H1), Bergville Ugwayi wesiZulu (B1), Natal (B2) and Iswazi (B3), and Msinga Ugwayi wesiZulu (M1) and Iswazi (M2). The cannabis seeds were subjected to germination tests under different temperature regimes of 20/15 °C, 30/25 °C, and 40/35 °C. The germination characteristics, such as seed viability and germination percentage and rate, were measured. The amino acids, carbohydrates, and fatty acids methyl esters (FAMEs) of the resultant seedlings, were also measured. The results revealed a significant effect ($p$<0.001) of temperature in the germination percentage and rate as well as in carbohydrates, amino acids, and FAMEs of the cannabis landraces. There was a general decreasing trend in germination percentage and rate with increasing temperature; however, this trend varied among genotypes. Specifically, genotypes such as ‘L1’, ‘B1’, ‘H1’, ‘B2’, and ‘M1’ exhibited higher germination at 20/15 °C, while ‘B3’, ‘M2’, and ‘L2’ performed better at 30/25 °C. The germination rate index was generally higher at 30/25 °C. Similarly, the concentration of the carbohydrates, amino acids, and FAMEs varied with temperature and genotypes. Amino acid content

increased with elevated temperature and showed no correlation with the germination indexes ($p \geq 0.05$). Contrarily, the concentration of the carbohydrates and FAMEs decreased with the rising temperature, with FAMEs peaking at 30/25 °C. The FAMEs strongly correlated with germination indexes ($p < 0.05$), indicating a link between lipid composition and seed performance. Amongst the carbohydrates, sorbitol positively correlated with the germination percentage ($p = 0.58$), while glucose and fructose showed an indirect correlation. These findings highlight the importance of considering temperature, genotype, and biochemical composition in selecting and improving cannabis genotypes and determining planting dates.

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**Keywords**: Physiology, amino acids, membrane fluidity, fatty acids, carbohydrates

### 8.1. Introduction

Temperature is one of the most important environmental factors regulating seed germination by influencing imbibition and the mobilization of storage reserves and, ultimately, modifying germination percentage and rate (Hills and Van Staden, 2003; Sikder *et al*., 2009; Kumar *et al*., 2014; Zucareli *et al*., 2015; Laghmouchi *et al*., 2017; Singh *et al*., 2018; Reis *et al*., 2020). During imbibition, the appropriate restructuring of membranes is influenced by temperature by altering their permeability and fluidity characteristics, thereby either facilitating or limiting the leakage of cellular components (Cabrera-Santos *et al*., 2021). On the other hand, temperature influences the supply of substrates necessary for seed reserve mobilization, including water, and consequently affects plant growth and development (Sikder *et al*., 2009; Langa *et al*., 2024a).

Generally, carbohydrates such as sucrose, glucose, and fructose are mobilized during germination from seed storage tissues to organs such as the stem and radicle, where they support growth and maintain the osmotic homeostasis of cells (Rosa *et al*., 2009). These carbohydrates tend to decrease with higher temperatures but accumulate in response to low-

temperature stress (Ribeiro *et al*., 2014; Wani *et al*., 2018). Conversely to carbohydrates, amino acid levels tend to increase with rising temperatures due to their role in stabilizing membranes and regulating cellular osmotic pressure (Ribeiro *et al*., 2014; Aidoo *et al*., 2016; Wei *et al*., 2020). On the other hand, plant lipid composition adjusts to maintain membrane fluidity, making membranes more rigid at low temperatures to enhance seed performance, while higher temperatures increase membrane fluidity (Van Wagenen *et al*., 2012; Belo *et al*., 2014; Dhaliwal and Angeles-Shim, 2022). Thus, the mobilization of seed reserves and the process of seed germination differ under various temperature conditions, making temperature a crucial factor influencing germination (Rosa *et al.*, 2009; Sikder *et al*., 2009; Belo *et al.*, 2014; Langa *et al*., 2024a).

Temperature also plays a significant role in determining the consistency of seed germination and distribution and adaptation of species beyond their area of origin (Laghmouchi *et al*., 2017; Singh *et al*., 2021). The effect of temperature on germination usually varies with species, and genotype (Kumar *et al*., 2014; Kumar *et al*., 2016). However, the optimum germination temperature promotes the highest germination percentage and rate within the shortest period of time (Kumar *et al*., 2014; Kumar *et al*., 2016). On the other hand, temperatures outside the normal limits, such as chilling and high temperatures, may cause secondary dormancy in the seed when conditions are not favourable for seedling establishment, ultimately negatively affecting the germination percentage and germination rate index (Hills and Van Staden, 2003; Singh *et al*., 2018; Malabarba *et al*., 2021).

*Cannabis sativa* L., commonly known as marijuana, dagga, or hemp, is grown worldwide for its oily seeds and long, durable fiber (Decorte *et al*., 2011; Salentijn *et al*., 2015; Chaohua *et al*., 2016). Marijuana or dagga is also grown for its drug effects caused by a compound called (-)-trans-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), which led to this crop being banned for many centuries (Decorte *et al*., 2011; Citti *et al*., 2018). This crop is known to germinate well under

temperatures ranging from 10 to 30 °C (Qin *et al*., 2014; Humphries and Florentine, 2019; Geneve *et al*., 2022; Bajwa *et al*., 2023). However, the optimal germination temperature varies with genotype and geographical location (Byrd, 2019). For instance, 'Georgina', 'Victoria' and Northern European hemp genotypes thrive in temperatures up to 30°C while the germination of 'Bama' fire hemp and Canadian cultivars declined at temperatures above 25°C (Qin *et al*., 2014; Byrd, 2019; Geneve *et al*., 2022).

Although previous studies have emphasized the influence of temperature on physiological aspects during cannabis germination, there is very little information on the effect of alternating temperature regimes on the biochemical processes. (Hills and Van Staden, 2003; Kumar *et al*., 2014; Laghmouchi *et al*., 2017; Singh *et al*., 2018). Furthermore, cannabis seeds are known for being rich in polyunsaturated fatty acids (PUFA), specifically linoleic acid (LA, omega-6) and α-linolenic acid (ALA, omega-3) (Galasso *et al*., 2016; Farinon *et al*., 2020). Therefore, it is critical to evaluate the variations in the composition of these fatty acids and other biochemicals during seed germination in response to different temperature regimes.

Additionally, there are limited studies on the effect of temperature on the germination of South African genotypes due to the previous legal restriction attributed to the intoxicating effects of $\Delta^9$-THC. However, public perception and laws in many countries like the United States, Canada, South Africa, and Germany have recently begun acknowledging cannabis as a plant of both medical and agricultural value (Decorte *et al*., 2011; Sawler *et al*., 2015; Gouws, 2017; Citti *et al*., 2018; Adebisi and Olaoye, 2022). Therefore, information such as optimal germination temperature as well as the physiological and biochemical responses of the cannabis towards the germination temperature is vital for genotype selection and improvement, planting date, and management practices for specific locations and seed lots (Wang *et al*., 2020). Therefore, the current study aimed to investigate the effect of different temperature

regimes on the physiological factors of various landraces during seed germination and the biochemical factors of the seedlings.

## 8.2. Materials and methods

### 8.2.1. Genotype collection

Seeds of eight cannabis genotypes (landraces) were collected from local growers in four regions of KwaZulu-Natal Province in South Africa, namely, Bergville, Ladysmith, Hammersdale, and Msinga. The collected cannabis landraces were assigned pseudonyms to ensure uniform naming of landraces for this experiment, as detailed in Table 8.1.

**Table 8.1:** Geographic location information on the source of landraces and the pseudonyms assigned to them

| Location | Geographic information | | | | | Landrace name | Pseudonym: |
|---|---|---|---|---|---|---|---|
| | Latitude: | Longitude: | Elevation | Average rainfall | Average temperature | | |
| Ladysmith | -28.57533 | 29.85948 | 1009.53 m | 108.7 mm | 23.3 °C | Ugwayi wesiZulu | 'L1' |
| | | | | | | Iswazi | 'L2' |
| Bergville | -28.667679 | 29.034111 | 1137.86 m | 109.4 mm | 23.5 °C | Natal | 'B2' |
| | -28.677544 | 29.125553 | | | | Ugwayi wesiZulu | 'B1' |
| | | | | | | Iswazi | 'B3' |
| Hammersdale | -29.870903 | 30.633519 | 597 m | 110.3 mm | 23.7 °C | Durban Poison | 'H1' |
| Msinga | -28.679887 | 30.274728 | 559 m | | 23.0 | Ugwayi wesiZulu | 'M1' |
| | | | | | | Iswazi | 'M2' |

The collected seed samples were stored for five days at room temperature in the Horticultural Sciences Laboratory of the University of KwaZulu-Natal, Pietermaritzburg in South Africa (latitude: -29.636311 and longitude: 30.409060), for further analysis. Prior to germination studies, the cannabis seeds were sorted based on colour. Dark-coloured seeds were associated with maturity, whereas greenish-coloured seeds were associated with immaturity (Miraji *et al*., 2021). Therefore, dark-coloured seeds were used for the experiment. Seeds showing defects were removed from the seed lot.

### 8.2.2. Experimental design

The experiment was structured using a factorial completely randomized design consisting of 8 genotypes and 3 temperature levels. Seeds were subjected to different alternating temperature levels of 20/15 °C, 30/25 °C, and 40/35 °C.

### 8.2.3. Seed viability test

A seed viability test was conducted before seed germination using 2,3,5-triphenyltetrazolium chloride (TCC) (Sigma Aldrich, Merck KGaA, 64271 Darmstadt, Germany), following the procedures by Mandizvo and Odindo (2019). Seed samples were preconditioned by soaking in distilled water for 18 h in an incubator set at 25 °C, and then excised with a scalpel blade through the embryo. The excised seeds were immersed in a 1% TCC for 12 h at room temperature. The experiment was replicated three times, and each replicate consisted of 25 seeds. Seed viability evaluation was done by placing the seeds in categories as viable and non-viable according to the coloration of the embryonic axis. Viable seeds were characterized by embryos showing a uniform shiny pink colour, and non-viable seeds were characterized by embryos showing a totally white colour (Paiva *et al*., 2017). Only the percentage of viable seeds was recorded.

### 8.2.4. Germination test

Seeds were soaked in distilled water for 24 h to promote germination. Thereafter, seeds were air dried and placed in 9 cm sterile Petri dishes containing cotton pads soaked with distilled water. Each Petri dish had 25 seeds, and each experiment was replicated three times for the different alternating temperatures of 20/15, 30/25, and 40/35 °C. The seeded Petri dishes were then transferred into a germination chamber (Micro-Clima Arabidopsis Chamber, ECD01E, Snijders, Netherlands) maintained at different alternating temperatures of 20/15, 30/25, and 40/35 °C, and a light cycle of 16/8 h. The germination progress was monitored daily, and the number of germinated seeds was recorded for each replicate for 10 days. A seed was considered to have germinated when the emerging radical elongated to a length of 2 mm or more. The germination percentage (GP) was calculated according to Khalaki *et al*. (2019) using Eq. 1.

$$\mathrm{GP} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100 \quad (1)$$

The germination rate index (GRI) was calculated according to Awasthi *et al*. (2016) using Eq. 2 and was expressed as a percentage per day (%/day).

$$\mathrm{GRI} = \frac{\text{Percentage of germinated seeds}}{\text{Day of first count}} + \cdots + \frac{\text{Percentage of germinated seeds}}{\text{Day of final count}} \quad (2)$$

### 8.2.5. Metabolite profiling

Four genotypes, representing landraces with specific names, were randomly selected for metabolic profiling of seedlings. These included ‘B1’ for ‘Ugwayi wesiZulu’, ‘B2’ for ‘Natal’, ‘H1’ for ‘Durban Poison’, and ‘L2’ for ‘Iswazi’. The seedlings were individually frozen at -70 °C for 24 h, and then they were freeze-dried for four days at -126.5 ºC in a Larry Virtis 255L. The dried samples were ground into a fine powder using mortar and pestle and stored in a chest freezer at -5 ºC until further analysis.

#### 8.2.5.1. Extraction and quantification of carbohydrates

The carbohydrates were extracted and quantified using a method previously described by Zhang *et al*. (2019) with some modifications. A 50 mg freeze-dried and powdered samples were weighed, followed by adding 1 mL of 70% (v/v) methanol (MeOH). The samples were extracted at 60 °C for 3 h. An extract (130 µL) was transferred into a 2 mL polypropylene tube and dried at 35 °C overnight in a speed vac (Model number: 7810010, Labconco, Kansas City, Missouri, USA) at 825 rpm. The dried samples were derivatized by adding 100 µL of methoxyamine at 50 °C for 2 h, followed by the addition of 30 µL of N,O-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and further derivatized at 70 °C for 30 min. The samples were transferred into an insert, positioned in a 2 mL gas chromatograph (GC) vial, and 1 µL was injected into the Gas Chromatography-Mass Spectrometry (GC-MS) (6890N, Agilent technologies network and 5975, Agilent Technologies Inc., Palo Alto, CA) in splitless mode.

Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to Agilent technology inert XL EI/CI Mass Selective Detector (MSD) (5975, Agilent Technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler. The carbohydrates were separated on a non-polar ZB-5MS (30 m, 0.25 mm ID, 0.25 µm film thickness) capillary column. Helium was used as the carrier gas at a 1 mL/min flow rate. The injector temperature was maintained at 250 °C. The oven temperature was programmed at 80 °C for 1 min, ramped up to 300 °C at a rate of 7 °C/min, and then held for 2 min. The detection process was performed in the full scanning mode from 50–550 (m/z). The chromatograms were analyzed by checking the MS database and GC/MS database of NIST 14. lib. The analytical performances were evaluated by calculating the relative standard deviation (RSD) of peak area and count of metabolites. The final results were expressed in milligrams per gram sample dry weight.

#### 8.2.5.2. Extraction and quantification of amino acids

The amino acids were extracted and quantified using a method previously described by Stenerson (2011) and Pérez-Palacios *et al*. (2015), with some modifications. A 4 mL sample of 6 M hydrochloric acid (HCl) was added to 100 mg of freeze-dried and powdered samples. The samples were hydrolyzed for 24 h at 110 °C, cooled to room temperature, and diluted at 1:9 with 70% methanol (v/v). 130 µL was transferred into a 2 mL polypropylene tube and dried completely under a gentle stream of nitrogen. The samples were reconstituted and derivatized with 30 µL N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) and 100 µL acetonitrile at 100 °C for 1 h. After cooling at room temperature, the samples were injected into the GC-MS instrument. Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to Agilent technology inert XL EI/CI Mass Selective Detector (MSD) (5975, Agilent Technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler. Separation of amino acids was performed on a ZB-5MS (30 m, 0.25 mm ID, 0.25 µm film thickness) capillary column. Helium was used as the carrier gas at a 1 mL/min flow rate. The injector temperature was maintained at 250 °C. A 1 µL of the sample was injected in splitless mode. The oven temperature was programmed at 100 °C for 5 min and ramped up to 325 °C at a rate of 20 °C/min for 4 min and thereafter held for 15 seconds. The MSD was operated in scan/sim mode, and the source and quad temperatures were maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 250 °C. The mass spectrometer was operated under electron impact mode at an ionization energy of 70 eV, scanning from 40 to 650m/z. Free amino acids were identified both by their retention time and by comparison of their characteristic m/z ions with those published by Pérez-Palacios *et al*. (2015). The quantification was carried out in the selected ion monitoring (SIM) mode. The final results were expressed in milligrams per gram sample dry weight.

#### 8.2.5.3. Extraction and quantification of fatty acid methyl esters (FAMEs)

The extraction and quantification of FAMEs were done using a method previously described by Duminy *et al*. (2023) with some modifications. Briefly, 2 mL of 2:1 chloroform: methanol (v/v) was added to 100 mg of freeze-dried and powdered samples. The samples were vortexed using a Fisherbrand™ ZX3 vortex mixer (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, UK) and sonicated using a Fisherbrand™ Q705 Sonicator with Probe (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, UK) at room temperature for 30 min, after which, the samples were centrifuged at 3000 rpm for 1 min. The bottom layer (chloroform), measuring 200 µL, was then dried completely using a gentle stream of nitrogen. Subsequently, it was reconstituted and vortexed with 170 µL of methyl tert-butyl ether (MTBE) and 30 µL of trimethylsulfonium hydroxide (TMSH). A 1 µL of the derivatized samples was injected in a 5:1 split ratio onto the GC-FID.

Separation was performed on a gas chromatograph (6890N, Agilent Technologies) coupled to a flame ionization detector (FID). The FAMEs were separated on a polar RT-2560 (100 m, 0.25 mm ID, 0.20 µm film thickness) (Restek, USA) capillary column. Hydrogen was used as the carrier gas at a 1.2 mL/min flow rate. The injector temperature was maintained at 240 °C. Exactly 1 µL of the sample was injected in a 5:1 split ratio. The oven temperature was programmed as follows: 100 °C for 4 min, ramped to 240 °C at a rate of 3 °C/min for 10 min. Compounds were identified by comparing retention times and mass fragmentation patterns to standards and the NIST library. Peaks were matched with known FAME standards. Quantitation of fatty acids used response factors from TG standards in mixtures, with TG-9:0 as the internal standard (ISTD). The final results were expressed in milligrams per gram sample dry weight.

### 8.2.6. Data analyses

The data was analyzed using the analysis of variance (ANOVA) on GenStat®, 20.1 Edition (VSN International, Hamel Hampstead, UK, 2020) at the 5% significance level. The means of the variables were separated using Tukey's test. Additionally, the statistical data analysis for multivariate analysis, such as correlation and Principal Component Analysis (PCA), was conducted using Statistical Computing Environment, R software, version 4.4.0 using the RStudio platform (Posit Software, PBC, 250 Northern Ave Suite 420 Boston, MA 02210). The *corrplot* package was used for correlation, whereas the FactoMineR package (version 2.11) was used for PCA.

## 8.3. Results

### 8.3.1. Seed viability

There were no significant differences between landraces in relation to seed viability ($p$=0.06). For instance, 'B1', 'B2', 'L2', and 'M1' were significantly different from 'M2'. Amongst the landraces, 'B1' and 'M2' had the highest and lowest seed viability at 82.00 and 97.67%, respectively (Figure 8.1).

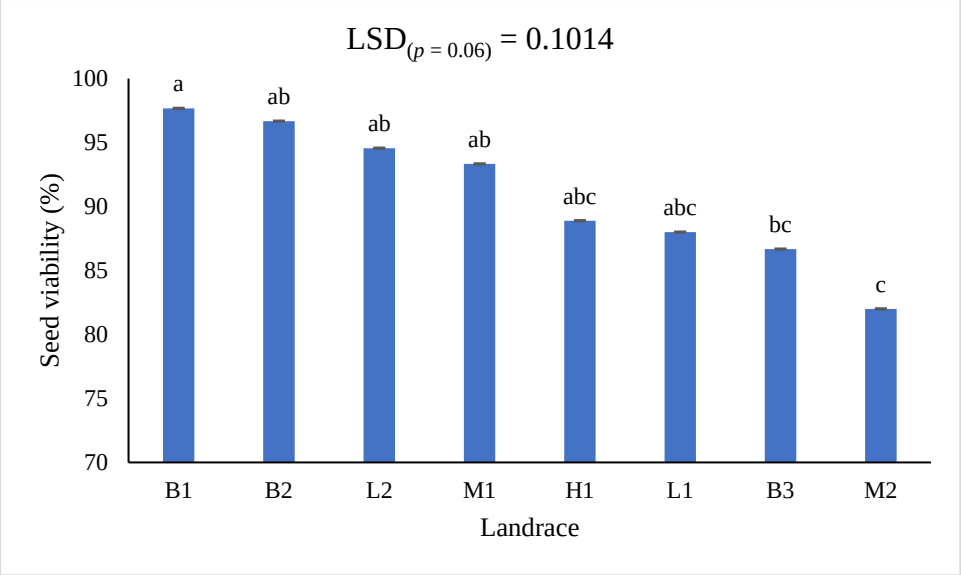



**Figure 8.1:** Seed viability of eight cannabis landraces before they were subjected to different temperature regimes ($p$=0.06; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 8.3.2. Germination

#### 8.3.2.1. Germination percentage

The landraces, temperature regimes, and their interactions showed significant differences ($p$<0.001) in germination percentage. The results revealed that ‘B3’, ‘L2’, and ‘M2’ had the highest germination percentages of 82.78, 89.44, and 77.78% under 30/25 °C (Figure 8.2). Under 20/15 °C, the genotypes ‘B2’, ‘B1’, ‘M1’, and ‘L1’, respectively, exhibited the germination percentage of 93.93, 86.67, 80.56, and 60.00%. ‘H1’ had the highest germination of 62.78% under 20/15 and 40/35 °C. Notably, the germination percentages for ‘B2’ decreased with increasing temperature.

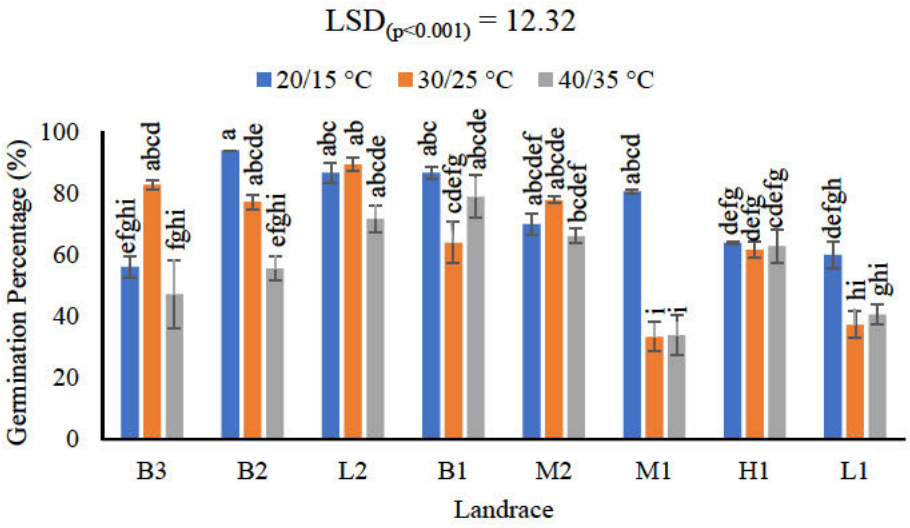



**Figure 8.2:** The germination percentage for the interaction effect between three different temperature levels and eight cannabis landraces ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

#### 8.3.2.2. Germination rate index

The landraces, temperature levels, and their interactions showed significant differences ($p$<0.001) in germination rate index. The germination rate index decreased with increasing temperature in landrace 'M1', as it had the lowest of 20.98%/day under 20/15 °C compared to other temperature regimes (Figure 8.3). Under 30/25 °C, 'B2', 'H1', 'M2', 'B3', 'L1', and 'L2' had the highest germination rate indexes of 44.70, 34.48, 41.05, 32.92, 14.77, and 57.12%/day, respectively, compared to other temperature regimes. Landraces 'B2' and 'L2' had the lowest germination rate indexes under 40/35 °C at 27.43 and 28.08%/day, respectively. 'B3', 'H1', 'M2', and 'L1' had the lowest germination rate indexes under 20/15 °C at 12.91, 24.36, 22.04, and 12.25%/day, respectively. On the other hand, the germination rate index for landrace 'B1' increased with increasing temperature, exhibiting a germination rate index of 47.95%/day under 40/35 °C compared to 34.38 and 36.50%/day recorded under 20/15 and 30/25 °C, respectively.

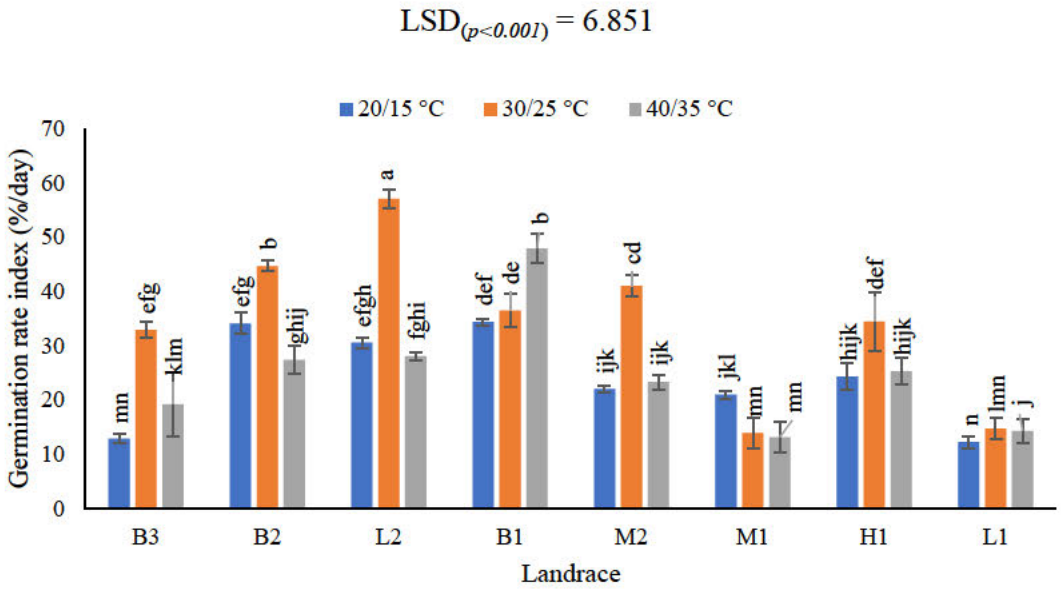



**Figure 8.3:** Germination rate index for the interaction effect between three different alternating temperature levels and eight cannabis landraces ($p$<0.001; number of replicates = 3). LSD 5% = least significance difference at 5 percent.

### 8.3.3. Biochemical profiling

#### 8.3.3.1. Carbohydrate concentration and composition

The landraces, temperature levels, and their interactions showed significant differences ($p$<0.001) in the sugar composition of the cannabis seedlings. The concentration of glucose, fructose, sucrose, mannitol, myo-inositol, and sorbitol varied with landrace and temperature levels. For instance, glucose concentration was generally higher compared with the other sugars in seeds of cannabis landraces at all temperature levels, followed by fructose concentration (Table 8.2). Conversely, the concentration of mannitol was generally lowest under the 20/15 °C regime, whereas the concentration of sucrose was generally lowest under the 30/25 and 40/35 °C regimes. 'B2' exhibited the highest glucose concentration of 30.21 mg/g DW under 20/15 °C and 31.06 mg/g DW under 30/25 °C. Similarly, 'B2' had the highest mannitol concentration of 6.57 mg/g DW under 40/35 °C. In contrast, 'B2' exhibited the lowest

mannitol concentration of 1.61 mg/g DW under 20/15 °C and the lowest sucrose concentrations of 2.21 and 0.73 mg/g DW under 30/25 and 40/35 °C, respectively.

The genotypes ‘H1’, ‘L2’, and ‘B1’ had the highest glucose concentrations of 5.14, 33.17, and 33.59 mg/g DW under 20/15 °C, 26.12, 15.49, and 23.25 mg/g DW under 30/25 °C, and 29.45, 5.75 and 6.42 mg/g DW under 40/35 °C, respectively. On the other hand, ‘H1’ had the lowest mannitol concentration at 1.08 mg/g DW under 20/15 °C and the lowest sucrose concentrations at 1.74 and 1.18 mg/g DW under 30/25 and 40/35 °C, respectively. The concentration of sucrose was lowest in the ‘B1’ genotype, where 1.50 and 0.84 mg/g DW were observed under 20/15 and 40/35 °C, respectively. Additionally, ‘B1’ showed the lowest sorbitol concentration of 1.59 mg/g DW under 30/25 °C. ‘L2’ had the lowest mannitol concentrations of 1.05 and 0.99 mg/g DW under 20/15 and 30/25 °C, respectively. Under 40/35 °C, ‘L2’ had the lowest sucrose concentration of 0.73 mg/g DW.

**Table 8.2:** The interaction effect between three different temperature levels and four cannabis landraces on the concentration of sugars (sucrose, glucose, sorbitol, myo-inositol, mannitol, and fructose) (number of replicates = 3)

| Landrace x Temperature | | | | | | | |
|---|---|---|---|---|---|---|---|
| Landrace | Temperature (°C) | Sucrose (mg/g DM) | Glucose (mg/g DM) | Sorbitol (mg/g DM) | Myo-inositol (mg/g DM) | Mannitol (mg/g DM) | Fructose (mg/g DM) |
| B2 | 20/15 | 3.31a | 30.21bc | 8.03a | 2.98d | 1.61g | 14.19d |
| | 30/25 | 2.21bc | 31.06b | 5.21b | 4.78c | 2.34f | 19.69b |
| | 40/35 | 0.73f | 5.94g | 2.21de | 1.97ef | 6.57b | 4.00f |
| H1 | 20/15 | 2.62ab | 5.14g | 1.71ef | 1.62f | 1.09h | 2.79f |
| | 30/25 | 1.74cd | 26.12d | 3.60c | 3.17d | 2.97e | 20.60b |
| | 40/35 | 1.18def | 29.45c | 3.99c | 6.45b | 6.29c | 21.36b |
| B1 | 20/15 | 1.50cdef | 33.59a | 4.26c | 7.68a | 7.16a | 26.45a |
| | 30/25 | 2.12bc | 23.25e | 1.59ef | 4.71c | 2.59f | 16.88c |
| | 40/35 | 0.84ef | 6.42g | 2.76d | 1.98ef | 5.49d | 4.27f |
| L2 | 20/15 | 1.57cde | 33.17a | 3.89c | 2.06ef | 1.05h | 13.67d |
| | 30/25 | 1.52cdef | 15.49f | 2.56d | 2.61de | 0.99h | 10.48e |
| | 40/35 | 0.73f | 5.75g | 1.44f | 1.71f | 5.54d | 4.28f |
| *p*-value | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| LSD | | 0.462 | 0.807 | 0.381 | 0.453 | 0.150 | 1.270 |

* Means with different letters are significantly different according to Fisher's test ($p<0.05$). LSD 5% = least significance difference at 5 percent; DW = dry weight.

#### 8.3.3.2. Amino acids concentration and composition

The landraces, temperature levels, and their interactions showed significant differences ($p<0.001$) in the amino acids of the cannabis seedlings. Seventeen amino acids were identified in the cannabis landrace seedlings grown at varying temperatures (Table 8.3). Notably, the concentration of amino acids increased with the increasing temperature in 'H', 'B1', and 'L2'. In 'B2', the concentration of amino acids was the highest under 20/15 °C, reduced to the lowest under 30/25 °C, and then increased at 40/35 °C. Amongst the amino acids, cysteine concentration was the lowest in all landraces and under all temperature levels, whereas lysine, leucine, and valine concentrations were generally higher. The leucine concentration in 'B1' increased from 10.78 to 11.97 and 17.11 mg/g DW under 20/15 °C, 30/25 °C, and 40/35 °C, with the corresponding valine concentration having increased from 7.92 to 9.79 and 16.01 mg/g DW, respectively. Additionally, cysteine concentration increased with the increasing

temperature, except in ‘B2’ as it recorded 6.25 mg/g DW under 20/15 °C, which was higher compared to 0.05 and 0.50 mg/g DW recorded under 30/25 and 40/35 °C, respectively.

**Table 8.3:** The interaction effect between three different temperature levels and four cannabis landraces on the composition and concentration of amino acids (number of replicates = 3)

| Landrace | Temperature (°C) | Amino acids (mg/g DM) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Alanine | Aspartic | Cysteine | Cystine | Glutamic acid | Glycine | Histidine | Isoleucine | Leucine | Lysine | Methionine | Phenylalanine | Proline | Serine | Threonine | Tyrosine | Valine |
| B2 | 20/15 | 11.96a | 12.76b | 6.26a | 11.25a | 12.82a | 12.88a | 17.52a | 15.31a | 18.37a | 28.00a | 7.83a | 14.80a | 15.59a | 7.81ab | 12.88a | 10.41a | 16.99a |
| | 30/25 | 4.11i | 2.85h | 0.05d | 2.19f | 2.56h | 3.87g | 0.00h | 7.66h | 10.91g | 3.47h | 2.38h | 6.64j | 4.28j | 2.38f | 1.62i | 3.45h | 8.43g |
| | 40/35 | 8.03f | 10.63de | 0.50d | 2.89e | 6.97f | 7.99e | 4.06f | 11.22e | 15.10d | 9.64f | 4.09f | 11.05g | 7.81f | 6.35d | 4.20f | 7.55e | 13.72d |
| H1 | 20/15 | 5.84g | 4.53g | 0.07d | 2.09fg | 4.42g | 5.29f | 3.30g | 9.37f | 12.75e | 4.64g | 2.97g | 8.70h | 6.21g | 3.87e | 2.26h | 4.90f | 10.29e |
| | 30/25 | 10.34c | 10.68d | 0.31d | 3.27d | 10.11e | 9.73cd | 3.97f | 14.90b | 18.48a | 18.93c | 5.23e | 13.73b | 11.47b | 7.46abc | 5.04e | 9.07c | 17.13a |
| | 40/35 | 10.61b | 13.21a | 1.99c | 3.95c | 11.69b | 9.84c | 5.61d | 14.60b | 18.41a | 16.53d | 5.67d | 13.09c | 11.36b | 7.86a | 9.18c | 10.50a | 17.16a |
| B1 | 20/15 | 3.94i | 2.63h | 0.05d | 2.13f | 2.66h | 3.68g | 0.00h | 7.05i | 10.78g | 3.01h | 2.29h | 6.81j | 5.00i | 2.27f | 1.34ij | 3.19h | 7.92h |
| | 30/25 | 5.56h | 5.05f | 0.18d | 2.10fg | 4.27g | 5.17f | 0.00h | 8.59g | 11.97f | 5.05g | 2.87g | 7.97i | 5.73h | 4.20e | 2.69g | 4.43g | 9.79f |
| | 40/35 | 9.59e | 11.89c | 1.81c | 3.71c | 10.44de | 9.43d | 4.88e | 13.17c | 17.11b | 15.39e | 5.67d | 12.22e | 9.04e | 7.33abc | 7.44d | 9.55b | 16.01b |
| L2 | 20/15 | 2.95j | 1.82i | 0.05d | 1.80g | 1.91i | 2.67h | 0.00h | 5.46j | 8.10h | 2.54i | 1.64i | 5.18k | 3.61k | 2.04f | 1.09j | 2.38i | 6.35i |
| | 30/25 | 9.94d | 10.29e | 5.38b | 8.36b | 11.06c | 10.66b | 13.40c | 12.96c | 16.33c | 23.34b | 7.01b | 12.62d | 10.48c | 7.29bc | 10.89b | 8.57d | 14.14c |
| | 40/35 | 9.92d | 10.95d | 5.38b | 8.07b | 10.67d | 10.67b | 15.10b | 12.25d | 15.34d | 23.41b | 6.08c | 11.52f | 9.37d | 7.17c | 11.04b | 8.81cd | 13.97cd |
| *p*-value | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| LSD | | 0.252 | 0.376 | 0.464 | 0.307 | 0.351 | 0.297 | 0.624 | 0.399 | 0.394 | 0.462 | 0.312 | 0.304 | 0.332 | 0.540 | 0.414 | 0.287 | 0.313 |

*Means with different letters are significantly different according to Fisher's test ($p<0.05$). LSD 5% = least significance difference at 5 percent; DW = dry weight.

#### 8.3.3.3. Fatty acid methyl esters concentration and composition

The landraces, temperature levels, and their interactions significantly ($p$<0.001) affected the FAMEs in the cannabis seedlings. The concentration of FAMEs generally decreased with increasing temperature. However, in 'B2' and 'L2', the concentrations of FAMEs were higher under 30/25 °C compared to 20/15 °C (Table 8.4). Amongst the FAMEs, linoleic acid, which is a polyunsaturated fatty acid, was the most abundant fatty acid in all landraces under all temperature regimes. Oleic acid, which is a monounsaturated fatty acid, was the second-highest FAME, and it was followed by palmitic acid, which is a saturated fatty acid. Notably, linoleic, oleic, and palmitic acid concentrations in landrace 'B2' and 'L2' were higher under 30/25 °C and lower under 40/35 °C compared with the other temperature levels. On the other hand, linoleic, oleic, and palmitic acid concentrations increased with the increasing temperature in 'H1' and 'B1'.

'B2' had linoleic acid concentrations of 96.28, 107.90, and 47.74 mg/g DW under 20/15, 30/25, and 40/35 °C, with a corresponding oleic acid of 21.09, 23.52, and 9.98 mg/g DW and palmitic acid of 12.02, 14.74 and 6.97 mg/g DW, respectively. 'H1' showed a linoleic acid concentration of 129.61, 100.02, and 76.62 mg/g DW under 20/15, 30/25, and 40/35 °C, with a corresponding oleic acid of 25.55, 20.56, and 15.99 mg/g DW and palmitic acid of 16.48, 13.15 and 9.81 mg/g DW, respectively. 'B1' showed a linoleic acid concentration of 107.13, 103.46, and 87.71 mg/g DW under 20/15, 30/25, and 40/35 °C, with a corresponding oleic acid of 25.53, 23.62, and 18.90 mg/g DW and palmitic acid of 12.65, 12.12 and 12.03 mg/g DW, respectively. On the other hand, 'L2' had a linoleic acid concentration of 102.79, 160.26, and 67.87 mg/g DW under 20/15, 30/25, and 40/35 °C, with corresponding oleic acid of 23.95, 38.10, and 15.83 mg/g DW and palmitic acid of 12.94, 20.73 and 9.77 mg/g DW, respectively.

**Table 8.4:** The interaction effect of three different temperature levels and four cannabis landraces on FAMEs concentration and composition (number of replicates = 3). C14 (myristic acid), C15 (pentadecaenoic acid), C16.1 (palmitoleic acid), C16 (palmitic acid), C17 (heptadecaenoic acid), C18.1 (oleic acid), C18.2 (linoleic acid), C18.3n6 (gamma-linolenic acid), C18 (stearic acid), C20.1 + C18.3n3 (cis-11-eicosenoic acid+ gamma-linolenic acid), C20.2 (eicosadienoic acid), C20.3n3 (cis-11.14.17-eicosatrienoic acid), C20.5n3 (cis-5.8.11.14.17-eicosapentaenoic (EPA)), C20 (arachidic acid), C21 (heneicosaenoic acid), C22.1 (erucic acid), C22.2 (cis-13.16-docosadienoic acid), C22 (behenic acid), C23 (tricosaenoic acid), C24.1 (nervonic acid), C24 (lignoceric acid)

| | | **FAMEs (mg/g DM)** | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Landrace | Temperature (°C) | C14 | C15 | C16.1 | C16 | C17 | C18.1 | C18.2 | C18.3n6 | C18 | C20.1 + C18.3n3 | C20.2 | C20.3n3 | C20.5n3 | C20 | C21 | C22.1 | C22.2 | C22 | C23 | C24.1 | C24 |
| B2 | 20/15 | 0.19c | 0.05de | 0.25abc | 12.02e | 0.14dcd | 21.09d | 96.28h | 0.87e | 6.79c | 22.70e | 0.154bcde | 0.027g | 0.085c | 1.52bcd | 0.07bc | 0.032ef | 0.038d | 0.761f | 0.095d | 0.0345de | 0.238f |
| | 30/25 | 0.18cd | 0.07bc | 0.23bc | 14.74c | 0.16c | 23.52c | 107.90c | 0.92e | 7.82b | 25.09c | 0.175bc | 0.055b | 0.037g | 1.80b | 0.09b | 0.037b | 0.044c | 1.121b | 0.134bc | 0.0381bc | 0.355b |
| | 40/35 | 0.10g | 0.04e | 0.09e | 6.97g | 0.08e | 9.98h | 47.74l | 0.44g | 3.81g | 9.11j | 0.078f | 0.032f | 0.034g | 0.83f | 0.05c | 0.035bcde | 0.084a | 0.513g | 0.065e | 0.0274f | 0.170g |
| H1 | 20/15 | 0.14ef | 0.07cd | 0.24abcc | 16.48b | 0.14cd | 25.55b | 129.61b | 1.35ab | 6.84c | 29.78b | 0.178b | 0.046cd | 0.045f | 1.49bcd | 0.07bc | 0.035bcd | 0.027g | 0.776ef | 0.098d | 0.0386bc | 0.275e |
| | 30/25 | 0.23b | 0.06cde | 0.17d | 13.15d | 0.13cd | 20.56e | 100.02g | 1.19cd | 5.82d | 21.37f | 0.157bcd | 0.043d | 0.074d | 1.27cde | 0.07bc | 0.031f | 0.034f | 0.766f | 0.163a | 0.0343de | 0.288e |
| | 40/35 | 0.28a | 0.10a | 0.18d | 9.81f | 0.12d | 15.99g | 76.62j | 0.92e | 4.50f | 11.27i | 0.147bcde | 0.037e | 0.085c | 1.15e | 0.06bc | 0.034cdef | 0.034f | 1.066c | 0.117cd | 0.0368cde | 0.318cd |
| B1 | 20/15 | 0.13f | 0.05de | 0.17d | 12.65d | 0.14cd | 25.53b | 107.13d | 1.14d | 7.08c | 23.98d | 0.183b | 0.045cd | 0.097b | 1.57bc | 0.14a | 0.034cde | 0.037de | 0.770ef | 0.094d | 0.0360cde | 0.291de |
| | 30/25 | 0.13f | 0.07bcd | 0.22c | 12.12e | 0.13cd | 23.62c | 103.46e | 1.28bc | 6.25d | 16.62g | 0.154bcde | 0.043d | 0.074d | 1.40cde | 0.07bc | 0.032ef | 0.038d | 0.830d | 0.095d | 0.0362cde | 0.341bc |
| | 40/35 | 0.23b | 0.12a | 0.27ab | 12.03e | 0.26a | 18.90f | 87.71i | 0.96e | 5.27e | 13.56h | 0.130de | 0.049c | 0.067e | 1.25de | 0.05c | 0.033def | 0.034ef | 1.161b | 0.129c | 0.0411b | 0.323c |
| L2 | 20/15 | 0.18cd | 0.06cde | 0.176d | 12.94d | 0.15cd | 23.95c | 102.79f | 0.95e | 7.20c | 24.14d | 0.156bcd | 0.045cd | 0.084c | 1.55bcd | 0.06bc | 0.036bc | 0.036def | 0.871d | 0.096d | 0.0378bcd | 0.284e |
| | 30/25 | 0.22b | 0.09b | 0.28a | 20.73a | 0.23b | 38.10a | 160.26a | 1.43a | 10.27a | 34.63a | 0.227a | 0.073a | 0.109a | 2.48a | 0.07bc | 0.041a | 0.056b | 1.360a | 0.154ab | 0.0523a | 0.443a |
| | 40/35 | 0.16de | 0.07bcd | 0.23bc | 9.77f | 0.13d | 15.83g | 67.87k | 0.66f | 4.92ef | 11.12i | 0.125e | 0.036ef | 0.047f | 1.15e | 0.07bc | 0.033def | 0.034ef | 0.821de | 0.095d | 0.0342e | 0.262ef |
| *p-value* | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| LSD | | 0.0147 | 0.0103 | 0.02 | 0.2836 | 0.0182 | 0.28 | 0.340 | 0.0636 | 0.2640 | 0.37 | 0.017 | 0.0025 | 0.00277 | 0.1813 | 0.0175 | 0.0 | 0.002 | 0.0299 | 0.0143 | 0.00 | 0.0160 |

*Means with different letters are significantly different according to Fisher's test ($p<0.05$). LSD 5% = least significance difference at 5 percent; DW = dry weight.

### 8.3.4. Multivariate analysis

#### 8.3.4.1. The Pearson's correlation between the germination indexes and biochemical composition of cannabis landraces

Pearson's correlation showed no correlation between the germination indexes and the different carbohydrates ($p$>0.05), except between the germination percentage and sorbitol, which had a weak positive correlation (r = 0.58, $p$<0.05) (Figure 8.4). Notably, sorbitol also had a positive correlation with glucose (r = 0.69, $p$<0.05). Furthermore, fructose had a strong positive correlation with glucose (r = 0.90, $p$<0.001) and myo-inositol (r = 0.87, $p$<0.001). Glucose also had a positive correlation with myo-inositol (r = 0.68, $p$<0.05). Additionally, sucrose had a negative correlation with mannitol (r = -0.69, $p$<0.05).

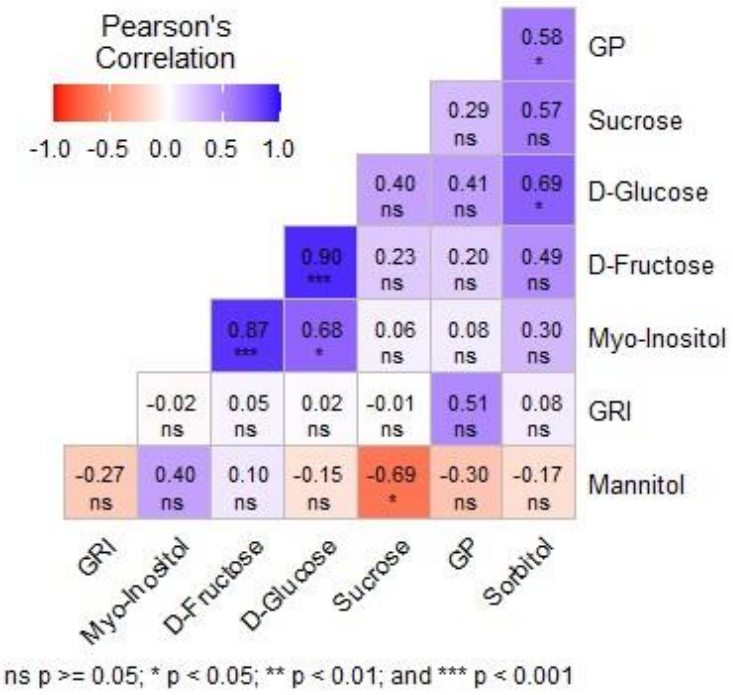



**Figure 8.4:** The Pearson's correlation between the germination percentage, germination rate index, and carbohydrates for four cannabis landraces subjected to three different temperature levels. **GP** (germination percentage), **GRI** (germination rate index).

Notably, Pearson's correlation also showed no correlation between the germination indexes and the amino acids ($p$>0.05) (Figure 8.5). However, there was a strong positive correlation among the amino acids.

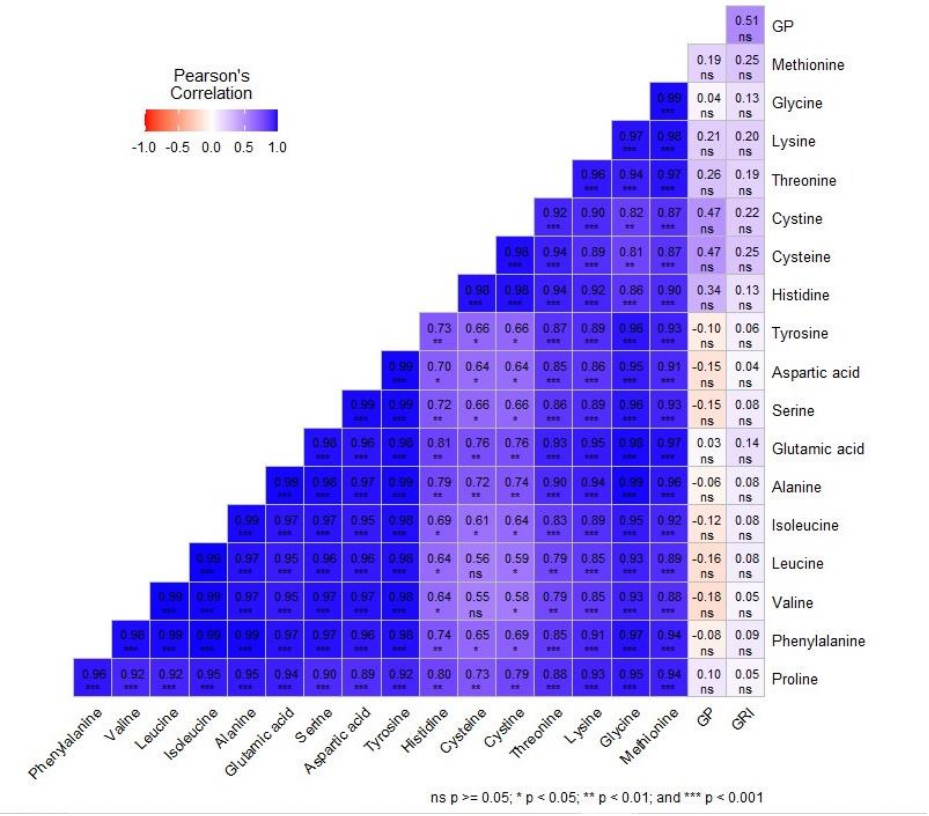



**Figure 8.5:** The Pearson's correlation between the germination percentage, germination rate index, and amino acids for four cannabis landraces subjected to three different temperature levels. **GP** (germination percentage), **GRI** (germination rate index).

On the other hand, GRI had a strong positive correlation with FAMEs such as C17 (r = 0.82, p < 0.01), C24.1 (r = 0.75, p < 0.01), C20 (r = 0.72, p < 0.01) and C20.3n3 (r = 0.78, p < 0.01), as well as C24 and C22 (r = 0.76, p < 0.01) (Figure 8.6). GRI also had a positive correlation with C16.1 (r = 0.59, p < 0.05), C18 (r = 0.66, p < 0.05), C18.1 (cis) (r = 0.63, p < 0.05), C16 (r = 0.62, p < 0.05), and C23 (r = 0.64, p < 0.05). Germination percentage had a positive correlation with C20.5n3 (r = 0.60, p < 0.05) and C20 and C18 (r = 0.66, p < 0.05).

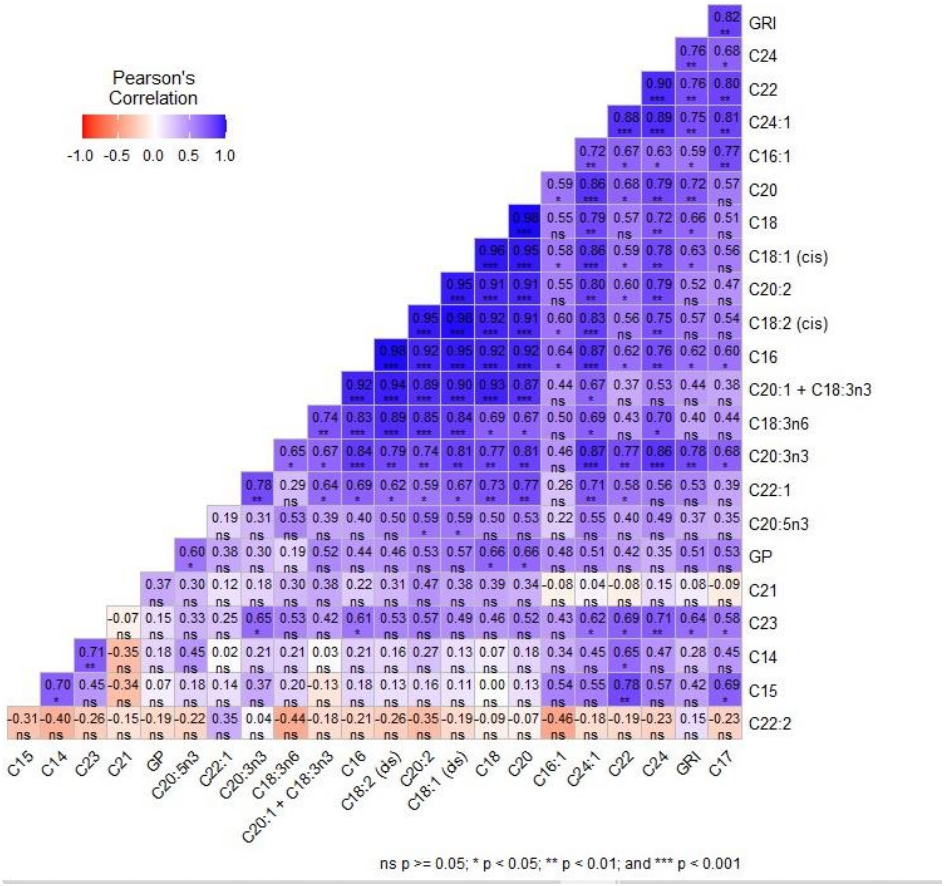



**Figure 8.6:** The Pearson's correlation between the germination percentage, germination rate index, and fatty acids methyl esters (FAMEs) for four cannabis landraces subjected to three different temperature levels. **GP** (germination percentage), **GRI** (germination rate index), **C14** (myristic acid), **C15** (pentadecaenoic acid), **C16.1** (palmitoleic acid), **C16** (palmitic acid), **C17** (heptadecaenoic acid), **C18.1** (oleic acid), **C18.2** (linoleic acid), **C18.3n6** (gamma-linolenic acid), **C18** (stearic acid), **C20.1 + C18.3n3** (cis-11-eicosenoic acid+ gamma-linolenic acid), **C20.2** (eicosadienoic acid), **C20.3n3** (cis-11.14.17-eicosatrienoic acid), **C20.5n3** (cis-5.8.11.14.17-eicosapentaenoic (EPA)), **C20** (arachidic acid), **C21** (heneicosaenoic acid), **C22.1** (erucic acid), **C22.2** (cis-13.16-docosadienoic acid), **C22** (behenic acid), **C23** (tricosaenoic acid), **C24.1** (nervonic acid), **C24** (lignoceric acid).

### 8.3.4.2. Principal component analysis for the biochemical composition of cannabis landraces

The principal component analysis was conducted separately based on different biochemical characteristics in response to temperature levels. Under carbohydrates, PCA identified two principal components (PCs), also referred to as dimensions or Dims in short throughout the study, with an eigenvalue of ≥ 1, which accounted for 84.90% of the total variation (Table 8.5). PC1 accounted for 53.16% of the total variation and positively correlated to fructose, glucose, sorbitol, and myo-inositol. PC2 accounted for 31.75% of the total variation and it positively correlated to mannitol and negatively correlated to sucrose.

**Table 8.5:** Summary of factor loadings, eigenvalue, percent, and cumulative variation for carbohydrates assessed in four cannabis genotypes subjected to different temperature regimes

| **Carbohydrates** | **Dim.1** | **Dim.2** |
|---|---|---|
| Fructose | 0.916 | 0.304 |
| Glucose | 0.954 | 0.0137 |
| Mannitol | -0.109 | 0.915 |
| Sorbitol | 0.758 | -0.290 |
| Myo-inositol | 0.760 | 0.560 |
| Sucrose | 0.525 | -0.759 |
| **Eigenvalue** | 3.190 | 1.905 |
| **Variability (%)** | 53.159 | 31.746 |
| **Cumulative (%)** | 53.159 | 84.905 |

Under amino acids, PCA identified two PCs with an eigenvalue of ≥ 1, which accounted for 98.13% of the total variation (Table 8.6). PC1 accounted for 89.09% of the total variation and positively correlated to all the amino acids. PC2 accounted for 9.04% of the total variation and positively correlated to cysteine.

**Table 8.6:** Summary of factor loadings, eigenvalue, percent, and cumulative variation for amino acids assessed in four cannabis genotypes subjected to different temperature regimes.

| **Amino acids** | **Dim.1** | **Dim.2** |
|---|---|---|
| Alanine | 0.991 | -0.129 |
| Glycine | 0.998 | 0.011 |
| Valine | 0.939 | -0.341 |
| Leucine | 0.939 | -0.328 |
| Isoleucine | 0.960 | -0.259 |
| Proline | 0.960 | -0.034 |
| Methionine | 0.987 | 0.120 |
| Serine | 0.964 | -0.212 |
| Threonine | 0.946 | 0.280 |
| Phenylalanine | 0.973 | -0.203 |
| Aspartic acid | 0.952 | -0.233 |
| Cysteine | 0.804 | 0.588 |
| Glutamic acid | 0.992 | -0.067 |
| Lysine | 0.969 | 0.190 |
| Histidine | 0.856 | 0.495 |
| Tyrosine | 0.970 | -0.214 |
| Cystine | 0.817 | 0.563 |
| **Eigenvalue** | 15.145 | 1.537 |
| **Variability (%)** | 89.089 | 9.040 |
| **Cumulative (%)** | 89.089 | 98.129 |

Under FAMEs, PCA identified four PCs with an eigenvalue of ≥ 1, which accounted for 87.75% of the total variation (Table 8.7). PC1 accounted for 58.71% of the total variation and positively correlated to the majority of FAMEs, including C16, C16.1, C17, C18, C18.1, C18.2, C20, C18.3n6, C20:1 + C18:3n3, C20.2, C22, C22.1, C20.3n3, C23, C24 and C24.1. PC2 accounted for 15.71% of the total variation and positively correlated to C14 and C15 and negatively correlated to C21. PC3 accounted for 8.49% of the total variation and positively correlated to C22.2 and C22.1. PC4 accounted for 4.84% of the total variation and positively correlated to C20.5n3.

**Table 8.7:** Summary of factor loadings, eigenvalue, percent and cumulative variation for FAMEs assessed in four cannabis genotypes subjected to different temperature regimes. **C14** (myristic acid), **C15** (pentadecaenoic acid), **C16.1** (palmitoleic acid), **C16** (palmitic acid), **C17** (heptadecaenoic acid), **C18.1** (oleic acid), **C18.2** (linoleic acid), **C18.3n6** (gamma-linolenic acid), **C18** (stearic acid), **C20.1 + C18.3n3** (cis-11-eicosenoic acid+ gamma-linolenic acid), **C20.2** (eicosadienoic acid), **C20.3n3** (cis-11.14.17-eicosatrienoic acid), **C20.5n3** (cis-5.8.11.14.17-eicosapentaenoic (EPA)), **C20** (arachidic acid), **C21** (heneicosaenoic acid), **C22.1** (erucic acid), **C22.2** (cis-13.16-docosadienoic acid), **C22** (behenic acid), **C23** (tricosaenoic acid), **C24.1** (nervonic acid), **C24** (lignoceric acid).

| **FAMEs** | **Dim.1** | **Dim.2** | **Dim.3** | **Dim.4** |
|---|---|---|---|---|
| C14 | 0.379 | 0.754 | -0.194 | 0.363 |
| C15 | 0.384 | 0.848 | 0.075 | -0.033 |
| C16 | 0.951 | -0.185 | 0.020 | -0.152 |
| C16.1 | 0.692 | 0.333 | -0.111 | -0.516 |
| C17 | 0.723 | 0.456 | 0.127 | -0.209 |
| C18 | 0.904 | -0.364 | 0.063 | -0.018 |
| C18.1 | 0.947 | -0.291 | -0.051 | -0.002 |
| C18.2 | 0.938 | -0.262 | -0.101 | -0.103 |
| C20 | 0.937 | -0.246 | 0.117 | 0.035 |
| C18.3n6 | 0.805 | -0.140 | -0.379 | -0.055 |
| C20:1 + C18:3n3 | 0.821 | -0.466 | -0.073 | -0.109 |
| C21 | 0.235 | -0.617 | -0.344 | 0.198 |
| C20.2 | 0.932 | -0.234 | -0.215 | 0.071 |
| C22 | 0.799 | 0.502 | 0.221 | 0.078 |
| C22.1 | 0.674 | -0.247 | 0.621 | 0.072 |
| C20.3n3 | 0.878 | -0.015 | 0.335 | 0.038 |
| C23 | 0.677 | 0.396 | -0.065 | 0.199 |
| C22.2 | -0.258 | -0.292 | 0.818 | 0.272 |
| C20.5n3 | 0.559 | 0.006 | -0.349 | 0.584 |
| C24 | 0.898 | 0.227 | 0.054 | 0.108 |
| C24.1 | 0.953 | 0.180 | 0.152 | -0.001 |
| **Eigenvalue** | 12.328 | 3.298 | 1.784 | 1.016 |
| **Variability (%)** | 58.706 | 15.706 | 8.495 | 4.840 |
| **Cumulative (%)** | 58.706 | 74.412 | 82.906 | 87.746 |

## 8.4. Discussion

### 8.4.1. Germination

Seed germination may vary with temperature and generally occurs within certain temperature limits, with the maximum germination efficiency occurring in a particular temperature range (Akinnuoye and Modi, 2015; Zucareli *et al*., 2015). In the current study, germination varied depending on the temperature and genotype, but it generally reduced as the temperature increased. Notably, there were no variations in germination associated with the origin of the genotype. Contrary to the findings of the current study, Byrd (2019) reported that the germination percentage of hemp cultivars from Canada declined at temperatures above 25 °C while Northern European cultivars were able to withstand temperatures above 25 °C as the germination started to decline once the temperature reached 30 °C. The findings of the current study on germination variations were associated with the landraces having optimal germination at a specific alternating temperature regime. For instance, 'L1', 'B1', 'M1', 'H1', and 'B2' demonstrated higher germination under 20/15 °C and lower germination under 30/25 °C. On the other hand, the 'B3', 'M2', and 'L2', which are genotypes with the 'Iswazi' common name, had higher germination under 30/25 °C.

Generally, it is recommended that a temperature range of 15- 27 °C is suitable for cannabis cultivation (Humphries and Florentine, 2019). Geneve *et al*. (2022) also found that 'Georgina' and 'Victoria' hemp genotypes had the highest seed germination percentage and rate between 19 and 30 °C. Thus, the findings of the current study corroborate with the findings by Humphries and Florentine (2019) and Geneve *et al*. (2022). Additionally, Qin *et al*. (2014) reported that the optimal germination temperature for the fire hemp genotype 'Bama' ranged between 20 and 25 °C. Furthermore, Bajwa *et al*. (2023) reported that the germination percentage between 10 and 30 °C was not significantly different, while it declined by 20%

under 35 °C compared to 30 °C. Notably, all the other genotypes had the lowest germination under 40/35 °C, except for 'L1', 'B1', and 'M1', which showed the lowest germination capacity under 30/25 °C. These findings suggest that high temperatures might have caused some damage to the seed and negatively affected the process of cell division and favour oxidative damage (Fahad *et al*., 2017), consequently affecting the germination of seeds in cannabis landraces. The results of the current study conform with the findings by Byrd (2019), who found that the germination percentage declined rapidly for all hemp cultivars as the temperature reached 40 °C and displayed limited germination capacity at temperatures near 45 °C. Bajwa *et al*. (2023) suggested that the germination of hemp seeds is more sensitive to high temperatures compared to low temperatures. According to Fahad *et al*. (2017), high temperatures can reduce the germination potential of seeds by causing severe damage to proteins, disrupting their synthesis, inactivating major enzymes, and damaging membranes, which ultimately leads to poor germination and stand establishment (Fahad *et al*., 2017).

### 8.4.2. Germination rate index

The germination rate index varied with temperature and cannabis genotype. However, it was generally higher under 30/25 °C and lower under 20/15 °C. These findings were contrary to the germination percentage, which was generally higher under 20/15 °C and the lowest under 40/35 °C. However, the findings were associated with a temperature of 30/25 °C speeding the initial phase of germination, shortening the germination time, whereas a low temperature of 20/15 °C was associated with a prolonged initial phase of germination leading to the extended germination time (Cafaro *et al*., 2023). Thus, the accelerated germination at 30/25 °C could be due to quicker seed imbibition at this temperature. Additionally, according to Sharma *et al*. (2022), the optimum temperature for germination rate is typically higher than the temperature required to achieve the maximum percentage of germination in partially dormant seeds. Thus,

the trend of the germination percentage and rate enhanced at different temperature levels was also associated with seed dormancy since cannabis is known to have certain residual dormancy (Jovičić *et al*., 2019; Elias *et al*., 2020).

### 8.4.3. Biochemical profiling

#### 8.4.3.1. Carbohydrates

Carbohydrates provide carbon sources which are critical in regulating development and adaptation to environmental challenges (Li *et al*., 2012). The findings of the current study on the response of carbohydrates of different cannabis landraces to different temperature regimes showed that the landraces consisted of a similar composition of carbohydrates. However, the concentration of carbohydrates varied with different temperature levels and genotypes. For instance, the sugar content generally decreased with the increasing temperature in 'B1' and 'L2', while they increased with the increasing temperature in "H1'. In 'B2', the content of the sugar increased with temperature reaching the highest under 30/25 °C and was the lowest under 40/35 °C. Nonetheless, the concentration of the carbohydrates generally decreased with the increasing temperature in the cannabis landraces. The results of the current study correlated with the findings by Ribeiro *et al*. (2014), who reported that glucose, fructose, galactose, sorbose, sucrose, and xylose levels decreased with temperature in *Ricinus communis* seedlings.

In the current study, the variations in the concentration of carbohydrates were associated with differences in adaptation mechanisms to elevated temperatures. Carbohydrates can play a protective role against stress factors, either as osmoprotectants, donors of carbon skeletons, or signaling molecules in the regulation of many defensive reactions (Ciereszko, 2018). Thus, the findings on 'B1', 'L2', and 'B2' were associated with the recommendation by Wani *et al*. (2018) that low-temperature stress influences sugar metabolism and sugar accumulation, while the sugars usually decrease in the warmer season.

Pearson's correlation analysis showed no significant correlations between the germination indexes and most carbohydrates. Except for sorbitol, which displayed a weak positive correlation with germination percentage, suggesting that it may have played a role in enhancing germination, although the effect was relatively small. Notably, sorbitol also showed a positive correlation with glucose, which also had a strong correlation with fructose, indicating that these two carbohydrates may be co-regulated during the germination process. Thus, the findings suggest that fructose and glucose may also indirectly play a role in enhancing germination. Furthermore, the first dimension (PC1) of the PCA showed a positive association between fructose, glucose, sorbitol, and myo-inositol, suggesting their collective influence on the overall metabolic landscape during germination. Hence, glucose was the most abundant carbohydrate component in the genotypes, followed by fructose, while sucrose and mannitol were the least abundant sugar. This trend could be associated with sucrose being converted into glucose and fructose to support cannabis germination and seedling growth, as suggested by Xu *et al*. (2010).

Generally, sucrose, glucose, and fructose are important carbon sources for germinating seeds (Xu *et al*., 2010). They are fast-use substrates for respiration and adenosine triphosphate (ATP) production for growing seedlings (Ciereszko, 2018; Thakur *et al*., 2020; Zhong *et al*., 2020). Glucose is also essential in plant development and gene expression (Hu *et al*., 2012). Thus, its abundance in plants was associated with various germinative processes, including germination, elongation of hypocotyls, the greening of cotyledon and expansion, and the development of true leaves (Hu *et al*., 2012). On the other hand, sucrose is the translocatable form of sugar required by emerging seedlings and is essential in regulating photosynthesis and respiration before the seedlings attain the capability for photosynthesis (Rognoni *et al*., 2007; Xu *et al*., 2010; Thakur *et al*., 2020).

#### 8.4.3.2. Amino acids

The current study found that the content of the amino acids varied with genotypes and germination temperature. For instance, the content of amino acids increased with the elevated temperature in ‘H1’, ‘B1’, and ‘L2’. Contrarily, the amino acids were the highest under 20/15 °C in ‘B2’, which then decreased to the lowest under 30/25 °C and increased under 40/35 °C. However, there was a general accumulation of amino acids, including valine, leucine, and lysine, with the elevated temperature, which was associated with the cannabis genotypes responding to increased temperatures. The results of the current study corroborate the findings by Aidoo *et al*. (2016), who reported that there was generally an accumulation of amino acids in response to increased temperature from 28 to 38 °C by 1.4- to 3.0-fold in foxtail millet. The results of the current study also corroborate the findings by Ribeiro *et al*. (2014), who reported that the levels of almost all identified amino acids in *Ricinus communis* seedlings were higher at 35 °C compared to 20 °C.

Additionally, Pearson’s correlation analysis showed no significant correlations between the germination indexes and the amino acids. However, there was a strong positive correlation among the amino acids themselves, indicating a tightly regulated network of amino acid metabolism during germination. On the other hand, the PCA showed that the first principal component collectively explained the majority of amino acid variation at 89.09%. These findings conform with the recommendation that amino acids play a role as osmoprotectants to resist abiotic stress by increasing the content of certain amino acids (Wang *et al*., 2018; Hu *et al*., 2022). Generally, the increase in amino acids and some carbohydrates at higher temperatures is associated with their role in stabilizing membranes and maintaining cellular osmotic pressure (Wei *et al*., 2020). Furthermore, Dubey and Rani (1989) suggested that different plant species differ in their capacity to accumulate specific amino acids under abiotic stress conditions, with proline being the most predominantly accumulated in various species

grown in saline conditions. In the current study, the cannabis landraces generally accumulated the essential amino acids, such as leucine, lysine, and valine, with increasing temperature. Thus, the accumulation of these essential amino acids was associated with the response of cannabis landraces to the elevated temperature. This is because metabolic processes such as the catabolism of lysine via the saccharopine pathway primarily drive lysine involvement in numerous forms of the plant stress response, demonstrating significant influence on both abiotic and biotic stress responses (Yang *et al*., 2020).

#### 8.4.3.3. Fatty acid methyl esters

Fatty acids play a critical role in seed germination and seedling development, both physiologically and anatomically (Kim *et al*., 2012; Özel *et al*., 2022). In the current study, the content of FAMEs varied with landrace and temperature. For instance, the content of FAMEs decreased with the increasing temperature in 'H1' and 'B1', while it increased under 30/25 °C compared to 20/15 °C in 'B2' and 'L2'. However, the content of FAMEs was generally the highest under 30/25 °C and lowest under 40/35 °C. Notably, the germination rate was also generally the highest under 30/25 °C. Additionally, Pearson's correlation analysis revealed that the germination percentage and germination rate index had strong positive correlations with several FAMEs, indicating a strong relationship between lipid composition and seed germination performance. The PCA results also revealed that PC1 represented a wide range of FAMEs associated with optimal germination performance, explaining most of the variation and showing a positive connection with a genotype of FAMEs. Therefore, the findings of the current study on the increased content of FAMEs under 30/25 °C were associated with energy metabolism in seeds, reflecting the role of lipids as energy sources in seeds during embryo development (Wei *et al*., 2020).

On the other hand, the reduced content of FAMEs with the elevated temperatures of 40/35 °C was associated with temperature regulating some of the growth chemical activity responsible, such as lipid composition, for the cannabis landraces to adapt under elevated temperature during germination. Temperature generally influences the amount of oxygen available, making it less soluble in the cytoplasm as the temperature increases, thereby directly affecting the lipid profile by destabilizing the desaturase enzyme (Wolf *et al*., 1982; Schulte *et al*., 2013). Oxygen enables the resumption of respiration and reactivation of metabolism during seed imbibition, leading to the production of reducing power and ATP required for seed germination (Corbineau, 2022; Rodrigues *et al*., 2022). However, the oxygen requirement for seed germination varies based on the species, dormancy level, and temperature (Corbineau, 2022).

According to Van Wagenen *et al*. (2012), many plants self-regulate lipid composition to maintain membrane fluidity at different temperatures to maintain structural integrity. Low temperature is known to result in membrane rigidification by inducing close packing of membrane lipids, while high temperature leads to fluidization (Belo *et al*., 2014; Dhaliwal and Angeles-Shim, 2022; Sharma *et al*., 2023). Thus, the membrane fluidity in plants decreases and increases to adapt to low and high temperatures, respectively (Sharma *et al*., 2023).

The seedlings of cannabis landraces were composed of saturated, monounsaturated, polyunsaturated, and unsaturated fatty acids. Linoleic acid was the most abundant FAME, followed by oleic and palmitic acid in all landraces under all temperature levels. The results of the current study corroborate the findings of Van Wagenen *et al*. (2012), who reported that EPA content decreased with increasing temperature in *Nannochloropsis Salina*. It is suggested that a high proportion of polyunsaturated fatty acids helps maintain membrane fluidity under low temperatures, thereby improving seed performance (Cabrera-Santos *et al*., 2021). Hence, the germination of cannabis landraces was generally higher under 20/15 °C compared to 30/25 and 40/35 °C. Furthermore, the abundance of linoleic acid and oleic conforms with the

recommendations that cannabis seed oil comprises 90% polyunsaturated fatty acids and desirable quantities of omega-6 and omega-3 lipids (Schluttenhofer and Yuan, 2017).

### 8.5. Conclusion

The current study highlights the effect of temperature on the seed germination and seedling metabolic factors of cannabis genotypes. It was concluded that cannabis genotypes have superior germination under specific alternating temperatures, but notably, elevated temperature decreased the germination of cannabis landraces. 'L1', 'B1', 'H1', 'B2', and 'M1' exhibited higher germination rate indexes under cooler conditions of 20/15 °C, while genotypes 'B3', 'M2' and 'L2' thrived under warmer temperatures of 30/25 °C. Additionally, it was concluded that germination temperature affects the biochemical factors during cannabis germination and seedling development. This was attributed to the observed decrease in sugars and FAMEs and increase in amino acids, which was associated with the adaptation mechanism of cannabis landraces towards the elevated temperatures of 40/35 °C. The findings of the current study, therefore, highlight the complex relationship between temperature and biochemical composition in cannabis germination. This knowledge can help growers make better decisions concerning the planting season for achieving a superior seedling establishment, which might translate to a better harvest. Furthermore, this knowledge will be useful when selecting genotypes better suited for specific regions and in developing heat-resistant genotypes, which is crucial given that heat stress has become very common in recent years. Based on the current findings, it is recommended that further research should assess the effect of different temperature regimes, particularly elevated temperatures, on physiological and biochemical processes, including changes in structure and storage substances during seed germination.

## 8.6. References


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# 9. CHAPTER 9: OVERALL DISCUSSIONS AND CONCLUSIONS

## 9.1. Introduction

*Cannabis sativa* L., commonly known as marijuana, dagga, or hemp, is an annual dioecious and dicotyledonous plant in the Cannabaceae family (Flores-Sanchez *et al*., 2009; Chaohua *et al*., 2016; Lata *et al*., 2016). Historically classified as an illicit drug due to its psychotropic component, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) (Citti *et al*., 2018), cannabis is now gaining recognition for its pharmaceutical applications and as a versatile agricultural crop (Decorte *et al*., 2011; Sawler *et al*., 2015; Gouws, 2017; Citti *et al*., 2018; Adebisi and Olaoye, 2022).

Currently, there are hundreds of cannabis genotypes worldwide, each with varying chemical compositions resulting from a long history of hybridization (Farag and Kayser, 2015; Jin *et al*., 2017; Langa *et al*., 2024a). These genotypes are categorized by chemotype and non-chemical characteristics, with distinct groups identified based on cannabinoid composition and plant morphology. However, the historical illegality of cannabis has hindered further taxonomic research and delayed industrial development, particularly in countries like South Africa.

## 9.2. Discussions

### 9.2.1. Cannabis seed genotype classification

The current study examined the seed physical properties, mineral element composition, and short tandem repeats (STR) markers of eleven different cannabis genotypes to categorize landraces into ‘High-CBD’, ‘High-THC’, or ‘Hemp’ and provide information on physical parameters for processing optimization (Chapter 3). The genotypes collected from local growers in four regions of KwaZulu-Natal Province in South Africa, namely, Bergville (B), Hammersdale (H), Ladysmith (L), and Msinga (M) were used to model landraces. These landraces included Ladysmith Ugwayi wesiZulu (‘L1’) and Iswazi (‘L2’), Durban Poison (‘H1’), Bergville Ugwayi wesiZulu (‘B1’), Natal (‘B2’) and Iswazi (‘B3’), and Msinga Ugwayi

wesiZulu (‘M1’) and Iswazi (‘M2’). The physical properties (length, width, thickness, geometric mean diameter, surface area) and mineral element composition (carbon, iron, potassium, oxygen, phosphorus, sulfur, silicon) showed significant differences. Contrarily, sphericity and aspect ratio showed no significant differences, indicating that a selected drying and milling technique could be suitable for processing any genotype.

Categorizing landraces, such as ‘L1’, ‘L2’, ‘H1’, ‘B1’, ‘B2’, ‘B3’, ‘M1’, ‘M2’, into ‘High-CBD’, ‘High-THC’, or ‘Hemp’ revealed a complex nature of the diversity of the genotypes. This followed that the physical properties, mineral element composition, and STR marker traits revealed positive correlations between ‘L1’, ‘L2’, ‘H1’, ‘B1’, ‘B2’, ‘B3’, ‘M1’, ‘M2’, ‘Hemp’, ‘High-CBD’ and ‘High-THC’, indicating similarities among the cannabis genotypes. These results were inconsistent with the findings of the previous studies, which reported that hemp seeds are larger than those of marijuana or oilseed genotypes because hemp often produces fewer seeds and, hence, concentrates more energy into the seeds (de Meijer, 1994; Naraine *et al*., 2020). Regarding mineral element composition, previous studies have demonstrated in between genotypes. For instance, Alonso-Esteban *et al.* (2022) found that the ‘Carmagnola’ hemp genotype had higher sodium, calcium, manganese, iron, and zinc, and low magnesium and phosphorus, while ‘Kompolti’ had higher potassium and copper and lower calcium, manganese, and iron.

Furthermore, Köhnemann*et al.* (2012) and Marcińska *et al.* (2022) distinguished fibrous and narcotic genotypes using leaves or inflorescence based on the STR markers. Additionally, although not based on specifically STR markers but genetic markers, Cascini *et al*. (2019) successfully distinguished between the seeds of drug-type and fiber-type cannabis with a perfect discrimination level of Area Under the Curve (AUC) 100% based on genetic markers through sequencing of tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS). However, the inconsistency of the results on the STR markers, specifically

with Köhnemann*et al.* (2012) and Marcińska *et al.* (2022), suggested tissue specificity as they used different plant materials, such as leaves and inflorescence. Thus, this could imply that there is still a need for the development and validation of a unique identification technique for cannabis seed genotype using the STR system.

Moreover, further analysis of genotypes revealed that clustering based on mineral composition grouped the landraces differently compared to clustering based on physical traits. Similarly, clustering with STR markers resulted in distinct groupings that differed from those based on physical traits and mineral element composition. Based on these findings, it was concluded that clustering based on these factors indicated complex genotype diversity, making it challenging to categorize landraces as 'High-CBD', 'High-THC', or 'Hemp' solely based on the measured parameters. These findings suggested that the physical parameters, mineral element composition, and STR markers were insufficient for comprehensive genotype classification. Therefore, in terms of seed physical and morphological properties, the observation from this study suggests integrating near-infrared hyperspectral imaging (NIR-HSI) and scanning electron microscope (SEM) into the classification of cannabis seed genotypes. These technologies have the potential to effectively differentiate genotypes and determine seed sex, with SEM also reviewing phenetic linkages of various crops (Kaya *et al*., 2011; Candan *et al*., 2016; Rashid *et al*., 2018; Majeed *et al*., 2023).

### 9.2.2. Effect of intrinsic and extrinsic factors on cannabis seed germination and seedling growth

Cannabis has varying stress tolerance depending on environmental conditions and genotype (Cole and Zurbo, 2008; Williams and Mundell, 2018; Humphries and Florentine, 2019; Adesina *et al*., 2020). Despite the critical roles of germination and seedling establishment in crop production, the physiology of these processes in South African cannabis genotypes remains poorly understood. Germination is influenced by factors such as temperature,

moisture, seed dormancy, seed quality, and light (Hu *et al*., 2018; Čanak *et al*., 2020). Chapters 4 to 8 revealed that seed dormancy, accelerated aging, different temperature regimes (particularly elevated temperatures), salinity, and drought stress had a negative influence on the germination and seedling growth of cannabis genotypes.

**Seed dormancy and germination response to dormancy-breaking treatments**

The adverse effect of dormancy on the germination and seedling growth of cannabis genotypes such as 'B2', 'B1', 'H1', 'L1', and 'L2' was observed and was largely associated with abscisic acid and gibberellins (ABA/GA) ratio. Gibberellic acid ($GA_3$) and prechilling, along with potassium nitrate ($KNO_3$), significantly improved both germination rates and seedling growth. On prechilling, the current study was consistent with Elias *et al*. (2020), who reported that they could break with wet prechilling treatment at 10 °C within 5 days in 'Merlot' and 'Berry Blossom' hemp genotypes. The study observed that the treatments that improved germination and seedling growth are known for physiological dormancy breaking. $GA_3$ and prechilling, particularly, are known to increase the synthesis of $GA_3$ in the embryo. An increased GA biosynthesis reduces the ABA/GA ratio, resulting in physiological dormancy breaking, ultimately aiding seed germination (Xu *et al*., 2020). Thus, the findings of the current study were consistent with Önol and Yildirim (2021), who reported physiological dormancy in cannabis. Although cannabis seeds have hard seed coats that might restrict germination, no physical dormancy was observed, and treatments for physical dormancy-breaking, including HCL, $HNO_3$, and $H_2SO_4$, were ineffective in improving seed germination and seedling growth.

**Effect of accelerated aging on cannabis seed germination**

Accelerated aging significantly reduced germination performance and seedling growth in 'L1', 'L2', 'B1', 'M1', and 'B2'. However, germination varied with genotypes. Previous studies have associated reduced germination with mitochondrial membrane degradation, which decreases the energy required for germination (Hussein *et al*., 2012). This was evident from

the observed linear increase in seed electrolyte leakage in the current study. Solute leakage from seeds during the membrane reorganization stage after rehydration reflects the extent of cell membrane damage and repair caused by aging (Bijanzadeh *et al*., 2017). The findings of the current study emphasized the importance of storing seeds under optimal conditions to maintain viability and ensure high-quality seed production. This practice is crucial for selecting superior genotypes in breeding programs, especially in regions prone to high temperatures and humidity during seed maturation. However, it is worth noting that it is essential to regularly assess the seed quality for high-oil crops like cannabis due to lipid instability, requiring non-destructive and efficient techniques. Therefore, in future studies, it is recommended to integrate advanced non-destructive techniques, including machine learning, spectrometry, X-ray imaging, Fourier transform near-infrared (FT-NIR), near-infrared NIR spectroscopy, and hyperspectral imaging, into seed quality assessment due to their ability to collect complex trait data and reduce analysis time (Rahman and Cho, 2016; Medeiros *et al*., 2020).

**Effect of sodium chloride (NaCl)-induced salinity and polyethylene glycol (PEG)-induced drought stress in cannabis seed germination and seedling growth**

NaCl-induced salinity and PEG-induced drought stress significantly reduced the germination and seedling growth of cannabis genotypes such as ‘L1’, ‘L2’, ‘H1’, ‘B1’, ‘B2’, ‘B3’, ‘M1’, ‘M2’. The findings on drought stress were consistent with Du *et al*. (2022), who reported that 20% PEG reduced germination rate and potential and delayed radicle and hypocotyl growth in ‘Yunma 1’ YM and ‘Bamahuoma’ BM hemp seedlings. On the other hand, the findings on salinity stress were consistent with Hu *et al*. (2018), who reported that salt stress inhibited seed germination and seedling development of the ‘Yunma 5’ and ‘Bamahuoma’ hemp genotypes.

The findings in salinity stress were associated with osmotic stress inhibiting water uptake essential for seed imbibition and the accumulation of $Na^+$ and $Cl^-$ in the embryo, which affects the breakdown and translocation of seed reserves, structural organization of protein and radicle

protrusion, thereby inhibiting seed germination (Voigt *et al*., 2009; da Silva *et al*., 2019; Saberali and Moradi, 2019; Desheva *et al*., 2020; Podder *et al*., 2020). The observed decrease in germination with increasing drought stress was attributed to the low water potential induced by osmotic stress from PEG 8000, which hindered water absorption, disrupted metabolic processes, and inactivated essential enzymes, ultimately lowering the germination percentage (Vijay *et al*., 2018; Bukhari *et al*., 2021; Violita and Azhari, 2021; Zia *et al*., 2021).

Notably, 'L2' consistently showed resilience to saline and drought conditions in the laboratory, while 'H1' was more resilient in greenhouse conditions. Increased drought stress completely inhibited the germination of 'M1' and 'L1', indicating limited tolerance in these genotypes. These findings suggested that 'L2' is suitable for growth under abiotic stress if environmental conditions such as temperature and humidity are controlled. Furthermore, the study indicated that 'H1' and 'L2' could be better selections for planting under saline conditions and for farmers with limited access to climate adaptation resources like irrigation technologies. Additionally, these landraces may be suitable for further study for breeding programs targeted at the development of saline- and drought-resistant genotypes.

**Germination and biochemical response to various temperature regimes in cannabis landraces**

Different temperature regimes significantly influenced the germination of 'L1', 'L2', 'H1', 'B1', 'B2', 'B3', 'M1', and 'M2' genotypes. The germination varied depending on the temperature and genotype, indicating that a specific temperature range is optimal for germination efficiency. However, the germination generally reduced as the temperature increased, suggesting that germination occurs within certain temperature limits. The results of the current study were consistent with Byrd (2019) and Bajwa *et al*. (2023), who reported that the germination of hemp seeds is more sensitive to high temperatures ranging between 40 and 45 °C compared to low temperatures. High temperatures usually reduce seed germination

potential by causing severe protein damage, disrupting protein synthesis, inactivating major enzymes, and damaging membranes, ultimately resulting in poor germination and stand establishment (Fahad *et al*., 2017).

Additionally, different temperature regimes significantly influenced the biochemical composition during germination, such as carbohydrate, amino acid, and fatty acids methyl ester (FAME) levels, with higher temperatures increasing amino acid content. Similar to germination, the concentration of the carbohydrates, amino acids, and FAMEs varied with temperature and genotypes. Amino acid content increased with elevated temperatures as an adaptive strategy to stabilize cellular structures and regulate osmotic pressure. However, this increase showed no correlation with germination indexes, indicating that higher amino acid levels do not directly influence the germination process. In contrast, carbohydrate and FAME concentrations decreased with rising temperatures in response to stress to maintain membrane fluidity. FAMEs peaked at 30/25°C and strongly correlated with germination indexes, indicating a link between lipid composition and seed performance. Some carbohydrates, such as sorbitol, glucose, and fructose, showed a correlation with the germination indexes. The results regarding temperature regimes can assist growers in optimizing planting seasons for improved seedling establishment and harvest quality. This knowledge is valuable for selecting genotypes adapted to specific regions and developing heat-resistant genotypes, which is essential for addressing the increasing prevalence of heat stress in recent years.

Overall, the observed variability in response to applied treatments was linked to environmental conditions rather than genetic makeup, as the genotypes exhibited similarities in physical properties, mineral element composition, and STR marker traits. Additionally, the observations showed that both physiological and biochemical processes played a significant role in germination and seedling development. Generally, biochemical processes play a significant role as healthy seedling development is dependent on the stored proteins, lipids, and

carbohydrates (Alencar *et al*., 2012; Erbas *et al*., 2016; Ali and Elozeiri, 2017; Zhao *et al*., 2018; Reis *et al*., 2020). For instance, carbohydrates such as sucrose, glucose, and fructose are generally mobilized during germination from seed storage tissues to organs such as the stem and radicle, where they support growth and maintain the osmotic homeostasis of cells (Rosa *et al*., 2009).

In the current study, it was observed that accelerated aging might have resulted in the degradation of the mitochondrial membrane, leading to a reduction in the energy supply necessary for germination. Additionally, osmotic stress reduced water uptake for seed imbibition and likely affected seed reserve breakdown, protein structure, and radicle protrusion, thus inhibiting germination. It was evident in the evaluation of different temperature regimes that there was a correlation between biochemical composition and germination indexes. However, the study did not document the effect of dormancy-breaking treatments, accelerated aging, salinity, and drought stress on biochemical processes during seed germination. Additionally, the study focused on seed germination and seedling growth, specifically from radicle protrusion to the 14-day-old seedling stage. Thus, it lacks information on how factors influencing seed germination and seedling development impact seed imbibition and various stages of plant growth, including changes in plant morphology, anatomy, and physiology. Therefore, further research is needed to understand the effects of these germination-limiting factors on seed imbibition and different growth stages of cannabis in the genotypes studied, which is essential for proper cultivation practices.

Moreover, seed germination is a complex process influenced by not only various external factors such as water, temperature, and light but by many internal factors, including reactive oxygen species (ROS), reactive nitrogen species (RNS), hormone production, and signaling, and epigenetic regulation, that play vital roles in the regulation of dormancy (Ma *et al.*, 2017; Wang *et al.*, 2024). Seed germination and dormancy regulation involve interactions between

water availability, temperature, light, phytohormones (ABA and GA), reactive oxygen and nitrogen species, metabolic pathways (including Tricarboxylic acid (TCA) and glyoxylate cycles), morphological transformations, and gene expression dynamics (Ma *et al.*, 2017; Puglia, 2024). Understanding these factors provides a comprehensive view of the mechanisms underlying seed germination and dormancy regulation for refining germination protocols, which is lacking in the current study. Therefore, future studies should consider the interplay of molecular, biochemical, and physiological responses to both intrinsic and extrinsic factors. This knowledge is crucial for improving cultivation practices, enhancing seedling establishment, and developing strategies to mitigate environmental stresses that affect cannabis germination performance.

### 9.3. Conclusions

In conclusion, the physical properties of the cannabis seeds provided crucial information optimization of processing techniques, such as milling and drying. However, the classification of cannabis seed genotypes using physical properties, together with mineral element composition and STR markers, proved to be challenging as the observation showed similarities in genotypes, and clustering revealed different groupings for each measured parameter. This revealed complex genotype diversity. Consequently, categorizing landraces as 'High-CBD', 'High-THC', or 'Hemp' solely based on the measured parameters was unsuccessful. Thus, these findings suggested improvement in the techniques used and exploring previously used methods such as gene sequencing. On the other hand, the findings of the current study highlighted the vulnerability of genotypes to environmental stressors. The study observed that seed dormancy, accelerated aging, elevated temperatures, salinity, and drought stress had adverse effects on the germination and seedling growth of cannabis genotypes. The observed cannabis variability in response to applied treatments was linked to environmental conditions rather than genetic makeup, as the genotypes exhibited similarities in physical properties,

mineral element composition, and STR marker traits. However, the study lacked insights into the molecular, biochemical, and physiological responses during seed germination. Nonetheless, this study provides valuable insights for genotype classification, aiding licensed producers and plant breeders in optimizing genotype selection for medicinal cannabis and hemp. These insights support licensed producers in ensuring regulatory compliance and maximizing yield in their cultivation practices. Additionally, the study provides detailed insights into the physiological and biochemical processes involved in cannabis germination and seedling development. These findings lay the groundwork for future research, including clinical studies and the optimization of production strategies. This research is also essential for enhancing cannabis cultivation practices and breeding programs. This is crucial as cannabis research progresses, overcoming historical legal challenges.

### 9.4. Recommendations and future research prospects

- Using NIR-HSI technology in future studies could significantly enhance cannabis classification by effectively differentiating genotypes and determining sex at the seed level. Complementarily, SEM can detect ultrastructural characteristics such as exo- and endomorphic features, macro- and micromorphological traits, seed coat morphology, and microsculpturing features. This capability enhances the ability to review phenetic linkages and classify crop species.
- Developing unique techniques for identifying seed genotypes using the STR system could significantly enhance the characterization of cannabis at the seed level. This advancement is crucial for selecting genotypes suitable for developing plant material for industrial applications or medicinal purposes. Additionally, it is recommended to characterize South African genotypes through gene sequencing, leveraging successful methodologies from previous research studies.

- Integrating advanced non-destructive techniques, including machine learning, spectrometry, X-ray imaging, FT-NIR, NIR spectroscopy, and hyperspectral imaging, could significantly improve the collection of detailed information on cannabis seed quality while reducing analysis time.
- Investigating the effects of seed dormancy-breaking growth hormones or chemicals at various concentrations and exposure durations, as well as exploring the cell signaling mechanisms and metabolic regulation involved in the germination and dormancy of cannabis seeds, is critical for optimizing cannabis cultivation, improving germination performance, and advancing the overall understanding of plant developmental biology.
- Future research should also investigate the effects of accelerated aging on seed germination in cannabis, considering the interplay of molecular, biochemical, and physiological responses.
- Furthermore, future studies should investigate the effects of salt stress and drought stress on cannabis landraces, considering interplay of molecular, biochemical and physiological responses in different stages of plant development.
- Moreover, future studies should assess the effects of different temperature regimes, especially elevated temperatures, on physiological and biochemical processes. This includes examining changes in seed structure, storage substances, and molecular responses during germination.

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