Physiological and molecular characterization of black pepper genotypes subjected to limited water availability

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By

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DECLARATION

I hereby declare that the work presented in the thesis entitled **"Physiological and molecular characterization of black pepper genotypes subjected to limited water availability"** is based on the original work done by me under the guidance of Dr. K. S. Krishnamurthy and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate® software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

(Theertha. A. P)

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Place : Kozhikode

Date :

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Theertha. A. P

Dedicated
to

My Family

CONTENTS

List of Figures

List of Tables

ABBREVATIONS AND SYMBOLS

%	-	Percentage
µg	-	Microgram
µl	-	Microlitre
µM	-	Micromolar
ABA	-	Abscisic acid
am	-	Ante Meridiem
amu	-	atomic mass unit
ANOVA	-	Analysis of Variance
APX	-	Ascorbate peroxidase
AsA	-	Ascorbate peroxidase
ASTA	-	American Spice Trade Association
BLAST	-	Basic Local Alignment Search Tool
BSA	-	Bovin serum albumin
C_{18}	-	Octyldecylsilane
Ca	-	Calcium
CAT	-	Catalase
cDNA	-	Complementary DNA
CDNB	-	1-chloro-2,4-dinitrobenzene
Chl	-	Chlorophyll
cm	-	Centimeter
cm^2	-	Centimeter square
CTAB	-	Cetyltrimethylammonium bromide
Cu	-	Copper

$CuSO_4$ - Copper sulphate

DEPC - Diethylpyrocarbonate

DHAR - Dehydroascorbate reductase

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DNase - Deoxyribonuclease

dNTP - Deoxynucleotide triphosphate

EC - Electrical conductivity

EDTA - Ethylenediamine tetra acetic acid

F - Factor

FC - Field capacity

FCR - Folin–Ciocalteau reagent

Fe - Iron

GCMS - Gas Chromatography Mass Spectrometry

GR - Glutathione reductase

GR - Glutathione reductase

GSH - Reduced glutathione

GSSG - Oxidised glutathione

GST - Glutathione S Transferase

H_2O - Water

H_2O_2 - Hydrogen peroxide

HCl - Hydrochloric acid

$HClO_4$ - Perchloric acid

HNO_3 - Nitric acid

HPLC - High-performance liquid chromatography

ICAR - Indian Council of Agricultural Research

IISR - Indian Institute of Spices Research

K	-	Pottassium
K_2SO_4	-	Potassium sulphate
Kb	-	Kilobase
KI	-	Potassium iodide
LCMS	-	Liquid Chromatography Gas Spectrometry
M	-	Molar
mAU	-	Milli-Absorbance Units
MDHAR	-	Monodehydroascorbate reductase
Mg	-	Milligram
Mg	-	Magnesium
MgCl2	-	Magnesium chloride
Min	-	Minute
Ml	-	Millilitre
mm	-	Millimeter
mM	-	Millimolar
Mn	-	Manganese
MRM	-	multiple reaction monitoring
mRNA	-	Messenger RNA
N	-	Normal
Na_2CO_3	-	Sodium carbonate
NAGS	-	National Active Germplasm Site
Na_2HPO_4	-	Disodium hydrogen phosphate
Na2SO4	-	Sodium sulphate
NaCl	-	Sodium chloride
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NaH_2PO_4	-	Sodium dihydrogen phosphate
$NaHPO_4$	-	Sodium bisulphate

NaOAC	-	Sodium Acetate
NaOH	-	Sodium hydroxide
NBT	-	nitro blue tetrazolium chloride
NBT	-	Nitro Blue Tetrazolium
NCBI	-	Sodium chloride
nm	-	Nano meter
ºC	-	Degree Celcius
OD	-	Optical density
ORF	-	Open reading frame
P	-	Phosphorus
PCA	-	Principal component analysis
pH	-	Potential of hydrogen
POD	-	Peroxidase
ppb	-	Parts per billion.
ppm	-	Parts Per Million
PPO	-	Polyphenol oxidase
PSA	-	Primary Secondary Amine
PVP	-	Polyvinylpyrrolidone
qRT-PCR	-	Quantitative real time PCR
RNA	-	Ribonucleic acid
Rnase	-	Ribonuclease
ROS	-	Reactive oxygen species
rpm	-	Revolutions per minute
rRNA	-	Ribosomal RNA
RT	-	Room temperatur
RT-PCR	-	Revrese transcription PCR
RWC	-	Relative water content

S	-	Second
SNP	-	Single nucleotide polymorphism
SOD	-	Superoxide dismutase
TAE	-	Tris acetate EDTA
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
U	-	Unit
UPGMA	-	Unweighted pair group method with arithmetic mean
UV-VIS	-	Ultraviolet- Visible
V	-	Volume
Viz.,	-	Namely
Zn	-	Zinc
β-ME	-	β-mercaptoethanol
ε	-	Molar extinction coefficient

Physiological and molecular characterization of black pepper genotypes subjected to limited water availability

ABSTRACT

The cultivation of black pepper (*Piper nigrum* L.) requires specific weather conditions, such as warm temperatures, high humidity, and uniform rainfall, which are typical of tropical climates. However, its cultivation faces significant impacts under drought stress. Hence, the present study ("Physiological and Molecular Characterization of Black Pepper Genotypes Subjected to Limited Water Availability.") focused on the identification of black pepper genotypes with more drought-tolerant characteristics from a selected pool of forty genotypes, which were maintained at the National Active Germplasm Site of ICAR-IISR.

Physiological and biochemical assessments, coupled with transcriptomic studies, revealed the adaptive mechanisms employed by drought-tolerant genotypes. Based on our experience and observation, morphological and physiological parameters, lower leaf length, leaf width, leaf area, petiole length, internodal length, and stomata number, and higher wax content were considered favorable traits for drought tolerance. Further evaluation of 21 genotypes from the mentioned forty genotypes was characterized by yield attributing traits and quality traits employing principal component analysis which indicated that 73.88% cumulative variation across five PCs is notably influenced by yield-related traits. The genotypes assumed to be drought-tolerant exhibited distinct trait patterns, especially in leaf area, stomatal density, and wax content. The overall yield, determined by berry size, spike length, and test weight, was emphasized in the findings of Pearson correlation analysis. Accessions 7211 (cluster 2), 1495, 1343, and 4132 (cluster 3) exhibited drought tolerance traits based on extensive research conducted at IISR over many years. . Accessions, 5717 and 4064 (cluster 1) exhibited drought-susceptible characteristics.

In the next step, moisture stress was imposed in the genotypes which showed drought tolerant and susceptible traits (7211, 1495, 1343, 4132, 5717, and 4064) by withholding irrigation for 28 days to decipher the mechanism of drought tolerance. The desirable physiological traits, such as higher relative water content, photosynthetic pigments, and lower membrane leakage and stomatal opening, helped the plants to tolerate water stress, as demonstrated by the accessions 7211, 1495, 1343, and 4132, in comparison to the accessions 5717 and 4064. Accessions 7211, 1495,

1343, and 4132 showed higher proline, minerals, ABA content and sugar accumulation, lower hydrogen peroxide and malondialdehyde levels, decreased starch and protein degradation, and improved antioxidant enzymatic activity.

When evaluated for yield, yield attributing traits, and quality characteristics, genotypes 1343, 1495, 4064, and 4132 exhibited lower yield reductions, while 5717 and 7211 had the highest reductions. These genotypes also showed comparatively lesser reduction in major yield-related traits such as spike length, number of berries per spike, berry size, and 100-berry fresh weight. GC-MS profiling revealed significant variations in elemental composition under imposed stress, particularly in the 7211, 1495, 1343, 4132, and 5717 genotypes, aiding in discrimination in terms of tolerance. Morphological characterization of shoots and roots demonstrated that genotypes 1343 and 1495 had minimal reductions in biomass and efficient resource allocation.

A comparative gene expression analysis of sixteen drought-responsive genes, including DHN, OSM, DREB, GST, HSP70, PX5, PX12, NAC, and SOD CuZn, specificallydiscriminates genotypes 7211, 1495, 1343, and 4132 as possessing drought tolerance traits compared to 5717 and 4064 under different field capacity levels of 25%, 50%, and 100%. At the same moisture levels, 7211 and 1343 exhibited the highest root-to- shoot ratio, followed by 4132 and 1495, as supported by molecular findings. Overall, genotypes 1343 and 4132 consistently showed desirable traits for drought tolerance compared to other genotypes while the genotypes 4064 and 5717 showed the least desirable traits among the genotypes.

Keywords: Water stress, Black pepper, Physiology, Morphology, Drought tolerance

പരിമിതമായ ജലലഭ്യതയ്ക്ക് വിധേയമായ കുരുമുളക് ജനിതകരൂപങ്ങളുടെ ശാരീരികവും ആണവികവുമായ വിശകലനം

സംഗ്രഹം

കുരുമുളക് (പൈപ്പർ നൈഗ്രം എൽ.) കൃഷിക്ക് ചൂടുള്ള താപനില, ഉയർന്ന ഈർപ്പം, സമാനമായ മഴവർഷം എന്നിവയുള്ള പ്രത്യേക കാലാവസ്ഥാ വ്യവസ്ഥകൾ ആവശ്യമാണ്, ഇവ സാധാരണയായി ട്രോപിക്കൽ കാലാവസ്ഥയിലെ സവിശേഷതകളാണ്. എങ്കിലും, വരൾച്ച സമ്മർദ്ദം കാരണം ഇതിന്റെ കൃഷി വലിയ പ്രത്യാഘാതങ്ങൾ നേരിടുന്നു. അതിനാൽ, നിലവിലെ പഠനം ("പരിമിത ജല ലഭ്യതയ്ക്ക് വിധേയമായ കരിമുളക് ജീനോട്ടൈപ്പുകളുടെ ശാരീരികവും ആണവികവുമായ വിശകലനം") വരൾച്ച പ്രതിരോധ സ്വഭാവങ്ങളുള്ള കുരുമുളക് ജീനോട്ടൈപ്പുകൾ തിരിച്ചറിയുന്നതിനായി, ഐസിഎആർ-ഐഐഎസ്ആറിന്റെ നാഷണൽ ആക്കീവ് ജെർംപ്ലാസം സൈറ്റിൽ സംരക്ഷിച്ചിട്ടുള്ള 40 ജീനോട്ടൈപ്പുകളിൽ നിന്ന് തെരഞ്ഞെടുത്ത ജീനോട്ടൈപ്പുകളുടെ ഒരു കൂട്ടത്തിൽ നിന്ന് നടത്തിയത്.
ഫിസിയോളജിക്കൽ, ബയോകെമിക്കൽ അസസ്മെൻ്റുകൾ, കൂടാതെ ട്രാൻസ്ക്രിപ്റ്റോമിക് പഠനങ്ങൾ, വരൾച്ച പ്രതിരോധ ജീനോട്ടൈപ്പുകൾ സ്വീകരിക്കുന്ന അനുയോജ്യമായ മാർഗങ്ങൾ വെളിപ്പെടുത്തി. രൂപപരമായും ശാരീരികമായും പ്രാഥമിക സ്ക്രീനിംഗിൽ, കുറഞ്ഞ ഇല നീളം, ഇല വീതി, ഇല വിസ്തീർണം, ഇലത്തണ്ടിനീളം, ഇന്റർനോഡൽ നീളം, സ്റ്റോമാറ്റയുടെ എണ്ണം, കൂടിയ മെഴുക് ഉള്ളടക്കം എന്നിവ വരൾച്ച പ്രതിരോധത്തിന് അനുകൂലമായ ഗുണങ്ങൾ ആയി കണക്കാക്കി. പരാമർശിച്ചിരിക്കുന്ന 40 ജീനോട്ടൈപ്പുകളിൽ നിന്ന് 21 ജീനോട്ടൈപ്പുകളുടെ ഫലപ്രദമായ വിലയിരുത്തൽ ഫലസ്വഭാവങ്ങളും ഗുണപരമായ സ്വഭാവങ്ങളും ഉപയോഗിച്ച് നടത്തപ്പെട്ടു.
പ്രധാന ഘടക വിശകലനത്തിൽ, 73.88% സംയോജിത വ്യത്യാസം അഞ്ച് പിസികളിൽ മിക്കവാറും ഫലവുമായി ബന്ധപ്പെട്ട സവിശേഷതകളാൽ സ്വാധീനിക്കപ്പെടുന്നു. വരൾച്ച പ്രതിരോധ സ്വഭാവങ്ങളുള്ള ജീനോട്ടൈപ്പുകൾ പ്രത്യേക സവിശേഷതാ മാതൃകകൾ പ്രദർശിപ്പിച്ചു, പ്രത്യേകിച്ച് ഇല വിസ്തീർണം, സ്റ്റോമാറ്റൽ ഡെൻസിറ്റി, മെഴുക് ഉള്ളടക്കം എന്നിവയിൽ. ബെറി വലിപ്പം, സ്പൈക്ക് നീളം, ടെസ്റ്റ് വെയ്റ്റ് എന്നിവയാൽ നിശ്ചയിച്ച മൊത്തം ഉൽപ്പാദനം പിയേഴ്സൺ കോറലേഷൻ വിശകലനത്തിലെ കണ്ടെത്തലുകളിൽ പ്രാധാന്യം നൽകിയിരുന്നു. വരൾച്ച പ്രതിരോധ ഗുണങ്ങളുടെ അടിസ്ഥാനത്തിൽ, ഡെൻഡ്രോഗ്രാം ജീനോട്ടൈപ്പുകൾ ക്ലസ്റ്റർ ചെയ്തു, ജന്യവും പരിസ്ഥിതിയും ഘടകങ്ങളുടെ പരസ്പര ബന്ധം വെളിപ്പെടുത്തി. ആക്സഷനുകൾ 7211 (ക്ലസ്റ്റർ 2), 1495, 1343, 4132 (ക്ലസ്റ്റർ 3) വരൾച്ച പ്രതിരോധം സൂചിപ്പിക്കുന്ന സവിശേഷതകൾ പ്രദർശിപ്പിച്ചു. ആക്സഷനുകൾ 5717, 4064 (ക്ലസ്റ്റർ 1) വരൾച്ചയ്ക്ക് വലുതായി പരിഗണിക്കാവുന്ന ജീനോട്ടൈപ്പുകൾ ആയിരിക്കാം.
തിരഞ്ഞെടുത്ത ജീനോട്ടൈപ്പുകൾ (7211, 1495, 1343, 4132, 5717, 4064) 28 ദിവസത്തേക്ക് ജലസേചനം നിർത്തി ഈർപ്പം സമ്മർദ്ദം ഏർപ്പെടുത്തിയിരുന്നു. പിന്നീട് ശാരീരികവും ജൈവ രാസപരവുമായ മാനദണ്ഡങ്ങൾ വിശകലനം ചെയ്തു. ഉയർന്ന ബന്ധപ്പെട്ട ജല ഉള്ളടക്കം, ഫോട്ടോസിന്തറ്റിക് പിഗ്മെന്റുകൾ, കുറഞ്ഞ മെംബ്രേൻ ലീക്കേജ്,

സ്റ്റോമാറ്റൽ ഓപ്പണിംഗ് എന്നിവ പോലുള്ള അഭികാമ്യമായ ശാരീരിക സവിശേഷതകൾ, 7211, 1495, 1343, 4132 ആക്സഷനുകൾ ജലസമ്മർദ്ദം സഹിക്കാൻ സഹായിച്ചപ്പോൾ, 5717, 4064 ആക്സഷനുകൾക്ക് താരതമ്യേന ഇത്രയും കരുത്തുണ്ടായിരുന്നില്ല. 7211, 1495, 1343, 4132 ആക്സഷനുകൾ ഉയർന്ന പ്രോളിൻ, ധാതുക്കൾ, എബിഎ ഉള്ളടക്കം, പഞ്ചസാര സമാഹരണം, കുറഞ്ഞ ഹൈഡ്രജൻ പെറോക്സൈഡ്, മലോൺഡയാൾഡീഹൈഡ് അളവ്, കുറഞ്ഞ സ്റ്റാർച്ച്, പ്രോട്ടീൻ ഇടിവുകൾ, മെച്ചപ്പെട്ട ആന്റിഓക്സിഡന്റ് എന്റൈമാറ്റിക് പ്രവർത്തനക്ഷമത എന്നിവ കാണിച്ചു.

ഉൽപ്പാദനം, ഫലസ്വഭാവങ്ങൾ, ഗുണനിലവാര സവിശേഷതകൾ എന്നിവക്കായി വിലയിരുത്തുമ്പോൾ, 1343, 1495, 4064, 4132 ജീനോട്ടൈപ്പുകൾ കുറവായ ഉൽപ്പാദന കുറവുകൾ പ്രദർശിപ്പിച്ചപ്പോൾ, 5717, 7211 ജീനോട്ടൈപ്പുകൾക്ക് ഉയർന്ന കുറവുകൾ ഉണ്ടായതായി കണ്ടെത്തി. ഈ ജീനോട്ടൈപ്പുകൾ പ്രധാന ഫലസ്വഭാവങ്ങളിൽ, സ്പൈക്ക് നീളം, ഓരോ സ്പൈക്കിലുമുള്ള മുളകുകളുടെ എണ്ണം, മുളക് വലുപ്പം, 100 ബെറി ഫ്രഷ് ഭാരം എന്നിവയിൽ താരതമ്യേന കുറവായ കുറവ് കാണിച്ചു. ജിസി-എംഎസ് പ്രൊഫൈലിംഗിൽ സമ്മർദ്ദത്തിൽ പ്രത്യേകിച്ച് 7211, 1495, 1343, 4132, 5717 ജീനോട്ടൈപ്പുകളിൽ മൂലക ഘടനയിൽ കാര്യമായ വ്യത്യാസങ്ങൾ വെളിപ്പെടുത്തി, പ്രതിരോധപരമായി വ്യത്യാസം വരുത്തുന്നതിനായി സഹായിച്ചു. തണ്ട്, വേർ എന്നിവയുടെ രൂപപരമായ വിശകലനത്തിൽ 1343, 1495 ജീനോട്ടൈപ്പുകൾ കുറഞ്ഞ ബയോമാസ് കുറവുകളും കാര്യക്ഷമമായ വനം വിഭജനം ഉള്ളതായും തെളിയിച്ചു.

DHN, OSM, DREB, GST, HSP70, PX5, PX12, NAC, SOD CuZn എന്നിവ ഉൾപ്പെടുന്ന പതിനാറ് വരൾച്ച-പ്രതിക്രിയാ ജീനുകളുടെ താരതമ്യ ട്രാൻസ്ക്രിപ്റ്റോമിക് വിശകലനം 7211, 1495, 1343, 4132 ജീനോട്ടൈപ്പുകൾക്ക് 5717, 4064 ജീനോട്ടൈപ്പുകളുമായി താരതമ്യം ചെയ്യുമ്പോൾ വരൾച്ച പ്രതിരോധ സവിശേഷതകൾ ഉള്ളവയെന്ന് തിരിച്ചറിഞ്ഞു. 25%, 50%, 100% ഇങ്ങനെ വ്യത്യസ്ത ഈർപ്പതലങ്ങളിൽ, 7211, 1343 ജീനോട്ടൈപ്പുകൾ ഏറ്റവും ഉയർന്ന വേർ-തണ്ട് അനുപാതം പ്രദർശിപ്പിച്ചു, തുടർന്ന് 4132, 1495, എന്നീ ജീനോട്ടൈപ്പുകൾ, ആണവ കണ്ടെത്തലുകൾ കൊണ്ട് പിന്തുണയ്ക്കപ്പെട്ടു. ഇപ്പഴത്തെ അന്വേഷണത്തിൽ, പഠനജീനോട്ടൈപ്പുകളുടെ ബഹുവിജ്ഞാനങ്ങൾ 1343, 4132 ജീനോട്ടൈപ്പുകളിൽ മാത്രമാണ് സുസ്ഥിരവും അഭികാമ്യവുമായ ഗുണങ്ങളെ പ്രാധാന്യമാർന്നതായി ചൂണ്ടിക്കാട്ടിയത്. മറ്റ് ചിലവക്ക് പ്രതിരോധവും അഭികാമ്യതയും പ്രകടമാക്കുന്ന വ്യത്യാസം കാണിച്ചു. ഇവയിൽ, 7211 ജീനോട്ടൈപ്പ് താരതമ്യേന കൂടുതൽ അനുകൂലമായതായി തെളിഞ്ഞു, തുടർന്ന് 1495, 4064, 5717 എന്നിവയുണ്ടായിരുന്നു.

പ്രധാനപ്പെട്ട വാക്കുകൾ: ജലസമ്മർദ്ദം, കുരുമുളക്, ശാരീരികവിശകലനം, രൂപപരമായ വിശകലനം, വരൾച്ച പ്രതിരോധം.

CHAPTER 1
INTRODUCTION

Piper, the largest and exemplary genus in the Piperaceae family, holds economic, medicinal, and commercial significance to its species, with 'black gold' spice, commonly known as King of spices (*Piper nigrum* L.), functioning as an exquisite example of its significance to the global economy. The family Piperaceae encompasses 4000 species, which were categorised into 5 genera. Among the five genera in Piperaceae, Piper stands out as exceptionally significant, boasting a total of 2000 species (Tebbs, 1993). In 1753, Linnaeus, in his seminal work "Species Plantarum," initially recognized 17 species within this botanical family (Yuncker, 1958). Piper genus comprises with herbs, shrubs, creepers and climbers. Plants in the Piper genus originated from the wet evergreen forests in southern India, gradually spread across the pantropical regions due to their demand.

Black pepper is believed to have originated around 110 million years ago in the early cretaceous period and populated across peninsular region of India 5 times independently, began during the Oligocene period roughly between 33.9 million and 23 million years ago (Sen *et al*., 2019). The humid tropics and subtropics, with their heavy precipitation, are favourable for their predominant growth and development. Black pepper requires a relative humidity of around 60-95%, an annual precipitation of 2000–3000 mm, and should be located between 20º North and 20º South of the Equator, ranging from almost sea level to an elevation of about 1500 m above mean sea level. They are particularly widespread in Central Asia, especially in India, and also in Central America (Mitra *et al*., 2021). Leading producers of black pepper in India are southern states viz. Kerala, Karnataka and Tamil Nadu.

Piper nigrum, *Piper longum*, and *Piper betle* are well known for their significance, making them the most prominent members of their genus (Vasavirama & Upender, 2014). The most recognized and popular culinary *Piper* species is *Piper nigrum*

which has a traditional history of uses as a remedy for anti-inflammatory, analgesic, anti-oxidant, anti-microbial, anti-cancer, anti-parkinsonian, anti-stress, nootropic, anti-epileptic, anti-hyperglycemic, hepatoprotective, anti-hyperlipidemic, anti- platelet, anti-angiogenic, immunomodulatory, anti-arthritic, anti-ulcer, anti- asthmatic, anthelmintic action, anti-amebic, anti-fungal, mosquito larvicidal and anti- snake venom. Many phytochemicals have been discovered thus far, including piperine, as well as essential oils, flavonoids, and steroids, have all been discovered as phytochemicals (Yadav, 2020).

Piperine was discovered to increase oral bioavailability by blocking a number of metabolising enzymes, which improves the therapeutic effectiveness of numerous drugs, vaccines, and nutrients (Chitlange *et al*., 2016). Pepper has been used for domestication purposes since thousand years ago. Hence, its cultivation is imperative, given the significant importance it holds.

Kerala was the leading producer in India, but its production significantly deteriorated during the late 2000s, in contrast to the steady increase seen in Karnataka and Tamil Nadu (Mon & Scaria, 2019). Pepper plants that are immature and frail, lacking adequate care, grown continuously in the same fields, and exposed to unfavourable environmental conditions, particularly drought, experience stunted growth and spike shedding (Negi *et al*., 2021) ultimately leading to yield loss, resulting in significant economic loss. In the present context, biggest challenge for farmers cultivating black pepper is the drought and elevatsed temperatures. The period of 2016-2018 witnessed the highly severe drought in southern India in the past 150 years, predominantly attributed to a significant shortage in the north eastern monsoon. According to the SPI (Standardized precipitation index), there has been an increase in the frequency of drought in Kerala over the past few decades (Abhilash *et al*., 2019). However, the sudden decrease in precipitation in 2016 was worse than that in 2023 (Shainu Mohan, 2023).

Hence, drought appears to be one of the major environmental constraint for crop production including black pepper production. Fluctuations in adequate

environmental conditions may negatively affect the productivity of black pepper (Anuradha, 2004). According to the Indian research reports, black pepper plants requires 2000- 3000 mm water for their development in reproductive stage (Yudiyanto *et al*., 2014). Equilibrium status under adequate conditions leads to a series of changes at morphological, physiological, biochemical, and molecular levels, which detrimentally affect the growth, development, and potential yield (Kumar *et al*., 2019; Krishnamurthy *et al*., 2000). Instead, these changes are employed to help the plants adapt and endure in such an environment, whether through stress escape, stress avoidance or stress tolerance between different species (Aslam *et al.*, 2015).

Physiological characteristics that have evolved as a result of exposure to drought conditions can serve as valuable indicators of stress, aiding in the decision-making process (Li *et al*., 2006) for selecting genotypes with tolerance characteristics (deeper root systems, longer root length, higher root-shoot ratio, stomatal closure control, reduced stomatal density, reduced leaf area, higher wax content, reduced transpiration and photosynthesis, proline accumulation, ABA accumulation, inhibition of chlorophyll degradation, balanced water status and ionic distribution, carbon distribution and consumption) (Pinheiro *et al.*, 2005; Kanavi *et al*., 2020; Krishnamurthy *et al*., 1998). Physiological traits are influenced by a combination of multiple genes and their expression is shaped by gene-specific factors as well as environmental cues that activate gene-specific activity. The expression and suppression of multigenes may significantly impart drought tolerance in black pepper. The degree of drought had an impact on biochemical parameters such as proline and carbohydrates, chlorophyll levels and growth parameters (Ghasemi *et al*., 2021).

Excessive production of reactive oxygen species (super oxide radicals, hydrogen peroxide and hydroxyl radicals) can cause damage to various cellular components, leading to oxidative damage and destructive effects on plant growth and development. Plants defend against this damage through both enzymatic and non-enzymatic activities, which are triggered by water stress (Anjum *et al*., 2016; Sanchez-Rodriguez *et al*., 2012). Hence, plants with increased antioxidant levels might be said to have drought resistance. The activities of antioxidant enzymes, such as superoxide

dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR), glutathione S-transferase (GST), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX), and polyphenol oxidase (PPO), are positively correlated with water stress in plant genotypes exhibiting drought tolerance (Csiszár *et al*., 2007; Chakhchar *et al*., 2015; Vanacker *et al*., 2018; Khaleghi *et al*., 2019).

Considering the current economic importance of black pepper and the forthcoming challenges posed by drought, it is crucial to identify drought-tolerant varieties through a series of morphological, physiological, biochemical (Anjum *et al*., 2011), and molecular studies and develop novel ones through selective breeding and cultivation methods. Only a limited number of reports are available for black pepper regarding the identification of genotypes with drought tolerance, and these concern to just a small fraction of the thousands of repositories. Thus, this study aims to identity a relatively drought tolerant genotype under water limited environment through comprehensive research, achieving the following objectives,

1. To study the genetic variation for morphological and yield attributing traits in a few selected drought tolerant accessions.

2. To study the physiological and molecular mechanism in selected drought tolerant genotypes.

3. To investigate influence of water stress on partitioning of drying matter, yield and quality parameters in selected drought tolerant genotypes.

CHAPTER 2
REVIEW OF LITERATURE

India has long held the position of the foremost producer of spices in the world. The most important spices include black pepper, cardamom, ginger, turmeric, chili, coriander, cumin, cloves, cinnamon, mustard seeds, fenugreek, asafetida, curry leaves, nutmeg, mace, carom seeds, bay leaves, and tamarind, cultivated among more than 60 distinct species. The inherent quality of Indian spices makes them outshine in contrast to other spices regarding flavor, aroma and texture. Black pepper known scientifically as *Piper nigrum* L., also is referred to as 'black gold' due to its inestimable economic demand in the world among these spices. Although it is currently cultivated in many tropical locations, it is originated from South India. Indian pepper, often known as "Malabar pepper", is well-known for its exceptional flavor and pungency and has been recognized as the best in the world. Peppercorn is an important spice in commerce between India and Europe for millennia due to its intrinsic quality and wide range of uses in medicines, culinary purposes, aesthetic industry, domestic consumption and the flavoring industry (Parry, 1969). India's leading position in the production and trading of black pepper is in jeopardy because other nations that have only started growing the "king of spices" using Indian root stocks are generating better results by implementing contemporary agricultural techniques (Jacob, 2017).

India occupies 38% of the total area used for growing spices among all the countries that produce them, only pepper is grown here, in 18% area (Anandaraj, 2013). Kerala and Karnataka are the only states that contribute more than 90% of both black pepper production and the cultivation area in India (Tripathi *et al*., 2018). According to a recent study reported by Paul (2023), Karnataka alone produced 21,000 tons (in 1.48 lakh hectares), 39,000 tons (in 1.90 lakh hectares), and 36,000 tons (in 1.80 lakh hectares) during the periods 2018-19, 2021-22, and 2022-23, respectively. Kerala cultivated black pepper during the same periods, producing 17,000 tons (in 0.83 lakh

hectares), 21,000 tons (in 0.76 lakh hectares), and 21,000 tons (in 0.82 lakh hectares) for the periods 2018-19, 2020-21, 2021-22, and 2022-23. The average yield for black pepper between 2018-19 and 2022-23 showed a decreasing trend in Tamil Nadu, Kerala, and Karnataka, with yields of 383 kg/ha, 256 kg/ha, and 200 kg/ha, respectively. However, black pepper productivity increased in Kerala and Karnataka, going from 205 kg/ha to 256 kg/ha and 142 kg/ha to 200 kg/ha, respectively, while it decreased from 539 kg/ha to 328 kg/ha in Tamil Nadu. Andhra Pradesh, Orissa, West Bengal, Tripura, Maharashtra, Manipur, Arunachal Pradesh, and the Andaman and Nicobar Islands are some of the other states in India that cultivate black pepper (Saji *et al*., 2007; Saji & krishnamoorthy, 2012).

The productivity of black pepper varies across different agro-ecological conditions and may be limited by the influence of environmental extremes, especially drought conditions, temperatures, and excessive rainfall (Yudiyanto *et al*., 2014). The drought, characterized by declining rainfall and elevated temperatures, detrimentally affects the yield of thermo-sensitive crops such as black pepper, based on the evaluation of rainfall and temperature data spanning the past 60 years (Kandiannan, 2014). The detrimental effect may be mitigated by irrigating at fortnightly intervals, which enhances productivity of black pepper by 90 to 100 percent compared to unirrigated crops in the summer season (Anandaraj & Kumar, 2004).

Morphological characterization and drought tolerance

Black pepper is an evergreen climbing plant that may reach a height of more than 10 metres. The leaves are simple, alternately arranged on stem, leaf surfaces were dark green on the adaxial face and pale green on the abaxial face. Although they are smooth to slightly textured, such variation may be influenced by age and environmental conditions. The leaf shapes - vary as ovate, elliptical, acuminate, oblong and lanceolate (Shainu Mohan, 2023).

The leaf shape of black pepper may vary considerably due to the influence of genetic factors, such as specific varieties or cultivars, and environmental factors. The primary stem, known as the orthotropic stem, grows vertically and upward, often climbing

rampantly with the support of a trellis. The orthotropic stem bears horizontally growing branches, which carry the fruits and flowers and are known as plagiotropic stems. The combined formulation of farm yard manure (FYM), neem cake, Azospirillum, and phosphobacteria, which was used as a biofertilizer, had a positive impact on plant growth and development. It significantly increased morphological characteristics such as vine length, leaf area, and internodal length when compared to the control (Bala *et al.*, 2022).

Black pepper plants produce both unisexual flowers and hermaphroditic (both male and female) flowers on same plant. Black pepper flowers are typically minuscule, approximately 2-3 millimetres in diameter. According to Figuerido & Sazima (2000), black pepper flowers were creamy, yellowish, or white in color. The inflorescence of black pepper has a raceme-like shape, with each bloom closely packed together and positioned centrally on the stem. The oldest flowers are positioned towards the bottom, followed by the youngest. Effective pollination and subsequent fruit development are facilitated by this arrangement. Black pepper inflorescence develops from the leaf axils of pepper vine along the climbing stem. The number of blooms on each spike ranged between 48 and 98, and this variation displayed a positive correlation with spike lengths of up to 20 cm (Pooja *et al*., 2022). Black pepper requires rainwater to dislodge the pollen grains from the anthers of male flowers to the stigma, a receptive organ of female flowers and external factors such as wind and tiny insects facilitate the external pollination. Sufficient rainfall ensures that both male and female flowers remain viable and this synchronization is essential for successful pollination. Drought condition reduces the pollination efficiency. Severe drought stress during the flowering to berry development stage significantly reduces black pepper yield (Rao *et al*., 2016).

Considerable variation was observed in the morphological characteristics due to reductions in leaf length, leaf area, root length, and shoot height in cowpea cultivars subjected to PEG induced drought treatment (Lakshmi & Srujana, 2018). Gomathi (2011) conducted a study on sugarcane clones by subjecting them to various drought treatments, which revealed a substantial reduction in the number of millable canes,

single cane weight, and intermodal length as dryness increased. In such conditions, leaf area and leaf area index were correlated with the maintenance of leaf water potential. Clones with drought-tolerant traits exhibited a positive correlation between leaf area index and yield under dry conditions. Morphological characteristics such as leaf length, leaf width, leaf area, petiole length, petiole diameter, and leaf mass per unit area serve as indicators of water use efficiency in all research works related to drought (Himes *et al*., 2021).

Seleiman *et al*. (2021) reported that water scarcity impacts plant height and leaf area, but leaf area growth is most significantly affected. The reduction in leaf development rate in plants is the first response to drought stress by reducing absorption of radiation and transpiration, occurring before the soil reaches dangerously low water levels at a critical stage (Belaygue *et al*., 1996; Pugnaire & Haase,1996). This implies that selecting black pepper genotypes based on leaf developmental rate may not be an effective strategy (Ramadasan & Vasantha, 1994). Either leaf abscission or downsizing mechanisms facilitated leaf area reduction in black pepper (Teles *et al*., 2023; Rasanjali *et al*., 2019). Comparable results have been documented in tomato (Heuvelink *et al*., 2005) and pepper cultivation, suggesting a decrease in leaf surface area as a consequence of water shortage (Koch *et al*., 2019; Cemek *et al*., 2020).

Physiological characterization and drought tolerance

Black pepper might display changing physiological activities in response to fluctuating environmental conditions like, drought, heat, cold, salt and heavy metals. Drought is one of the major environmental constraints that severely affects various morpho-physiological activities. It leads to deeper root systems, elongated root growth, higher root-shoot ratio, root hair expansion, root hydraulic conductance, stomatal closure control, leaf area reduction, increased wax coating, decreased transpiration and photosynthesis, proline accumulation, ABA (Abscisic Acid) accumulation, inhibition of chlorophyll degradation, balanced water status, ionic distribution, and carbon distribution and consumption (Kanavi *et al*., 2020; Pinheiro *et al*., 2005; Rivero *et al*., 2007; Beard & Sifers, 1997).

Drought stimulated leaf surface area reduction is attributed to the reduction in photosynthesis (Rucker *et al*., 1995). Loss of guard cell turgidity, together with a reduction in cell size, assists in stomatal closure and lowers transpiration rate. Schletz (2008) reported that developing black pepper leaves with more stomata tend to enhance growth potential by achieving a higher gas exchange rate compared to matured leaves. Instead, mature leaves help in regulating water loss, demonstrating a regulatory effect on stomatal development. Stomata in black pepper are distributed only on the abaxial surface, making it adequate to survive in hot climates by reducing the loss of water vapour (Ravindram, 2005). Stomatal density increases gradually with the exponential progression of maturity states, suggesting that epidermal cell development and stomatal growth are governed by distinct physiological mechanisms (Muyang *et al*., 2019). A strong correlation was discovered among stomatal number, stomatal area, and the index of stomatal shape across varying environmental conditions. Stomatal density was negatively correlated with stomatal area, even though it tends to have a flat structure, suggesting that plants adopted certain regulatory mechanisms at the stomatal level to adapt to specific environments (Zhu *et al*., 2018). In addition to stomata, trichomes, the cuticle, and cuticular waxes are vital structures on the epidermis that play a significant role in restricting water loss (Guo *et al*., 2016; Ichie *et al*., 2016; Bi *et al*., 2017; Zeisler-Diehl *et al*., 2018). Function of the wax layer on the cuticle surrounding stomata in *Arabidopsis thaliana* in preventing excessive water loss and potentially enhancing water use efficiency *(*Bertolino *et al*., 2019). Along with the cuticular wax content and trichomes, other leaf elements as callose and silicon deposition also imparts resistance to water stress (Fürstenberg-Hägg *et al*.,2013; Ellinger *et al*., 2014; Lewandowska *et al*., 2020).

Mineral nutrient deposition is of prime physiological function in drought tolerance. Mineral elements are necessary for plant physiological processes such as photosynthesis, respiration, and protein synthesis. The negative impact of drought stress can be mitigated through optimum nutrient supplementation and nutrient optimization (Ahanger *et al*., 2016). Plants require 17 essential elements, including both micronutrients and macronutrients, for their growth and development (Waraich *et al*., 2011). Furthermore, these elements enhance drought tolerance by maintaining

mechanisms that ensure the plant's ability to thrive in unfavorable environments. Deficiency in mineral nutrients can lead to stress in plants (Naik *et al*., 2022).

A plant treatment involved reducing water availability from 100% field capacity to induce increasing drought stress. This treatment aimed to evaluate mineral uptake, deposition, and distribution in the various organs of Chinese fir. Upon drought stress, phosphorus (P), potassium (K), calcium (Ca), iron (Fe), and aluminum (Al) levels increase in the phloem, while magnesium (Mg), sodium (Na), P, K, and Al increase in the xylem in stems. In branches, concentrations of P, Ca, K, Fe, and Al increased in the phloem, while concentrations of P, Mg, and manganese (Mn) increased in the xylem (Li *et al*., 2023).

In beech seedlings, elemental concentrations under drought showed a 94% decrease in K, a 94% decrease in Mg, a 75% decrease in Mn, and an 85% decrease in Zn compared to the control. Conversely, the concentration of chloride (Cl) increased by 115% to 125% in all parts of the plants (Peuke *et al*., 2011)

Drought stress imposed on *Brassica napus* and *Triticum aestivum* indicated downregulation of genes encoding transporters, leading to reduced absorption of Fe, Zn, and Mn (Courbet *et al*., 2022). White pine underwent a severe drought stress treatment to analyze leaf mineral nutrient levels and their correlation with leaf chlorophyll content. A significant reduction in mineral nutrients was observed for nitrogen (N), magnesium (Mg), phosphorus (P), and potassium (K), while a non-significant reduction was recorded in leaf iron (Fe), and an increase in leaf zinc (Zn). Chlorophyll pigment b showed a positive correlation with N, P, K, Mg, and Fe, while chlorophyll a exhibited a similar correlation with the element Zn within the leaf (Chandrasekaran *et al*., 2023).

The formation of reactive oxygen species due to the persistence of water deficit leads to lipid peroxidation, ultimately resulting in the reduction and degradation of chlorophyll (Shivakrishna *et al*., 2018). The indices of Chlorophyll a, Chlorophyll b, and total chlorophyll in black pepper plants subjected to water deficiency exhibited negative changes when compared to the control under well-watered conditions (Ambrozim *et al*., 2022). However, Ouma (2008) reported in mango (*Mangifera*

indica) rootstock seedlings that chlorophyll a and total chlorophyll levels gradually increase with rising stress levels, while the chlorophyll b content remains relatively constant. Reduction in total chlorophyll and chl a/b ratio reported in drought sensitive (Giymatli-2/17) wheat genotype, but not evident in tolerant one (Azamatli-95) (Huseynova *et al*., 2007). Similar reports was found in pea (Moran *et al*., 1994), drum wheat (Loggini *et al*., 1999) and *Boea hydroscopica* (Navari-Izzo *et al*., 2000). Based on drought screening studies conducted on onions, the 'Perama' and 'Seyhan' genotypes exhibited higher drought tolerance, as evidenced by favourable physiological traits, including relatively increased chlorophyll (a and b) content, carotenoids, chlorophyll index, and relative water content as compared to other genotypes across different drought levels (Chaudhry *et al.*, 2020).

Scientific reports have consistently demonstrated that assessing relative water content and cell membrane leakage are simple yet fundamental physiological strategies employed to examine drought tolerance. Relative water content is regarded as a useful alternative measurement for monitoring the internal plant water status, offering insights into their overall moisture levels. It serves as a measuring index for assessing drought tolerance, reflecting the metabolic processes in plant tissues and dehydration tolerance (Flower & Ludlow, 1987). Relative water content was negatively correlated and cell membrane leakage was positively correlated with escalating drought condition in black pepper genotypes (George, 2016; Krishnamurthy, 2005). Drought-tolerant black pepper genotypes exhibited higher relative water content and lower cell membrane leakage (Krishnamurthy *et al*., 1998) compared to other genotypes. These parameters can be used to identify drought-tolerant genotypes within the black pepper germplasm. . Similar results were reported in sugarcane genotypes and onion cultivars (Chaudhry *et al*., 2020) respectively. Chandravathi (2014) reported that African oil palm germplasm ZS-1 exhibited higher relative water content and lower electrolyte leakage, indicating it as more drought-tolerant than other genotypes.

Over the years, various analytical techniques have been employed to quantify plant hormones, including immunoassays, electrochemistry, mass spectrometry, and chromatography. Among the chromatographic methods, LC-MS (Liquid

Chromatography-Mass Spectrometry)-based hormone detection effectively quantifies hormones in plants, thanks to its high sensitivity, specificity, and ease of manipulation (Chu et al., 2017). The simultaneous determination of multiple hormones (cytokinins, abscisic acid, gibberellic acid, indole 3 acetic acid, jasmonic acid, brassinosteroids, salicylic acid, and strigolactones) efficiently facilitates the investigation of signaling networks controlling specific developmental pathways and physiological responses (Müller & Munne-Bosch, 2011).

The stress-responsive hormones abscisic acid (ABA), jasmonic acid (JA), indole-3-acetic acid (IAA), and salicylic acid (SA) have been reported in both *Arabidopsis thaliana* and the fruit crop *Citrus sinensis* using an Iontrap mass spectrometer (Trapp *et al*., 2014). Among these hormones, only ABA significantly responds to stress-induced conditions, enabling plants to adapt to such environments by initiating various plant growth mechanisms, including inhibition of germination, shoot and root growth retardation as well as ABA-controlled stomatal closure to reduce water loss (Aslam *et al*., 2022; Hossain *et al*., 2016). ABA synthesis, stress-induced gene expression, and metabolite composition are crucial factors that enable plants to adapt to drought stress in various tissues throughout the entire plant. ABA is primarily synthesized in leaves prior to receiving molecular signals from the roots and then transported to the shoots in response to drought stress (Takahashi *et al*., 2020). Advanced molecular genetics and genome-wide technologies have deepened our understanding and improved the breeding of stress-tolerant transgenic plants (Sah *et al*., 2016).

Biochemical characterization and drought tolerance

Drought stress triggered cascade of biochemical responses as osmotic adjustment through proline, mannitol, soluble proteins, soluble sugar and starch accumulation and antioxidant defense mechanisms are activated to counteract reactive oxygen species (ROS), such as hydrogen peroxide. Under prolonged water-scarce environments, the synthesis of secondary metabolites, such as phenolic compounds, alkaloids, and terpenoids, is considered a non-enzymatic antioxidant response aimed at protecting plants from oxidative damage. Other non-enzymatic antioxidants, including

glutathione, ascorbic acid, α-tocopherol, carotenoids, flavonoids, and tannins (collectively referred to as polyphenols), as well as melatonin and metal chelators, collectively work together to neutralize ROS and protect the integrity of cell membranes.

Peroxidase (POD), catalase (CAT), super oxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione S transferase (GST), polyphenol oxidase (PPO) are enzymatic antioxidants (Hossain *et al*., 2013; Rajan & Pushpa, 2015; *Ma et al.*, 2019). The enzymatic defense system within plants is the highly effective internal mechanism for detoxifying, preventing damages induced by ROS, and protecting the uninterrupted functioning of cells (Horvath *et al*., 2007). Environmental stress, like drought may increase the intracellular concentration of reactive oxygen species, which consequently are converted to H2O2 (Niu & Liao, 2016).

SOD (EC 1.15.1.1) is a metalloprotein that catalyzes the disproportionation of superoxide radicals (O^{2-}) into less reactive products (H2O2 and molecular oxygen) (Leonowicz *et al*., 2018; Assady *et al*.,2011),

$$2H^{+} + 2O_2^{-} \longrightarrow H_2O_2 + O_2$$

SOD is synthesized within various cellular compartments in plants including the cytosol, chloroplasts, mitochondria, apoplast and peroxisomes (Tripathy & Oelmuller, 2012). There are different isozyme forms of SOD present in plants, each with distinct specificity within the various organelles where activated unpaired oxygen is produced due to stress. Typically, three distinct isozyme variants of SOD have been reported in plants such as copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD) and iron SOD (Fe-SOD) (Racchi *et al*., 2001). Fe-SOD is present in chloroplasts and Mn-SOD is synthesized in mitochondria. Cu/Zn-SOD is found within organelles in three distinct isoforms, which were synthesized in chloroplasts, mitochondria, and peroxisomes (Ruth *et al*., 2002). Superoxide anion (O_2^{-}), singlet oxygen ($1O_2$), and hydrogen peroxide are primarily produced in the chloroplast, and they can have an impact on the photosynthetic apparatus. Eukaryotic Cu/Zn-SOD is known to be

cyanide-sensitive and exists in a dimeric form, whereas Fe-SOD and Mn-SOD can exist as either dimeric or tetrameric forms and are insensitive to cyanide (Leonowicz *et al*., 2018). Chloroplasts, housing two major reaction centers (Photosystem I and Photosystem II) connected by a series of electron carriers or transporters, function as significant centers for the production of ROS, with ROS (superoxide anion radical (O2 •-), singlet oxygen (1 O2) and hydrogen peroxide) formation occurring due to electron leakage resulting from electron overflow during periods of water stress (Janku *et al*., 2019). SOD can shield photosystem II from the damaging effects of these oxygen free radicals generated during both oxidative and water stress (Deeba *et al*., 2012). However, extended periods of drought can result in the complete destruction of the photosynthetic apparatus. Typically, the SOD activity response is elevated under unfavorable conditions such as dryness and metal toxicity. Krishnamurthy *et al*. (2016) reported that the enzymatic reactivity of both SOD and catalase in black pepper genotypes decreases with increasing lipid peroxidation and membrane leakage in response to worsening water scarcity. Several research reports have indicated differential SOD activity in different plants, which may be attributed to genetic variation, variations in gene expression, differences in the intensity and duration of water stress, nutrient availability, and variations in water tolerance mechanisms (Osakabe *et al*., 2014). In *Triticum aestivum*, SOD activity was either higher or maintained at a constant level during early drought conditions but decreased during chronic water stress (Deeba *et al*., 2012). In tobacco cultivars, the roots exhibited higher levels of SOD and catalase activity in comparison to peroxidase activity, while in the shoots, SOD and catalase activity were lower than that of peroxidase (Xu *et al*., 2010). Upregulated SOD expression in transgenic plants resulted in drought and salt tolerance (Badawi *et al*., 2004), demonstrating that SODs play an important role in plant survival under environmental conditions.

Catalases (EC 1.11.1.6) were the first discovered and characterized enzymes (Loew, 1990). They are highly efficient and ubiquitous enzymes that protect cells from oxidative damage by rapidly decomposing hydrogen peroxide into water and oxygen,

Catalase, located within aerobic entities, protects cells from environmental stress through energy-efficient enzymatic reactions. They are found in cellular organelles such as chloroplasts, mitochondria, cytosol, and peroxisomes in higher plants, but they are predominantly located in the peroxisome. Catalase exists in multiple isozyme subtypes, such as CAT 1, CAT 2, and CAT 3, which are encoded by the protein-coding genes Cat1, Cat2, and Cat3, respectively. These genes were selectively expressed in photosynthetic tissues, vascular tissues, and reproductive tissues, respectively (Sharma & Ahmad, 2014), but they are predominantly found in peroxisomes. Structural and functional versatility is found in multiple catalase isozymes across distinct plant varieties. The expression of these three genes varies in response to light intensity. Cat2 mRNA inhibits its translocation during prolonged dark periods but is activated when exposed to visible or UV light in the range of 290-400 nm (Sharma & Ahmad, 2014). ROS-induced stress activates catalase catalytic activity, which is further influenced by plant defense mechanisms, aging, and senescence. In higher plants, catalase mediates the degradation of hydrogen peroxide synthesized during mitochondrial electron transport, oxidation in photorespiration, and fatty acid β-oxidation (Yang & Poovaiah, 2002). Higher catalytic activity has been observed in drought-tolerant varieties, which helps prevent oxidative damage due to drought stress in plants. This has been reported in many research studies in various crops, including *Triticum aestivum* L. (Zhang & Kirkham, 1994), maize (Ge *et al*., 2006), *Solanum lycopersicum* (Çelik *et al*., 2017) and *Amaranthus tricolor* (Sarker & Oba, 2018).

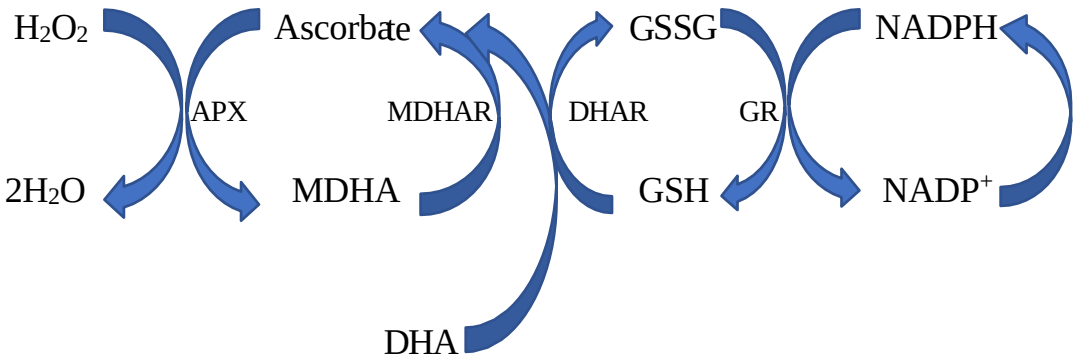
Peroxidases (EC 1.11.1.7) or peroxide reductases, heme-containing monomeric glycoproteins, reduce hydrogen peroxide into water through a mechanism similar to catalase, coupling the reaction with the oxidation of organic and inorganic substrates by hydrogen peroxide (Mathkor *et al*., 2019).

Electrochemical reduction signal of hydrogen peroxide can be considered as an indicator to quantify the peroxidase in the herbs (Yue *et al*., 2021). There are several peroxidase isozymes, encoded by multiple gene families, that can typically be

distributed among the vacuole, cell wall, cytosol, and apoplast, with their quantity and reactivity varying significantly among tissues and organs in different plant varieties (Yue *et al*., 2021). Peroxidase has significant potential for hydrogen peroxide scavenging, resistance to herbivory, detoxification, polymerization of lignin precursors and plant physiological role like growth, development and differentiation. Peroxidase activity in the crop plants increased when they were exposed to a 48-hour infestation by chewing insects such as Spodoptera (Singh *et al*., 2013). Increasing POD activity was reported in tomato leaves along with the elevated salt stress (Wu *et al*., 2022). Peroxidase activity was measured in pre-incubated crude enzyme extracts from the peels of various plants, including melon, watermelon, lemon, green beans, peas, and orange, at different temperature levels of 60°C, 70°C, and 80°C, revealing an exponential increase in peroxidase activity exclusively in melon peel, showcasing its resilience to higher temperature conditions (Mathkor *et al*., 2019). POD plays an essential role in the enzymatic defense mechanism of plants during dry conditions, where it cooperatively synergizes with SOD and CAT (Sun *et al*., 2018). This notion was corroborated by Mathkor *et al*. (2019), affirming the constitutive activity of these antioxidant enzymes in such unfavorable conditions.

Ascorbate-glutathione cycle

The Ascorbate-Glutathione pathway (ASC-GSH pathway) also known as the Foyer-Halliwell-Asada pathway, is a connected antioxidant pathway that detoxifies hydrogen peroxide in plant cells when they experience challenging environmental circumstances. The metabolic pathway carries antioxidant metabolites such as ascorbate, glutathione, and NADH/NADPH, along with enzymes like ascorbate dependent ascorbate peroxidase (EC 1.11.1.11), glutathione dependent dehydroascorbate reductase (EC1.8.5.1), NADH dependent monodehydroascorbate reductase (EC 1.6.5.4) and NADPH dependent glutathione reductase (EC 1.6.4.2).



Antioxidant metabolites were found to be localized in the nucleus, cytosol, chloroplast, mitochondria, and peroxisome, with each enzyme persisting in multiple isoforms based on intracellular organization to facilitate their enzymatic activity (Hasanuzzaman *et al*., 2017; Bartoli *et al*., 2017). The figure effectively describes each step, with ascorbate donating an electron to hydrogen peroxide in the first step, and this catalysis is mediated in the presence of APX. The oxidized ascorbate or monodehydroascorbate (MDHA) further reconstituted by MDHAR enzyme. Nonetheless, unless promptly reduced, MDHA undergoes a disproportionation process that may lead to the formation of ascorbate and DHA (dehydroascorbate). DHAR facilitates the reduction of dehydroascorbate (DHA) to ascorbate by consuming reduced glutathione (GSH) and generating oxidized glutathione (GSSG). Ultimately, the reduction reaction is carried out by GR at the expense of NADPH, donating electrons to GSSG to yield GSH (Sakhno *et al*., 2019).

Laxa *et al*. (2019) observed the significant efficiency of GR activity in a water-scarce environment among the four enzymes that participated in the ASC-GSH cycle, and these enzymes synergistically interacted within different cell organelles. Leaf rolling, a morphological adaptation to drought, is substantially positively correlated with the ASC-GSH cycle enzymes, especially GR and DHAR, which are distributed in both the apoplastic and symplastic areas of the leaf, potentially controlling the leaf rolling (Saruhan *et al*., 2009). A similar increment was reported in mung bean treated under salt stress, as evidenced by the reduced ascorbate (AsA) content and a higher GSH/GSSG ratio, along with a further increase in the activities of APX, MDHAR, DHAR, GR, GST (glutathione S-transferase), SOD, and CAT. Thus, exogenous GSH

application down regulates the synthesis of MDA, H2O2 synthesis as well as upregulates leaf RWC and chlorophyll synthesis (Nahar *et al*., 2015). The role of the ASC-GSH cycle in senescence is depicted by the soluble fractions of senescent pea leaves, which exhibited higher DHAR activity and lower GR, APX, and MDHAR activities. The variable response in the mitochondrial as well as peroxisomal ASC-GSH cycle indicated a prolonged cellular redox mechanism during leaf senescence in peroxisomes compared to mitochondria (Jiménez *et al*., 1998). Among chilling-induced developing maize lines, a relatively increased activity of ASPX, MDHAR, GR, and CAT was observed in sensitive lines compared to the tolerant line. This suggests that chilling-induced oxidation and aging were exhibited with less sensitivity (Hodges *et al*., 1997). The ASC-GSH cycle can enable the plant to adapt to unfavorable environmental conditions by reducing ROS induction through the recycling of ascorbate (Valero *et al*., 2016). Several studies have reported stage-specific increments in enzymatic antioxidant activities in the ASC-GSH pathway in plants, such as *Artemisia annua* (Sharma *et al*., 2017), *Brassica campestris* (Hossain *et al*., 2013), Prunus hybrids (Sofo *et al*., 2005), barley and triticale (Żur *et al*., 2021), *Oryza sativa* (Sharma & Dubey, 2005), castor bean (Amario *et al*., 2023), and *Maclura pomifera* (Khaleghi *et al*., 2019), with increments that occur as dehydration progresses.

GSTs (EC 2.5.1.18) are multifunctional enzymes encoded by large and heterogeneous gene families in plants, predominantly localized in the cytosol, participating in normal cellular metabolism for plant growth and development. They are also capable of regulating apoptotic cell death, oxidative stress tolerance (Rezaei *et al*., 2013), neutralizing xenobiotic compounds, and toxic heavy metals (Dixon *et al*., 2002), as well as inducing a systemic response to pathogenic attack (Gullner *et al*., 2018). GST functions by catalyzing the conjugation with GSH of xenobiotic compounds and stress-induced ROS, resulting in the formation of non-reactive compounds that can be readily removed (Roth *et al*., 2010). Hasanuzzaman *et al*. (2017) reported that both GST and GPX (Glutathione peroxidase) utilize GSH as a substrate, with GST responsible for detoxifying xenobiotics and GPX responsible for detoxifying ROS. Overexpression of the AsA-GSH cycle under prolonged unfavorable conditions may

lead to increased GSH and AsA production, thereby enhancing the chances of GST activity persisting to help the plants adapt to such an environment. Highly conserved protein structures of GSTs play multiple functions despite their highly diversified sequences (Dixon *et al*., 2002).

PPOs (EC1.10.3.1) are copper containing enzymes, primarily function by oxidizing phenolic compounds into quinones in the presence of oxygen, which are highly reactive compounds. As quinones react with proteins, they can further form protein complexes that are resistant to both plant and microbial enzymes. PPO plays a significant role in regulating physiological mechanisms and enhancing tolerance to environmental stress. The study on walnuts by Araji *et al*. (2014) suggested that PPO plays a pioneering and fundamental role in secondary metabolic activity, indirectly influencing cell death. Moderate to severe drought conditions elevates PPO activity (Surendar *et al*., 2015; Chakhchar *et al*., 2015) in parallel with the previously discussed antioxidant enzymes.

In earlier studies, hydrogen peroxide was identified as a toxic reactive oxygen species (ROS) capable of damaging cellular functions and entire cellular structures (Petrov & Van Breusegem, 2012). Normal plant cell metabolism generates hydrogen peroxide (H2O2) from a variety of sources, such as mitochondria, chloroplasts, peroxisomes, glyoxysomes, the plasma membrane, and the cell wall. It also produces H2O2 as a byproduct of cellular metabolism. Hydrogen peroxide displays relatively low reactivity at the cellular level. Nevertheless, it can still damage proteins containing iron and iron-sulfur complexes and oxidize methionine residues (Smirnoff & Arnaud, 2019). Higher plants can produce hydrogen peroxide (H2O2) under normal environmental conditions as a result of metabolic activity (Quan *et al*., 2008).

Recent investigations have revealed that an optimal concentration of H2O2 is required for physiological, biochemical reactions in plants and, resistance to environmental stresses and its acclimatization (Khan *et al*., 2018). H2O2 plays a principal role in various physiological processes, including photosynthesis and photorespiration (Foyer & Noctor, 2000), stomatal movement, and modulation of stomatal aperture. Additionally, it contributes to cell differentiation and growth, regulates root

development and controls gravitropism (Ma *et al*., 2014; Hernandez-Barrera *et al*., 2015), is involved in adventitious root development (Dunand *et al*., 2007), influences the cell cycle (Mittler, 2002), participates in cell wall formation which involves reinforcing processes such as lignification, oxidative cross-linking of hydroxyproline-rich proteins, and the assembly of cell wall complexes (Kuźniak & Urbanek, 2000; Carol & Dolan, 2006), triggers leaf senescence (Bhattacharjee, 2005), and plays a role in programmed cell death (Van Breusegem & Dat, 2006; Cheng *et al*., 2015; Vavilala *et al*., 2015). Moreover, H2O2 serves as an efficient signal molecule in pathogen defense mechanisms (Urszula & Rozalska, 2005). Plants experienced a negative correlation between H2O2 content and chlorophyll pigments such as chlorophyll a and chlorophyll b (Asaeda *et al*., 2022).

The research reports on tomato suggests that the primary long-distance signaling of H2O2 under drought conditions originates from the roots and moves to the shoots, inducing stomatal closure and resulting in a reduced transpiration rate (Reis *et al*., 2022). In an experiment with exogenous application of H2O2, nutrient uptake, which includes sodium (Na), potassium (K), calcium (Ca), iron (Fe), antimony (Sb), and arsenic (As), were elevated in the leaves and syconium of *Ficus deltoidea* (Nurnaeimah *et al*., 2020). Hydrogen peroxide triggers the activation of both enzymatic (catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione peroxidases (GPX), and ascorbate peroxidases (APX)) and non-enzymatic antioxidant system defense mechanisms, which are essential for defending plants against the oxidative stress (Bhardwaj *et al*., 2021). To attain a deeper comprehension of the mechanism underlying the role of hydrogen peroxide (H2O2) in biological processes, further research is required.

Highly reactive oxygen species severely damage plants through processes such as lipid peroxidation, protein depletion, and DNA degradation, and they may also lead to complete cell death (Yamamoto *et al*., 2001). Malondialdehyde content as a byproduct, the indicator of lipid peroxidation was discovered in black pepper (Purthur & Vijayakumari, 2014), Indian mustard (Saha *et al*., 2016), soyabean (Cakmak & Horst, 1991; Du *et al*., 2010), pea (Yamamoto *et al*., 2001), rice (Ma *et al*., 2012 ;

Awasthi *et al*., 2017) and Salvinia (Mandal *et al*., 2013). To a great extent, lipids are essential for maintaining the integrity of plant cells and their organs during drought stress, serving as a hydrophobic barrier to the external environment (Ohlrogge *et al*.,1995; Li-Beisson *et al*., 2017). The African oil palm genotype 'ZS-1' exhibited lower lipid peroxidation compared to other genotypes, showcasing its drought-tolerant traits, which are supported by additional physiological characteristics such as higher relative water content and lower electrolyte leakage (Chandravathi *et al*., 2014).

Lipids, encompassing a heterogeneous mixture of biomolecules, form membranes possessing a hydrophilic, polar head connected to a glycerol backbone and a hydrophobic tail composed of two fatty acids. The study by Noblet *et al*. (2017) discusses on reconstitution of fatty acids and lipids under different stress conditions, and the degree of reconstitution depending on the intensity of the stress. The qualitative and quantitative variations in lipid composition stimulate membrane fluidity and its ability to regulate physiological functions, enabling it to withstand unfavorable environments. Hence, it could be considered as a plant physiological marker in the current environmental status. The unfavorable conditions those effecting the lipid membrane composition as nutritional deficiency, temperature stress (heat, cold, and freezing), salinity and drought (Reszczyńska & Hanaka, 2020).

Proline, an amino acid, acts as an excellent osmolyte and plays a vital role in osmotic regulation by maintaining cell integrity and turgor when facing unfavorable environmental conditions such as drought (Hayat *et al*., 2012). An increase in proline levels was observed in the moisture-deficit-tolerant sugarcane genotype as compared to the susceptible one (Zhang *et al*., 2020). Proline maintains the integrity of DNA, proteins, the cell membrane complex, and cellular compartments, protecting them from drought stress damage (Kishor *et al*., 2005). The drought-tolerant sugarcane genotype, Yunzhe 05–51, maintains a higher proline concentration as drought conditions increase compared to the sensitive sugarcane genotype, Yuetang 93–159 (Zhang *et al*., 2020).

Application of silicon nanoparticles to drought-stressed plants increased the levels of proline and soluble sugars due to higher ROS (Desoky *et al*., 2021), ultimately leading to improved enzymatic and non-enzymatic antioxidant activities, thereby reducing ROS (Khaleghi *et al*., 2019). The influence of nano-silicon particles and their mechanism-of action in plants is still not clear. The interaction of nanoparticles with plants can regulate physiological and biochemical mechanisms. Studies on the interaction between nano-silicon and faba beans have elucidated favorable changes in productivity, even in water-scarce environments. However, there is a lack of a comprehensive scientific explanation on its ability to increase growth and yield.

Carbohydrates are organic compounds, serving as a principal source of energy for photosynthesis and plant development. Krishnamurthy *et al.* (2013) reported that the total carbohydrate level in the stem was higher during both on and off years at flowering and harvest, respectively. Starch content exhibited a similar pattern. These fluctuations in carbohydrate and starch content may play a significant role in the flowering pattern and productivity of black pepper. Generally, starch content is higher in the stems than in the leaves of black pepper. Carbohydrate reserves may have a limited impact on fruit set in sweet oranges and pecans (Monerri *et al*., 2011; Smith *et al*., 2007). The reducing sugar content in water-stressed black pepper plants was higher compared to well-watered ones (Krishnamurthy *et al*. 1998). The leaf soluble sugar content of rice genotypes, as well as the starch content, significantly decreased while proline increased with increasing drought stress levels. This trend may be attributed to the lower relative water content, stomatal conductance, and chlorophyll content (Jasmine *et al*., 2022). Similarly, decrease in sugar and starch content in rice grains was observed when artificial stress was applied (Chen *et al*., 2023).

Many investigators have suggested that accumulated soluble sugars and non-soluble starch during drought conditions can help maintain plant development and enhance resistance to drought (Li *et al*., 2020; La *et al*., 2019). The regulation of water management through the interaction of the accumulated sugar-to-starch ratio under water scarcity conditions is still not clear. The study on millet plants in dry environments revealed efficient deposition of photosynthetic soluble sugars into the

roots, facilitated by the activity of beta amylase. This process prevents the accumulation of excess soluble sugars as starch, aiding in drought tolerance and subsequent growth resumption when water becomes available again (Yang *et al*., 2023).

According to several studies, the total phenolic content in plants increases with rising temperatures and water scarcity conditions (de Abreu & Mazzafera, 2005; Jaafar *et al*., 2012). Secondary metabolites, especially total phenolic content, are highly distributed in xerophytic plants (Carrera & Bertiller, 2010; Campanella & Bertiller, 2011). These plants exhibit several adaptations, including reduced leaf area, thick and succulent leaves, deep-root systems, limited shoot growth, hard bark, leaf abscission, hairy leaves, a waxy cuticle, sunken stomata, and employ CAM (Crassulacean Acid Metabolism) photosynthesis, which reduces water usage (https://www.quora.com/What-are-some-characteristics-of-xerophytes). The highly accumulated phenolic compounds may play a significant role in these adaptations. Therefore, they are considered as favorable characteristics for plants that live in drought-stressed environments The total phenolic content in the pericarp exceeded that of black pepper, green pepper, and the methanolic extract of black pepper (Lee *et al*., 2020). A comparison was made between the total phenolic and flavonoid content in two Piper genus plants: *P. trichostrachyon* and *P. nigrum*. It was found that *P. trichostrachyon* had higher levels of these compounds compared to *P. nigrum*. Piper petioles served as a good source of phenolic compounds. (Al-Khayri *et al*., 2022). The observational findings regarding *Capsicum annuum* seeds and pulp illustrated a significant correlation between their total phenolic content and antioxidant activities, highlighting the significance of phenolic compounds in these plant components (Yalçin *et al*., 2021). Nano-SiO_2 treated faba beans under different irrigation regimes showed elevated levels of total phenol and flavonoids with reduction in water application (Desoky *et al.*, 2021). Predominantly arid climate enhanced the production of secondary metabolites in medicinal plants (Albergaria *et al*., 2020).

Molecular characterization

Studies on molecular markers such as single nucleotide polymorphisms (SNPs), microsatellites (SSRs), and DNA sequencing in black pepper help in molecular characterization and functional genomics. RNA sequencing, as a transcriptomic analysis, elucidates the gene expression profiles of plants under varying environmental conditions, aiding in the identification of gene expression patterns in the plant.

Raghavan *et al.* (2010) screened 20 black pepper accessions to identify the extent of genetic diversity at the molecular level using RAPD and SSR markers. SSRs provide genotype-specific bands, which are helpful for the identification and discrimination of genotypes within genetic diversity. The morphological differentiation among genotypes is supported by the presence of a unique DNA pattern within a specific set of genotypes that share common morphological characteristics, particularly under drought conditions.

The interaction between Calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) in plants profoundly affects the plant's response to environmental stress like drought. Real time PCR (qRT-PCR) transcriptomic data demonstrated the involvement of Ca mediated CIPK3 (CaCIPK) in responding to the drought stress. Overexpression of CaCIPK, in conjunction with the regulation of methyl jasmonate (MeJA) signaling, enhanced antioxidant activity in pepper genotypes (Ma *et al*., 2021).

The drought-tolerant PRS-64 (Angamali) and susceptible PRS-44 were identified among ten black pepper genotypes through physiological analysis, and further analysis was conducted at the molecular level to investigate the underlying basis. Gene expression studies were conducted to analyze the transcriptome by employing differentially displayed RT-PCR. Through this method, cDNA was obtained from re-amplified arbitrary primers. Prior to this, the amplification of the first strand cDNA was achieved using HT11G. The resulting transcript-derived fragments, ranging in size from 400 to 900 bp, were cloned using the pGEMT vector. Subsequent sequencing of these clones revealed homology with copper-containing amine

oxidases, which have various functions related to the oxidation of polyamines in peroxisomes and cell signaling. Polyamines play a significant role in growth, development, and the response to abiotic stress (Pallavi & Abida, 2018).

Negi *et al.* (2021) conducted a study aimed to identify candidate genes regulating the drought stress response in the leaf transcriptome of black pepper, and to identify potential genomic markers such as SSRs, SNPs, and InDels through the design and development of drought-responsive primers. The identification of transcription factors and their associated pathways in the drought tolerance mechanism could also be revealed. The Black Pepper Drought Transcriptome Database (BPDRTDb), accessible via http://webtom.cabgrid.res.in/bpdrtdb for academic purposes, contains valuable information that will enhance genetic initiatives for breeding drought-tolerant black pepper varieties.

For a comprehensive understanding of black pepper at the molecular level, it is essential to explore its significant role in gene regulation through the characterization of long non-coding RNAs (lncRNAs) and circular RNAs, especially under drought conditions. A total of 6406 lncRNAs and 4621 circRNAs (circRNAs) were identified through the comprehensive RNA-sequence evaluation, and their regulatory functions in multiple black pepper genes were elucidated via complex miRNA-lncRNA-mRNA and miRNA-circRNA-mRNA networks. These findings can help to refine breeding programmes for black pepper varieties, eventually leading to improved cultivation and yield management in the face of varied challenges (Kumar *et al*., 2023).

A drought-based study on black pepper genotypes, involving eleven drought-responsive genes (Basic leucine zipper protein (bZIP), NAC transcription factor, Aquaporin (AQUA), Betaine aldehyde dehydrogenase (BADH), Myeloblastosis oncogene (MYB), Dehydration-responsive element-binding protein (DREB), Mitogen-activated protein kinase (MAPK), Heat-shock protein (HSP70), Apetala 2(AP2), Osmotin (OSM), Dehydrin (DHN)) in correlation with its physiological parameters (RWC and cell membrane leakage), revealed the relationship between gene expression and physiological parameters. Higher RWC and lower membrane leakage have been associated with drought-tolerant genotypes, which exhibited

upregulated expression of DHN and OSM genes, as well as downregulated expression of AQUA and bZIP genes when compared to susceptible genotypes. It may help in screening black pepper genotypes based on drought tolerance conferred through the upregulation or downregulation of these genes accompanied by the identification of promoters and single nucleotide polymorphisms of these genes to further elucidate the molecular processes underlying drought tolerance (George *et al*., 2017).

In terms of biotic resistance, Suraby *et al*. (2020) identified 23 nucleotide-binding site (NBS) regions in black pepper, named PnRGAs, with conserved motifs similar to non-TIR NBS-LRR R genes. PnRGA24 in Piper colubrinum showed a 22-fold increase in expression as a resistant response to the pathogen Phytophthora capsici. The analysis of gene expression stability identified the most stable reference genes for qRT-PCR, PnGAPDH and PnUBCE, confirmed by multiple methods, yielding consistent results in the expression profiling of PnBGLU under pathogen stress (Umadevi *et al*., 2019). A multiplex PCR assay efficiently detected the pathogens Phytophthora, Pythium, and Fusarium in black pepper using genus-specific primers from the ITS region and including black pepper 18S rRNA gene-specific primers as an internal control (Jeevalatha *et al*., 2022).

Yield and quality parameters

The yield of black pepper primarily relies on four key factors: its inherent genetic constitution, the age of the pepper vine, the surrounding environmental conditions, and the fertility of the soil (Menon, 1949). Temperature extremities imply a significant impact on black pepper production compared to annual precipitation and relative humidity, emphasizing the importance of maintaining an optimum temperature range while implementing agronomic practices such as reflective mulching and proper irrigation to mitigate reduced productivity (Nair *et al.*, 2021).

Several mitigating strategies were adopted for black pepper production under drought stress, such as improving the timing of summer irrigations, spraying lime (1.5%) or kaolin (2%) with 0.5% muriate of potash, managing crop canopies, implementing drip irrigation, reducing spike shedding through improved irrigation frequency and timing followed by shade regulation, applying fertilizers through drip irrigation, optimizing

soil nutrient movement through drip fertigation, and monitoring evapotranspiration using methods like Bowen Ratio-Energy Balance (BREB) and Vapour Diffusion Model (VDM) to provide efficient guidance in irrigation scheduling (Krishnamurthy *et al*., 2020).

Prolonged drought stress in summer has led to the cessation of elongation in spike length and reduced berry development. Mixed cropping enhances black pepper growth more effectively when combined with coffee cultivation compared to mono-crop cultivation under the same circumstances (Rao, 2016).

Molecular technology provides epigenetic tools that offer promising solutions for improving crop yield and resilience to environmental stresses via DNA methylation, histone modifications, and RNA interference, facilitating the development of stress-resistant crops with increased productivity and an improved comprehension of genetic pathways for crop improvement (Sun *et al.*, 2021).

Many yield-contributing traits were positively correlated with each other and with overall yield, including lateral branch length and the number of nodes per lateral branch, juvenile leaf length and adult leaf length, juvenile leaf length and leaf width, petiole length and spike length, spike length and the number of matured berries per spike, the number of spikes per plant and the number of berries per plant, as well as fresh berry weight and dry weight, and fresh spike weight and dry weight. Hence, morphological characters such as leaf width, petiole length, and lateral branch length, along with yield-contributing traits like the number of spikes per lateral branch, number of spikes per vine, fresh spike yield, fresh berry yield, 100 fresh berry weight, 100 fresh berry volume, and dry recovery, can serve as key indicators for selecting genotypes with higher yields (Reshma *et al*., 2022).

Shango *et al.* (2020) found correlations between morphological characteristics and black pepper yield, both positive and negative, focusing on local varieties like Babu kubwa, Babu ndogo, Babu kati, and Ismailia. Babu kubwa displayed superior traits such as longer spike and petiole length, broader leaves, and larger berries compared to other varieties. Babu kubwa and Ismailia stood out for higher flower production and heavier spikes, while Ismailia and Babu ndogo had compactly arranged spikes.

Ismailia also had the highest weights for both 100 fresh spikes and 1000 fresh berries. Babu kati, despite lighter spikes, had the highest spike density. Notably, longer spikes were positively correlated with yield, while higher yields were associated with fewer spikes per kilogram and lighter 1000 berries, which might seem counterintuitive initially.

A study was conducted on two black pepper varieties, 'Kuching' and 'Semongok Aman', where different fertilization methods, including chemical fertilizer, foliar fertilizer, and integrated fertilizer, were applied to investigate the variations in yield formation when compared to the control group. A 72% higher yield in black pepper was observed with the integrated fertilizer treatment compared to foliar fertilizer, and it also demonstrated a 15% increase in yield compared to chemical fertilizer. These positive changes in physical characteristics, such as stem growth rate, photosynthetic pigment efficiency, and improved leaf gas exchange, contributed to the overall growth and yield increase when the integrated fertilizer was applied to the soil (Ann, 2019).

The influence of nitrogen level variations, encompassing very deficient N, deficient N, and conventional N, on muskmelon cultivation in Almeria, Spain, was evident in the remarkable increase in yield upon applying 8.2 mmol per liter of nitrogen. Conversely, the application of deficient nitrogen resulted in the formation of misshapen muskmelon fruit. Importantly, the reduction in nitrogen application did not entail any compromise in either yield or fruit quality for muskmelon (Grasso *et al*., 2022).

A rod-shaped, Gram-negative strain, *Bacillus* species 'NII-0943', was isolated from a bacterial culture obtained from the Western Ghats. This strain has the capability to solubilize phosphorus into a form that is readily available to plants. NII-0943 can grow without nitrogen, produce indole acetic acid (IAA) and siderophores, and solubilize tri-calcium phosphate. It serves as a promising plant growth-promoting inoculant, as evidenced by the increased root initiation, as well as enhanced soil nitrogen and phosphorus levels, when black pepper cuttings were inoculated with it. Hence, the pot experiment demonstrated that the NII-0943 strain has the potential to

enhance black pepper growth and production, serving as a biofertilizer (Dastager *et al*., 2011).

Coconut-based high-density multi-species cropping systems consisting of various crops, including vegetables, fruits, and spices, with black pepper as the vital spice crop were assessed for yield and quality parameters over four years. Significantly improved yields and quality were observed for both black pepper and coconut under various nutrient management regimes (Maheswarappa *et al*., 2016).

To optimize nutrient management for black pepper cultivation, a phased approach is recommended. In the first year, one-third of the dosage should be applied, followed by two-thirds in the second year, and the full dose from the third year onwards, while avoiding direct contact with the roots. The study also revealed that among the micronutrients, zinc and magnesium enhance both quality and yield (Ravindran & Kallupurackal, 2005)

Application of magnesium and boron in laterite soil increases the levels of these nutrients in leaves at all stages of growth. This enhanced availability of magnesium and boron, when combined with exogenous application, improves berry formation, spike length, the number of spikes per plant, as well as the quality parameters viz. oleoresin and piperine (Gladis & Nagula, 2016). For sustainable black pepper production, integrated fertilizer application combining both organic and inorganic components is required to ensure viability (Ann, 2012).

Exogenous application of plant growth hormones (NAA, GA3, BA, and 2,4-D) to evaluate their influence on yield and yield-attributing traits in the black pepper variety Panniyur-1 revealed that among these hormones, the synthetic auxin hormone NAA significantly contributed in improving yield, as well as the number of berries per spike, volume, and weight of berries (Kumar *et al*., 2002).

Naik *et al.* (2013) reported that analysis of four years of pooled data from an experiment with selected landraces of black pepper genotypes in a mixed crop system with arecanut which were used to screen for high-yielding varieties and disease tolerance among six promising black pepper genotypes showed that the cultivars

Ademane and Kudragutta performed better with lower PDI and higher sustainable yields. They could be considered as new lines for mixed cultivation with arecanut.

Hailemichael *et al.* (2011) reported that the sufficient oleoresin yield ranged from 13.63% to 16.01%, and essential oil yield ranged from 3.18% to 3.53% at the appropriate stage of harvest. The optimum yield of both oleoresin and oil can be obtained from berries harvested at the 5.5-month stage after fruit set. The highest yield of oil (4.95%) and oleoresin (19.41%) was obtained at the earliest harvest, which occurred 3.5 months after fruit set, with decreased yields observed from delayed harvest. Black pepper berries with a thick pericarp yield more oil and oleoresin than those with a thin pericarp, and little to negligible decrease in yield was reported for the latter (Somashekar *et al*., 2021).

Evaluation of black pepper varieties for growth and yield parameters in bush pepper showed that Panniyur-5 exhibited superiority in terms of internodal length (9.02 cm), bush length (103.52 cm), number of branches (63.8), primary (3.6), secondary (28), and tertiary (32.2) branches, as well as the number of spikes per bush (17), spike length, spike weight (21g), spike yield (357g), and number of berries per spike (32.67) over Panniyur-2 and Panniyur-4. This observation underscores a significant disparity in performance among the varieties across various agro-ecological conditions (Bhattacharya & Bandyopadhyay, 2017).

A comparative study conducted on the Ciinten variety to assess the variations in black pepper productivity across different agro-ecological locations in Sukabumi, Purwakarta, and Ciamis districts over two harvesting seasons Revealed that Ciinten variety in Sukabumi district exhibited considerably higher yield than in other two districts. The elevation and annual rainfall in Sukabumi nearly meet the conditions essential for black pepper cultivation (Bermawie *et al*., 2019).

In a study conducted in Uttara Kannada, Karnataka, 22 black pepper genotypes were evaluated for yield and quality parameters. Panniyur-1, (considered as check), recorded the highest fresh spike yield, fresh berry yield, and dry berry yield. Genotype SV-21 exhibited significant black and white pepper recovery, while SV-7 had higher

oleoresin content and substantial piperine content, and SV-15 had the highest bulk density (Hussain., 2017).

Narayanpur *et al*., (2021) also reported that Panniyur-1 (as check) produced highest number of spikes, fresh berry yield and dry berry yield. Whereas, the Cv. Kurimale yielded longer spikes as well as highest number of berries per spike and spike weight. Cv. Sigandini showed highest black pepper and white pepper recovery, highest piperine (5.1%) and bulk density (633.47 g/l) and Panniyur-1 had the highest essential oil (2.95%) and oleoresin content (8.78%).

Kurian *et al*. (2002) reported that there was a non-significant negative correlation between black pepper yield and quality parameters. However, a significant correlation existed among yield and yield-attributing traits. Thus, the number of spikes per plant and the number of berries per spike correlated positively with yield.

The research conducted in Vietnam compared four black pepper genotypes across three different locations, demonstrating that agroecological variations can influence the yield and peppercorn quality of black pepper. The "Vinh Linh" and "Lada" varieties performed better, with the highest yield harvested in Gia Lai and Dak Lak over the course of three years of evaluation (Oanh *et al*., 2021).

Vietnamese cultivated green pepper berries were subjected to hydrodistillation to yield seven essential oil compounds out of a total of 19 components, with the following proportions: 3-carene (35.21%), D-limonene (21.54%), β-caryophyllene (10.05%), β-pinene (9.17%), sabinene (7.37%), α-pinene (4.45%), and elemene (4.09%) (Dao *et al*., 2020).

The Nigerian Piper guineense berries from Lagos is dominated by linalool at 52.2%, making it the major contributing component that characterizes this new chemotype of the variety (Owolabi *et al*., 2013). Distribution of piperine among leaves, petioles, and fruits can be considered as the marker for identification of species (Al-Khayri *et al*., 2022).

A higher oil content, comprising major components such as α-pinene, β-pinene, sabinene, β-caryophyllene, and limonene, as well as δ 3-carene and oleoresin,

accumulated in dried matured black pepper berries. As a result, the protein content decreased by a factor of seven compared to fresh berries. This decrease in protein content may be attributed to the accumulation of oil displacing the protein (Thomas, 2014).

The potent antimicrobial and anti-proliferative activities of black pepper essential oil and oleoresin, when combined, can act as bio preservative agents (Morsy & Abd El-Salam, 2017). Piperine, when combined with pepper oil, can function together to inhibit or retard the growth and proliferation of Staphylococcus aureus, Bacillus subtilis, Salmonella species, and E. coli. This inhibition was measured using the agar well diffusion method (Hikal, 2018).

Upon analyzing the essential oil from Gia Lai Province, Vietnam using GC-MS, the major constituents, 3-carene (29.21%), D-limonene (20.94%), caryophyllene (15.05%), and β-pinene (9.77%), were found to be suitable for manufacturing insecticides and air deodorizers *(*Tran *et al*., 2019).

If the average piperine content is about 6%, piperine content in the form of oleoresin will be in the range from 25.74 to 48.32% (Dang *et al*., 2014). Piperine comprises approximately 98% of the total alkaloids (Gorgani *et al*., 2017). The black pepper ideotype should have a minimum of 5% piperine (Krishnamurthy *et al*., 2010).

Ashokkumar *et al.* (2021), reported β-caryophyllene, limonene, sabinene, α-pinene, β-bisabolene, and α-copaene as the major essential oil components and have the potential to serve as organic sources of precursors in the food, cosmetics, and pharmaceutical industries.

According to Lomarat *et al*. (2015), black pepper essential oil's major components, including δ-3-carene, limonene, (-)-β-pinene, α-pinene, and caryophyllene, were identified among 33 components through GC-MS analysis. Limonene displayed strong inhibitory activity against β-amyloid accumulation, while caryophyllene, along with the oil, exhibited COX-2 inhibition. These findings suggest that black pepper oil, with its AChE (acetylcholinesterase) inhibition and anti-inflammatory actions via COX-2 inhibition, may effectively reduce the risk of Alzheimer's disease.

Organic compounds such as, monoterpenes and sesquiterpenes from the oleoresin could support the immune system. Pepper essential oil functions as a natural antioxidant, displaying a wide range of efficacy against active free radicals. The ratio of monoterpenes to sesquiterpenes is a critical element in determining the quality of both oil and oleoresin. The essential oil components α- pinene, sabinene, β-pinene, δ-3-carene, limonene, and β-caryophyllene make a significant contribution to the overall composition (Dosoky *et al*., 2019). A higher proportion of sesquiterpene hydrocarbons was extracted through supercritical carbon dioxide extraction compared to the hydrodistillation method, possibly due to the increased temperature and pressure (Kumoro *et al*., 2010).

An investigation comparing the intracellular and *in vitro* antioxidant properties of essential oil extracted from white and black pepper (*Piper nigrum* L.) revealed that these pepper essential oils possess a significant antioxidant impact mainly attributed to 3-carene, which displayed more potent antioxidant activity than the caryophyllene found in white pepper (Wang *et al*., 2021).

A comparison of the antioxidant potential between mustard and black pepper oil and oleroresin revealed that black pepper exhibited higher activity, as determined by analyzing the oil-oleoresin constituents of mustard using peroxide, p-anisidine, and thiobarbituric acid tests. The GC-MS analysis of black pepper volatile oil revealed that the major component was β- caryophylline (29.9%), followed by limonene (13.2%), β-pinene (7.9%), sabinene (5.9%), and numerous other minor constituents. Antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) exhibited a slight decrease in activity compared to the major constituents in pepper oil and oleoresin (Kapoor *et al*., 2009). Various factors such as genetic makeup, nutrition, environmental conditions and the stage of development of berries are known to influence essential oil yield as well as composition.

CHAPTER 3
MATERIALS AND METHODS

The present research, entitled 'Physiological and molecular characterization of black pepper genotypes subjected to limited water availability,' was aimed at understanding the morphological, physiological, biochemical, and molecular changes due to water stress in black pepper genotypes to identify those with more drought-tolerant characteristics. The experimental locations for the current study were selected at ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode, Kerala and its experimental farm at Peruvannamuzhi and the experiments were conducted during 2020 to 2024. The field experiment conducted at Peruvannamuzhi, is situated in the foothills of the Western Ghats in Kerala, located at an altitude of 60 meters above sea level. It experiences an average annual rainfall of 4300 to 5300 mm and a temperature range of 23°C to 40°C. The soil at this location is acidic, with a pH range of 4.0-4.7. Sunshine hours vary from 45 to 260 hours per month.

Four genotypes exhibiting drought-tolerant characteristics and two genotypes displaying susceptibility were selected from the screened black pepper germplasm. These selected genotypes underwent further evaluation under water stress treatments, which encompassed assessments of physiological, biochemical, and quality parameters. Eight replicates of each accession were maintained at the Indian Institute of Spices Research under different moisture levels achieved through manual irrigation in grow bags. Irrigation was withheld for up to 28 days, and readings were taken at intermittent intervals, specifically at 7, 14, 21 and 28 days after stress (DAS). Properly irrigated plants were used as the control group with four replications. The experiment followed a factorial randomized block design with a total of 8 replicates. The same genotypes were characterized at the molecular level regarding drought-responsive genes while being maintained at different moisture levels, specifically 25%, 50%, and 100% of field capacity. Drought-specific primers were designed to identify SNPs in

those accessions, distinguishing genotypes with drought-tolerant and susceptible characteristics.

3.1 OBJECTIVES

To study the genetic variation for morphological and yield attributing traits in a few selected drought tolerant accessions.

Forty black pepper (*Piper nigrum* L.) germplasm accessions from the National Active Germplasm Site of ICAR-IISR, Kozhikode, Kerala, India, were selected to fulfill this objective. Morphological traits, including leaf length, leaf width, leaf area, petiole length, and internodal length and physiological traits, such as wax content and stomatal density were recorded.

List of accessions used: 4064, 4137, 1315, 6720, 1439, 5621, 1218, 5083, 1491, 1487, 1495, 931, 4132, 1093, 5642, 8060, 807, 6707, 1476, 1368, 4095, 6786, 7211, 5623, 8052, 4177, 1248, 971, 5717, 4226, 4216, 4152, 891, 1086, 5691, 1390, 6774, 1638, 1343, 813.

Among the forty genotypes, 21 were chosen, for evaluating their yield-attributing traits (number of spikes plant-1(NSP), spike length (SL), peduncle length (PL), berry size (BS), number of matured berries spike-1 (NMB), number of immature berries spike-1(NIB), test weight (TW), 100 berry fresh weight (BFW), 10 spiked berries weight (SBW) and 10 rachis weight (RW)) and quality parameters (starch, sugar, essential oil, piperine and oleoresin).

To study the physiological and molecular mechanism in selected drought tolerant genotypes.

To achieve this objective six genotypes viz 7211, 1495, 1343, 4132, 5717 and 4064 were used. The parameters studied were RWC, membrane leakage and leaf photosynthetic pigments.

To investigate influence of water stress on partitioning of dry matter, yield and quality parameters in selected drought tolerant genotypes.

Six genotypes viz 7211, 1495, 1343, 4132, 5717 and 4064 were used. Various parameters such as root length, root fresh weight and dry weight, root volume and number, root diameter, shoot volume and number, shoot diameter, shoot fresh weight and dry weight, root to shoot ratio and quality parameters such as piperine, oil, oleoresin etc. were determined.

3.2 MATERIALS

3.2.1 Instruments/apparatus used for the study

Weighing balance	:	Precisa XB220A electronic weighing balance
	:	Laboratory balance digits (Safferon) MSN-9016
Moisture balance	:	Precisa moisture analyser, XM 60-HR
Electrical conductivity meter	:	Eutech CON 700 meter
UV-VIS Spectrophotometer	:	Thermoscientific Gene SYS- 50
	:	UV- 1800 Shimadzu UV Spectrophotometer
Nano-drop spectrophotometer	:	Denovix DS II
pH meter	:	Eutech
Refrigerated centrifuge	:	REMI- C24 PLUS
Mechanical shaker	:	Rocker shaker 300B7, MACRO SCIENTIFIC works
Rotary evaporator	:	Model RT05025, Aditya Scientific made in china
Clevenger trap apparatus	:	ROTE, Soxhlet extraction mantle
Water bath	:	ROTEK
	:	Wise bath 300B8
Ultra deep freezer (-70^{0}C)	:	SANYO Ultra low deep freezer
Deep freezer (-20^{0}C)	:	ARCTICO Deep freezer 18593
Hot air oven	:	LABLINE
Autoclave	:	Equitron, SLEFA
GCMS	:	Shimadzu, QP2010

HPLC	:	Shimadzu preparative HPLC
LCMS	:	Shimadzu 8045
Nitrogen evaporator	:	Athena tech
Sonicator	:	Ultrasonic cleaner- Sonica
Leica compound microscope	:	Leica DM5000B microscope
PCR	:	Invitrogen proflex 3x32- well PCR system E
	:	IGENE g6 GEP thermocycler
Real time PCR	:	Qiagen Rotor gene Q
Multina analyser	:	Shimadzu MCE-202 MultiNA
Electrophoretic unit	:	GeNei ™
Gel doc	:	Bio-RAD Chemi DOC™ XRS with image Lab™software
Ice flaker machine	:	BREMA ice maker
Magnetic stirrer	:	SPINOT HOTPLATE
Vortexer	:	SPINIX
Ultrapure (Type 1) water	:	Direct Q3

3.3 OBSERVATIONS RECORDED

Experiment 1

Forty selected black pepper accessions were used for initial characterization.

3.3.1 Biometrical recordings

The biometric observations of 17 quantitative traits were monitored and recorded including, leaf length (LL), leaf width (LW), leaf area (LA), petiole length (PL), internodal length (IL), wax content (WC),number of stomata (NS), number of spikes plant-1(NSP), spike length (SL), peduncle length (PL), berry size (BS), number of matured berries spike-1 (NMB), number of immature berries spike-1(NIB), test weight (TW), 100 berry fresh weight (BFW), 10 spiked berries weight (SBW) and 10 rachis weight (RW).

3.3.1.1 Analysis of morphological characteristics

Morphological traits, including leaf length, leaf width, leaf area, petiole length, and internodal length, were measured using a centimeter ruler. Four replicates of two random leaves were taken from each accession for the measurements. All the remaining morphometric measurements were taken with the same number of replications. A ruler divided into centimeter increments was used to make all the measurements in the present study.

3.3.1.1.1 Leaf length (cm)

Leaf length was measured from the point where the petiole joins the leaf base to the leaf apex (leaf tip). The measurement was taken from the third leaf from the top of the plant.

3.3.1.1.2 Leaf width (cm)

The two points on the leaf blade edges that formed the widest distance were considered as the leaf width, which is perpendicular to the leaf length axis.

3.3.1.1.3 Leaf area (cm^2)

The product of measured leaf length and leaf width was multiplied by constant (0.67) was calculated to estimate the leaf area.

3.3.1.1.4 Petiole length (cm)

The petiole length was assessed by measuring the distance starting from the leaf base and ending at the point where the petiole is connected to the stem.

3.3.1.1.5 Internodal length (cm)

Internodal length measurement was assessed between the consecutive nodes, with the node being the point to which the leaf is attached.

3.3.1.2 Analysis of physiological characteristics

3.3.1.2.1 Leaf wax content

To quantify the epicuticular wax load, the colorimetric approach developed by Ebercon *et al.* (1977) was used. A 4.84 square centimeter area of a black pepper leaf was removed from the central region of the leaf, excluding the midrib portion, and prepared for wax extraction. The excised leaf piece was immersed once for 10 seconds in a 15 ml beaker containing 5 ml of chloroform. The chloroform was allowed to evaporate by boiling the content in a water bath at 70 ^{0}C until it completely evaporated. Prepared 5 ml wax reagent was added to the chloroform-evaporated beaker and boiled in a steaming water bath for 30 minutes. After the leaf wax- wax reagent mixture had cooled, 12 ml of deionized water was poured into it. The colour intensity was determined by measuring the spectrophotometric reading of the collected filtrate at 590 nm. The leaf wax content of each genotype was evaluated using four different sample sets to ensure accuracy.

Preparation of wax reagent: 20 g of powdered potassium dichromate and 40 ml of distilled water were blended to create a slurry, which was then mixed with 1 liter of 98% sulphuric acid to prepare the wax reagent. Heated the resultant slurry until a clear solution was obtained. Carnauba wax was used as the standard to create the graph.

3.3.1.2.2 Leaf stomatal density

A viscous slurry was prepared by melting thermocol in xylene to take stomatal imprints (Pawar & Gadakh, 2018). Prepared liquid suspension was thinly applied to both the abaxial and adaxial surfaces at the center portion of each leaf. The dried coat of slurry was gently peeled off from the leaf after 10-15 minutes. The dried transparent layer was placed at the center region of the slide, which was covered by a cover slip. The prepared glass slide was viewed under the compound microscope from LEICA, Wetzlar, Germany. The stomatal density was counted from three randomly selected microscopic fields within each of the four replications for each genotype. This counting was done under 10X magnification with an image size of 391.634 µm x 522.517 µm.

3.3.1.3 Analysis of yield attributing traits

The pepper vines harvested during November 2022-23 underwent detailed observations and were recorded.

3.3.1.3.1 Number of spikes per plant

Spike counts from each plant were collected and counted separately in four replications for each accession.

3.3.1.3.2 Spike length (cm)

The distance from the point at peduncle to the tip of spike was measured

3.3.1.3.3 Peduncle length (cm)

The peduncle length was measured from the primary or secondary stem to the peppercorns, and it represents the slender stalk supporting the inflorescence.

3.3.1.3.4 Berry size (mm)

The dimensions of 5 black pepper matured berries from each variety were determined by using a Vernier caliper with a precision of ±0.01 mm. The measurements were taken along both the axial and transverse axes.

Following mathematical formula was used to calculate the size of the berries.

Berry size (mm) = main scale division + (vernier scale division × least count)

3.3.1.3.5 Number of matured berries per spike

The number of matured berries was separately counted from each spike in four replications, with five spikes in each replication.

3.3.1.3.6 Number of immature berries per spike

The remaining number of immature berries was counted among the mature berries harvested from 5 spikes per plant across four replications.

3.3.1.3.7 Number of berries per spike

The number of berries in a spike, including both matured and immature berries, was counted from 5 spikes per plant in each of the four replications.

3.3.1.3.8 Test weight or 100 berry fresh weight (g)

One hundred detached mature berries were counted and their weight measured. This process was repeated in five replicates, with each replicate involving 100 fresh mature berries from four different plants.

3.3.1.3.9 Ten spiked berries weight

The weight of ten fully developed spiked berries was recorded separately in four replications per plant.

3.3.1.3.10 Ten rachis weight

Berries were removed from ten fully developed spikes and the weight of these ten rachises was measured separately in four replications.

3.3.1.4 Quality parameters

The quality parameters studied were oleoresin, essential oil, piperine, berry sugar and berry starch content.

3.3.1.4.1 Oleoresin

The ASTA (1975) method was employed to estimate the oleoresin content in black pepper berries. A glass column was filled with cotton at the bottom and 10 g of finely powdered black pepper berries. 50 ml of acetone was added to this column and left overnight undisturbed. The next day, the oleoresin-acetone blend was collected in a pre-weighed empty beaker by opening the tap of the column. Another 30 ml of acetone was added to the column, and was incubated for 1 hour for complete extraction. This process was repeated until the brown colour washed off from the cotton. The total solvent obtained was evaporated to dryness by placing the beaker over a boiling water bath. The weight of the empty beaker was subtracted from the weight of the beaker

containing oleoresin, and the oleoresin percentage was calculated using the following equation.

Oleoresin (%) = weight of the residue (g) / weight of the sample (g) × 100.

3.3.1.4.2 Essential oil

Extraction

The essential oil was extracted from black pepper berries in accordance with ASTA (1968) using a modified Clevenger apparatus. The Clevenger apparatus consists of a 1000 ml round-bottom flask, trap, and condenser. Half of the volume of the round-bottom flask was filled with distilled water, along with 30 g of powdered berry. Subsequently, the flask, trap, and condenser were positioned properly. The power was turned on for a 3-4 hour distillation, and the extraction time was noted when the first drop of condensed essential oil started collecting over the water. The vapour mixture of water and oil, freed from the oil gland due to boiling, was condensed through indirect cooling with water. Due to the immiscibility and lightness of the oil, a distinct layer of oil on the water layer was collected in the trap. The base layer of water was removed, and the remaining oil was collected in a 5 ml Eppendorf tube containing anhydrous sodium sulfate to remove residual moisture. The oil was kept in the refrigerator until the GC-MS (Gas Chromatography Mass Spectroscopy) analysis was completed. The essential oil content was calculated and expressed as a percentage.

Essential oil (%) = volume of oil extracted (ml) / weight of sample (g) × 100.

GC-MS analysis of essential oil volatiles

The Shimadzu QP-2010 instrument was used to analyse the essential oil, employing an Rtx-5 column with a length of 30 m, a diameter of 0.25 mm, and a thickness of 0.25 µm. The column temperature was initially set at 60°C for 5 minutes, then increased to 110°C at the rate of 5°C/min, further raised to 200°C at the rate of 30°C/minute, and finally reached 240°C at the rate of 5°C/min. The components were detected based on the electron ionization principle with an ionization energy of 70 eV. The carrier gas used here was helium, with a flow rate of 1 ml/min. The final analysis

took 55 minutes, with mass scanning ranging from 60 to 400 amu and a 3-minute solvent delay. Then 1 µl oil sample was injected for the analysis. The oil components were identified and were compared with the mass spectra of compounds listed in the National Institute of Standards and Technology library (www.nist.gov) and the WILEY library (sciencesolutions.wiley.com). Subsequently, these comparisons were further assessed against the components detailed in Adam's book (Adams, 2005). The proportion of each component was expressed as a relative composition of cumulsative peak area and calculated accordingly,

Relative % of peak area = (specific peak area / total peak area) × 100

3.3.1.4.3 Piperine

Extraction

Filtered denatured alcohol in the proportion of 1 (Methanol): 20 (Ethanol) was used for piperine extraction. 0.5 g of powdered black pepper berries was weighed and placed in a 250 ml round bottom flask with 70 ml of denatured alcohol. The mixture was refluxed for 1 hour and then allowed to reach room temperature. The extract was filtered using Whatman No. 1 filter paper into a 100 ml standard flask and finally made up to the mark with denatured alcohol. Further dilution was performed by making up to a 25 ml standard flask with 0.5 ml of the extracted sample. The second diluted solution was used for absorbance reading at 340 nm using the Shimadzu UV—Visible spectrophotometer (UV-1800) against denatured alcohol as the blank. The piperine content in the sample was estimated using the standard graph and expressed as a percentage.

Standard stock preparation: A first stock was prepared at a concentration of 1 mg/ml in the required volume. The second stock was then prepared at a concentration of 0.1 mg/ml. Further dilutions from the second stock were made in a series of 1, 2, 3, 4, and 5 µg/ml concentrations. The corresponding absorbance readings were used to plot the standard graph.

HPLC analysis of piperine

The previously mentioned first diluted sample in piperine extraction was used in the HPLC for the determination of piperine. This filtered extract was further filtered through a 0.25 µm membrane filter and collected in 1.5 ml microcentrifuge tubes. Twenty µl sample was injected directly to the HPLC system for the analysis.

HPLC conditions

The instrument used was the Shimadzu High Performance Liquid Chromatography (HPLC) with an SPD-10A UV-visible detector, employed to analyze and calculate the piperine content in the sample. The column used for the separation of the compound was Purospher® STAR RP-18 end-capped, with a 5 µm particle size and dimensions of 250 mm length x 4.6 mm diameter. The mobile phase consisted of acetonitrile: water: acetic acid in the ratio of 60:39.5:0.5, respectively. The mobile phase solvents were separately pre-filtered, mixed in the mentioned proportion, and sonicated just before the run. The instrument parameters were conditioned with isocratic elution at a flow rate of 1 ml/min; run time was set at 20 minutes, and the detection wavelength was established at 340 nm.

$$\text{Piperine Content}(\%) = \frac{\text{Absorbance of sample} \times \text{Factor} \times \text{Volume}}{\text{Weight of Sample} \times 10^6} \times 100$$

Where,

F (Factor) = 1/Absorbance of 1 ppm standard piperine at 343 nm

V = Volume of solution (100 ml) and 0.5 ml diluted to 25 ml

Standard stock preparation: Quantification of piperine was carried out by comparing it with the constructed calibration graph of standard piperine. The standard piperine solutions were injected at five concentration levels of 0.03125, 0.0625, 0.125, 0.25, and 0.5 mg/ml.

Experiment 2

3.3.2 Analysis of physiological characteristics

3.3.2.1 Soil moisture content

The estimation of soil moisture content was accomplished by employing both moisture balance and gravimetric methods to ensure accuracy and confirmation. Soil moisture content was measured at intervals ranging from 7 to 28 days after stress treatment. Physiological and biochemical parameters described below were evaluated to assess changes in black pepper genotypes at intervals of 7, 14, 21 and 28 days.

Moisture balance method:

The aluminum plate was weighed on the moisture balance, and its weight was tared. Freshly taken fine soil particles were placed on the aluminum plate, and the weight was adjusted to 5g by adding soil. Subsequently, the soil was uniformly spread around the plate before the reading was taken. The soil moisture content appeared on the moisture balance screen after a few minutes, expressed as a percentage. Three replicates were taken to determine the moisture percentage.

Gravimetric method:

As per the method described by Arnold (1995), the collected soil sample was weighed and kept in a hot air oven at 85 °C until the dry weight of the soil remained constant. The following formula was used to calculate the soil moisture content,

$$\text{Water Weight Ratio (\%)} = \frac{\text{Weight of moist soil} - \text{Weight of dry soil}}{\text{Weight of moist soil}} \times 100$$

3.3.2.2 Relative water content

Relative water content was estimated in accordance with the Fletcher *et al*. (1988). 0.1 g of a fresh leaf sample was weighed and incubated in 15 ml of deionized water for 3 hours. Then, the water was drained off and the leaf pieces were gently blotted with tissue paper to completely remove the water and weighed to determine its turgid weight. The squeezed leaf pieces were then dried in an oven at 85 °C for 72 hours,

and the dry weight was recorded. Relative water content was determined using the formula,

$$\mathrm{RWC}(\%) = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Turgid Weight} - \text{Dry Weight}} \times 100$$

3.3.2.3 Electrical conductivity

A conductivity meter was used to measure the electrical conductivity (EC) of 15 ml of deionized water in which 0.1g of leaf pieces was incubated for 3 hours to determine the initial conductivity (EC 1). The leaf sample, which was incubated in 15 ml of water, was boiled in a water bath at 100°C for 30 minutes. The electrolytes released from the leaf into the boiled water were used to measure the final conductivity (EC 2). The electrolyte leakage was determined using the formula provided by Dionisio-Sese and Tobita (1998) and expressed as a percentage.

$$\mathrm{EC}\ (\%) = (\mathrm{EC1}/\mathrm{EC2})\ \mathrm{X}\ 100$$

3.3.2.4 Leaf photosynthetic pigment

The Hiscox & Israelstam (1979) method was utilized to determine the chlorophyll pigments (a and b), total chlorophyll content, and carotenoids in a 0.1g leaf sample. Dimethyl sulfoxide (DMSO) preheated to 65°C was prepared to incubate the excised leaves for 48 hours in dark conditions. The completely decolorized leaf samples were subjected to spectrophotometric readings at different wavelengths, including 480 nm, 510 nm, 645 nm, 652 nm, and 663 nm. The chl a, chl b, total chlorophyll, a/b ratio and carotenoid contents were determined as per the following formula and expressed in mg/g fresh weight.

$$\text{Chl. a} = 12.7\ (A_{663}) - 2.69\ (A_{645}) \times 25/\ (1000 \times W)$$

$$\text{Chl. b} = 22.9\ (A_{645}) - 4.68\ (A_{663}) \times 25/\ (1000 \times W)$$

$$\text{Total chlorophyll} = [(OD_{652} \times 1000)/34.5]\ [25 \times 1000 \times W]$$

$$\text{Chl. a/b ratio} = \text{chl a/chl b}$$

$$\text{Carotenoids} = [(OD_{480} \times 7.6) - (OD_{510} \times 1.49) \times V/ (1000 \times W)]$$

Where, W = Weight of leaf sample taken (g)

V = Volume of solvent taken (ml)

3.3.2.5 Leaf stomatal aperture measurements

The previously described method of Pawar & Gadakh (2018) was employed to obtain stomatal imprints from the abaxial surface for aperture measurements. Stomatal imprints of fully developed leaves (third leaf from the tip) were taken for each replication between 10:30 am and 11:30 am. Stomatal apertures (pore length and pore aperture) were measured using a calibrated compound microscope from LEICA, Wetzlar, Germany. Measurements were taken from three randomly selected stomatal images for each genotype in three replicates. The microscope was set to 40 x magnification with a selected image size of 391.634 µm x 522.517 µm for the measurements.

3.3.3 Analysis of biochemical characteristics

3.3.3.1 Leaf protein

The total protein was extracted and estimated as per the method of Lowry *et al.* (1951).

Reagents

- Extraction buffer:

A- 0.2 M NaH_2PO_4 was prepared by dissolving 5.99 g in 250 ml of solvent

B- 0.2 M Na_2HPO_4 was prepared by dissolving 7.098 g in 250 ml of solvent

Solutions A and B were combined in proportions of 39 ml and 68 ml, respectively. The pH was adjusted to 7 with 0.1N HCl, and finally, the mixture was made up to 200 ml with a 0.2 M solution.

- Reagent A: 1 g of Na_2CO_3 with a 2% concentration was dissolved in 50 ml of 0.1 N NaOH.

- Reagent B: 0.25 g of $CuSO_4 \cdot 5H_2O$ with a 0.5% concentration was dissolved in 50 ml of potassium sodium tartrate with a 1% concentration.
- Reagent C: It was obtained by combining 50 ml of reagent A with 1 ml of reagent B
- Reagent D: Folin–Ciocalteau reagent (FCR) was prepared in 1:1 ratio.
- Standard: Bovin serum albumin

Extraction

A 0.1 g leaf sample was weighed and ground in 5 ml of extraction buffer using a mortar and pestle. The mixture was then centrifuged at 10,000 rpm for 15 minutes at 40 °C, and the supernatant was used for protein estimation.

Estimation

A 0.1 ml leaf protein extract was pipetted out using a micropipette into a test tube and then adjusted to a total volume of 1 ml. A 5ml of reagent C was added and left for a 10-minute incubation. Then 0.5 ml of reagent D was added into the test tube, vortexed the reaction mixture, and incubated the test tube at room temperature in the dark. A blue color developed indicating protein and the protein content was detected at 660 nm using UV-visible spectrophotometer against a reagent blank without having protein extract.

Standard stock preparation: For the first stock preparation, 50 mg of bovine serum albumin (BSA) was weighed dissolved in distilled water, making up the volume to 50ml in a standard flask (1 mg/ml). Working standards were prepared within a concentration range from 40 µg/ml to 200 µg/ml. The amount of protein in the sample was calculated by plotting a standard curve with BSA concentrations on the X-axis and their corresponding absorbance values on the Y-axis and using it as a reference.

3.3.3.2 Starch

Starch was extracted from both black pepper leaves and berries and estimated using the method of Hodge *et al*. (1962).

Reagents

- Extraction solvent: 80% hot ethanol
- Anthrone reagent: Weighed 200 mg of anthrone and dissolved it in 100 mL of ice-cold 95% sulfuric acid.
- 52% perchloric acid
- Standard: Glucose

Extraction

Samples (0.1 g) were homogenised with 5 mL of hot ethanol and centrifuged at 6000 rpm and 30°C for 5 minutes and the supernatant was collected which contained the sugars. The retained residue was repeatedly treated with the same solvent 2-3 times until the washings no longer gave color with the anthrone reagent. The residue was dried in a 60°C water bath until complete evaporation. Then, 5 ml water and 6.5 ml of 52% perchloric acid were added to the residue and vortexed well. Then the extract was kept at 4 °C for 20 minutes and later centrifuged at 6000 rpm for 20 minutes. The supernatant was collected and retained. The extraction was repeated with fresh perchloric acid 2-3 times, pooled the supernatants, and finally made up the volume to 100 ml in a standard flask.

Estimation

The supernatant (0.1 ml) was pipetted out to the test tube and made up the volume to 1ml with water. To this, 4 ml anthrone reagent was added and heated in boiling water bath for 8 minutes. The reaction mixture was cooled rapidly and the absorbance of the green to dark green color was read at 630 nm using a UV-visible spectrophotometer against a reagent blank without the starch extract.

Standard stock preparation: A standard glucose solution, with a concentration of 1 mg/mL, was prepared by diluting 100 mg of standard glucose to a total volume of 100 mL in a standard flask as a primary stock. 10 ml of the stock solution was pipetted to make the volume in a 100 ml standard flask, resulting in a concentration of 0.1mg/ml. The working standards ranged from 20 µg/mL to 100 µg/ml. The amount of starch present in the sample was calculated using the standard graph.

3.3.3.3 Proline

Bates *et al.* (1973) provided the protocol for estimating proline in the leaf sample.

Reagents

- Extraction solvent: 3% aqueous sulphosalicylic acid
- Acid ninhydrin: 1.25 g of ninhydrin was melted with agitation in 30 ml of glacial acetic acid and 20 mL of 6 M orthophosphoric acid. Stored at 4^0C and used within 24 hours.
- Glacial acetic acid
- Toluene
- Standard: Proline

Extraction

The sample material was weighed at 0.1 g and homogenized in 10 ml of 3% sulphosalicylic acid using a mortar and pestle. The blended sample was centrifuged at 5000 rpm for 10 minutes and the supernatant was collected for the estimation of proline.

Estimation

Two ml leaf extract was boiled at 100 ^{0}C for 1 hour with 2 ml acid ninhydrin and 2 ml glacial acetic acid in test tube. A brick red colour was developed in test tube which was placed immediately in an ice bath to terminate the reaction. Later, 4 ml toluene

was added and vortexed vigorously for 20-30 seconds and the toluene layer was separated. The OD value of the colored component was measured at 520nm using a UV-visible spectrophotometer against a blank that did not contain proline content after the sample reached room temperature.

Standard stock preparation: 1 mg of proline was weighed and made up the volume to 10 mL in a standard flask, resulting in a concentration of 0.1 mg/ml. From the first stock, working standards were prepared from range between 20 µg/ml to 100 µg/ml. The proline concentration in the leaf was estimated from the standard graph plotted with proline concentrations on the X-axis and absorbance on the Y-axis.

3.3.3.4 Sugar

Sugar estimation was conducted according to the Phenol-sulphuric method by Dubois *et al.* (1956).

Reagents

- Extraction solvent: 80% ethanol
- 5% phenol: 50 g phenol was dissolved in distilled water and made up to 1 liter
- 96% reagent grade sulphuric acid
- Standard: Glucose

Extraction

The 0.1 g leaf sample (and berry sample) was homogenized in 10 ml of 80% ethanol as an extraction solvent and then further centrifuged for 20 minutes at 2000 rpm. The resulting supernatant was used for the estimation of sugar content.

Estimation

1 ml of phenol solution and 1 ml of supernatant were mixed thoroughly, added 5 ml of sulphuric acid rapidly and agitated the mixture gently during the addition. It was incubated in a water bath at 30 °C for 20 minutes. The development of the yellow to

orange colour was monitored at 490 nm using a UV-visible spectrophotometer against a blank that did not contain sugar content.

Standard stock preparation: Standard glucose solution was prepared by dissolving 100 mg glucose in 100 ml of distilled water and from this, working standards of 50, 100, 150, 200, and 250 µg/ml were prepared. Sugar content present in the sample was measured using the graph plotted with standard concentrations on the X-axis and absorbance on the Y-axis.

3.3.3.5 Reducing sugar

A classical method of Nelson-Somogyi was used for the extraction and quantified the reducing sugar present in the black pepper berries.

Reagents

- Alkaline Copper Tartrate reagent:

A- The reagent was prepared by dissolving 2.5 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartarate and 20 g anhydrous sodium sulphate in 80 ml water and made up to 100 ml with distilled water.

B- The reagent was prepared by dissolving 15 g copper sulphate in a small volume of distilled water and 1 drop of sulfuric acid was added and volume is made up to 100 ml with distilled water.

The solutions A and B were combined in the volumes of 96 ml and 4 ml respectively.

- Arsenomolybdate reagent:

First, 2.5 g of ammonium molybdate was dissolved in 45 ml of distilled water, and then 2.5 ml of sulfuric acid was added and homogenized well. Next, 0.3 g of disodium hydrogen arsenate dissolved in 25 ml of water was added. The solution was then mixed well and incubated at 37°C for 24-48 hours.

Extraction

Weighed 0.1 g of powdered berries and homogenized them with hot 80% ethanol three times. The collected supernatant was pooled and evaporated by keeping it in a water bath at 80°C. After completely evaporating the ethanol, the retained sugar was dissolved in 10 ml of distilled water.

Estimation

A 0.2 ml extract was pipetted into a test tube and made up to 2 ml with distilled water. Then, 1 ml of alkaline copper tartrate reagent was added to it. The tubes were then placed in boiling water for 10 minutes and cooled to room temperature. Next, 1 ml of arsenomolybdic acid reagent was added to this mixture and incubated for 10 minutes. Finally, the volume was made up to 10 ml with distilled water, and the absorbance of the blue color developed was read at 620 nm.

Standard stock preparation: The first stock solution was prepared at a concentration of 1 mg/ml using standard D-glucose. Different dilutions were prepared from the stock solution and made up to 2 ml with distilled water. The reducing sugar content of the sample was calculated by comparing the absorbance of the sample with that of a calibration curve constructed using the standard, and expressed as mg/g.

3.3.3.6 Phenol

Phenol content estimated from the leaves of black pepper as per the method of Malick *et al.* (1980).

Reagents

- Extraction solvent: 80% Ethanol
- 1:1 Diluted Folin-Ciocalteau Reagent (FCR)
- 20% Na_2CO_3: 20g Sodium carbonate was dissolved in 100 ml distilled water
- Standard: Gallic acid

Extraction

0.1g leaf material was ground with 80% of 5 ml ethanol and centrifuged at 10,000 rpm for 10 minutes at room temperature. Supernatant was collected and repeated the same procedure. The supernatants were pooled for the phenol estimation.

Estimation

0.1 ml of the supernatant was pipetted in to a test tube, and 3 ml of distilled water was added to it. Equally proportioned, 0.5 ml of FCR was added to it and incubated at room temperature for 3 minutes. Then, 2 ml of 10% Na_2CO_3 was added, and the mixture was kept in the dark for 3 hours. The absorbance was measured at 650 nm using a UV-visible spectrophotometer against the blank.

Standard stock preparation: A second stock (0.025 mg/ml) was prepared from the primary stock (1 mg/ml). Working standards were prepared at concentrations of 4, 8, 12, 16, and 20 µg/ml. The phenol content was calculated from the plotted graph of standard gallic acid concentrations and their corresponding absorbance values.

3.3.3.7 Hydrogen peroxide content

Loreto & Velikova (2001) proposed method was employed for the extraction and estimation of hydrogen peroxide.

Reagents

- Extraction solvent: 0.1% (w/v) Trichloroacetic acid (TCA)
- 10 mM Potassium phosphate buffer: 1.36 g of potassium dihydrogen phosphate and 1.742 g of dihydrogen potassium phosphate were dissolved in distilled water, and adjusted the pH to 7 to make a solution with a total volume of 1 liter.
- 1 M Potassium iodide (KI)
- Standard: 30% (w/v) H_2O_2

Extraction

0.1 g of black pepper leaf sample was ground with 5 ml of ice-cold 0.1% TCA, and the mixture was then centrifuged at 12,000 g for 15 minutes at 40°C. Supernatant was used for the estimation of H2O2.

Estimation

The leaf extract (0.5 ml) was mixed with an equal volume of 10 mM potassium phosphate buffer (pH 7). The resulting mixture was properly homogenized and then mixed with 1 M potassium iodide. Absorbance at 390 nm was then taken using a UV-visible spectrophotometer against the blank.

Standard stock preparation: Hydrogen peroxide in plants was found in the range of concentrations between 0.171 to 10.26 µg/ml, along with its corresponding absorbance values obtained from standard hydrogen peroxide.

3.3.3.8 Lipid peroxidation

The protocol for lipid peroxidation, as outlined by Heath and Packer (1968), was employed to measure the Malondialdehyde content in black pepper leaves.

Reagents

- Extraction solvent: 5% (w/v) Trichloroacetic acid (TCA)
- 0.5% Thiobarbituric acid (TBA)
- 20% TCA
- Standard: Malondialdehyde (TBA)

Extraction

About 0.1 g fresh leaf material weighed and ground with 5 ml of 5% TCA. Centrifuged the homogenized sample at 12,000 g at 4^0C.

Estimation

A 5 ml reaction mixture was consisted of 4 ml of 0.5% TBA was prepared in 20% TCA and 1 ml of tissue homogenate. The homogenate was heated at 95°C in a boiling water bath for 30 minutes and immediately cooled by placing it in an ice bath. The supernatant was obtained from the centrifugation of the reaction mixture at 12,000 g for 10 minutes. The absorbance of the cooled solution was measured spectrophotometrically at 532 nm.

Standard stock preparation:

Working standards (0.2, 0.8, 1.6, 2.4, 3.2 and 4 µg/ml) of MDA was pipetted from the stock solution of MDA. The MDA content in the leaf sample was calculated from the standard graph that was plotted using the concentrations and absorbance of standard MDA.

3.3.3.9 Enzyme assays

Enzyme extraction

100 mg of freshly weighed black pepper leaves were ground with liquid nitrogen using a frozen mortar and pestle. The finely powdered leaf sample was then homogenized and immediately treated with 5 ml of sodium phosphate buffer at pH 7 and a pinch of Polyvinylpyrrolidone (PVP). Then, 30 µl of 1% ethylenediaminetetraacetic acid (EDTA) was added to it, and the mixture was centrifuged at 16,000 g for 20 minutes at 4°C. The enzyme extract obtained was further preserved at -20°C for enzyme assays. Heat killed enzyme extract served as control in all the enzyme assays.

3.3.3.9.1 Ascorbate peroxidase (APX)

Nakano & Asada (1981) proposed the APX activity assay. The 3 ml reaction mixture consisted of 0.3 ml of 1.5mM ascorbate, 1.6 ml of 0.1 M phosphate buffer at pH 7, and 0.3 ml of 1mM EDTA. A solution of 50mM H_2O_2 in phosphate buffer (pH 7) was prepared, and 0.7 ml was transferred and mixed well. Then, 100 µl of enzyme extract was immediately added to the cuvette containing the mentioned reactants. The cuvette was promptly placed in the UV-Visible spectrophotometer, and the oxidation of AsA

at 290 nm (ε = 0.0028 $M^{-1}cm^{-1}$) was monitored at 0.5 minute intervals for 3 minute reaction. The enzyme activity of APX was expressed as mol/min mg enzyme.

3.3.3.9.2 Monodehydroascorbate reductase (MDHAR)

MDHAR activity was performed in black pepper leaves as per the method of Hossain *et al.* (1984). A 3 ml reaction was prepared by combining 1.9 ml of 90 mM phosphate buffer at pH 7, 0.35 ml of 0.2 mM NADH, 0.15 ml of 0.125% Triton X-100, 0.35 ml of 2.5 mM AsA, and 0.15 ml of AsA oxidase in a test tube. The mixture was vigorously mixed and then transferred to a cuvette. Immediately taken the absorbance at 340 nm for 3 minute reaction at 0.5 minute intervals. The molar extinction coefficient (ε) of MDHAR at 340 nm is 0.0062 $M^{-1}cm^{-1}$ and its activity was expressed in terms of mol/min mg enzyme.

3.3.3.9.3 Dehydroascorbate reductase (DHAR)

DHAR activity was measured at 265 nm (ε= 0.014) for 3 minute reaction at 0.5 minute intervals (Doulis *et al*., 1997), The reactants consisted of 1.8 ml of 90 mM phosphate buffer, 0.3 ml of 1 mM EDTA, 0.5 ml of 15 mM reduced glutathione (GSH) and 0.3 ml of 2 mM Dehydroascorbate (DHA) resulting in a total volume of 2.9 ml. The remaining 0.1 ml enzyme extract was combined immediately and monitored the increasing absorbance of 3 ml reaction mixture. The DHAR activity was expressed as mol/min mg enzyme.

3.3.3.9.4 Glutathione reductase (GR)

The GR activity was measured in a 3 ml reaction mixture (Rai *et al*., 2013). This mixture consisted of 2.68 ml of 0.1 M phosphate buffer (pH 7), 0.1 ml of 0.1 M oxidised glutathione (GSSG), 0.1 ml of 15mM EDTA, 20 µl of 10 mM NADPH (prepared in 1% $NaHPO_4$). A 100 µl enzyme extract was immediately pipetted into the reaction mixture in the cuvette, which brought the total volume to 3 ml. The activity was subsequently recorded at 340 nm (ε=$5.03x10^{-9}$). The increase in activity was measured at every 0.5 minute interval during the 3 minute reaction.

3.3.3.9.5 Glutathione S Transferase (GST)

The GST activity (Habig *et al*., 1974) was assessed by monitoring the increase in absorbance at 340 nm (ε= 0.0096 $M^{-1}cm^{-1}$), which was due to the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). The reaction mixture, which had a total volume of 3 ml, consisted of 2.7 ml of 0.1 M phosphate buffer at pH 6.5, 0.1 ml of 75 mM GSH, 0.1 ml of 30 mM CDNB, and the final 0.1 ml enzyme extract was added to make up the remaining volume. The absorbance was measured at 0.5 minute intervals up to a duration of 3 minutes immediately after the reaction started. The activity was expressed in terms of mol/min mg enzyme.

3.3.3.9.6 Polyphenol oxidase (PPO)

Esterbauer *et al*. (1977) method was followed for the determination of PPO activity in a 3 ml reaction mixture consisting of 1.5 ml of 0.1 M phosphate buffer (pH 7), 0.5 ml of 120 mM Catechol, 0.9 ml of distilled water, and 0.1 ml of enzyme extract. The enzyme extract was pipetted immediately into the mixture and the absorbance was recorded at 420 nm (with an extinction coefficient of 3.4 M-1cm-1). The PPO activity was then expressed as mol/min mg enzyme.

3.3.3.9.7 Peroxidase (POX)

The POX activity was measured following the method described by Putter (1974). A 3 ml reaction mixture was prepared, consisting of 2.5 ml of 0.1 M phosphate buffer at pH 7, 0.1 ml of 12.3 mM H2O, 0.3 ml of 20 mM Guiacol, and 0.1 ml of enzyme extract. The enzymatic activity was measured at 436 nm with the molar extinction coefficient of 11300 M-1cm-1. The activity was expressed as mM/min mg enzyme.

3.3.3.9.8 Catalase (CAT)

The method proposed by Luck (1965) was used to estimate catalase activity at 240 nm (ε= 4.36 M-1cm-1). A 3 ml reaction mixture was prepared by adding 1.9 ml of 0.1 M phosphate buffer at pH 7, 1 ml of 150 mM H2O2 and 0.1 ml of leaf extract. The change in absorbance was spectrophotometrically measured at 0.5 minute intervals for a total reaction time of 3 minutes.

3.3.3.9.9 Superoxide dismutase (SOD)

Enzyme extraction

The leaf sample (100 mg) was ground using a pre-chilled mortar and pestle with 50 mM potassium phosphate at pH 7.8. The homogenate was transferred to a centrifuge tube and centrifuged at 10,000 rpm at 40^0C. The collected supernatant was stored at -20^0C.

Estimation

A 3 ml reaction mixture was prepared consisting of 2.3 ml 50 Mm potassium phosphate buffer, 100 µl 1.5 M Na_2CO_3, 100 µl 3 Mm EDTA, 200 µl 200 mM Methionine, 100 µl 2 µM Riboflavin, 100 µl 2.25 mM nitro blue tetrazolium chloride (NBT) and 0.1 ml enzyme extract. The riboflavin was added to the tubes last and was vortexed properly. Then, the mixture was incubated in sunlight for 1 minute. The reaction was initiated by exposure to sunlight and stopped by incubating it in the dark. The reaction mixture kept in the dark did not produce color and served as a control. The reaction mixture without enzyme extract developed the maximum color, and this decreased as the volume of the enzyme extract increased. Absorbance measurements of the incubated reaction mixtures were taken immediately at 560 nm, and the percentage inhibition of the interaction between riboflavin and NBT in the presence of methionine, established as one unit of SOD activity, was estimated. Enzyme activity was expressed in terms of Units/mg fresh weight.

3.3.3.10 Mineral estimation

The black pepper leaf samples (youngest fully mature leaf from the top) from control as well as 28 days after stress were used for mineral analysis.,

Plant sample processing

Decontamination: The collected leaf samples were first washed with running tap water and subsequently treated in the following order: 0.1% detergent solution, 0.1 N HCl, and double-distilled water. This process effectively removed adhered dust particles and insecticides on the surface, thereby decontaminating the plant tissues.

This sequential washing approach minimized excessive rinsing, preventing nutrient loss.

Sample drying: Dried the washed samples by affixing them to the surface using blotting paper and placing them in a labelled paper cover. Subsequently, the samples were dried in a hot air oven at 70°C until a constant weight was achieved.

Sample powdering: Oven-dried samples were ground in a mixer grinder with a stainless steel jar. Subsequently, the digestion was performed on the sample.

3.3.3.10.1 Nitrogen

Kjeldahl method was used for the estimation of nitrogen in black pepper leaves.

Reagents

- Concentrated sulphuric acid (98%)
- 40% Sodium hydroxide solution
- Catalytic mixture: 20 g copper sulphate. Anhydrous ($CuSO_4.5H_2O$), 100 g potassium sulphate (K_2SO_4) and 1 g selenium were mixed, homogenized and ground to fine powder.

Digestion

The previously mentioned processed sample, weighing 0.5 g, was transferred to a 250 ml Kjeldahl tube. Approximately 10 ml of concentrated H_2SO_4 was added to the digestion mixture, which was left overnight. The tubes were heated at a temperature of 410°C for 2 hours in a digestion block until a bluish-green colour appeared. Afterwards, the digestion block was removed, and the tubes were allowed to cool.

Distillation

The flask containing the residue was placed into the Kjeldahl distillation unit, programmed for further distillation, neutralized, and steam was passed for about 5 minutes. Subsequently, the distillate, approximately 100 ml, was collected in a 25 ml boric acid-double indicator solution in a 250 ml conical flask. The contents of the

flask were diluted to 100 ml with distilled water, and a 10 ml aliquot was taken into the Kjeldahl distillation unit. Then, 10 ml of 40% NaOH solution was added, and distillation was carried out. The distillate was collected in a 10 ml boric acid solution in a 100 ml conical flask.

The distillate was titrated against 0.1 N H_2SO_4 from an automatic burette to restore the original color of the boric acid-mixed indicator solution. The nitrogen content in the leaf sample was then calculated from the titre value (Jackson, 1973).

3.3.3.10.2 Preparation of sample

Wet oxidation method was used for the processing of plant sample for the estimation of P, K, Mg, Ca, Copper, Mn, Fe and Zn.

Reagents

- An acid mixture (HNO_3:$HClO_4$; 9:4): The diacid mixture was prepared by taking 1.8 litres of concentrated nitric acid in a 3 liter bottle and transferring 800 ml of concentrated perchloric acid. The mixture was thoroughly mixed and then stored for analysis.

Extraction

One gram weighed powdered leaf sample was transferred to a volumetric flask. The volumetric flask was sequentially rinsed with 0.1 N HCl, distilled water, double-distilled water, and finally dried before taking the sample. Then, 10 ml of the prepared diacid mixture was added and allowed to undergo cold digestion for 2 hours, avoiding frothing. A clear white solution was observed at the bottom of the flask while being heated slowly by regulating the thermostat of the hot plate. The flask was then allowed to cool, and the volume was adjusted to 100 ml for use in the analysis.

3.3.3.10.3 K, Ca, Mg, Fe, Cu, Zn and Mn

The diacid digest prepared from the wet oxidation method was utilized for the measurement of potassium, calcium, magnesium, iron, copper, zinc, and manganese

using an atomic absorption spectrophotometer (AAS) with appropriate standards (Dinesh *et al*., 2006).

1 ml of the diacid digest was diluted in a 50 ml volumetric flask. The potassium and calcium concentrations were determined from this digest through AAS using standard solutions of 1, 2, 3, 4, and 8 ppm for potassium and calcium. By using standard solutions of 0.5, 1.2, 4, and 6 ppm, magnesium was determined from the same solution using AAS.

The elements such as iron, zinc, copper, and manganese were measured using an atomic absorption spectrophotometer by preparing a clear solution of the diacid digest of leaf samples. Mg, Fe, Cu, Zn and Mn were measured using the absorption mode using hollow cathode lamp at wavelengths of 285.2 nm, 248.3 nm, 324.7 nm, 213.9 nm and 279.5 nm respectively. While, potassium and calcium was estimated via emission mode at 766.5 nm and 422.7 nm respectively.

3.3.3.10.4 Phosphorus

The vanado-molybdate method was employed to determine the phosphorus content in the digested leaf sample obtained (Jackson, 1973).

Reagents

- HNO_3- Vanado molybdate reagent preparation:
- Solution A: 25 g ammonium molybdate was dissolved in 400 ml distilled water
- Solution B: 1.25 g of ammonium metavanadate was dissolved in 300 ml of boiling water, cooled, and then added 250 ml of concentrated H2SO4. The mixture was allowed to cool until it reached room temperature.
- Composed solutions A and B and adjusted the volume to 1 liter.

Estimation

5 ml of the diacid extract of the leaf sample was taken and adjusted the volume to 25 ml in a volumetric flask. Then, 10 ml of water was added, followed by 5 ml of Vanado molybdate reagent, and brought it to the mark. The spectrophotometric reading was recorded at 470 nm using a blue filter.

Standard stock preparation: The standard concentrations ranged from 1 ml to 6 ml aliquots of a 10 ppm standard phosphorus solution to achieve concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 ppm phosphorus solutions. A graph was plotted, and the phosphorus content was calculated as per the standard graph.

3.3.3.11 Phytohormone quantitation

Extraction

Abscisic acid (ABA) as a phytohormone was followed a modified Quenchers (Quick-Easy-Cheap-Effective-Rugged-Safe) method for its extraction.

Reagents

- Extraction solvent: HPLC grade water and 0.1% formic acid in acetonitrile
- Anhydrous Na_2SO_4
- $NaCl_2$
- Octyldecylsilane (C_{18})
- Primary Secondary Amine (PSA)
- HPLC grade Methanol
- Standard: synthetic ABA

Extraction

The freshly collected leaf samples were wrapped in aluminium foil and immediately placed in ice flakes. Just before extraction, a 10 g leaf sample was weighed and ground

with liquid nitrogen in an ice-cold mortar and pestle along with 10 ml of 0.1% formic acid in acetonitrile. The blended sample was transferred into a 50 ml centrifuge tube containing 8 g anhydrous Na_2SO_4 and 2 g NaCl, and vigorously vortexed for 1 min. The blended sample was then centrifuged in a pre-cooled centrifuge (Remi refrigerated centrifuge, CPR-24 plus) at 10,000 rpm for 10 minutes. The supernatant was carefully transferred into the clean-up kit containing reagents with 1.52 g Na_2SO_4, 50 mg C_{18}, and 50 mg PSA, and it was vortexed properly. The homogenized sample was then centrifuged at 10,000 rpm for 5 minutes. The supernatant was transferred into individual containers and placed in the nitrogen evaporator, facilitating the evaporation of the volatile solvent while nitrogen gas was passed over the surface of the liquid samples. The solvent was evaporated, and the remaining residue was dissolved in a 1 ml equal volume of ice-cold water and methanol using a sonicator (Sonica ultrasonic cleanser) with 30 minutes of ultrasonication under cold conditions. The solution was then filtered using a 0.25 µm membrane filter into the sample-carrying vials, which were inserted into the autosampler.

LCMS Conditions

The Shimadzu LCMS 8045 version was employed for the performance analysis, equipped with a 3 µm C_{18} Shimadzu analytical column with the dimensions of 100 mm x 2.1 mm. The LC conditions consisted of mobile phase A, containing 0.1% formic acid in water, and mobile phase B, composed of methanol, for the isocratic elution of phytohormone with a flow rate of 0.35 mL/min.

The optimized working conditions for MS included an oven temperature of 40°C, interphase voltage of -3 kV, collision energy for two daughter ions set at 11 and 17 V, desolvation temperature of 250°C, heating gas flow of 10 L/min, nebulizing gas flow of 2.8 L/min, and drying gas flow of 10 L/min, utilizing Lab solutions software.

The specific precursor-to-product ion (mass-to-charge) transition was monitored, and the target phytohormone (ABA) was quantified using multiple reaction monitoring (MRM).

Standard stock preparation: From the primary stock solution of 1 mg/ml standard ABA (1000 ppm), 10 µl was pipetted and made up to 1000 µl using ice-cold water-methanol (50:50) solvent, resulting in a secondary stock solution of 1 ppm. Further dilutions were prepared at concentrations of 5, 10, 25, 50, 100, 100, 150, and 200 ppb. A standard graph was plotted using these dilutions, and it was used to determine the ABA content in the plant sample.

3.3.4 Analysis of yield and quality parameters of water stressed plants

The berries of both drought induced as well as control plants from selected genotypes (accessions 7211, 1495, 1343, 4132, 5717 and 4064) were harvested after maturity, dried and dried berry samples were used to analyse their quality parameters (piperine, oleoresin, essential oil, berry sugar and berry starch). The previously mentioned procedures for sugar estimation (Dubois et al., 1956), starch estimation (Hodge et al., 1962), piperine estimation, oleoresin extraction (ASTA 9.0, 1997) and essential oil extraction (ASTA, 1968) were followed.

3.3.5 Shoot and root biometrics with dry matter partitioning

After harvest, the grow bags grown plants were irrigated sufficiently, then the bags were torn using a knife, and the soil was carefully removed and washed with running tap water under constant pressure and the plants were uprooted carefully. The root systems of all plants were washed in a similar manner, and the water was drained using a cotton cloth. The roots were then placed on a white plastic sheet, and the root length was measured. Finally, the plants were partitioned in to root and shoot and the following parameters were measured.

3.3.5.1 Root length (cm)

The root length the plant (in four replicates) was measured from the base of the shoot till the tip of root using centimetre scale.

3.3.5.2 Root diameter (mm)

The vernier caliper was used to measure the primary root diameter of the plants (in four replicates). The measurements were noted using the following formula.

Root diameter (mm) = main scale division + (vernier scale division × least count)

3.3.5.3 Number of primary roots

The number of primary roots originating directly from the shoot was counted and tabulated for all genotypes. Four replications were maintained.

3.3.5.4 Number of secondary roots

Number of secondary roots that emerged from the main root or primary root were counted and four replications were maintained.

3.3.5.5 Root fresh weight (g)

The roots were gently blotted to remove excess water, and then the roots were separated from the shoots, and their fresh weight was measured for all the genotypes.

3.3.5.6 Root dry weight (g)

After weighing the roots were dried in a hot air oven at 75°C for 5 days to attain constant weight and the dry weight was measured.

3.3.5.7 Shoot fresh weight (g)

Immediately weighed the partitioned shoots from the roots and recorded the fresh weight for all the replicates of the genotypes.

3.3.5.8 Shoot dry weight (g)

The freshly partitioned weighed shoots were placed in a hot air oven set at 75°C for 5 days of incubation. The weight of their dry matter was then measured using a weighing balance and recorded.

3.3.5.9 Primary stem diameter (mm)

The stem thickness of the above-ground stem was measured using Vernier caliper for all the replicates of both control and stress treatments.

3.3.5.10 Secondary stem diameter (mm)

The width or thickness of the stem at the basal region, which originated from the main or primary stem of the black pepper was measured for all the replicates.

3.3.5.11 Root to shoot ratio (%)

Partitioned root and shoot (stem, leaves and reproductive part) were separately dried in a hot air oven at 75°C for 5 days to attain a stable weight. The following formula was used for the calculation of the root-to-shoot ratio (Anbumalarmathi, 2008).

$$\text{Root to shoot ratio} = \frac{\text{Root dry weight}\,(g)}{\text{Shoot dry weight}\,(g)} \times 100$$

Experiment 3

3.3.6 Molecular characterisation

Isolation of genomic DNA and sequence analysis

The extraction of genomic DNA was performed from the youngest fully mature leaves of 40 black pepper genotypes (third or fourth leaf from the tip) mentioned earlier. The CTAB (Cetyltrimethylammonium bromide) method was employed for DNA extraction.

Reagents

- Extraction buffer: The CTAB buffer was prepared with the following composition: 100 ml of 0.1 M Tris-HCl at pH 8, 40 ml of 20 mM EDTA, 81.8g of 1.4 M NaCl, and 20 g of 2% CTAB were made up to 1000 ml with autoclaved double-distilled water.

- Electrophoretic tank buffer: The 50x TAE (Tris-acetate-EDTA) buffer was formulated with the following composition: 242.2 g of 2 M Tris base, 57.1 ml of glacial acetic acid, and 100 ml of 0.5 M EDTA were combined and brought up to 1 litre with autoclaved double-distilled water. Then, 20 ml was pipetted

out, and the volume was adjusted to 1 litre to form a 1x concentration using autoclaved double-distilled water.

- Polyvinylpyrrolidone (PVP)
- β-mercaptoethanol (β-ME)
- Chloroform and Isoamyl alcohol was prepared in the proportion of 24:1.
- Ice cold Isopropanol
- 3M Sodium acetate (NaOAC)
- 70% Ethanol
- 2% Agarose gel
- Ethidium bromide

Extraction

A 100 mg leaf sample was ground with liquid nitrogen using a mortar and pestle. Subsequently, a pinch of PVP and 1 ml of extraction buffer were added, and the mixture was thoroughly homogenized. Following this, 10 µl of β-ME was introduced into the homogenate and vortexed thoroughly. The homogenate was then incubated in a 65°C water bath for 1 hour. The homogenate was immediately plunged into ice flakes and brought to room temperature. An equal volume of 1000 µl of chloroform and isoamyl alcohol, proportioned as 24:1, was added and mixed by inverting the mixture 50 times. The mixture was incubated at room temperature for 10 minutes and then centrifuged for 10 minutes at 12,000 rpm. The aqueous phase was transferred to a new vial, to which 500 µl of ice-cold isopropanol and 100 µl of 3 M NaOAC were added. The mixture was then mixed by inverting it 50 times. The vial was incubated overnight at -20°C. The next day, it was centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the remaining pellet was washed with 70% ethanol. The mixture was centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was removed, and the pellet was air-dried. Subsequently, it was dissolved in 50 µl of nuclease-free water.

Estimation of quantity and quality of extracted DNA

The nanodrop micro volume spectrophotometric (Denovex) quantification of the extracted DNA was expressed in ng/µl at 260 nm against the blank, using nuclease-free water. The purity of DNA was confirmed within the range of 1.8 by calculating the absorbance at 260/280 nm. The absorbance ratio at 260/230 nm was calculated to determine the presence of polysaccharide contamination. After loading the sample onto an agarose gel containing ethidium bromide dye, the gel was electrophoresed in 1x TAE buffer at 85 volts. Subsequently, the size and purity of the extracted DNA became visible as stained bands on the agarose gel, in the gel documentation system, and were compared with a known standard DNA ladder. Isolated DNA was stored at -20^0C for further work.

Primer synthesis

Eleven gene-specific (drought-specific) primers were designed using the Primer Quest tool. Gradient polymerase chain reaction (PCR) was performed in a total volume of 10 µl reaction mixture to identify the annealing temperature of the primers for amplification, and the composition was as follows:

PCR components	Volume (µl)
Forward primer	0.5
Reverse primer	0.5
EmeraldAMP PCR Master Mix (Combined DNA polymerase, optimized reaction buffer, dNTPs, and a density reagent)	5
Nuclease free water	3
Extracted DNA	1

The optimum annealing temperature of these primers was determined to perform the gradient PCR:

Sl No.	Gene	Primer name	Forward (5'-3') Reverse (5'-3')	T_a[a] (^{0}C)
1	LRR receptor like kinase	AMP5	ATTGATTCAGGTGGCTGGGG AATAAGGTGGGTTGGGCTCG	60
2	Defensin	AMP10	CGTCGCTGCTTTTGTGTGAA CAATTCACGTCAGGTGCACG	55.5
3	Ascorbate peroxidase 1 cytosolic	DAPC1	CCTCCAAGCAGCAAAGAAATG GTGCGCAAACCAGATGAAAG	55.2
4	Ascorbate peroxidase 6 cytosolic	DAPC6.I	CTAGTTGTTGGCGTGATTGTTG GAGTGCTGGATAACACCTTCTT	55.6
		DAPC6.II	GGTGTTATCCAGCACTCTACTATC AAATACTCTTTGCTGCCCTTTG	63.5
5	Glutathione S tranferase F13 like A	DGST	CAGAGAAGTACGCCGATCAAG TGGTTACAACTCAGCGAAGATAA	55.4
6	Peroxidase 5 like	DPX5	AAGGAGAAAGACAGATGGGTAAG CTCCCTACTTGAGATGCATAAGG	54.4
7	Peroxidase 12	DPX12	GTTGATGTGTGTGACGTGATTC CTCATCCACCACAGAGTACAAG	64.5
8	Peoxisomal (S)-2-hydroxy-acid oxidase GLO1 like	DPHAO	TAGCAGGATGTGAAGTGGTTG GAGCATTTGAAGCACCTTTCTC	64.1
9	Superoxide dismutase – CuZn	DSODCu	GATGACTCTGTATGGTATCGTTTCT TTTGTGCACTACCTGGCTATC	60.5
10	Superoxide dismutase – FeMn	DSOCFe	GCCACTCACCACTGACTTAC TAGTTTGATCTCGACGCCAAC	53.8

Note: Ta a (^{0}C) refers to annealing temperature

Different annealing temperatures were identified, ensuring good amplification for each primer (Fig 3.1 to 3.11), while common PCR conditions were maintained with varying annealing temperatures:

Step	Temperature	Time	Number of cycles
Initial denaturation	94^{0}C	5 min	
Denaturation	94^{0}C	1 min	35 cycles
Primer annealing	Varying temperatures	1 min	
Extension	72^{0}C	35 sec	
Final extension	72^{0}C	10 min	

PCR conditions

PCR reaction was performed under the conditions set up in a thermocycler as follows: initial denaturation for 5 min at 94^0C followed by 35 cycles of 1min denaturation at 94^0C, 50 sec at 60^0C and 2 min at 72^0C with final extension at 72^0C for 10 min and a hold at 4^0C temperature. Some modifications were made in the PCR condition in two primers, AMP 5 and AMP 10 out of eleven primers. The primers that generated reproducible amplified DNA products (Figure. 3.12) from 40 black pepper leaves were carefully selected for Multina Microchip Electrophoresis analysis.

3.3.6.1 MultiNA Microchip Electrophoresis analysis

Analysed the PCR products using the DNA 1000 kit on the Shimadzu MCE-202 MultiNA. Reagents used in Multina analysis from DNA 1000 kit: Seperation buffer, DNA marker regent and 100bp DNA ladder. The samples were loaded into the MultiNA instrument alongside the reagents. Samples and reagents were automatically blended on the chip, viewed in MultiNA Viewer software, and executed against MultiNA Control.

3.3.6.2 DNA Sequencing and sequences analysis

The forty black pepper accessions mentioned earlier were selected for PCR product sequencing by Sanger method. The amplified PCR products from each primer were further confirmed through sequence analysis. Selected amplicons were sequenced, and their sequences were determined. The raw sequences of the desired drought-specific genes were aligned using both forward and reverse sequences for each genotype. The identities of the genes were confirmed through BLAST analysis against the black pepper whole genome assembly. BLAST searches were performed on NCBI (www.ncbi.nlm.nih.gov) using nucleotide sequences for similarity searches. Open reading frames (ORFs) were identified on NCBI (http://www.ncbi. nlm.nih.gov/gorf/gorf.html) using the ORF finder. The software BioEdit version 7.2.5 was used to align the DNA sequences and detect single nucleotide polymorphisms (SNPs).

3.3.6.3 Isolation of RNA and gene expression analysis

The selected black pepper accessions, including Acc 7211, Acc 1495, Acc 1343, Acc 4132, Acc 5717, and Acc 4064, were screened at the molecular level through RNA expression analysis. They were maintained in grow bags under different moisture levels of 25%, 50%, and 100% field capacity. Five replications were maintained for each treatment, resulting in a total of fifteen replications for each accession. The youngest fully mature leaf was collected for RNA extraction.

Reagents

- Qiagen RNeasy® Plant Mini kit
- 0.1% DEPC (Diethyl pyrocarbonate) was prepared in double distilled water
- DEPC treated nuclease free water
- RNase zap
- 1x TAE (Tris-acetate-EDTA) buffer prepared in 0.1% DEPC water
- 2% Agarose was prepared in DEPC treated TAE buffer
- Ethidium bromide
- RNA loading dye
- 2x QuantiFast SYBR green

Extraction

Freshly collected samples were immediately washed thoroughly with autoclaved double-distilled water, wrapped in aluminium foil paper, labelled, and kept on ice without any delay. Leaf tissue was immersed in liquid nitrogen, ground in a frozen RNase-free autoclaved mortar and pestle.

The Qiagen RNeasy® Plant Mini kit was used for RNA isolation. Powdered leaf samples were weighed into pre-cooled 2 ml Eppendorf centrifuge tubes. 500 µl of RLT buffer, combined with 5 µl of β-mercaptoethanol (β-ME), was pipetted into the tube and vortexed vigorously. The QIAshredder spin column was placed in a 2 ml collection tube, and the lysate was transferred directly to the spin column. It was then centrifuged for 2 minutes at full speed. The supernatant was carefully transferred to a newly taken centrifuge tube without disturbing the pellet. A 0.5 volume of ethanol

was added to the cleared lysate and mixed immediately by pipetting. The RNeasy Mini spin column (pink color) was placed in a 2 ml centrifuge tube, and approximately 650 µl of the lysate was added to it. The lid was closed, and it was centrifuged for 15 seconds at ≥10,000 rpm. The flow-through was then discarded. 700 µl of RW1 buffer was added to the RNeasy spin column. Then, it was centrifuged for 15 seconds at ≥10,000 rpm, and the flow-through was discarded. Next, 500 µl of RPE buffer was pipetted into the RNeasy spin column, followed by centrifugation for 15 seconds at ≥10,000 rpm. Once again, 500 µl of RPE buffer was added to the RNeasy spin column, and it was centrifuged for 2 minutes at ≥10,000 rpm. The RNeasy spin column was placed in a new 1.5 ml collection tube. Then, 50 µl of RNase-free water was pipetted directly onto the spin column membrane. After allowing it to sit for 10 seconds, the sample was centrifuged for 1 minute at ≥10,000 rpm. The RNA was then eluted into the centrifuge tube.

Estimation of quantity and quality of extracted RNA

The eluted RNA was quantified using a Nano drop micro volume spectrophotometer (Denovex) with 1 µl of RNA. The concentration (ng/µl) of the sample was recorded based on the 260/280 nm ratio and 260/230 nm ratio. The quality of RNA was assessed by loading 3 µl of RNA, along with an equal proportion of RNA loading dye, onto a 2% agarose gel. The remaining RNA was then stored at -80°C for further studies.

cDNA synthesis

The Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was utilized for cDNA synthesis. The components required for cDNA preparation were thawed, vortexed, briefly spun, and stored at -20°C. 1 µl of template RNA with a random hexamer primer and 12 µl of nuclease-free water were pipetted into the sterile, nuclease-free 2 ml Eppendorf tube. Then, 4 µl of 5X reaction buffer, 1 µl of Ribolock RNase inhibitor, 2 µl of 10 mM dNTP mixture, and 1 µl of RevertAid M-MuLV Reverse Transcriptase were added sequentially to the same tube, combined, and centrifuged briefly. Incubated this mixture in the ProFlex PCR system at 25°C for 5 minutes, followed by 60 minutes at 42°C; finally, the reaction was terminated by incubating at 70°C for 5 minutes.

Primer synthesis

Primer quest tool was used to design drought- specific RT-PCR primers (purchased from Eurofins Genomics India Private Limited) with the following parameters: an amplicon length of 88-120 bp, a melting temperature range of 52-59°C, and a GC content of 40-60%. The lyophilized form of the purchased primers (Table 1) was prepared by suspending them in DEPC-treated nuclease-free water, and a 10 µM working concentration was prepared by diluting the 100 µM stock concentration.

Table 9. qRT-PCR primer sequences for full length

Sl No.	Gene	Primer name	Forward (5'-3') Reverse (5'-3')
QPCR primers for reference genes			
1	Ubiquitin (UBI)	QUBI	CGTGGAGGAATGCAGATTTT CCTAGAAACCACCACGGAGA
2	RNA-binding protein (RNABP)	QRNABP	ACCTTTTACGCTGGGTTCCT GTCACCCACCACACCTCTCT
3	Elongation factor 1A (EFIA)	QEFIA	AAAGGTGACGACCATTCCAG TCCCATCTCAGGTTTTGAGG
QPCR primers for candidate genes			
4	Ascorbate peroxidase 6 cytosolic	QAPC6	GGTTCCTTCTGATTGCTCTCTT TGTTTCTGCTTCTCGGTGTT
5	Glutathione S tranferase F13 like A	QGST	CAGAGAAGTACGCCGATCAAG GTTCTGTGACTCCACTTCCAA
6	Peroxidase 5 like	QPX5	TGAACCGCTCAAGTATCTTCTC TGGGTCTAGATAGGCAAATTGTT
7	Peroxidase 12	QPX12	CGGACTCAACTTTGCAACTC CCAAGTTCTTCGTGGCTAATG
8	Superoxide dismutase - CuZn	QSODCU	ATACGCCTTCAAGCACCAA CAATTGTCGATAGCCAGGTAGT
9	Superoxide dismutase - FeMn	QSODFE	GGCCTAAATTGAGCCTTACAAC CTTATTGTTGGTAGCATGGATGTG
10	Dehydrin (DHN)	QDHN	AGCAGATCAGCTGGAAGGAA ATCAGTGGCACATTGTTCA
11	Osmotin (OSM)	QOSM	ACCGTGTTTAAGACCGACCA ACCATTTCATGGGCAAAAGA
12	NAC transcription factor	QNAC	TGGGCTTTTGCTCTGTCTTT CTCACCCTTCTTCCCATTGA

Sl No.	Gene	Primer name	Forward (5'-3') Reverse (5'-3')
13	Dehydration-responsive element-binding protein	QDREB	GCAACCGAATTTCTCCGATA AGCACCGTTTCCTTTTCTGA
14	Drought responsive genes and transcription factors Basic leucine zipper protein	QbZIP	ACTCATGGTCTTCGGCATTC ACCGGCGTGGTCTGTATATC
15	Heat-shock protein	QHSP	GTCCCCCACGAGTAACTTGA GCAGAAATTGGGAGATGAGC
16	Apetala 2	QAP2	ATGAGCTCGAGGGTAGACGA CTATACCGGCTTTCCCACCT
17	Myeloblastosis oncogene	QMYB	GTGGCCTCCAATAAAAGCAA ATCCATTCATCCCACCAAAA
18	Aquaporin	QAQUA	TGAATCCTGCCGTGACATTA CGTGTTGTGCCGTATGAGAC
19	Peoxisomal (S)-2-hydroxy-acid oxidase GLO1 like	QDPHAO-I	TGGAGATGGCCTTACAAAGATG GTCGGGTTCCAGTGTTTCTT
		QDPHAO-II	GCTGTGAATCAGACCCTCTTC CAGAACAATTTGCCCGAATCTC

qPCR-based transcript profiling

The stability of reference genes and the relative expression of drought-specific primers were analyzed using qRT-PCR, which was carried out on the Rotor-Gene Q (Qiagen) with 72-well rotors. The 20 µl reaction mixture consisted of 2x QuantiFast SYBR green (Qiagen), 10 µM of both forward and reverse primers, cDNA, and nuclease-free water in the following composition:

PCR components	Volume (µl)
2x QuantiFast SYBR green	13
10 µM Forward primer	1
10 µM Reverse primer	1
cDNA	0.5
Nuclease free water	4.5

Each reaction consisted of three technical replicates. The absence of genomic DNA and non-specific amplification was confirmed by observing the reaction mixture, which included a reverse transcription negative control (without reverse transcriptase), and a reaction mixture with a non-template negative control. The PCR conditions were programmed as follows:

Step	Temperature	Time	Number of cycles
Initial denaturation	95^0C	5 min	
Denaturation	95^0C	10 sec	35
Primer annealing	60^0C	30 sec	

The specificity of PCR products was assessed using a melt curve program, with the temperature increasing by 1°C in the range between 65°C and 99°C. Three technical replicates of Ct values were maintained after setting the threshold value to 0.1, which corrected the slope and baseline. The relative gene expression was calculated and transformed to the log2 scale using the method of 2ΔCt (Livak & Schmittgen, 2001). Transcripts with equal expression were observed, indicating a log2-ratio of zero. Upregulated transcripts had values greater than zero, while downregulated transcripts had values less than zero.

3.3.7 Dry matter partitioning

The uprooted whole plant was separated into shoot and root after conducting the gene expression-studies with differentially imposed stress levels (25%, 50% and 100% FC moisture levels) for the assessment of dry matter production and partitioning. The root-to-shoot ratio for 25%, 50%, and 100% FC levels for each accession with five replicates, was calculated by dividing shoot dry matter by root dry matter. The previously mentioned calculation method, as described by Anbumalarmathi *et al.* (2008), was utilized.

3.4 Statistical analysis

3.4.1 Analysis of variance (ANOVA)

ANOVA was performed for both randomised block design (RBD) and factorial RBD using the R software.

3.4.2 Principal component analysis

Principal component analysis (PCA) was applied to the correlation matrix of morpho-physiological and biochemical traits, yield-attributing traits, and gene expression of selected genotypes to identify the parameters that best reflect the tolerance characteristics of the genotypes. Past 4.03 software was used for PCA, including the analysis of Eigenvalues, Eigenvectors, and 2D biplot visualization. PC1 and PC2 were used to categorize the genotypes, as they accounted for the most variance in the characteristics studied.

3.4.3 Pearson coefficient analysis

Pearson coefficient analysis was done to determine the degree of correlation between these variables using R- software

3.4.4 UPGMA cluster analysis

Using R- software, UPGMA cluster analysis was carried out to group the genotypes into clusters.

3.4.5 Heatmap analysis

Heatmap analysis, a powerful data visualization tool, was used to represent the intensity of data points across two dimensions using R software. The heatmap was generated with a specific color gradient, and hierarchical clustering was applied to both rows and columns to identify patterns.

3.4.6 Scoring

The drought tolerance evaluation of the selected genotypes, based on desirable drought tolerance traits, was ranked using a scoring system. The highest score of 30 was assigned to the most tolerant category, followed by a score of 20 for moderate

tolerance, and the lowest score of 10 was given to the susceptible category. Each parameter was assigned a weight based on its importance in drought tolerance, with higher weights indicating greater importance. The weights were distributed as 0.15, 0.125, 0.1, 0.05, and 0.025 for the important parameters, differentiating the genotypes based on their drought tolerance. The total weight of the parameters, RWC (0.15), EL (0.15), stomatal closure (0.1), proline (0.05), phenol (0.025), H2O2 content (0.025), MDA (0.025), sugar (0.125), starch (0.05), enzyme (0.15), and ABA content (0.15) was equal to one. The weighted score for each genotype was determined by calculating the product of each parameter's score and weight, and then added these products together.

CHAPTER 4
RESULTS AND DISCUSSION

The present study, entitled 'Physiological and Molecular Characterization of Black Pepper Genotypes Subjected to Limited Water Availability,' was conducted at the IISR experimental farm at Peruvannamuzhi and the Indian Institute of Spices Research, Kozhikode, from 2019 to 2023. In the first experiment, 40 black pepper accessions (Fig. 1), thirty of which were identified as drought tolerant and ten as susceptible after preliminary screening for drought tolerance by withholding irrigation for 15 days were used for further confirmatory studies to identify genotypes with the most tolerant characteristics.

Experiment 1

Some common morphological characters (leaf length, leaf width, petiole length, and internode length) and physiological characters (wax content and stomatal number) parameters were selected for the study. Based on black pepper plant's response to drought, the following assumptions were made for assessing their drought tolerance.

Morphological characteristics for drought tolerance		
	Desirable types	Undesirable types
Leaf length	Low to medium	High
Leaf width	Low to medium	High
Leaf area	Low to medium	High
Petiole length	Low to medium	High
Internodal length	Low to medium	High
Physiological characteristics for drought tolerance		
	Desirable types	Undesirable types
Wax content	High	Low to medium
Stomata number	Low to medium	High

These parameters were recorded in the forty genotypes planted in the field.

4.1.1. Morphological characters

4.1.1.1. Leaf length

Significant variation in leaf length (LL) was observed among forty black pepper accessions, ranging between 6.1 cm and 11.9 cm. Acc 6720 exhibited the lowest leaf length, followed by Acc 931, Acc 807, etc., while the highest leaf length was recorded in Acc 813. These genotypes were categorized into three groups (low, medium, and high) based on leaf length, as tabulated below.

	Low LL (< 7.8 cm)	Medium LL (7.8 cm – 8.8 cm)	High LL (> 8.8 cm)
Genotypes	Acc 6720, Acc 931, Acc 807, Acc 971, Acc 6707, Acc 1086, Acc 4152, Acc 1487, Acc 5621, Acc 1390, Acc 6786, Acc 5717	Acc 1315, Acc 4137, Acc 1343, Acc 1439, Acc 4177, Acc 1368, Acc 4132, Acc 6774, Acc 1495, Acc 1248, Acc 5642, Acc 891, Acc 7211, Acc 4095, Acc 8060, Acc 1315, Acc 5717	Acc 1491, Acc 5623, Acc 8052, Acc 1218, Acc 1638, Acc 1476, Acc 5691, Acc 4064, Acc 1093, Acc 5083, Acc 4226, Acc 4216, Acc 813

4.1.1.2. Leaf width

The leaf width (LW) of the leaves varied from 4.6 cm to 8.65 cm, and this variation was found to be significant at $P < 0.001$. Acc 1638 displayed the highest leaf width, while the lowest value was observed in Acc 5623. The forty genotypes were categorized into three groups based on their leaf width: less than 6.2 cm, 6.2-7.2 cm, and greater than 7.2 cm in their respective groups.

	Low LW (< 6.2 cm)	Medium LW (6.2 cm – 7.2 cm)	High LW (> 7.2 cm)
Genotypes	Acc 5623, Acc 6720, Acc 971, Acc 971, Acc 1086, Acc 1439, Acc 807, Acc 4095, Acc 5621, Acc 6786. Acc 1343, Acc 931, Acc 1315, Acc 4226, Acc 6707, Acc 813	Acc 7211, Acc 4152, Acc 1248, Acc 1476, Acc 4177, Acc 1495, Acc 5717, Acc 1390, Acc 1368, Acc 6774, Acc 5083, Acc 4216, Acc 4132	Acc 5642, Acc 891, Acc 4137, Acc 5691, Acc 1218, Acc 8060, Acc 1093, Acc 8052, Acc 1491, Acc 4064, Acc 1487, Acc 1638

4.1.1.3. Leaf area

A significant variation among black pepper genotypes was observed in leaf area (LA), ranging from 29.3 cm^2 to 80.6 cm^2. The maximum leaf area was noted in Acc 4064, while the lowest was found in Acc 6720. Based on leaf area, these genotypes into low leaf area (< 50 cm^2), medium leaf area (50 – 62 cm^2) and high leaf area (> 62 cm^2).

	Low LA (< 50 cm^2)	Medium LA (50 cm^2 – 62 cm^2)	High LA (> 62 cm^2)
Genotypes	Acc 6720, Acc 971, Acc 1086, Acc 931, Acc 807, Acc 5623, Acc 6707, Acc 1439, Acc 5621, Acc 6786, Acc 4152, Acc 1315, Acc 1343	Acc 4095, Acc 5717, Acc 1248, Acc 7211, Acc 1390, Acc 4177, Acc 1495, Acc 1368, Acc 1476, Acc 6774, Acc 1495, Acc 4132, Acc 4137	Acc 5642, Acc 4226, Acc 1487, Acc 8060, Acc 891, Acc 1218, Acc 8052, Acc 5083, Acc 1491, Acc 813, Acc 5691, Acc 4216, Acc 1638, Acc 1093, Acc 4064

4.1.1.4. Petiole length

Significant variability was observed in petiole length (PL), ranging from 2.6 to 5.4 cm. The highest value was recorded in Acc 1439, while the lowest value was noted in Acc 1248. Based on petiole length, the genotypes were grouped in to low PL (<3.5 cm), medium PL (3.5 cm – 4.2 cm) and high PL (> 4.2 cm) as shown below.

	Low PL (< 3.5 cm)	Medium PL (3.5 cm – 4.2 cm)	High PL (> 4.2 cm)
Genotypes	Acc 1218, Acc 971, Acc 7211, Acc 1495, Acc 931, Acc 6720, Acc 8052, Acc 4132, Acc 1093, Acc 807, Acc 4177, Acc 6786, Acc 1368	Acc 891, Acc 813, Acc 1343, Acc 4226, Acc 5691, Acc 1476, Acc 5623, Acc 6707, Acc 6774, Acc 1390, Acc 4095, Acc 1487, Acc 1638, Acc 4137, Acc 4152, Acc 5717, Acc 4064	Acc 8060, Acc 1315, Acc 5083, Acc 1491, Acc 5621, Acc 5642, Acc 1218, Acc 1086, Acc 4216, Acc 1439

4.1.1.5. Internodal length

Internodal length (IL) significantly differed ($P < 0.001$) among the genotypes, ranging from 2.2 cm to 10.05 cm. The longest internodal length was associated with Acc 4152, while the lowest internodal length was observed in Acc 1093, and the genotypes were categorized into low, medium, and high internodal length groups as shown below.

	Low IL (< 4 cm)	Medium IL (4 cm – 5.2 cm)	High IL (> 5.2 cm)
Genotypes	Acc1093, Acc 1343, Acc 1476, Acc 6707, Acc 4177, Acc 8052, Acc 1638, Acc 4064, Acc 5717, Acc 807, Acc 5642, Acc 891, Acc 4095	Acc 971, Acc 1248, Acc 813, Acc 7211, Acc 5691, Acc 6786, Acc 1390, Acc 931, Acc 5623, Acc 1495, Acc 1487, Acc 1368, Acc 4132	Acc 6774, Acc 6720, Acc 8060, Acc 4226, Acc 4137, Acc 4216, Acc 1086, Acc 1315, Acc 1218, Acc 1439, Acc 5621, Acc 5083, Acc 1491, Acc 4152

4.2. Physiological characters

4.1.2.1. Wax content

The wax content (WC) varied significantly ($P < 0.001$) in forty black pepper accessions, ranging from 2.20 to 15.90 µg/cm^2. The highest wax content was observed in Acc 1343, while the lowest was observed in Acc 1487. The genotypes were grouped into < 4.2 µg/cm^2, 4.2 – 9 µg/cm^2 and > 9 µg/cm^2 wax content as shown below.

	Low WC (< 4.2 µg/cm2)	Medium WC (4.2 – 9 µg/cm2)	High WC (> 9 µg/cm2)
Genotypes	Acc 1487, Acc 1476, Acc 8052, Acc 4064, Acc 1491, Acc 8060, Acc 1093, 6707, Acc 971, Acc 807, Acc 4226, Acc 1086, Acc 4226, Acc 1086, Acc 5717	Acc 4177, Acc 5083, Acc 891, Acc 931, Acc 5623, Acc 5642, Acc 1390, Acc 4095, Acc 4095, Acc 1638, Acc 5691, Acc 813, Acc 1439, Acc 4137, Acc 1218	Acc 1248, Acc 6774, Acc 7211, Acc 6720, Acc 4152, Acc 4216, Acc 1495, Acc 4132, Acc 5621, Acc 6786, Acc 1368, Acc 1315, Acc 1343

4.1.2.2. Stomatal frequency

Acc 5083 had the maximum stomatal frequency (94 per 204 mm^2), whereas Acc 4132 had the lowest stomatal frequency (41 per 204 mm^2). Genotypes differed significantly ($P < 0.01$) for stomatal frequency. The following table shows the grouping for stomatal frequency.

	Low SF (< 57)	Medium SF (57 – 70)	High SF (> 70)
Genotypes	Acc 4132, Acc 7211, Acc 1495, Acc 1343, Acc 1368, Acc 1638, Acc 1439, Acc 1086, Acc 1218, Acc 1476, Acc 891, Acc 5623, Acc 1248	Acc 4216, Acc 813, Acc 5717, Acc 5691, Acc 6707, Acc 807, Acc 4095, Acc 1491, Acc 1487, Acc 4137	Acc 4177, Acc 6774, Acc 4226, Acc 5621, Acc 971, Acc 1390, Acc 1093, Acc 1315, Acc 4152, Acc 8060, Acc 6720, Acc 6786, Acc 931, Acc 8052, Acc 5642, Acc 4064, Acc 5083

Figure 1. Black pepper genotypes planted in the field at Peruvannamuzhi experimental farm of ICAR-IISR

Table 2. Morphological and physiological characters of forty black pepper accessions

Genotype	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Petiole length (cm)	Internodal length (cm)	Wax content ($\mu g/cm^2$)	Number of stomata
Acc 8052	8.9 bcd	8.2 ab	73.0 ab	3.20 def	3.40 gh	2.47 gh	82 bc
Acc 5083	10.3 a	7.1 bcde	73.2 ab	4.43 abcd	9.20 a	4.20 fgh	94 a
Acc 8060	7.9 def	8.1 ab	63.67 bc	4.20 abcd	5.87 bcd	2.67 gh	74 cd
Acc 1491	8.8 bcd	8.4 ab	73.4 ab	4.43 abcd	9.20 a	2.67 gh	64 efg
Acc 4064	9.6 abc	8.4 bc	80.6 ab	4.03 bcde	3.50 gh	2.50 gh	87 ab
Acc 1487	7.5 def	8.4 ab	63.395 bc	4.07 bcde	5.17 cdef	2.20 h	67 def
Acc 1093	9.8 ab	8.2 ab	80.1 a	3.27 def	2.23 i	2.83 gh	73 cde
Acc 5717	7.8 cdef	6.7 bcd	51.8 cd	3.50 cdef	3.60 gh	4.33 fg	60 fghi
Aacc 971	6.9 fg	5.0 fg	34.9 gh	2.67 f	4.17 fg	3.53 gh	72 de
Acc 7211	8.6 bcde	6.2 cdef	53.8 cde	2.77 ef	4.57 efg	10.83 cd	51 ijk
Acc 5691	9.4 abc	7.8 ab	73.6 a	3.87 bcdef	4.60 efg	8.07 e	61 fgh
Acc 1248	8.5 bcde	6.3 cdef	53.8 cde	2.60 f	4.33 efg	9.13 de	57 ghij
Acc 6720	6.1 g	4.8 g	29.3 h	3.17 def	5.50 cde	11.23 c	76 cd
Acc 1086	7.2 efg	5.1 efg	37.1 fg	4.87 ab	6.23 bc	3.90 gh	47 kl
Acc 1218	9.0 abcd	8.1 a	72.9 a	4.83 abc	6.70 b	8.93 e	48 jkl
Acc 5621	7.7 def	5.8 cdefg	44.5 ef	4.77 abc	6.97 b	13.90 b	72 de
Acc 1343	8.2 cdef	6.0 defg	48.7 ef	3.73 bcdef	2.77 hi	15.90 a	52 hijk

Genotype	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Petiole length (cm)	Internodal length (cm)	Wax content ($\mu g/cm^2$)	Number of stomata
Acc 5623	8.9 bcd	4.6 defg	40.9 def	3.87 bcdef	4.90 def	5.90 f	55 ghijk
Acc 1439	8.3 cde	5.2 cdef	43.6 def	5.43 a	6.77 b	8.40 e	46 kl
Acc 1495	8.5 bcde	6.6 cde	55.9 cde	2.83 ef	5.13 cdef	13.77 b	57 ghij
Acc 4132	8.4 bcde	6.9 bcde	58 cd	3.23 def	5.20 cdef	13.83 b	41 l
Acc 813	11.90 a	6.18 jklm	73.60 ab	3.68 efghijk	4.43 jklmno	8.14 fghi	59.17 jk
Acc 4216	10.67 b	7.17 efgh	76.69 a	5.22 ab	6.07 bcdef	12.25 bc	58.50 jk
Acc 4226	10.38 bc	6.08 klmn	63.19 cd	3.82 efghij	5.95 cdefg	3.74 lmno	71.50 ef
Acc 1476	9.20 efgh	6.37 hijklm	58.60 cdefg	3.87 defghij	2.97 rs	2.30 no	50.00 mn
Acc 1638	9.10 efgh	8.65 a	78.78 a	4.08 cdefgh	3.48 pqr	7.00 ghij	43.67 op
Acc 4095	8.72 fghij	5.80 mnop	50.60 ghij	4.03 defghij	4.00 lmnop	6.82 hij	63.80 hij
Acc 891	8.60 fghijk	7.53 bcdef	64.77 bc	3.65 efghijk	3.98 lmnopq	4.98 jkl	52.33 lmn
Acc 5642	8.53 fghijkl	7.40 cdefg	63.10 cde	4.83 abc	3.80 mnopqr	6.17 ijk	85.83 b
Acc 6774	8.47 ghijkl	7.03 efghij	59.54 cdef	3.93 defghij	5.33 efghij	10.42 cde	70.33 efg
Acc 1368	8.40 ghijkl	6.90 efghijk	57.86 cdefg	3.50 fghijkl	5.20 efghijk	14.11 ab	41.20 p
Acc 4177	8.37 ghijklm	6.53 ghijklm	54.51 defghi	3.40 ghijkl	3.03 qrs	4.07 klmno	70.20 efg
Acc 1315	8.03 ijklmn	6.03 lmn	48.65 hijk	4.22 cdef	6.35 bcd	14.40 ab	73.75 ef
Acc 4137	8.03 ijklmn	7.65 bcde	61.48 cde	4.18 cdefg	6.00 cdefg	8.90 efgh	68.83 fgh

Genotype	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Petiole length (cm)	Internodal length (cm)	Wax content ($\mu g/cm^2$)	Number of stomata
Acc 1390	7.78 klmno	6.88 fghijk	54.01 efghi	4.02 defghij	4.75 ijkl	6.18 ijk	72.17 ef
Acc 6786	7.63 lmno	5.85 mnop	44.60 jkl	3.43 ghijkl	4.68 ijklm	14.04 ab	79.33 cd
Acc 4152	7.35 mnop	6.28 ijklm	46.10 ijkl	4.19 cdefg	10.05 a	11.25 c	73.83 ef
Acc 6707	6.97 opq	6.13 jklmn	42.48 jklm	3.87 defghij	2.97 rs	3.40 lmno	61.25 ijk
Acc 807	6.90 opq	5.70 mnopq	39.31 klmn	3.37 hijkl	3.70 nopqr	3.54 lmno	63.30 ij
Acc 931	6.47 pq	6.00 lmno	38.66 klmn	3.13 jkl	4.87 hijkl	5.50 jkl	81.33 bc
General mean	8.51	6.69	57.36	3.89	5.17	7.41	64.74
SE	0.46	0.34	40.35	0.32	0.42	1.25	20.70
CV	7.93	8.71	11.07	14.50	12.57	15.11	7.03

The mean followed by different alphabet indicate significant difference between the genotypes

These morphological and physiological parameters were further used in principal component analysis (PCA) to identify genetic variability for drought tolerance and to identify the tolerant ones based on the desired traits for drought tolerance which has been indicated earlier. Yield parameters were also considered in PCA.

4.2. Analysis of yield attributing traits

The yield-attributing traits, such as the number of spikes per plant, spike length, peduncle length, berry size, number of matured berries per spike, number of immature berries per spike, test weight/ number of berries per spike, 100-berry fresh weight, 10-spiked berry weight, and 10-rachis weight were recorded in selected 21 accessions only as the yield attributing traits could not be recorded in the remaining genotypes. Genotypes exhibited significant variability ($P < 0.001$) for each trait.

4.2.1. Number of spikes per plant

The number of spikes per plant (NSP) varied from 18 to 93. The genotypes were grouped into low, medium and high based on the number of spikes per plant (a favorable characteristic for the increased yield) as follows.

	Low NSP (< 55)	Medium NSP (55 –70)	High NSP (> 70)
Genotypes	Acc 5717, Acc 1491, Acc 4064, Acc 5083, Acc 8052, Acc 7211, Acc 1439	Acc 1093, Acc 1487, Acc 5621, Acc 5623, Acc 1343, Acc 4132	Acc 6720, Acc 8060, Acc 1086, Acc 1495, Acc 1248, Acc 1218, Acc 5691, Acc 971

4.2.2. Spike length

The spike length (SL) of the genotypes ranged from 5.62 cm (Acc 8060) to 13.07 cm (Acc 971). The greater spike length is expected to result in an increased number of berries. The range of spike length for grouping of genotypes into low, medium and high spike length is shown below.

	Low SL (< 8 cm)	Medium SL (8-9 cm)	High SL (> 9 cm)
Genotypes	Acc 8060, Acc 5083, Acc 5717, Acc 5621, Acc 1093, Acc 1248, Acc 8052, Acc 1439	Acc 1495, Acc 1491, Acc 4064, Acc 1218, Acc 1487, Acc 1086	Acc 4132, Acc 6720, Acc 5623, Acc 5691, Acc 1343, Acc 7211 Acc 971

4.2.3. Peduncle length

The peduncle length (PL) varied within the range of 0.63 cm (Acc 5083) to 2.63 cm (Acc 5623) among the genotypes. The genotypes were grouped into three categories based on peduncle length viz. low (< 1.3 cm), medium (1.3-1.8 cm), and high (> 1.8 cm) which is shown below.

	Low PL (< 1.3 cm)	Medium PL (1.3-1.8 cm)	High PL (> 1.8 cm)
Genotypes	Acc 5083, Acc 6720, Acc 1218, Acc 5717, Acc 1093, Acc 1495, Acc 4064, Acc 1343	Acc 4132, Acc 1491, Acc 1439, Acc 1248, Acc 1086, Acc 8052	Acc 8060, Acc 1487, Acc 7211, Acc 5621, Acc 5691, Acc 971, Acc 5623

4.2.4. Berry size

The genotypes were categorized into three levels based on the berry size viz. low (< 6.7 cm), medium (6.7-7.2 cm), and high (> 7.2 cm). The minimum berry size was 5.81 mm (Acc 1218), and the highest was 8.81 mm (Acc 4132). A larger berry size is considered a favorable trait for better yield.

	Low BS (< 6.7 cm)	Medium BS (6.7- 7.2 cm)	High BS (> 7.2 cm)
Genotypes	Acc 1218, Acc 4064, Acc 5083, Acc 6720, Acc 1487, Acc 1439, Acc 5691, Acc 1495	Acc 1491, Acc 7211, Acc 1343, Acc 5623, Acc 8060, Acc 5621	Acc 1086, Acc 1093, Acc 8052, Acc 1248, Acc 5717, Acc 971 Acc 4132

4.2.5. Number of matured berries per spike

The number of matured berries (NMB) per spike ranged from 31 (Acc 8060) to 78 (Acc 5691).. Genotypes were grouped in to low, medium, and high based on the number of matured berries per spike as shown below.

	Low NMB (< 45)	Medium NMB (45-60)	High NMB (>60)
Genotypes	Acc 8060, Acc 1495, Acc 1093, Acc 4132, Acc 5717, Acc 1439, Acc 1487, Acc 8052	Acc 1491, Acc 5623, Acc 5083, Acc 4064, Acc 1343	Acc 1086, Acc 1218, Acc 5621, Acc 6720, Acc 1248, Acc 971, Acc 7211, Acc 5691

4.2.6. Number of immature berries per spike

The minimum value of 6 immature berries per spike was recorded in Acc 1248 while the maximum of 33 was recorded in Acc 1343. The accessions with low, medium and high number of immature berries per spike are given in the table below.

	Low NIB (< 11)	Medium NIB (11-13)	High NIB (>13)
Genotypes	Acc 1248, Acc 5621, Acc 1491, Acc 4064, Acc 6720, Acc 1439, Acc 5717	Acc 5691, Acc 1218, Acc 5623, Acc 4132, Acc 1487, Acc 7211, Acc 1086	Acc 8052, Acc 5083, Acc 1495, Acc 971, Acc 8060, Acc 1093, Acc 1343

4.2.7. Test weight (g)

Test weight (TW) of the genotypes ranged from 60 g (Acc 8052) to 92 g (Acc 971). The 21 genotypes were grouped into low (< 55), medium (55-70), and high (>70) test weight groups as shown bleow.

	Low TW (< 55)	Medium TW (55-70)	High TW (>70)
Genotypes	Acc 5717, Acc 4132, Acc 8060, Acc 1439, Acc 1495, Acc 1487, Acc 1093	Acc 1491, Acc 8052, Acc 4064, Acc 5623, Acc 1343, Acc 5083	Acc 1218, Acc 5621, Acc 1086, Acc 6720, Acc 1248, Acc 5691, Acc 7211, Acc 971

4.2.8. 100-berry fresh weight

The 100-berry fresh weight (BFW) of 21 black pepper accessions ranged from 7.33 g (Acc 1218) to 20.60 g (Acc 4132). The accessions were grouped in to low (< 10 g), medium (10-12 g), and high (>12g) 100 berry fresh weight as shown below. .

	Low BFW (< 10 g)	Medium BFW (10-12 g)	High BFW (>12g)
Genotypes	Acc 1218, Acc 1491, Acc 1495, Acc 5083, Acc 8060, Acc 4064, Acc 1487	Acc 1248, Acc 1439, Acc 5691, Acc 6720, Acc 5623, Acc 5717, Acc 5621, Acc 7211	Acc 1343, Acc 1093, Acc 1086, Acc 971, Acc 8052, Acc 4132

4.2.9. 10-spiked berry weight

The weight of 10 spiked berries was measured for the 21 genotypes and categorized into low (<55 g), medium (55-85 g), and high (>85 g) berry weight. The least weight was recorded in Acc 5621 (27.20 g) and the highest in Acc 4132 (121 g).

	Low SBW (<55 g)	Medium SBW (55-85 g)	High SBW (>85 g)
Genotypes	Acc 5621, Acc 5717, Acc 5083, Acc 1093 Acc 4064, Acc 8060 Acc 1487	Acc 1495, Acc 1439, Acc 1491, Acc 5691, Acc 5623, Acc 1343, Acc 1086	Acc 7211, Acc 8052, Acc 1218, Acc 1248, Acc 6720, Acc 971, Acc 4132

4.2.10. 10-rachis weight

The 21 black pepper genotypes were grouped based on 10 rachis weight into low (≤4.5 g), medium (4.5-5.7 g), and high (>5.7 g) categories. The minimum 10 rachis weight of 2.83 g was recorded in Acc 8060, while the maximum of 7.99 g was recorded in the Acc 4132.

	Low RW (<4.5 g)	Medium RW (4.5-5.7 g)	High RW (>5.7 g)
Genotypes	Acc 8060, Acc 1248, Acc 1093, Acc 8052, Acc 5621, Acc 5083, Acc 5691, Acc 5717	Acc 4064, Acc 1218, Acc 5623, Acc 1086, Acc 1343, Acc 7211	Acc 6720, Acc 1495, Acc 1491, Acc 1487, Acc 1439, Acc 971, Acc 4132

Table 3. Yield attributing traits of selected 21 black pepper genotypes

Genotypes	Number of berries/spike	Spike length (cm)	Number of spikes /plant	Peduncle length (cm)	Berry size (mm)	Number of matured berries/ spike	No. of immature berries/ spike	100 berry fresh weight (g)	10 spiked berries weight (g)	10 rachis weight (g)
Acc 8052	60 e	7.63 cdef	49 fg	1.70 bcdef	7.41 bcd	45 efg	14 cdef	13.50 b	94 b	3.97 gh
Acc 5083	67 d	5.90 ef	47 g	0.63 g	6.00 efg	50 ef	16 bcde	8.88 cd	37.16 h	4.19 fg
Acc 8060	49 fg	5.62 f	75 b	1.82 bcdef	7.05 bcdefg	31 i	18 bc	8.89 cd	51.53 f	2.83 h
Acc 1491	57 e	8.17 bcd	25 h	1.50 cdef	6.72 bcdefg	47 ef	9 fgh	8.68 cd	66.59 de	5.86 bcd
Acc 4064	60 e	8.23 bcd	30 h	1.27 efg	5.93 fg	51 de	9 fgh	8.97 cd	46.62 fg	4.62 defg
Acc 1487	54 ef	8.67 bcd	59 d	1.90 bcde	6.49 cdefg	42 efgh	12 defg	9.16 cd	53.10 f	6.05 bc
Acc 1093	54 ef	7.50 cdef	58 de	1.17 fg	7.22 bcde	34 hi	20 b	12.19 bc	42.67 gh	3.94 gh
Acc 5717	46 g	6.80 def	18 i	1.13 fg	7.73 abc	36 ghi	10 fgh	11.22 bcd	27.72 i	4.43 efg
Acc 971	92 a	13.07 a	93 a	2.27 ab	7.95 ab	75 a	17 bcd	13.20 b	99.17 b	7.59 a
Acc 7211	90 a	12.20 a	52 efg	2.03 abcd	6.80 bcdefg	77 a	12 defg	11.54 bc	85.13 c	5.63 bcde
Acc 5691	89 a	11.73 a	88 a	2.23 ab	6.57 cdefg	78 a	11 efgh	10.26 bcd	67.23 de	4.37 efg
Acc 1248	80 b	7.50 cdef	78 b	1.63 bcdef	7.57 bcd	74 ab	6 h	10.20 bcd	98.50 b	3.82 gh
Acc 6720	75 bc	9.47 bc	74 b	1.10 fg	6.33 defg	65 bc	9 fgh	10.52 bcd	99.13 b	5.76 bcd
Acc 1086	74 bc	8.73 bcd	75 b	1.67 bcdef	7.21 bcde	61 cd	13 cdef	13.06 b	83.33 c	5.52 bcde
Acc 1218	72 cd	8.37 bcd	81 b	1.10 fg	5.81 g	61 cd	11 efgh	7.33 d	94.95 b	4.77 cdefg
Acc 5621	72 cd	6.80 def	60 cd	2.17 abc	7.16 bcdef	64 c	7 gh	11.37 bc	27.20 i	4.07 gh

Genotypes	Number of berries/spike	Spike length (cm)	Number of spikes /plant	Peduncle length (cm)	Berry size (mm)	Number of matured berries/ spike	No. of immature berries/ spike	100 berry fresh weight (g)	10 spiked berries weight (g)	10 rachis weight (g)
Acc 1343	66 d	12.03 a	66 c	1.27 efg	6.83 bcdefg	52 de	33 a	12.17 bc	71.42 d	5.53 bcde
Acc 5623	60 e	9.77 b	60 cd	2.63 a	6.97 bcdefg	49 ef	11 efgh	10.69 bcd	71.36 d	5.35 bcdef
Acc 1439	50 fg	7.90 bcde	55 def	1.52 cdef	6.56 cdefg	40 fghi	9 fgh	10.24 bcd	64.77 de	6.16 b
Acc 1495	50 fg	8.10 bcd	75 b	1.23 efg	6.58 cdefg	33 hi	16 bcde	8.75 cd	62.57 e	5.77 bcd
Acc 4132	46 g	9.33 bc	66 c	1.43 def	8.81 a	34 hi	11 efgh	20.60 a	121.00 a	7.99 a
General mean	64	8.738	61	1.59	6.93	52	13	11.01	69.76	5.153
SE	12.82	1.17	14.46	0.14	0.42	31.78	8.23	3.96	17.53	0.46
CV	5.52	12.40	6.22	23.38	9.38	10.77	21.97	18.07	6.00	13.16

Yield parameters are very important in assessing the yield of the vines. Generally, black pepper starts flowering during second year and the yield parameters were recorded during the year. Yield stabilises only after five years of planting and hence, only yield parameters were considered. Yield parameters provide information on yielding ability of the genotypes and hence we will be able to assess the variability for yield among the genotypes. Genotypes with increased spike length, increased number of berries per spike, more number of filled berries per spike, genotypes with bigger berries etc. (Theertha *et al*., 2023) yield better compared to others. In the present study, accessions 1086, 971, 1218, 5621, 6720, 1248, 7211, 1495, 5691, 4132 and 1343 show such characters and hence, they are likely to yield better. Accessions 7211, 6720, 1495, 4132, 971, 1343, and 1086 showed desirable drought tolerance traits such as low stomata, high wax, low leaf area and internodal length etc. Accessions 4132, 7211, 1343, 1495, 971, 1086 and 6720 showed better yield parameters as well as desirable drought tolerance traits which can be targeted for sustainable yield under moisture stress conditions.

Principal component analysis (PCA) of 21 selected accessions based on morpho-physiological and yield attributing traits

The PCA study revealed the genetic diversity among the 21 black pepper genotypes in terms of their morpho-physiological and yield attributing traits. A cumulative variation of 73.88% for the first five components (Table. 3) of the quantitative traits was achieved out of seventeen components, according to the criterion of having eigenvalues greater than one. The observed traits within the five axes had a significant impact on the selected accessions. Similar findings among the black pepper genotypes in their quantitative characteristics reveal significant diversity accounted for by six components (Reshma *et al*., 2022). The performance of black pepper genotypes cultivated in lowland and high-altitude regions varies significantly due to differences in both genetic makeup and environmental factors (Sainamole *et al*., 2002). Genetic diversity and environmental variables have led to substantial heterogeneity in the chosen black pepper genotypes for the quantitative parameters identified in the present investigation.

The highest variability was noticed in the first principal component, accounting for 30.87%. This substantial loading was attributed by spike length (0.753), 10 spiked berries weight (0.693), 10 rachis weight (0.623), number of spikes per plant (0.592), 100 berry fresh weight (0.537), test weight (0.527), number of matured berries per spike (0.523), wax content (0.506), berry size (0.486), and peduncle length (0.468), all contributing in a positive direction. While, leaf area (-0.742), leaf width (-0.705), leaf length (-0.587), number of stomata (-0.530), and petiole length (-0.414) contributed negatively to PC1 (Fig 4.1). The highest variability was explained by PC1 in the yield attributing traits and physiological trait of wax content. Bhor *et al.* (2021) similarly observed that traits related to yield had the highest amount of variability. Yield-attributed contributions had a greater influence on the variance captured by PC1 than on the variation described by the other principal components (Reshma *et al*., 2022). PC1 clearly experienced the highest variation and was followed by others, as reported in all research that used principal component analysis.

The second component accounted for 14.90% of total variance and was positively impacted by the number of matured berries spike-1 (0.752), test weight (0.730), and internodal length (0.355). The remaining parameters, 100 berry fresh weight (-0.654) and berry size (-0.648), contributed to a negative direction. The yield components of black pepper genotypes were positively associated with their actual yield (Shivakumar *et al*., 2020). This suggests that the variables determining yield are important when assessing the total variance observed in the dataset. The third principal component, which accounted for 12.27% of the total variance, was positively attributed with leaf area (0.505), leaf width (0.443), and leaf length (0.411), while negatively associated with internodal length (-0.652) and petiole length (-0.627). The number of immature berries spike-1 (0.566) and wax content (0.555) were found to be closely associated in PC4, explaining 8.26% variability.

The scatterplot was helpful for illustrating genotype categorization based on similarities and differences influenced by characteristics. Except for the number of immature berries spike-1, yield attributing characters such as number of spikes plant-1, test weight, spike length, peduncle length, berry size, number of matured berries spike-1, 100 berry fresh weight, 10 spiked berries weight, and 10 rachis weight, as

well as wax content, were primarily attributed to the first axis as positive levels (Fig. 4). Shivakumar *et al*. (2022) discovered that berry weight and dry seed weight were highly significant for diverse black pepper genotypes regarding spike and berry features, with a strong positive association utilizing principal component analysis.

The genotype 971, closely followed by 4132, 7211, 1343, 1495, 6720, and 1086, were drought-tolerant genotypes that exhibited substantial contributions to the primary component through their traits with drought tolerance characteristics. Among all the genotypes studied, Acc 4132 had the highest positive value in the first component and the lowest negative value in the second principal component, indicating that it was the most tolerant. It is assumed that drought-tolerant black pepper genotypes will tend to have reduced leaf area, shorter internodal and petiole lengths, lower stomatal density, and increased wax content.

As a result, it can be concluded that morphological characteristics and stomatal density (Fig. 2), which were distributed across the second and third quadrants, had both significant and non-significant negative associations with the traits that contribute to the yield and wax content of black pepper genotypes when compared to those in the first and fourth quadrants. The traits with sensitive features were demonstrated by the accessions 5083, 4064, 1491, 8060, and 1093, providing a significant negative influence. Genotypes with drought-prone characteristics were placed in the second and third quadrants.

A Pearson correlation analysis (Fig. 5) provides an excellent visual representation of the connection between variables in a dataset. Pearson correlation analysis found a substantial association between the observed morphological features. The current study observed a strong positive correlation ($P < 0.001$) between leaf area and leaf width ($r = 0.93$), similar findings reported by Preethi *et al*. (2018) indicated that leaf length ($r = 0.80$) and internodal length and petiole length ($r = 0.60$) were also significant ($P < 0.01$). Leaf area exhibited a similar association with leaf breadth (Jayarathna *et al*., 2016).

Larger leaf area did not correspond with increased cultivar production, although flower intensity spike-1, inflorescence size, and berry spike did (Chen *et al*., 2018).

Lack of irrigation reduces both leaf size and quantity in black pepper plants (Rasanjali *et al*., 2019; Teles *et al*., 2023). Similar results have been observed in tomato (Heuvelink *et al*., 2005) and pepper crops, demonstrating a decrease in leaf area due to water scarcity (Cemek *et al*., 2020; Koch *et al*., 2019). As a result, a reduction in leaf area might be viewed as a positive trait for drought tolerance since it minimizes water loss through transpiration, as demonstrated by the current study.

Leaf length and breadth were found to have a homogenous correlation (r=0.54). Stomata had a non-significant positive connection ($P \geq 0.05$) with leaf length (0.20), breadth (0.33), and area (0.36). Wax content had a significant negative connection with stomata number (-0.49) ($P<0.05$), followed by leaf width (-0.42), area (-0.39), and length (-0.18). Paulus and Sim (2011) found that black pepper types with broader leaf bases contain more stomata, as evidenced by the leaf length to breadth ratio. Elevated stomatal density might be associated with leaf area growth and cell division. Enhancing stomatal regulatory competence through selective breeding methods could enable plants to adapt more efficiently to environmental constraints such as drought (Xu & Zhou, 2008). During drought and high radiation, a decrease in transpiration rate and an increase in leaf wax load improve the yield index (Sanchez *et al*., 2001). The wax content showed non-significant positive relationship with the characteristics that contribute to yield. The positive association between leaf wax content and yield-attributing features sheds light on a plant's ability to save water content in the highest yielding genotypes, particularly when water is scarce. This knowledge might be employed in breeding methods to increase productivity in water-limited environments.

There was a substantial positive association ($P<0.001$) between berry size and 100 berry fresh weight, as well as test weight and the number of matured berries/spike (r=0.83). Fresh yield has been shown to be positively correlated with the quantity of berries per spike (Sainamole *et al*., 2002; Bermawie *et al*., 2019). The correlation between berries and test weight might be an indicator of total yield, emphasizing their significance in determining yield.

Positive relationships ($P < 0.01$) were found between test weight and spike length ($r = 0.61$), 10 rachis weight and spike length ($r = 0.59$), and number of developed berries per spike and spike length ($r = 0.57$) significantly. Genetic and environmental variables can influence the quantity of berries per spike (Ibrahim *et al*., 1985) and spike length (Krishnamurthy *et al*., 2010). The berry quantity is more sensitive to changes in environmental conditions than the length of the spike (Ibrahim *et al*., 1987). The current study emphasized the interplay of genetic and environmental factors, particularly those determining berry size, test weight, spike length, and the quantity of developed berries per spike. The number of berries in a spike is directly proportional to its length, a pattern that has been consistently observed across states such as Kerala (Maheswarappa *et al*., 2012; Sujatha & Namboothiri, 1995; Reshma *et al*., 2022), Karnataka (Tripathi *et al*., 2018), and Assam (Deka *et al*., 2016; Nath *et al*., 2021). Rachis weight, spiked berry weight, 100-berry fresh weight, spike length, number of spikes per plant, and peduncle length all showed a non-significant but positive correlation. The number of berries per spike favorably affects pepper production.

According to Shivakumar *et al*. (2022), the Panniyur-1, Agali, and Narayakodi genotypes had high PC1 values, as well as higher values for yield-attributing traits (berry weight, dry seed weight, and fresh pericarp weight), indicating a strong positive correlation, as observed in the present research. These qualities are relevant to both principal component analysis and genotype clustering.

The results of the morpho-physiological and yield contributing trait-based dendrogram (Fig. 6) in the current study revealed genotypes with the most favourable features for drought tolerance in clusters 2 and 3, whereas genotypes with sensitive characteristics were grouped in cluster 1. Accessions 7211, 971 (cluster 2), 4132, 1495, and 1343 (cluster 3) possessed drought-tolerant characteristics. The current study is consistent with prior findings in wheat genotypes that were classified based on morphological attributes using the resulting dendrogram into high homogeneity groups within the clusters (Pasandi *et al*., 2016). Wheat yield variables such as spikes plant $^{-1}$, number of grains spike $^{-1}$, grain weight spike $^{-1}$, grain yield plant^{-1}, and spike

density all contributed significantly to the first main component and were clustered together in the same cluster (Fouad, 2020).

The integration of both PCA and cluster analysis offered insights into the performance of genotypes subjected to altered environmental conditions, aiding in the selection of superior genotypes in drought-prone regions. The variance within black pepper genotypes for yield contributing factors as determined by PCA, as well as the subsequent categorization of genotypes by dendrogram in the current study, may reflect both genotypic differences and environmental impacts.

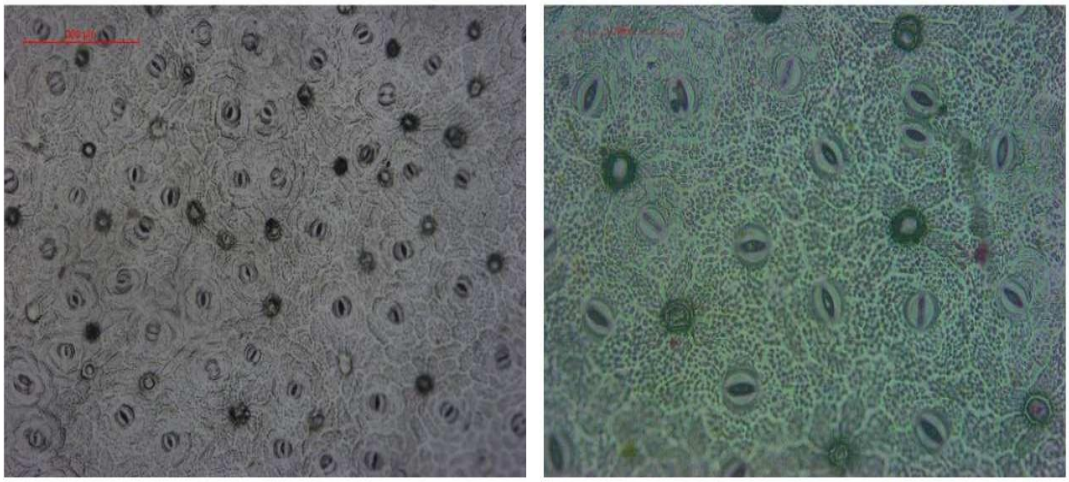


Figure 2. Stomata on the ventral side of black pepper leaves (10X and 20X, image size: 391.634x 522.517 µm)

Table 4. Eigen values and percentile variance of selected black pepper genotypes

	Principal Component				
	PC 1	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Eigen value	5.24	2.53	2.08	1.4	1.28
% Variance	30.87	14.9	12.27	8.26	7.56
Variable	**Eigen vectors**				
Leaf length (cm)	-0.587	0.022	**0.411**	0.232	**0.379**
Leaf width (cm)	-0.705	-0.095	**0.443**	0.026	**0.349**
Leaf area (cm^2)	-0.742	-0.04	**0.505**	0.119	**0.403**
Petiole length (cm)	-0.414	0.255	-0.627	0.144	0.154
Internodal length (cm)	-0.255	**0.355**	-0.652	0.078	**0.348**

	Principal Component				
	PC 1	**PC 2**	**PC 3**	**PC 4**	**PC 5**
No. of stomata	-0.53	0.227	**0.31**	-0.284	-0.358
Wax content (µg/cm^2)	**0.506**	-0.065	-0.17	**0.555**	-0.036
Number of spikes plant^{-1}	**0.592**	0.279	0.14	0.219	-0.043
Test weight (gm)	**0.527**	**0.73**	**0.332**	-0.039	0.036
Spike length (cm)	**0.753**	0.171	**0.356**	0.202	0.077
Peduncle length (cm)	**0.468**	0.239	0.074	-0.51	0.065
Berry size (mm)	**0.486**	-0.648	0.084	-0.429	0.07
Number of matured berries spike^{-1}	**0.523**	**0.752**	0.224	-0.092	0.113
Number of immature berries spike^{-1}	0.061	-0.276	**0.35**	**0.566**	-0.476
100 berry fresh weight (g)	**0.537**	-0.654	0.104	-0.145	0.203
10 spiked berries weight (g)	**0.693**	-0.082	0.138	0.101	**0.439**
10 rachis weight (g)	**0.623**	-0.289	-0.226	**0.158**	**0.305**

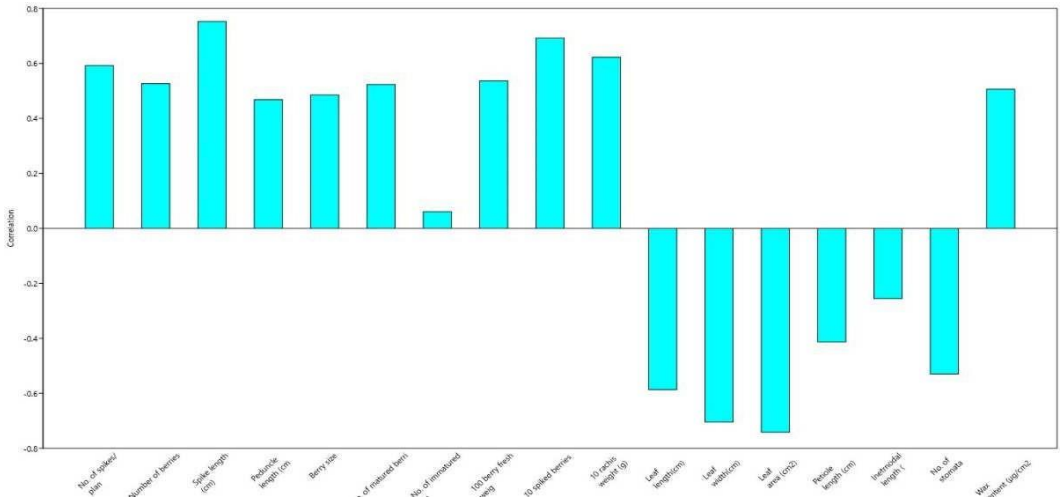


Figure 3. Loading plot of first principal component with variables

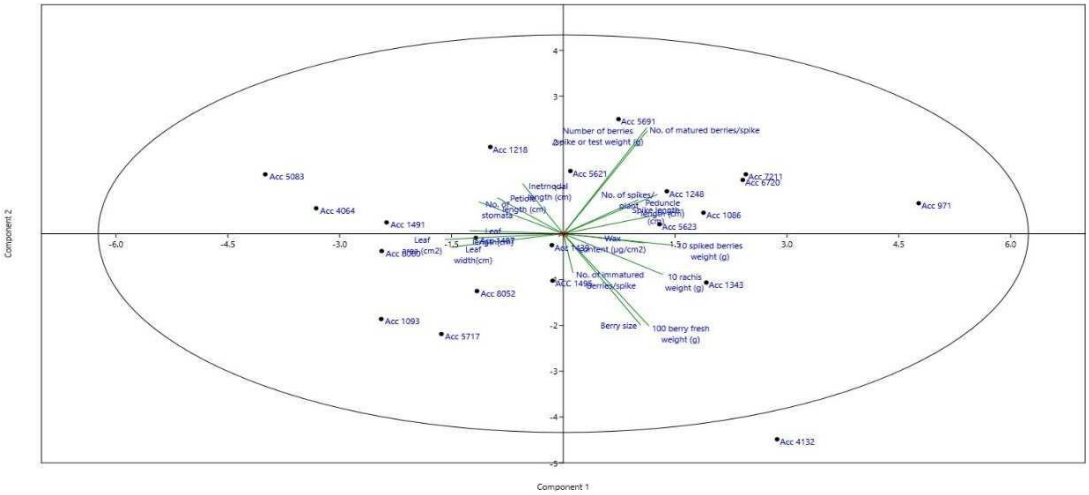


Figure 4. Black pepper genotypes distribution across the first two primary components

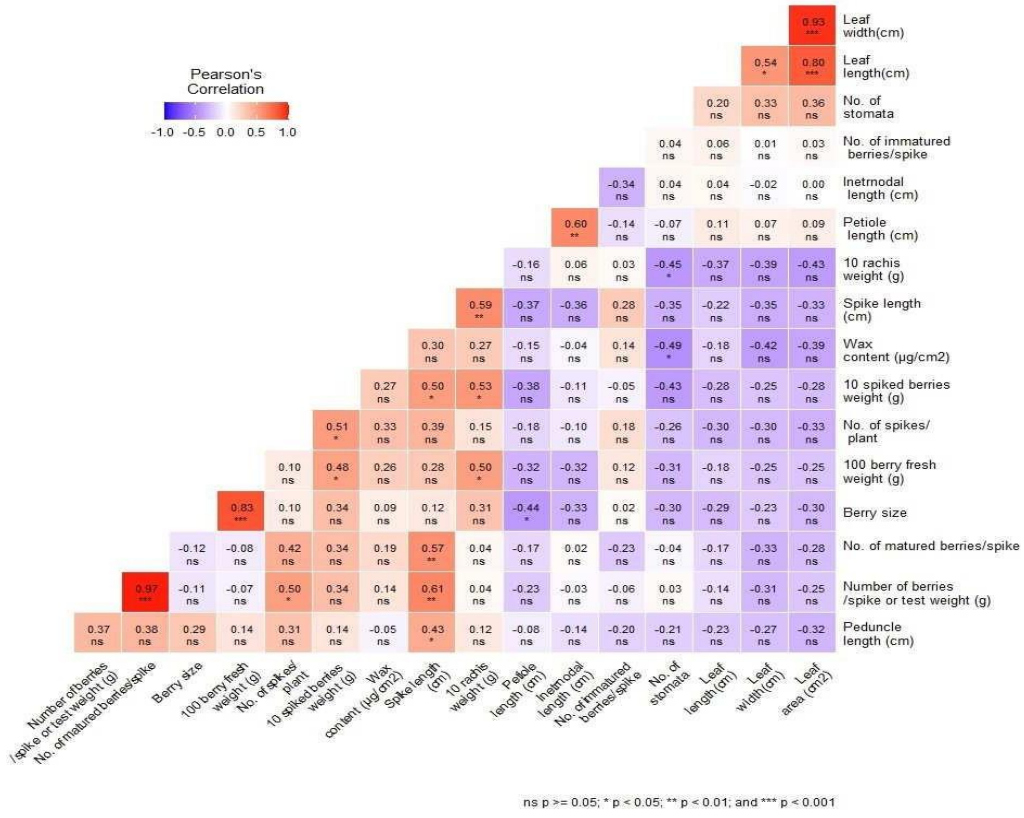


Figure 5. Pearson correlation matrix of 17 quantitative traits to profile 21 black pepper genotypes

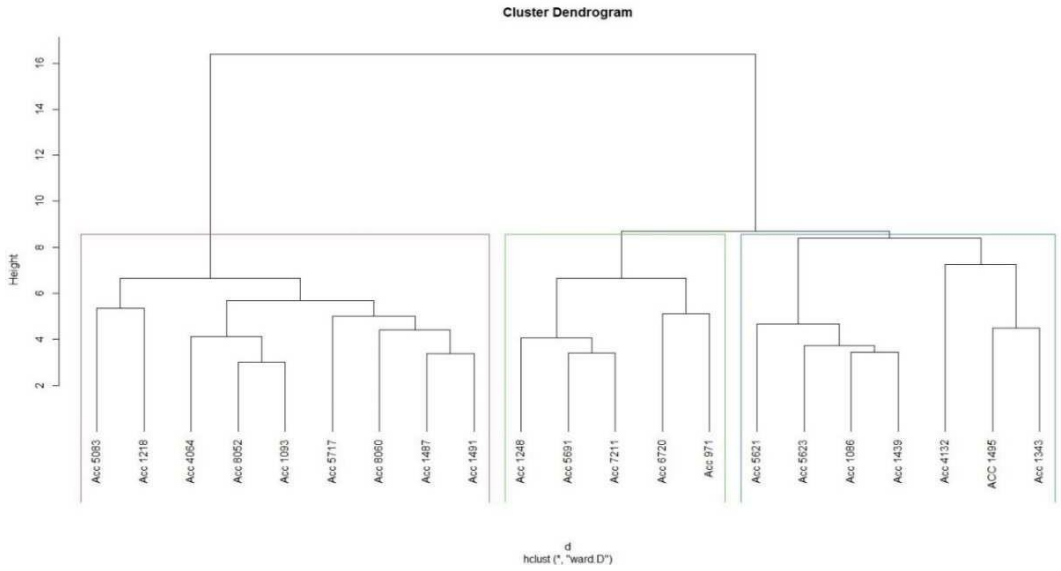


Figure 6. Clustering of black pepper genotypes

PCA results suggested that accessions 1343, 1495, 4132, 1439, 7211, 971, 5623 and 1086 can be considered for drought tolerance, accessions 7211, 6720, 1495, 971, 4132, 5691, 5621 and 1343 for yield attributing traits and accessions 1343, 4132, 7211, 1495 and 971 for both drought tolerance and yield attributing traits.

4.3. Quality parameters

The quality parameters of black pepper genotypes like oleoresin, volatile oil and piperine were estimated from dried berries of 21 accessions.

4.3.1. Oleoresin (%)

The oleoresin, varied significantly ($P<0.001$) among 21 accessions, ranging from 7.56% (Acc 1495) to 13.71% (Acc 5621). The significantly highest oleoresin content was recorded in Acc 5621 (13.71%) followed by accessions 6720 (13.58%), 1086 (13.4%), 5691 (13.4%), and 7211 (13.3%). Lowest content was exhibited by Acc 1495 (7.56%) followed by accessions, 1093 and 4064 (8.45% and 8.49% respectively). The remaining accessions showed intermediate values between these extremes.

	Low oleoresin (< 10 %)	Medium oleoresin (10-13%)	High oleoresin (> 13%)
Genotypes	Acc 1495, Acc 1093, Acc 1487, Acc 5623, Acc 8052, Acc 5717, Acc 971, Acc 5083, Acc 4064	Acc 1248, Acc 1439, Acc 1343, Acc 8060, Acc 1218, Acc 1491, Acc 4132	Acc 7211, Acc 5691, Acc 1086, Acc 6720, Acc 5621,

The maximum yield of black pepper oleoresin obtained from optimal conditions ranged from 11 to 13% when using ethanol as the extraction solvent, compared to hexane and acetone (Gafar, 2022). However, the present study utilized acetone as the extraction solvent. The choice of solvent significantly influence the yield and quality. Typically, black pepper, rather than white pepper, is used as the raw material for oleoresin extraction to achieve a better yield, which aligns with our current study.

Microcapsules prepared from gum arabic exhibited better encapsulation ability for piperine in black pepper oleoresin, enhancing its stability, masking unpleasant characteristics, and protecting it from interactions (Shaikh *et al*., 2006).

A better strategy for improving quality attributes in black pepper is through a selection program, as it significantly correlates positively with both oleoresin and oil (Kurian *et al*., 2002), paralleling the present study's implications for optimizing the yield of oleoresin. The range of oleoresin obtained through hydro distillation was 4.27% to 12.73% among black pepper seeds, whereas it was 10.6% through supercritical fluid extraction (Ashokkumar *et al*., 2021).

4.3.2. Essential oil

The essential oil in black pepper berries significantly differed ($P<0.001$) among the 21 accessions which ranged between 3.15% to 6.25%. Accessions with higher oil contents were Acc 5691 (6.25%), Acc 4132 (6.19%), Acc 6720 (6.17%), Acc 1093 (6.17%) and Acc 1439 (6.15%). The lowest oil content was recorded in the Acc 5083 (3.15%) followed by Acc 5717 (3.24%).

	Low EO (< 4.5%)	Medium EO (4.5- 6%)	High EO (> 6%)
Genotypes	Acc 5083, Acc 5717, Acc 4064, Acc 971, Acc 1487	Acc 5691, Acc 5623, Acc 8060, Acc 1343, Acc 1495, Acc 8052, Acc 1491, Acc 1248, Acc 5621, Acc 1218, Acc 1093, Acc 7211	Acc 1439, Acc 6720, Acc 4132, Acc 1086

4.3.2.1. GC-MS characterization of volatile compounds

The GCMS analysis was employed to identify the volatile oil constituents of 21 different black pepper accessions. A total of 60 essential oil components were identified. The major components identified were Myrcene (1.02 to 2.62%), alpha-Thujene (0.07 to 3.16%), Linalool (0.13 to 0.92%), and Camphene (0.05 to 0.49%). However, the predominant contributions were made by the constituents alpha-Pinene (2.86 to 12.87%), sabinene (0.32 to 21.35%), beta-Pinene (5.22 to 10.83%), D-Limonene (10.03 to 20.64%), Caryophyllene (7.6 to 37.97%), and alpha-Phellandrene (0.15 to 11.2%).

Among the accessions, the highest content of alpha-Thujene was noticed in Acc 1086 (3.16%), while the lowest was found in Acc 8060 (0.07%). Acc 5621 had the highest levels of alpha-Pinene, alpha-Phellandrene, Camphene, and Myrcene, and the lowest levels of Sabinene, gamma-Terpinene, Linalool, and beta-Bisabolene. Acc 4132 showed the highest (21.35%) and Acc 5621 showed the lowest (0.32%) sabinene content in. Acc 4132 exhibited the maximum beta-Pinene (10.83%), and the highest D-Limonene content (20.64%). The lowest D-Limonene content was recorded in Acc 6720 (10.03%) in, while Acc 6720 exhibited the least value for Myrcene. Acc 4064 recorded the highest linalool (0.92%), and caryophyllene (40%) contents. The range for different oil constituents in different accessions is given below.

The genotypes 4132 and 4064 showed better chemical composition. Genotype 4132 had high contents of sabinene, beta-Pinene and D-Limonene, indicating its resistance ability. Genotype 4064 possessed the highest levels of linalool and caryophyllene, essential components in black pepper volatile oil. The increase in oil components even under drought stress demonstrates their resilience, which is crucial for the flavor industry.

Myrcene (%)		
	Low to medium (1 to 1.73%)	Medium to high (1.73 to 2.7%)
Genotypes	Acc 6720, Acc 8060, Acc 1248, Acc 5083, Acc 1218, Acc 4064, Acc 5717, Acc 1491, Acc 8052 Acc 971	Acc 1495, Acc 1093, Acc 7211, Acc 1439, Acc 5691, Acc 1086, Acc 1487, Acc 5623, Acc 1343, Acc 5621, Acc 4132
alpha-Thujene (%)		
	Low to medium (0.05 to 1.5%)	Medium to high (1.5 to 3.2%)
Genotypes	Acc 8060, Acc 5621, Acc 5083 Acc 971, Acc 1248, Acc 5717, Acc 4064, Acc 6720, Acc 1093, Acc 8052	Acc 5623, Acc 1218, Acc 1495, Acc 1491, Acc 1343, Acc 1439, Acc 7211, Acc 5691, Acc 1487, Acc 1086, Acc 4132
Linalool (%)		
	Low to medium (0.1 to 0.6%)	Medium to high (0.6 to 1%)
Genotypes	Acc 5621, Acc 971, Acc 1248, Acc 8052, Acc 5623, Acc 6720, Acc 5691, Acc 1086, Acc 1487	Acc 1218, Acc 1495, Acc 1491, Acc 5083, Acc 1439, Acc 4064, Acc 1343, Acc 7211, Acc 4132
Camphene (%)		
	Low to medium (0.05 to 0.1%)	Medium to high (0.1 to 0.5%)
Genotypes	Acc 5083, Acc 1491, Acc 1093, Acc 5717, Acc 4064, Acc 8060, Acc 971, Acc 1487, Acc 1248 Acc 1439	Acc 1343, Acc 5623, Acc 8052, Acc 1086, Acc 1495, Acc 1218, Acc 5691, Acc 7211, Acc 5621, Acc 4132
alpha-Pinene (%)		
	Low to medium (2 to 4.6%)	Medium to high (4.6 to 7%)
Genotypes	Acc 5083, Acc 971, Acc 5717 Acc 6720, Acc 1248, Acc 1093, Acc 4064, Acc 1487, Acc 8060	Acc 8052, Acc 7211, Acc 1491, Acc 5623, Acc 1439, Acc 1495, Acc 1218, Acc 1086, Acc 5691, Acc 1343, Acc 5621, Acc 4132
Sabinene (%)		
	Low to medium (0.3 to 13%)	Medium to high (13 to 22%)
Genotypes	Acc 5621, Acc 8060, Acc 5083, Acc 971, Acc 5717, Acc 1248, Acc 4064, Acc 6720, Acc 1093 Acc 8052	Acc 1218, Acc 5623, Acc 1491, Acc 1495, Acc 7211, Acc 1439, Acc 1343 Acc 5691, Acc 1487, Acc 4132

beta-Pinene (%)		
	Low to medium (5 to 7.5%)	Medium to high (7.5 to 11%)
Genotypes	Acc 1487, Acc 4064, Acc 6720 Acc 1439, Acc 5717, Acc 1491, Acc 5623, Acc 5083, Acc 1093, Acc 971	Acc 1248, Acc 8052, Acc 7211, Acc 1495, Acc 1218, Acc 1086, Acc 5691, Acc 8060, Acc 5621, Acc 1343, Acc 4132
D-Limonene (%)		
	Low to medium (10-15%)	Medium to high (15 to 21%)
Genotypes	Acc 6720, Acc 4064, Acc 5083, Acc 5717, Acc 8052, Acc 971, Acc 1093, Acc 1248, Acc 1218	Acc 1439, Acc 5623, Acc 7211, Acc 1491, Acc 1495, Acc 1343, Acc 5621, Acc 5691, Acc 1086, Acc 1487, Acc 4132
Caryophyllene (%)		
	Low to medium (7 to 23 %)	Medium to high (23 to 40%)
Genotypes	Acc 1487, Acc 1218, Acc 5691, Acc 8052, Acc 1491, Acc 5621, Acc 1439, Acc 5083, Acc 1093	Acc 1086, Acc 5623, Acc 5717, Acc 7211, Acc 971, Acc 1495, Acc 1248, Acc 1343, Acc 4064, Acc 4132
alpha-Phellandrene (%)		
	Low to medium (0.1 to 0.6%)	Medium to high (0.6 to 12%)
Genotypes	Acc 1248, Acc 6720, Acc 8052 Acc 1218, Acc 4064, Acc 5691, Acc 5083, Acc 1487, Acc 1086	Acc 1491, Acc 7211, Acc 1343, Acc 1439, Acc 5717, Acc 1093, Acc 971, Acc 1495, Acc 5623, Acc 8060, Acc 5621, Acc 4132

Typically, the essential oil content ranged from 0.4 to 7% in dried black pepper berries (Menon *et al*., 2002). Among *Piper* species, the predominant volatile component of monoterpenes account for 87.6% of black pepper profiled using GCMS, followed by *P. cubeba* (38.3%) and *P. longum* (10.5%). The proportional range of remaining dominated components includes sesquiterpenes (12.3%), α-pinene (6.6%), β-pinene (15.9%), 3-carene (17.6%), limonene (35.6%), and β-caryophyllene (9.5%) (Al-Sayed *et al*., 2021).

According to Asadi and History (2022), the chemical profiling of shadow-dried black pepper berries predominantly revealed five components out of nineteen. They were selected and proportioned as follows: trans-caryophyllene bicyclo (36.43%),

limonene cyclohexene (6.75%), 3-carene (4.97%), cyclohexene, 1-methyl-4-(5-methyl) (4.93%), and 2-beta-pinene bicyclo (4.18%). These findings are consistent with the present study, which found higher caryophyllene in Acc 4064.

A comparative quantitative analysis of GCMS profiling on black pepper essential oil (EO) and its hydrolate was conducted, identifying 55 components from EO and 12 compounds solely from the hydrolate. The higher proportional contributions of EO were (E)-caryophyllene (41.6%), limonene (9.7%), and sabinene (8.6%). In contrast, α-terpineol (34.7%), borneol (17.3%), and terpinen-4-ol (13.9%) were identified as the abundant compounds from the hydrolate (Milenkovic *et al*., 2022). These findings support the present study, which also identified higher percentages of caryophyllene and limonene as major components in black pepper. The black pepper oil profiling in the present study demonstrated its consistency across different studies and emphasized its potential for optimizing these components through stress resilience and selective breeding.

Table 5. Chemo-profiling of volatile oils from selected 21 accessions using GC-MS.

Compound	RI	1218	1495	4132	1086	7211	6720	5621	971	1248	1343	5691	5623	1439	8060	4064	1093	8052	5717	5083	1487	1491
alpha.-Thujene	924	1.82	1.84	1.84	3.16	2.48	1.02	0.20	0.40	0.67	2.02	2.58	1.75	2.44	0.07	0.79	1.05	1.50	0.71	0.38	2.76	1.87
alpha.-Pinene	932	5.30	5.17	6.45	6.43	5.00	3.40	12.87	3.01	3.58	7.00	6.90	5.03	5.10	4.58	3.69	3.61	4.66	3.18	2.86	3.71	4.69
Camphene	946	0.14	0.13	0.40	0.13	0.16	-	0.49	0.09	0.12	0.12	0.15	0.12	0.12	0.09	0.08	0.07	0.12	0.07	0.05	0.10	0.06
Sabinene	969	13.38	14.82	21.35	-	17.21	7.75	0.32	4.24	6.21	18.92	20.04	13.43	18.10	0.41	6.72	9.90	12.20	6.08	3.64	17.95	14.67
beta.-Pinene	974	8.80	8.65	10.83	9.34	6.71	5.81	10.72	7.44	7.69	5.22	10.45	6.91	6.31	10.58	5.80	6.99	8.13	6.56	6.96	9.44	8.32
Myrcene	988	1.44	1.74	1.85	2.10	1.85	1.02	2.62	1.73	1.43	2.32	1.99	2.23	1.90	1.21	1.53	1.81	1.73	1.60	1.43	2.11	1.73
alpha.-Phellandrene	1002	0.29	2.90	2.23	0.57	0.93	0.17	11.20	2.20	0.15	1.58	0.43	2.94	1.68	3.52	0.35	2.18	0.28	1.88	0.46	0.48	0.61
3-Carene	1008	-	-	8.20	0.02	2.68	-	11.17	7.70	-	-	-	10.27	5.89	13.20	10.20	7.68	-	7.56	6.37	0.03	1.04
alpha.-Terpinene	1014	0.48	0.17	0.22	0.39	0.53	0.15	-	-	-	0.16	-	0.21	0.33	-	0.10	-	-	-	-	0.29	0.47
p-Cymene	1020	-	-	-	-	-	-	-	-	-	-	-	-	-	0.26	-	0.13	-	-	-	-	-
o-Cymene	1022	0.11	-	-	0.15	0.62	-	1.13	0.16	-	0.26	-	0.27	0.37	16.36	0.20	-	-	0.23	0.16		0.12
D-Limonene	1024	14.98	17.04	20.64	19.59	15.63	10.03	18.35	13.86	14.49	17.19	19.03	15.37	15.35	-	11.74	14.24	13.82	12.92	12.84	19.57	16.61
.beta.-cis-Ocimene	1044	0.17	0.23	0.10	0.43	0.18	0.55	-	-	0.37	0.63	0.23	0.41	0.30	0.12	0.21	0.12	0.24	0.10	0.18	0.28	0.25
gamma.-Terpinene	1054	0.79	0.29	0.39	0.65	0.96	0.26	0.11	0.11	-	0.29	0.16	0.36	0.56	-	0.20	0.13	0.14	0.15	0.12	0.49	0.79
cis-Sabinene hydrate	1065	-	0.20	-	0.40	0.46	0.10	-	-	-	0.39	0.20	-	0.31	0.77	-	-	-	-	-	0.24	0.27
Terpinolene	1086	0.30	0.23	0.16	0.30	0.44	0.15	0.66	0.55	0.18	0.17	-	0.70	0.53	0.57	0.69	0.15	0.21	0.47	0.40	0.29	0.37
Linalool	1095	0.61	0.65	0.78	0.55	0.73	0.41	0.13	0.23	0.26	0.68	0.51	0.37	0.77	-	0.92	-	0.31	-	0.76	0.58	0.76
trans-Sabinene hydrate	1098	0.11	-	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.13
L-camphor	1141	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpinen-4-ol	1174	1.77	0.63	0.69	1.45	2.21	0.57	-	0.10	0.16	0.11	0.32	0.51	1.28	0.20	0.24	0.16	0.25	0.23	0.10	0.82	1.79

Compound	RI	1218	1495	4132	1086	7211	6720	5621	971	1248	1343	5691	5623	1439	8060	4064	1093	8052	5717	5083	1487	1491
alpha.-Terpineol	1186	0.16	-	0.14	0.19	0.17	0.11	0.15	0.13	-	-	-	-	0.16	-	0.12	-	0.14	0.15	0.25	0.10	0.15
2-Undecanone	1293	-	-	-	-	-	-	-	-	-	-	-	-	-	0.60		-	0.10	-	-	-	-
delta.-EIemene	1335	0.43	0.44	0.11	1.46	0.70	1.23	-	0.86	4.64	2.30	0.46	1.25	1.03	-	1.50	0.12	1.04	0.31	1.00	0.33	0.67
.alpha.-Cubebene	1345	-	-	-	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	0.44	-	-
Cyclosativene	1369	-	-	0.14	-	-	0.12	-	0.20	-	-	-	-	-	-	0.10	0.15	-	0.15	-	-	-
isoledene	1374	-	-	-	-	-	-	-	0.29	-	-	-	-	-	-	-	-	-	-	-	-	-
.alpha.-Copaene	1374	-	-	5.49	0.38	1.57	7.14	0.11	6.10	6.68	0.11	-	0.53	0.19	2.65	0.11	5.02	0.11	5.09	0.71	-	-
beta.-Elemene	1389	0.16	0.16	0.38	0.26	0.75	-	1.10	1.01	1.12	1.00	0.13	0.60	1.22	-	0.47	-	1.05	0.46	3.56	0.11	0.47
trans-Caryophyllene	1408	-	-	-	-	-	-	-	-	-	0.51	-	-	-	0.20	-	-	-	-	-	-	-
alpha.-Gurjunene	1409	-	-	0.11	0.37	-	0.58	-	0.12	0.15	0.13	-	-	0.16	-	-	0.10	-	-	0.72	-	-
cis-.alpha.-Bergamotene	1411	2.18	-	-	-	-	-	-	-	-	-	-	-	-	23.37	0.14	-	-	-	-	1.61	1.69
Caryophyllene	1417	9.02	30.15	32.42	24.65	27.83	-	18.21	29.87	34.99	37.97	9.04	25.90	19.56	-	40.00	22.47	10.40	25.90	22.18	7.60	11.49
.beta.-Copaene	1430	-	-	0.15	-	0.10	0.66	0.33	0.27	-	1.75	-	0.29	-	-	-	1.07	-	0.19	-	-	-
trans-.alpha.-Bergamotene	1432	3.08	3.17	-	-	0.21	-	-	-	-	-	2.86	-	-	0.60	0.20	-	-	-	-	2.24	2.31
.alpha.-Guaiene	1437	-	-	-	-	-	0.37	-	-	-	0.28	-	0.97	0.17	-	0.27	-	-	-	0.15	-	-
.beta.-trans-Farnesene	1440	-	-	-	-	-	-	-	-	-	-	-	-	-	0.23	-	-	-	-	-	-	-
.alpha.-Humulene	1452	0.62	0.81	1.64	1.68	1.55	2.96	1.57	2.44	3.03	1.59	0.54	1.71	1.96	-	2.87	1.54	2.07	-	3.33	0.46	0.96
(E)-.beta.-Farnesene	1454	2.16	2.47	-	0.11	0.21	-	-	-	-	-	2.01	0.10	-	2.67	0.32	0.46	0.19	0.50	0.36	1.61	1.71
.gamma.-Gurjunene	1475	-	-	-	-		0.12	-	-	-	-	-	-	-	-	-	0.70	-	0.81	0.16	-	-
.gamma.-Muurolene	1478	-	-	-	-	0.12	-	-	0.13	-	-	-	-	-	-	-	-	-	0.10	-	-	-
Germacrene D	1484	-	-	0.44	-	7.17	-	-	0.42	0.39	-	-	-	-	-	1.41	0.36	1.09	-	0.21	-	-

Compound	RI	1218	1495	4132	1086	7211	6720	5621	971	1248	1343	5691	5623	1439	8060	4064	1093	8052	5717	5083	1487	1491
.beta.-Selinene	1489	-	0.25	0.65	-	0.49	1.44	2.40	2.35	2.73	2.04	0.23	1.08	4.12	-	0.15	0.56	0.64	0.63	8.75	0.15	1.41
alpha.-Zingiberene	1493	-	3.67	0.31	-	0.15	0.47	-	-	0.20	0.34	2.88	-	-	4.55	0.58	0.19	0.18	0.19	-	2.52	2.66
Epicubebol	1493	-	-	0.13	-	0.16	0.52	-	1.43	-	-	-	-	0.42	-	-	1.02	0.15	1.26	0.26	-	-
alpha.-Selinene	1498	-	0.12	-	-	0.33	-	-	1.96	1.95	-	-	-	3.06	-	-	-	-	-	6.89	-	1.03
beta.-Bisabolene	1505	-	4.41	2.90	-	0.32	1.09	0.13	-	0.71	-	3.80	-	-	-	1.22	2.75	1.66	3.02		3.04	3.42
.alpha.-Farnesene	1505	-	-	-	0.17	-	0.57	-	-	0.19	0.14	-	-	-	-	-	-	-	-	0.13	-	-
trans-.gamma.-Bisabolene	1514	-	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
delta.-Cadinene	1522	-	-	2.60	0.30	0.87	3.20	-	2.62	2.65	-	-	0.51	0.27	-	-	2.16	-	2.29	-	-	-
.alpha.-Cadinene	1537	-	-	0.57	-	0.17	-	-	1.00		-	-	0.22	-	-	-	0.71	-	0.70	-	-	-
Elemol	1548	-	3.04	6.47	-	0.16	-	-	-	-	0.19	1.55	-	-	-	-	5.51	0.17	4.44	-	1.85	2.14
Germacrene B	1559	-	-	-	0.85	-	-	-	-	-	7.96	-	-	-	1.71	0.40	-	6.88	-	-	-	-
E-Nerolidol	1561	-	-	1.17	0.79	-	0.22	-	0.44	-	-	-	-	1.62	-		0.98	-	1.52	3.17	-	-
Spathulenol	1577	-	-	-	-	0.47	0.44	-	-	0.46	0.82	-	-	0.55	-	0.29	-	0.29	-	-	-	-
Caryophyllene oxide	1582	-	-	0.23	0.45	0.93	0.64	-	0.25	0.47	1.26	0.97	-	0.41	-	0.58	0.17	0.96	0.60	-	0.69	0.89
epi-Cubenol	1627	-	-	0.23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
alpha.-epi-Cadinol	1638	-	-	-	-	-	-	-	0.15	-	-	-	-	-	-	-	-	-	-	-	-	-
.alpha.-epi-Muurolol	1640	-	-	0.46	-	0.15	0.19	-	0.49	-	-	-	-	0.17	-	-	0.34	-	0.44	-	-	-
delta.-Cadinol	1655	-	-	1.62	-	0.52	0.62	0.17	2.26	-	0.11	-	-	0.84	-	-	1.56	0.10	2.08	-	-	0.21
.alpha.-Bisabolol	1685	-	3.74	-	-	0.13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.02	3.43
Total number		24	27	38	30	42	34	22	37	27	33	24	27	34	23	34	35	31	35	32	30	34
Total percentange		68.30	107.12	128.73	77.42	104.11	54.08	94.14	96.62	95.67	115.56	87.46	94.04	97.25	88.52	94.19	96.16	70.81	92.57	88.98	89.33	89.19

RI- Retention Indices

4.3.3. Piperine (%)

The piperine content showed significant variation (P<0.001) among the 21 black pepper accessions, ranging from 2.69% (Acc 5623) to 4.44% (Acc 5691). Accessions with higher piperine content included Acc 5691 (4.44%), Acc 8060 (4.06%), and Acc 4132 (4.01%). Accessions 4064 (2.69%), 5717 (2.8%), 1487 (2.81%) and 8052 (2.89%) had the lowest piperine content. The remaining accessions recorded intermediate piperine content which is shown below.

	Low piperine (< 3.3 %)	Medium piperine (3.3-4.0)	High piperine (>4.0)
Genotypes	Acc 5623, Acc 5717, Acc 1487, Acc 8052, Acc 1248, Acc 1093, Acc 6720	Acc 1495, Acc 1439, Acc 4064, Acc 1218, Acc 5621, Acc 1491, Acc 1343, Acc 971, Acc 1086, Acc 7211, Acc 5083	Acc 4132, Acc 8060, Acc 5691

The flavour of black pepper is derived from the chemical piperine (Ahmad *et al*., 2012). Black pepper plays a beneficial role in aromatherapy, alleviating stressful conditions in humans through the inhalation of its aroma (Dilrukshi *et al*., 2023), as well as serving as a neuroprotective agent (Sharma *et al*., 2023). In dried black pepper, piperine generally ranges from 4 to 6% and 5 to 9% (Tiwari *et al*., 2020). The piperine content of most of the selected accessions in the present study also showed around 4 % piperine but slightly below the previously reported values. This emphasizes the scope for enhancing piperine content through breeding techniques.

Table 6. The arrangement of black pepper accessions based on quality parameters

Sl No	Number of accessions	Accessions	Characteristics
1	3	Acc 5623, Acc 5717, Acc 1487	Low piperine Low oil Low oleoresin
2	3	Acc 1218, Acc 1491, Acc 1343	Medium piperine Medium oil Medium oleoresin
3	1	Acc 5621	Medium piperine Medium oil Low oleoresin
4	1	Acc 8060	High piperine Medium oil medium oleoresin
5	2	Acc 971, Acc 5083	High piperine Low oil Low oleoresin
6	3	Acc 1086, Acc 7211, Acc 4132	High piperine High oil High oleoresin

4.3.4. Berry starch (%)

The starch content varied significantly among the genotypes ($P<0.001$). The maximum starch content was recorded in Acc 1343 (45.61%), while the minimum was recorded in Acc 8052 (10.94%). Accessions with low (< 8 %), medium (8-12.5%), and high (> 12.5%) starch contents are listed below.

	Low starch (< 15 %)	Medium starch (15-25%)	High starch (> 25%)
Genotypes	Acc 8052, Acc 1093, Acc 1491, Acc 4064, Acc 8060, Acc 5691, Acc 1495	Acc 5621, Acc 1086, Acc 1487, Acc 5717, Acc 6720, Acc 7211, Acc 4132	Acc 5083, Acc 5623, Acc 1439, Acc 971, Acc 1248, Acc 1218, Acc 1343

The total starch content was higher in white pepper (52.4%) than in black pepper (36.2%), as the white pepper is devoid of skin (Zhu *et al*., 2017). Pericarp thickness had a beneficial influence on both berry starch content and production (Somashekar *et al*., 2021). The higher starch content in pericarp was reported in Narayakodi and Chumala. It varied from 11.76 to 28.52 % (Praveena *et al*., 2021). Berries with higher starch content possess elevated levels of essential oils and piperine, making them popular despite being susceptible to mold due to their high moisture content (Mathai, 1981). A significant variation was observed in 26 black pepper genotypes in terms of starch and protein (Zachariah *et al*., 2010). The variation obtained in starch content ranged from 28% to 49% (Murthy & Bhattacharya, 2008). These report suggest that wide variation exists for berry starch content among black pepper genotypes.

The range (10.94%-45.61%) of starch content observed in the present study indicates significant genetic variability among different genotypes which can be exploited for various purposes.

4.3.5. Berry reducing sugar (%)

The sugar content in the berries of different genotypes varied significantly ($P < 0.001$), ranging from 0.21% (Acc 4064) to 3.3% (Acc 5623). The genotypes showing low, medium, and high sugar content are shown below.

	Low sugar (< 0.7%)	Medium sugar (0.7 – 1.5 %)	High sugar (> 1.5 %)
Genotypes	Acc 4064, Acc 5621 Acc 1487, Acc 8060, Acc 5691, Acc 1491, Acc 1248, Acc 5717	Acc 1093, Acc 4132, Acc 1218, Acc 1439, Acc 5083, Acc 1495	Acc 971, Acc 6720, Acc 8052, Acc 1343, Acc 7211, Acc 1086, Acc 5623

The higher reducing sugar content was recorded in IISR Shakthi (9.90%), followed by Narayakodi. The minimum value obtained was 2.74% (Somashekar *et al*., 2021). Two sweet pepper genotypes grown conventionally or organically over three years showed significant variations in quality attributes, with organic cultivation resulting in higher sugar content and agronomic yield, particularly in the RTV genotype, as well as differences in other phytochemicals influenced by meteorological variations (Lo Scalzo *et al*., 2020).

The present study, along with previous studies on reducing sugar content, exhibited significant variation among black pepper genotypes. Additionally, the study on sweet pepper reported that cultivation practices and environmental factors increase the sugar content. This correlation underscores the vitality of considering both genetic variability and cultivation practices in breeding techniques to improve sugar content of berries.

Based on the mentioned quality parameters (volatile oil, oleoresin, piperine, starch and reducing sugar), the genotypes 7211, 1495, 1343, 4132, 1086, 6720, 5621, and 5691 are showing better characteristics.

Table 7. Quality parameters of 21 selected black pepper accessions

Genotype	Berry starch (%)	Berry sugar (%)	Essential oil (%)	Oleoresin (%)	Piperine (%)
Acc 8052	10.94 o	1.7 4d	5.05 efg	8.76 g	2.89 n
Acc 5083	25.04 g	1.36 ef	3.15 j	9.98 de	3.92 d
Acc 8060	13.44 m	0.52 jk	4.9 b	12.03 bc	4.06 b
Acc 1491	11.95 n	0.61 ij	5.17 ef	12.88 bc	3.69 h
Acc 4064	12.13 n	0.21 l	4.12 i	8.49 d	3.51 q
Acc 1487	17.19 l	0.50 k	4.23 k	8.57 h	2.81 o
Acc 1093	11.6 n	0.75 ij	5.17 b	8.45 g	3.28 l
Acc 5717	21.77 i	0.66 ij	3.24 j	9.25 g	2.80 o
Acc 971	34.25 d	1.78 d	4.18 i	9.75 de	3.73 g
Acc 7211	24.44 g	2 bc	5.57 c	13.3 de	3.88 e
Acc 5691	14.21 m	0.60 ijk	4.58 a	13.4 a	4.44 a

Genotype	Berry starch (%)	Berry sugar (%)	Essential oil (%)	Oleoresin (%)	Piperine (%)
Acc 1248	36.99 c	0.61 fg	5.17 ef	10.46 f	3.09 m
Acc 6720	23.13 h	1.65 d	6.17 b	13.58 d	3.30 l
Acc 1086	18.97 j	2.21 b	6.25 b	13.4 d	3.77 f
Acc 1218	39.13 b	0.98 gh	5.4 cd	12.76 c	3.59 i
Acc 5621	17.48 kl	0.37 k	5.25 de	13.71 d	3.67 h
Acc 1343	45.61 a	1.90 cd	4.92 g	10.75 d	3.70 gh
Acc 5623	26.46 f	3.30 a	4.58 h	8.75 b	2.69 p
Acc 1439	31.1 e	1.15 ef	6.15 b	10.58 bc	3.49 j
Acc 1495	11.06 jk	1.48 e	4.98 fg	7.56 e	3.39 k
Acc 4132	24.8 g	0.83 hi	6.19 b	12.96 bc	4.01 c
General mean	11.33	1.22	5.27	13.31	3.42
SE	0.1291	0.0182	0.0149	0.244	0.0004
CV	3.18	11.02	2.32	3.71	0.61

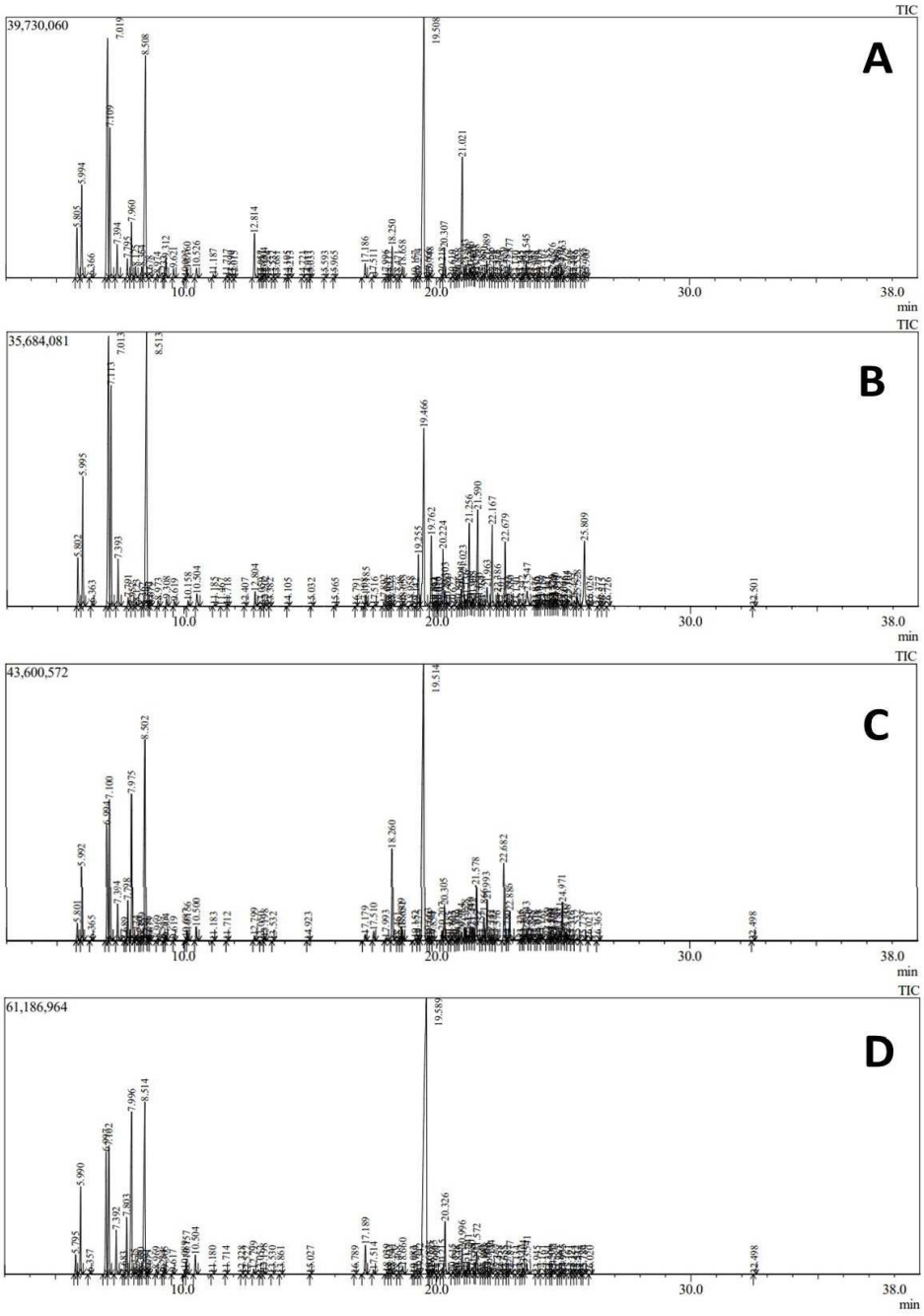


Figure 7. Chemo profiling of volatile oil from selected black pepper genotypes: (A) 5717, (B) 4064, (C) 7211, and (D) 1495. The X-axis represents retention time, and the Y-axis represents peak intensity.

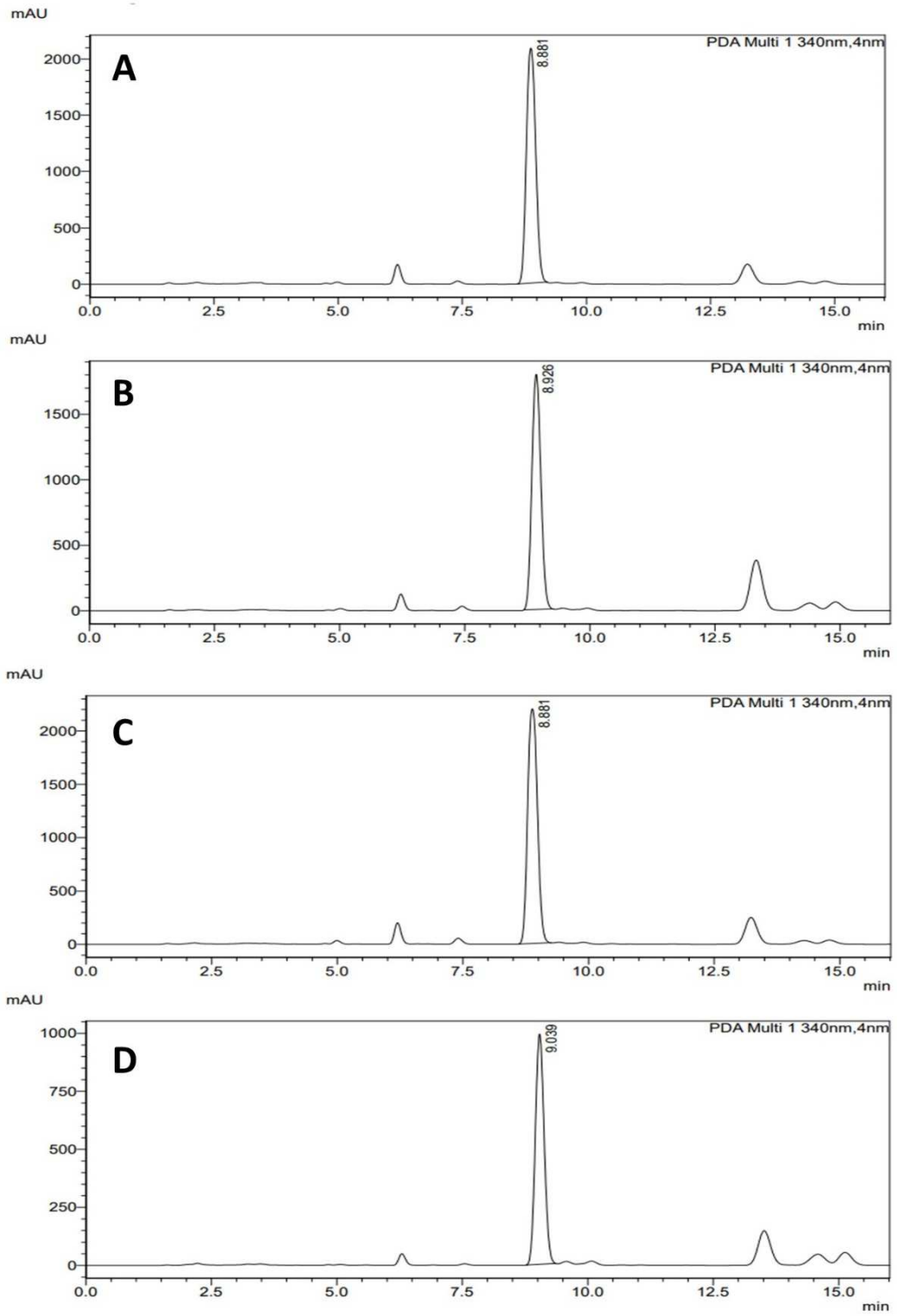


Figure 8. HPLC chromatogram of piperine extracted from selected black pepper genotypes: (A) 1495, (B) 4132, (C) 4064, and (D) 1343. The X-axis represents retention time, and the Y-axis represents milli Absorbance Units.

Experiment 2

4.4. Physiological and biochemical characterization under drought conditions

4.4.1 Physiological response to drought

The genotypes 7211, 1495, 1343, and 4132 (drought tolerant), 5717 and 4064 (drought susceptible) were chosen to study the mechanism involved in drought tolerance using morpho-physiological data from 40 black pepper genotypes and further characterized at the physiological and biochemical levels. The genotypes were grown in big containers (90 cm height x 60 cm width) for 2 years and the plants were maintained near field capacity (21% soil moisture content). Moisture stress was imposed for one month (from 1 December to 31 December) by withholding irrigation for one set of plants (4 replications) and another set of plants (4 replications) was maintained as control. Soil moisture and other physiological (Table. 7) and biochemical (Table. 10) parameters were determined at 7 days interval in both the sets till 28 days after stress (DAS), where plants started showing severe wilting symptoms in stressed plants.

4.4.1.1. Soil moisture content (%)

The soil moisture levels were measured at 7-day intervals till 28 DAS. The soil moisture percentage reduced to 17.20% after 7 days of stress (DAS). Further observations were recorded after 14, 21, and 28 DAS, during which the soil moisture content reduced to 15.40%, 12.8%, and 10.5%, respectively. Wilting symptoms started after14 DAS, which was more pronounced after 21 DAS and became more severe in the later stage at 28 DAS.

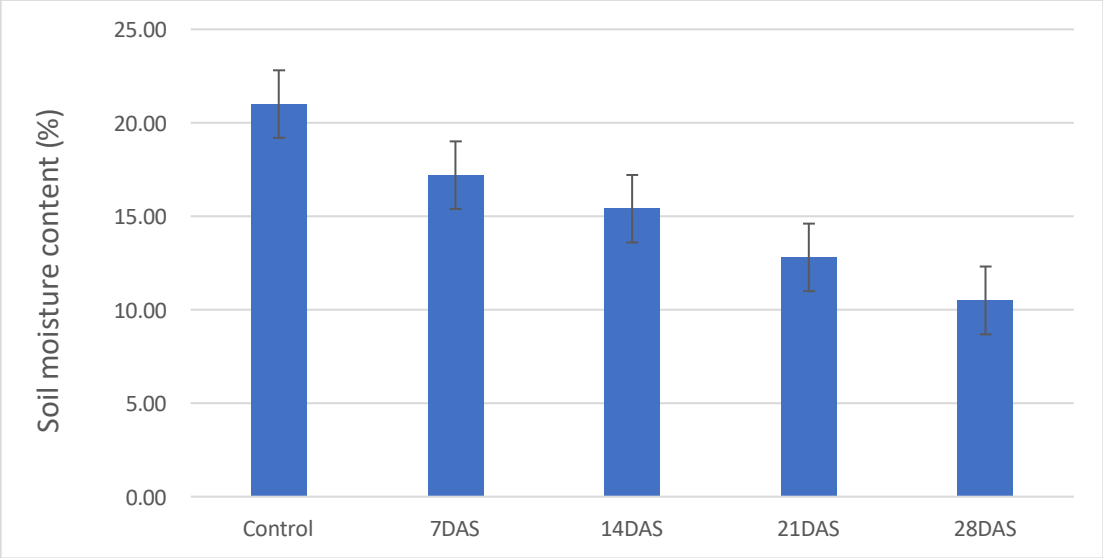


Figure 9. Influence of water stress on moisture content of selected genotypes

4.4.1.2. Relative water content (%)

The relative water content (RWC) significantly decreased ($P < 0.001$) in all accessions in response to increasing water stress levels in plants from 7 to 28 DAS. RWC was typically high in well-watered plants, ranging between 90.52% and 92.62%. At each stress level, a gradual decrease in RWC was noticed in all the accessions. The order of reduction in RWC among the accessions was 60.94% (Acc 4132), 61.41% (Acc 1495), 71.99% (Acc 7211), 78.24% (Acc 1343), 80.85% (Acc 4064) and 87.45% (Acc 5717) at 28 DAS respectively. The highest reduction in RWC (considered as susceptible trait to drought) was exhibited by accessions 5717 and 4064. In contrast, comparatively less reduction in RWC, indicative of a favorable trait for drought tolerance, was observed for Acc 4132, Acc 1495, Acc 7211 and Acc 1343.

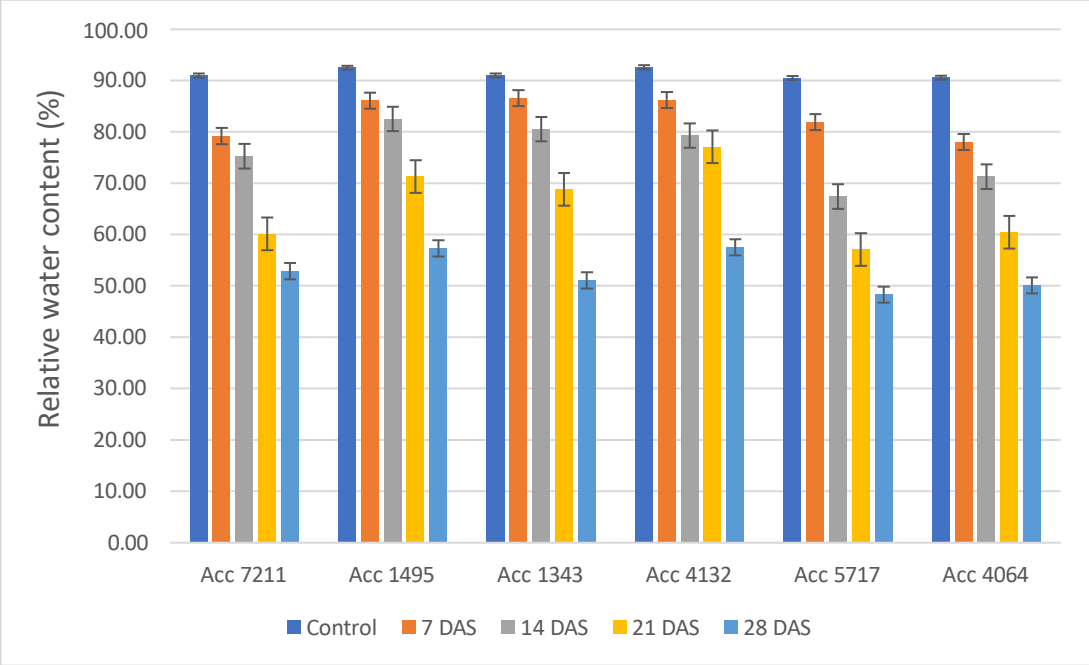


Figure 10. Relative water content variation in selected black pepper genotypes with increasing drought stress intensity.

Relative water content is an effective physiological measure utilized to assess the water status of plant tissues using leaves to screen drought-tolerant varieties (Nir Sade, 2015). In a similar drought-based study aimed at identifying black pepper varieties, it was discovered that genotypes 931, 1495, and Acc 813 exhibited the least wilting symptoms under 2 months of drought stress, despite having 14% soil moisture, and had higher relative water content than wilted genotypes, with percentages of 80.6%, 82.5%, and 83%, respectively (Rao *et al.*, 2016a). Panniyur 5 has been identified as a resistant black pepper variety, exhibiting a lesser reduction in relative water content (53%) compared to the sensitive Panniyur 1 (72%) over the 15-day drought period of stress (Puthur & Vijayakumari, 2014). Irrigation and RWC showed a positive association in *Capsicum annuum* (Yildirim *et al.*, 2019).

A potential drought-resistant variety of pomato, 'Wauseon,' has been identified based on minimal yield loss and the lowest drop in RWC, making it an effective indicator for further genetic and breeding studies aimed at improving drought tolerance in potatoes (Soltys-Kalina *et al*., 2016)

The present study is supported by previous findings which consistently show RWC as a reliable physiological parameter to assess plant water status, essential for screening drought tolerance. Additionally, *Capsicum annuum* and pomato showed a positive association between irrigation and RWC, reinforcing the present findings that decreasing RWC correlates with decreasing moisture levels, establishing it as a critical indicator of drought tolerance.

4.4.1.3. Electrolyte leakage (%)

Membrane stability declined gradually due to electrolyte leakage at progressive stress levels compared to well-watered (control) plants ($P < 0.001$). It varied from 7.36% to 10.01 % in control plants. The accession 1343 exhibited the lowest in electrolyte leakage (43.25% increase) among all genotypes, followed by accessions 7211 (57%), 4132 (64%), 5717 (68.7%), 1495 (76.6%) and 4064 (77.1%) after 28 DAS. Lower membrane leakage is considered as a drought-tolerant trait and vice versa.

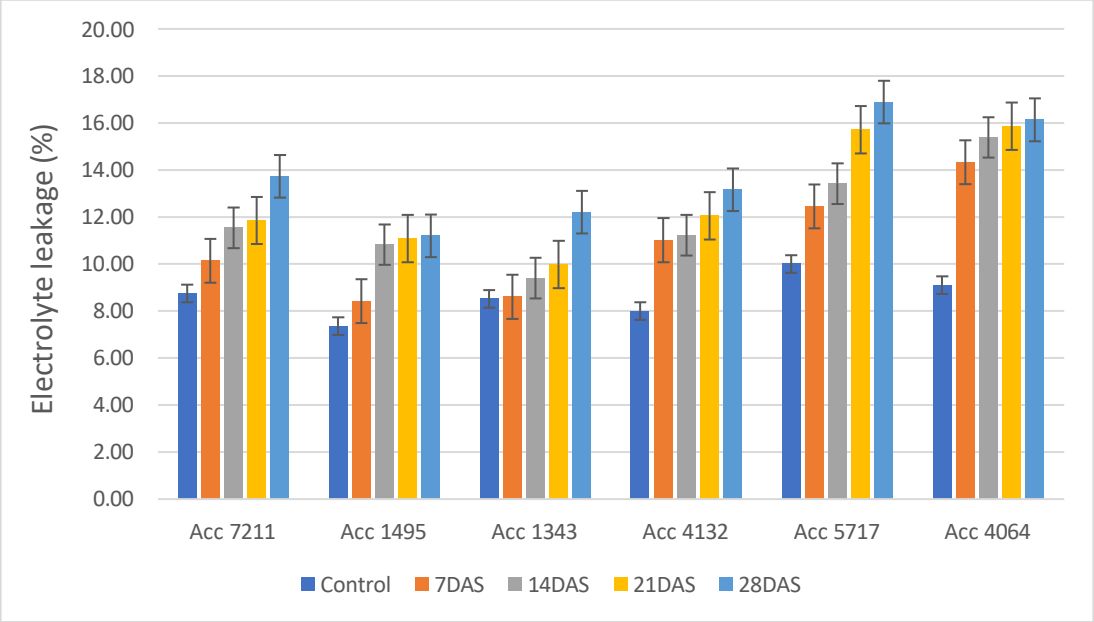


Figure 11. Variation in electrolyte leakage of selected black pepper genotypes with increasing drought stress intensity.

The severity of drought on the plants could be quantified by electrolyte leakage, which serves as a clear marker (Keerthi *et al*., 2023). Water stress increases the permeability of the cell membrane, enhancing the efflux of potassium and calcium ions from the

cells (Hniličková *et al.*, 2019). Contrastingly, flooded environmental constraints led to higher electrolyte leakage among the genotypes of the grass *Miscanthus* species, specifically Msac-G1, Msin-G2, Mxg-G5, and Hyb-G6, while control and drought conditions influenced relatively lower leakage, especially for the genotypes Msac-G3 and Mxg-G5. Comparable results were observed in winter wheat (Khalvandi *et al*., 2021), pepper (Meng *et al*., 2021; Ghahremani *et al*., 2023), and *Brassica* seedlings (Khan *et al*., 2017).

The present research, along with the aforementioned studies, establishes that electrolyte leakage is a key parameter in determining the influence of drought in plants. It illustrates water stress influence in increasing cell membrane permeability, leading to the efflux of potassium and calcium ions. This mechanism is consistent across various plant species under different environmental conditions, including grass *Miscanthus* species, winter wheat, pepper, and Brassica seedlings. These findings reaffirm the association between electrolyte leakage and drought stress, emphasizing the significance of membrane integrity as a vital indicator for assessing drought tolerance in plants.

4.4.1.4. Leaf photosynthetic pigment

The knowledge of the mechanism underlying drought-induced changes in chlorophyll pigments is essential for developing improved strategies to enhance plant tolerance to water scarcity and further reduce the negative impacts of drought on crop yield (Vanaja *et al*., 2006), as chlorophyll serves as a feasible vital indicator of photosynthesis (Houborg *et al*., 2015). Carotenoid pigment may have photoprotective properties and protect the photosystems (PS I and PS II) from photooxidative damage (Swapnil *et al*., 2021).

The leaf photosynthetic pigments—chlorophyll a, b, and carotenoids in black pepper leaves found to vary significantly among the genotypes ($P < 0.001$). The principal chlorophyll pigment a ranged from 0.99 (Acc 4064) to 1.57 mg/g FW (Acc 1343) in control plants. The concentration of chlorophyll a initially increased and reached a peak at 14 DAS in accessions 7211 (34.72% increase), 4132 (11.42%), 1343 (9.97%), and 1495 (2.55%). Then it started to decrease drastically from 14 to 28 DAS compared

to the control levels. Chlorophyll a maintained almost the same level up to 14 DAS in Acc 1495. The concentrations of chlorophyll a in accessions 5717 and 4064 exhibited a drastic decrease, even initially from 7 DAS, followed by a gradual reduction at 14, 21, and 28 DAS. The highest reduction was recorded at 28th day in all accessions: 114.9% (Acc 5717), 120.9% (Acc 1343), 109.2% (Acc 1495), 89.9% (Acc 4064), 62.5% (Acc 4132), and 60.1% (Acc 7211). The lower reduction indicates favorable trait for drought tolerance.

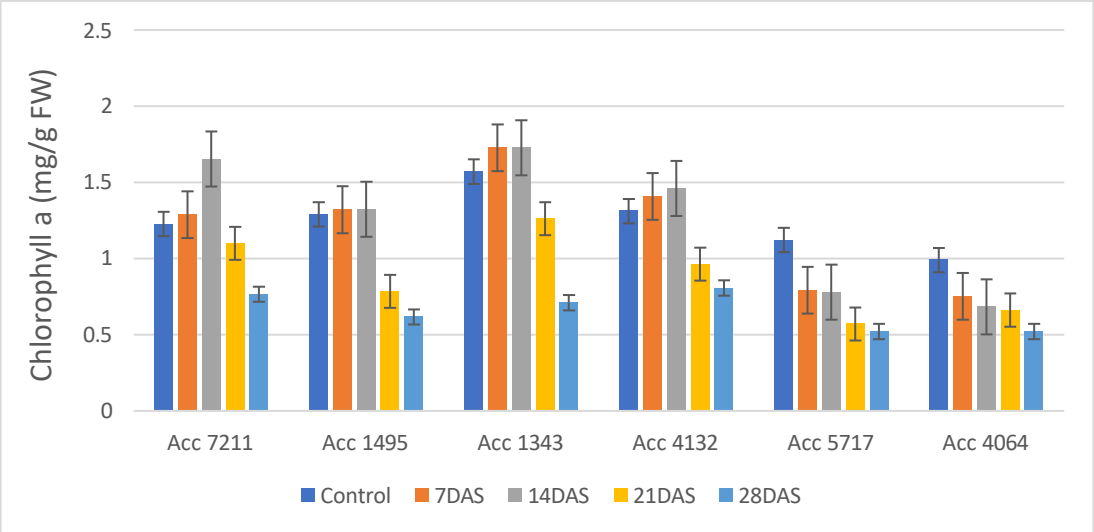


Figure 12. Chlorophyll a content variation in selected black pepper genotypes with increasing drought stress intensity.

The drought-tolerant soybean genotype 'Speeda' maintained higher chl a pigment levels throughout the drought stress experiment, with the recovery treatment showing no significant effect, whereas the decreased chl a pigment of the drought-susceptible 'Coraline' genotype was boosted by foliar application of H2O2 (Basal *et al*., 2024). During a 15-day water stress period, the promising drought-tolerant black pepper variety Panniyur 5 experienced a comparatively lesser reduction (37%) in chl a than the drought-sensitive Panniyur 1 (Puthur & Vijayakumari, 2014).

The review supports the present study findings by bringing out the relevance of chlorophyll as a vital indicator of drought tolerance in plants by maintaining the higher chl a content in drought tolerant soybean and black pepper varieties and conversely to drought sensitive types. These reports highlighted the vital role of chl a dynamics in

plant's tolerance to drought through the stabilization of photosyntheitic efficiency under water limited condtions thereby improving the agricultural productivity.

Typically, higher chlorophyll b pigment was observed in well-watered plants, ranging between 0.257 (Acc 5717) and 0.436 mg/g FW (Acc 1343) compared to water stressed plants. A gradual decrease in chlorophyll b with increasing stress intensity was noticed in all accessions, with the highest decrease observed in Acc 5717 (180.46%) and Acc 4064 (175.96%), representing an unfavorable trait for drought tolerance. Relatively lower decrease was observed in Acc 7211 (144.8%), Acc 1343 (125.5%), Acc 1495 (92.2%), and Acc 4132 (83.3%).

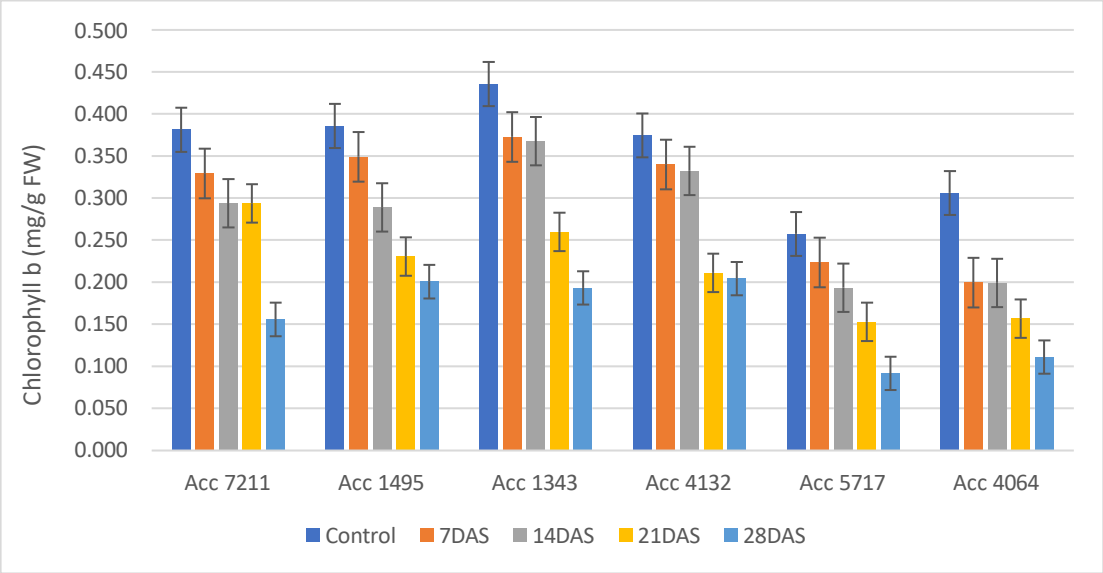


Figure 13. Chlorophyll b content variation in selected black pepper genotypes with increasing drought stress intensity.

Across all sample dates under drought stress treatment, the 'Speeda' genotype (drought-tolerant) had a higher chlorophyll b content than the 'Coraline' genotype (drought-susceptible), despite the fact that foliar H2O2 spray was ineffective in reducing this decline (Basal *et al*., 2024). This is similar to the present findings, where there was a lower reduction in chlorophyll b content in genotypes with drought-tolerant characteristics compared to those with susceptible characteristics.

Under *in vitro* drought stress, the addition of PEG to the growth medium of tomato plantlets decreased chlorophyll b content more than chlorophyll a, leading to

imbalances in PSII photochemical activity and potential photodamage (Suminar *et al*., 2022). Similarly, chlorophyll a and chlorophyll b contents in seedlings of Chorispora bungeana drastically declined at various time points under both moderate and severe drought stress, with chlorophyll b consistently lower than chlorophyll a (Yang *et al*., 2016). A similar pattern was found in the present study of selected black pepper genotypes, thereby increasing the chlorophyll a/b ratio.

A contrasting trend was found in bean plants, where chlorophyll b (Chl b) levels were typically higher than in irrigated plants under drought stress, except in the fourth trifoliate leaf. The exogenous application of ZnO nanoparticles in such conditions increased Chl b content in the leaves, indicating a positive effect on pigment synthesis under stress (Babanli, 2024).

The total chlorophyll content among the selected accessions varied from 1.296 (Acc 4064) to 2.006 mg/g FW (Acc 1343). It increased from the control to 14 DAS in accessions 7211, 4132, and 1343, showing the same trend as chlorophyll a. However, the total chlorophyll content then declined for the remaining days. Acc 1495 exhibited a slight decrease for the 7th and 14th days after stress, followed by a sharp decline at 21 and 28 DAS, 65% and 105%, respectively. In contrast, accessions 5717 and 4064 showed sharp decrease up to 28 DAS with 124.7% and 105%, respectively.

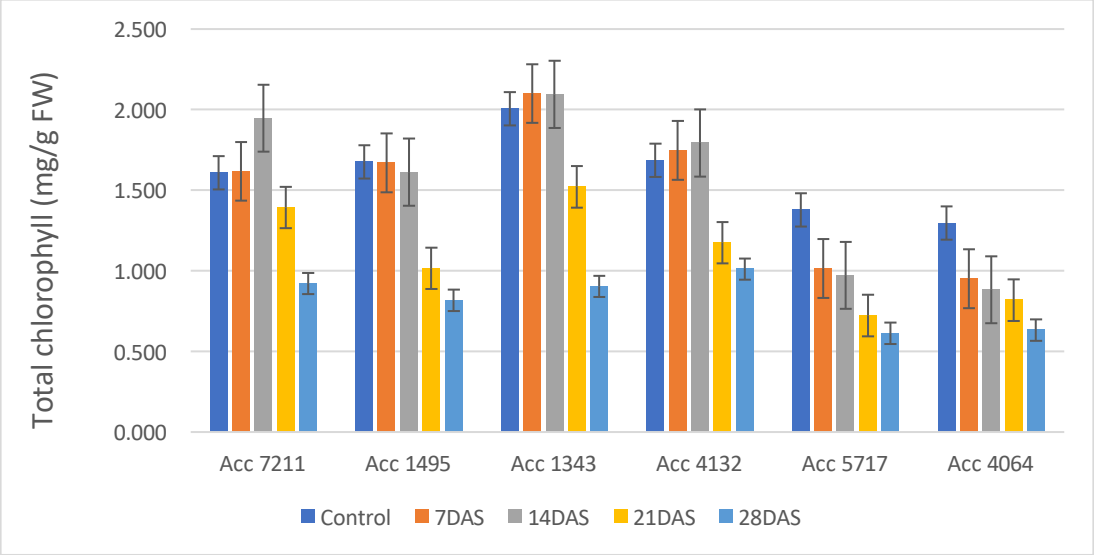


Figure 14. Total chlorophyll content variation in selected black pepper genotypes with increasing drought stress intensity.

The chlorophyll pigments (a and b) of lettuce seedlings remained highest until day 4 of the drought stress experiment, which could be attributed to the efficiency of the chlorophyll apparatus even in such an environment. Further quantification in the prolonged treatment showed their decrement, suggesting elevated chlorophyllase activity and photodestruction of chlorophyll (Shin *et al*., 2021). A slight increase in the total chlorophyll content of wheat cultivars was reported on the 3rd and 5th days of drought, but it decreased by 13 to 15% on the 7th day. The decline was not compatible with changes in the chlorophyll a/b ratio (Nikolaeva *et al*., 2010).

These studies support the present findings, illustrating an initial increase in total chlorophyll, which reflects the initial resilience and efficiency of the photosynthetic apparatus. The subsequent reduction might be due to chlorophyllase activity and photodestruction of chlorophyll under prolonged stress conditions. Such a dynamic response of chlorophyll pigments to drought stress highlights the relevance of monitoring these changes for developing strategies to enhance drought tolerance in plants.

The a/b ratio showed an increase at 28 DAS for Acc 7211. A progressive increase was observed in accessions 7211 and 1495 up to 14 DAS, as well as for 1343 and 4132 up to 21 DAS, compared to the control. Further observations revealed a subsequent decrease in levels, except for Acc 7211. However, no similar pattern of variation was found for accessions 5717 and 4064.

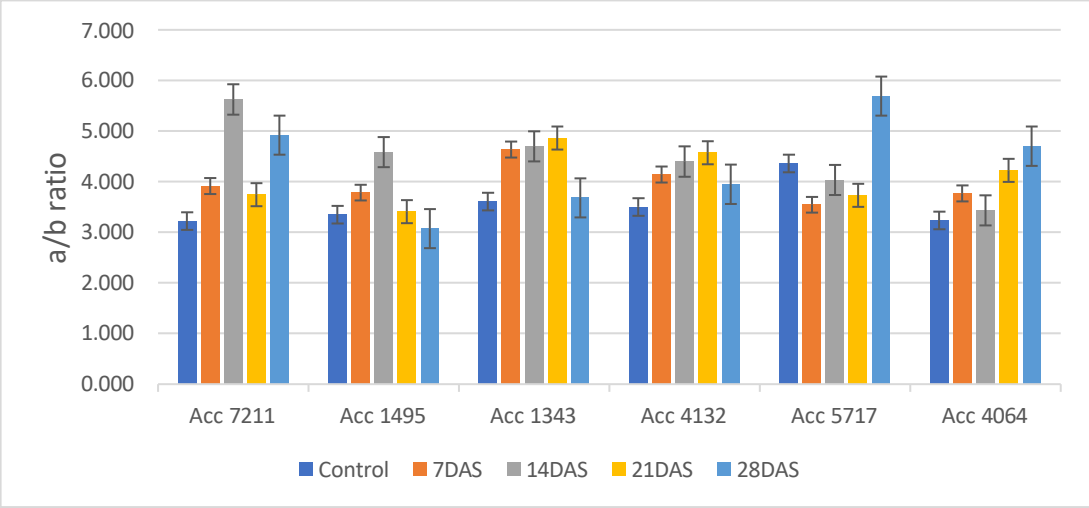


Figure 15. Variation in a/b ratio among selected black pepper genotypes with increasing drought stress intensity

The synergistic action of chlorophyll pigments and carotenoids together improves adaptive strategies to maintain photosynthetic activity and enhance stress tolerance. The present study's findings on drought-tolerant genotypes were consistent with the theory that demonstrates the adverse effect of drought on the photosynthesis mechanism, as supported by Flexas *et al*. (2004) and Chaves *et al*. (2009), particularly in higher plants.

Under drought stress, carotenoid pigment variation exhibited a similar trend in accessions 7211, 1495, 1343, and 4132. Carotenoid levels increased up to 21 DAS in these accessions by 50.78%, 43.83%, 77.36%, and 60.8%, respectively, and then decreased at 28 DAS. Acc 4132 showed a very low decrease at 28 DAS, more favorable for drought tolerance. Meanwhile, accessions 5717 and 4064 followed a similar pattern, experiencing a rise on the 7th day and a gradual reduction until 28 DAS, with a decrease of 38% and 30.18%, respectively, compared to the control, which is an unfavorable trait under drought stress.

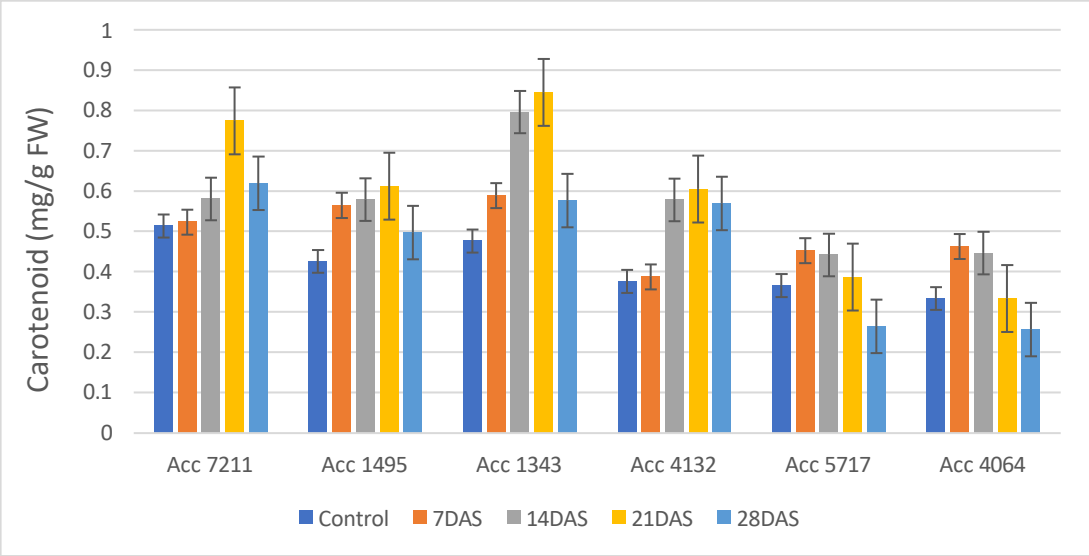


Figure 16. Carotenoid content variation in selected black pepper genotypes with increasing drought stress intensity

The foliar H2O2 application on both genotypes ('Speeda' as tolerant and 'Coraline' as susceptible), which had initially decreased carotenoid levels, began to elevate them in response to the recovery treatment (Basal *et al*., 2024). However, a higher carotenoid to chlorophyll ratio was reported in Panniyur 1 (black pepper variety), which,

subjected to a 15-day drought duration, exhibited its characteristic tolerance to drought through antioxidation (Purthur & Vijayakumari, 2014).

Likely, Chl a and total Chl rose with increasing limited water supply, but Chl b remained nearly constant at all moisture levels, except for when reduced moisture content showed a slight increase, as reported in *Mangifera indica* (Ouma, 2008). Whereas ascorbic acid was required to restore the reduced chlorophyll pigments (chlorophyll a, b, total, and carotenoid) of pepper (*Capsicum annuum* L.) plants, increasing them under drought stress (Khazaei *et al*., 2020). A positive correlation exists between total carotenoids and both total chlorophyll (r = 0.57) and chlorophyll b (r = 0.64) (Jang *et al*., 2022).

A significant variation was visible in chlorophyll content (Chlorophyll a, b, and total chlorophyll) at the beginning and end of each growth stage of *Capsicum* spp (*C. annuum, C. chinense* and *C. frutesense*) under the influence of specific drought treatments. A notable reduction in chlorophyll levels occurred under severe drought stress conditions in *C. annuum* during the vegetative stage, with fluctuations observed at the flowering and fruiting stages under different levels of drought treatment. In contrast, significant variations could not be observed in *C. chinense* and *C. frutesense* during the flowering and vegetative stages under all drought conditions. However, they demonstrated the highest total chlorophyll content during the fruiting stage under particular drought treatments (Okunlola *et al*., 2017).

The carotenoid pigment variation trend in the genotypes with tolerant characteristics mirrors the responses in 'Speeda' and 'Coraline' genotypes of soybean and the black pepper variety Panniyur 1, where an initial increase was associated with improved drought tolerance. The initial increase and subsequent decrease indicated the antioxidant response to mitigate the photodamage due to prolonged drought stress, as reported in *Mangifera indica* and *Capsicum annuum.* Carotenoids and chlorophyll pigments being positively associated, as reported in several research reports, further supports the present findings. This explains the interdependence of these pigments in maintaining photosynthetic efficiency under stress conditions.

The selection of superior black pepper genotypes in drought tolerance, based on simultaneous analysis of multiple physiological traits such as chlorophyll content, chlorophyll stability, relative water content, and membrane stability index, identified IC 598869 and IC 598890 as superior to the established drought-tolerant variety P5, having lower rankings for most characters. This demonstrates their efficiency in drought tolerance, as they withstood imposed drought for 20 days, highlighting the importance of physiological traits in screening (Prakash *et al*., 2023).

Moreover, the identification of superior black pepper genotypes IC 598869 and IC 598890 based on multiple physiological traits, including chlorophyll and carotenoid content, underscores the importance of comprehensive screening for drought tolerance.

4.4.1.5. Leaf stomatal aperture measurements

In stomatal aperture measurements (Table. 8), stomatal length did not follow a particular trend among the selected genotypes. However, stomatal width exhibited a decreasing trend in all genotypes measured at 14 and 28 days after stress (DAS), ranging between 5.39 µm and 6.78 µm among control plants. The reduction was more prominent at 28 DAS compared to 14 DAS. The extent of stomatal width reduction varied among different genotypes. At 28 DAS, a greater proportional decrease was identified in genotype 4132, followed by genotypes 1343, 7211, 1495, 4064, and 5717 with reductions of 221.25%, 184.08%, 137.41%, 108.66%, 87.96%, and 85.03%, respectively. This higher degree of decrement could be interpreted as a favorable trait indicating more efficient closure of stomata, thereby reducing the risk of water loss through transpiration and conserving water for metabolic processes.

In *Arabidopsis thaliana*, drought stress resulted in stomatal closure via H_2S-mediated ion fluxes (Jin *et al*., 2017). *Arabidopsis* CYP707A1 and CYP707A3 possess distinct functions in regulating ABA levels and stomatal conductance in response to humidity, with CYP707A1 predominantly responsible for ABA catabolism within guard cells, which influences ABA-mediated stomatal closure (Okamoto *et al*., 2009). Transcription factor HsfA1a enhances tomato drought tolerance by influencing stomatal closure sensitivity to endogenous ABA (Wang *et al.*, 2015). Stomatal closure

mediated by ABA catabolism is influenced by both CO2 levels and light conditions, leading to reductions in leaf stomatal density (Movahedi *et al*., 2021). Treatment with NO donors (sodium nitroprusside and S-nitroso-N-acetylpenicillamine) in drought-stressed wheat plants tends to enhance water retention, minimize transpiration rate, induce stomatal closure, and reduce ion leakage, potentially conferring enhanced tolerance to drought (Mata & Lamattina, 2001). Collectively, all these observations support the present study, which demonstrates the regulation of stomatal aperture by the action of ABA under water-limited conditions in plants.

Table 8. Stomatal aperture size as influenced by stress intensity

	Stomatal length (µm)			Stomatal width (µm)		
	Control	14 DAS	28 DAS	Control	14 DAS	28 DAS
7211	16.86 c	14.66 ef	16.15 cd	6.78 a	5.61 bc	2.85 f
1495	16.99 c	13.93 fg	13.12 g	5.89 b	5.00 c	2.82 f
1343	16.62 c	16.19 cd	16.23 cd	5.76 b	3.98 d	2.03 g
4132	20.51 b	20.71 b	21.11 ab	5.69 bc	3.45 def	1.77 g
5717	20.77 b	20.78 b	20.01 b	5.67 bc	4.16 d	3.06 ef
4064	22.14 a	16.34 cd	15.23 de	5.39 bc	3.80 de	2.87 f

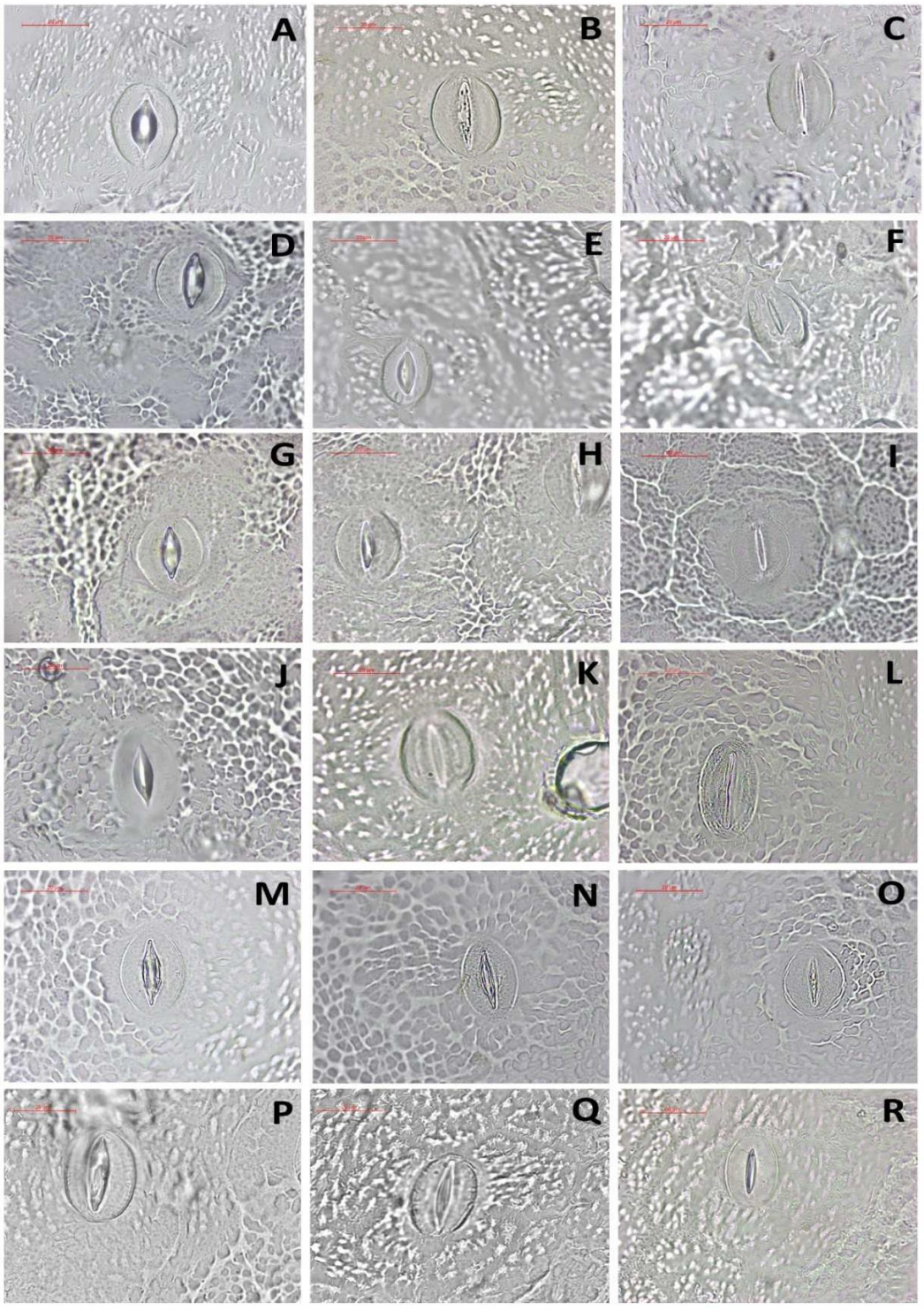


Figure 17. Variations in stomatal aperture size among different genotypes (7211, 1495, 1343, 4132, 5717, and 4064) at corresponding stress levels (control, 14 DAS, and 28 DAS), each represented by letters (A-R) on the image. The illustrations depict changes in stomatal aperture size under varying moisture conditions observed using a Leica compound microscope (×400) (scale bars = 20 µm).

4.4.2. Biochemical response to drought

The biochemical parameters viz. proline, protein, phenol, H2O2, lipid peroxidation, antioxidant activity (catalase, peroxidase, superoxide dismutase, DHAR, MDHAR, ascorbate peroxidase, glutathione S transferase, polyphenol oxidase and glutathione reductase), sugar and starch were determined at 7 days intervals till 28 DAS in stressed plants as well as under control.

4.4.2.1. Proline (µg/g)

A lower quantity of proline (30.70 µg/g to 66.83 µg/g) was recorded in the well-watered control plants compared to the stressed plants. Increase in proline content with the increase in stress intensity was observed in all the accessions. A higher percentage of increase was noticed in accessions 4064 (145.8 µg/g), 1495 (152.79 µg/g), 1343 (206.32 µg/g), 4132 (217.16 µg/g), and 7211 (166.04 µg/g), which was 192.2%, 201.3%, 226.3%, 224.9%, and 150.7% increase respectively, at 28 DAS compared to the control.

Greater proline accumulation was detected in accessions (1343 and 4132) exhibiting drought-tolerant traits, in contrast to other accessions. The proline content of Acc 5717 (128.2 µg/g) initially increased by 131.52%, but decreased to the control level at 28 DAS (100.3 µg/g) indicating its inability to accumulate proline under severe stress conditions.

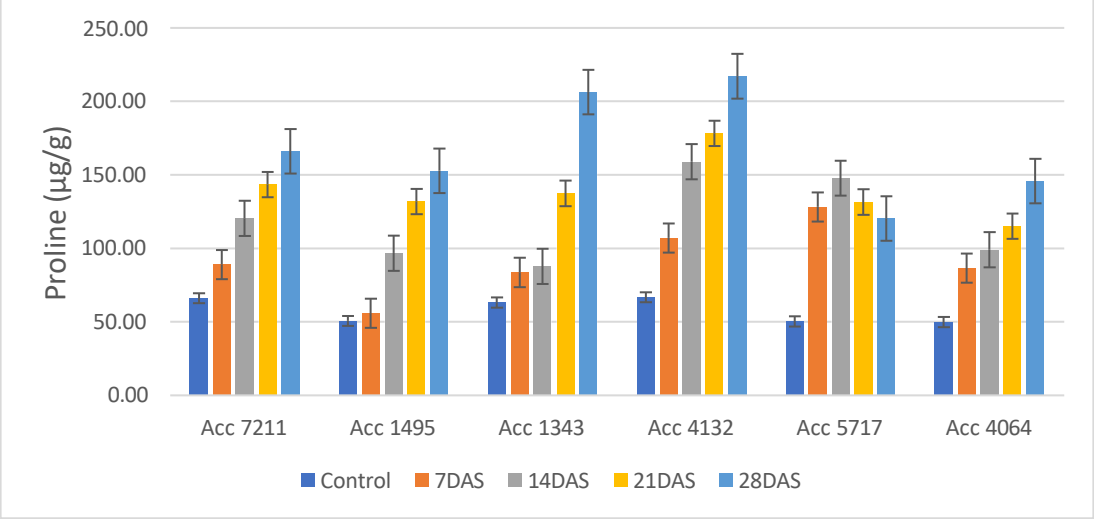


Figure 18. Proline content variation in selected black pepper genotypes with increasing drought stress intensity

In pepper, 41.34% increase in proline accumulation for osmoregulation under the foliar application of salicylic acid in drought-stressed plants was noticed (Ghahremani *et al*., 2023; Escalante-Magaña *et al*., 2019). Fruit quality was also improved (Fiasconaro *et al*., 2019). The exogenous application of proline at lower amounts alleviates drought stress. However, higher amounts lead to toxic effects on plants despite the drought stress (Hayat *et al*., 2012). Heavy metal pollution in soil water due to industrialization, weathering of rocks and urbanization as well as salinity and heat stress negatively impact crop growth. This could be mitigated through development of thermo-tolerant genotypes. Proline serves as multifunctional defense mechanism against various environmental stresses (Siddique *et al*., 2018). Similarly, several studies have been conducted to identify the positive role of proline in protecting plants under drought stress, aiming to identify tolerant varieties for targeted breeding and cultivation in black pepper (Purthur & Vijayakumari, 2014), pepper (Mahmood *et al*., 2021), and chickpea cultivars (Mafakheri *et al*., 2010) and red pepper (Wassie *et al*., 2023). Variations in proline content in previous studies in response to drought stress showed a similar trend to the present study, with an increase in proline levels providing osmotic protection to the plants under such conditions.

4.4.2.2. Phenol (mg/g)

The total phenol content in control plants ranged from 4.77 mg/g (Acc 5717) to 7 mg/g (Acc 7211), with a higher mean value compared to stressed plants. In the present study, total phenol was found to be negatively associated with progressive stress levels. The mean values of phenol observed for accessions 7211, 1495, 1343, 4132, 4064, and 5717 were 7 mg/g, 6.95 mg/g, 6.60 mg/g, 6.11 mg/g, 5.38 mg/g, and 4.77 mg/g, respectively. Initially, phenol content dropped significantly and then showed a slow increase till 28 DAS in the accessions 7211, 1495, 1343, and 4132. The tendency of increased phenol content is a positive trait for drought tolerance. Conversely, a decrease with progressive stress levels was noticed in the accessions 5717 and 4064. At 28 DAS, the proportion of reduction observed for the accessions was 74.9% (Acc 7211), 70.3% (Acc 1495), 64.7% (Acc 1343), 52.12% (Acc 4132), 75.4% (Acc 5717), and 76.3% (Acc 4064).

The initial decrease followed by further increase under progressive stress levels indicated the adaptive response to drought stress, which was demonstrated by 7211, 1343, 4132, and 1495 as the better ones in phenol content dynamics.

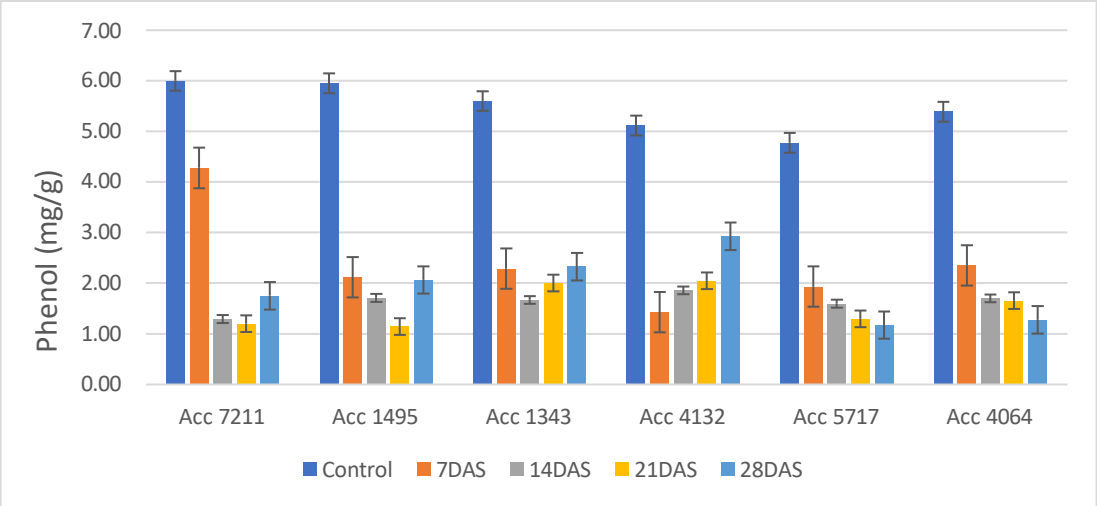


Figure 19. Phenol content variation in selected black pepper genotypes with increasing drought stress intensity

Drought-resistant tea genotypes had higher phenol content compared to susceptible ones, suggesting of its role in drought tolerance (Rahimi *et al*., 2021). Upon re watering phenolic content increased (3.39 fold) after 20 days of stress treatment had started to reduce phenol content in Saharan plant *Oudeneya africana* (Lee *et al*., 2021). The enhanced synthesis of polyphenolic content mitigates the injurious effect of drought stress on plants (Lone *et al*., 2023). Cold stress, an antagonistic counterpart to drought, highlights the significance of phenolic acid in the phenolic pathway, crucial for cold tolerance, with UV-absorbing phenolics possessing potent antioxidant properties (Rácz *et al*., 2023). Similar to the present study, the total phenolic content increased by 22.57%, 44.12%, and 47.11% respectively in the red pepper cultivars of Hagerew, Markofana, and Mitmita due to drought imposition (Wassie *et al*., 2023).

The initial decline and subsequent rise in phenol content as an adaptive response to drought in the black pepper genotypes with drought-tolerant characteristics were supported by previous findings reported in tea genotypes, Saharan plant Oudeneya africana, and red pepper cultivars of Hagerew, Markofana, and Mitmita. This response is facilitated by the multi-functional activity of phenolic compounds via signal

transduction, osmotic adjustment, antioxidant defense, membrane stability, and interactions with other stress-related metabolites.

4.4.2.3. H_2O_2 content (µmol/g FW)

Hydrogen peroxide content varied significantly ($P<0.001$) among the selected accessions. Typically, the hydrogen peroxide content was lower in well-watered plants compared to water stressed plants and it increased with stress intensity. A gradual increase in hydrogen peroxide was found in all the accessions. Accessions 7211, 1495, 4132 and 5717 exhibited a steady increase up to 21 days, then maintained almost the same level, whereas in Acc 1343, it increased up to 14 days, then steadily dropped at 21 DAS and maintained the same level at 28 DAS also. The higher increment was seen at 28 DAS in Acc 5717 with 133.5%. The medium level of increment was observed in the accessions 4064, 1495 and 4132 at 73%, 78.14% and 84.71% respectively. A lower increase was observed in the accessions 7211 and 1343 at 48.6%, 54.39% respectively, compared to control.

Thus, the moderate and controlled levels of hydrogen peroxide increase in the accessions 7211 and 1343 help them maintain lower membrane damage under stress conditions, where a balanced H2O2 level contributes to maintaining lower membrane damage and higher enzymatic activities of antioxidants like APX and CAT (Helena & Carvalho, 2008).

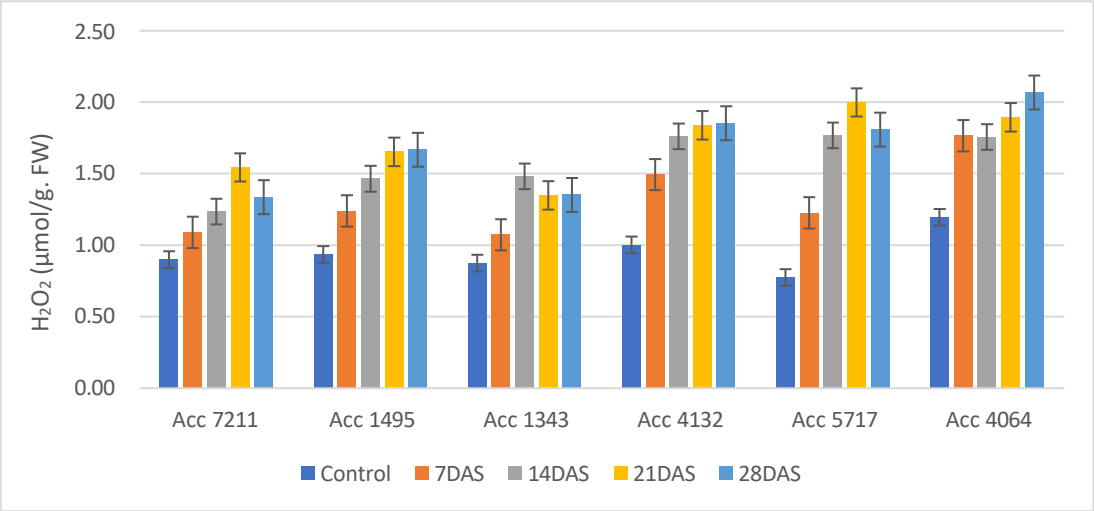


Figure 20. H_2O_2 content variation in selected black pepper genotypes with increasing drought stress intensity

The multifaceted role of hydrogen peroxide in plants subjected to environmental constraints serves as both a signaling molecule and a mediator of stress responses, growth, and development. Both biotic and abiotic stress factors induce the generation of hydrogen peroxide as ROS, which could activate the coordinated expression of genes related to stress, such as SAR (Systemic Acquired Resistance), in terms of biotic stress in pepper plants (Hyong *et al*., 2007). A rapid decrease in soil water content in response to severe water stress increased the signaling molecule, H2O2 content in leaves of wheat genotypes (Luna *et al.*, 2005). The H2O2 content remained relatively unchanged in drought-tolerant maize genotypes compared to sensitive genotypes under stress, which maintained low MDA content and higher enzymatic activities of APX, CAT, and SOD (Chugh *et al*., 2011). Similar results were reported in cotton roots (Zhang *et al*., 2014), maize plants (Demiralay *et al*., 2022), and *Brassica juncea* leaves (Naveen *et al*., 2021).

The observation in the present study of black pepper genotypes, showing variation and an increase in hydrogen peroxide content, was consistent with previous research findings on the role of H_2O_2 in plant stress responses observed in various plants like wheat, maize, and *Brassica juncea.*

4.4.2.4. Lipid peroxidation (µmol/g FW)

The MDA content, representing lipid peroxidation was lower in control plants compared to stress plants. MDA content in the control plants of Acc 7211, Acc 1495, Acc 1343, Acc 4132, Acc 5717, and Acc 4064 wass 13.94, 17.25, 11.74, 16.29, 21.29, and 12.09 µmol/g FW, respectively. A gradual increase in MDA content was observed in these accessions with progressive stress levels. Under severe water stress conditions (28 DAS), the MDA content increased by 127.9%, 147.44%, 226.16%, , 251.48%, 251.5%, and 273.3% in the accessions 1343, 7211, 1495, 4132, 5717, and 4064 respectively. A relatively lower increase was observed in accessions (7211 and 1343) that could be regarded as those showing the drought-tolerant trait.

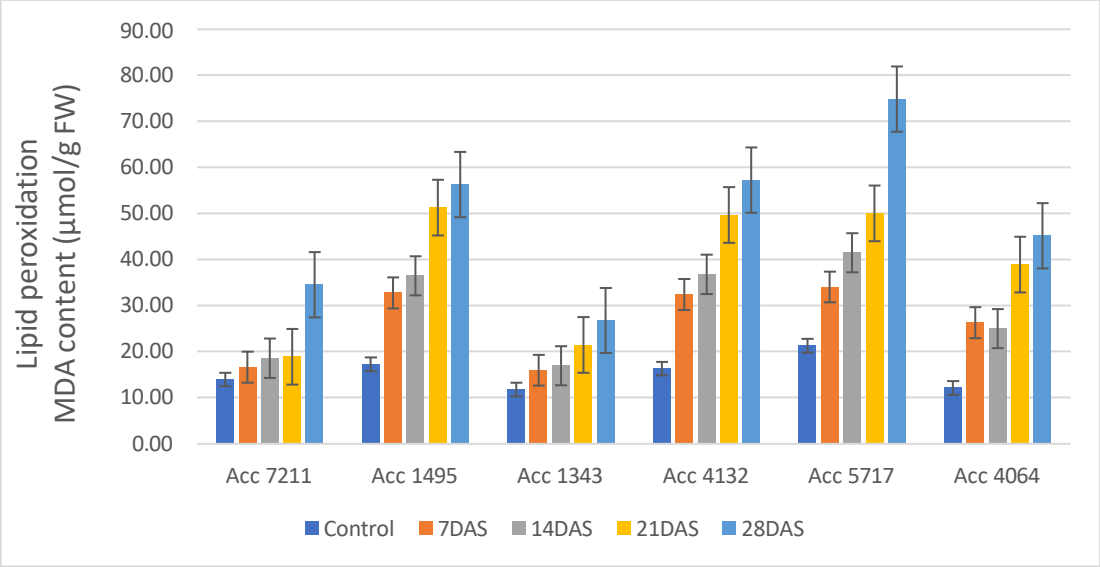


Figure 21. MDA content variation in selected black pepper genotypes with increasing drought stress intensity

Lipid peroxidation affects membrane integrity and disrupts cellular activity, causing damage to crops and reducing crop drought tolerance (Rao *et al*., 2016; Ambrozim *et al*., 2022). Similar physiological changes are observed under other environmental stresses such as salinity stress, chilling stress, nutrient deficiency, and heavy metal toxicity (Kusvuran *et al*., 2016). After 10 days of non-irrigated conditions, pennyroyal (*Mentha pulegium* L.), showed a significant increase (294%) in lipid peroxidation (Ulusu *et al*., 2022). However, exogenous application of ABA recovered the plants from lipid peroxidation while they were suffering from water scarcity, resulting in a considerable increase in relative water content as well as a decrease in electrolyte leakage (Mahadi Hasan *et al*., 2021).

Puthur & Vijayakumari (2014), observed higher MDA accumulation in the drought-sensitive Panniyur 1, with a 211% increase after 15 days of drought, compared to the drought-tolerant P5, which exhibited a comparatively reduced increment of MDA with a 111% increase. This corroborates the findings of the present study.

4.4.2.5. Total sugar (%)

The total sugar content varied significantly ($P < 0.001$) among the accessions, ranging from 0.64% to 1.01% in control plants, further increased to 1.98 (Acc 1343), 1.85

(Acc 4132), 1.81 (Acc 1495), 1.67 (Acc 7211), 1.22 (Acc 4064) and 1.05% (Acc 5717) at 28 DAS. The highest increase was observed in Acc 1343 (132.41%), demonstrating the most favorable trait for drought tolerance, followed by Acc 4132 (98.67%), Acc 1495 (79.2%), Acc 7211 (78.23%), Acc 5717 (63.76%), and Acc 4064 (57.54%). Elevated sugar content is a trait associated with drought tolerance.

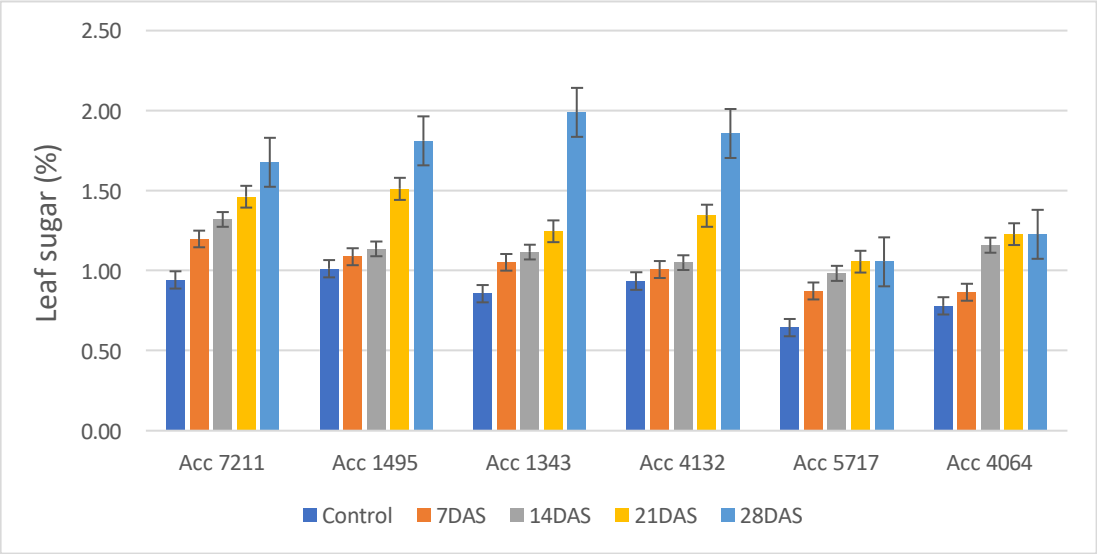


Figure 22. Leaf sugar content variation in selected black pepper genotypes with increasing drought stress intensity

Under the influence of environmental factors, sugar accumulation varies significantly among plants, especially for drought-responsive species. Strategic deficit irrigation practices positively influence plant development, most likely due to a considerable overproduction of beneficial metabolites such as sugars, organic acids, and antioxidant chemicals (Bogale *et al*., 2016; Farooq *et al*., 2009). The similar trend of sugar accumulation in grapevine leaves is attributed to the reduction in sugar assimilation (Muller *et al*., 2011). Anuradha (2004) observed a progressive rise in sugar levels in *Piper nigrum* leaves in response to the severity of the drought. As a compatible solute, sugar maintained adequate water potential in the drought-tolerant Panniyur 5 variety of *Piper nigrum*, thereby achieving better osmotic adjustment (Puthur & Vijayakumari, 2014). Transgenic lines of APX, DREB2A, and DREB2A-APX in rice are capable of enhancing reducing sugar levels to maintain cellular integrity through osmotic adjustment under the influence of regulated water deficit treatment compared to wild type *Oryza sativa* (Sandhya *et al*., 2021). The parallel

findings from previous studies were also observed in the present study, providing collective evidence that underscores the significance of sugar metabolism as a drought resilience response across different plant species.

4.4.2.6. Starch (%)

The starch content varied significantly ($P < 0.001$) among the accessions and showed diminishing levels with increasing stress intensity. Relatively lower and steady reductions in starch were observed in accessions Acc 1495 (18.35%), Acc 4132 (1.56%), and Acc 4064 (1.6%), suggesting they possess better drought tolerance compared to Acc 7211 (21.77%), Acc 1343 (82.56%), and Acc 5717 (91%).

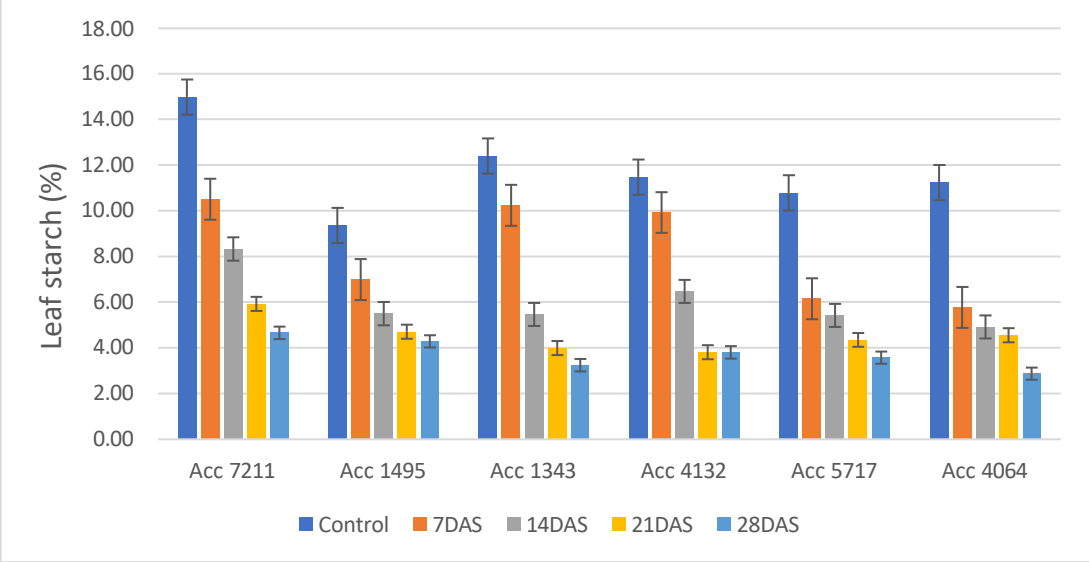


Figure 23. Leaf starch content variation in selected black pepper genotypes with increasing drought stress intensity

The present study was supported by previous studies showing that, under severe drought conditions in soybean leaves, starch content declined due to the retarded rate of photosynthesis, which could have been caused by decreased activity of the cytochrome pathway (Ribas-Carbo *et al*., 2005). A comparative study of the starch and sugar content in black pepper stems and leaves revealed that it was greater in the off year than in the on year (Krishnamurthy *et al*., 2013). Additionally, ABA serves as a crucial regulator, and starch content and its conversion to soluble sugars play a role in the physiological changes that enable stomatal closure, particularly in response

to environmental stressors like drought, thus increasing drought tolerance (Daszkowska-Golec & Szarejko, 2013).

4.4.2.7. Total protein (%)

The total leaf protein content was found to reduce under water-stressed plants compared to well-watered control plants. However, plants with tolerant characteristics exhibited relatively higher protein content compared to susceptible plants. Among control plants, it ranged from 5.12% to 7.39%. Higher reduction was found in Acc 4064 (92.1%) and 4132 (87.64%), and a medium level of reduction was observed in Acc 5717 (76.5%) Acc and Acc 1343 (63.56%). Meanwhile, a low-level reduction was noted in Acc 1495 (41.60%) and Acc 7211 (30.99%) which is desirable for drought tolerance, followed by 1343 and 5717.

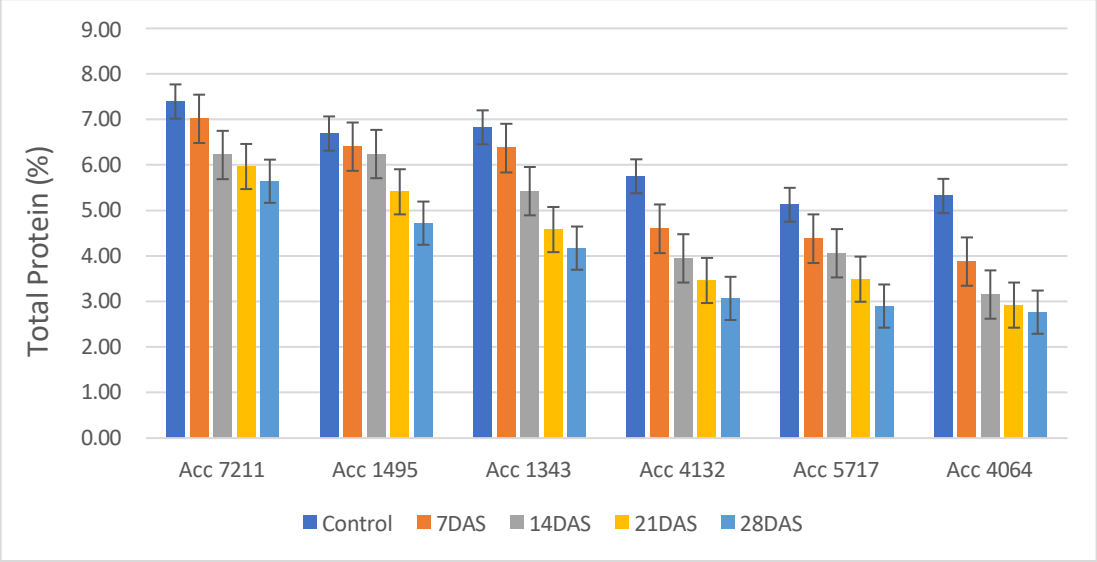


Figure 24. Total protein content variation in selected black pepper genotypes with increasing drought stress intensity

The total soluble protein decreased with increasing drought stress intensity in *Piper* species such as *P. nigrum*, *P. longum*, *P. chaba*, *P. colubrinum*, and *P. hymenophyllum*. The relatively highest protein content was recorded in *P. nigrum* accessions 1618, 1567, Panniyur 1, and Sreekara. Panniyur 1 exhibited the highest protein content, while *P. colubrinum* showed the least (Krishnamurthy, 2005).

A significant decrease in total soluble protein was noticed from FC to -0.6 MPa to -1.0 MPa (52, 49 and 9 μM/g FW respectively), which was negatively correlated with increased aridity (Akhzari & Pessarakli, 2016).

Similar reduction in total proteins with stress intensity as found in the current study was reported in maize (Mohammadkhani & Heidari, 2008); wheat (Parchin & Shaban, 2014); tomato (Ghorbanli *et al*., 2009) and banana (Surendar *et al*., 2013).

Contrasting to the present study, induced drought stress initially increased the total protein in groundnut genotypes (Vanaja, 2015) and wheat (Parchin & Shaban, 2014). These proteins may act as stress response proteins to mitigate its effect, but further reduction occurred, which might be attributed to lower photosynthesis under prolonged stress conditions (Havaux *et al*., 1987).

These findings, including those from the present study, reveal both an initial reduction and an initial increase followed by a decrease under prolonged stress, suggesting a complex relationship between total protein content and drought tolerance. The maintenance of higher protein levels or lower reduction emphasizes drought resilience in plants.

4.4.2.8. Antioxidant enzyme activity

The antioxidant enzymes activity studied showed significant variation ($P < 0.001$) among the accessions for all the enzymes (catalase, peroxidase, superoxide dismutase, DHAR, MDHAR, ascorbate peroxidase, glutathione S-transferase, polyphenol oxidase, and glutathione reductase).

4.4.2.8.1. Peroxidase (μmol/min mg enzyme)

Peroxidase activity gradually increased from control and reached highest levels at 21 DAS in accessions 7211 (918.58%), 1495 (563.12%), 1343 (713.32%), and 4132 (993.35%). The dramatically higher peroxidase activity with increasing stress levels may indicate better drought tolerance mechanism in those accessions. Subsequently, the activity decreased compared to the previous level. However, accessions 5717 and 4064 showed lower increase compared to the other accessions under stress which indicate their lower ability to cope with stress.

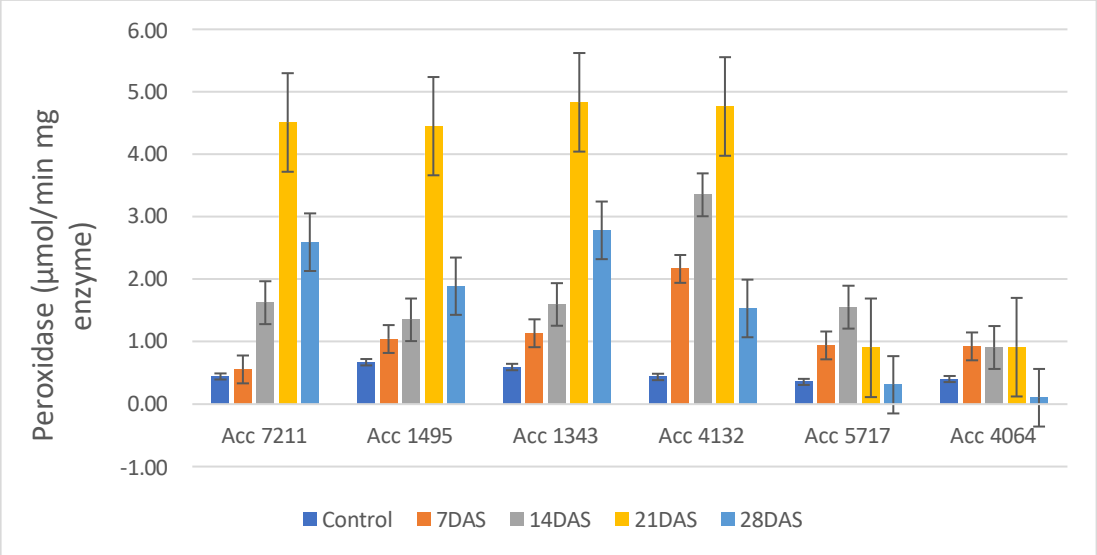


Figure 25. Variation in peroxidase activity of selected black pepper genotypes with increasing drought stress intensity

Under water stressed condition, peroxidase exhibited increased activity across all selected genotypes within the same species of *Piper* (Krishnamurthy *et al*., 2000) as well as in different species of *Piper* (Krishnamurthy, 2005) also. Similarly, in the shoots and leaves of canola cultivars, higher POD activity was observed, especially in SLM046 when exposed to PEG application. Under control conditions, higher activity was observed in roots than in shoots (Mirzaee *et al*., 2013). Similar results were reported in *Pistacia khinjuk* seedlings (Mirzaei & Yousefzadeh, 2013), and also in wheat (Tale Ahmad & Haddad, 2011).

The observations from the present and previous studies highlight the correlation between POD activity and drought tolerance across different plant species and conditions. An identical trend was observed under salt stress, with a positive correlation between POD activity and tolerance in the cotton cultivar Pora (Meloni *et al*., 2003).

4.4.2.8.2. Catalase (µmol/min mg protein)

The catalase activity significantly increased with stress intensity in all the accessions. In control plants, the observed catalase activities for the respective accessions were as follows: 0.11 (Acc 7211), 0.10 (Acc 1495), 0.10 (Acc 1343), 0.20 (Acc 4132), 0.10 (Acc 5717), and 0.17 µmol/min mg protein (Acc 4064). Lower activity under well-

watered conditions was observed in all the accessions. However, the relatively lowest increase was noticed in Acc 4064 (161.74%), followed by Acc 5717 (450.5%) and Acc 4132 (475.5%) compared to the control. Comparatively increased activity was noticed in Acc 1343 (992.2%), followed by Acc 1495 (943.7%) and Acc 7211 (756.7%) which is associated with drought tolerance

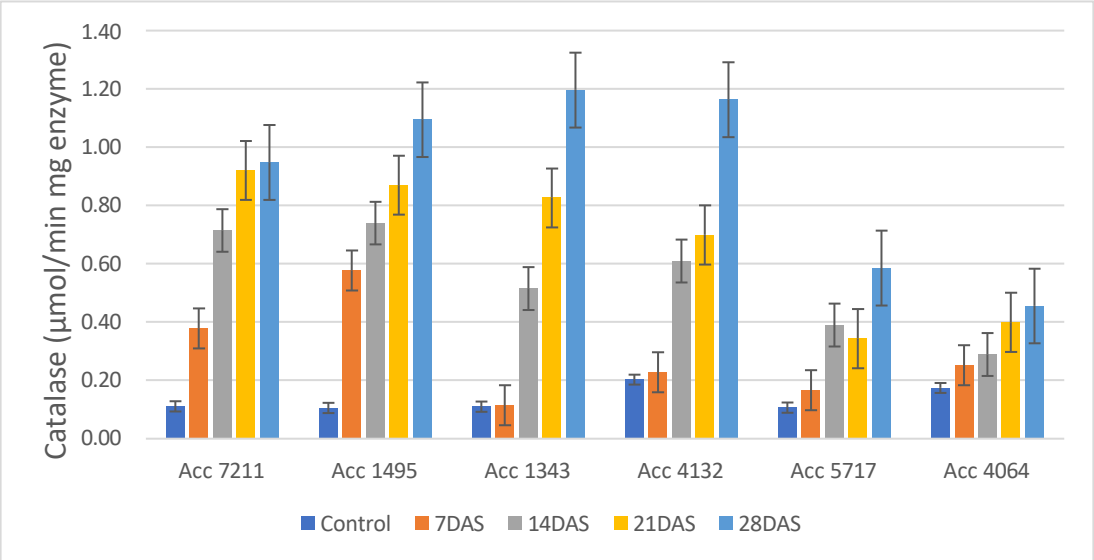


Figure 26. Variation in catalase activity of selected black pepper genotypes with increasing drought stress intensity

During the early drought period, catalase activity increased significantly in pennyroyal plants, yet subsequently rapidly decreased with sustained drought stress, reflecting the plant's sensitivity to severe stress conditions (Ulusu *et al*., 2022). Under chilling stress, there was an increase initially in catalase activity and a decrease at a later stage, with a relatively higher rate of activity in tolerant genotypes (185%) compared to sensitive ones (86%) in pumpkin genotypes.

Such trend was not observed in both drought tolerant and susceptible black pepper genotypes in the current study. Tolerant accessions generally maintained higher activity specifically, Acc 828 exhibited higher activity compared to others (Krishnamurthy, 2005). In *Pistacia khinjuk* seedlings, activity was increased during shoot in drought phase (Mirzaei & Yousefzadeh, 2013).

Catalase activity showed significant positive correlation with stress intensity and the tolerant genotypes maintained a higher activity than the susceptible genotypes in the

present study as well as in previous research findings, demonstrating that by reducing ROS levels, catalase contributes to the maintenance of membrane integrity, preventing lipid peroxidation, and maintaining the functionality of cellular membranes.

4.4.2.8.3. Superoxide dismutase (Units/mg fresh weight)

The SOD activity ranged between 1.84 (Acc 4064) and 3.04 (Acc 4132) units/mg FW in control plants. Activity slowly increased with increasing stress level up to 21 DAS in all the accessions, with 20%, 16.8%, 16.7%, 12.2%, 3.8%, and 8.57% increase in accessions 7211, 1495, 1343, 4132, 5717, and 4064, respectively. Then, it decreased at 28 DAS. Accessions 5717 and 4064 showed minimum activity compared to the remaining accessions.

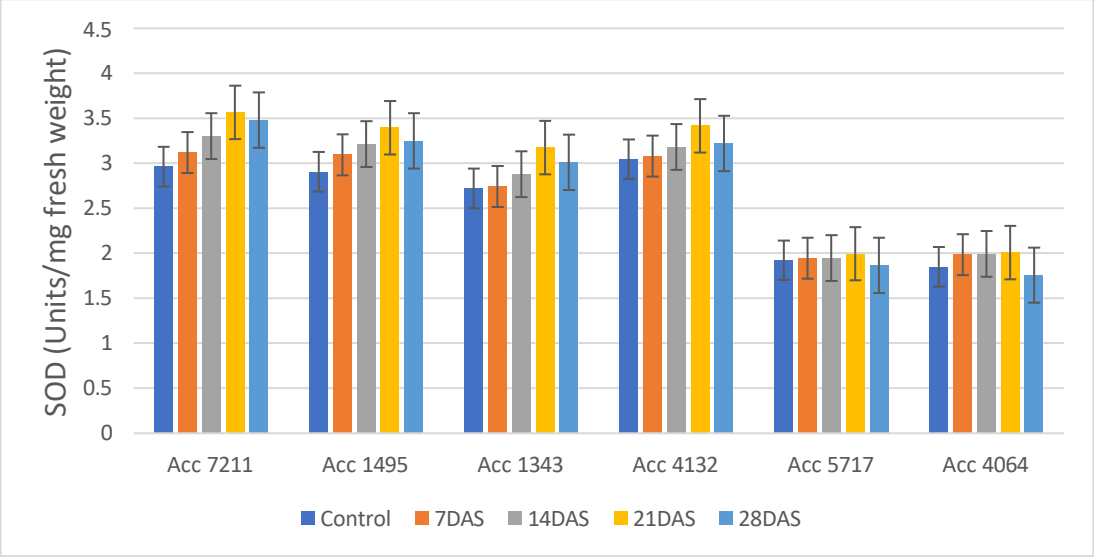


Figure 27. Variation in SOD activity of selected black pepper genotypes with increasing drought stress intensity

Higher SOD activity under imposed drought conditions was observed in *Piper nigrum*, specifically for Acc 1618, than for Panniyur-1 and Acc 1567, which was negatively correlated with membrane leakage and catalase activity compared to other *Piper* species such as *P. chaba*, *P. longum*, *P. hymenophyllum*, and *P. colubrinum* (Krishnamurthy, 2005).

Puthur and Vijayakumari (2014) reported that the drought-sensitive variety Panniyur-1showed higher SOD activity under control and did not show an increment under drought stress; further decline was observed under severe drought stress. Meanwhile, the tolerant Panniyur-5 experienced a steady increase in SOD activity with progressive stress levels.

Generally, several reports are available on SOD activity being positively correlated with drought stress, especially in drought-resistant varieties, serving as a marker (Mirzaee *et al*., 2013; Ge *et al*., 2006; Mirzaei & Yousefzadeh, 2013; Mombeini *et al*., 2021; Ahmad & Haddad, 2011).

Likewise, chilling stress also significantly enhanced SOD activity in resistant genotypes of pumpkin calli, AB-44 and Iskenderun-4, (187.29 $U \cdot min^{-1} \cdot mg^{-1}$ FW and 172.89 $U \cdot min^{-1} \cdot mg^{-1}$ FW, respectively), compared to sensitive genotypes such as A-24 (101.55 $U \cdot min^{-1} \cdot mg^{-1}$ FW) and CU-7 (76.69 $U \cdot min^{-1} \cdot mg^{-1}$ FW) after 8 days of stress exposure (Kusvuran *et al*., 2013). A similar trend of enzymatic activity was displayed under salt stress (Meloni *et al*., 2003).

SOD activity functioned as a reliable marker for stress tolerance, consistently demonstrating findings from both present and previous investigations. Increased activity was observed in genotypes with drought-tolerant characteristics, reflecting its role in mitigating oxidative stress by detoxifying superoxide radicals (ROS) into hydrogen peroxide and molecular oxygen, as shown in the present study. Further detoxification of hydrogen peroxide was done by catalase, as well as the Ascorbate-Glutathione Cycle, which worked together to convert H_2O_2 into water and molecular oxygen.

4.4.2.8.4. Polyphenol oxidase (µmol/min mg enzyme)

Polyphenol oxidase activity in control plants ranged between 22.06 (Acc 1495) and 29.61 (Acc 1343) µmol/min per mg of enzyme. The enzyme activity steadily increased up to 14 DAS for accessions 7211, 1343, 4132, decreased at 21 DAS, then reaching its maximum activity again at 28 DAS.

In contrast, in Acc 1495, the activity increased at 7 DAS, then decreased at 14 DAS, and increased again up to 28 DAS. Similar pattern of increment till 14 DAS was observed in accessions 5717 and 4064, then decreased to control levels at 21 DAS, and further declined even lower than the control at 28 DAS.

Relatively higher activity was recorded in accessions 1495 (82.5%), 7211 (67.4%), 4132 (61.05%), and 1343 (55.5%) which could help in water stress tolerance.

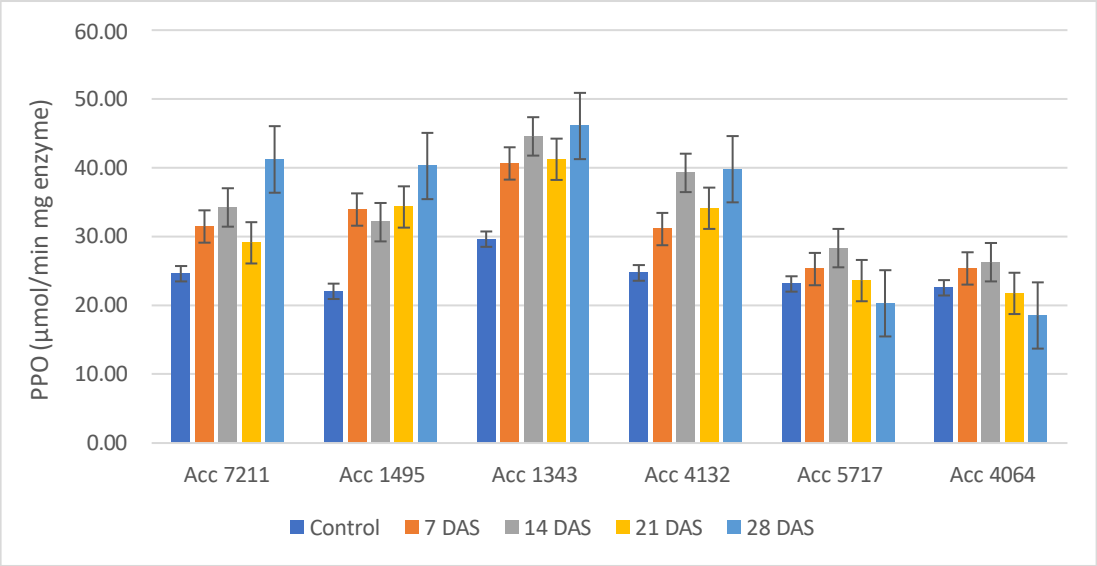


Figure 28. Variation in polyphenol oxidase activity of selected black pepper genotypes with increasing drought stress intensity

An increase in PPO activity was noted initially on the 3rd day, with a 1.5-fold increment compared to the control, but it started to decrease to 1.05 and 1.1 times on the 6th and 10th days of drought stress, respectively, in pennyroyal plants. This indicates the dynamic response of PPO as an antioxidant to prolonged stress (Ulusu *et al*., 2022). In black pepper genotypes, activity increased with stress intensity in both drought-tolerant and susceptible genotypes until the end of the treatment, at which point the tolerant ones showed a marked increase (Krishnamurthy, 2005; Krishnamurthy *et al*., 2000). Green peppers are ideal for storage compared to red and yellow peppers because they have reduced PPO activity, which helps reduce enzymatic browning, delayed expression, and better retention of ascorbic acid and phenols, enhancing both quality and nutritional value (Barbagallo *et al*., 2012).

This variability in PPO activity highlights its significance in stress adaptation and quality retention in different plant species, as demonstrated in previous studies and the present investigation. By catalyzing the oxidation of phenolic compounds, PPO reduces ROS levels and mitigates oxidative stress.

4.4.2.8.5. Glutathione S transferase (µmol/min mg enzyme)

GST activity exhibited a gradual increase in all accessions up to 14 DAS compared to the control. In the control, the activity level ranged between 1.89 (Acc 4064) and 3.09 (Acc 4132). Subsequently, it decreased with progressive stress levels and dropped below the control level in Accessions 5717 and 4064 after 21 DAS. Genotypes with greater resilience to drought, such as 1495 and 1343, showed slower decreases in activity, followed by 4132 and 7211.

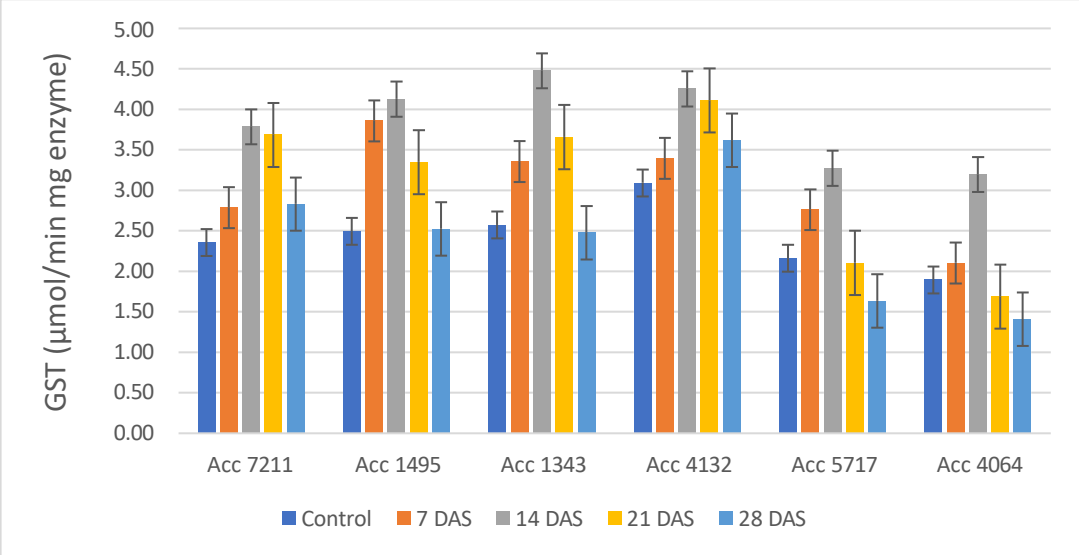


Figure 29. Variation in glutathione S transferase activity of selected black pepper genotypes with increasing drought stress intensity

The anti-oxidative activity contributed by one of the antioxidant enzymes, GST, in *Allium* species subjected to moisture deficiency, showed increased activity which helps in mitigating oxidative damage caused by stress (Csiszár *et al*., 2007). It had also the antifungal activity which was positively correlated with increasing infection in wheat plants observed after 48, 72 and 96 hours of inoculation (Debona *et al*., 2012). A drought-influenced variation study on barley genotypes illustrated a similar elevation of GST activity in the tolerant genotype, Yusof, indicating its greater

tolerance compared to the sensitive genotypes, Moroc9-75 and HS1 (Rezaei *et al*., 2013).

At the molecular level, the downregulation of the gene ZmGST26 in maize roots led to susceptibility and affected various physiological and biochemical parameters in Arabidopsis, thereby downregulating drought stress-related genes (Jiang *et al*., 2022). Unfavorable environmental conditions induce the production of salicylic acid, which acts as a signal for GST induction to mitigate and protect the plants (Cetinkaya *et al*., 2014).

These studies support the present findings, showing elevated activity of GST in drought-tolerant Allium species and barley genotypes, as well as its contribution to antifungal defense in wheat. Furthermore, GST helps maintain physiological and biochemical stability under stress at the molecular level.

4.4.2.8.6. Ascorbate-glutathione cycle (AGC)

The activities of MDHAR, DHAR, APX, and GR in the Ascorbate-glutathione cycle increased significantly with stress intensity up to 21 DAS in accessions 7211, 1495, 1343, and 4132. However, under severe water stress conditions at 28 DAS, the activities began to decrease compared to the levels observed at 21 DAS. A similar pattern of minimal increase was observed in accessions 5717 and 4064, where the activities at 28 DAS reached the same level of control level or dropped below the control level. Consequently, these accessions could be considered as having susceptible traits.

Table 9. Percentage increase in activity of enzymes in the ascorbate-glutathione cycle over the control at 21 DAS

Genotypes	MHAR	DHAR	APX	GR
Acc 7211	97.8	100.9	98.18	98.6
Acc 1495	88.3	92.3	92.7	87.8
Acc 1343	135.1	135.9	132	131.2
Acc 4132	124.6	125.8	129.3	125.4
Acc 5717	50.7	46.6	53.4	49.8
Acc 4064	50.7	44.6	51.7	46.6

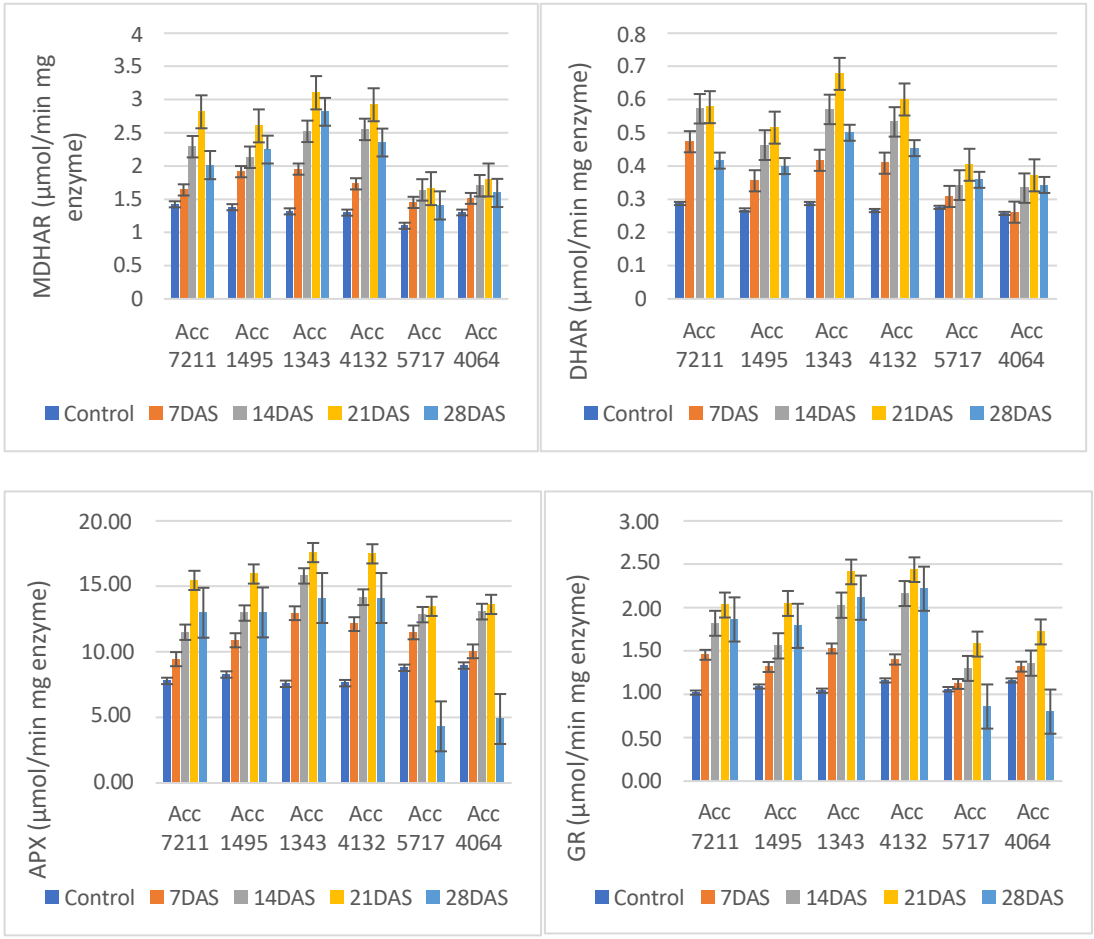


Figure 30. Variation in ascorbate glutathione activity of selected black pepper genotypes with increasing drought stress intensity

Typically, the AsA-GSH cycle among antioxidant systems has been proven to be a stress tolerance marker in several research studies, while upregulated activity in those enzymes has conferred better tolerance to abiotic stresses, influenced by the stress severity, duration, and developmental stage, thereby reducing the generated ROS (Hasanuzzaman *et al*., 2019; Hossain *et al*., 2016).

Akin to the present study, the components in the AsA-GSH cycle reduce the excess hydrogen peroxide generated through high sunlight irradiance and heat stress exposure in wheat genotypes, particularly in drought-tolerant ones with higher intensity (Aliyeva *et al*., 2020). The activities of components such as APX, GR, DHAR, and MDHAR remained almost stable in control plants, whereas in water-stressed genotypes, the activity of these four components increased synergistically (Lou *et al*., 2018).

These findings are consistent with previous research, which has shown the relevance of the AsA-GSH cycle in maintaining cellular redox balance and overall plant health under various abiotic stressors, particularly in drought-resistant genotypes.

Table 10. Physiological and biochemical parameters of selected genotypes under control conditions.

	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
RWC	90.99 a	92.53 a	91.04 a	92.62 a	90.52 ab	90.61 a
EC	8.750 mnop	7.360 p	8.523 nop	8.002 op	10.010 jklmno	9.110 lmnop
Chl a	1.23 f	1.29 ef	1.57 b	1.31 ef	1.12 g	0.99 h
Chl b	0.381 abc	0.386 ab	0.436 a	0.375 bc	0.257 fg	0.306 def
Total chl	1.608 f	1.676 def	2.006 b	1.686 de	1.379 h	1.296 i
a/b ratio	3.22 u	3.35 t	3.61 p	3.50 r	4.36 h	3.23 u
Carotenoids	0.514 gh	0.426 kl	0.476 hij	0.376 mn	0.366 mn	0.334 no
H2O2	0.899 q	0.936 p	0.876 q	1.003 o	0.774 r	1.756 f
Proline	66.23 rs	50.71 u	63.22 s	66.83 r	50.39 u	99.12 o
Phenol	6.00 a	5.95 a	5.60 b	5.12 d	4.78 e	1.70 opq
MDA	13.95 mn	17.26 l	11.74 n	16.29 lm	21.29 k	25.00 j
Leaf sugar	0.94 pq	1.01 mno	0.86 r	0.93 q	0.64 t	1.16 ij
Leaf starch	14.99 a	9.37 i	12.40 b	11.48 c	10.79 e	4.91 r
Protein	7.40 a	6.69 d	6.83 c	5.75 h	5.13 l	3.16 u
CAT	0.11 p	0.10 p	0.11 p	0.20 no	0.11 p	0.29 l
POX	0.44 r	0.67 p	0.59 q	0.44 rs	0.36 t	0.91 o
SOD	2.97 jk	2.91 k	2.72 l	3.05 hij	1.92 mno	1.99 m
GST	2.36 p	2.49 o	2.57 n	3.09 k	2.16 q	3.20 j
PPO	24.62 ijk	22.06 lmn	29.62 efg	24.72 ijk	23.13 jkl	26.26 hi
APX	7.80 ij	8.27 i	7.58 j	7.63 j	8.79 n	13.08 kl
DHAR	0.29 j	0.27 j	0.29 j	0.27 j	0.28 mno	0.34 klmno
MDHAR	1.43 k	1.38 k	1.32 k	1.30 k	1.1 n	1.7 lm
GR	1.02 h	1.09 h	1.04 h	1.16 gh	1.06 kl	1.36 jk

Table 11. Physiological and biochemical parameters of selected genotypes at 7 DAS

	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
RWC	79.21 efgh	86.12 cd	86.59 bc	86.24 cd	81.95 ef	78.03 fgh
EC	10.140 ijklmno	8.430 op	8.610 nop	11.019 hijklm	12.460 efghi	14.340 abcde
Chl a	1.29 ef	1.32 de	1.73 a	1.41 cd	0.79 ij	0.75 ijkl
Chl b	0.329 cde	0.349 bcd	0.373 bc	0.340 bcde	0.223 gh	0.200 hijk
Total chl	1.617 ef	1.669 ef	2.100 a	1.748 cd	1.015 k	0.952 klm
a/b ratio	3.91 l	3.78 m	4.63 f	4.14 j	3.54 q	3.77 mn
Carotenoids	0.523 fg	0.565 ef	0.589 cde	0.387 lm	0.452 jk	0.463 ijk
H2O2	1.090 n	1.240 l	1.073 n	1.494 i	1.226 l	1.766 f
Proline	88.97 p	55.99 t	83.69 q	107.17 n	128.24 k	86.64 pq
Phenol	4.28 f	2.12 j	2.29 i	1.43 s	1.94 m	2.35 h
MDA	16.61 lm	32.74 i	15.97 lm	32.42 i	34.03 hi	26.29 j
Leaf sugar	1.20 hi	1.09 kl	1.05 lm	1.01 no	0.87 r	0.86 r
Leaf starch	10.51 f	6.99 k	10.24 g	9.93 h	6.16 m	5.77 o
Protein	7.02 b	6.40 e	6.37 e	4.60 n	4.38 o	3.88 s
CAT	0.38 jk	0.58 f	0.11 p	0.23 mn	0.17 o	0.25 lm
POX	0.56 q	1.04 n	1.14 m	2.16 h	0.94 o	0.92 o
SOD	3.12 fgh	3.10 ghi	2.74 l	3.08 ghi	1.95 mn	1.98 m
GST	2.79 lm	3.86 d	3.36 h	3.40 h	2.76 m	2.10 q
PPO	31.46 def	33.95 cd	40.64 b	31.10 ef	25.29 ij	25.39 ij
APX	9.45 h	10.89 g	12.95 d	12.12 e	11.49 lm	10.05 mn
DHAR	0.47 ef	0.36 i	0.42 gh	0.41 h	0.3 lmno	0.26 no
MDHAR	1.64 j	1.92 hi	1.95 gh	1.73 ij	1.46 lmn	1.52 lm
GR	1.46 ef	1.32 fg	1.53 e	1.40 ef	1.12 kl	1.32 jk

Table 12. Physiological and biochemical parameters of selected genotypes at 14 DAS

	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
RWC	75.29 hi	82.53 de	80.54 efg	79.29 efgh	67.43 j	71.29 ij
EC	11.540 fghijk	10.830 hijklmn	9.411 klmnop	11.226 ghijkl	13.420 cdefg	15.390 abcd
Chl a	1.65 ab	1.32 de	1.73 a	1.46 c	0.78 ij	0.68 klm
Chl b	0.294 def	0.289 ef	0.368 bc	0.332 bcde	0.193 hijk	0.199 hijk
Total chl	1.947 b	1.612 ef	2.095 a	1.793 c	0.972 kl	0.883 mn
a/b ratio	5.62 b	4.58 g	4.70 e	4.40 h	4.03 k	3.43 s
Carotenoids	0.581 cde	0.579 cde	0.796 b	0.578 cde	0.442 jk	0.447 jk
H2O2	1.236 l	1.465 j	1.482 ij	1.761 f	1.769 f	1.756 f
Proline	120.42 l	96.84 o	87.90 p	159.01 e	147.81 g	99.12 o
Phenol	1.30 t	1.71 op	1.67 pq	1.86 n	1.60 r	1.70 opq
MDA	18.56 kl	36.45 gh	16.94 lm	36.77 fgh	41.48 e	25.00 j
Leaf sugar	1.32 g	1.14 j	1.12 jk	1.05 lmn	0.98 op	1.16 ij
Leaf starch	8.33 j	5.50 p	5.47 p	6.47 l	5.42 q	4.91 r
Protein	6.22 f	6.24 f	5.43 j	3.95 r	4.06 q	3.16 u
CAT	0.71 e	0.74 e	0.51 g	0.61 f	0.39 ijk	0.29 l
POX	1.62 j	1.35 l	1.60 j	3.35 e	1.55 k	0.91 o
SOD	3.30 cd	3.21 def	2.88 k	3.18 efg	1.95 mn	1.99 m
GST	3.78 e	4.13 c	4.48 a	4.25 b	3.27 i	3.20 j
PPO	34.25 c	32.10 cde	44.57 a	39.26 b	28.33 gh	26.26 hi
APX	11.50 f	12.97 d	15.79 b	14.17 c	12.84 kl	13.08 kl
DHAR	0.57 bc	0.46 ef	0.57 bc	0.53 cd	0.34 klmn	0.34 klmno
MDHAR	2.29 ef	2.13 fg	2.53 cd	2.56 cd	1.64 lm	1.7 lm
GR	1.82 d	1.56 e	2.03 c	2.16 bc	1.3 jk	1.36 jk

Table 13. Physiological and biochemical parameters of selected genotypes at 21 DAS

	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
RWC	60.13 k	71.3 ij	68.8 j	77.11 gh	57.1 k	60.45 k
EC	11.860 fghij	11.090 ghijklm	9.984 jklmno	12.053 efghij	15.720 abc	15.870 ab
Chl a	1.10 g	0.79 ij	1.26 ef	0.96 h	0.57 nop	0.66 lmn
Chl b	0.294 def	0.231 gh	0.260 fg	0.211 ghi	0.153 jkl	0.157 ijkl
Total chl	1.393 h	1.016 k	1.521 g	1.175 j	0.723 o	0.819 n
a/b ratio	3.74 mn	3.40 s	4.86 d	4.57 g	3.73 n	4.22 i
Carotenoids	0.774 b	0.612 cd	0.845 a	0.605 cde	0.387 lm	0.334 no
H2O2	1.544 h	1.652 g	1.349 k	1.838 d	1.998 b	1.894 c
Proline	143.45 h	131.86 j	137.49 i	178.22 c	131.59 jk	115.21 m
Phenol	1.20 u	1.15 v	2.01 l	2.05 kl	1.30 t	1.66 q
MDA	18.87 kl	51.29 c	21.45 k	49.68 cd	50.00 cd	38.92 efg
Leaf sugar	1.46 f	1.51 e	1.25 h	1.34 g	1.06 lm	1.23 h
Leaf starch	5.92 n	4.70 s	3.99 x	3.81 y	4.35 v	4.56 u
Protein	5.97 g	5.41 j	4.58 n	3.46 t	3.49 t	2.92 w
CAT	0.92 c	0.87 d	0.83 d	0.70 e	0.34 k	0.40 ij
POX	4.51 c	4.45 d	4.83 a	4.77 b	0.90 o	0.91 o
SOD	3.57 a	3.40 bc	3.18 efg	3.42 b	2.00 m	2.01 m
GST	3.69 f	3.35 h	3.66 fg	4.11 c	2.10 q	1.69 s
PPO	29.08 fg	34.32 c	41.23 b	34.11 c	23.59 jkl	21.74 lmn
APX	15.46 b	15.95 b	17.58 a	17.49 a	13.47 k	13.62 k
DHAR	0.58 b	0.52 d	0.68 a	0.60 b	0.4 k	0.38 kl
MDHAR	2.82 b	2.61 c	3.11 a	2.92 ab	1.66 lm	1.8 l
GR	2.03 c	2.05 c	2.41 a	2.44 a	1.58 ij	1.72 i

Table 14. Physiological and biochemical parameters of selected genotypes at 28 DAS

	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
	28 DAS	28 DAS	28 DAS	28 DAS	28 DAS	28 DAS
RWC	52.9 l	57.32 k	51.08 lm	57.55 k	48.29 m	50.1 lm
EC	13.740 bcdef	11.200 ghijkl	12.210 efghij	13.167 defgh	16.890 a	17.300 a
Chl a	0.77 ijk	0.62 mno	0.71 jkl	0.81 i	0.52 p	0.50 p
Chl b	0.156 ijkl	0.201 hij	0.193 hijk	0.204 ghij	0.092 m	0.120 klm
Total chl	0.922 lm	0.817 n	0.904 lm	1.011 k	0.614 p	0.640 op
a/b ratio	4.92 c	3.07 v	3.68 o	3.95 l	5.69 a	4.72 e
Carotenoids	0.619 c	0.497 ghi	0.577 cde	0.570 de	0.265 p	0.280 op
H2O2	1.336 k	1.668 g	1.353 k	1.852 d	1.808 e	2.090 a
Proline	166.04 d	152.80 f	206.33 b	217.17 a	120.37 l	147.63 gh
Phenol	1.75 o	2.07 k	2.33 hi	2.93 g	1.17 uv	1.31 t
MDA	34.52 hi	56.29 b	26.77 j	57.26 b	74.84 a	40.90 ef
Leaf sugar	1.68 d	1.81 c	1.99 a	1.86 b	1.05 lm	1.25 h
Leaf starch	4.66 t	4.29 w	3.24 A	3.81 y	3.58 z	2.90 B
Protein	5.65 i	4.73 m	4.17 p	3.07 v	2.90 w	2.79 x
CAT	0.95 c	1.09 b	1.20 a	1.16 a	0.59 f	0.48 gh
POX	2.59 g	1.89 i	2.78 f	1.53 k	0.31 u	0.11 v
SOD	3.48 ab	3.25 de	3.01 ij	3.22 def	1.87 no	1.78 op
GST	2.83 l	2.52 no	2.48 o	3.62 g	1.63 s	1.390 t
PPO	41.22 b	40.28 b	46.07 a	39.81 b	20.32 mn	16.32 o
APX	13.00 d	13.02 d	14.11 c	14.11 c	4.32 o	4.89 o
DHAR	0.42 gh	0.40 h	0.50 de	0.45 fg	0.36 klm	0.35 lmno
MDHAR	2.02 gh	2.25 ef	2.82 b	2.35 de	1.4 lmn	1.46 lmn
GR	1.86 d	1.79 d	2.11 bc	2.22 b	0.86 l	0.8 l

4.4.2.9. Mineral elements

The major elemental composition analyzed in the well-watered soil predominantly contained organic carbon, followed by calcium, potassium, nitrogen, and phosphorus, with respective concentrations of 2.36%, 1208.07 ppm, 854.37 kg/ha, 206.98 kg/ha, and 46.49 kg/ha while under stress (28 DAS) except for potassium which showed an increase, the concentration of all the other elements were lesser than control (1.97%, 765.43 ppm, 929.47 kg/ha, 181.89 kg/ha, and 41.48 kg/ha respectively). Potassium showed an increase of 8.79% over control. The highest decrease (57.83%) was noticed in calcium, and the lowest decrease was observed in phosphorus (12.10%) over control.

Moist soil typically contains organic carbon in the range of 2 to 10% (source: https://www.agric.wa.gov.au/measuring-and-assessing-soils/what-soil-organic-carbon). Organic carbon and nitrogen were found to be at decreased levels in water-drained soil compared to well-watered soil (Zhang *et al*., 2022). Hence, minerals are more readily available for plant uptake in well-moistured soil due to its solubility, while they are less available to plants in dry soil conditions. Dry soil conditions reduce soil microbe activity, decreasing nitrogen mineralization and organic matter decomposition, while trapping potassium between mineral layers, resulting in decreased plant uptake and potential nutrient imbalances. Thus, soil analysis during dry years is crucial for determining remaining nutrients for future crops (source: https://onfruit.ca/2016/07/25/dry-soil-conditions-impact-on-nutrient-availability/).

Minerals were assessed in six accessions, four accessions (Acc 7211, Acc 1495, Acc 1343, and Acc 4132), showcasing drought-tolerant traits, while two accessions (Acc 5717 and Acc 4064) exhibited susceptible traits. Mineral levels such as nitrogen, phosphorus, potassium, calcium, magnesium, iron, copper, manganese, and zinc were determined at 28 DAS, in comparison to the control. Iron and manganese showed clear-cut discrimination between drought tolerant (7211, 1495, 1343, and 4132) and susceptible genotypes (5717 and 4064). An increased iron accumulation, as compared to the control, was observed in accessions 7211, 4132, 1495, and 1343 (45.45%, 30.5%, 13.88%, and 1.53% increase, respectively). While, a decreased accumulation

was noticed in genotypes 5717 (27.46%) and 4064 (90.81%), which were considered as drought susceptible. A similar trend was observed in manganese, where the highest increase was noted in 4132 (243.57%), followed by 1495 (224.9%), 7211 (203.95%), and 1343 (83.02%). Comparatively lower increase was recorded in accessions 4064 (58.09%) and 5717 (81.19%).

Except for 5717, all the accessions followed a uniform pattern of increment in their potassium levels under water stress compared to the respective control plants. The genotypes 1343, 7211, 4132, and 1495 showed drought tolerance traits, with higher accumulation of iron and manganese under drought stress conditions.

The utilization of nanocapsule-potassium at high temperatures mitigates stress indices and improves pepper growth and resistance qualities compared to conventional potassium application (Halaji *et al*., 2023). Potassium is essential for plant growth and survival in unfavorable environmental conditions such as drought, aiding stress resistance through biochemical functions by mitigating ROS production and activating the antioxidant defense mechanism (Hasanuzzaman *et al*., 2018). However, a specific trend could not be identified in the mineral elements nitrogen, phosphorus, calcium, magnesium, copper, and zinc in leaf samples in the current study.

The micronutrients Zn, Si, and Mg improve antioxidant activity and promote drought tolerance, while enhanced water absorption through improved root development is achieved by the influence of P, K, and Mg (Naik *et al*., 2022). For plant growth and development, as well as crop yield, the essential requirements include major minerals (N, P, and K), secondary minerals (Ca and Mg), and microelements, especially Zn (Ahmed *et al*., 2023). The plant mineral composition is altered under the influence of varied geographical and climatic conditions, as well as the application of fertilizers and irrigation practices (Jabeen *et al*., 2019). Variability in mineral composition was also observed in *Piper* species such as *P. nigrum, P. longum, P. chaba* and *P. colubrinum* (Rajan, 2020)

Under water restriction, tolerant black gram genotypes showed a reduction in leaf nitrogen relative to sensitive genotypes (Kumar, 2021

). The nutrient deficiency led to the dysfunction of chlorophyll pigments, thereby disturbing the metabolic functioning of plants (Chandrasekaran *et al*., 2023).

A significant elevation in phosphorus and calcium concentrations in leaves was observed at 15 days of drought stress, followed by a slight increase in potassium accumulation, which further peaked at 30 days of stress before declining. Initially, there was an increase in manganese concentration, but the level subsequently declined to control levels over the stress period. However, the iron showed an increase of 575.17% at 45 days, compared to control conditions (Li *et al*., 2023).

Improved nutrient intake and utilization potential may have the ability to reduce the negative effects of drought stress. The availability of nutrients in both the soil and the plant influences the growth, development, and yield of black pepper plants. Among the macro and microelements studied, the highest uptake was observed for nitrogen, followed by potassium and calcium. Iron exhibited the highest uptake among the microelements (Srinivasan *et al*., 2007).

Overall, the study emphasizes the importance of adequate mineral accumulation, particularly iron, potassium and manganese, in improving drought resistance and maintaining plant health under stressful conditions. This finding is consistent with other studies that suggest improved nutrient intake and utilisation can reduce the negative effects of drought stress, emphasising the importance of maintaining an optimal balance of macro and microelements for plant health and productivity under environmental stress conditions.

Table 15. Mineral composition of soil

Soil mineral	Control	28 DAS
Organic carbon (%)	2.36±0.044	1.97±0.016
N (kg/ha)	206.98±3.62	181.89±3.621
P (kg/ha)	46.49±4.80	41.48±3.52
K (kg/ha)	854.37±22.27	929.47±3.65
Ca (ppm)	1208.07±32.73	765.43±17.86
Mg (ppm)	256.78±11.66	198.68±0.40
Fe (ppm)	38.8±0.21	36.44±0.041
Cu (ppm)	11.45±0.211	7.98±0.041
Mn (ppm)	26.19±2.96	25.23±0.215
Zn (ppm)	4.26±0.29	3.36±0.011

Table 16. Mineral composition of selected black pepper genotypes under control condition

Genotype	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Fe (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)
Acc 7211	3.094 b	0.209 bc	2.51 b	2.81 b	0.303 e	123.33 bc	11.33 ab	151.67 c	32.67 c
Acc 1495	3.318 a	0.306 a	2.52 b	2.17 c	0.367 de	142.33 bc	11.67 a	257.00 c	28.33 de
Acc 1343	2.492 d	0.156 d	1.82 de	4.09 a	0.423 d	108.67 bc	5.67 f	292.67 c	24.33 e
Acc 4132	3.108 b	0.211 bc	2.39 bc	3.97 a	0.527 c	82.00 c	10.67 abc	153.00 c	26.33 de
Acc 5717	2.268 ef	0.143 de	2.03 cd	4.02 a	0.750 a	160.00 bc	6.67 ef	278.33 c	26.67 de
Acc 4064	2.464 de	0.190 c	1.88 de	3.96 a	0.547 bc	413.67 a	10.00 bc	529.00 b	84.00 a

Table 17. Mineral composition of selected black pepper genotypes under 28 days after drought stress

Genotype	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Fe (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)
Acc 7211	2.730 c	0.194 c	3.15 a	2.58 bc	0.296 e	179.40 bc	11.33 ab	461.00 b	42.33 b
Acc 1495	3.155 ab	0.136 de	2.64 b	4.04 a	0.598 bc	162.10 bc	8.17 de	835.00 a	24.33 e
Acc 1343	2.590 cd	0.233 b	2.43 bc	2.39 c	0.308 e	110.33 bc	10.00 bc	535.67 b	28.67 cd
Acc 4132	3.052 b	0.216 bc	2.71 b	4.22 a	0.613 b	107.01 bc	9.33 cd	525.67 b	24.33 e
Acc 5717	2.520 d	0.123 e	1.53 e	4.13 a	0.616 b	125.53 bc	8.33 d	504.33 b	25.67 de
Acc 4064	2.240 f	0.197 c	2.49 b	4.03 a	0.398 d	216.80 b	9.67 cd	836.33 a	46.00 b

4.4.2.10. Abscisic acid (ABA)

LC-MS was employed to quantify the phytohormone ABA, a stress hormone in plants, based on its mass-to-charge transition and MRM in leaves, stems, and roots of the accessions 7211, 1495, 1343, 4132, 5717, and 4064.

Under well-watered conditions, ABA concentration in leaves was low in all the genotypes, ranging from 6.52 ppb (4132) to 16.79 ppb (7211). Significant difference in ABA content was observed among the genotypes. As stress levels increased from the control condition to 14 DAS and further to 28 DAS, ABA content also increased in all the genotypes, with the highest value being 418.78 ppb (1343), followed by 301.42 ppb (7211), 104.17 ppb (1495), 50 ppb (4132), 21.87 ppb (5717), and 14.89 ppb (4064). Compared to the control, the amount of ABA increased very significantly under severe stress (28 DAS), and the tolerant genotypes 1343 (3861.78%), 7211 (1694.78%), 4132 (681.65%), and 1495 (631.37%) recorded huge increase. Relatively lower increase was recorded in susceptible genotypes 5717 (126.14%) and 4064 (96.37%).

The ABA content in the stems was in the range of 8.60 ppb (1495) to 29.79 ppb (4132), under control. At 28 DAS, except for Acc 5717 and Acc 1343, all other genotypes showed significant increase compared to their controls. The highest proportion of increase was shown by Acc 1495 (469.29%), followed by Acc 4132, Acc 7211, and Acc 4064 with 64.21%, 43.11%, and 33.02% increase respectively. Meanwhile, Acc 1343 maintained almost the same ABA content both under control and stress, with a slight increase of 2.63% under severe stress while in Acc 5717there was a 10% decrease compared to the control.

The genotype 5717 which is drought susceptible did not show increased synthesis of ABA compared to the drought tolerant genotypes which showed an increase in ABA content under stress. This suggests that an insufficient ABA-regulated response may lead to inability to conserve water, a reduction in growth rate, and drought-induced damage in plants as reported by Harb *et al*., (2010). Contrary to this finding, Acc 4064

did not show decrease in ABA content, despite being susceptible to water stress compared to the remaining genotypes with tolerant characteristics. This observation is supported by the finding that the endogenous hormone ABA present in cowpea plants was higher in the sensitive line, similar to the drought-tolerant ones, but both of which were lower than in their well-watered condition (Lei *et al*., 2016).

A very significant increase in ABA content in roots was recorded among the treatments within the same genotypes, and also among the genotypes both under control as well as under stress (<0.001) compared to leaves and stem. The control plants showed lower ABA content than the plants that experienced drought stress at 28 DAS. ABA content ranged between 12.98 ppb (Acc 4064) to 167.94 ppb (Acc 1343) among control plants. Severe stress of 28 days led to a significant rise in ABA levels, with values of 210.94, 81.14, 71.64, 62.94, 61.42, and 61.29 ppb recorded in the genotypes 1343, 7211, 4132, 4064, 1495, and 5717 respectively which amounts to 25.61%, 61.25%, 221.77%, 384.93%, 110.35%, and 55.25% increase over their respective controls. The highest increases were found for the genotypes 4132, 4064, 1495 and 7211, while the least was shown by the genotypes 1343 and 5717. A consistent accumulation of ABA was identified in genotypes 4132 and 1495 across leaf, stem, and root tissues, suggesting that they possess superior drought tolerance compared to others.

Drought stress strengthened the quantity and activity of ABA content in pepper plants, higher in leaves than in roots, which alleviates drought stress (Padilla *et al*., 2023). This corroborated the findings of the present study, as higher ABA content with progressive stress levels was observed in the leaves especially in tolerant genotypes (7211, 1495 and 1343). But contrary to the earlier finding, roots showed the highest ABA content followed by leaves and least in stem in other genotypes.

Researchers investigated the effects of altering the expression of the CaAIR1 gene, responsible for hypersensitivity to drought, which may regulate the ABA response in hot pepper (*Capsicum annuum*) (Park *et al*., 2014). It was reported that ABA treatment

influenced the pepper plant to tolerate and survive drought stress by potentially reducing water loss through transpiration, as observed during the water withholding treatment used to impose drought stress (Muhammad *et al*., 2022; Daszkowska-Golec & Szarejko, 2013). Similar to the findings of the current study, HPLC-based detection in *Oryza sativa* showed enhancement of ABA with the progression of 7, 14, and 28 days of stress, with increases of 74.6%, 82.8%, and 99.4%, respectively, along with the synergistic action of salicylic acid, which will together improve drought tolerance and crop productivity (Verma *et al*., 2022).

Table 18. ABA content in leaf, stem and root

	Leaf ABA content (ppb)			Stem ABA content (ppb)		Root ABA content (ppb)	
Genotype	Control	14 DAS	28 DAS	Control	28 DAS	Control	28 DAS
Acc 7211	16.79 g	24.23 f	301.42 b	26.97 c	38.60 b	50.32 f	81.14 c
Acc 1495	14.24 ghi	16.48 g	104.17 c	8.60 f	48.97 a	29.20 h	61.42 e
Acc 1343	10.57 hij	15.33 gh	418.78 a	18.60 d	18.12 d	167.94 b	210.94 a
Acc 4132	6.52 j	29.74 e	50.93 d	29.79 c	48.91 a	22.26 i	71.64 d
Acc 5717	9.67 ij	14.68 gh	21.87 f	17.29 d	15.71 de	39.48 g	61.29 e
Acc 4064	7.58 j	15.87 g	14.89 gh	12.98 e	17.27 d	12.98 j	62.94 e

4.4.3. Ranking of genotypes

The genotypes were scored based on their desirable characteristics for drought tolerance in terms of physiological (relative water content, electrolyte leakage and stomatal aperture measurements) and biochemical (proline, phenol, H_2O_2 content, MDA content, sugar, starch protein, peroxidase, catalase, SOD, polyphenol oxidase, glutathione S transferase, and enzymes in ascorbate-glutathione cycle) parameters mentioned above as depicted in table 7 and table 10 respectively. The sum of weighed scores for each accession was ranked, and the accessions with higher rank have better drought tolerance ability.

Based on scoring of physiological and biochemical parameters, accessions 1343, 4132, 1495 followed by 7211 (≥48.25) exhibited higher score, indicating favorable traits compared to the remaining genotypes.

Table 19. Ranking of selected black pepper genotypes based on physiological biochemical parameters related to drought tolerance

Parameters ↓	1343	1495	4132	7211	5717	4064
RWC	3	4.5	4.5	3	1.5	1.5
EL	4.5	1.5	3	4.5	3	1.5
Stomatal closure	2	2	3	2	1	1
Proline	1.5	1.5	1.5	0.5	0.5	1
Phenol	0.5	0.25	0.75	0.25	0.25	0.25
H2O2 content	0.75	0.5	0.5	0.75	0.25	0.5
MDA	0.75	0.5	0.25	0.75	0.25	0.25
Sugar	3.75	2.5	2.5	2.5	1.25	1.25
Starch	0.5	1.5	1	1	1	0.5
enzyme	25.5	22.5	21	22.5	12	10.5
ABA content	9	12	10.5	10.5	6	7.5
Total score	51.75	49.25	48.5	48.25	27	25.75
Rank	1	2	3	4	5	6

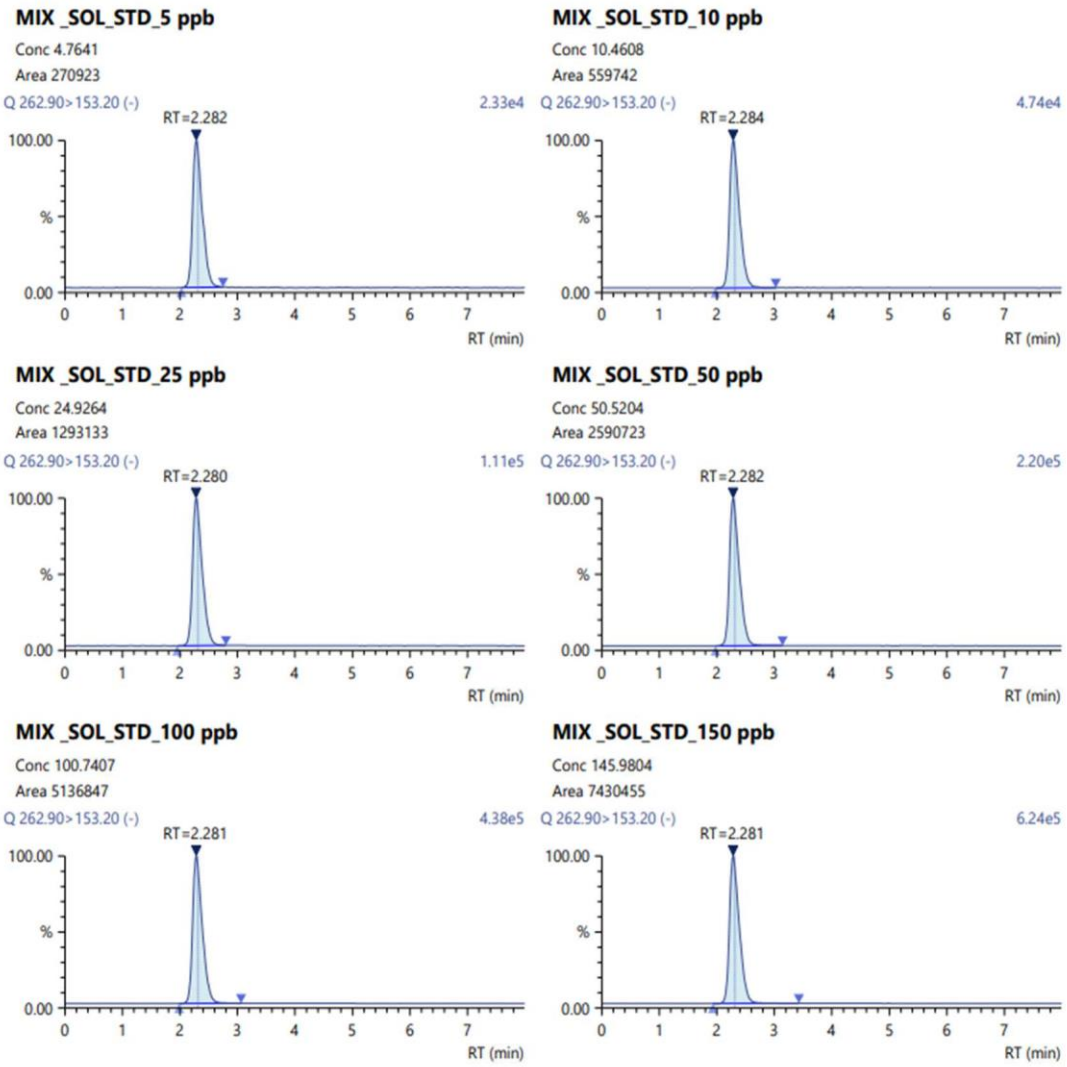


Figure 31. LC-MS calibration curve for ABA.

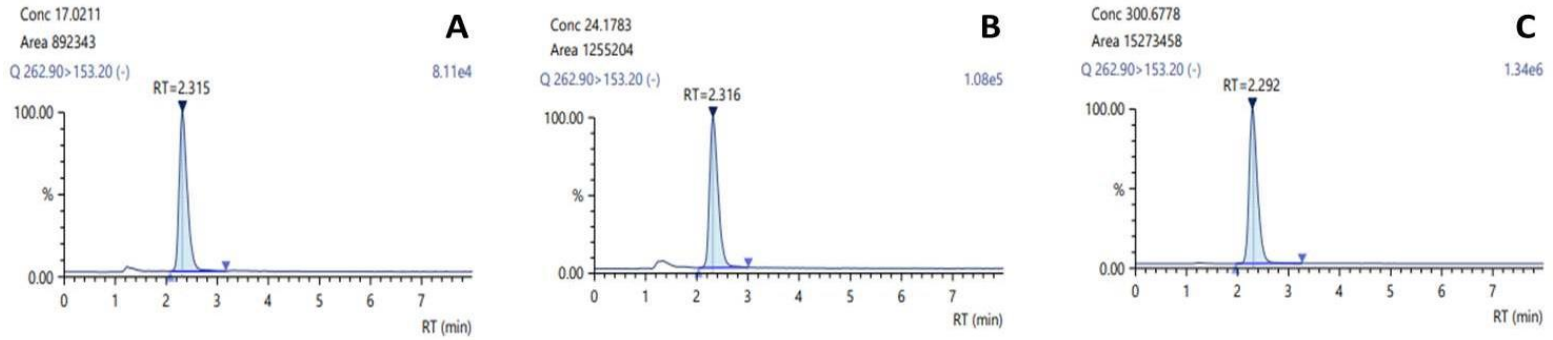


Figure 32. LC-MS chromatogram of leaf ABA at control, 14 DAS and 28 DAS (A-C respectively) for the genotype 7211

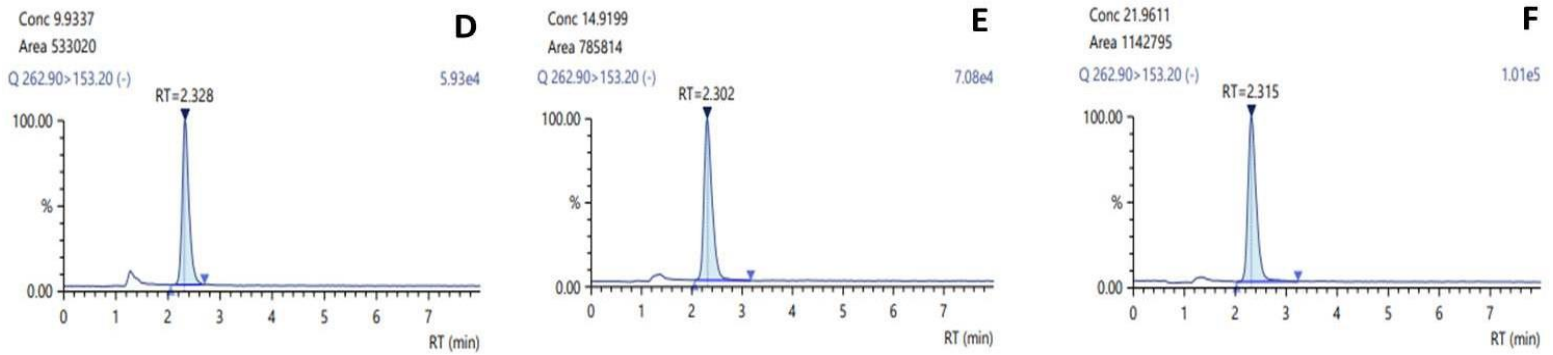


Figure 33. LC-MS chromatogram of leaf ABA at control, 14 DAS, and 28 DAS (D-F respectively) for the genotype 5717.

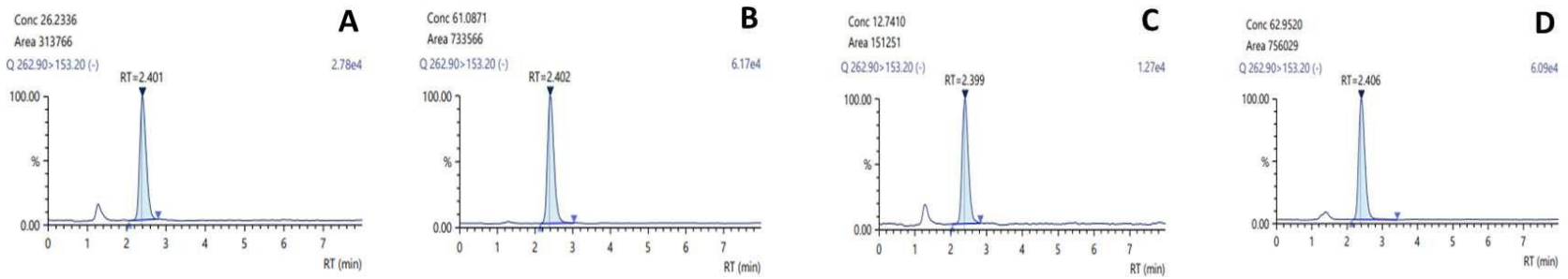


Figure 34. LC-MS chromatogram of root ABA for control (A and C) and 28 DAS (B and D) for genotypes 1495 (A and B) and 4064 (C and D) respectively.

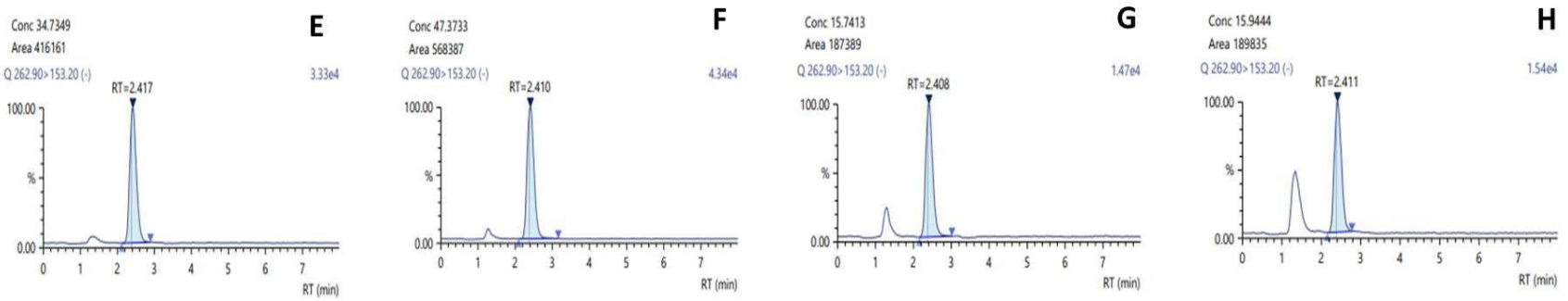


Figure 35. LC-MS chromatogram of stem ABA for control (E and G) and 28 DAS (F and H) for genotypes 4132 (E and F) and 5717 (G and H) respectively

Experiment 3

4.5. Analysis of yield and quality parameters

After maturity, the spikes were harvested from the selected six black pepper genotypes which were used in the earlier experiment and their yield and yield attributing traits were recorded in both control and drought stress-induced (28 DAS) plants of each accession. Quality analysis such as estimation of sugars, starch, oleoresin, piperine, and essential oil components was under taken.

4.5.1. Yield and yield attributing traits

4.5.1.1. Yield (kg)

The deficit moisture condition greatly affected the yield compared to their respective controls. The fresh weight of berries under control varied from 0.301 kg (Acc 7211) to 1 kg (Acc 1495). Though there was yield reduction under stress, percentage reduction was very less compared to control in tolerant genotypes, 2.17% (1343), 6.19% (1495), 6.66% (4064) and 6.89% (4132). In contrast, greater yield reduction was noticed in susceptible ones (16.85% in 5717 and 14.28% in 7211).

4.5.1.2. Spike length (cm)

The maximum spike length recorded among both control and water-stressed treatments was 12.78 cm and 11.78 cm, respectively, both in the Acc 4064. Whereas the minimum value recorded from both control and water-stressed treatments was 7.18 cm and 6.10 cm, respectively, both in the Acc 5717. Reduction in spike length was observed in all genotypes subjected to drought stress (2.48%, 8.49%, 8.64%, 11.71%, 12.18%, and 17.62% for genotypes 4132, 1495, 7211, 1343, 4064, and 5717, respectively). The length of the spike is a key feature that contributes to pepper yield. Minimal spike length reduction under water stress is a favorable trait for tolerance, which was found in genotype 4132, followed by 1495 and 7211.

4.5.1.3. Number of berries per spike

The number of berries per spike ranged from 43 (Acc 1495) to 78 (Acc 7211) among control plants. In water-stressed plants, the maximum was shown by the same

genotype 7211 (69), and the least by genotypes 1495 and 5717, with an average value of 37 berries each. Withholding water for 28 days resulted in lower number of berries compared to their respective control plants. Among water-stressed plants, genotypes 1343 (10.1%), followed by 7211 (13%), 4132 (15.8%), 1495 (16.2%), and 5717 (18.9%) showed lesser decrease compared to the genotype 4064 which showed 28% decrease.

4.5.1.4. Berry size (mm)

Berry size was less in treated plants compared to their respective control in all the genotypes. A greater reduction was noticed in Acc 7211, with a decrease of 22.9%, followed by acc 5717 and 4064 with reductions of 19% and 17%, respectively, relative to their control plants. However, the least percent reduction was observed in Acc 1495, followed by 4132 and 1343, (9.04%, 11.3%, and 11.8%, respectively). Better berry size under stress was noticed in the genotypes 1495, 4132, and 1343 compared to other genotypes.

4.5.1.5. Peduncle length (cm)

The peduncle length varied from 1.23 mm (Acc 4064) to 1.6 mm (Acc 4132) in control and it decreased in general under stress. Comparatively higher per cent decrease was shown by 1343 (8.93%), followed by 4064 and 5717 (7.14% and 7.7%, respectively).

4.5.1.6. Hundred berry fresh weight (g)

The maximum fresh weight recorded for 100 berries was 17.9 g in Acc 4132, and the minimum of 9.6 g in Acc 4064 under control. The highest percent decrease was noticed in Acc 1343 (23.91%), followed by Acc 4064 and 5717 (23.24% and 21.89%, respectively). The least decrease (7%) was noted in Acc 4132.

4.5.1.7. Ten spiked berry weight (g)

The genotypes under control recorded higher 10 spiked berries weights, ranging between 30.32 g (5717) and 91.58 g (4132) than their respective water-stressed plants. The genotype 4064 showed the highest variation (23.8%). The genotypes 4132 and

1343, with 5.15% and 7%, respectively showed the least decrease in ten spiked berry weight under stress.

4.5.1.8. Ten rachis weight

In control treatment, 10 rachises weight ranged between 3.58 g and 7.34 g, displayed by the genotypes 5717 and 4132, respectively. Compared to the control, genotype 1495 recorded 24.23% reduction, which is the maximum reduction among the genotypes. This was followed by the genotypes 5717, 4064, and 7211, with decrease of 20.5%, 15% and 12.2%, respectively. Comparatively favorable response were observed in the genotypes 4132 and 1343, with reductions of only 8.94% and 9.82%, respectively.

Genotypes 1343 and 4132 exhibited superior drought tolerance traits across multiple yield attributing parameters, followed by others. They were able to maintain or minimize reductions in these yield-related parameters under drought stress, indicating their adaptive mechanisms to cope with water scarcity.

An earlier morphogenetic variability study by Gedebo *et al*. (2017) on black pepper genotypes indicated that the genotypic and phenotypic variances among the yield-attributing traits, such as leaf length, leaf width, fruiting spike length, and hundred fruit weight, showed positive correlations with fresh and dry yield, suggesting that these traits are reliable selection criteria for improving yield. However, peduncle length and number of berries per spike did not show a direct correlation with yield. A study on the Panniyur 1 variety found a high positive correlation between spike length and yield, as well as between berries per spike and spike length, indicating that these traits positively influence overall yield. Additionally, the positive association between green spike yield and quality parameters like piperine and oleoresin content suggests potential for simultaneous improvement in both yield and quality (Pradeepkumar *et al*., 2003). An inter-correlation was found between black pepper yield and yield-contributing traits, specifically the number of spikes per lateral and spike length, and path analysis revealed that the green black pepper yield per vine directly and positively affects the dry yield per vine (Thanuja, 2003).

The variations in yield-attributing traits such as spike yield, spike number, and spike length influenced the yield of black pepper (Hussain., 2017). As per Prakash (2019), black pepper vines with a dry weight greater than 500 g per vine demonstrated superior performance in yield-contributing traits. The selection of a high-yielding variety based on the number of berries per spike, 100-berry volume, and 100-berry weight led to the identification of Karimunda, which was propagated in higher altitudinal areas of Idukki (Preethy *et al*., 2018). Altitudinal variation led to changes in such traits, resulting in spike shedding at lower altitudes while stem wilting was visible at higher altitudes (Shango *et al*., 2020). The black pepper cultivars significantly exhibited diversity in all morphological as well as floral traits, such as spike length, peduncle length, spiking intensity, number of spikes per lateral branch, number of spikes per vine, number of berries in 10 spikes, fresh weight of 100 berries, and volume of 100 berries. This diversity arises due to their adaptability to various environmental conditions and altitudinal variation (Reshma *et al.*, 2022).

Under prolonged drought stress, heavy rainfall as well as irregular rain precipitation condition led to failure of berry set and severe berry shedding (Krishnamurthy *et al*. 2016). In wheat genotypes, grain weight per spike, grain yield per plot, grain number per spike, grain number per spikelet, and 1000-grain weight were the yield-related traits limited under drought stress conditions, thereby resulting in yield loss (Xu *et al*., 2023). Significant genetic variability was discovered among oat genotypes for morphological, physiological, and yield-contributing traits. The heritability and genetic advance of observed traits, such as chlorophyll content, panicle length, and spikelets per panicle, suggest predominant additive gene effects, making these traits reliable criteria for selection and yield improvement (Dubey *et al*., 2014).

The present study underscores the significant impact of morphogenetic variability and environmental conditions on yield and yield-attributing traits in black pepper, particularly highlighting the role of traits such as spike length, number of berries per spike, and fruit weight, which show a strong positive correlation with yield. The findings emphasize the adaptability of black pepper genotypes to diverse environmental conditions, including altitudinal variation, and their potential for sustainable productivity. While previous studies have noted the importance of these traits in crop improvement, the current study provides specific insights into black

pepper, reinforcing the value of targeted breeding strategies to enhance yield and quality under challenging environmental conditions.

4.5.2 Quality parameters

4.5.2.1 Oleoresin (%)

The oleoresin content of selected genotypes decreased under stress compared to the control for all genotypes. The minimum and maximum oleoresin content observed in control plants was between 8.52% and 12.45%, respectively. The highest percentage decrease was observed in genotype 5717 (7.29%), followed by 7211 (4.45%). Genotypes 4064, 1495, and 1343 showed a lower reduction percentage, indicating a favorable trait.

The non-volatile component of black pepper, oleoresin, potentially alters the sensory qualities of black pepper due to drought stress, influencing the composition and concentration of volatile compounds (Gafar, 2022). Similarly, fungal infection on berries during storage leads to a weakening of berry quality and loss of aroma (Gafar, 2022). Oleoresin extracted through the maceration method had the beneficial characteristic of having the highest phenolic as well as antioxidant activity compared to those extracted through ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and ultrasound-MAE (UMAE) (Zhang *et al*., 2022). Oleoresin extracted through supercritical carbon dioxide and oleoresin prepared by a conventional method appear to have greater beneficial antioxidant properties than essential oils alone (Tipsrisukond *et al*., 1998). In the advanced study, non-encapsulated oleoresin with hydroxypropyl beta-cyclodextrin (HPBCD) exhibited effective antioxidant activity and antibacterial effects against *Escherichia coli* K12 and *Salmonella enterica* serovar Typhimurium LT2, which could have important applications in the food industry *(*Teixeira *et al*., 2013).

4.5.2.2 Essential oil (%)

The essential oil content of control plants ranged from 3.04% (Acc 7211) to 6.08% (Acc 4132). Drought-stressed plants exhibited reduced oil content across all

genotypes compared to control plants. The genotype 4064 showed the most unfavorable trait with a higher reduction of 20%, followed by genotypes 1495 (13%) and 5717 (11.46%). In contrast, genotypes 1343 (8%), 7211 (5.38%), and 4132 (2.63%) exhibited a favorable trait with lower reductions in oil content.

Imposed drought stress on three *Ocimum* species revealed significant variations in oil content in *O. basilicum* and *O. americanum*, but not in O. *x africanum*. The oil composition was not affected by drought stress, except for the chemical constituent camphor present in *O. x africanum* oil, which reduced with increased water stress. Mulugeta & Radácsi (2022) reported that drought alters EO content differently among species.

The similar study related to drought stress reported species-specific variations in six *Lamiaceae* species, revealing that drought stress decreased the essential oil content by 36% (expressed in mL kg−1 DW) in *S. sclarea* plants, yet did not affect the other species studied (García-Caparrós *et al.*, 2019).

Species specific variation, variation among varieties, geographical locations, and different methods of extraction procedures, environmental conditions, and different developmental stages of same variety influenced the EO content of black pepper which is helpful for the selection of high yielding varieties (Preethy *et al*., 2018; Kumar *et al*., 2021; Shango *et al*., 2021)

4.5.2.2.1 GCMS characterization of volatile compounds

The major compounds identified during the GCMS analysis of selected black pepper genotypes under both well-watered and drought-stressed conditions included alpha-Thujene, alpha-Pinene, Camphene, Sabinene, beta-Pinene, Myrcene, Linalool, D-Limonene, Caryophyllene, and alpha-Phellandrene.

The 28 days drought stress altered the essential oil composition of genotype 7211, specifically affecting some of these compounds. The compound Camphene was not detected under water-stressed conditions and a decrease of 24% was observed in alpha-Phellandrene. Alpha-Pinene, Sabinene, and Beta-Pinene showed notable

proportional increases (of around 10%), indicating adaptive changes in response to drought stress.

The compounds Linalool and D-Limonene exhibited the highest proportional decreases in genotype 1495, with 111.11% and 84.62%, respectively, suggesting a significant response to water scarcity. Other compounds displayed a slight increase or decrease in response to drought stress.

The oil composition of genotype 1343 revealed decrements (>10%) in alpha-Phellandrene, Caryophyllene, and Camphene, (11%, 24%, and 33.3% respectively) under water stress while other compounds demonstrated comparatively lower variations as increments. Increases of less than 10% were observed in beta-Pinene (9.21%), alpha-Thujene (8.67%), Myrcene (8.98%), D-Limonene (7.79%), and alpha-Pinene (6.46%).

The oil profile of genotype 4132 under stress differed from the control plants, showing decreases in D-Limonene, alpha-Thujene, and caryophyllene by 16.88%, 10.40%, and 8.63%, respectively. Relatively smaller variations in increments, considered favorable, were observed in Camphene (3.45%), Sabinene (4.88%), beta-Pinene (3.01%), and Linalool (2.56%). Negligible variation was detected only in Myrcene (0.7%).

The oil profile of genotype 5717 under stress demonstrated increased concentration in only one constituent, alpha-pinene, with a proportional variation of 5.23%. The constituent D-Limonene was not detected in both well-watered and drought-stressed conditions. Similarly, Camphene was detected in the control in trace amount (0.06%) but was not detected in the drought-stressed plant. The highest reductions were recorded in Linalool (151.95%), followed by alpha-Thujene (21.82%), beta-Pinene (15.20%), Myrcene (14.93%), and Caryophyllene (9.97%).

It was noted that the same constituent, camphene, was detected in trace amount (0.07%) in the genotype 4064 under control, but was not detected under stress, similar to the genotype 5717. Likewise, Linalool was also not present in the water-stressed plant, whereas it was identified at 0.41% in the control. Compared to the control, the highest decrease was reported in D-limonene (52.27%), Caryophyllene (45.73%), and

Myrcene (14.10%). The increase in the components of alpha-Thujene, alpha-Pinene, and beta-Pinene was proportionately less than 10% (9.23%, 8.93%, and 8.21%, respectively).

Genotype 7211 appears to be the most desirable, followed by 4132 and 1343. Comparatively, these genotypes maintain volatile oil compound levels that illustrate their adaptive changes in response to water scarcity, making them more favourable. The remaining genotypes 5717, 1495 and 4064 exhibited potentially non-desirable traits in relation to drought tolerance compared to other genotypes.

Zachariah & Parthasarathy (2008) reported that the essential oil constituents vary due to the variability in black pepper cultivars, agroclimatic variations and locations, maturity of berries, oil extraction methods, and other factors.

The chemical composition of the essential oil from the same variety from two regions greatly varied. The Bangladeshi pepper oil constituted 18 chemical constituents, consisting of 65.42% total monoterpenes and 34.58% sesquiterpenes. Meanwhile, Indian black pepper had 77.67% total monoterpenes and 14.20% sesquiterpenes (Rahman *et al.*, 2017). Geographical condition-dependent variations in essential oil (EO) were also reported in the eastern coastal region of Madagascar and the Amazon region of Brazil, showing qualitative similarities but having quantitative differences among the same black pepper varieties (Dosoky *et al*., 2019).

According to the optimized various distillation methods, the oil content and constitution significantly varied, as reported by Dinh *et al*. (2020), from picked black pepper berries from Vietnam. The highest yield was obtained from hydrodistillation, steam distillation, and microwave-assisted hydrodistillation, at 2.19%, 1.57%, and 1.4% respectively. The major constituents identified from these distillation methods were α-Pinene, β-pinene, δ-3-Carene, Limonene, β-caryophyllene, β-Myrcene, α-Phellandrene, and Caryophyllene oxide.

In addition to the distillation method, the developed efficient and precise extraction method, known as MD–HS-SPME, under optimal conditions, confirmed its relative standard deviation (RSD) value to be less than 10% from six replicates of *Piper nigrum* (Liu *et al*., 2007). Improved essential oil with antioxidant and antibacterial

activity was achieved through the nano emulsification process by encapsulation (Nie *et al*., 2023).

An evaluation of the effect of seasonal variation on the essential oil (EO) of four promising black pepper cultivars (Kottanadan, Ottaplackal, Kuthiravally, and Cheriakaniakadan) revealed significant variations in their quantities. Variations were observed in the identified major compounds over three consecutive years in Kottanadan oil, which included sabinene (11.2-22.6%), limonene (12.7-23.8%), β-caryophyllene (8.9-24.1%), and β-pinene (7.5-15.4%). Ottaplackal oil contained β-caryophyllene (15.5-21.7%), myrcene (0-18.6%), β-pinene (3.8-11.7%), sabinene (0.1-26.8%), and limonene (15.5-21.7%). Kuthiravally oil contained limonene (9.0-16.9%), β-pinene (3.8-10.9%), and β-caryophyllene (29.0-46.0%), while Cheriakaniakadan comprised β-pinene (7.7-11.2%), limonene (14.7-17.8%), sabinene (9.7-22.3%), and β-caryophyllene (17.4-23.1%) (Menon *et al*., 2002).

4.5.2.3 Piperine (%)

The piperine content under control treatment was highest in genotype 4132 (4.38%), followed by 1343 (3.99%), 7211 (3.98%), 1495 (3.65%), 4064 (3.34%), and 5717 (2.93%). However, the piperine levels decreased after 28 DAS for all genotypes. The highest reduction was observed in genotype 4064 with a decrease of 16.40%. Genotypes 1495 (10.06%) and 5717 (10.03%) exhibited the second highest reduction, followed by 1343 (8.13%). The lowest reduction in piperine was recorded in the genotypes 4132 (4.78%) and 1495 (4.59%).

Comparatively higher piperine content was demonstrated in the genotypes 1343 and 4132, indicating that these genotypes maintain piperine content under water stressed condition, slightly above control level which suggests that industries can utilize such genotypes even under water stress condition to obtain better piperine output.

Zachariah *et al*. (2010) reported that piperine content of black pepper cultivars was positively correlated to the oleoresin content present in it. Black pepper varieties from Kerala showed higher content than those from North Kanara (Pruthi, 1993), which was supported by Sruthi *et al*. (2016). Oleoresin showed more variability than piperine among 42 black pepper genotypes (Pradeepkumar *et al*., 2003). Varying location as

well as climatic condition led to the variability in turmeric yield and curcumin content (Srinivasan, 2009; Anandaraj *et al*., 2014)

Indian black pepper cultivars of Kottanadan, Kuthiravally, Kumbakodi, Nilgiri and Kuthiravally were rich in piperine and oleoresin, whereas high essential oil yielding varieties were Balanakotta, Kumbakodi and Kaniyakadan (Krishnamoorthy & Parthasarathy, 2010).

The South Indian promising high-yielding, drought-tolerant black pepper variety, Panniyur 9, exhibits high-yielding attributes with piperine (6.11%), oleoresin (12.71%), and essential oil (5%) (Kumar & Soni, 2015).

4.5.2.4 Berry starch (%)

The range of berry starch among the control plants varied between 2.93% and 4.38%, with genotypes 5717 and 4132 showing these respective values. Under drought stress, genotype 7211 maintained the highest content with the lowest reduction at 4.59%, followed by genotype 4132 at 4.78%, 1343 (8.13%), 5717 (10.03%), and 1495 (10.06%). Genotype 4064 showed highest reduction (16.40%).

Starch, a complex carbohydrate, serves as the primary storage form of energy in plants and plays a vital role in the growth, development, and metabolic processes of entire plant species. The chloroplast apparatus, where starch is synthesized through photosynthesis (the process by which plants convert light energy into chemical energy), produces sugars that are subsequently stored as starch. Under unfavorable environmental stress conditions, including drought stress in plants, starch develops strategies to enhance drought tolerance (Stagnari *et al*., 2016).

An evaluation of the correlation between starch and sugar content in water-stressed leaves of four different plant species subjected to drought stress revealed a negative relationship. Higher sugar content was maintained in contrast to starch content, possibly due to lower photosynthesis (Quick *et al*., 1992).

4.5.2.5 Berry sugar (%)

The genotypes under control exhibited higher sugar content ranging from 16.07% (4064) to 44.66% (1343). However, under drought stress conditions, the berry sugar content decreased in all genotypes. The genotype 7211 showed a relatively lower reduction (9.94%) in sugar content followed by 4132 (17.41%). Significantly higher reductions were noticed in genotypes 4064 (27.33%), 1343 (30.67%), 1495 (30.99%), and 5717 (35.13%).

The induction of water deficiency in red wine grapes primarily results in reduced berry weight due to water loss, which elevates the levels of quality-related moieties, including sugars (Gambetta *et al.*, 2020). A shift in water transport from xylem to phloem is accompanied by significant sugar deposition in the berry (Greer & Rogiers, 2009).

Water-stressed black pepper plants accumulated more sugar than well-irrigated plants (George, 2016). The organic osmolyte trehalose in pepper plants, accumulated during drought, protects the structural and functional integrity of heterogeneous molecules, shielding them from denaturation (Bhat *et al*., 2017).

In a similar treatment with prolonged duration, it has been suggested that the higher yield of the maize variety Dong Dan 8 is attributed to increased sugar production, which serves as a signal for metabolic regulation, along with higher photosynthetic activity (Anjum *et al*., 2016).

The results on quality parameters revealed that in terms of oil and oil constituents, the genotypes 4132, 1343, and 7211 seems to be better while the genotype 4132 maintained oleoresin under drought. Piperine, the main constituent which gives characteristic taste to black pepper showed reduction under water stress and the genotypes 4132 and 1495 showed lesser reduction. Considering both the yield and quality parameters under water stress, 4132 and 1343 maintained sustainable yield and quality and these genotypes can be further promoted for evaluation under rainfed conditions followed by 7211 and 1495.

Table 20. Yield and quality parameters of selected black pepper genotypes under control conditions.

Parameters ↓	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
yield (kg)	0.301 f	1.040 a	0.989 b	0.770 c	0.171 g	0.539 e
Spike length (cm)	10.31 de	12.775 a	11.45 bc	10.65 cd	7.18 h	8.75 fg
No. of berries per spike	78.75 a	43.25 efg	65.25 bc	44.00 e	44.50 e	54.50 d
Berry size (mm)	6.43 c	5.97 cd	5.75 de	7.89 a	7.50 ab	5.63 def
Peduncle length (cm)	1.60 a	1.23 cd	1.53 ab	1.40 abcd	1.40 abcd	1.50 abc
10 siked berries weight (g)	69.61 b	50.44 d	63.58 c	91.58 a	30.32 g	44.94 e
10 rachis weight (g)	5.15 c	6.23 b	5.42 c	7.34 a	3.58 e	4.58 d
100 berry weight (g)	11.60 c	10.25 d	11.55 c	17.90 a	11.10 c	9.59 ef
Oleoresin (%)	12.45 a	7.11 j	10.84 d	12.41 a	9.57 f	8.52 h
Essential oil (%)	3.04 fg	3.27 de	4.61 b	6.08 a	3.13 ef	4.12 c
Piperine (%)	3.98 c	3.65 e	3.99 c	4.38 a	2.93 g	3.34 f
Total sugar (%)	1.22 g	2.80 b	3.42 a	2.88 b	2.42 d	3.39 a
Starch (%)	31.09 c	28.40 d	44.66 a	27.92 d	23.97 ef	16.07 h

Table 21. Yield and quality parameters of selected black pepper genotypes at 28 DAS.

Parameters ↓	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
yield (kg)	0.264 f	0.979 b	0.968 b	0.720 d	0.146 g	0.505 e
Spike length (cm)	9.49 ef	11.78 b	10.25 de	10.39 de	6.10 i	7.80 gh
No. of berries per spike	69.5 b	37.00 g	59.75 cd	38.50 efg	37.25 fg	43.75 ef
Berry size (mm)	5.23 efg	5.48 def	5.14 fg	7.08 b	6.30 c	4.80 g
Peduncle length (cm)	1.50 abc	1.175 d	1.40 abcd	1.38 abcd	1.30 bcd	1.40 abcd
10 siked berries weight (g)	59.18 c	43.80 e	59.38 c	87.09 a	26.03 g	36.30 f
10 rachis weight (g)	4.59 d	5.02 cd	4.94 cd	6.74 b	2.97 f	3.98 e
100 berry weight (g)	9.84 de	8.69 g	9.32 efg	16.73 b	9.10 fg	7.78 h
Oleoresin (%)	11.92 c	6.87 k	10.52 e	12.05 b	8.93 g	8.21 i
Essential oil (%)	2.89 gh	2.89 gh	4.27 c	5.92 a	2.81 h	3.43 d
Piperine (%)	3.80 d	3.31 f	3.69 de	4.18 b	2.66 h	2.87 g
Total sugar (%)	1.11 g	2.14 e	2.62 c	2.45 d	1.79 f	2.51 cd
Starch (%)	24.93 e	20.71 g	38.64 b	22.74 f	15.83 h	12.20 i

Experiment 4

4.6 Analysis of shoot and root characteristics and dry matter partitioning

The black pepper genotypes after 28 days of moisture stress were used for destructive sampling and were partitioned in to roots and shoots to determine both shoot (primary stem diameter, secondary stem diameter, shoot fresh weight, and shoot dry weight) and root characteristics (root length, primary root diameter, number of primary roots, number of secondary roots, root fresh weight, and root dry weight). Furthermore, the dry weight of both shoots and roots was used to calculate the root-to-shoot ratio. Lesser reduction in root and shoot parameters and higher root to shoot ratio under drought are the desirable characteristics for drought tolerance.

4.6.2 Shoot characters

4.6.2.1 Primary stem diameter (cm)

Under well watered condition, all the genotypes maintained a significantly higher primary stem diameter ($P<0.001$) ranging from 9.60 mm to 22.97 mm compared to their respective water-stressed plants. The highest value was exhibited by Acc 4064 and the lowest by Acc 7211. Simultaneously, a higher reduction in diameter (55.86%), was observed in Acc 4064, followed by 5717, 7211, 1343, 1495, and 4132, with reductions of 24.4%, 17.43%, 12.15%, 11.05%, and 10.19%, respectively. The higher reduction in stem diameter indicates susceptibility due to lower water content as well as low resource allocation to minimize metabolic energy expenditure.

4.6.2.2 Secondary stem diameter (cm)

Under well water condition, similar to the primary stem diameter, the highest secondary stem diameter was noticed in Acc 4064 (11.35 mm), while the lowest in 1343 (6.30 mm) and showed a reduction at 28 DAS in general. A contrasting trend was identified in 5717 at 28 DAS, which retained its control level. The decrease was 17.75%, 13.82%, 7.2%, 6.51%, and 3.03% for the accessions 7211, 4064, 4132, 1495, and 1343, respectively.

4.6.2.3 Shoot weight (g)

Genotypes showed significant variation in shoot fresh weight among them under both control and water-stressed condition ($P < 0.001$). Shoot fresh weight ranged between 723.33 g (Acc 7211) and 1613.27 g (Acc 5717) under well-watered condition. A greater reduction in shoot fresh weight was observed in the genotypes 4064 (46.2%), 7211 (43.6%), 4132 (33.9%), and 5717 (26.1%) compared to the accessions 1495 (6.77%) and 1343 (2.13% increase). This reduction might be due to reduced water absorption and increased transpiration, leading to retarded plant development. Shoot dry weight varied from 88.43 g to 458.57 g and showed a similar trend as that of shoot fresh weight.

4.6.3 Root characters

4.6.3.1 Root length (cm)

Under well-watered conditions, the measured root length ranged widely from 36.6 cm to 51.17 cm among the genotypes ($P<0.001$). The maximum root length was produced by the genotype 4064 and the minimum by 7211. Accessions 7211, 1495, 4132, 1343, 5717, and 4064 after 28 days of stress exhibited increased root growth (62.27%, 54.62%, 54.19%, 50.09%, 33.08%, and 17.26%, respectively) compared to their controls. The genotypes 7211, 1495, 4132, and 1343 with higher root growth exceeding 50%, in response to drought stress (28 DAS), which is due to exploration in to deeper soil layers to absorb water from deeper layers of soil for essential physiological processes. Whereas 5717 and 4064 though showed increased root length under stress, could not extend their roots in to deeper soil layer.

4.6.3.2 Primary root diameter (mm)

The primary root diameter varied significantly among the genotypes, ranging from 4.44 mm (Acc 7211) to 8.37 mm (Acc 5717) under stress. Genotype 1343 showed a very high increase in root diameter (94.98%), followed by accessions 4132 (32.34%), 4064 (30.15%), and 7211 (26.97%) at 28 DAS compared to control. A relatively lower reduction, which was less than 20%, was recorded in accessions 5717 (12.99%) and 1495 (18.83%).

4.6.3.3 Number of primary roots

The number of primary roots ranged from 7 (Acc 4064) to 14 (Acc 7211 under fully irrigated conditions. The genotypes 7211, 1343, 1495, and 4132 exhibited 71.43%, 58.33%, 53.85%, and 20% increase in primary roots respectively at 28 DAS. These genotypes with increased primary roots under stress may have the advantage to absorb more water. In contrast a 9% reduction over control was observed genotype 5717 which would have hampered its water extraction ability under stress, thus making it susceptible.

4.6.3.4 Number of secondary roots

The number of secondary roots differed significantly among the genotypes under control, varying between 29 (Acc 4064) and 64 (Acc 4132). Very high increase of 250% was observed genotype 1343, followed by 7211, 1495, 4064, 4132, and 5717, with corresponding increases of 98.21%, 85.19%, 55.17%, 50%, and 50% was noticed under stress. The response of genotypes was similar as that of primary roots and the order of genotypes was also same.

4.6.3.5 Root fresh weight (g)

A significant variation among the genotypes was noticed in root fresh weight under well-watered condition, ranging from 60 g to 190 g. Root weight was significantly reduced at 28 DAS when compared to the control, with reductions of 52.49%, 27.39%, 22.37%, and 20.10% for the genotypes 7211, 5717, 4064, and 4132 respectively. Conversely, minimal reductions of 3.44% and 18.56% were observed for the genotypes 1343 and 1495, respectivelywhich indicate their drought tolerance ability. The root weight of control plants ranged widely, from 12 g (genotype 1343) to 44 g (genotype 4132). Similar trend as that of root fresh weight was observed for root dry weight also.

4.6.2.6.1. Root-to-shoot ratio

Significant difference among the genotypes was noticed for root to shoot ratio under control conditions which, ranged from 8.31% (Acc 5717) to 21.77% (Acc 4132). In comparison to the control, the increase in root to shoot ratio in genotypes 4132 and

1495 was 28.98% and 25.26%, respectively under stress. The minimum increase was noticed in accessions 4064 (5.87%) and 7211 (4.64%). Thus, the more favourable trait for drought tolerance was elucidated by the accessions 4132 and 1495, followed by 1343 and 5717. These relatively elevated root-shoot ratios under stress indicate a greater allocation of resources to the root system in these genotypes compared to others, which is helpful in extracting more water from soil, thus help in tolerating the drought conditions.

The genotypes were evaluated based on their shoot and root characteristics. Genotype 1343 demonstrated the highest resilience to drought, followed by accessions 1495 and 4132. In comparison, genotypes 7211, 5717, and 4064 exhibited relatively lower resilience.

Contrary to the observed measurements of root length, root width, root fresh weight, and root dry weight which generally increased in black pepper in the current study, decreased values were observed in drought-imposed curly pepper (*Capsicum annuum*) compared to well-watered conditions (Ridho *et al*., 2022). Moreover shoot characteristics were tended to weaken under the same environmental extreme to make the plants sustain in that condition (Reinelt *et al*., 2023). Santos *et al*. (2020) reported that, cowpea genotypes exhibited higher root development and reduced shoot weight, resulting in a higher root-to-shoot ratio, indicating drought tolerance under water-scarce conditions as observed in the present study.

Geneticists and plant breeders are targeting yield enhancement under drought stress by understanding the underlying mechanism, which involves higher fine root growth and deep root systems, along with reduced xylem width and xylem pit anatomy in seminal roots for water conservation. These traits have been identified as significant factors linked to elevated drought tolerance (Comas *et al*., 2013).

Drought-induced root and shoot plasticity helped differentiate the plants according to their response to drought (Reinelt *et al*., 2023). The higher root-to-shoot ratio as observed in the current study served as an indicator to identify drought-resistant varieties Ethiopian red pepper cultivars, Markofana and Mitmita showed 27.91% and 50.92% increase in root to shoot ratiorespectively under drought. Hence, Mitmita was

considered more tolerant than Markofana as it showed a higher increase (Wassie *et al*., 2023). Several drought-imposed experiments reported a positive correlation between the root-to-shoot ratio and drought conditions (Kou *et al*., 2022; Kim *et al*., 2020; Nejad, 2011; Reinelt *et al*., 2023).

The evaluation of genotypes based on shoot and root morphological parameters in the current study demonstrated that genotype 1343, followed by 4132, then 1495 and 7211, exhibited more favorable tolerance traits to drought.

Table 22. Root and shoot characters of six selected black pepper accessions under control condition

Parameters ↓	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
Maximum root length (cm)	36.67 f	43.33 def	47.25 de	48.87 d	39.00 ef	51.17 cd
Primary root diameter (mm)	4.44 e	5.49 de	5.31 de	7.81 bc	8.37 abc	6.56 cd
Number of primary root	14 c	13 cd	12 cde	10 ef	12 cde	7 g
Number of secondary root	56 cd	54 cd	30 f	64 c	36 ef	29 f
Root fresh weight (g)	66.67 g	184.17 ab	60.00 g	190.00 a	173.23 b	142.57 de
Root dry weight (g)	12.05 e	44.12 bc	17.87 e	39.01 cd	38.09 cd	30.58 d
Shoot fresh weight (g)	723.33 de	1141.11 c	400.00 g	1063.33 c	1613.27 a	680.12 e
Shoot dry weight (g)	118.33 f	252.08 c	88.43 g	179.21 d	458.57 b	172.48 d
Root to shoot ratio (%)	10.19 e	17.50 d	20.21 bcd	21.77 bcd	8.31 e	17.73 cd
Primary stem diameter (mm)	9.60 de	13.30 bc	10.82 cde	13.33 bc	14.81 b	22.97 a
Secondary stem diameter (mm)	6.85 de	9.99 ab	6.30 e	8.68 bc	8.74 bc	11.35 a

Table 23. Root and shoot characteristics of six selected black pepper accessions after 28 days of stress

Parameters ↓	Acc 7211	Acc 1495	Acc 1343	Acc 4132	Acc 5717	Acc 4064
Maximum root length (cm)	59.50 bc	67.00 ab	70.97 a	75.30 a	51.90 cd	60.00 bc
Primary root diameter (mm)	5.64 de	5.52 de	10.36 a	10.33 a	9.45 ab	8.53 abc
Number of primary root	24 a	20 b	19 b	12 cde	11 de	8 fg
Number of secondary root	111 a	100 ab	105 ab	96 b	54 cd	45 de
Root fresh weight (g)	43.72 h	155.33 cd	58.00 gh	158.20 c	135.98 e	116.50 f
Root dry weight (g)	14.62 e	54.75 a	34.44 d	49.58 ab	45.44 bc	32.76 d
Shoot fresh weight (g)	503.63 f	1068.78 c	408.52 g	793.85 d	1278.75 b	465.00 fg
Shoot dry weight (g)	137.14 e	249.73 c	146.50 e	176.62 d	480.49 a	174.56 d
Root to shoot ratio (%)	10.66 e	21.92 bc	23.51 b	28.07 a	9.46 e	18.77 cd
Primary stem diameter (mm)	8.18 e	11.98 bcd	9.65 de	12.10 bcd	11.90 bcd	14.74 b
Secondary stem diameter (mm)	5.82 e	9.38 bc	6.12 e	8.10 cd	8.70 bc	9.97 ab

Figure 36. Genotypes 7211, 1495, 1343, 4132, 5717, and 4064, maintained in plastic bags.

Figure 37. Root morphology of selected genotypes 7211, 1495, 1343, 4132, 5717, and 4064 under corresponding control conditions (A-F) and after 28 days of drought stress treatment (G-L).

Figure 38. Selected genotypes under control (A) and water stress (28 DAS) treatments (B).

Evaluation of morpho-physiological and yield-attributing traits of selected black pepper genotypes under control and drought stress conditions using principal component analysis

The analysis of the selected genotypes under well-watered conditions with selected physiological, yield, and root-to-shoot characteristics revealed five components that captured a significant cumulative variability of 100%. These components had eigenvalues greater than one, highlighting their significance. The first principal component, with an eigenvalue of 6.64, displayed the highest variability, accounting for 34.97% of the total variability. The second highest variability (31.33%) was associated with an eigenvalue of 5.95. The cumulative variance of the remaining third, fourth, and fifth principal components was 33.70%, with respective eigenvalues of 3.02, 2.13, and 1.26.

The contribution of each variable, either positive or negative, to the principal components with its numerical values varied. For PC 1, the variability was contributed by parameters like spike length (0.887), total chlorophyll (0.862), piperine (0.841), carotenoids (0.786), and starch (0.754). In contrast, negative contributions were highlighted by traits such as electrical conductivity (-0.781) and stomatal width (-0.526). Collectively, these characteristics significantly impact yield and physiological responses to drought tolerance.

For PC 2, the highest variability was contributed by loadings of root length (0.920), total sugar (0.894), root-to-shoot ratio (0.874), and essential oil (0.719), indicating carbohydrate storage, root growth, and essential oil content. Conversely, traits such as stomatal width (-0.794), which indicates water conservation by reducing stomatal opening, number of primary roots (-0.670), and number of berries per spike (-0.620) were negatively correlated.

Positive contributions attributed to PC 3 included berry size (0.945) and the number of secondary roots (0.737), reflecting fruit development and root architecture. Conversely, the major negative loading was observed in total sugar.

Oleoresin and the number of berries per spike, with higher loadings of 0.696 and 0.532, made major contributions to PC 4. However, negative contributions were

attributed to traits such as RWC (-0.459), number of secondary roots (-0.347), and spike length (-0.323), indicating balanced moisture content and growth traits.

The fifth component had positive attributions of starch (0.520), number of primary roots (0.410), and total chlorophyll (0.407). Negative contributors included the number of berries per spike (-0.319) and stomatal width, highlighting that adaptation helps the plant conserve water and survive adverse conditions but at the cost of reduced reproductive success.

According to the analyzed scatterplot (Fig. 68), the distribution of genotypes across the first two principal components (PCs) showed that genotype 4132 had strong positive values for both PC1 (1.20) and PC2 (2.559). Acc 1495 displayed positive values in PC1 (1.763) and PC2 (1.023). The highest positive contribution in PC1 (2.366) was shown by Acc 1343, with a positive contribution in PC2 (0.925) as well. Collectively, these findings indicate a comparatively higher positive correlation with spike length, piperine, essential oil, starch, total sugar, carotenoids, total chlorophyll, root length, and root-shoot ratio. These traits reflect the growth, yield, and quality of the genotype, as well as its inherent drought tolerance.

Acc 7211 had a higher value for PC1 (1.867) but a negative value for PC2 (-4.522), influenced by its proximity to carotenoids, which protect against oxidative stress, and starch, which serves as an energy reserve. Genotypes 7211, 1495, 1343, and 4132 all positively contributed to PC1. Except for 7211, the remaining genotypes also had positive values for PC2. Therefore, Acc 7211 showed moderate drought-tolerant characteristics.

Conversely, genotype 5717 displayed negative values in both PC1 (-3.985) and PC2 (-1.839). This suggests that the negative PC1 values for the parameters related to lower RWC, carotenoids, starch, piperine, total chlorophyll, spike length, and yield, as well as unfavorable traits of higher EC and primary root diameter. Negative PC2 values indicated the lack of positive traits related to total sugar, essential oil, maximum root length, and root-to-shoot ratio, and were influenced negatively by traits like stomatal width, lower carotenoids, and fewer berries per spike. Hence, genotype 5717 appeared to have drought-susceptible characteristics.

Similar to genotype 5717, genotype 4064 also showed a strong negative PC1 (-3.216) value, illustrating lower RWC, total chlorophyll content, carotenoids, spike length, and reduced yield, which are unfavorable for drought tolerance. However, the positive contribution to PC2 (1.852) indicated favorable traits as compensatory effects, such as higher sugar content, essential oil, root-to-shoot ratio, and root length. Hence, while it possesses lower water retention and photosynthetic efficiency, it also has adaptive responses that could provide moderate drought tolerance traits.

Overall, the genotypes 4132, 1343, and 1495, with their strongest positive contributions to the PCs, demonstrated robustness in growth, yield potential, and drought tolerance traits. Conversely, genotype 5717, with negative values in the PCs, exhibited vulnerability to drought due to lower key physiological traits. However, the lower water retention ability of genotype 4064 demonstrated compensatory drought tolerance mechanisms through favorable traits in PC2, highlighting the importance of specific traits in influencing inherent drought tolerance and growth performance under well-watered conditions.

Under the similar control conditions, significant variation was observed in morpho-physiological and yield traits such as plant height, chlorophyll content, pod length, pods per plant, and seed yield per plant, with genotypes G8 (VBG-11011), G7 (VBG-10010), and G10 (VBG-12062) attaining high principal component scores. However, genotype G9 (VBG-12005), having a higher score, showed drought-tolerant traits under water-stressed conditions (Mohanlal *et al*., 2020).

Thus, the prominent characters grouped within different principal components, which account for the variability, tend to remain together (Mahendran *et al*., 2015). The knowledge of desired characteristics required for efficient selection strategies enables the exploitation of genetic resources (Nachimuthu *et al*., 2014).

The selected genotypes subjected to drought stress conditions (28 DAS) were analyzed by PCA, which revealed significant genetic variability with a cumulative variance of 97.25%. Only the first four principal components (PCs) had eigenvalues

greater than 1, with values of 9.69, 4.39, 2.57, and 1.82, respectively. These PCs accounted for 51.02%, 23.11%, 13.51%, and 9.60% of the total variability.

The key traits with high loading values on PC1 were total chlorophyll (0.934), root length (0.924), piperine (0.903), carotenoids (0.895), spike length (0.895), and the number of secondary roots (0.861). These traits are crucial for drought resilience, indicating the plant's capability to cope with drought stress through improved photosynthetic efficiency, nutrient uptake, and root growth and development. Negative contributions on PC1 also highlighted key physiological traits related to drought tolerance, including electrical conductivity (EC) (-0.890) and stomatal width (-0.877).

The significant positive contributions of PC 2 were found in traits such as total sugar (0.868), primary root diameter (0.700), root-to-shoot ratio (0.632), and essential oil content (0.583). These traits contribute to improved drought tolerance by enhancing the plant's carbohydrate metabolism and root growth, which are crucial for water uptake and stress response. In contrast, the negative loadings of PC 2, number of primary roots (-0.816) and number of berries per spike (-0.781), indicate reduced reproductive ability and root development under drought stress conditions.

The traits oleoresin (0.852), primary root diameter (0.572), essential oil content (0.485), and berry size (0.436) were positively related to PC 3, indicating contributions to drought resilience through enhanced secondary metabolite production and root growth. In contrast, the negative contributions to PC 3 by the traits yield (-0.521) and spike length (-0.415) suggest reduced productivity under unfavorable environmental conditions.

The fourth principal component included positive contributions from starch (0.561) and number of berries per spike (0.490) facilitated the carbohydrate storage and yield. However negative loadings had berry size (-0.687) and RWC (-0.587) which explained water retention ability and fruit development under drought stress conditions.

The genotype distribution across two principal components (PCs) (Fig. 69) helped identify that genotype 4132 had higher positive values for PC1 (3.51) and PC2 (2.31), followed by genotype 1343 with strong positive values for PC1 (2.92) and PC2 (0.21). This indicates better drought-resilient performance under water stress, as these genotypes exhibit lower reductions in photosynthetic pigments, quality and yield traits, and higher root architecture.

The genotypes 7211 and 1495 expressed moderate drought-tolerant traits, as they showed positive values for the first principal component (0.29 and 1.51, respectively) and negative values for the second component. In contrast, the genotypes 5717 and 4064 exhibited more susceptible traits to drought stress due to their high negative impact on the first component (-4.78 and -3.46, respectively). This analysis was based on the first two principal components, which capture a significant portion of the variance that is highly associated with drought tolerance traits.

In the case of the maize genotypes, significant variability was observed with PCA under controlled irrigation treatment to identify drought-tolerant genotypes by correlating morpho-physiological traits such as kernel weight, grain yield, RWC, and POD activity with drought resistance. Drought-tolerant genotypes, like crosses P1×P5 and P4×P6, exhibited higher scores for these traits under water-stressed conditions as found in the present findings, indicating their suitability for cultivation in drought-prone environments (Hefny *et al*., 2017).

Similarly, the application of PCA analysis on cotton cultivars identified key drought-tolerant indicators encompassing morphological, physiological, and yield-related traits. Traits such as transpiration rate (Tr), chlorophyll (Chl), effective fruit branch number (EFBN), and single boll weight (SBW) exhibited significant variation in response to drought stress and contributed substantially to the first two principal components (PCs), which accounted for 65% of the variability. These traits characterized drought-resistant varieties like Zhong R 2016 and Xin lu zao 45 (Sun *et al*., 2021).

PCA analysis together with cluster analysis identified drought tolerant safflower genotypes based on yield-attributing traits (yield potential (Yp), yield stability (Ys), and related indices), similar to our present study. The analysis illustrated genotypes with high yield potential (PC1) and low susceptibility to drought stress (PC2), such as Kermanshah47, IL, Hamedan38, Syrian, and Kordestan5 (Bahrami *et al*., 2014).

Upon drought stress in wheat (Ghosh *et al*., 2020; Ahmed *et al*., 2019; Faisal, 2016) and barley seedlings (Hellal *et al*., 2018) through the application of PEG treatment, decreases were observed in root traits such as length, fresh weight, and tissue water content. The drought-based screening of wheat genotypes with PCA analysis as similar to the present study has also been reported in other studies, such as those by El-mohsen *et al*. (2015) and Ahmad *et al*. (2017).

In identifying the drought-tolerant soybean genotypes using PCA analysis, traits associated with quantitative yield-attributing characteristics, such as the number of seeds, number of pods with seeds, number of pods without seeds, leaf dry matter, and shoot dry matter, showed a significant positive correlation with yield under stress conditions. These characteristics indicate the cultivars' ability to sustain higher values of these key traits under drought stress (Giordani *et al*., 2019).

The color-based heatmap was used to visualize the magnitude of the morpho-physiological and yield-attributing traits of selected genotypes under well-watered conditions (Fig. 70 A). The two-dimensional visualization depicts that the horizontal axis corresponds to the parameters, and the vertical axis represents the genotypes. The scale bar on the right side interprets the values depicted in the heatmap, with dark blue indicating lower values and lighter blues transitioning through a gradient to yellow and red signifying higher values. Therefore, in the present study under control conditions, higher values represent genotypes with drought-tolerant traits, while lower values indicate drought-susceptible traits.

The more positive values were contributed by the traits RWC, followed by starch, root-to-shoot ratio, number of berries per spike, maximum root length, and number of secondary roots. The horizontal clustering of the heatmap grouped these traits into

clusters 2, 3, and 4. The genotypes 1343 (vertical cluster 4) and 7211 (vertical cluster 2) showed that positive traits as more drought-favorable, followed by 4132 and 1495 (vertical cluster 1). The least positive values were exhibited by the genotypes with the least favorable traits, 5717 and 4064 (vertical cluster 3).

Remaining parameters (number of primary roots, spike length, oleoresin, EC, primary root diameter, berry size, stomatal width, piperine, essential oil, total sugar, yield, carotenoid and total chlorophyll) included in horizontal cluster 1 having lower range did not show any differentiation between the genotypes for each parameter especially, total chlorophyll, carotenoids, and yield.

The heatmap analysis on drought-stressed selected genotypes clustered them into two groups. The genotypes belonging to cluster 1 (7211, 1495, 1343, and 4132) and cluster 2 (5717 and 4064) shared proximal positive characteristics among themselves. The genotypes in cluster 1 exhibited higher numbers of secondary roots, greater root length, higher relative water content (RWC), more starch, and longer spike lengths, indicating drought tolerance. In contrast, cluster 2 genotypes demonstrated susceptibility to drought.

The traits that were highly influential on genotypes under drought conditions, as per this analysis, included maximum root length, RWC, the number of berries per spike, and the number of secondary roots, which were placed in horizontal cluster 2.

Under both control and drought-stressed conditions, the most influential and prominent traits for determining drought tolerance were maximum root length, RWC, starch content, the number of berries per spike, and the number of secondary roots. These traits further solidified the drought-tolerance ability of the genotypes 1343, 7211, 4132, and 1495.

This kind of large dataset can be converted into an easily understandable form using PCA, which reduces the dimensionality of the variables. Similar to the present study, drought-tolerant wheat genotypes were identified by examining morphological traits (shoot length, root fresh weight, root dry weight) and yield-related traits (seedling

vigor index), both PCA and heatmap analyses were used. These analyses co-clustered 127 genotypes into four different clusters, noting the genotypes in cluster 4 as the most tolerant under PEG-induced drought conditions (Mohi-Ud-din *et al*., 2021). A comparable result was reported by Khan *et al*., (2018) in wheat genotypes subjected to drought stress.

Table 24. Eigen values and percentile variance of selected black pepper genotypes with physiological and yield attributing traits under control condition

Control	PC 1	PC 2	PC 3	PC 4	PC 5
eigenvalue	6.64	5.95	3.02	2.13	1.26
% Variance	34.97	31.33	15.87	11.20	6.62
Cumulative percentage of variance	34.97	66.30	82.17	93.38	100.00
Variable	**Eigen vectors**				
RWC	**0.592**	0.472	0.464	-0.459	-0.025
EC	-0.781	-0.426	-0.050	0.417	0.177
Total.chl	**0.862**	0.146	-0.133	0.229	**0.407**
Carotenoids	**0.786**	-0.561	-0.205	0.098	0.124
Stomatal width	-0.526	-0.794	0.064	-0.003	-0.299
yield	**0.640**	0.684	-0.269	-0.184	0.129
Spike length	**0.887**	0.230	-0.224	-0.323	-0.083
Number of berries per spike	0.378	-0.620	-0.298	**0.532**	-0.319
Berry size	-0.093	-0.127	**0.945**	0.045	0.284
Oleoresin	0.357	-0.315	0.534	**0.696**	-0.063
Essential oil	0.238	**0.719**	0.437	0.484	-0.031
Piperine	**0.841**	0.207	0.337	0.302	-0.212
Total sugar	-0.154	**0.894**	-0.344	0.146	0.193
Starch	**0.754**	-0.104	-0.208	0.326	**0.520**
Maximum root length	-0.055	**0.920**	-0.134	0.285	-0.227
Primary root diameter	-0.671	0.352	0.562	0.040	0.329
Number of primary root	0.542	-0.670	0.038	-0.297	**0.410**
Number of secondary root	0.525	-0.094	**0.737**	-0.347	-0.229
Root to shoot ratio	0.438	**0.874**	-0.028	0.174	-0.111

Table 25. Eigen values and percentile variance of selected black pepper genotypes with physiological and yield attributing traits under drought stressed condition at 28 DAS

Treatment	PC 1	PC 2	PC 3	PC 4	PC 5
eigenvalue	9.69	4.39	2.57	1.82	0.52
% Variance	51.02	23.11	13.51	9.60	2.75
Cumulative percentage of variance	51.02	74.13	87.64	97.25	100.00
Variable	**Eigen vectors**				
RWC	0.756	0.076	-0.254	-0.587	0.115
EC	-0.890	0.151	0.419	0.004	0.100
Total.chl	**0.934**	-0.186	0.262	-0.140	0.070
Carotenoids	**0.895**	-0.427	0.129	-0.017	0.010
Stomatal width	-0.877	-0.374	0.069	-0.245	0.161
yield	0.739	0.354	-0.521	0.237	-0.036
Spike length	**0.895**	-0.100	-0.415	-0.129	0.022
Number of berries per spike	0.186	-0.781	0.287	**0.490**	0.181
Berry size	0.275	0.318	**0.436**	-0.687	-0.401
Oleoresin	0.444	-0.246	**0.852**	0.001	0.132
Essential oil	0.635	**0.583**	**0.485**	-0.059	0.136
Piperine	**0.903**	-0.138	0.345	-0.132	**0.171**
Total sugar	0.265	**0.868**	-0.181	0.371	0.077
Starch	0.726	-0.238	0.144	**0.561**	-0.284
Maximum root length	**0.924**	0.363	-0.016	0.024	0.120
Primary root diameter	0.050	**0.700**	**0.572**	0.369	-0.210
Number of primary root	0.516	-0.816	-0.204	0.061	-0.149
Number of secondary root	**0.861**	-0.493	-0.012	-0.031	-0.123
Root to shoot ratio	0.744	**0.632**	-0.139	0.030	**0.161**

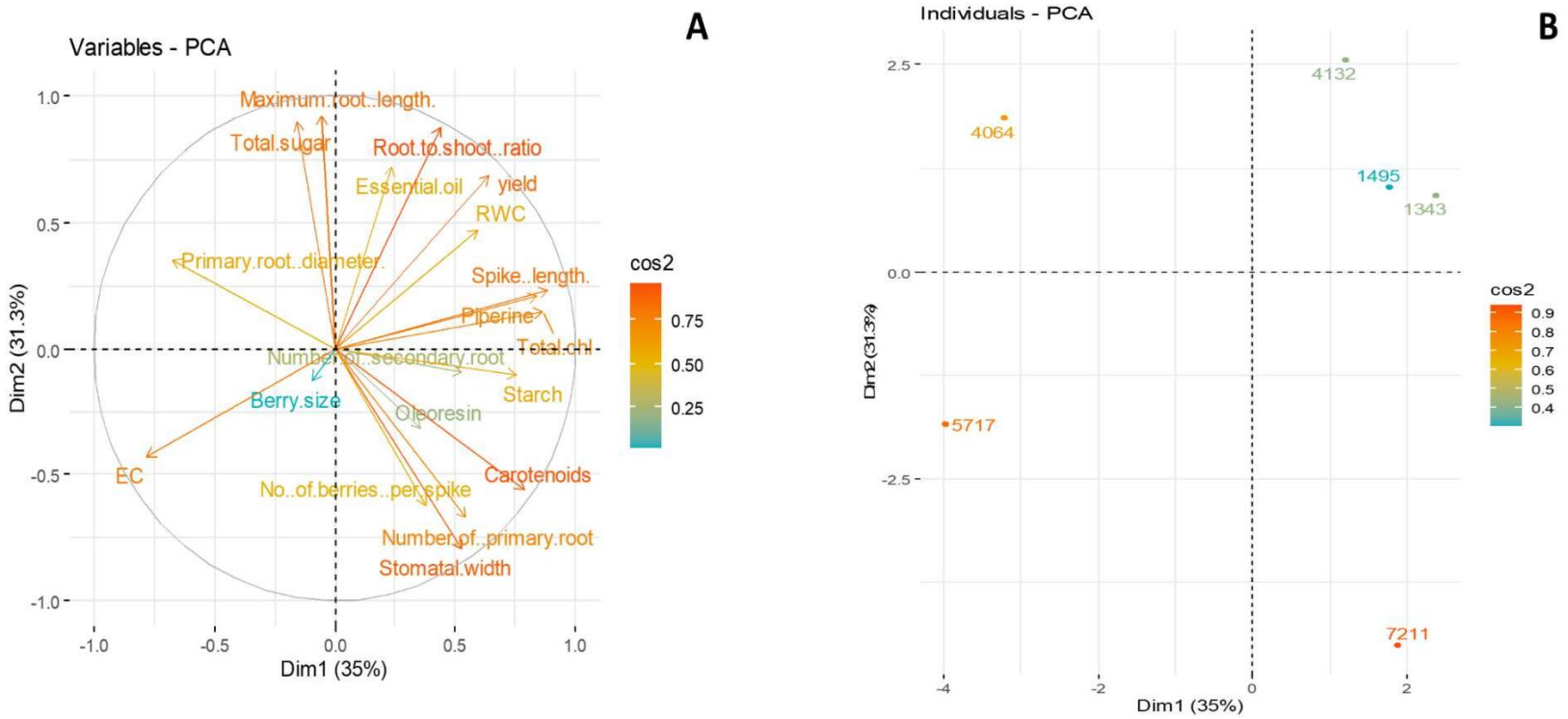


Figure 39. Distribution of black pepper morpho-physiological and yield trait variables (A) and genotypes (B) across the first two primary components in control conditions

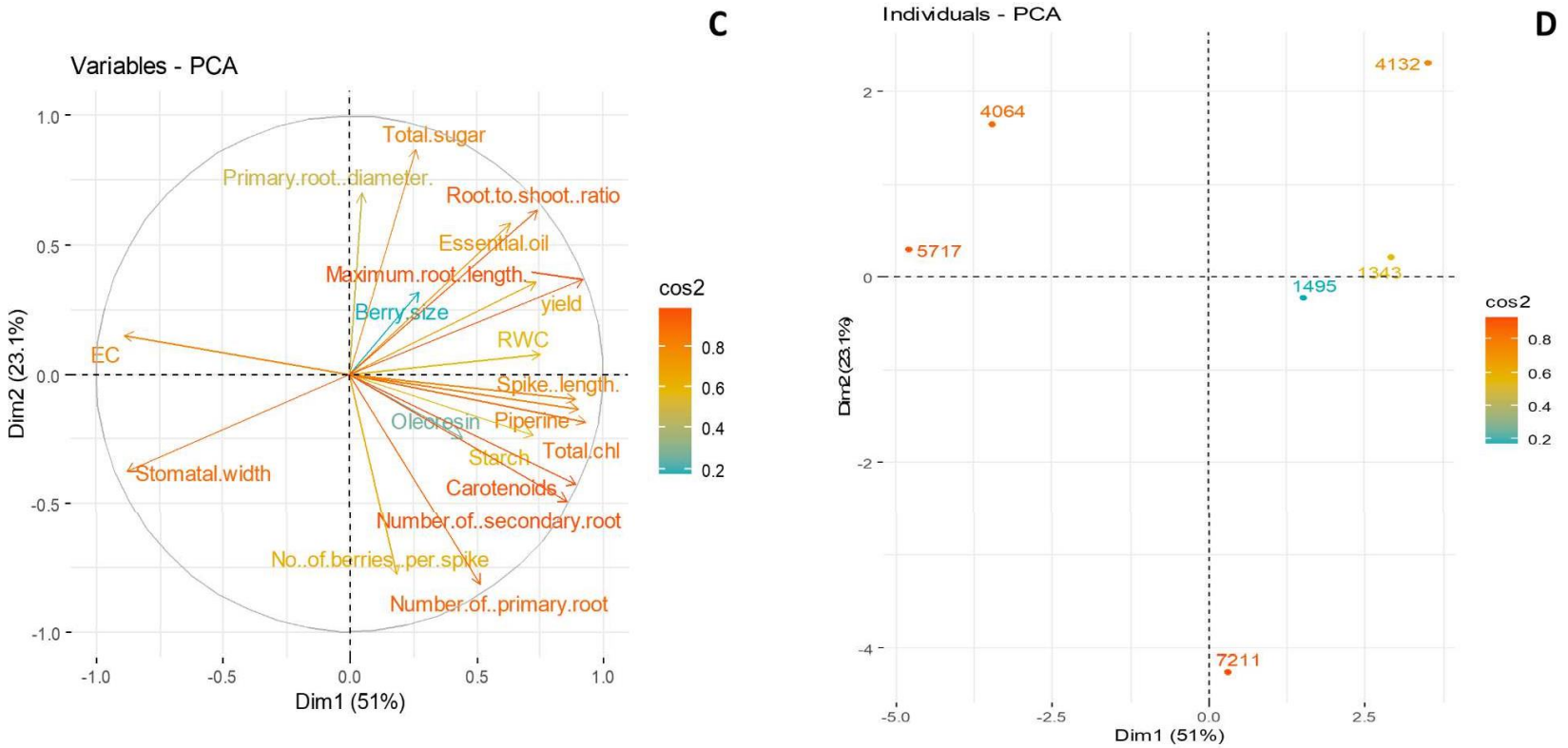


Figure 40. Distribution of black pepper morpho-physiological and yield trait variables (C) and genotypes (D) across the first two primary components in drought stress condition (28 DAS)

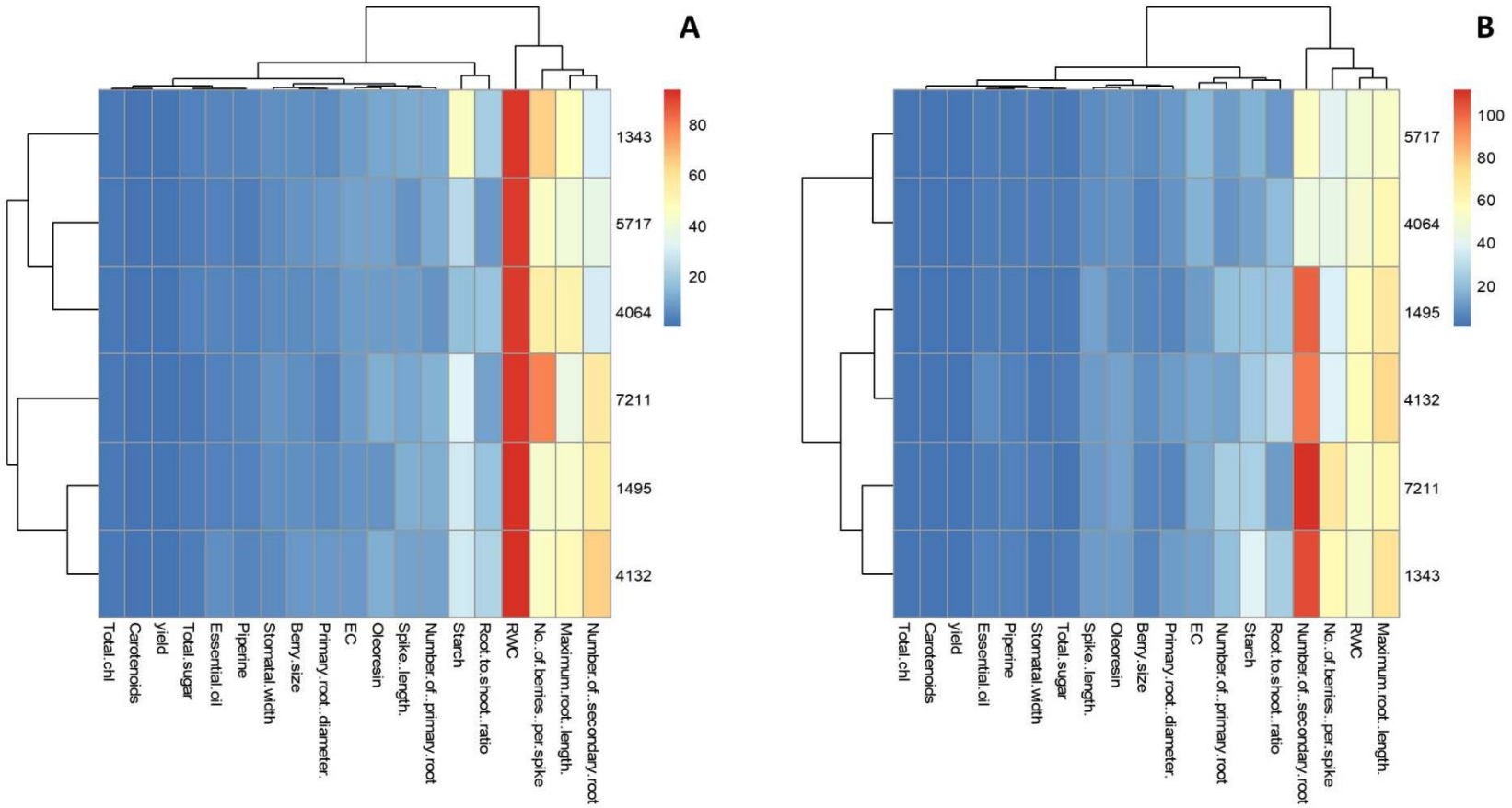


Figure 41. Heatmap of morpho-physiological and yield traits among the selected black pepper genotypes under control (A) and drought stress conditions (B)

Evaluation of biochemical traits of selected black pepper genotypes under control and drought stress conditions using principal component analysis

The PCA biplot, in terms of biochemical parameters of the selected genotypes (7211, 1495, 1343, 4132, 5717, 4064) under control conditions, showed significant genetic variability on PC 1 (41.08%), followed by PC 2 (18.68%), PC 3 (17.42%), PC 4 (13.95%), and PC 5 (8.68%). The respective decreasing eigenvalues are 6.98, 3.18, 2.96, 2.37, and 1.51.

The first principal component accounted for significant positive contributions from APX (0.913), total protein (0.907), SOD (0.899), proline (0.748), leaf ABA (0.653), and phenol (0.638). These traits, which include increased antioxidant activity, osmoprotectant accumulation, and stress hormone synthesis, are positive indicators of drought tolerance. The negative contributions to PC 1 were from Mn (-0.794), Fe (-0.793), and H2O2 (-0.427), which indicate an intrinsic resistance to oxidative stress.

The second principal component revealed that the traits stem ABA (0.851), CAT (0.680), proline (0.644), and leaf starch (0.450) showed positive contributions, which help in maintaining overall health, growth, and resilience. PC 2 was negatively associated with POX and phenol, with values of -0.627 and -0.533, respectively. The third principal component displayed positive contributions from phenol (0.330), polyphenol oxidase (PPO) (0.589), root ABA (0.614), and Mn (0.550), revealing traits associated with drought resistance through stress signaling and protective enzyme activities. However, malondialdehyde (MDA) (-0.902) had a negative contribution, indicating lipid peroxidation and cell membrane damage.

Crespo *et al.* (2011) stated in their study that supported the present study that the biochemical parameters positively correlated to PC1 include proline, carotenoids, catalase, peroxidase, and reducing sugars. In contrast, chlorophyll a, chlorophyll b, and total chlorophyll showed an inverse result to the present study's observation, as they negatively contributed to PC1 but positively to PC2. Thus, the *Gmelina arborea* genotypes distributed in the first quadrant, being positively correlated to both PC1 and PC2, demonstrated their ability to tolerate drought.

The fourth and fifth components include the traits H2O2 (0.784), CAT (0.541), and K (0.501) in PC 4, as well as POX (0.536), CAT (0.337), and APX (0.328) in PC 5, which are positively associated with antioxidant regulation. However, root ABA (-0.622) showed a strong negative contribution to PC 4, and leaf starch (-0.662) contributed negatively to PC 5, indicating their roles in carbohydrate stabilization.

The genotypes 7211 (3.33, 0.284), 1343 (1.97, 0.297), and 4132 (0.608, 2.74) were clustered in the first quadrant, indicating that these genotypes, having positive values for both PCs, shared drought-tolerant characteristics. The genotype 1495 (0.997, -3.35) conferred moderate drought tolerance traits because it had a positive contribution to PC 1 and a negative contribution to PC 2. In contrast, the genotypes 5717 (-2.54, 0.144) and 4064 (-4.36, -0.117) were more susceptible, as illustrated by 4064 having the highest negative values on both PCs and 5717 having the second highest negative value on PC 1.

The same analysis with selected genotypes under drought stress (28 DAS) helped identify the better genotypes with favorable biochemical traits. The first four components explained the highest variability, accounting for 96.53% of the total variance, with eigenvalues greater than one. PC 1, with an eigenvalue of 9.54, accounted for 56.12% of the total variance and had substantial positive contributions from key traits, including CAT (0.924), POX (0.975), SOD (0.945), PPO (0.989), APX (0.971), leaf ABA (0.775), proline (0.743), phenol (0.743), and total protein (0.731). As per Alscher *et al.* (2002), oxidative stress induces the activation of SOD, which in turn leads to the activation of other antioxidant enzymes. This observation supports the findings of our present study.

This component was associated with antioxidant enzymatic activity, osmoprotection, ABA signaling, and secondary metabolite production for defense mechanisms against environmental stress. Major negative contributions were made by essential physiological traits related to drought tolerance, including H2O2 (-0.815) and MDA content (-0.615).

The significant positive contributions of PC 2 were observed in stem ABA (0.791), leaf starch (0.651), K (0.427), Fe (0.372), and Mn (0.345). These traits enhance

drought resilience by improving carbohydrate metabolism and nutrient absorption, which are crucial for the plant's water uptake and stress response in water-limited environments.

Biochemical (malondialdehyde, superoxide dismutase, and peroxidase) and Physiological (relative water content, relative water loss) indexes of *Iris germanica* genotypes illustrated a major positive contribution of PC2 with moisture-related traits such as RWC and RWL, while PC2 was positively contributed to by SOD and POD activities. These comprehensive indexes are required for a reliable evaluation of drought tolerance (Bo *et al*., 2017). The desired physiological and biochemical characteristics of the chilli genotypes like KCa-5, KCa-7, and KCa-10 exhibited higher relative water content (RWC), chlorophyll content, total phenolics, proline content, superoxide dismutase (SOD), and catalase activities, which were attained higher principal component score as observed in the present study indicated enhanced physiological tolerance and antioxidant potential under water stress conditions (Lakshmi Sahitya *et al*., 2018).

PC 3 recorded 14.10% of the total variability, explained by higher positive loadings of phenol (0.605), MDA content (0.512), and proline (0.440), while protein (-0.577), leaf ABA (-0.385), and K (-0.320) showed negative loadings. This component facilitated secondary metabolite production and stress markers and also indicated a decline in protein synthesis and potassium content under water-limited conditions.

Compared to morpho-physiological indicators, the biochemical osmoprotectant proline plays an important role in drought tolerance, along with the regulatory role of MDA content (Chen *et al*., 2016; Fang & Xiong, 2015). The same was noticed in present study also. At the same time, there was a positive correlation between the proline content and total chlorophyll content of drought tolerant cultivars in drought prone conditions (Hura *et al*., 2007) supported to the current study.

The fourth component accounted for 9.54% of the total variation and exhibited major positive loadings for Mn (0.638) and K (0.416), indicating improved nutrient assimilation and stress response. In contrast, MDA (-0.442) and leaf starch (-0.494) had significant negative contributions to PC 4, reflecting their role in carbohydrate

storage and controlling MDA accumulation in drought-prone regions. Drought stress exerts a transformative influence across multiple dimensions of morphological, physiological, biochemical, and yield characteristics (Praveen *et al*., 2021).

The coordinates of the genotypes illustrate their distribution in the PCA space (Fig. 71 F). The genotypes 7211 (2.80, 0.978), 1495 (0.994, 2.16), and 4132 (1.48, 0.934) have positive scores on both PC1 and PC2. As they possesses antioxidant enzyme activities, osmoprotectant accumulation, efficient hormone regulation, ROS degradation, secondary metabolite production and robust root systems. These traits collectively enhance the plants' ability to withstand water stress, maintain productivity, and ensure survival under drought conditions.

The highest positive score on PC1 was obtained by genotype 1343 (3.19, -3.15). However, it was negatively correlated with PC2. The genotypes 5717 (-4.08, -0.778) and 4064 (-4.39, -0.138) were negatively scored for the first two PCs, revealing the highest negative correlation with PC1 and indicating susceptibility to drought.

PCA was used to differentiate drought-tolerant soybean genotypes from sensitive genotypes based on several indexes with higher performance in both normal and stressed conditions (Rahi *et al*., 2020) as was done in our study.

One of the previous reports observed in a PCA biplot that the cosine of the angle between trait vectors, when acute (<90°), indicated a positive correlation, whereas angles (>90°) indicated a negative correlation between the traits. Traits with angles equivalent to 90° were independent of each other (Abdi & Williams, 2010). In the present study, it was illustrated that the highest positive correlation (<90°) exists among the morpho-physiological and yield traits like root to shoot ratio, yield, RWC, and spike length as well as biochemical parameters such as stem ABA, leaf starch, PPO, and SOD in first quadrant.

A similar drought-based PCA study on maize genotypes examined morpho-physiological and biochemical traits to identify the most drought-tolerant genotypes, including TZBR COMP 2-YC1S1 280 and TZEI 161. These results suggested significant variations in morphological traits such as the number of leaves, leaf area,

plant height, and stem diameter compared to others (Olawuyi *et al*., 2013). The differentiation of drought-tolerant genotypes from susceptible ones and others was indicated by the high photosynthetic rate (Singh *et al*., 1995).

The heatmap analyses on the both control and drought stressed (Fig. 73) conditions on genotypes regarding biochemical analyses did not give a clear cut differentiation between genotypes with tolerant (7211, 1495, 1343, 4132) and susceptible (5717 and 4064) characteristics.

Numerous studies have utilized principal component analysis (PCA) to identify drought-tolerant genotypes across various plant varieties under drought-stressed conditions (Wang *et al*., 2020; Silvente *et al*., 2012; Mahdi, 2012; Sreenivasa *et al*., 2019; Khayatnezhad *et al*., 2011). These research efforts aim to enhance our understanding of the mechanisms that enable certain genotypes to withstand water scarcity. The insights gained from these studies contribute to the development of more resilient crop varieties, ensuring better productivity and sustainability in water-limited environments.

Table 26. Eigen values and percentile variance of selected black pepper genotypes with biochemical traits under control condition

Control	PC 1	PC 2	PC 3	PC 4	PC 5
eigenvalue	6.98	3.18	2.96	2.37	1.51
% Variance	41.08	18.68	17.42	13.95	8.86
Cumulative percentage of variance	41.08	59.76	77.18	91.14	100.00
Variable	**Eigen vectors**				
H2O2	-0.427	0.059	0.421	**0.784**	0.148
Proline	**0.748**	**0.644**	0.110	0.118	0.007
Phenol	**0.638**	-0.533	**0.330**	0.419	-0.158
MDA	-0.196	-0.115	-0.902	-0.366	0.027
Leaf starch	0.520	**0.450**	0.289	0.078	-0.662
Protein	**0.907**	-0.269	0.249	0.117	-0.174
CAT	-0.363	**0.680**	-0.007	**0.541**	**0.337**
POX	0.520	-0.627	0.222	0.003	**0.536**
SOD	**0.899**	0.062	-0.138	0.285	0.295

Control	PC 1	PC 2	PC 3	PC 4	PC 5
PPO	0.507	0.387	**0.589**	-0.463	0.179
APX	**0.913**	-0.092	-0.002	0.223	**0.328**
Leaf ABA	**0.653**	-0.558	-0.078	0.032	-0.505
Stem ABA	0.449	**0.851**	-0.182	0.117	-0.166
Root ABA	0.463	0.056	**0.614**	-0.622	0.138
K	0.549	-0.191	-0.639	**0.501**	-0.040
Fe	-0.793	-0.177	0.402	0.344	-0.244
Mn	-0.794	-0.248	**0.550**	0.073	-0.028

Table 27. Eigen values and percentile variance of selected black pepper genotypes with biochemical traits under drought stressed condition at 28 DAS

Treatment	PC 1	PC 2	PC 3	PC 4	PC 5
eigenvalue	9.54	2.85	2.40	1.62	0.59
% Variance	56.12	16.77	14.10	9.54	3.47
Cumulative percentage of variance	56.12	72.89	86.98	96.53	100.00
Variable	**Eigen vectors**				
H2O2	-0.815	0.257	0.364	0.364	-0.068
Proline	**0.743**	-0.203	**0.440**	0.392	-0.244
Phenol	**0.743**	0.078	**0.605**	0.273	-0.038
MDA	-0.615	0.373	**0.512**	-0.442	0.157
Leaf starch	0.546	**0.651**	-0.181	-0.494	-0.030
Protein	**0.731**	0.261	-0.577	-0.227	0.111
CAT	**0.924**	0.020	0.328	0.030	0.192
POX	**0.975**	-0.094	-0.171	-0.068	0.081
SOD	**0.945**	0.322	0.028	-0.049	0.000
PPO	**0.989**	0.007	0.074	0.025	0.125
APX	**0.971**	0.142	0.153	0.093	0.066
Leaf ABA	**0.775**	-0.497	-0.385	-0.061	0.025
Stem ABA	0.543	**0.791**	0.275	0.055	0.023
Root ABA	0.543	-0.813	-0.046	0.134	0.158
K	0.670	**0.427**	-0.320	**0.416**	-0.307
Fe	-0.392	**0.372**	-0.741	0.385	-0.099
Mn	-0.385	**0.345**	-0.179	**0.638**	**0.542**

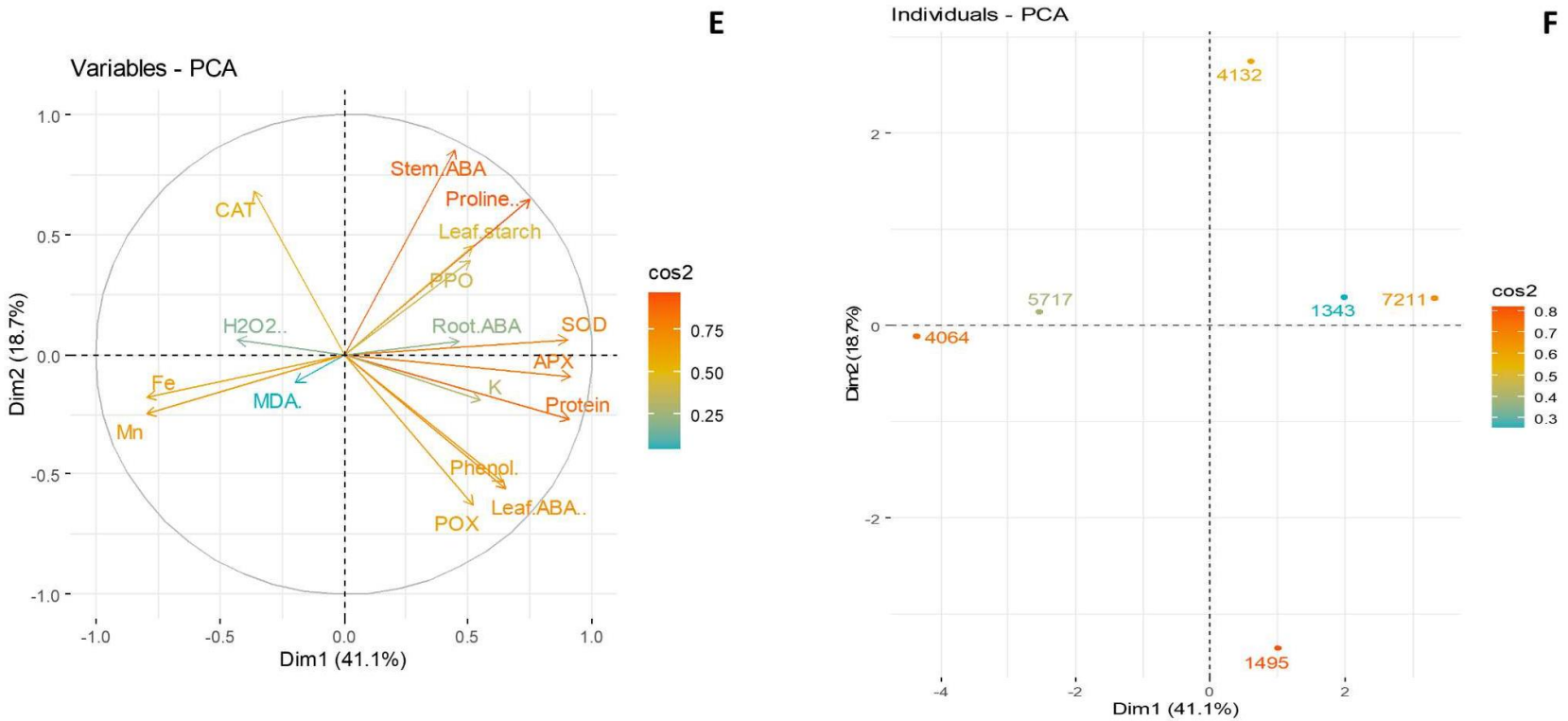


Figure 42. Distribution of black pepper biochemical variables (E) and genotypes (F) across the first two primary components in control conditions

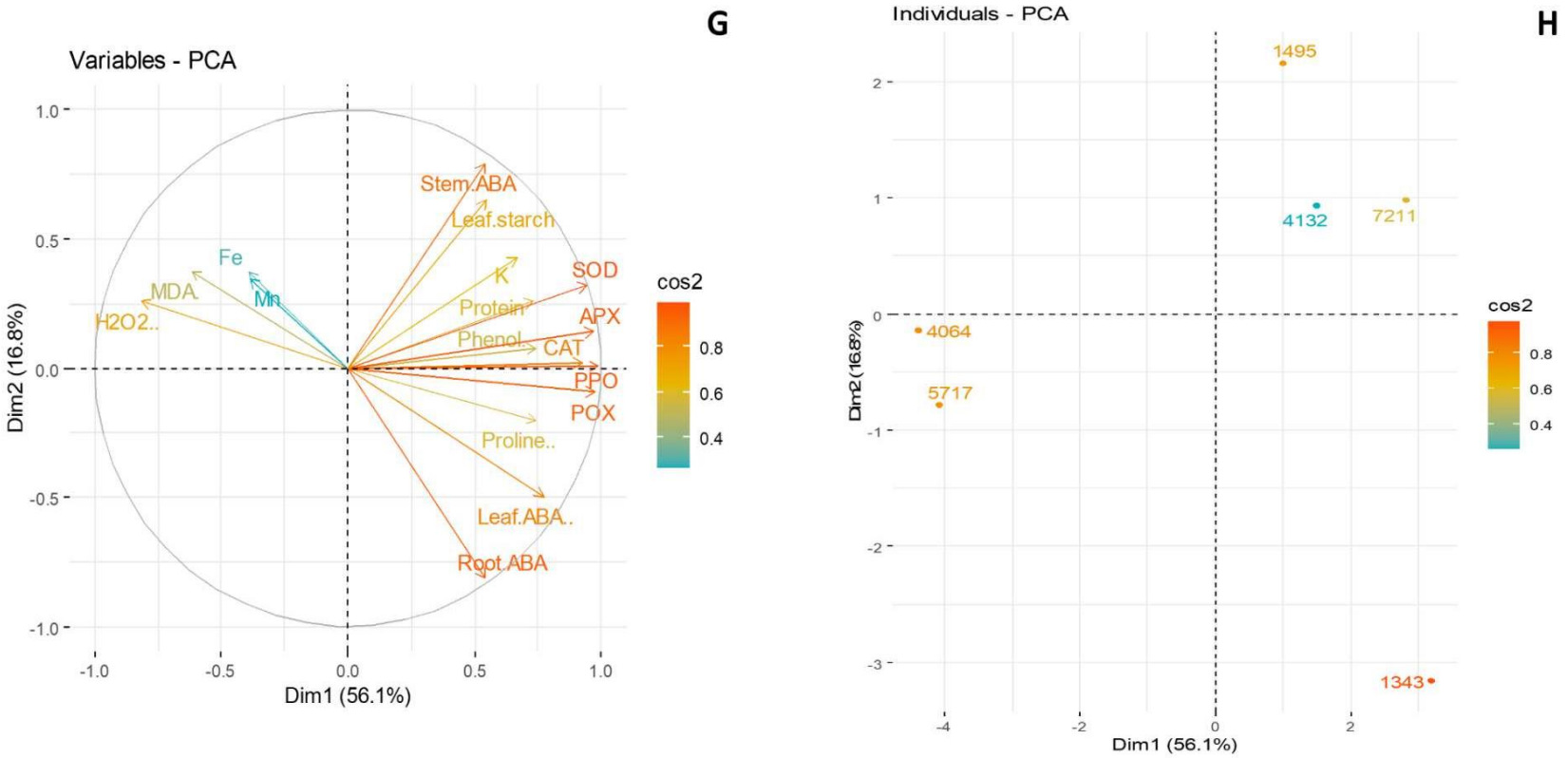


Figure 43. Distribution of black pepper biochemical variables (G) and genotypes (H) across the first two primary components in drought stress conditions

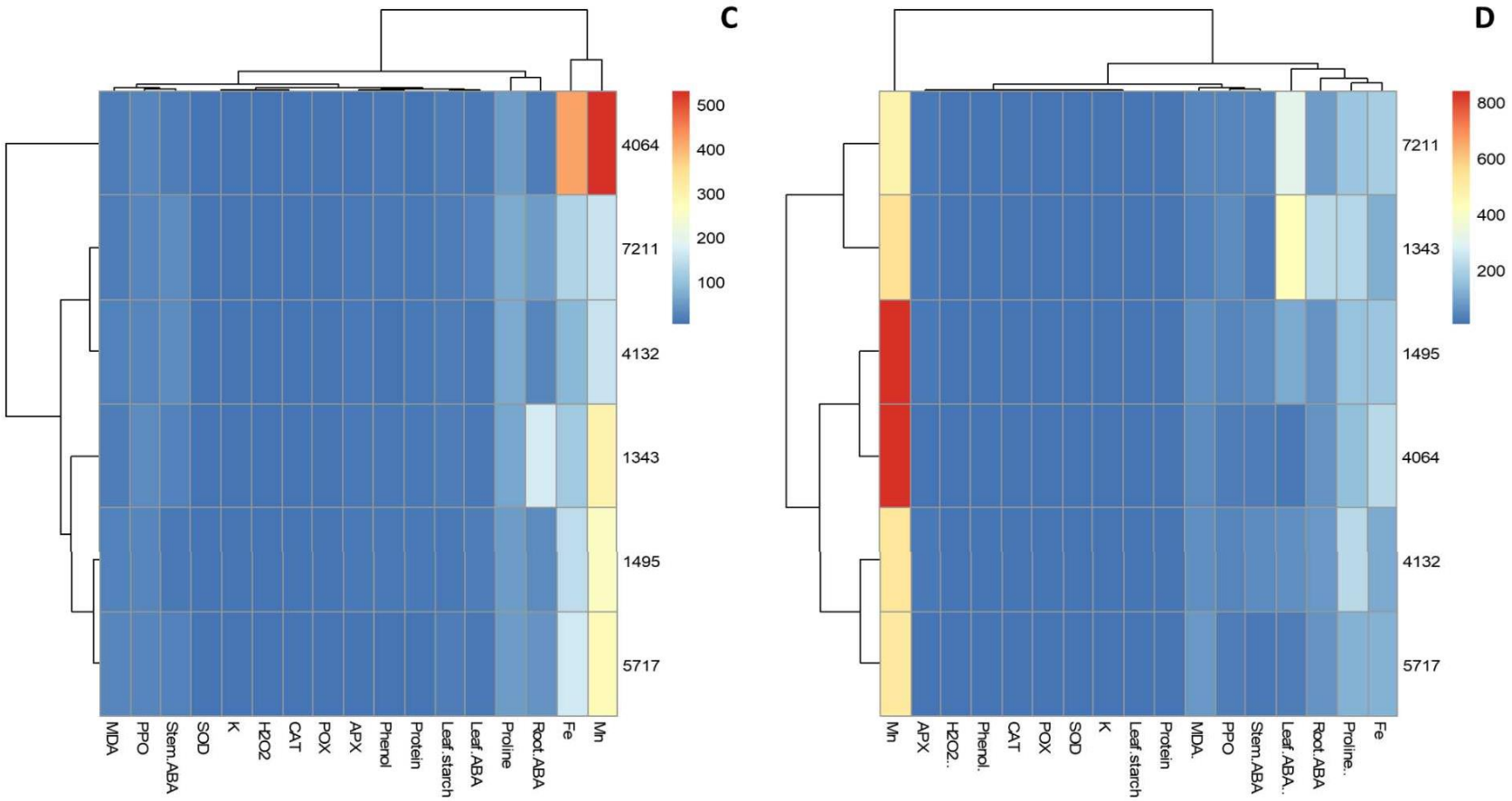


Figure 44. Heatmap of biochemical traits among the selected black pepper genotypes under control (C) and drought stress conditions (D)

Experiment 5

4.7 Molecular characterisation under drought condition

4.7.2 MultiNA Microchip Electrophoresis analysis

MultiNA Microchip Electrophoresis is a cutting-edge technique that offers significant advantages over conventional gel electrophoresis, providing higher resolution and more sensitive analysis of nucleic acids. This technique reveals a more complex banding pattern than the bands observed in traditional agarose gel electrophoresis. In the MultiNA analysis of the present study on the AMP5 primer, in addition to the double bands observed in the agarose gel image (Fig. 39), several other bands with varying amplicon sizes were noted. The presence of multiple bands could potentially be attributed to gene duplication events, wherein several copies of the genes or alleles have formed. As part of the current study, the variable banding pattern was analysed and quantified the concentration of amplicons.

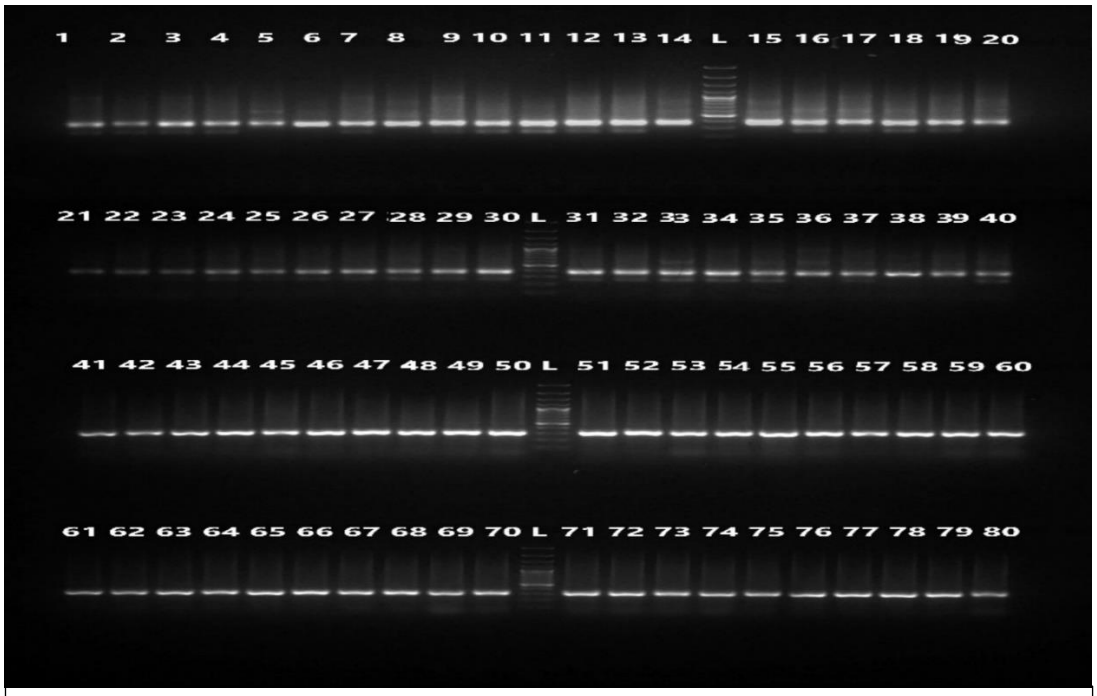


Figure 45. Amplified products of AMP5 primer with accessions, lane (1-40): Acc 4226, Acc 807, Ac 1495, Acc 4216, Acc 7211, Acc 1248, Acc 1368, Acc 6707, Acc 6786, Acc 1476, Acc 4095, Acc 4177 Acc 971, Acc 8052, Acc 5623, Acc 5083, Acc 5621, Acc 1439, Acc 8060, Acc 5642, Acc 4132, Ac 6720, Acc 1315, Acc 4137, Acc 1491, Acc 4064, Acc 1487, Acc 931, Acc 1093, Acc 1218, Acc 5717 Acc 4152, Acc 1638, Acc 1343, Acc 813, Acc 1086, Acc 5691, Acc 1390, Acc 6774 and Acc 89 respectively
Amplified products of AMP10 primer with accessions, lane (41-80): Acc 4226, Acc 807, Acc 1495 Acc 4216, Acc 7211, Acc 1248, Acc 1368, Acc 6707, Acc 6786, Acc 1476, Acc 4095, Acc 4177, Ac 971, Acc 8052, Acc 5623, Acc 5083, Acc 5621, Acc 1439, Acc 8060, Acc 5642, Acc 4132, Acc 6720 Acc 1315, Acc 4137, Acc 1491, Acc 4064, Acc 1487, Acc 931, Acc 1093, Acc 1218, Acc 5717, Ac 4152, Acc 1638, Acc 1343, Acc 813, Acc 1086, Acc 5691, Acc 1390, Acc 6774 and Acc 89 respectively.

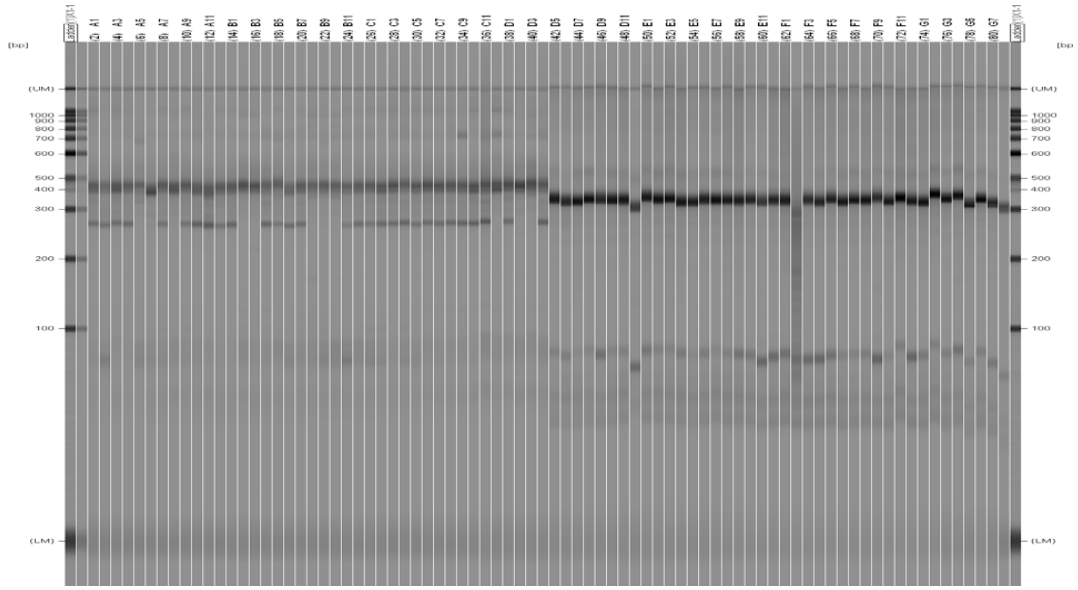
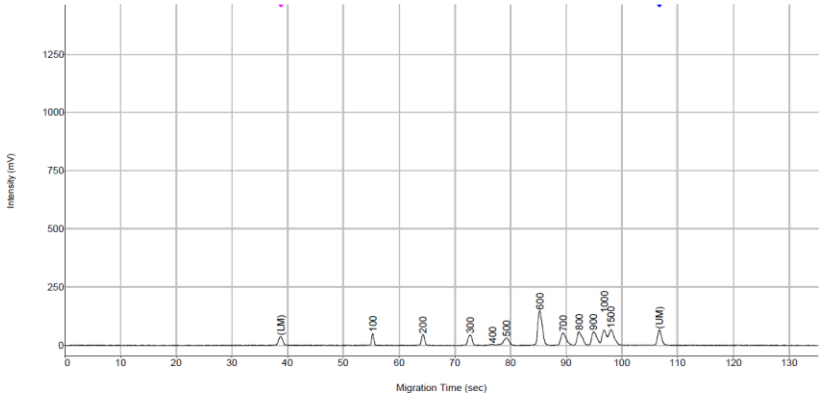


Figure 46. Banding pattern of AMP5 and AMP 10 in MultiNA analysis



No.	Size (bp)	Conc. (ng/µL)
1	(LM)	-
2	100	-
3	200	-
4	300	-
5	400	-
6	500	-
7	600	-
8	700	-
9	800	-
10	900	-
11	**1000**	-
12	1500	-
13	(UM)	-

Figure 47. Graphical representation of the ladder

4.7.3 Identification of SNPs

Four genotypes, two drought tolerant (Acc 7211 and Acc 1495) and two susceptibie (Acc 5717 and Acc 4064) were selected from the previously chosen 40 black pepper genotypes to check for the presence of SNPs. Out of the eleven gene-specific primers (AMP5, AMP 10, DAPC1, DAPC6 I, DAPC6 II, DGST, DPX 5, DPX 12, DPHAO, DSOD-Cu and DSOD-Fe), only the peroxisomal (S)-2-hydroxy-acid oxidase GLO1 (DPHAO)-like gene was observed to have single nucleotide polymorphism, differentiating drought tolerance and susceptibility traits. The same gene was further used for sequence analysis in the remaining 36 accessions as well. However, no SNPs were found to differentiate tolerant and susceptible genotypes.

This finding parallels previous studies that utilized SNPs to distinguish between drought-tolerant and susceptible genotypes in other crops. For instance, genome-wide association sequencing in grape rootstocks identified SNPs with significant associations with drought tolerance traits, while deleterious mutations in some SNPs were linked to susceptibility (Trenti *et al*., 2021).

Similarly, SNPs were effectively used to differentiate drought tolerance and susceptibility in wheat (Nouraei *et al*., 2024), barley (Elbasyoni *et al*., 2022), and maize (Liu *et al*., 2021). These studies collectively underscore the critical role of SNPs in identifying genetic variations that contribute to drought tolerance, although the present study suggests that in black pepper, the DPHAO-like gene may be a key but not a sole determinant of drought tolerance.

Experiment 6

4.8.1. Validation of Drought-Responsive Candidate Genes from Transcriptome Data through qPCR

A comparative transcript abundance analysis was employed for the identification of potential candidate genes involved in the drought tolerance mechanism. The expression profiles of identified genes under well watered condition were compared with those maintained under 25% and 50% field capacity. The reference genes Ubiquitin (UBI), RNA-binding protein RNABP, and Elongation factor 1A (EF1A) were analyzed among the selected accessions (7211, 1495, 1343, 4132, 5717, and 4064), which were maintained at 25%, 50%, and 100% field capacity. The analysis aimed to identify the most stable gene. Among the evaluated reference genes, a comparatively more stable Ct value was observed in the RNABP gene. The available analytical tools, such as ΔCt, Best Keeper, NormFinder, and Genorm, identified a stable gene as a reference for gene expression analysis (Prashina, 2021). The ΔCt method, NormFinder, and Genorm identified RNABP as a stable reference gene under diverse water regimes in black pepper (George *et al*., 2017).

The relative expression of sixteen drought-responsive genes and transcription factors like CuZn Superoxide dismutase –(SOD CuZn), FeMn Superoxide dismutase –(SOD FeMn), Dehydrin (DHN), Osmotin (OSM), Peroxidase 5 like (PX5), Peroxidase 12 (PX12), Glutathione S tranferase F13 like A (GST), Ascorbate peroxidase 6 cytosolic (APC6), Heat-shock protein (HSP70), Aquaporin (AQUA), Peoxisomal (S)-2-hydroxy-acid oxidase GLO1 like (PHAO), NAC transcription factor (NAC), Basic leucine zipper protein (bZIP), Myeloblastosis oncogene (MYB), Dehydration-responsive element-binding protein (DREB) and Apetala 2 (AP2) followed a similar pattern of expression in the accessions 7211, 1495, 1343, and 4132, which showed drought-tolerant characteristics, contrasting with the patterns observed in 5717 and 4064.

A progressively similar pattern of FeMn SOD expression was observed among the selected genotypes as the moisture levels decreased to 50% and 25%. The Acc 1343 exhibited the highest fold increase (13-fold), followed by Acc 4064 with an 11.5-fold

increase under 25% field capacity. No drought-associated variation was observed in FeMn SOD.

CuZn SOD- was highly expressed in accessions 7211 (2.75-fold), followed by 4132 (2.17-fold), 1343 (2-fold) and 1495 (1.16-fold) while its expression was down regulated in susceptible accessions 5717 (1.5-fold) and 4064 (2.1-fold).

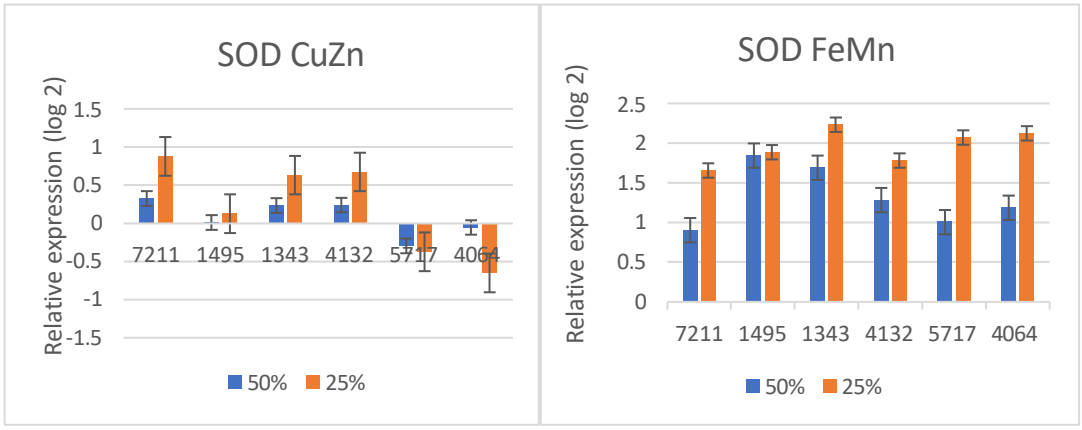


Figure 48. Differential expression of SOD-CuZn and SOD-FeMn genes in response to drought stress under 50% and 25% FC.

As found in the present study, Mohammadi *et al.* (2020) reported upregulated SOD-CuZn expression in drought-tolerant common bean plants compared to susceptible ones. Similarly, the association between drought tolerance and both transgenic and non-transgenic tobacco plants was evident, with the SOD CuZn expression significantly increased in transgenic plants—30 times higher than in non-transgenic plants.

Furthermore, the selection criterion undertaken for triticale grain under drought stress differentiated between the drought-tolerant genotype 28 and the susceptible genotype 3 through gene expression studies conducted with SOD CuZn, SOD-Fe, and SOD Mn. Higher expression levels were demonstrated by SOD-CuZn and SOD Mn for genotype 28, indicating tolerance, conversely to genotype 3, which was susceptible. However, no substantial difference could be found between the two genotypes with SOD Mn (Saed-Moucheshi *et al.*, 2021), as similar to the present study. A drought tolerance-based comparison of two upland rice genotypes, Douradão and Primavera, under the same water withholding conditions, demonstrated that the genes

CuZnSOD3, CuZnSOD4, MnSOD, and FeSOD2 were upregulated in Douradão genotype compared to Primavera, with fold changes of 1.94, 1.45, 1.59, and 1.62, respectively (de Deus *et al*., 2015). Involvement of the cofactor CuZn in plants under stressed conditions has a significant role in ROS detoxification (Xie *et al.*, 2019) as evidenced in the present study.

The drought-responsive gene, DHN, was positively regulated with higher expression in accessions 7211, 1343, 4132, and 1495, at higher stress levels (25% field capacity). Meanwhile, accessions 5717 and 4064 exhibited relatively much lower fold changes, with 10 and 8, respectively.

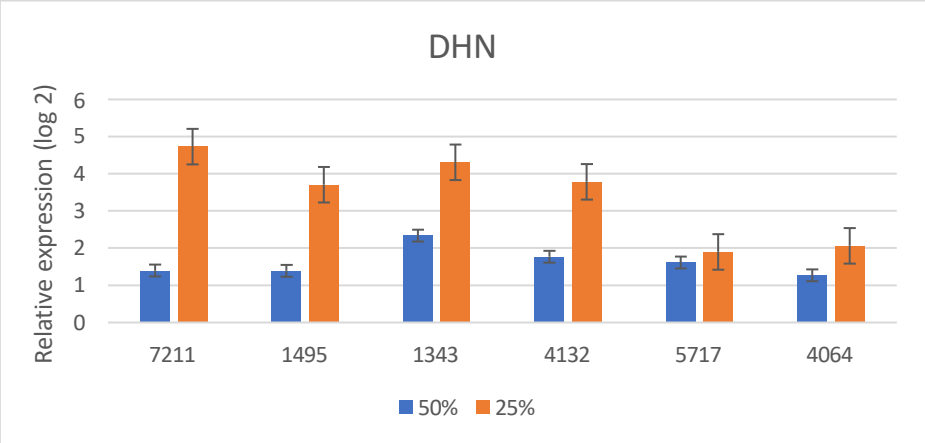


Figure 49. Differential expression of dehydrin gene in response to drought stress under 50% and 25% FC.

The regulatory functions of ABA, MAPK, calcium, and proteins involved in the regulation, along with transcription factors, actively participate in the complex process of dehydrin expression under abiotic stress. This finding aligns with previous research that underscores the regulatory influence of ABA, MAPK, calcium, and proteins, along with transcription factors, in modulating dehydrin expression during abiotic stress. Specifically, Sun *et al.* (2021) highlighted the positive correlation of the DHN gene with environmental stress factors such as drought, cold, and salinity. Additionally, the study by Li *et al.* (2023) demonstrated that overexpression of CaDHN2 in Arabidopsis, which interacts with CaGDP-L-galactose phosphorylase, enhances drought tolerance by boosting antioxidant enzymatic activities and reducing ROS content. Similarly, Chiappetta *et al.* (2015) reported that the upregulation of

OesDHN in oleaster is modulated under progressing drought conditions. Collectively, these studies support the pivotal role of DHN genes in enhancing drought tolerance through complex regulatory networks and stress-responsive pathways.

Osmotin was first discovered in tobacco, *Nicotiana tabacum* var. Wisconsin 38, and its identical proteins, known as osmotin-like proteins (OLPs), were subsequently found to be ubiquitous throughout the entire plant kingdom. These proteins serve as functional markers, acting as defense proteins against pathogens and inducing stress tolerance to both biotic and abiotic stresses (Manghwar & Hussain, 2022). The osmotin gene encodes the osmotin protein, enabling plants to cope with water scarcity as part of their adaptive response. This adaptation is imparted through the multifaceted functions of osmotin, including osmoregulation, cellular protection, and its potential role in defense mechanisms (Chowdhury *et al*., 2017).

The relative expression of the gene OSM in the present study showed an increase in accessions 4132, 1343, 1495, and 7211 with 10, 9, 8, and 5 fold change respectively under 25% field capacity. Accessions 5717 and 4064 also exhibited the same level of fold change, with values of 4.9 and 4.3, respectively.

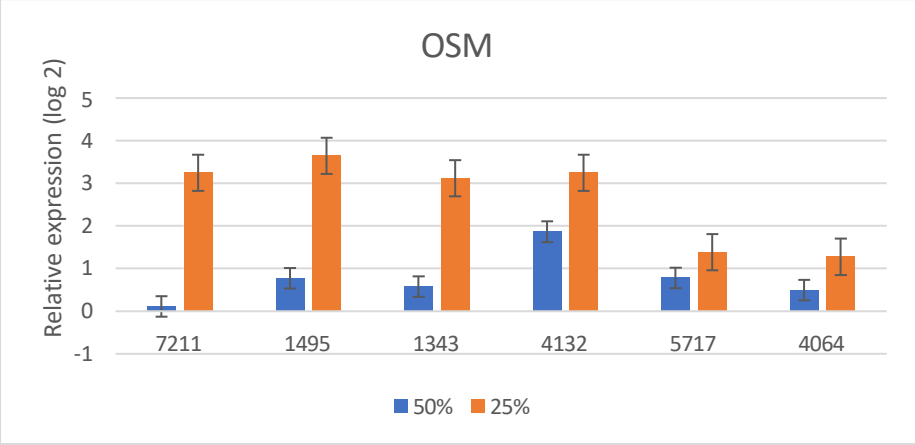


Figure 50. Differential expression of osmotin gene in response to drought stress under 50% and 25% FC.

These results are akin to the study, where the introduction of the osmotin gene from tobacco into tomato plants (*Solanum lycopersicum*) via Agrobacterium-mediated transformation enhanced resistance to salt and drought stresses (Goel *et al*., 2010). The transgenic tomato plants demonstrated increased relative water content,

chlorophyll content, proline content, and leaf retraction compared to wild-type plants under stress conditions, corroborating the role of osmotin in improving drought tolerance. The similarity between the present study and the above cited study underscores the pivotal function of the OSM gene in mediating plant responses to drought stress, highlighting its potential for enhancing drought resilience through genetic and biotechnological approaches.

Withholding irrigation and maintaining field capacity at 50% and 25% led to a significant reduction in the CT values for the respective moisture levels and a substantial elevation in the relative gene expression of GST in the accessions 1343 (8.6-fold), followed by 4132 (5.17-fold), 1495 (4.4-fold), and 7211 (3.9-fold). The higher CT values compared to their control indicated reduced relative expression in 5717 (1.8-fold) and 4064 (1.3-fold). Genotype 5717 maintained comparable similarity in gene expression at both 50% and 25% field capacity levels, while genotype 4064 exhibited decreased expression at 25% (1.3-fold) compared to 50% field capacity (1.9-fold), which was relative to their control.

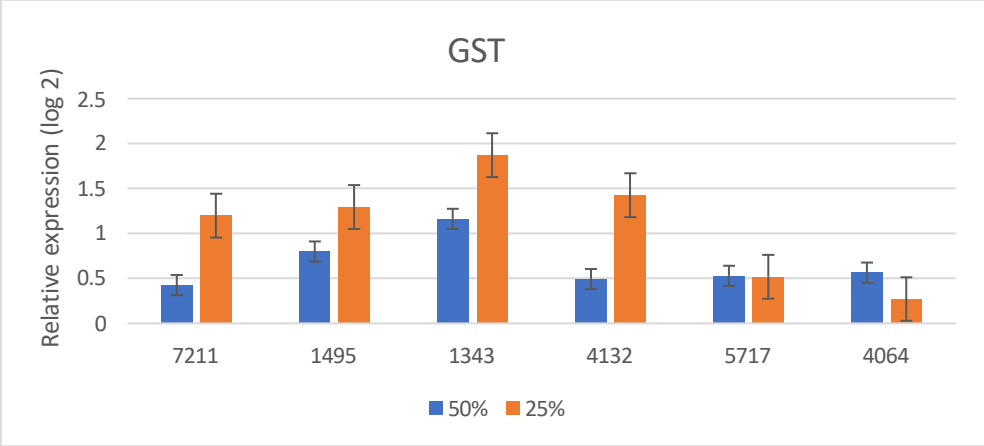


Figure 51. Differential expression of glutathione S transferase gene in response to drought stress under 50% and 25% FC.

These findings are consistent with the study by Kang *et al*., (2013) where exogenous application of salicylic acid upregulated GST and alleviated drought-induced growth retardation. Similarly, Islam *et al*. (2019) identified upregulation of GST transcripts in *Capsicum annuum* under various abiotic stresses, while Ahmad *et al*. (2020) observed differential GST expression in *Phaseolus vulgaris* under drought and salt

stress. The similar variation found in a study related to drought tolerance in maize plants, where ZmGST26 was downregulated in key tissues, implying a potential role in mitigating the impact of drought, possibly through regulatory mechanisms to conserve resources or minimize stress-induced damage (Jiang *et al*., 2022). Gene expression in plants may vary based on the type of plant tissue, varieties, and environmental factors. This variability is evident in the present study and the related works discussed here.

The expression of Peroxisomal (S)-2-hydroxy- acid oxidase GLO 1 like under 50% and 25% field capacity was investigated among the selected genotypes, and the highest increase of 60.5 fold change in the expression was noticed in the genotype 1495 followed by the genotypes 7211 (34.3-fold), 1343 (37.2-fold), 4132 (25.4-fold) and 5717 (22.9-fold). The minimum fold change in the expression of PHAO-I was shown by the genotype 4064 (3.2 and 3.4 fold changes at 50% and 25% field capacity compared to the control.

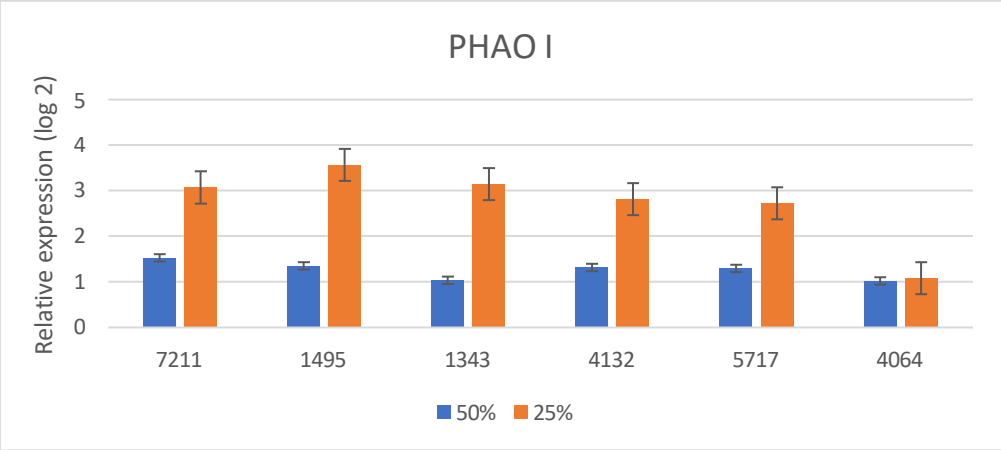


Figure 52. Differential expression of peroxisomal (S)-2-hydroxy- acid oxidase 1 gene in response to drought stress under 50% and 25% FC.

These findings align with the reference study where metal toxicity, such as manganese stress, enhanced the regulation of GLO1, aiding in oxidative damage recovery (Liu *et al*., 2019). The correlation between PHAO-I transcript levels and varying light intensities, as reported by Libao *et al*. (2020), further supports the notion that GLO1 expression is responsive to different environmental stressors. Together, these studies emphasize the crucial role of GLO1 in mediating stress responses, demonstrating its

potential as a key factor in enhancing drought tolerance by mitigating oxidative damage and adapting to various abiotic stresses.

Ascorbate peroxidase detoxifies reactive oxygen species, which are predominantly generated under unfavourable abiotic as well as biotic stress conditions. Moreover, it plays a vital role in plant growth and development. The subcellular compartments—chloroplasts, mitochondria, peroxisomes, and the cytosol have been identified as sites for ascorbate peroxidase.

The APX 6 gene was upregulated in all genotypes viz. 7211 (4.2-fold), 4132 (4.6-fold), 1495 (3.5-fold), 1343 (3.4-fold), 5717 (2.9-fold), and 4064 (3.5-fold) and all the genotypes showed almost similar fold change in expression. Tolerant and susceptible genotypes showed similar expression levels.

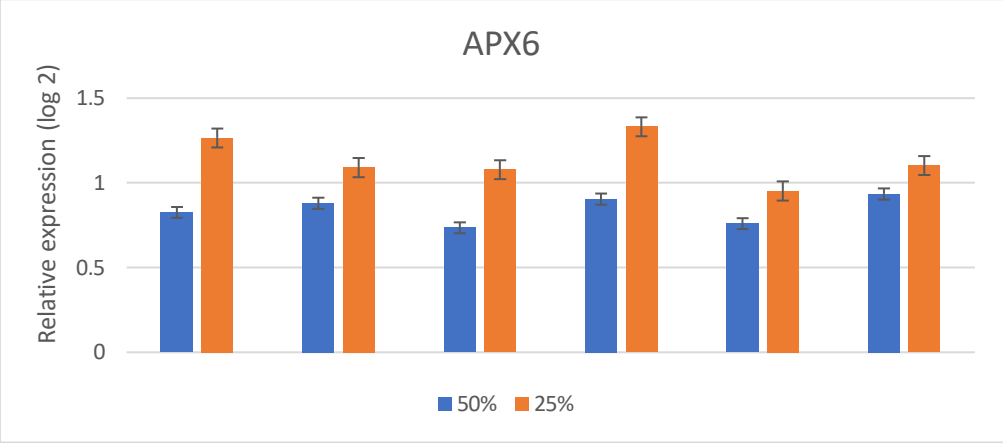


Figure 53. Differential expression of ascorbate peroxidase 6 cytosolic gene in response to drought stress under 50% and 25% FC.

This aligns with earlier findings showing differential transcript accumulation of APX isoforms in various wheat genotypes under mild water stress, where cytosolic APX1 was upregulated in both tolerant and susceptible genotypes (Sečenji *et al.*, 2010). The study on *Capsicum annuum* L. also supports the differential expression of APX in response to various environmental stresses, highlighting the gene's role in stress response mechanisms (Pang *et al.*, 2023).

The distinct expression trends of APX isoforms in different organelles, such as the cytosolic APX6 in sugarcane linked to immunity against pathogens *Pseudomonas*

solanacearum and *Fusarium solani* var. coeruleum (Liu *et al*., 2018), further corroborate the present findings. Additionally, the overexpression of ascorbate peroxidase isoforms in coffee progenies under water scarcity, enhancing gas exchange and photochemical activity (de Oliveira Santos *et al*., 2021). These references collectively validate the present study's results, demonstrating the crucial role of APX genes in mediating drought stress responses across different plant species and conditions.

The investigation on the HSP70 gene, subjected to drought stress, explored mitigating the impact of water scarcity in plants by protecting cellular integrity, enhancing water retention, being involved in stress signaling pathways, osmotics adjustment, and antioxidant defense mechanisms. The gene HSP70 was expressed at almost the same level, upregulated under 50% FC compared to the control in the genotypes 7211 (12.7-fold), 1495 (13-fold), 1343 (17-fold), and 4132 (16.6-fold). Subsequent increases in its expression under 25% FC was noticeable only in the genotypes 7211, 1495, and 4132, with 26.9-fold, 32-fold, and 32.6-fold, respectively. However, the genotype 1343 showed no increase at 25% FC compared to its expression at 50% FC. A similar trend with somewhat lower gene expression was detected in genotype 5717, with 5.6-fold and 6.2-fold at 50% and 25% FC, respectively, as well as in genotype 4064, with 6.5-fold and 7.4-fold for the respective moisture levels.

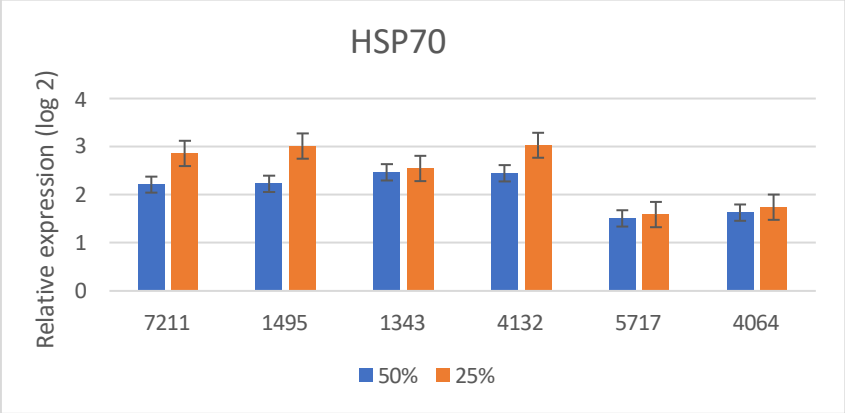


Figure 54. Differential expression of heat-shock protein gene in response to drought stress under 50% and 25% FC.

These results are supported by other studies, such as the investigation on drought-tolerant tomato cultivar *Solanum lycopersicum* L, which demonstrated that silencing

HSP70 led to increased cell membrane leakage and reduced relative water content, indicating the gene's role in maintaining cellular stability under stress (Aghaie & Tafreshi, 2020).

Moreover, the study on perennial ryegrass found that combined heat and drought stress invoked higher HSP70 expression than when each stress was applied separately, highlighting the gene's responsiveness to multiple stress factors (Rahman *et al*., 2022). Additionally, the significant expression of HSPs in response to various stresses, including drought, cold, hypoxia, and UV light, suggests that HSP70 serves as a chaperone, protecting other proteins involved in plant defense mechanisms (Khan *et al*., 2019; Hamdin *et al*., 2020). These findings collectively validate the present results, emphasizing the crucial role of HSP70 in drought stress tolerance and its potential as a target for enhancing stress resilience in plants.

The drought-responsive genes peroxidase 5 and peroxidase 12 were gradually upregulated at 50% and 25% FC, with an increased PX5 expression observed in all accessions. However, PX12 was downregulated in accessions 5717 and 4064, with fold changes corresponding to 1.3 and 1.5, respectively. Comparatively, higher fold changes with upregulation were demonstrated by the accessions 4132 (3.29-fold), 7211 (2.3-fold), 1343 (1.69-fold), and 1495 (1.49-fold). However, there was a substantial rise in expression of PX5 in Acc 1343 (19.29-fold), followed by 1495 (5.9-fold) and 7211 (4.79-fold). Significant upregulation was not observed in the accessions 4064 (2.19-fold), 5717 (3.46-fold), and 4132 (3.68-fold).

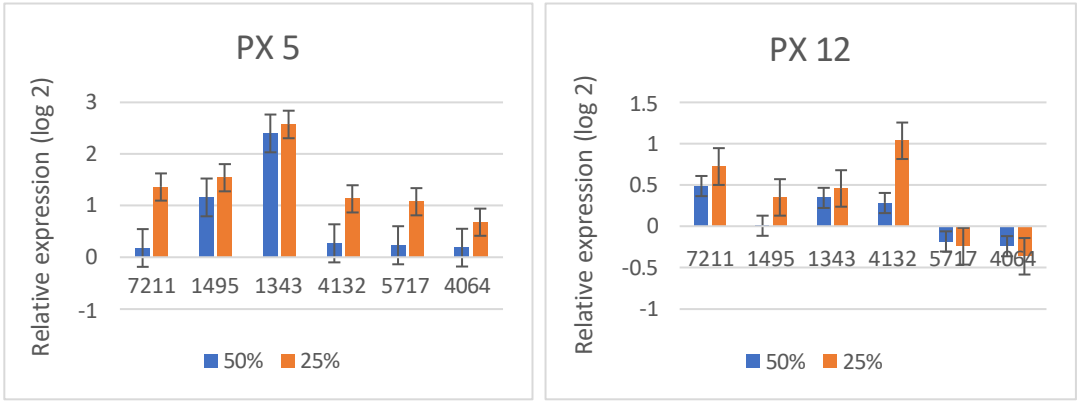


Figure 55. Differential expression of peroxidase 5 and peroxidase 12 genes in response to drought stress under 50% and 25% FC.

This is consistent with findings from other studies that underscore the critical role of peroxidases in plant stress responses. For instance, the overexpression of peroxidase 5 in black pepper roots has been associated with enhanced plant signaling for growth and development, as well as adaptation to oxidative stress (Hamdin *et al*., 2020). The peroxidase 5 and peroxidase 12 are specific members of the peroxidase gene family. Both of them play a significant role in antioxidant defense and plant stress responses. Their transcript accumulation can vary based on the type of stress, its intensity, plant species, and environmental factors.

Similarly, peroxidase 12 is recognized for its role in inducing resistance against the fungus *Ustilago maydis* in maize, contributing to the plant's antioxidant defense mechanisms (Hemetsberger *et al*., 2012). The peroxidase gene family in the rice cultivar displayed distinct regulation, with induced expression observed in members such as POX5, POX8, and POX22 when subjected to *Xanthomonas oryzae* infection. Notably, only two genes, POX8 and POX22, were predominantly expressed during both pathogen interaction and mechanical stress, in contrast to POX5 (White, 1997). The large multigene class III family encodes proteins in higher plants, such as barley, which are triggered by infectious rusts and mildew, catalyzing the hydrogen peroxide present during the infection (González *et al*., 2010). Moreover, wheat peroxidase genes like TaPRX-2A have shown enhanced expression under drought, salt stress, and ABA treatments, indicating their significant role in stress tolerance (Su *et al*., 2020). A microarray experiment has also shown that the wheat peroxidase was significantly expressed under drought-stressed conditions (Lendvai *et al*., 2010).

These reports collectively support the present findings, demonstrating that the upregulation of peroxidase genes, particularly PX5, is a crucial adaptive response to drought stress, enhancing the plant's ability to cope with adverse environmental conditions.

The gene AQUA exhibited down regulation, in the accessions 7211 (18.12-fold), 1495 (9.5-fold), 1343 (6.6-fold), and 4132 (9.6-fold). In comparison, lesser downregulation was seen in accessions 5717 (4-fold) and 4064 (5-fold).

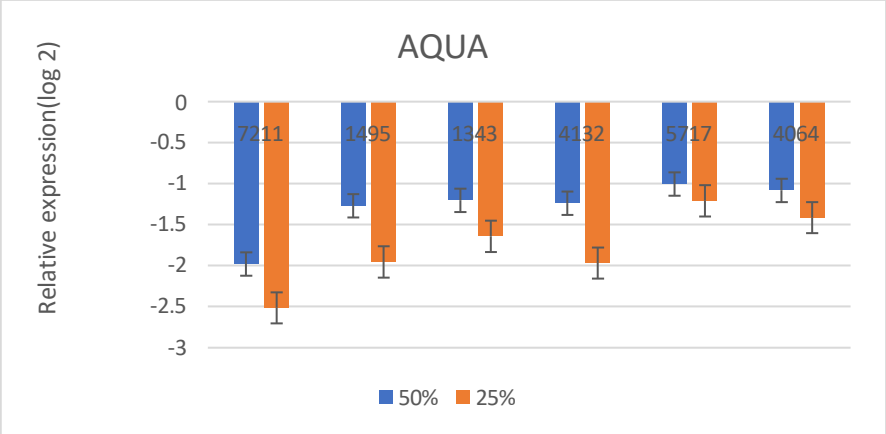


Figure 56. Differential expression of aquaporin protein in response to drought stress under 50% and 25% FC.

Aquaporins are integral membrane proteins that facilitate the movement of water molecules and other small molecules across the cellular membrane. A complex regulatory mechanism of aquaporins was observed under drought stress conditions. The expression of the candidate gene varied among different plants under various environmental conditions. The predominant drought-induced expression was shown by the members of subfamilies of the AQP family, consisting of plasma membrane intrinsic protein (PIP) and tonoplast intrinsic protein (TIP) (Shivaraj *et al*., 2021). Other members of the aquaporin subfamily, such as SIPs, NIPs, and XIPs, have not been well-studied to exploit their drought tolerance functions in plants, as compared to the PIPs and TIPs (Patel & Mishra, 2021).

The present study supports the report that that timely and sufficient downregulation of specific aquaporins contributes to water conservation during drought stress (Zupin *et al.*, 2017). Under the same environmental stress conditions, a similar pattern of expression was found, where arbuscular mycorrhizal plants regulate PIP genes, downregulating them and significantly reducing water loss to minimize membrane permeability (Porcel *et al*., 2006).

The NAC transcription factor plays a crucial role not only in plant growth and development but also in responding to abiotic stresses. Understanding the nuances of NAC transcription factor (TF) gene expression in drought-tolerant plants provides insights into the molecular mechanisms that contribute to their resilience. This

knowledge can be leveraged in breeding programs and biotechnological approaches to enhance drought tolerance in crops (Nakashima *et al*., 2012).

The variation in the expression of the transcription factor NAC was observed in the present study with both upregulation and downregulation, differentiating the genotypes with drought-tolerant and susceptible traits. The upregulated expression, was found in the accessions 1343, 1495, 4132, and 7211, with fold changes of 57.6, 28, 18.9, and 12.3, respectively, at 25% FC. The accessions 5717 (3.6 fold) and 4064 (1 fold) showed downregulation under 25% FC.

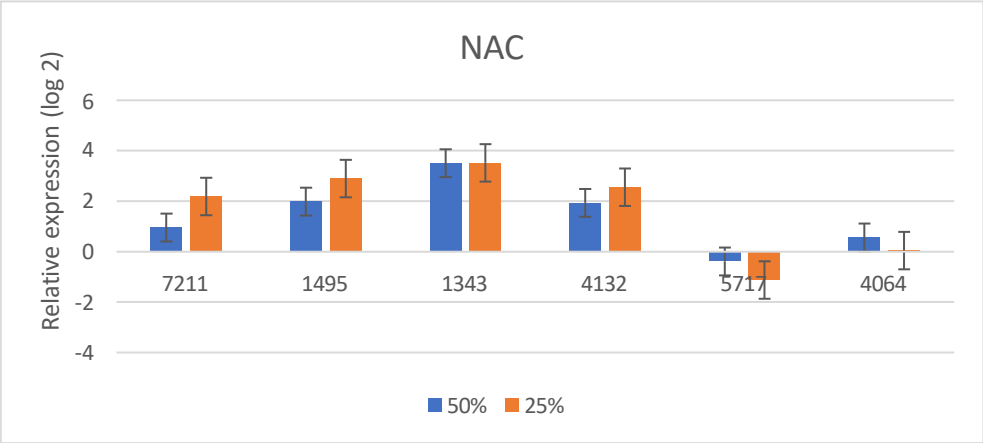


Figure 57. Differential expression of NAC transcription factor in response to drought stress under 50% and 25% FC.

NAC transcription factor, TaNAC29 from bread wheat (*Triticum aestivum*). Showed enhanced expression, thereby conferring tolerance to salt and drought stresses, and elicited an ABA hypersensitive response at both the vegetative and reproductive periods (Huang *et al*., 2015). The NAC transcription factor, GmNAC12, isolated from the soybean (*Glycine max*), showed a 10-fold upregulation under drought stress conditions compared to its control (Yang *et al*., 2022). Li *et al*. (2023) reported a negative regulation of MdNAC29 in apple plants, calli, and tobacco. This regulation, which modulates the repression of drought resistance genes, leads to a reduction in drought tolerance. The NAC transcription factor confined to the nucleus in the rice cultivar named *Nicotiana benthamiana* is ONAC066, which has demonstrated a positive correlation with drought and oxidative stress, thereby enhancing tolerance

through the upregulation of stress-responsive genes and the modulation of physiological processes. (Yuan *et al*., 2019).

The findings of the present study align with the general consensus that NAC transcription factors are crucial for drought tolerance, though the specific regulatory roles may vary depending on the plant species and environmental conditions. The observed upregulation in tolerant genotypes indicates the complex regulatory mechanisms of NAC transcription factors in mediating drought stress responses.

In plants, like in all other eukaryotes, bZIP (basic leucine zipper) transcription factors constitute a family of proteins that participate in transcriptomic regulation. These factors are characterized by a conserved DNA-binding domain known as the bZIP domain, which consists of a basic region followed by a leucine zipper motif. An antagonistic expression to the NAC is the bZIP transcriptional factor, as identified by upregulation in Acc 5717 (1.95-fold) and Acc 4064 (2.22-fold). The expression in the remaining accessions was downregulated with fold changes of 11.5 (7211), 2.7 (1495), and 2.3 (1343) at 25% FC. While the Acc 4132 maintained the expression same as control.

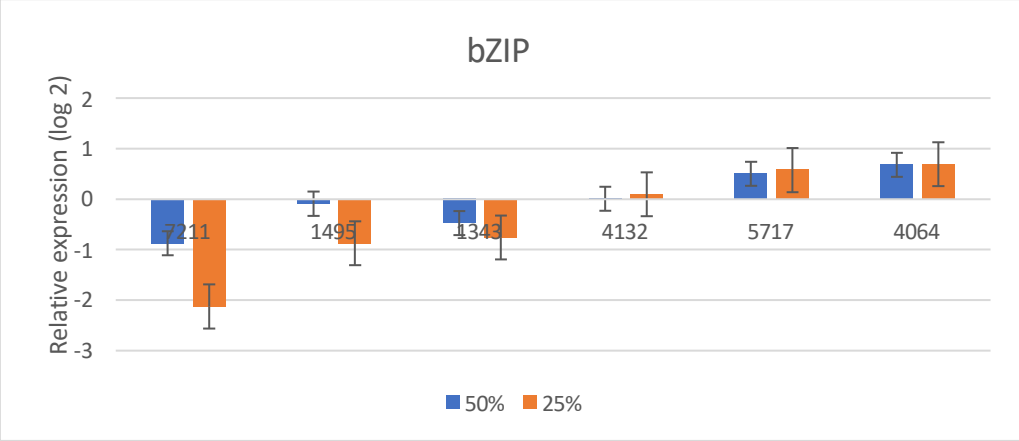


Figure 58. Differential expression of basic leucine zipper transcription factor in response to drought stress under 50% and 25% FC.

Contrary to these findings, previous research has consistently shown that bZIP transcription factors are associated with improved stress tolerance. For instance, in *Chrysanthemum grandiflora*, bZIP overexpression imparted drought and salt stress

tolerance, highlighting favorable morpho-physiological traits (Liu *et al*., 2022). Diverse bZIP transcription factors (191) were discovered in wheat (*Triticum aestivum*), exhibiting differential expression under drought, heat, and salinity. Similarly, they enhanced gene expression to confer tolerance under such conditions (Agarwal *et al*., 2019). The overexpression of GhABF2, a bZIP transcription factor in cotton (*Gossypium hirsutum* L.), led to significant tolerance in drought and salt stress by regulating genes associated with abscisic acid (ABA). A similar trend was identified in *Arabidopsis thaliana* as well (Zhang *et al*., 2020). These recent findings do not align with the present study as tolerant accsession didn't show increased expression and the differentiation based on the expression of bZIP was more pronounced in susceptible ones, in contrast to these studies.

This suggests a complex regulatory mechanism of bZIP transcription factors in drought tolerance, potentially influenced by specific genotype-environment interactions. Thus, while bZIP transcription factors are generally linked to enhanced stress tolerance, the present study highlights the need for further investigation into their differential expression and role in specific genotypes under varying drought conditions.

The gene apetala 2 (AP2) is a transcription factor, a member of the AP2/ERF (Apetala2/Ethylene Response Factor) family involved in the developmental regulation of plants. It also plays a crucial role in various environmental stresses, including drought stress. The AP2 gene was upregulated in both all the genotypes. No variation in expression levels associated with drought tolerance could be found in the AP2 gene. Genotype 1495 showed the highest expression (7.89-fold increase), followed by 4064 (5.86-fold), 5717 (5.7-fold), 1343 (5.35-fold), 7211 (5.17-fold), and 4132 (5.1-fold).

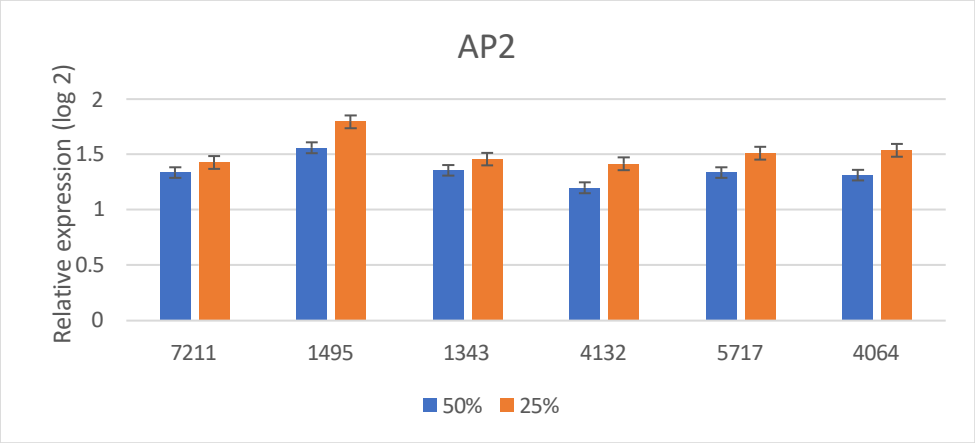


Figure 59. Differential expression of apetala 2 transcription factor in response to drought stress under 50% and 25% FC.

The AP2 gene expression was reported to be upregulated in response to water scarcity in several research reports. It can modulate various processes such as the regulation of stomatal opening, increased root development, and the induction of stress-responsive genes. RT-qPCR successfully elucidated the significant increase in AP2 gene expression in maize leaves during their vegetative stage under the withholding irrigation regime (Abdul Mohsin, 2023). An effective environmental stress response as resistant confirmed for the AP2/ERFs genes in the root and shoot tissues of sunflower (Najafi *et al*., 2018).

The AP2 transcription factor functions in the context of stress-responsive pathways in rice varieties, enabling tolerance in challenging environments. The regulatory significance of AP2 in orchestrating the adaptive responsive plants to water deficit conditions, shedding light on its potential as a molecular marker for drought tolerance. The AP2 factor is detected in sunflower (*Helianthus annuus* L.) under different conditions like drought, heat, salinity, and cold stress, characterized by increased transcript levels (Najafi *et al*., 2018). A comparable pattern was noted in the rice (*Oryza sativa*) variety (Zhang *et al*., 2020); transgenic *Trifolium alexandrinum* L (Abogadallah *et al*., 2011); timber tree species, *Pinus massoniana* Lamb (Sun *et al*., 2022). The present study's findings on AP2 gene upregulation across different genotypes further substantiate the gene's pivotal role in mediating plant resilience to drought stress, aligning well with the existing literature.

The DREB (Dehydration Responsive Element-Binding protein) is a transcription factor, part of the major AP2/ERF (APETALA2/Ethylene Response Factor) family, and it plays a vital role in the regulation of the environmental stress response. DREB proteins are capable of binding to specific target DNA sequences, known as DRE/CRT (Dehydration Responsive Element/C-Repeat), in the promoter regions of target genes. Upon binding to the regulatory region, they activate the genes that are involved in enhancing the plant's tolerance to dehydration, osmotic regulation, and protection of cellular structures (Dossa *et al*., 2016).

The DREB gene expression was upregulated in all the genotypes. The elevated gene expression with decreasing moisture levels (50% and 25% FC) was noticed. The peak expression with a 69.5-fold change was observed in the genotype 1343, followed by 4064 (47.5-fold), 1495 (26.5-fold), 7211 (25.1-fold), 4132 (23.2-fold), and 5717 (16.2-fold). Drought tolerance-associated gene expression could not be observed in the selected genotypes.

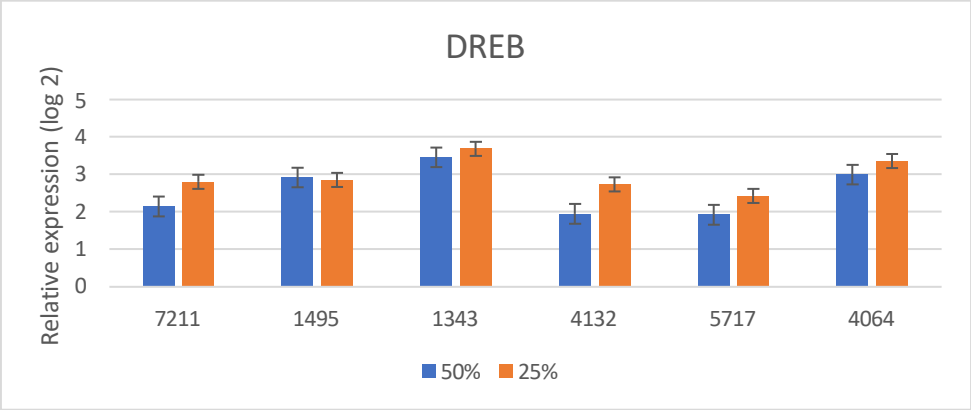


Figure 60. Differential expression of dehydration-responsive element-binding protein in response to drought stress under 50% and 25% FC.

The tissue-specific expression of PsDREB2A exhibited distinct patterns in the leaves and roots of dehydrated pea plants. The highest elevation of expression, with a 2.2-fold change, was found in the roots during prolonged drought conditions, whereas no significant change was observed in the leaves under prolonged stress conditions compared to the control (Herrmann & Bucksch, 2014). The DREB subfamily includes 576 genes with the AP2 domain, incorporating 32 new proteins. Molecular simulations revealed TraesCS2B02G002700 as a stable DREB interacting with DNA,

and the study predicted six target genes regulated by this transcription factor, offering insights into potential salt stress response mechanisms in plants (Hassan *et al*., 2022). An investigation into the qPCR transcription analysis of both DREB and APX, separately and in a synergetic manner, revealed that their overexpression in an indica rice cultivar subjected to drought stress enhanced its drought tolerance (Sandhya *et al.*, 2021). Typically, biotic and abiotic stresses enhance gene regulation among Arabidopsis (under salt and drought stresses) (Ma *et al*., 2015), *Sorghum bicolor* (under cadmium and salt stresses) (Akbudak *et al*., 2018), and wheat species (under drought stress) (Rustamova *et al*., 2022).

The MYB gene is distributed widely in both prokaryotes and eukaryotes. It encodes transcription factors, which are proteins that regulate the expression of target genes. MYB proteins specifically bind to the target DNA sequence, thereby regulating the transcription of candidate genes. The MYB gene plays a crucial role in plant growth and development, as well as in the mediation of regulatory responses to biotic and abiotic stress (Chen *et al.*, 2018; Wang *et al*., 2021; Li *et al*., 2019). The transcriptional factor MYB expression icnreased with decreasing soil moisture content (50% and 25% FC, respectively). The genotype 1343 under 25% FC showed highest expression (70.5-fold over control) which was on par with its expression at 50% FC. This was followed by accessions 1495 (47.8-fold) and 7211 (47.5-fold) at 25% FC. Almost equivalent gene expression at 50% FC with fold changes of 7.1-fold (Acc 4132), 9.8-fold (Acc 5717), and 8.6-fold (Acc 4064), and further upregulation for the respective genotypes at 25% were 33.8-fold, 25.2-fold, and 12.2-fold was observed.

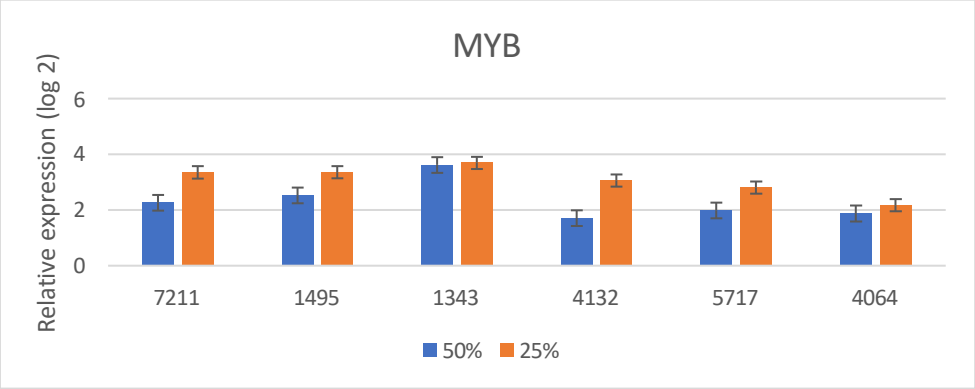


Figure 61. Differential expression of myeloblastosis oncogene in response to drought stress under 50% and 25% FC.

The present study underscores the pivotal role of the MYB gene in enhancing drought tolerance in plants. Widely distributed in both prokaryotes and eukaryotes, the MYB gene encodes transcription factors that regulate the expression of target genes, playing a critical role in plant growth, development, and responses to biotic and abiotic stresses (Chen *et al*., 2018; Wang *et al*., 2021; Li *et al*., 2019). The study revealed that MYB gene expression increased with decreasing soil moisture content, with genotype 1343 showing the highest expression (70.5-fold over control) at 25% field capacity (FC), followed by accessions 1495 (47.8-fold) and 7211 (47.5-fold). Similar trends were observed at 50% FC and further upregulation at 25% FC for genotypes 4132, 5717, and 4064. These findings correlate with previous research where the overexpression of MYB transcription factors, such as MYB5 in tobacco, LcMYB2 in Arabidopsis, and OsMYB6 in rice, significantly enhanced drought tolerance by increasing proline accumulation, reducing oxidative damage, and upregulating stress-related gene expression (Chen *et al*., 2015; Zhao *et al*., 2019; Tang *et al*., 2019). Additionally, the overexpression of PdMYB2R089 and PdMYB2R151 in Arabidopsis improved root and shoot growth, reduced oxidative stress, and regulated stomatal movement and seed germination under drought conditions, further supporting the present study's findings (Zhang *et al*., 2023). Collectively, these studies highlight the MYB gene's crucial role in mediating plant responses to drought stress, validating the observed upregulation and drought tolerance traits in the selected genotypes.

Figure 62. Genotypes maintained under (A) 25% field capacity, (B) 50% field capacity, and (C) 100% field capacity

4.8.2. Root to shoot ratio

The root-to-shoot ratio was determined for the genotypes 7211, 1495, 1343, 4132, 5717 and 4064, which were maintained under 25%, 50%, and 100% field capacity. Root-to-shoot ratios varied significantly among the genotypes under all the three moisture levels. An increase in the root-to-shoot ratio was observed with progressive water deficit in all the genotypes. The highest ratio was recorded under 25% FC, which ranged from 0.55 (Acc 4064) to 0.80 (Acc 7211) followed by the ratio under 50% FC which varied from 0.30 (Acc 5717) to 0.47 (Acc 4132). The lowest ratios were observed at 100% field capacity ranging from 0.25 (Acc 5717) to 0.31 (Acc 4132). Higher root growth must have contributed to drought tolerance, especially in genotypes 7211 and 1343, followed by 4132, which was supported by gene expression results also.

This is consistent with drought based screening of cultivars with deeper root system could be suggested as one of the inexhaustible strategies to mitigate and maintain the crop yield (Fukai & Cooper, 1995; O'Toole & Bland, 1987). Root growth of pepper seedlings was unaffected under different water withholding conditions, while the application of ABA showed reduced leaf growth (Leskovar & Cantliffe, 2019). However, higher lateral root growth and density were observed in hot peppers subjected to similar water-limited levels, with higher xylem density particularly noted in tolerant cultivars (Kulkarni & Phalke, 2009).

The rice genotypes Dular, Browngora, Aditya, and IR36 exhibited superior root-to-shoot ratios, making them tolerant due to their extensive root systems, thereby reducing yield loss (Hijam *et al*., 2012). This finding is consistent with the present study, which observed similar results in genotypes 7211, 1495, 1343, and 4132. A study on cotton varieties in terms of drought demonstrated that the genotypes with extensive root systems and improved growth traits under PEG treatment exhibited a higher root-to-shoot ratio (Mahmood *et al*., 2022). Drought stress significantly affected the root-to-shoot ratio in wheat genotypes, elevating it by 155.55% under severe drought stress compared to control conditions (Pouri *et al*., 2019). This is similar to the findings of the current study, which showed similar results in genotypes

with tolerant characteristics. Nahakpam (2020) found similar trends in rice genotypes, suggesting that genotypes with higher root-to-shoot ratios possess better adaptability to drought conditions.

Collectively, these studies support the present findings, indicating that genotypes with increased root growth relative to shoot growth, as evidenced by higher root-to-shoot ratios, are better equipped to withstand drought stress. This correlation between enhanced root development and drought tolerance provides a valuable strategy for selecting and breeding drought-resistant cultivars.

The genotypes 1343 and 4132 displayed better molecular mechanism associated with drought tolerance, indicating higher adaptive responses to water constraints. Genotypes 7211 and 1343 exhibited the highest drought tolerance ability, followed by 4132 and 1495. Consequently, these genotypes with better drought tolerance ability makes them promising candidates for further research and cultivation in water-limited environments.

Table 28. Effect of water stress on root to shoot ratio under 25%, 50% and 100% field capacity

Genotype ↓	25% FC	50% FC	100% FC
7211	0.804 a	0.452 ef	0.287 h
1495	0.588 cd	0.374 g	0.269 h
1343	0.720 b	0.416 efg	0.264 h
4132	0.638 c	0.468 e	0.307 h
5717	0.574 d	0.297 h	0.254 h
4064	0.547 d	0.403 fg	0.266 h

Evaluation of genetic diversity and gene expression traits in black pepper genotypes using principal component analysis

PCA analysis to the log_2 transcripts of sixteen drought-responsive genes at 25% FC of drought-stressed selected black pepper genotypes revealed significant genetic diversity, with 95.06% of the cumulative variance accounted for by the first four principal components, each having eigenvalues greater than 1 (Table. 29).

PC 1 (61.94%) was dominated positively by DHN (0.983), SOD CuZn (0.964), OSM (0.946), HSP70 (0.920), PX12 (0.906), NAC (0.888), GST (0.882), MYB (0.857), and root-to-shoot ratio (0.827), indicating a higher drought response under water-scarce conditions to mitigate the drought stress effects. In contrast, AQUA (-0.847), bzip (-0.834), and SOD FeMn (-0.562) showed significant negative contributions.

The significant positive association was displayed in PC 2 (16.58%) with SOD FeMn (0.791), PX5 (0.779), and DREB (0.666). The higher negative value was noted in the trait APC6 (-0.586). Hence, PC 2 emphasizes the traits that are associated with stress tolerance and developmental processes.

Principal Component 3 (10.20%) was significantly and positively influenced by DREB (0.542), APC6 (0.344), and the root-to-shoot ratio (0.328). Similarly, Principal Component 4 was significantly influenced by APC6 (0.329), DREB (0.424), NAC (0.350), and HSP70 (0.305). This reflects the roles of antioxidant defense, secondary metabolite production, and regulatory mechanisms in response to drought.

Regarding the genotypes, the highest positive correlation with PC1 was observed in genotype 7211, followed by 1343, 4132, and 1495, with values of 3.52, 2.17, 1.91, and 1.35, respectively. These genotypes exhibit upregulated expression of drought-tolerant genes such as DHN, SOD CuZn, OSM, HSP70, PX12, NAC, and GST. This upregulation helps protect cells from dehydration, regulate oxidative stress, maintain osmotic balance, and stabilize proteins. Additionally, these genotypes have a higher root-to-shoot ratio, allowing them to optimize water uptake from deeper soil layers, which further contributes to their drought resilience.

Similarly, a gene expression study conducted to identify drought-tolerant maize genotypes using PCA for differential expression analysis revealed that genes associated with osmoregulation, sugar metabolism, antioxidant activity, and phenylpropanoid biosynthesis pathways played an essential role in enhancing drought tolerance in the H21 line, which exhibited high principal scores (Gillani *et al*., 2023).

The PCA results clearly separated the dataset into two categories based on tissue type and stress responses. Drought-responsive miRNAs, such as miR2878-5p, miR159f, miR408-3p, and miR528-5p, target key copper-requiring proteins and showed

significant differential expression. Specifically, these miRNAs were up-regulated in drought-tolerant genotypes and down-regulated in sensitive genotypes as supported our present study's findings. They play a crucial role in regulating oxidative stress and stomatal closure, thereby enhancing drought tolerance (Balyan *et al*., 2017).

The highest positive coordinate for PC2 was achieved by genotype 1343, with a value of 3.21, followed by genotype 1495 with a value of 0.616. These genotypes demonstrated higher expression of SOD FeMn, PX5, and DREB, which play essential roles in drought tolerance. In contrast, genotypes 5717 (-4.11, -0.027) and 4064 (-4.85, -0.322) showed strongly negative coordinates for both PC1 and PC2. This indicates their susceptibility under 25% FC.

The heatmap (Fig. 75) clearly distinguished between variables, identifying drought stress-responsive genes and genotypes under drought stress at 25% FC regarding drought tolerance. This allows for the quick identification of genotypes 7211, 1495, 1343, and 4132 (vertical cluster 1), indicated in red, which have higher upregulated expression in their drought-responsive genes, especially in DHN, NAC (cluster 4), DREB, OSM, HSP70, MYB, and PHAO I (cluster 2). Iturriaga *et al*. (1996) reported that the expression of Myb-motif composed with three helix-turn-helix repeats that showed the upregulation under drought stress and ABA treatment.

A moderate level of higher expression was found in SOD CuZn, PX12 (cluster 3), and GST. In contrast, lower positive values were noted in genotypes 5717 and 4064 (vertical cluster 2). The genes AP2, APC6, PX5, and SOD FeMn (cluster 5) did not show differentiation between drought-tolerant and susceptible genotypes but still exhibited upregulation, as indicated by positive values. The blue color indicates the downregulation of all genotypes in the bZIP and AQUA (cluster 1) genes. However, a contradictory result was found in the transcription factor homeodomain-leucine zippers when overexpressed in drought-tolerant wheat genotypes (Cabello & Chan, 2012). There are several comparable studies corroborating present study's observations, which have reported identification of drought-tolerant genotypes at the molecular level through expression analysis (Martins *et al*., 2023; Xu *et al*., 2024; de Oliveira Santos *et al*., 2021).

PCA studied eleven standard drought-responsive genes of wheat genotypes that were consistently and differentially expressed under various experimental conditions. The key genes contributing to drought tolerance include those encoding auxin-responsive family proteins, NAD(P)H-dependent oxidoreductase 1, and glycosyltransferase. This was supported by a heatmap analysis in our study, demonstrating differential gene expression patterns in response to drought stress, which helped differentiate the genotypes as tolerant or susceptible. (Aqeel *et al*., 2022).

The same analysis differentially identified the genes dehydrin and glutathione peroxidase, which were significantly upregulated in response to water stress. These factors contribute to stabilizing membranes and improving yield under drought conditions, highlighting their critical role in maintaining stress responses (Gupta *et al*., 2012). PCA analysis revealed dehydrin gene upregulated expression in the genotypes 1343, 4132, 7211, and 1495, as reported earlier in the present study

Overall, the heatmap analysis effectively illustrated the key differences in gene expression patterns among genotypes, facilitating the identification of drought-tolerant genotypes such as 7211, 1495, 1343, and 4132, which have a higher root-to-shoot ratio, and providing valuable insights for future research and breeding programs.

Table 29. Eigen values and percentile variance of selected black pepper genotypes with drought-responsive genes under 25% FC

	PC 1	PC 2	PC 3	PC 4	PC 5
eigenvalue	10.53	2.82	1.73	1.08	0.84
% Variance	61.94	16.58	10.20	6.35	4.94
Cumulative percentage of variance	61.94	78.51	88.71	95.06	100.00
Variable	**Eigen vectors**				
SOD_FeMn	-0.562	**0.791**	0.230	0.024	0.065
SOD_CuZn	**0.964**	-0.066	0.184	-0.149	0.104
DHN	**0.983**	0.051	0.110	0.013	-0.137
OSM	**0.946**	0.056	-0.242	0.201	0.060
NAC	**0.888**	0.295	0.024	**0.350**	0.037
PX5	0.601	**0.779**	0.066	-0.165	0.027

	PC 1	PC 2	PC 3	PC 4	PC 5
PX12	**0.906**	-0.243	0.106	0.077	**0.322**
GST	**0.882**	0.394	0.052	0.032	0.252
APC6	0.642	-0.586	**0.344**	**0.329**	0.134
DREB	0.091	**0.666**	**0.542**	**0.424**	-0.273
bzip	-0.834	0.046	0.080	0.190	**0.509**
HSP70	**0.920**	-0.132	-0.182	**0.305**	0.103
AP2	-0.141	0.244	-0.834	0.401	-0.254
MYB	**0.857**	0.411	-0.168	-0.253	0.061
AQUA	-0.847	0.431	0.026	-0.070	**0.301**
PHAO I	0.746	0.206	-0.523	-0.313	0.170
Root to shoot ratio	**0.827**	-0.101	**0.328**	-0.338	-0.288

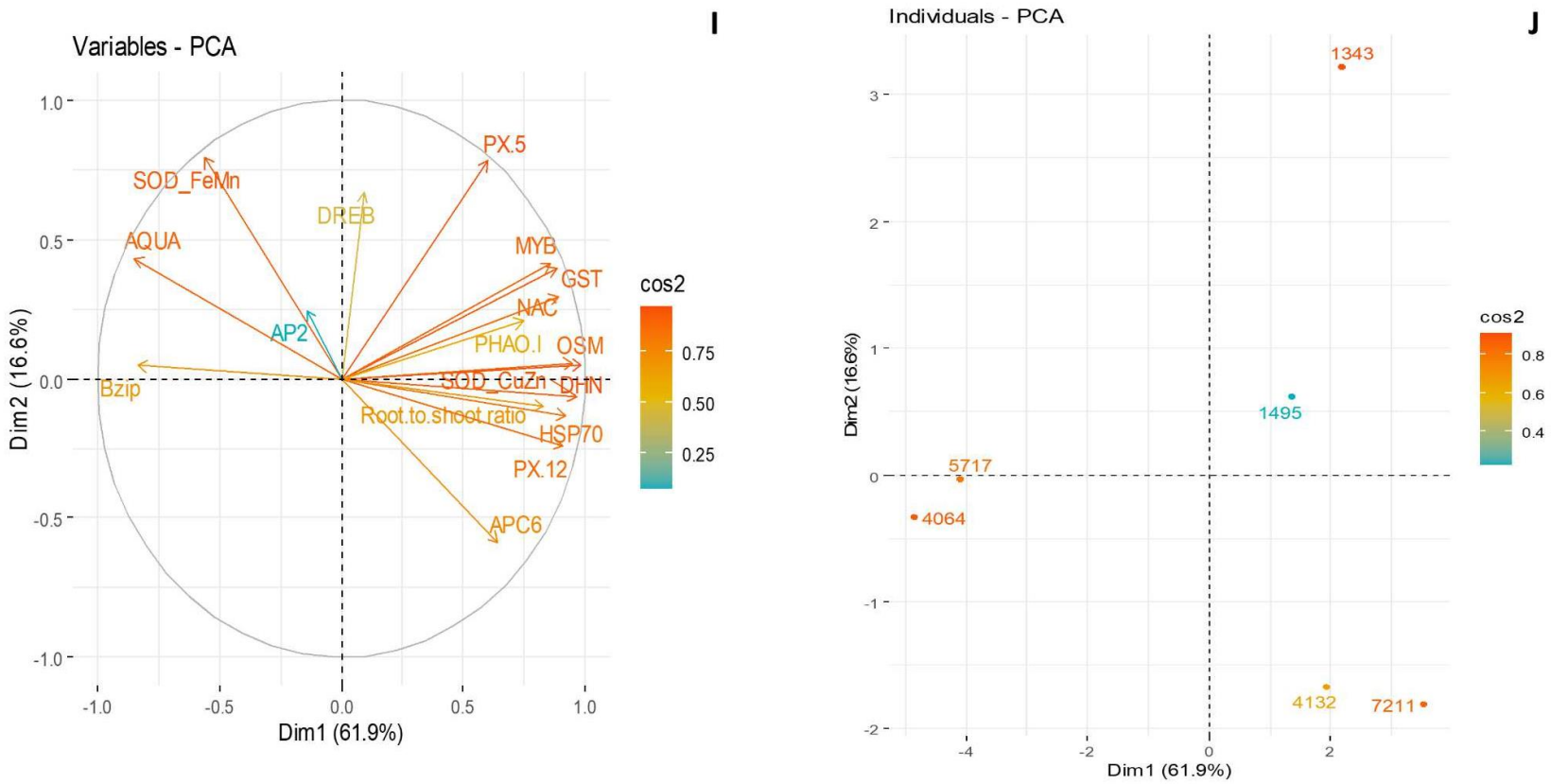


Figure 63. Distribution of black pepper drought responsive genes (I) and genotypes (J) across the first two principal components under 25%FC

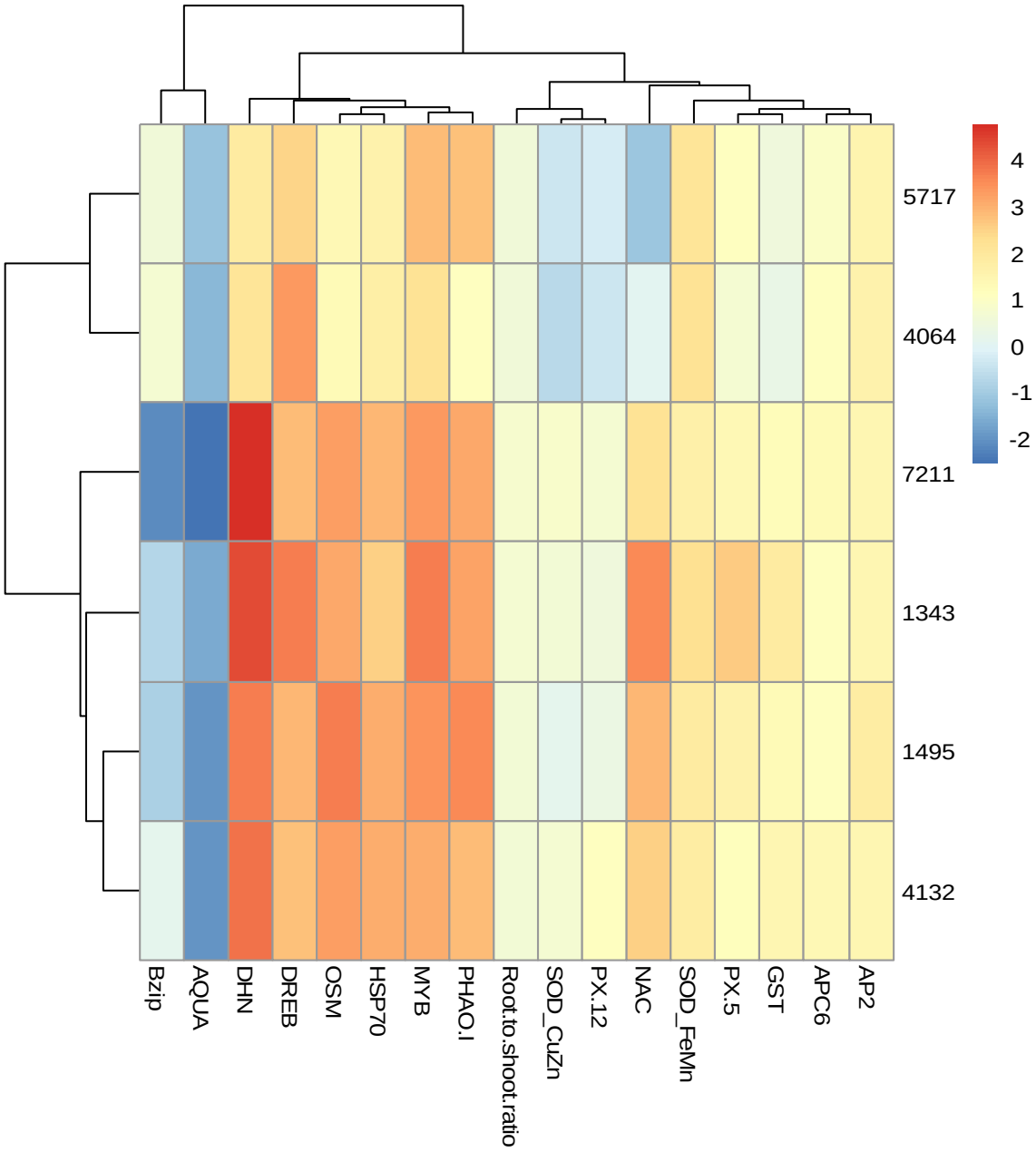
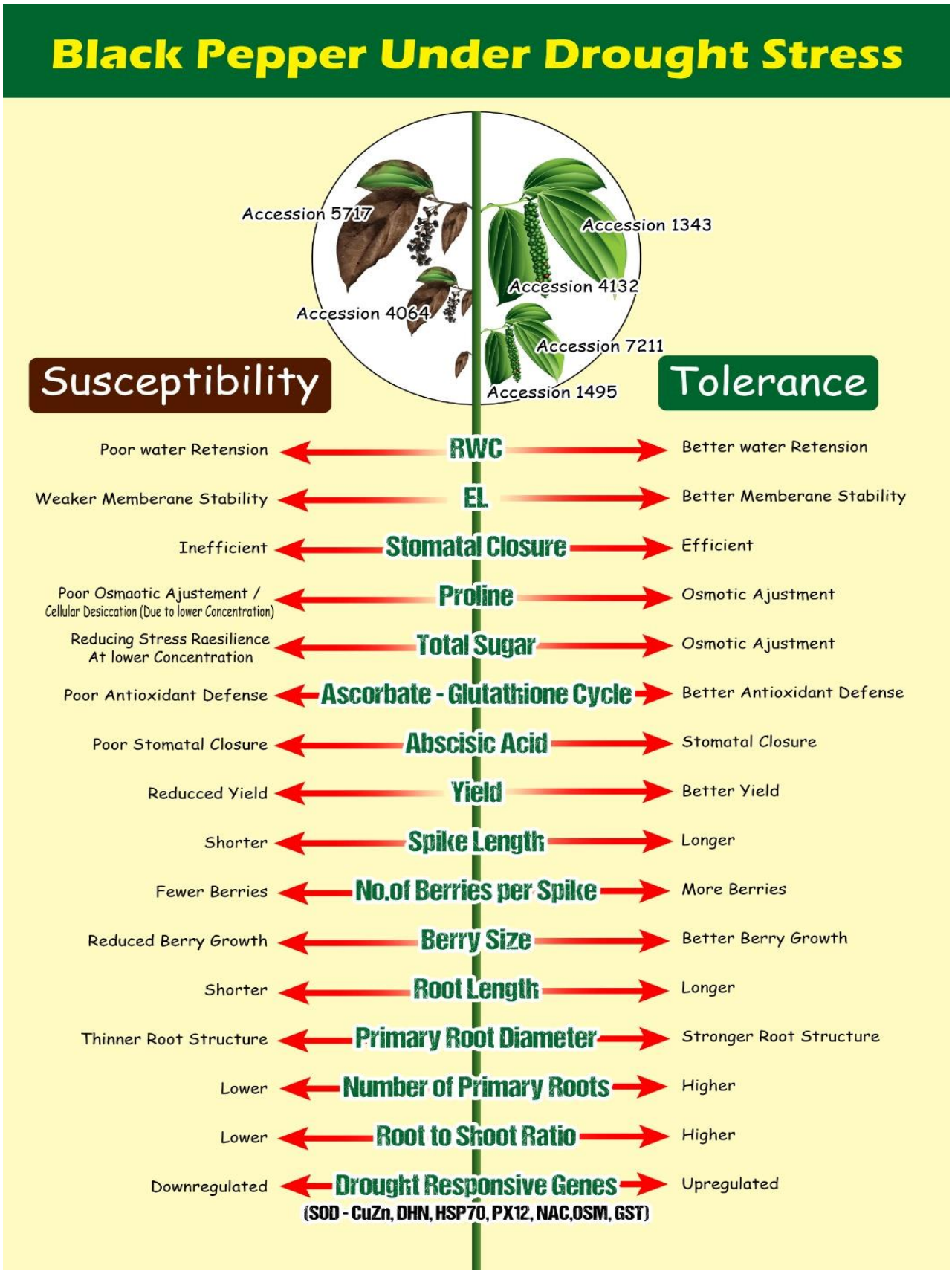


Figure 64. Heatmap analysis of differentially expressed drought-responsive genes among the selected black pepper genotypes under 25% FC


Black Pepper Under Drought Stress
Accession 5717
Accession 1343
Accession 4132
Accession 4064
Accession 7211
Accession 1495
Susceptibility
Tolerance
Poor water Retension
RWC
Better water Retension
Weaker Memberane Stability
EL
Better Memberane Stability
Inefficient
Stomatal Closure
Efficient
Poor Osmaotic Ajustement / Cellular Desiccation (Due to lower Concentration)
Proline
Osmotic Ajustment
Reducing Stress Raesilience At lower Concentration
Total Sugar
Osmotic Ajustment
Poor Antioxidant Defense
Ascorbate - Glutathione Cycle
Better Antioxidant Defense
Poor Stomatal Closure
Abscisic Acid
Stomatal Closure
Reducced Yield
Yield
Better Yield
Shorter
Spike Length
Longer
Fewer Berries
No.of Berries per Spike
More Berries
Reduced Berry Growth
Berry Size
Better Berry Growth
Shorter
Root Length
Longer
Thinner Root Structure
Primary Root Diameter
Stronger Root Structure
Lower
Number of Primary Roots
Higher
Lower
Root to Shoot Ratio
Higher
Downregulated
Drought Responsive Genes
(SOD - CuZn, DHN, HSP70, PX12, NAC,OSM, GST)
Upregulated

CHAPTER 5
SUMMARY AND CONCLUSION

The present study, entitled “Physiological and molecular characterization of black pepper genotypes subjected to limited water availability,” characterized forty black pepper genotypes to identify those exhibiting drought tolerance. The genotypes, 7211, 1495, 1343, 4132, 5717, 4064, 8052, 5083, 8060, 1491, 1487, 1093, 971, 5691, 1248, 6720, 1086, 1218, 5621, 5623 and 1439 were short listed from the pool of mentioned 40 black pepper genotypes in terms of morphological (leaf length, leaf width, leaf area, petiole length and internodal length) and physiological (stomata number and wax content) parameters.

According to the principal component analysis of 21 selected genotypes, a cumulative variation of 73.88% across five principal components was significantly impacted by yield-related traits. Genotypes with drought tolerance displayed unique trait patterns, particularly in leaf area, stomatal density, and wax content. The genotypes 7211, 1495, 1343, and 4132 demonstrated desirable morpho-physiological traits such as lower leaf length (< 7.8 cm), leaf width (< 6.2 cm), leaf area (< 50 cm^2), petiole length (< 3.5 cm), internodal length (< 4 cm), lower stomatal number (< 57), and higher wax content (> 9 $\mu g/cm^2$). These genotypes also exhibited yield-attributing traits, including a higher number of spikes per plant, spike length, peduncle length, berry size, number of matured berries per spike, number of immature berries per spike, test weight, 100 berry fresh weight, 10 spiked berry weight, and 10 rachis weight. In contrast, genotypes 5717 and 4064 exhibited the most susceptible traits to drought.

The dendrogram based on morpho-physiological and yield attributing traits, classified genotypes with similar traits into the same cluster. Genotypes with better drought-tolerant traits were part of clusters 2 (7211) and 3 (1495, 1343, and 4132), while cluster 1 contained genotypes showing susceptible traits. Pearson correlation analysis,

revealed positive correlation between morphological traits and stomata number, both of which were negatively correlated with yield-contributing traits and wax content.

The quality evaluation of 21 genotypes, involving the volatile oil and characterization of its compounds, oleoresin, piperine, sugar, and starch resulted in the categorization of genotypes into different ranges for each parameter. The low oleoresin content (<10%) was observed in genotypes 1495, 5717, and 4064; medium content (10-13%) was found in 1343 and 4132, while the maximum content (>13%) was observed in genotype 7211. Essential oil content was lower (<4.5%) in genotypes 5717 and 4064, while medium range (4.5% - 6%) was found in 7211, 1495, and 1343, and higher content (>6%) was observed in genotype 4132. Similarly, piperine content was categorized into lower range (<3.3% genotype 5717), medium range (3.3%-3.7% genotypes 4064 and 1495), and higher range (>3.7% genotypes 7211, 1343, and 4132). Low sugar content (<0.8 mg/g FW) was observed in genotypes 4064 and 5717, medium (0.8-1.5 mg/g FW) in 4132 and 1495, and higher sugar (>1.5 mg/g FW) in 7211 and 1343. Genotype 4064 exhibited low starch (<8%), while medium starch (8-12.5%) was found in 7211, 1495, and 4132, and higher starch (>12.5%) in 1343.

In conclusion, in terms of quality genotypes, 1343 and 4132 emerged as comparatively better performers and 4064 as poor performer under drought. These genotypes (1343 and 4132) exhibited medium oleoresin content, medium to high essential oil content, high piperine content, medium sugar content, and medium to high starch content. These findings suggest that these genotypes possess desirable characteristics in terms of their chemical composition, potentially making them suitable for cultivation under limited water conditions..

The identified major components, such as Myrcene, alpha-Thujene, Linalool, Camphene, alpha-Pinene, Sabinene, beta-Pinene, D-Limonene, Caryophyllene, and alpha-Phellandrene, were present in the medium to higher range of 1.73-2.7%, 1.5-3.2%, 0.6-1%, 0.1-0.5%, 4.6-7%, 13-22%, 7.5-11%, 15-21%, 23-40%, and 0.6-12%, respectively. Genotypes 5623, 1439, 7211, 1495, 1343, 1487, and 4132 consistently displayed comparatively higher proportions of these terpene compounds. Comparatively higher concentrations of these multiple compounds suggest that they

may be useful for diverse purposes such as medicinal/nutraceutical use. Likewise, genotypes 8060, 5717, 4064, 971, 1248, 5621, and 5083 had relatively lower concentrations these compounds. However, they could contribute to the overall chemical diversity within the chemical profile.

As the part of second objective, the selected genotypes (7211, 1495, 1343, 4132, 5717 and 4064) were further characterized at the physiological (RWC, EL and leaf photosynthetic pigments) and biochemical (proline, phenol, H^2O^2 content, lipid peroxidation, total sugar and starch, total protein, antioxidant enzyme activity, mineral elements and ABA) level. These parameters were measured after the stress imposition at 7days interval till 28 days after stress (DAS).

At 28 DAS, all genotypes experienced the highest decrease in relative water content (RWC) compared to control, with a relatively higher reduction observed in 5717 and 4064 compared to other genotypes, indicating higher drought susceptibility. However, genotype 1343 showed greater tolerance, which had the lowest electrolyte leakage at 43.25%. Chlorophyll content exhibited a genotype-specific response to drought stress, with some genotypes maintaining higher content, suggesting favorable traits. However, the highest decrease in chlorophyll b was reported in 5717 and 4064. The chlorophyll a/b ratio and carotenoid levels also varied significantly among genotypes, with a slower decrement in carotenoid levels observed in 4132 under drought stress, indicating its drought tolerance ability.

Comparatively higher accumulation of proline and sugar, reduced accumulation of hydrogen peroxide and malondialdehyde, lower degradation of starch, protein and minerals, and increased antioxidant enzymatic activity and ABA response were the biochemical mechanisms displayed by the accessions 7211, 1495, 1343, and 4132. These findings indicate their drought-resilient ability. Based on scoring of physiological and biochemical parameters, accessions 1343, 4132, 1495 and followed by 7211 (≥48.25) exhibited higher score, indicating favorable traits compared to the remaining genotypes.

The evaluation of selected genotypes based on yield and yield-related attributes indicated significant reduction due to drought stress. The genotypes 5717 and 7211

showed relatively lower yield compared to genotypes 1343, 1495, 4064, and 4132, which showed less reduction. Similarly, spike length, a crucial trait contributing to pepper yield, was reduced in all genotypes. However, genotype 4132 exhibited the least reduction, suggesting it as a favorable trait.

The number and size of berries per spike were negatively affected in all the drought-stressed genotypes, with lesser reduction observed in genotypes 1343, 7211, 4132, and 1495, showcasing their potential in drought tolerance. Other traits such as peduncle length, berry size, rachis, and berry weight also showed similar trends of reduction with varying levels among the genotypes. However, a more favorable response was consistently demonstrated by genotypes 4132 and 1343 in maintaining these attributes under drought stress conditions. Identification of genotypes with good yielding ability under drought condition is crucial for selecting and breeding drought-tolerant varieties with sustainable yield which ultimately leads to sustainable production.

The study of quality parameters in the selected genotypes revealed that genotype 7211 emerged as the most preferred one under drought. This genotype exhibited a lower reduction in volatile oil quantity (5.38%) and its constituents, thereby enhancing its adaptive response in such circumstances. Following closely behind, genotypes 4132 and 1343 displayed adaptive responses to drought tolerance by showing lower reductions in volatile oil content and its constituents. In contrast, genotypes 5717, 1495, and 4064 exhibited higher reductions in oil and its components, making them relatively less desirable in terms of drought tolerance. Furthermore, the same trend was not observed in berry starch and sugar content, which varied among the genotypes, except for 7211, which maintained higher starch and sugar content. Meanwhile, genotypes 4064, 1343, 1495, and 5717 exhibited higher reductions, indicating unfavorable traits in response to drought.

The variation in the volatile oil composition, investigated via GC-MS profiling, was significant in response to drought stress. The oil composition of genotype 7211 indicated small increase in certain chemicals and a large drop in alpha-Phellandrene under drought, which indicates better stability. Genotype 4132 showed moderate

stability, while genotype 1343 exhibited decrease in some key chemicals while maintaining increase in others. However, genotypes 5717, 1495, and 4064 responded with significant decrease in various volatile chemicals, making them less desirable for drought tolerance.

Root studies indicated that genotypes 4132 and 1495 exhibit characteristics such as reduced reduction in primary stem diameter and favorable secondary stem diameter under drought. They also demonstrated a higher root-to-shoot ratio, reflecting increased resource allocation to the root system as an adaptation to drought stress. The enhanced relative increase in root characteristics of 1495 and 1343 indicates their ability to absorb water from deeper soil layers. In contrast, genotypes 4064, 5717 and 7211 show lower drought tolerance compared to the aforementioned genotypes.

A comparative gene expression study was conducted in selected black pepper genotypes subjected to varying soil water levels (25%, 50%, and 100% field capacity). Results showed consistent upregulation of several genes under drought stress, including DHN, OSM, NAC, GST, HSP70, PX5, PX12, AQUA and SOD CuZin the genotypes 7211, 1495, 1343, and 4132, which could indicate their drought tolerance ability.Genotypes 5717 and 4064 showed lower expression levels of these genes compared to other genotypes. SOD FeMn showed elevated expression in genotypes 1343 and 4064 under water-scarce conditions. Meanwhile, transcription factor bZIP was upregulated in 4132, 5717 and 4064 only. AP2 gene expression was consistently upregulated in all the genotypes.

The genotypes 1343 and 4132 displayed better molecular mechanism associated with drought tolerance in terms of gene expression patterns, especially overexpression of NAC and other genes, indicating possibly higher adaptive responses to water shortage.

Significantly varied root-to-shoot ratios were observed across six genotypes (7211, 1495, 1343, 4132, 5717, 4064) subjected to different soil moisture levels (25%, 50%, 100% FC), with the highest ratio (0.55 to 0.80) observed under 25% field capacity, indicating an increase in root-to-shoot ratio under water deficit conditions. Genotypes 7211 and 1343 exhibited the highest drought tolerance, followed by 4132 and 1495, as supported by molecular findings.

In conclusion, the genotypes 1343 and 4132 consistently displayed desirable traits across multiple dimensions, including morpho-physiological biochemical and molecular responses to drought conditions, compared to other genotypes evaluated in the study, such as 7211 and 1495, which also exhibited relatively better drought tolerance ability than 5717 and 4064. These findings emphasize the potential for future cultivation and breeding initiatives to yield drought-resilient variants.

Future studies can build upon the targeted transcriptome analysis conducted in the present study by incorporating advanced techniques such as gene editing to gain additional insights and enhance the application of findings toward developing drought-tolerant black pepper varieties. These approaches will enable precise trait improvement, accelerating breeding efforts for better adaptation and higher yield under water-limiting conditions.

CHAPTER 6
RECOMMENDATIONS

Drought stress is a significant environmental limiting factor for crop productivity worldwide, posing challenges, especially for essential spices like black pepper (*Piper nigrum* L.), which is highly sensitive to drought. In the present scenario, droughts have become more frequent and severe in traditionally grown tropical regions such as India, Vietnam, and Indonesia. These conditions have profoundly affected both the yield and quality of the spice. To tackle this, it is essential to understand and enhance drought tolerance in black pepper genotypes to ensure sustainable agriculture and food security. The aim of the current study was to screen black pepper genotypes to identify genotypes superior in drought tolerance and produce sustainable yield. Hence, the ongoing attempts to cultivate drought-resistant cultivars offer promise for preserving black pepper production in increasingly dry areas.

The phenotypic and genotypic traits were analyzed to pinpoint the genotypes that exhibit drought tolerance traits. Furthermore, quality evaluations of these genotypes highlighted improved volatile oil content, oleoresin, piperine, sugar, and starch levels, providing insights into their chemical composition under drought conditions. The findings lay the framework for future studies and practical applications in drought-resistant crop development, helping to stabilize and increase the productivity of black pepper production in drought-prone areas. Future research might focus on utilizing these genotypes in breeding programs to develop new black pepper cultivars with improved drought resistance. Marker-assisted selection (MAS) can be used to incorporate the discovered drought-responsive genes into other cultivars, expediting the breeding process.

Agronomists could develop specific management practices that optimize water use efficiency through deficit irrigation strategies based on the physiological responses observed in the desirable genotypes (1343, 4132, followed by 7211 and 1495) in the current study. Insights from this study on yield-related attributes under drought stress

provide valuable knowledge for crop management practices. Maintaining optimal soil moisture levels to reduce drought stress in crop plants, especially during critical growth phases, is an essential approach to maximizing yield. Under drought stress, genotypes exhibiting minimal reduction in yield-related traits, such as spike length and berry size, should be integrated into crop management plans to ensure stable yields under drought conditions.

Consistent upregulation of drought-responsive genes (DHN, OSM, DREB, GST, HSP70, PX5, PX12, and SOD CuZn) in certain genotypes suggests that these genes could be targeted in genetic engineering approaches to improve drought tolerance in other black pepper varieties. The CRISPR/Cas9 platform could be used to edit these genes in other cultivars, potentially enhancing their tolerance to drought. This knowledge may be utilized to develop high-quality black pepper products that satisfy market needs despite adverse environmental conditions.

The higher concentrations of essential volatile components, such as terpenes, in certain black pepper genotypes make them crucial for the essential oil industry, as analyzed by GC-MS. Selective breeding of genotypes with higher myrcene, linalool, and caryophyllene content can cater to the pharmaceutical and fragrance industries.

Genotypes that maintain a higher root-to-shoot ratio can adapt better to water-scarce environments by enhancing water uptake under such conditions. These genotypes ensure sustainable production in extreme climatic conditions and can be utilized in breeding programs aimed at improving the overall resilience of black pepper crop.

The long-term performance of the identified genotypes under field conditions needs to be further studied across different agro-climatic zones. Exploring the interaction between soil microorganisms and genotypes under drought stress could provide insights into biological means of improving drought tolerance. The sustainability and resilience of the genotypes identified in the present study can be integrated into future perspectives and practical applications to improve black pepper cultivation.

Training programs can be developed to educate farmers about the special requirements and advantages of these genotypes, leading to better adoption and crop resilience at the grassroots level. Policymakers should fund research and development

programs aimed at improving agricultural drought tolerance. Funding for breeding programs, as well as incentives for farmers who adopt drought-tolerant cultivars, can help accelerate the transition to more resilient agricultural systems.

CHAPTER 7
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CHAPTER 8

PUBLICATIONS

- Theertha, A. P., Shivakumar, M. S., & Krishnamurthy, K. S. (2023). Genetic variability analysis in elite black pepper genotypes using morpho-physiological and yield-attributing traits. *Journal of Spices and Aromatic Crops, 32*(2), 107–119. https://doi.org/10.25081/josac.2023.v32.i2.8742

Poster presentation

- Theertha, A. P., & Krishnamurthy, K. S. "Genetic variability for morphophysiological traits in elite black pepper accessions" SYMSAC-X during 09-12 February 2021, ICAR-Indian Institute of Spices Research.

- Theertha, A. P., & Krishnamurthy, K. S., Shivakumar, M. S., Umadevi, P., Rajesh, M. K., Shelvy, S., & Fayad, M. A. "Genetic variability among black pepper accessions for drought tolerance traits" International Plant physiology virtual symposium on Physiological Interventions for Climate Smart Agriculture (IPPVS) during 11-12 March 2021, ICAR- Sugarcane Breeding Institute, Coimbathore.

- Theertha, A. P., Krishnamurthy, K. S., & Shivakumar, M. S. "Principal component and cluster analyses for morpgo-physiological traits in elite black pepper accessions" International Conference on Physiological and Molecular Mechanisms for Abiotic Stress Tolerance in Plants during 26th- 28th October 2022, University of calicut

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Genetic variability analysis in elite black pepper genotypes using morpho-physiological and yield-attributing traits

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Abstract

The genetic variability in selected 21 black pepper accessions was analyzed based on desirable drought-tolerant and susceptible characteristics using principal component and cluster analyses. The experiment was conducted at ICAR-Indian Institute of Spices Research, Experimental farm, Peruvannamuzhi, Kozhikode using a randomized block design with four replications. Morphological, physiological and yield contributing traits were studied. The traits examined showed a comprehensive range of variability. The principal component and UPGMA clustering analyses were employed to assess the proportional contribution of various traits and grouped the genotypes, respectively. The first principal component was responsible for the highest variation (30.87%) in the yield-related characteristics, which were positively correlated with each other and correlated negatively with the morphological characteristics and stomatal frequency. Separate clusters were formed for the genotypes that displayed drought-tolerant characteristics (cluster 2 and 3) and those that showed susceptible characteristics (cluster 1). The results indicated that the analysed black pepper genotypes have significant genetic variability among them which may be helpful for identification of genotypes with desirable drought tolerant characteristics. Accessions 7211 (cluster 2), 1495, 1343 and 4132 (cluster 3) showed characteristics that make them potentially drought tolerant while the accessions 5717 and 4064 (cluster 1) showed drought susceptible traits.

Keywords: Drought tolerance, cluster analysis, principal components, pearson correlation, stomatal frequency

Introduction

Drought emerges as a primary environmental constraint for black pepper (*Piper nigrum* L.) production, especially considering its cultivation as a rain-fed crop in the southern part of India (Krishnamurthy *et al.*, 2016). Despite its widespread cultivation in countries like Vietnam, Malaysia, and Indonesia (Ambrozim *et al.*, 2022), black pepper remains sensitive to drought, requiring between 2,000 and 3,000 mm of water during the reproductive stage (Yudiyanto *et al.*, 2014).

In response to drought stress, black pepper plants undergo morphological, physiological, and biochemical changes to adapt to water-scarce conditions, as reported by Krishnamurthy *et al.* (2000). Traits such as high leaf area, elevated stomatal density, high stomatal conductivity, low wax content, and lower root growth make pepper plants susceptible to water stress due to increased water loss (Suleiman *et al.*, 2021).

Drought-tolerant black pepper genotypes exhibit increased leaf wax content as a response to water scarcity, serving as a means of conserving water to survive in arid environments, with the cuticle playing a role in limiting water evaporation from the leaves (Thankamani and Ashokan, 2002). Futhermore, plants have developed various mechanisms to tolerate drought encompassing deeper root systems, a higher root-shoot ratio, reduced transpiration and photosynthesis, proline accumulation, ABA accumulation, inhibition of chlorophyll degradation, and balanced water status and ionic distribution, as well as carbon distribution and consumption (Kanavi *et al.*, 2020; Pinheiro *et al.*, 2005).

In the present study, we tried to identify drought tolerant black pepper genotypes from a group of germplasm accessions based on some assumptions using principal component analysis (PCA), pearson correlation analysis and cluster analysis, elucidating the correlation between drought tolerance characteristics and the crop drought tolerance while avoiding the bias of a single indicator (Huseynova *et al.*, 2007). Numerous drought-resistant indicators are challenging to take into account for screening purposes. PCA and cluster analysis are considered among the most crucial multivariate analysis techniques (Oyelola *et al.*, 2004). Additionally, cluster analysis can be employed to evaluate genetic similarity and dissimilarity in datasets by grouping the genotypes based on the characteristics under study. The implementation of PCA and cluster analysis together will improve the accuracy and usefulness of the screening of crop varieties for stress response. The objective of the current study was to identify black pepper genotypes with drought-tolerant characteristics using dendrogram-based cluster analysis and PCA.

Materials and methods

Plant material

Twentyone black pepper genotypes were selected based on drought tolerance response. The experiment was conducted in a randomised block design with four replications at the Peruvannamuzhi

experimental farm of ICAR- Indian Institute of Spices Research, Kozhikode during 2021-22. Seventeen quantitative traits were monitored and recorded, which includes leaf length (LL), leaf width (LW), leaf area (LA), petiole length (PL), internodal length (IL), wax content (WC), number of stomata (NS), number of spikes plant^{-1} (NSP), spike length (SL), peduncle length (PL), berry size (BS), number of matured berries spike^{-1} (NMB), number of immature berries spike^{-1} (NIB), test weight (TW), 100 berry fresh weight (BFW), 10 spiked berries weight (SBW) and 10 rachis weight (RW).

Analysis of morphological characteristics

To determine the leaf area, four replicates of two random leaves were collected from each accession. The length and width of each leaf were measured using a centimetre ruler, and the leaf area was determined as a product of length X width and a constant (0.71) as per Mohankumar and Prabhakaran (1980). The length of the petiole was assessed by measuring the distance starting from the leaf base and ending at the point where the petiole is connected to the stem. In a comparable manner, the length of both the petiole and internode were gauged with similar number of replications for each accession. A ruler marked with centimetre increments was used to determine all of the measurements of length and width in this study.

Analysis of yield related characteristics

The dimensions of five black pepper matured berries from each variety (in three replications) were determined by using a Vernier caliper with a precision of ±0.01 mm. The measurements were taken along both the axial and transverse axes and the berry size was determined as follows: Berry size (mm) = main scale division + (Vernier scale division × least count)

Other morphometric measurements of yield related traits (NSP, TW, SL, PL, NMB, NIB, BFW, SBW and RW) were collected from four replicates of each accession, with the mean of two readings for each replication.

Determination of leaf stomatal density

In order to take stomatal impressions, a viscous solution was prepared by dissolving thermocol in xylene. The prepared liquid suspension was layered uniformly on the abaxial and adaxial surfaces at the center of each leaf. The dried coat of viscous solution was gently peeled off from the leaf after 10-15 minutes. Then the transparent layer was mounted on a clean glass slide and a cover slip was placed over it. The prepared glass slide was viewed under the compound microscope, LEICA, Wetzlar, Germany. Stomatal density (number per μm^2) was counted from three microscopic fields chosen at random from four replications for each genotype, using 10X magnification with an image size of 391.634 μm x 522.517 μm (Fig. 1).

Determination of leaf wax content

The colorimetric approach developed by Blum and Ebercon (1976) was used to quantify the epicuticular wax load. The leaf area (4.84 cm^2) was cut from the centre portion of the leaf, excluding the midrib, for the wax extraction. The leaf pieces were immersed one at a time, each for 10 s, in 5

ml chloroform in a 15 ml beaker. The solvent was concentrated by evaporating it in a water bath set at 70^0 C until it became dry. Then, 5 ml of wax reagent was added to this dried content and boiled in a steaming water bath for 30 minutes, cooled and then 12 ml of deionized water was added. The filtrate was collected and the intensity of colour was read at 590 nm using Shimadzu UV-Visible Spectrophotometer (UV-1800). The amount of wax in the leaves of each genotype was carefully evaluated using four different sample sets to ensure the precision.

Wax reagent: Powdered potassium dichromate (20 g) and 40 ml of distilled water were blended to create a slurry, which was then mixed with 1 litre of strong sulfuric acid to prepare wax reagent. A clear solution was prepared by heating the resultant slurry. A wax standard graph was developed by using carnauba wax.

Statistical analysis

Principal component analysis (PCA) was applied to the correlation matrix of the seventeen variables and twentyone genotypes, in order to find the parameters that best reflect the tolerance to response variables. Past 4.03 software was used for the analysis of principal components, Eigen values, Eigen vectors and 2D biplot visualization of PC1 and PC2. Pearson coefficient analysis was used to assess the strength of the correlation between these parameters. UPGMA cluster analysis was used for grouping the genotypes into clusters using R- software.

Results and discussion

Morpho-physiological and yield attributing characters studied revealed genetic diversity among the genotypes, which was explained by the findings of PCA. As per this criterion, the first five components in the current study were responsible for 73.88% of total variation for the quantitative traits (Table 1). PCA revealed seventeen components, out of which five principal components (PCs) showed eigenvalues greater than one, suggesting a significant impact on selected accessions. Reshma *et al.* (2022) reported a similar result among the black pepper genotypes in their quantitative traits with significant diversity accounted for by the six primary components. Considerable variation in the performance of black pepper genotypes, which were located in lowland and high-altitude areas arise from the changes in both genetic composition and environmental influences (Sainamole *et al.*, 2002). Genetic variability and environmental factors led to the significant variation in the selected black pepper genotypes for the observed quantitative traits in the current study.

The first principal component accounted for the highest variability of 30.87% with substantial loadings recorded for spike length (0.753), 10 spiked berries weight (0.693), 10 rachis weight (0.623) and number of spikes plant^{-1} (0.592), which contributed in a positive direction while the leaf area (-0.742), leaf width (-0.705), leaf length (-0.587), number of stomata (-0.530) and petiole length (-0.414) contributed negatively to PC1 (Fig. 2). The yield attributing characters and wax content

mainly contributed to the highest variability in PC1. Bhor *et al.* (2021) also reported that traits associated with yield displayed the highest level of variability. Yield related contribution had a great impact on variation captured by PC1 than on the variation explained by remaining principal components (Reshma *et al.*, 2022). It is evident that PC1 experienced the highest variance and was followed by others, as reported in all the studies that employed principal component analysis.

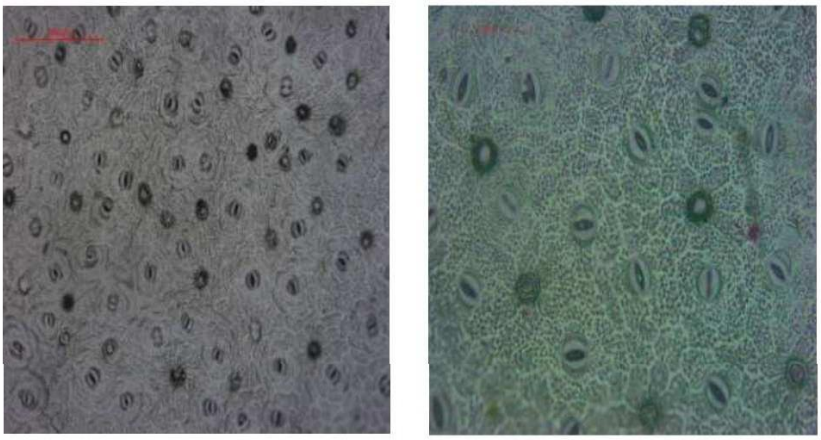

Fig. 1. Stomata on the ventral side of black pepper leaves (10X and 20X, image size: 391.634x 522.517 μm).

The second component accounted for 14.90% of total variation, contributed positively by number of matured berries spike^{-1} (0.752), test weight (0.730) and internodal length (0.355). The remaining traits *viz.* 100 berry fresh weight (-0.654) and berry size (-0.648) contributed in the negative direction. Characteristics that constitute the yield components of black pepper genotypes were positively correlated with the actual yield (Shivakumar *et al.*, 2020). This proposes that the variables contributing to yield are relevant in determining the overall variation identified in the dataset. The third principal component accounted for 12.27% of the total variation, associated positively with leaf area (0.505), leaf width (0.443) and leaf length (0.411), and negatively associated with internodal length (-0.652) and petiole length (-0.627). Number of immature berries spike^{-1} (0.566) and wax content (0.555) were shown to be associated positively in PC4 which explained 8.26% variability.

Table 1. Eigen values and percentile variance of selected black pepper genotypes

	Principal Component				
	PC 1	PC 2	PC 3	PC 4	PC 5
Eigen value	5.24	2.53	2.08	1.4	1.28
% Variance	30.87	14.9	12.27	8.26	7.56
Variable	Eigen vectors				
Leaf length (cm)	-0.587	0.022	0.411	0.232	0.379
Leaf width (cm)	-0.705	-0.095	0.443	0.026	0.349
Leaf area (cm^2)	-0.742	-0.04	0.505	0.119	0.403
Petiole length (cm)	-0.414	0.255	-0.627	0.144	0.154
Internodal length (cm)	-0.255	0.355	-0.652	0.078	0.348
No. of stomata	-0.53	0.227	0.31	-0.284	-0.358
Wax content ($\mu g/cm^2$)	0.506	-0.065	-0.17	0.555	-0.036
Number of spikes $plant^{-1}$	0.592	0.279	0.14	0.219	-0.043
Test weight (gm)	0.527	0.73	0.332	-0.039	0.036
Spike length (cm)	0.753	0.171	0.356	0.202	0.077
Peduncle length (cm)	0.468	0.239	0.074	-0.51	0.065
Berry size (mm)	0.486	-0.648	0.084	-0.429	0.07
Number of matured berries $spike^{-1}$	0.523	0.752	0.224	-0.092	0.113
Number of immature berries $spike^{-1}$	0.061	-0.276	0.35	0.566	-0.476
100 berry fresh weight (g)	0.537	-0.654	0.104	-0.145	0.203
10 spiked berries weight (g)	0.693	-0.082	0.138	0.101	0.439
10 rachis weight (g)	0.623	-0.289	-0.226	0.158	0.305

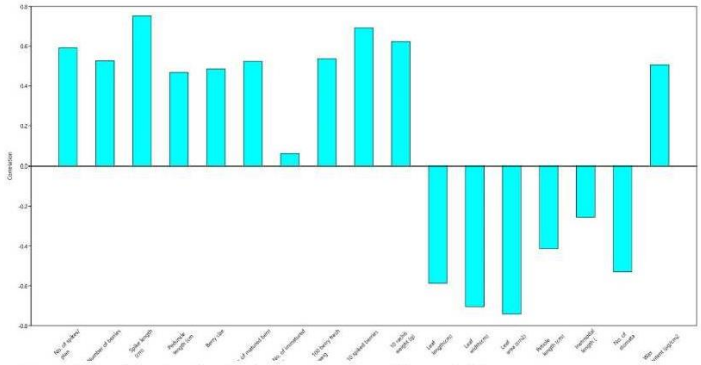

Fig. 2. Loading plot of first principal component with variables

Scatterplot helped to visualize the genotypes grouping based on similarities and differences according to the influence of traits. Except the number of immature berries spike^{-1} in yield attributing characters, number of spikes plant^{-1}, test weight, spike length, peduncle length, berry size, number of matured berries spike^{-1}, 100 berry fresh weight, 10 spiked berries weight and 10 rachis weight including the wax content mainly attributed to the first axis as positive levels (Fig. 3). Shivakumar *et al.* (2022) observed that berry weight and dry seed weight were highly significant among various black pepper genotypes for spike and berry traits, displaying a strong positive correlation through principal component analysis.

The traits that made the most significant contribution to the first principal component were genotypes having drought tolerant characteristics *viz.* accession 971, closely followed by accessions 4132, 7211, 6720, 1343, and 1086. Whereas negative contribution was made by the traits, which highly correlated with the accessions (5083, 4064, 1491, 8060 and 1093) with susceptible characteristics. Among all the genotypes considered, accession 4132 displayed the positive value in first component and most negative value in the second principal component, indicating that it possessed the most tolerant characteristics. Malek *et al.* (2021) observed that the genotypes could be distinguished based on the key contributing features using the biplot described by the first two PCs.

Hence, it can be concluded that morphological characteristics and stomatal density, which were distributed across the second and third quadrants, exhibited both significant and non-significant negative associations with the traits that contribute to the yield and wax content of black pepper genotypes compared to those in the first and fourth quadrants. It is assumed that drought tolerant black pepper genotypes will have reduced leaf area, reduced internodal and petiole lengths, lower stomatal density and higher wax content. Genotypes displaying traits that make them vulnerable to drought were placed in the second and third quadrants.

An effective visual depiction of the relationship between variables in a dataset can be obtained by using a Pearson correlation analysis. Pearson correlation analysis revealed a significant relationship among observed morphological traits. According to the present study, there was a strongest positive correlation ($P<0.001$) between the leaf area and leaf width ($r= 0.93$) which is in consistent with the findings reported by Preethi *et al.* (2018), followed by leaf length ($r= 0.80$) as well as between internodal length and petiole length ($r= 0.60$) ($P<0.01$). Leaf area showed a consistent relationship with the leaf width (Jayarathna *et al.*, 2016).

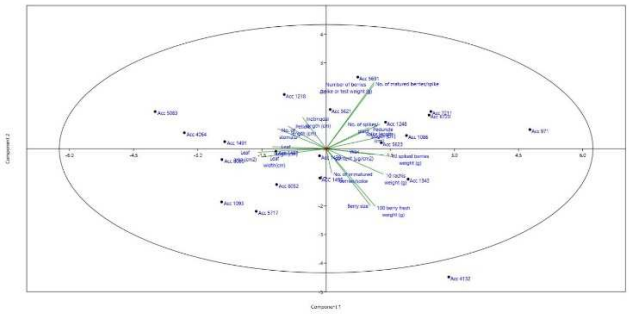

Fig. 3. Black pepper genotypes distribution across the first two primary components

Larger leaf area was not positively correlated to the improved cultivar production, although the flower intensity spike^{-1}, inflorescence size and berry spike have positive correlation with fruit development (Chen *et al.*, 2018). Water deficiency lead to reduction in both leaf size and quantity in black pepper plants (Rasanjali *et al.*, 2019; Teles *et al.*, 2023). Similar findings have been reported in tomato (Heuvelink *et al.*, 2005) and pepper crops, indicating a reduction in leaf area due to water shortage (Cemek *et al.*, 2020; Koch *et al.*, 2019). Hence, a reduction in leaf area can be regarded as a favourable characteristic for drought tolerance as reduced leaf area reduces the water loss through transpiration, supported by the present study.

A homogeneous association was shown between leaf length and width (r= 0.54). While non- significant positive correlation (P≥0.05) was showed by stomata with leaf length (0.20), leaf width (0.33) and leaf area (0.36). Wax content exhibited high negative correlation with stomata number (-0.49) (P<0.05), followed by leaf width (-0.42), leaf area (-0.39) and leaf length (-0.18). Black pepper varieties with broader and larger leaf bases typically have more stomata (Paulus & Sim, 2011) as shown by the leaf length to width ratio. Elevated stomatal density could be associated with the leaf area expansion and cell division. Enhancing regulatory competence of stomatal traits through an approach of selective breeding strategies might help plants to respond more efficiently to environmental constraints like drought (Xu & Zhou, 2008). While experiencing drought and excessive radiation, a decrease in transpiration rate and an increase in leaf wax load have a positive impact on the yield index (Sanchez *et al.*, 2001). The wax content demonstrated the non- significant positive relationship with the traits that contribute to yield. The positive correlation between leaf wax content and yield-attributing traits provides valuable insights into a plant's ability to conserve water content in the best yield-producing genotypes especially under water deficit. This information may be used in breeding strategies to enhance production under water limited environment.

Among yield attributing traits, highly significant and positive correlation (P<0.001) was found between berry size and 100 berry fresh weight as well as test weight and number of matured berries/ spike (r= 0.83). The fresh yield was found to have a positive correlation with the number of berries per spike (Sainamole *et al*., 2002; Bermawie *et al*., 2019). The association between berry and test weight could be an indication of overall yield, emphasizing their importance in determining the yield.

Significant positive correlations (P<0.01) were identified between test weight and spike length (r= 0.61), 10 rachis weight and spike length (r= 0.59) and number of matured berries/spike and spike length (r= 0.57). The number of berries spike^{-1} (Ibrahim *et al*., 1985) as well as spike length (Krishnamurthy *et al*., 2010) can be affected by both genetic and environmental factors. The quantity of berries shows greater responsiveness to changes in environmental conditions compared to the length of the spike, further supporting and extending the understanding provided by Ibrahim *et al*. (1987). The current study focused on the interaction of genetic and environmental variables, especially influencing berry size, test weight, spike length, and the number of matured berries per spike. The number of berries in a spike is directly associated with the length of the spike, a correlation observed consistently across different states such as Kerala (Maheswarappa *et al*., 2012; Sujatha and Namboothiri, 1995; Reshma *et al*., 2022), Karnataka (Tripathi *et al*., 2018), and Assam (Deka *et al*., 2016; Nath *et al*., 2021). Rachis weight, spiked berries weight, 100 berry fresh weight, spike length, number of spikes per plant and peduncle length were non- significantly and positively correlated with each other. Number of berries per spike directly and positively contributes to the pepper yield.

Shivakumar *et al*. (2022) reported that Panniyur-1, Agali, and Narayakodi genotypes exhibited high values on PC1, and these genotypes displayed higher values for yield-attributing traits (berry weight, dry seed weight, and fresh pericarp weight), indicating a strong positive correlation as observed in the present study. These traits hold relevance in both principal compont analysis and clustering of genotypes.

Results of the morpho-physiological and yield contributing traits based dendrogram (Fig. 4) in the present study displayed genotypes with most preferable traits for the drought tolerance in clusters 2 and 3, while genotypes with susceptible characteristics were grouped in cluster 1. The accessions 7211, 971 (cluster 2), 4132, 1495, and 1343 (cluster 3) had traits suited for drought tolerance. The present study is in conformity with previous findings in wheat genotypes that were categorized based on morphological traits by the resultant dendrogram into high homogeneity clusters within the clusters (Pasandi *et al*., 2016). The attributes responsible for wheat yield like spikes plant $^{-1}$, number of grains spike $^{-1}$, grain weight spike $^{-1}$, grain yield plant^{-1}, and spike density made substantial contributions to the first principal component and were grouped together in the same cluster (Fouad, 2020). The variation among black pepper genotypes for yield

contributing traits as per the PCA and further classification of genotypes by dendrogram in the present study might reflect their genotypic variations as well as the environmental influences.

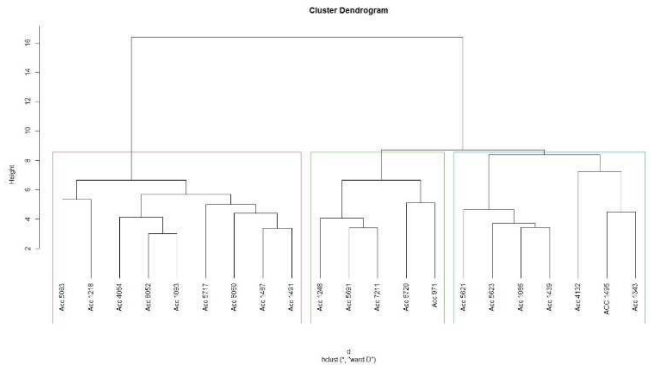

Fig. 4. Clustering of black pepper genotypes

In conclusion, the current study confirmed significant genetic variability among the selected black pepper genotypes which is useful to select genotypes with desirable drought-tolerant characteristics. The identification of factors that contribute significantly to PC1 can be helpful in identifying black pepper genotypes with drought-tolerant characteristics with sustainable yield. The genotypes 4132, 7211, 1343, 1495 and 971 were identified as having better drought-resilient traits. This knowledge can also be used to develop targeted breeding and cultivation strategies aimed at selecting genotypes with specific drought-tolerant traits. Ultimately, this can lead to the development of black pepper variety tolerant to drought, resulting in improved yields and sustainable black pepper production.

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Genetic variability for morpho physiological traits in elite black pepper accessions

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Black pepper is the most commonly used spice, throughout the world and its extracts have been used in folk medicine. It is a perennial woody evergreen climbing vine, native to evergreen forests of the Western Ghats of South India and is extensively cultivated in tropical regions. Plant requires a long rainy season, fairly high temperature and partial shade for its best growth. By opening and closing of stomata, plants can regulate the amount of water loss, when the environmental conditions are unfavorable. Drought stress modifies the physiological parameters such as stomatal count and wax. Ultimately, it destabilizes the membrane structure and permeability, protein structure and function. Here we have studied the genetic variability for morphology (leaf length, leaf width, petiole length and internodal length), stomatal density and wax content of 40 black pepper genotypes identified for their drought tolerance in preliminary screening. The study was carried out in ICAR-IISR Experimental Farm, Peruvannamuzhi. The experiment was laid out in RBD with four replications. The data analysis revealed a significant genetic variability among these accessions for morphological characters, stomatal count and epicuticular wax. The leaf area ranged from 99.37 cm2 to 29.36 cm2 and highest value was observed in the accession 1083. Accession 6720 had the maximum wax content (25.96 µg/cm2) and the accession 1218 showed the minimum value (0.38 µg/cm2). Stomatal density ranged from 94 (accession 1086) to 41 (accession 1368) per 20X microscopic field.

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Principal component and cluster analyses for morpho-physiological traits in elite black pepper accessions

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The genetic variability of selected 40 black pepper accessions was analyzed by principal component and cluster analyses, based on the desirable drought tolerant and susceptible characteristics. The experiment was carried out at ICAR-Indian Institute of Spices Research, Experimental farm, Peruvannamuzhi, Kozhikode using a randomized block design with four replications. Morphological (leaf length, leaf width, petiole length, and internodal length) characters and physiological (wax content and number of stomata) characters were studied. A wide range of variability was observed for all the traits under study. The proportional contribution made by various features to overall variability was assessed by applying the principal component analysis with the seven axes (PC1 to PC7) using Past 4.03 software. Out of seven, only three principal components (PC1, PC2 and PC3) accounted for a maximum variability of 76.97% with Eigen values more than unity. PC1 accounted for 44.91% of the total variability in attributes like leaf area, leaf width, petiole length, and leaf length. Characters such as the number of stomata, petiole length, and leaf width belonged to component 2 which was responsible for 17.46% of the total variation. PC3 with 12.45% of the total variation and traits like wax content, internodal length, the number of stomata, and petiole length led to the variation. The genotypes were grouped into four clusters based on UPGMA clustering analysis using R software. Cluster III and IV had the highest number of genotypes (12) followed by cluster II with 9 genotypes and cluster I with 7 genotypes. The genotypes with the most desirable drought-tolerant traits were found in Cluster IV. The results showed that the analyzed forty black pepper genotypes have significant genetic variability that can be helpful for the identification of the genotypes with the most desirable drought-tolerant characteristics.

Keywords: Cluster, Genetic variability, Genotype, Pepper, Wax content

CERTIFICATE

This certificate is presented to ~~Mr./~~Ms/Mrs. Theertha A.P. who has attended and presented an oral/poster presentation entitled Principal component and cluster analyses for morphophysiological traits in elite black pepper accessions in the theme Water relation & mineral nutrition under Research Scholar/~~Student~~ category at the International Conference on **'Physiological and Molecular Mechanisms of Abiotic Stress Tolerance in Plants'** from 26th - 28th October 2022 jointly organized by the Department of Botany, University of Calicut, Kerala and Indian Society for Plant Physiology (South Zone).

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GENETIC VARIABILITY AMONG BLACK PEPPER ACCESSIONS FOR DROUGHT TOLERANCE TRAITS

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In this study, the genetic variability among 40 black pepper accessions was studied based on morphological and physiological characters with the objective of identification of genotypes with desirable traits for drought tolerance conditions. The data analysis revealed considerable genetic heterogeneity among these accessions for stomatal count and epicuticular wax and leaf area. The leaf area ranged from 29.36 cm^2 to 80.1 cm^2. Accession 1343 had the maximum wax content (15.96 $\mu g/cm^2$). Stomatal density ranged from 41 (accession 1368) to 94 (accession 1086) per 10X microscopic field. Results indicated that the accessions 7211, 1495, 1343 and 4132 were the most desirable genotypes for drought tolerance. Molecular response of these forty genotypes was also attempted based on four gene (drought) specific primers for molecular validation. MultiNA Microchip Electrophoresis analysis yielded few bands with varying amplicon size for each accession with the respective primers.

ICAR-Sugarcane Breeding Institute
In collaboration with
Indian Society for Plant Physiology (ISPP), New Delhi,
National Academy of Agricultural Sciences (NAAS) - Coimbatore Chapter
Society for Sugarcane Research and Development (SSRD)

"Physiological Interventions for Climate Smart Agriculture"

Participation Certificate

This is to certify that **Theertha A.P*., Krishnamurthy K.S., Shivakumar M.S., Umadevi P., Rajesh M.K. Shelvy S. and Fayad M.A** Participated and presented poster paper on "Genetic variability among black pepper accessions for drought tolerance traits" in the session PHYSIOLOGICAL INTERVENTIONS FOR HORTICULTURAL CROPS in the International Plant Physiology Virtual Symposium on **"Physiological Interventions for Climate Smart Agriculture (IPPVS 2021)"** held during 11-12th March 2021 at ICAR-Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, in collaboration with Indian Society of Plant Physiology(ISPP), New Delhi, India.

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