Improvement of red ginger (*Zingiber officinale* Rosc.) through *in vitro* and *in vivo* studies

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By

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CERTIFICATE

This is to certify that the thesis entitled **'Improvement of red ginger (*Zingiber officinale* Rosc.) through *in vitro* and *in vivo* polyploidy induction'** submitted to the University of Calicut by **Ms. Neenu Maria George** for the award of degree of **Doctor of Philosophy in Botany** is the result of research work carried out by her in the Division of Crop Improvement and Biotechnology, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India during the period 2019 to 2024. This has not been submitted for the award of any other degree or diploma of this or any other university. The plagiarism has been checked at CMHK library, University of Calicut and the values are within the acceptable limits. The corrections and suggestions recommended by the adjudicators have been incorporated in the thesis.

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DECLARATION

I hereby declare that the work presented in the thesis entitled "**Improvement of red ginger (*Zingiber officinale* Rosc.) through *in vitro* and *in vivo* studies**" is based on the original work done by me under the guidance of Dr. D. Prasath 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.

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Neenu Maria George

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ABBREVIATIONS AND SYMBOLS

µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometre
%	Percentage
α	Alpha
β	Beta
γ	Gamma
Acc.	Accession
ANOVA	Analysis of Variance
APM	Amiprophos-methyl
ASTA	American Spice Trade Association
BA	6-benzyladenine
BAP	6-Benzylaminopurine
Bp	Base pair
CD	Critical Difference
cm	Centimeter
CO_2	Carbon Dioxide
Coll. No.	Collection Number
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of Variation
DAP	Days After Planting
DASD	Directorate of Arecanut and Spices Development
DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DUS	Distinctiveness, Uniformity, and Stability
EDTA	Ethylenediaminetetraacetic acid
et al.,	And his coworkers
etc.,	And so on
FCM	Flow cytometry
Fig.	Figure
FYM	Farm Yard Manure

g	Gram
g L^{-1}	Gram per litre
GA	Genetic advance
GAM	Genetic Advance as per cent Mean
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GCV	Genotypic Coefficient of Variation
H	Hour
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HPLC	High-performance liquid chromatography
i.e.,	That is
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
ICAR	Indian Council of Agricultural Research
IISR	Indian Institute of Spices Research
ISSR	Inter Simple Sequence Repeat
Kg cm^{-2}	Kilogram per Centimeter Square
Kg ha^{-1}	Kilogram per Hectare
LAF	Laminar Air Flow
LSD	Least Significant Difference
M	Molar
mg	Milligram
mg L^{-1}	Milligram per litre
Min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimeter
MS	Murashige and Skoog
N	Normal
NAA	1-Naphthaleneacetic acid
NaCl	Sodium chloride
NAGS	National Active Germplasm Site
ng	Nanogram
nm	Nanometer
ºC	Degree Celcius

PAST	PAleontological STatistics
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PCV	Phenotypic Coefficient of Variation
pH	Potential of hydrogen
PI	Propidium Iodide
PIC	Polymorphism Information Content
PPM	Parts per Million
PPV&FRA	Protection of Plant Varieties and Farmer's Rights Act
PVP	Polyvinylpyrrolidone
RB	Round Bottom
RCBD	Randomized Complete Block Design
RHS	Royal Horticultural Society
rpm	Revolutions per minute
S	Seconds
SSR	Simple Sequence Repeat
t ha^{-1}	Tonne per Hectare
TAE	Tris-Acetate EDTA
TDZ	Thidiazuron
UPGMA	Unweighted Pair Group Method with Arithmetic mean
UV	Ultraviolet
viz. ,	Namely

ABSTRACT

Improvement of red ginger (*Zingiber officinale* Rosc.) through *in vitro* and *in vivo* studies

Red ginger (*Zingiber officinale* Rosc.) stands out among ginger types, garnering global recognition for its attractive red rhizome and potent medicinal attributes. Because of its slender rhizome and comparatively lower yield, it has yet to gain popularity among ginger cultivators. Crop improvement strategies, such as polyploidy induction, hold the potential to enhance the rhizome size, yield, and quality traits of red ginger. The study commenced with the morphological and biochemical characterization of 17 red ginger genotypes, comprising 14 Indian, two exotic lines, and the released variety IISR Varada. The results uncovered the significant difference in the growth, yield and quality parameters between the red ginger genotypes and IISR Varada. The study facilitated the identification of morphological and biochemical diversity among both exotic and indigenous red ginger genotypes. Among the genotypes, the highest yield per plant was recorded in Coll. No. 9073 (807.50 g) followed by Acc. 838 (625.00 g). Biochemical characterization of red ginger genotypes revealed that the highest essential oil and oleoresin contents were recorded in G9, at 4.30 % and 10.34 %, respectively. Among the exotic red ginger genotypes, the highest essential oil was recorded in Acc. 850 (3.89 %); the highest oleoresin content was observed in Acc. 899 (7.19 %). Among the genotypes, the lowest crude fibre content was observed in IISR Varada (4.90 %), whereas the highest was in G1 (10.47 %). The maximum percentages of 6-gingerol, 8-gingerol, and 6-shogaol were recorded in the red ginger genotypes Coll. No. 9073 (1.33 %), Acc. 844 (0.17 %), and G9 (0.18 %), respectively. The major compound identified in the essential oil was α-zingiberene, with the highest content reported in Acc. 845 (30.46 %). The high yielding genotype of Indian and exotic red ginger i.e., Coll. No. 9073 and Acc. 899 were selected as best performing genotypes. The second objective was to genetically improve red ginger through *in vitro* and *in vivo* polyploidy induction using colchicine. An efficient *in vitro* regeneration system was standardized for Indian and exotic red ginger genotypes. The maximum shoot multiplication was observed at a concentration of 5.0 mg L^{-1} BAP in exotic red ginger, while in Indian red ginger, it was observed at a BAP concentration of 3.0 mg L^{-1}. For *in vitro* polyploidy induction, colchicine concentrations of 0.00, 0.025, 0.50, 0.75 and 0.10 % at 24 and 48 h was used. The *in vitro* induction of polyploidy was found to be more effective compared to the *in vivo* method, resulting in a total of five tetraploids (2n=44). The highest tetraploidy induction was observed in the treatment using 0.10 % colchicine for 48 h, under *in vitro* treatment. For *in vivo* polyploidy induction, colchicine concentrations of 0.00, 0.05, 0.10, 0.15 and 0.20 % at 24 and 48 h was used and a single tetraploid (2n=44) was identified in Indian red ginger for 0.15 % at 24 h. The induced tetraploids exhibited increased vigor,

improved morphology and stomatal parameters, and enhanced yield. The identified polyploids need to be tested for yield under multi-environment before commercial scale adoption.

Keywords: Red ginger, *In vitro* regeneration, Colchicine, Polyploidy induction, Tetraploidy

സംഗ്രഹം

ഇൻ വിട്രോ/ഇൻ വിവോ പഠനങ്ങളിലൂടെ ചുവന്ന ഇഞ്ചിയുടെ (സിഞ്ചിബർ ഓഫീസിനെൽ റോസ്ക്) മെച്ചപെടുത്തൽ

ചുവന്ന ഇഞ്ചി (സിഞ്ചിബർ ഓഫീസിനെൽ റോസ്ക്) മറ്റ് ഇഞ്ചി താരങ്ങളിൽനിന്നും, അതിന്റെ ആകർഷകമായ ചുവന്ന നിറം കൊണ്ടും, ഔഷധഗുണങ്ങൾ കൊണ്ടും വേറിട്ട് നില്കുന്നു. അതുകൊണ്ട് തന്നെ ചുവന്ന ഇഞ്ചി ആഗോള തലത്തിൽ ശ്രദ്ധിക്കപ്പെട്ടിട്ടുണ്ട്. എന്നാൽ, ചുവന്ന ഇഞ്ചിയുടെ വണ്ണം കുറഞ്ഞ ഭൂകാണ്ഡവും കുറഞ്ഞ ഉല്പാദനക്ഷമതയും കാരണം, ചുവന്ന ഇഞ്ചിക്ക് കർഷകരുടെ ഇടയിൽ വേണ്ടത്ര പ്രചാരം ലഭിച്ചിട്ടില്ല. പോളിപ്ലോയ്ഡി ഇൻഡക്ഷൻ പോലെയുള്ള വിള മെച്ചപ്പെടുത്തൽ തന്ത്രങ്ങൾ അവലംബിച്ച് ചുവന്ന ഇഞ്ചിയുടെ വലുപ്പം, വിളവ്, ഗുണമേന്മ എന്നിവ വർധിപ്പിക്കാനുള്ള സാദ്ധ്യതകൾ ഉണ്ട്. പതിനാല് ഇന്ത്യൻ തരങ്ങളും രണ്ടു വിദേശിയതരങ്ങളും ഉൾപ്പെടെ പതിനാറു ചുവന്ന ഇഞ്ചിയുടെ തരങ്ങളും ഐഐസ്ആർ പുറത്തിറക്കിയ ഐഐസ്ആർ വരദ എന്ന ഇഞ്ചി ഇനവുമാണ് ഈ പഠനത്തിന് ഉപയോഗിച്ചത്. ഈ പതിനേഴു തരങ്ങളുടെയും രൂപഘടനയും ബയോകെമിക്കൽ സ്വഭാവസവിഷേഷതകളും വിശകലനം ചെയ്തു. ചുവന്ന ഇഞ്ചിയും ഐഐസ്ആർ വരദയും തമ്മിലുള്ള വളർച്ച, വിളവ്, ഗുണമേന്മ എന്നിയവയിലുള്ള വ്യത്യാസം ഈ പഠനത്തിന്റെ ഫലമായി കണ്ടെത്തി. വിദേശീയവും തദ്ദേശീയവും ആയ ചുവന്ന ഇഞ്ചി തരങ്ങളുടെ രൂപഘടനയിലും ബയോകെമിക്കൽ ഘടനയിലും ഉള്ള വൈവിധ്യം തിരിച്ചറിയാൻ പഠനം സഹായിച്ചു. ചുവന്ന

ഇഞ്ചികളിൽ ഏറ്റവും ഉയർന്ന വിളവ് കിട്ടിയത് കളക്ഷൻ നമ്പർ 9073 (807.50 g) ക്കും തുടർന്ന് Acc.838 (625.00 g) ആണ്. ചുവന്ന ഇഞ്ചിയുടെ ബയോകെമിക്കൽ പഠനങ്ങളെ അടിസ്ഥാനമാക്കി G9 ൽ ഏറ്റവും ഉയർന്ന സുഗന്ധതൈലവും (4.30%) ഒലേറെസിൻ (10.34%) ശതമാനവും രേഖപ്പെടുത്തി. വിവിധ ചുവന്ന ഇഞ്ചി ഇനങ്ങളിൽ, Acc. 850 (3.89 %) യിൽ ഏറ്റവും ഉയർന്ന സുഗന്ധ തൈലത്തിന്റെ അളവും Acc. 899 ൽ ഏറ്റവും ഉയർന്ന ഒലിയോറെസിന്റെ അളവും (7.19%) രേഖപ്പെടുത്തി. ഐഐഎസ്ആർ വരദയിലാണ് (4.90 %) അസംസ്കൃത നാരിന്റെ അളവ് ഏറ്റവും കുറവായി കണ്ടത്. അതേസമയം ഏറ്റവും ഉയർന്നത് G1 ൽ (10.47 %) ആയിരുന്നു. 6-ജിഞ്ചറോൾ, 8-ജിഞ്ചറോൾ, 6-ഷോഗോൾ എന്നിവയുടെ പരമാവധി ശതമാനം യഥാക്രമം ചുവന്ന ഇഞ്ചി ഇനങ്ങളായ 9073 (1.33 %), 844 (0.17 %), G9 (0.18 %) എന്നിവയിലാണ് കണ്ടെത്തിയത്. ചുവന്ന ഇഞ്ചിയുടെ അവശ്യ എണ്ണയിൽ കണ്ടെത്തിയ പ്രധാന സംയുക്തം α-സിഞ്ചിബറിൻ ആയിരുന്നു, ഈ സംയുക്തത്തിന്റെ ഏറ്റവും ഉയർന്ന അളവ് Acc. 845 (30.46%) ൽ ആണ് രേഖപ്പെടുത്തിയത്. പഠനങ്ങളുടെ അടിസ്ഥാനത്തിൽ ചുവന്ന ഇഞ്ചിയുടെ തദ്ദേശീയ ഇനമായ കളക്ഷൻ നമ്പർ 9073 യെയും വിദേശ ചുവന്ന ഇഞ്ചി ഇനമായ Acc. 850 നെയും മികച്ച ചുവന്ന ഇഞ്ചി ഇനമായി തിരഞ്ഞെടുത്തു. കോൾചിസിൻ ഉപയോഗിച്ച് ഇൻ വിട്രോ/ഇൻ വിവോ പോളിപ്ലോയിഡി ഇൻഡക്ഷനിലൂടെ ചുവന്ന ഇഞ്ചി ജനിതകപരമായി മെച്ചപ്പെടുത്തുക എന്നതായിരുന്നു രണ്ടാമത്തെ ലക്ഷ്യം. തദ്ദേശീയവും, വിദേശിയവുമായ ചുവന്ന ഇഞ്ചി ഇനങ്ങൾക്കായി കാര്യക്ഷമമായ ഇൻ വിട്രോ റീജനറേഷൻ സംവിധാനം രൂപപ്പെടുത്തി. വിദേശിയ ചുവന്ന ഇഞ്ചിയിൽ 5.0 മില്ലിഗ്രാം/ലിറ്റർ ബെൻസൈൽ അമിനോ പ്യൂരിന്റെ (ബിഎപി) സാന്ദ്രതയിൽ പരമാവധി ചിനപ്പുപൊട്ടൽ നിരീക്ഷിക്കപ്പെട്ടു, ഇന്ത്യൻ ചുവന്ന ഇഞ്ചിയിൽ ഇത് 3.0 മില്ലിഗ്രാം/ ലിറ്റർ എന്ന ബിഎപി സാന്ദ്രതയിലാണ്. ഇൻ വിട്രോ പോളിപ്ലോയിഡി പ്രേരിപ്പിക്കാനായി 24-48

മണിക്കൂറിൽ വിവിധ കോൾചിസിൻ സാന്ദ്രതകൾ (0.00, 0.025, 0.50, 0.75, 0.100 %) ഉപയോഗിച്ചു. ഇൻ വിവോ രീതിയുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ പോളിപ്ലോയിഡിയുടെ ഇൻ വിട്രോ ഇൻഡക്ഷൻ കൂടുതൽ ഫലപ്രദമാണെന്ന് കണ്ടെത്തി. ഇൻ വിട്രോ രീതിയിലൂടെ ആകെ അഞ്ച് ടെട്രാപ്ലോയിഡുകൾ (2n=44) ആണ് ലഭിച്ചത്. ഇൻ വിട്രോ രീതിയിൽ 48 മണിക്കൂറിനുള്ളിൽ 0.10% കോൾചിസിൻ ഉപയോഗിച്ചപ്പോഴാണ് ഏറ്റവും ഉയർന്ന ടെട്രാപ്ലോയിഡി ഇൻഡക്ഷൻ നിരീക്ഷിക്കപ്പെട്ടത്. ഇൻ വിവോ പോളിപ്ലോയിഡി ഇൻഡക്ഷനായി, 24, 48 മണിക്കൂറിൽ വിവിധ കോൾചിസിൻ സാന്ദ്രതകളാണ് (0.00, 0.05, 0.10, 0.15, 0.20 %) ഉപയോഗിച്ചത്. ഇതിൽ തദ്ദേശീയ ചുവന്ന ഇഞ്ചിയിൽ മണിക്കൂർന്നേരം 0.15 % എന്ന നിരക്കിൽ ഉപയോഗിച്ചപ്പോളാണ്. ഇതിൽ തദ്ദേശീയ ചുവന്ന ഇഞ്ചിയിൽ മണിക്കൂർന്നേരം 0.15 % കോൾചിസിൻ എന്ന നിരക്കിൽ ഉപയോഗിച്ചപ്പോൾ ഒരൊറ്റ ടെട്രാപ്ലോയിഡ് (2n=44) കണ്ടെത്തുകയുണ്ടായി. ഇത്തരത്തിൽ വികസിപ്പിച്ച പ്രേരിത ടെട്രാപ്ലോയിഡുകൾ വർധിച്ച വീര്യവും മെച്ചപ്പെട്ട രൂപശാസ്ത്രവും മെച്ചപ്പെടുത്തിയ വിളവും പ്രദർശിപ്പിച്ചു. ഇവയുടെ ആസ്യരന്ധ്രങ്ങളുടെ വിന്യാസവും സ്വഭാവവിശേഷവും മെച്ചപ്പെട്ടതായിരുന്നു. വികസിപ്പിച്ചെടുത്ത പോളിപ്ലോയിഡുകൾ വാണിജ്യാടിസ്ഥാനത്തിൽ ഉപയോഗപ്പെടുത്തുന്നതിനുമുമ്പ് വ്യത്യസ്തമായ പരിസ്ഥിതികളിൽ വിളവ് പരിശോധിക്കേണ്ടതുണ്ട്.

സൂചകപദങ്ങൾ: ചുവന്ന ഇഞ്ചി, ഇൻ വിട്രോ റീജനെറേഷൻ, കോൾച്ചിസിൻ, പോളി പ്ലോയ്ഡി രൂപപ്പെടുത്തൽ, ടെട്രാപ്ലോയ്ഡി

Dedicated to
My Beloved Parents and Husband

CHAPTER 1
INTRODUCTION

Zingiber officinale Rosc., commonly known as ginger, is a versatile and widely recognized spice belonging to the family Zingiberaceae. Throughout the annals of history, ginger has played a pivotal role in various cultures across tropical Asia, where its myriad benefits have been harnessed for medicinal, culinary, and beverage purposes since ancient times (Ballester *et al*., 2022).

The rhizome extracts are utilized extensively in traditional medicine, herbal remedies, and dietary supplements. It offers a range of health advantages, including anti-emetic, anti-inflammatory, anti-diabetic, antioxidant, and anti-cancer actions. Additionally, several studies have shown that ginger can prevent and treat several illnesses, including diabetes mellitus, respiratory problems, obesity, cardiovascular diseases, chemotherapy-induced nausea and emesis, and neurodegenerative diseases (Arcusa *et al*., 2022; Zhang *et al*., 2020). The presence of secondary metabolites in ginger, especially in essential oils and oleoresins, is thought to be responsible for its beneficial properties. Therefore, phenolics and terpenes in the ginger rhizome are primarily involved in its pharmacological properties. The main polyphenols found in fresh ginger are gingerols including 6-, 8-, and 10-gingerol. 6- Gingerol is the major one among the various gingerols, imparting pungency to the fresh ginger rhizome (Zhang *et al*., 2017). Gingerols are converted into corresponding shogaols by heating and drying or by storing them for long periods. 6- shogaol is reported to be the main pungent principle of dried ginger and has been utilized extensively to alleviate a variety of ailments (Kou *et al*., 2018). α-Zingiberene, β-sesquiphellandrene, α-curcumene, α-farnesene, β-bisabolene, camphene, *etc*., are identified as the significant terpenes providing characteristic aroma, nutritional, and medicinal properties (Kiyama, 2020; Mao *et al*., 2019).

Red ginger is one of the ginger genotypes that belongs to the Zingiberaceae family. Although red ginger bears some resemblance to common white ginger in

terms of morphology, it is distinguished by its smaller and more pungent rhizomes (Gnasekaran *et al*., 2021; Suciyati & Adnyana, 2017). Furthermore, the rhizome of red ginger is adorned with scarlet-coloured scaly leaves. Additionally, the pulvinus of the leaf sheath exhibits a striking scarlet stain. This plant species is predominantly found in Peninsular Malaysia as well as in neighboring Southeast Asia countries, including the northeastern states of India (Kizhakkayil & Sasikumar, 2011; Weiss, 2002). Red ginger is particularly valued for its medicinal properties, in contrast to common ginger, which is used as a culinary spice. Red ginger is superior to other ginger varieties due to the presence of higher essential oil and phenolic compounds such as gingerols and shogaols (Rinanda *et al*., 2018). It holds a significant role in traditional medicine among Indonesians and Malaysians. It has been traditionally employed as a remedy for various health conditions such as rheumatism, stomach ailments, respiratory problems, and postpartum recovery assistance (Ghasemzadeh *et al*., 2015). According to scientific research, red ginger has been demonstrated to possess antioxidant, anticancer, anti-inflammatory, antimicrobial, antifungal, and antihypertensive properties (Shimoda *et al*., 2010; Sivasothy *et al*., 2011). The characterization and comparison of available Indian and exotic red ginger germplasm may unveil the true potential of these genotypes.

Genetic diversity forms the foundation of every crop improvement initiative and the success of selection hinges on its type and extent within the genetic resources accessible to plant breeders. The lack of pollen fertility and self-incompatibility are the main constraints to sexual reproduction in ginger, resulting in limited genetic diversity for breeding (Jatoi & Watanabe, 2013). Understanding the levels and patterns of genetic diversity holds significant importance for various applications within the field of plant breeding. Hence, a crucial determinant of the effectiveness of a breeding initiative lies in the presence of genetic diversity within the targeted population. The characterization and agronomic assessment of ginger have been extensively researched on a global scale, with a primary focus on countries where ginger holds significant value, such as India, China, Japan, Thailand, and others. Numerous investigations have identified varying degrees of variability and furnished valuable insights into the correlations between plant traits,

yield, and their collective impact on genetic diversity (Chongtham *et al*., 2013; Jatoi & Watanabe, 2013; Islam *et al*., 2017; Rana *et al*., 2019; Anargha *et al*., 2020). The research conducted on the genetic variability of ginger in India revealed that there is a moderate to high variability in terms of quantitative and qualitative traits (Akshitha *et al*., 2019; Basak *et al*., 2019). The North Eastern states of India and the Western Ghats, where ginger cultivation takes place, are renowned for ideal agro-climatic conditions and a rich reservoir of germplasm (Sasikumar *et al*., 1995). It is crucial to undertake a comprehensive investigation and documentation of the diverse range of ginger genotypes cultivated in these regions, especially in North Eastern states. This will enable us to fully understand and appreciate the richness and diversity of these plants in that area.

Ginger essential oil and oleoresins extracted from the rhizome have gained global recognition due to their significant biological properties (antimicrobial, antioxidative, anticancer, and anti-ulcer). These properties are of great interest in the pharmaceutical, cosmetics, and food sectors (Uddin *et al*., 2023). Due to its smaller size, the red ginger rhizome is unable to adequately fulfil the ever-increasing need for bioactive compounds that are extracted from rhizomes, including oils and phenolics. Consequently, it is essential to improve ginger productivity and optimize the production of its secondary metabolites. Conventional breeding methods have proven ineffective due to the absence of seed set. Significant advancements in breeding can be achieved by selecting superior varieties from the available germplasm and employing *in vitro*-based approaches such as tissue culture, somaclonal variations, mutation, and polyploidy breeding.

Polyploidy, which refers to the presence of more than two sets of chromosomes, is a prevalent phenomenon in flowering plants and plays a key role in angiosperm evolution and diversification (Salma *et al*., 2017). The whole genome duplications provide organisms with the ability to produce new phenotypes, enhancing their adaptability and resilience in response to environmental challenges (Rutland *et al*., 2021). The common ways that polyploids are generated include gametic non-reduction and somatic doubling, a less frequent mechanism (Ramsey &

Schemske, 1998; Levin, 2002; Madlung, 2013). Polyploidy can be intentionally induced primarily through the application of antimitotic chemicals such as colchicine, oryzalin, trifluralin, and related substances, which inhibit the spindle fibre formation and result in the cells with doubled chromosomes (Sattler *et al*., 2016). To embark on this endeavour, effective polyploidization systems are essential. The two main approaches for achieving polyploidization involve *in vivo* and *in vitro*. The *in vivo* method involves the direct application of chemicals to various parts of the plant. Whereas the *in vitro* method is the most widely adopted method for polyploidization, enabling accelerated polyploid development within a controlled and confined environment (Nasirvand *et al*., 2018).

An efficient *in vitro* culture protocol is a fundamental requirement for achieving successful *in vitro* polyploid induction. Limited work has been reported on the standardization of tissue culture media and micropropagation techniques for red ginger genotypes (Zuraida *et al*., 2016; Karyanti *et al*., 2021). Hence, it is essential to develop a reliable and standard *in vitro* regeneration system for red ginger. Plantlets can be generated *in vitro* by cultivating suitable explants in nutrient-rich media under ambient culture conditions. The effectiveness of the tissue culture method is influenced by numerous factors, encompassing the source and maturity of the explant, sterilization procedures, the composition of the culture medium, the presence of plant growth regulators, incubation conditions (Seran, 2013; Ravindran *et al*., 2016). *In vitro* regeneration systems offer a potent tool for manipulating ploidy, thus facilitating the breeding and development of novel crop varieties (Touchell *et al*., 2020).

Polyploidy breeding has garnered significant attention among the array of crop improvement methods, emerging as a versatile and pivotal technique in the realm of plant breeding. At present, polyploid breeding techniques are applied to a wide range of agricultural and horticultural crops, such as watermelon (Khan *et al*., 2023), wheat (Walkowiak *et al*., 2020), bananas (Amah *et al*., 2019), and citrus (Ollitrault *et al*., 2020). Organisms with higher ploidy levels frequently display morphological characteristics that either differ from or are more apparent than those

of their diploid counterparts. Polyploid organisms often manifest an array of improved traits, such as increased leaf thickness and darker pigmentation, larger and longer-lasting flowers with robust petals, increased vigor, enhanced tolerance to environmental stressors, pests, and pathogens, augmented metabolite production, and the potential to restore fertility in otherwise sterile hybrids (Adams & Wendel, 2005; Comai, 2005). The most widely observed consequence of polyploidization in plants is the enlargement of cell size. Subsequently, these increments can be observed in the enlargement of individual organs and may even extend to the entire plant's size (Dhooghe *et al*., 2011). Polyploids can directly result in morphological and anatomical modifications and affect various physiological and biochemical processes.

The increased rhizome vigour resulting from tetraploidy, can be effectively utilized in ginger cultivation to create genotypes with robust rhizomes that are well-suited for the processing industry. Artificial polyploidy for the increased production of essential oils and secondary metabolites has been successfully achieved in *Citrus limon* (Bhuvaneswari *et al*., 2020), *Z. officinale* (Prasath *et al*., 2022), and *Mentha spicata* (Bharati *et al*., 2023a). Genome duplication can lead to changes in the biochemical composition and metabolic profile of the plant (Madani *et al*., 2021).

It is crucial to uncover the genetic variability of exotic ginger genotypes to guide future conservation and genetic enhancement studies. Comparative studies between the Indian genotypes with the exotic type will add to ginger improvement. The red ginger genotypes displaying superior quality traits can serve as valuable parent plants in hybridization endeavours. Considering the discoveries and the promising possibilities of synthetic polyploidization, it would be reasonable to suggest that it could serve as an exceptional method for producing genotypes characterized by enhanced morphology and increased essential oil contents in red ginger. Nevertheless, given its medicinal and economic significance, it is unexpected that there have been no prior investigations into the potential of synthetic polyploidization in red ginger to enhance rhizome vigour and essential oil yield. Therefore, this study aimed to generate polyploids of red ginger genotypes

through *in vivo* and *in vitro* polyploidization using colchicine and to evaluate its effect on selected horticultural characteristics.

Objectives

- Comparative characterization of red ginger accessions based on morphological characters and chemical fingerprints.
- To genetically improve red ginger through *in vitro* and *in vivo* polyploidy induction using colchicine and its effect on selected horticultural characteristics and essential oil composition.

CHAPTER 2
REVIEW OF LITERATURE

Ginger (*Z. officinale* Rosc.), the most popular spice crop, belongs to the family Zingiberaceae, which is broadly distributed in tropical regions. Zingiberaceae is the largest family within the Zingiberales order, comprising 53 genera and more than 1300 species, with about 150 species under the genus *Zingiber* (Ravindran & Babu, 2016; Unuofin *et al*., 2021).

Ginger has a rich history of cultivation in India and China and is believed to have originated in Southeast Asia (Kumari *et al*., 2020). Later, it was introduced to various other regions across the world. Now, it is cultivated and found in various tropical and subtropical regions across the globe (Fig. 1), including countries such as Jamaica, Taiwan, Sierra Leone, Brazil, Nigeria, Fiji, Japan, Mauritius, Indonesia, Sri Lanka, Costa Rica, Ghana, Hawaii, Malaysia, Bangladesh, Philippines, Thailand, Solomon Islands, Trinidad and Tobago, Uganda, Guatemala, as well as numerous islands in the Pacific Ocean. Ginger production in India is contributed by the 15 states, namely, Andhra Pradesh, Assam, Karnataka, Odisha, Meghalaya, Mizoram, Arunachal Pradesh, Uttaranchal, Bihar, Telangana, Madhya Pradesh, West Bengal, Maharashtra, Kerala, and Gujarat. India, the world's largest producer of ginger, produced 2.43 million tons from 20.59 lakh ha in 2022-2023. Among the various ginger-growing states, Madhya Pradesh produced the highest quantity of 5.45 lakh tons, followed by Karnataka (5.00 lakh tons) and Odisha (2.27 lakh tons) during the year 2022-2023. India exported about 50885 tons with an export value of 432.46 crores (Spices Board of India, 2023).

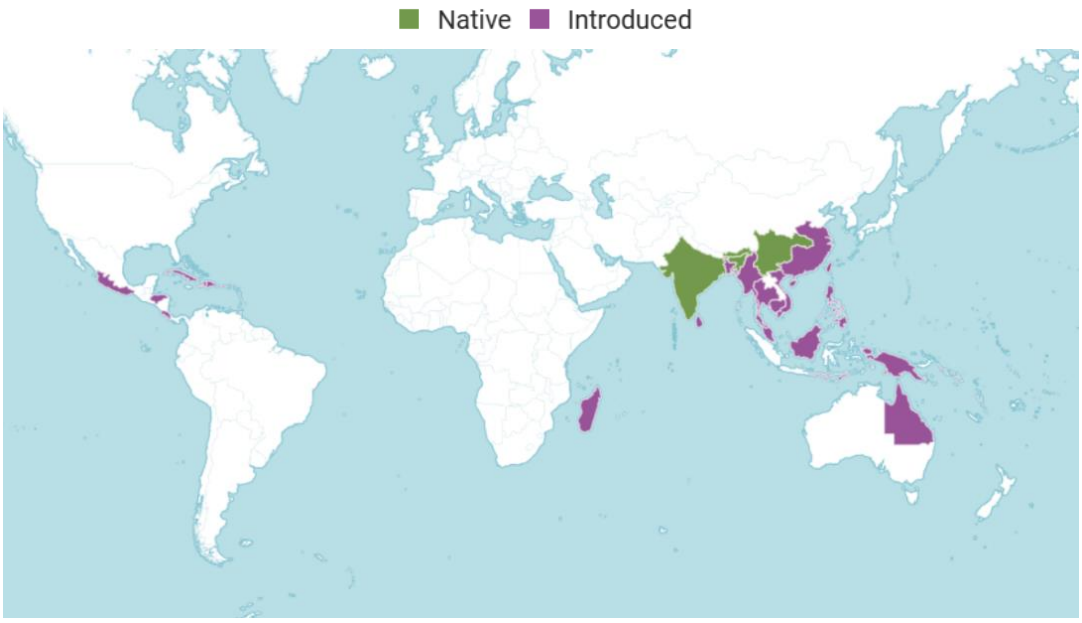

Fig. 1. Distribution of ginger across the world (Source: https://powo.science.kew.org/)

2.1 Red ginger (*Z. officinale* Rosc.; Synonym: *Z. officinale* var. *rubrum*)

Red ginger is primarily cultivated in Indonesia, China, and Malaysia. In Indonesia, it is commonly referred to as 'Jahe Merah', while in Malaysia, it is known as 'Halia Bara'. The North-Eastern regions of India exhibit immense variability in ginger genotypes. Red ginger is also commonly found in the north-eastern states of India, particularly in Nagaland (Yadav *et al*., 2004).

The red ginger is characterized by its red colour, both on the outer skin of the rhizomes and at the base of the shoot. It is an annual plant that typically reaches a height of 50-100 cm. The rhizomes have a reddish-brown colour and are thick. The morphology of red ginger is comparable to common ginger, but the rhizome is smaller in size and has a more pungent flavour. The leaves are lancet-shaped and narrow, ranging from 5-25 cm in length and 0.8-2 cm in width. In contrast to common ginger, the petiole appears reddish in young red gingers. The flowers are born on a cone-shaped spike with a stem length of 10-25 cm and green overlapping bracts at the base of the flower. The corolla is tubular, trilobed, yellowish, and about

2-2.5 cm long. The labellum is trilobed and dark purple with creamy yellow spots (Suciyati & Adnyana, 2017; Zhang *et al*., 2022).

Red ginger has a complex chemical composition, comprising approximately 169 different chemical constituents. These constituents consist of monoterpenes, diterpenes, sesquiterpenes, flavonoids, vanilloids, and other compounds (Sivasothy *et al*., 2011; Kusumavathi *et al*., 2017). The biological properties exhibited by red ginger are attributed to the combined or cumulative effects of its various constituents. Additionally, red ginger comprises amino acids, vitamins, and trace elements such as iron, zinc, manganese, copper, chromium, nickel, and strontium. According to a study by Ghasemzadeh *et al*. (2010), the overall content of flavonoids and phenolic compounds in red ginger is higher compared to common ginger. The various active compounds present in ginger can grouped as volatiles and non-volatiles. The volatile fraction includes the essential oils actively contributing to the aroma. The dried rhizome contains about 1-4% essential oil, with sesquiterpene derivatives being the main constituent, such as (-)-zingiberene, (+)-curcumene, (-)-β-sesquiphellandrene, and β-bisabolene. Furthermore, ginger also contains derivatives of monoterpenes, which include α-pinene, β-pinene, borneol, camphene, linalol, bornyl acetate, cineol, limonene, ρ-cymene, curcumene, citral, β-elemene, geraniol, farnesene, β-phellandrene, myrcene, and sabinene. The non-volatile compounds present in ginger are found in the form of oleoresin, which typically ranges from 4.0% to 7.5% of the ginger's composition. This oleoresin contains various components such as gingerol, shogaol, gingediol, gingediasetat, gingerdion, and gingerenon, which collectively contribute to the spicy flavour associated with ginger (Hernani & Hayani, 2001; Kusumawati *et al*., 2017; Prasad & Tyagi, 2015).

2.2 Cytology

Ginger is reported to have a chromosome number of 2n = 2x = 22, with a basic chromosome number suggested as x = 11 (Sugiura, 1936; Raghavan & Venkatasubban, 1943; Chakravorti, 1948; Sato, 1960; Ramachandran, 1969; Ratnambal, 1979; Omanakumari & Mathew, 1985; Rai *et al*., 1997; Das *et al*., 1998; Dhamayanthi & Zachariah 1998; Eksomtramage *et al*., 2002; Nayak *et al*., 2005;

Wang *et al*., 2014). Karyomorphological studies by Bhadra and Bandyopadhyay (2015) also indicated the chromosome number of ginger as 2n = 22. Subsequently, certain workers have reported variations in chromosome numbers. Raghavan and Venkatasubban (1943) conducted an extensive cytological study involving 25 Zingiberaceae species, including *Z. officinale*, revealing the occurrence of two basic chromosome numbers (x = 11 and 12) in ginger. However, Etikawati and Setyawan (2000) reported that all the varieties of *Z. officinale*, in Indonesia including, red ginger had the same chromosome number (2n = 32), with a basic chromosome number of x = 16. These findings contrast with those of Daryono *et al*. (2012), who demonstrated that *Z. officinale* var. *officinale* and *Z. officinale* var. *amarum* shared a similar diploid chromosome number of 2n = 2x = 30, while red ginger exhibited a different diploid chromosome number (2n = 2x = 22). Analysis of somatic chromosome numbers in 21 ginger germplasm collections unveiled that the two accesions exhibiting high pollen fertility (Acc. 195 and Acc. 821) were tetraploids with 2n = 44. While most other genotypes displayed the normal somatic chromosome number of 2n = 22. Additionally, one collection was found to possess aneuploid chromosomes, with a count of 2n = 24 (Nair, 2016).

2.3 Pharmacological activities and medicinal uses

The rhizome of *Z officinale* has been recognized to hold numerous medicinal properties. These include anti-nausea/antiemetic, anti-inflammatory, antioxidant, antifungal, antibacterial activity, anticancer, and antidiabetic properties (Fahrurrozi & Wirawan, 2019; Levita *et al*., 2018; Suciyati & Adnyana, 2017). In traditional medicine, red ginger is utilized to alleviate various conditions including headaches, bloating, indigestion, nausea, vomiting, rheumatism, and respiratory issues (Gnasekaran *et al*., 2021; Prasetyo *et al*., 2022). Moreover, it is commonly employed in the management of bacterial infections, hypertension, atherosclerosis, hyperuricemia, hypercholesteremia, rubella, tuberculosis, autoimmune diseases, growth disorders, and cancer (Nordin *et al*., 2015; Razali *et al*., 2020). The pharmaceutical value of red ginger is associated not only with its essential oils and terpenoids but also with phenolic compounds such as gingerol and shogaol. These

compounds play a substantial role in the medicinal properties of red ginger (Lukiati *et al*., 2020).

Ginger finds widespread and safe use in the medicine, pharmaceutical, and food industries. For more than 25 centuries, ginger has held significant importance in traditional Indian, Chinese, and Japanese medicine. The rhizome, or underground stem, is particularly in high demand as a traded product. Its pungent taste and stimulating aroma are the defining characteristics that make ginger an indispensable ingredient in global cuisines and the food processing industry. The essential oil extracted from ginger finds application in various sectors, such as flavouring beverages, confectionery, cosmetics, perfumes, and pharmaceuticals (Babu *et al*., 2016). In Western countries, ginger is utilized in a wide range of culinary creations including gingerbread, cakes, soups, puddings, biscuits, pickles, wine, and beer (Kumari *et al*., 2020). Various red ginger products are available in the market, including food items like pickled ginger and dried ginger, as well as red ginger essence found in instant beverages like coffee and tea. Moreover, red ginger extracts are incorporated into creams, ointments, body lotions, capsules, herbal formulations, and cosmetics (Jayanudin *et al*., 2015; Zhang *et al*., 2022).

The main emphasis of this investigation is to explore the genetic diversity among red ginger genotypes and to improve red ginger through *in vitro* and *in vivo* polyploidy induction. In relation to the earlier research conducted, a brief review of the ginger crop improvement is outlined under the following subheadings in this chapter.

1. Morphological characterization and genetic variability studies

2. Biochemical characterization

3. Direct *in vitro* regeneration of red ginger

4. Polyploidy breeding

2.4 Morphological characterization and genetic variability studies

Flowering and pollination are rare occurrences in ginger. Natural seed set has not been observed thus far due to low pollen fertility and self-incompatibility (Dhamayanthi *et al*., 2003). Hence, this leads to limited genetic diversity, which poses challenges for crop improvement initiatives. As a result, the scope for crop improvement programs in ginger is limited to clonal selection, mutational breeding, polyploidy breeding, and other biotechnological methods (Babu *et al*., 2016). To ensure meaningful breeding, protection, and sustainable utilization of available resources, it is crucial to understand the current state of diversity within the specific taxonomic group. This understanding serves as the primary step in effectively managing and utilizing genetic resources in a responsible and informed manner (Jan *et al*., 2011).

Germplasm is a pool of available plant genetic resources that can be used as seed material for crop improvement and could help boost output. Identifying unique genotypes, understanding genetic connections, including parentage, and the effective management and use of germplasm all depend on the characterization and evaluation of diversity (Pathirana & Carimi, 2022). Morphological characterization remains the fundamental and first stage in diversity evaluation before using other cutting-edge techniques. It enables the clustering of accessions, the creation of core collections, the finding of collection gaps, and the selection of germplasm for breeding programmes (Rohini *et al*., 2020). Agronomic assessment is necessary for plant breeding to determine the level of variability existing in the genetic material. These studies revealed the available genetic diversity among the ginger genotypes, thus providing relevant information on the levels of agricultural and economic traits of plants (Deme *et al*., 2021).

Ginger has been studied extensively for its yield, quality traits, and rhizome characters, with numerous researchers investigating this variability. Studies conducted by Khan (1959), Thomas (1966), Krishnamurthy *et al*. (1972), Muralidharan and Kamalam (1973), Mohanty and Sharma (1979), Nybe and Nair (1979), Nybe *et al*. (1980), Kumar *et al*. (1980), Mohanty *et al*. (1981), Arya and

Rana (1990), Saikia and Shadeque (1992), Sasikumar *et al*. (1992), Ravindran *et al*. (1994), Pandey and Dobhal (1993), Sasikumar *et al*. (1999, 2003), Chandra and Govind (1999), Singh *et al*. (1999), Singh *et al*. (2000), Das *et al*. (2000), Tiwari (2003), Abraham and Latha (2003), Rana and Korla (2007), Medhi *et al*. (2007) and Islam *et al*. (2008) have collectively contributed to our understanding of the variability observed in ginger regarding these aspects.

Ravindran *et al*. (1994) attempted the characterization of about 100 accessions maintained in the National Active Germplasm site, ICAR-IISR, Peruvannamuzhi, based on the data collected on morphology, yield, and quality parameters. The number of tillers per plant showed maximum variability (2.75-35.50) followed by yield per plant (55-770 g). Shogaol content (2.70-7.50 %) exhibited the highest variability among the quality traits.

Genetic variability of 36 ginger accessions was done by Aragaw *et al*. (2011) at two locations in Ethiopia. The highest genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) were documented for the number of plants per plot (29.37 %, 31.92 %), fresh rhizome yield (38.78 %, 38.79 %), and dry rhizome yield (39.20 %, 39.96 %). High heritability and genetic advance were observed in oleoresin (94.96 %, 38.28 %), essential oil (82.28 %, 35.46 %), fibre content (99.10 %, 40.74 %), and fresh (99.90 %, 46.61 %) and dry rhizome (99.21 %, 48.44 %) yield. The findings indicated a broad range of variability among the ginger accessions.

Jyotsna *et al*. (2012) examined the growth, yield, and quality of four types of organically produced ginger growing in Manipur under rain-fed conditions, namely Manipur local, Bhaisey, Gorubathan, and Nadia. The findings of the study indicated that Bhaisey exhibited the highest yield and dry matter content, measuring 20.46 t ha^{-1} and 20.4 %, respectively. Gorubathan ranked second in terms of yield (19.13 t ha^{-1}) and dry matter content (19.7 %) while also displaying favourable quality characteristics compared to the other varieties.

In a study conducted by Chongtham *et al*. (2013) in southern West Bengal, 10 ginger genotypes were evaluated for their yield and quality characteristics. The

genotypes exhibited significant variations in all yield attributes, except for the length of primary finger. Among the genotypes, Gorubathan had the highest values for yield attributes, such as length of primary fingers, diameter of secondary fingers, and rhizome yield per plant. On the other hand, Sambuk had the highest dry recovery rate at 33.48 %.

High heritability was observed for plant height, number of shoots, number of mother and finger rhizomes, and yield per plant in five ginger varieties (Lakshmi & Rajasekhar, 2013). The genotype Suprabha demonstrated significantly higher productivity in terms of fresh rhizome yield. There was a positive correlation observed between all the characters and rhizome yield.

The genetic diversity among ginger genotypes from eastern parts of India was analysed by Ravishanker *et al*. (2013). Knowledge of the genetic variation among elite germplasm is critical for finding potential lines for a trait of interest. They used cluster analysis and Principal Component Analysis (PCA) to screen out unique parents for breeding high-yielding ginger rhizomes. Among that, 25 genotypes were categorized into five clusters using cluster analysis. These clusters had the highest level of divergence. This result is consistent with that of Jatoi *et al*. (2006) who found significant genetic variations in an Asian collection of ginger.

Jatoi and Watanabe (2013) gave an insight into diversity and relationship among 19 accessions of ginger landraces by examining 32 morphological traits. Plant height, tillers per plant, sheath length, leaf length, rhizome weight and rhizome thickness and all showed high to moderate variation in the germplasm. Principal component analysis was used to identify the variability and distribution pattern of gingers and all yield-associated traits showed high contribution to PC1.

Rajyalakshmi and Umajyothi (2014) conducted a study on various genotypes of ginger, focusing on yield-attributing characteristics such as tillers per plant, number of mother and finger rhizomes per plant, and yield per plant. The researchers found significant variations among the genotypes for these traits. The variety Suprabha exhibited the highest fresh rhizome yield at 21.71 t ha^{-1}, while IISR Varada produced a significantly lower rhizome yield of 6.67 t ha^{-1}. The PCV

values was higher than the GCV values for all the studied traits. Notably, high GCV, PCV, heritability, and genetic advance as a percentage of the mean were recorded for the number of tillers per plant, number of mother and finger rhizomes per plant, and fresh rhizome yield. Rhizome yield displayed the highest GCV value at 72.46%.

Kallappa *et al*. (2015) conducted a study in the hill sector of Karnataka to assess yield and quality characteristics among 10 ginger varieties. The research revealed significant variations in these traits among the varieties. Maran exhibited the highest fresh rhizome yield, measuring 29.37 t ha^{-1}, followed by Rio-de-Janeiro with 28.04 t ha^{-1} and Karkal Local with 25.84 t ha^{-1}. The study concluded that Maran, Rio-de-Janeiro, and Karkal Local performed exceptionally well in terms of both yield and quality parameters in rainfed conditions within the hilly region of Karnataka.

For yield and quality parameters in 16 ginger germplasm lines, Bategari (2016) analysed genetic diversity, heritability, and genetic advance as a percentage of mean. Significant genetic variability was noted among all the genotypes for plant height, number of leaves per plant, and fresh yield per plant. The highest GCV was observed for the number of leaves (28.64 %), oleoresin content (20.64 %) and fresh yield per plant (18.12 %). In all cases, the PCV exceeded the GCV. Traits such as yield per plant (89.20 %), oleoresin content (80.30 %), stem girth (79.39 %), number of leaves per plant (75.69 %), and plant height (67.93 %) exhibited high heritability and were considered superior.

Kumar *et al*. (2016) performed a multivariate analysis on ginger germplasm from North Eastern India. A total of 82 ginger accessions were collected and subjected to morphological characterization, utilizing 12 quantitative and seven qualitative characters. The study revealed significant variations among all 12 quantitative characters studied, as well as considerable variations in the qualitative characters.

The qualitative and quantitative features of ginger from Burkina Faso germplasm exhibited modest genetic diversity (Nandkangre *et al*., 2016). The morphometric characterization of 56 landraces revealed significant variation for

traits such as rate of emerged shoots, plant height, leaf length, number of leaves, rhizome length, and width. The number of leaves, leaf length, and rhizome width all had a significant impact on plant height.

In a study conducted in the high rainfall regions of Tamil Nadu, 24 ginger genotypes were examined for their yield and quality. Among them, Sengottai local (Z.O4) demonstrated the highest fresh rhizome yield, recorded at 227.47 g plant^{-1}, and the highest dry rhizome yield, measuring 48.57 g plant^{-1}. The genotype Z.O-1 (IISR Varada) followed closely with a rhizome yield of 224.16 g plant^{-1} and a dry rhizome yield of 47.73 g plant^{-1}. The dry recovery percentage varied from 16.68 % to 22.47 %, with Z.O-6 achieving the highest dry recovery of 22.47 %, followed by Sengottai local (22.33 %) (Balakumbahan & Joshua, 2017).

Blanco and Pinheiro (2017) conducted a study in Brazil to assess the genetic variability of ginger genotypes based on agronomic characters. The study revealed significant variations among 61 accessions across the agronomic traits. Additionally, the research highlighted the significance of genetic aspects in the expression of phenotypes such as plant height, rhizome thickness, and yield traits, as indicated by the high CVg/CVe ratio (>1) and substantial heritability (>80%). Notably, several accessions were identified as potential clones based on their favourable agronomic traits. Identification and characterization of available germplasm may be useful for future breeding programmes.

Umar *et al*. (2017) studied the genetic variability, heritability, and genetic advance of eight quantitative characters among 25 ginger accessions. Significant genetic variability was noted across all accessions for the number of tillers, plant height, leaf length, number of primary fingers per rhizome, number of secondary fingers per rhizome, and fresh rhizome yield.

Twenty ginger germplasm samples were subjected to genetic diversity investigations by Islam *et al*. (2017) to identify promising germplasm for the release of a variety. Analysis of variance and D^2 analysis was performed, and the lines were grouped into five clusters.

In a study by Ravi *et al*. (2017) in the Sirsi and Uttara Kannada area of Karnataka, Humnabad Local was identified as a superior genotype based on growth and yield parameters. Among the various genotypes evaluated, Humnabad Local exhibited higher values for several parameters. These included plant height, number of leaves, and leaf area index. Additionally, Humnabad Local displayed superior yield attributes, such as a length of primary fingers of 7.41 cm, length of secondary fingers of 5.78 cm, and rhizome yield per plant of 360.20 g. The projected fresh rhizome yield was recorded as 21.55 t ha^{-1}, and the dry recovery percentage was the highest at 27.35 % for Humnabad Local.

Chakraborty *et al*. (2018) evaluated some important ginger genotypes to measure suitability and performance in Terai region of West Bengal. Out of the eight genotypes, GCP-49 showed the highest rhizome yield per plot (11.19 kg $plot^{-1}$). GCP-49 exhibited the highest dry matter content of 21.7 % compared to other varieties. GCP-49 was identified as the superior genotype among the investigated genotypes.

Similarly, Basak *et al*. (2019) conducted a study to characterize and evaluate 18 ginger genotypes in the Terai region of West Bengal. The findings indicated that various morphological and rhizome traits of ginger had interdependencies and influenced the overall yield. The yield and number of shoots exhibited high GCV values of 43.26 % and 32.4 %, respectively. The yield also showed a high PCV value of 45.2%. Regarding heritability, rhizome thickness recorded the highest value at 94.26%, followed by yield at 91.36 %. Significant genetic gains were observed for rhizome yield (85.07 %) and number of shoots (55.04 %). Based on the evaluation, the genotypes GCP-39, SE-8640, SEHP-9, SE-8681, SG-2640, and Acc. 247 were identified as suitable for cultivation in the Terai region of West Bengal.

Among the 10 DUS characters studied, 27 ginger genotypes exhibited maximum variation for growth habit, shoot diameter, number of tillers, rhizome thickness, shape, and dry recovery (Akshitha *et al*., 2019).

Rana *et al*. (2019) found substantial variability in yield and yield contributing factors of 22 germplasm lines collected from important ginger-growing

regions of Himachal Pradesh and Manipur. The characters such, as number of leaves, weight of primary fingers, and yield per plant all had high PCV and GCV.

Das *et al*. (2020) conducted a study aiming to eliminate duplicate entries in the ginger germplasm collection in Odisha by utilizing morphological and molecular characterization. The objective was to identify and remove any existing synonyms from the ginger germplasm conservatory. Among the total of 60 accessions, 45 exhibited significant differences in their morphological traits.

Morphological characters, both vegetative and generative organs, of 13 ginger genotypes of Zingiberaceae from Serang District, Indonesia, were observed and documented by Windarsih *et al*. (2021). Based on the results, there were variations in vegetative and generative characteristics including plant growth habit, petiole, leaf margin, leaf venation, leaf blade shape, colour of the pseudo-stem base, leaf blade base, leaf blade apex, rhizome surface roughness, colour of rhizome flesh, rhizome shape by transversal section, and anthocyanin colour of the bud of the rhizome.

Dev (2022) evaluated the genetic variability of 40 distinct ginger genotypes sourced from various regions of the country. The study revealed high heritability and significant genetic gains for yield per plot and the weight of mother, primary, and secondary rhizomes. It is suggested that these traits hold significance for selection and can be effectively enhanced through breeding efforts. The overall assessment demonstrated substantial variability among ginger genotypes, indicating the potential for improving yield and quality attributes through breeding efforts.

The kinship of local ginger in Pandeglang Regency, Banten Province, Indonesia was conducted based on morphologic traits (Prasetyo *et al*., 2022). According to the findings, two types of gingers, namely small white ginger, and red ginger, were discovered in various locations throughout Pandeglang. Both ginger genotypes exhibited similarities in their morphological characteristics, such as stem shape, stem colour, leaf shape, leaf tip, leaf base, and root shape. These results indicate a significant kinship connection among ginger genotypes in the Pandeglang Regency.

Chukwudi (2022) attempted classification of ginger germplasm and identification of morphological markers associated with rhizome yield. The pseudostem diameter, plant height, number of leaves per plant, and leaf width exhibited the strongest correlation coefficients with ginger rhizome yield.

Soni *et al*. (2022) conducted an experiment to evaluate the performance of seven ginger genotypes under Mizoram condition. The study concluded that Bold Nadia, Bhaise, PGS 102, and Gorubathani exhibited superior performance in terms of high fresh weight of clump, rhizome yield, and other yield-contributing traits. Notably, Bold Nadia stood out as particularly well-suited for dry ginger purposes.

The GCV, PCV, heritability, correlation, and path analysis were evaluated for the 150 germplasm collected across North East India. The ANOVA result indicated substantial variations across the germplasm for the observed traits, suggesting a considerable level of variability within the collections (Begum *et al*., 2023).

2.5 Biochemical characterization

The assessment of genetic diversity relies heavily on the characterization of biochemical constituents, as it is an essential and necessary component of the process. The analysis of ginger genotypes to determine the levels of chief biochemical constituents such as essential oil, oleoresin, and crude fibre has enabled the identification of cultivars that are abundant in specific constituents (Kizhakkayil & Sasikumar, 2011). The composition of plant phytoconstituents varies and is influenced by environmental factors such as humidity, rainfall, and temperature, as well as geographic elements like altitude, soil type, and duration of sunlight (Chaudhary *et al*., 2023). Hence, biochemical characterization holds importance when the genotypes were obtained from various geographical locations, along with morphological markers. Conducting a quality profiling of accessions within the germplasm collection can be valuable in identifying genotypes that are well-suited for different end uses (Ravindran & Babu, 2004).

2.5.1 Essential oil

The distinct aroma and flavour of ginger can be attributed to the presence of its essential oil (Zachariah, 2008). The yield of essential oil ranges from 1.0% to 3.0% in dry ginger (Govindarajan, 1982). Diverse extraction procedures, such as conventional hydro distillation, microwave-assisted hydro distillation, solvent-free microwave hydro distillation, and improved solvent-free microwave extraction with three microwave-absorption media, were used to obtain the essential oil from ginger (Sharifi-Rad *et al*., 2017). The extraction of essential oils from ginger is influenced by several factors including the variety and origin of the plant, cultivation practices, humidity during harvest, extraction methods, and, the age of the plant (Kamaliroosta *et al*., 2013). The composition of ginger essential oils has been analyzed and measured using GC-MS or GC with flame ionization detector techniques (Sultan *et al*., 2005; Singh *et al*., 2008). The analysis of the obtained essential oils revealed that α-zingiberene was the most prevalent compound (17.4 to 25.4 %), ar-curcumene (14.1 % to 16.4 %), β-bisabolene (9.9 % to 12.5 %), and β-sesquiphellandrene (9.7 % to 13.4 %) (Wang *et al*., 2006; Uddin *et al*., 2023).

Several studies were conducted to investigate the isolation methods, chemical composition, and pharmacological properties of ginger essential oil (Nigam *et al*., 1964; Krishnamurthy *et al*., 1970; Smith & Robinson, 1981; Van Beek *et al*., 1987; Erler *et al*., 1988; Ekundayo *et al*., 1988; Saikia & Shadeque, 1992; Afzal *et al*., 2001; Onyenekwe & Hashimoto, 1999; Figueiredo *et al*., 2008; Singh *et al*., 2008)

The steam distillation method was employed to extract essential oils from fresh rhizomes of 17 different clones of Australian ginger, which comprised both commercially cultivated varieties and experimental tetraploid clones. The analysis of the obtained oils was conducted using GC-MS after a 7-week post-harvest period. The essential oils obtained from these samples were notable for their significantly high levels of citral, ranging from 51 % to 71 %. In contrast, they exhibited lower levels of sesquiterpene hydrocarbons, which are typically found in ginger oil (Wohlmuth *et al*., 2006).

GC-MS analysis was conducted to determine the chemical composition of the essential oil derived from ginger rhizomes collected from Nahan, Himachal Pradesh, India. A total of 51 compounds, accounting for 95.1 % of the oil, were recognized. The essential oil exhibited high quantities of various monoterpenoids, including geraniol (14.5 %), geranial (9.5 %), 1,8-cineole (10.9 %), neral (8.1 %), geranyl acetate (6.3 %), borneol (5.6 %), trans-dimethoxy citral (5.0 %), linalool (4.8 %), and alpha-terpineol (3.6 %). Additionally, five compounds, namely trans-linalool oxide, trans-linalool oxide acetate, (Z)-dimethoxy citral, (E)-dimethoxy citral, and epi-zingiberenol, were noted in ginger oil for the first time (Gupta *et al*., 2011).

The essential oils derived from the rhizomes and leaves of *Z. officinale* var. *rubrum* Theilade were examined using capillary GC and GC-MS. The oil extracted from the leaf showed 46 constituents, while 54 different constituents were identified from the rhizome oil. The dominant compound in the leaf oil was β-caryophyllene, accounting for 31.7 % of the composition. On the other hand, the rhizome oil was composed of monoterpenoids, such as camphene (14.5 %), geranial (14.3 %), and geranyl acetate (13.7 %) as the three major compounds (Sivasothy *et al*., 2011).

Sasidharan *et al*. (2012) isolated and characterized the essential oil derived from two common cultivars of ginger, Bhaise, and Majulay, cultivated in Sikkim, using analytical GC and GC-MS techniques. In total, 60 compounds were recorded. The major compounds found in oil isolated from Bhaise were geranyl acetate (18.8 %), zingiberene (16.3 %), and geranial (8.2 %), while zingiberene (19.8 %) and geranial (16.5 %) were the dominant compounds in oil extracted from Majulay. Notably, oil isolated from Bhaise exhibited a higher proportion of oxygenated compounds (43.1 %) compared to other ginger cultivars. This study represented the first report on the essential oils obtained from ginger cultivars in Sikkim.

A total of 46 ginger germplasm accessions were analysed using GC/MS to profile their volatile oil constituents. Additionally, HPLC was used to identify the pungent principles present. The essential oil analysis identified 60 compounds. The accessions were grouped into 14 clusters based on Jaccard's and Sorensen-Dice

similarity coefficients, which exhibited similar forms and content. However, the third similarity coefficient, simple matching, resulted in slightly different groupings. Notably, the study revealed that three accessions, namely Brazil, Kintoki, and Pink Ginger, were distinct and unique. HPLC analysis showed that 6-gingerol was the principal pungent compound in most ginger accessions, except for the exotic ginger Oman, where 8-shogaol was the principal compound (Kizhakkayil & Sasikumar, 2012).

In a study conducted by Choudhari and Kareppa (2013), the methanol extract of dried ginger rhizomes was subjected to GC/MS analysis. The results revealed the presence of various compounds, with zingiberene (23.69 %), α-bergamotene (23.69 %), and Ar-curcumene (23.69 %) being the most abundant constituents. Other identified compounds included gingerol (14.31 %), zingerone (10.07 %), β-sesquiphellandrene (9.94 %), (Z)-β-farnesene (9.94 %), caryophyllene (9.94 %), and β -elemene (0.72 %).

Pandotra *et al*. (2013) evaluated the essential oils constituents of 18 ginger cultivars from the Northwest Himalayan region using GC analysis. The analysis identified 31 major components in the oils. Moderate variations in the oil profiles were observed by employing the Pair-wise Euclidean Dissimilarity index and hierarchical analysis of molecular and oil profiles. The results indicated that all the genotypes could be grouped into two clusters. The study disclosed that the different genotypes from different regions exhibited distinct genetic and chemical characteristics.

According to a study conducted in the hilly region of Karnataka by Kallappa *et al*. (2015), the variety Rio-de-Janeiro exhibited the maximum essential oil content at 2.31 %, while the variety Humanabad Local had the lowest content at 1.06 %.

Goudar *et al*. (2017) conducted an experiment to evaluate the quality traits in 12 ginger genotypes. The quality parameters exhibited significant variations across different ginger genotypes. The essential oil content of ginger genotypes varied between 1.32 % and 2.25 %. Maximum essential oil content was recorded in the Suravi genotype, which was comparable to the Humnabad Local, Rejatha, and

Suruchi genotypes, with percentages of 2.25 %, 2.13 %, 2.04 %, and 1.93 %, respectively. On the other hand, the Himachal genotype exhibited the lowest essential oil content of 1.32 %.

In a study conducted by Bhattarai *et al*. (2018), the chemical constituents of ginger essential oils from three distinct regions in Nepal was identified through hydro distillation. The oils obtained from these regions were found to be abundant in derivatives of monoterpenes and sesquiterpenes. The oil yield varied between 1.1 % and 1.9 %.

Widayat *et al*. (2018) assessed the yield of essential oil obtained from dried red ginger waste using the steam distillation process. The red ginger waste is obtained from fresh red ginger, which is juiced and then dried under sunlight for durations of 1, 2, 3, and 4 days. Subsequently, the dried red ginger waste is exposed to steam distillation for 3 and 7 h to determine the amount of red ginger essential oil extracted. The most significant yield (1 %) was achieved when steam distillation was applied to red ginger waste that had been dried for three days.

Oforma *et al*. (2019) noted a decrease in the number of sesquiterpenes and monoterpenes in stored ginger compared to the components found in freshly harvested and dried samples. However, this reduction did not significantly impact the overall average percentage composition. They identified only ten sesquiterpene compounds, whereas previously reported studies on freshly harvested ginger had identified 29 compounds.

Akshitha *et al*. (2020) examined 28 different genotypes of ginger in India to assess variations in various quality parameters. The highest oil content, measuring 6 %, was found in red ginger. Arunachal local recorded the highest oil content of among ginger genotypes, at 3 %. Analysis of the oil profiles in these varieties revealed that zingiberene was identified as the main constituent in ginger essential oil, with the highest concentration observed in the Maran genotype.

Anargha *et al*. (2020) evaluated the essential oil content in selected 20 ginger genotypes, of which the maximum essential oil percentage was noticed in cultivar

Thalavur (2.42 %). The essential oil percentage was recorded between 0.90 % to 2.42 %.

Babu *et al*. (2021) observed a significant level of variability in several quality traits across 13 different genotypes. In the present study, the essential oil content across different genotypes varied between 1.62 % and 2.44 %. The highest oil content of 2.76 % was observed in the Rio-de-Janeiro, with exotic accessions Acc. 869 (2.44 %) and Acc. 393 (2.42 %) following closely behind.

Kamal *et al*. (2023) examined the variations in yield and chemical composition of essential oils from two ginger cultivars (Chinese and Thai) found locally in Pakistan. The essential oil extracted from fresh, oven-dried, and sun-dried samples of two distinct ginger cultivars underwent analysis using gas chromatography-mass spectrometry (GC-MS), revealing noteworthy variations in composition. Inter-varietal differences and pretreatment methods significantly influenced both the yield and chemical composition.

2.5.2 Oleoresin

Oleoresins consist of the non-volatile pungent compounds of ginger, along with essential oils and other non-volatile compounds like carbohydrates and fatty acids (Varakumar *et al*., 2017). Commercially available dried ginger typically yields between 3.5 % and 10 % of oleoresin (Zachariah, 2008). Oleoresin extraction from ginger rhizome involves extracting it using a solvent, followed by the evaporation of the solvent to obtain the final product. The ginger oleoresin is a dark brown, thick liquid that possesses the distinctive flavour and pungency of ginger (EFSA FEEDAP Panel *et al*., 2020). The quality of ginger is determined by the proportion of gingerols and shogaols present in it (Kamaruddin *et al*., 2023). Gingerols are the primary pungent compounds found in the rhizomes of ginger (*Z. officinale*). These compounds are well-known for their significant role in promoting human health and providing nutritional benefits. Gingerol analogues are sensitive to heat and readily undergo dehydration reactions, leading to the formation of shogaols (Jayanudin *et al*., 2019). These shogaols are responsible for the distinct pungent flavour found in dried ginger (Semwal *et al*., 2015). The conventional method for preparing ginger

oleoresin involves extracting dried ginger powder using organic solvents such as acetone or ethanol, ethylene dichloride, or ethyl acetate. After extraction, the solvent is completely removed. The yield of the oleoresin and the gingerol content can vary depending on factors such as the source and variety of ginger, the solvent used, and the extraction method employed. Among various ginger varieties, red ginger contains the highest concentration of oleoresins. These oleoresins are primarily responsible for the pharmacological effects associated with red ginger (Pratoko, 2018).

According to Saikia and Shadeque (1992), ginger varieties such as Moran and Jorhat hard exhibited favourable characteristics for the extraction of oil and oleoresin. These varieties yielded higher percentages of acetone extract, specifically 9.7% for Moran and 9.5% for Jorhat hard.

Landraces exhibited superior quality attributes, despite their lower yields compared to improved varieties, as noted by Kizhakkayil and Sasikumar (2009). Among the 46 accessions, Kozhikkalan demonstrated the highest oleoresin content at 8.2%, followed by Vizagapatnam-1 at 7.1% and Pulpally at 7%.

In a study conducted by Jyotsna *et al*. (2012), significant variations in quality characteristics were observed among the genotypes. Among the genotypes the oleoresin content ranged from 4.28% to 5.12%, with the highest content recorded in Baise (5.12 %) and the second highest in Gorubathan (4.88 %). These findings were observed under organic cultivation in rainfed conditions in Manipur.

In a study conducted by Chongtham *et al*. (2013), it was observed that Suravi exhibited a high oleoresin content of 10.25%, followed by Suruchi with 6.50% in the southern part of West Bengal. These values were higher than those recorded for the improved varieties, which had an oleoresin content of 4.00 %. These findings suggest that the local cultivars are well-adapted and acclimatized to the agroclimatic conditions of the state.

In a study carried by Singh *et al*. (2013), the oleoresin content of 11 clones collected from various locations in Manipur ranged from 1.00 % to 8.51 %. The

clones with the highest oleoresin content were Nadia (8.50 %) and IBSD=Z-41d (8.51 %), while the clone with the lowest percentage was IBSD=Z-41o (1.00 %).

Murthy *et al*. (2015) isolated oleoresin from the ginger rhizome and its chemical composition was estimated using high-performance liquid chromatography (HPLC). Gingerol is the primary constituent of oleoresin, representing approximately 12.8 % of the extract.

In a study conducted by Kallappa *et al*. (2015) in the hill zone of Karnataka, the variety Rio-de-Janeiro exhibited the maximum oleoresin percentage of 9.06%, followed by Maran with 8.47% and Suprabha with 8.27 %. On the other hand, the variety Himagiri recorded the lowest oleoresin content at 4.20 %. These results highlight the variations in oleoresin content among different ginger varieties under the specific conditions of the hill zone in Karnataka.

Balakumbahan and Joshua (2017) reported that the genotype Z. O-5 exhibited a higher oleoresin content of 9.56 % in the high rainfall zone of Tamil Nadu. This finding indicates that Z. O-5 genotype has a comparatively higher concentration of oleoresin in that specific geographical area.

In a study led by Goudar *et al*. (2017), the oleoresin content of 12 ginger genotypes was found to range from 3.69% to 7.35 %. Among the genotypes, Humnabad Local exhibited the highest oleoresin content at 7.35 %, which was comparable to Rejatha with a content of 6.12 %. On the other hand, Jorhat-2 recorded the lowest oleoresin content at 3.69 %.

Among the eight ginger genotypes, the highest oleoresin content was detected in the genotype GCP-49 (4.1 %) in Teri region of West Bengal (Chakraborty *et al*., 2018).

The research led by Akshitha *et al*. (2020), it was observed that red ginger (*Z. officinale* var. *rubrum*) exhibited a high oleoresin content of approximately 12.18 %. It was followed by Arunachal local with an oleoresin content of 8.55 %. These findings highlight the variability in oleoresin content among different ginger varieties, with red ginger showing the highest content in the study.

Anargha *et al*. (2020) evaluated the variability in oleoresin content of 20 ginger genotypes collected from the Western Ghats regions of Kerala. Oleoresin percentage in this investigation varied between genotypes from 4.00 to 12.50 %. Irinjalakkuda (T12) (12.50%) and Pozhuthana (T16) (10.47 %) genotypes were found to have significantly higher oleoresin contents.

The oleoresin content across the 13 genotypes, varied from 3.4 % (Acc. 736) to 6.69 % (Rio-de-Janeiro). Exotic accessions, namely Acc. 869 (5.88 %), Acc. 874 (5.63 %), Acc. 873 (5.34 %), and Acc. 393 (5.28 %), exhibited notably higher oleoresin content compared to the released variety IISR Varada (4.01 %) (Babu *et al*., 2021).

Influence of ginger cultivars and maturity stages on oleoresin and 6-gingerol was investigated by Vedashree and Madhava (2023). The ING 5 variety exhibited the highest oleoresin yield at 11.05%, with ING 6 following closely at 10.5%. ING 6 displayed the highest [6]-gingerol content at 7.59%,

Rongpipi *et al*. (2023) analysed the qualitative parameters of various ginger genotypes in Nagaland. A significant disparity in oleoresin content was observed across the seventeen ginger genotypes, ranging from 3.26 % to 6.59 %. The maximum oleoresin content was found in BGG-8 at 6.59 %, followed by CBG-1 at 5.21 % and CBG-2 at 4.83 %. Conversely, the lowest oleoresin content was recorded in BGG-1 at 3.26 %.

2.5.3 Crude Fibre

Ginger with low fibre content is preferred for industrial applications, for instance, food products, and medicinal purposes. (Das *et al*., 2014). Crude fibre content varies from 3 to 8% in the ginger rhizome (Kiyama, 2020). Fibre content tends to change with geographical regions and climatic conditions (Sanwal *et al*., 2012). In the Kerala climatic condition, Goudar *et al*. (2017) observed the lowest fibre content in the varieties Varada (3.34 %) and Suravi (3.62 %).

Saikia and Shadeque (1992) conducted a study on the yield and quality of ginger grown in Assam, where they reported that Moran had the highest crude fibre

content at 8.05%, followed by Jorhat hard at 7.86 %. Additionally, varieties such as Wynad, Thinladium, Riode-Janerio, Ernad, and Singmakhir also exhibited higher fibre content ranging from 6.00 % to 7.21%.

In a study focused on the variability of quality characters in a global germplasm collection of ginger, Kizhakkayil and Sasikumar (2009) found that the primitive types or landraces exhibited lower fibre content compared to the improved varieties.

Jyotsna *et al*. (2012) conducted a study on ginger varieties under rainfed conditions in Manipur and observed a range of crude fibre content from 5.17 % to 7.68 % among the studied varieties. The lowest crude fibre content was found in Nadia at 5.17 %, followed by Bhaise at 5.71 %, while the highest crude fibre content was observed in Manipur local at 7.68 %.

Sanwal *et al*. (2012) reported a positive and significant correlation between yield, dry recovery, fibre, and starch content with the maturity of ginger rhizome. Among the 33 genotypes studied, the average crude fibre content at 150 days was 2.9 %, which augmented to 6.41 % at 240 days. Lower fibre content was observed in genotypes such as China, Tura Local, Mahima, Kachai Ginger, and Nadia.

Singh *et al*. (2013) detected significant variation in the crude fibre content among 11 traditional cultivars grown in various locations of Manipur. The crude fibre percentage ranged from 6.20% to 13.97%. The variety IBSD=Z-41e had the least crude fibre content at 6.20%, while the varieties IBSD=Z-41g, IBSD=Z-41t, and IBSD=Z-41k had the highest crude fibre content.

In the hill zone of Karnataka, Kallappa *et al*. (2015) found that the variety Karkal Local had the highest crude fibre content at 6.73 %, while variety IISRVarada had the lowest crude fibre content at 3.70 %.

In a study conducted by Pattnaik *et al*. (2016) on 20 elite ginger germplasms at HARS, Pottangi, it was observed that the total fibre content varied among the cultivars. The fibre content ranged from 4.45% to 9.85%. The highest fibre content was found in Suprava (9.85 %), and was also high in Banspal (9.45 %), No.12 (8.55

%), and Subhada (8.50 %). On the other hand, the lowest fibre content was recorded in Sargiguda (4.45 %), followed by China (5.56 %), Kalinga Local (5.48 %), and Pottangi Local (5.86 %).

In a study conducted by Balakumbahan and Joshua (2017) on 24 genotypes under a high rainfall zone in Tamil Nadu, the fibre content was evaluated. The finding showed that the genotype Suprabha had the highest fibre content of 9.56 %, followed by V1S1-8 from Pottangi with 7.41 % fibre content. Interestingly, the genotype Sengottai local, which had the highest fresh rhizome yield, exhibited a lower fibre content of only 5.42 %.

An experiment was undertaken to assess the quality parameters of 16 ginger genotypes under Soppinabetta ecosystem in Karnataka (Ravi *et al*., 2018). Among the genotypes studied, the genotype IISRVarada exhibited a lower crude fibre content of 3.20 %, while Suruchi had a slightly higher content of 3.79 %. The highest crude fibre content of 4.87 % was observed in the genotype Himagiri.

In a study conducted by Akshitha *et al*. (2020) on 28 genotypes in India, it was observed that Acc. 578, Nadia, and Aswathy exhibited lower fibre content compared to the other genotypes.

Crude fibre content varied between 1.20 % and 5.75 % among the ginger genotypes. The T16 (Pozhuthana) genotype had the highest crude fibre content (5.75 %), whereas the T7 (Kothamangalam) genotype had the lowest crude fibre content (1.20 %) (Anargha *et al*., 2020).

Significant variations were observed in crude fibre content (ranging from 5 % to 11 %) among three ginger genotypes, namely Varada, Suprabha, and Suravi grown in nine different agro-climatic regions of Odisha. The variety Varada was noted to be lowest in two regions, North Western region (5.2 %) and North Central region (5.1 %), whereas the highest was observed for Suprabha in North West region (7.2 %) and Suravi in North Central region (8.1 %), respectively (Das *et al*., 2022).

The qualitative characteristics of different ginger varieties in Nagaland was examined by Rongpipi *et al*. (2023). There was a significant variation in the fibre content across the various genotypes, with values ranging from 3.36 % to 6.04 %. CBG-2 exhibited the highest fibre content at 6.04 %, followed by BGG-5 at 4.77 %, CBG-5 at 4.66 %, and CBG-6 at 4.61 %. Conversely, BGG-2 displayed the lowest fibre content at 3.36 %.

2.6 Direct *in vitro* regeneration of red ginger

Traditional breeding methods are not feasible in ginger due to factors such as self-incompatibility and reduced fertility of pollen (Rana *et al*., 2019). The plant tissue culture system enables the cultivation of entire plants, organs, tissues, or cells in a laboratory setting under carefully controlled and sterile conditions. *In vitro* regeneration and micropropagation have emerged as valuable techniques for the commercial propagation of numerous horticultural crops, including ginger (Miri, 2020b). This method is particularly beneficial for crops that are vegetatively propagated or exhibit slow growth, as it overcomes limitations associated with fertility issues and low seed sets, which hinder conventional breeding programs (Ahmar *et al*., 2020; Ravindran & Babu, 2005).

In vitro plantlets can be produced by culturing various explants in nutrient media. The success of the tissue culture technique is influenced by several factors including explant source, type, maturity, sterilization procedure, culture media, plant growth regulators, incubation conditions, *etc.*

2.6.1 Explant sources and types

While every plant cell has the potential to regenerate into complete plants, the degree of expression of this capacity can vary among plant species and varieties, and even among cells within the same plant. The developmental stage of explants is a determining factor in the success of plant regeneration in tissue culture (Ikeuchi *et al*., 2016). Irrespective of the type of explant used, the initial cell division occurs in a young region near the cambium and vascular bundles. Explants in the early developmental stage exhibit greater regenerative capacity and higher totipotency

compared to mature explants (Long *et al*., 2022). Several types of explants, such as meristem, axillary buds, aerial pseudostems, and shoot tips, are utilized in the micropropagation of ginger and related species. Among these, rhizome buds and shoot tips are frequently employed as explants, as they exhibit high responsiveness to produce pathogen-free propagules in copious quantities (Kavyashree, 2009; Mohammed & Quraishi, 1999; Gezahegn *et al*., 2023). The research conducted by Olivier (1996) supports the notion that axillary buds are the most suitable explants for the successful clonal propagation of ginger. Babu *et al*. (1992) described the direct regeneration of plantlets from the immature inflorescence of ginger. Ikeda and Tanabe (1989) reported on the *in vitro* establishment of ginger utilizing *in vitro* pseudostem explants. Multiple shoot induction from the *in vitro* aerial stem of ginger was also observed by Lincy *et al*. (2004).

The size of rhizome explants plays a role in the initiation of shoot buds and the morphogenic response observed under *in vitro* conditions. Explants that are too small may not survive well in culture, while larger explants may pose challenges in terms of effective decontamination or handling (George *et al*., 2007). Sathyagowri and Seran (2011) conducted an experiment to identify the suitable explant size for culture establishment. Among the many sizes (0.5, 1.0, and 2 cm) selected, 0.5 cm long rhizome bud was found to be best in culture initiation.

2.6.2 Sterilization of explant

Sterilization of ginger rhizome is crucial due to its association with soil-borne pathogens. Various research studies have documented diverse sterilization methods to establish cultures free from contamination. The selection of disinfectants, their concentrations, and exposure durations vary based on the nature of the explants. In general, ethanol, sodium hypochlorite, and mercuric chloride are employed as disinfectants (Seran, 2013). Usually, rhizome buds have been sterilized using a 0.1% solution of mercuric chloride ($HgCl_2$) for a duration of 10 to 20 minutes, to establish sterile cultures in ginger (Babu *et al*., 1996; George *et al*., 2022; Kambaska & Santilata, 2009). In certain cases, underground rhizome buds are subjected to a combination of appropriate detergents and sterilizing agents to ensure

effective sterilization, primarily due to the presence of significant bacterial and fungal contamination. Rout *et al*. (2001) established a suface protocol for young emerging buds of ginger, which involved treating them with a 2% (v/v) Teepol solution for 15 minutes, followed by subsequent treatment with a 0.2 % (w/v) aqueous solution of $HgCl_2$. Shaik and Kanth (2018) treated the rhizome buds with 0.1 % chloramphenicol for 30 minutes to avoid bacterial contamination.

2.6.3 Culture media and plant growth regulators

The Murashige and Skoog (1962) basal medium is widely favoured for the *in vitro* regeneration of ginger. Furthermore, the choice of carbon source plays a crucial role in influencing plant regeneration in the culture medium. Generally, 3 % sucrose is used as a carbon source for micropropagation of ginger (Kambaska & Santilata, 2009). George *et al*. (2022) cultured emerging buds in basal MS medium with sucrose as a carbon source. The axenic cultures were selected for further multiplication.

Plant growth regulators, particularly cytokinins and auxins, play a crucial role in regulating cell division and differentiation processes. These plant growth regulators have a significant impact on the pattern and rate of cell division, as well as the subsequent differentiation of cells into various tissue types (Long *et al*., 2022). In ginger, the effective initiation and multiplication of shoot tips and the subsequent production of plantlets are typically accomplished using MS basal medium supplemented with 6-Benzyl- amino-purine (BAP) alone or in combination with α- Naphthalene acetic acid (NAA).

According to the findings of Inden *et al*. (1988), a culture medium with a high concentration of BAP and a low concentration of NAA was found to be efficient in promoting shoot proliferation. The explants were cultured on MS medium containing various concentrations and combinations of BAP and NAA to stimulate both shoot and root growth. The maximum rate of shoot multiplication was recorded when the explants were cultured on MS basal medium enriched with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (Kambaska & Santilata, 2009). Similarly, Gezahegn *et al*. (2023) used 2.0 mg L^{-1} BAP and 1.0

mg L^{-1} Kinetin for the shoot initiation and multiplication in ginger. According to the research conducted by Zuraida *et al*. (2016), the maximum shoot multiplication was achieved from the rhizome buds of *Z. officinale* var. *rubrum* when cultured on MS medium enriched with 5.0 mg L^{-1} BAP. The study conducted by Mohammed and Quraishi (1999), observed the highest multiplication of plantlets in common ginger when cultured on MS medium supplemented with 3.0 mg L^{-1} BAP in conjunction with 0.5 mg L^{-1} NAA. Despite being a commonly used cytokinin for shoot multiplication, the high concentration of BAP in turmeric hinders the rate of shoot proliferation (Naz *et al*., 2009). Kun-Hua *et al*. (2011) achieved the multiplication of clump buds by culturing shoot tips in a nutrient medium supplemented with 2.5 mg L^{-1} BAP and 0.2 mg L^{-1} NAA under *in vitro* conditions. Similarly, Zhou *et al*. (2020) used 1.0 μM BAP and 0.5 μM NAA to induce bud clump in ginger. The impact of various sucrose concentrations and growth regulators on the *in vitro* multiplication of red ginger was assessed by Inderiati *et al*. (2023). The combination of 1 mg L^{-1} of BA and 30 g L^{-1} of sucrose resulted in the maximum number of shoots per explant (6.33) and the highest number of leaves per shoot (3.72). Syahid *et al*. (2023) attempted micropropagation of red ginger through *in vitro* culture on MS medium enriched with BA of 0.1, 0.3, 0.5, and 1.0 mg L^{-1}. The findings revealed that the most effective rate of shoot induction occurred with 1.0 mg L^{-1} of BA. During the multiplication process, the application of 2.0 mg L^{-1} of BA yielded the optimal number of shoots.

While BAP and kinetin are widely utilized cytokinins in micropropagation, the cytokinin TDZ (thidiazuron) has also been examined for its efficacy in shoot proliferation and elongation (Deepa *et al*., 2018). TDZ was utilized to improve the formation of adventitious shoots from aerial stem explants of ginger, resulting in enhanced regeneration (Lincy & Sasikumar, 2010). In this study, adventitious shoots and roots were successfully regenerated from pseudostem explants of two ginger cultivars, namely 'Jamaica' and 'Varada'. The highest rates of shoot and root regeneration were noticed in cultures supplemented with a combination of TDZ and IBA at a ratio of 1:1 or

1:0.5 mg L^{-1} in both varieties. In a study conducted by Karyanti *et al*. (2021), diverse types of cytokinins and the effect of different concentrations were assessed, and it was found that the most effective concentration for promoting the growth of red ginger shoots *in vitro* was 1 ppm TDZ.

2.6.4 Rooting and acclimatization

Rooting in ginger typically occurs naturally, but it can also be induced on culture media with or without the presence of auxins such as NAA, IAA, or IBA (Abbas *et al*., 2011; Seran, 2013). Research findings indicate that successful *in vitro* plantlet development, with healthy shoots and roots, has been achieved using MS medium supplemented with BAP (1-5 mg L^{-1}) either alone or in combination with a low concentration (0.5 mg L^{-1}) of NAA. Prolific shoot multiplication was observed from shoot tip cultured in 2.5 mg L^{-1} and 0.5 mg L^{-1} NAA. Vigorous rooting was also obtained in the same medium (Khatun *et al*., 2003). Zahid *et al*., (2021) evaluated the influence of auxins namely, IAA, IBA, and NAA at 2.5, 5.0, and 7.5 µM, respectively. The number of roots and root length of 'Bentong' ginger varied significantly depending on the type and concentration of auxins used. The maximum number of roots per plantlet (15.44 ± 0.8) was observed with a concentration of 7.5 µM NAA. In certain instances, following the multiplication of shoots, the plantlets are transferred to a secondary medium to promote shoot elongation (Sathyagowri & Seran, 2011), or to facilitate the rooting of microshoots (Bhagyalakshmi & Singh, 1988; Dogra *et al*., 1994; Babu *et al*., 1997; Rout *et al*., 1998; Kambaska & Santilata, 2009; Shaaban *et al*., 2023). In contrast to previous studies suggesting NAA as a suitable auxin for ginger rooting, the study conducted by Miri (2020b) found that IBA resulted in the highest number and length of roots.

Tissue culture plants are grown in an artificial nutrient medium under a controlled sterile environment. When transferred to an external environment, the plants need to adjust to several factors, such as hot temperature, low humidity, high light intensity, nutrient stress, and pathogen attack. The success of the plant tissue culture technique relies on the successful establishment of the plants in the

natural soil environment (Deb & Imchen, 2010). Myriad studies have indicated that *in vitro* plantlets of ginger can be successfully acclimatized when transferred to field conditions. According to the findings of Samsudeen *et al*. (2000), a success rate of over 80% was achieved by transplanting ginger plants into a potting mixture consisting of soil, sand, and vermiculite in equal proportions (1:1:1). The transplanted plants were then kept in a mist chamber under high humidity for maintenance. *In vitro* acclimatization of ginger plantlets under high relative humidity and CO_2 enrichment resulted in a robust root and shoot system and exhibited a significantly high survival rate to *ex vitro* conditions (Cha-um *et al*., 2005). Furthermore, the *ex-vitro* acclimatization process of *in vitro* regenerated ginger plantlets helped in the acclimatization phase, resulting in an increased survival rate (Zhou *et al*., 2022).

2.7 Polyploidy breeding

Polyploidy, which refers to the presence of more than two sets of chromosomes, is a frequent phenomenon in angiosperms. The deliberate induction of polyploidy through synthetic methods has been instrumental in overcoming issues of hybrid sterility and incompatibility, contributing significantly to the evolution and diversification of flowering plants (Sattler *et al*., 2016). This approach widens the range of opportunities for crop improvement in spice crops by introducing greater heterozygosity and genetic enhancements.

The discovery by Blakeslee and Avery in 1937 that colchicine treatment may promote chromosomal doubling in plant cells was a milestone in plant breeding and resulted in the development of synthetic polyploidy. This method has proven to be highly effective in altering the chromosome count of plants and is now widely utilized in plant breeding processes (Chen *et al*., 2020). Polyploidy induction leads to the duplication of chromosomes, which subsequently triggers various changes at the genomic, epigenetic, and gene expression levels (Chen & Ni, 2006). These alterations can have a broad impact on phenotypic traits, as they not only increase the number of existing genes but also influence other aspects of gene function and regulation (Adams & Wendel, 2005). Changes in ploidy levels in plants have

notable effects on various morphological, physiological, and biochemical traits. These alterations can be observed in various aspects of plant morphology, including plant height, growth habit, shoot and root numbers, as well as leaf characteristics such as leaf count, shape, and length-to-width ratio. Additionally, modifications in ploidy levels can influence the number and size of flowers, seeds, and pollen, as well as impact the composition of the cell wall and other related traits (Miri, 2020a; Trojak-Goluch *et al*., 2021). Polyploid plants have been found to exhibit physiological changes, including variations in the size and density of stomata (McGoey *et al*., 2014). Research studies have indicated that polyploid plants may exhibit delayed flowering and slower growth rates. However, they also demonstrate improved tolerance to nutrient and mineral deficiencies, enhanced resistance to both biotic and abiotic stress factors, and consequently, better adaptation to disturbances in their habitat (Corneillie *et al*., 2019; Doyle & Coate, 2019; Tossi *et al*., 2022; Van de Peer *et al*., 2021; Vichiato *et al*., 2014). These effects of polyploidization can be exploited for economic purposes to obtain desirable levels of metabolite production, yield and other aspects of plants (Gantait & Mukherjee, 2021; Farhadi & Moghaddam, 2023).

2.7.1 Pathways of Polyploidy induction

The generation of polyploids can occur through natural or artificial means. Natural processes that contribute to the formation of new polyploid lineages in plants include the spontaneous doubling of chromosomal sets within somatic cells and the fusion of unreduced gametes (Tayalé & Parisod, 2013). The infrequency of these events poses a challenge to the breeding process. Efficient systems are essential to artificially induce polyploidy, primarily using antimitotic chemicals. This method serves as a powerful tool for crop improvement purposes.

The two primary techniques for polyploidization are *in vitro* and *in vivo* pathways (Nasirvand *et al*., 2018). The *in vitro* pathway is widely recognized as the most popular method for polyploidization. This approach allows for accelerated polyploid development within a controlled and confined environment. Establishing an efficient *in vitro* culture system is essential for achieving successful polyploid

induction through the *in vitro* method (Touchell *et al*., 2020). *In vitro* polyploidization can be achieved through two approaches. The first involves treating the explant with antimitotic chemicals before introducing it into the culture medium. Alternatively, an aqueous solution of anti-mitotic agents can be directly added to either liquid or solid culture media, allowing it to interact with the explant. There are reports on the successful *in vitro* polyploidy induction in *Z. officinale* Rosc. (Adaniya & Shirai, 2001; Kun-Hua *et al*., 2011; Smith & Hamill, 1997; Zhou *et al*., 2020). Polyploidization techniques have been applied to various spice crops, including *Aframomum corrorima* (Wannakrairoj & Tefera, 2013), *Thymus vulgaris* (Navrátilová *et al*., 2021), *Trachyspermum ammi* (Noori *et al*., 2017), *Allium cepa* (Yun *et al*., 2021), *Punica granatum* (Shao *et al*., 2003), and *Allium sativum* (Wen *et al*., 2022) *Cuminum cyminum* (Sanaei-Hoveida *et al*., 2023). These studies have successfully induced polyploidy in these spice crops using the mentioned techniques. On the other hand, *in vitro* systems necessitate specialized skills and costly laboratory equipment to conduct these procedures.

In vivo polyploidization involves applying anti-mitotic agents to various parts of a whole plant or plant parts such as rhizome buds, seeds, nodal segments, corms, etc (Hassanzadeh *et al*., 2020; Samadi *et al*., 2022). Chemicals can be applied using various methods, such as immersion (Omidbaigi *et al*., 2010; Talebi *et al*., 2017), syringe injection (Mishra *et al*., 2010), cotton plug method (Kulkarni & Borse, 2010), and dropwise application to the apical meristem (Talebi *et al*., 2017). Tetraploid ginger was developed by Ramachandran (1982), Ramachandran and Nair (1992), and Prasath *et al*. (2022), via *in vivo* polyploidization. In contrast to *in vitro* protocols, *in vivo* methods do not require highly skilled workers or fully equipped laboratories. However, it typically involves a longer duration and exhibits a lower rate of polyploidy induction (Salma *et al*., 2017). The main drawback of this system is the extended time required to establish and multiply a polyploid population.

2.7.2 Factors influencing artificial polyploidy induction

2.7.2.1 Plant parameters

The genotype of plants is the key and most influential factor in the development of polyploidy. It is evident that different genotypes and ecotypes of the same plant species exhibit varying degrees of responsiveness to polyploidy induction (Niazian & Nalousi, 2020). Adaniya and Shirai (2001) successfully induced tetraploidy in *Z. officinale* Rosc. cv. Sanshu through *in vitro* treatment with 0.2% colchicine over a duration of eight days. Similarly, Kun-Hua *et al*. (2011) achieved the highest percentage of tetraploidy in *Z. officinale*, obtained from Laiwu city, China by subjecting it to 0.2% colchicine treatment for 30 hours. Besides plant genotypes, the choice of plant parts or explants plays a vital role in polyploidy induction. Explants that exhibit active cellular division, such as somatic embryos, callus, nodal segments, apical buds, juvenile root tips, immature inflorescence, and germinated seeds, are considered the most effective in promoting polyploidy formation. According to Fu *et al*. (2019), the ability to induce chromosomal duplication varies across different types of explants. Selection of the explant is also influenced by the mode of polyploidy induction. Prasath *et al*. (2022) used emerging rhizome buds of ginger for polyploidy induction in the *in vivo* method, whereas Zhou *et al*. (2020) selected the *in vitro* raised shoot tips as explants for *in vitro* treatment.

2.7.2.2 Anti-mitotic chemicals

Antimitotic compounds play a key role in polyploidy induction by inhibiting the formation of spindle fibers during the metaphase stage. This inhibition leads to the nondisjunction of chromosomes and subsequent increase in ploidy level. Several factors, including the type of compound, dosage, exposure time, and mode of application, are considered as contributing factors to the effectiveness of polyploidy induction (Gantait & Mukherjee, 2021). Colchicine is widely employed as the primary antimitotic drug in studies related to chromosomal duplication (Fig. 2). This well-known mitotic arresting alkaloid can bind to -tubulin, thereby impeding the formation of tubulin dimers and disrupting the cytoskeleton (Salma *et al*., 2017). To

mitigate the toxic effects of colchicine on plants, it is commonly believed that using moderate doses of the compound along with extended exposure times can be effective in increasing the production rate of polyploids (Manzoor *et al*., 2019). Alternative substances, such as oryzalin, trifluralin, amiprophos-methyl (APM), flufenacet, pronamide, and nitrous oxide gas, have been suggested as substitutes for colchicine due to their reduced harmful effects (Afshari *et al*., 2009; Alavi *et al*., 2022). APM and oryzalin, in particular, are considered superior to colchicine because they exhibit higher selectivity for plant tubulins and do not possess affinity for animal cells (Jakše *et al*., 2003; Rauf *et al*., 2021; Bharati *et al*., 2023a). 1.0-2.0% Dimethyl Sulfoxide (DMSO) is commonly used as wetting agent in aqueous solutions of anti-mitotic chemicals to enhance the permeability and penetration of chemicals into cells (Eng & Ho, 2019). Apart from DMSO, detergents like Tween 20 or Triton X-100 have been employed to enhance cell permeability in the process of chromosome doubling. For instance, in the chromosome doubling of mint, Moetamedipoor *et al*. (2022) utilized Tween 20, while Jakše *et al*. (2003) employed Triton X-100 for onion. The use of shaker also insures the maximum penetration of chemicals in the cells.

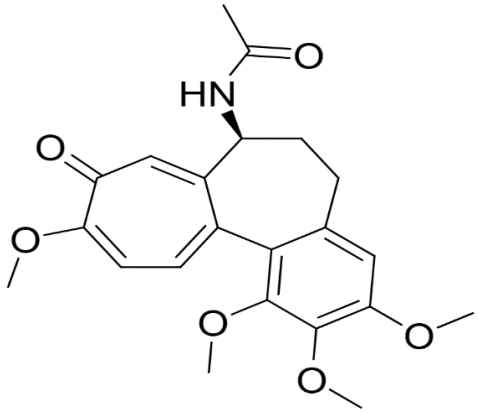

Fig. 2. Structure of colchicine (Source: https://cb.imsc.res.in/imppat/phytochemical-detailedpage/IMPHY003176)

Standardization of the ideal concentration of an antimitotic drug is crucial for successful polyploidy induction. Treating explants beyond the threshold concentration can be harmful, whereas lower exposure levels may not be effective.

Therefore, determining the minimal optimal dose of the antimitotic agent is essential (Podwyszyńska *et al*., 2018). Ramachandran and Nair (1992) produced autotetraploids of ginger (cultivar 'Maran') by treating the rhizome buds with 0.25% colchicine solution for 4h. Successful tetraploidy induction in IISR Rejatha was achieved by treating with 0.10 % colchicine for 48h. *In vitro* investigations conducted by Smith and Hamill, (2002) on *Z. officinale* revealed that the highest level of polyploidization was achieved when exposed to 0.50 % colchicine for a duration of 2 h, whereas Adaniya and Shirai, (2001) reported maximum polyploidy at 0.20 % colchicine for 8 days.

2.7.3 Identification of putative and its verification

Polyploids can be distinguished using either direct or indirect methods. In most experiments, indirect identification strategies are commonly employed initially to identify potential polyploids. Subsequently, direct identification methods are used to verify and confirm the ploidy of these individuals.

2.7.3.1 Indirect identification methods

Indirect strategies for identifying polyploids are convenient and fast, but their accuracy may be compromised as they depend on morphological, physiological, and anatomical traits. This approach typically involves an initial screening process to select a smaller subset of individuals from a large population. Commonly observed morphological characteristics such as leaf parameters, shoot diameter, plant height, and floral traits are utilized to identify potential polyploid plants (Salma *et al*., 2017). Polyploid leaves often display various distinctive characteristics, including increased width, thickness, greener color, and changes in the leaf margin (Cheng *et al*., 2012). This method has been utilized as a means of identifying ploidy levels during the early screening of populations in plants such as *Z. officinale* (Zhou *et al*., 2020), *Allium cepa* (Yun *et al*., 2021), and *Allium sativum* (Yousef & Elsadek, 2020). The application of anti-mitotic chemicals can also result in changes in the floral morphology, including modifications in size, length, coloration and the shape and size of the pollen grains (Dijkstra & Speckmann, 1980; Omezzine *et al*., 2012; Samadi *et al*., 2022 Sanaei-Hoveida *et al*., 2023).

Stomatal traits such as stomatal length, width, frequency, and the number of chloroplasts in stomatal guard cells have proven to be useful in identifying mutated plantlets (Beck *et al*., 2003; Hodgson *et al*., 2010). Multiple studies have provided evidence to support the observation that polyploids possess larger stomata with lower frequencies compared to diploids (Kun-Hua *et al*., 2011; Mishra *et al*., 2010; Nasirvand *et al*., 2018; Noori *et al*., 2017; Borges *et al*., 2023).

Nowadays, the determination of plant ploidy is commonly carried out using flow cytometric (FCM) analysis, which offers a significantly faster and more accurate alternative to traditional methods. Flow cytometric (FCM) analysis entails the precise estimation of nuclear DNA content and ploidy status (Doležel *et al*., 2007). Among various indirect methods, flow cytometric (FCM) analysis is extensively favored by researchers in synthetic polyploidy due to its ability to provide highly accurate results in a significantly shorter time frame (Keshtkar *et al*., 2019; Hassanzadeh *et al*., 2020; Prasath *et al*., 2022).

2.7.3.2 Direct identification methods

Chromosome counting is considered the most effective direct method for accurately determining the ploidy level of a plant. While chromosome counting can be performed on various plant parts, root tips have been commonly used and widely employed for this purpose (Eng & Ho, 2019). The process of chromosome counting is typically consisting of three main steps: (1) preparation and pre-treatment of the sample, (2) fixation of the sample, and (3) staining of the sample. Even though chromosome counting allows the visual identification of chromosome number, it is a time-consuming process that requires skilled operators (Niazian & Nalousi, 2020). Researchers continue to employ this fundamental method of chromosome counting to determine the ploidy of induced polyploids (Alavi *et al*., 2022; Bharati *et al*., 2023a; Prasath *et al*., 2022).

2.7.4 Effect of induced polyploidy in spices

Polyploidization, a well-known phenomenon, leads to significant genomic effects. The primary consequence is the expansion of the organism's genome size,

which subsequently triggers various alterations in the genetic makeup and gene expression patterns, thereby modifying the overall genetic architecture (Wang *et al*., 2021). The profound genomic transformations resulting from polyploidization can have significant implications for the cellular and biochemical interactions that govern morphogenesis. Therefore, the morphological characteristics of the plant may be influenced, as these genomic changes can disrupt or modify the processes underlying the development of its physical attributes (Madlung, 2013).

Several studies have investigated the effects of altering the ploidy level in various spice crops, and these investigations have revealed notable changes in morphology, biochemistry, and physiology.

2.7.4.1 Morphological effects

Synthetic polyploidy is usually results in an enlargement in organ size especially in size of the leaves, root, flower and fruits. This phenomenon is called 'gigas effect' (Sattler *et al*., 2015). In ginger, research by Adaniya & Shirai (2001), Prasath *et al*. (2022), Ramachandran & Nair (1992), Smith & Hamill (1997), and Zhou *et al*. (2020) has highlighted the presence of larger and thicker leaves in polyploid ginger plants. Furthermore, apart from exhibiting larger green leaves, polyploid plants also demonstrate gigas characteristics in various other aspects. These include increased plant height (Navrátilová *et al*., 2021), larger stem size (Noori *et al*., 2017; Shambulingappa *et al*., 1965; Smith & Hamill, 1997; Zhou *et al*., 2020), floral characters (Noori *et al*., 2017; Bharati *et al*., 2023b), the presence of larger rhizomes with higher yield (Smith & Hamill, 1997; Zhou *et al*., 2020), variations in the size and density of trichomes (Zhao *et al*., 2022), larger bulb size (Hailu *et al*., 2020), elongated peduncles and seeds (Dijkstra & Speckmann, 1980; Gupta *et al*., 2021), among others. Additionally, the spadices, flowers, and floral parts in tetraploid ginger were larger in size. The rhizomes of tetraploids were thicker, had longer internodes, and exhibited less branching compared to diploid plants (Ramchandran & Nair, 1992). The tetraploid gerbera exhibited delayed growth but increased vigour, characterized by thicker and broader leaves (Mahanta *et al*., 2023).

2.7.4.2 Physiological and biochemical effects

Numerous studies have documented changes in stomatal size following polyploidization across various plant species. In general, these studies have consistently reported a decrease in stomatal density in polyploid plants when compared to their diploid counterparts. This reduction in stomatal density is primarily attributed to the larger size of individual stomata relative to the leaf area (Foschi *et al*., 2013; Hailu *et al*., 2021; Moetamedipoor *et al*., 2022). Zhou *et al*. (2020) reported an increment in the length and width of the guard cells in ginger. According to Takizawa *et al*. (2008), tetraploid capsicum exhibited enhanced photosynthetic capacities in comparison to its diploid counterpart. Polyploidization has been found to impact physiological processes such as flowering and pollen germination. In the case of saffron, polyploidization has been associated with delayed emergence of flowers and leaves (Zaffar *et al*., 2003). However, other studies have reported polyploid plants with restored and high pollen fertility, reaching levels of more than 80% (Ramachandran & Nair, 1992), as well as increased pollen viability (Adaniya & Shirai, 2001; Jakše *et al*., 2003). Ploidy changes in plants have been linked to various morphological, anatomical, and physiological characteristics that contribute to enhanced tolerance to abiotic stresses. For example, tetraploid fenugreek has been found to exhibit improved salt tolerance according to research conducted by Marzougui *et al*. (2010). The effects of polyploidization on biochemical traits have been extensively researched. Most of these traits frequently show changes in the pattern of secondary metabolite synthesis in plants (Bagheri & Mansouri, 2015; Cao *et al*., 2018; Madani *et al*., 2021; Omezzine *et al*., 2012; Mohammadi & Talebi, 2023). Besides, few research has examined the effects of polyploidization on the output of essential oil as well as the content of the oil ((Bertea *et al*., 2005; Dijkstra & Speckmann, 1980; Prasath *et al*., 2022; Sanaei-Hoveida *et al*., 2024). The yield of secondary metabolites, including alkaloids, phenolics, and flavonoids which have pharmacological and therapeutic benefits, can be increased (Marzougui *et al*., 2012).

2.7.5 Applications of polyploidy breeding

The demand for spices is influenced by the quantity and quality of essential oils, oleoresins, and other secondary metabolites. Numerous factors, including as biotic and abiotic stress factors, climatic conditions, and manuring techniques, can have a substantial impact on these variables (Askary *et al*., 2018). Colchicine induced polyploids have proven to be superior to normal diploids. The synthesis of plant essential oil will thus rise because of polyploidization. The induced tetraploids of *Tymus presicus*, generated two times abundant secondary metabolites (Tavan *et al*., 2015). In recent decades, polyploids horticultural and ornamental crop have been effectively created. Variation in colour, structure, and shelf life are valuable economically in the decorative plant sector (Manzoor *et al*., 2019). Currently, gingers are in high demand as ornamentals and have immense potential for landscaping and cut flower bouquets. Prabhukumar *et al*. (2015) attempted induced mutation studies in three selected ginger species using colchicine, acridine and ethylmethanesulphonate. Mutation studies produced plants with attractive variegated leaves and increased the ornamental value of the plants.

The initial investigation on induced polyploidy in ginger was published in 1982 by Ramachandran. subsequently, Ramachandran and Nair (1992) created ginger autotetraploids by soaking rhizome buds in a solution containing 0.25% colchicine. In tetraploids, they noted more ferocious growth, greater yield, and larger rhizomes. Induced polyploidy in ginger, however, has not been the subject of many investigations (Adaniya & Shirai, 2001; Kun-Hua *et al*., 2011; Lindayani *et al*., 2018; Prasath *et al*., 2022; Ratnambal & Nair, 1982; Smith & Hamill, 1997; Zhou *et al*., 2020). In order to improve the knob size of the ginger rhizome in Queensland, Australia, Smith and Hamill (1997) have started an innovative systematic effort. This team created autotetraploids with large-rhizome, and after several seasons of field testing, one of the autotetraploids was selected for commercial distribution as "Buderim Gold" (Smith & Hamill, 2002; Smith *et al*., 2004).

The effect and influence of polyploidization on the production of primary and secondary metabolites has also been explored by several researchers (Dixit & Chaudhary, 2014; Gantait & Mukherjee, 2021; Hailu *et al*., 2020; Samadi *et al*., 2022; Mondal *et al*., 2024). The thymol content of ajowan (*Trachyspermum ammi*), the primary bioactive component of this medicinal spice, can be accelerated using this methodology since polyploidy induction has increased the thymol content of the induced tetraploids compared to the diploids (Noori *et al*., 2017). In *Thymus vulgaris* (Shmeit *et al*. 2020) and *Mentha piperita* (Zhao *et al*., 2022) authors found a rise in the essential oil yield. Prasath *et al*. (2022) recorded higher essential oil percentage in induced tetraploids of ginger. Conversely, the gingerol content of the tetraploid ginger and its diploid progenitor did not change much (Wohlmuth *et al*., 2005). In *Nigella sativa* (Gupta *et al*., 2021), *Salvia officinalis* (Hassanzadeh *et al*., 2020), *Trigonella foenum-graecum* (Marzougui *et al*., 2009) and *Andrographis paniculata* (Priya & Pillai, 2023), increased ploidy improved enzyme activity as well as flavonoid and phenolic levels.

Induced tetraploids are viable and may combine with another diploid species to create hybrid-triploids (Aleza *et al*., 2009; Urwin, 2014). The results suggest that polyploids generated by antimitotic agents can function as a progenitor for plant breeding programmes. The formation of polyploids will expand the gene pool available for use in creating superior genotypes with higher yield and better quality.

CHAPTER 3
MATERIALS AND METHODS

The present study entitled "Improvement of red ginger (*Z. officinale* Rosc.) through *in vitro* and *in vivo* studies" was conducted at ICAR-Indian Institute of spices Research, Kozhikode, Kerala during 2019-2023.

3.1 Morphological characterization of red ginger genotypes

Morphological characterization and genetic diversity studies of red ginger genotypes were carried out at ICAR-Indian Institute of Spice Research (ICAR-IISR), Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, during 2021–2023.

3.1.1 Geographical location and climatic condition of the experimental site

The experimental site is located at 11º 36'34" North latitude and 75º 49'12" East longitude. The meteorological data collected from May 2021 to January 2022 and May 2022 to January 2023 is given in Appendix.

3.1.2 Experimental material

Two types of red ginger genotypes were used for the study such as exotic red ginger and Indian red ginger. The 16 red ginger accessions selected for the study, comprised of two exotic red ginger genotypes, i.e., Acc. 850 and Acc. 899, and 14 Indian red ginger genotypes collected from the North-Eastern states of India and propagated in the National Active Germplasm Site (NAGS), ICAR-IISR, Peruvannamuzhi, Kerala were used for the morphological and biochemical characterization (Table 1). IISR Varada, a released variety of ginger, was used as a control in the characterization studies.

Table 1. Details of the red ginger genotypes used in the study and their location of collection

Genotype	Source
Acc. 838	North Eastern states
Acc. 842	North Eastern states
Acc. 844	North Eastern states
Acc. 845	Kerala
Acc. 848	Kerala
Coll. No. 9040	North Eastern states
Coll. No. 9046	North Eastern states
Coll. No. 9073	North Eastern states
Coll. No. 9076	North Eastern states
Coll. No. 9078	North Eastern states
G1	Nagaland
G3	Nagaland
G6	Nagaland
G9	Nagaland
Acc. 850	Malaysia
Acc. 899	Malaysia
IISR Varada	ICAR-IISR

3.1.3 Test plot design

The morphological characterization was conducted following the Distinctiveness, Uniformity and Stability (DUS) guidelines (PPV&FRA, 2007).

Design : Randomized Complete Block Design (RCBD)

No. of accessions : 17

Bed size : 3 m^2 (3m x 1m)

Plants per plot : 30

Number of replications : 2

Seasons : 2 (2021-2022 & 2022-2023)

3.1.4 Field preparation and planting

The land was kept ready for planting, and a bed size of $3m^2$ was prepared. A piece of rhizome with one or two emerging buds was used as planting material. Rhizomes were placed in small pits with a spacing of 30 x 25 cm, and a thin layer of soil was spread on top of the rhizomes. Green leaves were used to mulch each bed immediately after planting. The crop was cultivated as rainfed crop. Application of fertilizer was done in accordance with the standard package of practice of ICAR-IISR (Jayashree *et al*., 2015) which was 70:50:50 Kg ha^{-1}. Manuring was repeated at 45 and 90-day intervals. The ICAR-IISR ginger micronutrient mixture was applied twice as foliar spray, 60 and 90 days after planting (dosage at 5 g l^{-1}). Weeding was done at appropriate time intervals. Earthing up was done to cover the exposed rhizomes after every manuring and weeding. Disease control and pest management was done at appropriate time intervals (Jayashree *et al*., 2015). The overall view of the experimental field is represented in the Fig. 3.

Fig.3. General field view of the Experimental Farm, ICAR-IISR in Peruvannamoozhi, Kozhikode, Kerala

3.1.9 Observations recorded

Observations were recorded for the two consecutive years as per DUS guidelines of PPV&FRA (2007). Morphological traits were recorded 150 days after planting, i.e., at the end of the vegetative growth phase, and rhizome characters were measured after harvest. Data were collected from three randomly selected plants from each replication.

3.1.9.1 Qualitative characters

Qualitative morphological traits including, growth habit, leaf colour, shoot colour and shape of the rhizome were recorded in the form of multiscale score described as per DUS guidelines (PPV&FRA, 2007) (Table 2). Visual assessment was used to measure the qualitative morphological traits. The Royal Horticultural Society (RHS) colour chart was used to assess the colour characteristics.

Table 2. DUS descriptors for qualitative characters

Characteristics	States	Score	Stage of observation
Plant: Growth habit	Erect	1	At the end of the growing phase
	Semi-erect	3	
	Spreading	5	
Shoot: Intensity of green colour	Light green	1	At the end of the growing phase
	Green	3	
	Dark green	5	
Leaf: Intensity of green colour	Light green	1	At the end of the growing phase
	Green	3	
	Dark green	5	
Rhizome: Shape	Straight	1	At the time of harvest
	Curved	3	
	Zigzagged	5	

3.1.9.2 Quantitative characters

3.1.9.2.1 Plant height (cm)

Plant height from soil level to the tip of the top leaf of the main pseudostem was recorded.

3.1.9.2.2 Number of shoots

Total number of shoots in each clump was counted and recorded.

3.1.9.2.3 Shoot diameter (cm)

The diameter of the shoot was measured at a height of five centimetres above the soil level of the tallest shoot in each clump.

3.1.9.2.4 Number of leaves on main shoot

Number of leaves on main shoot was counted and recorded.

3.1.9.2.5 Leaf length (cm)

Leaf length was measured from the upper fourth leaf of the main shoot.

3.1.9.2.6 Leaf width (cm)

Upper fourth leaf was selected to measure the leaf width.

3.1.9.2.7 Rhizome thickness (cm)

Rhizome thickness was measured from the primary rhizomes after harvest, and the diameter was determined through cross-sectional measurements of the rhizomes.

3.1.9.2.8 Yield per plant (g)

Yield per plant was estimated by weighing individual clump after harvest.

3.1.9.2.9 Dry recovery (%)

Dry recovery (%) was recorded after peeling and sun drying of the fresh rhizomes. Freshly peeled rhizomes were exposed to sunlight until their moisture content reached 10-11% (10- 15 days). The dry recovery percentage was determined by calculating the weight loss observed in the fresh rhizomes following the drying process.

3.2 Chemo profiling

3.2.1 Essential oil (%)

Essential oil (%) was determined as per the procedure recommended by ASTA (1997) using Clevenger apparatus. 30 g of the powdered sample was taken in a round bottom flask (1000 ml), and water was added to half its volume. The RB flask was set in a heating mantle, the oil collecting trap and condenser were placed in position, and distillation was performed for three hours. Oil released from the sample formed a separate layer on the top of the water in the trap. Amount of the oil was noted and collected in an Eppendorf tube (1 ml); a pinch of anhydrous sodium sulphate was added to remove the traces of water. The oil was stored in refrigerator for GC-MS analysis.

$$\text{Essential oil } (\%) = \frac{\text{Amount of the oil collected}}{\text{Amount of the sample used}} \times 100$$

3.2.2 Oleoresin (%)

The percentage of oleoresin in the red ginger samples were estimated by ASTA (1997) method. About 10 g of the dried and powered sample was transferred to a glass column (18×500 mm) with non-absorbent cotton at the bottom. 50 ml of acetone was poured into the column and left to stand overnight. The extract was collected in a pre-weighed beaker. 30 ml of acetone was added to the sample and kept for an hour. The filtrate was collected to the same beaker. The beaker containing the extract was kept on water bath to evaporate the solvent. Then, the beaker was kept in the warm air oven until the weight is constant.

$$\text{Oleoresin (\%)} = \frac{\text{Weight of the residue}}{\text{Weight of the sample}} \times 100$$

3.2.3 Crude fibre (%)

Crude fibre analysis was conducted based on the ASTA (1997) method. Dried and crushed ginger samples were used for the procedure. The experiment was conducted with Fibra Plus FES 6 (Pelican equipment). 1g of the homogenized ginger sample was transferred to crucible and fixed in the instrument. 1.25 % H_2SO_4 was taken in a flask and boiled on a hot plate. Then acid was poured into each crucible and allowed to boil for 45 minutes, for acid digestion. Then drained the acid solution and washed 2-3 times with distilled water. After that, the sample was digested with pre-heated 1.25% NaOH for 45 minutes. Thereafter, the alkali solution was drained and rinsed with distilled water for 2–3 times. After all these processes, the crucibles were kept in the warm air for drying. The weight of the crucibles was recorded on a regular basis until a consistent weight was obtained (W1). The, the crucible were placed inside the muffle furnace and burned fibre at 500ºC for 5 h and weight of the crucible containing ash was recorded after cooling (W2). Ashing was repeated until a white ash was obtained and the weight remained constant. The difference in the weight after burning in muffle furnace giving the crude fibre content (W3). Fibre content was calculated as follows,

Crude fibre (W3) = W1-W2

$$\text{Crude fibre (\%)} = \frac{\text{W3}}{\text{Weight of the sample}} \times 100$$

3.2.4 High Performance Liquid Chromatography (HPLC) for the estimation of non-volatile fractions

Gingerols and shogaols in the dried red ginger rhizomes were estimated by ISO method (1997) as given below:

3.2.4.1 HPLC Conditions

The extract was analysed on a Shimadzu preparative model LC20AP HPLC system fitted with a PDA detector. Acetonitrile and 1% acetic acid were used as the mobile phase. The solvents were filtered through a filter of pore size 0.45 µm (Merck, USA) and followed by 30 minutes sonication. A Purospher STAR RP-18 column with 250 × 4.6 mm dimension and 5 µm particle size was used. The separation process was achieved through an isocratic elution program with a flow rate of 1 ml min^{-1} (65:35) and a run time of 20 minutes. The detector was set at 280 nm.

3.2.4.2 Preparation of standards and determination of response factor

Authentic standards of gingerols and shogaols were purchased from Sigma Aldrich. Standard stock solutions were prepared from the synthetic standards at a concentration of 1 mg ml^{-1} by dissolving 10 mg standard in methanol and made up to 10 ml in a standard flask. Four different working concentrations were prepared from the stock solution, *viz.* , 0.2, 0.4, 0.6 and 0.8 mg/ml and calibration curve were plotted for each standard. The response factor of the standards is calculated as follows:

$$K = \frac{C \times 100}{A}$$

K = Response factor

C = Concentration of standard

A = Area of standard peak

3.2.4.3 Sample preparation

One gram of ginger powder was weighed and transferred to 100 ml standard flask. It was made up with methanol and mixed thoroughly for about 2 h and kept undisturbed overnight. 20ml of the supernatant was pipetted out to 50 ml round bottom flask without disturbing the solution. Then, it was concentrated to about 1ml using rotary evaporator at 50ºC and the residue was transferred to 5ml standard flask

and made up with HPLC grade methanol. The extract was filtered to 1.5 ml Eppendorf tubes using 0.22 µm syringe driven filter. 20 µl of the extract was injected to the HPLC and area of each peak was noted. The percentage of gingerols and shogaols were calculated as follows:

$$6\text{- Gingerol (\%)} = \frac{A \times K}{C}$$

A = Area under the peak corresponding to (6)- gingerol

K = Response factor of standard

C = Concentration of sample

3.2.5 Gas chromatography-Mass spectrometry (GC-MS)

The essential oil of ginger was analysed using Shimadzu GC-2010 instrument equipped with QP 2010 mass spectrometer. RtX-5 column (30 m long and 0.25 mm diameter, film thickness 0.25 µm) was used. Helium was used as the carrier gas at a flow rate of 1ml min^{-1}. The injection port was maintained at 240ºC and the detector temperature was 220ºC. The oven temperature was set according to the following program: initially at 60ºC for 5 minutes, then raised to 110ºC at a rate of 5ºC per minute, further increased to 200ºC at a rate of 3ºC per minute, and finally raised to 220ºC at a rate of 5ºC per minute, where the column was held for 5 minutes. To perform the analysis, 0.1µl of the sample was injected. The split ratio was 1:40. Mass range was 600-450 amu. The total run time was 37 minutes. By comparing the mass spectra of compounds found in the Wiley library and National Institute of Standards and Technology library (Stein, 2008), the components of the oil were determined. Adams' book (Adams, 2001) was then used to validate the identification.

The percentage of each compound was expressed as relative percentage of peak area and calculated as follows:

$$\text{Relative percentage of peak area} = \frac{\text{Area of specific peak}}{\text{Total peak area}} \times 100$$

3.3 Statistical analysis

3.3.1 Analysis of variance

Analysis of variance for various morphological and biochemical traits was conducted following the methodology described by Panse and Sukhtame (1954), and the variability among the red ginger genotypes was assessed at a 5% level of significance.

3.3.2 Estimation of genetic variability parameters

3.3.2.1 Genotypic and phenotypic variance

The genotypic and phenotypic variances were esimated according to Johnson *et al*. (1955) and Comstock and Robinson (1952).

$$\text{Genotypic variance } (\sigma^2_g) = \frac{\text{Mean sum of squares due to genotypes} - \text{Error mean sum of squares}}{\text{Replication}}$$

$$\text{Phenotypic variance } (\sigma^2_p) = \sigma^2_g + \sigma^2_e$$

$$\text{Environmental variance } (\sigma^2_e) = \text{Error mean sum of squares / Replication}$$

3.3.2.2 Genotypic and phenotypic coefficient of variation

Genotypic and phenotypic coefficient of variation were calculated by using the formula of Singh and Chaudhary (1999).

$$\text{Genotypic Coefficient of Variation (GCV\%)} = \frac{\sqrt{\text{Genotypic variance}}}{\text{General mean}} \times 100$$

$$\text{Phenotypic Coefficient of Variation (PCV\%)} = \frac{\sqrt{\text{Phenotypic variance}}}{\text{General mean}} \times 100$$

PCV and GCV were categorized according to Sivasubramanian and Menon (1973).

PCV and GCV	Category
<10 %	Low
10-20 %	Moderate
>20 %	High

3.3.2.3 Heritability in broad sense

Heritability in broad sense (h^2) was calculated according to the formula by Allard (1960).

$$\text{Heritability in broad sense } (h^2) = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}} \times 100$$

The categorization of broad-sense heritability was performed based on the provided ranges as outlined by Robinson *et al*. (1949).

Heritability	Category
<30 %	Low
31-60 %	Moderate
>60 %	High

3.3.2.4 Genetic advance (GA)

Genetic advance (GA) was computed using the formula outlined by Johnson *et al*. (1955).

$$\text{Genetic advance (GA)} = h^2 \times \sigma p \times K$$

Where,

h^2 = Heritability in broad sense

σp = Phenotypic standard deviation

K = 2.06 (selection differential at 5% selection intensity)

3.3.2.5 Genetic advance as percent of mean (GAM)

The calculation of genetic advance as a percentage of mean was carried out by applying the equation provided by Johnson *et al*. (1955), utilizing the genetic advance values.

$$\text{Genetic advance as percent mean (GAM \%)} = \frac{\text{Genetic advance}}{\text{Mean}} \times 100$$

The range of genetic advance as a percentage over the mean (GAM) was provided by Johnson *et al*. (1955).

PCV and GCV	Category
<10 %	Low
10-20 %	Moderate
>20 %	High

Statistical analyses were conducted using R version 4. 3. 0 developed by the R Core Team (2022). ANOVA across all parameters was performed using the Agricolae package developed by De Mendiburu (2021) and the Doebio research package by Popat and Banakara (2020). Principal Component Analysis (PCA) for volatile compounds was conducted using "Metaboanalyst 5.0" developed by Xia and Wishart (2011).

3.4 Standardization of *in vitro* regeneration protocol for red ginger genotypes and genetic fidelity assessment using ISSR and SSR markers

3.4.1 Materials

3.4.1.1 Plant material

Two red ginger (*Z. officinale* Rosc.) genotypes, exotic red ginger (Acc. 899) and Indian red ginger (Coll. No. 9073), were used in the study. Rhizomes of these genotypes were obtained from National Active Germplasm Site (NAGS), ICAR-IISR, Kozhikode, Kerala, India. Young vegetative buds of 1.5 to 3.0 cm were used as the explant. Both apical and axillary buds were used in the experiments.

3.4.1.2 Culture medium

MS (Murashige & Skoog, 1962) basal medium, the most used culture medium for plant tissue culture was employed for the present study. In all the experiments, MS medium in full strength was used in all the experiments (Table 3).

Table 3. Composition of Murashige and Skoog* basal medium

Composition	Molecular formula	Concentration (mg L^{-1})
Macronutrients		
Ammonium nitrate	$NH_4 NO_3$	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2.2H_2O$	440.00
Potassium di hydrogen orthophosphate	KH_2PO_4	170.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Micronutrients		
Sodium EDTA	Na_2EDTA	37.30
Ferrous sulphate	$FeSO_4.7H_2O$	27.80
Boric acid	H_3BO_3	6.20
Manganese sulphate	$MnSO_4.4H_2O$	22.30
Potassium iodide	KI	0.83
Zinc sulphate	$ZnSO_4.7H_2O$	8.60
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
Copper sulphate	$CuSO_4.5H_2O$	0.025
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Vitamins		
Myo-inositol	$C_6H_{12}O_6$	100.00
Thiamine HCl	$C_{12}H_{17}CIN_4OS.HCl$	0.10
Nicotinic acid	$C_6H_5NO_2$	0.50
Pyridoxine HCl	$C_6H_{11}NO_3.HCl$	0.50
Amino acid		
Glycine	$C_2H_5NO_2$	2.00

*Murashige and Skoog, 1962

The chemicals used for macro and micronutrients and vitamins were obtained from 'Himedia Laboratories', Mumbai, India.

3.4.1.3 Carbon source

Sucrose (Sisco Research Laboratories, Mumbai, India) was used as the carbon source in all the experiments, at a concentration of 30 g L^{-1}.

3.4.1.4 Plant Growth Regulators

Cytokinins: 6- Benzylaminopurine (BAP) (Himedia Laboratories, Mumbai, India) was used for the shoot multiplication studies of red ginger genotypes at five different concentrations (0.0, 1.0, 2.0.3.0,4.0 and 5.0 mg L^{-1}).

3.4.1.5 Gelling agent

Plant tissue culture grade agar (Himedia Laboratories, Mumbai, India) was used for solidifying the culture medium at a concentration of 0.6 %.

3.4.1.6 Glassware

Culture tubes of 25×150 mm was used for shoot initiation (Borosil Limited, Mumbai, India). For shoot multiplication, 250 ml phyta jars (Himedia Laboratories, Mumbai, India) were used.

3.4.1.7 Plugging the culture vessels

The culture tubes and phyta jars were closed tightly with cotton plugs made up of non-absorbent cotton enclosed with mesh cloth and polypropylene caps, respectively.

3.4.1.8 Sterilization of culture medium

The culture medium was sterilized by autoclaving for 15 minutes at 121°C with a pressure of 1.06 kg cm^{-2}.

3.4.1.9 Incubation condition

The cultures were incubated at 25±2°C with a 14 h photoperiod at 3000 lux light intensity. The temperature inside the incubation room was maintained with the help of air conditioners. Whereas the light intensity was adjusted with white, fluorescent tubes.

3.4.1.10 Distilled water

Double distilled water was used for rinsing, surface sterilization procedures, preparation of stocks, media, growth regulators and antibiotics stocks *etc.*

3.4.1.11 Instruments

Horizontal autoclave was used for sterilizing the media, glassware, forceps, scalpel *etc*. Double distilled water was produced with vertical double distillation unit. Chemicals were measured using the weighing balance. Rotary shaker was utilized to properly dissolve the component chemicals in stock solutions. Eutech pH meter was used to measure the pH of the culture media. Inoculation was carried out under aseptic condition inside – Laminar Air Flow (LAF) chamber (Klenzaids, Mumbai, India).

3.4.2 Procedures

3.4.2.1 Preparation of explant

Healthy harvested rhizomes collected, cleaned, and planted in trays containing sterilized coir pith, to reduce the incidence of soil borne pathogens. The emerging buds were collected after two weeks and used as explants.

3.4.2.2 Surface sterilization of explant

The outer layer of the rhizome was delicately peeled away, and the buds underwent a thorough 30-minute rinse under a continuous stream of tap water. Following that, the rhizome was immersed in a 5% (v/v) solution of teepol (Himedia Laboratories, Mumbai, India) for a duration of 30 minutes, after which it was washed with distilled water. The explants were treated with 0.3 % (w/v) bavistin (Crystal Crop Protection Pvt. Limited, Delhi, India) for one-h and were subsequently washed with distilled water three to four times. Subsequently, the explants underwent a 10-minute treatment with 0.2% (w/v) streptomycin (Himedia Laboratories), followed by three rinses using distilled water. Thoroughly cleaned explants were transferred to a laminar air flow chamber, treated with 70% (v/v) ethanol for 60 seconds and washed again in sterile distilled water, followed by 0.1% (w/v) mercuric chloride ($HgCl_2$) (Himedia Laboratories) for 10 min. Following this, the explants underwent three to four rinses with sterile distilled water. Once

sterilized, the explants were trimmed to sizes ranging from 1.0 to 1.5 cm in preparation for inoculation.

3.4.2.3 Preparation of culture media

MS (Murashige & Skoog, 1962; Table 3) medium was used as the nutrient medium for entire experiment. Separate stock solutions were prepared for macronutrients, micronutrients, vitamins, and amino acids. Details of the stock solutions are given in the Table 4. Among the different macronutrients, calcium chloride, was prepared as separate stock solution. Sodium EDTA and ferrous sulphate of micronutrients were dissolved separately and mixed to final stock. For each of the used hormones, separate stocks were made.

Table 4. Details of the stocks used for MS medium

Stock	Composition	Stock strength	Quantity for 1L medium
A	**Macronutrients** $NH_4 NO_3$ KNO_3 KH_2PO_4 $MgSO_4.7H_2O$	X40	25ml
B	**Macronutrients** $CaCl_2.2H_2O$	X200	5ml
C	**Micronutrients** H_3BO_3 $MnSO_4.4H_2O$ $ZnSO_4.7H_2O$ $Na_2MoO_4.2H_2O$	X1000	1ml
D	**Micronutrients** Na_2EDTA $FeSO_4.7H_2O$	X200	5ml
E	**Micronutrients** $CoCl_2.6H_2O$ $CuSO_4.5H_2O$	X5000	0.2ml
F	**Vitamins & amino acid** Thiamine HCl Nicotinic acid Pyridoxine HCl Glycine	X1000	1ml
	Growth regulators BAP	100 mg/100ml	

Stocks of required quantity was taken, sucrose and Myo-inositol were added at concentration of 30 g L^{-1} and 100 mg L^{-1} respectively. All the components were mixed properly, and pH of the medium was adjusted to 5.75. Agar was added to the medium and then boiled. After cooling, medium was poured to different culture vessels and autoclaved at 121°C with a pressure of 1.06 kg cm^{-2} for 15 minutes.

3.4.2.4 Inoculation

Inoculation process was carried out under laminar air flow chamber (Klenzaids, Mumbai, India). Before inoculation, the LAF was wiped with 70 % alcohol and UV sterilized for 20 minutes. All the equipment using for inoculation including culture media were kept inside LAF for UV sterilization. The surface-sterilized explants were trimmed to sizes ranging from 1 to 1.5 cm using sterile blade and then placed into the culture medium for shoot initiation with sterile forceps. The cultures were incubated under controlled conditions. The cultures were regularly monitored for shoot initiation.

3.4.2.5 Shoot multiplication

After four weeks, buds that exhibited signs of shoot initiation were subcultured to MS medium with five different concentrations of BAP for shoot multiplication. The response of the explants in different medium were observed and recorded regularly. Five replications were maintained for each treatment, with three shoots in each phyta jar. After 30 days, the response of the buds in different medium was documented, including the number of roots and shoots per explant and the length of roots and shoots per explant.

3.4.2.6 Subculturing

Every four weeks, well developed plantlets were separated and transferred to freshly prepared media. The plantlets were regularly sub cultured to maintain the vigour.

3.4.2.7 Hardening

Well-developed and well-rooted plantlets were taken out of the phyta jar and separated carefully from the clump without any damage. Unhealthy and damaged roots and leaves were removed, and the remaining roots were cleaned using a soft brush to get rid of the agar. The plantlets were transplanted to pro-trays filled with sand: coir pith: vermicompost (1:1:1) and kept inside a mist chamber for rooting and acclimatization. After three-four weeks, established plantlets were taken out from the chamber and transplanted to pots filled sand: soil: farmyard manure and later established in the field.

3.4.3 Clonal fidelity assessment

3.4.3.1 DNA isolation

DNA was isolated from 10 randomly selected fresh red ginger leaves following the protocol of Doyle and Doyle (1987) with slight modification.

1. 100 mg of leaf sample was weighed and grinded in liquid nitrogen by using a pre chilled mortar and pestle.

2. Then a pinch of polyvinylpyrrolidone (PVP) and 1200 µl of CTAB extraction buffer, comprising of 2% CTAB, 1.4M NaCl, 100mM Tris and 20 mM EDTA, at pH 8, was added to it and ground thoroughly. Then, it was transferred to a 1.5 ml Eppendorf tube and incubated at 65ºC for one hour.

3. 800 µl of chloroform (24): isoamyl alcohol (1) mixture was added. Then, vortexed and centrifuged at 12000 rpm and 23ºC for 15 minutes.

4. After centrifugation, the pellet, which contained the cellular debris and proteins, was discarded, and the supernatant was collected in an Eppendorf tube.

5. The supernatant was again treated with 800 µl of chloroform (24): isoamyl alcohol (1) mixture, vortexed and centrifuged at 12000 rpm and 23ºC for 15 minutes.

6. The supernatant was collected and transferred to an Eppendorf tube. 500 µl chilled isopropanol was added.
7. It was then left at 4ºC overnight to precipitate the DNA.
8. It was then centrifuged at 12000 rpm and 4ºC for 10 minutes to pellet down the DNA.
9. After removing the supernatant, the pellet was washed with 100µl of 70% ethanol, centrifuged at 120000 and 4ºC for 5 minutes.
10. After centrifugation, ethanol was removed from pellet and air dried to remove completely. Finally, the pelleted DNA was dissolved in 30 µl of nuclease free water and stored at -20ºC.

3.4.3.2 Quantification and quality checking of isolated DNA

Quality and quantity of the extracted DNA was estimated using spectrophotometer and agarose gel electrophoresis.

3.4.3.2.1 Quantification and quality assessment using spectrophotometer

Quality and quantity of the DNA was analysed using DeNovix Nanodrop (DeNovix, Wilmington, DE). One µl nuclease free water used as blank to calibrate the instrument. Then one µl DNA sample, diluted 50 times with nuclease free water, was placed to measure the quantity and quality (A260/230 and 260/280). The values were noted down. After each sample, the platform was wiped with tissue paper to avoid contamination and finally with nuclease free water.

3.4.3.2.2 Quality checking using agarose gel electrophoresis

The quality of the extracted DNA was confirmed by visualizing it on 0.8 % agarose gel. The procedure is as follows.

1. The gel casting tray was kept ready, comb was placed and aligned properly.
2. 0.8 % agarose gel was prepared by dissolving 0.8 g of agarose in 100ml TAE buffer (1X).

3. Agarose was boiled in a microwave oven until it was fully dissolved to produce a clear solution.

4. The melted agarose was allowed to cool to about 50-60ºC, then add 1-2 µl of ethidium bromide, mixed thoroughly and poured to gel tray. The tray was kept undisturbed for 30-40 minutes.

5. After complete solidification, the gel was separated from the tray without any damage and transferred to electrophoresis chamber (Genei, India) and TAE buffer (1X) was poured to immerse the gel.

6. The PCR product was loaded into the wells of agarose gel along with a standard DNA marker.

7. Gel run was kept at 80-90 volts.

8. After the maximum separation of the DNA fragments, the bands were visualized using the gel documentation system (Syngene Gel Doc; Syngene Synoptics Ltd, Cambridge, UK) under UV light.

3.4.3.3 Polymerase chain reaction

Ten ISSR (Giridhari *et al*., 2020) and 12 SSR primers (Vidya *et al*., 2021) were used to evaluate the genetic fidelity (Table 5 & Table 6) among the clones. The PCR reaction was performed with the Invitrogen Pro Flex PCR system, 19105 (Applied Biosystems, Waltham, MA).

Table 5. List of ISSR primers with sequence and annealing temperature

Primer	Primer sequence (5′-3′)	Annealing temperature (T_a) °C
ISSR 5	CTCTCTCTCTCTCTTG	46.5 °C
ISSR 10	ACACACACACACACACG	50.4 °C
ISSR 12	CACCACCACGC	45.0 °C
UBC 816	CACACACACACACACAT	58.0 °C
UBC 826	ACACACACACACACACC	54.8 °C
UBC 827	ACACACACACACACACG	51.4 °C
UBC 835	AGAGAGAGAGAGAGAGYC	41.5 °C
UBC 851	GTGTGTGTGTGTGTGTYG	54.0 °C
UBC 856	ACACACACACACACACYA	50.4 °C
UBC 897	CCGACTCGAGNNNNNNATGTGG	50.4 °C

3.4.3.2.1 PCR reaction mixture for ISSR analysis

PCR components	:	Volume (µl)
Nuclease free water	:	8
Primer (10 mM μl^{-1})	:	1
PCR Master mix	:	10
Template DNA (50 ng μl^{-1})	:	1

A total volume of 20 µl reaction mixture was used to perform polymerase chain reaction.

3.4.3.2.2 Reaction conditions for ISSR markers

- Initial denaturation step at 94°C for five minutes
- Thirty-five cycles of the steps (denaturation at 94°C for one minute; one minute at corresponding annealing temperature; extension at 72°C for two minutes)
- Final extension at 72°C for 10 minutes

3.4.3.2.3 PCR reaction mixture for SSR analysis

PCR components	:	Volume (µl)
Nuclease free water	:	8
Forward primer (10 mM μl^{-1})	:	0.5
Reverse primer (10 mM μl^{-1})	:	0.5
PCR Master mix	:	10
Template DNA (50 ng μl^{-1})	:	1

A total volume of 20 µl reaction mixture was used to perform polymerase chain reaction.

3.4.3.2.4 Reaction conditions for SSR analysis

- Initial denaturation step at 94°C for 3 minutes

- Thirty-five cycles of the following steps (denaturation at 94°C for 45 seconds, annealing at the specific annealing temperature for 45 seconds, and extension at 72°C for one minute),
- Final extension at 72°C for 10 minutes

Table 6. List of SSR primers with sequence and annealing temperature

Primer	Primer sequence (5′-3′)	Annealing temperature (T_a) °C
ZO SSR 2	F-TGATTCCGTATCAACTCCAT R-CAAGGAAGACTTCAACTCCAT	61.4 °C
ZO SSR 16	F-ATCAAGGAAAAGACCTCAAAG R-CATTATCATCGTCTTCCTCTG	56.3 °C
ZO SSR 21	F-TCCTCCTCTTTTCTCCTCTC R-GCTAGAAGCGAGGGATTT	57.8 °C
ZO SSR 25	F-CTGAGTCCAGTGCTGTATAGG R-GTTCTTGCTCGCTAGATCAC	60.4 °C
ZO SSR 35	F-GGTCCAAGGTCTTTAAGCAT R-ACGAAGACAACGATATCAGC	52.2 °C
ZO SSR 36	F-GAGGACTACTCCGATATGAGC R-GGAGTTAGGGTTAGGGATTG	60.0 °C
ZO SSR 38	F-GAAGGAGGCTCTCGAAGT R-GCACCTGCTTACAGTTACAAT	48.5 °C
ZO SSR 64	F-TCCAGAGGTCTCTCAGCTT R-ACTGCGACGACTCAGGTC	57.6 °C
ZO SSR 73	F-GCTCTCCCTTCGAAAAAC R-GCGTAGGTGCAGAAGTAGTTA	54.4 °C
ZO SSR 91	F-CTCCATCCTATCAACTGTCAC R-ACATTCTGAAGCTCTTGCAT	51.1 °C
ZO SSR 111	F-CTAAGGGGCTCCTTCTTC R-CAGCTGGAAGCAGCTATG	60.1 °C
ZO SSR 108	F-GATCTCCTGCTTGTTATCTCTC R-TGTTCTAGGTGTTGTGGAAG	60.1 °C

3.4.3.4 Agarose gel electrophoresis

The PCR products were examined in 2 % agarose gel for ISSR primers and 3.5 % agarose gel for SSR primers.

3.4.4 Statistical analysis

Analysis of Variance was performed and followed by the post hoc analysis, Duncan's multiple range test (DMRT), to distinguish statistically significant differences among the means at a significance level of 1% ($P \leq 0.01$). The statistical analysis was performed using R package Doebioresearch by Popat and Banakara (2020).

The binary matrix was used to score discrete repeatable bands. The presence of band was indicated by 1 and its absence by 0, respectively. Polymorphism Information Content (PIC) value was calculated for the molecular markers. It was calculated using the formula (Roldan-Ruiz *et al*., 2000),

$PIC = 2f (1-f)$

where, f is the frequency of the band in the gel. The genetic similarities among the *in vitro*-raised plantlets were assessed by the Jaccard's similarity coefficient (Jaccard, 1908). The similarity matrix was produced using PAST (PAleontological STatistics) 4.03 software (Hammer *et al*., 2001). Then a dendrogram was made by Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis based on similarity coefficients.

3.5 Polyploidy induction

3.5.1 Ploidy estimation of the mother plants

Ploidy level of selected red ginger genotypes i.e., Coll. No. 9073 and Acc. 899 was estimated by chromosome counting using the root tip squashing technique, followed by flow cytometric analysis.

3.5.1.1 Chromosome counting

The somatic chromosomes were prepared by root tip squashing method proposed by Saha *et al*., (2020) with slight modifications. The rhizomes were planted in trays with clean sand and irrigated on a regular basis. Root tips were collected for analysis when sprouting and root emergence began. Actively growing

root tips (about 1cm) were collected between 11.20-11.30 am. The root tips were pre-treated with a mixture of saturated paradichlorobenzene solution and 2 mM 8-hydroxyquinoline (1:1) at 4–5°C for 6 h. The root tips were then thoroughly cleaned with distilled water and treated with a mixture of 1N HCl and absolute alcohol (1:1) for an hour. Following that, root tips were left in 45 % acetic acid for 20 minutes. Afterwards, the washed root tips were treated for 2 h at 37ºC in a combination of 5 % cellulase (Sigma) and 5% pectinase (Sigma) (1:1) in citrate buffer at pH 4.8. Then, toot tips were rinsed 3-4 times with distilled water and stained with a mixture 9:1 of 2 % aceto-orcein and 1N HCl for overnight and then squashed in 45% acetic acid. Metaphase plate with well spread chromosomes were captured using Leica microscope.

3.5.1.2 Flow cytometric analysis

IISR Varada (2n = 22) was used as the reference standard for the whole flow cytometric analysis. Small portion of tender leaf samples from unknown samples and reference standard were chopped with a blade in a Petri plate containing 1 ml hypotonic propidium iodide lysis buffer (Krishan, 1975) with slight modification. The buffer was made up of 2 mg ml^{-1} RNase A (Sigma-Aldrich), sodium citrate tribasic dehydrate (Sigma-Aldrich), 50 µg ml^{-1} PI (Sigma-Aldrich), and 0.3 % (v/v) Tween-20 (Sigma-Aldrich). The buffer was additionally supplemented with β-mercaptoethanol (1%) and PVP-40 (1%) to reduce the effects of cytosolic and phenolic substances on propidium iodide fluorescence. Subsequently, the suspension was filtered through a 10 µm cell strainer (CellTrics, Sysmex) to separate the nuclei, which were then gathered into 1.5 ml Eppendorf tubes. The nuclei suspension was incubated for 15 minutes. Then, the suspension of isolated nuclei was injected to a Cytoflex flow cytometer (Beckman Coulter), fitted with 488 nm laser and 585/42 Band Pass filter. Reference control was used as an external standard for ploidy analysis.

About 500-2000 nuclei were collected and analysed for each sample. Using a diploid control, the detector gain was calibrated, and the same gain settings were applied for running the samples. The findings were represented through histograms

displaying a single parameter, with the G0/G1 peak depicted on a linear scale. The fluorescence intensity, represented by the median value, was documented for both the reference control and samples, and subsequently processed using FCS Express Software (DENOVO software, USA). The ploidy level of the unknown samples was calculated using the formula.

$$\text{Ploidy level of the sample} = \frac{\text{Median value of sample G0/G1 peak}}{\text{Median value of standard G0/G1 peak}} \times \text{Reference ploidy}$$

3.5.2 *In vitro* polyploidy induction

3.5.2.2 Establishment of *in vitro* cultures

Rhizome buds of red ginger genotypes, *viz.*, Indian (Coll. No. 9073) and exotic (Acc. 899) red ginger, were used as explants for culture initiation. The explants were surface sterilized properly and inoculated to basal MS medium for initiation. After that, shoots were subcultured to shoot multiplication medium. Sufficient *in vitro* plantlets were produced using the already standardized *in vitro* protocol.

3.5.2.4 Experimental material

In vitro raised plantlets were used as the starting material for polyploidy induction. The shoot was trimmed 4-5 mm above the meristem, and the roots were also removed and used as an explant for polyploidization. The explants were cultured in basal MS medium for 7-8 days for initiation of the growth. The anti-mitotic chemical colchicine (Sigma Aldrich) was used to induce polyploidy.

3.5.2.5 Determination of the optimum colchicine concentration for polyploidy induction

3.5.2.5.1 Stock preparation

Colchicine was used as the antimitotic agent. One percentage stock solution was prepared by dissolving 1g of colchicine powder (Hi-Media laboratories) in 2–5

ml of absolute alcohol and making up to 100 ml by adding distilled water with constant stirring.

3.5.2.5.2 Working concentrations

Four different concentrations *viz.*, 0.025, 0.050, 0.075 and 0.100 % were prepared out from the stock. Distilled water was used as the control in the experiment.

3.5.2.6 Colchicine treatment

The technique for inducing polyploidy *in vitro* followed the approach outlined by Smith *et al*. in 2004. The entire experiment was conducted aseptically inside a laminar air flow chamber. All the equipment used for inoculation and colchicine treatment was autoclaved and UV sterilized. Hormone-free liquid MS medium was prepared and autoclaved. The aqueous solution of colchicine was filter sterilized and added to liquid MS basal medium in the 250 ml conical flask and mixed thoroughly. The explants that showed signs of growth in the basal MS medium were selected and transferred to the conical flask with 50 ml liquid MS containing different concentrations of colchicine. Conical flasks were pugged with cotton pugs and sealed properly with phyta wrap to avoid contamination. Treatments were incubated for 24 and 48 hours at 25 °C under dark conditions with constant shaking at 110 rpm in a shaker. After treatment, the explants were taken out and rinsed three to four times with sterile distilled water and inoculated to MS solid medium supplemented with BAP for shoot multiplication.

A factorial experiment was conducted utilizing a completely randomized design, consisting of four replicates. The study encompassed three factors:

1. The first factor considered various concentrations of colchicine (0%, 0.025%, 0.05.%, 0.075% and 0.100 %)

2. The second factor involved two red ginger genotypes, *viz.*, Indian red ginger (Coll. No. 9073) and exotic red ginger (Acc. 899)

3. The third factor included varying soaking times (24 and 48 h).

In the experiment, 18 explants were treated for Indian red ginger, and 12 explants were treated for exotic red ginger in each replication. A total of 72 explants were treated for each treatment of Indian red ginger and 48 explants for exotic red ginger.

3.5.2.7 Regeneration

The colchicine treated explants were reinoculated to respective regeneration media for regeneration and multiplication. The explants of Indian red were inoculated to MS medium supplemented with 3 mg L^{-1} BAP. Whereas the explants of exotic red ginger were inoculated to MS medium supplemented with 5 mg L^{-1} BAP. The cultures were incubated at 25±2°C with a 14 h photoperiod at 3000 lux light intensity for about 120 days.

3.5.2.8 Hardening

The explants were allowed to regenerate and grow under aseptic condition for about three months. Well-developed plantlets were deflasked, solid agar attached to roots washed out properly, and matured roots were removed. Cleaned plantlets were allowed to dry on blotting paper and prepared for planting. Plantlets were transplanted to protrays filled with autoclaved coir pith: sand: vermicompost (1:1:1). The planted trays were kept inside a mist chamber encased in a semi-transparent plastic sheet for two weeks for rooting and acclimatization. Humidity inside the chamber decreased gradually over two weeks by putting small holes in the sheet. The number and size of the holes increased day by day and acclimatized the plants to the outer environment. After that, the plants were taken out and replanted in larger pots and maintained under greenhouse conditions, and their growth responses were carefully observed.

3.5.3 *In vivo* polyploidy induction

3.5.3.1 Plant material

Healthy rhizomes of exotic (Acc. 899) and Indian red ginger (Coll. No. 9073) genotypes were obtained from National Active Germplasm Site (NAGS),

ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India. The rhizomes were spread in fine sand for sprouting and watered regularly for sprouting. Young emerging buds were selected for the study.

3.5.3.2 Determination of the optimum colchicine concentration for polyploidy induction

3.5.3.2.1 Stock preparation

An aqueous solution of colchicine (1%) was prepared by dissolving 1g of colchicine powder in 2-5 ml absolute alcohol and making up to 100 ml by adding distilled water with constant stirring. Different concentrations of colchicine were attempted to find out the optimum range of concentration for polyploidy induction.

3.5.3.2.1 Working concentrations

Five different concentrations (0.00 %, 0.05 %, 0.10 %, 0.15 %, and 0.20 %) of colchicine solution were used for the experiment.

3.5.3.3 Colchicine treatment

Rhizomes were taken out from the sand and washed thoroughly to get rid of the sand particles, and young emerging buds were excised from it. Actively dividing buds were placed in a Petri dish with a thin layer of blotting paper and immersed in colchicine solution for 24 and 48 h. Using a Pasteur pipette colchicine solution was dropped over each bud at a 2 h interval. After the incubation, buds from each treatment were transferred to a conical flask washed 3-4 times with water. Subsequently, the buds were planted in pro-trays filled with coir pith and FYM and watered properly. Later, the sprouted plantlets were transplanted into pots and maintained under greenhouse conditions (Prasath *et al*., 2022).

Experimental design was completely randomized with four replications in each treatment. The experiment involved three factors:

1. The first factor considered various concentrations of colchicine (0.00 %, 0.05 %, 0.10 %, 0.15 % and 0.20 %)

2. The second factor involved two red ginger genotypes, *viz.*, Indian red ginger (Coll. No. 9073) and exotic red ginger (Acc. 899)

3. The third factor included varying soaking times (24 and 48 h).

Twelve explants of exotic red ginger and 18 explants of Indian red ginger were treated in each replication of the experiment. As a result, 48 explants were treated for exotic red ginger and 72 explants of Indian red ginger were exposed to each concentration of colchicine.

3.5.4 Observations recorded from *in vitro* and *in vivo* treated population of plants

3.5.4.1 Survival rate (%)

The survival rate of explants after *in vitro* colchicine treatment was recorded following 4 weeks of incubation.

$$\text{Survival rate (\%)} = \frac{\text{Number of explants survived}}{\text{Total number of explants treated}} \times 100$$

3.5.4.2 Sprouting rate (%)

The number of the buds germinated after the *in vivo* colchicine treatment was recorded periodically and after 60 days the final figures were determined.

$$\text{Spouting rate (\%)} = \frac{\text{Number of bud germinated}}{\text{Total number of bud treated}} \times 100$$

3.5.4.3 Morphological parameters recorded (150 DAP)

3.5.4.3.1 Plant height (cm)

Plant height was measured from the soil level to the tip of the topmost leaf on the main shoot.

3.5.4.3.2 Number of shoots

The total number of shoots in a clump was recorded for estimating the number of shoots.

3.5.4.3.3 Shoot diameter (cm)

Shoot diameter was recorded about five centimetres above the soil using a non-stretchable string and scale.

3.5.4.3.4 Number of leaves

Total number of leaves on the main shoot was recorded from the tallest plant in the clump.

3.5.4.3.5 Leaf length (cm)

Length of the leaf was recorded from the upper fourth leaf of the main shoot. Length was measured from the base to the pointed tip.

3.5.4.3.6 Leaf width (cm)

Width of the leaf was measured from the upper fourth leaf of the main shoot from the maximum widest point.

3.5.4.4 Physiological parameters recorded (150 DAP)

Physiological parameters like stomatal density, stomatal length, and width were measured from the treated plants after 150 days of planting. Stomatal measurements were recorded according to the method of Smith and Hamill (2002). The measurements were taken only from the abaxial surface of the upper fourth leaf.

3.5.4.4.1 Stomatal length (µm)

Stomatal length was measured under a light microscope at 40X magnification. Stomatal length was estimated using the Motic Image Plus 2.0 software. Observations were recorded from a total of 50 stomata for each treatment.

3.5.4.4.2 Stomatal width (µm)

Stomatal width was recorded at 40X magnification, under a light microscope connected with a camera. The measurements were recorded using the Motic Image Plus 2.0 software. For each treatment, observations were taken from a total of 50 stomata.

3.5.4.4.3 Stomatal density (stomata mm^{-2})

A thin film of transparent nail polish was applied on the abaxial surface of the leaf and allowed to dry. After 10 minutes, the impression was collected using transparent adhesive tape. Then, it is attached to a clear microscopic slide and observed under Leica DM 5000 B research microscope. Stomatal density was recorded at 10X magnification. A total of 50 fields were viewed for each treated group.

$$\text{Stomatal density} = \frac{\text{Number of stomata in the field of view}}{\text{Area of the field of view}}$$

3.5.5 Identification and selection of putative polyploids

Since a large population of plants was produced after the treatment, an initial screening was conducted to select a small population of putative polyploids. The plants with some detectable anomalies in growth patterns like growth habit, leaf arrangement, leaf shape, and morphological variations such as dark green and thick leaves, robust and stunted growth, *etc.*, were selected for further screening. Variations in the stomatal parameters were also employed as criteria for the identification of potential polyploids.

3.5.6 Confirmation of the ploidy level of putative polyploids and screening for polyploids

Ploidy level of the selected putative polyploids was further assessed through flow cytometric analysis. Further cytological studies were conducted to confirm the exact ploidy status and chromosome number.

3.5.6.1 Flow cytometry

The ploidy level of putative polyploids were checked using flow cytometric technique.

3.5.6.2 Chromosome counting

Somatic chromosome number of the confirmed polyploids were identified using conventional root tip squashing method (Saha *et al*., 2020).

3.5.7 Comparison between diploids and *in vitro* induced tetraploids in M1V1 generation

The confirmed polyploids were selected from the treated population and maintained separately. The basic morphological characters such as plant height (cm), number of shoots, shoot diameter (cm), number of leaves, leaf length (cm), leaf width (cm), leaf colour, and leaf area (cm) were recorded four months after planting and compared with the diploid control.

3.5.7.1 Leaf area

Leaf area of the diploids and tetraploids were calculated using the formula developed by Kandiannan *et al*. (2009).

$$\text{Leaf area} = -0.0146 + 0.6621 \times L \times W$$

L = Leaf length

W = Leaf width

Physiological parameters including stomatal length and width (µm), density (stomata/mm^2), and the number of chloroplasts in stomatal guard cells, were also recorded and compared between the diploid control plants and induced tetraploids.

3.5.7.1 Scanning electron microscopy (SEM)

Variations in the stomatal size and density of the diploids and induced tetraploids were studied under Scanning Electron Microscope (VEGA3 TESCAN).

The upper third leaf of the plant was chopped to 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.5 and incubated overnight at 4ºC. Then, the samples were rinsed twice with phosphate buffer and dehydrated with 50, 60, 70, 80, 90 and 100 % ethanol solutions for 5 minutes in each concentration and observed under Scanning Electron Microscope.

3.5.7.2 Chloroplast count

The number of chloroplasts in the stomatal guard cells of both diploids and polyploids were recorded and compared. The lower epidermis was peeled off from the tender leaf and placed in a microscopic slide with a drop of distilled water (Rao *et al*., 2019). The chloroplast count was taken at 100X magnification using a Leica DM 5000 B research microscope. 50 stomata from diploids and polyploids were chosen at random for observation.

3.5.7.3 Fluorescence microscopy

The chloroplast in the guard cells of stomata of both diploids and polyploids were visualized under fluorescence microscopy (Leica DM 5000 B research microscope) at 100X magnification.

3.5.8 Comparison between diploids and *in vivo* induced tetraploids in M1V2 generation

Treated plants were harvested at maturity, and the confirmed polyploid and diploid mother plants (M1V1) were further multiplied clonally, and their morphological characteristics were compared in the second generation (M1V2). Morphological characteristics, including plant height (cm), number of shoots, shoot diameter (cm), number of leaves, leaf length and width (cm), leaf area (cm^2), and leaf colour were recorded four months after planting. Following the harvest, the yield per plant was also documented.

Physiological parameters, including stomatal length and width (µm), density (stomata/mm^2) and number of chloroplasts in stomatal guard cells, were also recorded and compared between the diploids and tetraploids.

3.5.9 Comparison between diploids and induced tetraploids in M1V3 generation

The rhizomes of the induced polyploids were harvested at maturity and planted along with corresponding diploids in pots. The vegetative characteristics, including plant height (cm), number of shoots, shoot diameter (cm), number of leaves on the main shoot, leaf length (cm), leaf width (cm), leaf area (cm^2), and leaf colour, were documented 150 days post-planting. Spike formation was observed in both diploids and tetraploids at the end of the vegetative phase. Spike length was measured from the soil level to tip of the inflorescence. Measurements of different flower parts such as, number of bracts, length of bract (cm), width of bract (cm), length of bracteole (cm), width of bracteole, length of flower (cm), length of calyx (cm), width of calyx (cm), length and width of dorsal petal (cm), length and width of lateral petal (cm), length and width of labellum (cm), length of anther crest (cm), length of anther (cm), length of pistil (cm), and pollen diameter (µm) were collected and subjected to comparison.

3.5.10 Statistical analysis

3.5.10.1 Analysis of variance

Data on sprouting rate and survival rate was subjected to analysis of variance (Two-way ANOVA) using R studio, and means were separated through Least Significance Difference (LSD) test at a significance level of $p \leq 0.05$. The percentage data were analysed after angular transformation. Two - Way Analysis of Variance was performed for all the morphological and physiological parameters recorded from the treated population of plants after *in vitro* and *in vivo* polyploidy induction, followed by LSD ($p \leq 0.05$) test.

3.5.10.2 t- test

The comparative characterization of diploids and induced tetraploids were done using unpaired t- test ($p \leq 0.01$). The analysis was performed using Microsoft Excel.

CHAPTER 4
RESULTS AND DISCUSSION

The results of the present experiments entitled "Improvement of red ginger (*Z. officinale* Rosc.) through *in vitro* and *in vivo* studies" were presented and discussed in detail in this chapter.

OBJECTIVE 1

COMPARATIVE CHARACTERIZATION OF RED GINGER ACCESSIONS BASED ON MORPHOLOGICAL CHARACTERS AND CHEMICAL FINGERPRINTS

4.1 Morphological and biochemical characterization of red ginger genotypes

4.1.1 Analysis of variance for various characters

Analysis of variance for morphological characters is given in the Table 7. The results revealed that there is a significant difference in all morphological parameters including plant height, number of shoots, shoot diameter, number of leaves on main shoot, leaf length, leaf width, yield parameters and quality traits. The selected genotypes were significantly different from each other. All observations were significant at the 1% significance level ($p<0.01$).

4.1.2 *per se* performance of red ginger genotypes for different morphological characters

Per se performance of 16 red ginger genotypes and IISR Varada for 12 characters was investigated and given in Table 8-10.

4.1.2.1 Plant height (cm)

A significant difference was observed in the plant height of different genotypes (Table 8). In the 2021-2022 season, significantly highest plant height was recorded in Coll. No. 9046 (72.50 cm) and was on par with G6 (70.50 cm), Coll. No. 9078 (69.30 cm) and IISR Varada (70.00 cm) and the lowest was observed in Acc. 848 (43.00 cm). The grand mean was 63.39 cm. The genotypes Coll. No. 9040 (68.20 cm), Coll. No. 9046 (72.50 cm), Coll. No. 9078 (69.30 cm), G6 (70.50 cm), and IISR Varada (70.00 cm) were significantly above grand mean.

Table 7. Analysis of variance for morphological and quality traits in red ginger genotypes

Characters	Year 1			Year 2			Pooled		
	Replication	Genotypes	Error	Replication	Genotypes	Error	Replication	Genotypes	Error
Degrees of freedom	1	16	16	1	16	16	1	16	16
Plant height	0.04	110.66***	3.36	1.88	103.25***	6.07	36.86	90.59***	16.36
Number of shoots	0.26	29.44***	1.58	0.03	54.97***	1.40	3.56	37.54***	4.67
Shoot diameter	0.001	0.02***	0.00	0.00	0.02***	0.00	0.00	0.02***	0.00
Number of leaves on main shoot	10.62**	26.38***	0.74	7.07*	18.60***	1.16	13.91	19.12***	3.37
Leaf length	0.13	20.68***	1.14	1.09	23.59***	0.63	0.00	20.46***	1.68
leaf width	0.00	0.37***	0.02	0.01	0.23***	0.02	0.01	0.27***	0.03
Rhizome thickness	0.002	0.08***	0.00	0.00	0.08***	0.003	0.00	0.08***	0.00
Yield per plant	1569.4	27112.9***	1325.7	1360	44794***	3253	0.70	31184.6***	4769.1
Dry recovery	0.01	4.16***	0.56	0.23	7.40***	1.29	8.30**	4.84**	0.94
Essential oil	0.02	1.64***	0.01	0.07	1.14***	0.02	1.82***	1.22***	0.11
Oleoresin	0.00	9.27***	0.09	0.38	5.68***	0.20	31.32***	6.44***	0.89
Crude fibre	0.01	4.21***	0.29	0.02	2.47***	0.08	5.57***	3.03***	0.31

***Significant at 0.1% level, ** significant at 1% level, * significant at 5% level

In the 2022-2023 season, the highest plant height was documented in Coll. No. 9073 (82.50 cm), while the lowest was in Acc. 845 (53.00 cm). The grand mean was 65.47 cm, with genotypes Coll. No. 9046 (73.50 cm), Coll. No. 9073 (82.50 cm), G6 (71.00 cm), and IISR Varada (72.50 cm) significantly surpassing it.

A notable difference in plant height among genotypes was observed in the combined analysis across two seasons. Significantly high plant height was observed in Coll. No. 9073 (74.75 cm), which was on par with Coll. No. 9040 (69.10 cm), Coll. No. 9046 (73.00 cm), G6 (70.75 cm) and Coll. No. 9078 (67.65 cm). The lowest height was recorded in Acc. 848 (51.25 cm). The grand mean value was recorded at 64.43 cm. The genotype Coll. No. 9073 (74.75 cm) exhibited significantly higher plant heights than the grand mean.

4.1.2.2 Number of shoots

In the first season, the maximum number of shoots was observed in Acc. 844 (22.50) and was on par with Coll. No. 9076 (22.00), G6 (22.00), Coll. No. 9078 (20.50), Coll. No. 9046 (20.00), Acc. 838 (20.00). The average number of shoots in the first season was 17.74. The genotypes Acc. 844 (22.50), Coll. No. 9076 (22.00), Coll. No. 9078 (20.50) and G6 (22.00) were significantly different from the grand mean. In the second season, Coll. No. 9073 recorded the highest number of shoots (30.00), followed by Coll. No. 9076 (27.50). Over the two seasons, the lowest number of shoots was produced by IISR Varada (9.50 in the first season and 10.50 in the second season). The genotypes Acc. 844 (22.00), Coll. No. 9073 (30.00), Coll. No. 9076 (27.50), and G6 (22.50) exhibited mean number of shoots significantly greater than the grand mean of 18.38. After the pooled analysis, highest number of shoots was recorded in two genotypes i.e.., Coll. No. 9073 (24.75) and Coll. No. 9076 (24.75) and that was on par with Acc. 844 (22.25), G6 (22.25), Acc. 838 (20.25) and Collo. No. 9046 (20.25). Out of the 17 genotypes, the lowest number of shoots was recorded in IISR Varada (10.00). Among the red ginger genotypes, the lowest number of shoots was observed in Acc. 850 (11.50). The grand mean was 18.06. The genotypes Coll. No. 9073 (24.75) and Coll. No. 9076 (24.75) were significantly different from the grand mean.

Table 8. *Per se* performance for morphological traits in the red ginger genotypes

Genotypes	Plant height (cm)			Number of shoots			Shoot diameter (cm)		
	Year 1	Year 2	Pooled	Year 1	Year 2	Pooled	Year 1	Year 2	Pooled
Acc. 838	53.10	55.75	54.43	20.00*	20.50	20.25*	1.00	0.88	0.94
Acc. 842	60.50	61.75	61.13	17.00	17.50	17.25	0.82	0.83	0.82
Acc. 844	65.00	65.50	65.25	22.50*	22.00*	22.25*	0.97	0.96	0.96
Acc. 845	54.00	53.00	53.50	15.50	14.50	15.00	0.89	0.88	0.88
Acc. 848	43.00	59.50	51.25	18.50	16.00	17.25	0.86	1.02	0.94
Coll. No. 9040	68.20*	70.00	69.10*	17.50	16.50	17.00	0.93	0.93	0.93
Coll. No. 9046	72.50*	73.50*	73.00*	20.00*	20.50	20.25*	0.99	0.93	0.96
Coll. No. 9073	67.00	82.50*	74.75*	19.50	30.00*	24.75*	0.88	0.98	0.93
Coll. No. 9076	65.75	62.50	64.13	22.00*	27.50*	24.75*	0.88	0.82	0.85
Coll. No. 9078	69.30*	66.00	67.65*	20.50*	19.00	19.75	1.07*	0.93	1.00
G1	65.00	66.00	65.50	19.00	18.50	18.75	0.97	0.97	0.97
G3	61.50	64.00	62.75	14.50	13.50	14.00	1.11*	1.13*	1.12*
G6	70.50*	71.00*	70.75*	22.00*	22.50*	22.25*	0.93	0.92	0.93
G9	63.50	65.75	64.63	19.50	18.50	19.00	0.93	0.96	0.94
Acc. 850	63.25	57.75	60.50	11.50	11.50	11.50	1.03*	1.03	1.03
Acc. 899	65.50	66.00	65.75	12.50	13.50	13.00	0.99	0.90	0.94
IISR Varada	70.00*	72.50*	71.25	9.50	10.50	10.00	1.18*	1.25*	1.22*
Grand Mean	63.39	65.47	64.43	17.74	18.38	18.06	0.96	0.96	0.96
CV (%)	2.92	3.76	6.28	7.08	6.45	11.96	2.57	3.80	5.50
CD (5%)	3.88	5.22	8.58	2.66	2.51	4.58	0.05	0.08	0.11

*Indicates values above grand mean+CD (5%)

4.1.2.3 Shoot diameter (cm)

Over the two seasons, the significantly highest shoot diameter was produced by IISR Varada (1.18 cm in the first season and 1.25 cm in the second season) and pooled analysis recorded a shoot diameter of 1.22 cm. Among the red ginger types, G3 showed a significantly higher shoot diameter (1.12 cm). The grand mean recorded was 0.96 cm. In the first season, the genotypes Coll. No. 9078 (1.07 cm), G3 (1.11 cm), Acc. 850 (1.03 cm), and IISR Varada (1.18 cm) exhibited significantly different compared to the grand mean of 0.96 cm. The lowest shoot diameter (2021-2022) was recorded in Acc. 842 (0.82cm) among the different genotypes. During the second season, Coll. No. 9076 recorded the lowest shoot diameter of 0.82 cm.

In the second season, the genotypes G3 (1.13 cm) and IISR Varada (1.25 cm) were significantly different from the grand mean (0.96 cm). Pooled analysis revealed the lowest shoot diameter in Acc. 842 (0.82 cm). The genotypes G3 (1.12 cm) and IISR Varada (1.22 cm) were significantly different from the grand mean (0.96 cm) after the pooled analysis as well. Shoot diameter of 17 genotypes were represented in the Table 8.

4.1.2.4 Number of leaves on main shoot

A significant variation was observed within the red ginger genotypes for number of leaves on main shoot ranging from 12.50 to 25.50 (Table 9). The maximum number of leaves was recorded in Coll. No. 9040 (25.50) and was on par with G6 (24.50), G9 (24.50) and Acc. 844 (24.00) while the lowest number of leaves was observed in Acc. 848 (12.50), in the first season. The genotypes Acc. 844 (24.00), Coll. No. 9040 (25.50), Coll. No. 9046 (23.50), G6 (24.50), and G9 (24.50) were significantly different from the grand mean (20.26). During the second season, the highest number of leaves (25.50) was recorded in Coll. No. 9073 and G6, which was found to be on par with Acc. 842 (23.50), Acc. 844 (24.50), Coll. No. 9040 (24.75), Coll. No. 9046 (23.50), Coll. No. 9073 (25.50) and G9 (24.50). The lowest number of leaves (16.50) was recorded in Acc. 838 and Acc. 845. The genotypes Acc. 844 (24.50), Coll. No. 9040 (24.75), Coll. No. 9073 (25.50), and G6 (24.50),

G9 (24.50) showed significant deviation from the grand mean of 21.54. The pooled analysis showed significantly high number of leaves in Coll. No.9040 (25.13) and was statistically on par with G6 (25.00), G9 (24.50), Acc. 844 (24.25), Coll. No. 9046 (23.50) and 9073 (23.00), Acc. 842 (22.50) and IISR Varada (22.25). The genotypes that showed significant difference for the grand mean (20.90) in the pooled analysis were Coll. No. 9040 (25.13) and G6 (25.00). The lowest number of leaves was recorded in Acc. 848 (16.00).

4.1.2.5 Leaf length (cm)

The leaf length ranged from 18.50 cm – 29.00 cm. In year 1 and year 2, the maximum leaf length was recorded in IISR Varada (28.65 cm, 29.00 cm), while the minimum leaf length in Acc. 842 (18.75 cm, 18.50 cm), respectively. The grand mean of the first season was 23.20 cm and genotypes Acc. 845 (25.75 cm), G3 (27.25 cm), Acc. 850 (28.00 cm), and IISR Varada (28.65 cm) were significantly higher than the grand mean in leaf length. In the second season, the genotypes Acc. 848 (26.75 cm), G3 (27.75 cm), Acc. 850 (27.45 cm), Acc. 899 (26.15 cm), and IISR Varada (29.00 cm) were recorded leaf length significantly above the grand mean (23.19 cm).

Significant difference among the genotypes for leaf length was recorded in the pooled analysis over two seasons. IISR Varada (28.83 cm) recorded the significantly highest leaf length and was on par with Acc. 850 (27.73 cm), and G3 (27.50 cm), and on the other hand lowest leaf length was observed in Acc. 842 (18.63 cm). After the pooled analysis, the leaf lengths of genotypes G3 (27.50 cm), Acc. 850 (27.73 cm), and IISR Varada (28.83 cm) significantly exceeded the grand mean of 23.20 cm.

Table 9. *Per se* performance for morphological traits in the red ginger genotypes

Genotypes	Number of leaves on main shoot			Leaf length (cm)			Leaf width (cm)		
	Year 1	Year2	Pooled	Year 1	Year2	Pooled	Year 1	Year2	Pooled
Acc. 838	21.00	16.50	18.75	19.40	23.50	21.45	2.70	2.75	2.73
Acc. 842	21.50	23.50*	22.50*	18.75	18.50	18.63	2.55	2.60	2.58
Acc. 844	24.00*	24.50*	24.25*	20.75	20.25	20.50	2.55	2.60	2.58
Acc. 845	17.00	16.50	16.75	25.75*	24.75	25.25	2.70	2.75	2.73
Acc. 848	12.50	19.50	16.00	22.65	26.75*	24.70	2.40	2.55	2.48
Coll. No. 9040	25.50*	24.75*	25.13*	19.90	19.40	19.65	2.35	2.55	2.45
Coll. No. 9046	23.50*	23.50*	23.50*	19.60	18.50	19.05	2.60	2.60	2.60
Coll. No. 9073	20.50	25.50*	23.00*	24.75	21.90	23.33	3.50*	2.65	3.08
Coll. No. 9076	18.50	20.50	19.50	24.95	23.25	24.10	3.15*	2.90	3.03
Coll. No. 9078	19.00	20.50	19.75	24.85	24.50	24.68	3.25*	3.15*	3.20*
G1	21.50	20.50	21.00	23.20	22.25	22.73	3.00	3.05	3.03
G3	16.50	17.50	17.00	27.25*	27.75*	27.50*	3.55*	3.55*	3.55*
G6	24.50*	25.50*	25.00*	19.25	18.95	19.10	2.58	2.70	2.64
G9	24.50*	24.50*	24.50*	22.50	21.50	22.00	3.45*	3.40*	3.43*
Acc. 850	16.50	19.50	18.00	28.00*	27.45*	27.73*	2.95	2.85	2.90
Acc. 899	16.50	20.50	18.50	24.25	26.15*	25.20	2.10	2.15	2.13
IISR Varada	21.50	23.00	22.25*	28.65*	29.00*	28.83*	2.60	2.70	2.65
Grand Mean	20.26	21.54	20.90	23.20	23.19	23.20	2.82	2.79	2.81
CV (%)	4.25	5.00	8.79	4.60	3.42	5.59	4.70	4.89	5.96
CD (5%)	1.83	2.28	3.89	2.26	1.68	2.75	0.28	0.29	0.36

*Indicates values above grand mean+CD (5%)

4.1.2.6 Leaf width (cm)

The leaf width ranged from 2.10 cm to 3.55 cm across the two seasons. The grand mean for the first season was 2.82 cm and the genotypes Coll. No. 9073 (3.50 cm), Coll. No. 9076 (3.15 cm), Coll. No. 9078 (3.25 cm), G3 (3.55 cm), and G9 (3.45 cm) recorded significantly above the grand mean. The highest leaf width was observed in G3 (3.55 cm), and the lowest leaf width was observed in Acc. 899 (2.10 cm, 2.15 cm) for the two seasons. During the second season, the leaf widths of Coll. No. 9078 (3.15 cm), G3 (3.55 cm), and G9 (3.40 cm) were significantly higher than the grand mean of 2.79 cm. Pooled analysis recorded maximum leaf width in G3 (3.55 cm), and significantly high leaf width was also recorded in G9 (3.43 cm) and Coll. No. 9078 (3.20 cm). The lowest leaf width was recorded in Acc. 899 (2.13 cm). After pooled analysis, the leaf width of genotypes Coll. No. 9078 (3.20 cm), G3 (3.55 cm), and G9 (3.43 cm) significantly surpassed the grand mean of 2.81 cm.

4.1.2.7 Rhizome thickness (cm)

Significant variation in rhizome thickness was observed among different genotypes, ranging from 1.30 cm to 2.35 cm (Table 10). The genotype IISR Varada exhibited the highest rhizome thickness (2.29 cm and 2.35 cm), while the lowest was recorded in Acc. 850 (1.30 cm and 1.43 cm) for the two respective seasons. During 2021-2022, the grand mean was 1.83 cm, and the genotypes Acc. 845 (1.95 cm), Coll. No. 9046 (1.90 cm), G1 (1.89 cm), G3 (2.11 cm), and IISR Varada (2.29 cm) were recorded the rhizome thickness above the grand mean. During the 2022-2023 period, Acc. 845 (1.96 cm), Coll. No. 9046 (1.95 cm), G3 (2.04 cm), and IISR Varada (2.35 cm) surpassed the grand mean. In the pooled analysis, IISR Varada demonstrated the maximum rhizome thickness at 2.32 cm, whereas Acc. 850 exhibited the minimum at 1.36 cm. Following pooled analysis, the rhizome thickness of genotypes Acc. 845 (1.95 cm), G3 (2.07 cm), and IISR Varada (2.32 cm) significantly exceeded the grand mean of 1.83 cm.

Table 10. *Per se* performance for morphological traits in the red ginger genotypes

Genotypes	Rhizome thickness (cm)			Yield per plant (g plant^{-1})			Dry recovery (%)		
	Year 1	Year2	Pooled	Year 1	Year2	pooled	Year 1	Year2	Pooled
Acc. 838	1.87	1.67	1.77	720.00*	530.00	625.00*	21.25	21.52	21.38
Acc. 842	1.71	1.70	1.70	410.00	370.00	390.00	22.11	22.58	22.35
Acc. 844	1.71	1.75	1.73	505.00	470.00	487.50	24.64*	25.94*	25.29*
Acc. 845	1.95*	1.96*	1.95*	390.00	375.00	382.50	23.94*	24.99	24.46*
Acc. 848	1.83	1.78	1.81	570.00	560.00	565.00	22.01	24.64	23.32
Coll. No. 9040	1.84	1.79	1.82	580.00	525.00	552.50	23.49*	23.55	23.52*
Coll. No. 9046	1.90*	1.95*	1.92	425.00	465.00	445.00	22.13	21.49	21.81
Coll. No. 9073	1.87	1.91	1.89	695.00*	920.00*	807.50*	20.43	22.42	21.43
Coll. No. 9076	1.82	1.87	1.84	535.00	605.00	570.00	20.99	23.11	22.05
Coll. No. 9078	1.84	1.92	1.88	440.00	512.50	476.25	22.89	27.94*	25.41*
G1	1.89*	1.87	1.88	630.00*	685.00*	657.50	21.34	21.68	21.51
G3	2.11*	2.04*	2.07*	615.00*	570.00	592.50	24.59*	25.11	24.85*
G6	1.86	1.83	1.84	420.00	385.00	402.50	23.99*	23.97	23.98*
G9	1.81	1.80	1.81	580.00	590.00	585.00	22.44	22.42	22.43
Acc. 850	1.30	1.43	1.36	425.00	255.00	340.00	20.38	21.72	21.05
Acc. 899	1.53	1.59	1.56	307.50	420.00	363.75	21.99	21.91	21.95
IISR Varada	2.29*	2.35*	2.32*	600.00*	605.00	602.50	20.28	20.70	20.49
Grand Mean	1.83	1.83	1.83	520.44	520.15	520.29	22.28	23.27	22.78
CV (%)	1.47	2.87	2.89	7.00	10.96	13.27	3.37	4.89	4.26
CD (5%)	0.06	0.11	0.11	77.19	120.92	146.40	1.59	2.41	2.06

*Indicates values above grand mean+CD (5%)

Fig. 4. Rhizome characters of red ginger genotypes; a) Acc. 838; b) Acc. 842; c) Acc. 844; d) Acc. 845; e) Acc. 848; f) Coll. No. 9040; g) Coll. No. 9046; h) Coll. No. 9073; i) Coll. No. 9076; j) Coll. No. 9078

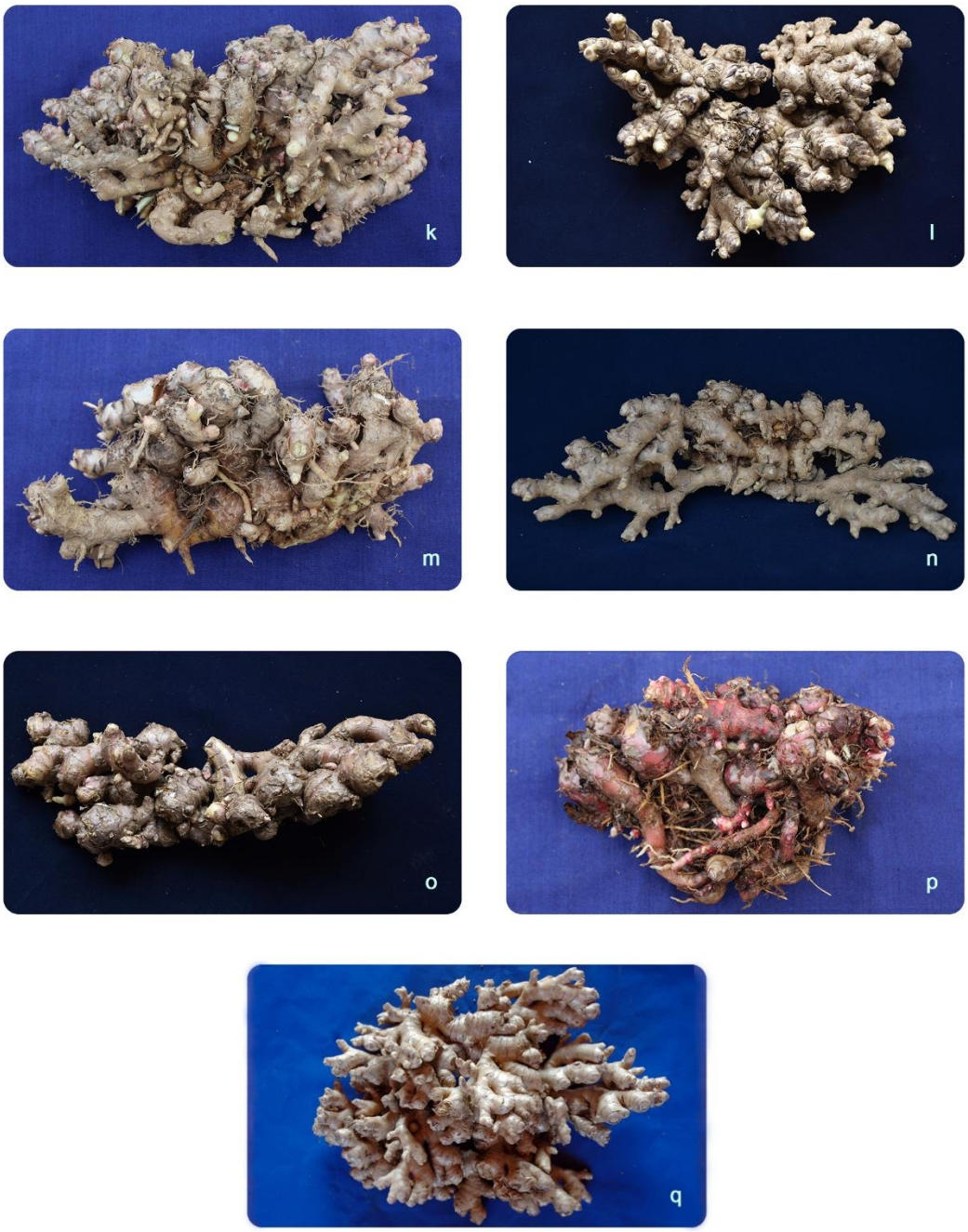

Fig. 5. Rhizomes characters of red ginger genotypes; (k) G1; (l) G3; (m) G6; (n) G9; (o) Acc. 850; (p) Acc. 899; (q) IISR Varada

4.1.2.8 Yield per plant (g)

Yield per plant ranged from 255.00 g to 920.00 g among the genotypes for two seasons (Table 10). Fresh rhizomes of the various red ginger genotypes and IISR Varada are depicted in the Fig. 4 and 5. The mean yield per plant for the year 2021-2022 showed significant variation among the genotypes, of which a significantly higher yield per plant was exhibited by Acc. 838 (720.00 g), followed by Coll. No. 9073 (695.00 g). The lowest yield per plant was recorded in Acc. 899 (307.50 g). The yield per plant of genotypes Acc. 838 (720.00 g), Coll. No. 9073 (695.00 g), G1 (630.00g), G3 (615.00 g), and IISR Varada (600.00 g) in the first season significantly surpassed the grand mean of 520.44 g. For the year 2022-2023, the maximum yield of 920.00 g was observed in Coll. No. 9073, and the minimum yield of 255.00 g was recorded in Acc. 850. The grand mean recorded for the year was 520.15 g, with genotypes Coll. No. 9073 (920.00 g) and G1 (685.00 g) significantly exceeding it. From the pooled analysis, maximum yield per plant was recorded in Coll. No. 9073 (807.50 g) followed by Acc. 838 (625.00 g) and minimum in Acc. 850 (340.00 g) followed by Acc. 899 (363.75 g). The genotypes Coll. No. 9073 surpassed the grand mean of 520.29 g.

4.1.2.9 Dry recovery (%)

A significant variation was observed for dry recovery percentage during 2021-2023 (Fig. 6), which ranged from 20.28 % - 27.94 %. G3 (24.59 %) produced the highest dry recovery percentage in the first season and was on par with Acc. 844 (24.64 %), Acc. 845 (23.94 %), Coll. No. 9040 (23.49 %), and G6 (23.99 %) and surpassed the grand mean was of 22.28 %. In the second season, Coll. No. 9078 (27.94 %) followed by Acc. 844 (25.94 %) recorded significantly high dry recovery and surpassed the grand mean of 23.27 %. The lowest dry recovery percentage was observed in IISR Varada (20.70 %). Dry recovery percentage after pooled analysis ranged from 20.49 % to 25.41%. Significantly high dry recovery was noticed in Coll. No. 9078 (25.41 %), Acc. 844 (25.29 %), G3 (24.85 %), Acc. 845 (24.46 %), G6 (23.98 %), and Coll. No. 9040 (23.52 %). Overall, lowest dry recovery was recorded in IISR Varada (20.49 %). Following pooled analysis, genotypes Acc. 844 (25.29 %), Coll. No. 9078 (25.41 %), and G3 (24.85 %) significantly deviated from the grand mean of 22.78%.

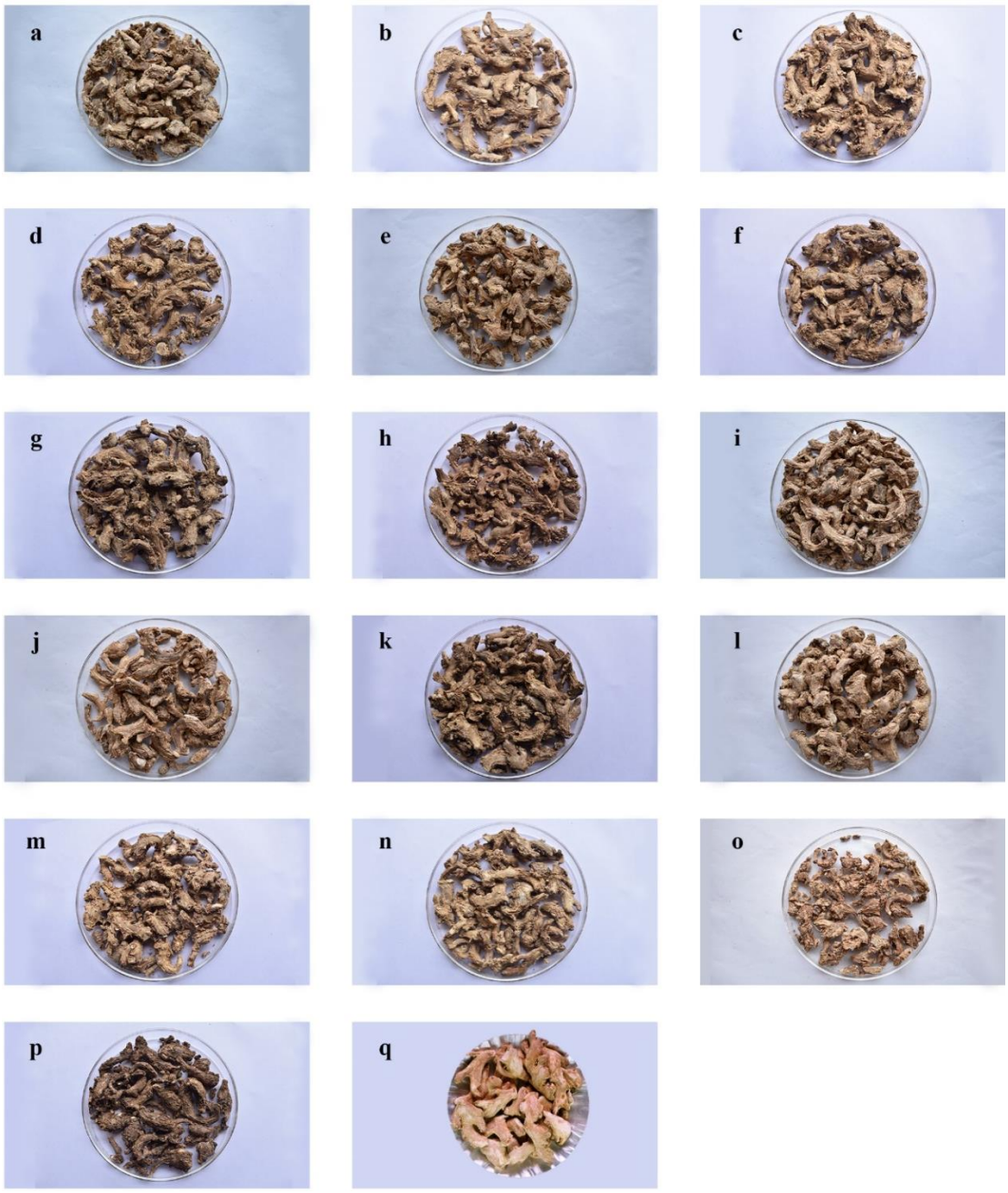

Fig. 6. Dried rhizomes of red ginger genotypes; (a) Acc. 838; (b) Acc. 842; (c) Acc. 844; (d) Acc. 845; (e) Acc. 848; (f) Coll. No. 9040; (g) Coll. No. 9046; (h) Coll. No. 9073; (i) Coll. No. 9076; (j) Coll. No. 9078; (k) G1; (l) G3; (m) G6; (n) G9; (o) Acc. 850; (p) Acc. 899; (q) IISR Varada

In this research, a total of 16 red ginger genotypes and IISR Varada (released variety) were examined to assess six morphological characteristics. Analysis of variance revealed a statistically significant difference in the six quantitative morphological characteristics, including plant height, number of shoots, shoot diameter, number of leaves, leaf length, and leaf width among the genotypes. Pooled data analysis showed the maximum plant height (74.75 cm), and number of shoots (24.75) in Coll. No. 9073, among the 21 genotypes. IISR Varada demonstrated superiority in shoot diameter (1.22 cm), and leaf length (28.83 cm). The maximum number of leaves (25.13) and leaf width (3.55 cm) was recorded in Coll. No. 9040 and G3, respectively. These studies unveiled the existing genetic diversity within red ginger genotypes, thereby furnishing valuable insights into the agricultural and economic traits of the plant. These variances are attributed to the genotype and adaptability of these genotypes across diverse agroclimatic conditions. The presence of genetic variability within the targeted population is crucial for the success of any breeding program (Salgotra & Chauhan, 2023). Sasikumar *et al*. (1992) and Ravindran *et al*. (1994) reported a similar finding in India, while more recent research by Windarsih *et al*. (2021) in Indonesia also reported variability in vegetative and generative characteristics. Similar to our findings, Kumar *et al*. (2016) identified considerable variability in qualitative and quantitative characters among the 82 ginger accessions collected from the different North-eastern states of India. Significant variability was observed in all the studied growth parameters of ginger by Ravi *et al*. (2016). Conversely, grouping of genotypes revealed limited variability across most morphological characteristics in ginger (Akshitha *et al*., 2019). Umar *et al*. (2017), Islam *et al*. (2017), and Basak *et al*. (2019) also reported similar findings consistent with the present study.

ANOVA result revealed a statistically significant difference in the six quantitative morphological characteristics, including rhizome and yield characters. Pooled mean data analysis showed the maximum yield per plant (807.50 g) in Coll. No. 9073 and rhizome thickness (2.32 cm) in IISR Varada. The highest dry recovery percentage was observed in Coll. No. 9078 (25.41 %). The findings are consistent with the results reported by Martini and Paramita (2021) and Das *et al*. (2022)

across various genotypes. Rana *et al*. (2019) identified substantial variability in both yield and yield-contributing factors among 22 germplasm lines gathered from key ginger-growing regions in Himachal Pradesh and Manipur. While under Mizoram condition, Bold Nadia, Gorubathani, and PGS 102 showed significantly similar fresh weight of clump (Soni *et al*., 2022). Ravishanker *et al.* (2013), Rajyalakshmi and Umajyothi (2014), Blanco and Pinheiro, (2017), Chakraborty *et al*. (2018), and Dev (2022) also documented variability in growth and yield parameters. Variations in yield could be attributed to the genotypic composition, growth characteristics, and yield attributes, along with factors such as weather conditions, soil composition, and other management practices. Based on the present study, the genotypes such as Coll. No. 9073 and Acc. 899 with good rhizome characters specifically, yield per plant were shortlisted for further crop improvement studies.

4.1.3 Quality traits

The chemo profiling of 16 red ginger genotypes and IISR Varada included major quality traits such as essential oil, oleoresin, and crude fibre contents, as outlined in Table 11.

4.1.3.1 Essential oil (%)

Essential oil percentages among the genotypes varied from 1.50 % to 5.10 %. During the 2021-2022 season, the highest essential oil percentage was recorded in G9 (5.10 %) and was on par with Coll. No. 9076 (4.35 %) and Acc. 844 (4.12 %). The grand mean recorded for the year was 3.52 %, with genotypes Acc. 842 (4.03 %), Acc. 844 (4.12 %), Coll. No. 9040 (4.02 %), Coll. No. 9073 (3.75 %), Coll. No. 9076 (4.35 %), Coll. No. 9078 (3.98 %), G6 (4.05 %), G9 (5.10%), Acc. 850 (3.92 %), and Acc. 899 (3.84 %) significantly surpassed it. For the year 2022-2023, Coll. No. 9040 (4.01 %) recorded the maximum essential oil percentage surpassing all other genotypes and was statistically on par with Acc. 850 (3.87 %). The lowest essential oil percentage for both seasons was recorded in IISR Varada (1.50%, 1.53%). During this period, with a grand mean of 3.13 %, the essential oil percentage of genotypes Acc. 838 (3.60 %), Acc. 842 (3.50 %), Coll. No. 9040 (4.01 %), Coll. No. 9073 (3.50 %), G9 (3.50 %), and Acc. 850 (3.87 %) exceeded the

grand mean. After pooled analysis, the essential oil percentage was recorded maximum at 4.30 % and minimum at 1.52 % in G9 and IISR Varada, respectively. Significantly high essential oil was recorded in Coll. No. 9040 (4.01 %), Acc. 850 (3.89 %), Coll. No. 9076 (3.83%), Acc. 842 (3.76 %), Acc. 844 (374 %), G6 (3.73 %), Coll. No. 9078 (3.65 %), and Coll. No. 9073 (3.63 %) also. Following pooled analysis, genotypes Coll. No. 9040 (4.01 %), and G9 (4.30 %) significantly deviated from the grand mean of 3.31%.

The essential oil content exhibited a wide range among the genotypes, as illustrated in Table 11. A significant difference in essential oil content was observed between the common ginger IISR Varada and red ginger genotypes. Among the Indian red ginger types, G9 (4.30 %) reported the maximum essential oil content, while among the exotic types, Acc. 850 demonstrated superiority with a value of 3.89%. IISR Varada recorded the least essential oil percentage at 1.52% after pooled analysis. In dry ginger, the essential oil yield typically falls within the range of 1.0% to 3.0% (Govindarajan, 1982). The present study revealed high essential oil content in red ginger genotypes ranging from 2.03% to 4.30% after pooled analysis. Like the current study, Akshitha *et al*. (2020) and Babu *et al*. (2021) reported that exotic ginger genotypes exhibited superior essential oil yields compared to Indian ginger cultivars. Notably, recent studies have reported even higher essential oil content, reaching up to 6.0% in red ginger (Akshitha *et al*., 2020). Previous reports also documented a lower essential oil content in IISR Varada (Akshitha *et al*., 2020; Kizhakkayil & Sasikumar, 2009; Ravi *et al*., 2018; Das *et al*., 2022). Similar findings were reported by Goudar *et al*. (2017), Bhattarai *et al*. (2018), and Anargha *et al*. (2020) in common cultivars of ginger. Hence, red ginger accessions with high essential oil yields, such as G9, Coll. No. 9040, Acc. 850, Coll. No. 9076, Acc. 844, and Acc. 842, hold significant potential for commercial exploitation in essential oil production for industries involved in food, nutraceutical, pharmaceutical, cosmetic, and perfume applications.

4.1.3.2 Oleoresin (%)

Oleoresin percentage varied significantly among the 17 ginger genotypes, ranged from 3.13 % - 11.75 %. It was observed that G9 (11.75 %, 8.93 %) recorded

the maximum oleoresin percentage for two seasons. The grand mean for the first season was 8.34 % and the genotypes Acc. 838 (10.23 %), Acc. 842 (9.08 %), Acc. 844 (9.62 %), Coll. No. 9073 (10.08%), Coll. No. 9076 (10.97 %), Coll. No. 9078 (9.41 %), G1 (9.42 %), G6 (9.00 %) and G9 (11.75 %) recorded significantly above the grand mean. Acc. 845 (4.10 %) recorded the minimum oleoresin percentage for the first season and G3 (3.13 %) for the second season. The grand mean for the second season was 6.75 %, with genotypes Acc. 842 (97.73 %), Coll. No. 9073 (8.48 %), G1 (8.09 %), and G9 (8.93 %) significantly exceeding it. Pooled analysis revealed significantly higher oleoresin percentage in G9 (10.34%) and was on par with Coll. No. 9073 (9.28 %), Coll. No. 9076 (8.70 %), G1 (8.75 %), Acc. 838 (8.59 %), and Acc. 842 (8.41 %) and lowest in Acc. 848 (4.10 %). Following pooled analysis, the grand mean was recorded at 7.43%, with the genotype G9 (10.34%) significantly exceeded it.

The quality of ginger is significantly influenced by the presence and characteristics of oleoresin. In the current study, increased oleoresin content was documented in red ginger genotypes, ranging from 4.10 % (Acc. 848) to 10.34 % (G9). Remarkably, within the category of exotic types, Acc. 899 recorded the highest oleoresin content (7.19%), which was higher than IISR Varada (6.55 %). Aligned with the current study, Akshitha *et al*. (2020) reported the highest oleoresin percentage, approximately 12.02%, in red ginger. Variation in the oleoresin content with respect to genotypes and geographical origin were reported earlier by Anargha *et al*. (2020), Balakumbahan and Joshua (2017), Kallappa *et al*. (2015), Singh *et al*. (2013), and Chongtham *et al*. (2013). These observations align with the findings of the current study. In a study conducted by Goudar *et al*. (2017), the oleoresin content of 12 ginger genotypes was found to range from 3.69 % to 7.35 %. The current study, the observed variability in oleoresin among the genotypes could be attributed to both genetic factors, place of origin and the interaction between genotype and environment. Red ginger genotypes with high oleoresin yield, such as G9, G1, Coll. No. 9073, Coll. No. 9076 and Acc. 838, hold significant potential for commercial exploitation.

4.1.3.3 Crude fibre (%)

Significant variation was observed in crude fibre percentage among the genotypes. It varied from 5.00 % to 11.00 %. IISR Varada recorded the least crude fibre value of 4.79 % and 5.00 for the two seasons, respectively. The red ginger genotype G1 exhibited the highest crude fibre percentage (11.00 % and 9.93 %), while the lowest fibre content in the red ginger genotypes was recorded in Acc. 899 (6.43 % and 5.87 %) for the two respective seasons. The grand mean for the first season was 7.53 % and the genotypes Acc. 838 (9.22 %), and G1 (11.00 %) recorded the fibre percentage above the grand mean. In the second season, genotypes Acc. 838 (7.35 %), Coll. No. 9046 (7.98 %), G1 (9.93 %), and G9 (7.41 %) recording fibre percentages above the grand mean of 6.72 %. After pooled analysis, the lowest crude fibre content was observed in IISR Varada (4.90%), followed by Acc. 848 (5.43 %). Conversely, the significantly highest crude fibre content was recorded in G1 (10.47 %), exceeding the grand mean of 7.13 %.

The pooled analysis has indicated that most of both Indian and exotic red ginger genotypes exhibit high fibre content (above 6.0 %). Significant difference in crude fibre content were recorded for ginger genotypes across diverse agro-climatic regions, as documented by Sanwal *et al*. (2012). In the current study, the crude fibre percentage ranged from 4.90% (IISR Varada) to 10.47% (G1). Likewise, Das *et al*. (2022) and Goudar *et al*. (2017) reported the lowest fibre content in the Varada, 3.34% in Kerala and 5.20% in the north-western region of India. Jyotsna *et al*. (2012) conducted a study in Manipur under rainfed conditions and observed a variation in crude fibre content ranging from 5.17% to 7.68% among the investigated ginger varieties. Consistent with these findings, Akshitha *et al*. (2020) reported a high crude fibre percentage in red ginger (9.51%). These high-fibre types are valuable to produce dry ginger. Kizhakkayil and Sasikumar (2009), Singh *et al*. (2013), Ravi *et al*. (2018), Anargha *et al*. (2020), and Babu *et al*. (2021) have also documented variability in crude fibre percentage based on ginger genotypes.

Table 11. *Per se* performance for quality traits in the red ginger genotypes

Genotypes	Essential oil (%)			Oleoresin (%)			Crude fibre (%)		
	Year 1	Year 2	Pooled	Year 1	Year 2	Pooled	Year 1	Year 2	Pooled
Acc. 838	3.59	3.60*	3.59	10.23*	6.95	8.59*	9.22*	7.35*	8.28
Acc. 842	4.03*	3.50*	3.76*	9.08*	7.73*	8.41*	7.70	6.62	7.16
Acc. 844	4.12*	3.35	3.74*	9.62*	7.03	8.33	8.36	6.22	7.29
Acc. 845	2.68	2.65	2.67	4.10	4.61	4.35	7.00	6.48	6.74
Acc. 848	2.04	2.02	2.03	4.55	3.66	4.10	5.49	5.36	5.43
Coll. No. 9040	4.02*	4.01*	4.01*	8.59	7.55	8.07	6.91	6.18	6.54
Coll. No. 9046	3.03	2.84	2.93	7.33	6.62	6.97	8.40	7.98*	8.19
Coll. No. 9073	3.75*	3.50*	3.63*	10.08*	8.48*	9.28*	7.82	6.80	7.31
Coll. No. 9076	4.35*	3.32	3.83*	10.97*	6.43	8.70*	8.33	6.99	7.66
Coll. No. 9078	3.98*	3.32	3.65*	9.41*	5.82	7.62	7.65	6.15	6.90
G1	3.62	3.02	3.32	9.42*	8.09*	8.75*	11.00*	9.93*	10.47*
G3	2.69	1.65	2.17	5.49	3.13	4.31	6.80	6.74	6.77
G6	4.05*	3.42	3.73*	9.00*	7.41	8.20	6.15	7.08	6.62
G9	5.10*	3.50*	4.30*	11.75*	8.93*	10.34*	8.22	7.41*	7.81
Acc. 850	3.92*	3.87*	3.89*	7.92	5.13	6.52	7.82	6.17	6.99
Acc. 899	3.84*	3.35	3.59	8.57	5.82	7.19	6.43	5.87	6.15
IISR Varada	1.50	1.53	1.52	6.50	6.60	6.55	4.79	5.00	4.90
Grand Mean	3.52	3.13	3.31	8.34	6.75	7.43	7.53	6.72	7.13
CV (%)	2.69	4.49	9.96	3.64	6.69	12.71	7.09	4.17	7.83
CD (5%)	0.20	0.30	0.70	0.64	0.96	2.00	1.13	0.60	1.18

*Indicates values above grand mean+CD (5%)

4.1.4 Estimation of non-volatile fractions in the red ginger genotypes

Non-volatile compounds in the dried rhizomes of red ginger genotypes were estimated with High Performance Liquid Chromatography (HPLC). The major non-volatile compounds identified in the dried rhizome were 6-gingerol, 8-gingerol, and 6-shogaol. Notably, 6-gingerol emerged as the prominent pungent principle across the genotypes. The percentage of 6-gingerol, 8-gingerol, and 6-shogaol in the red ginger genotypes were estimated using the authentic standards and the respective HPLC chromatograms are represented in the Fig. 7. Analysis of variance revealed significant variation in the gingerols and shogaol percentage among the studied genotypes (Table 12). The representative chromatograms are depicted in the Fig. 8. The percentage of gingerols and shogaol is presented in the Table 13.

Table 12. Analysis of variance for gingerols and shogaol content in red ginger genotypes

Compounds	Replication	Genotypes	Error
Degrees of freedom	1	16	16
6-Gingerol	0.00	0.26***	0.00
8-Gingerol	0.00	0.003***	0.00
6-Shogaol	0.00	0.003***	0.00

***Significant at 0.1%

4.1.4.1 6-Gingerol (%)

Highly significant variation was observed in the 6-gingerol percentage between the genotypes (Table 13). Highest mean 6-gingerol percentage was recorded in the Coll. No. 9073 (1.33 %), followed by Acc. 844 (1.23 %), Acc. 842 (1.19 %) and the lowest percentage was recorded in G3 with a value of 0.26 %. The grand mean was 0.79 % and the genotypes Acc. 842 (1.19 %), Acc. 844 (1.23 %), Coll. No. 9040 (1.16 %), Coll. No. 9046 (0.82 %), Coll. No. 9073 (1.33 %), G1 (1.08 %), G6 (0.96 %) and G9 (1.15 %) recorded significantly above the grand mean.

4.1.4.2 8-Gingerol (%)

Significant difference was observed in the 8-gingerol percentage among the red ginger genotypes. Maximum 8-gingerol content was observed in Acc. 844 (0.17 %), followed by G1 (0.15 %). Among the 17 genotypes, G3 (0.04 %) exhibited the lowest 8-gingerol percentage. The genotypes Acc. 842 (0.13 %), Acc. 844 (0.17 %), Coll. No. 9040 (0.14 %), Coll. No. 9046 (0.10 %), Coll. No. 9073 (0.14 %), G1 (0.15 %), and G9 (0.13 %) significantly exceeding the grand mean of 0.09 %.

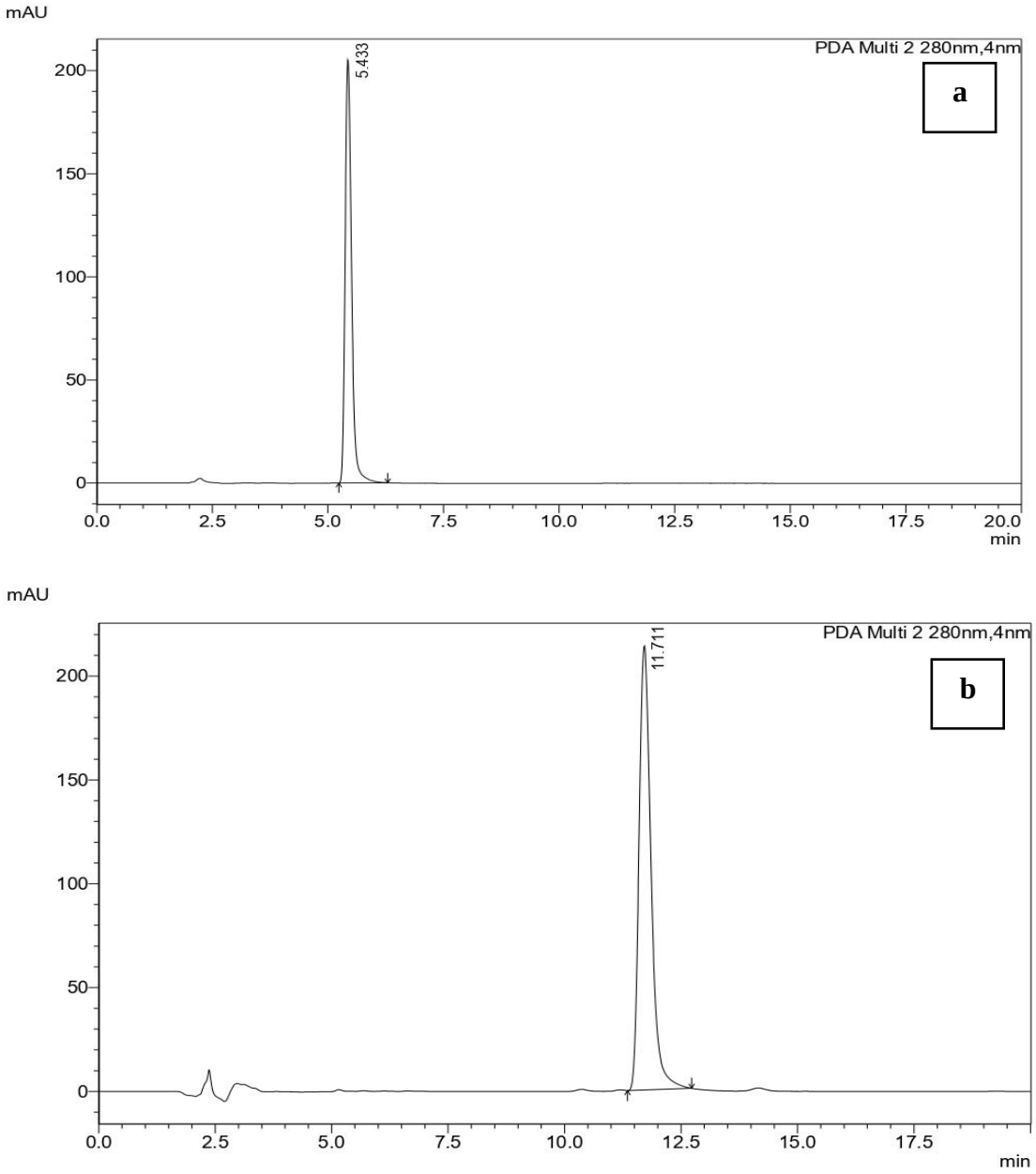

Fig. 7. HPLC chromatogram of standards: (a) 6-Gingerol; (b) 6-Shogaol

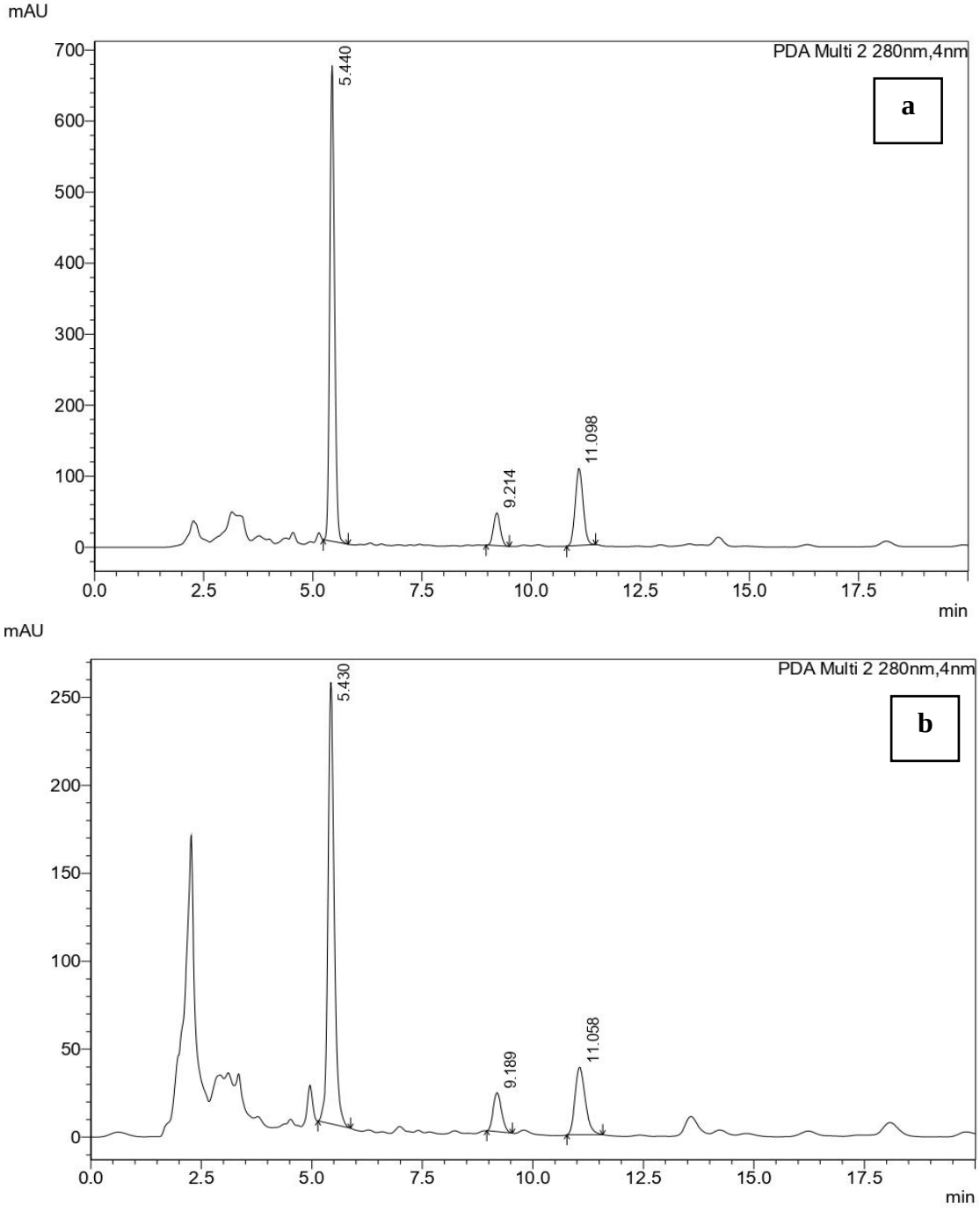

Fig. 8. HPLC chromatogram of representative genotypes. (a) Acc. 899 (exotic red ginger); (b) Coll. No. 9073 (Indian red ginger)

Table 13. *Per se* performance for gingerols and shogaol contents in the red ginger genotypes

Genotypes	6-Gingerol (%)	8-Gingerol (%)	6-Shogaol (%)
Acc. 838	0.79	0.07	0.17*
Acc. 842	1.19*	0.13*	0.13
Acc. 844	1.23*	0.17*	0.16*
Acc. 845	0.35	0.06	0.09
Acc. 848	0.37	0.04	0.09
Coll. No. 9040	1.16*	0.14*	0.14*
Coll. No. 9046	0.82*	0.10*	0.12
Coll. No. 9073	1.33*	0.14*	0.17*
Coll. No. 9076	0.72	0.06	0.17*
Coll. No. 9078	0.57	0.05	0.13
G1	1.08*	0.15*	0.13
G3	0.26	0.04	0.06
G6	0.96*	0.09	0.11
G9	1.15*	0.13*	0.18*
Acc. 850	0.46	0.06	0.09
Acc. 899	0.65	0.09	0.08
IISR Varada	0.35	0.09	0.17*
Mean	0.79	0.09	0.13
CV (%)	1.59	6.08	3.06
CD (5%)	0.03	0.01	0.01

*Indicated values above grand mean+CD (5%)

4.1.4.3 6-Shogaol (%)

The 6-shogaol percentage varied among the genotypes from 0.06 % to 0.18 %. G9 exhibited the highest 6-shogaol percentage with a value of 0.18 % and was statistically on par with Acc. 838 (0.17 %), Coll. No. 9073 (0.17 %), Coll. No. 9076 (0.17 %), and IISR Varada (0.17 %). The lowest 6-shogaol content was observed in G3 (0.06 %). The grand mean was 0.13%, with genotypes Acc. 838 (0.17%), Acc. 844 (0.16%), Coll. No. 9040 (0.14%), Coll. No. 9073 (0.17%), Coll. No. 9076 (0.17%), G9 (0.18%), and IISR Varada (0.17%) significantly surpassed it.

Gingerols and shogaols, the major phenolic compounds present in ginger, are responsible for the pungency and spicy taste (Zhang *et al*., 2022). The present study identified 6-gingerol as the most abundant among the gingerol derivatives. Previous reports supported the findings (Masuda *et al*., 2004; Murthy *et al*., 2015). Among the Indian red ginger types, Coll. No. 9073 reported the highest percentage of 6-gingerol at 1.33%, while the exotic type Acc. 899 recorded the highest value of 0.65%. The released variety IISR Varada (0.35 %) was found to have lower gingerol content compared to red ginger genotypes. The present study revealed that Indian red ginger genotypes have superior gingerols and shogaols content compared to exotic types. Those superior genotypes such as, Coll. No. 9073, Acc. 844, Acc. 842, Acc. 838 and G9 can be exploited for the industrial purposes. According to Kizhakkayil and Sasikumar (2012), 6-gingerol percentage in fresh rhizome ranged from 0.49% to 3.11%, 6-shogaol percentage varied from 0.05% to 0.41%, followed by 8-gingerol percentage (0.02% - 0.08%) in various ginger genotypes. Like present study, Soni *et al*. (2023) reported an average 6-gingerol content of 1.94 % among the seven ginger genotypes. Similarly, Hamid *et al*. (2023) also estimated the content of 6-gingerol as 1.012% and 6-shogaol as 0.0005%. Pawar *et al*. (2011) reported that common ginger has a gingerol content ranging from 0.1% to 0.2%. The current study, employing HPLC, observed minimal levels of shogaol (0.06-0.18 %) in all ginger accessions, consistent with the findings reported by Jolad *et al*. (2005). Only a few studies have reported on the gingerol and shogaol content in red ginger (Kusumawati *et al*., 2017; Nishidono *et al*., 2018). Ghasemzadeh *et al*. (2015) optimized the protocol for the extraction of 6-gingerol and 6-shogaol from Malaysian red ginger, recording 2.92 mg g^{-1} and 1.88 mg g^{-1}, respectively. The extraction of gingerols and shogaols in various ginger genotypes were attempted by several researchers (Hawlader *et al*., 2006; Li *et al*, 2008; Pawar *et al*., 2015; Azizah *et al*., 2019). The variation in gingerols and shogaol content among various ginger genotypes can be attributed to factors such as genotype, environmental conditions, harvesting time, drying methods, and extraction procedures etc (Jayashree *et al*., 2014; Ghasemzadeh *et al*., 2016; Vedashree *et al*., 2020).

4.1.5 Characterization of genotypes based on qualitative morphological characters

The 16 red ginger genotypes and IISR Varada were characterized for different qualitative morphological characters based on the DUS parameters. The characters including plant growth habit, intensity of green colour of shoot and leaf and rhizome shape are listed in the form of multiscale scores given in the DUS guidelines Table 14.

4.1.5.1 Plant: Growth habit

Among the 16 genotypes, the exotic red ginger genotypes, i.e.., Acc. 850 and Acc. 899, were observed to have an erect growth habit. The semi-erect growth habit was seen in all other genotypes.

4.1.5.2 Shoot: Intensity of green colour

Acc. 848, G6, Acc. 850 and Acc. 899 were identified with green coloured shoot with a colour code of G137C. All the other red ginger genotypes and IISR Varada belonged to the dark green category (G137A and G137B).

4.1.5.3 Leaf: Intensity of green colour

IISR Varada was categorized under green group for leaf colour (G137C). All the red ginger genotypes were grouped under dark green (G137A, GN137A, GN137B).

4.1.5.4 Rhizome: Shape

Out of the 16 red genotypes, 11 genotypes were noticed with straight rhizomes. Curved rhizomes were observed in Acc. 838, Acc. 850, Acc. 899, G3 and G6. IISR Varada showed straight rhizome.

The findings indicated distinct variations among the exotic and Indian red ginger genotypes. Exotic red ginger genotypes Acc. 850 and Acc. 899 exhibit an erect growth habit, with green-coloured shoots and dark green leaves, along with curved rhizomes. In contrast, most Indian types and IISR Varada displayed a semi-erect growth habit, with dark green shoots and green leaves, as well as straight rhizomes. Notably, IISR Varada produced straight rhizomes, while Acc. 838, G3, and G6 produced curved rhizomes.

Similarly, Akshitha *et al*. (2019) conducted the grouping of 27 ginger genotypes based on DUS descriptors and observed variations in the morphological characters.

Table 14. Grouping of red ginger genotypes based on DUS descriptor

Characteristics	State	Score	Genotypes
Plant: Growth habit	Erect	1	Acc. 850, Acc. 899
	Semi-erect	3	Acc.838, Acc. 842, Acc. 844, Acc. 845, Acc. 848, Coll. No. 9040, Coll. No. 9046, Coll. No. 9073 Coll. No. 9076, Coll. No. 9078, G1, G3, G6, G9, IISR Varada
	Spreading	5	Nil
Shoot: Intensity of green colour	Light green	1	Nil
	Green	3	Acc. 848, G6, Acc. 850, Acc. 899
	Dark green	5	Acc.838, Acc. 842, Acc. 844, Acc. 845, Coll. No. 9040, Coll. No. 9046, Coll. No. 9073, Coll. No. 9076, Coll. No. 9078, G1, G3, G9, IISR Varada
Leaf: Intensity of green colour	Light green	1	Nil
	Green	3	IISR Varada
	Dark green	5	Acc. 838, Acc. 842, Acc. 844, Acc. 845, Acc. 848, Coll. No. 9040, Coll. No. 9046, Coll. No. 9073, Coll. No. 9076, Coll. No. 9078, G1, G3, G6, G9, Acc. 850, Acc. 899
Rhizome: Shape	Straight	1	Acc. 842, Acc. 844, Acc. 845, Acc. 848, Coll. No. 9040, Coll. No. 9046, Coll. No. 9073, Coll. No. 9076, Coll. No. 9078, G1, G9, IISR Varada
	Curved	3	Acc. 838, G3, G6, Acc. 850, Acc. 899
	Zigzagged	5	Nil

4.1.6 Variability parameters for different quantitative traits

4.1.6.1 Genotypic coefficient of variance (GCV) and phenotypic coefficient of variance (PCV)

Genetic variability study among the 16 red ginger genotypes and one ginger genotype (IISR Varada) for 12 traits was done based the pooled data (Table 15). High GCV coupled with high PCV was observed in number of shoots (22.45 %, 25.44 %), yield per plant (22.09 %, 25.77 %), essential oil (22.48 %, 24.59 %), oleoresin (22.43 %, 25.78 %), 6-gingerol (42.93 %, 42.95 %), 6-shogaol (29.05 %, 29.11%), respectively.

Moderate GCV coupled with moderate PCV was recorded in number of leaves on main shoot (13.42 %, 16.04 %), leaf length (13.21 %, 14.34 %), leaf width (12.46 %, 13.82 %), rhizome thickness (10.88 %, 11.26 %) and crude fibre (16.34 %, 18.12 %). Low GCV combined with moderate PCV was observed in plant height (9.46 %, 11.35 %) and shoot diameter (8.96 %, 10.51 %). Low GCV combined with low PCV was recorded in dry recovery (6.13 %, 7.46 %).

The examination of variability parameters disclosed high genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) for number of shoots, yield per plant, essential oil, oleoresin, gingerols, and shogaol percentage. In the present study, magnitude of GCV estimates ranged from 6.13 % to 42.93 %, while the magnitude of PCV ranged from 7.46 % to 42.95 %. The estimates of PCV were slightly higher compared to GCV, implies slight influence of environment on the studied characters. Likewise, Aragaw *et al*. (2011), Ravishanker *et al*. (2013), Karthik *et al*. (2017) and Zambrano and Baldin (2017) reported minimal environmental impact on the phenotypic expression of various traits in ginger. The high GCV and PCV estimates suggest considerable variability, providing significant opportunities for enhancing these traits through selection. High GCV and PCV was reported for yield per plant by Rajyalakshmi and Umajyothi (2014), Bategari (2016), Karthik *et al*. (2017), Rana *et al*. (2019). Similarly, Aragaw *et al*. (2011), and Basak *et al*. (2019) reported high GCV and PCV for number of shoots in ginger genotypes. Conversely, Umar *et al*. (2017) recorded moderate GCV

and PCV for number of shoots. Like the present study, moderate GCV and PCV was observed for leaf length, leaf width, and fibre content by Aragaw *et al*. (2011). Ravi *et al*. (2016) reported high GCV and PCV for oleoresin and essential oil content. The findings pertaining to oleoresin have been documented in prior research by Anargha *et al* (2020). Soni *et al*. (2023) reported low GCV along with low PCV for plant height and gingerol percentage, while moderate GCV coupled with moderate PCV was observed for dry recovery (%). The dry recovery exhibited low GCV and low PCV, indicating less variability for the trait and selection will not be effective. Ravi *et al*. (2016) reported low GCV and moderate PCV for dry recovery percentage. The presence of moderate to low variability implies a requirement for improving the base population.

4.1.6.2 Heritability and genetic advance as per cent mean (GAM)

Heritability and genetic advance as per cent mean is given in the Table 15. High heritability combined with high GAM was recorded in number of shoots (77.88 %, 40.81 %), number of leaves on main shoot (69.99 %, 23.13 %), leaf length (84.81 %, 25.05 %), leaf width (81.37 %, 23.16 %), rhizome thickness (93.40 %, 21.66 %), yield per plant (73.47 %, 39.00 %), essential oil (83.59 %, 42.35 %), oleoresin (75.69 %, 40.20 %), crude fibre (81.33 %, 30.36 %), gingerols (99.88 %, 88.38 %), and 6-shogaol (99.61 %, 59.72 %). High heritability coupled with moderate GAM was recorded in plant height (69.40 %, 16.23 %), shoot diameter (72.67 %, 15.74 %) and dry recovery (67.47 %, 10.37 %).

The estimates of GCV and PCV alone are insufficient to unveil the extent of influence from genetic and environmental factors on overall phenotypic variability. Therefore, it is crucial to consider heritability as a measure of inheritance of the traits. In addition to heritability, genetic advance proves more valuable in envisaging the potential gains under selection. In the current investigation, among the 14 traits assessed, there was notable occurrence of high heritability combined with high GAM for number of shoots, number of leaves on the main shoot, leaf length, leaf width, rhizome thickness, yield per plant, essential oil content, oleoresin content, crude fibre content, as well as percentages of gingerols and shogaols. Traits with

high heritability indicated a reduced influence of environmental factors on their phenotypic expression, owing to additive gene effects. Therefore, simple selection methods are effective for improving these specific traits, as these traits were under genetic control. Whereas high magnitude of heritability combined with moderate GAM was recorded for plant height, shoot diameter and dry recovery. Hence, these traits can be subject to effective selection, as observed in studies by Mohanty and Sharma (1979), Rao *et al*. (2004), Islam *et al*. (2008) and Blanco and Pinheiro (2017).

Table 15. Variability parameters for different quantitative characters in red ginger

Characters	Genotypic Coefficient of Variance (%)	Phenotypic Coefficient of Variance (%)	Heritability in Broad Sense (%)	Genetic Advance as per cent Mean (%)
Plant height	9.46	11.35	69.40	16.23
Number of shoots	22.45	25.44	77.88	40.81
Shoot diameter	8.96	10.51	72.67	15.74
Number of leaves on main shoot	13.42	16.04	69.99	23.13
Leaf length	13.21	14.34	84.81	25.05
leaf width	12.46	13.82	81.37	23.16
Rhizome thickness	10.88	11.26	93.40	21.66
Yield per plant	22.09	25.77	73.47	39.00
Dry recovery	6.13	7.46	67.47	10.37
Essential oil	22.48	24.59	83.59	42.35
Oleoresin	22.43	25.78	75.69	40.20
Crude fibre	16.34	18.12	81.33	30.36
Gingerols	42.93	42.95	99.88	88.38
6-Shogaol	29.05	29.11	99.61	59.72

Similarly, Soni *et al*. (2023) noted that traits like the number of leaves per plant, weight of primary rhizome fingers, and yield per plant displayed both high heritability and significant genetic advance. The traits demonstrating high

variability, heritability, and moderate to high GAM are valuable for plant breeders, facilitating direct selection and subsequent improvement.

4.1.7 Characterization of essential oil based on GC-MS profile

The essential oil extracted through hydro-distillation using Clevenger apparatus was further characterized using GC-MS (Shimadzu QP-2010). A total of 53 compounds were initially identified, and from this, 29 compounds-primarily major and highly variable-were specifically chosen for subsequent statistical analysis. The percentage of essential oil constituents is enlisted in the Table 16-17. The representative GC-MS chromatograms are depicted in the Fig. 9. The relative abundance of compounds among 17 genotypes, accompanied by hierarchical cluster analysis, is portrayed as a heat map (Fig.10). This representation facilitates the easy visualization of variations in the compounds. Zingiberene, β-sesquiphellandrene, Ar-curcumene, β-bisabolene, and α-farnesene were the main chemical components found in ginger oil. The analysis of variance indicated significant variance in the essential oil constituents among the various genotypes (Fig. 11).

Zingiberene was the major compound in the essential oil that had the highest peak area across the ginger genotypes. The Acc. 845 had the highest proportion of zingiberene (30.46 %), followed by Coll. No. 9046 (28.82 %). Zingiberene content in the red ginger genotypes varied from 20.74 % (Acc. 899) to 30.46 % (Acc. 845).

The second major compound was β-sesquiphellandrene. The content ranged from 11.40 % (Coll. No. 9078) to 14.60% (Coll. No. 9046). The amount of Ar-curcumene in each genotype varied significantly, with G9 having the greatest amount (12.70 %), followed by G6 and Coll. No. 9073 (12.51 % and 12.28 %, respectively). IISR Varada recorded the lowest level of Ar-curcumene, with 6.63 %.

The concentration of α-farnesene, one of the various sesquiterpene hydrocarbons, varied extremely between genotypes, ranging from 3.87 % (G6) to 11.86 % (G3), with most of them displaying 4-6 %. β - bisabolene, whose concentrations ranged from 3.72 % (IISR Varada) to 6.83 % (Acc. 845).

Out of the various monoterpene hydrocarbons reported, camphene content showed a variation from 1.30 % (G3) to 5.39 % (Acc. 899). The lowest (0.34 %) and highest (1.82 %) α-pinene content was reported in Acc. G3 and Coll. No. 9078 respectively. Similarly, β-pinene was seen the range of 0.1-2 % in the selected genotypes. Whereas β-phellandrene was recorded in a range of 1.58 % (Acc. 845) to 4.03 % (G9). β-Phellandrene content was not detected in Acc. G3. The highest of β-Myrcene content was reported in Acc. 899 (1.08 %) and Acc. 850 (1.06 %), whereas the lowest concentration was noticed in genotype G3 (0.38 %).

Geranial varied from 0.84 % (IISR Varada) to 5.93 % (Acc. 899). The lowest content of neral was identified in IISR Varada (0.99 %), whereas the highest neral content was reported in Acc. 899 (3.85 %). Mean percentage of linalool varied from 0.29 % (Acc. 845) to 3.16 % (Coll. No. 9073) in the genotypes. Endo-borneol was also recorded in a range of 0.53 % (G3) to 2.09 % (Acc. 899). 1,8 - cineole varied significantly in the selected genotypes from very trace amount (0.03 %) to 4.81 % (IISR Varada). γ-cadinene and β-eudesmol was noticed in range of 0.46-3.62 % and 0.44-1.83 % respectively. Zingiberenol was identified in all genotypes at approximately 1%. γ-muurolene was present only in exotic red ginger genotypes i.e., Acc. 850 and Acc. 899. An average peak area of 0.13 % (Acc. 848) to 1.15 % (Coll. No. 9078) was observed for β-ocimene, in the studied genotypes. Citronellol was present only in a few genotypes: Acc. 845, Acc. 848, G1, G3, Acc. 850, Acc. 899, and IISR Varada, ranging from 0.03% to 0.47%. Bornyl acetate was observed in a range of 0.15% to 0.55% but was absent in G3. Geranyl acetate varied from 0.01% (Acc. 848) to 0.74% (Acc. 899).

D-limonene was exclusively present in the red ginger genotype G3, which was found to be absent in rest of the 16 genotypes. All the other reported compounds were observed at extremely low concentration i.e., <1% in the ginger genotypes.

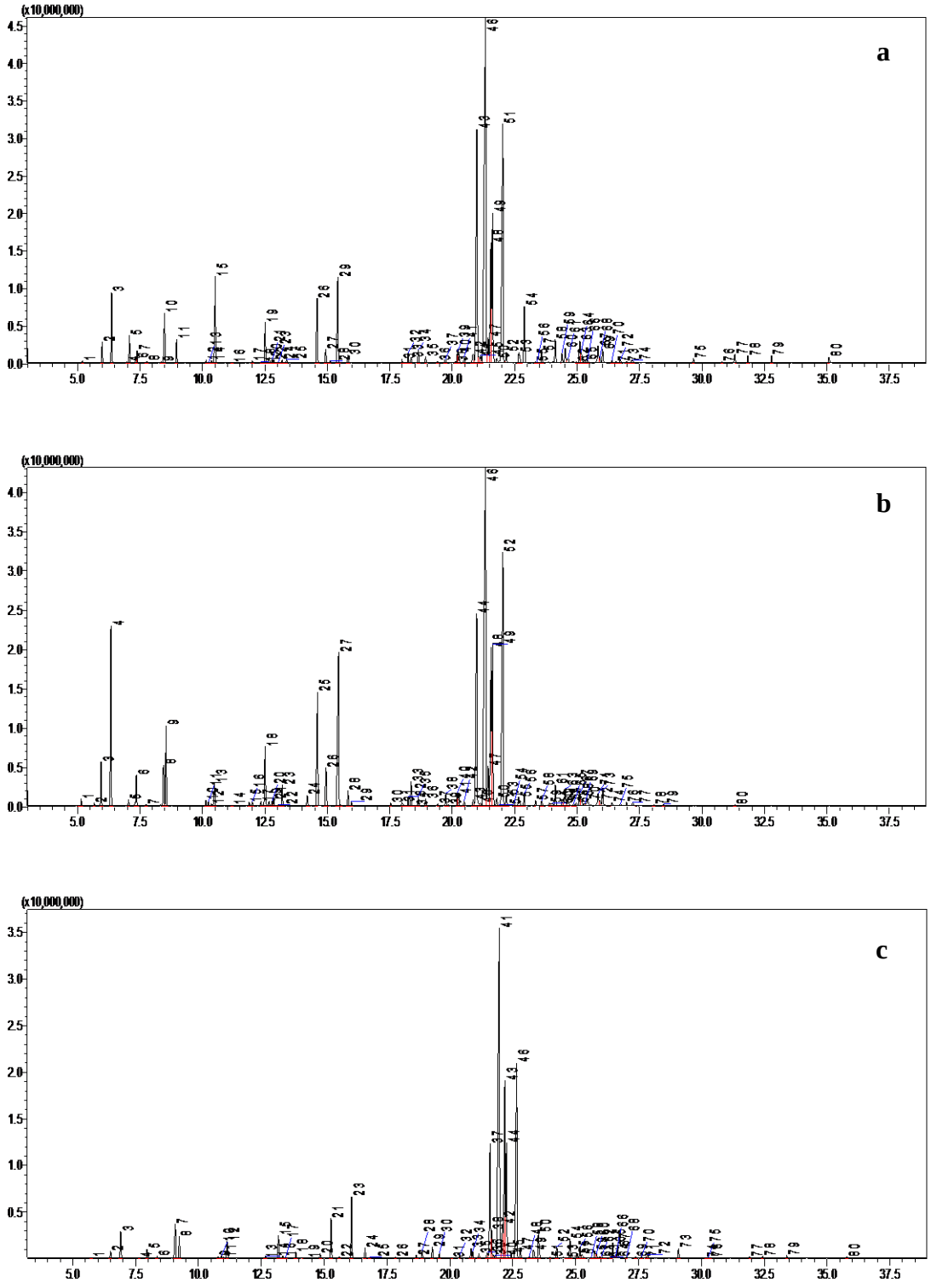

Fig. 9. GC-MS chromatogram of representative genotypes indicating variation in the essential oil constituents; (a) Coll. No. 9073; (b) Acc. 899; (c) IISR Varada

Table 16. Variation in the major essential oil constituents of 16 red ginger genotypes and IISR Varada

Compounds	Kovats index	Acc. 838	Acc. 842	Acc. 844	Acc. 845	Acc. 848	Coll. No. 9040	Coll. No. 9046	Coll. No. 9073
α-Pinene	939	1.16	0.92	1.40	0.63	1.19	1.20	0.65	1.04
Camphene	954	3.57	2.77	3.93	2.08	4.03	3.48	2.00	3.15
β-Pinene	979	0.72	1.17	1.45	0.16	0.26	1.59	1.16	1.12
β-Myrcene	990	0.70	0.49	0.84	0.42	0.71	0.71	0.46	0.59
D-Limonene	1029	-	-	-	-	-	-	-	-
β-Phellandrene	1029	3.37	1.86	3.59	1.58	2.33	3.21	1.97	2.75
1,8 Cineole	1031	-	-	-	1.57	2.77	-	-	-
β-Ocimene	1050	1.06	0.34	0.99	-	0.13	0.91	0.61	0.90
Linalool	1096	1.97	1.93	2.39	0.29	0.64	2.11	1.87	3.16
endo-Borneol	1169	1.66	1.40	1.62	1.24	1.48	1.54	1.29	1.59
Myrtenal	1195	0.26	0.23	0.22	0.08	0.11	0.22	0.18	0.21
Citronellol	1225	-	-	-	0.15	0.15	-	-	-
Neral	1238	2.49	1.78	2.84	2.06	2.41	2.22	1.55	2.95
cis-Geraniol	1252	0.57	0.69	0.86	0.64	-	0.92	0.59	0.59
Geranial	1267	3.65	2.69	4.54	2.96	3.37	3.62	2.31	4.36
Bornyl acetate	1288	0.27	0.29	0.29	0.15	0.12	0.27	0.23	0.27
Geranyl acetate	1371	0.13	0.33	0.42	0.11	0.01	0.28	0.17	0.16
trans-α-Bergamotene	1434	0.24	0.26	0.20	0.17	0.18	0.19	0.21	0.24
γ-Muurolene	1479	-	-	-	-	-	-	-	-
Ar-Curcumene	1480	10.18	11.75	10.81	7.30	7.58	9.71	10.96	12.28
α-Zingiberene	1493	24.91	25.18	22.84	30.46	26.32	25.98	28.82	22.53
α-Farnesene	1505	4.86	5.03	4.14	7.18	6.66	4.82	5.18	4.60
β-Bisabolene	1505	5.64	6.30	5.81	6.83	5.85	6.01	6.66	5.81
γ-Cadinene	1513	1.03	1.21	1.01	2.50	2.62	1.15	1.09	1.07
β-Sesquiphellandrene	1522	12.79	13.55	12.51	14.51	12.83	13.02	14.60	12.72
Elemol	1548	0.57	0.68	-	0.53	0.62	0.63	0.51	0.46
Nerolidol	1561	-	-	-	-	-	-	-	-
Zingiberenol	1606	0.92	1.00	0.94	1.08	1.08	0.91	1.02	0.94
β-Eudesmol	1650	1.15	1.30	0.94	0.67	0.75	1.06	1.06	0.99

Table 17. Variation in the major essential oil constituents of 16 red ginger genotypes and IISR Varada using GC-MS

Compounds	Kovats index	Coll. No. 9076	Coll. No. 9078	G1	G3	G6	G9	Acc. 850	Acc. 899	IISR Varada
α-Pinene	939	1.27	1.82	1.29	0.34	1.40	1.45	1.26	1.26	1.80
Camphene	954	3.93	5.11	3.69	1.30	4.20	4.30	4.88	5.39	3.48
β-Pinene	979	1.21	1.71	1.48	0.08	1.42	1.29	0.26	0.19	0.33
β-Myrcene	990	0.8	0.98	0.74	0.38	0.72	0.87	1.06	1.08	1.64
D-Limonene	1029	-	-	-	0.37	-	-	-	-	-
β-Phellandrene	1029	3.12	3.89	3.15	-	2.64	4.03	2.08	1.95	3.48
1,8 Cineole	1031	-	0.03	0.03	1.59	-	-	1.95	2.52	4.81
β-Ocimene	1050	1.01	1.15	0.51	-	-	0.94	-	-	-
Linalool	1096	2.27	2.08	2.53	0.41	2.47	2.65	1.00	0.91	2.77
endo-Borneol	1169	1.47	1.49	1.48	0.53	1.88	1.61	1.64	2.09	1.92
Myrtenal	1195	0.23	0.28	0.20	0.03	0.27	0.21	0.06	-	0.16
Citronellol	1225	-	-	0.03	0.18	-	-	0.32	0.47	0.40
Neral	1238	3.01	3.26	2.77	2.45	3.10	2.33	3.57	3.81	0.99
cis-Geraniol	1252	0.79	1.13	0.80	0.36	-	0.69	1.60	1.30	0.72
Geranial	1267	4.34	4.82	4.38	3.68	4.71	3.31	5.33	5.93	0.84
Bornyl acetate	1288	0.28	0.29	0.25	-	0.18	0.28	0.44	0.55	0.18
Geranyl acetate	1371	0.26	0.28	0.28	0.07	0.15	0.21	0.63	0.74	0.11
trans-α-Bergamotene	1434	0.21	0.21	0.20	0.18	0.19	0.23	-	-	0.23
γ-Muurolene	1479	-	-	-	-	-	-	0.19	0.34	-
Ar-Curcumene	1480	10.30	9.77	12.07	8.35	12.51	12.70	9.06	9.33	6.63
α-Zingiberene	1493	23.95	21.27	22.12	25.83	22.05	22.11	22.05	20.74	24.26
α-Farnesene	1505	5.02	4.36	4.33	11.86	3.87	4.34	6.89	6.89	4.48
β-Bisabolene	1505	5.68	5.08	5.69	4.93	5.94	5.97	5.56	5.13	3.72
γ-Cadinene	1513	1.05	0.86	1.06	3.62	1.04	1.09	1.46	1.79	0.46
β-Sesquiphellandrene	1522	12.59	11.40	12.29	12.95	12.47	12.87	12.56	12.20	12.04
Elemol	1548	0.55	0.54	0.43	0.75	0.63	0.58	-	-	1.05
Nerolidol	1561	-	-	-	-	-	-	-	-	2.40
Zingiberenol	1606	0.92	0.91	0.94	1.21	0.98	0.89	1.05	0.95	1.28
β-Eudesmol	1650	1.09	1.09	1.01	0.89	1.12	0.93	0.59	0.44	1.83

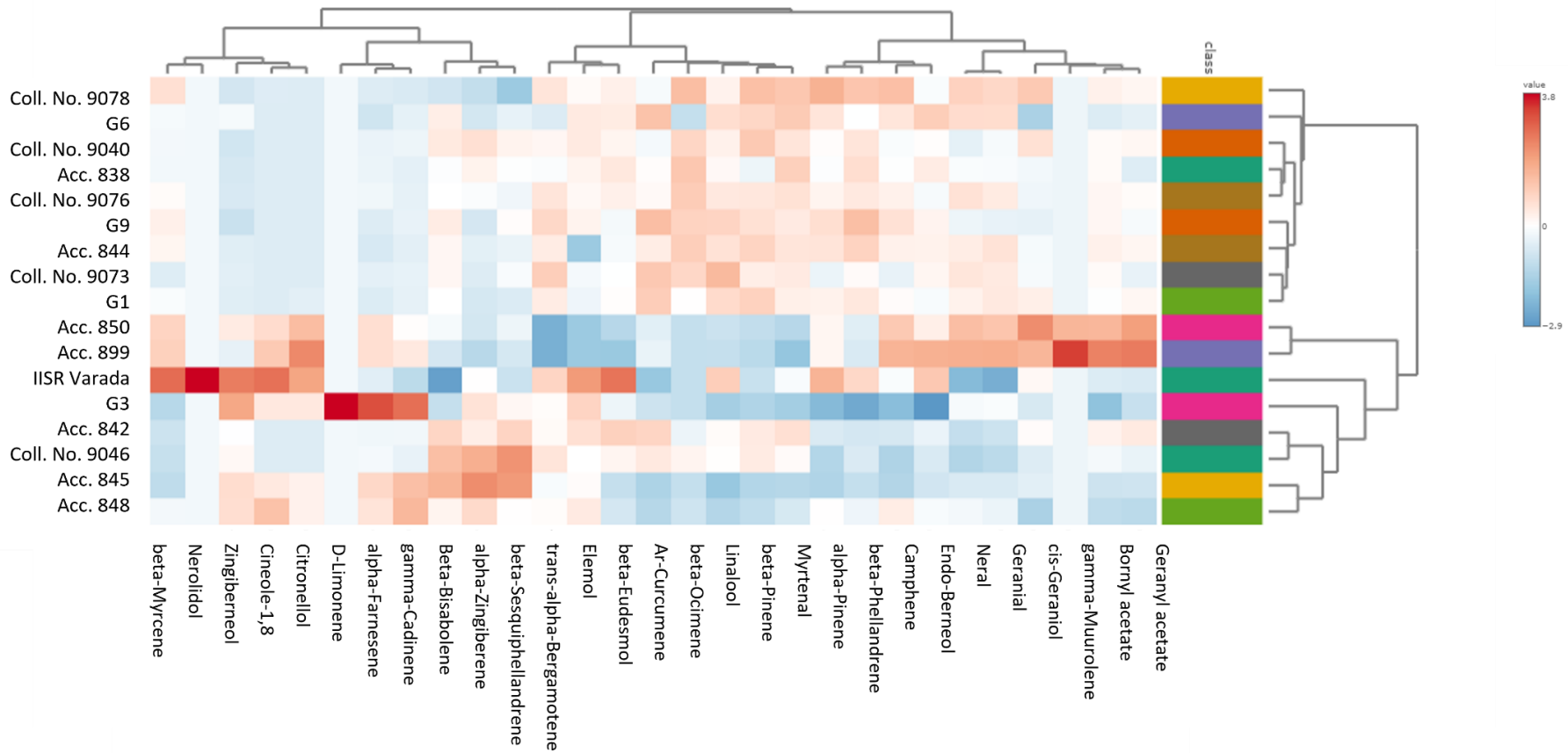

Fig. 10. Heat map indicating variation of major essential oil compounds among 16 red ginger genotypes and IISR Varada

The essential oil of exotic red ginger genotypes exhibited distinguishable compounds when compared to Indian types and IISR Varada. The oil extracted from dried rhizomes was rich in sesquiterpenoids, primarily comprising zingiberene, β-sesquiphellandrene, ar-curcumene, β-bisabolene, and α-farnesene. However, the relative abundance of these compounds was lower compared to Indian red ginger types and common ginger. On contrary to the present study, Sivasothy *et al*. (2011) reported that oil from red ginger rhizome is rich in monoterpenoids (81.9 %), comprising camphene (14.5 %), geranyl acetate (13.7 %), geranial (14.3 %), neral (7.7 %), geraniol (7.3 %), and 1,8-cineole (5.0 %). An earlier study conducted by Malek *et al*. (2005), also reported high concentration of monoterpenoids (64.6 %) with significantly elevated quantities of neral (14.2 %), geranial (28.4 %), and β - sesquiphellandrene (9.9 %). High percentages of monoterpenoids (60.6 %) was also reported recently by Rinanda *et al*. (2018). The substantial variations in the rhizome oil composition, noted in previous research compared to the outcomes of the present study, could be attributed to factors including cultivation conditions, maturity level, growing season, and methods utilized for extraction (Sharifi-Rad *et al*., 2017; Jayasundara & Arampath, 2021; Yu *et al*, 2022). Like their finding, the percentage of neral, geraniol, geranial, bornyl acetate and geranyl aetate was found to be high in exotic type compared to Indian type in the present study. An increased percentage of camphene, ranging from 4.88 to 5.39, was recorded, as reported by Widayat *et al*. (2018). The presence of γ-muurolene was identified as a distinctive compound found exclusively in Acc. 850 and Acc. 899. β-ocimene was found to be absent in exotic types. The percentage of citronellol was notably high (ranging from 0.32 % to 0.47 %) in the exotic type, while it was found in only a few genotypes within a range of 0.03 % to 0.18 %. A slight increased β-myrcene content was reported in Acc. 899 (1.08 %) and Acc. 850 (1.06 %) compared other Indian types, while IISR Varada recorded the highest value (1.64 %). α-bergamotene and elemol were completely absent in the exotic red ginger types, whereas it was present in all other genotypes. The amount of β-eudesmol was extremely low in Acc. 850 (0.59 %) and Acc. 899 (0.44 %).

Sesquiterpene hydrocarbons constituted most of the essential oil, followed by monoterpene hydrocarbons. The present findings align with earlier studies that identified zingiberene as the predominant component in dry ginger oils followed by β-sesquiphellandrene, ar-curcumene, β-bisabolene, and α-farnesene (Kizhakkayil & Sasikumar, 2012; Choudhari & Kareppa, 2013; Feng *et al*., 2018; Babu *et al*., 2021). The ginger genotype IISR Varada exhibited significant variations compared to the red ginger types in the percentage of essential oil constituents. The presence of nerolidol in IISR Varada (2.40 %) alone represented a distinguishable difference between common ginger and red ginger genotypes. Babu *et al*. (2021) reported similar findings. β-myrcene content was high in IISR Varada (1.64 %). Additionally, 1,8-cineole was found to be remarkably high in IISR Varada (4.81 %), whereas it was absent or present only in trace amounts in red ginger types, except exotic types. The percentage of neral and geranial was found to be low in IISR Varada (0.99 %, 0.84 %, respectively), while in red ginger genotypes, it ranged from 1.55% to 3.81% and 2.31% to 5.93%, respectively. A slight increase in the amount of elemol, and β-eudesmol was also observed in IISR Varada. The ar-curcumene content in red ginger genotypes varied from 7.30 % to 12.70 %, whereas IISR Varada showed only 6.63%. In IISR Varada, percentage of β - Bisabolene and γ-cadinene was low compared to red ginger genotypes. Sasidharan *et al*. (2012); Oforma *et al*. (2019) and Akshitha *et al*. (2020) reported comparable results.

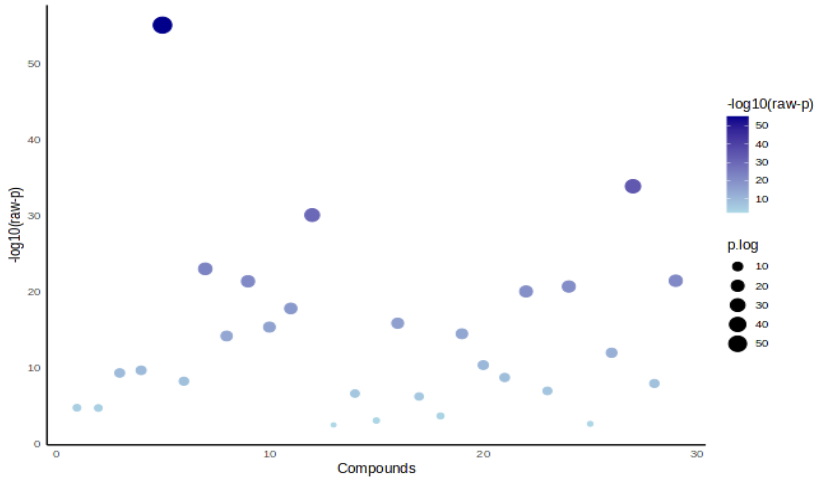

Fig. 11. One way ANOVA for essential oil constituents in 16 red ginger genotypes and IISR Varada

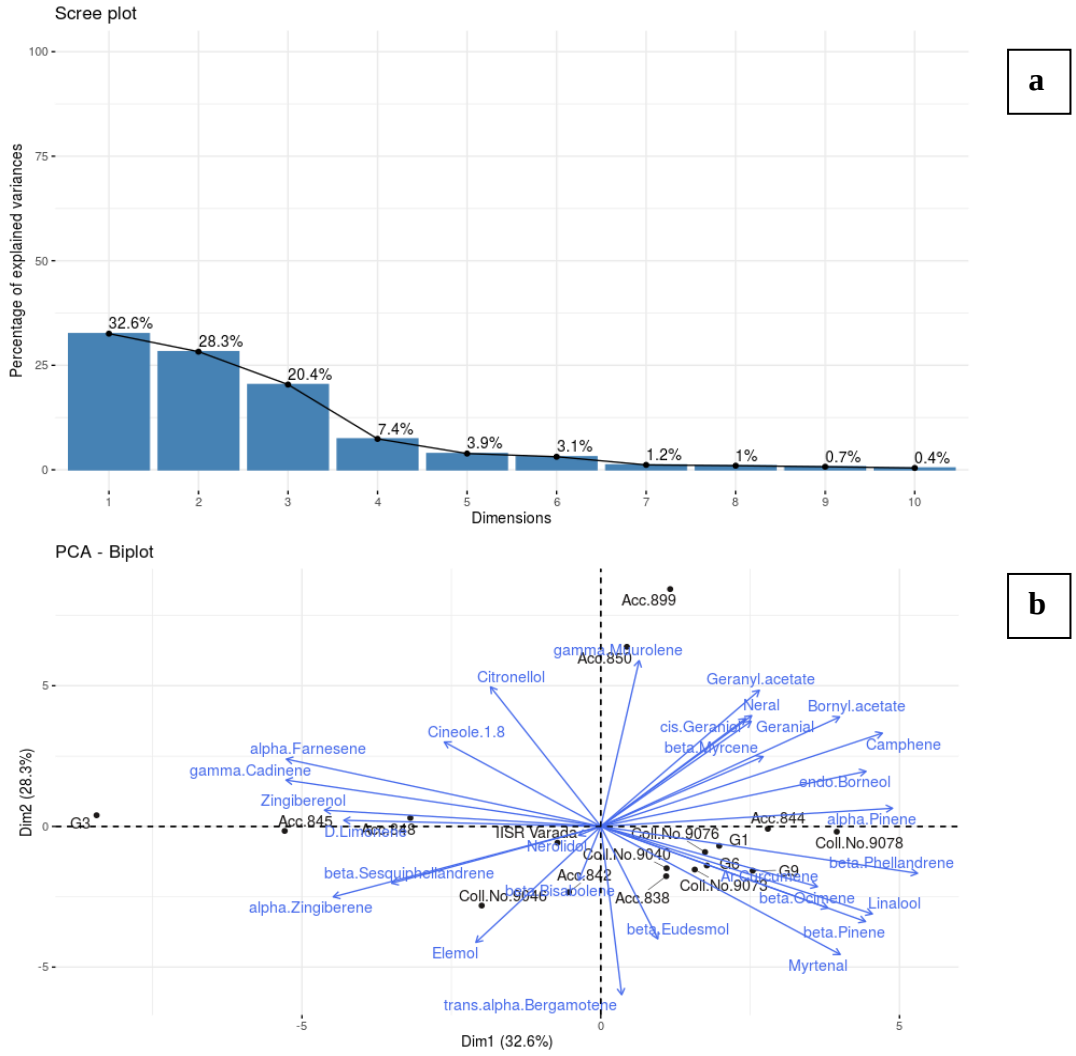

Fig. 12. Principal component analysis major essential oil constituents. (a) Scree plot; (b) PCA biplot of both genotypes and essential oil constituents

4.1.7.1 Principal component analysis (PCA)

Principal component analysis was performed to evaluate the comprehensive pattern of variation and interrelationships among the 17 genotypes and 29 volatile constituents. The scree plot illustrated (Fig. 12. a) the extent of variance explained by each principal component (PC). The analysis revealed that the first two principal components explained 32.60 % and 28.30 % of the total variance in the data. Collectively, these components accounted for a cumulative contribution of 60.90 %, considered reasonable for expounding the fundamental connections between genotypes and compounds. The PCA biplot (Fig. 12. b) clearly depicted the distribution of genotypes based on the variation in the essential oil components. Percentage contribution of variables to different PCs are given in the Table 18. Further analysis of the primary contributors to each principal component more clearly defined these associations. The volatile compounds alpha-pinene (6.43 %), β -phellandrene (7.58 %), α -farnesene (7.47 %) and gamma-cadinene (7.48 %) contributed maximum towards PC1. For PC2, γ -muurolene (10.79 %) exerted most considerable influence, followed by citronellol (7.65 %), geranyl acetate (7.28 %) and myrtenal (6.44 %). Notably, the major candidates of essential oils, namely α -zingiberene, β-sesquiphellandrene, ar-curcumene, and β-bisabolene, did not contribute significantly to first two PCs, indicating that their concentrations do not vary much with genotypes. The exotic red ginger genotypes Acc. 850 and Acc. 899 were distinct for γ -muurolene and plotted at the upper right quadrant. In contrast, Acc. 848, Acc. 845 and G3 located toward the middle-left quadrant, coinciding with higher citronellol, 1,8-cineole, γ -cadinene, α -farnesene, zingiberenol, and D-limonene (only in G3) percentage. The lower region of the quadrant, where most Indian red ginger genotypes and IISR Varada were concentrated, may account for the limited variation observed in essential oil constituents.

The PCA have demonstrated their significance as valuable tools for comprehending the interconnections among characteristics and genotypes. As indicated by the PCA results, the exotic red ginger genotypes (Acc. 850 and Acc. 899) were distinct and unique in terms of specific volatile oil constituents compared

to Indian types. The grouping observed across the four quadrants of the plot elucidates the genetic variability within genotypes, and the grouping of certain genotypes into clusters signifies their genetic similarity. This grouping could potentially be attributed to the geographic proximity of the genotypes to their respective areas of origin. Similarly, Akshitha *et al*. (2020) explained the extent of genetic diversity contributed by essential oil constituents of different ginger genotypes and its association with PCA. Similar findings were reported by Aragaw *et al*. (2011); Ravishanker *et al*. (2013); Yu *et al*. (2022) in ginger, Shukurova *et al*. (2020) in *Z. barbatum*; Mishra *et al*. (2018) and Qiang *et al*. (2021) in turmeric.

Table 18. Percentage contribution of variables to different PCs

Variables	PC1	PC2	PC3	PC4	PC5
α -Pinene	6.43	0.13	4.73	0.71	3.36
Camphene	5.99	3.42	0.23	0.39	7.67
β -Pinene	5.30	3.56	1.14	0.55	1.36
β -Myrcene	1.99	1.92	10.64	0.00	0.06
D-Limonene	4.99	0.02	0.13	18.68	7.49
β -Phellandrene	7.58	0.86	1.29	0.07	1.22
1.8, Cineole	1.85	2.80	9.26	0.43	2.00
β -Ocimene	3.90	2.62	1.22	0.73	4.45
Linalool	5.57	3.01	0.99	0.25	2.78
endo-Borneol	5.32	1.19	2.19	4.81	7.35
Myrtenal	4.33	6.44	0.01	0.30	0.14
Citronellol	0.92	7.65	4.37	0.55	0.07
Neral	1.72	4.82	3.75	6.78	2.70
cis-Geraniol	1.59	4.57	0.01	1.50	27.86
Geranial	1.71	4.34	5.22	5.81	1.36
Bornyl acetate	4.31	4.71	0.34	4.75	3.98
Geranyl acetate	1.90	7.28	0.62	1.30	9.65
trans alpha-Bergamotene	0.03	11.12	0.27	0.55	0.07
γ -Muurolene	0.11	10.79	0.06	1.13	0.47
Ar. Curcumene	3.55	1.42	4.20	0.40	0.93
α -Zingiberene	5.41	1.95	0.14	10.97	0.07
α -Farnesene	7.47	1.78	0.41	4.27	1.55
β -Bisabolene	0.04	1.14	10.17	11.58	2.92
γ -Cadinene	7.48	0.84	1.51	2.99	3.05
β -Sesquiphellandrene	3.30	1.27	2.54	18.94	0.51
Elemol	1.18	5.28	4.54	0.988	0.43
Nerolidol	0.04	0.03	16.06	0.41	1.50
Zingiberenol	5.77	0.10	6.37	0.06	0.28
β -Eudesmol	0.25	4.96	7.61	0.10	4.73

To conclude, significant variability was identified among the red ginger genotypes and between IISR Varada. Statistical analysis revealed significant differences in the studied morphological parameters and quality traits. In terms of rhizome parameters, particularly thickness, red ginger genotypes were notably lower compared to IISR Varada. Among the 17 genotypes, the highest rhizome yield (807.50 g) was recorded for Acc. 9073. The exotic red ginger types exhibited low rhizome yield compared to both Indian red ginger types and IISR Varada. The red ginger genotypes demonstrated superiority over IISR Varada regarding biochemical aspects such as essential oil content, oleoresin concentration, crude fibre content, and gingerol percentage. The genetic variability studies indicated high heritability and genetic advance percent mean for growth parameters, rhizome characters, yield, and quality traits, serving as a valuable guideline for plant breeders to improve ginger through simple selection. Essential oil characterization also revealed variation in the composition of constituents between the red ginger genotypes and IISR Varada. The red ginger genotypes were distinguished as a superior selection of ginger due to their high essential oil yield and oleoresin content, as well as significant percentages of gingerols and shogaol. Moreover, they exhibited a higher fibre content compared to IISR Varada. PCA results indicated the distinctiveness of the exotic types from other red ginger genotypes.

OBJECTIVE 2

TO GENETICALLY IMPROVE RED GINGER THROUGH *IN VITRO* AND *IN VIVO* POLYPLOIDY INDUCTION USING COLCHICINE AND ITS EFFECT ON SELECTED HORTICULTURAL CHARACTERISTICS AND ESSENTIAL OIL COMPOSITION

4.2 Standardization of *in vitro* regeneration protocol for red ginger genotypes and genetic fidelity assessment using ISSR and SSR markers

The genotypes demonstrating superiority in morphological and biochemical characteristics were identified and selected for tissue culture. Among the Indian red ginger genotypes, Coll. No. 9073 was chosen, while for the exotic type, Acc. 899 was selected.

4.2.1 *In vitro* culture establishment

Young rhizome bud explants were subjected to *in vitro* culture initiation to facilitate plant regeneration in both Indian and exotic red ginger varieties. An optimization process was employed for the surface sterilization of sprouting buds to attain maximum axenic cultures. Notably, the regeneration of buds was achieved successfully on a basal MS medium, devoid of any exogenous plant growth regulators. The explants showed growth signs about five to seven days after the inoculation. It is noteworthy that each bud produced a single shoot during this regeneration process. Subsequently, the *in vitro*-established shoots were further subcultured onto MS medium containing various concentrations of BAP after a four-week incubation period.

4.2.2 The effect of 6-Benzylaminopurine (BAP) on shoot multiplication of red ginger genotypes

The impact of different concentrations of BAP (1, 2, 3, 4, and 5 mg L^{-1}) on *in vitro* shoot multiplication was evaluated (Table 19). Shoot multiplication was observed at all five BAP concentration levels starting from the second week of the experiment, and the response was documented after four weeks. The two red ginger

genotypes under investigation exhibited distinct responses to varying hormone concentrations in the current study. The response of exotic red ginger to various concentrations of BAP is depicted in the Fig. 13. The mean number of shoots in exotic red ginger ranged from 1.50 to 3.30. Whereas the mean number of roots ranged from 3.9 to 10.10. The exotic red ginger (Acc. 899) exhibited a significantly higher number of shoots (3.30 ± 0.12) and roots (10.10 ± 0.40) at 5.0 mg L^{-1} BAP. Consistent with the findings in this study, Pandey *et al*. (1997) and Sathyagowri and Seran (2011) also observed that the most successful induction of adventitious shoots occurred when the MS basal medium was supplemented with 5.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA.

Similarly, Zuraida *et al*. (2016) documented the highest number of micro shoots originating from rhizome buds of *Z. officinale* var. *rubrum* when they were cultured on MS medium supplemented with 5.0 mg L^{-1} of BAP. On contrary to the present study, Inderiati *et al*. (2023), reported the maximum number of microshoots of red ginger at 1 mg L^{-1} BA, whereas Syahid *et al*. (2023) observed the optimum number of shoots at 2 mg L^{-1}. In addition, Karyanti *et al*. (2021) conducted experiments using various types and concentrations of cytokinins and found that 1 ppm thidiazuron was the best cytokinin concentration for the *in vitro* growth of red ginger shoots.

For exotic red ginger, the average shoot length ranged from 2.97 to 3.97 cm, and the root length varies from 3.84 to 6.98 cm. BAP at 2.0 mg L^{-1} resulted in significantly greater shoot and root lengths, measuring 3.97 ± 0.25 cm and 6.98 ± 0.38 cm, respectively. As the BAP concentration increased, the shoot and root length decreased. Among the various concentrations of BAP tried, the lowest shoot and root length was observed at BAP 5 mg L^{-1}. Sathyagowri and Seran (2011) also documented an inverse relationship between the concentration of BAP and shoot length. Whereas the growth response in terms of shoot and root multiplication and elongation was recorded as significantly lower for the control group (0.00 mg L^{-1}). Increased levels of BAP resulted in enhanced shoot development and decreased shoot elongation and rooting.

Table 19. Effect of BAP concentrations on shoot multiplication of exotic and Indian red ginger after four weeks of incubation

BAP concentration (mg L^{-1})	Mean no. of shoots		Mean shoot length (cm)		Mean no. of roots		Mean root length (cm)	
	Exotic red ginger	Indian red ginger	Exotic red ginger	Indian red ginger	Exotic red ginger	Indian red ginger	Exotic red ginger	Indian red ginger
Control (MS)	1.50 ± 0.16^{c}	1.40 ± 0.29^{c}	2.97 ± 0.11^{c}	2.75 ± 0.06^{b}	3.90 ± 0.80^{d}	4.30 ± 0.56^{c}	3.84 ± 0.47^{c}	3.04 ± 0.15^{b}
1 mg L^{-1}	1.80 ± 0.12^{c}	2.30 ± 0.26^{bc}	3.81 ± 0.2^{ab}	2.77 ± 0.18^{b}	4.80 ± 0.82^{cd}	7.10 ± 0.86^{ab}	5.70 ± 0.19^{b}	3.13 ± 0.37^{b}
2 mg L^{-1}	2.00 ± 0.52^{bc}	3.60 ± 0.40^{ab}	3.97 ± 0.25^{a}	3.37 ± 0.25^{b}	5.00 ± 0.52^{bcd}	7.90 ± 0.71^{a}	6.98 ± 0.38^{a}	4.40 ± 0.49^{a}
3 mg L^{-1}	2.68 ± 0.13^{ab}	3.80 ± 0.72^{a}	3.46 ± 0.22^{abc}	4.40 ± 0.40^{a}	6.40 ± 0.62^{bc}	8.10 ± 0.58^{a}	4.43 ± 0.27^{c}	4.99 ± 0.41^{a}
4 mg L^{-1}	2.90 ± 0.29^{a}	2.80 ± 0.41^{ab}	3.27 ± 0.22^{bc}	3.29 ± 0.13^{b}	7.30 ± 1.42^{b}	6.00 ± 0.61^{bc}	4.13 ± 0.37^{c}	4.37 ± 0.46^{a}
5 mg L^{-1}	3.30 ± 0.12^{a}	2.30 ± 0.60^{bc}	3.18 ± 0.05^{c}	3.05 ± 0.17^{b}	10.10 ± 0.40^{a}	5.20 ± 0.51^{c}	3.89 ± 0.16^{c}	4.61 ± 0.36^{a}

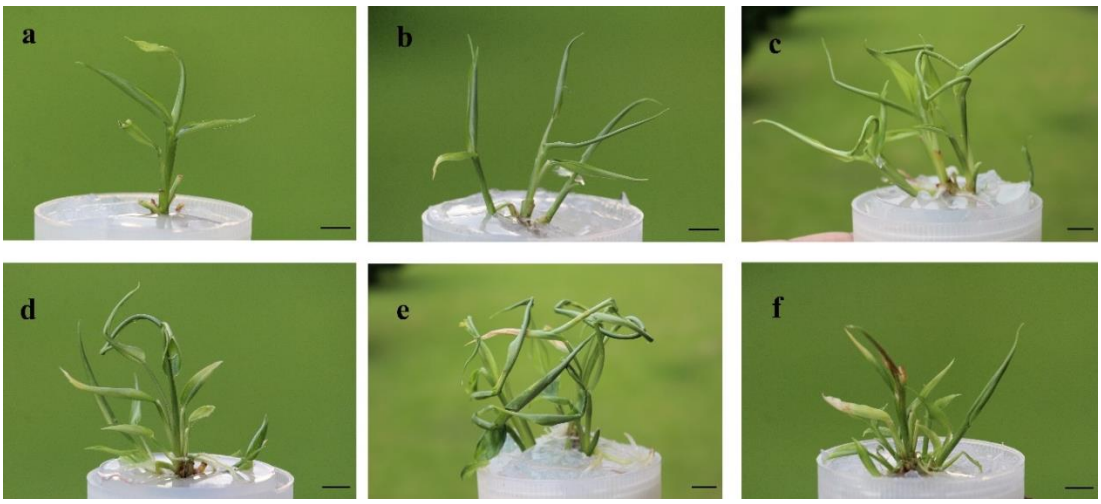

Fig. 13. Direct *in vitro* multiplication of exotic red ginger after 4-weeks culture on the medium: (a) without growth regulators (control); supplemented with: (b) 1 mg L^{-1} BAP; (c) 2 mg L^{-1} BAP; (d) 3 mg L^{-1} BAP; (e) 4 mg L^{-1} BAP; (f) 5 mg L^{-1} BAP. Scale bar = 1cm

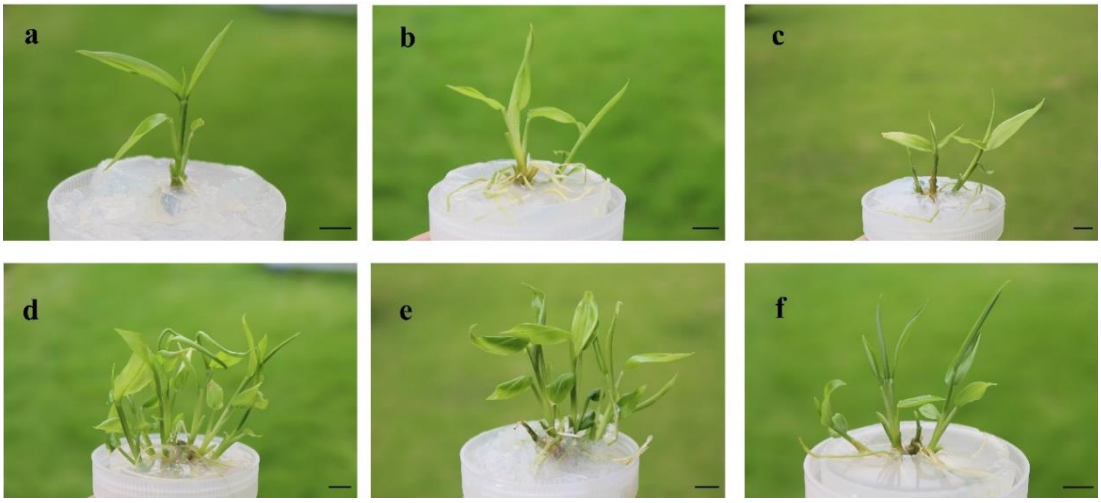

Fig. 14. Direct *in vitro* multiplication of Indian red ginger after 4-weeks culture on the medium: (a) without growth regulators (control); supplemented with: (b) 1 mg L^{-1} BAP; (c) 2 mg L^{-1} BAP; (d) 3 mg L^{-1} BAP; (e) 4 mg L^{-1} BAP; (f) 5 mg L^{-1} BAP. Scale bar = 1cm

The response of Indian red ginger to various concentrations of BAP is depicted in the Fig. 14. For Indian red ginger, the most favourable shoot multiplication was observed when utilizing MS media supplemented with BAP concentrations ranging from 2-4 mg L^{-1}. However, BAP at a concentration of 3.0 mg L^{-1} in the MS medium is the most effective plant growth regulator, yielding the

highest number of green shoots (3.80 ± 0.72) and roots (8.10 ± 0.58), as well as the shoot (4.40 ± 0.40 cm) and root (4.99 ± 0.41 cm) lengths. The lowest shoot and root initiation was observed in explants that were inoculated to MS medium devoid of BAP (control). This finding aligns with the observations made by Mohammed and Quraishi in 1999, where they documented the highest multiplication of common ginger plantlets in an MS medium containing 3.0 mg L^{-1} of BAP along with 0.5 mg L^{-1} of NAA. The results also clearly indicate that exceeding the ideal BAP levels led to a decrease in the number of shoots and a reduction in plant height. Several studies have noted that the highest production of multiple shoots from turmeric rhizome buds occurred when using MS media supplemented with 3.0 mg L^{-1} of BAP (Balachandran *et al*., 1990; Naz et a., 2009). While BAP is a commonly employed cytokinin for promoting shoot proliferation, elevated concentrations of BAP in turmeric inhibit the rate at which shoot multiplication occurs, as indicated by Naz *et al*. (2009). In contrast to these findings, Zuraida *et al*. (2016) reported that the use of MS medium containing 5.0 mg L^{-1} of BAP was effective for promoting shoot multiplication in exotic red ginger.

The outcomes of the current research distinctly demonstrated that the *in vitro* response is significantly affected by the genotype. Both the genotypes, Indian and exotic red ginger, produced maximum shoot multiplication at different concentrations of plant growth regulators.

4.2.3 Rooting and acclimatization

In the present study, spontaneous rooting was observed in rhizome bud explants after 10-15 days. Significant root development was noted in both exotic and Indian red ginger when cultured on a MS medium containing only BAP. The fully matured plantlets were taken out of the phyta jars and transferred to mist chamber for hardening. The survival rate of *in vitro*-raised plantlets from both Indian and exotic red ginger genotypes was 77.78% and 81.25%, respectively (Fig. 15).

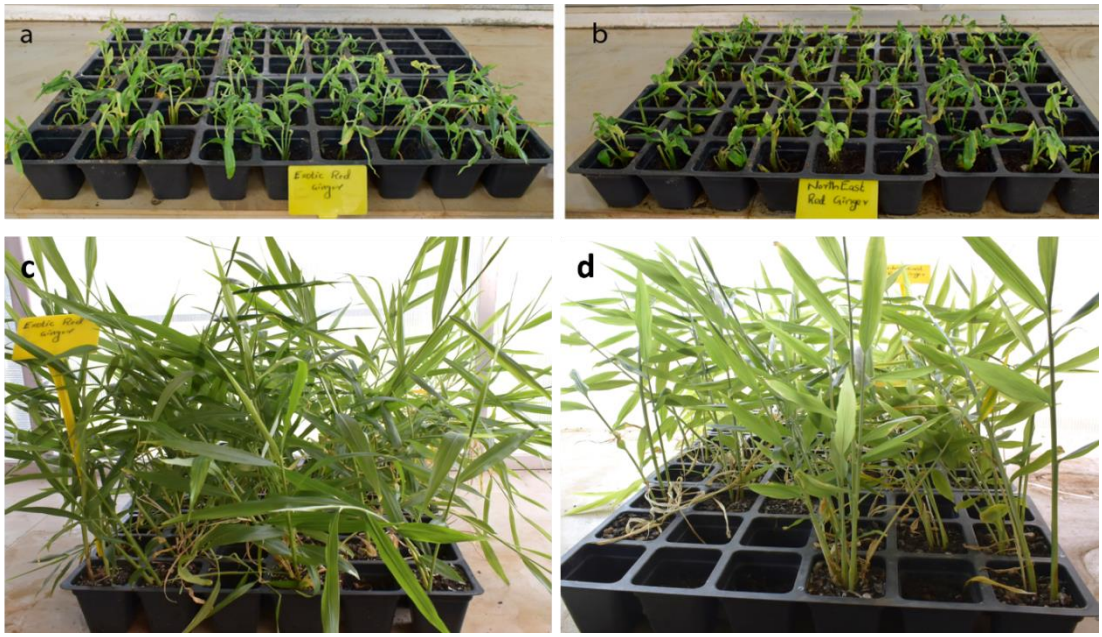

Fig. 15. *In vitro* raised plantlets at initial stage of hardening (a) exotic red ginger; (b) Indian red ginger; (c & d) after four months of hardening

4.2.4 Genetic fidelity assessment using ISSR and SSR markers

Genetic homogeneity of the regenerated clones was estimated from the 10 randomly selected plants after hardening. Ten ISSR primers produced a total of 51 bands for Indian red ginger, and 50 bands for exotic red ginger. All the primers generated monomorphic bands, similar to those of the mother plants, within the range of 250 to 2500 base pairs for both genotypes (Table 20). Representative ISSR profiles generated for the two genotypes displayed in the Fig. 16. a. For SSR primers, both genotypes produced a total of 15 bands out of the 12 primers assessed, falling within the 100 to 200 base pair range (Table 21). The *in vitro* raised plantlets exhibited a uniform banding pattern that closely resembled that of their respective mother plants for all the primers, as depicted in Fig. 16. b. Nonetheless, the primer known as ZO SSR 108 revealed a polymorphic band in one of the regenerated plants of exotic red ginger (Fig. 16. c). Among the ten ISSR primers, UBC 816, UBC 827, UBC 851, UBC 856 and UBC 897 displayed polymorphism between Indian and exotic red ginger. In the case of twelve SSR primers that were assessed, ZO SSR 16, ZO SSR 73, ZO SSR 91, and ZO SSR 108 exhibited variation between the genotypes. These markers are suitable for distinguishing between the genotypes.

Table 20. Genetic fidelity assessment among mother plant and tissue-cultured plants using ISSR primers

Primer name	Total bands		No. of polymorphic bands		Amplicon range (bp)		PIC value	
	Indian red ginger	Exotic red ginger	Indian red ginger	Exotic red ginger	Indian red ginger	Exotic red ginger	Indian red ginger	Exotic red ginger
ISSR 5	5	5	0	0	250-2500	250-2500	0	0
ISSR 10	3	3	0	0	500-100	500-1500	0	0
ISSR 12	10	10	0	0	250-2000	250-2000	0	0
UBC 816	5	6	0	0	250-750	250-2000	0	0
UBC 826	2	2	0	0	1000-2000	1000-2000	0	0
UBC 827	3	4	0	0	750-2000	750-2000	0	0
UBC 835	7	7	0	0	250-2000	250-2000	0	0
UBC 851	8	7	0	0	500-2000	500-2000	0	0
UBC 856	6	5	0	0	750-1500	750-1500	0	0
UBC 897	2	1	0	0	1000-1500	1000-1500	0	0

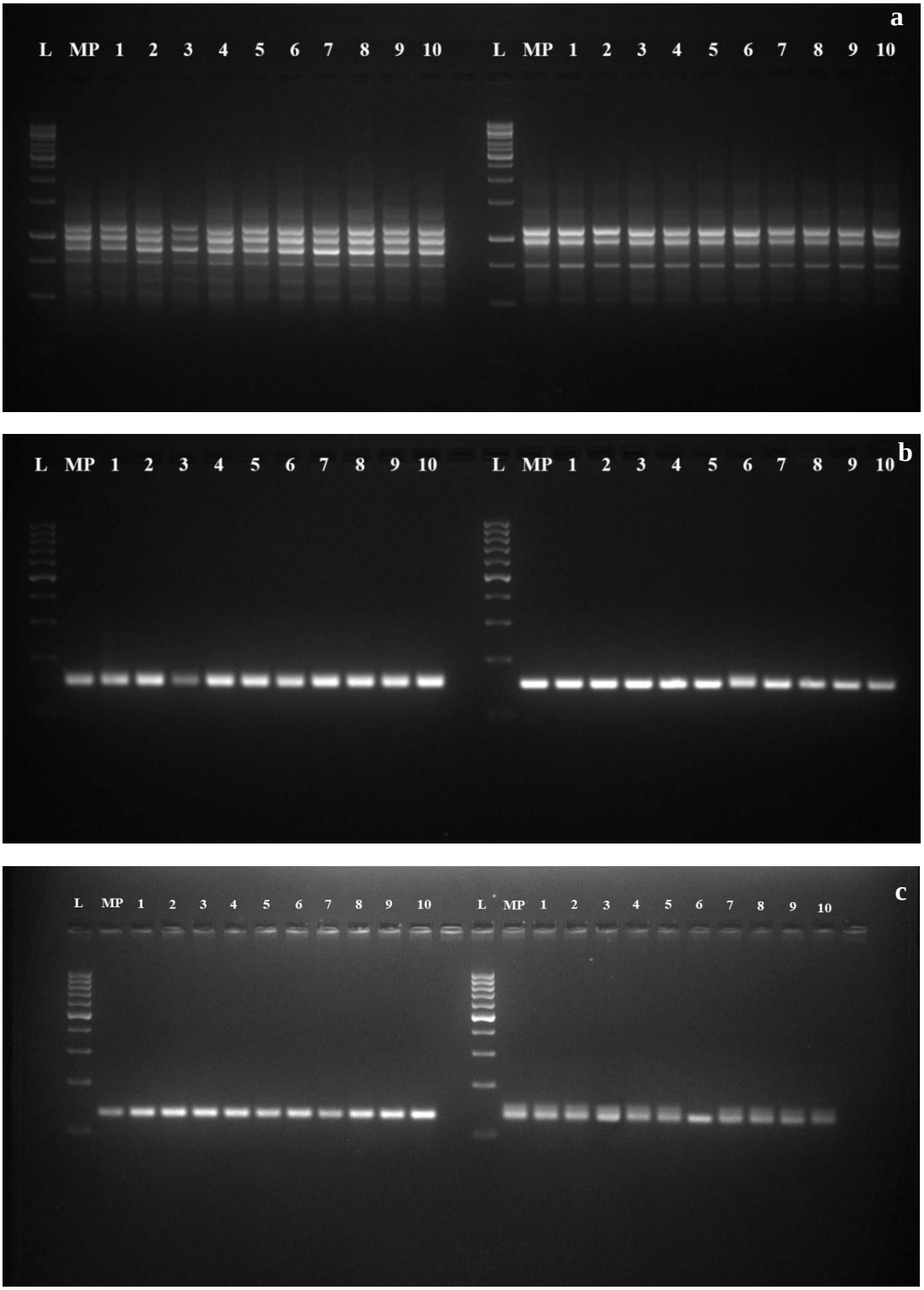

Fig. 16. Banding profile of red ginger genotypes using primers: a) UBC 851; b) ZO SSR 25; c) ZO SSR 108: L − DNA ladder, MP- Indian red ginger mother plant, 1 to10: micropropagated plants of Indian red ginger, L − DNA ladder, MP- exotic red ginger mother plant, 1 to10: micropropagated plants of exotic red ginger

ISSR and SSR markers have demonstrated their efficacy in assessing the genetic purity of *in vitro* propagated plants. Numerous studies have reported findings related to the evaluation of genetic uniformity between *in vitro* propagated plants and their parent plants using molecular markers in various plant species within the Zingiberaceae family, including *Curcuma. angustifolia* Roxb. (Jena *et al*., 2018), *C. longa* L. (Pittampalli *et al*., 2022), *Z. officinale* Rosc. (Mohanty *et al*., 2008), and *Z. moran* and *Z. zerumbet* (L.) Smith (Das *et al*., 2013). In the current research, a complete similarity of 100% was observed between the *in vitro* cultured Indian red ginger plants and their donor plants, while a 93% similarity was noted between the *in vitro* propagated exotic red ginger plants and their parent plants. These slight genetic differences may have arisen from naturally occurring mutations influenced by various factors, including the *in vitro* regeneration process and its duration, the concentration of plant growth regulators, stress induced by *in vitro* conditions, the impact of MS media additives, and repetitive subculture, all of which have been associated with somaclonal variation or genetic diversity (Razaq *et al*. 2013). As a result, the plants from both the genotypes were exact clones of the respective mother plants. There are reports on the generation of polymorphism in micropropagated plants. For example, in the case of bananas, Devi *et al*. (2017) reported a 9.6% polymorphism rate. The results align with the studies of Thakur *et al*. (2016), who observed a 97% similarity in the DNA banding profiles between micropropagated *Pittosporum eriocarpum* Royle and the mother plant. This finding indicates the genetic purity of the micropropagated clones.

The similarity between micropropagated plants and the mother plant of exotic red ginger was determined using Jaccard's similarity coefficient for ISSR and SSR markers. The similarity values ranged from 0.929 to 1.00 among the 10 plants examined. The dendrogram produced via UPGMA analysis indicated a 93% similarity between the *in vitro* cultivated plantlets and their respective donor mother plants.

Table 21. Genetic fidelity assessment among mother plant and tissue-cultured plants using SSR primers

Primer name	Total bands		No. of polymorphic bands		Amplicon size range (bp)	PIC value	
	Indian red ginger	Exotic red ginger	Indian red ginger	Exotic red ginger		Indian red ginger	Exotic red ginger
ZO SSR 2	1	1	0	0	117-154	0	0
ZO SSR 16	2	1	0	0	115–163	0	0
ZO SSR 21	1	1	0	0	164–182	0	0
ZO SSR 25	1	1	0	0	143–178	0	0
ZO SSR 35	1	1	0	0	129–195	0	0
ZO SSR 36	1	1	0	0	162–189	0	0
ZO SSR 38	1	1	0	0	158–188	0	0
ZO SSR 64	1	1	0	0	122–149	0	0
ZO SSR 73	2	1	0	0	154–178	0	0
ZO SSR 91	1	2	0	0	126–181	0	0
ZO SSR 111	2	2	0	0	124–160	0	0
ZO SSR 108	1	2	0	1	117–145	0	0.09

4.3 Polyploidy induction

4.3.1 Ploidy estimation of the mother plants

4.3.1.1 Somatic chromosome count estimation of mother plants

Somatic chromosome number of the parent plants were identified using root tip squashing method. Indian red ginger (Coll. No. 9073) was found to be diploid with a chromosome number 2n = 22 (Fig. 17. a). Exotic red ginger (Acc. 899) exhibited a chromosome number of 2n = 33 (Fig. 17. b). This is the first report of triploidy in ginger.

Understanding the ploidy level of genetic resources is fundamental and essential for the development of an efficient breeding programme. Earlier reports on the chromosome number of *Z. officinale* showed the diploid number to be 2n = 22 (Ramachandran & Nair, 1992; Eksomtramage *et al*., 2002; Saensouk & Saensouk,

2004; Nayak *et al*., 2005; Joseph, 2010; Bhadra & Bandyopadhyay, 2015; Nair, 2016). Wang *et al*. (2014) reported that the basic chromosome number of ginger in China is x = 11. In addition to this, variations in the chromosome numbers were also documented by Sharma and Bhattacharya (1959), Dhamayanthi and Zachariah (1998), and Nair (2016) with a diploid chromosome number of 2n = 24. Etikawati and Setyawan (2000) observed the diploid chromosome count of red ginger as 2n = 32, whereas Daryono *et al*. (2012) observed the chromosome number as 2n = 22 in red ginger and 2n = 30 in *Z. officinale* Rosc. var. *officinale* and *Z. officinale* Rosc. var. *amarum*.

4.3.1.2 Confirmation of ploidy level of mother plants through flow cytometry

The ploidy status of the parent red ginger plants was analysed using the flow cytometric technique. The diploid control (IISR Varada) demonstrated the G0/G1 peak at channel 5.3×10^5 (Fig. 17. c). In Indian red ginger (Coll. No. 9073), the distribution of DNA exhibited a prominent peak corresponding to the G0/G1 nuclei, located at channel 5.1×10^5 (Fig. 17. d), confirming the ploidy level as diploid. In exotic red ginger (Acc. 899), the G0/G1 peak was observed around channel 8.0×10^5 (Fig. 17. e), indicating triploidy. Most histograms displayed a low coefficient of variation (<3%), suggesting high reliability in the obtained results.

Researchers widely employ this method of ploidy verification in ginger, favouring it over conventional root tip squashing method methods due to its ability to provide more accurate results in a much shorter time (Wang *et al*., 2014; Zhou *et al*., 2020; Prasath *et al*., 2022).

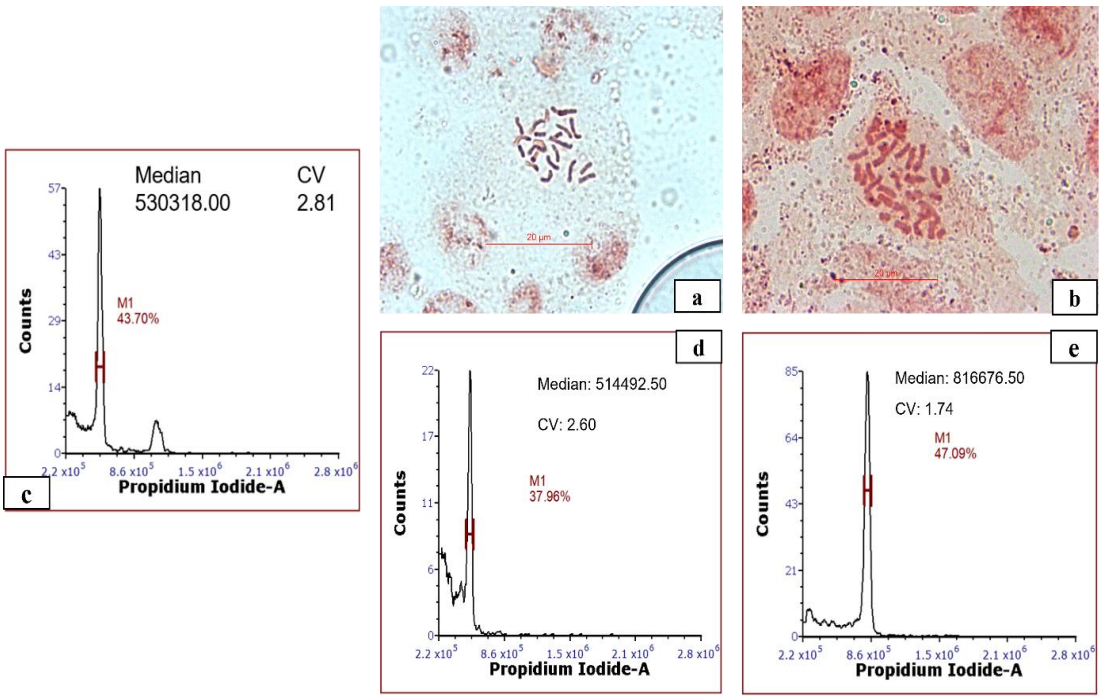

Fig. 17. Mitotic metaphase plate of (a) Indian red ginger showing 2n=22, (b) Exotic red ginger showing 2n=33; Flow cytometry histogram of nuclei of (c) IISR Varada, (d) Indian red ginger, (e) Exotic red ginger

4.3.2 *In vitro* polyploidy induction

4.3.2.1 Effect of colchicine treatment on survival rate of red ginger genotypes

The red ginger genotypes were successfully established under *in vitro* condition after treating with different colchicine concentrations (0.025, 0.050, 0.075 and 0.1%) for 24 and 48 h. The initial visible effect of colchicine treatment was the browning and necrotic symptoms. It was very prominent in the high dosages like 0.075% and 0.1%. The regeneration of the explant noticed after 3-4 weeks of treatment. Sprouting of the explants were observed even after the fourth week of inoculation. It was noticed that the survival rate exhibited a notable decrease as both the concentration and duration of exposure to colchicine increased. The survival rate of the explants, following treatment with various concentrations of colchicine, was recorded under *in vitro* conditions after 45 days of incubation.

Table 22. Analysis of variance of the survival rate of Indian and exotic red ginger at different concentrations of colchicine and durations after *in vitro* colchicine treatment

Responses	Degree of freedom	Mean squares	
		Indian red ginger	Exotic red ginger
Concentration	4	2997.45 ***	3406.30***
Duration	1	504.04***	584.30***
Concentration x Duration	4	66.79***	104.00*
Error	30	10.44	31.20
CV (%)		5.98	10.99

The two-way ANOVA result clearly established the significant ($p<0.001$) influence of various colchicine concentrations and time durations on the survival rate of Indian red ginger (Table 22) (Fig. 18). The control group for 24 h and 48 h recorded a maximum survival rate of 98.61 % and 95.83 %, respectively (Table 23). The treated groups exhibited a survival rate ranged from 37.50 % to 90.27 %. Among the various treatments, the highest mean survival rate was recorded with colchicine concentrations of 0.025 % for 24 h (90.27 %). At the highest concentration of colchicine, which was 0.1% for 48 h, the lowest survival rate was recorded, measuring 37.50 %.

The ANOVA findings indicated a highly significant impact ($p < 0.001$) of varying colchicine concentrations and durations on the survival rate of exotic red ginger, as presented in table 22. The control group demonstrated the highest survival rates, recording 97.92 % for both the 24 h and 48 h durations (Table 23) (Fig. 19). Following this, a survival rate of 81.25 % was noted in the 0.050% colchicine treatment group for the duration of 24 h. A sudden decrease in the survival rate was observed when the colchicine concentration increased from 0.075 % to 0.100 %. The lowest survival rate, which was 14.58 %, was recorded after treating with a 0.100 % colchicine solution for 48 h. Rhizome buds exposed to 0.1% colchicine solution for 48 hours exhibited extremely low germination rates and resulted in shoots with abnormal appearances that quickly perished.

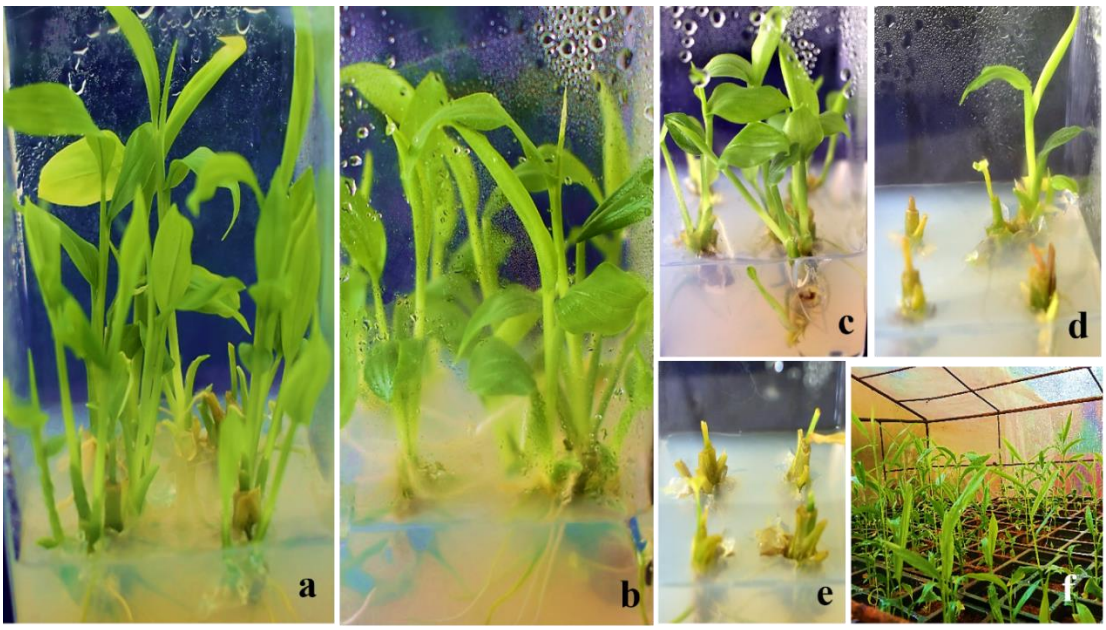

Fig. 18. Effect of colchicine treatment on Indian red ginger regeneration: (a) Control; (b) 0.025%; (c) 0.050%; (d) 0.075%; (e) 0.100%; (f) Hardening

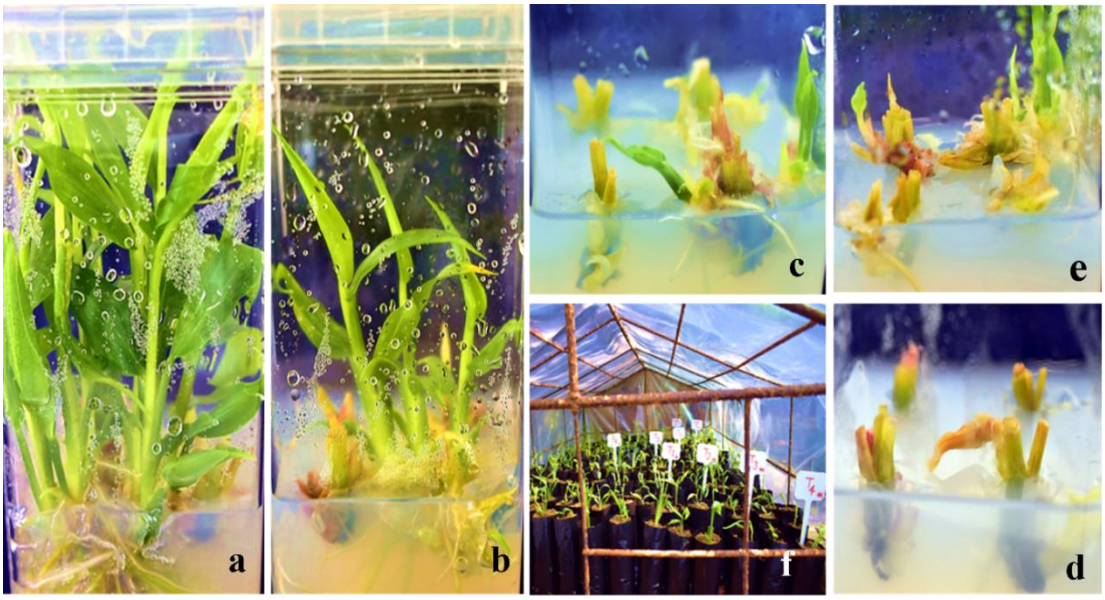

Fig. 19. Effect of colchicine treatment on exotic red ginger regeneration: (a) Control; (b) 0.025%; (c) 0.050%; (d) 0.075%; (e) 0.100%; (f) Hardening

Table 23. Effect of colchicine concentrations and time durations on the survival rate of Indian and exotic red ginger

Colchicine concentration (%)	Duration (h)	Survival rate (%)	
		Indian red ginger	Exotic red ginger
Control (0)	24	98.61 (77.40) ± 2.78^{a}	97.92 (78.33) ± 4.17^{a}
	48	95.83 (75.60) ± 5.32^{ab}	97.92 (78.33) ± 4.17^{a}
0.025	24	90.27 (71.18) ± 6.99^{abc}	81.25 (64.43) ± 4.17^{b}
	48	80.56 (64.96) ± 13.22^{c}	79.17 (63.47) ± 10.76^{b}
0.050	24	84.72 (67.51) ± 6.99^{bc}	66.67 (54.82) ± 6.81^{c}
	48	55.56 (48.30) ± 12.00^{d}	39.58 (38.82) ± 14.23^{e}
0.075	24	77.78 (61.96) ± 4.54^{c}	52.08 (46.20) ± 7.96^{d}
	48	50.00 (45.00) ± 9.07^{de}	29.17 (32.63) ± 4.81^{ef}
0.100	24	62.50 (52.63) ± 14.60^{d}	25.00 (29.60) ± 11.79^{fg}
	48	37.50 (37.63) ± 12.32^{e}	14.58 (21.91) ± 7.98^{g}

Colchicine treatment adversely impacted the survival rate of treated explants in both red ginger genotypes. In the present study, the treated explants exhibited a notably slower sprouting response compared to the untreated control. Similarly, Zhou *et al*. (2020) reported delayed sprouting as the initial observable impact of colchicine following the inoculation of ginger explants. As the concentration and duration of colchicine treatment increased, the survival rate proportionately decreased. The observed effect may be attributed to the toxicity of colchicine, leading to a decreased rate of cell division and, in some cases, explant death (Sullivan & Castro, 2005). Within the treated population, the survival rate of Indian red ginger ranged from 37.50 % to 90.27 %, while the survival rate of exotic red ginger varied from 14.58 % to 81.25 %. A substantial decline in the survival rate of red ginger genotypes was found to be negatively correlated with both the concentration of colchicine and the duration of treatment. Consistent with the findings of the present study, Lindayani *et al*. (2010) investigated the effect of colchicine on the *Z. officinale* and *Z. officinale* var. *rubrum*. They observed that a few plants failed to survive at higher colchicine concentrations. Kun-Hua *et al*.

(2011) observed a significant decrease in survival rate of *in vitro* shoot tips of ginger with increasing colchicine concentration and treatment time. Adaniya and Shirai (2001) examined the impact of different treatment durations on *in vitro* shoot tips of ginger using a 0.2 % colchicine solution, noting a significant decrease in the survival rate with increasing treatment time. The sprouting rate for exotic red ginger was significantly lower compared to Indian red ginger. Different genotypes or ecotypes of the same plant species may react differently to different colchicine concentrations (Niazian & Nalousi, 2020). Our study proved the genotypic influence of antimitotic agents on the survival rate of explants under *in vitro* condition. Babil *et al*. (2016) similarly observed variations in the survival rates of two Dioscorea species, namely *D. rotundata* and *D. cayenensis*, following treatment with colchicine during *in vitro* seedling experiments. In alignment with our research, when explants of *Manihot esculenta* were exposed to colchicine at a concentration of 0.1 g L^{-1}, most of the explants did not survive, and all the explants died above 0.25 g L^{-1}. Navrátilová *et al*. (2021) observed the lethality associated with elevated doses of anti-mitotic agents in the *in vitro* polyploidization process of *Thymus vulgaris.* The influence of colchicine on the survival rate was evident in various plants, such as *Mentha piperita* (Zhao *et al*., 2017), *Trigonella foenum-graecum* (Keshtkar *et al*., 2019), and *Allium sativum* (Wen *et al*., 2022).

4.3.2.2 Effect of colchicine treatment on morphological characteristics of red ginger genotypes

A large population of plants was established in the greenhouse after undergoing *in vitro* colchicine treatment and proper hardening (Fig. 20). Noticeable variations in morphological traits were detected when comparing the control group with the treated group of Indian red ginger plants, and the ANOVA results are presented in Table 24. The substantial impact of colchicine concentration on the morphological characteristics was evident in Indian red ginger, including parameters such as plant height, number of shoots, shoot diameter, number of leaves, and leaf length, with statistical significance at a 5% level. The treatment duration had a significant effect at the 5% probability level, but it only significantly impacted the

plant height. The combined influence of colchicine concentration and treatment duration produced significant effects ($p<0.01$) on the plant height. The table 25 displayed the differentiation of mean values following LSD analysis at a 5% significance level.

According to the analysis of variance at a 5% significance level, the concentration of colchicine exerted a significant influence on all the observed morphological characteristics in exotic red ginger (Table 24). However, the duration of the treatment was found to be significant only for certain traits, including plant height, number of shoots, and the number of leaves. The combined effect of concentration and duration was significant only for plant height and the number of shoots, while the rest of the characters showed no significant variation. The separation of mean values was illustrated in the Table. 25 after conducting LSD at a 5% significance level.

Fig. 20. Acclimatization of *in vitro*-treated plants under greenhouse condition; (a-b) Hardening inside mist chamber; (c-d) Plants under greenhouse condition

Table 24. Analysis of variance for the selected morphological characters of Indian and exotic red ginger genotypes after *in vitro* colchicine treatment

Characters	Indian red ginger					Exotic red ginger				
	Concentration	Duration	Concentration x Duration	Error	CV (%)	Concentration	Duration	Concentration x Duration	Error	CV (%)
Degrees of freedom	4	1	4	20		4	1	3	18	
Plant height	368.05***	85.68*	67.60**	10.76	9.41	378.18***	472.59**	108.40*	32.59	16.20
Number of shoots	3.55***	0.53^{ns}	0.78^{ns}	0.50	25.87	2.38*	5.04**	1.82*	0.59	27.71
Shoot diameter	0.05*	0.001^{ns}	0.01^{ns}	0.01	18.76	0.11**	0.004^{ns}	0.03^{ns}	0.02	22.33
Number of leaves on main shoot	20.70***	2.13^{ns}	3.80^{ns}	2.27	12.01	23.46**	104.17***	12.17^{ns}	4.48	16.62
Leaf length	22.87**	0.18^{ns}	3.63^{ns}	4.19	14.52	73.49***	0.01^{ns}	6.27^{ns}	8.34	16.13
Leaf width	0.36^{ns}	0.03^{ns}	0.14^{ns}	0.16	17.67	0.41**	0.35*	0.03^{ns}	0.06	14.34

4.3.2.2.1 Plant height (cm)

In Indian red ginger, the *in vitro* colchicine treatment demonstrated a highly significant variation in plant height, which was associated with the concentration and duration of the treatment. The maximum plant height was observed in the control group for both 48 h (46.60 cm) and 24 h (43.17 cm), followed by 0.025 % colchicine for 24 h (39.33 cm), which was statistically on par with 0.05 % colchicine for 24 h (39.17 cm). The minimum plant height was recorded at 0.1% colchicine for 48h (18.83 cm).

The exotic red ginger exhibited significant variation in plant height compared to the control, with a range spanning from 16.33 to 45.67 cm. The control group displayed the highest mean plant height at 45.67 cm, while the lowest mean height was observed in the 0.1% colchicine treatment for 24 h, measuring 16.33 cm. Notably, among the various treatments, the maximum height was recorded at 0.025 % colchicine for 48 h (45.67 cm), which was comparable to the height at 0.05 % for 48 hours (42.00 cm). A significant reduction in plant height was evident among the treated groups from the 0.075 % concentration onwards.

4.3.2.2.2 Number of shoots

A significant difference in the number of shoots was observed with the colchicine treatment in Indian red ginger. The treatment with 0.10 % colchicine for 48 hours recorded the lowest number of shoots, measuring 1.33. This value was statistically equivalent to the results obtained with 0.10 % colchicine for 24 h (2.33) and 0.075 % colchicine for 48 h (1.67). In contrast, the control group recorded the maximum number of shoots, with a count of 4.00.

Among the exotic type, the highest number of shoots was noted in the 0.075 % treatment for 48 h (4.33), and this result was statistically comparable to the 0.025 % (4.00) treatment for the same duration. The lowest number of shoots was recorded in the 0.1% colchicine treatment for 24 h (1.33). The remaining treatments showed no statistically significant difference compared to the control.

4.3.2.2.3 Shoot diameter (cm)

In Indian red ginger, shoot diameter showed significance with varying colchicine concentrations (Table 25). The highest shoot diameter was seen in the control for 24 h (0.78 cm) and 48 h (0.74 cm) and the lowest shoot diameter was observed with 0.1% colchicine for 48 h (0.42 cm). The duration of the treatment did not exert a significant impact on the shoot diameter of the plants.

In comparison to the treated plants of exotic type, the control group exhibited a higher shoot diameter. The highest average shoot diameter, measuring 0.87 cm, was recorded in the control group after 48 h. This result was statistically comparable to both the control group after 24 h (0.74 cm) and the 0.075 % treatment for 24 h (0.68 cm). Conversely, the lowest shoot diameter was observed in the 0.05 % treatment for 48 h, measuring 0.45 cm. Overall, a slight decrease in shoot diameter was observed in the treated plants.

4.3.2.2.4 Number of leaves

The concentration of the colchicine played significant effect on the number of India red ginger type. The control group exhibited the highest mean number of leaves, which were 15.67 (for 48 h) and 13.67 (for 24 h). On the other hand, the mean number of leaves did not show significant variations for colchicine concentrations of 0.025 %, 0.050 %, and 0.075 % when compared to the control. A reduction in the average number of leaves was noted with the application of 0.075 % colchicine for 48 h and 0.100 % colchicine for both 24 and 48 h. A colchicine concentration of 0.100% yielded the lowest average number of leaves, recorded at 9.67 for a duration of 48 h.

A notable disparity in the number of shoots was evident between the treatments and the control group in exotic red ginger. Plantlets treated with 0.025% colchicine for 48 h exhibited a significantly higher number of leaves on the main shoot (18.00), which was statistically comparable to the control for 48 h (16.67). No statistically notable differences were detected among the remaining treatments. The lowest number of leaves on the main shoot was noted in the 0.100 % colchicine treatment lasting for 24 h (8.00).

Table 25. Interactive effect of different colchicine concentrations and durations on selected morphological characteristics of the Indian and exotic red ginger genotypes

Concentration (%)	Duration (h)	Indian red ginger						Exotic red ginger					
		Plant height (cm)	No. of shoots	Shoot diameter (cm)	No. of leaves on main shoot	Leaf length (cm)	Leaf width (cm)	Plant height (cm)	No. of shoots	Shoot diameter (cm)	No. of leaves on main shoot	Leaf length (cm)	Leaf width (cm)
Control	24	43.17±3.79^{ab}	3.33±0.58^{ab}	0.78±0.04^{a}	13.67±1.03^{ab}	16.40±2.35^{ab}	2.33±0.29^{ab}	40.50±7.47^{ab}	3.00±0.58^{bc}	0.74±0.24^{ab}	11.67±2.08^{cd}	23.57±2.07^{a}	2.30±0.27^{a}
	48	46.60±3.25^{a}	4.00±1.00^{a}	0.74±0.05^{a}	15.67±0.58^{a}	18.03±0.56^{a}	2.57±0.12^{a}	45.67±4.04^{a}	2.67±0.58^{c}	0.87±0.08^{a}	16.67±0.58^{ab}	23.00±2.00^{ab}	1.93±0.06^{ab}
0.025	24	39.33±2.08^{bc}	3.33±0.58^{ab}	0.62±0.07^{ab}	14.33±0.58^{ab}	13.23±1.10^{bc}	2.07±0.21^{ab}	30.83±9.93^{bc}	2.33±0.58^{cd}	0.49±0.22^{cd}	10.33±2.08^{de}	17.63±5.09^{c}	1.57±0.49^{bc}
	48	37.67±2.89^{bcd}	3.33±0.58^{ab}	0.64±0.16^{a}	13.33±2.08^{ab}	14.17±3.62^{b}	2.33±0.29^{ab}	45.67±3.06^{a}	4.00±1.00^{ab}	0.50±0.08^{cd}	18.00±3.61^{a}	15.17±3.22^{cd}	1.43±0.12^{c}
0.05	24	39.17±1.04^{bc}	2.67±0.58^{bc}	0.70±0.10^{a}	13.00±2.65^{ab}	13.77±0.64^{bc}	2.50±0.50^{a}	25.17±7.69^{cd}	2.33±0.58^{cd}	0.49±0.12^{cd}	11.33±1.16^{cde}	14.73±2.19^{cd}	1.63±0.12^{bc}
	48	35.00±4.36^{cd}	2.67±0.58^{bc}	0.62±0.09^{ab}	12.67±1.16^{bc}	14.33±1.35^{ab}	2.20±0.27^{ab}	42.00±3.97^{a}	2.67±0.58^{c}	0.45±0.04^{d}	14.33±3.06^{bc}	17.00±1.00^{cd}	1.53±0.25^{bc}
0.075	24	28.00±4.09^{e}	2.67±1.16b^{c}	0.62±0.22^{ab}	12.33±1.53^{bcd}	13.67±3.51^{bc}	2.50±0.87^{a}	36.17±4.31^{ab}	2.33±0.58^{cd}	0.68±0.10^{abc}	11.67±2.08^{cd}	18.57±0.81^{bc}	1.90±0.17^{ab}
	48	27.83±2.26^{e}	1.67±0.58^{cd}	0.72±0.13^{a}	10.00±1.00de	13.73±1.97^{bc}	2.40±0.36^{a}	30.83±4.19^{bc}	4.33±1.52^{a}	0.46±0.04^{d}	12.67±0.58^{cd}	16.00±4.58^{cd}	1.53±0.25^{bc}
0.1	24	33.17±4.37^{de}	2.33±0.58^{bcd}	0.58±0.05^{ab}	10.67±1.16^{cde}	13.03±0.56^{bc}	2.07±0.31^{ab}	16.33±1.53^{d}	1.33±0.58^{d}	0.58±0.09^{bcd}	8.00±1.73^{e}	12.33±1.76^{d}	1.40±0.17^{c}
	48	18.83±3.01^{f}	1.33±0.58^{d}	0.42±0.19^{b}	9.67±1.53^{e}	10.60±1.74^{c}	1.67±0.29^{b}	-	-	-	-	-	-
LSD 5%		5.59	1.2	0.21	2.56	3.49	0.68	9.79	1.32	0.22	3.63	4.95	0.42

4.3.2.2.5 Leaf length (cm)

Apart from 0.1% colchicine for 48 h, there were no differences in the average leaf length observed among treated plants when compared to the control plant. 0.1% colchicine for 48 h had a significantly lower leaf length of 10.60 cm, while control had a significantly higher leaf length of 18.03 cm.

The leaf length of exotic red ginger varied between 12.33 and 23.57 cm. Colchicine-treated plants exhibited a significant reduction in leaf length compared to the control. The control group for 24 h recorded the highest leaf length (23.57 cm), whereas the lowest leaf length was observed in the plantlets treated with 0.1% colchicine for 24 h (12.33 cm).

4.3.2.2.6 Leaf width (cm)

No significant difference was observed in the mean leaf width among the treated plants of Indian red ginger. The leaf width ranged from 1.67 cm to 2.57 cm. The maximum leaf width was recorded in the control group for 48 h, while the minimum leaf width was observed with 0.1% colchicine treatment for 48 h.

In the exotic type, a significant difference in the average leaf width was recorded compared to the control, with the leaf width ranging from 1.40 to 2.30 cm. The plantlets displayed a maximum leaf width of 2.30 cm in the control for 24 h, and this result was statistically comparable to the control for 48 hours (1.93 cm). Among the colchicine-treated plantlets, the mean leaf width was highest for 0.075% for 24 h (1.90 cm). The lowest leaf width was observed at 0.1% colchicine for 24 h (1.40 cm), and this was statistically comparable to 0.025% for 48 h (1.43 cm). The variation in leaf width was not proportional to the concentration and duration of the treatment.

The impact of colchicine on morphological characteristics was evident in our study. Indian red ginger displayed significant differences in plant height, number of shoots, shoot diameter, number of leaves, and leaf length. However, the variation in leaf width was found to be insignificant among the various treatments. For exotic type, all the morphological characters were found to be significant for different

colchicine concentrations. The control group exhibited the highest mean values for all the morphological characters studied. A notable reduction in morphological parameters was observed with an increase in colchicine concentrations, although the decrease was not proportional. Furthermore, the impact of duration alone and the combined effect of duration and concentration on the treated population were significant for only a few characters. The adverse impact of colchicine on plant morphology was prominently evident at higher concentrations. Specifically, the treatment with the highest concentration of 0.10% colchicine for 48 h resulted in the lowest values for plant height, number of shoots, shoot diameter, number of leaves, leaf length, and width in Indian red ginger. Additionally, some of the surviving plantlets of the exotic type succumbed during the growth stage at this high concentration. Kazemi and Kaviani (2020) investigated the effect of colchicine on the morphology of *Catasetum pileatum.* The researchers observed that the surviving plantlets experienced mortality during growth stages. Despite this observation, there was no discernible difference in selected morphological characteristics, including leaf length, leaf width, *etc.* Lindayani *et al*. (2010) observed a reduction in the height of *Z. officinale* and *Z. officinale* var. *rubrum* plants with increasing colchicine concentrations. In a similar study involving *Neolamarckia cadamba,* seeds and *in vitro*-derived nodal segments treated with colchicine exhibited a noteworthy increase in stem diameter and a reduction in plant height. Additionally, no apparent trend was observed in the number of leaves with varying concentrations of colchicine (Eng *et al*., 2021). Several articles have examined the morphological traits of regenerants originating from explants treated with colchicine in comparison to control plants. The experiments assessed a range of plant morphological features, including plant height, stem diameter, number of shoots, number of leaves, leaf colour, and the presence of abnormal leaves (Amiri *et al*., 2010; Kerdsuwan, & Te-chato, 2012; Baig *et al*., 2016; El-Latif *et al*., 2018; Singh *et al*., 2020; Boonyawiwat *et al*., 2023). These morphological parameters are used to detect the potential polyploids from a large, treated population. The degree of variation observed in plant morphological characteristics may indicate genetic alterations and serve as an indicator of the efficacy of mutagenic treatments.

4.3.2.3 Effect of colchicine treatment on physiological parameters of red ginger genotypes

The ANOVA result clearly depicted the influence of colchicine on the stomatal parameters like stomatal length, stomatal width, and stomatal density. Indian red ginger exhibited highly significant differences in all parameters with varying concentrations of colchicine and time durations. After the colchicine treatment, exotic red ginger also exhibited significant variations in stomatal characteristics. The result of the analysis of variance for the selected stomatal parameters are depicted in the Table 26. In exotic red ginger, the stomatal parameters were significantly affected by the concentration of colchicine. The duration of the treatment was significant for all parameters except for stomatal width. The combined influence of both the concentration and duration of the treatment was evident in the observed stomatal length and width. However, no major influence on stomatal density was identified. Interactive effect of different colchicine concentrations and duration of treatments in the selected stomatal parameters of Indian and exotic red ginger under *in vitro* conditions is depicted in the Table 27.

4.3.2.3.1 Stomatal length (µm)

In Indian red ginger, significant variations in stomatal length were observed at different colchicine concentrations, while no significant effects were observed for varying treatment durations.

Table 26. Analysis of variance of stomatal parameters of the Indian and exotic red ginger genotypes after *in vitro* colchicine treatment

Characters	Indian red ginger					Exotic red ginger				
	Concentration	Duration	Concentration x Duration	Error	CV (%)	Concentration	Duration	Concentration x Duration	Error	CV (%)
Degrees of freedom	4	1	2	90		4	1	3	81	
Stomatal length	6.55***	0.55^{ns}	2.26*	0.83	6.55	2.22***	8.19***	4.70***	0.27	3.23
Stomatal width	1.94***	2.43***	1.89***	0.11	4.58	0.40**	0.35^{ns}	1.07***	0.10	4.19
Stomatal density	6.33**	8.42***	1.55**	0.40	11.83	9.12***	1.31***	0.18^{ns}	0.08	6.84

Highest stomatal length was observed at 0.025 % colchicine for 24h (14.82 µm) and it was statistically on par with 0.05 % for 24 h (14.37 µm) and 48h (14.69 µm) and 0.075 % for 48 h (14.41 µm). The lowest stomatal length was reported in the control group at 48 h (12.86 µm) and was statistically similar to the control group at 24 h (13.47 µm) and the 0.1% colchicine treatment for both 24 h (13.59 µm) and 48 h (13.42 µm).

In exotic red ginger, the highest mean stomatal length was recorded at 0.025 % for 48 h (16.75 µm), and it was statistically on par with control at 24 h (16.53 µm) and 48 h (16.42 µm), as well as 0.05 % (16.40 µm) and 0.075 % (16.44 µm) for 48 h. The lowest mean stomatal length was observed in the 0.025% colchicine treatment for 24 h (14.68 µm). No specific trend was observed in the results of stomatal length after the treatment.

4.3.2.3.2 Stomatal width (µm)

The effect of colchicine concentration and treatment time on stomatal width was found to be highly significant in Indian red ginger. the maximum stomatal width was reported at 0.025 % colchicine treatment for 24 h (8.23 µm). the minimum st0matal width was identified at 0.1 % colchicine for 48 h (6.82 µm).

The stomatal width exhibited high significance in exotic red ginger in response to various colchicine concentrations. The highest mean stomatal width, measuring 8.00 µm, was recorded in the 0.1 % concentration for 24 h. Conversely, the lowest mean stomatal width was observed in the 0.05 % treatment for 48 h, measuring 7.16 µm.

4.3.2.3.3 Stomatal density (stomata mm^{-2})

A significant variation in stomatal density was observed among the different treated populations following treatment with various colchicine concentrations and durations. The maximum stomatal density was reported in control for 48 h (6.47mm^{-2}) and 24 h (6.14mm^{-2}). The minimum stomatal density was found to be when treated with 0.1 % colchicine for 48 h, measuring 4.31 mm^{-2}, followed by 0.05 % (4.67mm^{-2}) and 0.075 % (4.80 mm-2) for 48 h.

Table 27. Interactive effect of different colchicine concentrations and duration of treatments in the selected stomatal parameters of Indian and exotic red ginger

Concentrations (%)	Duration (h)	Indian red ginger			Exotic red ginger		
		Stomatal length (µm)	Stomatal width (µm)	Stomatal density (mm^{-2})	Stomatal length (µm)	Stomatal width (µm)	Stomatal density (mm^{-2})
Control	24	13.47 ± 0.48^{de}	7.25 ± 0.25^{cd}	6.14 ± 0.70^{ab}	16.53 ± 0.57^{ab}	7.67 ± 0.55^{bc}	4.49 ± 0.27^{a}
	48	12.86 ± 0.93^{e}	6.91 ± 0.33^{ef}	6.47 ± 0.46^{a}	16.42 ± 0.45^{ab}	7.62 ± 0.29^{c}	4.60 ± 0.46^{a}
0.025	24	14.82 ± 1.36^{a}	8.23 ± 0.56^{a}	5.53 ± 0.85^{bc}	14.68 ± 0.44^{d}	7.59 ± 0.31^{c}	3.69 ± 0.18^{c}
	48	13.85 ± 0.56^{bcd}	7.18 ± 0.27^{de}	5.03 ± 0.55^{de}	16.75 ± 0.53^{a}	7.68 ± 0.31^{bc}	4.12 ± 0.33^{b}
0.050	24	14.37 ± 1.55^{abc}	7.15 ± 0.30^{de}	5.41 ± 0.51^{cd}	16.08 ± 0.52^{bc}	7.96 ± 0.24^{ab}	3.95 ± 0.37^{b}
	48	14.69 ± 0.47^{a}	7.77 ± 0.24^{b}	4.67 ± 0.48^{ef}	16.40 ± 0.52^{ab}	7.16 ± 0.28^{d}	4.36 ± 0.27^{a}
0.075	24	13.72 ± 0.64^{bcd}	7.49 ± 0.43^{bc}	5.70 ± 0.57^{bc}	16.15 ± 0.43^{b}	7.43 ± 0.20^{cd}	4.38 ± 0.25^{a}
	48	14.41 ± 0.68^{ab}	6.91 ± 0.20^{ef}	4.80 ± 0.79^{ef}	16.44 ± 0.46^{ab}	7.67 ± 0.32^{bc}	4.46 ± 0.14^{a}
0.100	24	13.59 ± 1.13^{cde}	7.02 ± 0.38^{def}	5.41 ± 0.62^{cd}	15.65 ± 0.71^{c}	8.00 ± 0.25^{a}	2.37 ± 0.17^{d}
	48	13.42 ± 0.57^{de}	6.82 ± 0.18^{f}	4.31 ± 0.68^{f}	-	-	-
LSD (5%)		0.81	0.30	0.56	0.46	0.29	0.25

The stomatal density of exotic red ginger genotypes exhibited highly significant variation in response to different concentrations and treatment durations. The maximum average stomatal density was observed in the control group for 48 h (4.60 mm^{-2}), followed by 24 h (4.49 mm^{-2}). The stomatal density of the control group was statistically comparable to 0.075 % for 48 h (4.46 mm^{-2}) and 24 h (4.38 mm^{-2}), followed by 0.05% for 48 h (4.36 mm^{-2}).

The lowest mean stomatal density was observed in the 0.1% colchicine treatment for 24 h (2.37 mm^{-2}). A significant decrease was observed at the higher concentration of colchicine compared to the control.

The stomatal parameters were significantly influenced by colchicine treatment under *in vitro* condition, and detailed measurements are provided in the table 27. In the current study, the control group exhibited the highest stomatal density for both genotypes. Indian red ginger displayed the lowest stomatal density, with 4.31 stomata mm^{-2} recorded at 0.10 % colchicine for 48 h. The exotic type exhibited the lowest stomatal density at 0.10 % colchicine for 24 h, recorded 2.37 stomata mm^{-2}. However, no specific trend was observed in stomatal length and width compared to the control. The stomatal density was identified as a stable physiological parameter, aiding in the identification of potential polyploids within the extensive treated population. The previous results indicated that plants subjected to colchicine treatment exhibited a notable increase in stomatal size, particularly evident in *Glycyrrhiza glabra* plantlets treated with 0.10 % colchicine for 24 h and *Carthamus tinctorius* plantlets treated with 0.03 %, 0.05 %, and 0.10 % colchicine (Moghbel at al., 2015). Kazemi and Kaviani (2020) similarly noted a significant difference in the number of stomata and guard cell length within the treated population of *Catasetum pileatum*. Consistent with our findings, they observed that colchicine treatment generated no specific trend in stomatal variations. Comparable findings of enlarged stomata and reduced stomatal density have been observed in horticultural and agronomic crops induced by colchicine, including *Stevia rebaudiana* (Azizan *et al*., 2021), *Gladiolus grandifloras* (Manzoor *et al*., 2018), and *Tagetes erecta* (Sajjad *et al*., 2013).

4.3.2.4 Identification and selection of putative polyploids

A total of 17 putative polyploids of Indian red ginger and 11 exotic red ginger plants were selected based on the altered morphology and stomatal parameters such as increased stomatal size and decreased stomatal density. These chosen plants underwent additional scrutiny to validate their ploidy status.

4.3.2.5 Ploidy determination

4.3.2.5.1 Ploidy determination by flow cytometry

The determination of the ploidy level of the newly generated putative polyploids was conducted using flow cytometry. Among the 17 putative polyploids identified from the Indian red ginger, six plants were detected as tetraploids. The results demonstrated that when analysing the relative nuclear DNA content of the diploid control via linear histograms, conspicuous G0/G1 peaks were observed at channel 5.1 x 10^5 (Fig. 21. A). In contrast, the induced polyploid plantlets exhibited an analogous peak at channel 10 x 10^5, which was identified as potential tetraploids (Fig. 21. B-F). The putative polyploids identified from the exotic red ginger genotype were evaluated using flow cytometry to determine their ploidy, and none of them were found to be polyploids.

Table 28. The influence of different colchicine concentrations and treatment durations on polyploidy induction in Indian red ginger

Colchicine concentration (%)	Duration (h)	Total number of explants treated	Number of explants regenerated	Number of tetraploids	Tetraploid induction rate (%)
0.025	24	72	65	1	1.54
	48	72	58	0	0
0.050	24	72	61	2	3.28
	48	72	40	0	0
0.075	24	72	56	0	0
	48	72	36	0	0
0.100	24	72	45	1	2.22
	48	72	27	2	7.41

The effect of various concentrations of colchicine and treatment time on the polyploidy induction of Indian red ginger was illustrated in the table 28. All the induced polyploids were confirmed as tetraploids after flowcytometric analysis. The highest tetraploidy induction was observed in the treatment using 0.100 % colchicine for 48 h (7.41 %), followed by 0.050 % colchicine for 24 h (3.28 %). The lowest polyploidy induction rate was recorded at a concentration of 0.025% for 24 h (1.54 %). Conversely, colchicine treatment of 0.075 % proved ineffective, displaying a 0% polyploid induction rate for both the time durations. Among the six polyploids, one failed to develop a viable root system and subsequently perished. Smith and Hamill (1997) developed autotetraploids of ginger by immersing *in vitro* shoots in 0.5 % colchicine for 2 h. *In vitro* polyploidy induction in ginger was attempted by Adaniya and Shirai (2001) and identified 0.2 % colchicine for eight days was effective in tetarploidy induction. Kun-Hua *et al*. (2011) reported maximum tetraploidy induction in ginger at 0.2% colchicine for 30h under *in vitro* condition. Recently, Zhou *et al*. (2020) achieved tetraploidy induction in ginger at 150 mg L^{-1} colchicine for seven days. This is the first report of *in vitro* tetraploidy induction in Indian red ginger genotype.

4.3.2.6 Comparative characterization of diploids and tetraploids in the MIV1 generation

The impact of the colchicine treatment was evident in the induced tetraploids, characterized by delayed and stunted growth. The confirmed tetraploids of the Indian red ginger genotype were compared with the diploid control in the first generation (Fig. 21. a-f). The morphological characteristics such as plant height, number of shoots, shoot diameter, number of leaves, leaf length, leaf width, leaf area and leaf colour were recorded. Furthermore, the effect of colchicine treatment on the stomatal parameters such as, stomatal length, width and density was evaluated.

4.3.2.6.1 Morphological parameters

The studied morphological parameters of diploids and tetraploids are enlisted in the table 29. T-test revealed significant variations in the studied morphological parameters of diploids and tetraploids.

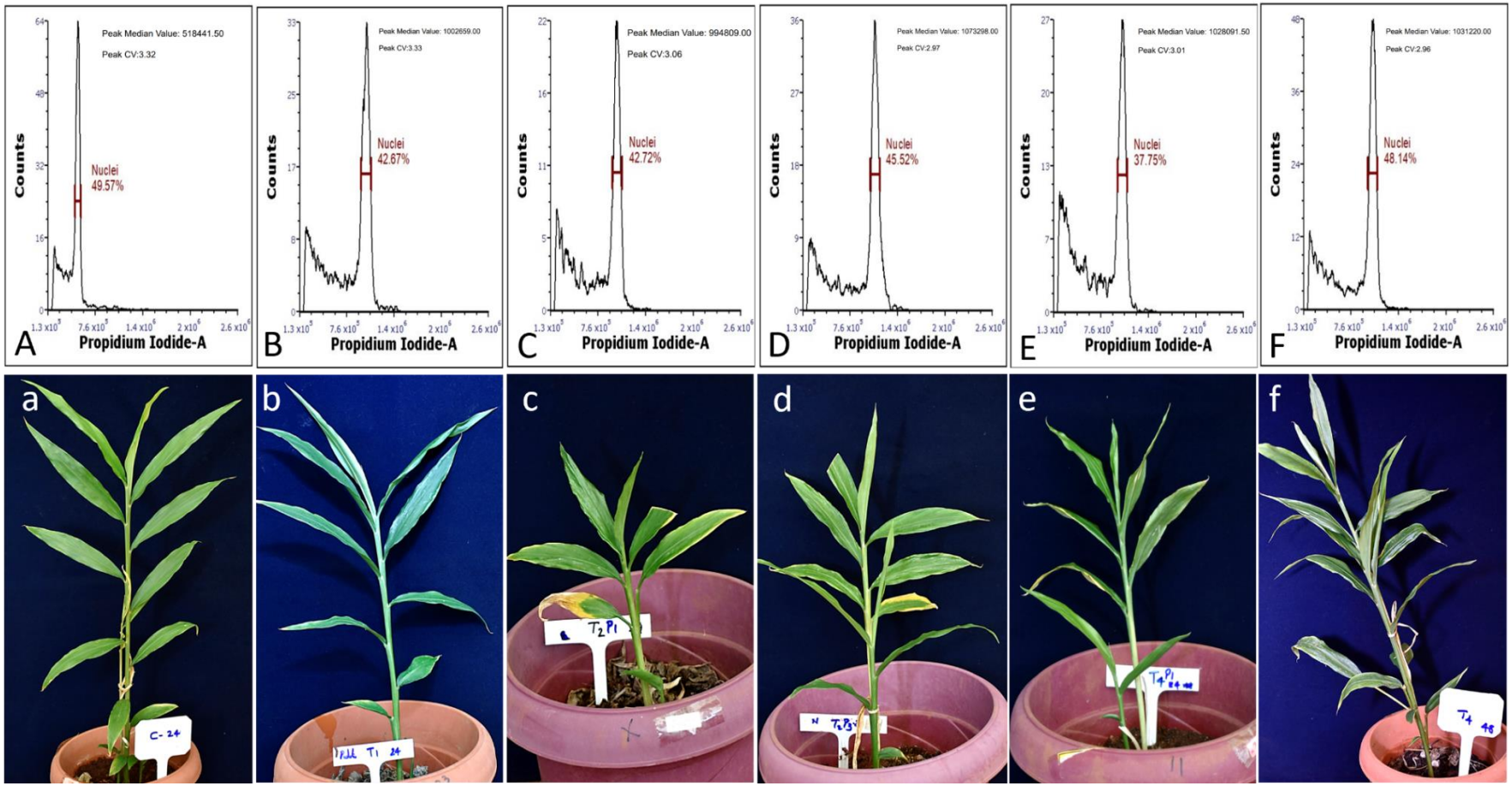

Fig. 21. Flow cytometric histogram of (A) diploid control and (B-F) confirmed tetraploids after *in vitro* colchicine treatment; (a-f) Diploid control and induced tetraploids of M1V1 generation, (a) Diploid control (Coll. No. 9073), (b) Tetraploid (0.025%, 24 h) (c) Tetraploid (0.05%, 24 h), (d) Tetraploid (0.05%, 24 h), (e) Tetraploid (0.10%, 24 h), (f) Tetraploid (0.10%, 48 h)

Table 29. Comparison of morphological characteristics of diploids and tetraploids of M1V1 generation

Ploidy level	Plant height (cm)	No. of shoots	Shoot diameter (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Yield per plant (g)
Diploids	47.27± 2.16	4.00± 0.00	0.69± 0.09	16.00± 2.00	16.93± 0.51	2.37± 0.32	25.98± 2.81	13.67± 2.08
Tetraploids	47.67± 5.51	2.67± 0.33	0.97± 0.14	15.00± 2.00	21.83± 2.02	3.17± 0.29	44.74± 2.52	24.33± 4.04
t- value	0.12	4.00	2.80	0.61	4.07	3.21	8.60	3.18
p- value	0.91	0.02	0.05	0.57	0.06	0.03	0.00	0.02

Data represents mean±standard deviation.

4.3.2.6.1.1 Plant height (cm)

The plant height of tetraploids was recorded as 47.67 cm, while diploids exhibited a height of 47.27 cm, indicating any significant difference between the two.

4.3.2.6.1.2 Number of shoots

A significant difference was observed in the number of shoots between diploids and tetraploids. Diploids (4.00) exhibited a significantly higher mean number of shoots compared to tetraploids (2.67).

4.3.2.6.1.3 Shoot diameter (cm)

The induced tetraploids exhibited a significantly higher shoot diameter of 0.97 cm, whereas the diploids exhibited a significantly lower shoot diameter of 0.69 cm.

4.3.2.6.1.4 Number of leaves

No significant difference was observed in the mean number of leaves between the diploids and induced tetraploids. The mean number of leaves in the diploids was 16.00, while in tetraploids, it was 15.00.

4.3.2.6.1.5 Leaf length (cm)

A slight increase in leaf length was observed in the induced tetraploids, but it was not statistically significant. The diploids recorded an average leaf length of 16.93 cm, whereas the tetraploids showed an average length of 21.83 cm (Fig. 22).

4.3.2.6.1.6 Leaf width (cm)

A significant increase in leaf width was observed in the tetraploid Indian red ginger compared to corresponding diploids. The average leaf width of induced tetraploids was recorded at 3.17 cm, while diploids exhibited a mean leaf width of 2.37 cm.

4.3.2.6.1.7 Leaf area (cm^2)

The leaf area was highly significant for induced tetraploids and diploids. Tetraploids recorded an average leaf area of 44.74 cm^2, whereas diploids exhibited an average leaf area of 25.98 cm^2.

Fig. 22. Variation in the leaf parameters of induced tetraploid ginger compared to diploid control: (a) Diploid control; (b) Tetraploid (0.025%, 24 (h); (c) Tetraploid (0.050%, 24 h); (d) Tetraploid (0.050%, 24 h); (e) Tetraploid (0.100%, 24 h); (f) Tetraploid (0.100%, 48 (h)

4.3.2.6.1.8 Yield per plant (g)

The yield per plant exhibited significant variation between the induced tetraploids compared to diploids (Table 29, Fig. 23). Tetraploids showed a notably substantial rise in yield per plant, with a fresh weight of 24.33 g, whereas diploids yielded an average of 13.67 g.

Fig. 23. Variation in the fresh rhizome yield of (a) Diploid Indian red ginger (control); (b) *In vitro* raised tetraploid Indian red ginger

4.3.2.6.1.9 Leaf colour

Significant variation was observed in the leaf colour of diploids and induced tetraploids of Indian red ginger (Fig). The diploid control exhibited normal green colour with a colour code of G137C. While induced tetraploids showed dark green coloured leaves (GN137A).

Morphological variations observed in induced polyploids are commonly referred to as the "gigas effect" (Balao *et al*., 2011). A common outcome of polyploidy is the enlargement of cell size, attributed to a greater number of gene copies. Nevertheless, this enlargement in cell size does not necessarily lead to a proportional increase in the size of the entire plant or its organs, as polyploids may exhibit a reduction in the number of cell divisions (Sattler *et al*., 2016). The process of induced tetraploidization in Indian red ginger has resulted in a spectrum of morphological variations, including a decrease in the number of shoots alongside increases in shoot diameter, leaf width, and leaf area, and resulted in the improved rhizome yield (Table 29, Fig. 22 & 23). On the other hand, the tetraploids showed no significant variation in plant height, number of leaves, and leaf length compared to the diploids Conversely, Adaniya and Shirai (2001) observed increased plant height in tetraploid ginger resulting from *in vitro* colchicine treatment. A notable enhancements in leaf area, width, and thickness were evident in induced polyploids of *Thymus vulgaris* (Shmeit *et al*., 2020) and *Digitalis lanata* (Bhusare *et al*., 2021). The differences in morphological parameters were discernible solely under greenhouse conditions. Similar to Kun-Hua *et al*. (2011), who observed an increase in leaf length and width following *in vitro* colchicine treatment in ginger, these

changes became significant when the plants were grown in greenhouse conditions rather than *in vitro*. In consistent with Zhou *et al*. (2020), the leaves of the tetraploid ginger were greener and wider compared to diploids. In a recent study, the polyploid variants of *Mentha spicata* demonstrated a notable increase in leaf area, as well as thickness of leaves and stems, nearly doubling in comparison to the control genotype (Bharati *et al*., 2023a). Hence, leaf morphological characteristics, such as increased width, thickness, and darker green coloration, can serve as criteria for pre-screening tetraploids. In alignment with the findings of the current study, Smith *et al.* (2004) noted that tetraploid ginger exhibited a reduced number of shoots and leaves, despite being larger in size. Artificially induced tetraploid plants of *Catasetum pileatum* (Kazemi & Kaviani, 2020), *Hyoscyamus reticulatus* (Madani *et al*., 2015), *Tetradenia riparia* (Hannweg *et al*., 2016), *Allium cepa* (Ren *et al*., 2018), and *Eclipta alba* (Salma *et al*., 2018) exhibited superior phenotypic traits. Improved morphological traits of induced tetraploids can enhance the yield and quality attributes (Smith & Hamill, 1997; Zhou *et al*., 2020). Enhancing the yield of rhizomes is a key objective in ginger breeding. Therefore, the rhizome yields of both diploid and tetraploids were assessed. The findings revealed that induced tetraploids (24.33 g) exhibited higher rhizome yields compared to the control (13.67 g). Similar to the present findings, Adaniya and Shirai (2001) observed larger rhizomes in tetraploids than the diploids. There was an augmentation in the fresh weight of the rhizome observed in *Z. officinale* var. *rubrum* raised *in vitro* following colchicine treatment. (Lindayani *et al*., 2010). Smith *et al.* (2004) demonstrated that *in vitro* colchicine treatment also resulted in the development of a robust tetraploid ginger line. Similar observation was also reported by Kun-Hua *et al*. (2011).

4.3.2.7 Physiological parameters

T-test revealed significant difference in the studied physiological parameters such as stomatal length, stomatal width, stomatal density, and chloroplast count with the help of light microscopy imaging and detailed results are given in the table 30 (Fig. 24). Scanning electron microscopy imaging confirmed the identified variations in the stomatal size and density (Fig.25)

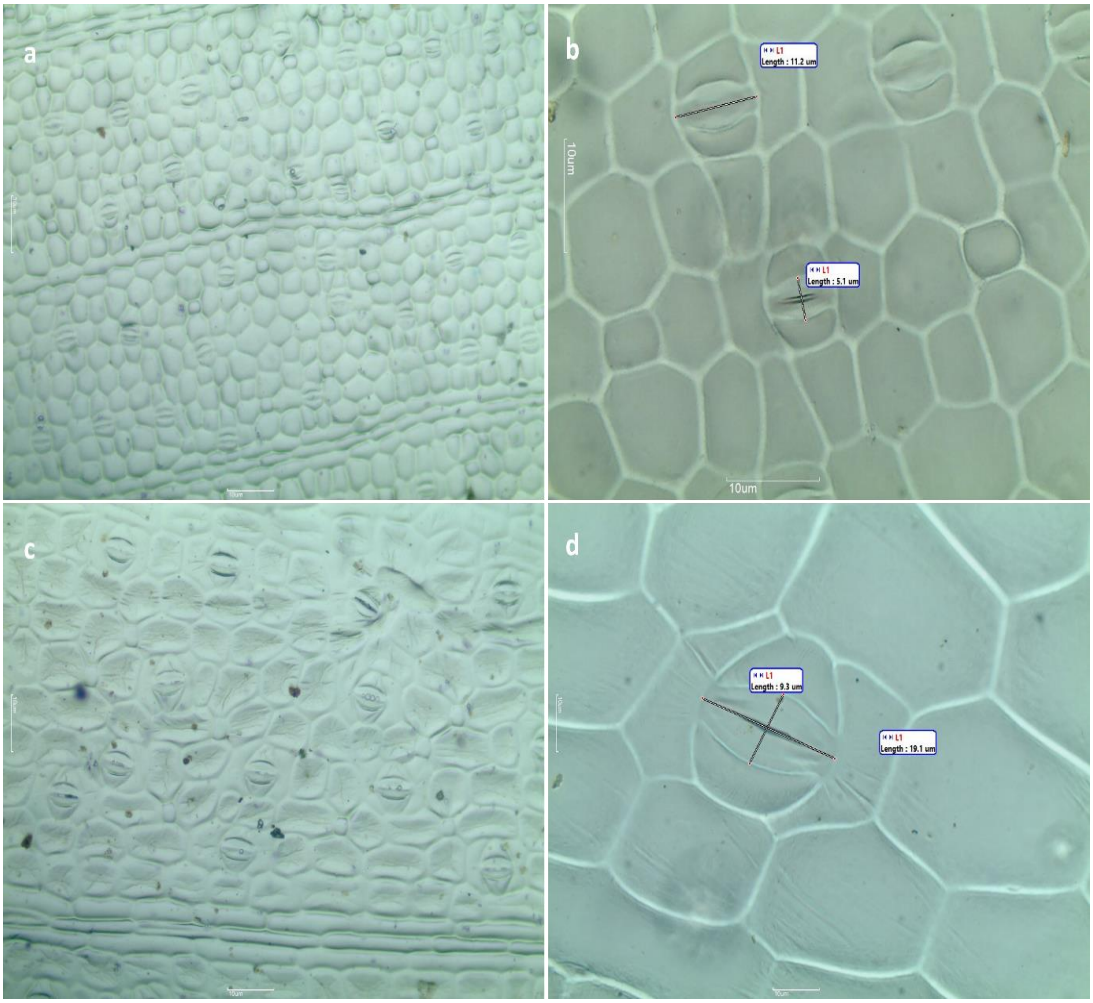

Fig. 24. Variations in the stomatal parameters of diploids and induced tetraploids (M1V1): (a) Stomatal density of diploid Indian red ginger at 10x magnification; (b) Stomatal size of diploid Indian red ginger at 40x magnification; (c) Stomatal density of tetraploid Indian red ginger at 10x magnification; (d) Stomatal size of tetraploid Indian red ginger at 40x magnification, Scale bar = 10 µm

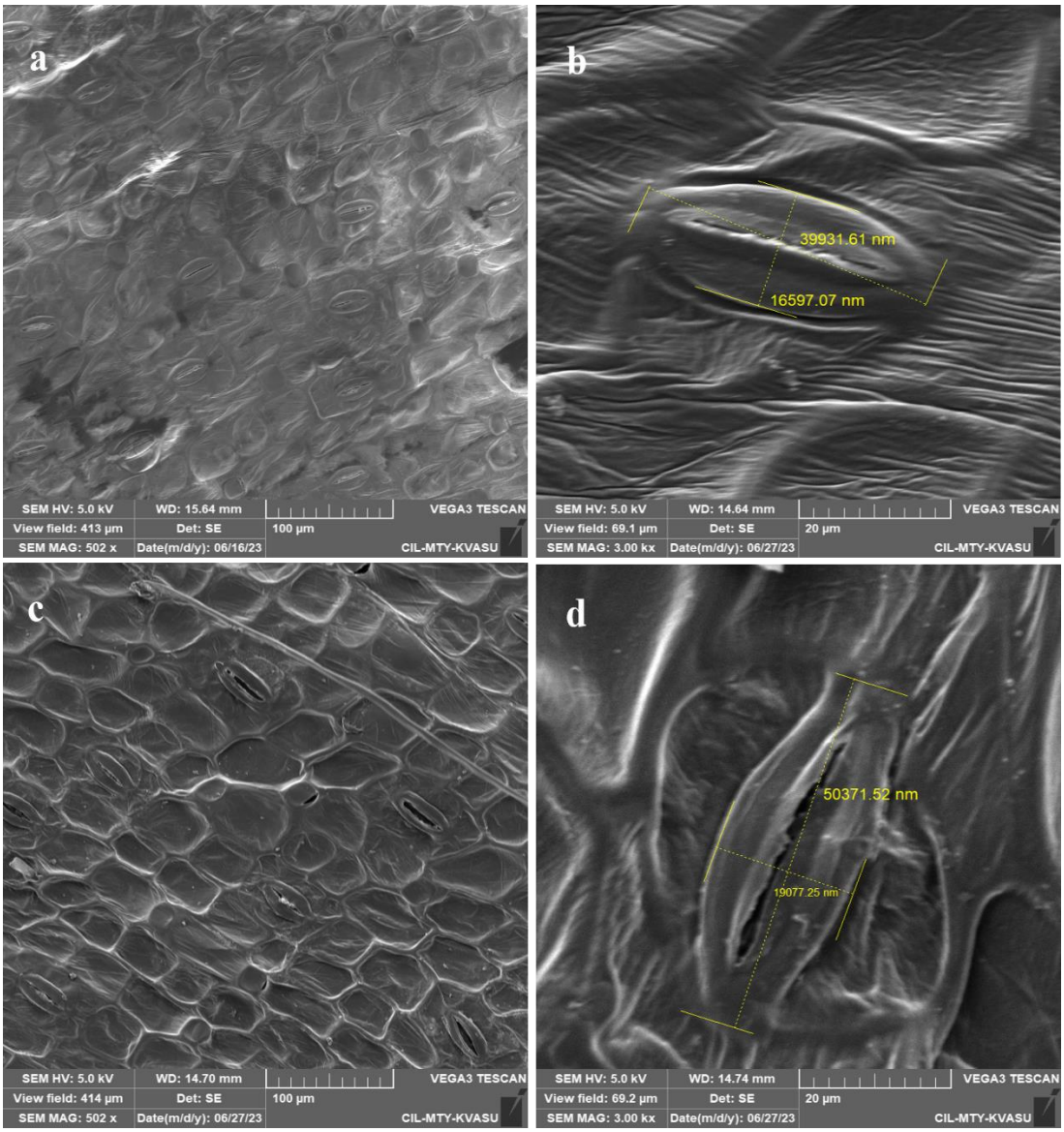

Fig. 25. Scanning Electron Microscopy images of diploid and tetraploid Indian red ginger; a) Lower epidermis of diploid; b) Enlarged single stomata of diploid; c) Lower epidermis of tetraploid; b) Enlarged single stomata of tetraploid

Table 30. Comparison of physiological characteristics of diploids and tetraploids of M1V1 generation

Ploidy level	Stomatal length (µm)	Stomatal width (µm)	Stomatal density (stomata mm^{-2})	Chloroplast count
Diploids	13.47±0.47	7.25±0.25	6.43±0.63	24.52±1.83
Tetraploids	20.58±0.65	9.24±0.30	2.83±0.29	45.88±1.53
t- value	23.96	16.16	16.47	28.37
p- value	0.00	0.00	0.00	0.00

4.3.2.7.1 Stomatal length (µm)

A highly significant difference was observed in the stomatal length of diploid Indian red ginger and induced tetraploids. Tetraploids recorded an average guard cell length of 20.58 µm, whereas the diploid counterpart exhibited an average guard cell length of only 13.47 µm.

4.3.2.7.2 Stomatal width (µm)

The stomatal width showed a highly significant difference between diploid Indian red ginger and induced tetraploids. The mean width of stomatal guard cells was recorded at 7.25 µm for diploids, while tetraploids exhibited an average stomatal width of 9.24 µm.

4.3.2.7.3 Stomatal density (stomata mm^{-2})

Significant variation was observed for the stomatal density between diploids and tetraploids. Induced tetraploids exhibited significantly lower stomatal density of 2.83 stomata mm^{-2} compared to diploids (6.43 stomata mm^{-2}).

4.3.2.7.4 Chloroplasts count

The chloroplasts count of induced tetraploids significantly varied from diploids. Tetraploids exhibited a twofold increase in chloroplast count, with a value of 45.88 compared to diploids' count of 24.52.

Stomatal density and size are crucial physiological traits influenced by alterations in ploidy levels (Lattier, *et al*., 2019). Previous research indicated a positive correlation between ploidy and stomata size, as well as a negative correlation between ploidy and stomata density across a broad spectrum of angiosperms (Beaulieu *et al*., 2008). The outcome of the present study similarly established a connection between ploidy level and stomatal parameters. Significant difference in the stomatal parameters including size, density and chloroplast count were illustrated as box plots in the Fig. 27. In the present study, induced tetraploids exhibited enlarged stomatal length and width compared to their diploid counterparts (Table. 30, Fig. 24). A similar effect was observed in citrus upon polyploidization (Xue *et al*., 2017). The stomata on tetraploid leaves exhibited greater length and width compared to those on diploid leaves (Kun-Hua *et al*., 2011).

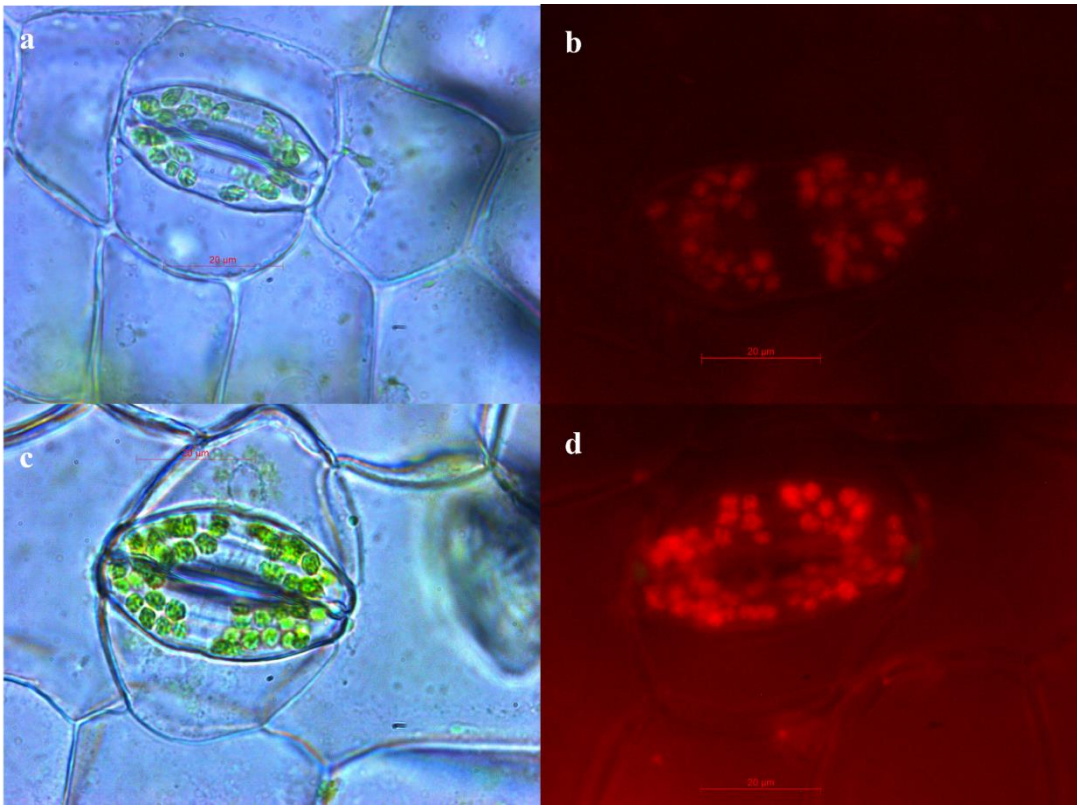

Fig. 26. Variation in chloroplast count of diploid and tetraploid stomatal guard cells; a) Light microscopy image of diploid stomata; b) Fluorescence microscopy image of diploid stomata; c) Light microscopy image of diploid stomata; d) Fluorescence microscopy image of diploid stomata

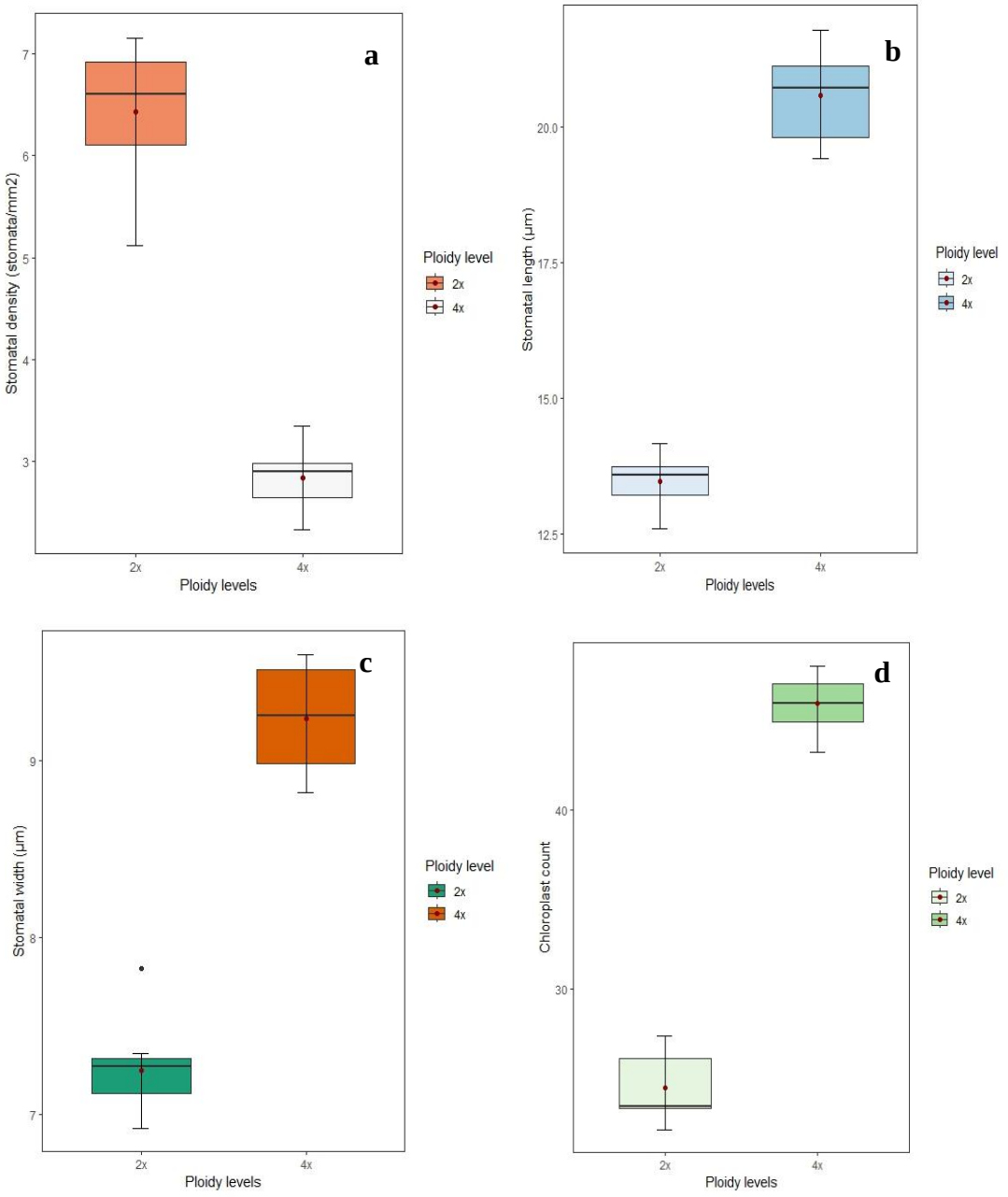

Fig. 27. Box plot of stomatal parameters in diploids (2x) and induced tetraploids (4x); (a) Stomatal density; (b) Stomatal length; (c) Stomatal width; (d) Chloroplast count

Consistent findings were observed in similar studies investigating *in vitro* polyploidy induction in ginger, as documented by Smith and Hamill (1997), Smith *et al*. (2004), and Zhou *et al*. (2020). Tetraploid cassava leaves possessed stomatal guard cells that are fewer in number but larger in size compared to those found in diploids (Mondin *et al*., 2018). The statistical analysis of stomatal parameters unveiled a significant decrease in the stomatal density of tetraploid Indian red ginger. This finding aligns with the research conducted by Ren *et al*. (2018), who observed a similar trend in tetraploid onion. Several other studies have also reported a notable reduction in stomatal density following polyploidization. Notably, Wen *et al*. (2022), Noori *et al*. (2017), Wannakrairoj and Tefera (2013), and Gantait *et al*. (2011) have documented such decreases in their respective investigations. A distinct contrast in chloroplast numbers within guard cells was evident between diploid and tetraploid plantlets, as seen in *Lilium* (Jeloudar *et al*., 2019). Furthermore, the number of chloroplasts in stomatal guard cells of tetraploids is twice that of diploids (Fig. 26). Similar observation was also reported in *Pogostemon cablin* (Yan *et al*., 2016), *Manihot esculenta* (Zhou *et al*., 2017), and *Lycium ruthenicum* (Rao *et al*., 2019). Significant rise in the number of chloroplasts within stomatal guard cells was utilized as a marker for detecting whole-genome duplication in *Lagerstroemia indica* (Zhang *et al*., 2010).

4.3.3 *In vivo* polyploidy induction

4.3.3.1 Effect of colchicine treatment on sprouting rate of red ginger genotypes and polyploidy induction efficiency

A small piece of the rhizome with a single bud was treated with different concentrations (0.00, 0.05, 0.10, 0.15, 0.20 %) of colchicine for 24 and 48 h. The initial effect of the colchicine treatment was delayed sprouting. The buds in the control showed the sign of germination after one week. Whereas in treated buds the emergence of green shoot was noticed even after 3-4 weeks (Fig. 28). The ANOVA result (Table 31) exhibited highly significant difference between the various colchicine concentrations and treatment durations on the sprouting rate in Indian red ginger ($p<0.001$). The sprouting rate after treatment is given in the Table 32.

Maximum sprouting was observed in the control for 24h (97.22 %) and 48h (94.44 %). Among the treated groups, the highest sprouting rate was observed when treated with 0.05 % (84.72 %) colchicine for 24 h. The lowest sprouting of 11.11 % was observed at colchicine concentration 0.20% for 48 h. As the concentration of colchicine and duration increased the sprouting rate decreased significantly (Table 32).

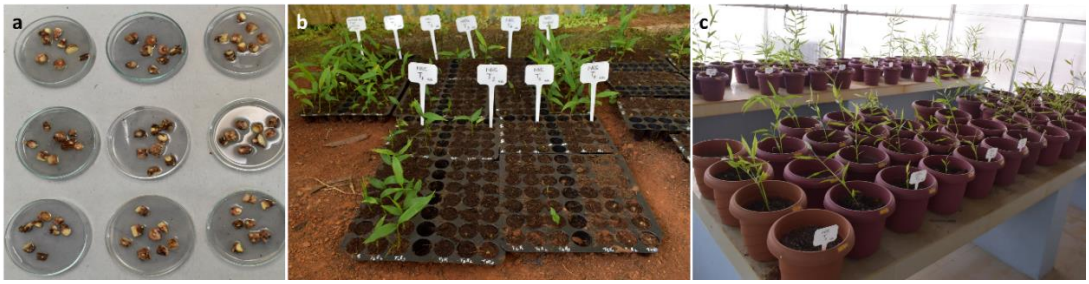

Fig. 28. (a) Colchicine treatment of single bud rhizome under *in vivo* condition; (b) Germinated plantlets after two months; (c) Survived plantlets after four months under greenhouse condition

Table 31. Analysis of variance of different concentrations of colchicine and durations on the sprouting rate of Indian red ginger and exotic red ginger

Responses	Degree of freedom	Mean squares	
		Indian red ginger	Exotic red ginger
Concentration	4	1210.34***	5741.7 ***
Duration	1	1210.34***	1522.9 ***
Concentration x Duration	4	111.39*	177.6 ***
Error	30	40.36	22.40
CV (%)		10.99	12.57

The ANOVA results indicated that varying concentrations of colchicine and durations significantly impacted the percentage of sprouting in exotic red ginger ($p<0.001$) (Table 31). The highest sprouting was recorded in control for 24 h (93.75 %) and 48 h (91.67 %) (Table 32). The sprouting rate in the treated groups varied from 0.00 to 70.83 %, with the highest percentage of sprouting observed at the lowest colchicine concentration of 0.05 %. Rhizome buds exposed to higher

concentrations of colchicine showed lower sprouting rate. No sprouting was noted in the treated buds following exposure to a 0.15 % colchicine solution for 48 h. A similar occurrence was observed in the treatment with a 0.20 % colchicine solution for 48 h. The 24 h treatment with 0.20 % colchicine resulted in a very low sprouting rate of 12.50 %. It can be concluded that excessively high concentrations of colchicine may be toxic to the rhizome buds, leading to cell death. As per the findings, the sprouting rate of exotic red ginger decreased as the colchicine concentration and the duration of treatment increased. Polyploidy induction was noticed at a colchicine concentration of 0.15 % for 24 h; however, the efficiency of polyploidy induction was found to be low (16.67 %).

Table 32. Effect of colchicine concentrations and time durations on the sprouting rate of Indian and exotic red ginger

Colchicine concentration (%)	Duration (h)	Sprouting rate (%)	
		Indian red ginger	Exotic red ginger
Control (0)	24	97.22 (77.06) ± 3.21^{a}	93.75 (74.79) ±7 .98^{a}
	48	94.44 (75.25) ± 4.54^{a}	91.67 (73.09) ± 6.81^{a}
0.05	24	84.72 (67.21) ± 5.32^{b}	70.83 (57.01) ± 10.76^{b}
	48	83.33 (66.05) ± 4.54^{b}	47.92 (43.80) ± 7.98^{c}
0.10	24	73.61 (59.17) ± 5.32^{c}	45.84 (42.60) ± 4.81^{c}
	48	62.50 (52.27) ± 5.32^{d}	39.58 (38.93) ± 7.98^{c}
0.15	24	59.72 (50.64) ± 5.32^{d}	16.67 (23.74) ± 6.81^{d}
	48	40.28 (39.36) ± 5.32^{e}	0.00 (0.83) ± 0.00^{e}
0.20	24	30.56 (33.53) ± 3.21^{f}	12.50 (20.44) ± 4.82^{d}
	48	11.11 (19.17) ± 4.54^{g}	0.00 (0.83) ± 0.00^{e}

Data represents mean ± standard deviation.

Polyploidization heavily relies on the interaction of colchicine concentration and the duration of treatment. The sprouting rate was proportional to the colchicine concentration and treatment time for both the genotypes. Higher concentrations of colchicine had a negative impact on the sprouting of rhizome buds. Based on the current findings, increased levels of colchicine and prolonged treatment duration led to reduced sprouting. The delay for sprouting is associated with the toxic effect of colchicine. The sprouting rate of exotic red ginger was very lower compared to Indian red ginger. One potential reason for this variation is that colchicine may be

able to penetrate rhizome buds due to the soft texture of exotic red ginger, potentially causing acute toxicity and cell death. All the treated buds were unable to germinate and some of the germinated buds were unable to complete its growth phase at the concentration of 0.15 % and 0.20 % for 48 h. Colchicine exhibited the greatest adverse impact on exotic red ginger (Acc. 899), leading to the lowest survival rate of 0.00 %. The lethality of colchicine has been demonstrated in various studies, with studies also reporting the lack of surviving plants after treatments. No individual seeds of *Petroselinum crispum* survived exposure to 0.10 % and 0.20 % colchicine for 48 h (Nasirvand *et al*., 2018). Similarly, treating garlic cloves with a 0.6% colchicine solution for 48 h resulted in a 0% survival rate (Yousef & Elsadek, 2020). Consistent with the current study, elevated concentrations of colchicine led to a reduced sprouting rate in ginger (Prasath *et al*., 2022). The effect of colchicine on the sprouting rate of colchicine was clear on some other plants like *Ricinus communis* (Baghyalakshmi *et al*., 2020), *Lagerstroemia indica* (Ye *et al*., 2010), *Crocus sativus* (Samadi *et al*., 2022), *Mentha suaveolens* (Moetamedipoor *et al*., 2022), and *Petroselinum crispum* (Nasirvand *et al*., 2018). Therefore, it is understood that employing moderate doses with prolonged exposure times or vice versa can be efficacious in mitigating its toxic effects and enhancing the rate of polyploid production.

Various researchers provided distinct explanations for the diminished growth observed at higher doses. Disruption in the synthesis of enzymes crucial for the germination process may represent a physiological consequence induced by colchicine, resulting in a reduction in germination rates (Roychowdhury & Tah, 2011). Decreased synthesis of auxins and brassinosteroids or the inhibition of signal transduction pathways may contribute to delayed sprouting (Ma *et al*., 2016).

4.3.3.2 Effect of colchicine treatment on morphological characteristics of red ginger genotypes

Morphological parameters such as plant height, number of tillers, shoot diameter, number of leaves, leaf length and leaf width were recorded from the germinated plantlets after colchicine treatment. Variations in the morphological

characteristics were used for the early selection of putative polyploids. The ANOVA results indicated that varying concentrations of colchicine had a significant effect ($p<0.05$) on some selected morphological characteristics. The results from the variance analysis indicated a significant influence of colchicine concentration on the observed morphological traits, including plant height, number of shoots, and number of leaves in Indian red ginger, at a 5% significance level (Table 33). Only the number of leaves showed a significant response to treatment time at the 1% probability level. The interactive effect of colchicine concentration and duration of treatment was insignificant for all the morphological traits.

At a 5% significance level, the analysis of variance showed that the concentration of colchicine had a significant impact on the observed morphological features in exotic red ginger, such as plant height, number of shoots and number of leaves (Table 33).

Whereas only the number of leaves was influenced by duration of the treatment. There was no statistically significant interactive effect observed between concentration and time duration on the morphological traits. Interactive effect of different colchicine concentrations and duration on selected morphological characteristics of Indian and exotic red ginger after LSD test is given in the Table 34.

4.3.3.2.1 Plant height (cm)

In Indian red ginger genotype, highly significant difference was observed for plant height at different colchicine concentrations. The values ranged from 26.00 to 50.75 cm. The highest plant height was displayed by the control group (24 h), measuring 50.75 cm. With increasing concentration, a slight reduction in plant height was observed. The lowest plant height was recorded at 0.2 % colchicine concentration for 24 h (26.00) and was statistically on par with 0.20 % for 48 h (30.90 cm), 0.15% for 24 h (27.50 cm) and 48 h (30.10 cm), and 0.10 % for 48 h (32.50 cm).

Table 33. Analysis of variance for the selected morphological characters of Indian and exotic red ginger genotype after *in vivo* colchicine treatment

Character	Indian red ginger					Exotic red ginger				
	Concentration	Duration	Concentration x Duration	Error	CV (%)	Concentration	Duration	Concentration x Duration	Error	CV (%)
Degrees of freedom	4	1	4	10		4	1	4	10	
Plant height	277.838***	5.83^{ns}	20.61^{ns}	12.92	10.05	509.47*	25.52^{ns}	13.08^{ns}	0.69	24.12
Number of shoots	3.63*	0.20^{ns}	0.33^{ns}	0.8	29.81	4.30*	0.75^{ns}	0.25^{ns}	0.002	6.62
Shoot diameter	0.003^{ns}	0.006^{ns}	0.001^{ns}	0.002	5.89	0.01^{ns}	0.00^{ns}	0.01^{ns}	5	15.16
Number of leaves on main shoot	25.16***	22.05**	4.93^{ns}	1.65	9.55	27.06*	70.08**	6.33^{ns}	5.2	12.69
Leaf length	3.79^{ns}	5.72^{ns}	1.26^{ns}	1.91	9.83	2.86^{ns}	13.02^{ns}	8.08^{ns}	0.07	15.52
Leaf width	0.14^{ns}	0.10^{ns}	0.02^{ns}	0.07	12.53	0.06^{ns}	0.33^{ns}	0.00^{ns}	0.69	24.12

Plant height significantly varied across different concentrations, ranging from 31.75 to 64.25 cm. But there was no significant variation was observed between the durations. The plants in the control group (48 h) produced significantly maximum height (64.25 cm), followed by control 24 h (59.50 cm) and 0.05 % 48h (53.00 cm). The lowest was recorded in 0.10 % 48 h (31.75 cm) and was statistically on par with 0.15 % and 0.20 %.

4.3.3.2.2 Number of shoots

In Indian red ginger, significant difference was noticed for number of shoots at various colchicine concentrations and varied from 1.5 to 4.5. Maximum number of shoots was observed at treatment 0.05 % for 24 h (4.5) and 48 h (4.5) followed by control for 48 h (3.5) and 24 h (3.0), and 0.15 % for 24h (3.0). The lowest number of shoots was recorded at 0.2 % for 48 h (1.5).

Exotic red ginger showed a statistically significant difference in the mean number of shoots among the treatments. The values varied from 2.5 to 5.5. Significantly maximum number of shoots was recorded for control 48 h (5.5) and was statistically on par with control 24 h (4.5) and 0.05 % for 48 h (4.0). The number of shoots decreased as the concentration of colchicine increased. Nevertheless, there was no statistically significant distinction between the treatments involving 0.10 %, 0.15 % and 0.20 % colchicine concentrations.

4.3.3.2.3 Shoot diameter (cm)

In Indian red ginger, no significant variation in shoot diameter was observed with respect to the concentration and duration of treatment. The values ranged from 0.62 to 0.72 cm. The height shoot diameter was recorded in the control group incubated for 24 h (0.72 cm). The lowest shoot diameter, measuring 0.62 cm, was observed at 0.15% and 0.20% colchicine concentrations after 48 h treatment.

There was no significant difference in shoot diameter of exotic red ginger treated with the different colchicine concentrations**.** The shoot diameter varied from 0.64 to 0.78 cm. The highest shoot diameter was recorded at control 24 h (0.78 cm) and was statistically on par with control 48 (0.70 cm), 0.05% 24 h (0.68 cm) and 0.05 % 48 h (0.75 cm). The lowest shoot diameter was observed at 0.20 % colchicine for 24 h (0.64 cm). It was significantly on par with 0.10 % (0.65 cm) and 0.15 % (0.67 cm).

Table 34. Interactive effect of different colchicine concentrations and durations on selected morphological characteristics of Indian and exotic red ginger

Concentration (%)	Durations (h)	Indian red ginger						Exotic red ginger					
		Plant height (cm)	No. of shoots	Shoot diameter (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)	Plant height (cm)	No. of shoots	Shoot diameter (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)
Control	24h	50.75± 1.77^{a}	3.00± 1.41^{abc}	0.72± 0.14^{a}	20.00± 1.41^{a}	16.45± 2.19^{a}	2.35± 0.49^{a}	59.50± 13.43^{ab}	4.50± 0.71^{ab}	0.78± 0.07^{a}	15.50± 2.12^{ab}	19.25± 0.35^{a}	1.90± 0.14^{a}
	48h	46.35± 1.91^{ab}	3.50± 0.70^{ab}	0.70± 0.14^{ab}	14.50± 0.71^{b}	14.50± 1.41^{ab}	2.10± 0.28^{ab}	64.25± 1.06^{a}	5.50± 0.71^{a}	0.70± 0.09^{abc}	20.00± 1.41^{a}	19.50± 1.41^{a}	1.60± 0.14^{a}
0.05	24h	41.50± 0.70^{bc}	4.50± 0.70^{a}	0.68± 0.21^{ab}	14.50± 2.12^{b}	15.50± 0.71^{ab}	2.30± 0.14^{ab}	47.75± 3.89^{abcd}	3.50± 0.71^{bc}	0.68± 0.02^{abc}	13.00± 0.00^{b}	18.25± 1.06^{a}	1.75± 0.42^{a}
	48h	36.25± 4.59^{cd}	4.50± 0.70^{a}	0.68± 0.07^{ab}	14.00± 1.41^{b}	13.45± 0.35^{ab}	2.05± 0.07^{ab}	53.00± 2.12^{ab}	4.00± 1.41^{abc}	0.75± 0.02^{ab}	20.50± 2.12^{a}	17.00± 2.83^{a}	1.35± 0.07^{a}
0.1	24h	35.75± 2.48^{cd}	2.50± 0.70^{bc}	0.70± 0.14^{ab}	13.50± 0.71^{bc}	13.60± 0.57^{ab}	2.20± 0.28^{ab}	33.00± 3.54^{cd}	2.50± 0.71^{c}	0.65± 0.02^{bc}	11.00± 1.41^{b}	19.75± 0.35^{a}	1.75± 0.07^{a}
	48h	32.50± 3.54^{de}	2.50± 0.70^{bc}	0.65± 0.07^{ab}	12.00± 1.41^{bcd}	14.25± 0.35^{ab}	2.05± 0.07^{ab}	31.75± 1.77^{d}	2.50± 0.71^{c}	0.65± 0.02^{bc}	13.50± 0.71^{b}	14.50± 0.00^{a}	1.45± 0.21^{a}
0.15	24h	27.50± 3.54^{e}	3.00± 1.41^{abc}	0.68± 0.07^{ab}	14.00± 1.41^{b}	14.00± 0.71^{ab}	2.00± 0.14^{ab}	36.75± 3.89^{cd}	2.50± 0.71^{c}	0.67± 0.04^{bc}	11.00± 1.41^{b}	17.75± 3.89^{a}	1.95± 0.35^{a}
	48h	30.10± 5.16^{de}	2.50± 0.70^{bc}	0.62± 0.07^{b}	11.00± 1.41^{cd}	12.60± 0.57^{b}	1.90± 0.14^{ab}	-	-	-	-	-	-
0.2	24h	26.00± 5.66^{e}	2.50± 0.70^{bc}	0.67± 0.28^{ab}	10.50± 0.71^{d}	13.35± 3.04^{b}	1.75± 0.07^{b}	41.55± 20.58^{bcd}	2.50± 0.71^{c}	0.64± 0.00^{c}	13.50± 4.95^{b}	17.75± 3.89^{a}	1.70± 0.57^{a}
	48h	30.90± 5.52^{de}	1.50± 0.70^{c}	0.62± 0.07^{b}	10.50± 0.71^{d}	12.75± 1.06^{b}	1.80± 1.80^{ab}	-	-	-	-	-	-
LSD 5%		8.01	1.99	0.09	2.86	3.08	0.57	20.87	1.91	0.11	5.16	5.26	0.60

4.3.3.2.4 Number of leaves

A highly significant difference was observed in the number of leaves of Indian red ginger after colchicine treatment for both 24 and 48 h. The number of leaves in Indian type varied from 10.50 to 20.00. The maximum number of leaves was observed in the control group for 24h (20.00). As the colchicine concentration and duration increased, a decrease in the number of leaves was observed. The lowest number of leaves was observed at the 0.20 % concentration for both 24 and 48 h (10.50). It was statistically on par with 0.10 % and 0.15 % for 48 h.

In exotic red ginger, there was significant difference in the number of leaves on main shoot with the colchicine concentrations and duration of treatments. The maximum number of leaves was recorded at colchicine 0.05 % for 48 h (20.50) and control 48 h (20.00) and it was statistically on par with control 24 h (15.50). The lowest number of leaves was observed in the treatment of 0.10 % and 0.15 % colchicine for 24 h (11.00) and was not significantly differ from the colchicine concentrations 0.05 % (13.00) and 0.20 % for 24 h (13.50) and 0.10 % for 48 h (13.50).

4.3.3.2.5 Leaf length (cm)

The variation in leaf length was found to be insignificant for both the concentration and the duration of colchicine treatment for the Indian type. The maximum leaf length, measuring 16.45 cm, was observed in the control group. The minimum length of the leaf was recorded at the 0.15 % concentration for 48 h, which measured 12.60 cm.

In exotic red ginger, there was no significant variation in the leaf length with different colchicine concentrations and treatment durations. The highest leaf length was recorded at 0.10 % (19.75 cm) colchicine for 24 h and the lowest leaf length was reported at 0.10 % (14.50 cm) for 48 h.

4.3.3.2.6 Leaf width (cm)

Leaf length did not show any significant differences among the different colchicine concentrations and treatment durations in Indian red ginger. A colchicine

concentration of 0.20 % for 24 h recorded the minimum leaf width, measuring 1.75 cm. In contrast, the maximum width of the leaf was observed in the control group for 24 h, measuring 2.35 cm.

The analysis of leaf width revealed no significant difference among the various treated populations of exotic types. The highest average leaf width was recorded at colchicine concentration 0.15 % for 24 h (1.95 cm) while the lowest average leaf width was measured at 0.05 % colchicine for 48 h (1.35 cm).

In this study, morphological variations caused by colchicine were found to be significant compared to the control group for both genotypes, as given in the table 34. The control group was found to be superior for the various characters studied. For the treated population, minimal variation was observed at the lowest concentrations of colchicine and treatment times. However, significant alterations were noticed in plant height, number of shoots, and number of leaves for both Indian and exotic red ginger genotypes from 0.1 % onwards. Nevertheless, no significant differences were observed in shoot diameter, leaf length, and width between the treated and control groups. Retardation in the growth parameters of red genotypes may be attributed to the toxic effect of colchicine. Colchicine treatment may not be always coupled with chromosome doubling, but also, it is associated with various genetic alterations such as chromosomal breakage, transposons activation, gene silencing and activations etc (Niazian & Nalousi, 2020). Several studies propose a connection between subtle modifications in gene expression and phenotypic changes (Balao *et al*., 2011; Li *et al*., 2012). Prolonged colchicine treatment resulted in the reduction of plant height, stemming from disruptions in mitosis and consequential tissue damage. Similar to our results, Feng *et al*. (2016) observed significant decrease in morphological characteristics, such as plant height and shoot diameter, following the application of antimitotic agents in two *Rosa* sps. It was also noticed in ornamental gingers (Zingiberaceae) that varying concentrations of chemical mutagens could lead to a reduction in plant height, number of leaves, leaf size etc (Prabhukumar *et al*., 2015). On the contrary, the application of colchicine was documented to result in an elevation in the height of *Pennisetum purpureum* (Kamwean *et al*., 2016). Colchicine increased the plant height of *Capsicum annuum*, and there were no observed differences in leaf length and width (Tammu *et al*.,

2021). Likewise, there was no notable alteration in the leaf characteristics observed in *Impatiens balsamina* following the application of colchicine (Wiendra & Pharmawati, 2019). This outcome indicated that altering the concentration of colchicine could lead to diverse effects on each characteristic of red ginger genotypes, influencing both the enhancement and reduction of their overall quality.

4.3.3.3 Effect of colchicine treatment on physiological characteristics of red ginger genotypes

Physiological parameters, such as stomatal length, stomatal width, and stomatal density, were measured from all the survived plants within the treated population. The analysis of variance of the exotic red ginger genotype revealed highly significant ($p<0.001$) variation for all the selected physiological parameters at different concentrations of colchicine under study (Table 35). The duration of the treatment also had a significant effect ($p<0.01$) on the stomatal parameters, except for stomatal width. Additionally, the interactive effect of concentration and time duration was highly significant ($p<0.001$).

The ANOVA result of Indian exotic red ginger genotype indicated that there was a highly significant level of variation ($p< 0.001$) in all the assessed physiological parameters at the various concentrations of colchicine that were examined (Table 35). A significant variation was also noted between the different treatment durations. Morevover, the combined impact of concentration and duration of treatment exhibited a remarkably significant effect, with a p-value less than 0.001. The means were compared using LSD test and given in the table 36.

4.3.3.3.1 Stomatal length (µm)

In Indian red ginger, stomatal length varied from 13.00 to 16.87 µm. The lowest stomatal length was seen in the control group for 24 h (13.00 µm), while the highest length was recorded at 0.2 % colchicine treatment for 24 h (16.87 µm). No significant difference in stomatal length was observed across the treatment durations.

Table 35. Analysis of variance of stomatal parameters of Indian and exotic red ginger after *in vivo* colchicine treatment

Character	Indian red ginger					Exotic red ginger				
	Concentration	Duration	Concentration x Duration	Error	CV (%)	Concentration	Duration	Concentration x Duration	Error	CV (%)
Degrees of freedom	4	1	4	90		4	1	2	72	
Stomatal length	19.32***	17.32*	35.93***	4.4	14.49	45.79***	3.16**	4.50***	0.43	3.79
Stomatal width	2.90***	3.22***	4.04***	0.1	4.17	6.29***	0.00^{ns}	2.02***	0.1	4.05
Stomatal density	8.32***	1.46***	7.64***	0.12	5.3	3.55***	1.08**	2.93***	0.14	8.5

In exotic type, highly significant variation was found between the different treated population. The stomatal length varied from 15.78 to 21.28 µm. The maximum length, measuring 21.78 µm, was observed at 0.20 % colchicine treatment for 24 h. In contrast, the minimum length was observed in the control group for 48 h, measuring 15.78 µm.

4.3.3.3.2 Stomatal width (µm)

Indian red ginger exhibited the highest stomatal width at a colchicine concentration of 0.20 % for 24 h, measuring 8.73 µm. This was followed by 0.10 % colchicine, which yielded a width of 7.96 µm, and 0.15% colchicine for 48 h, resulting in a width of 7.88 µm. The lowest stomatal width was observed in the control (6.81 µm) for 24 h.

At different colchicine concentrations, stomatal width varied significantly among the treated groups of the exotic type. Colchicine concentration at 0.20 % for 48 h (9.30 µm) showed the maximum stomatal width, while the minimum stomatal width was observed in the control group for 48 h (7.15 µm). Additionally, the effect of treatment duration was found to be insignificant for stomatal width. However, the interactive effect of both concentration and time duration was highly significant.

4.3.3.3.3 Stomatal density (stomata mm^{-2})

A significant variation was observed in the stomatal density of the treated plants of Indian red ginger. The stomatal density varied from 4.55 to 7.94 mm^{-2}. The maximum stomatal density was recorded in the control group for 24 h (7.94 mm^{-2}). Whereas the minimum stomatal density was seen in the plants treated with 0.20 % colchicine for 24 h (4.55 mm^{-2}).

Stomatal density was found to be highly significant for all the concentrations and time durations in exotic red ginger. The values ranged from 3.44 to 4.96 mm^{-2}. Maximum stomatal density was observed in the control group for 48 h (4.96 mm^{-2}). The minimum stomatal density was recorded at 0.10 % colchicine for 48 hours (3.44 mm^{-2}) and it was statistically on par with 0.20 % for 24h (3.56 mm^{-2}).

Table 36. Interactive effect of different colchicine concentrations and duration of treatments in the selected stomatal parameters of Indian and exotic red ginger after *in vivo* colchicine treatment

Concentration (%)	Durations (h)	Indian red ginger			Exotic red ginger		
		Stomatal length (µm)	Stomatal width (µm)	Stomatal density (mm^{-2})	Stomatal length (µm)	Stomatal width (µm)	Stomatal density (mm^{-2})
Control	24	13.00 ± 0.39^{f}	6.81 ± 0.39^{g}	7.94 ± 0.37^{a}	17.11 ± 0.23^{bc}	7.88 ± 0.31^{b}	4.59 ± 0.33^{b}
	48	13.72 ± 0.49^{de}	7.76 ± 0.22^{bc}	7.14 ± 0.22^{b}	15.78 ± 0.51^{e}	7.15 ± 0.22^{e}	4.96 ± 0.37^{a}
0.05	24	14.08 ± 0.22^{bcd}	7.05 ± 0.43^{efg}	6.61 ± 0.31^{de}	17.10 ± 0.61^{bc}	7.42 ± 0.39^{de}	4.67 ± 0.51^{ab}
	48	14.41 ± 0.84^{bc}	7.29 ± 0.31^{de}	6.56 ± 0.35^{de}	16.50 ± 0.56^{d}	7.81 ± 0.29^{bc}	4.61 ± 0.30^{b}
0.10	24	14.05 ± 0.71^{cd}	7.10 ± 0.17^{ef}	6.77 ± 0.54^{cd}	16.53 ± 0.32^{cd}	7.54 ± 0.16^{cd}	4.55 ± 0.24^{b}
	48	14.42 ± 0.42^{bc}	7.96 ± 0.22^{b}	6.50 ± 0.34^{de}	17.09 ± 0.66^{bcd}	7.87 ± 0.30^{b}	3.44 ± 0.21^{c}
0.15	24	14.49 ± 0.21^{bc}	6.98 ± 0.38^{fg}	6.41 ± 0.28^{e}	$17.380\pm.42^{b}$	7.70 ± 0.30^{bc}	4.46 ± 0.35^{b}
	48	14.52 ± 0.58^{b}	7.88 ± 0.19^{b}	6.34 ± 0.29^{e}	-	-	-
0.20	24	16.87 ± 6.45^{a}	8.73 ± 0.45^{a}	4.55 ± 0.34^{f}	21.28 ± 1.32^{a}	9.30 ± 0.47^{a}	3.56 ± 0.53^{c}
	48	13.26 ± 0.43^{ef}	7.56 ± 0.21^{cd}	6.94 ± 0.34^{bc}	-	-	-
LSD 5%		1.86	0.28	0.31	0.59	0.28	0.33

Data represents mean±standard deviation.

The examination of stomatal size (length and width) and density within the treated population of red ginger genotypes revealed a significant variation compared to the untreated control (Table 36). There was no proportional increase observed in the stomatal length and width across the treatments. The result suggested that the treated plants of both Indian and exotic red ginger genotypes exhibited highest stomatal length and width at 0.20 % colchicine for 24h.

Similar variations in stomatal size and density resulting from colchicine treatment have been documented in *Ricinus communis* (Baghyalakshmi *et al*., 2020). A decrease in stomatal density was observed in the colchicine-treated plantlets. However, a consistent decrease was not observed in both genotypes. The lowest stomatal density was recorded at 0.20 % after 24 h treatment for Indian red ginger, while for exotic red ginger, it occurred at 0.10 % after 48h. In line with our findings, the potential polyploids of *Lagerstroemia indica* exhibited enlarged stomata and reduced stomatal density (Ye *et al*., 2010). The current findings align with those reported by previous researchers (Ayu *et al*., 2019; Manzoor *et al*., 2018; Kazemi & Kaviani, 2020). The application of colchicine typically results in a reduction in stomatal density, attributed to the enlargement of both stomata and epidermal cells (Gantait *et al*. 2011). An increase in ploidy consistently correlates with an increase in stomatal size and a decrease in stomatal density. Therefore, it can be used as an indicator of ploidy change for the early detection of the putative polyploids.

4.3.3.4 Identification and selection of putative polyploids

Putative polyploids were selected based on the altered morphological characteristics, such as changes in plant height, number of shoots, leaf colour, delayed growth. Additionally, variations in stomatal parameters were observed. A total of 21 putative polyploids of Indian red ginger were identified within the treated population. Only six putative polyploids were selected from the exotic red ginger population. These selected plants underwent further analysis to confirm their ploidy status.

4.3.3.5 Ploidy determination

4.3.3.5.1 Ploidy determination by flow cytometry

The determination of the ploidy level of the newly generated putative polyploids was conducted using flow cytometry. Among the 21 putative polyploids identified from the Indian red ginger, only a single plant was detected as tetraploid. The results demonstrated that when analysing the relative nuclear DNA content of the diploid control via linear histograms, conspicuous G0/G1 peaks were observed at channel 4 x 10^5 (Fig. 29. a). In contrast, the induced mutant plantlets exhibited an analogous peak at channel 8 x 10^5, which was identified as a potential tetraploid (Fig. 29. c). Tetraploid was formed from the treatment of 0.15 % colchicine for 24 h. The putative polyploids identified from the exotic type were evaluated using flow cytometry to determine their ploidy, and none of them were found to be polyploids.

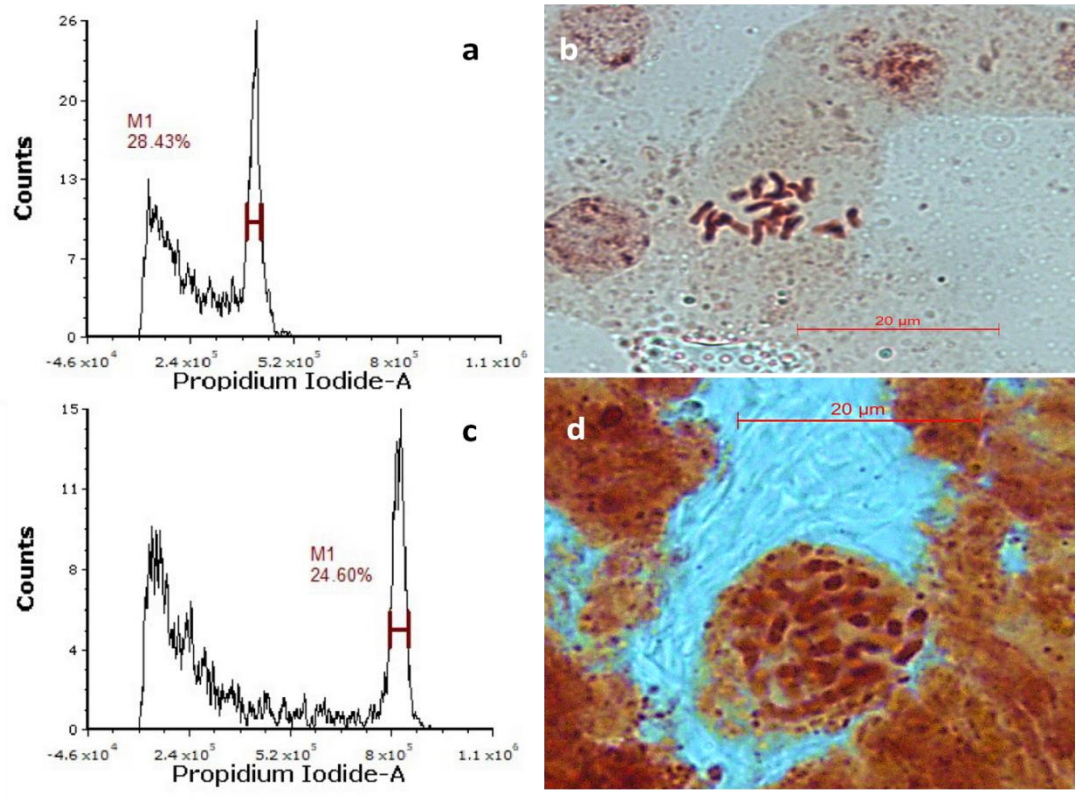

Fig. 29. Flow cytometry histogram of nuclei of (a) Diploid Indian red ginger; Mitotic metaphase plate of (b) Indian red ginger showing 2n=22; Flow cytometry histogram of nuclei of (c) tetraploid Indian red ginger; Mitotic metaphase plate of (b) *in vivo* induced tetraploid Indian red ginger showing 2n=44

Flow cytometry is a frequently used technique for ploidy detection, which is much faster and more precise compared to traditional methods. This method is widely employed by researchers in studies on polyploidy induction for the convenient detection of putative polyploids from large, treated population of plants (Bharati *et al*., 2023b; Jaskani *et al*., 2005; Kulkarni & Borse, 2010; Ye *et al*., 2010; Zhang *et al*., 2010). Additionally, it serves to confirm the ploidy status of the selected plantlets after chromosome counting (Prasath *et al*., 2022).

4.3.3.5.2 Ploidy determination by chromosome counting

Cytological examination of the root tip cells revealed the exact chromosome number of the putative polyploids. The cells exhibited a chromosome number of 44 (2n=4x=44). Hence, tetraploidy was confirmed in the Indian red ginger (Fig. 29. d).

Only one plant from the treated Indian red ginger was identified as a tetraploid mutant in the present study. No change in the ploidy level of exotic red ginger was observed after colchicine treatment. A probable reason for the failure of polyploidy induction in the exotic type is that increased colchicine penetration into the tissues negatively affects the sprouting rate, due to the smaller size and thickness of the rhizomes. Subsequently, this leads to a small population of putative polyploids, all of which were unconverted diploids with significant variations in the morphological and stomatal parameters. Podwyszynska *et al*. (2015) reported comparable findings, noting variations in flower morphology among a set of treated diploid daylilies exposed to antimitotic agents. The researchers proposed that these variations were not solely a result of chromosome doubling but also a consequence of induced chromosomal and gene mutations by antimitotic agents. Similar observation was reported in *Crocus sativus* (Samadi *et al*., 2022), *Ocimum basilicum* (Omidbaigi *et al*., 2010) and *Papaver somniferum* (Mishra *et al*., 2010).

Tetraploid induction was successfully achieved through a 0.15 % colchicine treatment over a 24 h period. However, the highest concentrations of colchicine and longer treatment durations proved ineffective for polyploidy induction, as they resulted in a more pronounced lethal effect on the rhizome buds. Ramachandran (1982) published the initial study on induced polyploidy in ginger. Subsequently, Ramachandran and Nair (1992) developed autotetraploids of ginger by subjecting

rhizome buds to a 0.25 % colchicine solution for 4h. Prasath *et al*. (2022) successfully induced polyploidy in ginger through *in vivo* treatment with a 0.10 % colchicine solution for 48 h. There were only a few reports on the *in vivo* polyploidy induction in ginger. This is the first report of *in vivo* polyploidy induction in red ginger genotype.

4.3.3.6 Comparative characterization of diploids and induced tetraploids (M1V2)

In the first season, the growth of the induced tetraploids was more stunted than that on the diploid control. The identified tetraploid plant was harvested at maturity, and its rhizomes were planted separately alongside diploid controls to evaluate stability in subsequent generations. However, in the second season, the tetraploids exhibited significantly more vigorous growth compared to the diploids (Fig. 30). Various morphological characteristics, including plant height, number of shoots, shoot diameter, number of leaves, leaf length, leaf width, leaf area, leaf colour, and yield per plant, were recorded and analysed. Additionally, physiological parameters such as stomatal length, stomatal width, stomatal density, and chloroplast count were measured and compared.

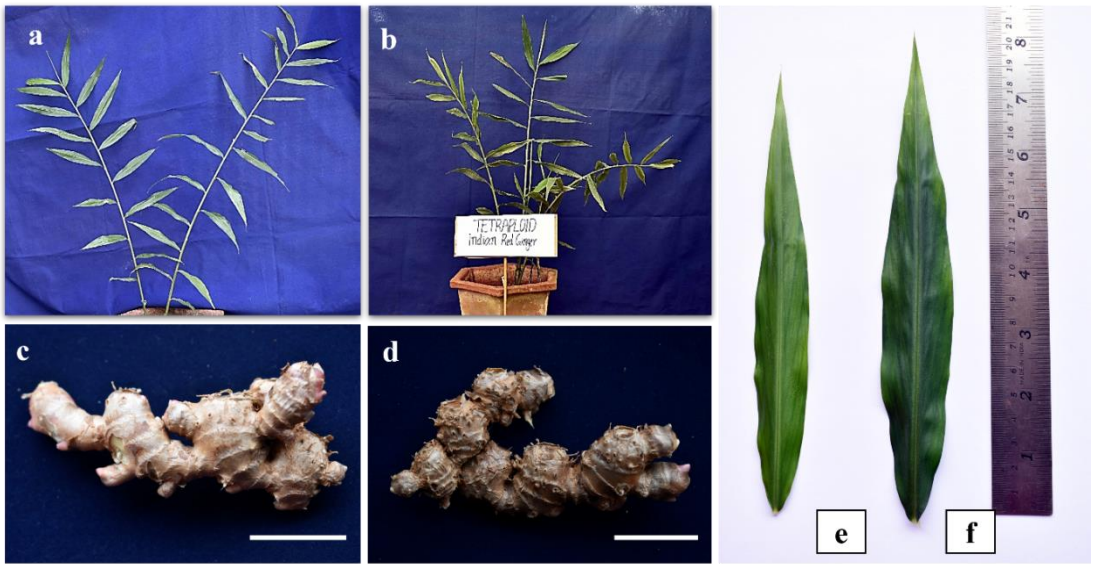

Fig. 30. Diploid and tetraploid Indian red ginger in M1V2 generation; a) Habit of diploid Indian red ginger; b) Habit of induced tetraploid Indian red ginger; c) Rhizome of diploid control; d) Rhizome of induced tetraploid; Variation in the leaf size of (e) diploid and (f) tetraploid

4.3.3.6.1 Morphological parameters

The t- test revealed significant difference in the studied morphological parameters (Table 37).

Table 37. Comparison of morphological characteristics of diploids and tetraploids of M1V2 generation

Ploidy levels	Plant height (cm)	No. of shoots	Shoot diameter (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Yield per plant (g)
Diploid	63.25± 14.50	2.50± 0.70	0.72± 0.02	20.00± 1.41	18.90± 0.14	2.75± 0.07	33.79± 1.12	18.63± 0.53
Tetraploid	59.50± 7.07	3.50± 0.70	0.84± 0.07	18.00± 3.24	21.55± 0.07	3.45± 0.07	48.32± 0.83	38.75± 0.35
t- value	0.33	1.41	2.39	0.63	23.70	9.89	14.72	44.65
p- value	0.77	0.29	0.14	0.59	0.00	0.01	0.00	0.00

Data represents mean±standard deviation.

4.3.3.6.1.1 Plant height (cm)

No significant difference was observed in the plant height between diploids and tetraploids. The diploids recorded an average plant height of 63.25 cm, while the tetraploids recorded a mean plant height of 59.50 cm.

4.3.3.6.1.2 Number of shoots

The number of shoots did not exhibit any significant variation between the diploids and tetraploids. The average number of shoots in diploids was 2.50, whereas in tetraploids, it was 3.50.

4.3.3.6.1.3 Shoot diameter (cm)

There was no significant variation observed in the shoot diameter between diploids and tetraploids. The diploids recorded an average shoot diameter of 0.72 cm, while the tetraploids showed an average of 0.84 cm.

4.3.3.6.1.4 Number of leaves

Colchicine treatment did not result in any significant variation in the number of leaves between induced tetraploids and diploids. The diploids had a mean number of leaves of 20.00, whereas the tetraploids exhibited an average of 18.00 leaves.

4.3.3.6.1.5 Leaf length (cm)

Significant variation was observed between diploids and induced tetraploids. The mean leaf length in diploids was 18.90 cm, while the tetraploids exhibited an increased leaf length of 21.55 cm.

4.3.3.6.1.6 Leaf width (cm)

A significant difference was observed in the leaf width of diploids and tetraploids. A noticeable increase in the leaf width of tetraploids (3.45 cm) was evident from the observation, while diploids recorded an average leaf width of 2.75 cm.

4.3.3.6.1.7 Leaf area (cm^2)

Colchicine had a significant impact on the leaf area of induced tetraploids. A notable variation was observed between tetraploids and diploids, with the leaf area of tetraploids (48.32 cm^2) being higher compared to diploids (33.79 cm^2).

4.3.3.6.1.8 Yield per plant (g)

Significant variation was observed in the yield per plant between induced tetraploids and diploids. Tetraploids exhibited a highly significant increase in yield per plant, with a fresh weight of 38.75 g, whereas diploids recorded an average yield of 18.63 g.

4.3.3.6.1.9 Leaf colour

Diploids exhibited leaves of a normal green colour with a colour code of G137B, while the induced tetraploids displayed leaves of a darker green shade, distinguished by the colour code GN137A.

4.3.3.6.2 Physiological parameters

Stomatal length, width, and density, as well as chloroplast count, were assessed using light microscopy imaging (Fig. 31). The t-test revealed a significant difference in stomatal size, density, and chloroplast count between induced tetraploids and diploids, as shown in bar diagrams (Fig. 32). Furthermore, scanning electron microscopy imaging corroborated the observed variations (Fig. 33).

4.3.3.6.2.1 Stomatal length (µm)

A marked statistically significant difference was noted in the stomatal length between diploid Indian red ginger and induced tetraploids. Tetraploids displayed an average guard cell length of 20.05 µm, while their diploid counterparts exhibited a markedly lower average guard cell length of only 14.35 µm.

4.3.3.6.2.2 Stomatal width (µm)

A markedly significant difference was observed in the stomatal width between diploid Indian red ginger and induced tetraploids. Diploids had a recorded mean width of stomatal guard cells at 6.57 µm, while tetraploids exhibited an average stomatal width of 8.41µm.

4.3.3.6.2.3 Stomatal density (stomata mm^{-2})

A significant variation in stomatal density was noted between diploids and tetraploids. Induced tetraploids exhibited a markedly lower stomatal density of 3.06 stomata mm^{-2}compared to diploids, which had a stomatal density of 5.35 stomata mm^{-2}.

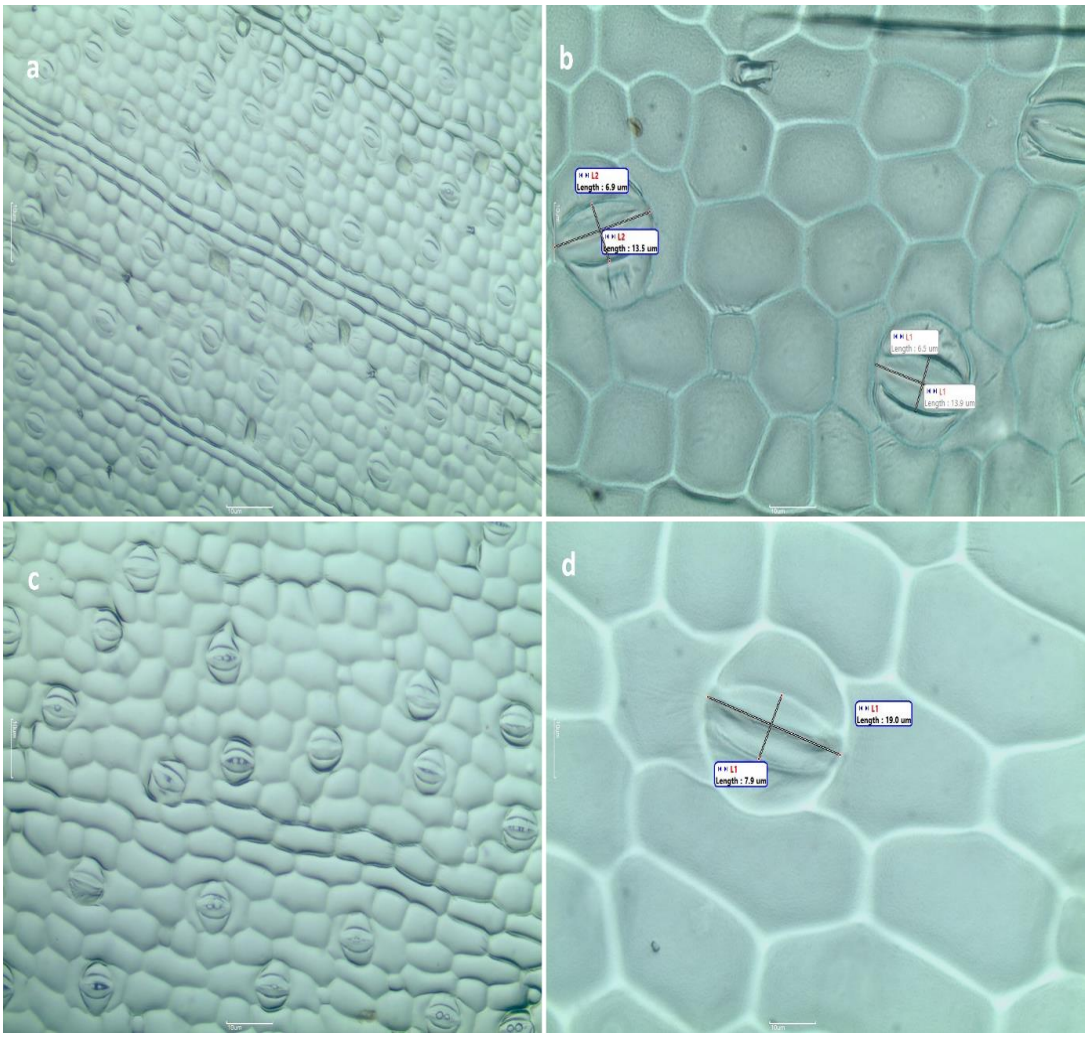

Fig. 31. Variations in the stomatal parameters of diploids and induced tetraploids (M1V2): (a) Stomatal density of diploid Indian red ginger at 10x magnification; (b) Stomatal size of diploid Indian red ginger at 40x magnification; (c) Stomatal density of tetraploid Indian red ginger at 10x magnification; (d) Stomatal size of tetraploid Indian red ginger at 40x magnification, Scale bar = 10 µm

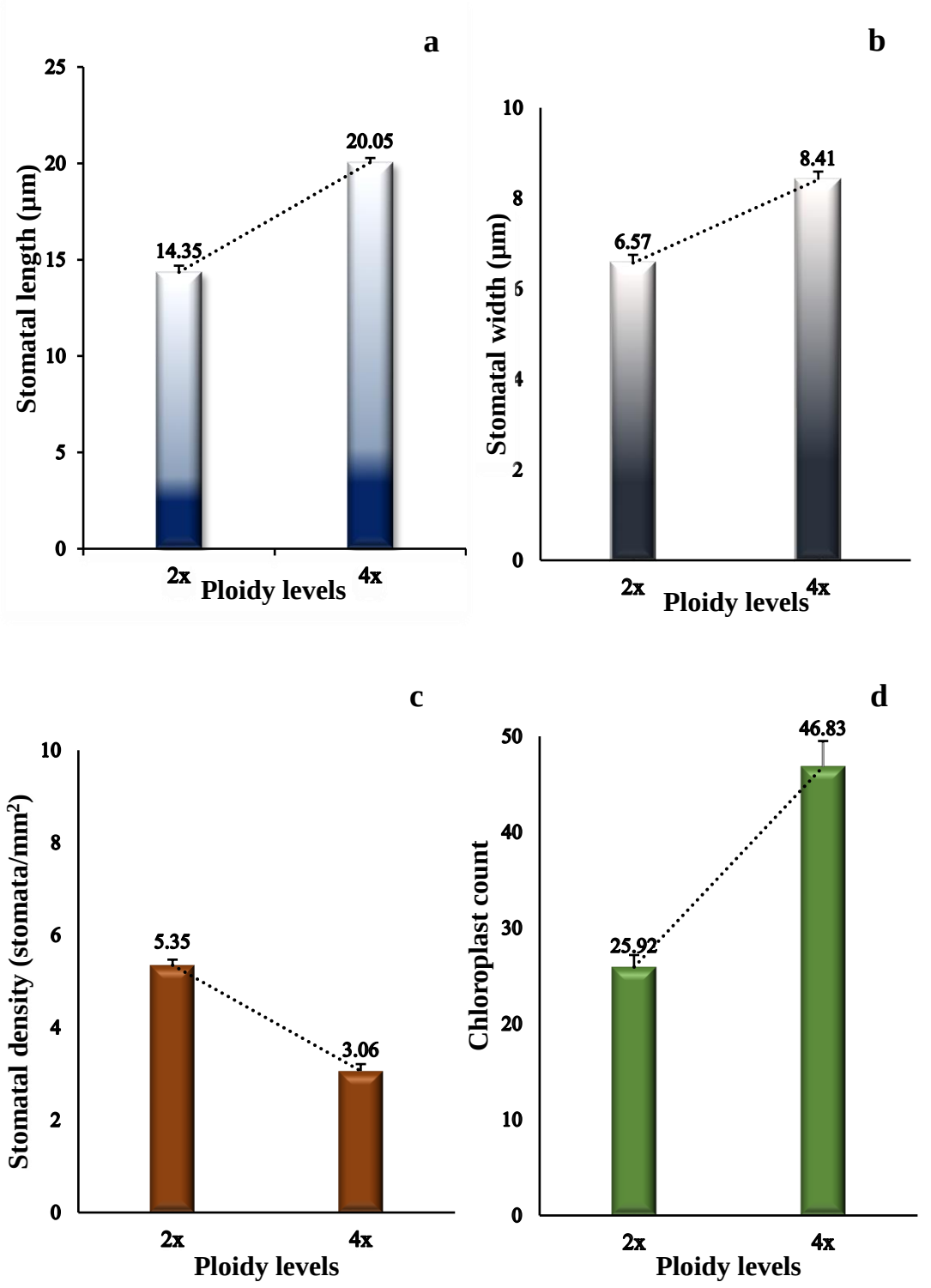

Fig. 32. Variations in the stomatal parameters of diploids and induced tetraploids (M1V2), (a) Stomatal length; (b) Stomatal width; (c) Stomatal density; (d) Chloroplast count

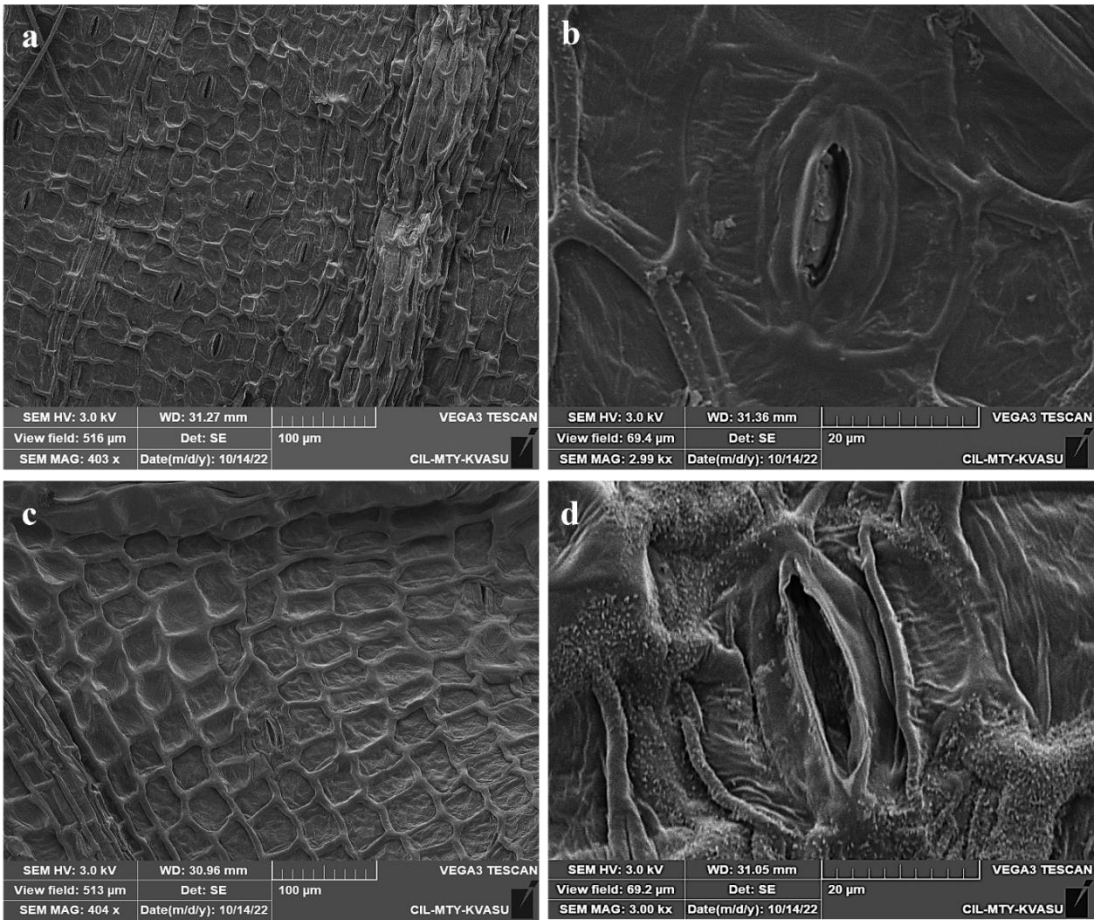

Fig. 33. Scanning Electron Microscopy imaging of abaxial surface of upper fourth leaf of diploid and tetraploid Indian red ginger; a) Lower epidermis of diploid; b) Enlarged single stomata of diploid; c) Lower epidermis of tetraploid; b) Enlarged single stomata of tetraploid

4.3.3.6.2.4 Chloroplasts count

There was a significant variation in chloroplast count between induced tetraploids and diploids. Tetraploids demonstrated a twofold increase in chloroplast count (46.83) compared to diploids (25.92) (Fig. 34).

4.3.3.7 Comparison between diploids and induced tetraploids of M1V3 generation

The induced tetraploids and diploids exhibited much more evident variations in the third generation (M1V3) compared to second generation (M1V2). The selected morphological and physiological parameters were studied. Floral

morphology of both diploids and tetraploids of Indian red ginger genotype were compared and recorded.

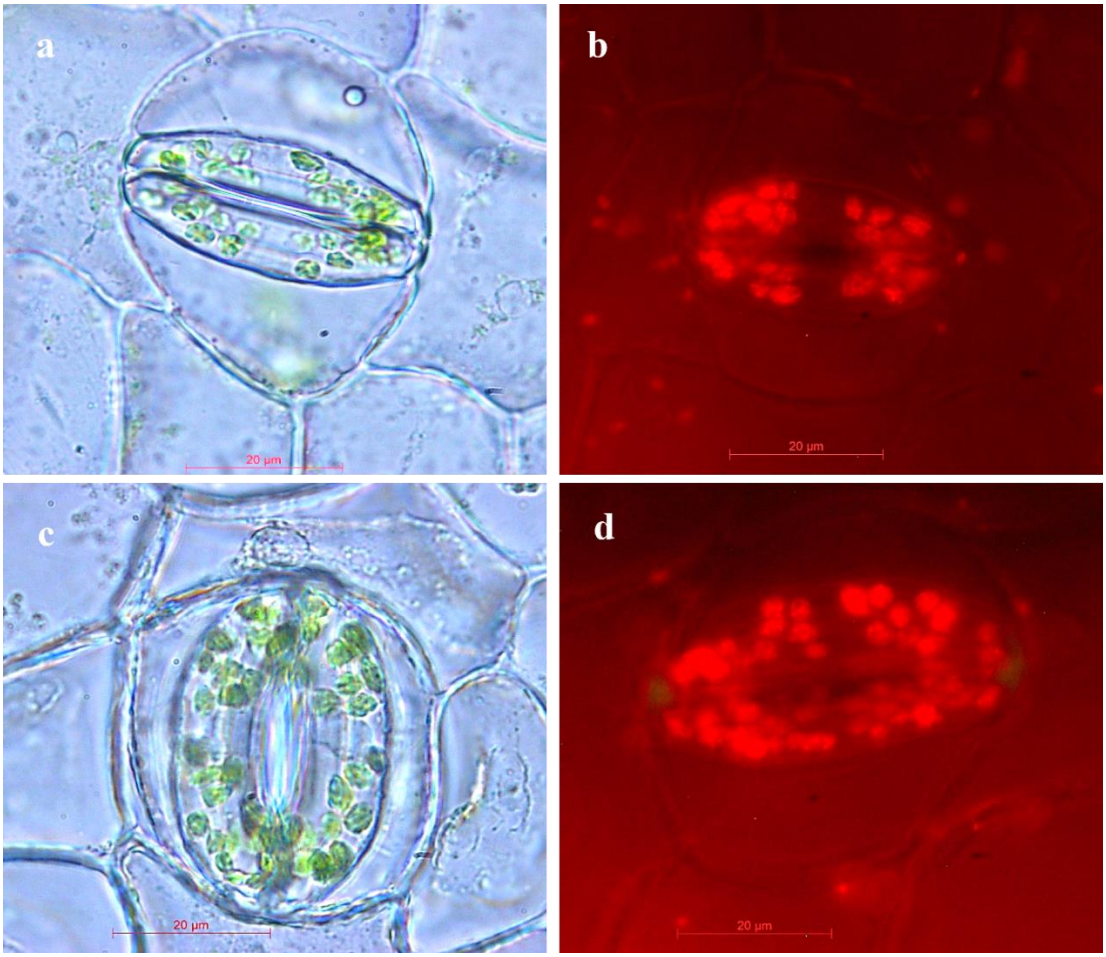

Fig. 34. Variation in chloroplast count of diploid and induced tetraploid stomatal guard cells a) Light microscopy image of diploid stomata; b) Fluorescence microscopy image of diploid stomata; c) Light microscopy image of diploid stomata; d) Fluorescence microscopy image of diploid stomata; scale bar = 20 µm

4.3.3.7.1 Morphological parameters

The comparative characterization of morphological parameters of diploids and induced tetraploids of M1V3 generation are enlisted in the Table 38. The variations in the plant habit and rhizome size are clearly depicted in the Fig. 35.

4.3.3.7.1.1 Plant height (cm)

In the comparative analysis of induced tetraploid and diploid plants, a modest elevation in plant height was observed among the tetraploids, with a mean height of 75.50 cm compared to 73.50 cm for diploids. Despite this observed disparity, statistical evaluation utilizing a significance threshold of p = 0.05 did not indicate a statistically significant difference in plant height between the two groups.

Table 38. Comparison of morphological characteristics of diploids and tetraploids of M1V3 generation

Ploidy level	Plant height (cm)	No. of shoots	Shoot diameter (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Yield per plant (g)
Diploid	73.50± 0.71	7.50± 0.71	0.81± 0.01	21.00± 0.71	20.40± 0.14	2.45± 0.07	32.49± 1.16	147.00± 4.24
Tetraploid	75.50± 14.85	7.00± 1.41	1.28± 0.01	24.00± 2.12	24.70± 0.28	3.45± 0.07	55.40± 1.77	228.00± 2.83
t- value	0.19	0.45	41.59	1.89	19.23	14.14	15.30	4.30
p- value	0.88	0.70	0.00	0.31	0.00	0.00	0.00	0.00

Data represents mean±standard deviation.

4.3.3.7.1.2 Number of shoots

There was no significant difference in the number of shoots between diploids and tetraploids. Diploids showed an average number of shoots of 7.50, whereas induced tetraploids produced an average number of shoots of 7.00.

4.3.3.7.1.3 Shoot diameter (cm)

Statistical analysis revealed a highly significant increase in the shoot diameter of induced tetraploids. The average shoot diameter of tetraploids was recorded at 1.28 cm, whereas diploids exhibited an average shoot diameter of 0.81 cm in the M1V3 generation.

4.3.3.7.1.4 Number of leaves

In comparing induced tetraploid and diploid plants, a slight increase in the number of leaves among tetraploids was noted, with an average of 24.00 leaves compared to 21.00 for diploids. Despite this observed difference, statistical evaluation using a significance threshold of p = 0.05 did not reveal a significant disparity in the number of leaves between the two groups.

4.3.3.7.1.5 Leaf length (cm)

Statistical analysis unveiled a notably significant increase in the leaf length of induced tetraploids. Tetraploids displayed an average leaf length of 24.70 cm, while diploids showed an average leaf length of 20.40 cm in the M1V3 generation.

4.3.3.7.1.6 Leaf width (cm)

A highly significant difference was observed in the leaf width between diploids and induced tetraploids. A significant increase in the mean leaf width was recorded for induced tetraploids (3.45 cm) compared to diploids (2.45 cm).

4.3.3.7.1.7 Leaf area (cm^2)

In the M1V3 generation, there was a highly significant increase in the leaf area of induced tetraploids compared to diploids. The average leaf area of tetraploids was recorded at 55.40 cm^2, whereas diploids exhibited a mean leaf area of 32.49 cm^2.

4.3.3.7.1.8 Leaf colour

The disparity in leaf coloration between diploids and induced tetraploids was pronounced. The induced tetraploids exhibited dark green leaves, whereas diploids displayed a normal green colour with a colour code of GN137A and G137B, respectively.

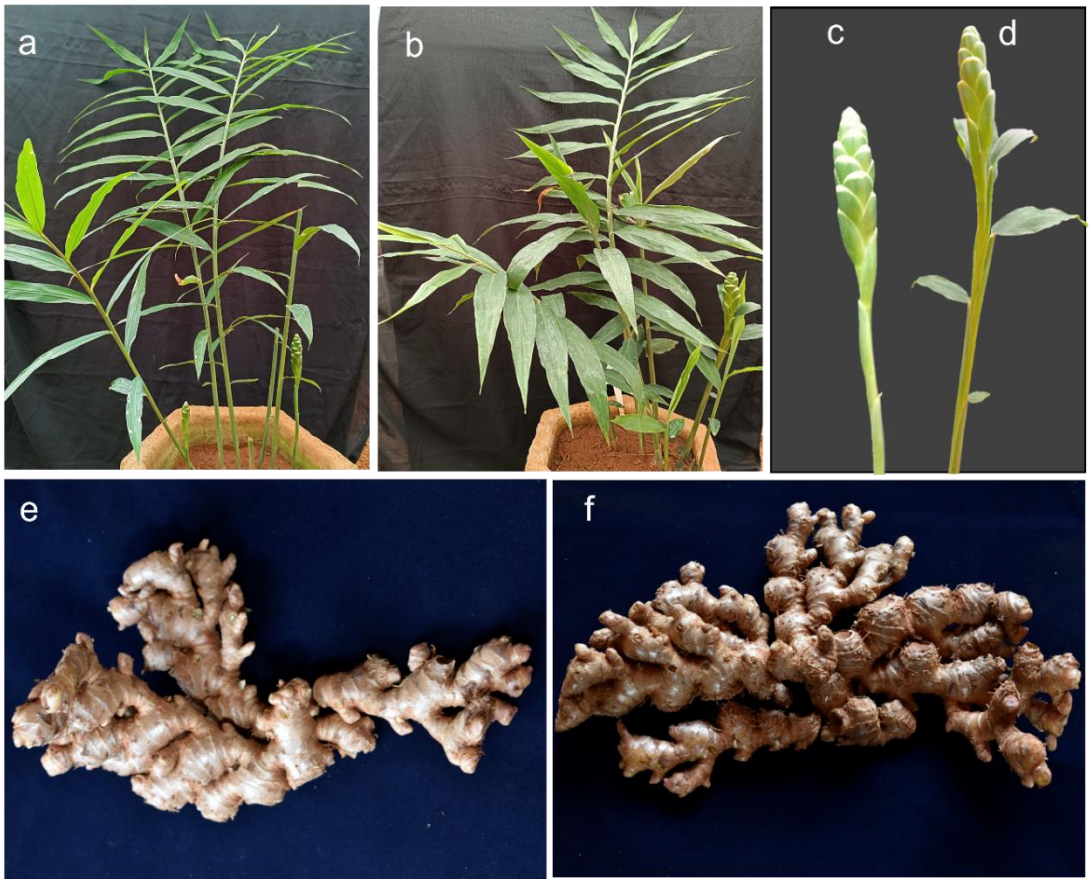

Fig. 35. (a) Diploid control of Indian red ginger of M1V3 generation; (b) Tetraploid Indian red ginger; (c) Spike of normal diploid; (d) Spike of induced tetraploid; (e) Rhizome of diploid; f) Rhizome of induced tetraploid

In this experiment, the induction of polyploidy in Indian red ginger genotypes led to notable and significant changes in their morphology when compared to the diploid control. The induced tetraploids exhibited large, dark green leaves with increased leaf area. Polyploidization has resulted in larger and thicker green leaves in ginger (Prasath *et al*., 2022; Ramachandran & Nair, 1992), garlic (Yousef & Elsadek, 2020), fenugreek (Marzougui *et al*., 2011; Shambulingappa *et al*., 1965), chilli (Kulkarni & Borse, 2010; Tammu *et al*., 2021), mint (Moetamedipoor *et al*., 2022) and watermelon (Khan *et al*., 2023). Furthermore, the tetraploid red ginger genotypes displayed vigorous growth, characterized by significantly larger shoot diameter. Comparable results were previously reported by Prasath *et al*. (2022), and Talebi *et al*. (2017). In addition to that, tetraploids also exhibited gigas characteristics, including larger rhizomes and higher yield. Recently, Prasath *et al*. (2022) reported an augmentation in rhizome size in tetraploid ginger

induced through *in vivo* colchicine treatment. Ramachandran and Nair (1992) also noted thicker and more robust rhizomes in tetraploid ginger compared to diploids. Whole genome duplication leads to dynamic changes in the genetic landscape, which in turn alter the genetic architecture and gene expression profiles (Wang *et al*., 2021). Such dramatic changes can lead to alterations in morphogenesis and developmental events, consequently giving rise to novel phenotypes (Madlung, 2013). Polyploidization is consistently associated with gigas effects, resulting in increased cell size and overall enlargement of the plant (Balao *et al*., 2011; Trojak-Goluch *et al*., 2021).

No significant difference was observed in plant height, number of shoots, and number of leaves over three seasons. However, an increase in both plant height and number of leaves was noted in induced tetraploids during the M1V3 generation. Overall, induced tetraploids displayed a superior phenotype, particularly in terms of rhizome yield compared to corresponding diploids. These superior genotypes hold promise for exploitation in the commercial production of ginger rhizome.

4.3.3.7.1.10 Floral morphology

Spike formation was observed in both diploids and induced tetraploid Indian red ginger during the month of October to November. Flowering started during the end of November in both diploids and tetraploids. In diploid plants, the time of anthesis was observed to occur between 3-4pm, whereas in tetraploids, it was noted to happen between 5-6pm. Another notable observation was the presence of spikes with leaves in tetraploids, which is an unusual phenomenon in ginger (Fig. 35. c & d). Variation in the floral characteristics is given in detailed in the table 39. T-test revealed significant variation in the floral characteristics of diploids and induced tetraploids.

Table 39. Comparison of floral characteristics between diploids and tetraploids of Indian red ginger (M1V3)

Floral morphology	Diploid	Tetraploid	p value
Spike length (cm)	27.20±3.11	46.75±2.47	0.02
No. of bracts	22.50±0.71	14.50±0.71	0.01
Length of bract (cm)	2.52±0.16	3.22±0.08	0.00
Width of bract (cm)	1.80±0.07	2.61±0.12	0.00
Length of bracteole (cm)	2.48±0.08	2.78±0.08	0.00
Width of bracteole (cm)	1.28±0.08	1.74±0.09	0.00
Flower length (cm)	4.20±0.10	4.50±0.12	0.00
Length of calyx (cm)	0.94±0.09	1.24±0.05	0.00
Width of calyx (cm)	0.70±0.07	1.21±0.05	0.00
Length of dorsal petal (cm)	1.60±0.07	2.12±0.04	0.00
Width of dorsal petal (cm)	0.58±0.04	0.78±0.08	0.00
Length of lateral petal (cm)	1.59±0.05	1.86±0.05	0.00
Width of lateral petal (cm)	0.38±0.04	0.59±0.02	0.00
Length of labellum (cm)	1.42±0.08	1.82±0.04	0.00
Width of labellum (cm)	0.92±0.08	1.58±0.08	0.00
Length of anther crest (cm)	1.56±0.05	1.86±0.11	0.00
Length of anther (cm)	0.80±0.07	0.92±0.04	0.01
Length of pistil (cm)	3.98±0.08	4.42±0.11	0.00
Pollen diameter (µm)	89.25±1.50	100.27±3.14	0.00

4.3.3.7.1.10.1 Spike length (cm)

A highly significant increase was observed in the spike length of induced tetraploid Indian red ginger. Tetraploid red ginger exhibited an average spike length of 46.75 cm, whereas diploid red ginger produced only 27.20 cm.

4.3.3.7.1.10.2 Number of bracts

The number of bracts decreased significantly in induced tetraploids compared to diploids. Tetraploids exhibited a mean number of 14.50 bracts, whereas diploids produced 22.50 bracts on the spike.

4.3.3.7.1.10.3 Length of bract (cm)

Induced tetraploids had an average bract length of 3.22 cm, while diploids had a bract length of 2.52 cm. The difference in the length of bract was statistically significant.

4.3.3.7.1.10.4 Width of bract (cm)

A highly significant increase in bract width was recorded in tetraploids, with an average width of 2.61 cm compared to 1.80 cm in diploids.

4.3.3.7.1.10.5 Length of bracteole (cm)

Statistical analysis revealed a significant difference in bracteole length between diploids and tetraploids. The average bracteole length in diploid red ginger was 2.48 cm, while induced tetraploids exhibited a longer length of 2.78 cm.

4.3.3.7.1.10.6 Width of bracteole (cm)

An evident increase in bracteole width was observed in induced tetraploids, measuring 1.74 cm, compared to their diploid counterparts, which had a width of 1.28 cm.

4.3.3.7.1.10.7 Flower length (cm)

t-test revealed a highly significant difference in flower length between diploids and tetraploids. Diploids had a recorded length of 4.20 cm, while tetraploids produced a length of 4.50 cm.

4.3.3.7.1.10.8 Length of calyx (cm)

Statistical analysis indicated a significant disparity in bracteole length between diploids and tetraploids. Diploid red ginger had an average calyx length of 0.94 cm, whereas induced tetraploids displayed a longer length of 1.24 cm.

Fig. 36. Variations in the different floral parts of diploid and induced tetraploid Indian red ginger; (a) Flower - diploid (left), tetraploid (right); (b) Bract; (c) Bracteole; (d) Calyx; (e) Dorsal petal; (f) Lateral petal; (g) Labellum; (h) Anther crest; (i) Pistil

4.3.3.7.1.10.9 Width of calyx (cm)

Induced tetraploids exhibited a significantly greater calyx width, measuring 1.21 cm, compared to diploids, which had a calyx width of only 0.70 cm.

4.3.3.7.1.10.10 Length of dorsal petal (cm)

Induced tetraploids exhibited a significantly greater dorsal petal length, measuring 2.12 cm, compared to diploids, which showed a smaller dorsal petal length of only 1.60 cm.

4.3.3.7.1.10.11 Width of dorsal petal (cm)

A statistically significant increase in the width of the dorsal petal was observed in the induced tetraploids, measuring 0.78 cm, whereas diploids exhibited a slightly narrower dorsal petal of 0.58 cm.

4.3.3.7.1.10.12 Length of lateral petal (cm)

The diploids displayed a lateral petal length of 1.59 cm, significantly lower compared to induced tetraploids, which measured 1.86 cm.

4.3.3.7.1.10.13 Width of lateral petal (cm)

The induced tetraploids exhibited a lateral petal width of 0.59 cm, which was significantly higher than that of diploids, measuring 0.38 cm.

4.3.3.7.1.10.14 Length of labellum (cm)

Length of labellum varied significantly between diploid Indian red ginger and induced tetraploid red ginger, with a notable increase observed in the tetraploids (1.82 cm) compared to the diploids (1.42 cm).

4.3.3.7.1.10.15 Width of labellum (cm)

There was a significant difference in the width of the labellum between diploid Indian red ginger and induced tetraploid red ginger, with a marked increase seen in the tetraploids (1.58 cm) compared to the diploids (0.92 cm).

4.3.3.7.1.10.16 Length of anther crest (cm)

Induced tetraploids exhibited a notable increase in the length of the anther crest compared to diploids, with tetraploids measuring at 1.86 cm and diploids at 1.56 cm.

4.3.3.7.1.10.17 Length of anther (cm)

Induced tetraploids displayed a statistically significant increase in anther length, measuring 0.92 cm, while diploids exhibited slightly smaller anthers, with a length of 0.80 cm.

4.3.3.7.1.10.18 Length of pistil (cm)

A statistically significant variation was noted in the length of the pistil between diploids and tetraploids. Diploids exhibited a pistil length of 3.98 cm, whereas tetraploids showed a longer length of 4.42 cm.

4.3.3.7.1.10.19 Pollen diameter (µm)

A statistically significant difference in pollen diameter was noted between diploids and tetraploids. Diploids exhibited a pollen diameter of 89.25 µm, whereas tetraploids displayed larger pollen grains, measuring 100.27 µm in diameter (Fig. 37).

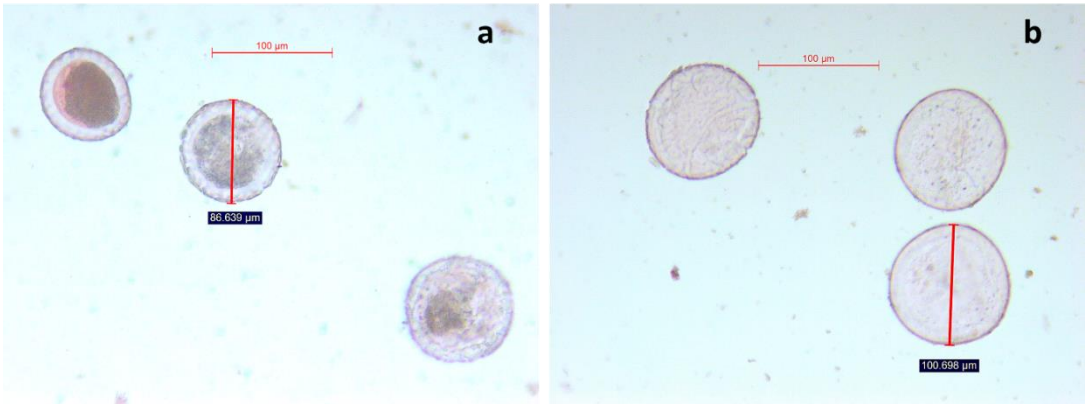

Fig. 37. Variation in the pollen diameter of (a) diploid and (b) tetraploid

The present investigation revealed a significant increase in spike length, along with the size of various floral components and pollen diameter, in diploid Indian red ginger upon induction of tetraploidy (Fig. 36 & Fig. 37). The observed gigantism in both the spike and flower was directly associated with the polyploidization event. This finding is consistent with the earlier research conducted by Ramachandran and Nair (1992), who also documented an enlargement in spike size, flower dimensions, and floral parts in tetraploid ginger. Previous studies have also documented the same occurrence in chilli (Panda *et al*., 1984), anise hyssop (Talebi *et al*., 2017), ajowan (Noori *et al*., 2017), coriander (Purbiya *et al*., 2021), An intrinsic trait of polyploids is the enlargement of cell size, often attributable to the presence of extra chromosomes within the nucleus. Frequently, this increase in

cell size correlates with a general expansion in the size of the organism or various parts (Adams & Wendel, 2005). Abdoli *et al*. (2013) similarly documented larger flowers in colchicine-induced tetraploid plants of *Echinacea purpurea*, compared to diploid control plants. In tetraploid *Melissa officinalis*, there was a consistent increase in the average length of the flower, width of the calyx, and width of the corolla, with respective increments of 10.90%, 55.30%, and 30.04% (Bharati *et al*., 2023b). Pollen grain size serves as a widely utilized indicator of ploidy level in plants, given the positive correlation observed between pollen grain size and ploidy level (Sanders, 2021). Most pollen grains in tetraploids exhibited larger size with increased pollen diameter compared to those from diploid counterparts, mirroring findings reported in studies of *Carum carvi* (Dijkstra & Speckmann, 1980), *Helianthus annuus* (Srivastava & Srivastava, 2002), and *Ocimum basilicum* (Omidbaigi *et al*, 2010). Recently, Sanaei-Hoveida *et al*. (2023) also reported significant difference in the pollen grain shape and size in tetraploid *Cuminum cyminum* compared to diploid control.

4.3.3.7.2 Physiological parameters

The physiological parameters of diploids and induced tetraploids from the M1V3 generation are graphically represented in Fig 38.

4.3.3.7.2.1 Stomatal length (µm)

A significant and noticeable difference in stomatal length was observed between diploid Indian red ginger and induced tetraploids. Tetraploids showed an average guard cell length of 20.47 µm, whereas their diploid counterparts exhibited a notably lower average guard cell length of only 15.47 µm.

4.3.3.7.2.2 Stomatal width (µm)

Statistical analysis revealed significant difference in stomatal width between diploid Indian red ginger and induced tetraploids. Diploids had a recorded mean width of stomatal guard cells at 7.15 µm, while tetraploids exhibited an average stomatal width of 9.02 µm.

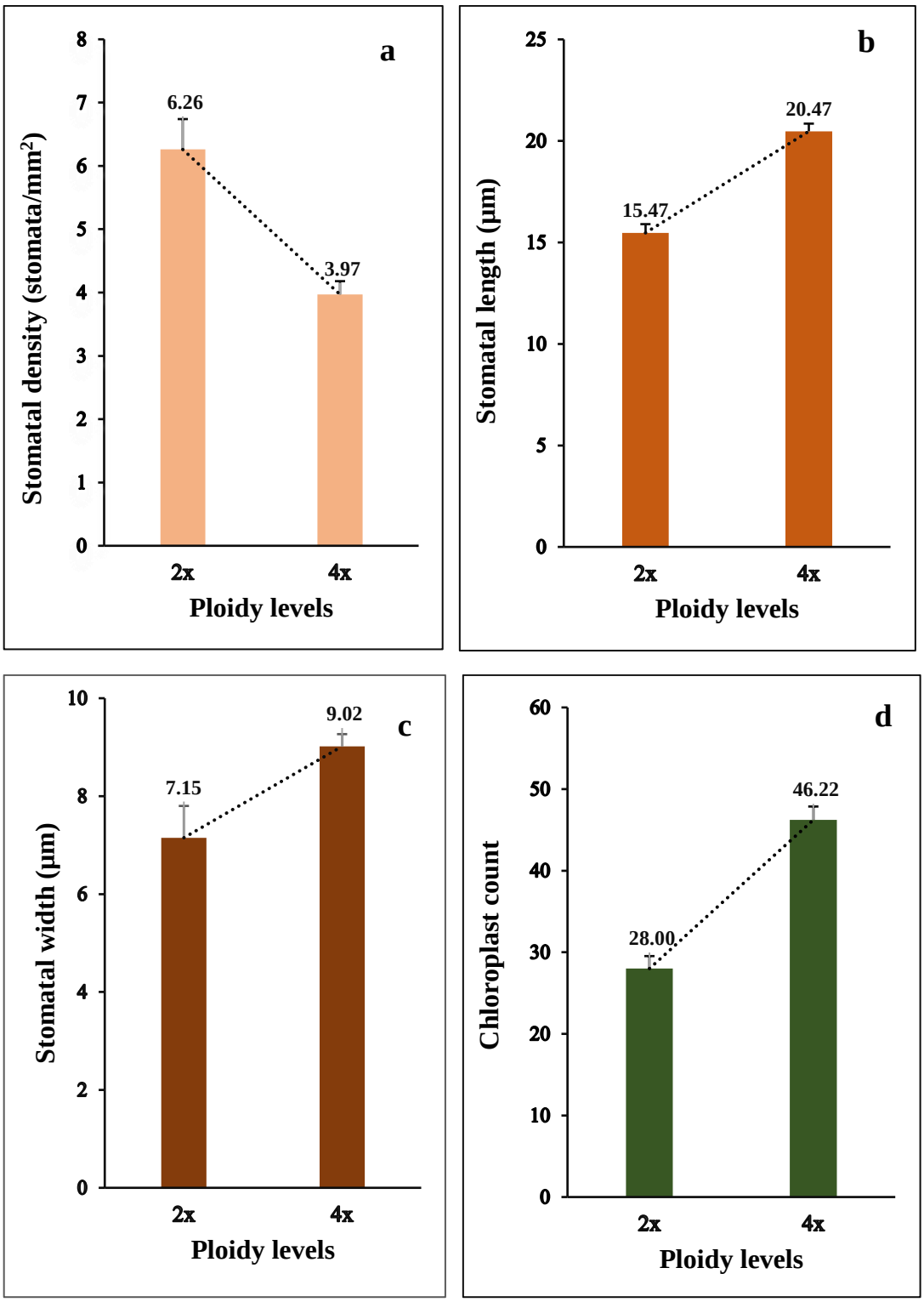

Fig. 38. Variations in the stomatal parameters of diploids and induced tetraploids (M1V3); (a) Stomatal length; (b) Stomatal width; (c) Stomatal density; (d) Chloroplast count

4.3.3.7.2.3 Stomatal density (mm^{-2})

The comparative study revealed a significant difference in stomatal density between diploids and induced tetraploids. Diploids recorded a significantly higher number of stomata per mm^2 (26.93 mm^{-2}), whereas induced tetraploids exhibited a significantly lower number of stomata per mm^2 (17.07 mm^{-2}).

4.3.3.7.2.4 Chloroplast count

Induced tetraploids exhibited a highly significant increase in chloroplast count compared to diploids. The average chloroplast count of induced tetraploids was recorded at 46.22, whereas diploids exhibited a count of 28.00.

The examination of stomatal size (both length and width) and density in induced tetraploid red ginger genotypes revealed a significant variation compared to the diploid control across M1V2 and M1V3 generations. It is graphically portrayed in the Fig. 38. The induced tetraploids of Indian red ginger showed significant increase in the stomatal length and width and significant decrease in the stomatal density compared to diploids. The findings of the current study underscore the impact of elevated ploidy levels on the physiological characteristics of plants. Changes in stomatal parameters upon polyploidization have been widely documented across many plant species. In most studies, a recurring observation is a decrease in stomatal density among polyploids as compared to their diploid counterparts. This reduction is associated with the enlargement of individual stomata in relation to leaf area and increased cell size (Hassanzadeh *et al*., 2020). Decreased stomatal density in tetraploid plants has been documented in parsley (Nasirvand *et al*., 2018), castor (Baghyalakshmi *et al*., 2020), and *Catharanthus roseus* (Xing *et al*., 2011), indicating a consistent trend across various species. Furthermore, the presence of large stomata in polyploids may enhance water uptake and facilitate efficient gaseous exchange in these lines. Increment in the stomatal length and width followed by polyploidy induction was also reported by Pliankong *et al*. (2017), Yousef and Elsadek (2020), Khan *et al*. (2023), Sanaei-Hoveida *et al*. (2024). In the current investigation, an increase in ploidy was associated with a rise in the number of chloroplasts in stomatal guard cells, aligning with earlier findings in basil

(Omidbaigi *et al*., 2010), anise hyssop (Talebi *et al*., 2017), and sage (Hassanzadeh *et al*., 2020). Significant differences were observed in stomatal characteristics and the number of chloroplasts in guard cells between triploid and hexaploid mojito mint, as reported by Moetamedipoor *et al*. (2022). The increased size of stomata, chloroplasts, and stomatal guard cells in polyploid plants may enhance photosynthetic efficiency, leading to inherent adaptability to environmental factors (Marzougui *et al*., 2010; Gupta *et al*., 2021; Šmarda *et al*., 2023).

In this experiment, induced tetraploids consistently exhibited enlarged stomata with reduced frequency and a higher count of chloroplasts in the stomatal guard cells across two consecutive generations. Stomatal characteristics, encompassing stomatal density, length, and width, along with chloroplast count within the stomatal guard cells, serve as valuable indicators for identifying mutated plantlets (Hodgson *et al*., 2010; Tang *et al*., 2010). Numerous studies offer gripping evidence to support these findings (Nasirvand *et al*., 2018; Mishra *et al*., 2010; Rao *et al*., 2019; Ansari *et al*., 2022).

An *in vitro* regeneration system for multiplication and *in vitro* colchicine treatment was successfully developed for both Indian and exotic red ginger genotypes. Triploidy was detected in the exotic red ginger types with a chromosome number of 2n = 33, whereas the Indian type was identified as diploid (2n = 22). The toxicity of colchicine on the plant tissues was evident from the low germination rate exhibited by the rhizome buds, especially in exotic types. The survival rate of explants was proportional to the colchicine concentration and treatment time. The influence of colchicine on the morphological and physiological parameters was clearly demonstrated in the treated population. Polyploidy induction was achieved only in the Indian red ginger type (Acc. 9073), whereas polyploidy induction was not identified in the exotic type (Acc. 899), through *in vitro* and *in vivo* polyploidy induction. A total of seven polyploids were developed through both pathways. One among them perished during growth. Comparative characterization indicated significant enhancement in phenotypes, especially in vigour and yield, and physiological parameters such as stomatal size and chloroplast count.

CHAPTER 5
SUMMARY AND CONCLUSION

The study entitled "Improvement of red ginger (*Z. officinale* Rosc.) through *in vitro* and *in vivo* studies" was conducted at ICAR-Indian Institute of spices Research, Kozhikode, Kerala during the period of 2019 to 2023. The current investigation commenced with a comprehensive morphological and biochemical characterization of various red ginger genotypes (14 indigenous and two exotic), compared with the released variety 'IISR Varada'. The characterization led to identification of superior genotypes for yield and quality. The shortlisted red ginger genotypes were subjected to genetic enhancement through *in vitro* and *in vivo* polyploidy induction using colchicine, with subsequent assessment of its impact on selected horticultural characteristics.

5.1 Morphological and biochemical characterization of red ginger genotypes

The genotypes exhibited significant differences across all morphological traits, including plant height, number of shoots, shoot diameter, number of leaves on the main shoot, leaf length, leaf width, rhizome thickness, yield per plant, and dry recovery. Among the genotypes, highest plant height (74.75 cm) and number of shoots (24.75) was recorded in Coll. No. 9073. Highest shoot diameter (1.22 cm), leaf length (28.83 cm), and rhizome thickness (2.32 cm) was exhibited by IISR Varada. Maximum number of leaves on main shoot (25.13) and leaf width (3.55 cm) were recorded in genotypes Coll. No. 9040 and G3, respectively. Dry recovery was maximum in Coll. No. 9078 (25.41 %). Out of the 17 genotypes, highest yield per plant (807.50 g) was recorded in the Indian red ginger Coll. No. 9073. Among the exotic types, Acc. 899 exhibited highest yield per plant (363.75 g). The yield was found to be very low in exotic red ginger types compared to Indian red ginger types and IISR Varada.

The biochemical characterization unveiled notable variations in the major quality traits, including essential oil, oleoresin, crude fibre, gingerols and shogaol

contents among the red ginger genotypes. The higher essential oil and oleoresin content of 4.30 % and 10.34 % observed in Indian red ginger, G9. Among the genotypes, low fibre content was observed in IISR Varada (4.9 %). All the red ginger genotypes exhibited high fibre content, ranging from 5.43% to 10.47%, making them valuable to produce dry ginger. The pungent principle, 6-gingerol was recorded maximum in Coll. No. 9073 (1.33 %). Among the exotic red ginger, Acc. 899 (0.65 %) was superior in terms of gingerols percentage. Acc. 844 exhibited the highest 8-gingerol percentage (0.17 %) among the 17 ginger genotypes, while Indian red ginger G9 had a high concentration of 6-shogaol at 0.18 %. Exotic types demonstrated lower concentrations of 8-gingerol and 6-shogaol, ranging from 0.06% to 0.09% and 0.08% to 0.09%, respectively. The red ginger genotypes displayed superiority over the released variety 'IISR Varada' concerning biochemical attributes such as essential oil, oleoresin, crude fibre, and gingerol. Therefore, the study highlighted the potential of these red ginger collections to expand the existing range of varieties, enabling their utilization in commercial ventures across various processing and pharmaceutical industries.

The study concluded that the impact of the environment on the examined traits is minimal, as indicated by the negligible difference between the genotypic coefficient of variation (GCV) and the phenotypic coefficient of variation (PCV) for all component traits analysed. A significant finding of the study was the observation of a high GCV coupled with a high PCV for several traits, including the number of shoots, yield per plant, essential oil, oleoresin, gingerols, and 6-shogaol. This underscores the substantial variability inherent in these characteristics. The traits such as the number of shoots, number of leaves on the main shoot, leaf length, leaf width, rhizome thickness, yield per plant, essential oil, oleoresin, crude fibre, gingerols, and 6-shogaol exhibited both high heritability and a high genetic advance per cent mean (GAM). This suggests that these traits can be effectively improved through simple selection methods, given their limited environmental influence on phenotypic expression.

Based on GC-MS analysis, the primary chemical constituents identified in ginger oil included zingiberene, β-sesquiphellandrene, Ar-curcumene, β-bisabolene, and α-farnesene. The predominant component in the essential oil across ginger genotypes was zingiberene, with Acc. 845 displaying the highest proportion at 30.46%. Significant disparities were observed in both the essential oil constituents and their compositions between Indian red ginger genotypes and exotic types, as well as with IISR Varada. In the present study, the percentage of camphene, citronellol, neral, geraniol, geranial, bornyl acetate, and geranyl acetate was notably higher in exotic types. γ-muurolene was present only in exotic red ginger gentypes i.e, Acc. 850 and Acc. 899. Nerolidol was exclusively detected in IISR Varada. Conversely, neral and geraniol were found to be in lower concentrations, while 1,8 cineol and myrcene exhibited higher levels in IISR Varada.

The study identified the existence of diversity among the 14 Indian red ginger genotypes and two exotic types with respect to morphological and quality characters. The red ginger genotypes were superior to IISR Varada in terms of quality traits. The high yielding genotype of Indian and exotic red ginger i.e., Coll. No. 9073 and Acc. 899 were identified as best performing genotypes for yield and quality.

5.2 Standardization of *in vitro* regeneration system for red ginger genotypes and improvement of red ginger through *in vitro* and *in vivo* polyploidy induction

A successful direct *in vitro* regeneration system was standardized for both Indian (Coll. No. 9073) and exotic red ginger (Acc. 899) genotypes. In this study, the responses of two red ginger genotypes to varying levels of BAP were compared. In exotic red ginger, the highest level of shoot multiplication occurred at a BAP concentration of 5.0 mg L^{-1}, while in Indian red ginger, optimal shoot multiplication was observed at 3.0 mg L^{-1} of BAP. Furthermore, this study confirmed genetic fidelity between the *in vitro* propagated plants and the donor mother plants using ISSR and SSR markers. The developed *in vitro* regeneration system holds promise

for the creation of high-yielding elite red ginger polyploids via *in vitro* polyploidy breeding techniques.

In vitro polyploidy induction was conducted using varying concentrations of colchicine on selected Indian and exotic red ginger genotypes, namely, Coll. No. 9073 and Acc. 899, respectively. The findings from somatic chromosome number analysis indicated that Indian red ginger is diploid (2n = 22), while exotic red ginger is triploid (2n = 33). The survival rate of treated explants in both red ginger genotypes was adversely affected by colchicine treatment. In this study, the treated explants showed a significantly delayed sprouting response compared to the untreated control. The survival rate was proportional to the treatment concentration and time duration. The application of 0.10 % colchicine for 48 h led to necrosis and complete loss of treated explants in the exotic type. Significant variations were observed in the morphological characters such as plant height, number of shoots, shoot diameter, number of leaves, and leaf length, between the treated population and control under greenhouse conditions. The colchicine treatment also influenced the stomatal parameters like stomatal length, width, and density. Flow cytometric analysis identified six polyploids (2n = 44) within the treated population of Indian red ginger. On the other hand, no polyploids of the exotic kind were effectively created. The most effective induction of tetraploidy was observed in the treatment involving 0.10% colchicine for 48 h, resulting in a rate of 7.41%. The morphological characterization disclosed a significant increase shoot diameter, leaf width and leaf area among the induced tetraploids. A statistically significant enhancement was observed in both the stomatal length (20.58 µm), stomatal width (9.24 µm) and the number of chloroplasts (45.88) within the stomatal guard cells of the induced tetraploids in comparison to the diploids (13.47 µm, 7.25 µm, and 24.52 respectively). The stomatal density was found to be low in tetraploids (2.83 stomata/mm^2). Chromosome doubling led to an increased yield per plant in induced tetraploids (24.33 g) compared to diploids (13.67 g).

The initial response to the *in vivo* colchicine treatment was a delay in bud germination. The highest germination rate was observed in the non-treated controls.

Conversely, the lowest survival rate was noted in buds treated with 0.20 % colchicine for 48 h. With increasing colchicine concentration and exposure time, the germination rate decreased. Notably, for the exotic type, none of the treated buds germinated at colchicine concentrations of 0.15 % and 0.20 % for 48 h. Considerable variation was noted in the selected morphological and physiological traits among the treated plants with the control. Out of the 21 putative polyploids of Indian red ginger, only one was confirmed to be tetraploid (2n = 44) following flow cytometric analysis and validated the duplication of genetic material through chromosome counting. Conversely, none of the six putative polyploids of exotic red ginger were found to be polyploid. Tetraploidy was induced through the treatment with 0.15 % colchicine for 24 h. Significant improvements in both morphology and physiology were observed in the induced tetraploids across the second and third generations. Our findings observed an initial period of sluggish growth in tetraploids. Nevertheless, by the following season, tetraploids demonstrated a substantial increase in size compared to diploids. After three generations, the tetraploid ginger exhibited greater vigour in growth, with significantly darker and larger leaves compared to the diploid ginger. Increased stomatal length (20.47 µm), stomatal width (9.02 µm) and decreased stomatal density (3.97) was observed in the tetraploid ginger. Chloroplast count was significantly higher in the tetraploid red ginger (46.22) compared the diploids (28.00). The tetraploid Indian red ginger was promising in terms of rhizome size and fresh yield (228.00 g).

A successful protocol for inducing tetraploid lines in red ginger has been established through both *in vivo* and *in vitro* methods using colchicine. Our findings suggested that reducing the concentration of colchicine or shortening the treatment duration can improve the survival rate of plants. However, this approach also resulted in a noticeable decrease in the induction rate of tetraploidy. This highlighted the significance of carefully selecting the duration and concentration of colchicine treatment, considering the tolerance of the specific plant species, to achieve optimal tetraploid plant development. The induced tetraploids exhibited enhanced morphological and physiological attributes, including vigorous growth, increased leaf size, enhanced floral parts, yield, thicker rhizomes, enlarged stomatal size, and

improved chloroplast count, thereby broadening the genetic diversity of red ginger. The tetraploid variant of Indian red ginger displayed promising characteristics, particularly in terms of rhizome size and fresh yield.

It was observed that the *in vitro* induction of polyploidy proved to be more efficient compared to the *in vivo* method, resulting in the development of five tetraploids. The attributes observed in the identified tetraploid lines, including increased yield and other desirable characteristics, hold considerable commercial potential for the processing industry. These enhanced genotypes offer promising prospects for adoption on a commercial scale, leading to substantial economic benefits. To conclude, our study established an effective method for inducing polyploidy in red ginger, laying the foundation for the development of new varieties in ginger.

CHAPTER 6
RECOMMENDATIONS

Red ginger (*Z. officinale* Rosc.) is significant as a medicine across Southeast Asian countries and has garnered widespread global appeal for its distinctive aroma, flavour, and colour, making it a desired ingredient in various cuisines. The study aimed to identify genetic variability within red ginger genotypes and to improve it through in vitro and in vivo polyploidy induction. The present study revealed the diversity in red ginger genotypes with high essential oil content (4.30 %) and high gingerols (1.47 %) and shogaols (0.18 %), providing a spicy taste and pungent smell, rendering these genotypes valuable reservoirs for extracting essential oils and harvesting gingerols and shogaols. The essential oil will be utilized as natural preservatives in foods and serve as an active ingredient in medicines, cosmetics, and perfumes. The presence of high gingerols and shogaols will also elevate its demand in the pharmaceutical and medicinal industries. The wealth of genetic diversity present within the basal population presents promising prospects for future crop improvement initiatives in ginger. The red ginger genotypes exhibiting superior quality traits can serve as valuable genotypes for crop improvement. This approach empowers researchers to meticulously choose and refine cultivars possessing specific characteristics that align with the dynamic needs of both agriculture and market requirements. Molecular characterization with a broader range of polymorphic primers will need to be conducted to thoroughly explore the diversity at the molecular level within the red ginger genotypes.

Polyploidy has emerged as a pivotal tool in plant breeding, serving as a valuable technique for introducing genetic variation and developing superior plant varieties. The current study has paved the way for polyploidy breeding of red ginger genotypes. Induced polyploidization through *in vitro* and *in vivo* colchicine treatment has yielded a total of seven polyploids. The present study has revealed increased vigour and promising yields in induced polyploids. The identified polyploids have to be evaluated for stability, adaptability, and yield across various

environments before considering commercial-scale adoption. Furthermore, comprehensive characterization of quality traits will be imperative to fully harness the potential of these tetraploids, with the aim of developing high-yield and high-quality red ginger varieties. Further investigation into the pollen viability and seed-setting ability of the tetraploid ginger is also necessary, especially considering its improved floral features. The present study has unveiled that polyploidy induction events exhibit species-specific traits, contingent upon variables like the concentration of colchicine and the duration of treatment. Consequently, revisiting and refining these factors will be crucial to achieve successful polyploidy induction in exotic red ginger varieties. The result indicated that the ploidy is strongly associated with morphological and stomatal parameters.

Polyploidization could involve intricate molecular and epigenetic mechanisms that lead to either the enhancement or repression of gene expression, consequently altering the phenotype. The connection between polyploidization and plant traits needs to be evaluated. Therefore, gaining a deeper comprehension of these mechanisms, coupled with advancements in experimental techniques for polyploidy induction systems and protocols, allows us to utilize the effects of polyploidization more effectively. The rapid progress in molecular techniques, including CRISPR genome editing, high-throughput biology, and multi-omics, is poised to accelerate our comprehension of artificial polyploidy. These techniques enable targeted modifications, facilitate screening, and hence, unveil the molecular mechanisms underlying polyploidization. This, in turn, will yield novel mechanistic insights and pave the way for the development of new polyploids crops for cultivation.

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APPENDIX

Monthly meteorological data of ICAR-IISR experimental farm, Peruvannamuzhi, Kozhikode, Kerala during 2021-2022 and 2022-2023

Season	Month	Rainfall (mm)	Rainy days	Temperature (ºC)		Relative humidity (%)	
				Maximum	Minimum	Maximum	Minimum
1st season (2021-2022)	January	119.00	6.00	33.48	21.53	95.65	74.03
	February	0.00	0.00	35.39	21.18	93.64	62.43
	March	23.90	4.00	35.52	23.15	95.29	59.03
	April	141.80	14.00	34.90	24.18	95.47	64.73
	May	799.30	21.00	32.89	23.25	96.00	72.77
	June	508.00	22.00	32.73	24.05	94.34	72.64
	July	868.30	27.00	29.98	23.35	95.35	87.29
	August	649.90	27.00	29.94	23.60	95.48	89.00
	September	438.30	18.00	31.02	23.80	95.30	84.77
	October	583.00	17.00	32.06	23.76	94.45	67.00
	November	455.00	18.00	32.53	23.87	93.37	72.53
	December	42.00	4.00	34.02	23.11	94.97	70.39
2nd season (2022-2023)	January	0.00	0.00	34.34	21.89	75.29	95.61
	February	0.00	0.00	35.36	23.50	70.88	92.56
	March	56.40	5.00	38.67	26.89	68.40	86.55
	April	114.50	11.00	34.59	24.30	62.06	95.26
	May	563.00	23.00	32.16	24.38	71.18	99.44
	June	650.20	25.00	31.55	23.77	73.85	99.99
	July	1382.50	28.00	30.16	23.53	78.84	99.29
	August	895.20	22.00	24.50	18.80	61.92	79.28
	September	524.60	13.00	30.74	23.42	68.56	89.26
	October	354.30	15.00	31.69	22.77	72.97	93.03
	November	245.20	12.00	31.90	21.55	72.14	92.93
	December	36.60	3.00	32.48	19.69	83.42	76.65

PUBLICATIONS AND PRESENTATIONS

- George, N. M., Raghav, S. B., & Prasath, D. (2022). Direct *in vitro* regeneration of medicinally important Indian and exotic red-colored ginger (*Zingiber offcinale* Rosc.) and genetic fidelity assessment using ISSR and SSR markers. *In Vitro Cellular & Developmental Biology – Plant, 58,* 551–558.

- Neenu, M. G., Aswathi, P., & Prasath, D. (2023). Synthetic polyploidy in spice crops; A review. *Crop Science,* 1-22.

Oral presentation

- Neenu Maria George and D. Prasath. (2022). Effect of colchicine induced polyploidy on morphological and physiological parameters of red ginger genotypes (*Zingiber officinale* Rosc.). National conference on Enhancing Competitiveness in Horticulture Through Technology Innovations, 17-18 November 2022, ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala.

Poster presentations

- Neenu Maria George and D. Prasath. 2021. Direct *in vitro* regeneration of red ginger (*Zingiber officinale* Rosc.) through rhizome buds. SYMSAC X 2021, 09-12 February 2021, International Symposium on Spices as Flavours, Fragrances & Functional Foods, Indian Society for Spices, Kozhikode, Kerala, India.

- Neenu Maria George and D. Prasath. 2023. Effect of colchicine on *in vitro* polyploidy induction and stomatal characteristics of red ginger genotypes (*Zingiber officinale* Rosc.). International Seminar on Gingers, 01-03 March 2023, KSCSTE – Malabar Botanical Garden and Institute for Plant Sciences, Kozhikode, Kerala.