ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

PLANTLETS AND MICRO-RHIZOME REGENERATION, GENETIC DIVERSITY AND EVALUATION OF GINGER (*Zingiber officinale* Roscoe) AGAINST BACTERIAL WILT IN ETHIOPIA

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Plantlets and Micro-Rhizome Regeneration, Genetic Diversity and Evaluation of Ginger (*Zingiber Officinale* Roscoe) for Bacterial Wilt in Ethiopia

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A Thesis Submitted to the Institute of Biotechnology, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

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By

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Declaration

I, Genene Gezahegn Konobo, hereby declare that this dissertation and its entirety is my all work and has been submitted in partial fulfillment of the requirements for a PhD degree at Addis Ababa University. The dissertation and its parts has not been submitted to other University anywhere for any degree award. All reference sources and materials used for the entire work have been acknowledged well.

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List of Acronyms

AARC	Areka Agricultural Research Center
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of Molecular Variance
BAP	Benzyl Amino Purine
CBT	Center of Biotechnology
CPG	Casamino acid-Peptone-Glucose
CTAB	Ctyl Triacetate Bromide
EBI	Ethiopian Biodiversity Institute
EDTA	Ethylene Diamine Tetra Acetate
EST	Expressed Sequence Tag
FAO	Food and Agriculture Organization
GBW	Ginger Bacterial Wilt
GUS	β-glucuronidase
IBA	Indol Butyric Acid
IRAP	Inter- Retrotransposon Amplified Polymorphism
ISSR	Inter Simple Sequence Repeat
MEGA	Molecular evolutionary genetic analysis
NA	Nutrient Agar
NAA	α-Naphthalene Acetic Acid
NBS	Nucleotide Binding Site
PAG	Polyacrylamide Gel
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis

List of Acronyms (Continued)

PRs	Pathogen Resistant Proteins
RAPD	Randomly Amplified Polymorphic DNA
SARI	Southern Agricultural Research Institute
SCAR	Sequence Characterized Amplified Region
SNNPRS	Southern Nations, Nationalities And Peoples Regional State
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
TAE	Tris-acetate EDTA
TEDTA	Tris-HCl Ethylene Diamine Tetra Acetate
TZC	Tetrazolium Chloride
UNJ	Unweighted Neighbor Joining

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Plantlets and Micro-Rhizome Regeneration, Genetic Diversity and Evaluation Of Ginger (*Zingiber officinale* Roscoe) Against Bacterial Wilt in Ethiopia

Genene Gezahegn Konobo

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Abstract

Ginger (*Zingiber Officinale* Roscoe) is an important crop used for many purposes and is a major spice across Ethiopia since 13th century. Ginger production, productivity, and product quality were limited due to different biotic and abiotic factors. Among the biotic factors, it was challenged primarily due to bacterial wilt disease eruption in the last decade. The use of clean tissue culture generated seed rhizome as part of integrated management with other cultural practices was an option to reduce the disease pressure. However, the disease still poses a threat to ginger cultivation in the country. In the absence of efficient strategies to reduce the disease impact, the use of tolerant cultivars appears to be the best disease control strategy. Hence, this study aimed to enhance disease-free planting materials generation and ginger diversity analysis. The first and second experiments targeted in vitro disease-free plantlets generations and micro-rhizome induction respectively. Experiments three, and four are with the objectives of genetic diversity assessment among ginger accessions using morphological and molecular markers consecutively. The last objective of the study focused on evaluating the study materials for resistance against the pathogen. In the course of in vitro regeneration, alternative nitrogen source salts replaced 1.65 g/l ammonium nitrate in Murashige and Skoog medium.

Ammonium chloride at 1.0 g/l, potassium nitrate (3.8 g/l), and urea (3.0 g/l) are the best alternatives. Micro-rhizome induction resulted in viable *in vitro* generated micro-rhizomes that are planted directly in the soil. Murashige and Skoog medium supplied with elevated levels of sucrose (80 g/l) and benzyl amino purine (6.0 mg/l) was the best treatment among thirteen treatment combinations for the micro-rhizome induction. For genetic diversity analysis, both by morphological traits and SSR markers, 100 ginger accessions including two released varieties (Boziab and Yali) and wild species (mango ginger) were collected from different agro-ecologies of south, central, and southwestern parts of Ethiopia. The morphological trait-based analysis was done by using 24 quantitative and qualitative traits. The analysis has revealed the availability of genetic diversity via high values of genetic coefficient of variation, heritability, and genetic advance as mean. Cluster analysis also grouped the 100 accessions into four clusters. The study materials assigned to four sub populations; Southern, Central, Southwest and Oromia based on the area of the collection were also assessed for genetic diversity using twelve polymorphic SSR markers. The SSR based molecular diversity population structure anlysis has grouped the 100 accessions into three clusters unlike morphological-based analysis which resulted in four clusters. AMOVA has showed 96% among individuals and 4% variation was among the four populations. Sprouted buds were found better for quality DNA extraction in ginger, which is the first of its kind. In both morphological and SSR-based diversity analysis, the wild type (JW89) was unique due to some traits like rhizome size and unique fragment bands. The diversity analysis based on morphological traits and SSR markers, in general, revealed that there is high genetic diversity among ginger accessions. Evaluation of the 100 study materials against bacterial wilt disease pathogen under the protected conditions in the screen house and further tested in the field also revealed that there is response variation among the accessions.

Disease severity, disease incidence, days to severe disease symptoms, fresh and dry yield losses showed high variations among accessions. the evaluation experiment revealed that (12) accessions were grouped as very tolerant accessions. The majority, 88 accessions also showed different response levels from tolerant to very susceptible based on disease scores and yield loss analysis results. Four accessions (BASP19, BSSB47, BSSB49, and OKW63) and wild type (JW89) have shown consistent variations being grouped to very tolerant in disease screening, in similar clusters during morphological and molecular genetic diversity analysis. The study results of the three separate experiments' morphological traits, SSR markers, and screening against pathogen gave clue that there might be potential markers linked to disease tolerance and high rhizome yield, which needs further verification.

Keywords: ammonium nitrate, disease free, markers, plantlets, diversity, ginger, micro-rhizome, tolerance, sucrose, sprouts.

CHAPTER 1

1. Introduction

1.1. General Introduction

Spices are the dry parts of plants, which are used to flavor food, as pungent stimuli, and in some cases as medicine around the world. They can be obtained from seeds, berries, buds, leaves, bark, and roots of plants growing mainly in the tropical, subtropical, and temperate zones. Spices have various effects when used in foods: not only do they impart flavor, pungency, and color characteristics, but they also have antioxidant, antimicrobial, pharmaceutical, and nutritional properties. In addition to these direct effects, complex or secondary effects can be achieved by using spices for cooking (Herman, 2015). Many of the spices in production and utilization across the world are also produced and used as part of the Ethiopian food system, of which ginger is the major one (Kifle *et al*., 2023).

Ginger (*Zingiber officinale* Roscoe) is an important delicacy, medicine, spice and monocotyledonous perennial herb belonging to the family *Zingiberaceae*. It was reported as a diploid with somatic chromosome number of 22 (n=11) and genome size of 23.618 mega base pair (Wahyuni *et al*., 2003). Edible ginger was grouped into two divisions: *Zingiber officinal* cultivar group officinale "ginger" and *Zingiber officinale* cultivar group "rubrum" with small, pungent tasting rhizomes. Ginger originated in Island Southeast Asia and was likely domesticated first by the Austronesian peoples. Currently ginger is produced in South and Southeast Asia, tropical Africa, Latin Americas and the Caribbean's.

Traditionally, ginger uses as an acrid bitter to strengthen and stimulate digestion. Modern uses include prophylaxis for nausea and vomiting, dyspepsia, lack of appetite, anorexia, colic, bronchitis and rheumatic complaints (Herba, 2018).

The cultivation and utilization of ginger in Ethiopia started during 13th Century when Arabs introduced it from India to East Africa (Jansen, 1981; Endrias and Asfaw, 2011). The economic part rhizome, itself is the most commonly used planting material for ginger propagation across the world. The requirement of large amount of seed rhizome (2-2.5 tons) to plant one hectare of land has impacted product availability (Girma and Kinde, 2008). The potential and importance of this spice crop has been expressed via different conditions like total area covered and productivity per hectare farmland. The top five ginger-producing nations globally include India, Nigeria, China, Indonesia and Nepal. Ethiopia ranks 15th globally and fourth in Africa next to Nigeria, Cameroon and Mali (FAOSTAT, 2022). Its production at large scale was mostly limited in the hot humid areas of Southern and Southwestern parts of Ethiopia. Among all spice crops grown in Ethiopia, ginger has been the main source of income for those farmers who grow it and was well recognized in improving the livelihood of many ginger growers (Asfaw and Derbew, 2021). Ginger had been a leading export spice crop from 2005-2011 in terms of average exported volume and hard currency value. Being the largest export spice crop in Ethiopia, ginger contributed about 66% (7199 tons) to the spice export value in 2011. However, this was declined to 8.75% (1183 tone) of total spice product exported in 2013 (Ethiopian spice authority annual report, 2013) which is due to devastation by ginger bacterial wilt disease.

Before 2012, no disease at all in Ethiopia was reported in ginger. However, sudden nationwide shocking disease complex of ginger bacterial wilt and ginger leaf spot diseases have shortly devastated the crop within one year of cropping season in 2012 (Bekele *et al*., 2016). Moreover, during storage and cultivation, rhizomes for planting as seed are susceptible to diseases particularly Pythium aphanidermatum and Ralstonia solanacearum that causes rhizome rot and bacterial wilt respectively leading to a substantial reduction in field performance and yield (Kifle *et al.,* 2021). The national spices research program center accomplished some research experiments and achieved results with possible recommendations. Collections of more than 90 ginger accessions from domestic and introduction from foreign sources, development varieties (Yali and Boziab) with practical crop management recommendations were some of the achievements. Harvesting and postharvest management practices, seed multiplication and distribution of released varieties have been demonstrated for producers (Zakir *et al.,* 2018). Moreover, spacing, time of planting and irrigation frequency recommendations were recently reported (Asfaw and Deribew, 2021).

In contrary to many agronomic recommendations, much limed research works were conducted in genetic diversity analysis and variety improvement. Moreover, there is no report in biotechnology side for improvement of ginger crop production and productivity in the country. *In vitro* propagation protocol was optimized using shoot buds as explants on MS media supplemented with different levels and combinations of growth regulators (Ayenew *et al.,* 2012). Production of ginger has significantly reduced in the last ten years due to various production constraints. Among the major challenges, bacterial wilt disease, lack of improved varieties, limitations of improved crop management practices, harvesting and postharvest technologies are few of many. Moreover, weak technology transfer system to popularize available ginger technologies, weak value chain,

limitation of value added products and product market adulterations were also causes for low production. The imact of ginger bacterial wilt disease since 2012 production season for the last ten years has become the leading ginger production constraint (Zakir *et al*., 2018; Kifle *et al.,* 2023). On the other hand, the national spices program has been struggling to generate improved varieties. However, due to low attention given for the crop only two varieities were registered as varieties (Girma *et al*., 2022).

Therefore, this research work was conducted with general hypothesis of potential establishment of low cost in vitro regeneration protocols for shoot and micro-rhizome induction. Another target was Ethiopian ginger genetic resource diversity assessment to understand the range of genetic variation towards ginger economical traits, including resistance to bacterial wilt disease. Molecular marker based diversity analysis to verify the genetic variability and project potential markers linked to quantitative and qualitative traits was also addressed. Evaluation of the study materials for resistance to bacterial wilt disease pathogen under protected and open field conditions is the final experiment of the entire study.

1.2. Research Hypotheses

The following hypotheses have been tested

1. Ammonium nitrate in MS growth media can be re adjusted to minimum level or omitted and replaced by other salt type as alternative source to achieve higher number of ginger plantlets in vitro.
2. Micro rhizome can be induced, and produced in vitro and used as disease free initial planting material by adjusting levels of media components.
3. There are plenty of diverse genotypes among Ethiopian ginger accessions that can be revealed by morphological traits and exploited for ginger genetic improvement.

4. SSR markers differentiate ginger accessions to different classes that can be revealed and used for further ginger variety improvement programs.
5. There are ginger accessions that can better tolerate or resist ginger bacterial wilt disease

1.3. Research Objectives

1.3.1. General Objective

To evaluate alternative nitrogen sources and media components on *in vitro* planting materials production, assessment of the range of genetic diversity among ginger accessions and evaluate for resistance against ginger bacterial wilt disease.

1.3.2. Specific Objectives

1. To identify alternative source of nitrogen nutrient in MS media as replacement of ammonium nitrate.
2. To evaluate the effect of media components at different concentration level and combinations on optimal number of in vitro micro-rhizome induction.
3. To analyze ginger genetic diversity in Ethiopia using morphological traits
4. To evaluate genetic diversity among Ethiopian ginger accessions using molecular markers.
5. To test performance of ginger germplasm for resistance against bacterial wilt.

CHAPTER 2

2. Literature Review

2.1. Origin and Distribution of Ginger

Ginger is believed to have originated in Southeast Asia, probably in India, and is cultivated in several parts of the world now. Globally, the leading ginger producing countries are India, Nigeria, China, Indonesia, Nepal, Thailand, Cameroon, Bangladish, Japan and Mali (FAO, 2022). In the thieteenth century, the Arabs brought ginger to East Africa and ginger has perhaps been known since then in Ethiopia (Jansen, 1981). Previous reports indicated that ginger was little grown or used in Ethiopia (Kostlan, 1913). Ethiopia is now well known for ginger fresh and dry rhizome product supply in local and foreign markets (Endrias and Asfaw, 2011). The production was mainly in the wetter regions of Kaffa, Illubabor, Gamo-Gofa, Sidamo, and Wollega, mostly in gardens before large-scale production was commenced (Peter, 2001). Largescale production of ginger started around 2005 following the introduction of new cultivars by World Vision to the Wolaita and Kambata areas. However, the large-scale production was mainly limited to these parts of Ethiopia and very recently expanded to other parts even if challenged by bacterial wilt disease. Farmers around Kambata, Wolaita, Hadiya, and Bench Maji, which were part of the former southern nations, nationalities and peoples region (SNNPR), are now the major source of ginger (Tadesse and Asfaw, 2015; Girma *et al.*, 2022).

2.2. Botany and Morphology of Ginger

Ginger is a monocotyledon plant, which consisting a large number of species in 53 genera belonging to *Zingiberaceae* family in the order of *Zingiberales*. The ginger plant is an erect herbaceous perennial growing from one to three feet in height. The stem is surrounded by leaf

sheathe bases of parallel or alternative leaves (Fig. 1). The plant is erect, with aerial shoots (pseudo stem), leaves and the underground stem (rhizome). The roots of ginger have unlimited growth from the base of the sprouts. These fibrous roots and the number of such roots keep on increasing with the growth of tillers. A club-like spike of yellowish, purple-lipped flowers have showy greenish-yellow bracts beneath rarely occurs in cultivation (Bhatt *et al*., 2013). The ginger economic part is the modified stem known as a rhizome, having nodes with scale leaves and internodes. Except for the first few nodes, all the nodes have axillary buds. There may be one or more apical buds on the rhizome but only one bud becomes active for sprouting and growth in the ginger plant.

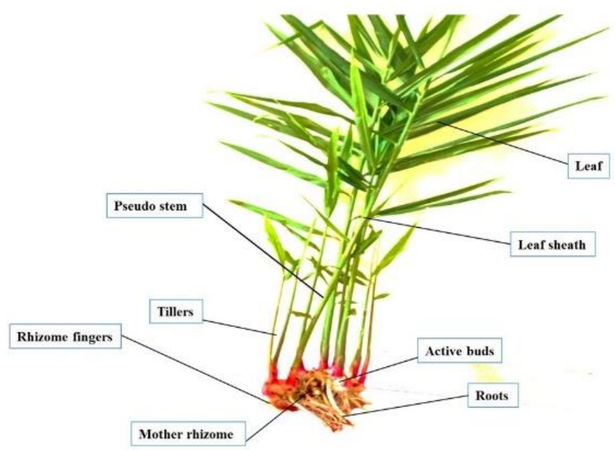

Figure 2.1. Morphological structure of complete ginger plant (picture taken during experiment)

2.3. Dietary Use and Health Benefits of Ginger

Both fresh and dried ginger rhizomes are used worldwide as a spice. Ginger extracts are extensively used in the food, beverage and confectionary industries for the production of marmalade, pickles, chutney, ginger beer, ginger wine, liquors, biscuits, ginger tea, and other bakery products (Mesomo *et al.*, 2013). In Ethiopia, it is among the major spices used in every kitchen to flavor stew, tea, bread, and local alcoholic drinks (Nigist and Berhanu, 2003).

Medicinally, it is used mainly to relieve stomachache, fever, influenza, headache, coughs, and toothache (Asfaw and Demisse, 2009). It is also used to treat many diseases like bleeding, cancer, chest congestion, chicken pox, cholera, chronic bronchitis, cold extremities, common cold, cough, and diarrhea. Ginger is also used during difficulty in breathing, to prevent motion sickness and vomiting during pregnancy (Haniadka *et al.*, 2013).

2.4. Nutrient Composition

The nutrient composition of ginger includes 80% moisture, 12.3% carbohydrates, 2.4% fiber 2.3% protein, 1.2% minerals, and 0.9% fat. The minerals present in ginger are iron, calcium, and phosphorous. It also contains vitamins such as thiamine, riboflavin, niacin, and vitamin C. The flavoring properties of ginger are due to the combination of pungency and aromatic essential oils. A series of homologous phenolic ketones known as gingerols are sources of pungency in fresh ginger. These gingerols are thermally unstable and can be converted under high temperatures to shogaol (after shoga, the Japanese word for ginger) (Mishra, 2009). Research reports also revealed there are essential and non-essential metal elements in ginger. Ethiopian ginger cultivar analysis for some metals reported Mg followed by Ca are the most abundant macronutrients.

Whereas, Mn followed by Fe, Zn, Co, and Cu are micronutrients in Ethiopian ginger (Wagesho and Chandravanshi, 2015). It was also reported that the composition of all components varies with the type of variety, agronomic conditions, maturity, curing methods, drying and storage conditions as well as agro ecological conditions (Bhatt *et al*., 2013).

2.5. Genetic diversity of ginger

Genetic variability refers to the tendency of individual genetic characteristics in a population to vary from one another. It refers to potential of a genotype to change or deviate when exposed to environmental or genetic factors. Genetic variability is the tendency to genetically vary from one another but genetic diversity refers to the diversity within a species. The greater genetic diversity, the higher is the chance of a long-term survival. Genetic variability may be high or low. High variability is when there is a high tendency to vary from one another in a population. Conversely, a low genetic variability is when that tendency is relatively low. When the genetic variability is high, the chance of selecting better genotypes is greater. Genetic variability is variation in their genetic make-up of an organism which arises due to the genotypic difference and the base for selection is the main concern of plant breeders (Islam *et al*., 2002).

It provides the basis of effective selection and it plays a vital role in the breeding program. Possibility of achieving improvement in any plants depends heavily on the amount of genetic variability. The most diverse the plants, there is the greatest chance to generate productive recombinants and in segregating generations during genetic improvement (Verma *et al*., 2014).

Genetic diversity is essential to meet the diversified goals of plant breeding such as breeding for increasing yield, wider adaptation, desirable quality, pest and disease resistance. Genetic variability in a population can be partitioned into heritable and non-heritable variation with the aid of genetic parameters such as variance, genotypic coefficient of variation, heritability and genetic advance, which serve for selection of some outstanding genotypes from existing ones (Othieno & Shinyekwa, 2011).

Edwards, (2000) indicated the three ways of assessing the existence of variability in breeding population; (1) by using simple measures of variability, such as range, mean, variance, standard deviation, coefficient of variability and standard error (2) by estimating the components of variance and (3) by measuring the genetic diversity e.g. D^2 statistics. Genotypic and phenotypic coefficients of variation are used to measure the variability that exists in a population (Burton and Devence, 1953).

Phenotypic and genotypic coefficients of variation values had classified as high (>20%), medium (10-20%) and low (<10%). The narrow difference between phenotypic coefficients of variation (PCV) and genotypic coefficients of variation (GCV) of the traits shows low environmental influence. The lower value of PCV generally depicts low variability among the tested sample while a high proportion GCV to the PCV is desirable in breeding works. So, the high values of GCV and PCV suggested there is a possibility of improvement through direct selection for the traits. While both GCV and PCV < 10% that can be low and hence these traits provide almost less chance for selection (Burton and Devence, 1953).

2.6. Ecology of ginger

Ginger needs warm, humid climate for good yield and cultivated well at an altitude of 1500 meters above sea level. It grows well in a climate with moderate rainfall with well-drained soil like sandy loam rich in humus. It is recommended not to grow ginger in same soil year after year, but to have rotation of crop. It is best to plant ginger in the pre-monsoon period, after burning the surface soil for the higher yield and reduce disease incidence. Edible or culinary ginger is the fat, knobby, aromatic rhizome of *Zingiber officinale*, a tender herbaceous perennial plant in the large ginger family (Zingiberaceae) native to humid, partly-shaded habitats in moist tropical and subtropical forests of Southeast Asia. Ginger loves hot, humid conditions and rich soil with lots of nutrients. In our cool climate the plants do well in full sun; in more southern locations the plants may need partial shade (Kifle *et al*., 2023).

Fertilize regularly during the growing season unless planted in very fertile soil. If planting in the ground, amend it first with lots of compost, rotted manure, or other rich organic matter. Mulch in ground plants to keep soil warmth and moisture, and prevent competition from weeds. Water regularly but do not allow the soil or planting medium to remain soggy. Container grown plants should not be watered at all when leafless and dormant; resume watering when new shoots appear (Kifle *et al*., 2023).

2.7. Ginger Production and Marketing in Ethiopia

Ginger *(Zingiber officinale* Rosco*)* 'Zingible' in the Amharic language has been known in Ethiopia since the beginning of the 13th century. It is cultivated in wider environments across the country than any other spices for flavoring a variety of foods, and local drinks (Borget, 1993). In the past, Ethiopia has been importing a large quantities of ginger from India and Jamaica (Anand, 1982), but later besides large domestic use, export has been made to Egypt, Saudi Arabia, Yemen, Kenya, and Djibouti. Cultivation was under sub-optimal conditions with rainfall often less than 1500 mm per year and at lower temperatures with reasonable yield records for local consumption for a long time before the start of large-scale production (Jansen, 1981; Endira and Asfaw, 2011).

Ginger research and production in Ethiopia has been going on as part of coffee diversification, since the inception of coffee research in 1969 (Girma *et al*., 2008). The potential and importance of this spice crop could be expressed via different conditions like total area covered and productivity per unit area of farmland. Ethiopia was at 15th position globally and fourth in Africa next to Nigeria, Mali and Cameroon according to FAO crop production estimation report (FAOSTAT, 2022). The report of FOA indicated also 10,394 ton was harvested from 3633 hectar of farmland in the ryear 2021. Its production at large scale was mostly limited in the hot humid areas of Southern and Southwestern parts of Ethiopia. Among all spice crops grown in Ethiopia, ginger has been the main source of income for producers and traders (Asfaw and Derbew, 2021).

According to Ethiopian external trade statistics report, 22.6 million USD was earned from ginger in 2011. However, in the last decade the export quantity and price fluctuated year to year due to bacterial wilt disease eruption (Kifle *et al.,* 2023). Ginger production, processing and

marketing assessment reports indicates producers provide ginger product in three different forms. The first one is fresh ginger, which is usually supplied to market in rainy season for immediate cash demand and sometimes in dry season mainly by farmers. The second type of marketable ginger product is dried ginger. Dried ginger is favorite ginger product exchanged in large volumes by all market participants at different stages of marketing from local assembling to export market. The third type of marketable ginger products are extracted ginger products. These include ginger powder, essential oils, oleoresin and others (Endrias and Asfaw, 2011).

2.8. Ginger Research and Development in Ethiopia

The national spices research program based at Tepi spice research center accomplished different research experiments and achieved milestone results with possible recommendations. Collections of more than 90 ginger accessions from domestic and introduction from foreign sources, development of suitable varieties (Yali and Boziab) with practical crop management recommendations were some of the achievements. Research on intercropping of ginger with associated crops, identification of major pests, and possible management practices were also reported. Harvesting and postharvest management practices, seed multiplication and distribution of released varieties have been demonstrated for producers. Evaluations of the quality standards of the ginger varieties were also conducted and compared with international standards (Zakir *et al.,* 2018). Edossa (1998) recommended 20 cm between rows and 15 cm between plants spacing claiming the highest fresh rhizome yield (20 t/ha).

However, practical experiences in field indicated that the rhizomes planted with this spacing combination resulted in congested and intermingled rhizomes, and this finally created problem to harvest quality rhizomes. Accordingly, spacing of 30 cm X 15 cm between rows and between

plants was recommended later (Girma *et al*., 2008). Moreover, spacing, time of planting and irrigation frequency recommendations were recently reported (Asfaw, and Deribew, 2021). The amount of seed rhizome or seed setts required to sow one hectare of land varies based on seed type used. Seed rhizome with 2.5 - 5 cm length having at least 1 active sprouting bud in amount of 2.5-3 t/ha was reported as optimum rate to get high fresh rhizome yield at average of 16 t/ha (Girma and Kinde, the 2008). Due to dynamic climate change and different rain seasons in ginger producing areas planting time was suggested to be based on the availability of moisture.

In contrary to many agronomic recommendations, much limed research works were conducted in genetic diversity analysis and variety improvement. Moreover, there is no report in biotechnology side for improvement of ginger crop production and productivity in the country. In vitro propagation protocol was optimized using shoot buds as explants on MS media supplemented with different levels and combinations of growth regulators (Ayenew *et al.,* 2012). This protocol was adapted in government and private tissue culture laboratories with little modifications for micro-propagation and disease cleaning in ginger (Berihu, 2018; Genene *et al*., 2019).

2.9. Ginger Production Constraints in Ethiopia

Production of ginger has significantly reduced in the last ten years due to various production constraints. Among the major challenges, bacterial wilt disease, lack of improved varieties, limitations of improved crop management practices, harvesting and postharvest technologies are few of many. Moreover, weak technology transfer system to popularize available ginger technologies, weak value chain system, limitation of value added producta and product market adulterations were also causes for low production. The imact of ginger bacterial wilt

disease since 2012 production season for the last ten years has become the leading ginger production constraint (Zakir *et al*., 2018; Kifle *et al.,* 2021). On the other hand the the variety improvement program based at Tepi research center has been struggling to generate improved varieties. However, due to low attention given and nature of the crop only two varieities were registered as varieties (Girma *et al*., 2022).

2.9.1. Ginger bacterial wilt

Bacterial wilt is the most important and devastating plant disease of many high value crops**.** Similarly**,** ginger bacterial wilt disease causative agent, *Ralstonia solanacearum* is one of the top ten bacterial species that affects crop plants causing high product loss to wide range of host plants. The pathogen induces ginger bacterial wilt disease (GBW) which has been resulting in huge economic losses (Mansfield *et al.*, 2012, Kifle *et al*., 2021). It was also reported as one of the most serious and devastating diseases of bacterial origins in the world affecting large number of plants. Most of the important Solanacearum crops such as tomato, potato, pepper and eggplant are affected by the pathogen each year (Alarm, 2013). It was widely distributed in different regions of the world from tropical to temperate areas causing severe wilting in large number of crop species at all growth stages (Nelson, 2013).

The pathogen was classified into five unique races based on the hosts it affects, and to five biovars, based on the use ability of hexose, alcohols and disaccharides. Race 1 includes strains under biovars 1, 3 and 4. These strains of the race cause disease to a broad range of host plants like, tomato, tobacco and peanut. The second race type consists biovar 1 and biovar 3 which are mainly affecting; banana (*Musa acuminata*), plantain (*Musa paradisiaca*), Heliconia (*Helthliconia* spp.)

and other plants in the Musaceae family. Race 3 has strains of biovar 2, and occurs in the upland tropic areas and causes wilting disease in crops like potato, tomato and geranium. Ginger infecting starin was recoginzed as race 4 and biovars 3 and 4. The last race 5 strains infect mulberry (*Morus alba*) (Mathew *et al*., 2008).

2.9.2. Epidemiology and Survival of the Pathogen

Ginger bacterial wilt disease pathogen, *Ralistonia solanacearum* inhibits plant growth, after entering to the roots via open areas during planting material preparation, field management, or certain nematodes and natural holes where secondary roots emerge (McCarter, 1991). After entering to the host, the pathogen has tendency for the vascular system, where it reporduce rapidly, then it filles the xylem with cells of the bacteria and secondary metabolites. Once the infection was developed it moves up through the xylem and finally blocks water transportation, which causes wilting (That and Sijam, 2010). Later it causes rapid wilting of the plant within few (5–10) days of the infection. The severity of the disease pressure became high when favourable environmental conditions like high rainfall, and warm weather are available (Kumar *et al.*, 2012). The bacterial cells persist for long time in soil by entering to new or adjacent plant roots and continuing its saprophytic life cycle. The pathogen has also long living nature under starved conditions freely in soil and pure water out of host plants (Denny, 2006; Habetewold *et al.*, 2015).

2.9.3. Symptoms of the Disease

Ginger bacterial wilt disease symptoms are of external and internal types. The common visually observable external symptoms in giner includes curling and bending of leaves because of turgidity loss. Wilting yellowing and drying of leaves, growth of adventitious roots, and the development

of narrow dark stripes in the infected vascular bundles beneath the epidermis are also among the symptoms. This results due to the multiplication of the pathogen cells in the vascular system that clogs the vessels (Álvarez *et al*., 2010; Kurabachew and Ayana, 2017).

Severe cases of the disease are characterized by breakout of pseudo stems from the infected plant by a gentle pull and can be broken off at the base. Early infections are identified by mild drooping and curling of lower leaf margins. Yellowing starts at the edges of the lower leaves then slowlly continues in the upper most leaves. In the pick disease stages, infected plants exhibit deep yellowiing and complete wilting symptoms. The pseudo stems develop dark streaks at the vascular tissues that leads to plant collaps before rhizome development. Moreover, infected plants leaf sheaths became yellowish to pale green, leaves curl up and at the end ginger plants dries up.
The junction between pseudo stems and the rhizome of infected plants emit foul smell, and rhizome extrudes milky ooze from the vascular strands while are pressed gently (Handiso *et al*., 2017).

2.9.4. Characteristics of the Pathogen

The specific pathogen to ginger is identified asa rod-shaped bacterium type having an average size range from 0.5 to 0.7 by 1.5 to 2.5 μm. The pathogen was catagorized as obligatory aerobic (Denny and Hayward, 2001). The basic biochemical characteristics are catalase positive, oxidase positive and nitrate reduction positive. It does not hydrolyze starch and can readily degrade gelatin at optimum environmental conditions. The bacterium is inhibited by concentrations of sodium chloride (NaCl) greater than 2% broth culture system. Both liquid and solid (agar) growth media can be used for pure culture of the bacterium. Individual colonies are most of the time visible after 36 to 48 hours of growth at 28°C during solid culture system. The typical method for isolation of the virulent pathogen is using Kelman"s tetrazolium chloride (TZC) suppiled nutrient agar medium

(Kelman, 1954). The virulent wild-type colonies are characterized by large, elevated, fluidal and either entirely white or with a pale red center after two days on TZC medium., the optimal growth temperature for most strains of ralistonia pathogen is 28-32°C; however, some strains that are pathogenic on potato have lower optimal temperature at 27°C.

Casamino acid-Peptone-Glucose (CPG) medium is also another media that can be used for in vitro morphological studies of the pathogen. Reports on this medium indicates the colonies were irregular white and fluidal after incubation period of 48-72 hours. Colony fluidity on CPG medium are the main differentiating characters of strains infecting only ginger than those affecting other hosts plants. Ginger strain colonies are highly fluidal having spiral pink center whereas, strains of ther crops fluidity and pink center was less conspicuous.

The pathogen colonies on sucrose peptone agar were round to irregular, creamy white and fluidal (Kumar and Sarma, 2004). Culture based identification of virulent isolates of the pathogen indicates the bacteria is fluidal, presents irregular shape and white with pink centered colonies on tetrazolium chloride (TZC) media and white on nutrient agar medium (Abdela and Tesfaye, 2017).

2.9.5. Economic importance of bacterial in Ethiopia

Ginger bacterial wilt disease prevalence study conducted in twelve major growing areas in the former Southern nations, nationalities and peoples and some parts of Oromia region reported the disease was widely distributed in all areas assessed in 2012 and 2014 production years. The disease incidences recorded and yield losses were extremely high in some areas. The disease incidence was highly increased in interval of 78.4 to 99% with average of 92% in all surveyed areas during

2014 production season irrespective of their geographic locations (Habtewold *et al*, 2015). Field surveys conducted also indicated the incidence of the disease was 100% with 78% severity in 2015 and similar patterns in consecutive years with little reduction, which may be due to effect of weather condition as the pathogen is highly affected by seasonal factors (Yuliar *et al.,* 2015; Said *et al.,* 2020). Some other reports on the other hand indicated there are potential fungal diseases fusarium wilt and ginger leaf spot with more or less equal incidence, prevalence and severity to bacterial wilt from the same fields surveyed (Said *et al*., 2020; Kifle *et al*., 2021). Integrated bacterial wilt disease management experiment conducted at Jimma and Tepi during 2017 cropping season reported more than 50% yield loss in untreated plots with some difference among cultivars and locations (Guji *et al*., 2019). Based on the survey reports both from federal and regional research centers, high economic loss has been reported. Survey result of 2014, which covered production areas (Dawro, Wolaita, Kenbatatenbaro, Hadiya, Gomogofa, Konta, Alaba, Sheka, Bench maji, and Gambella) revealed yield loss of up to 98%.

The prevailing ideal weather condition for the bacteria epidemics (average rain fall, 287.9 mm, maximum and minimum temperature of 27.8 °C and 17.1 °C) and use of infected seed rhizome were reported as aggravates (Habetewold *et al*., 2015)**.** Because of lack of internal quarantine regulations in seed system in the country, the disease has remained as threat to ginger production (Kifle *et al*., 2021; Girma *et al.,* 2022). Direct yield losses and incidence variation was also reported widely due to effect of the host, cultivar, climate, soil type, cropping pattern, geography and strain (Habetewold *et al*., 2015; Hunduma *et al*., 2016).

2.9.6. Management of Ginger Bacterial Wilt Disease

Disease occurring in plants due to infection by *Ralistonia solanacearum* bacterial strains is one of the major production constraints for ginger and other horticultural crops. Planting pathogen free planting materials, rhizome treatment by hot water, use of antibiotics to eliminate the pathogen, phyto-sanitations, and alternating ginger with non-host crops were among the recommended strategies for field control (Kumar and Hayward, 2005). A virulent mutant of the pathogen has been used as a bio-control agent for the virulent pathogen (Trigalet and Demery, 1990).

Inpsite of these all strategies, the disease still a threat to ginger global production. Hence, it is very important to look for efficient strategies to minimize the disease pressure. At this point the use of resistant cultivars appears to be the best strategy, which relies on exploitation of host resistance (Kumar, 2006; Gaelle, 2014). Various research trials were reported for resistance cultivar identification against different strains of the disease pathogen. The degree of susceptibility to tomato strain of the pathogen was reported there was significant variation among six tomato cultivars. The report also indicated additive genes were more important than the non-additive genes for resistance. Thus, in tomato breeding programs against bacterial wilt, selection of resistant genotypes can be potential solution (Abdalla and Abdulla, 1998). Bacterial cell proliferation in stems of resistant tomato plants was suppressed basically due to limitation of pathogen transfer from proxylem tissue to other xylem tissues (Nakaho *et al*., 2004).

2.10. Ginger genetic improvement

Poor flowering and seed set have seriously handicapped the breeding of ginger for any trait of interest. Hence, crop improvement programs were limitted to observation and discrimination of

naturally occurring narrow genetic bases. Successful plant breeding programs need wide genetic variation to search ideal genotypes during the process of evaluation (Nirmal *et al*., 2013).

Now a days traditional mutation breeding has lost its preeminent position, but induced mutations continue to be in great demand with various biotechnological tools. Methods of mutation induction and analyses of mutants have brought great changes. In vitro culture techniques also provide an alternative means of plant propagation and a tool for crop improvement (Vasil, 1988). Advanced tissue culture technologies enabled a piece of easily handled tissue explants for treatment. Excised stem tips or callus growing on standard nutrient mediums can grow to maturity and evaluated for useful mutations. Advanced *in vitro* techniques such as somatic embryogenesis and single cell culture are with low rate of chimer formatiom during mutations induction (Chopra, 2005). Varieties resistant to bacterial wilt disease developed through mutation induction have been reported in vegetables like tomato, chilli and eggplant.

Screening for the resistant gene analogues (RGAs) in edible ginger and related species like mango ginger resulted in the identification of mRNA clusters with similarity to known *R*-genes (Karthika *et al*., 2019). This screening protocolo for identifying resistant ginger cultivars for bacterial wilt developed and validated on large number of ginger germplasm under greenhouse condition revelead some test plants escaped the disease development (Kumar, 2006).

2.11. Ginger Breeding Experiences in Ethiopia

Preliminary research works on diversity analysis in Ethiopia indicated that both local and introduced ginger germplasms are variable in their morphological characters, rhizome yield,

volatile oil and oleoresin contents (Girma *et al*, 2008). Genetic variability study conducted based on morphological and some quality traits using 36 ginger accessions indicated that there was wide genetic variability among the study materials. The study then suggested for further verification experiments using large accessions and advanced tools (Momina *et al*., 2011).

Ginger variety development activities at national level by Tepi spice research center so far have been evaluating local and introduced germplasms for adaptability, yield, quality and disease resistance traits. So far, the program released two varieties and five cultivars were under evaluation for release but challenged due to bacterial wilt disease (Zakir *et al.*, 2018). Attempts towards development of integrated management strategies for ginger bacterial wilt, reported some variation between two cultivars tested (Guji *et al.,* 2019).

2.12. Biotechnology Applications on Ginger

Biotechnology with multi-definitions and applications has been enhancing the agricultural sector for the last century. Genetic improvement in plants with aid of biotechnology tools complemented with good agronomic practices, have helped billions of rural farmers across the world. Countries like China, India and Brazile where the agricultural research system is well established are in the front line benefited countries from biotechnology. Biotechnology companies in these countries have become the primary employers delivering useful new varieties of tropical crops (Gery *et al*., 2003; Jie *et al*., 2018).

The application of modern biotechnology was highly expressed in plants starting from micro-propagation and disease cleaning to genetically modified plants. These biotechnological tools have

played a pivotal role in the improvement of plant species. Some of these techniques such as micro-propagation and production of enhanced secondary metabolites using cell suspension cultures were highly applied (Yue *et al*., 2016). In vitro production of haploids, somatic embryogenesis, somatic hybridization (Kackar, 1993), germplasm conservation and storage, recombinant DNA technology, and transgenes are the main techniques used to improve crops and other organisms (Baskaran and Jayabalan, 2005). Many of these are in use recently also for the improvement of ginger.

2.12.1. Micro propagation of Ginger

Vegetative propagation in plants like ginger has risk of systemic infections by root knot nematodes and bacterial disease pathogens. Diseases caused by bacterial wilt, soft rot and rhizome rot pathogens has caused heavy yield losses in ginger over the world. The diseases are mainly transmitted by rhizome propagules and needs production of disease free clones. Hence, micro-propagation using tissue culture technique is used to get disease free large amount of uniform planting materials. It is also considered as an alternative means of safe plant propagation and as a tool for variety development programs (Nirma, 2016).

Micro-propagation was achieved either through direct organogenesis or indirect organogenesis (Seran, 2013). Clean culture establishment plays a very important role in micro propagation from new explants of ginger. Usually explants are used from the rhizome buds, leaf explants, internodes and roots. However, the adventitious buds have been widely reported as best explant source. The buds have shoot primordia for the direct organogenesis as nutrients are provided for the development of the shoots from these buds.

In indirect organogenesis, the explants are subjected to enter the callus phase and then dedifferentiated into plantlets mainly for rapid proliferation of plant cells (Nabarawya, 2015). However, contamination may be a problem in the in vitro initiation and regeneration of ginger explants. In order to achieve contamination free cultures explants need thorough washing with running water and detergents. These explants later should be exposed to surface sterilizing agents such ethanol followed by sodium hypochlorite, calcium hypochlorite or mercuric chloride followed by washing with distilled water and culture to media at appropriate size. Later, initiated shoots are subjected to multiple shoot formation steps that may need specific growth regulators in media. After rooting, the shoots are primarily acclimatized by transferring to the netted pots filled with sterilized peat mixture and keeping in the growth chamber or acclimatization tunnels by maintaining humidity at average of 80% and the light period 16 h (Shiva and Agrawal, 2014). Ginger micro propagation protocols have also been optimized in Ethiopia since 2012 and now used in different research laboratories and universities (Ayenew *et al.,* 2012; Berihu, 2018; Genene *et al*., 2021)

2.12.2. Micro-rhizome Induction and Production

Rhizomes that are modified stems, in ginger are the economical parts of the plant, and used as planting materials for production in the next growing season affecting its supply. Besides, most of the diseases are easily transmitted through vegetative propagation by fragmentation of rhizomes. Plant tissue culture, also improves crop productivity through germplasm conservation and variety development programs for crops like ginger. The efficiency of micro propagation techniques depend on establishing the in vitro raised plants in ex vitro conditions. Micro propagation through

in vitro shoot regeneration requires a proper rooting and acclimatizing of the in vitro raised plantlets to be successfully established in field conditions. Rooting and acclimatizing the in vitro-raised shoots adds extra cost and time to prepare them for field transplantation. Hence, micro-rhizomes are considered as alternative direct planting materials (Zahid *et al*., 2021). Micro-rhizomes are miniature rhizomes induced in vitro, harvested, can be stored and planted in greenhouse conveniently for further multiplication. Produced micro-rhizomes also provide an alternative means for propagating disease free planting material and a means for germplasm conservation of elite cultivars.

Researchers have attempted micro-rhizome induction of Zingiberaceae species mainly ginger. Elevated sucrose (>80 g/l) concentration than the control for standard micro propagation systems (20–30 g L-1) was found as the primary factor for micro-rhizome induction (Labrooy *et al*., 2020). High concentration of sucrose in MS media was frequently reported as inducer of swelling at ginger shoot explants (Mehaboob *et al*., 2019). These part through time increases in size and appears as micro rhizome with yellow-orange color having an aromatic odor (Abbas *et al*., 2014). Also, micro-rhizomes have got enough potential to be used by commercial growers as disease free planting material, produced in vitro irrespective of seasonal fluctuations, easily transferable and sown like seeds (Bhat *et al*., 1994; Sharma and Singh, 1995, 1997). Different works were reported for ginger by evaluating different media components like carbon sources, growth regulators, nitrogen concentrations and physical growth factors temperature and photoperiod.

2.13. Molecular Markers for Ginger Improvement

Molecular marker can be one of the macro molecules contained within a tissue sample taken from an organism or living entity. Markers are very important to indicate certain characteristics source of variation. These markers have played a big role in breeding and improvement of crop species including ginger (Prasanna *et al*., 2016). Phylogenetic analysis and metabolic profiling within and among *Zingiber* species were also rarely investigated. On the other hand, ginger accessions from different geographical locations were indistinguishable using above ground traits (Shivakumar *et al*., 2018). Hence, there are DNA markers used for crops in Zingiberaceae family including ginger for different research purposes.

Simple sequence repeat (SSR) markers are the most used powerful markers in genetic diversity studies to assess the level of genetic diversity within a germplasm accurately. Only few polymorphic SSR markers were reported in the ginger species *Zingiber officinale* and *Curcuma longa (*Kumar *et al*., 2009). Until now, eight DNA SSR markers and five EST-SSR highly polymorphic markers have been developed been reported in *Z. officinale*.

Comparative studies among markers showed SSR markers displayed better polymorphism results compared to ISSR and IRAP markers (Kaewsri *et al*., 2007; Nor *et al.,* 2019). Further assessment, development and characterization of more SSR markers for ginger species would be helpfull for future high density studies and evaluation of genetic resource for different economic traits. Hence, SSR markers could be used for ginger genetic improvement and conservation programs.

Another DNA marker used for different purposes is Inter-Simple sequence repeats (ISSR) which are semi-arbitrary markers. These markers can also be amplified by PCR, in the presence of one primer corresponding to a target region (Das *et al*., 2011). Similar to RAPD markers ISSR markers have the advantage of creating polymorphic fragments without genome sequence pre-information. Moreover, ISSR markers are also effective for differentiating genetic relationships among closely related ginger cultivars (Sigrist *et al.,* 2010). Genetic diversity study reports revealed that ISSR markers are capable of generating higher polymorphisms compared to RAPD and AFLP markers in Curcuma species (Pandotra *et al.,* 2013). However, ISSR markers found as less informative compared to AFLP in the case of wild ginger species, *Z. moran* and cultivars of Northwest Himalayan (Kumar *et al.,* 2009; Nor *et al.,* 2019). The differences in resolving power of these different markers mainly due to the difference parts of the whole genome they are placed in and distributed. Using combination of markers helps to exploit more polymorphic regions. Recent studies confirmed that the use of PCR based markers, SSR nad ISSR are effective to study the relationship or diversity among different species (Pandotra *et al*., 2013). SSR and ISSR markers are among pair of markers used in ginger and related wild type (torch ginger) to expose genetic relationship within and among species. Both ISSR and SSR are used mainly have been used to study the similarity between different populations and varieties of ginger.

Later these markers were effective to characterize and differentiate among the accessions of wild and edible ginger species. They were capable of detecting the polymorphic and monomorphic alleles within and among accessions. Moderate to high levels of genetic diversity wre detected by the combined use of the ISSR and SSR markers in ginger. Depending on their data generated from different experimental works, SSR marker was confirmed more informative compared to ISSR in

terms of gene diversity, polymorphism information content values and heterozygosity. The coodominance nature and rich allelic diversity also made SSR high ability in evaluating diversity, genetic structure and relationship studies in ginger (Nor *et al.,* 2019).

The molecular marker technology has also provided more advanced marker systems like Nucleotide Binding Site (NBS) that targets resistance genes and their analogues across plant genotypes. NBS markers work based on the principle of selective target region amplification, using primers designed from conserved motifs of cloned resistance genes. The fragments (markers) have high affinity to obtained resistance gene and gene analogue enriched regions across the plant genomes. The NBS technique has been previously applied in durum wheat to discriminate closely related genotypes within and among species (Mantovani *et al*., 2006; Caser *et al*., 2010). Later NBS profiling was applied to assess the genetic diversity among different genera of Zingiberaceae. Use of NBS primer enzyme combinations compared to SSR analysis of the same set of genotypes, indicated the effectiveness of NBS markers to detect diversity among different species. In the NBS analysis it was concluded that the resistance gene analogues are amplified by large and variable numbers in the genome (Kumar *et al*., 2012).

Other markers that have been used in ginger species include restriction fragment length polymorphism (RFLP), sequence characterized amplified region (SCAR) and single nucleotide polymorphism (SNP) that have been used to identify and characterize ginger species (Jatoi *et al.,* 2006).

CHAPTER 3

3. Replacement of Ammonium Nitrate by Alternative Nitrogen Sources for sustainable Ginger *(Zingiber officinale* Roscoe*)* production

Abstract

Ginger *(Zingiber officinale* Roscoe*)*, which is an important perennial herb, is used for many purposes and become a major spice crop across Ethiopia. Its production has been challenged primarily due to bacterial wilt disease eruption as of the 2012 production season. The use of disease-free tissue culture generated seed rhizome as part of integrated management was considered as the best option to reduce the challenge. However, attempts to produce large amount of tissue culture plantlets was challenged by the lack of major nitrogen source, ammonium nitrate. Hence, an experiment was designed to select a potential alternative source of nitrogen as replacement of ammonium nitrate. The study evaluated three nitrogen salts at different levels in MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l Kinetin using Boziab variety. The experiment was established in completely randomized design in five replications for each treatment, The result indicates the highest mean shoot number (9.33) was achieved from 1.0 g/l NH_4Cl followed by 3.8 g/l KNO_3 and 3.0 g/l urea with values of mean shoot numbers 7.33 and 7.00 respectively. Shoot growth, rooting and survival after acclimatization were affected negatively at higher levels of NH_4Cl. The highest mean number of roots (19) was observed on a medium containing 1.0 g/l NH_4Cl followed by control MS media (16). Survival after acclimatization was found to be 98% for plants derived from both media containing 4.5 and 3.8 g/l urea and KNO_3 respectively followed by 95% for plants from medium containing 1.0 g/l NH_4Cl. The finding from this experiment indicates low cost salts are alternative potential sources of nitrogen to enhance large-scale disease free ginger production.

Keywords: alternative, ammonium nitrate, disease-free, ginger, low-cost, urea

3.1. Introduction

The cultivation and utilization of ginger in Ethiopia started during 13th Century when Arabs introduced it from India to East Africa (Jansen, 1981; Endrias and Asfaw, 2011). Ginger has been used for many purposes, become a major spice in both the local, and export markets. Until 2012, ginger was produced throughout the whole country, primarily in the lowlands of SNNPR, Oromia and Gambela, and was Ethiopia's most exported spice, primarily to neighboring countries. However, since 2012, a disease complex of fungi and bacterial wilt, which spread across Ethiopia has been challenging its production (Titus and Wojtek, 2020). Fresh rhizome with active buds sprouting is the most commonly used planting material for production requiring large amount per unit area of land up to 2.5 tons per hectare of land in 40x15 cm spacing (Asfaw and Derbew, 2021).

Moreover, the ginger wilt disease complex that erupted in the 2012 production season has devastated the crop and almost halted its production and marketing (Tariku *et al*., 2016). The use of disease-free clean planting material associated with a clean planting medium has been suggested to be one of the best options to restore ginger production in Ethiopia (Kifle *et al.*, 2021). Many research works including in Ethiopia have reported rapid clonal propagation of ginger on Murashige and Skoog (1962) (MS) growth medium with varying concentration of plant growth regulators (Ayenew *et al.,* 2012).

The most common plant tissue culture medium, MS medium is characterized by high level of nitrogen in the form of two nitrate salts; ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) supplementing each other to supply balanced ions for explants. These nitrogen and potassium are macronutrients required in large amounts in plant tissue culture media for successful

regeneration and mass propagation. However, the explosive nature of ammonium nitrate makes it to be banned from the market and this resulted in unavailability and unaffordable cost up to $400/Kg in Ethiopia. Recently this has been challenging tissue culture research and large-scale propagation of strategic crops including ginger across the country.

Studies focused on the effect of nitrogen source in the form of ammonium nitrate and potassium nitrate indicates that MS media with nitrogen only from potassium nitrate but omitted ammonium nitrate resulted in significantly high number of shoots per explant (Cecilia, 2010). MS medium with high ammonium nitrate has been also reported to be inhibitory to the tuberization process in *Discorea alata* (Jean and Cappadocia, 1991). In another related work banana plantlets in vitro regenerated from MS medium supplemented with high concentration of potassium and nitrogen reported significant reduction of bacterial wilt disease incidence and increased incubation period of the disease (Atim *et al.,* 2013).

Recent study carried out to substitute NH_4NO_3 with Urea in potato plantlets regeneration MS media reported maximum shoot and leaf numbers with healthy and robust plantlets of potato over media with NH_4NO_3 (Bashura *et al.,* 2021). Hence, this research report focuses on the effectiveness of easily available three different nitrogen salts as an alternative source of nitrogen nutrient in MS media instead of NH_4NO_3 to enhance ginger *in vitro* mass propagation.

3.2. Materials and Methods

Plant material

Plantlets of nationally released ginger boziab variety (Zingiber officinale var. bozaib), were obtained from Areka Agricultural Research Center, were used as experimental materials. The in vitro plants were generated as per protocol developed by Ayenew *et al*. (2012) and optimized as per Areka tissue culture laboratory conditions (Genene *et al.,* 2019). The experiment was conducted from 2020 to 2021 at Areka agricultural research center plant tissue culture laboratory, which is located at 7 04' N latitude and 37 41' E longitude at altitude of 1790 meters above sea level.

Shoot induction and multiplication

Explants of 2-3 cm length from ginger rhizomes sprouted on sterile sand were collected for surface sterilization and initiation. Surface sterilization was done after thorough washing with tap water and home detergents. Then explants were sterilized by 70% ethanol for 10 minutes followed by washing with sterile distilled water before exposing it to 2% chlorinated detergent (local Gion berekina) for 15 minutes and repeated washing by distilled water four times. Then each explant was trimmed to 1-1.5 cm length before transferring to initiation Murashige and Skoog (1962) medium supplemented with 2 mg/l BAP and 1 mg/l kinetin keeping one explant per jar (Fig 3.1A and B; Appendix II). For multiplication, well-initiated explants with growing shoots and active nodes at the bottom of the explants on initiation media were transferred to fresh medium similar in growth hormones combination as initiation stage. Apical dominance was carefully removed to induce multiple shoot growth before transfer to this stage. Successive sub-culturing to fresh same media was done on average of 30 days interval (Ayenew *et al.,* 2012).

Experimental design

Three alternative nitrogen salts; ammonium chloride (NH_4Cl), potassium nitrate (KNO_3) and urea (N_2H_4CO) at four concentration levels (3x4) constituting 13 treatments including one standard MS medium (Table 3.1). All treatments were replicated 5 times and supplemented with growth regulators (2 mg/l BAP and 1 mg/l Kinetin). Culture jars of 250 ml capacity were randomly arranged in completely randomized design (CRD) on growth shelves after explants were transferred to a 40 ml semi solid media. Shoot buds as explants were cultured per jar in five replications for each treatment. In treatments where KNO_3 is omitted or minimized the comparable amount of Potassium di-hydrogen phosphate (KH_2PO_4); 1.9 g and 0.95 g/l for full and half-strength media respectively was added to the media during preparation in order to keep K^+ level constant. Sucrose (table sugar) at 30 g/l as carbon source and solidifying agent; agar at 6 g/ l were used across the experiments. The pH was adjusted to 5.8 using 1N of NaOH and HCl before addition of agar.

Rooting and Acclimatization

Rooting stage was avoided as per protocol in use at Areka plant tissue culture research laboratory in which spontaneous rooting was observed at the multiplication stage. For acclimatization, well-established plantlets with shoots, leaves and roots were carefully pulled out from the media using forceps. Clean tap water was used to wash out media and very long roots were trimmed to 5-6 cm before planting in pots containing soil mixture. The soil mix for acclimatization was prepared from decomposed coffee husk, red ash, and forest soil, in a 2:1:1 ratio respectively. The soil mix was sterilized in a barrel given fire at the bottom for 3 hours to avoid any nematodes and worms.

Data Analysis

The data collected for the number of explants survived, days to shoot induction, number of shoots, number of roots, shoot and root length, and numbers of plants established in soil during acclimatization was used for analysis. Analysis of variance (ANOVA) and the mean of each treatment were compared using Least significant distance (LSD) at a 5% significance level using statistical analysis system (SAS, 2011). Whereas, data of explants survival in vitro and plants that survived during acclimatization were stated as percentages. Cost-benefit analysis was also computed based on the current market price of chemicals used. The cost saved by different alternative N_2 sources used in this study was calculated with the formula:

$$\text{Cost saved in percent} = \frac{NH_4NO_3 \text{ cost} - \text{the cost of alternative multiplied by } 100\%}{NH_4NO_3 \text{ cost}}$$

3.3. Results

Surface sterilization and culture initiation

Surface sterilization with ethanol and chlorinated sterilant alone have not controlled the microbial contaminants; hence, 0.1 % of mercuric chloride for 10 minute after ethanol treatment later has led to 80% explants free of contamination. Among the contamination free explants, 90% have initiated an average of 2.5 shoot in MS initiation medium containing 2.0 mg BAP and 1.0 mg/l Kinetin. Contamination free plantlets transferred for multiplication to fresh MS standard media performed well with a record mean number of plantlets (8.5) per explant (Table 3.1 and Fig. 3.1C).

Effect of different alternatives on shoot multiplication

The ANOVA result for the in vitro regeneration variables among the 13 treatments showed highly significant difference among treatment means for all six measurements.

Among the test treatments three combinations; 3.8 g/l KNO_3, 1.0 g/l NH_4Cl and 3.0 g/l urea were promising as compared to check MS medium (13th treatment) and can be used as replacement for NH_4NO_3 (Table 3.1). MS medium with omitted NH_4NO_3 and replaced by 1.0 g/l NH_4Cl resulted in a comparable number and length of shoots with control MS media containing 1.65 g/l NH_4NO_3. Shoot proliferation resulted mean of (9.33) from 1 mg/l of NH_4Cl supplied media and 10.0 plantlets were recorded for control MS media (Table 3.1). MS medium supplemented with 3.8 g/l KNO_3 to compensate for N_2 from ammonium nitrate showed the second-highest mean number of shoots (7.33) per explant. For the third alternative tested urea, which is the common inorganic nitrogen fertilizer medium containing 3.0 and 4.5 g/l produced 7.0 mean numbers of shoots per explant. Another in vitro performance parameter recorded is the number of active buds at the base of shoot clumps that can grow to new shoots in the next sub-culturing. The highest mean number (7.33) for this was also recorded on the medium containing 1.0 g/l NH_4Cl followed by 4.5 g/l urea where 5.67 buds per explant were achieved (Table 3.1).

The result also revealed presence of significant differences among treatments for the number of active buds that indicated shoot proliferation continues with a similar trend in subsequent sub-culturing, as the number of buds is directly proportional to shoot number (Table 3.1). Significant mean shoot number was recorded on medium containing 1 mg/l NH_4Cl among the alternatives tasted followed by 3.8 g/l KNO_3 and urea at 3 and 4.5 g/l media. In contrary, a non-significant number of shoots and buds, very short shoots, and roots were recorded on a medium containing a high amount of NH_4Cl (>1 g/l) and urea above 4.5 g/l (Table 3.1).

Table 3. 1. Mean shoot number per explant on medium containing different nitrogen sources.

#Trt	Treatment combination	NS/exp	NB/exp	SL(cm)	LN	RN	RL(cm)
1	MS-NH_4NO_3 + 1.9 g/l KNO_3	7.33^{b}	5.00^{bc}	6.23^{cde}	4.33^{a}	10.33^{cde}	10.00^{b}
2	MS -0.825NH_4NO_3 +0.95 g/l KNO_3	6.33^{bc}	4.33^{cd}	7.90^{ab}	3.67^{a}	11.33^{cd}	8.96bc
3	MS -0.825NH_4NO_3 -0.95 g/l KNO_3	4.33^{ed}	4.00^{cd}	4.60^{fg}	4.33^{a}	7.33^{fgh}	8.13^{cde}
4	MS -0.825 g/l NH_4NO_3	5.00^{cd}	5.00^{bc}	5.60^{def}	4.33^{a}	8.33^{efg}	7.66^{de}
5	MS- NH_4NO_3 +1 g/l NH4Cl	9.33^{a}	7.33^{a}	8.67^{a}	5.00^{a}	19.66^{a}	12.56^{a}
6	MS- NH_4NO_3 + 1.325 g/l NH4Cl	2.66^{ef}	2.67de	3.26^{gh}	1.67^{c}	6.33^{ghi}	7.33^{ef}
7	MS- NH_4NO_3 + 1.65 g/l NH4Cl	2.00^{f}	2.66de	2.60^{h}	1.66^{c}	5.33^{hi}	4.73^{g}
8	MS- NH_4NO_3 + 2 g/l NH4Cl	1.67^{f}	3.00^{d}	2.06^{h}	2.00^{bc}	4.00^{i}	3.13^{h}
9	MS -NH4NO3 + 1.5 g/l urea	6.67^{bc}	6.33^{ab}	7.00^{bcd}	4.33^{a}	10.00^{cde}	8.36^{cde}
10	MS – NH4NO3 + 3 g/l urea	7.00^{b}	5.67^{abc}	7.36^{abc}	4.67^{a}	11.67^{c}	9.23^{bc}
11	MS – NH4NO3 + 4.5 g/l urea	7.00^{b}	5.67^{abc}	5.67^{def}	4.10^{a}	14.67^{b}	11.73^{a}
12	MS – NH4NO3 + 6 g/l urea	5.00^{cd}	4.33^{cd}	5.00^{ef}	3.5a^{b}	9.00^{def}	11.70^{a}
13	MS standard	10.00^{a}	5.67^{abc}	8.56^{a}	5.00^{a}	16.00^{b}	12.86^{a}
	CV (%)	19.17	22.77	14.54	24.89	14.69	10.26

NS/exp-number shoots per explant, NB/explant- number of active buds per explant, SL- shoot length, LN-leaf number per shoot, RN- root number per shoot, RL- root length, #Trt-number of treatments and CV- coefficient of variation.

Moreover, it was observed that supplying ammonium chloride beyond 1 g/l and urea above 4.5 g/l resulted in limited root induction and growth accompanied by shoot drying within three weeks of culturing (Fig. 3.1).

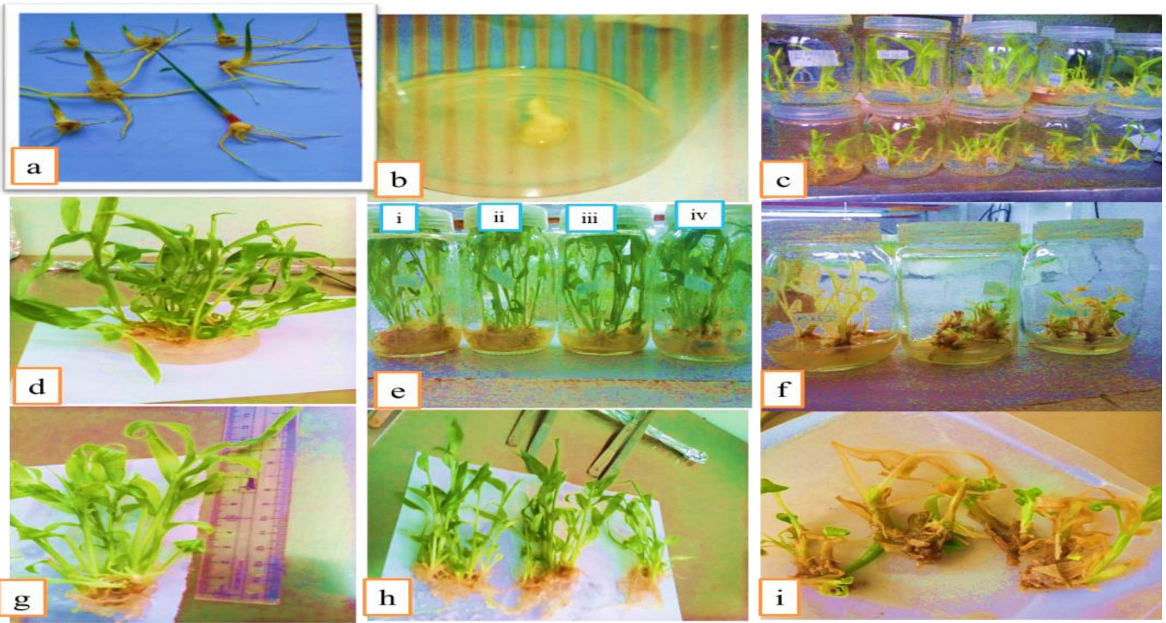

Figure 3. 1. Response of explants to nitrogen sources and concentrations. a. Explants collected from rhizome sprouts, b. Shoot induction, c. Shoot multiplication d. Well proliferated shoots, e. Response of explants in d/t alternatives (i, standard, ii, NH_4Cl, iii KNO_3 and iv urea), f. Poor response at elevated NH_4Cl, g. Shoot length, h. Shoot number and leaf number, i. Poor shoot growth from medium with 2 mg/l NH_4Cl.

Root growth and acclimatization

The shoots developed roots spontaneously at the multiplication stage on all medium combinations tested as treatments. The analysis result showed that there is a highly significant difference among treatments for root number and length. The highest mean number of root length were recorded from the medium of KNO_3 at 3.8 g/l concentration followed by standard MS medium with 1.65 g/l NH_4NO_3 and 3 g/l urea (Table 3.2 and Fig. 3.2). Whereas, the lowest mean number and length of roots were recorded from the medium in which shoot growth and development were also declined. Media containing 1.325 to 2 g/l of NH_4Cl showed very weak root growth with no significant difference. A similar trend was also observed in root length, which decreases when the amount of NH_4Cl increases from 1.325 to 2 g/l. The low number and length of roots have affected the growth of shoots in vitro and affected the acclimatization survival percentage of plants

acclimatized from these media. The root and shoot growth inhibition effect observed was directly proportional to the increase in ammonium chloride amount (Fig. 3.2b).

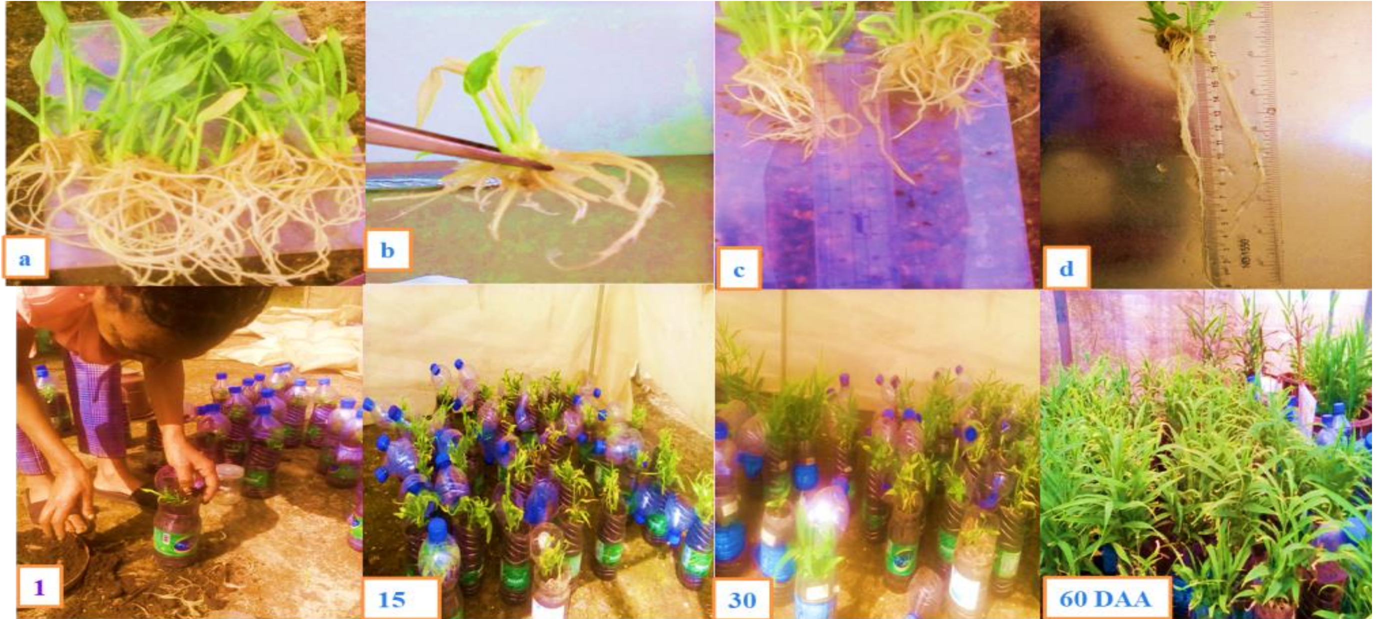

Figure 3. 2. Rooting and acclimatization performance.
a. Singled plantlets ready for acclimatization, b. Plants with poor root growth, c. Root length difference among treatments. Numbers; 1, 15, 30, and 60 are days after acclimatization (DAA) and respective pictures show acclimatized plantlets' performance after the days respectively.

Acclimatization was done after six weeks of culturing directly from the multiplication stage as the plantlets developed roots in multiplication. Plantlets proliferated together as shoot clumps are detached first after washing out media before acclimatizing. Plants with better shoot and root growth has resulted in 96% survival after 15 days of primary acclimatization (Figs 3.2). Whereas plants with small shoots and few and short roots failed to achieve a survival rate after fifteen days of the acclimatization process. In this regard, plants from a medium with 1.65 and 2 g/l NH_4Cl resulted in 15 percent acclimatization survival due to their weak regeneration. On the other hand, plantlets from 1.325 g NH_4Cl and 1.5 g/l urea resulted in 65 and 75% acclimatization survival after 15 days of acclimatization respectively. Plantlets from the remaining media combinations resulted in an average of 94% survival after 15 days. The mediums from which plants with the highest

survival rate were nearly equal with MS standard with and without growth regulators used as con trol checks (Fig. 3.3).

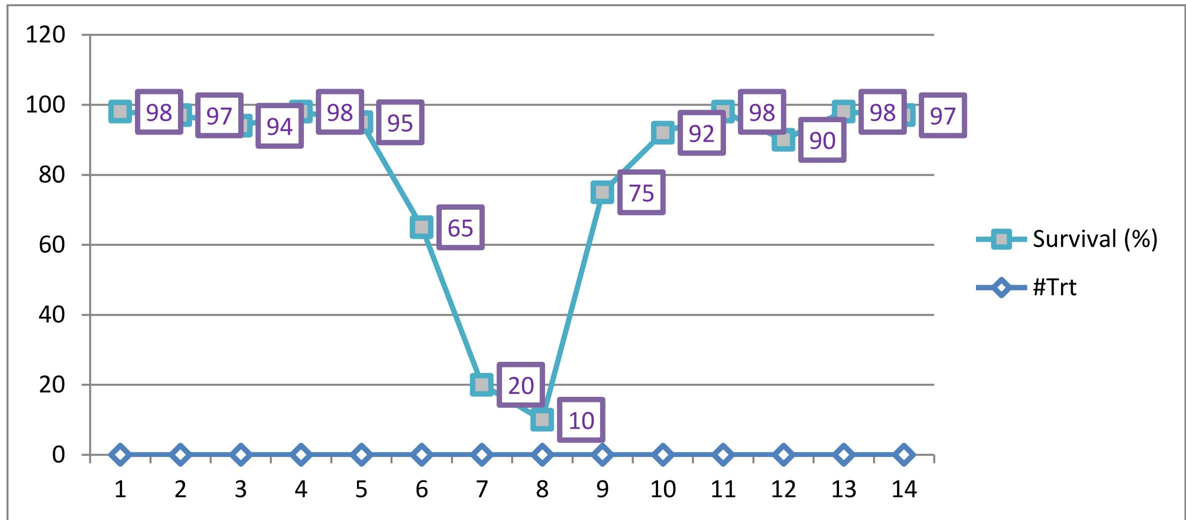

Figure 3. 3. Graphic representation of plantlets survival from all treatments (1-14) during acclimatization after 15 days of acclimatization

Cost-benefit analysis

The cost of alternative nitrogen sources has saved costs for the in vitro regeneration of ginger with a minimum number of plantlets penalty per batch culture, which can be compensated by subsequent sub-culturing. All the three alternative nitrogen sources tested have lower cost as compared to ammonium nitrate. (Table 3.2).

Urea at $0.88/kg current price to use 3 g/l amount in one liter of medium has saved 99.75% extra-cost from an equal amount of NH_4NO_3 at a pick price of $392/kg during experiment time in 2021 which might be a special case in Ethiopia due to import restriction. Maximum plantlets penalty (30%) per batch culture has also resulted from a medium of urea at a selected amount of 3 g/l that can be compensated by subsequent sub-culturing, as it is easily available at a very low cost. Whereas, a kg of ammonium nitrate rarely obtained by high cost ($392/Kg) has only a 30%

plantlets advantage over urea, 26.7% over KNO3, and 6.7 % to NH_4Cl medium. The second alternative was NH_4Cl; from which the highest mean shoot number, better growth, and also required in a small amount (1 g/l) resulting in statistically non-significant shoot number as compared to standard MS medium (Table 1).

Table 3. 2. Cost and plantlets comparison of alternative sources.

#Item	Alternative salts	Unit cost (USD/kg)	Cost saved (%)	Average plants/exp	Plantelts advantage	Economical cost/plantlets/birr
1	NH_4NO_3	392	0	10	0	100
2	KNO_3	47	88	7.33	26.7	15
3	NH_4Cl	27.45	93	9.33	6.7	8
4	Urea	0.88	99.75	7.00	30	5

3.4. Discussion

The high loss (>50%) of plantlets due to contamination was later minimized to 10% on average by clean lines control and subsequent sub-culturing. Previous researchers have also reported higher contamination rates at early initiation stages due to the endogenous latent contaminating microbes. Spontaneous root development is also reported previously due to enough endogenous auxin hormones to induce roots (Ayenew *et al.*, 2012; Berihu 2018; Genene et al. 2019).

Previous study conducted on ginger using double the amount (3.8 g/l) of KNO_3 in MS media as a sole source of nitrogen indicated that nitrogen in the form of KNO_3 significantly improved the proliferation rate of ginger in vitro in both full and half-strength media.

A similar study also reported leaf growth and root formation were also better in media devoid of NH_4NO_3 (Cecilia, 2010). Another experiment conducted to substitute NH_4NO_3 by urea for in vitro regeneration of potatoes indicated that 5 g/l urea resulted in better proliferation and regeneration of potato plantlets which is basically in line with our findings even if the crops are different (Bashara *et al.,* 2021). Ammonium nitrate was recently banned in many countries including Ethiopia.

Hence, tissue culture researchers are globally looking for alternative sources as a replacement especially to compensate ammonium part. Our experiment, which included ammonium chloride, was the first in its kind to the best of our knowledge, which can be adapted to other crops' in vitro regeneration protocols. Previous studies also reported that the uptake of nitrate ions by plant cells leads to attraction towards an alkaline pH, while NH4+ uptake results in a more rapid shift towards acidity and makes the balance of pH in plants (Hyndman *et al*., 1982; Edwin *et al.,* 2007).

These previous reports confirms the elevated amount of NH_4Cl and urea makes the media more acidic which finally limits shoot and root growth for in vitro ginger plantlets production. This might be due to the toxic effect of high chlorine on plants, which prevents the uptake of nutrients by the explants. This is probably due to the accumulation of NH^+_4 and Cl^- free ions which could make the media acidic and hence affect nutrient uptake and can act as toxic to plant shoots. Hence, applications of lower levels of ammonium chloride (1 g/l) and urea (3-4.5 g/l) were good enough for ginger. Research reports for the acclimatization survival rate of ginger in vitro regenerated plants are nearly similar to our findings. A study conducted using two cultivars to optimize the

protocol for Ethiopian ginger reported an average of 83.5% acclimatization survival (Ayenew *et al.,* 2012).

The acclimatization survival rate achieved in this specific study was also in line with recently reported protocol, which was 95% survival rate for ginger micro-propagation in a standard MS media (Zahid *et al*., 2021).

3.5. Conclusions

The experiment of evaluating alternative salts to replace ammonium nitrate was accompanied by extensive observations analysis at different stages and rounds. From the results, it was concluded that ammonium nitrate which is a major source of nitrogen but hardly accessible at local markets in Ethiopia can be fully replaced by one of the three nitrogen salts. Among the three salt types urea, which is a common inorganic nitrogen fertilizer was the best potential alternative for efficient and low-cost mass propagation of disease-free ginger plantlets. This research is the first in its kind on ginger for evaluating urea and ammonium chloride as alternative nitrogen sources in the world to the best of our knowledge. This can be adopted for other crops in vitro regeneration protocols. Reduction of contamination rate to a minimum level and upgrading acclimatization survival rate should be investigated in the future.

CHAPTER 4

4. Micro-Rhizome Induction and Production for Ginger (*Zingiber officinale* Roscoe)

Abstract

Ginger had been the leading spice crop in terms of production and income in Ethiopia until its production was interrupted due to ginger bacterial wilt disease. The disease is transmitted mainly through infected planting materials. Regeneration of healthy planting materials is believed to be the best option to raise clean seed rhizomes. The objective of this research is to induce and produce in vitro micro-rhizomes that can be directly planted to soil. MS medium containing a combination of sucrose, benzylaminopurine (BAP), ammonium nitrate, and silver nitrate at different concentrations were evaluated. The experiment was laid in completely randomized design and conducted in two phases in which each treatment is replicated five times. Data on micro-rhizome induction and viability was recorded and analyzed. The results indicated that 80.0 g/l sucrose at all concentrations of BAP was the best for the main parameters observed. On contrary, medium supplemented with ammonium nitrate and silver nitrate resulted in low rate of micro-rhizome induction. The viable mean number of micro-rhizome (5.67) and shoot number (10.33) per explant were obtained from a medium supplemented with 80.0 g/l sucrose and 6.0 mg/l BAP. The viability test also showed that up to 80% of the micro-rhizome sprouted after one month of planting to the soil. The micro-rhizome production potential observed in this experiment can enhance disease-free ginger production.

Keywords: disease free, ginger, in vitro, micro-rhizome, sucrose

4.1. Introduction

Ginger (*Zingiber officinale* Rosco) is an important delicacy, medicinal, and spice monocotyledonous perennial herb belonging to the family *Zingiberaceae*. The cultivation and utilization of the crop in Ethiopia started during the 13th century when Arabs introduced it from India to East Africa (Jansen, 1981). Its large production was mostly limited in the wetter regions of the south and southwestern parts of Ethiopia. The potential and importance of this spice crop have been expressed via different conditions like total area covered and productivity per hectare farmland. In terms of area harvested and total production, Ethiopia stood 10th and 14th respectively in 2011 among the 36 countries engaged in ginger production globally (FAO, 2013). Before 2012, no pest at all in Ethiopia was reported in ginger. The only bottlenecks reported for ginger had been a shortage of planting material for improved varieties and poor field and post-harvest management practices (Endrias and Asfaw, 2011).

The rhizome is the most commonly used material for ginger plant propagation and many rhizomes (2.2 t) are required for planting one hectare of land (Girma and Kinde, 2008). Moreover, during storage and cultivation, rhizomes used for vegetative propagation are susceptible to diseases particularly fungus (*Pythium)* and bacteria (*Ralstonia solanacearum)* which cause rhizome rot and bacterial wilt respectively, causing a substantial reduction in yield and quality. Bacterial wilt is now very important in Ethiopia mainly in major ginger-producing areas of South and South Western parts, where ginger has been widely produced for commercial purposes. Disease incidence in the field usually ranges from 10 to 40% globally, but the disease was also known to destroy the crop with the incidence of 80 to 100% in Ethiopia (Habtewold *et al*., 2015; Tariku *et al*., 2016).

Plant tissue culture improves crop productivity through the production of large amounts of uniform plantlets, disease cleaning, germplasm conservation, and variety development for crops like ginger in which it is difficult to get new varieties by crossbreeding. However, tissue culture-generated ginger seedlings are less efficient for direct field planting and need mini-rhizome production under a greenhouse, which takes an additional season to plant in a field (Berihu, 2018; Genene *et al.*, 2019; Zhao *et al*., 2023).

In vitro production of micro-rhizomes tackles this problem by increasing the micro-propagule's efficiency and saving time as the micro-rhizomes can be planted directly into the soil without any further acclimatization in the glasshouse. Micro-rhizomes are miniature rhizomes developed in vitro and can be stored and planted in pots or nursery beds conveniently for further multiplication (Archana *et al*., 2013).

Micro-rhizomes *in vitro* propagation may provide an alternative means for propagating disease-free planting materials and for germplasm conservation of elite cultivars. In addition, micro-rhizomes have enough potential to be used by commercial growers as disease-free planting material, produced in vitro irrespective of seasonal fluctuations, easily transferable, and sown like seeds (Zahid *et al*., 2021). Hence, this research has been conducted to optimize efficient protocol for *in vitro* micro-rhizome induction and enhance disease-free rhizome seed generation in Ethiopia.

4.2. Materials and Methods

Plant materials

Ginger shoot tips of Volvo cultivar; a popular farmer's variety was *in vitro* regenerated as per previous protocol (Ayenew *et al.,* (2012). Then well initiated shoots were transferred to growth regulators free MS medium to get physiologically similar experimental materials followed by two subsequent sub-culturing for multiplication of plantlets. Then plantlets growing on growth regulators free medium were then used as experimental materials as per treatments and design.

Stock solution and culture medium preparation

Stock solution for MS medium was prepared by salt type category for all nutrients, growth regulators, vitamins and organic additives and stored at 4°C until use. Agar at 6.0 g/l of medium was used to semi-solidify the media during preparation after pH was adjusted to 5.8. Semi-solid MS medium was prepared for all treatments for in vitro micro-rhizome induction and production throughout the experiment.

Experimental design

All experiments were conducted in two phases. In the first phase, there were four different concentrations of sucrose (30, 60, 80 and 100 g/l) and three concentrations of benzyl amino purine BAP (3.0, 6.0 and 9.0 mg/l). The second phase combined ammonium nitrate (NH_4NO_3) and silver nitrate ($AgNO_3$) each consisting three levels and selected combination of sucrose and BAP in the first phase was supplied to all treatments. For ginger micro-propagation, MS medium containing 30 g/l sucrose, 2.0 mg/l BAP and 1.0 mg/l kinetin were used as control.

Completely randomized design (CRD) structured in factorial arrangement by cross multiplication model and one control resulted in 13 treatments for first phase and 10 for second phase of the experiment. Each treatment was replicated five times using glass culture jars containing 40 ml medium as per the treatments and five shoots were cultured in each jar. The experiment at each stage was done in three separate rounds for verification of treatment effects.

Culture condition

All required materials including growth media were sterilized by autoclaving at 121°C temperature and 103.42 Kpa pressure for 20 minutes. The autoclaved materials and medium were stored in transfer room for four days to inspect media contamination and quality before use. During culturing, shoots were safely detached and separated by scalpels with surgical blade under laminar flow hood cabinet and transferred to culture jars containing 40 ml medium of respective treatment combinations**.** All cultures then were transferred to growth room and maintained at light intensity of 92.6.2 $\mu molm^{-2}s^{-1}$ (5,000 lux), temperature of 27±2°C and relative humidity of 80% under 16 h photoperiod. To get optimum number of well-established micro-rhizomes per shoot, cultures were directly transferred once after six weeks of first culturing to the same fresh medium and visually assessed in 15 days interval for three months.

Micro-rhizome performance

Micro-rhizomes were carefully collected from the base of shoots without harming the lower shoots and planted in pots containing sterile soil mix (2:1:1, topsoil, compost, and sand, respectively) and maintained in a greenhouse.

Whereas, shoots were transferred to a fresh medium of corresponding treatment for another round of culturing to the respective medium combination to verify the repeatability of the first observations.

Data recording

Data for date of micro-rhizome induction, rate of micro-rhizome induction, number of micro-rhizome per shoot, weight of micro-rhizome per shoot/jar, number of shoots per explant, number of roots per explant were recorded per each treatment at all stages. Data for days to sprouting and number of rhizomes sprouted as a percentage was also recorded during *in vivo* viability testing.

Data analysis

Data collected for growth and micro-rhizome parameters was subjected to analysis of variance (ANOVA) using the SAS version. 9.1.3 (SAS 2011) to differentiate the main and interaction effects of the factors at each stage of the experiment. Mean comparison among the treatments and significance test was computed using Fisher's protected least significant difference test at a 5% probability level. Linear correlation coefficient analysis to understand the association of in vitro shoot growth traits to micro-rhizome number and weight was done by Minitab 19.1.1 at a 95% confidence interval.

4.3. Results

Effect of sucrose and growth regulator on micro-rhizome induction

The result for in vitro micro-rhizome induction by combined effect of sucrose and BAP showed that there is a significant difference among treatments at a probability level of 5% (Table 4.1).

Shoot buds cultured in MS medium supplemented with 80 g/l sucrose and 6.0 mg/l BAP resulted in the best response in terms of most parameters recorded (Fig. 4.1a). One-way analysis of variance (ANOVA) also showed that 100 g/l sucrose-containing medium has no significant effect on the number of micro-rhizomes per explant (Table 4.1). On the contrary, medium with 30 g/l sucrose with 3, 6, and 9 mg/l BAP did not show a significant difference with standard check medium supplemented with 30 g sucrose and 2.0 mg/l BAP except for large root number and length at 9.0 mg/l (Fig. 4.1b). Maximum single fresh micro-rhizome weight (3.92 g) with a mean of four active buds was recorded at the selected medium (80 g/l sucrose with 6.0 mg/l BAP). The highest mean number of shoots (10.33) was also recorded from a medium containing 80 g/l and 6.0 mg/l correspondingly. However, micro-rhizome induction was not observed on a medium containing 30 g/l sucrose at all levels of BAP from 2 to 9 mg/l including control treatment.

On media containing the highest concentration of sucrose (100 g/l), induction was numerically better but their viability was poor due to low biomass and limited number of active buds to sprout. Root growth was also highly affected due to treatment effects in which the maximum number and length of roots was recorded from a medium supplemented by 60 g/l sucrose with 3, 6, and 9 mg/l BAP at the expense of shoot and micro-rhizome development (Table 4.1). Shoot growth and micro-rhizome induction revealed that shoot number; shoot length, leaf number, root number, and length were negatively affected as sucrose increased from 30 g to 100 g/l. However, the rate of micro-rhizome induction, micro-rhizome number, and total micro-rhizome fresh weight increased as the sucrose amount increased up to 100 g/l at all levels of BAP (Fig. 4.1).

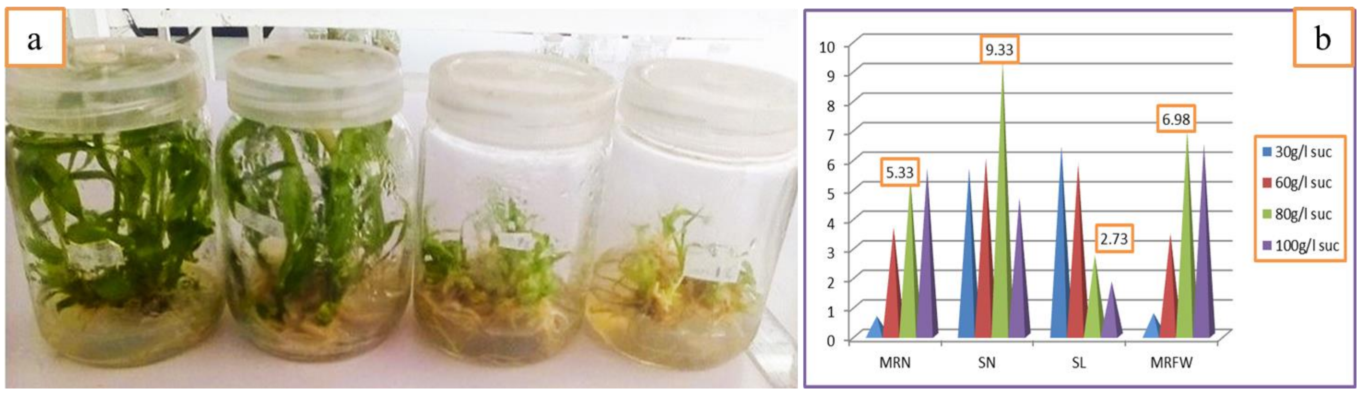

Figure 4. 1. Micro-rhizome growth on different combinations of sucrose and BAP a. On medium with 6 mg/l BAP and 30, 60, 80 &100 g/l sucrose (left to right). b. number of micro-rhizome as affected by sucrose at fixed BAP (6 mg/l).

Table 4. 1. Effect of sucrose and BAP on micro-rhizome induction

#Treatments	**Combinations**		**Recorded parameters**						
	Sucrose(g/l)	BAP	MRN/S	NB/R	NS/exp	SL(cm)	RN	RL(cm)	MRFW/S(g)
1	30	3	0.0	0.0	6.33	6.5	16.00	10.26	0.0
2	30	6	0.0	0.0	4.66	6.26	16.67	10.33	0.0
3	30	9	0.66	0.66	5.67	6.43	11.67	12.16	0.76
4	60	3	2.33	2.66	4.67	5.43	19.00	11.16	3.03
5	60	6	2.33	3.00	7.33	6.43	21.67	12.36	3.46
6	60	9	3.66	2.66	6.00	5.83	23.33	13.03	3.46
7	80	3	4.66	4.33	9.00	2.46	13.00	9.00	3.79
8	80	6	5.66	5.00	10.33	2.36	11.00	8.23	8.08
9	80	9	5.33	4.67	9.33	2.73	11.00	8.76	6.98
10	100	3	6.00	1.00	3.00	1.40	6.33	6.5	5.95
11	100	6	6.33	1.33	4.33	1.6	6.67	6.26	5.90
12	100	9	5.66	2.00	4.67	1.83	7.33	5.50	6.5
13	30	2	0.0	0.0	9.67	8.83	20.00	12.36	0.00
CV (%)			23.96	21.53	15.25	7.35	7.26	5.45	21.66
LSD (5%)			1.73**	1.09*	1.65**	0.57*	1.75*	0.88*	1.32**

MRN-micro-rhizome number per shoot, NB-number of node per rhizome, NS-number of shoot, SL-shoot length, RN-root number, RL root length, and MRFW- MRFW-micro-rhizome fresh weight

The maximum viable number of micro-rhizome (7) per explants and number of shoots (23) were produced from medium containing 80 g/l sucrose and 6 mg/l BAP. This result was achieved by direct transfer of induced micro-rhizome to respective medium once after six weeks of culturing (Fig. 4.2a). From this, we have learned that the combination of sucrose and growth regulator and elevating time of culturing can be used to produce large number of viable micro-rhizome. The technique also resulted in more than double number of shoots simultaneously from the same medium as compared to control micro-propagation media (Fig. 4.2b).

Figure 4. 2. Induced micro-rhizome records from different treatments a. Micro-rhizome in vitro b. maximum number of micro-rhizomes and shoots per jar achieved, c. Micro-rhizomes active buds to sprout and d. Single micro-rhizome fresh weight (3.92g).

Correlations among in vitro growth parameters

Linear correlation analysis conducted to associate growth parameters that have strong positive or negative significant correlation with viable in vitro micro-rhizome showed that micro-rhizome fresh weight has a negative significant linear correlation with shoot number, shoot length, root number, and root length (Table 4.2). Whereas, the fresh weight of rhizomes has a significant positive correlation with a number of micro-rhizome and a number of buds that can sprout in vivo per rhizome (Fig. 4.2c).

Table 4.2. Linear correlation coefficient of in vitro growth parameters

Traits	MRN/S	NB/R	NS/exp	SL(cm)	RN	RL(cm)
NN/R	0.612**					
NS/Exp	-0.042	0.546				
SL	-0.931**	-0.493	0.164			
RN	-0.641**	-0.065	0.247	0.813*		
RL	-0.751**	-0.150	0.267	0.889*	0.896*	
MRFW(g)	0.951**	0.711* *	0.096	-0.866**	-0.582*	-0.689**

MRN/S- number of rhizome per shoot, NN/R- number of nodes per rhizome, NS/exp- number of shoots per explant, SL- shoot length, RN- root number and RL- root length.

In the micro-rhizome sprouting test, rhizomes with large number of active buds and good biomass have a high rate of sprouting up to 80%. Whereas, those with low weight and less number of buds showed a low emergence rate in vivo up to zero level. Therefore, to achieve viable micro-rhizomes the shoot and root growth should be optimum by using an appropriate combination of sugar and growth regulators. The viability of micro-rhizomes with high fresh weight and enough active buds is better than others with less weight and a limited number of buds. Linear correlation coefficient at 95% confidence interval revealed that micro-rhizome fresh weight, number of micro-rhizome, and active buds have a significant positive correlation with each other (Table 4.2).

Effect of ammonium nitrate and silver nitrate on micro-rhizome induction

The second phase of the experiment, which involved ammonium nitrate (NH_4NO_3) and silver nitrate ($AgNO_3$) at different levels (Table 4.3) in combination with a medium with selected sucrose and BAP combination (80 g/l sucrose and 6 mg/l BAP).

However, all nine treatments have failed to result in better micro-rhizome induction and growth as compared to the medium supplied with 80 g/l sucrose and 6 mg/l BAP alone (Table 4.3). Analysis of variance for all in vitro parameters recorded was significant at 5% probability with a maximum mean number of micro-rhizome and weight observed from a medium with 80 g/l sucrose and 6 mg/l BAP. Whereas, the lowest mean was from medium supplemented with 0.55 g/l NH_4NO_3 and 1.9 g/l $AgNO_3$ in contrast to 80 g/l sucrose and 6 mg/l BAP for the corresponding parameters (Fig. 4.3 and Table 4.3). The result also revealed that maximum shoot and root growth was recorded from a control medium supplied with 30 g/l sucrose and 2 mg/l BAP as it was in the first phase (Table 4.1).

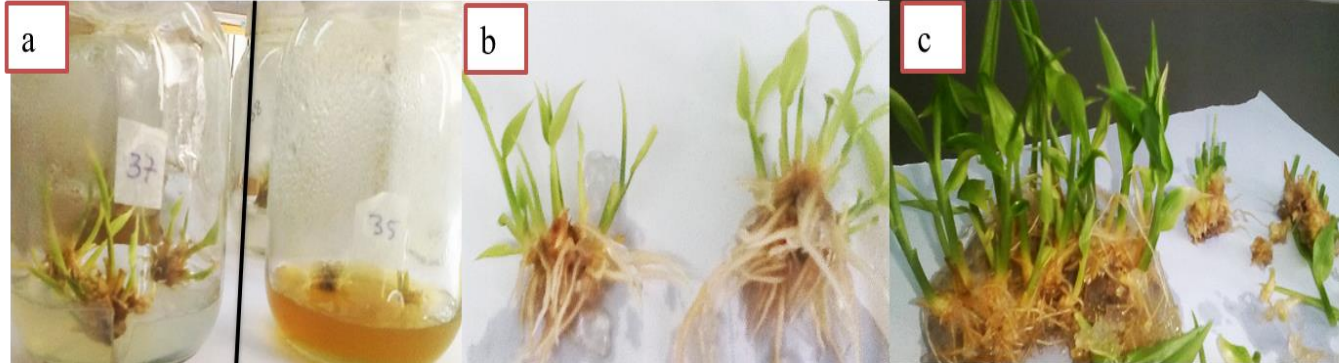

Figure 4. 3. Effect of silver nitrate and ammonium nitrate on micro-rhizome induction a. media without silver nitrate (left side) and media with 2.9 g/l silver nitrate (right side), b. Plantlets from low ammonium nitrate and high silver nitrate with poor micro-rhizome and growth, c. plantlets on medium containing sucrose and BAP alone with induced micro-rhizome.

Very low amount of ammonium nitrate (0.55 g/l (33%)) of MS standard (1.65 g/l) and high concentration of silver nitrate (1.9 g/l) has a negative effect on in vitro micro-rhizome induction as well as shoot and root growth.

Medium supplemented with $AgNO_3$ at 2.9 g/l level has resulted in media color change to orange, which might be due to side reaction with other media components and inhibition effect on explants that resulted in exudation of secondary metabolites (Fig 4.3).

Table 4. 3. Effect of ammonium nitrate and silver nitrate on micro-rhizome induction

#Treatment	Combinations		Measured in vitro traits					
	NH_4NO_3 (g/l)	$AgNO_3$ (g/l)	NR/Ex	RW (g)	NS	SL(cm)	RN	RL(cm)
1	1.65	0.9	2.00	2.50	6.33	4.7	11.00	3.03
2	0.825	0.9	2.33	1.70	3.33	4.67	15.00	3.20
3	0.55	0.9	1.67	0.60	3.33	1.93	4.67	4.03
4	1.65	1.9	0.67	1.77	3.00	2.83	5.00	1.87
5	0.825	1.9	0.35	0.70	3.67	3.97	8.00	1.90
6	0.55	1.9	0.33	0.33	2.00	2.90	3.33	1.97
7	1.65	0	5.67	6.00	9.00	5.83	11.33	6.63
8	0.825	0	4.00	3.67	5.67	3.27	14.33	2.70
9	0.55	0	2.33	1.43	1.67	1.07	2.67	1.50
10	1.65	0	0.33	0.23	10.33	8.73	18.33	11.43
CV (%)			22.26	20.21	11.95	9.10	8.23	19.91
LSD (5%)			0.75**	0.65**	0.98**	0.62**	1.32**	1.23**

NR- number of micro-rhizome per shoot, RW- rhizome weight, SN- shoot number, SL- shoot length, RN- root number, RL- root length and control in use for ginger micro-propagation.

***Ex vitro* performance of micro-rhizome**

Rhizome sprouting ability was tested in sterilized soil mixture (2:1:1) topsoil, sand, and coffee husk respectively. After one month of planting, 80% of micro-rhizome sprouted with vigorous growth from medium enriched with 80 g/l sucrose and 6 mg/l, BAP followed by 70% from medium supplemented with 60 g/l sucrose and the same amount of BAP (Fig. 4.4). On the contrary, the lowest percentage of planted micro-rhizomes emerged from media containing 100 g/l sucrose and 9 mg/l BAP.

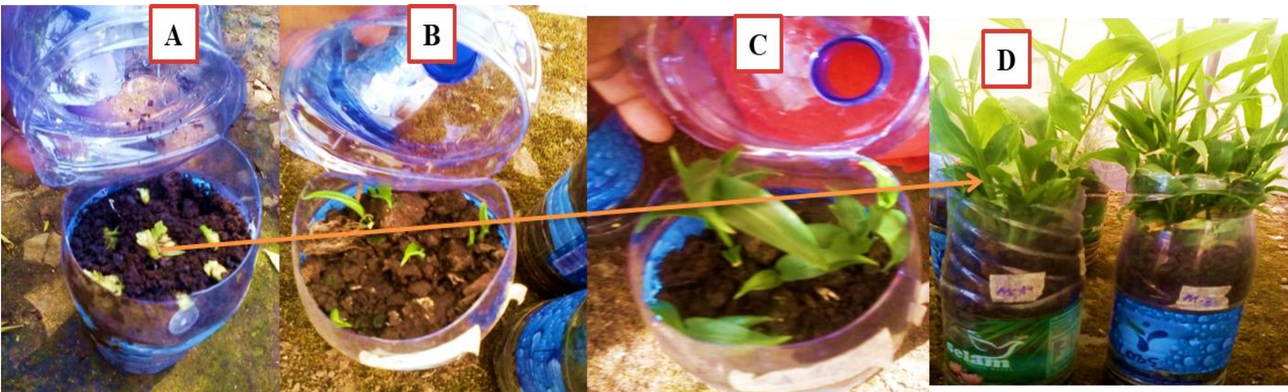

Figure 4. 4. In vivo performance of micro-rhizomes produced in vitro. a micro-rhizome at planting, B. sprouted micro rhizomes after a month; C. sprouts on growth after 15 days of emergence, and D. plants on vigorous growth after 2 months of emergence

4.4. Discussion

A report by Archana *et al*. (2013) indicated that sucrose from 80 to 90 g/l with different types and concentrations of growth regulators has better-induced micro-rhizome in ginger after one month of culturing. Another study also revealed that an increase in sucrose decreases plantlets' number and height due to high bulging at the base rather than leaf and stem growth (Zeng *et al.,* 2008). Our results in this experiment confirmed that a higher level of sucrose (80 g/l) with the optimum level of BAP (3-6 mg/l) is better for inducing and producing viable micro-rhizome in vitro.

The inverse proportionality of some shoot and root growth parameters to sucrose levels is also in line with the above reported findings. Similarly, Swarnathilaka *et al*. (2016) indicated that MS medium fortified with 4 mg/l BAP and 90 g/l sucrose was the best combination for maximum number and fresh weight of micro-rhizome that also binds to our observations. Moreover, our experiment showed that BAP concentrations above 6 mg/l did not affect the micro-rhizome induction instead it affected root number and length at 9 mg/l combined with 60 g/l sucrose. This indicates BAP has less effect on micro-rhizome induction in ginger as compared to sucrose. Zheng *et al*. (2008) reported the negative effect of sucrose at 110 g/l that it makes plantlets etiolate and die. The same author reported that it is sucrose that plays the main role in micro-rhizome induction and development.

The non-significant difference among BAP levels recorded here also further confirmed that it is sucrose that mainly influenced explants to induce micro-rhizome than BAP. An elevated amount of sucrose induces micro-rhizomes by increasing carbohydrate storage and creating bulges at the base of shoots, which later developed into rhizomes. This was further confirmed previously by nutrient composition analysis of ginger rhizomes in which carbohydrate takes the largest proportion (12.3%) next to 80% water in fresh rhizomes and 50% dry ginger (Bhatt *et al*., 2013). Swarnathilaka *et al*. (2016) also showed that shoot and root growth is inversely proportional to micro-rhizome number and weight. This might be due to plant cells' physiological response as affected by available nutrients in the medium. This is mainly due to enhanced development of storage organs like rhizome due to large carbon in growth media. In this case, a saturated amount of sucrose has promoted rhizome induction and growth rather than shoot and roots. Our findings

of the second phase, which included ammonium nitrate and silver nitrate partially is in contrast with the results reported by An *et al*. (2020).

An *et al*. (2020) stated that the maximum micro-rhizome weight on MS medium supplemented with low ammonium nitrate and high silver nitrate combined with nearly similar amounts of sucrose and BAP we used. However, in this experiment, both low concentrations of ammonium nitrate and high concentrations of silver nitrate have negatively affected micro-rhizome induction and mean fresh weight. Thingbaijam *et al*. (2014) also stated that silver nitrate at a very small level has improved micro-rhizome and shoot multiplication but was inhibitory at high concentrations (2.9 g/l). The same authors elaborated that the small amount of silver nitrate enhances induction by inhibiting the ethylene effect whereas at high concentrations it affects nutrient uptake to explants and negatively affects in vitro growth. Poor media quality and inhibition of micro-rhizome and shoot growth might be due to the nature of silver nitrate. Low ammonium nitrate was reported to enhance micro-rhizome development by limiting upper part growth due to low nitrogen in media that can serve for protein synthesis (Cecilia, 2010). However, this has not significantly affected micro-rhizome induction in this experiment that may be due to the genotype effect.

Most of the studies on ginger micro-rhizome production have not included the performance of the in vitro-produced micro-rhizomes except a few. Zhaid *et al.* (2021) reported that micro-rhizomes sprouted on moist sand after two weeks but did not report the maximum rate of sprouting. Micro-rhizome viability rate recorded here can be considered good but needs to be maximized by improving micro-rhizome quality during induction and production.

The low viability of micro-rhizomes in some treatments may be due to their small size and a small number of active buds that declined to emerge.

4.5. Conclusions

These experiments conducted to induce and produce viable micro-rhizome ginger seed generally indicated that micro-rhizomes induced and produced on MS medium supplemented with a high sucrose (80 g/l) and 6 mg/BAP can be used as alternative disease-free planting materials.

In the study, an optimum number of micro-rhizomes, a significant number of shoots that can be transferred to multiplication medium or can be acclimatized simultaneously were achieved. This finding of obtaining viable micro-rhizomes and plantlets at the same time was not yet reported to our knowledge. The in vitro-produced micro-rhizomes can also be used at seed multiplication nurseries and enhance ginger production at a large scale. A high concentration of sucrose, above 80 g resulted in a less viable small-sized but large number of micro-rhizomes that should be further investigated to increase their viability. Moreover, repeated experiments with modification should be conducted to get the maximum number of micro-rhizomes. Further work to increase the viability percentage under different in vivo growth conditions including open field is also required. Finally, in vitro produced micro-rhizomes can be used as seed sources for ginger production.

CHAPTER 5

5. Assessment of Ginger (*Zingiber officinale* Roscoe) Genetic Diversity using Morphological traits

Abstract

Production of ginger as a spice and medicinal plant at a small-scale level has a long history in Ethiopia using genetically undefined locally available and introduced materials. It became an important spice crop at the leading position until the pressure of ginger bacterial wilt disease eruption. The disease has challenged ginger production and productivity on top of a lack of improved varieties and poor management practices. For successful breeding and improvement of economically important traits in any crop, genetic diversity assessment is the first step. Hence, the experiment was executed by analyzing genetic diversity based on 24 morphological traits. One hundred accessions collected from different areas were evaluated under screen house. The 100 study materials were arranged in completely randomized design and each accession is replicated in three pots. Significant variation for 17 measurable traits resulted in a high genetic (GCV) and phenotypic coefficient of variations (PCV). Maximum values for GCV (25.4), PCV (25.93), heritability (95.8), and Genetic advance (GA) as a percent of the mean (61.73) were recorded for rhizomes yield per plant. Correlation analysis among traits revealed that there is a strong positive significant correlation between rhizome yield and some growth traits like plant height and leaf area. Cluster analysis based on Euclidian distance has partitioned the study materials into four clusters with high to medium distance from the average centroid. The distribution of accessions, across clusters was independent of collection areas. The study revealed high genetic diversity among the study materials which can be exploited for variety improvement strategies.

Key Words: accessions, diversity, heritability, phenotype, variety

5.1. Introduction

Ginger has been used for many purposes and become a major spice crop for local consumption as well as export markets in Ethiopia. The cultivation and utilization was started during the 13th century when Arabs introduced it from India to East Africa (Jansen, 1981; Endrias and Asfaw, 2011). The potential and importance of this spice crop has been expressed via different conditions; like total area covered, productivity per unit area and multiple use values. Ethiopia stood 10th and 14th in 2011 among 36 countries engaged in ginger production globally in terms of area harvested, and total production respectively (FAO, 2013). Its production at large scale was mostly limited in the hot humid areas of southern and southwestern parts of Ethiopia.

Ginger was the first ranked among spice crops and has been the main source of income and well recognized in improving the livelihood of many ginger growers and traders in Ethiopia (Kifle *et al*., 2021). It has been the leading export spice crop from 2005-2011 in terms of average volume exported and hard currency value. Being the largest export spice crop in Ethiopia, ginger contributed about 66% (7199 tons) to the spice export value in 2011. However, this was declined to 8.75% (1183 tone) of total spice product exported in 2013 and at the lowest level below 1000 tons until 2022 (Kifle *et al.,* 2023). This was mainly due to devastation by ginger bacterial wilt disease. Moreover, ginger germplasm improvement and genetic diversity information were limited due to low attention given to the crop in spite of its economic, social and health importance.

Ginger is among crops mainly propagated clonally from modified underground stems called rhizome. This asexual propagation system in ginger restricted its improvement mainly to clonal selection.

In such circumstances, a high variability and broad genetic base become imperative to have sound basis for worth full selection (Nisar and Ghafoor, 2011). Hence, for meaningful breeding, conservation and sustainable utilization of genetic resources is imperative to understand the existing diversity.

Ginger genetic diversity studies conducted before indicates that some morphological characters help in easy and quick identification of genotypes. Maximum variation was observed for growth habit, number of tillers, shoot diameter, rhizome thickness, rhizome shape and dry mater recovery (Akshita *et al*., 2019). Previous genetic variability study based on morphological and some quality traits like dry mater and oileorisen content in Ethiopia. The study reported wide genetic variability and leaved recommendations for further evaluation using a large number of accessions from geographically wider areas (Momina *et al*., 2011). Ginger variety development research at national level has been evaluating local and introduced germplasm for adaptability, yield, and quality but has released only two varieties so far. Moreover, ginger germplasm collection and conservation activities undertaken by the national program were challenged by ginger bacterial wilt disease in 2012 (Girma *et al*., 2022).

Southern and southwestern Ethiopia areas are often understood as major areas of ginger production and germplasm diversity (Tefera and Asfaw, 2015). Except preliminary research report, on a limited number of accessions intensive collection and diversity analysis had not yet been undertaken in Ethiopia. Hence, the present study report states the genetic diversity of 100 ginger accessions including released varieties (boziab and yali), promising genotypes (volovo), locally

available landraces, and wild species, mango ginger (Curcuma amada Roxb.) collected from wider production agro-ecologies.

5.2. Materials and methods

Experimental site

The experiment was conducted at Areka Agricultural Research Center under a protected screen house. Areka Research Center is located 300 km south of Addis Ababa at 7°4'12" N and 37°42'0" E at an elevation of 1774 meters above sea level. The protected screenhouse had an average daily temperature of 28.5 ^{0}C and relative humidity of 85 %.; in the year 2021/22 production season Areka and its surrounding received average monthly precipitation of 122.5 ml with a minimum of 21 in January, and a maximum of 189 ml in May. The outdoor monthly average humidity also ranged from 44 in Jan to 80 in July.

Experimental materials

Ginger rhizomes of accessions from different production areas of the Southern, Central, Southwestern and Oromia regional states of Ethiopia (Fig 5.1 and Appendix III) were collected as per germplasm collection guidelines (FAO, 1993; Ethiopian Biodiversity Institute, 2012). Then from the field samples, single seed rhizomes with at least three active buds were intentionally selected to raise experimental materials as clonal propagates. The materials were then used for morphological traits based diversity analysis and other experiments. Hundred ginger accessions including two released (Yali and Boziab), one farmers variety Volvo and a wild type, Jimma wild (JW89) were collected from different areas, Areka and Jimma agricultural research centers (Fig 5.1). Most of the accessions (60%) were from Southern and Central Ethiopia areas (Wolaita,

Gamo, Gofa, Kambata-Tambaro, Derashe, South-Omo, Basketo, Hadiya and Gurage). Among these areas, some were known for ginger diversity and product supply for local consumption, marketing and export from Ethiopia. Western Oromia which includes; Jimma, Illubabor and Wellega areas, from which 21% of study materials collected is also one of the potential diversity and production for ginger. The remaining 19% accessions were collected from Southwestern part of Ethiopia (Appendix II). The collection areas are from lowest altitude (1230 m.a.s.l) in Damba Gofa to high altitude (2050 m.a.s.l) in Damot Sore Damba zamine village of Wolaita. Areas addressed have different trend and habit of ginger production culture. Hadaro tunto of Kambata and Boloso Bombe of Wolaita are areas where ginger was a major cash crop and known as belts of ginger in Ethiopia.

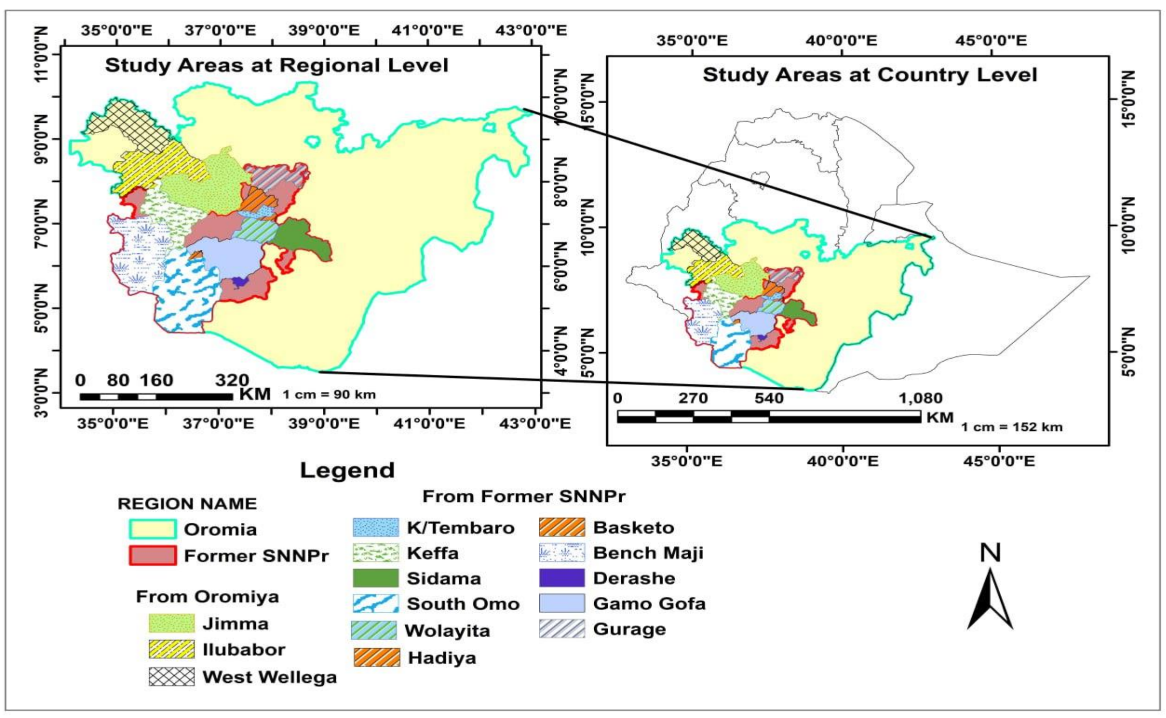

Figure 5. 1. Map of ginger accessions collection sites

Management of the experiment

Planting was done in black polyethylene pots with 25 cm height and 16 cm diameter, potted by solar sterilized soil mixture (2:1:1) of top soil, compost and sand respectively. Watering was done once in a week to a saturated level allowing drainage through small pores to prevent water logging. Weeding and soil filling were also done throughout the active growth period under the screen house.

Experimental design

Hundred accessions rhizome seed was planted to three pots placing three clonal mini rhizomes per pot for two consecutive years (2021 and 2022). Pots were randomly placed in fixed place after grouping 25 (5x5) pots in one place letting 50 cm between groups for ease of observation and data recording. Moreover, to have uniform emergence rhizomes with active buds were stored for one month at room temperature before planting. Experimental unit pots are arranged rondmly in three replication for each study accessions

Data collection

Data for 17 quantitative (plant height, tiller number, leaf number, leaf width, leaf length, leaf area, inter leaf length, tiler thicknes, days to harvesting, root number, root length, number of rhizome finger, number of buds, rhizome length, rhizome width, rhizome wight, and dry matter) was collected by measuring and counting observations. Moreover seven qualitative (leaf arrangement, leaf color, root type, rizome layer, rizome arrangement, root texture, and rhizome color) traits during growth and harvest time were also recorded after visual observation.

Study traits were selected intentionally based on their partitioning ability from previous ginger descriptor and diversity analysis reports (Shakeel *et al*., 2013; Akshitha *et al*., 2019). Growth traits

such as; plant height, tiller number, leaf number, leaf length, leaf width, leaf area (calculated), tiller thickness, inter leaf length, leaf arrangement and leaf color were collected after six months of planting which is the maximum growth time and onset of maturity (Akshitha *et al*., 2019).

Recording for days to maturity was started six months of planting in five days interval up to complete leaf senescence and stem collapse of all study materials. Post-harvest data; for rhizome yield per plant and pots, number of rhizome fingers per plant, number of buds per rhizome finger, rhizome finger length, rhizome finger width, root number, and related traits were taken during harvesting. Dry matter recovery after oven drying until permanent weight, was also calculated during and after harvesting.

Data analysis

Qualitative data recorded as categories were converted to binary digits and subjected to descriptive statistics such as frequency distribution as percentage by micro-soft office excel 2016 and statistical analysis software (SAS) version 9.3 (2011). Whereas, the data recorded for quantitative traits was analyzed using different statistical methods. Analysis of variance (ANOVA) to differentiate significant variation among means of all accessions for recorded each trait and other descriptive statistics like minimum and maximum, standard deviation and variance was done by SAS version 9.3 (2011).

Genotypic and phenotypic variances were computed based on their respective expectations using Microsoft excel 2016. Genotypic variance ($\delta^2 g$) was computed as the difference of genotypic mean square (MSG) and error mean square (MSE) divided by number of replications (r) (Singh and

Chaudhary 1979). While, phenotypic variance (δ^2p) is the sum of genotypic (δ^2g) and error variances (δ^2e). Coefficient of variations for both genotypic and phenotypic variances was also computed using methods suggested by Singh and Chaudhary (1979). Heritability at broad sense (h^2b %) was estimated as a ratio of genotypic variance to phenotypic variance using the formula; (h^2b %) = $\delta2g/\delta2p*100$ (Falconer, 1981). Genetic advance (GA) and genetic advance as percent of mean (GA%) were estimated by Johnson *et al*. (1955) method as follows: $GA = \delta^2g/\delta^2p*k*\delta p$, where δ^2g is genotypic variance, δ^2p is phenotypic variance, δp is standard deviation of phenotypic variance and k is the selection differential at a particular selection intensity, 2.06 at 5% selection intensity. Genetic advance as percent (GA%) = $GA/\chi*100$, where χ is the mean of a trait.
Pearson linear correlation coefficient among all traits paired was computed by Minitab 17 (2010) to identify traits with strong positive and negative correlation directly or indirectly with economically important traits.

Principal components and cluster analyses were performed to see the grouping pattern and clustering of ginger accessions. A correlation matrix based on the 17 quantitative characters was used to perform principal component analysis. Then distance matrix of the Euclidean dissimilarity coefficients between all pairs of entries was used to develop cluster dendrogram by K-means optimum cluster number determination method (Kakushadze *et al.,* 2017).

5.3. Results

Production trends in agro-ecologies

Before the outbreak of ginger bacterial wilt disease the commercial production was expanded to potential areas like Basketo and Tepi where private investors were involved. Most of the other places addressed were areas where ginger has been in production for home consumption as spice and medicinal value using locally available landraces for long time. Farmers in these areas are producing their own local seeds and planting rhizomes of unknown source purchased from markets. Ginger farmers in different areas have groups and local names for their varieties. Wolaita farmers have two groups; 'Maculine and Feminene'. Kambata and Hadiya communities have als o names like 'Hargame' for local varieties they were producing since immemorial time. Farmers in other areas also identified variations among the different cultivars for pre harvest requirements and postharvest characteristics as well as their distinctions in commercial values.

Mean squares and descriptive statistics values

Analysis of variance (ANOVA) for 17 quantitative traits showed that there was significant difference among mean values of the hundred accessions at p<5%. Descriptive statistics such as mean, standard deviation, coefficient of variation, minimum and maximum scores were summarized in Table 5.1. Plant height (cm) ranged from 24 to 69 cm. Other main traits which has linear positive correlation with rhizome yield per plant like leaf length, leaf area, tiller thickness, number of rhizome fingers, number of active buds, rhizome finger length and width have also high range between minimum and maximum values recorded (Table 5.1). The mean values for growth traits (1-9) from plant height (PH) to days to harvesting (DtH) were summarized in Table 5.2. Mean of 49.58 cm plant height, from soil surface to the pick of the plant, 3.81 number of tillers

per plant, 13.34 leaves per tillers, 2 cm width at the broadest part of leaves, and 17.61 cm leaf length from collar region to tip of leaves. Mean value of leaf area calculated from leaf length and width was 35.41 cm^2; distance between leaves of main tillers was 4.77 cm, circumstance of tiller thickness at 2.36 cm and mean of 196 days to rhizome harvesting (Table 5.2).

Rhizome yield and related traits measured and counted have also records from low to high range between minimum and maximum score values. Single rhizome fresh weight ranged from 7.7 to 134.7 g/rhizome with total mean value of 68.29 g/rhizome for the 100 study materials. Number of rhizome fingers per single rhizome was 1 and 10 for minimum and maximum scores with overall mean of 4.39. Dry matter recovery (DMR) as percentage has also wide range from 17.4 to 41.7 with total mean of 27.43% and standard deviation (SD) of 4.7. Root number and length were other traits with wide variance among test materials.

Maximum standard deviation (SD) was recorded for rhizome yield per plant (21.36) followed by days to harvesting (11) and the lowest (0.3) SD was recorded for leaf width. High coefficient of variation (CV) was observed for countable traits like number of roots, tillers, rhizome fingers and buds. Whereas, the lowest was for days to maturity and plant height (Table 5.1).

Table 5. 1. Summary of mean squares and descriptive statistics of 17 basic quantitative traits

Number	Trait	TMS (Df=99)	EMS (Df=200)	Mean	SD	CV	MIN	MAX
1	PH	256***	4.73	49.58	9.4	4.38	24	69
2	TN	3.4***	0.49	3.81	1.2	18.49	1	8
3	LN	15.79***	1.36	13.34	2.5	8.73	6	21
4	LW	0.21**	0.07	2	0.3	13.34	1	3
5	LL	15.89**	2.38	17.61	2.2	8.62	10	23
6	LA	121.81*	34.73	35.41	8	16.64	13	58.8
7	ILL	3.12***	0.6	4.77	1.2	16.32	2	9
8	TTK	0.45***	0.02	2.36	0.4	6.52	1.4	3.3
9	DtH	381.47***	6.73	196	11	1.73	175	225
10	RN	123.11**	6.19	10.35	6.7	24.02	1	43
11	RL	59.83**	2.59	13.12	4.64	12.27	2	24
12	NRF	3.5**	0.86	4.39	1.32	21.16	1	10
13	NB	4.9**	0.79	5.11	1.47	17.39	2	10
14	RiW	3.32**	1.14	5.77	1.36	18.49	2	9
15	RiL	6.27**	1.53	9.43	1.76	13.11	4.5	15
16	SRW	1338.74**	19.27	68.29	21.36	6.43	7.7	134.62
17	DMR	37.13**	14.32	27.43	4.7	13.79	17.4	41.7

Legend: PH-plant height in cm, TN- tiller number per plant, LN- leaf number per tillers, LW- leaf width in cm, LL- leaf length, LA- leaf area, ILL-inter leaf length. TTK- tiller thickness, DtH- days to harvest, RN- root number per plant, RL- root length in cm. NRF- number of rhizome fingers per rhizome, NB= number of active buds per rhizome finger, RiW- rhizome width in cm, RiL- rhizome length in cm, RW/P- rhizome weight per plant in gram, RY/P- rhizome fresh weight per pot in gram and DMR-dry mater recovery in percent.

Genotypic and phenotypic variances, heritability and genetic advances

Quantitative traits variance and related genetic and phenotypic computed values of variances, coefficient of variances, broad sense heritability, genetic advance and genetic advance as percent of mean were summarized (Table 5.2). Across the traits evaluated, the highest variance (δ^2), genetic variance (δ^2g) and phenotypic variances (δ^2p) were recorded for rhizome yield per plant

followed by plant height and leaf area. In contrast, the lowest records for the corresponding three values were for leaf width, tiller thickness and inter leaves length (ILL). The genetic coefficient of variance (GCV) varies from lowest for leaf width 1.53 to highest for rhizome yield per plant 25.4. Phenotypic coefficient of variance (PCV) also showed the highest for rhizome yield and the lowest for leaf width.

Heritability estimates at broad sense (h^2b) level as percent ranged from 34.68 for dry matter recovery to 95.8 for rhizome yield per plant. Genetic advance as percent of mean (GA%) was highest for root number (115.1%) and lowest (11.4) for days to harvesting (Table 5.2). Traits such as leaf length and days to harvest have low heritability and GA percentage have not additive effect and are not good to be used as selection traits. Whereas, traits like plant height, rhizome yield, root length, leaf number and number of buds per rhizome showed high heritability and GA percentage of mean. These traits have also strong linear correlation coefficients with each other might have additive effect and could be considered as selection traits in ginger breeding programs (Table 5.3).

Table 5. 2. Variances, Heritability and Genetic advance of 16 quantitative traits

S.N.	Trait	δ^2	δ^2g	δ^2p	GCV (%)	PCV (%)	h^2b	GA	GA (%)
1	PH**	87.92	83.8	88.49	13	13.36	94.65	18.27	36.85
2	TN**	1.45	0.97	1.46	5.05	6.19	66.44	1.656	43.47
3	LN**	6.14	4.81	6.17	6	6.801	77.96	3.967	29.74
4	LW*	0.12	0.05	0.117	1.53	2.415	40	0.28	14.01
5	LL**	4.95	1.82	4.96	3.21	5.307	36.69	1.678	9.529
6	LA*	63.57	29	63.76	9.05	13.42	45.53	7.475	21.11
7	ILL**	1.44	0.84	1.44	4.2	5.494	58.33	1.442	30.23
8	TTK***	0.16	0.14	0.163	2.46	2.631	87.76	0.723	30.64
9	DtH**	130.1	125	131.6	7.98	8.195	94.89	22.34	11.4
10	RN**	44.9	39	45.16	19.4	20.89	86.29	11.91	115.1
11	RL**	21.54	19.1	21.67	12.1	12.85	88.05	8.416	64.15
12	NRF/R**	1.73	0.88	1.74	4.48	6.296	50.57	1.375	31.33
13	NB/RF**	2.15	1.37	2.16	5.18	6.502	63.43	1.921	37.59
14	RFD**	1.86	0.73	1.867	3.55	5.688	38.93	1.091	18.9
15	RFL**	3.1	1.58	3.11	4.09	5.743	50.8	1.842	19.53
16	RW/p**	456.15	440	459.1	25.4	25.93	95.8	42.15	61.73
17	DMR**	21.87	7.6	21.92	5.26	8.94	34.68	3.336	12.16

Legend: δ2: variance, δ2g: genetic variance, δ2p: phenotypic variance, GCV (%): genetic coefficient of variance, PCV (%): phenotypic coefficient of variance, h2b: heritability at narrow sense, GA: genetic advance and GA (%): genetic advance as percent of mean.

Genetic diversity based on Qualitative traits

Among the seven qualitative traits used to discriminate 100 ginger accessions to different groups, only one trait; leaf arrangement on tillers failed to partition accessions to groups as 100% of the study materials were with alternative leaves (Fig 5.2A). The second trait, leaf color has grouped the hundred accessions into two groups (light green and dark green). Most of the accessions (85%) under the experiment have a light green color and only 15% have a deep green color (Fig 5.2A).

For the rooting system, 67% of accessions have taproots with few main and sub roots mostly on the bottom side of rhizomes. The remaining 33% have adventitious roots developed from all rhizome sides. A large number of roots, relatively short, small-sized rhizomes having few rhizome fingers and active buds, characterizes accessions with adventitious root types. The other rhizome-related qualitative trait, root surface has grouped the study materials into three categories (smooth, semi-hairy, and hairy types). A larger proportion of accessions (83%) has a smooth root surface, which is a typical characteristic of accessions with a few main taproots. The second category constituted 13% of the study materials, having hairs on secondary roots but not on main roots and were categorized as semi-hairy rooted. The rest 4% of the study materials are accessions with roots with full of hair-like structures on their surface mainly observed on accessions with adventitious root architecture (Fig. 5.2).

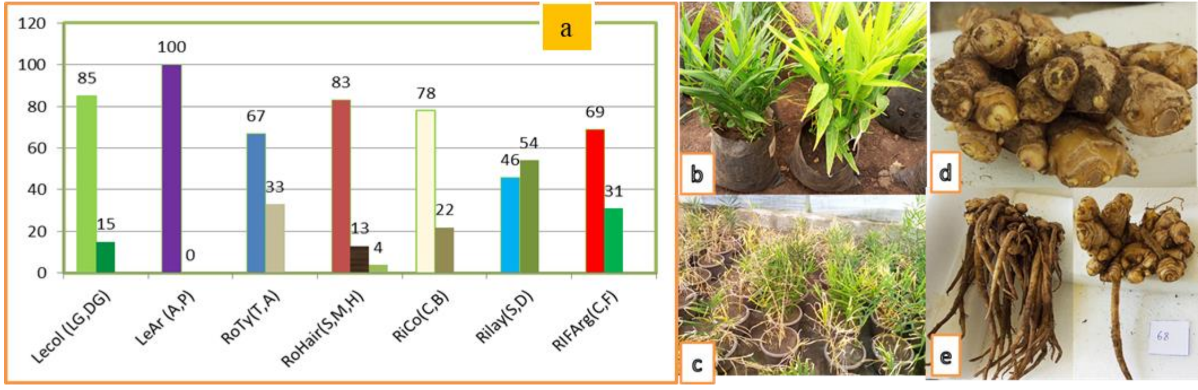

Figure 5. 2. Phenotypic variations based on qualitative and quantitative traits a. Bar graph of seven qualitative traits frequency. b. deep green (left)) and light green leaves (right), c. maturity variation among accessions, d. double layered rhizome fingers and e. different accessions with large number and single root rhizomes (left and right) respectively.

Correlation between traits

The result of pairwise correlation analysis summarized in table 3 revealed that there is a linear positive and negative relation among all the 24 quantitative and qualitative traits evaluated across the experiment.

The result showed that fresh rhizome yield per plant has a strong significant positive correlation at 95% confidence interval with nine different quantitative growth and yield traits. Among the traits, plant height, leaf length, tiller thickness, leaf area, root length, and number of rhizome fingers showed correlation coefficient of 0.253, 0.283, 0.31, 0.23, 0.229, and 0.496 respectively. Rhizome weight has also a strong positive linear correlation with rhizome finger width and length (Table 5.3). The analysis result also showed some traits have a negative linear correlation with rhizome yield. Days to harvesting (DtH) and rhizome finger growth pattern (RFGP) around the mother rhizome had a negative correlation with the economic part of fresh rhizome. Whereas, most of the categorical traits have failed to have a significant correlation to rhizome fresh weight (Table 5.3).

Table 5. 3. Pairwise Pearson Correlations among 17 quantitative traits

Traits	PH	TN	LN	LW	ILL	LL	TTK	LA	DtH	RN	RL	NRF	NBF	RFD	RFL	RW/P	DMR
PH																	
TN	0.08																
LN	0.44	0.03															
LW	0.48	-0.11	0.06														
ILL	0.38	0.01	0.06	0.22													
LL	0.41	0.01	0.10	0.27	0.57												
TTK	0.31	-0.03	0.11	0.54	0.04	0.17											
LA	0.55	-0.08	0.09	0.79	0.51	0.79	0.44										
DtH	-0.15	0.07	-0.05	-0.21	0.10	-0.04	-0.23	-0.16									
RN	0.063	0.154	-0.011	-0.130	-0.034	0.163	-0.240	0.046	0.036								
RL	0.193	0.215	-0.030	0.150	0.253	0.419	0.093	0.355	0.086	0.373							
NRF	-0.06	-0.09	0.05	-0.13	-0.04	0.07	-0.02	-0.03	-0.14	0.22	-0.05						
NBF	0.08	-0.06	0.25	-0.12	-0.08	-0.03	0.01	-0.08	-0.19	0.23	-0.03	0.66					
RFD	0.206	0.060	0.090	-0.069	0.152	0.157	0.016	0.063	-0.195	0.187	0.036	0.481	0.476				
RFL	0.163	0.076	0.126	0.062	0.171	0.200	0.199	0.172	-0.259	0.097	0.030	0.595	0.479	0.556			
RW/P	0.253	0.119	0.179	0.093	0.021	0.284	0.310	0.230	-0.089	0.134	0.229	0.496	0.482	0.444	0.47		
DMR	0.04	0.07	-0.13	0.06	-0.01	0.004	-0.04	0.05	-0.13	0.23^{*}	0.11	-0.26^{*}	-0.06	0.04	0.06	-0.10	

Legend: PH- plant height, TN- tiller number, LN- leaf number, LW- leaf width, LBL- length between leaves, LL- leaf length, TTK- tiller thickness, LA- leaf area, DtH- days to harvest, RN- root number, RL- root length, NRF- number of rhizome finger, NBF- number of buds per finger, RFD- rhizome finger diameter, RFL- rhizome finger length, RW/P- rhizome weight per plant. and DMR is dry matter recovery.

Multivariate analysis

Principal component analysis

Principal Component Analysis (PCA) conducted using Minitab 19.1.1 (2017) showed that the 17 quantitative traits evaluated were reduced to three PCA artificial traits with eigenvalues >1. These three components with high eigenvalues have cumulative variation proportion of 66.1% of the total variation among 100 accessions. PCA1, PCA2, and PCA3 have 37.1%, 15.6%, and 13.3% proportion of variation respectively (Table 5.4). PCA1 was loaded by rhizome yield and associated traits. Rhizome yield has the largest eigenvector value (0.376), followed by 0.316 for rhizome finger length, 0.276 for rhizome finger diameter, and 0.269 for plant height. The second component was highly dominated by leaf area (0.404) followed by rhizome fingers (0.355). The third is dominated by root number (-0.463), root length (-0.450), and tiller thickness (0.378).

Score plot of the first component (horizontal axis) and second component (vertical axis) drawn to understand grouping of accessions (Fig. 5.3). The distribution of accessions on scatter plot of the first two principal components has clearly separated the materials with high accumulation around the start points of the two axis plots. In the distribution, there is no correspondence between collection areas and groups on the plot. This indicates that there is variation within collection areas and the interrelation between different collection areas is strong. The first component was effective in separating ginger accessions with double rhizome (red color) finger layers from accessions with single layer (blue color) rhizome fingers in which most of accessions with single layer and doubled layered were clustered separately on the score plot quadrants (Fig. 5.3).

Table 5. 4. Eigen analysis (value and vector) of the correlation matrix

Quantitative Traits	PC1	PC2	PC3
Eigenvalue	3.34	1.41	1.20
Proportion (%)	37.1	15.6	13.3
Cumulative (%)	37.1	52.7	66.1
Eigenvectors	PC1	PC2	PC3
PH	0.269	-0.254	0.046
TN	0.033	0.026	-0.303
LN	0.146	-0.013	0.117
LW	0.186	-0.374	0.275
ILL	0.169	-0.270	-0.225
LL	0.280	-0.276	-0.257
TTK	0.204	-0.195	0.378
LA	0.294	-0.404	0.006
DtH	-0.129	-0.017	-0.356
RN	0.102	0.113	-0.463
RL	0.165	-0.162	-0.450
NRF	0.250	0.355	0.028
NBF	0.245	0.350	0.074
RFD	0.276	0.234	-0.037
RFL	0.316	0.200	0.081
RFW/Pl	0.376	0.175	-0.005

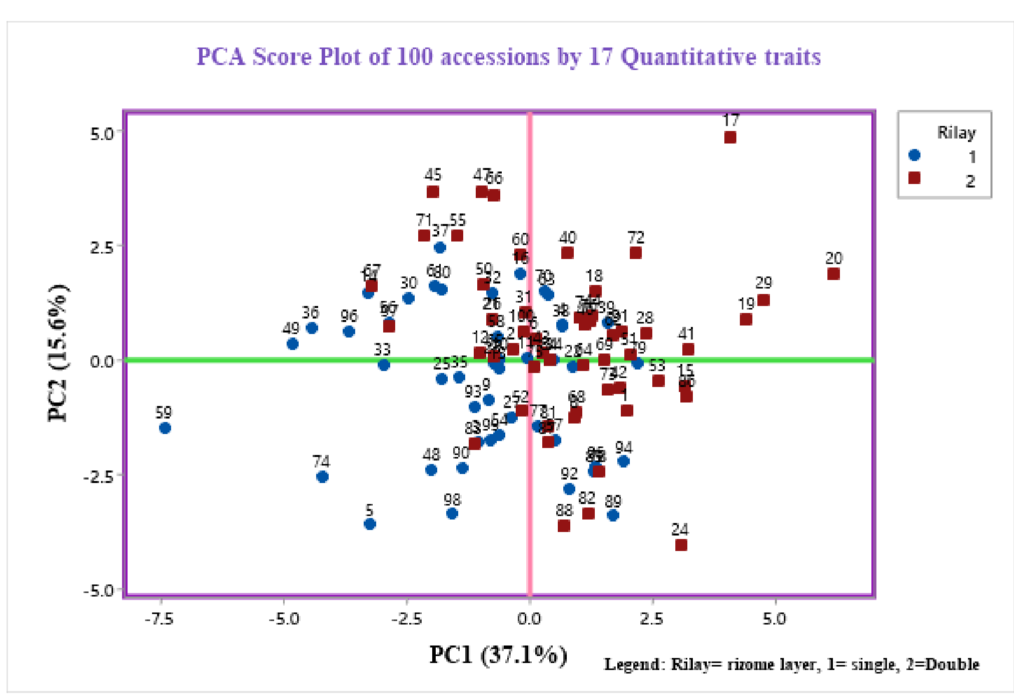

Figure 5. 3. Distribution of the 100 study accessions on PCA score plot

Clustering of ginger accessions

The cluster analysis based on the mean values of 17 traits evaluated has grouped 100 ginger accessions into four cluster groups (Fig 5.4). Cluster one, C-I (blue) is the largest cluster that accommodated 57% of accessions with the highest sum of squares and medium maximum distance from the centroid (38.05). This cluster is loaded by accessions with the largest mean values for days to harvesting and medium centroid values for most traits (Table 5.5 and 5.6). The second cluster (C-II) colored is the cluster with 14% of accessions with maximum distance from the centroid. The cluster is characterized by intermediate cluster mean values for most traits evaluated and high cluster mean values for plant height (54.64 cm). In the third cluster, C-III (green colored) which accommodated 24% of accessions, cluster mean values for most traits are low as compared to other clusters. The

only trait with the largest cluster mean value here is dry matter t, which had a weak linear positive correlation with other economic traits including rhizome fresh yield (Table 5.3).

Table 5. 5. Cluster partitioning and Centroids

Clusters	Number of accessions	List of accessions in clusters	Within cluster sum of squares	Average distance from centroid	Maximum distance from centroid
Cluster I	57	1,42,79,73,95,84,94,10,91,64,75,90,22,85,92,23,72,38,11,30,2,31,16,9,83,37,66,80,43,46,60,18,76,58,81,87,34,5,68,88,44,77,54,24,4,13,70,6,7,26,25,50.55,57,78,45,49	37445.6	24.83	38.05
Cluster II	14	3,8,27,28,39,41,51,53,62, 89,15,69,86,17	7895.8	22.92	36.89
Cluster III	24	12,32,14,40,61,21,93,65,71,48,99,52,82,36,59,33,35,96,74,56,97,67,98,100	14647.1	23.72	40.36
Cluster IV	5	19,20,29,47,63	5820.0	32.47	51.85

The fourth cluster, C-IV (brown colored) contained only 5% of evaluated accessions. The cluster was characterized by the highest mean values for plant height, tiller number, rhizome fresh yield per plant, and associated traits. Accessions in the cluster (19, 20, 29, 47, and 63) have large mean values for fresh yield in ANOVA.

Table 5. 6. Centroid values of each trait across clusters

Traits	C-I	C-II	C-III	C-IV	Grand Centroid
PH	49.01	54.64	46.32	52.93	49.59
TN	3.90	3.67	3.51	4.60	3.81
LN	13.45	14.31	12.61	13.00	13.35
LW	1.99	2.17	1.96	1.80	2.01
ILL	4.70	4.76	4.76	4.93	4.77
LL	18.00	18.45	17.14	18.73	17.89
TTK	2.41	2.57	2.13	2.49	2.37
LA	36.14	40.10	34.00	33.90	36.07
DtH	197.12	195.47	196.31	190.33	196.36
RN	10.73	11.78	8.87	9.27	10.36
RL	13.68	14.54	10.77	13.83	13.11
NRF	4.43	4.71	3.86	5.53	4.39
NBF	5.11	5.64	4.48	6.60	5.11
RD	5.84	5.97	5.27	7.00	5.78
RFL	9.37	9.88	9.02	10.83	9.43
RW/P	55.09	65.01	38.30	84.87	53.94
DMR (%)	27.30	27.60	28.07	25.52	27.44
Distance between cluster centroids					
C-I					
C-II	45.35				
C-III	51.22	96.24			
C-IV	111.38	66.99	162.25		

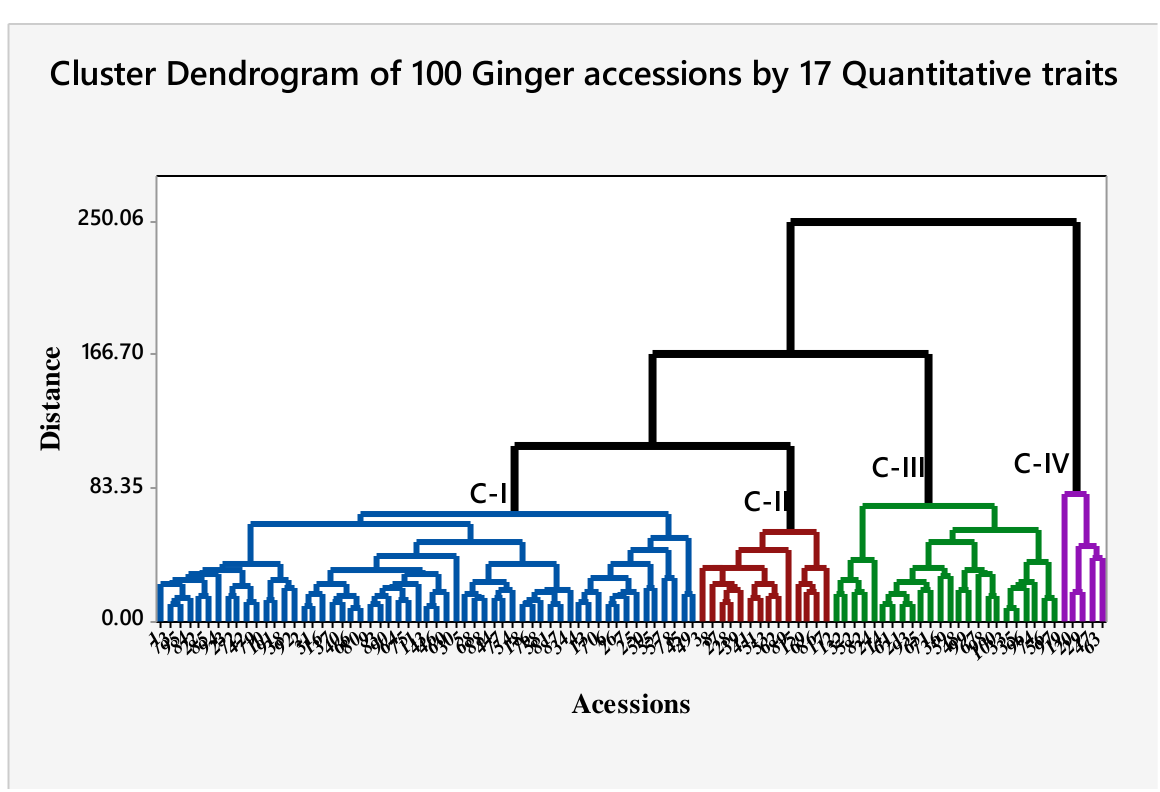

Figure 5. 4. Dendrogram of all 100 study accessions grouped to four clusters based on distance matrix

5.4. Discussions

Assessment and collection of ginger accessions confirmed that most farmers in Boloso-Bombe and Hadaro-Tunto districts in the Southern and Central Ethiopia regional states respectively were producing ginger as major crop. Previous assessment reports indicated that these areas are the ginger belts of the country. These areas contribute more than 90% of ginger product to the ginger value chain system (Endrias and Asfaw, 2011). South and Southwestern areas on the other hand were considered as the center for ginger diversity and product supply for local consumption, processing and export in Ethiopia (Girma *et al.*, 2022).

Western Oromia from which 21% of materials were collected is also one of the potential diversity and production area, which can be exploited to maximize ginger production and increase economic benefits. Some preliminary reports from ministry of agriculture also indicates that ginger has been in production for home consumption in Amhara regional state. Areas around Gondar, some parts of Gojam and north Shewa could be assessed for additional genetic diversity and variety improvement programs. Wider area assessed during the collection confirmed previous reports that ginger has wide adaptation range from very low to high altitude agro-ecologies with different weather conditions of Ethiopia (Asfaw *et al*., 2021). Farmers in these areas are mainly producing their own local landraces and unknown cultivars purchased from markets for home consumption.

Previous reports states ginger farmers in some collection areas have groups and local names for their varieties. Wolaita farmers have two groups; 'Maculine and Feminene'. Kambata-Tambro and Hadiya communities have also names like 'Hargame' for local varieties they were producing since immemorial time. Farmers in other areas also identified variations among the different cultivars for pre harvest requirements and postharvest characteristics as well as their distinctions in commercial values (Tefera and Asfaw, 2015).

The wider adaptation range, long history of production and various production and utilization habits of multi-cultural communities in Ethiopia might be the potential sources of ginger genetic diversity. Genetic diversity analysis using seventeen quantitative and seven qualitative morphological traits in this experiment confirmed that there is high genetic diversity among Ethiopian ginger accessions.

Highly significant variation for most quantitative traits and grouping by qualitative traits indicate that there is sufficient variability among study materials. Variability study result reported before a decade using 36 local and introduced ginger germplasm are in line with the finding of our experiment (Momina *et al.,* 2011).

High variance observed in this experiment for rhizome fresh yield per plant, dry matter recovery, and traits with significant positive linear correlation with rhizome yield such as plant height, leaf area, root length, rhizome finger number and layer of fingers indicated that there is a high opportunity to select best performing varieties. The observations of the experiment are in line with results reported for different numbers of ginger landraces used for diversity and relationship studies using standard traits in Japan and India (Shakeel *et al*., 2013; Akshitha *et al*., 2019). A very wide range between minimum and maximum values, high standard deviation, and low coefficient of variation for most traits evaluated also revealed that genotype effect is more contributor for the variations recorded. These are further strengthened by large value estimates of genotypic variance (GV) and phenotype variances (PV) and their coefficients. Genotypic coefficient of variation (GCV) and Phenotypic coefficient of variation (PCV) estimate values were classified as large (>20), medium (10-20), and low (<10) as suggested by Deshmukh *et al.* (1986). As expected in morphological trait based diversity analysis the PCV values are larger than GCV for all the significant quantitative traits. This indicate there was also the contribution of environmental effect for the variations recorded as PCV is derived from genetic variance and error variances. However, the difference between PCV and GCV is narrow for the corresponding traits, reflecting variation was not mainly due to environment.

Rhizome yield and its components had a low difference values between PCV and GCV. These traits has also high heritability and genetic advance as a percent of the mean. Hence, traits with high GCV and heritability (h^2b) can be considered good indicators for breeders to selection of genotypes as superior varieties from the study materials. Heritability greater than 30% was considered as high and below 10% is taken as low, which can be affected by breeding materials, the nature of the trait, and environmental conditions (Dabholker, 1992). Maximum heritability estimate values for plant height and rhizome yield are in agreement with a previous report by Momina *et al.* (2011). Similarily, Dev and Sharma (2022) also reported high heritability coupled with high and moderate genetic gain for yield per plot and weight of mother, primary and secondary rhizomes. Very high heritability records in this and previous reports indicates that there is a narrow gap between PCV and GCV, which may be due to low environment effect and high genotypic effect. In other words, these traits were very stable under different environments and seasons. The GCV and heritability estimate high values for rhizome yield and strongly correlated traits were supported by genetic advance (GA%) as a percent of mean, which is also very high for the corresponding traits. Heritability, GCV, and GA% alignment can give a guarantee for the researcher to use the phenotypic performance for the selection of genotypes for next step of the breeding process (Johnson *et al*., 1955).

Qualitative traits; leaf color, root system, root texture, rhizome surface color, rhizome layer and rhizome finger arrangement have partitioned the 100 accessions into two and three groups at different proportions. Grouping of accessions to the category was also in line with a previous report by Das *et al*. (2013) in the identification of duplicates among 60

ginger accessions using morphological and molecular markers. Among these traits, rhizome finger layer as double and single is highly correlated to rhizome fresh weight and total yield per unit area. In this regard, accessions with double and more layered rhizome primary fingers have a large number of fingers and rhizome width. Previous studies also reported more than 50% of accessions had single layered which was in line with the proportion recorded in this experiment, 54% of accessions are with double layered rhizome fingers.

Character association is an important analysis in diversity studies to identify which traits have direct relation to economic traits to be improved through selection. The pairwise linear correlation coefficient result in this work showed that rhizome yield per plant and unit area has a strong significant positive relation at a 95% confidence interval with nine quantitative growth and yield component traits and one categorical trait. This result is supported by findings of previous research reports (Abrahm and Leta, 2003; Das *et al*., 2013; Ravishanker *et al*., 2014). On the contrary, some traits have a negative linear correlation with rhizome yield. Days to harvesting (DtH) and rhizome finger growth pattern around mother rhizome (circular or flat) have a negative correlation with economic traits, rhizome weight, and dry matter recovery (DMR) which is also supported by former reports (Momina *et al.,* 2011; Ravishanker *et al*., 2014). Traits with significant positive correlation coefficients hence are best for selection of varieties to improve ginger productivity in Ethiopia.

Multivariate (PCA and cluster) analysis was proved as a valuable tool to understand the relationship between characteristics as well as between study materials (Khadivi *et al*., 2012). The correlation matrix-based PCA, has resulted in the distribution of accessions on a scatter plots of the first two principal components clearly in different dimensions. The result is in agreement with previous PCA analysis of ginger landraces with the same matrices and reported PC1 and PC2 shares large proportion of total variation (Das *et al*., 2013; Shankeel *et al*., 2013). The cluster analysis based on mean values of traits, which grouped the 100 ginger accessions in to four cluster groups contradicts and coincides with different previous investigations. This might be due to the range of genetic diversity, number of study materials and differentiating ability of study traits. Our result is in contradiction with number of clusters reported by Momina *et al*. (2011) using less number (36) of accessions. However, our result is in agreement with finding of Shakeel *et al*. (2013) who grouped 19 landraces of ginger to three clusters. Another study report on 60 ginger accessions grouped the materials to four clusters irrespective of their place of collection in similar manner as in our case (Das *et al*., 2013). The distribution across the scatter plot in PCA graph and cluster dendrogram was irrespective of collection area, and altitude range in similar pattern as previously reported. Hence, the distribution pattern of this study revealed that there is high genetic variability within collection areas, which implies there is high rate of exchange for ginger seed rhizome across the production areas addressed during sample collection.

5.5. Conclusions

Assessment of genetic diversity among 100 ginger accessions collected from different agro-ecologies of multi-cultural communities based on 24 morphological traits has revealed high diversity among the accessions. High values were observed for genetic and phenotypic coefficient of variations, heritability at broad sense and genetic advance as percent of mean. Strong positive correlation among some traits like rhizome fresh yield, plant height, tiller number, leaf area, number of rhizome finger and dry matter content has identified breeding traits that can be used for ginger variety improvement. Principal component and cluster analysis based on distance matrix revealed that the study materials are diverse across collection areas. From the subsequent observations and analysis results it was concluded that there is high diversity of ginger in Ethiopia that can be exploited for improvement for economic traits including disease tolerance.

CHAPTER 6

6. Genetic Diversity Analysis of Ethiopian Ginger Accessions using SSR Markers

Abstract

Understanding the existing genetic variation in the production system's germplasm pool is the first step in any program aimed at improving crop genetic diversity. Regardless of its economic and sociocultural values in Ethiopia, the limited research attention on ginger has limited its genetic improvement. Thus, the purpose of this study was to evaluate the genetic diversity among 100 ginger accessions collected from different areas by using 12 polymorphic SSR markers. The genomic DNA was extracted from sprouted buds using modified CTAB protocol. The polymorphism rate indicated that 97.2% of bands were found to be polymorphic out of the 139 distinct bands produced across all loci. The result also showed an average of 3.64 different alleles (Na), 1.53 number of effective alleles (Ne), and 0.55 Shannon information index (I). The observed heterozygosity was 0.13, and the expected heterozygosity was 0.28. AMOVA revealed a 4% variation between populations and a 96% variation within populations. The 100 accessions were grouped into three clusters based on population structure, cluster and PCoA analysis. Phylogenetic tree analysis has also created three major tree branches and identified a significant number of identical duplicates. The experiment moreover suggested that there might be potential markers associated with high rhizome yield and disease tolerance, but more research is necessary to confirm this. The experiment's findings reported can serve as a foundation for Ethiopia's efforts to improve the genetic conservation and improvement of ginger.

Key words: accessions, buds, diversity, duplicates, sprouts, SSR marker

6.1. Introduction

Ginger (*Zingiber officinale* Roscoe) is a popular spice and medicinal plant belonging to the family *Zingiberaceae*. It is a diploid (2n=22) with a 23.618 Mb genome (Wahyuni *et al.,* 2003). Ginger has two divisions; *Zingiber officinale* cultivar officinale "edible ginger" and *Zingiber officinale* cultivar "rubrum" with small, pungent-tasting rhizomes. It was originated in Southeast Asia's islands and first domesticated by the Austronesian people. Ginger is now grown in Southeast Asia, tropical Africa, Latin America, and the Caribbean's (Langner *et al.,* 1998). The top five ginger-producing nations globally include India, Nigeria, China, Nepal and Indonesia. Ethiopia ranks 15th globally and fourth in Africa next to Nigeria, Cameroon, and Mali (FAOSTAT, 2022).

Ginger has been used as a food flavoring, to strengthen and stimulate digestion, and as a medicinal plant in Ethiopia (Girma *et al.,* 2022). Numerous, biotic and abiotic factors, including ginger bacterial wilt disease, have contributed to a significant reduction in ginger production and productivity. Furthermore, despite its economic and socio-cultural values, its genetic improvement is limited due to little research attention given to it. The primary step in any genetic improvement program is to understand the existing genetic variation among germplasm pools in a production system. To this end, some preliminary research works conducted in Ethiopia indicated that both local and introduced ginger germplasms showed variability in their morphological characters, rhizome yield, volatile oil, and oleoresin contents (Girma *et al.,* 2008).

Earlier genetic variability studies using morphological and some quality traits of ginger accessions indicated that there is high genetic variability among ginger accessions in Ethiopia. These previous reports might be used as a source of information to improve ginger yield and other economic traits including disease resistance (Momina *et al*., 2011). However, molecular marker-based study reports in the country were not available in ginger for any trait.

In the era of agricultural technological advancement, molecular markers have played a tremendous role in the breeding and conservation of crop species (Prasanna *et al*., 2016). Many molecular markers including simple sequence repeat (SSR) were developed and used for genetic diversity analysis in the *Zingibiraceae* family. Among these markers, SSR was suggested as better markers due to their co-dominance, high polymorphic rate, and simplicity as compared to other markers (Kaewsri *et al.,* 2007; Neeta, 2019*)*. As a result, the SSR marker-based ginger genetic diversity assessment presented here can serve as a baseline for future genetic improvement and conservation efforts in Ethiopia. In this work, we analyzed 100 ginger accessions collected from different agro-ecologies. The study materials included released varieties, a wild type Mango ginger (*Curcuma amada* Roxb.), and landraces. The genetic diversity was conducted using twelve polymorphic SSR markers.

6.2. Materials and Methods

Plant materials

Study materials included one hundred ginger accessions, including wild, Mango ginger (*Curcuma amada* Roxb.) and released varieties, collected from various regions of Ethiopia. Based on the regions in which the accessions were collected, the accessions were grouped into four populations, the Southern (35), Central (25), Southwest (18), and Oromia (22) named populations I, II, III, and IV respectively. A high-quality rhizome with three or more active buds was chosen from a field sample of each accession to generate clonal plants. Mini rhizomes were produced from the selects and planted in pots containing solar-sterilized soil mixture of topsoil, sand, and compost in a 2:1:1 ratio respectively, under a screen house. After seven months, rhizomes from pots were harvested and stored to sprout. Subsequently, sprouting buds (Fig. 6.1a) were taken as a sample for the extraction of genomic DNA.

DNA extraction

Sprouted buds of 0.5–1.0 g were safely taken by surgical blade into 1.5 ml DNA extraction tubes (Fig. 6.1a). Extraction was done after minor adjustments to Devi *et al*. (2016) CTAB protocol developed for *Zingeberale* order plants, including ginger. After crushing each sample individually in 500 µl of CTAB buffer, the samples were incubated for 40 minutes at 60°C and centrifuged for 6 minutes at 10,000 revolutions per minute (rpm). After the first centrifugation, 60 µl chloroform-isoamyl alcohol (24:1) was added and vortexed before centrifugation similarly as in the first step. An equal amount of supernatant was then collected in new pre-labeled tubes before adding 60 µl chloroform-isoamyl alcohol (24:1)

for the second time followed by vortexing and centrifugation again. Seventy percent of the collected amount of cold isopropanol was added to fresh tubes containing the supernatant after it was collected once more. Following cautious inversions, the final centrifugation at 10,000 rpm for six minutes was carried out. Upon observing the DNA pellets at the bottom of the tubes, the upper solution was carefully decanted. After that, the pellets were cleaned twice with 500 µl of 70% ethanol, and they were left to dry overnight by inverting the tubes onto fresh tissue paper. The pellets were re-suspended in 100 µl TE and 5 µl RNase enzyme. DNA extract suspensions were then incubated for an hour at 37°C before being stored at 4°C until used.

Primer screening

The genetic diversity analysis among the study materials was done using 12 polymorphic SSR markers. Seven were from the CBT (Center of Biotechnology) series developed for ginger by Jatoi *et al.* (2006) and verified by Das *et al*. (2013). The remaining five were ZOF (*Zingiber officinale*) type markers developed from ginger EST known as the ZOF series from the Spice EST database (Chandrasekar *et al.,* 2009). These 12 markers were evaluated for DNA fragment band quality and polymorphism rate across sample study materials during annealing temperature optimization (Table 6.1).

PCR amplification

Throughout the experiment, PCR amplifications were carried out with a 25 µl total mix volume. The PCR mix included 10.5 µl of master mix, 8.5 µl of molecular grade water, 2 µl of each accession's genomic DNA, 2 µl of forward primer, and 2 µl of reverse primer.

The PCR was conducted at initial denaturation at 94 °C for five minutes, followed by denaturation at 94 °C for one minute, an annealing step at 50 to 63°C for one minute (Table 6.1), an extension step at 72 °C for two minutes, and a final extension step at 72 °C for ten minutes.

Gel electrophoresis

By standard protocols for gel electrophoresis, a molecular grade agarose gel was prepared using 2% (w/v) agarose and 2% (v/v) Tris-acetate EDTA (TAE) buffer to separate DNA fragments according to their size. Gel run was for an hour and forty minutes, the Voltage was set at 80 volt. However, 0.8% agarose was used for the DNA quality verification for 90 minutes. As per the Krickhouse Trust protocol (https://youtu.be/kcQoDcxSE6M), PAG electrophoresis using 40% Bi's acrylamide gel was also used to check very small fragments.

Table 6. 1. List of the primers used for amplification and the band size

No.	Primer	Sequence	Annealing (T ºC)	Band range (bp)	Resolution
1	SSR-CBT01	(CT)9	58/60	100-500	High
2	SSR-CBT02	(GCT)8	56/58	250-500	Medium
3	SSR-CBT03	(GA)21	60/67	200-1000	Very high
4	SSR- CBT04	(CGG)10	63	200-1000	High
5	SSR- CBT05	(GAA)9	51	150-1000	Medium
6	SSR- CBT06	(AG)7	60	100-1000	High
7	SSR- CBT07	(GA)21	50	100-500	Low
8	SSR-ZOF1	(TTC)8	51/53	250-1000	High
9	SSR-ZOF2	(CAG)12	53	300-1000	High
10	SSR-ZOF3	(TCC)7	53	300-1000	Very high
11	SSR-ZOF4	(CGA)9	51/53	200-1000	High
12	SSR-ZOF5	(CCG)9	53	300-1000	High

Marker data recording and analysis

By comparing the relative distances of reproducible DNA fragment bands with high resolution within the range of estimated band lengths for each SSR marker to a 100 bp DNA ladder, the bands were scored. Dual scoring in band length as well as present (1) and absent (0) was used because the SSR marker is co-dominant. Several molecular genetic diversity parameters, population structure, clustering, and phylogenetic tree were analyzed using GenAlEx6.5 (Peakall and Smouse 2006, 2012), Popgen32 (Yeh *et al.,* 1999), structure 2.3.4, and Darwin 6.0. Genetic distance, PCoA, gene and genotype frequency, Hardy-Weinberg Equilibrium, gene differentiation, gene flow, expected and observed heterozygosity and F-statistics among sub-populations and within the population were estimated using GenAlex and Popgen32. Population structure for partitioning the accessions to K number of clusters based on distance matrix was computed by Structure 2.3.4 (Pritchard *et al*., 2000) program based on Bayesian method and delta K was determined by structure harvester using structure analysis output online uploading. Unweighted neighbor-joining (UNJ) phylogenetic tree to estimate genetic evolutionary relationship among all study materials based on pairwise dissimilarity matrix using MEGA11 (Tamura *et al*., 2021) and DARWIN 6 (Perrier and Jacquemoud, 2006).

6.3. Results

DNA quality and marker selection

A spectrophotometer, electrophoresis, and visual inspection of the 100 study materials were used to test for DNA quantity, quality, and purity. The results showed pure DNA pellets (Fig. 1b), with an average of 216.6 ng/ml at 260A, and a 260/280 ratio of 1.86.

The quality test on 0.8% (w/v) agarose gel electrophoresis in 0.2% TAE buffer at 80 V for 90 minutes was also high being non-fragmented with clear bands (Fig 1c). DNA extracted from silica-dried and fresh leaves was of poor quality (Table 6.2). Among the 12 SSR markers tested for the study, 9 (75%) produced reproducible, clear, and high-quality polymorphic DNA fragments. Two (CBT2 and CBT5) were monomorphic across study materials, while the other (CBT7) was not used due to inconsistency in annealing temperature and low-quality bands (Table 6.1).

Table 6. 2. Sample source evaluation for quality DNA extraction from ginger

Sample type	DNA pellet	Qty at 260 (A)	260/280	DNA quality
Silica dried leaf	Not visible	<30	1.5	No bands
Fresh leaf	Very small	35	2.2	smeared
Sprouted buds	Very big	216.6	1.86	high

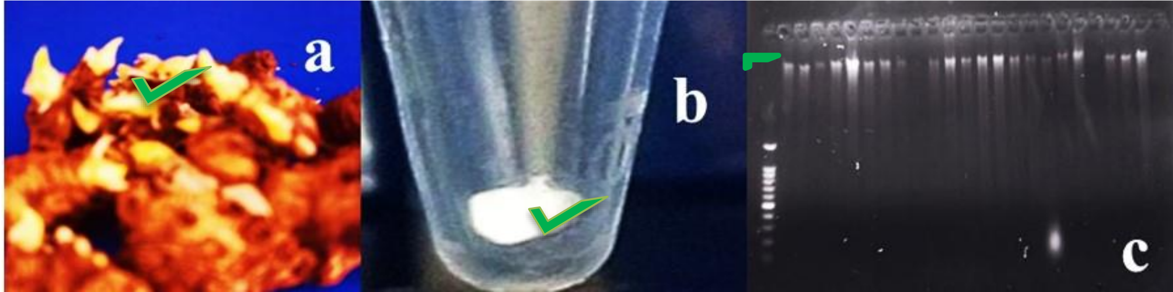

Figure 6. 1. Sample preparation and genomic DNA quantity and quality test

a, sprouted buds ready for DNA extraction, b. DNA pellet of one sample and c. DNA bands on agarose gel.

Genetic diversity analysis

The polymorphic markers selected and used for the genetic diversity analysis of 100 accessions have generated 139 clear and reproducible bands with fragment lengths from 100 to 1000 base pairs.

Among the total bands scored 135 (97.2%) were polymorphic across the 100 accessions in the four sub-populations. The largest and unique DNA fragments as alleles (900 and 1000 bp) were observed at the CBT4 marker locus in the wild-type ginger (JW89) (Fig 6.2a). The SSR markers showed an average of 3.64 different alleles (Na), 1.53 number of effective alleles (Ne), and 0.55 Shannon information index (I) values. Marker CBT4 had the maximum number of alleles (9) followed by ZOF1 (8) alleles and the largest effective number of alleles (2.78) was observed at locus ZOF2 accompanied by the largest value of I (1.13), as revealed by Popgen32 and GenAlex6.502 (Table 6.3).

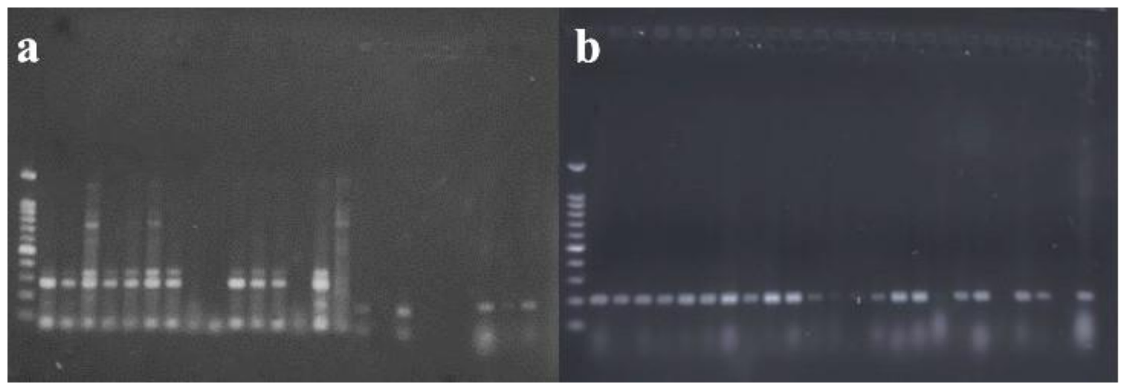

Figure 6. 2. SSR marker bands amplified by CBT4 and CBT2 markers a. highly polymorphic CBT4 marker and b. monomorphic CBT2 marker

Observed heterozygosity (Ho) and expected heterozygosity (He) across all loci resulted in different values. Maximum Ho was recorded for CBT4 (0.34) while maximum He (0.68) value was observed at ZOF2 (0.19) locus. The average values for Ho (0.13) and He (0.28) were observed across all loci. The average value of genetic distance (Nei D) from all genotypes across loci were 0.28. Relatively higher mean values for FIS (0.53) and FIT (0.55) were observed while the FST (0.05) was medium. Among the study markers, ZOF1 showed the highest FST value (0.125) followed by ZOF2 and CBT1 with 0.053 and 0.051 values respectively.

The lowest FST values, 0.01 and 0.016 were for ZOF5 and ZOF3 respectively when all accessions were considered as one population. The largest gene differentiation (Gst) value across all genotypes was recorded by locus ZOF1 at 0.097 value. The second Gst value was for CBT1 (0.031) and the lowest is for CBT4 at 0.002 value. However, the average gene flow (Nm) across all loci (4.75) is very high as revealed by the GenAlex program (Table 6.3).

Table 6. 3. Basic molecular analysis statistics average values of each locus across all populations

Locus	#Allele	Na	Ne	FIS	FIT	FST	Nm	Ho	He*	Nei**	Gst	I
ZOF1	8	4.00	2.09	0.82	0.84	0.13	1.75	0.10	0.58	0.58	0.097	0.90
ZOF2	5	3.75	2.78	0.74	0.75	0.05	4.49	0.19	0.68	0.68	0.024	1.13
ZOF3	8	4.25	1.20	0.35	0.36	0.02	15.78	0.10	0.16	0.16	-0.01	0.39
ZOF4	4	3.50	1.27	0.74	0.75	0.03	7.47	0.05	0.19	0.19	0.004	0.43
ZOF5	7	4.25	1.29	0.33	0.34	0.01	24.71	0.14	0.21	0.21	-0.012	0.49
CBT1	4	2.75	1.16	0.26	0.29	0.05	4.66	0.10	0.13	0.13	0.031	0.27
CBT3	6	3.50	1.19	0.19	0.22	0.03	8.55	0.09	0.15	0.15	0.009	0.35
CBT4	9	4.75	1.70	0.12	0.14	0.02	11.88	0.34	0.39	0.39	0.002	0.81
CBT5	4	2.00	1.07	0.12	0.15	0.03	7.55	0.06	0.07	0.07	0.013	0.14
Mean	6.11	3.64	1.53	0.41	0.43	0.04	9.65	0.13	0.28	0.28	0.029	0.55
SE		0.24	0.09	0.09	0.09	0.012	2.34	0.02	0.21	0.21	0.019	0.06

The Hardy-Weinberg equilibrium (HWE) test for allele and genotype frequency distribution across the four sub-populations was highly significant. However, markers CBT3 in population I (South), ZOF3, and CBT4 in populations II and III (Central) and (Southwest) respectively are non-significant at $p<5\%$ and are at HWE for their respective alleles and genotypes (Table 6.4).

The pairwise genetic diversity matrix resulted in the maximum genetic distance (Nei' D) value (0.055) was between South population and Central. On the other hand, Nei's I values between pair populations revealed Southwest and Oromia populations are 99.9% identical. The highest gene flow (15.43) was between populations I and III followed by between populations III and IV (12.56). The pairwise matrix also resulted in variations in the gene flow (Nm) genetic diversity parameter. The lowest Nm (5.59) value was recorded among populations I and II (Table 6.3). Similarly, the highest pairwise matrix value for FST was also recorded for population I and population II (0.076) at $p<0.05$ followed by 0.046 between populations II and III. Gene differentiation (Gst) value among the four populations paired also revealed a maximum (0.063) between pair populations I and II to a minimum (0.005) among populations III and IV (Table 6.4).

Table 6. 4. Pairwise genetic diversity pairwise matrix values among populations

Pop(#)	Pop(#)	FST	Nei D	Nei I	Nm	I	GST
Pop1(35)	Pop2(25)	0.076**	0.055	0.946	5.597	0.048	0.063
Pop1(35)	Pop3(18)	0.02ns	0.012	0.988	15.431	0.023	0.003
Pop2(25)	Pop3(18)	0.046*	0.033	0.968	8.465	0.036	0.027
Pop1(35)	Pop4(22)	0.033*	0.022	0.978	7.398	0.05	0.017
Pop2(25)	Pop4(22)	0.032ns	0.030	0.971	10.989	0.05	0.015
Pop3(18)	Pop4(22)	0.015ns	0.008	0.992	12.569	0.039	-0.005

Legend: F-statistics (FST), Genetic distance (Nei D), Genetic Identity (Nei I), gene flow (Nm), Shannon information index (I) and gene differentiation (GST)

Analysis of molecular variance (AMOVA) indicated that 96% of variation was within and among individuals across the 100 study materials and only 4% variation contributed to among the four populations.

This AMOVA result revealed the genotypes in the four populations across collection areas are evenly distributed, which might be due to high germplasm exchange between producers and clonal propagation system dependency in ginger (Table 6.5).

Table 6. 5. Summary of AMOVA table

Source	df	SS	MS	Est. Variance	%	Fst	Value	P(%)
Among Populations	3	14.051	4.684	0.051	4%	Fst	0.036	0.001
Among Individuals	96	209.989	2.187	0.804	56%	Fis	0.581	0.001
Within Individuals	100	58.000	0.580	0.580	40%	Fit	0.596	0.001
Total	199	282.040		1.435	100%	Nm	6.782	

Multivariate analysis

Principal coordinate analysis

Principal coordinate analysis (PCoA) based on genetic distance among all study materials result indicated 53.45% of total variation was contributed by the first three principal components. The distribution of accessions across the four quadrants showed three clusters irrespective of collection area (Fig. 6.3). Most of the materials were concentrated around the start points of the main axes in quadrants two and three. The first and fourth quadrants on the other hand contained evenly distributed materials from all the four populations. Regardless of the agro-ecologies from where the genotypes were collected, there were also a significant number of genotypes overlapping. Phylogenetic tree analysis further confirmed this by identifying a sizable number of accessions as identical duplicates. The wild relative mango ginger (JW89) has the highest eigenvector value (1.00) than the other

99 ginger genotypes. Moreover, phylogenetic tree analysis separated it as a unique genotype on a separate tree line (Fig. 6.5).

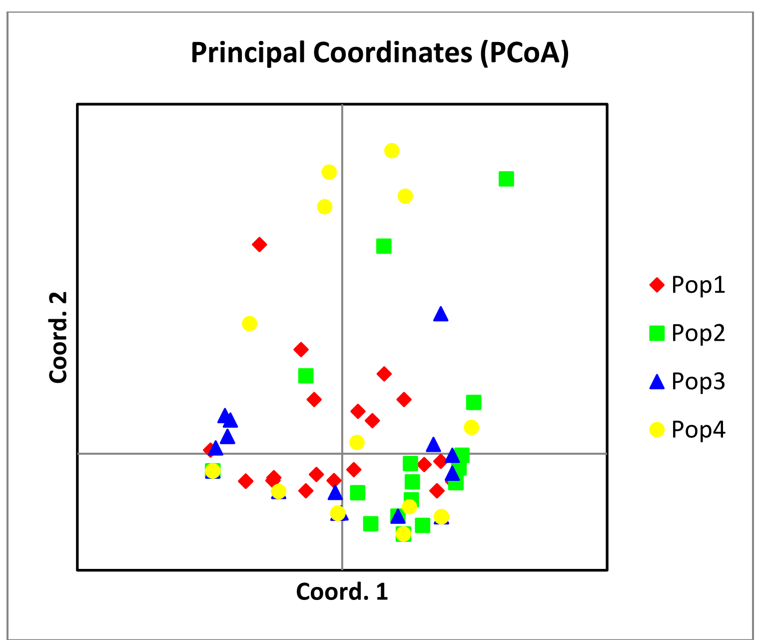

Figure 6. 3. PCoA distribution of 100 accessions across the four bi-plot quadrants

Population structure analysis

Dendrogram based on Nei genetic distance among the four populations by UPGMA method reduced the four populations to three. Other diversity analysis parameters between pairs of populations also indicated populations clustered together had low values of FST, genetic distance, and the highest gene flow values (Table 6.5).The maximum delta k (ΔK) value at K3 is 92.87 because of population structure analysis using the Bayesian method (Table 6.6). As a result, three clusters were formed by the clustering analysis from all 100 study materials in the four populations (Fig. 6.4a). Cluster I, which was colored blue, held 45% of the study accessions, Cluster II, which was colored yellow, contained 41%, and the third cluster, which was colored red, contained 14%.

Average distance (Heterozygosity) among individuals within a cluster was the highest for cluster I followed by the second cluster. However, the FST value was highest (0.87) for cluster II and lowest for cluster III (Fig. 6.4b and Table 6.7).

Table 6. 6. Delta K value summary as table from structure harvester

K	Mean LnP(K)	StdevLnP(K)	Ln'(K)	|Ln''(K)|	ΔK
1	-1144.09	0.056765	—	—	—
2	-940.04	3.092356	204.05	57.38	18.55
3	-793.37	1.261437	146.67	117.15	92.87
4	-763.85	1.732211	29.52	43.9	25.34
5	-778.23	10.49678	-14.38	17.18	1.64
6	-809.79	44.79793	-31.56	32.58	0.73
7	-808.77	25.7651	1.02	6.54	0.25
8	-801.21	17.27673	7.56	20.22	1.17
9	-813.87	29.42154	-12.66	5.86	0.19
10	-820.67	12.53281	-6.8	—	—

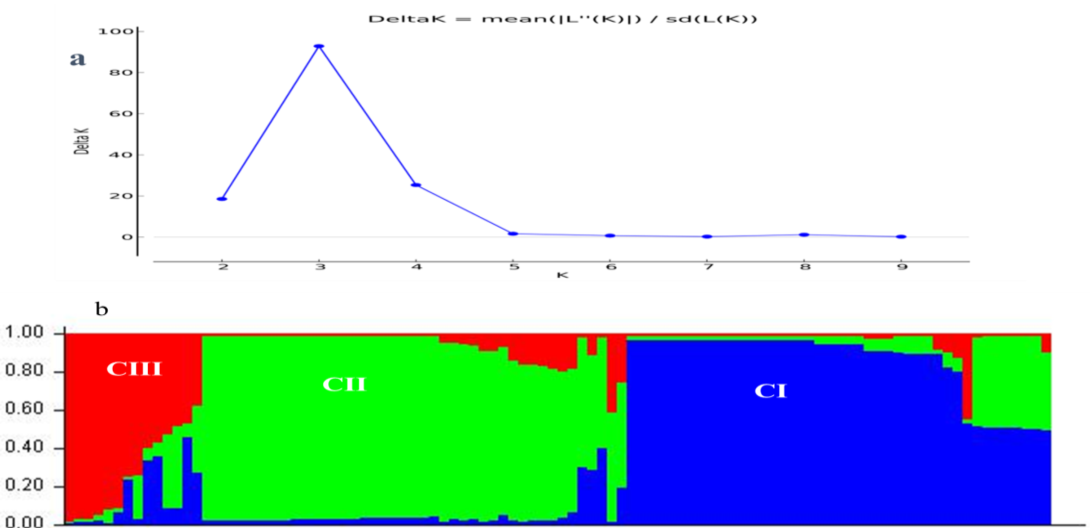

Figure 6. 4. Bayesian model-based clusters of the 100 ginger accessions a, K value determined by structure harvester, b. accessions distribution across clusters.

Table 6. 7. Cluster membership and distance between individuals in clusters

Cluster	1	2	3
Membership proportion (%)	14%	41%	45%
Average distances (He) between individuals in the same cluster	0.657	0.041	0.096
FST	0.0025	0.8702	0.7656

Molecular evolutionary analysis

Three primary phylogenetic tree branches have been identified by both MEGA 11 distance based, and DARWIN 6.0 dissimilarity matrix-based Unweighted Neighbor-Joining (UNJ) analysis. The phylogenetic tree was in consistence with the PCoA, population structure, and cluster analysis results. This analysis further confirmed the presence of potentially identical duplicates. Zero distance for more than 30% of the study accessions was observed in different tree branches. The phylogenetic tree contained also three main branches as determined by population structure and cluster analysis in which k and ΔK pick values were at three. The wild type (JW89) with high eigenvalue in PCoA and unique in some morphological traits was separated as unique in the phylogenetic tree dandrogram (Fig 6.5).

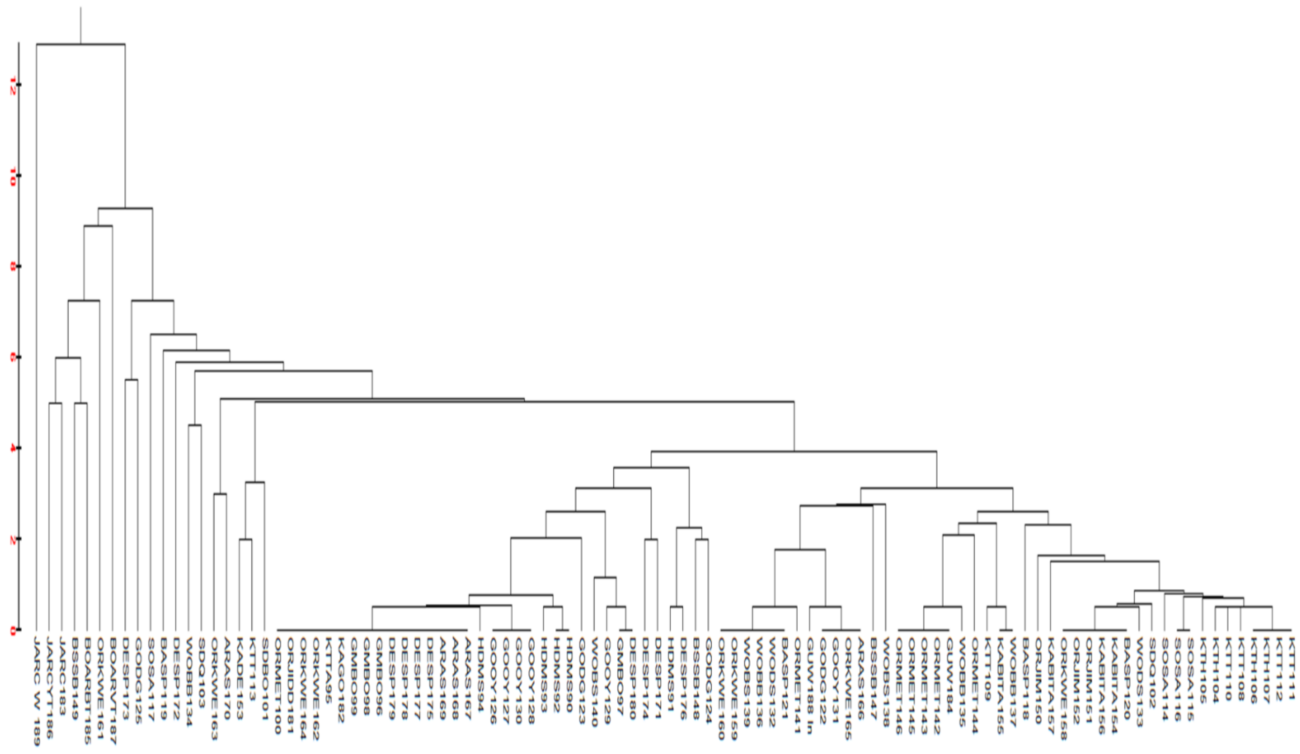

Figure 6. 5. Phylogenetic tree of the hundred accessions by MEGA 11 based on distance matrix.

Marker trait association

In independent experiments, the genetic diversity of the 100 accessions utilized in this molecular diversity analysis was also investigated based on 24 morphological traits and the reaction of the accessions to the ginger bacterial wilt disease. A few distinct characteristics were present in the wild type (JW89) and some ginger accessions in all three investigations (morphological, disease response, and SSR marker). In the morphological diversity analysis, the wild type was early maturing, big rhizome with high weight, and different smell as compared to others. During the assessment for disease resistance in next chapter (7), no signs of the bacterial wilt disease were observed.

Because of the distinct marker bands found, SSR marker diversity analysis has also produced a distinct phylogenetic tree (Fig. 6.5). During population structure and molecular evolutionary phylogenetic tree analysis, some accessions with better disease response at very tolerant level (KT13, BAS19, BSB49, ORW61, and ORW63) were grouped in similar cluster (C-I) with the disease resistant wild type (Fig. 6.6). Moreover, accessions, KT13, BAS19, and ORW63) recorded high rhizome yields during diversity analysis.

6.4. Discussion

Previous reports on the quantity and purity of genomic DNA extracted from ginger leaf samples were low (Mahdi *et al*., 2013). In this experiment, we used fresh ginger sprouted buds from mother rhizomes directly using modified CTAB protocol with minor modification (Devi *et al*., 2016). Total number of DNA fragments, average bands per locus, rate of polymorphic bands and genetic diversity parameters values all confirmed that there is high genetic diversity among in with in populations and over all study materials when considered as one population.

Analysis results of most genetic diversity indicators of this study supports previous reports. Our results, which showed that 135 (97.5%) of the 139 total fragments had polymorphic bands, are in agreement with an earlier study that found 99% of the fragments had polymorphic bands (Jatoi *et al*., 2006).

Our research, however, revealed more polymorphic bands than the earlier study result (63.29%) conducted by Das *et al*. (2013). Another diversity analysis study of 47 ginger genotypes using SSR markers also reported nearly similar result like our findings (Have *et*

al., 2017). Previous research reports also presented fragments with DNA size from 50 to 1000 bp to which our result coincides (Das *et al*., 2013; Harve *et al*., 2016).

Five primers from ginger EST designed by Chandrasekhar *et al.* (2009) showed better partitioning of the accessions. This experiment supports the earlier findings. Genetic diversity analysis values Na, Ne, Nei D and I were also in line with previous findings (Das *et al*., 2013; Nor *et al*., 2019). Heterozygosity, gene differentiation and gene flow within and among the four sub-populations from different area of collection showed there is low genetic diversity among sub populations and high with in populations among indvivduals. Similar study reports on ginger and other asexually propagating crops also achieved similar results suggesting there is more diversity within population than among (Wada *et al*., 2021; Ismail *et al.,* 2016).

Molecular variance analysis, which found 96% of diversity within populations, suggest that there is high gene flow among populations and that the evenly distributed production cultivars throughout Ethiopian collection areas might be the result of the exchange of planting materials. Pair wise distance matrix analysis among the populations generally reveals there are similar accessions in production across collection areas. From South population are collected from multi-cultural communities of Southern Ethiopia, which mainly produce their own local cultivars for home consumption rather than large scale.

In contrast to population I, areas from which study materials for populations II and III were gathered are acknowledged as ginger communities at the national level. The areas include southwest (Kaffa, Benchimaji, and Tepi areas) and the South central zones (Wolaita,

Kambata, and Hadiya). These two production areas cover 99% of Ethiopian ginger production and supply to local and international markets (Kifle *et al*., 2021; Grima *et al*., 2022). The fourth population group from Western Oromia covering Jimma, Iluababor and Wellega had medium heterozygosity and distance from all the three population groups. The narrow genetic distance between population II (Central) and Population III (Southwest) indicates there may be high exchange of planting materials among commercial growers and smallholder farmers.

A similar trend in population structure was also observed by PCoA and cluster multivariate analysis of all study materials as a single population. In PCoA analysis, the first two principal coordinates contributed 53.7% of the total variation. Significant number of accessions overlapping observed also indicated there are potential identical duplicate accessions collected from different areas. Population structure analysis, which determined the whole study materials to three clusters with different proportion of members, was also in agreement with previous reports. Three clusters with different number of accessions irrespective of collection area and sub-populations was in agreement with similar study reports (Jatoi *et al*., 2006; Das *et al*., 2013; Herve *et al*., 2017; Ismaili *et al*., 2016). However, the number of clusters by morphological traits has partitioned the same study materials to four clusters. Membership to each cluster based on both morphological and molecular markers was irrespective of collection areas and hence population groups.

Three main tree branches were formed by phylogenetic tree analysis based on the distance and dissimilarity matrices among all study materials for molecular genetic evolutionary

determination. This analysis also confirmed that the study ginger accessions were genetically diverse. Similar previous studies also reported nearly similar number of main phylogenetic tree branches (Harve *et al*., 2017). Moreover, the molecular evolutionary analysis also identified significant number of identical duplicates. Hence, some accessions are most likely hundred percent similar (Fig 6.5). This indicates that a large number of ginger genotypes across the country are identical duplicates but are considered unique genotypes by different communities. The result of this experiment showed similar trend with previous study result in the same crop in number of clusters and identical duplicates identification (Akshitha *et al.,* 2022). The population dendrogram, PCoA, cluster, and phylogenetic analysis consistency in this study verified the SSR marker based diversity study has laid milestone for ginger genetic improvement and conservation in Ethiopia. It was further confirmed that the SSR markers employed could distinguish between identical duplicates and variables when it came to identifying potential duplicates with zero genetic distance.

The relation of morphological traits based diversity, disease response and molecular marker analysis results pointed out that there is sufficient diversity among the ginger genotypes collected from major and minor producing areas in Ethiopia.

Hence, these genotypes with high rhizome yield, better disease tolerance clustered by SSR markers should be further evaluated at field condition for rhizome quantity and quality as well as disease under hotspot environments. High throughput molecular markers and sequencing platform based studies may verify the results reported in these experiments.

6.5. Conclusion

The genetic diversity among Ethiopian ginger accessions was identified by the molecular genetic diversity analysis study conducted. The population structure and diversity indicator parameter values have divided the study materials into three clusters in a way that is comparable to reports from other countries. Consequently, these findings may serve as baseline data for Ethiopian ginger genetic improvement and conservation initiatives. As far as we are aware, this is the first study of its kind conducted in Ethiopia, and the DNA that was taken from recently sprouted buds was the first of its kind in the ginger community. Additional high throughput markers and sequencing methods could confirm additional genetic markers and economically significant trait-linked markers to improve the genetics of ginger and mitigate significant issues such as bacterial wilt and leaf spot diseases that affect ginger production worldwide.

CHAPTER 7

7. Screening of Ginger (*Zingiber officinale* Roscoe) Accessions for Bacterial Wilt Disease under protected and Open Field Conditions

Abstract

Ginger bacterial wilt disease caused by *Ralistonia solanacearum* has been the main production constraint for a decade in Ethiopia. The disease has devastated all plantations across the country. Among different management options, the best way to control this disease is using genetically resistant ginger plants. To this end, an experiment has been designed to evaluate the response of 100 ginger accessions including released varieties and close wild relatives under protected and open field conditions. Disease severity, disease incidence, days to severe disease symptoms, and fresh and dry yield losses showed high variations among accessions. Combined analysis of these traits revealed twelve accessions were promising and are considered as very tolerant accessions. Fresh and dry matter yield losses showed a wide range between minimum and maximum from 1.65 to 77.73% for fresh yield loss; whereas the dry matter content loss is from zero to 55% during greenhouse screening. Evaluation at field condition also showed there is variation among the study materials for disease scores and yield difference. Few accessions showed consistent tolerance level while others showed contrasting response at field in greenhouse. Both fresh and dry rhizome yield variation was high in similar manner as in greenhouse. However, there is no accession that is completely resistant except the wild type mango ginger (JW89). This study laid the base for further evaluation of ginger accessions under different environment conditions.

Keywords**:** accessions, pathogen, tolerance, severity, yield loss.

7.1. Introduction

Ginger is a herbaceous rhizomatous, and perennial plant belonging to the family *Zingiberaceae*. The genus *Zingiber* includes about 85 species of aromatic herbs among which edible ginger (*Zingiber officinale*) is widely used as a spice and a folk medicine. Ginger was originated in maritime Southeast Asia and likely domesticated first by the Austronesian peoples who transported ginger. The crop was introduced to Ethiopia as early as the thirteenth century (Jansen, 1981). It is cultivated in the south, southwestern and northwestern parts of the country, and is among the most important spices used in every Ethiopian dish (Girma, *et al.,* 2008). Lack of improved high-yielding and disease-resistant varieties, poor pre and postharvest management, and disease complexes are the main production constraints (Endrias and Asfaw, 2011; Girma *et al*., 2022).

Bacteria-caused wilt disease is now a major hindrance to ginger production as of 2012 (Kifle *et al*., 2021). Direct yield losses and incidence by *Ralistonia solanacearum,* a pathogen causing the disease vary widely according to the host, cultivar, climate, soil type, cropping pattern, geography, and strain (Habtewold *et al*., 2015; Yuliar *et al.*, 2015). The integrated use of pathogen-free planting material, rhizome treatment by heat, antibiotics, phyto-sanitation, and rotation with non-host crops are among the recommended strategies for field control of the disease (Kumar and Hayward, 2005). However, the disease still poses a threat to ginger cultivation over the world. In the absence of efficient strategies to eradicate pathogens from infected soils and water, the use of resistant cultivars appears to be the best disease control strategy (Gaelle, 2014).

Varieties resistant to bacterial wilt disease caused by the same pathogen but different strains were reported in some solanaceous vegetables like tomato, pepper, and eggplant (Kurabachew and Ayana, 2016). Resistant gene analogs (RGAs) in edible ginger and related species (mango ginger) were identified using bioinformatics-driven methods. The result in the identification classified gene analogues with high similarity to known resistant genes based on the presence and organization of conserved domains (Karthika *et al*., 2019). Screening methodology for identifying resistant ginger germplasm against bacterial wilt was also developed using a large collection of ginger germplasm under greenhouse conditions reporting some plants escaped disease development (Kumar, 2006).

According to the integrated management of bacterial wilt disease, study report by Guji *et al*. (2019) there was some variation between two cultivars in Ethiopia. Moreover, farmers from different areas especially in marginal areas to ginger claim that their ginger is not yet infected which needs scientific investigation and analysis. Assessment reports indicated possible research intervention areas to manage ginger bacterial wilt on top of cultural practices in Ethiopia. Germplasm enhancement through collection of locally available accessions, production of clean rhizome seed and closely maintaining the ginger accessions were pointed out (Zakir *et al*. 2018). However, there is no experimental report for the response of ginger accessions to the ginger bacterial wilt disease in Ethiopia. Therefore, this experiment focused on the response of ginger accessions to ginger bacterial wilt pathogen under screen house and open field conditions.

7.2. Materials and Methods

Experimental site description

The experiment was conducted at Areka Agricultural Research Center under a screen house with an average daily temperature of 28.5 ^{0}C and humidity of 85 %. Field evaluation was also conducted in the Areka research center station which is located 300 km south of Addis Ababa at 7°4'12" north and 37°42'0" east at an elevation of 1774 meters above sea level. The area has an average minimum temperature of 15^{0}C and a maximum of 30 ℃ and it has an average annual precipitation of 122.5 ml with a minimum of 21 in January, and a maximum of 189 ml in May. Monthly average humidity also ranges from 44 in January to 80 in July as per meteorology data of 2022.

Experimental materials

Ginger accessions collected based on germplasm collection guidelines and used for genetic diversity analysis were used for screening. In the process, 100 accessions including released varieties (Yali and Boziab) and one wild type were obtained and used as experimental materials across the study. From all accessions, single seed rhizome with at least three active buds was selected from the field samples to get clonally propagated experimental material for artificial inoculation. Seed rhizome was pretreated with broad sense fungicide and bactericides, Cymocopy and hydrogen peroxide (H_2O_2) respectively to remove disease causing pathogen inoculum inside the seed for 6 hours. After discarding the chemical residues and washing with distilled water, seed rhizome was also treated with hot water at 50^{0}C for 50 minutes before planting in pots.

Soil preparation and plants management

To prevent disease development before artificial inoculation by inoculums in the seed or soil, the seed and soil were sterilized before planting and potting, respectively. The soil mixture was prepared from 50% of topsoil, 25% of decomposed coffee husk, and 25% of sand in a 2:1:1 ratio respectively. Then the soil mix was solar sterilized for 72 hours after spraying small water on the surface before covering with black plastic. The treated seed rhizome of each accession was then planted in one pot in March 2021 for assessment of experimental materials for bacterial wilt disease development by inoculum in the rhizomes (Fig 7.1C). In 2022, planting of seed rhizomes produced in 2021 from single mother rhizomes was done for all accessions in three plastic pots with 20x15 cm height and diameter respectively. Three hundred pots were potted with 6 kg of soil mixture as in 2021. Then three mini rhizome buds were planted to three pots from each 100 accession as a replication. Water was supplied twice weekly starting from the day of planting to seven months at which ginger stops active growth due to maturity. The experimental materials in pots were then inoculated with standard *Ralistonia solanacearum* pathogen isolate suspension at 45 days of emergence as recommended before (Kumar, 2006; Handiso *et al*., 2017).

Inoculum isolation and pure culture preparation

The bacteria (*Ralistonia solanacearum*) inoculum was isolated from ginger plant rhizomes collected from hot spot areas of Wolaita and Kambata administrative zones. Rhizomes for inoculum isolation were collected from disease prone areas by selecting those with the disease symptoms. Inoculum isolation was done at the plant pathology laboratory of

Hawaasa agricultural research center and safely transported to Areka for inoculation as per standardized protocol (Handiso *et al*., 2017). Rhizome discs of nearly 3.0 g and 1.0 cm^2 were taken from the sample rhizome. These discs were surface washed with tap water, sterilized with 70% ethanol for 5 minutes and then washed 3 times with distilled water. Then the discs were gently crushed by disinfected mortar before adding some amount of distilled water to make suspension. The crude suspension was transferred to the sterilized test tube and serially diluted by taking 1 ml suspension from 10^{-1} to 10^{-9} in separate clean test tubes with 9 ml distilled water. Then from each diluted suspension, a loop full sample was cultured by striking on two separate Petri-plates with 20 ml nutrient agar (NA) semi-solid medium.

The NA medium, containing 5 g peptone, 1.5 g HM peptone B, 1.5 g yeast extract, 5 g sodium chloride, and 15 g agar was prepared one day before culturing. After 48 hours of incubation at 28 $^{\circ}$ C, bacterial colonies that were clear and separate were picked from the plates. A loop of the picked colonies were cultured onto fresh NA media and incubated in the same manner to isolate pure culture. The single colony again from each plate was taken and cultured on 0.05 % (w/v) TTC (triphenyl tetrazolium di-chloride) supplied NA media for virulent *Ralistonia* pathogen type identification (Kelman, 1954). Colony morphology and growth habit were observed to select the most virulent strain for pathogenicity test before the actual experiment. A total of 12 isolates were plated separately in three replications to TTC media and checked for similarity among isolates and replications after 48 hours of incubation. Then bacterial colonies were collected from fresh culture plates of

the selected isolate from NA media and diluted with distilled water to get standard colony forming unit of 10^8 (cfu/ml) by spectrophotometer at 600 nm (Kumar, 2006).

Pathogenicity test and plant inoculation

Inoculum suspension prepared for artificial inoculation was stored at 4° C and the remaining culture plates were stored at the same temperature as backup. Pathogenicity test was done on ginger in vitro plantlets of two cultivars Volvo and Boziab by making small pricks at the leaf, and pseudo stem junction. Then 10 µl inoculum was placed on each plantlet carefully without dropping to the plant growth medium. After the pathogenicity test was observed ginger plants of all 100-study materials were inoculated. The inoculation was done 45 days after emergence with 100 µl inoculum suspension at the junction between the lower leaf and pseudo stem by making small pinpricks using syringe needle (Fig 7.3A). From each pot, three tillers were inoculated and six tillers for each accession were artificially inoculated in two pots whereas; the third pot plants were used as un-inoculated control checks.

Screening at open field

The study materials were also assessed in open field at Areka research center main station during the 2023 planting season. Planting was done in March for all accessions in plot size of 2.25 m^2 (1.5x1.5 m) using recommended spacing (30x15cm). All agronomic practices from land preparation to field management were applied as per recommendation (Girma and Kinde, 2008).

Data collection and analysis

Days to disease symptom development, number of plants infected, leaves with symptoms, disease incidence, and severity were collected as percent and disease scale index.

The disease severity was scored using an index of 0–4 according to Roberts *et al*. (1988). Index of zero was for no leaf with symptom and wilting per plant, 1 for 1-25% of leaves wilted, 2 for 26-50% plant leaves wilted, 3 for 51-75% portion of plant wilted. The last index 4 was for 76-100% plant part affected and completely dried were given score 5. Disease scoring was started 15 days after inoculation in seven days intervals for 90 days as recommended by Mathews *et al*. (2008).

Data was also collected during harvesting for rhizome yield and root growth traits. The number of roots and root length per plant, fresh rhizome yield per plant and pot, number of secondary rhizome fingers, number of active buds per finger, and dry matter content were finally compared to check plants. Accessions with a mean disease severity score of 1 and incidence less than 30% were categorized as very tolerant, those with score two and up to 40% incidence were tolerant, accessions with a severity score of 3 are moderately susceptible and those with a 4 and 5 scores were highly susceptible. Later this score and grading were associated with fresh yield and dry yield loss levels as also suggested by previous studies (Mathews *et al*., 2008).

The experimental data were subjected to different analysis packages and models to differentiate the response variation of accessions against the pathogen inoculum. Disease severity score and incidence were subjected to descriptive statistics frequency distribution

using SAS 9.1.3 (2011). Root growth and rhizome yield-related data were associated with disease scores using linear correlation models at a 95% confidence interval.

Yield loss due to disease effect was computed using the following formula to estimate the economic impact of the disease on each accession.

YL= (YC-YI)/YC*100 where; YC is the yield of control plants, YI is the yield of inoculated plants. Linear regression analysis at 0.05% probability was done using the Y= a+ bx+e model using Minitab 19.1.1 program. Yield loss represented by Y, a is the intercept, b is the regression coefficient, X is disease severity score and e is the error coefficient to estimate disease pressure effect on rhizome yield of each accession.

7.3. Results

Pathogen inoculum isolation

Isolation of the virulent pathogen of the bacteria causing ginger wilt from all serial dilution levels (10^{-1} to 10^{-10}) resulted in different types of colony growth on nutrient agar (NA) media after 48 hours of incubation under 28°C. Separate and contamination-free colonies of bacteria were observed from culture plates stricken with samples from dilution levels 10^{-6} and 10^{-7} (Fig 7.1A). Culture plates inoculated by suspensions from 10^{-1} to 10^{-5} were accumulated with aggregates of different overlapped colonies and fungal contaminations. Whereas, there was poor colony growth on the highly diluted suspension sample 10^{-8} to 10^{-10} cultures. Relatively good bacterial colonies were developed on plates cultured with samples from 10^{-6} and 10^{-7} dilution levels. Hence, single colonies were randomly picked from 10^{-6} and 10^{-7} plates for pure culture isolation and virulent type pathogen identification. Twelve colony isolates were transferred to fresh NA medium as pure culture. Virulent

strain identification from pure pathogen isolates on NA media supplied with tetrazolium chloride (TZC) has resulted in colonies characterized by unique morphology and color. Colony growth of the 12 isolates on TZC media showed that some isolates are duplicates as they showed similar growth patterns, shapes, and color intensity. Isolates labeled as 6 a nd 12 with fluidal, deep pink, compact colonies and irregular shapes (Fig 7. 1B and C) ar e similar to reported typical virulent *Ralistonia solanacirium* race 3 biovar 4.

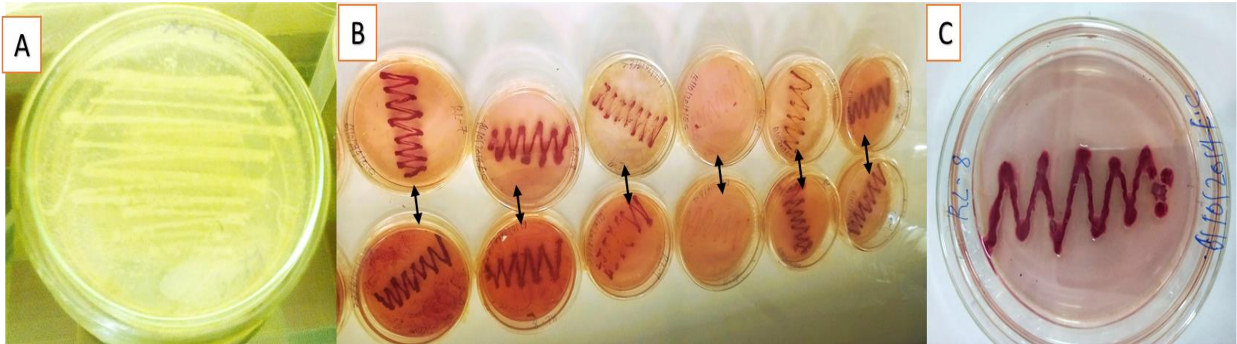

Figure 7. 1. Virulent pathogen identification process. A, pathogen isolated from pure single colony, B. Replicated pathogen isolates for virulent type identification on TTC supplied NA media , C. Selected isolate with typical virulent type character.

Figure 7. 2. Pathogenicity test on in vitro ginger plantlets. A. in vitro plantlets of two cultivars (Boziab left and Volvo right) during inoculation, B. response of plantlets after 10 days of inoculation and C. plantlets completely wilted after 25 days.

Pathogenicity test

Pathogenicity of the inoculum suspension tested on in vitro plantlets inoculated at leaf and stem junction by placing 10 µl of sample has resulted in disease symptoms on leaves after 8 days of inoculation, and wilting of the plantlets was observed after 10 days of inoculation. Complete wilting of all plantlets in the jars was also observed 25 days after inoculation (Fig 7.2)

Accessions response to pathogen inoculation

Response of all accessions to the pathogen after inoculation assessed as of 15 days after inoculation (15 DAI) in seven days intervals showed that there is variation among accessions irrespective of their collection area. Variation was observed for days to the first symptom, number of tillers infected, and number of leaves infected per tiller. Disease severity recorded as the scale of 0-4, and disease incidence as a percentage of tillers infected per pot indicated that there is high variation among accessions.

During the first recording after 15 DAI, 80% of the study materials have shown symptoms on the inoculated plant leaves at disease severity scale 1. Observations recorded in seven days intervals for disease development trend continued up to 90 DAI finally revealed that the disease development was expressed at sever level after 45 DAI in the large proportion of study plants. Some highly susceptible accessions showed complete wilting symptom and high disease incidence after 52 DAI (Fig 7.3 and 7.4). At the end of the disease score (90 DAI), plants of 83 study accessions are at complete wilting and sever disease score (3 to 4). The disease incidence increased from 40 to 100% on 80% of the accessions evaluated. The experiment revealed that there is no ginger accession with complete resistance to the

pathogen as all showed symptoms at different levels of disease severity and incidence except the wild type (JW89). Nine ginger accessions (KT13, BAS19, BSSB47, BSSB49, ORJ50, ORW59, ORW61, ORW63 and HDMS94) showed low disease severity index scale (1) and disease incidence from 16.5 to 30% at 90 DAI. Eight accessions, KT6, 44, KBI56, ORW58, ARHR67, DESP73, GBO98 and GBO99 (Appendix I) were with average severity index scale 2 (Fig 7.3 and 7.4).

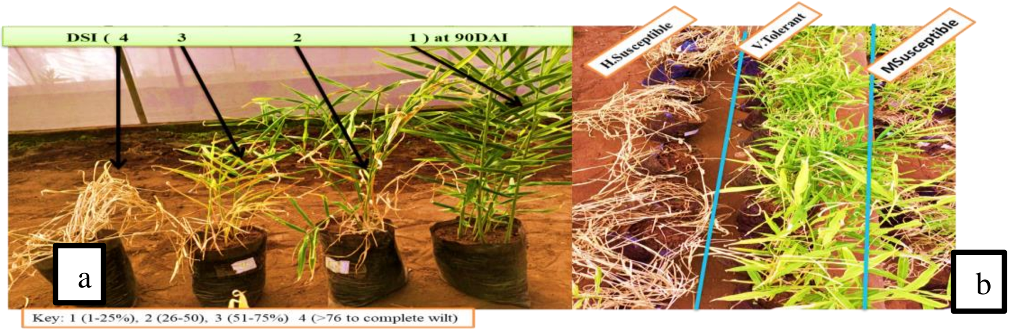

Figure 7. 3. Response variation among different accessions after 90 days of inoculation

a. sample plants from very susceptible to very tolerant accessions (left to right) and b. accessions under very susceptible and very tolerant categories

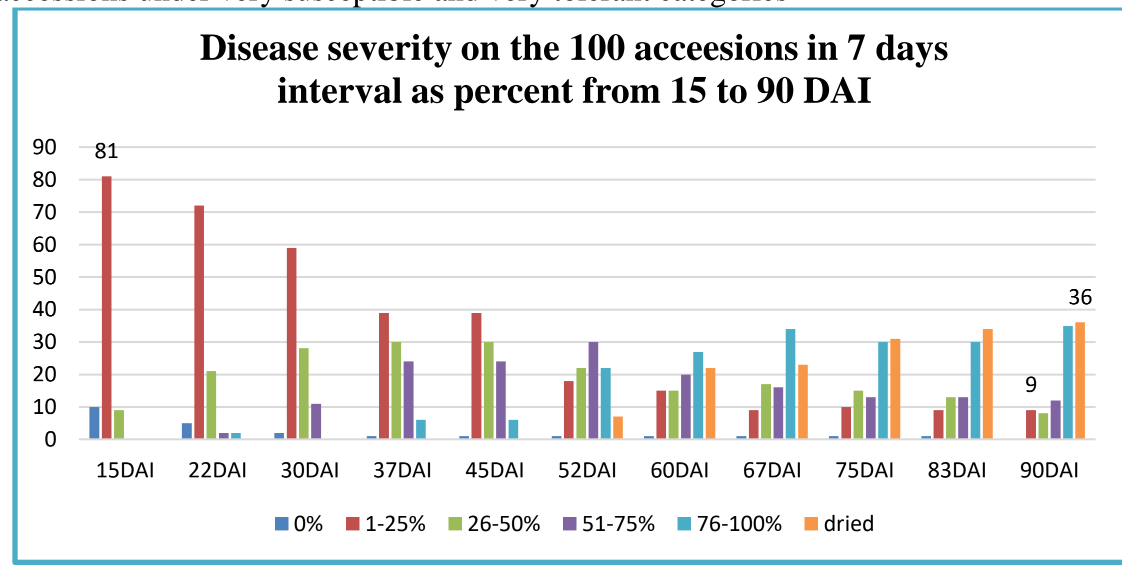

Figure 7. 4. Graphic representation of disease severity progress in seven days intervals

Disease effect on rhizome fresh yield

Rhizome yield components per plant recorded after harvesting were compared to the result recorded from check plants. The result showed that the yield loss as a percentage against the control plants ranged from 1.65 to 77.73%. Accessions with a disease severity iscale of (4), and incidence above 75% showed very high yield loss. On the other hand, the rhizome fresh yield of most accessions with disease severity scales of (1) and (2) was less affected. The lowest yield loss (1.65%) followed by 3.05% was shown by accession numbered (ORW59) and (KTT9), whereas the maximum yield loss (77.73%) was shown by accession WBB40 followed by accession WBS33 at 76% loss (Fig 7.6). The two accessions with the lowest yield loss were collections from Oromia and central Ethiopia areas with disease severity scores one and two at 90 DAI respectively (Table 1). On the contrary, the two accessions with maximum yield loss and highest disease severity were from Southern Ethiopian collections. Nine accessions with a disease severity scale (1) and relatively low disease incidence scored lower rhizome fresh yield loss from 1.65 to 12.92% and an average loss of 9.23%. Accessions with severity score index of (2) are eight with an average yield loss of 32.4%, whereas 12 accessions with a score scale index of (3) and 71 accessions completely wilted at index 4 have an average yield loss of 36.15 and 40.73% respectively (Fig 7.5). The overall yield loss due to disease as compared to rhizome yield harvested from uninfected plants was 36.02%. The 100 study materials have different levels of loss percentage, 30 accessions have high rhizome yield loss above 50% (50.75 to 77.37%), 43 have loss percentage between 20 and 50%, and 27 have loss less than 20% (Fig 7.5). Accessions with high yield loss were characterized by early disease symptom development at the severe level. Moreover, a large proportion of tillers per plant were diseased which

contributed to high disease incidence. This finally led to leaf drying and complete wilting of the whole plants before enough rhizome was initiated. Root growth was also among the highly affected characters. The average root number was reduced up to 90% for some accessions as compared to control plants. Root length which has a strong linear correlation to rhizome yield was also highly affected with record root length reduction of up to 74% for accession number SOSA15 (Appendix III).

The disease pressure difference between the study materials was also expressed on development of secondary rhizome fingers and active buds, which have a strong positive correlation with total rhizome yield. The result also revealed that some accessions with low disease severity and incidence were expected to have low yield loss as tolerant had high yield loss percentages like accession ARHR67 with a loss percent of up to 50.35%.

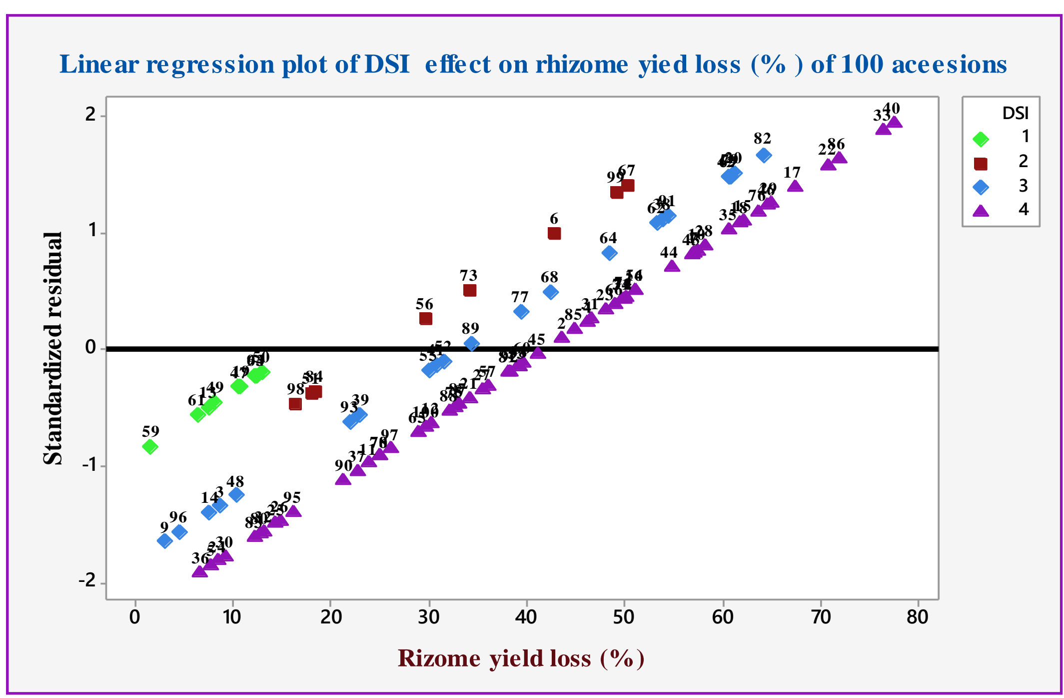

Figure 7. 5. Linear regression of disease severity with fresh yield loss against each severity scale change from 1 to 4.

Inversely some accessions under high disease pressure were with low yield loss. Accessions with codes WODS32 and GODG25, which were at complete wilting and 100% incidence after 90 DAI, had low yield loss percentages of 13.21 and 14.42% respectively. On the other hand, most of the accessions with disease severity index score of 3 had medium to high yield loss (20 to 50%) as expected.

Correlation of disease severity with rhizome yield and component traits

Linear correlation analysis result indicated that disease severity has significant negative correlation with main ginger growth and rhizome yield traits (Table 7.1). Total rhizome fresh weight, number of rhizome fingers, root number and root length have very high linear negative association with disease severity score. Whereas, rhizome yield loss and dry weight losses have positive correlation with the disease severity (Table 7.1). However, the disease pressure has not affected number of tillers per plant at significant level.

Table 7. 1. Correlation of disease severity with some traits at 95% confidence interval

Traits	DSI (1-4)	DI (%)	TN	RN	RL(cm)	NRF	NBF	RFW	RYL (%)
DI(%)	0.69***								
TN	0.002	0.03							
RN	-0.45***	-0.37***	0.09						
RL(cm)	-0.37***	-0.24*	-0.21*	0.51***					
NRF	-0.202*	-0.103	0.12	0.36***	0.17				
NBF	-0.41***	-0.24*	-0.01	0.52***	0.42***	0.59***			
RFW	-0.46***	-0.37***	-0.003	0.11	0.08	0.15	0.19*		
RYL (%)	0.42***	0.35***	0.022	-0.04	-0.08	-0.09	-0.26	-0.60	
DML (%)	0.22	0.22	0.01	-0.03	-0.14	-0.01	-0.12	-0.19	0.11

Legend: DSI- disease severity index, DI- disease incidence, TN- tiller number, RN- root number, RL-Root length, NRF- number of rhizome fingers, NBR- number of buds per rhizome , RFW- rhizome fresh weight, RYL- rhizome yield loss and DML-dry weight loss.

Disease effect on rhizome dry matter content

Dry matter content is among the economically important traits the disease negatively affects. Dry mater loss analysis showed that there was high variation among the accessions. The highest dry matter content loss (55%) was recorded for accession (GUW88) and the lowest (0.5%) was for accession number (ARHR70). Zero loss was observed for seven accessions (KTT9. KTT13, KTT14, BASP19, WOBS38, BSSB49, and JW89) when compared to check plants. Moreover, the dry matter content loss (DML) analysis result showed 25 of the 100 accessions showed losses below 10% and 46 accessions were with DML of 10-30%. The remaining 22 showed high losses above 31% and the overall average loss was 18.43% as compared to control-uninfected plants of all study materials. The regression analysis result also showed that there is a significant change in dry matter content loss at a 95% confidence interval with a change in disease severity scale.

Field evaluation of the study materials

The experimental materials evaluated at Areka agricultural research center main station during 2023 production season were evaluated for the bacterial wilt disease through natural infestation. Variation among the study materials was also observed which was expressed by days to disease symptom, disease severity, disease incidence and rhizome fresh yield. The field assessment result revealed some accessions with better performance during pot evaluation has also repeated at the field. Accessions like BSSP19, BSSB49 and ORW63 are highly promising during field evaluation with high rizeome fresh and dry yield.

In contrary, some accessions with poor performance during pot experiment had very tolerant level response during field evaluation. Accessions showed this nature are GOOY27, ARHR70, DESP73, DESP74 and ORJ81 (Fig 7.6 and Appendix III).

Disease severity and incidence recorded has significant positive correlation with rhizome fresh and dry mater recovery as it was in the greenhouse condition. The maximum fresh rhizome yield (18.5 t/h) was shown by accession DESP72 that was among accessions with low disease severity and incidence. In the contrary, highly susceptible accessions yielded very low rhizome yield (6 t/h) being the lowest record in accession SDQA03. The range of dry mater recovery among the genotypes due to disease impact was also very wide. The maximum dry matter recovery as percent (31.52%) was recorded for accession ORW63, which has low disease severity, and incidence scores both in greenhouse and field condition. In contrary the lowest dry matter recovery (12.53%) was recorded for accession GUW84, which is among the highly susceptible accessions in both evaluation conditions.

Figure 7. 6. Response variation among ginger accessions at field condition. A. accession with high tolerance level response, B. accession with moderate tolerance response and C. accession from highly susceptible accession group.

7.4. Discussion

The isolation and identification of virulent strain of the disease pathogen was successful as verified by pathogenicity test and disease development after inoculation of all experimental materials. Ginger bacterial wilt disease pathogen identifications in previous studies also indicated that *Ralistonia solanacirium* race 3 biovar 4 with a similar in vitro characters as to this study observation. Isolates with similar morphology further tested by (ELISA) kit in Ethiopia were reported as highly virulent strains of the pathogen (Tariku *et al*., 2016). Another research report by Handiso *et al*. (2017) was also in consistent with pathogen strains isolated in this experiment. The observation results both on TZC media, pathogenicity test and disease development after inoculation of experimental materials are consistent. Pathogen isolates with deep pink, fluidal, compact colony development pattern, and irregular shape was the most virulent strain.

Disease development trend scored by visual observation from all inoculated study materials from 15 days after inoculation up to 90 days indicated there is variations among the accessions. Based on the disease severity, incidence, days to symptom development, and yield losses, all 100 test materials were categorized into four groups (very tolerant, tolerant, susceptible, and very susceptible). Accessions that expressed the disease at a severe level after 52 days of inoculation relatively low disease incidence (<30%) and severity scores accompanied by low rhizome yield loss (<10%) were considered as very tolerant. In this regard, twelve accessions (SDQA3, KTH5, KTT9, KTT13, SOSA14, BASP19, GODG24, GOOY30, BSSB49, KBI56, ORW59 and HDMS94) were grouped under very tolerant accessions. Whereas, fifteen accessions with average severity scale index (2) and yield loss from 10 to 20% range were included under tolerant group.

The largest proportion of accessions (43 of 100) failed under the susceptible category are those with disease severity scores of 3 to 4 accompanied by fresh rhizome yield loss from 21-40%. The final category, very susceptible included accessions that showed severe levels early as of 22 days of inoculation, and high disease incidence (>60%) and yield loss (>40%). Plants of accessions in this group were completely wilted and dried out at the final disease score time. The wild type (JW89) on which disease symptom was not observed across the study time was resistant as also reported in previous studies (Karthika *et al*., 2019). Moreover, this wild type was reported as resistant by maintaining research center (Jimma Agricultural research center, Ethiopia) from which the experimental material was collected.

Previous research reports on the resistance of ginger cultivars and related species indicates there were variation among test materials but none of them indicated complete resistance for *Ralistonia solanaceous* (Mathew *et al*., 2008; Brehanu, 2020). In his screening protocol development for ginger against the pathogen, Kumar (2006) reported all study materials developed symptom and some have recovered later. Similar study of screening ginger accessions for rhizome root disease complex grouped 134 ginger accessions to six groups from very resistant to highly susceptible based on disease incidence (Senapati and Sugata, 2005). Complete resistance of crops for *Ralistonia solanaceous* and other bacteria is seldom except few reports on eggplant and tomato (Hatice *et al*., 2021; Marc *et al.,* 2020).

Variation among the study accessions response to pathogen inoculation was expressed by disease scores (severity and incidence), low root growth, negative impact on rhizome

component traits, and ultimately yield loss at different levels. The linear correlation coefficient of disease scores with rhizome yield and component traits indicated disease severity and incidence have a highly significant negative impact. Some traits like root number, root length, number of rhizome fingers, number of buds per rhizome, and rhizome fresh weight per plant were highly affected. Moreover, the disease scores have a highly significant positive correlation with rhizome yield loss.

Linear regression analysis of variance at 0.05% probability also showed that the disease effect on total fresh yield and loss was highly significant. Rhizome yield per pot is higher for accessions with low disease severity scores, which is due to low disease impact on rhizome yield. The experiment was less affected by environment, as the regression analysis error variance for most recorded traits is low. This is due to low error as the experiment was conducted under a protected screen house condition. Some accessions with low disease severity and incidence expected to have low yield loss because of tolerance were affected highly. This contradiction might be due to green wilting symptoms at a severe level but underrated because of green leaves rather than progressive wilting and plant collapse. Inversely some accessions, which were at complete wilting and 100% incidence, had low yield loss percentages might also be due to the long incubation period and early maturing nature of the accessions.

Another interesting response observed was accessions under high disease pressure have developed more tillers during growth. Moreover, most of the rhizome buds were sprouted during harvesting as compared to control plants and less affected accession. These

conditions probably happen as the developing rhizome was forced to replace itself while the growing tillers were collapsing before the full development of the daughter rhizome fingers. Thus, primordial stem buds were continually growing to tillers to replace early emerged but challenged to grow due to disease pressure. The continuous sprouting of rhizome buds to tillers were at the expense of rhizome which finally leads to low rhizome weight and hence, high yield loss per unit area.

7.5. Conclusion

The screening experiment observations and analysis results from disease scoring to yield losses revealed that the response variation among ginger accessions was highly contributed due to the genotype effect against the disease pathogen. The experiment also revealed that ginger accessions collected from different parts of the country have different reaction levels to the disease pathogen. All the ginger accessions, except wild type (Mongo ginger), there is no accession that is completely resistant. However, there were accessions with low disease severity and incidence accompanied by low fresh and dry yield losses. Tweleve accessions were identified as very tolerant and can be further evaluated under different environmental conditions. The wild type on which disease was not observed can be used as standard check and source of resistance gene for advanced breeding techniques. Further investigation through repeated assay and molecular-assisted selection of genotypes with better disease tolerance, high rhizome yield, and quality of ginger products are research agendas in Ethiopia.

CHAPTER 8

8. Conclusions and Recommendations

The study conducted in subsequent separate experiments accompanied by extensive observations, data collections, analysis and interpretations have derived conclusions and also forwarded recommendations. The first experiment was conducted to replace ammonium nitrate with low cost alternative nitrogen sources for enhancement of disease free ginger plantlets as part of integrated ginger bacterial wilt management strategy. Among the tested three salt types, urea a common inorganic nitrogen fertilizer at 3.0 g/l and ammonium chloride at 1.0 g/l are the best potential alternatives to ammonium nitrate in MS medium for efficient and low cost mass propagation of disease-free ginger plantlets.

The second experiment was conducted with the objective to induce and produce viable micro-rhizome ginger seed in tissue culture system. The result finally indicated, micro-rhizomes induced and produced on MS medium supplemented by elevated concentrations of sucrose (80 g/l) and 6.0 mg/BAP were best as means of alternative disease free seed micro-rhizome. The in vitro produced micro-rhizomes can also be used at seed multiplication nurseries and enhance ginger production at large scale. Moreover, repeated experiments with modification should be conducted to get the maximum number of micro-rhizomes. Further work to increase the viability percentage under different in vivo growth conditions including open field is also required to use in vitro produced micro-rhizomes as seed source for large scale disease free ginger production in non-infected farmlands.

Assessment of genetic diversity among 100 ginger accessions collected from different areas of multi-cultural communities in Southern and Southwestern Ethiopia based on morphological traits and farmers information has revealed significant variation for groowth and yield traits. High values were observed for genetic and phenotypic coefficient of variations, heritability and genetic advance as percent of mean. Strong positive correlation among traits revealed that there is significant correlation between rhizome yield and some traits like plant height, leaf area, tiller numbers and number of rhizome finger. The distribution of accessions across four clusters was independent of collection areas. Hence, it was concluded that there is high genetic diversity among Ethiopian ginger accessions. Thus, can be exploited for selection using traits that have strong correlation with rhizome yield and yield components.

The molecular genetic diversity study using SSR markers also indicated high diversity among experimental accessions. Genetic diversity, population structure and molecular evolution phylogenetic tree analysis consistently clustered the 100 accessions to three clusters. Some accessions and the wild type were separated to similar groups in disease screening, phenotype and molecular based analysis. Further high throughput markers and sequencing techniques may verify more genetic markers and economically important trait linked markers to enhance ginger genetic improvement and alleviate major challenges like ginger bacterial wilt disease in ginger production across the world.

The screening experiment results at different stages from disease scoring to yield loss analysis revealed that there is response variation among ginger accessions.

The disease response variation mainly due to the genotype effect against the disease pathogen as the experiment was under protected condition and open field. Ginger accessions collected from different parts of the country have different response levels to ginger bacterial wilt disease pathogen. Out of the hundred accessions tested, there is no ginger accession with complete resistance except the wild type. However, accessions were categorized to four levels; very tolerant, tolerant, susceptible and very susceptible based on disease scores and yield losses due to the disease pressure. Further evaluation and molecular assays to identify source of variation at genetic level were required. The wild type on which disease symptom was not observed can be used as standard check and source of resistance gene for advanced breeding techniques. Further evaluations under different environmental conditions against the pathogen and molecular-assisted selection of genotypes need be planned to verify the reported results.

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APPENDIXES

Appendix I. List of Published Manuscripts

1. Genene Gezahegn, Tileye Feyissa and Yayis Rezene (2023). Replacement of ammonium nitrate by alternative nitrogen sources in MS medium to enhance ginger (*Zingiber officinale* Rosc.) in vitro regeneration. Plant Cell, Tissue and Organ Culture (PCTOC) https://doi.org/10.1007/s11240-023-02513-7

2. Genene Gezahegn, Tileye Feyisa and Yayis Rezene (2023). Induction of micro-rhizomes for in vitro ginger (*Zingiber officinale* Rosco) disease-free planting materials regeneration. Biotechnology Reports 41. https://doi.org/10.1016/j.btre.2023.e0080

3. Genene Gezahegn, Yayis Rezene and Tileye Feyissa (2024). Genetic Diversity Analysis of Ethiopian Ginger (*Zingiber officinale* Roscoe) Accessions using SSR Markers. Genet Resour Crop Evol. https://doi.org/10.1007/s11240-023-02513-7

Appendix II. Media components required for standard MS media

Components	Chemicals/ Ingredients /	amount (mg /l)
Macroelemnts	Potassium nitrate	1900.00
	Ammonium nitrate	1650.00
	Calcium chloride anhydrous	332.16
	Magnesium phosphate anhydrous	180.69
	Potassium phosphate monobasic	170.00
	Sodium phosphate monobasic	221.00
Microelements	Manganese sulphate.H_2O	16.90
	Boric Acid	6.20
	Potassium iodide	0.83
	Molybdic acid (sodium salt).$2H_2O$	0.25
	Zinc sulphate.$7H_2O$	8.60
	Copper sulphate.$5H_2O$	0.03
	Cobalt chloride.$6H_2O$	0.03
	Ferrous sulphate.$7H_2O$	27.80
	Na_2.EDTA	37.26
Vitamins	Myo-Inositol	100.00
	Thiamine HCL	0.10
Carbohydrate	Sucrose	30000.00
Plant Growth Regulators	BAP	2
	Kinetine	1
Gelling agent	Agar	6000.00

Appendix III. Accessions Passport Data and sample code

Accession No.	CODE	COORDINATES			PLACE /AREA	Year of collection
		Longitude	Latitude	Altitude		
101	SDBOZ101	0382406	062651	1849	Loya	2021
02	SDQ102	0382114	062800	1734	Qarricha	2021
03	SDQ103	0382120	062849	1810	Qarricha	2021
04	KTH104	0374214	071245	1775	Lalo	2021
05	KTH105	0374214	071245	1775	Lalo	2021
06	KTH106	0374214	071245	1775	Lalo	2021
07	KTH107	0374128	071215	1721	Hadaro T.	2021
08	KTT108	0373304	071418	1736	Bohe	2021
09	KTT109	0373304	071418	1736	Bohe	2021
10	KTT110	0373304	071418	1736	Bohe	2021
11	KTT111	0372751	071740	1673	Qelexa	2021
12	KTT112	0373517	071437	1760	Soyame	2021
13	KTT113	0373517	071437	1760	Soyame	2021
14	SOSA114	0363521	061311	1583	Melorashia	2021
15	SOSA115	0363521	061311	1583	Melorashia	2021
16	SOSA116	0363521	061311	1583	Melorashia	2021
17	SOSA117	0363519	061512	1570	Melorashia	2021
18	BASP118	0363640	061513	1661	Gezima	2021
19	BASP119	0363536	061513	1418	Gara wolayta	2021
20	BASP120	0363536	061513	1418	Gara wolayta	2021
21	BASP121	0363536	061513	1418	Gara wolayta	2021
22	GODG122	0365651	062102	1302	Bola	2021
23	GODG123	0365655	062055	1308	Bola	2021
24	GODG124	0365749	062122	1230	Karza	2021
25	GODG125	0365749	062122	1230	Karza	2021
26	GOOY126	0365104	061404	1324	Shefite	2021
27	GOOY127	0365104	061404	1324	Shefite	2021

28	GOOY128	0365104	061404	1345	Shefite	2021
29	GOOY129	0365103	061418	1345	Shefite	2021
30	GOOY130	0365103	061418	1345	Shefite	2021
31	GOOY131	0365103	061418	1345	Shefite	2021
32	WODS132	0373929	065800	1998	D.Zamine	2021
33	WODS133			2050	D.Zamine	2021
34	WOBB134	0373639	070808	1593	Faraocha	2021
35	WOBB135	0373639	070808	1593	Faraocha	2021
36	WOBB136	0373609	070840	1560	B.zuria	2021
37	WOBB137	0373609	070840	1560	B.zuria	2021
38	WOBS138	0373738	070936	1617	M.Hembecho	2021
39	WOBS139	0373738	070936	1617	M.Hembecho	2021
40	WOBS140	0373919	071024	1646	T.Hembecho	2021
41	ORMET141	0352844	081932	1621	Adale Bise	2021
42	ORMET142	0352734	082014	1656	Adale Bise	2021
43	ORMET143	0353648	081303	1753	Kamise	2021
44	ORMET144	0351244	081244	1767	Kamise	2021
45	ORMET145	0355042	082105	1634	Yayo Bondao	2021
46	ORMET146	0355031	082101	1674	Yayo Ammuma	2021
47	BSSB147	0353656	070235	1505	Yali	2021
48	BSSB148	0353624	070241	1518	Wutsqin	2021
49	BSSB149	0353616	070238	1473	Wutsqin	2021
50	ORJIM150	0362923	072943	1814	Halo sebeka	2021
51	ORJIM151	0362922	072942	1801	Halo sebeka	2021
52	ORJIM152	0363211	073105	1959	Sabaka dabiye	2021
53	KADECHA153	0360707	070540	1860	Goba gecha	2021
54	KABITA154	0353807	071412	1522	Odda	2021
55	KABITA155	0353820	071425	1533	Odda	2021
56	KABITA156	0354146	071339	1453	Tugga	2021
57	KABITA157	0354141	071334	1416	Tugga	2021

58	ORKWE158	0344707	083202	1808	Soya-Shogo	2021
59	ORKWE159	0345112	083108	1792	Soya-samro	2021
60	ORKWE160	0345041	083218	1622	Soya-Galano	2021
61	ORKWE161	0345110	083201	1615	Soya-Galano	2021
62	ORKWE162	0345104	083204	1490	Soya-Galano	2021
63	ORKWE163	0345250	084112	1930	Hawa Harer	2021
64	ORKWE164	0345231	084121	1890	Hawa Moi	2021
65	ORKWE165	0344805	084503	1862	Hawa Botoso	2021
66	ARHRT166	0373755	070959	1610	M.Hembecho	2021
67	ARHRT167	0373540	070991	1521	Ajora	2021
68	ARHRT168	0373377	070832	1492	Kutoambe	2021
69	ARHRT169	0373640	070890	1519	Faraocha	2021
70	ARHRT170			1610	Hembecho	2021
71	DESP171	0372249	054102	1538	Shabale	2021
72	DESP172	0372330	054223	1408	Boshe	2021
73	DESP173	0372337	054113	1350	Albata	2021
74	DESP174	0372210	053854	2039	Aygida	2021
75	DESP175	0372255	054109	1499	Dahule	2021
76	DESP176	0372302	054123	1460	Harale	2021
77	DESP177	0372323	054131	1425	Boshe	2021
78	DESP178	0372334	054130	1398	Boshe	2021
79	DESP1179	0372341	053836	1695	Walesa	2021
80	DESP180	0372337	053838	1680	Walesa	2021
81	ORJIDD181	0370001	074167	1640	Twon	2021
82	KAGO182	0372117	072025	1705	Gojeb	2021
83	JARC183	0364709	074014	1753	Melko	2021
84	GUW184	0373438	081344	1280	Wolkite	2021
85	AARC185	0374122	070358	1752	Dubo BTC	2021
86	JARC186	0364709	074014	1753	Melko YTC	2021
87	AARC 187	0374122	070358	1752	Dubo VTC	2021

88	GUW188	0373441	081348	1350	Wolkite	2021
89	JARC189	0364709	074014	1753	Melko wild	2021
90	HDMS190	0373047	072110	1745	jacho	2021
91	HDMS191	0372920	072037	1665	jacho	2021
92	HDMS192	0373011	072026	1747	up.jacho	2021
93	HDMS193	0373016	072146	1717	Hageno	2021
94	HDMS194	0375041	072139	1704	Hageno	2021
95	KTTA195	0373417	071556	1992	S.Amuguna	2021
96	GMBO196	0375914	065354	1442	Zefano	2021
97	GMBO197	0373610	063146	1518	Z.sanko	2021
98	GMBO198	0376600	063156	1497	Z.Gidohera	2021
99	GMBO199	0373558	063156	1490	Z.Gidohera	2021
100	ORMET200	0355031	082101	1674	Hawa Moi	2021

Appendix. IV. Detail information of primers used

Name	Sequence(5'-3')	Length	MW	Tm	GC%	OD	Tube	nmol	water/Tube	Purity
SSR-CBT01_F	TCCTCCCTCCCTTCGCCCACT	21	6165.00	65.82	66.67%	5	1	26.76	267.64	OPC
SSR-CBT01_R	CGATGTCGCCATGGCTGCTCC	21	6374.16	65.82	66.67%	5	1	25.89	258.86	OPC
SSR-CBT02_F	ATCAGCAGCCATGGCAGCGACC	22	6714.40	65.66	63.64%	5	1	24.57	245.74	OPC
SSR-CBT02_R	AGGGGATCATGTGCCGAAGGCC	22	6825.48	65.66	63.64%	5	1	24.17	241.74	OPC
SSR-CBT03_F	ACCCTCTCCGCCTCGCCTCCTC	22	6479.19	69.39	72.73%	5	1	25.47	254.66	OPC
SSR-CBT03_R	CTCCTCCTCCTGCGACCGCTCC	22	6519.22	69.39	72.73%	5	1	25.31	253.10	OPC
SSR-CBT04_F	CTCTGTCTCCTCCCCCGCGTCG	22	6550.24	69.39	72.73%	5	1	25.19	251.90	OPC
SSR-CBT04_R	TCAGCTTCTGGCCGGCCTCCTC	22	6614.30	67.53	68.18%	5	1	24.95	249.46	OPC
SSR-CBT05_F	GCCTCGATCATCATCAG	17	5130.39	54.59	52.94%	5	1	32.16	321.61	OPC
SSR-CBT05_R	ATCAACCTGCTTGCCTGG	18	5450.59	57.30	55.56%	5	1	30.27	302.72	OPC
SSR-CBT06_F	CGATCCATTCCTGCTGCTCGCG	22	6638.33	65.66	63.64%	5	1	24.86	248.56	OPC
SSR-CBT06_R	CGCCCCCATGCATGAGAAGACG	22	6714.40	65.66	63.64%	5	1	24.57	245.74	OPC
SSR-CBT07_F	TTCCCTGTTAAGAGAGAAATC	21	6429.27	54.11	38.10%	5	1	25.66	256.64	OPC
SSR-CBT07_R	GTGTATTTGGTGAAAGCAAC	20	6196.12	53.70	40.00%	5	1	26.63	266.30	OPC
SSR-ZOF1_F	TAACTGGCCCTGGAACAAAC	20	6095.03	57.80	50.00%	5	1	27.07	270.71	OPC
SSR-ZOF1_R	GGTGCAGTAAACCAGGATGAA	21	6528.33	58.01	47.62%	5	1	25.27	252.74	OPC
SSR-ZOF2_F	CAGCGTCACTATCTCCACCA	20	5981.93	59.85	55.00%	5	1	27.58	275.83	OPC
SSR-ZOF2_R	ACGGGGATCTTAGGCTTGTT	20	6179.08	57.80	50.00%	5	1	26.70	267.03	OPC
SSR-ZOF3_F	AAGGCCCTCGACATCTGTAA	20	6086.02	57.80	50.00%	5	1	27.11	271.11	OPC
SSR-ZOF3_R	TACGGGAGGAGTGACCACA	19	5886.89	59.72	57.89%	5	1	28.03	280.28	OPC
SSR-ZOF4_F	ATCGAATCCATCCCACTGAA	20	6029.99	55.75	45.00%	5	1	27.36	273.63	OPC
SSR-ZOF4_R	CAGGTCGGAGGTGAAGTCTC	20	6198.08	61.90	60.00%	5	1	26.62	266.21	OPC
SSR-ZOF5_F	TTCGACACCACCAGAACAGA	20	6064.01	57.80	50.00%	5	1	27.21	272.10	OPC
SSR-ZOF5_R	GTCCATGGCGTACACCTTCT	20	6043.97	59.85	55.00%	5	1	27.30	273.00	OPC