SAFFLOWER GROWTH, DEVELOPMENT, YIELD AND OIL CONTENT AS INFLUENCED BY GENOTYPE AND ENVIRONMENT INTERACTION UNDER ON-FARM CONDITIONS

A thesis submitted in fulfillment of the requirements for the award of degree of Doctor of Philosophy in Crop Science (Horticulture Stream)

By

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September 2024

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DECLARATION

I declare that the work contained in this thesis is my original work and was compiled at the Botswana University of Agriculture and Natural Resources. To the best of my knowledge, it contains no material that has been previously submitted for any other degree in this or other University except where due acknowledgment has been made.

Author's signature ...M. Mogapiemang...... Date 25/04/2024

DEDICATION

This thesis is dedicated to my family and friends who have always been my source of strength and inspiration. In loving memory of my dearest mother.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my heavenly Father who has been my source of strength and wisdom. His grace and tender mercies enabled me to conquer every challenge that I faced during my study period.

I am deeply grateful to my supervisors Prof V. E. Emongor, Dr G. Malambane, and Dr R Mapitse for their mentorship, support, and belief in me. I would like to thank Prof Emongor for his spiritual and academic advice, patience, and experience that made the completion of my research work and thesis possible. I would like to acknowledge Dr Malambane for always reminding me that hard work and time management is the key to completion, which pushed me to complete my tasks on time. I would like to extend my sincere gratitude to RUFORUM and Mastercard Foundation through the CARP project awarded to Prof Emongor for funding my study.

I benefited greatly from Dr Mathapa's assistance with the fluorescence microscope. The hard work of the Chief Technician, Mr Mathowa during my fieldwork and purchasing of my reagents is highly appreciated. Many thanks to Agisanyang Tautsagae, Tebogo Stimela, and Galalea Gaonosi from the Tissue Culture Laboratory for their technical assistance and friendship, they made it possible. I would also like to thank Laboratory Technicians from Crop and Soil Sciences (CSS), Food Science and Technology, Animal Sciences and Production, and Biological Sciences Departments for their technical support. I also give thanks to the Department of Agriculture Research and Botswana International University of Science and Technology for allowing me to use their instruments and equipment. I am grateful to the CSS staff and my fellow graduate students who gave me advice and moral support. I would like to recognize Mrs Ramasedi and Mr Mathibe for hosting my study on their farms. I would be remiss in not mentioning my safflower-RUFORUM teammates, Bonolo Setshogela, Dineo Kereilwe, Keheng Basiame, Maatla

Koroneneo, Maduo Kolenyane, Dr Moatshe-Mashiqa, Pako Monyame, and Dr Tiroesele, it was a pleasure collaborating with you all.

I give thanks to my father Kealogile Mosupiemang, my brother Mogomotsi Mosupiemang and Moabi Tshelane for their encouragement. To Hilary Shoniwa I will not forget your prayers. Finally, I would like to give my sincere gratitude to my dearest friend Mogomotsi Molefe for the support and understanding throughout this journey.

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LIST OF SYMBOLS AND ABBREVIATIONS

AMMI	Additive main effects and multiplicative interaction
APX	Ascorbate Peroxidase
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
CEC	Cation exchange capacity
BUAN	Botswana University of Agriculture and Natural Resources
DNA	Deoxyribonucleic acid
DREB	Dehydration Responsive Element Binding
FAME	Fatty acids methyl esters
G×E	Genotype environment interaction
GGE	Genotype main effects and genotype × environment interaction effects
GR	Gluthathione Reductase
H_2O_2	Hydrogen peroxide
LSD	Least significance difference
LRWC	Leaf relative water content
NCBI	National center for biotechnology information
PCA	Principal component analysis
PCR	Polymerized chain reaction
pH	Potential hydrogen
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerized chain reaction
ROS	Reactive oxygen species
RWC	Relative water content
SOD	Superoxide dismutase

LIST OF PUBLICATIONS AND PRESENTATIONS

1. Mosupiemang, M., Emongor, V. E., Malambane, G., & Mapitse, R. (2023). Growth, development and yield of safflower genotypes in response to environmental variations. *Journal of Phytology*, *15*, 145-154.

2. Mosupiemang, M., Malambane, G., Mathapa, B. G., & Emongor, V. E. (2022). Oleosin expression patterns and size of oil bodies as a factor in determining oil content in safflower (*Carthamus tinctorius* L.) genotypes. *European Journal of Agriculture and Food Sciences*, *4*(5), 54-60A.

3. Mosupiemang, M., Emongor, V. E., & Malambane, G. (2022). A Review of drought tolerance in safflower. *International Journal of Plant & Soil Science* 34(10):140-149.

4. Mosupiemang, M., Malambane, G., & Emongor, V. E. (2022). Seed yield and oil yield of safflower genotypes in response to environmental variations. *A paper orally presented at the Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) 18th Annual General Meeting.* 12-16 December 2022, Harare, Zimbabwe.

5. Mosupiemang, M., Malambane, G., Mathapa, B. G., & Emongor, V. E. (2022). Oleosin expression patterns and size of oil bodies as a factor in determining oil content in Safflower Genotypes. *A paper orally presented at the Botswana University of Agriculture and Natural Resources, Science Week*. 15-17 August 2022.

6. Mosupiemang, M., Emongor, V. E., & Malambane, G. (2021). Drought stress effect on the leaf relative water content and proline content of safflower genotypes. *RUFORUM Working Document Series,* 19 (1): 101-104.

ABSTRACT

Safflower (*Carthamus tinctorius* L.) is amongst the neglected and underutilized oilseed crop that is adaptable to environmental conditions present in arid and semi-arid lands (ASALs). It has many uses like foods, textile, pharmaceutical, cosmetic, and industrial (paint, biodiesel). Safflower seed produce healthy and high-quality vegetable oil which is rich in vital linoleic and oleic fatty acids. The economic potential of this crop is very high and has been noticed in Botswana. Recently, the Botswana government has developed a new policy known as Temo-Letlotlo Programme in which the government subsidises inputs for farmers in growing 13 food crops of which safflower is one of them. The goal of this programme is to promote food security, commercialization, inclusivity in agricultural production, and social capital. Therefore, to increase the productivity of safflower in ASALs such as Botswana, genotypes that show greater adaptability and stability for growth, yield, and oil content need to be assessed and recommended to farmers, hence, the aim of this study. In the first study, an on-farm trial was conducted to assess the influence of season, location, and genotype and their interactions on the growth, phenological development, oil content, yield, and yield components of safflower. This study was conducted in summer and winter at three sites (Sebele, Ramonaka, and Molepolole) in the southern part of Botswana using five safflower genotypes (Turkey, Sina, PI537636, Kenya9819, and Gila). The findings demonstrated that winter planting delayed the phenological development (days to emergence (2.56 days), stem elongation (33.3 days), branching (47.8 days), flowering (50 days), and maturity (79.4 days)), and promoted the vegetative growth, yield, yield components, and oil content of safflower (plant height (38.7%), shoot biomass (218%), root biomass (239%), capitula number/plant (18.6%), capitula diameter (4.7%), capitula weight (30%), 1000-seed weight (17.8%), seed yield/ha (84.4%), oil content

(20.7%) and oil yield (114.2%)). Safflower planted in winter at Ramonaka had better vegetative growth (plant height (90.6 cm), root biomass (13.2 g/plant), and shoot biomass (132 g/plant)) than that of other locations planted either in winter or summer. The results further revealed variability amongst the studied genotypes for almost all the phenological development (days to emergence, stem elongation, branching, flowering, and maturity), growth (plant height and shoot biomass), oil content seed yield, and yield components traits studied (capitula number/plant, capitula diameter, capitula weight, and 1000-seed weight). In addition, location, season, and their interactions significantly affected these parameters (number of primary branches/plant, capitula/plant, capitula weight, 1000-seed weight, seed yield, oil content, and oil yield/ha). Genotype by environment interactions (GGE) biplots for seed yield demonstrated that Sebele showed greater representativeness and discriminative ability therefore, it was considered a perfect site for choosing genotypes that are adapted to the entire region. Genotype Kenya9819 was identified as the highest seed-yielding and stable genotype based on the GGE biplots. When evaluating genotypes based on overall superiority, the genotype by yield*trait combination (GYT) biplot showed that genotypes Turkey and Kenya9819 had an above-average seed yield-trait combination, hence superior, while genotypes Gila and PI537636 performed poorly (they were below-average for all studied traits except for yield*oil content). Therefore, genotypes Turkey and Kenya9819 were recommended to be grown in the southern part of Botswana based on their overall superiority. On the other hand, genotype Gila could be used for breeding purposes to improve the seed oil content of other genotypes due to its high seed oil content. The second study determined the relationship between oleosin genes and oil bodies in regulating the oil content of safflower seeds. This was achieved by isolation and quantifying the oleosin genes and oil bodies from the seeds of five (Gila, Turkey, Sina, PI537636, and Kenya9819) safflower genotypes using qPCR and fluorescence

microscope, respectively and assessed them against the seed oil content. The results revealed a strong inverse relationship where smaller oil bodies were exhibited by genotypes containing high oil content (Kenya9819 and Gila) and high relative expression of oleosin genes. The findings indicated that oleosin genes and oil bodies are important traits to consider when characterizing oil seed crops for oil content. In the third study, the response of safflower genotypes to drought stress was evaluated under a greenhouse and verified under field conditions. Drought stress was found to reduce the chlorophyll content, leaf relative water content (LRWC), and plant height irrespective of stage of development, genotype, and stress duration. For instance, at days 20 and 30, drought stress reduced chlorophyll content by 37.8% and 63.7%, respectively, during the branching stage under the greenhouse. Furthermore, the levels of ascorbate peroxidase (APX) and proline escalated in response to drought stress irrespective of genotype, developmental stage and stress duration. For example, drought stress increased the APX content by 1.03 and 15.6X higher in stressed plants after 10 and 20 days of drought stress, respectively than control plants during the flowering stage in the greenhouse. This trend was observed in both the greenhouse and field experiments. There was a substantial ($P<0.05$) genetic differences due to drought stress, duration of water stress and phenological stage of safflower as evidenced by different contents of chlorophyll content, LRWC, proline, and APX, and plant height. Additionally, the biplot analysis showed that the genotype Kenya9819 was the most superior and drought tolerant. While the genotype Gila ranked poorly and most susceptible to drought. The results showed that drought stress tolerance was very complex, and it involves several mechanisms either working synergistically or independently based on the traits involved. Therefore, more drought stress tolerance traits need to be studied to make more informed choices during genotype selection and evaluation. In overall, genotype Kenya9819 was found to be a superior genotype because of its greater yield stability (chapter 3),

better oil production (chapter 4) and drought tolerance (chapter 5) followed by genotype Turkey though it was low oil yielding. On the other hand, genotype Gila was the least stable in terms of seed yield and very susceptible to drought stress, however, it produced high oil content making it an ideal genotype for use in breeding for high oil yield.

Chapter 1

Introduction

1.1 Background of the study

Safflower (*Carthamus tinctorius* L.) is a minor oil seed crop that is highly valued for its multiple uses. It has oil of high quality because of unsaturated fatty acids, oleic and linoleic contained in the seeds (Maghsud et al., 2014) which aids in lowering cholesterol level in the blood and reduces the risk of human arteriosclerosis (Gautam et al., 2014). Safflower oil is flavourless, colourless and has minimal allergic responses compared to other functional oils making it suitable in many cosmetic products (Popov & Kang, 2011; Khalid et al., 2017).

Safflower is commonly grown in arid and semi-arid parts of the world since it is drought tolerant and has greater adaptability to low moisture conditions (Öztürk et al., 2008; Janmohammadi, 2015). It has greater adaptation capability in areas with cool winters and arid summer months than other oilseed crops (Erbas et al., 2016). High adaptability of safflower to low moisture conditions is mainly due to its effectual deep root and its many fine lateral roots system which can considerably tolerate periods of moisture shortage (Janmohammadi et al., 2016). Safflower is also adaptable to a wide range of soils, but the best for production are deep, fertile, well-drained soils (Armah-Agyeman et al., 2002). Therefore, safflower can be planted almost in many parts of the world in diversified environments because of its ability to withstand drought, strong winds, hail storms and flooding (Khalid et al., 2017).

1.2 Biology of safflower

Safflower is also known as false saffron because it resembles saffron. It comes from Asteraceae family, genus *Carthamus*, tribe Tubiflorae, sub-division Angiosperm of division Phanerogams (Paikara & Parihar, 2019). *Carthamus* genus comprises of 25 species, with only *C. tinctorius* being the cultivated type, having 2n = 24 chromosomes (Singh & Nimbkar, 2006). It is a herbaceous broad-leaved annual crop with many spines on bracts and leaves (Ekin, 2005; Golzar et al., 2012). Germination of safflower seed can occur at temperatures as low as 2-5º C (Dajue & Mundel, 1996) and it takes around 3-10 days varying with genotype, temperature and growing site (Emongor & Oagile, 2017). The rosette stage, which follows after germination, is a slow growing stage which varies from 20 to 35 days (Gautam et al., 2014; Emongor & Oagile, 2017; Moatshe & Emongor, 2019). At this stage, numerous leaves are produced and strong taproot develop (Emongor, 2010; Emongor & Oagile, 2017). Young safflower plants during the rosette stage are resistant to cold and frost (Dajue & Mundel, 1996; Li et al., 1997; El-Bassam, 2010) but the crop is frost sensitive after the initiation of stem elongation (Dajue & Mundel, 1996; Li et al., 1997; El-Bassam, 2010). The roots of safflower grows deeper, reaching to 3.7 m of the ground depth and at the same time generating several lateral roots (Asgharzadeh et al., 2013).

Following the rosette stage, the stems of safflower elongate quickly and then branch widely (Ekin, 2005). It can grow to height of about 1 m in poor, dry soils in full sun (Gautam et al., 2014). Normally, safflower plants tends to vary in plant height from 0.3-3.0 m with globular heads of different colour shades ranging from yellow to orange and red (Arslan, 2007a; Emongor, 2010; Khalid et al., 2017). The branching pattern of safflower is primary, secondary and tertiary each terminating in capitulum (Shinwari et al., 2014). The primary branches are believed to have less

effect on the yield traits, but the secondary branches are known to have the major role in influencing the safflower yield (Baloch et al., 2015). Each branch normally have from one to five flower heads (capitula) containing 13-71 seeds per head (Popov & Kang, 2011; Emongor & Oagile, 2017). Flower colour in safflower is broadly grouped into four classes: a) yellow in bloom, turning to red on drying, b) yellow in bloom, remaining yellow on drying, c) orange in bloom, turning to dark red on drying, and d) White in bloom, remaining white on drying (Singh & Nimbkar, 2006). Safflower petals have two pigments, carthamin (red) which does not dissolve in water and carthamidin (yellow) that dissolves in water and mainly used as a material for dye (Machewad et al., 2012). The florets are self-pollinated (90-94.5%) but seed set can be increased by bees or other pollinating insects (Kumari & Pandey, 2005; OGTR, 2019; OECD, 2020; Emongor & Emongor, 2023). The seeds are white or cream in colour. They may be with or without pappus and are four sided, having thick pericarp (Singh & Nimbkar, 2006; Emongor & Oagile, 2017). Lower leaves are mostly spineless, however, spines develop during the bud stage and become hard during flowering (Dajue & Mundel, 1996; Emongor & Oagile, 2017). Safflower reaches physiological maturity approximately 30 days past flowering and are harvested when majority of the plant is brownish or with a bit of green colour on the bracts (OECD, 2020). In general, safflower genotypes are divided into two categories based on oil quality, those high in linoleic acid (polyunsaturated fatty acid) and oleic acid (monounsaturated fatty acids) (Armah-Agyeman et al., 2012).

1.3 Global safflower production

Although safflower can be planted in many parts of the world, its cultivation began in the Middle East around 3 000 years ago (Dajue & Mundel, 1996; Erbas et al., 2016). This makes it one of

humanity's oldest flowering crops and was usually grown in small plots for a grower's personal use (Johnston et al., 2002). The 2021 data shows that Kazakhstan, Mexico, Russia, USA, India, Turkey, Argentina, China, Uzbekistan, and Tanzania are the top ten world producers of safflower seed (FAOSTAT, 2021). The major world producer of safflower was Mexico until 1980. However, the area and production of safflower in Mexico decreased significantly in later years (Gautam et al., 2014). India and Ethiopia have the oldest history of safflower cultivation as an oil crop (Ekin, 2005). Safflower was introduced into western countries, such as Italy, France, Spain, and the United States during the 5th to the 14th centuries (Delshad et al., 2018). In Africa, Ethiopia and Tanzania are currently the main producers of safflower seed. In overall, the production of safflower is low world-wide in comparison with that of other oil crops and thus, it has remained a minor oilseed crop (Emongor, 2010). Safflower seed production in the world is below 1 000 000 metric tonnes annually (Figure 1.1). Thereby, safflower oils correspond to 0.10% of total vegetable oil production (Christou & Alexopoulou, 2012). The minor status of safflower has caused accurate production statistics to be difficult if not impossible to acquire (Gilbert, 2008).

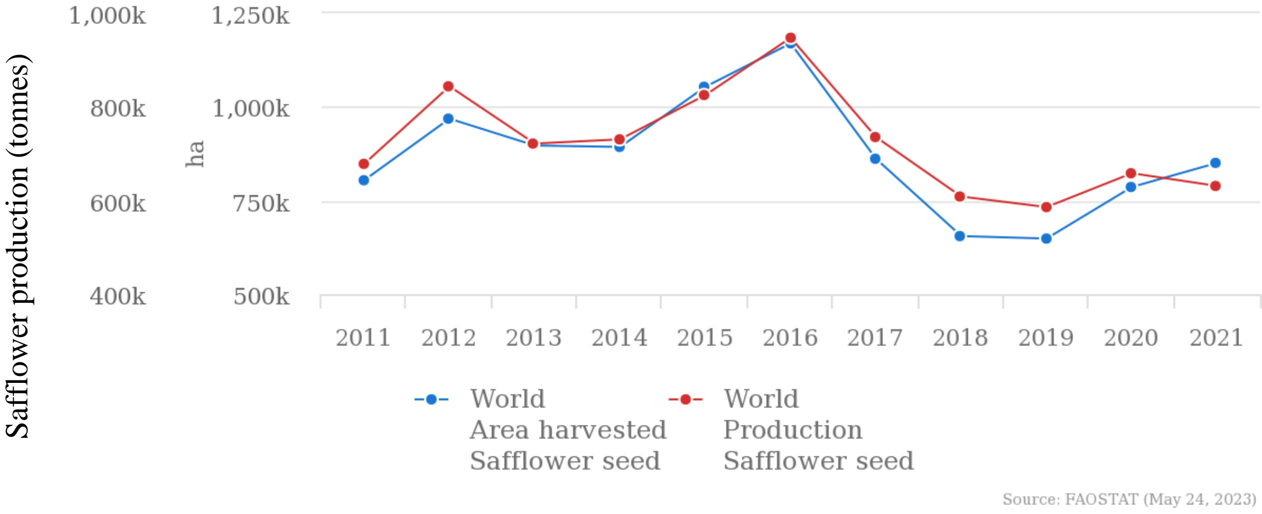

Figure 1.1 World production/yield quantities of safflower seed from 2011-2021. (FAOSTAT, 2023)

1.4 Uses of safflower

1.4.1 Food uses

Safflower vegetable oil is used for cooking, salad dressing, and making margarine and high-quality paints. Its use in frying is mainly because it is stable at high temperatures and thus does not produce odour during frying and it does not change during cold temperatures making it suitable for use in chilled foods (Ekin, 2005). Safflower oil is also used in infant foods and liquid nutrition formulations (Singh & Nimbkar, 2006). Oleic safflower varieties are now preferred as an ingredient in infant formulations because of its high oxidative stability (Dunford, 2012). The young leaves harvested from safflower are boiled and eaten as side dish in India, Pakistan and Burma (Gautam et al., 2014). Safflower shoots, and thinning are consumed as salad and pot herb of which they are high in phosphorus, iron, calcium, and vitamin A (Singh & Nimbkar, 2006).

1.4.2 Industrial uses

The red and yellow pigments from the florets are used as a colourant. Safflower petals are good for colouring cosmetics like body lotion or face cream, shampoo, and perfume (Al-Snafi, 2015), and for textile dye and food preparation (Baloch et al., 2015). Safflower oil of high linoleic acid type is commonly used in the preparation of alkyd resins for paints and varnishes (Liu et al., 2016) while oleic oils are used for cooking (Rehman et al., 2015). However, the recent production of inexpensive petroleum products and the use of water-based paints, reduced the use of safflower oil in the varnish and paint industry (Singh & Nimbkar, 2006). Now safflower is increasingly considered for use in the production of biofuel. Safflower use in biofuel is owed to the newly created varieties that produce oil with 80% monounsaturated oleic acid which is stable at high temperature making them superior for biodiesel production (Mihaela et al., 2013). However, safflower seed oil is presently too expensive for use as biofuel just like most vegetable oil (Popov & Kang, 2011). Safflower oil is also used in the manufacturing of bio-lubricants, surfactants, and alcohols. This oil is also used to prepare roghan, which is used to preserve leather and as a glass cement (Singh & Nimbkar, 2006).

1.4.3 Medicinal uses

Safflower has beneficial effects on medical conditions concerning cardiovascular system, musculoskeletal organ, digestive system (Delshad et al., 2018). Safflower has shown to have a promising antioxidant properties with different modes of action (Ebadi et al., 2014). For example, its antimicrobial as well as antioxidant effects not only can be widely used as a food protective agent, but also may inhibit or retard the progression of certain diseases, namely vitiligo and black spots, psoriasis and mouth ulcers (Delshad et al., 2018). The flowers of safflower are also used to

make herbal tea (Khalil et al., 2013). Since safflower tea has a bitter herbal taste, a non-bitter, sweet-smelling tea which contains amino acids, minerals and vitamins B_1, B_2, B_{12}, C and E have been developed in China (Dajue & Mundel, 1996). The extract of florets containing pigments are utilized in treatment of several ailments such as menstrual problem and swelling related to trauma (Machewad et al., 2012). Moreover, the flowers are known to contain medicinal properties that cure sterility in both men and women (Singh & Nimbkar, 2006). Safflower cultivars with high linoleic acid may be used as health care oil for making medicine for reducing cholesterol in prevention of atherosclerosis and heart disease (Arslan, 2007a).

1.4.4 Animal feed

The byproduct (meal) after oil extraction has protein that is used as an ingredient in animal feeds (Shinwari et al., 2014). This meal usually contains about 19.3-24.0% protein, and a lot of fibre (Popov & Kang, 2011). Safflower is also used as forage and silage for livestock feeding (Asgharzadeh et al., 2013) especially non-spiny types. A suitable livestock forage is achieved if harvested at or just after flowering stage (Christou & Alexopoulou, 2012). Safflower seed oil can be used as a supplement to improve milk fat composition of dairy goats (Shi et al., 2015) and dairy cows (Bell et al., 2006; Dschaak et al., 2010). The seeds can also be used as bird feed (Johnston et al., 2002), broilers (Daffa-alla et al., 2015) and layers (Oguz et al., 2007).

1.4.5 Other uses

In Egypt, safflower was used as ceremonial ointment in religious ceremonies and to anoint mummies prior to binding (Al-snafi, 2015). Safflower can also be used as a cut flower (Emongor, 2010) and it has gained importance in floriculture as a consequence of higher demand for dried

flowers (Uher, 2008). Safflower straw can be used in a similar way as cereal straw (Dajue & Mundel, 1996) thus it can be used in the production of biogas.

1.5 Problem statement

Botswana has a semi-arid climatic condition, thereby, the temperatures are extreme in both winter and summer with incidences of high evapotranspiration rates during summer. The temperatures can drop below freezing point in some parts of the country in winter and can rise above 37°C in some summer months (Jain et al., 2006; Statistics Botswana, 2016). The average precipitation is between 350 and 550 mm (Statistics Botswana, 2016) and it occurs mainly in summer often with dry spells for extended period. Even though safflower is known to do well under diverse environments, evaluation of its performance in Botswana climatic condition is still at an infancy stage. Very few genotypes have been evaluated in varying locations in Botswana. In addition to evaluating the productivity of genotypes across different locations, it is also important to know the performance of each genotype across different planting seasons (summer and winter). This is due to the fact that summer crop genotypes from temperate regions sown as a winter-crop in sub-tropical and tropical regions, tend to have a lengthy rosette stage, which greatly delays maturity (Dajue & Mundel, 1996). Thus, as part of introducing safflower in Botswana, it will be of major importance to know the interaction of genotype and environment on the productivity of safflower.

Currently, there is a growing interest in extracting seed oil in the form of oil bodies. This is mainly because oil bodies have the potential to be used in the food industry, in preparation of cosmetic products and medication hence makes safflower a perfect candidate for such technology because of its high-quality oil that also contain low allergic reactions. Oil bodies can be used as an

ingredient in salad dressings, beverages, dairy like food, sauces, edible films, coatings and hair products (Nikiforidis et al., 2014; Cai et al., 2018). Therefore, studies on oil body size and their associated oleosins proteins are of great importance in the establishment of this new technology. Studying the expression patterns of oleosin genes of different safflower genotypes may aid in engineering plants with high seed oil content and with small, stable, and non-coalescence seed oil bodies which are ideal for use in the food industry. However, studies comparing oleosin gene expressions among safflower genotypes in relation to oil body size and oil content are lacking in literature. Such studies may guide in selection of high oil yielding genotypes and breeding for high seed oil content by optimizing the oleosin level.

1.6 Justification

These harsh weather conditions prompt the cultivation of climate smart crops that can survive extreme conditions. One such climate smart crop is safflower which has a long tap root to scavenge for soil moisture and has also shown to tolerate other harsh conditions such as cold, salinity, alkali and has lower demands for fertilizers (Arslan, 2007b). The crop has shown high potential as an oil crop and thus it will also serve as an important economic crop in Botswana. Since safflower is a multipurpose crop, its cultivation may help in diversification of the economy and help to enhance the socioeconomic status of several smallholder farmers in Botswana. In addition, safflower has a great potential in the market share because all its parts (leaves, flowers, stems, and seeds) can be utilized for different purposes (medicinal, food, feed and industrial). Thus, the successful adoption of this crop in Botswana may offer numerous benefits across different fields; for example, the livestock sector especially the declining cattle production (Statistic-Botswana, 2019) may benefit more from safflower meal due to high protein content. Seed oil from safflower may reduce reliance

on the importation of cooking oils and promote local production of healthy vegetable oil. Information on how safflower genotypes interact with the environments will be useful in selection of the most stable and adaptable genotypes. This is because farmers in developing countries, use no or limited inputs and grow safflower under harsh and unpredictable environments, and they need more stable varieties (Mohammadi et al., 2008). Moreover, planting safflower on the farmers' fields will help farmers appreciate the crop and thereby, accelerating its adoption in Botswana. Although safflower is identified as a drought tolerant crop, studies have shown that genotypes of a similar crop respond differently to drought stress and thus, information on how different safflower genotypes respond to drought stress may help in selecting a genotype which is most tolerant to drought. Therefore, studying the growth and antioxidants activities may help in a better understanding of the drought tolerance mechanism of safflower.

1.7 Objectives

1.7.1 General objective

To evaluate different safflower genotypes across different sites in the Southern region of Botswana as a way of selecting the most stable and adaptable genotypes for the region and to evaluate the drought tolerance mechanisms of each genotype in order to promote their adoption, thereby contributing to food self-sufficiency in Botswana.

1.7.2 Specific objectives

I. To evaluate the effects of environment, genotype and their interaction on the growth, phenological development, and oil content in selection of the most adaptable and stable genotypes across all the study sites.

II. To assess the relationship between oleosin gene expression, oil body size and the oil content of safflower genotypes.

III. To determine the growth and the activities of drought related antioxidants (proline and ascorbate peroxidase) among safflower genotypes under drought stress.

1.8 Hypothesis

1.8.1 Hypothesis for objective one

Ho: The environment, genotype and their interactions does not have a significant effect on the growth, development, and oil content of safflower.

Ha: The environment, genotype and their interactions have a significant effect on the growth, development, and oil content of safflower.

1.8.2 Hypothesis for objective two

Ho: There is no relationship between oleosin gene expression, oil body size and the oil content of safflower genotypes.

Ha: There is a relationship between oleosin gene expression, oil body size and the oil content of safflower genotypes.

1.8.3 Hypothesis for objective three

Ho: There are no significant differences on the growth and on the activities of drought related antioxidants among safflower genotypes under drought stress.

Ha: There are significant differences on the growth on the activities of drought related antioxidants among safflower genotypes under drought stress

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Chapter 2

Literature Review

In relevance to evaluating safflower genotypes for their possible adoption in Botswana, this literature review focuses on three subjects. These subjects are: The growth, development, oil content, and yield of safflower as affected by genotype and environmental interaction, use of oleosin genes and oil bodies in selection of high oil yielding genotypes, and how safflower genotypes respond to drought stress conditions focusing on mechanism that help in tolerance to stress. The review will end with the identification of existing research gaps.

2.1 Safflower growth and development

The environment that influences plant productivity are relative humidity, rainfall, temperature, soil conditions, and agronomic practices. Thus, for the purpose of this study planting season, planting location, and year of planting will be collectively termed as environmental factors. The soil attributes such as pH, organic matter, gravel content, CEC, salinity and alkalinity, taken either alone or in combination, have an influence on the land suitability for safflower (Jafarzadeh et al., 2008). Safflower genotypes showed high sensitivity to growth in soil with physical impediments, except for only few of them which were more stable at soil density variations (Bonfim-Silva et al., 2018). Safflower may grow well under a wide range of soil types but during drought conditions, growing safflower on sandy soils without supplemental irrigation could result in significant yield loss while planting safflower on clay soil under similar conditions without supplemental irrigation may lead to stable yields. For example, Ferreira et al. (2017) found that in the sandy soil, the height of safflower plants under irrigation were higher than in the clayey soil, although it was lower in

drought conditions in sandy soil. Thus, it could be argued that in areas receiving a high amount of rainfall planting safflower in clayey soils should be avoided with preference being given to planting in a well-drained soil. This is because safflower plants are not tolerant to waterlogging and it predisposes the plants to root diseases (Emongor & Oagile, 2017; Emongor & Emongor, 2023).

Different planting seasons result in large variations in climatic conditions like rainfall and temperature as such seasonal variation plays a substantial role in the productivity of safflower. For instance, water deficit is known to disturb the balance of the nutritional characteristics of plants (Chakraborty et al., 2016). However, excess rainfall, especially after flowering begins, promotes the incidence of many leaf and capitula diseases, which reduce yields and even cause crop death (Dajue & Mundel, 1996; Emongor & Emongor, 2023). Furthermore, decrease in moisture content was found to increase the oil content during seed development in safflower (Gecgel et al., 2007). Just like moisture, temperature also influences the development of plants. Temperature is amongst the principal factors that control plant distribution and production (Fei et al., 2018). All physiological and morphological developments occurring in plants are markedly influenced by temperature (Sage & Kubien, 2007; Shabana et al., 2013). For example, emerging safflower plants need cool temperatures (15-20°C) for root development as well as rosette growth, and high temperatures (20-30°C) during stem growth, flowering and seed formation (Li et al., 1997; Carapetian, 2001; El-Bassam, 2010). Moreover, Singh et al. (2016) highlight that a significant decrease in percent germination was observed at a temperature lower than 5°C and higher than 30°C. They further found that there was no germination of safflower seeds at temperatures below 3°C and above 38°C. Although Mihaela et al. (2013) highlighted that safflower can survive during

summer temperature of 43°C even in conditions of prolonged drought, planting in such high temperatures is often associated with poor plant growth, development and consequently yield reduction. Thus, temperature is a yield determining factor in safflower which needs to be taken into consideration in breeding programs. Winter temperatures have been found to increase the rosette stage duration and consequently increasing plant cold hardiness (Yasari et al., 2016; Moatshe et al., 2020; Emongor & Emongor, 2023). Therefore, compared with spring sown types, genotypes with this prolonged rosette character tend to have increased cold tolerance and are considered as winter types (Landry et al., 2017). Among other seasonal variables, photoperiod also causes significant variations in the growth and phenology of safflower (Dajue & Mündel, 1996; Moatshe, 2019; Emongor & Emongor, 2023). For instance, increasing temperature and daylength towards the end of winter and spring, promotes rapid plant growth and the central stem begins to elongate and branch (Singh & Nimbkar, 2006; Wachsmann et al., 2010; OECD, 2020; Emongor & Emongor, 2023). In long day plants such as safflower, flowering rate accelerates as photoperiod increases and it flowers in photoperiods of above 12 hours per day (Gilbert, 2008; Torabi et al., 2020). Generally, the photoperiod decides the flowering time in accordance with the gene composition and the accumulated light hours during vegetative growth period (Gilbert, 2008; Sharma, 2009).

2.2 Effects of plant genotype, planting season and location on the growth and development

2.2.1 Days to emergence

Planting season has been identified as one of the factors that affects the number of days to emergence. Safflower grown in winter was found to have delayed emergence with an average of 9.32 days to emergence which was significantly longer than 8.99 days taken by seeds grown in

summer (Moatshe & Emongor, 2019). The variation in days to emergence in different planting season was explained by the differences in temperatures between seasons (Emongor et al., 2017; Moatshe & Emongor, 2019). Torabi et al. (2015) demonstrated that higher temperatures of 20-27°C decreased emergence of safflower seedlings. They further revealed that a lower temperature of 7°C increased the number of days to emergence. Wachsmann et al. (2010) and Emongor and Oagile (2017) further highlighted that emergence of safflower occurs at 2-5°C but at a slower rate.

2.2.2 Days to flowering

The impact of genotype on the number of days to flowering has been reported in safflower (Bagheri et al., 2001; Singh, 2007; Archibald, 2011; Golkar, 2014; Bahmankar et al., 2016; Moatshe et al., 2020a). Moatshe (2019) in Botswana revealed that safflower plants took 82-117 days after sowing to reach flowering depending on plant genotype and planting season. Shinwari et al. (2014) found that the mean value of the safflower genotypes for days to flower initiation was 175 days, with a range of 160 to 188 days in Islamabad, Pakistan. Another study in Egypt by Shabana et al. (2013) showed that safflower genotype G48 flowered earlier (113.11 days) than Giza1 (120.89 days). Ahadi et al. (2011) found that the number of days to flowering of safflower was inversely correlated to daylength because of environmental conditions. According to Wachsmann et al. (2010) flowering in safflower was influenced by daylength than by time of sowing in Australia. Kumar et al. (2016) attributed delayed safflower flowering in the second year to cooler temperatures than in the previous year in Delhi, India. While Dajue and Mundel (1996) and Singh et al. (2008) explained that the reason for higher genotypic differences in the number of days to flowering was probably because days to flowering is a quantitatively trait which is influenced by dominance, additive and epistatic gene effects. The importance additive and dominance influence

in the genetic control of early maturity in safflower was reported by Golkar (2011). Golkar (2011) further reported dominant gene effects in the control of days to flowering in safflower. However, Gupta and Singh (1988) reported partial dominance in genes that control days to flowering in safflower. While Kotecha (1979) and Shahbazi and Seadi (2007) reported predominant influence of additive gene action in the control of days to maturity in safflower. While Gupta and Singh (1988) reported over dominance of gene action on the same trait. The cause of variability in the above results could be attributed to genotype and environmental interactions due to different growing conditions. The number of days to flowering has been found to be affected by the interaction of safflower accession and planting location (Hussein et al., 2018; Sajid et al., 2021; Zhao et al., 2022). Sajid et al. (2021) explained that variations among locations in relation to days to flowering was possibly due to differences in the duration of photoperiod among the study locations.

2.2.3 Days to maturity

Past studies have shown that genotypes vary in time taken to reach physiological maturity in safflower (Bagheri et al., 2001; Singh, 2007; Archibald, 2011; Golkar, 2014; Bahmankar et al., 2016; Golkar et al., 2017). Moatshe et al. (2020a) found that safflower took 100-146 days after sowing to reach physiological maturity when grown in Botswana depending on genotype and season. Shinwari et al. (2014) found that days to maturity among safflower genotypes ranged between 208 to 229 days. While Shabana et al. (2013) also found that the means of days to maturity of safflower genotypes were significantly different with the genotype Entry48 having the greatest number of days to maturity 147.11 days followed by Giza 1, Entry 45 and Entry 21 with 156.44, 158.33 and 159.50 days, respectively. However, Arslan and Culpan (2018) revealed that the

highest number of days to maturity among the genotypes was 133.27 days while the shortest days to maturity was 118.61 days, suggesting existence of early maturing genotypes which can be a source of early maturing genes. Ahadi et al. (2011) showed that physiological maturity of safflower plants was delayed mainly because of declining mean temperature and shorter photoperiod which delayed the rosette stage. The number of days to maturity have been found to vary with planting location (Sajid et al., 2021). Temperature is amongst factors that contributes to differences in number of days to maturity across locations is temperature, with high temperatures being associated with earlier maturity (Emongor & Oagile, 2017). As a result, Zanetti et al. (2022) highlighted that thermal time a useful predictor of safflower maturity and harvest time. However, this was contrary to the findings of Hussein et al. (2018), who found that safflower plant maturity was not influenced by planting location. This is possibly because their study locations had less environmental variations and or their genotypes were very stable and adaptable to environmental variations.

2.2.4 Plant height

It has generally been found that safflower plant genotypes affects plant height (Vorpsi et al., 2010; Soleymani et al., 2011; El-Lattief, 2012; Killi et al., 2016). Some genotypes were found to exhibit higher plant height of 199.7 cm (El-Lattief, 2012), while other genotypes were found to show extremely lower values of 40.15 cm (Killi et al., 2016). The reason for variations among genotypes for the number of branches was probably due to additive gene effects in the genetic control of plant height (Golkar et al., 2012). Moatshe et al. (2016) found that winter sown safflower was noticeably tall in comparison to summer grown plants with differences of 46.25%, 39.22%, 29.20%, 40.06%, 37.77% and for the genotypes Kiama, PI-537636, Sina, Gila, and PI-527710 respectively, in plant

height. Similarly, Emongor et al. (2017) found that safflower plants grown in summer were remarkably shorter than winter grown plants, which were taller by 64.3% than safflower grown in summer. They further explained that this seasonal variation in vegetative growth was mainly attributed to climate especially variation in temperature between winter and summer. Naseri (2011) highlighted that winter grown safflower attained tall plant height possibly because of minimal temperatures and favourable moisture regime which led to a longer vegetative growth. Plant height has been found to be influenced by genetic and environmental interaction (Camas et al., 2007; Hussein et al., 2018; Sajid et al., 2021; Zhao et al., 2022). This was mainly attributed to variations in temperature, rainfall, soil type and fertility that were in different locations.

2.2.5 Primary branches per plant

Genotypic variation in the number of branches per plant of safflower is widely documented (Vorpsi et al., 2010; El-Lattief, 2012; Killi et al., 2016; Arslan & Culpan, 2018). Some genotypes have fewer number of branches per plant than other genotypes (Arslan & Culpan, 2018). The high variation in the number of branches per plant among safflower genotypes could be attributed to additive and non-additive genetic effects that are important in the inheritance of branches (Golkar et al., 2012). Emongor et al. (2017) found planting season to have a substantial impact on the number of primary branches per plant, with the greatest number of branches (16.1) being in the winter grown safflower while the lowest number of branches (9.5) being in the summer grown safflower. Similarly, Bell et al. (2012) reported a greater reduction in number of branches (41%) of safflower during spring/summer growing season than fall/winter season. Wachsmann et al. (2010) pointed out that early sowing in winter/autumn allows more time for a large rosette stage and an extensive branch structure to develop, creating high yield potential. Abbadi and Gerendas

(2012) hypothesized that cultivars which produce lower number of branches enhanced safflower plants to produce more achenes per capitulum and mature quickly avoiding the wet period at physiological maturity which reduces achene yield and achene quality. Planting location has also been found to cause a marked effect on the number of branches in safflower (Hussein et al., 2018; Sajid et al., 2021; Zanetti et al., 2022).

2.3.6 Number of capitula per plant

It is well established that the number of capitula per plant plays a crucial role in determining safflower grain yield (Bagheri et al., 2001; Emongor et al., 2015; Emongor & Oagile, 2017). Studies on the influence of safflower genotypes on the number of capitula per plant are many and varied (Chaundry, 1990; Vorpsi et al., 2010; El-Lattief, 2012; Arslan & Culpan, 2018). Arslan and Culpan (2018) reported genotypic variation in number of capitula per plant. The reason for genotypic differences in the number of capitula per plant was explained by the predominant non-additive gene effects of genes on capitula number per plant of safflower (Golkar et al., 2012; 2017; Golkar, 2014). Moatshe et al. (2016) found that all safflower genotypes under study developed more capitula number per plant in winter as compared to summer with exclusion of genotype PI-527710. Naseri (2011) reported that safflower planted earlier in winter tended to have longer vegetative period which resulted in the production of more branches and number of capitula within a plant. Number of capitula per plant has also been found to vary greatly with planting location (Hussein et al., 2018; Sajid et al., 2021). Climatic factors such as excess rainfall promotes development of capitula diseases and subsequently reducing the number of capitula within a plant. On the contrary, Zanetti et al. (2022) reported that the number of capitula per plant was not affected by planting location.

2.2.7 Thousand seed weight

Several studies revealed that there is important genotypic variation with respect to 1000-seed weight of safflower (Arslan et al., 2003; Mahasi et al., 2006; Hamza, 2015; Moatshe, 2019). Moatshe (2019) found that 1000-seed weight of safflower varied between 13.7-61.0 g differing with growing season, plant density, and genotype. While Emongor et al. (2017) found that 1000-seed weight of safflower varied between 34.0-51.8 g differing with genotype and growing season. Likewise, Abd El-Lattief (2012) found that there was a significant difference of 1000-seed weight of among safflower genotypes, with the maximum weight of 49.13 g observed from line 1687. The genotypic variation observed among safflower genotypes has been attributed to additive gene effects in controlling seed weight (Golkar et al., 2012; 2017; Golkar, 2014). While Shahbazi and Saeidi (2007) reported additive-dominance gene effects in 1000-seed weight of safflower. Planting location was reported to significantly contribute to the genetic variation of 1000-seed weight among safflower genotypes (Hussein et al., 2018; Zhao et al., 2022). Studies observed substantial genetic and environmental interaction for 1000-seed weight of safflower (Uysal et al., 2006; Camas et al., 2007; Beyyavas et al., 2011).

2.2.8 Seed yield

Research has shown that safflower genotypes have a large variation in seed yield (Mokhtassi-Bidgoli et al., 2007; Pahlavani et al., 2007; Beyyavas et al., 2011; Zareie et al., 2013; Asghar & Younes, 2015; Arslan & Culpan, 2018; Moatshe, 2019). Moatshe (2019) reported that safflower seed yield varied between 2140 to 578 kg/ha in Botswana differing with genotype, plant density and planting season (winter or summer). While Oarabile (2017) observed a significant genotype

and growing season effect on seed yield of safflower planted in Botswana. The safflower seed yield ranged between 888-3113 kg/ha varying with genotype and planting season with genotypes PI537598-SINA-USA and PI407616-BJ-2131-Turkey yielding highest and lowest, respectively in all growing seasons. Research in Turkey showed that safflower seed yield was 570-2515 kg/ha varying with genotype and growing conditions (Gur & Ozel, 1997; Ozel et al., 2002; Uysal et al., 2006; Beyyavas et al., 2011). Hamza (2015) in Egypt found seed yield of 512-2846 and 1978-2510 kg/ha, respectively, differing with genotype. Arslan and Culpan (2018) reported a genotype which produced a very low seed yield of 147.90 kg/ha. Killi et al. (2016) postulated that the high variation in seed yield of safflower was due to environmental and genetic interaction. Moatshe et al. (2016) found that Sina consistently yielded higher safflower seed yield than other genotypes under study in both winter and summer with an average of 5568 kg/ha and 5107 kg/ha, respectively, demonstrating existence of genotypes with stable yields across different growing seasons. This was supported by Hussein et al. (2018) who found that genotypes which had non-significant interaction with seasons had stable performance over seasons. Lower yields in summer sown genotypes might be because in cases of excessive rainfall during grain filing the seed heads can easily get saturated by rain causing the staining of seeds hence reducing its value and also sprouting seeds (GRDC, 2010). According to Christou and Alexopoulou (2012) yields of safflower are low under humid or rainy conditions because seed set was reduced by the occurrence of leaf spot and head rot diseases increases. Armah-Agyeman et al. (2002) also reported that rain during flowering and seed-filling increased disease outbreak and prevented proper seed set, which led to reduction in seed yield.

Planting location was also identified as one of the yield determining factors in safflower production (Alizadeh et al., 2008; Abdulahi et al., 2009; Hussein et al., 2018; Zanetti et al., 2022; Zhao et al., 2022). Zanetti et al. (2022) observed that weather conditions significantly affected seed yields of safflower, despite its high adaptability to different environments. Similarly, Camas et al. (2007), also emphasized that marked yield differences experienced among tested among locations could be ascribed to the climatic variations. In addition, de Oliveira Neto et al. (2022) highlighted that significantly low grain yield averages observed in one of their study sites was explained by the high rainfall in the region which caused waterlogging that stressed safflower plants.

Safflower seed yield is regulated by additive gene effects (Golkar et al., 2012; 2017; Golkar, 2014). Broad-sense heritability of seed yield of safflower was reported to be high (Falconer & Mackay, 1996; Camas & Esendal, 2006; Golkar, 2014). However, other types of genetic effects could be involved in genotypic variation of safflower seed yield (Mather & Jinks, 1982; Golkar et al., 2012; 2017; Golkar, 2014).

2.2.9 Seed oil content

Safflower seeds comprise of 16.1-64.6% oil content as influenced by genotypes, environmental conditions, and agronomic practices (Knowles, 1989; Dajue & Mündel, 1996; Killi et al., 2016; Khalid et al., 2017; Moatshe et al., 2020b; Emongor & Emongor, 2023). The seeds generally contain 55-65 % kernel and 33-44 % hull (Gecgel et al., 2007; Emongor & Emongor, 2023). Generally, safflower has less oil content compared to some important oilseed crops like peanut, rapeseed, sunflower, and sesame (Erbas et al., 2016), but its quality is high due to its fatty acid composition comprised mainly of polyunsaturated and monounsaturated fatty acids linoleic and

oleic, respectively. The amount of oil in a seed is a vital economic attribute for safflower varieties and arguably amongst the essential traits determining the success of safflower adoption by farmers in non-native locations (Bassil & Kaffka, 2002; Singh & Nimbkar, 2006; OGTR, 2015; AI Surmi et al., 2016). The hull content affects oil content, thus, a decrease in the hull content was reported to directly increase oil content in safflower (Knowles, 1989; Gautam et al., 2014). On the basis of genetic, physiological and morphological factors of pericarp structure (Denis et al., 1994; Figueiredo et al., 2013) and seed weight (Fernandez-Martinez, 2002; Ensiye & Khorshid, 2010; Figueiredo et al., 2013) of safflower differences in seed oil content and yield are observed among genotypes (Smith, 1996; Omidi et al.; 2009; Fernandez et al., 2012). Omidi et al. (2009) reported a positive relationship between kernel/pericarp percentage and oil content and recommended thin hull seeds as they produced a high oil content, hence are essential for selection in breeding. It was also observed that varieties of safflower with reduced or absent spines tends to have lower oil content than spiny varieties (Gautam et al., 2014; Emongor & Oagile, 2017; Moatshe, 2019).

Several studies have demonstrated existence of genotypic variation in the oil content of safflower (Soleymani et al., 2011; Emongor et al., 2017; Arslan & Culpan, 2018; Saisanthosh et al., 2018). Moatshe et al. (2020b) found that safflower genotypes had oil contents of 17.3-56.6% as influenced by plant density and growing season. The genotypes Sina, PI527710, and PI537635 had consistently high oil content, however, the genotype Gila produced the least oil content of 17.3% (Moatshe et al., 2020b). Saisanthosh et al. (2018) and Arslan and Culpan (2018) reported safflower genotypes which produced 43 and 15.6 % oil content, respectively. While Tobeh et al. (2012) found that there was no significant variation in oil content among safflower cultivars. The large variation in oil content among safflower genotypes could be due to genetic differences in hull

content, presence or absence of spines, and oil biosynthesis (Gautam et al., 2014; Moatshe et al., 2020b). Cosge et al. (2007) reported that safflower varieties with spines produced more oil than spineless varieties. Kose et al. (2018) postulated that the variation in oil content was possibly the result of selection made in terms of oil content along breeding programs and the difference between the lines and varieties was strongly due to the genotypes, together with environmental conditions. Genotypic variation in safflower oil content has been attributed to additive gene effects (Golkar et al., 2011; Golkar, 2014), allelic dominance (Ramachandran & Goud, 1981; Gupta & Singh, 1988), and epistatic effects (Pahlavani et al., 2007).

Studies on the influence of planting season on the oil content of safflower are many and varied (Cosge et al., 2007; Oz, 2016; Arslan & Culpan, 2018; Moatshe et al., 2020b). Some studies reported that safflower oil content was significantly affected by planting season with the oil content ranging from 24.53-28.47 %, 21.23-25.76% in winter and spring sowing, respectively (Cosge et al., 2007). Another study highlighted that a higher oil content was obtained from the autumn sown (27.42%) than spring sown (26.10%) safflower (Oz, 2016). Generally, safflower requires warm weather conditions for increased oil contents in the seed (Armah-Agyeman et al., 2002; Emongor & Oagile, 2017). A larger seasonal variation in oil content of safflower was mainly because of large differences in climatic conditions among planting seasons. The high oil content in winter planted safflower especially in areas experiencing mild winters is attributed to the longer growth period than summer grown safflower (Emongor et al., 2015; 2017; Moatshe, 2019). Safflower oil content has also been reported to vary with planting locations (Camas et al., 2007; Hussein et al., 2018; Zanetti et al., 2022; Zhao et al., 2022). Sajid et al. (2021), postulated that variations in safflower oil content among locations were attributed to differences in temperatures across

locations. Additionally, Zhao et al. (2022) observed that variations in oil content across various sites were due to water stress with higher rainfall leading to higher oil content.

2.2.10 Oil yield

Several literature have revealed existence of genotypic variation in the oil yield of safflower (Soleymani et al. 2011; Hamza, 2015; Arslan & Culpan, 2018; Moatshe, 2019). Moatshe (2019) reported that in Botswana the safflower oil ranged between 421-2990 and 538-2622 kg/ha in safflower planted in winter and summer, respectively, but influenced by genotype and plant density. Emongor et al. (2017) researching on nine safflower genotypes in Botswana established that oil yield varied between 226 to 1313 kg/ha varying with genotype. The genotype Sina had markedly high oil yield of 1313 kg/ha, followed by genotype PI537632 (692 kg/ha), and genotype PI30441 produced the least oil yield of 226 kg/ha (Emongor et al., 2017). Arslan and Culpan (2018) and Hamza (2015) reported safflower genotypes that produced 10.3 and 860 kg/ha of oil, respectively. While Kose et al. (2018) reported high oil yield was associated with high seed yield which was affected by genotype oil content and ecological conditions of planting site. Cosge and Kaya (2008) demonstrated that safflower oil yield from late-autumn sowing was approximately twice higher than the one from late-spring sowing. This was in conformity with the results of Sampaio et al. (2018) who found that the cultivation of safflower in the autumn in Brazil favoured the crop due to the larger photoperiod and long days which advanced the plant cycle, since the solar interception was greater. Moreover, lower yields in spring sown safflower harvested in summer, might be because of excess rainfall experienced during the planting season, especially after flowering begins resulting in increased incidence of plant diseases, which may reduce yields and even cause crop loss (Dajue & Mundel, 1996; Sampaio et al., 2018). Oil yield has been found to vary with planting location (Ebrahimi et al., 2016; Zanetti et al., 2022). Variations in oil yield

across locations was due to temperature stress, water stress, photoperiod and diseases that affected seed yield and oil content (Ebrahimi et al., 2016; Zanetti et al., 2022).

2.3 Genotype x environment interaction

Genotype environment interaction (G×E) is the variation in the response of a genotype grown in a different environment. It is very important in determining the stability and adaptation of many field crops. There are plant genotypes that are adapted to a broad range of environments, whereas some have restricted distribution and hence, having particular adaptation (Mohammadi et al., 2008). Thus, genotypes chosen from individual environment may not be able to keep their high performance in another environment because of G×E interaction effect (Li et al., 2017). Therefore, the stability and adaptability of a cultivar during growth is determined in part by the G×E interaction (Liu et al,. 2017). Both stability and adaptability appear to decrease as the effects of G×E interactions become larger (Liu et al., 2017). A genotype is considered to be stable if its G×E interaction variance is small or if its response to environments is parallel to the mean response of all genotypes in the trial and/or if the residual mean square from the regression model on the environmental index is small (Lin et al., 1985; Liu et al., 2017). The phenotype of an individual is controlled by its genotype, the environment and any interactions between the environment and genotype (Li et al., 2017). Therefore, the most stable genotypes alter their phenotypic responses to remain stable despite of environmental variations (Singh et al., 2014). Stable and adaptable genotypes are usually recommended to be grown under various environments and can also be used in breeding programs.

Multiple environmental trials are conducted to identify superior cultivars for the target regions (Alizadeh et al., 2008). Evaluation of G×E based on several years and locations is a good strategy used in breeding and selection programs to develop improved varieties (Tabrizi, 2006; Mohammadi et al., 2008). Yan and Frégeau-reid (2018) postulated that multi-year and multi-location data can be used to investigate whether there are any repeatable G×E patterns and if yes, the patterns can be used as a guide to divide the target region into meaningful sub-regions or mega-environments (ME). They further stated that if there are no repeatable G×E patterns, then the target region should be treated as a single ME. Generally, a high number of genotypes are tested in several locations and years, and mostly determining the pattern of genotypic responses among environments without the help of graphical display of the data is not easy (Yan et al., 2001). The basic reason for the difficulty is that a genotype's response to environments is multivariate (Lin et al., 1985). Thereby, a multivariate analysis makes it possible for the genotypes with similar responses to be clustered and thus the data can be summarized and analyzed more easily (Mohammadi et al., 2008).

Öztürk et al. (2008) found marked differences in genotype × year interactions for safflower seed yield, plant height, seed number per head and oil content in both irrigated and non-irrigated experiments indicating that genotypes behaved differently in terms of all variables across all years under study. Abdulahi et al. (2009) used AMMI model to study the G×E of 16 safflower genotypes planted under 18 environments on seed yield and their results revealed that the agro-climatic conditions of the environments were different, and that there was a differential response of the genotypes to environments. They further showed that the environmental effect on seed yield was higher than the G×E interaction, but the G×E effect was approximately three times higher than the

genotypic effect. The results of Abdulahi et al. (2009) were in conformity with those of Mohammadi et al. (2008). Mohammadi et al. (2008) found significant main effects of 17 genotypes, 26 environments, and G×E interaction on grain yield of safflower. Their results further revealed that 83.78% of the treatment sum of squares was mainly due to environmental effects with only 1.37% and 14.85% being attributed to genotype and G×E interaction effects, respectively. Similar findings in safflower have been reported (Moghaddam & Pourdad, 2009; Alivelu et al., 2015; Ebrahimi et al., 2016; Hussein et al., 2018). All these studies revealed that seed yield of safflower is largely affected by the environment.

2.4 Identification of the high oil yielding genotypes based on the expression patterns of oleosin gene.

2.4.1 Oil bodies and oleosin gene expression in seeds

Seeds store lipids in the form of small spherical intracellular organelles, called oil bodies, with size ranging between 0.5 and 2.0 µm diameter (Leprince et al., 1997). Lipids are stored as triacylglycerols (TAGs) in lipid droplets (Hsieh & Huang, 2004; Chapman et al., 2012; Chapman & Ohlrogge, 2012; Miquel et al., 2014). Lipids provide energy to germinating seeds and for seedling growth (Tzen et al., 1993; Murphy, 2004; Miquel et al., 2014; Lu et al., 2018). Oil bodies are amassed during seed maturation (Peng & Tzen, 1998; Murphy, 2004; Schmidt & Herman, 2008). In high oil seed crops, lipids fill much of the cytoplasmic space by onset of dormancy (Schmidt & Herman, 2008; Miquel et al., 2014). The seeds contain a large number of oil bodies and small amounts of water which ensures oil body stability (Cai et al., 2018).

Oleosins are low molecular weight alkaline proteins of approximately 15–26 kDa depending on plant species that accumulate on the surfaces of oil bodies (Lee et al., 1991; Peng & Tzen, 1998; Murphy, 2003). Oleosins are the most abundant proteins associated with oil bodies and are usually present as two or more isoforms (Tzen et al., 1990; 1993; Peng & Tzen, 1998; Murphy, 2004; Siloto et al., 2006). Oleosins are classified according to their relative molecular weight as either high or low M_r (Peng & Tzen, 1998). The oleosins do not have enzymatic domains and are rather inert structural proteins whose function is to stabilize the lipid bodies in developing seeds, mature seeds, and as the recognition signals for lipase biding in germinating seeds (Ling, 2007; Schoot et al., 2011). Oleosins also play an vital role in freezing tolerance of seeds (Shimada & Hara-Nishimura, 2010). They cover the whole surface of the oil body and represent 1-4% of the total mass of the oil body (Ting et al., 1997). Oleosins contain regions of highly conserved amino acid sequences which have facilitated the cloning of genes from many plant species and families (Capuano et al., 2007). They have three structural domains consisting of an amphipathic N-terminal domain, a central hydrophobic domain and a C-terminal amphipathic domain (Roberts et al., 2008). The central hydrophobic region constitutes the longest known continuous region of hydrophobic amino acids known in any protein and is unique to these proteins (Herman, 2009). The formation of oleosin results in the deposition of discrete oil bodies in plant tissues, stabilizes the oil body surface (Li & Fan, 2009) and prevent coalescence of the oil droplets (Alexander et al., 2002). The distribution of oleosins is, therefore, consistent with a role in the biogenesis and stabilization of oil bodies, ensuring the maintenance of an appropriate size and volume ratio to balance the conflicting needs for efficient storage and ease of mobilization (Capuano et al., 2007).

Oil bodies are found also in other plant tissues such as fruit but only pollen and seeds produce oleosins where the oil bodies are subjected to developmentally regulated desiccation and hydration (Schmidt & Herman, 2008). The expression of oleosin genes are tissue specific (Fang et al., 2014; Miquel et al., 2014) and it is commonly expressed in seeds and floral anthers (Shimada & Hara-Nishimura, 2010). Although oleosins appear to be universally present in seeds which store oil, this is not the case for oil storing fruit (Capuano et al., 2007).

The presence of oleosins enables oil bodies to be maintained as relatively small and discrete organelles with a relatively large surface area to volume ratio (Leprince et al., 1997). Siloto et al. (2006) found that accumulation of oleosins determines the size of oil bodies in Arabidopsis. Furthermore, Lu et al. (2018) also found that in safflower the accumulation of oleosin did not only determined the size of the oil bodies in seeds, but also regulated their oil content. Therefore, the size of the oil bodies is partially controlled by the oleosins synthesized during seed maturation (Huang, 1996). The results of Hu et al. (2009) showed that in rapeseed, transcript abundances of the oleosin1 gene was higher in three high oil cultivars than in two low oil cultivars. They further found that the low accumulation of oleosins resulted in the formation of unusually large oil bodies in low oil cultivars of rapeseed. Similarly, Ho et al. (2014) found that the mean size of lipid bodies of mesocarp tissue for high oil yield palm were significantly lower than the lipid bodies of mesocarp tissue for low oil yielding palms, indicating a negative correlation between oleosin and oil yield. A study on rice oleosin also confirmed that seed oil content was negatively correlated with oil body size and that oleosin participates in the formation of oil bodies and enlarges oil storage capacity (Liu et al., 2013). All these results suggest that the accumulation patterns of oleosins in oil seed crops may be used to determine whether genotypes are of high oil or low oil content.

2.5 The response of safflower to drought stress

Drought is amongst the most devastating events that hamper crop productivity. Adaptation to drought is a complex processes, involving numerous changes such as attenuated growth, increased expression or induction of genes, temporary increases in abscisic (ABA) levels, production of compatible solutes and protective proteins, increased levels of antioxidants and inhibition of energy-consuming pathways (Bartels & Sunkar, 2005). Plant tactics to manage drought usually involve multiple of stress avoidance and tolerance strategies that vary with genotypes (Chaves et al., 2002). Plant's adaptation to drought stress can be further grouped into morphological, physiological, biochemical, and molecular responses.

2.5.1 Physiological responses to drought stress

2.5.1.1 Chlorophyll content

Leaf chlorophyll content is an essential factor in the determination of photosynthesis rates and dry matter accumulation (Amini et al., 2013). Studies have shown that drought stress decreases leaf chlorophyll content of safflower (Thippeswamy et al., 2013; Canavar et al., 2014; Mohammadi et al., 2016; Bortolheiro & Silva 2017). Amini et al. (2013) revealed that drought stress diminished chlorophyll content of safflower depending on genotype suggesting existence of genetic variation in tolerance to drought. Similarly, Thippeswamy et al. (2013) showed that leaf chlorophyll content of safflower cultivars decreased with increasing water stress when compared to the unstressed plants, but the percent decrease was greater in cultivar Nira than cultivar A1. A decline in leaf chlorophyll content is prevalent in plants grown in short supply of water, therefore, it can be used

together with other drought tolerance indices to select drought tolerant genotypes (Mosupiemang et al., 2022a).

2.5.1.2 Photosynthesis rate

Photosynthesis is the mechanism by which plants collect sunshine energy and change it into complex organic compounds. Photosynthesis determines the rate of plant growth (Medlyn et al., 2002). Stressful growth conditions such as drought, salinity, and extreme temperatures, greatly hinder the process of photosynthesis in many plants by stomatal regulation, changing the ultrastructure of the organelles and the levels of several pigments, metabolites and enzymes that take place in this process (Ashraf & Harris, 2013). Stomatal shutdown is amongst the processes that plants use to avoid water stress. It is also among the early processes that plants take to adapt to conditions of limited water availability to preserve the water status (Servani et al., 2014; Mosupiemang et al., 2022a). By controlling stomatal opening and closure, plants can regulate the rate of transpiration and diffusion of carbon dioxide (Cruz De Carvalho, 2008). The reduction in intercellular Carbon dioxide due to stomatal shutdown lowers light use efficiency and the rate of photosynthesis (Chaves et al., 2002). Kazemeini et al. (2015) indicated that the photosynthetic rate of safflower was reduced by drought stress at vegetative and reproductive stages. Similarly, Amini et al. (2013) established that drought stress decreased photosynthesis rate in safflower and the rate of decrease was influenced by genotypes.

2.5.1.3 Leaf area

Leaf area is amongst the main factors that control the rate of photosynthesis and accumulation of photoassimilates. Plants with greater leaf area (LA) and chlorophyll content accumulate more

photoassimilates resulting in greater biological yield (Refay et al., 2013). Anjum et al. (2017) reported that a good sign of crop growth and development and soil conditions is leaf area index (LAI) because it influences crop yields. Small plant size, LA and LAI are main traits for regulating water use and minimizing injury to plants in conditions of limited water (Amini et al., 2013). Tayebi et al. (2012) highlighted that water stress reduced leaf number, size, colour, and vigor of safflower. While Salem et al. (2014) revealed that drought stressed safflower plants had smaller stems accompanied by fewer, dry, and smaller leaves than well-watered plants. Canavar et al. (2014) and Chavoushi et al. (2020) indicated that drought stress significantly reduced LA of safflower. Severe water stress can slow leaf growth and development and/or completely stop leaf growth and development in safflower (Emongor, 2010; Emongor & Emongor, 2023).

2.5.1.4 Relative water content

Relative water content (RWC) is commonly used as an indicator of plant water status. Studies in safflower have revealed a reduction in plant RWC with an rise in drought stress (Eslam, 2011; Hojati et al., 2011; Mohammadi et al., 2016). Hojati et al. (2011) indicated that RWC of safflower varieties remarkably decreased in response to drought stress and the more severe the drought was the greater the decrease in RWC. While Canavar et al. (2014) and Mohammadi et al. (2016) established that drought stress reduced safflower plant RWC in all genotypes under study. However, in severe drought stress situations there was no genetic variation in safflower plant RWC among genotypes investigated (Canavar et al., 2014; Mohammadi et al., 2016). Roudbari et al. (2012) attributed the genotypic variation for LRWC to differences in safflower genotype’s water-uptake capacity from the growth medium. The findings of Roudbari et al. (2012) showed that genotypes which had greater plant RWC had the least yield losses, longer stomata, and high LAI

in comparison to plants with low RWC. The above results suggest that RWC can be used as one of the drought tolerance indices to be considered in selecting tolerant safflower genotypes.

2.5.1.5 Seed yield

Crop yield losses resulting from drought stress is of primary concern to crop scientists due to limiting water supply to maintain crop production in arid and semi-arid lands (Chaves et al., 2002; Eslam, 2011; Mollasadeghi et al., 2011; Bahrami et al., 2014; Mosupiemang et al., 2022a). It is well established that drought stress causes significant seed yield losses in safflower (Majidi et al., 2011; Bahrami et al., 2014; Aeini et al., 2018; Joshan et al., 2019; Wei et al., 2020). Occurrence of drought stress at the seed filling phase of safflower was reported to have significantly reduced capitulum size, achene number per capitulum, 1000-seed weight, and seed yield (Eslam, 2011). Joshan et al. (2019) found that safflower plants subjected to drought stress had significantly lower seed yield of 17.2% than non-stressed plants. The genotype Parnian had stable seed yield under non-stressed and drought stressed conditions (Joshan et al., 2019). Joshan et al. (2019) highlighted the significance of growing or breeding of exceptional genotypes that perform well under drought stress for sustainable crop production in context of climate change.

2.5.1.6 Oil content

Safflower oil contains polyunsaturated and monounsaturated fatty acids (Moatshe et al., 2020b; Emongor & Emongor, 2023). Safflower oil content, oil yield, and fatty acids composition is reported in literature to be lowered by drought stress (Eslam, 2011; Kazemeini et al., 2015; Janmohammadi et al., 2017; Joshan et al., 2019; Emongor & Emongor, 2023). If drought stress

occurs at vegetative, flowering, and/or seed filling stages greatly reduces oil content of safflower (Eslam, 2011; Joshan et al., 2019). However, other studies have revealed that safflower oil content was increased (Bortolheiro & Silva, 2017) or not affected (Aeini et al., 2018) by drought stress. Bortolheiro and Silva (2017) explained that an increment in oil content of safflower oil caused by drought stress was as result of change in plant dynamics, which gave preeminence to the sectioning and flux of photosynthates to the seeds than to other parts of the plant.

2.6 Drought tolerance mechanisms

Plants have developed strategies of adapting, coping, escaping, or tolerating conditions of limited water by altering their total chemical reactions, phenological, physiological, molecular, and biochemical characteristics (Comas et al., 2013; Canavar et al., 2014; Mosupiemang et al., 2022a). In many countries, food security is at risk of being affected by climate change because the frequency of droughts is predicted to increase therefore affecting sustainable crop and animal production (IPCC, 2007; Mittler & Blumwald, 2010; Farooq et al., 2009; Challinor et al., 2014; Emongor & Emongor, 2023).

2.6.1 Biosynthesis of Osmoprotectants

Plants growing under drought stress environment preserve their water potential beneath that of the soil by accumulating compatible organic solutes to remain hydrated (Serraj & Sinclair, 2002; Hossain et al., 2016). These organic solutes accumulates in the cytoplasm to trigger the osmotic potential to decline underneath that of the soil to enable water uptake, sustainment of cell membrane integrity and water potential balance within the cells of drought stressed plants (Ahmad et al., 2008). The main osmoprotectants are sugars, betaines, and amino acids (Kantar et al., 2011).

2.6.1.1 Proline content

Proline is an osmolyte that has been widely researched in relation to abiotic stresses in plants (Gross et al., 2002; Wang, 2013; Emongor, 2015; Basu et al., 2016; Hussain et al., 2016; Chaudhary et al., 2017; Mosupiemang et al., 2022a). It is considered as a powerful antioxidant and effective preventer of programmed cell death (Dar et al., 2017). High levels of proline are correlated with heat shock proteins that help to protect against stresses by regulating correct folding and conformation of the cell membranes and enzymatic proteins (Sevillano et al., 2009; Aghdam, 2013; Tautsagae, 2020; Mosupiemang et al., 2022a). Proline and soluble proteins guard the plants from drought stress by osmoregulation, decreased biosynthesis of ROS, and maintenance of membrane integrity, structural characteristics of proteins and enzymes (Farooq et al., 2009; Sevillano et al., 2009; Aghdam, 2013). It is among the most explored osmolyte/ antioxidants with regard to abiotic stresses in plants. Several studies have reported that drought stress increased the proline content in safflower (Thippeswamy et al., 2013; Mohammadi et al., 2016; Aeini et al., 2018; Chavoushi et al., 2019; Farooq et al., 2020; Wei et al., 2020). Thippeswamy et al. (2013) found that proline concentration increased in stressed safflower cultivars and their levels of proline varied with cultivar. While Chavoushi et al. (2019) reported that the proline content in the leaves and roots of drought stressed safflower was five-fold greater than in non-stressed plants. The rise in proline levels in plant tissues under drought stress has been explained by the expression of specific genes controlling proline and pyrroline-5-carboxylate synthase synthesis and inhibition of proline dehydrogenase (Ishitani et al., 1995; Ueda et al., 2001; Wei et al., 2020). From literature cited above it suggests that proline is a potential indicator of drought stress tolerance among plant genotypes and plant species.

2.6.1.2 Reducing sugars

Reducing sugars as osmoprotectants regulate the osmotic adjustment, sustain cell membrane integrity, and scavenge toxic ROS in drought stressed safflower plants (Javed et al., 2013; Mohammadi et al., 2016; Aeini et al., 2018). Reports showed that during drought stress there is a greater production of reducing sugars in the roots and leaves of safflower (Javed et al., 2013; Mohammadi et al., 2016; Chavoushi et al., 2019). Furthermore, significant genotypic variation in the production of osmoprotectants in drought stressed safflower is well known (Thippeswamy et al, 2013; Mohammadi et al., 2016; Aeini et al., 2018; Chavoushi et al., 2019; Farooq et al., 2020). This indicates that genotypes that display low levels of osmoprotectants during stress can be enhanced by genetic engineering for high biosynthesis of osmoprotectants.

2.6.2 The role of enzymatic antioxidants in drought tolerance

Exposing plants to unfavourable environmental conditions like as excess water availability, drought, temperature extremes, heavy metals, air pollutants, nutrient deficiency, or salt stress increases the production of ROS such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (OH) that are very reactive and toxic and causes damage to proteins, lipids, carbohydrates, DNA which eventually results in cell death (Mittler et al., 2004; Gill & Tuteja, 2010). The ROS have double role in plants, small concentrations act as a signal for inducing abiotic stress responses towards adaptation processes, while the high concentrations cause oxidative damage (Hasanuzzaman et al., 2019). Under normal conditions, potentially toxic oxygen metabolites are produced in small quantities and there is an appropriate steady production and quenching of ROS (Sharma et al., 2012; Hasanuzzaman et al., 2019). However, some biotic or abiotic stresses such as drought may disturb the metabolic equilibrium of

cells, resulting in greater production of ROS (Mittler et al., 2004). During normal plant growth and development ROS accumulates in low concentrations in different cell compartments, however biosynthesis increases during stress (Mittler et al., 2004; Gill & Tuteja, 2010; Mohammadi et al., 2016; Aeini et al., 2018; Hasanuzzaman et al., 2019). Under limited water conditions crop plants increased the biosynthesis of ROS which oxidize cellular components like carbohydrates, lipids, DNA, and proteins (Mittler et al., 2004; Gill & Tuteja, 2010; Sharma et al., 2012; Mohammadi et al., 2016; Aeini et al., 2018). Mittler et al. (2004) and Gill and Tuteja (2010) reported that death of cells occurred in uncontrolled oxidation of cellular components.

Plants possess complicated antioxidant defensive methods comprising of enzymatic and non-enzymatic and components to scavenge ROS (Mittler et al., 2004; Sharma et al., 2012). The non-enzymatic antioxidants comprise of tocopherols, carotenoids, ascorbate, and glutathione which protect plant cells from oxidative injury (Hasanuzzaman et al., 2012; Malambane et al., 2018). The major ROS-scavenging antioxidant enzymes in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidases (POX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Blokhina et al., 2003; Mittler et al., 2004; Hasanuzzaman et al., 2012; Hassan et al., 2015; Wei et al., 2020). These antioxidant enzymes function to stop the cascades of uncontrolled oxidation (Noctor & Foyer, 1998; Hasanuzzaman et al., 2012). Drought stress enhanced activities of CAT, SOD, APX, and GR in safflower (Hojati et al., 2011; Sajedi et al., 2012; Amini et al., 2013; Kazemeini et al., 2015; Farooq et al., 2020; Wei et al., 2020). Safflower genotypes tolerant to drought have significant activity of SOD, CAT, APX, POX, and GR compared to less drought tolerant genotypes (Hojati et al., 2011; Sajedi et al., 2012; Amini et al., 2013; Wei et al., 2020). Under conditions of extreme water stress, ROS are not

scavenged, therefore they get amassed in plant cells becoming toxic and altering cellular metabolism and expression of new genes (Mittler et al., 2004; Gunes et al., 2008; Gill & Tuteja, 2010; Sajedi et al., 2012; Harb et al., 2015; Hasanuzzaman et al., 2019).

2.6.2.1 Ascorbate peroxidase

Ascorbate peroxidase (APX) (EC 1.11.1.11) is involved in scavenging ROS and protecting cells in higher plants, algae, euglena and other organisms (Gill & Tuteja, 2010). It is amongst the widely distributed antioxidant enzymes in plant cells (Wang et al., 1999). It catalyses the reduction of H_2O_2 to H_2O (Neto et al., 2010) thereby reducing H_2O_2 using ascorbate as an electron donor (Gunes et al., 2008). APX together with catalase (CAT) are two enzymes that scavenge H_2O_2 and prevent its accumulation to toxic levels (Harb et al., 2015). APX isoenzymes are expressed by distinct regulatory mechanisms in response to various environmental stresses or cell conditions, and play a cooperative role to protect each organelle and minimizes tissue injury (Shigeoka et al., 2002). Based on amino acid sequences, five chemically and enzymatically different isoenzymes of APX have been found at different subcellular localization in higher plants (Sharma et al., 2012). The enzymatic activities of APX in safflower have been reported to increase with drought stress. Khosrowshahi et al. (2018) found that the activities of APX increased in both safflower leaves and roots under water stress. Likewise, Amini et al. (2013) established that the activities of APX were enhanced under drought stress among different safflower genotypes. They further found that among 64 safflower genotypes studied, the genotype Hamedan 38 exhibited the greatest values of APX and CAT, leaf area index and high seed yield under drought stress conditions. This suggested that the activities of APX under drought stress correlates with plant tolerance to drought and hence the activities of APX can be used to distinguish between drought sensitive and tolerant genotypes.

2.6.2.2 Catalase

Catalase (CAT) (EC 1.11.1.6) detoxifies H_2O_2 accumulated under moisture stress, to form water and oxygen (Blokhina et al., 2003). This antioxidant is known to have a low affinity to H_2O_2 and it is responsible for scavenging most of the H_2O_2 (Harb et al., 2015). CAT is mainly found in the peroxisomes (Mittler, 2002). It is also localized in the cytosol and chloroplasts but it does not perform as efficiently as it would in peroxisomes (Banerjee & Roychoudhury, 2018). Therefore, it has been hypothesized that CAT is less susceptible as a scavenging enzyme than APX, therefore, the activity of CAT is only expressed under severe drought stress whereas under moderate drought stress H_2O_2 scavenging is preferably done ascorbic acid through the ascorbate/glutathione cycle (Cruz, 2008). The effective removal of H_2O_2 is attained by a high concentration of CAT in specific cellular compartments (Mittler & Poulos, 2005). Furthermore, the activation of the major scavengers APX and CAT are stronger in tolerant genotypes compared to their sensitive counterparts though sensitive genotypes activate GPX more than tolerant genotypes (Laxa et al., 2019). Farooq et al. (2020), found that the levels of CAT increased significantly under water stress conditions in safflower. Similarly, Hojati et al. (2011) also revealed that the levels of CAT increased in both the roots and leaves in drought stressed safflower compared to non-stressed plants. Conversely, Amini et al. (2013) revealed that drought stress caused remarkable increases in mean of activities of POX and APX with the exception of CAT. This was possibly because APX and POX have a very high affinity to H_2O_2 than CAT. Therefore, it can be concluded that peroxidases (APX and POX) provided major defense against drought stress than CAT (Amini et al., 2013). Amirkhiz et al. (2015) demonstrated that the activities of CAT in safflower leaves under drought stress were increased by Fe application. This suggests that antioxidant enzymes may provide a better defense system against drought stress in plants when plant nutrients are not

limiting. Thus nutrients/mineral supplements should be considered for use in activating or increasing the activities of antioxidants under abiotic stress conditions.

2.6.2.3 Glutathione reductase

Glutathione reductase (GR) (EC 1.6.4.2) is also a potential enzyme of the ascorbate glutathione (AsA-GSH) cycle and performs an crucial role in protection against ROS by maintaining the reduced level of glutathione (GSH) (Gill & Tuteja, 2010). It does so by removing H_2O_2 via the ascorbate glutathione cycle to maintain high levels of reduced ascorbate within chloroplasts (Jiang & Huang, 2001). Transgenic plants that produce GR were found to be abiotic stress tolerant (Gill & Tuteja, 2010), therefore GR can be a useful tool in determining plant tolerance to abiotic stresses. Glutathione reductase has not received attention in drought stress related studies in safflower. However, the function of this antioxidant in drought tolerance mechanism is well reported in other crops. In sunflower, at the early and middle stages of drought (when watering was stopped for 3-4 days), GR activities were not affected by drought, however, drought increased GR activities at the late stage of drought stress (when watering was stopped for 7-8 days) (Zhang & Kirkham, 1996). This implied that the activities of GR were affected by drought stress duration, the longer the drought duration the more activity of GR (Zhang & Kirkham, 1996). Masoumi et al. (2011) found that in soybean, the more water deficit stress, the higher the level of GR but the activity significantly differed with plant genotype. Other studies also have demonstrated an increase in the activities of GR under drought stress in rice (Shehab et al., 2010) and sunflower (Pourtaghi et al., 2011). In contrast, other studies revealed that the activities of GR were not significantly increased under drought stress in soybean roots (Porcel et al., 2003) and on the sunflower shoots (Baloğlu et al., 2012).

2.7 Conclusion

Studies examining the impact of genotype and environmental interaction on the phenological development, growth, yield, and oil content of safflower are many and variable. While some studies showed that some safflower genotypes are stable and adaptable to environmental fluctuations in temperate and Mediterranean type of climates, however, it is still unknown how these genotypes will perform under new environments or regions such as Southern Africa where safflower has not been planted or fully adopted. Therefore, it is important to select safflower genotypes based on the multi-environment analysis (multi season, location and year) for informed recommendations for different locations within a country. It is also apparent from literature that safflower genotypes vary in oil content. Therefore, to select high oil yielding genotypes it is important to examine the genes and cellular functions that relate to seed oil. However, such information is lacking for most crops including safflower, hence this study will contribute to filling this knowledge gap. Furthermore, studies have shown that safflower genotypes vary in response to drought stress depending on the duration and intensity of stress and phenological stages of safflower. Therefore, this study will contribute to the existing knowledge by underlying some of the drought tolerance mechanism safflower employs to mitigate the impacts of drought stress.

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Chapter 3

Growth, Development, and Yield of Safflower Genotypes in Response to Seasonal Variations

Abstract

The economic potential of safflower as a climate smart crop in Botswana has been noticed. Recently, the government of Botswana in 2022/2023 developed a policy to support farmer's endeavours of leveraging high profits from this multipurpose crop. To make sure farmers get the most out of safflower, genotypes that perform better and are stable across different environments need to be recommended. The present study evaluated the phenological development, growth, yield, and yield components of five safflower genotypes at three different sites (Ramonaka, Sebele, and Molepolole) in the southern part of Botswana under farmers' fields during winter and summer seasons. The experimental design in each location was a randomized complete block with three replications. The treatments in each site were five safflower genotypes Gila, Sina, Turkey, PI537636, and Kenya9819. The results showed that phenology, growth, yield, yield components, oil content, and oil yield of safflower were statistically ($P<0.05$) influenced by genotype, location, seasons, and their interactions. Safflower grown in winter at Ramonaka exhibited better vegetative growth than that of other locations planted either in winter or summer. The yield components (number of primary branches/plant, capitula number/plant, capitula diameter, capitula weight, and 1000-seed weight) were affected by main effects (genotype, season, and location) and the interaction of season x location. Safflower planted in winter had statistically ($P<0.05$) higher yield components than summer planting though influenced by genotype and location. Safflower grown in winter at Ramonaka produced greater seed yield of 3803.13 kg/ha than that obtained in other locations and seasons. Genotype Kenya9819 attained a seed yield of 2265.22 kg/ha which was

higher than other genotypes with exception of genotypes Turkey and Sina. In general, the seed yield/ha of safflower ranged between 821-3803.13 kg/ha depending on genotype, season, and location. Genotype Gila planted at Molepolole in winter produced seed with the greatest oil content of 42.27% compared to other genotypes planted at different locations and seasons. The seed oil content ranged between 11.8-42.3% varying with genotype, location, and season. The maximum oil yield of 1121 kg/ha was produced by plants grown at Ramonaka in winter. Genotype Kenya9819 planted at Ramonaka had the highest oil yield of 753 kg/ha. Genotype by environment interactions (GGE) biplots for seed yield demonstrated that Sebele showed high representativeness and discriminative ability and therefore, it was considered as a model location for selecting genotypes adapted to the whole region. Genotype Kenya9819 was identified as the highest seed-yielding and stable genotype. When evaluating genotypes based on overall superiority, the genotype by yield*trait combination (GYT) biplot revealed that genotypes ranked as Kenya9819 > Turkey > Sina > PI537636 > Gila. Therefore, genotypes Kenya9819 and Turkey had an above-average seed yield-trait combination, hence superior, while genotypes PI537636 and Gila had below-average values.

3.1 Introduction

Safflower (*Carthamus tinctorius* L.) is of the most drought and saline-tolerant oilseed crops that are commonly grown in arid and semi-arid lands (ASALs) (Dajue & Mundel, 1996). It is highly esteemed for its commercial utility as vegetable oil, animal feed, cut flower, leafy vegetable, pharmaceuticals, food colourants, textile dye, cosmetics, and biofuel production (Janmohammadi, 2015; Emongor & Emongor 2023). Safflower oil extracted from seeds is of high quality and is one of the most desirable vegetable oils because it contains monounsaturated fatty acid oleic and

polyunsaturated fatty acids α-linolenic and linoleic which cannot be biosynthesized by the human body hence, making them essential fatty acids (Piccinin et al., 2019; Nazir et al., 2021). Although safflower is multipurpose and adaptable to various climatic conditions, the crop is underutilized and has remained minor in terms of area of production (Ekin, 2005; Emongor, 2010). Therefore, to increase the area under safflower production, especially in new areas like Botswana that have not fully adopted this crop, more studies on genotype and environmental interactions are critical. Planting location has been identified as one of the important factors to be considered when selecting stable safflower genotypes. This is because Alivelu et al. (2015) found that planting location (environment) significantly affected safflower seed yield and contributed 51% of the total (G×E×GEI) variation, while G×E interaction contributed 14.9% of the total sum of squares. Similarly, Hamza and Abdalla (2015) and Hussein et al. (2018) demonstrated that safflower genotypes showed varied yield performance when planted at different locations. This highlights the need to select genotypes that are suitable for each location. Moreover, past studies have shown that the yield of safflower was significantly affected by the planting season with winter sowing being the most favourable and summer planting being the least favourable in Botswana (Emongor et al., 2017; Moatshe & Emongor, 2019). Other studies have reported that safflower does better when sown in autumn than in winter in countries that experience very low winter temperatures (Golzarfar et al., 2012; Sampaio et al., 2018). These discrepancies in past results prompt the need to conduct multi-environment/season and/or multi-location studies as they efficiently select the most stable, adaptable, and high-yielding genotypes. Thus, cultivars selected from one environment might not maintain their high performance in another environment due to the G×E interaction effect (Li et al., 2017). This is because some genotypes are adapted to a broader range of environmental conditions, while others are more limited in their potential distribution and have

specific adaptations (Mohammadi et al., 2008). Therefore, this study evaluated safflower genotypes in two different seasons and three locations. This was done to select safflower genotypes that produce stable yields across the study locations and seasons of Botswana. Moreover, it has been difficult to select superior genotypes considering all studied traits, therefore, a genotype by yield*trait (GYT) biplot model suggested by Yan and Frégeau-Rei (2018) was employed to evaluate genotypes based on yield*traits combination.

3.2 Materials and methods

3.2.1 Experimental site

The experiment was carried out under on-farm conditions at three locations namely Sebele, Molepolole, and Ramonaka in southern Botswana (Figure 3.1). The vegetation of these three sites is characterized as tree savanna and climate is characterized as hot semi-arid. The warmest month of the year in these sites is January and the coolest and driest month is July. These locations experience the most rainfall in summer, which commonly starts in late October and continues to April. Rainfall in this region is limited and highly erratic. During the 2020/2021 experiment the average minimum and maximum temperatures were 19.1 and 29.6°C, respectively for summer and 8.6 and 26.0°C, for winter in all three sites. While during the 2021/2022 the average minimum and maximum temperatures were 17.8 and 31.4°C, respectively for summer and 9.1 and 25.9°C for winter in all three sites (Data obtained from the Department of Meteorology). In Sebele the study was conducted at the Botswana University of Agriculture and Natural Resources (BUAN) Experimental Farm. This site is located at an altitude of 994 m above sea level and latitude of 24°33’ South and a longitude of 25°54’ East in Sebele, Gaborone in the Southeast district of Botswana.

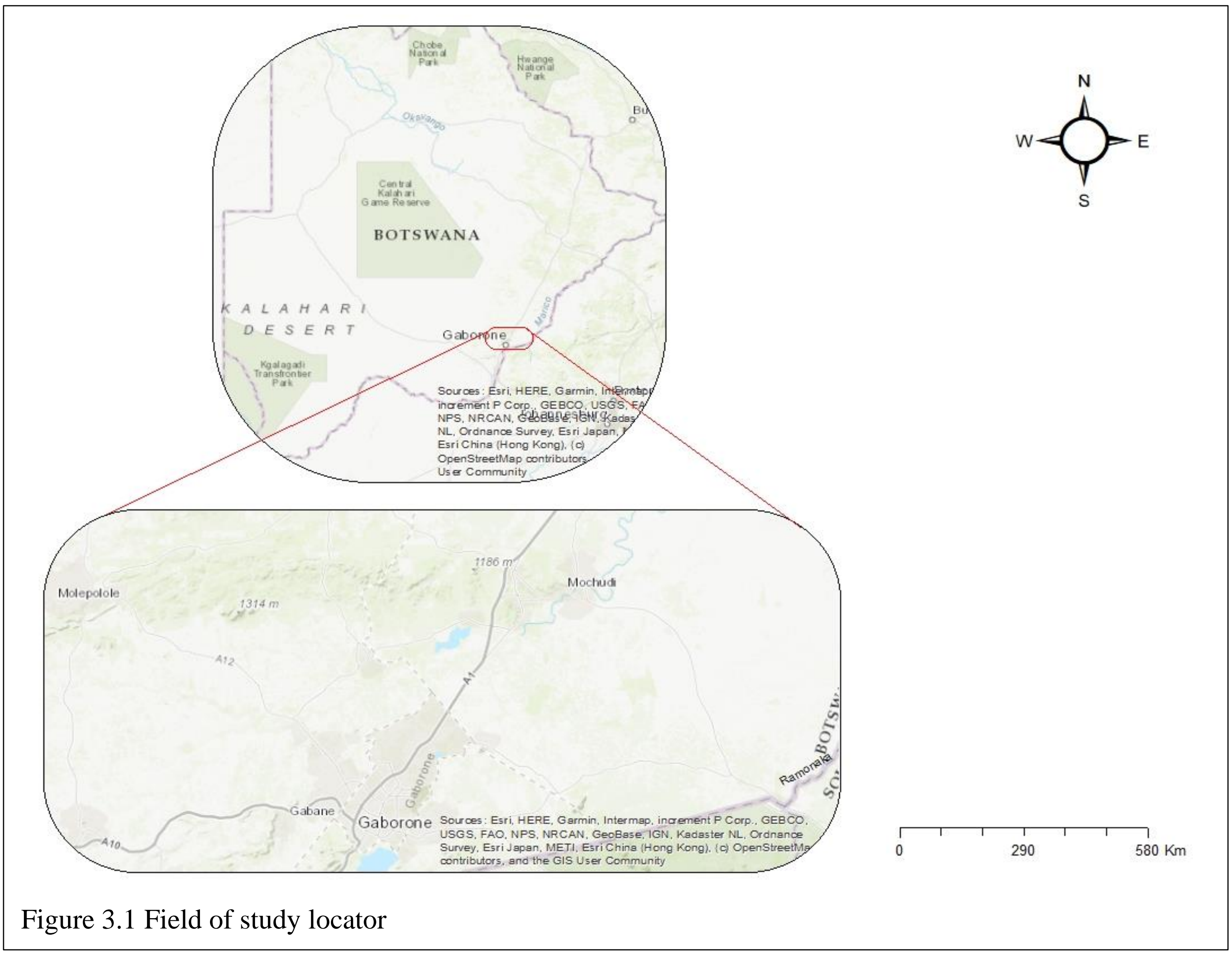

Figure 3.1 Field of study locator

Sebele received an average annual rainfall of 202.5 mm during the 2020/2021 season and 132 mm during the 2021/2022 season. The soils of Sebele are sandy loam in texture (Table 3.1). In Molepolole, the study was conducted under on-farm conditions and the coordinates of the site are 24.3966 S, 25.4970 E with an altitude of 1150.6 m above sea level. The site is in the Kweneng district of Botswana, and it received an average annual rainfall of 284.6 mm during the 2020/2021 season and 513.5 mm during the 2021/2022 season. The soil of this site was sandy clay loam in texture (Table 3.1). In Ramonaka, the study was also conducted under on-farm conditions and the coordinates of the site are 24.19704450 S and 26.23046160 E with an elevation of 936.25 m above

sea level. The site is in the Kgatleng district of Botswana, and it received an average annual rainfall of 205.3 mm during the 2020/2021 season and 194 mm during the 2021/2022 season. The soil of this site was a clay loam in texture. Generally, the farm in Ramonaka is located just around 30 m away from the Madikwe River which is along the South African border and the soils are generally pure greyish clay loam which is very fertile and contains high organic matter. When comparing the soil fertility of the three locations, generally, Ramonaka had more fertile soil followed by Molepolole and lastly Sebele which had poor soils (Table 3.1).

Table 3.1.Soil chemical and physical characteristics of the three experimental sites

Soil characteristic	Sebele	Molepolole	Ramonaka
pH ($CaCl_2$)	5.38	4.99	5.88
EC (µs/cm)	35.8	73.7	120.4
CEC (meq/100g)	4.0	4.6	14.0
Organic carbon (%)	0.1	0.23	0.30
Textural class	Sandy loam	Sandy clay loam	Clay loam
Soil colour	Brown to yellowish brown	Dark grayish brown	Dark gray
Sand (%)	78.3	65.6	39.2
Silt (%)	9.7	7.8	31.4
Clay (%)	12	26.6	29.4
Total N (%)	0.03	0.05	0.10
Available P (ppm)	12.08	45.3	52.8
Exchangeable K (cmol/kg)	0.11	0.21	0.29
Exchangeable Ca (cmol/kg)	1.23	2.22	4.42
Exchangeable Mg (cmol/kg)	1.02	2.00	4.1
Exchangeable Na (cmol/kg)	0.30	0.28	0.38

3.2.2 Experimental design

The experimental design in each site was a Randomized Complete Block Design (RCBD) with three replications. The treatments in each site were five safflower genotypes (Gila-PI537692 (USA), Sina-PI537598 (USA), Turkey-PI407616-BJ-2131 (Turkey), PI537636 (USA), and Kenya9819 (Kenya)). Each individual plot (experimental unit) measured 3 x 5 m. The experiments were carried out in summer (November to February) and winter (May to October) in both seasons.

3.2.3 Agronomic practices

Soil sampling was done as per Soil and Plant Analytical Laboratory, Department of Agricultural Research, Botswana, where the soil was sampled from five different spots in each field/farm using a zigzag pattern, and subsamples were mixed to make a composite sample. The soil was air-dried and sieved with a 2 mm mesh sieve. Soil analysis was done for pH, EC, CEC, P, K, Ca, Na, Mg, texture, and organic carbon using the standard procedures (Table 5.1). The experimental sites were disc ploughed using a tractor to have a smooth planting bed. Two seeds were sown per hill at a depth of 2.5 cm and two weeks after emergence plants were thinned to one plant per hill. The spacing was 40 cm between rows (inter-row spacing) and 25 cm within plants (intra-row spacing) (Moatshe et al., 2016; Emongor et al., 2015). Plants were mainly rainfed in summer and supplementary irrigation was provided in periods of prolonged dry spells where no rainfall was received within two weeks. Plants were irrigated at 6 mm per irrigation interval twice a week. Weeds were controlled manually by hoeing between plants and rows. Fertilizer was applied at a rate of 80 kg N/ha (calcium ammonium nitrate-28% N) and 50 kg P/ha (single super phosphate-10.5% P) two weeks after emergence.

3.2.4 Data collection

Days to emergence were recorded when 80% of the plants in a plot had emerged while days to onset of stem elongation, branching, flowering, and maturity were recorded as the number of days from seed sowing. Primary branches per plant (a total number of branches originating from the core stem) were counted at physiological maturity from 10 randomly selected plants per replication. Plant height was measured from the ground level to the apex of the main stem at physiological maturity from 10 randomly selected plants per replication. Root and shoot biomass were determined at 50% flowering by harvesting and oven drying the roots and the shoots at 65°C for 72 hours. The number of capitula per plant was recorded on 10 plants randomly selected from the inner rows of each plot per replication at physiological maturity. For seed yield and 1000-seed weight, the plants were harvested in an area of 9 m^2 in the centre of each experimental plot. Seeds were threshed and weighed to determine seed yield. The 1000-seed weight was determined by counting 1000 seeds using a seed counter (Contador, Pfeuffer GmbH, Germany) and the 1000 seeds were weighed using a digital balance (AS 60/C2 model, RADWAG Wagi Elektroniczne, Poland).

Oil content was determined using the Soxhlet n-hexane extraction method (Wrolstad et al., 2005). Approximately 15 g of safflower seeds were grounded using Polymix (Analysenmuhle A10 model, Kinematica, Switzerland). Then exactly 5 g of crushed safflower seed was weighed and inserted into a Soxhlet extractor connected to a round bottom flask containing 150 mL of n-hexane. The extraction was conducted as the solvent was heated up to a boiling temperature around of 70°C for six hours. After extraction, the solvent was evaporated using a rotary evaporator. The final weight of the oil was determined by weighing and expressed as a percentage of oil content.

$$Oil\ content\ (\%) = \frac{weight\ of\ oil\ extracted}{weight\ of\ seed\ sample}\ x\ 100$$

Oil yield (kg/ha) was calculated by multiplying seed oil content by seed yield.

3.2.5 Data analysis

Data were analyzed as a 2 x 3 x 5 factorial experiment to study the seasons (2), sites (3), safflower genotypes (5), and their interactions. Measured variables were analysed by three-way analysis of variance (ANOVA) using R-Software version 4.2.2 and the agricolae package version 1.3-5. Treatment means and interaction effects were compared using Fisher's least significant difference (LSD) procedure at a significance level of 5%. To select the most adaptable and high-yielding genotype, seed yield data was graphically analysed using a GGE biplot based on the principal component analysis (PCA) of environment data (Yan & Tinker, 2006). The genotype by yield*trait (GYT) biplot was generated by the combination of each trait and grain yield as developed by Yan and Frégeau-Reid (2018). Each trait was multiplied with grain yield. The GGE and GYT biplot analyses were executed on R-Software version 4.2.2 using the METAN package of Olivoto and Lúcio, (2020).

3.3 Results

3.3.1 Number of days to emergence

The interactions of season × location × genotype and location × genotype for number of days to emergence were not significant ($P > 0.05$) in this study (Appendix 1). However, a highly significant ($P < 0.001$) interaction of genotype × season was found for number of days to emergence (Appendix 1). The results demonstrated that genotypes Kenya9819 (10.3 days) and

Gila (10.1 days) grown in winter took a significantly (P < 0.05) longer time (average of 10.22 days) to emergence than other genotypes grown in different seasons (Figure 3.2A). On the other hand, genotypes Sina and PI537636 planted in summer took 5.7 days to emergence which was substantially shorter than the duration taken by other genotypes planted in different seasons. However, genotypes PI537636, Sina, Gila, and Turkey did not statistically (P > 0.05) vary in their days to emergence when grown in summer (Figure 3.2A). Generally, the genotype Kenya9819 took the longest time to emerge independent of planting season (Figure 3.2A).

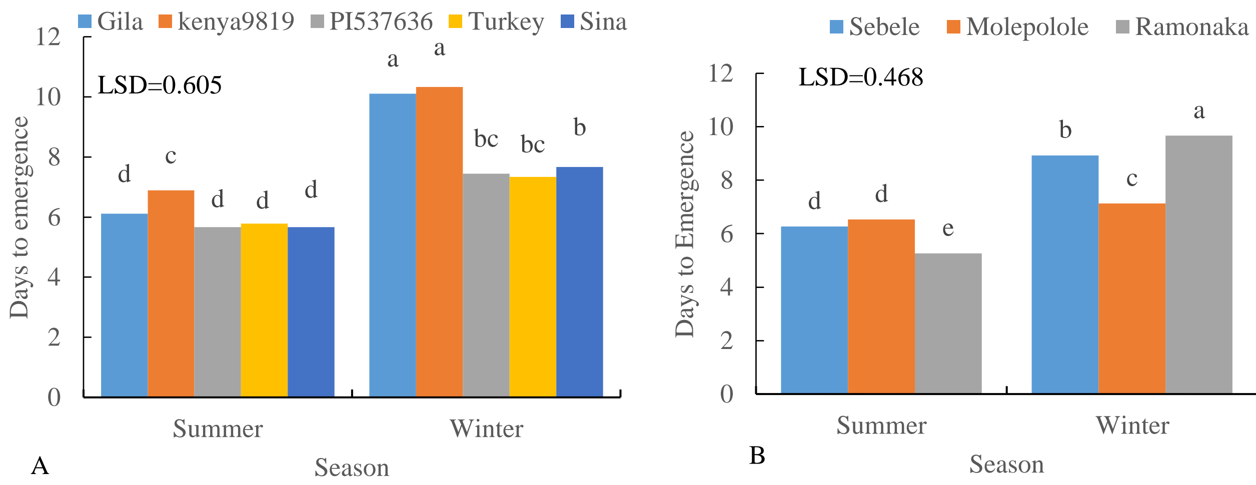

Figure 3.2. Effects of season, genotype and location on the number of days to emergence. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The number of days to emergence varied significantly (P < 0.001) due to season and location interaction (Appendix 1). The results revealed that winter (8.58 days) planted safflower significantly (P <0.05) delayed seedling emergence by 2.56 days as compared with summer (6.02 days) planted across the study locations (Figure 3.2B). In this regard, safflower planted at

Ramonaka in winter took 9.7 days to emergence which was markedly longer than in other locations and seasons. The second longest time of 8.9 days to emergence was registered in Sebele during winter. In contrast, safflower planted in Ramonaka took 5.3 days to emergence which was considerably shorter than in other locations and seasons (Figure 3.2B). In general, the number of days to emergence ranged between 5.3 to 9.7 days depending on the season and location (Figure 3.2B).

3.3.2 Number of days to stem elongation

A significant ($P < 0.01$) interaction of season × location × genotype was found for number of days to stem elongation (Appendix 1). The results revealed that winter planting statistically ($P <0.05$) delayed days to stem elongation of safflower by 33.3 days (106.3%) compared to summer planting (Figure 3.3). In winter, genotype Gila planted at Ramonaka took 69.7 days to reach stem elongation stage which was remarkably longer than days of other genotypes planted in different seasons and locations with exception for genotypes Sina, Kenya9819, and Turkey; Gila, and Turkey planted in winter at Ramonaka, Sebele, and Molepolole, respectively (Figure 3.3). On the contrary, genotypes Sina and PI537636 planted at Ramonaka in summer took 24.3 days to reach the stem elongation stage which was markedly shorter than days taken by other genotypes planted in different locations and seasons (Figure 3.3). In summer, genotypes took relatively ($P > 0.05$) similar number of days to reach stem elongation stage except in Ramonaka (Figure 3.3). On average, safflower planted at Ramonaka in winter took the longest days (67.8) to reach stem elongation stage as compared with that obtained in other locations and seasons. Generally, genotypes Sina and PI537636 took considerably similar number of days to reach stem elongation stage irrespective of location and

season. The number of days to reach stem elongation ranged between 24.3 to 69.7 days depending on the genotype, location, and season (Figure 3.3).

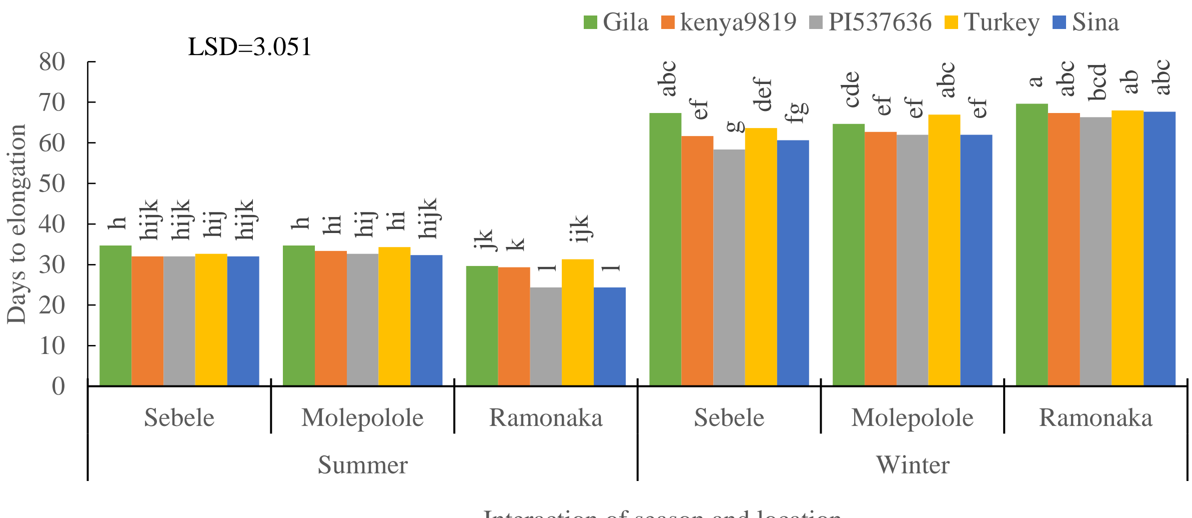

Figure 3.3. Effects of season, location and genotype on the number of days to elongation. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD

3.3.3 Number of days to branching

The results revealed that the interactions of season × location × genotype and location x genotype for number of days to branching were not significant ($P > 0.05$) (Appendix 1). However, a highly significant ($P < 0.001$) interaction of season × genotype for number of days to branching was observed in this study (Appendix 1). Figure 3.4A depicts that safflower planted in winter took statistically ($P < 0.05$) longer days to reach branching stage than safflower planted in summer. The genotype Gila planted in winter took 88.1 days to reach branching stage which was substantially longer than other genotypes planted either in winter or summer (Figure 3.4A). In contrast, the genotype Sina took 35.1 days to branching stage which was markedly ($P < 0.05$) fewer days than

those taken by other genotypes planted either in summer or winter (Figure 3.4A). In summer, genotypes Gila and Turkey took the longest period to reach branching stage compared to other genotypes (Figure 3.4A).

The interaction of season × location significantly ($P < 0.001$) influenced days to branching stage of safflower (Appendix 1). Branching started significantly ($P < 0.05$) earlier (34.6 days) in Ramonaka during summer and longer (91.3 days) in Sebele during winter than safflower planted in different locations and seasons (Figure 3.4B). In general, safflower planted in Ramonaka reached the onset of branching early independent of season. In summer, safflower planted at Sebele and Molepolole showed no marked differences in their number of days to the start of branching. Safflower took an average of 38 and 85.8 days in summer and winter, respectively to reach branching stage. Generally, the number of days to the onset of branching ranged between 34.6 to 91.3 days depending on the season and location (Figure 3.4B).

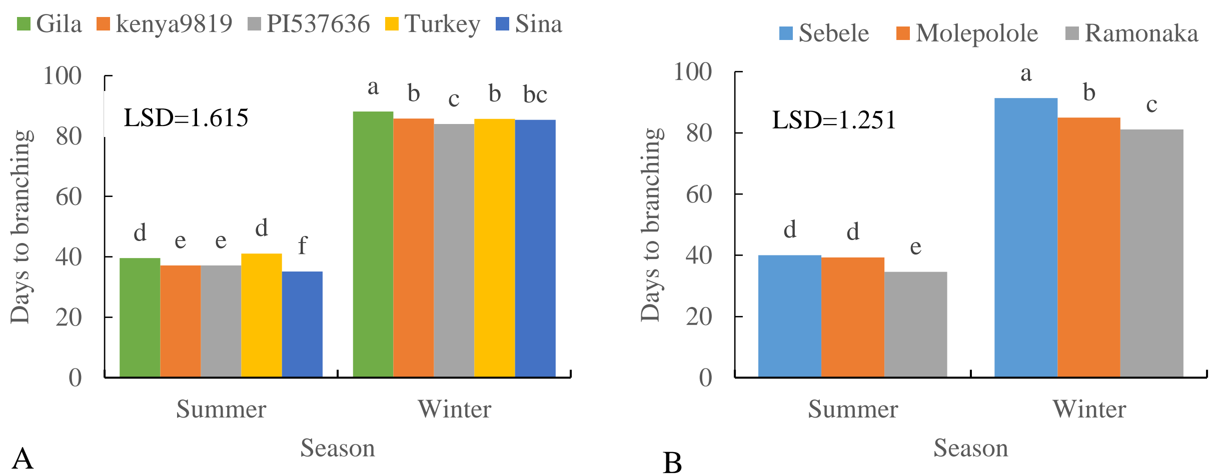

Figure 3.4. Effects of season, plant genotype, (A) and season, and location (B) on the number of days to branching in safflower.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.4 Number of days to flowering

A significant ($P < 0.05$) three-way interaction of season x location x genotype was found for number of days to flowering (Appendix 1). Safflower planted in winter took significantly ($P < 0.05$) more days to flowering independent of location and genotype (Figure 3.5). Genotype Gila planted at Ramonaka in winter took 128.3 days after sowing to reach flowering stage which was statistically ($P < 0.05$) longer than the number of days other genotypes planted at different locations and seasons took, except for genotype Turkey planted at Ramonaka and Molepolole in winter. On the contrary, genotypes Sina and PI537636 grown at Ramonaka in summer took 52 days after sowing to reach flowering stage which was substantially earlier than other genotypes planted in different locations and seasons (Figure 3.5). Furthermore, genotypes Sina and PI537636 consistently took relatively similar number of days to reach the flowering stage irrespective of location and season. Generally, the number of days to flowering ranged between 52 to 128.3 days depending on the genotype, season, and location (Figure 3.5).

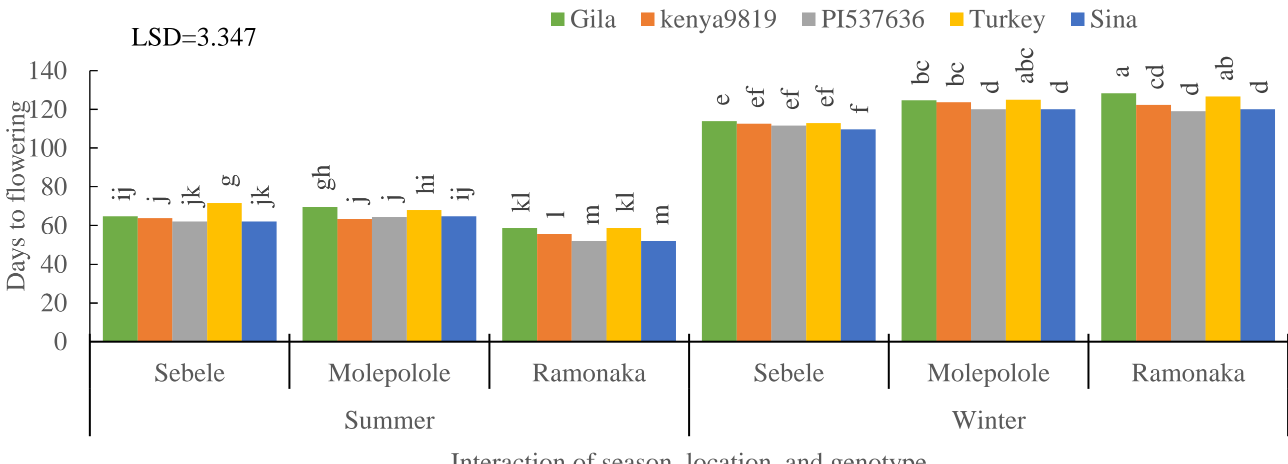

Figure 3.5. Interaction effect of season × location × genotype on the number of days to flowering. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.5 Number of days to maturity

There was no significant ($P > 0.05$) interaction effect for number of days to maturity, hence main effects are reported (Appendix 1). A highly significant ($P < 0.001$) main effect of season was found for number of days to maturity (Appendix 1). Figure 4.6A showed that safflower planted in summer took 93.3 days after sowing (DAS) to reach maturity which was noticeably shorter than that of winter (173 DAS) planted safflower took to reach physiological maturity. In general, planting safflower in winter delayed plant maturity by 79.4 DAS.

The main effect of location significantly ($P < 0.01$) influenced the number of days to maturity (Appendix 1). Safflower planted in Ramonaka took 125 DAS to reach maturity which was substantially shorter than DAS that safflower grown in either Sebele or Molepolole took (Figure 3.6B). Furthermore, safflower planted at Sebele and Molepolole had no marked differences in their number of days to maturity. On the other hand, there was a highly significant ($P < 0.01$) genotypic variation for number of days to maturity (Appendix 1). The genotype Sina took 123 DAS to reach maturity which was substantially shorter than DAS of other genotypes with exception for genotype PI537636 (Figure 3.6C). The genotypes Turkey took 141 DAS to reach maturity which was considerably longer than DAS that Sina took (Figure 3.6C). Genotypes Gila, Turkey, and Kenya9819 did not vary in their DAS to reach maturity (Figure 3.6C).

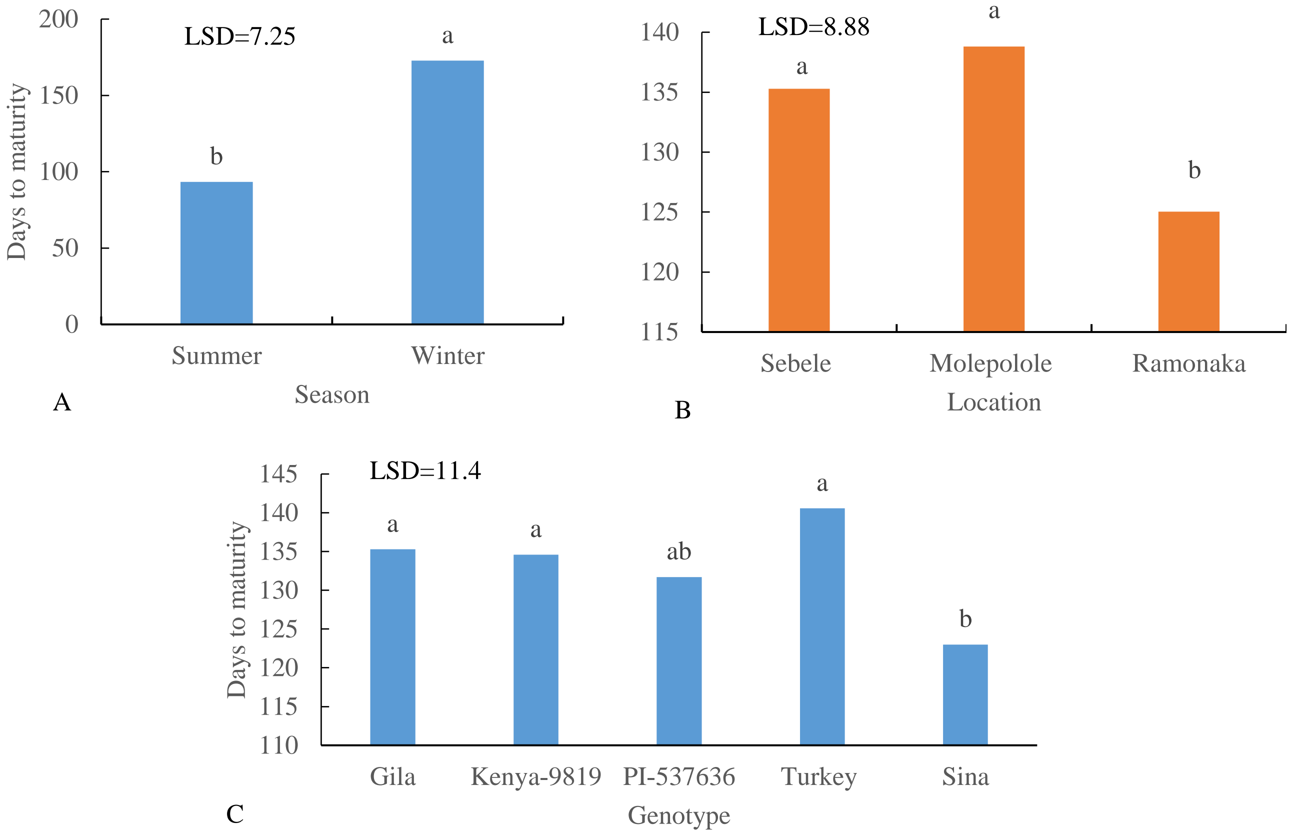

Figure 3.6. The effect of season, location, and genotype on the number of days to maturity. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.6 Plant height

The interactions of season × location ×genotype and location x genotype for plant height were not significant (P > 0.05) in this study (Appendix 2). However, plant height varied significantly (P < 0.001) with the interaction of season and location (Appendix 2). Generally, safflower planted in winter had outstandingly higher plant height than that planted in summer irrespective of location (Figure 3.7A). Thus, planting safflower in winter increased plant height by 38.67% compared to planting it in summer (Figure 3.7A). Moreover, safflower planted at Ramonaka in winter had noticeably taller plant height of 90.6 cm than safflower planted in other locations and seasons

(Figure 3.7A). In summer, no marked differences were observed among the locations for plant height (Figure 3.7A). However, in winter there were substantial variations in locations with respect to safflower plant height (Figure 3.7A).

The interaction effect of season × genotype significantly ($P < 0.001$) affected safflower plant height (Appendix 2). The genotype Turkey planted in winter attained a plant height of 97.06 cm which was outstandingly higher than plant height of any genotype and season (Figure 3.7B.). In contrast, the genotype Kenya9819 planted in summer had noticeably shorter (51.3 cm) plants than other genotypes planted in any season except for genotypes PI537636 and Sina planted in summer (Figure 3.7B). Overall, the height of genotypes PI537636 and Sina was statistically ($P > 0.05$) similar in winter and summer planting, though there was a substantial seasonal difference (Figure 3.7B). In general, safflower plant height ranged between 51.26 and 97.06 cm depending on the genotype and season (Figure 3.7B).

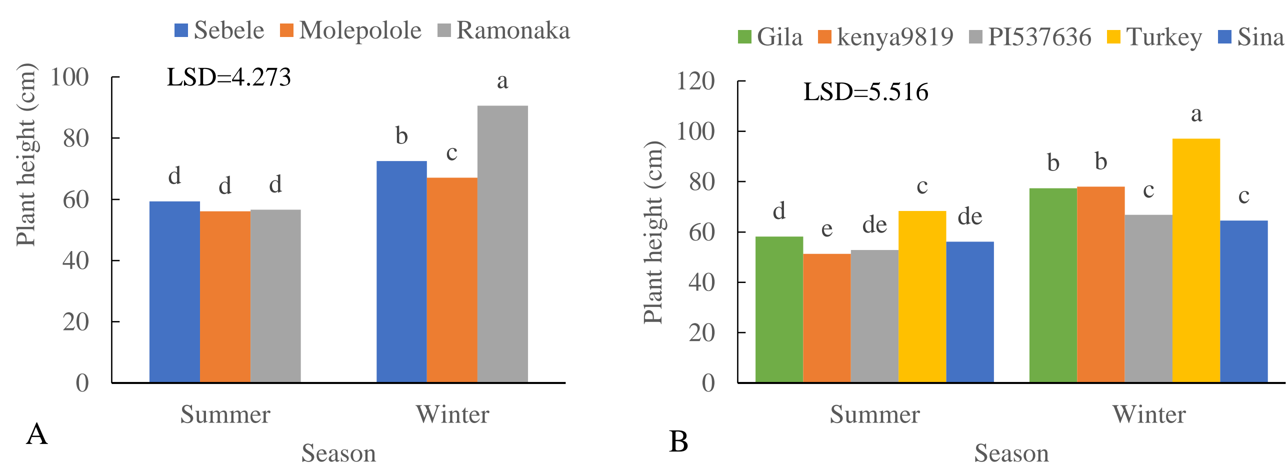

Figure 3.7. Effect of season × location (A) and season × genotype (B) on the plant height. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.7 Shoot biomass

The three-way interaction of season × location × genotype significantly ($P < 0.01$) influenced safflower shoot biomass (Appendix 2). The results demonstrated that on average, safflower shoot biomass was statistically ($P < 0.05$) higher in winter than summer independent of location and genotype (Figure 3.8). The genotype Sina planted at Ramonaka in winter produced markedly ($P < 0.05$) plants with higher shoot biomass of 177.43 g/plant than that of other genotypes planted in different locations and seasons except for Kenya9819 planted at the same location and season. The genotype Gila planted in summer at Sebele had plants with the least shoot biomass of 18.0 g/plant compared to other genotypes planted in other locations and seasons with exception of all genotypes planted in summer at all locations and genotypes PI537636 and Sina at Sebele, and Kenya9819 at Molepolole in winter (Figure 3.8). In summer, there were no significant ($P>0.05$) genotypic variation for shoot biomass in all locations (Figure 3.8). Also, there was no genotypic variation for safflower shoot biomass planted at Molepolole in winter. Overall, safflower shoot biomass ranged between 18.03 to 177.43 g/plant depending on genotype, season, and location (Figure 3.8).

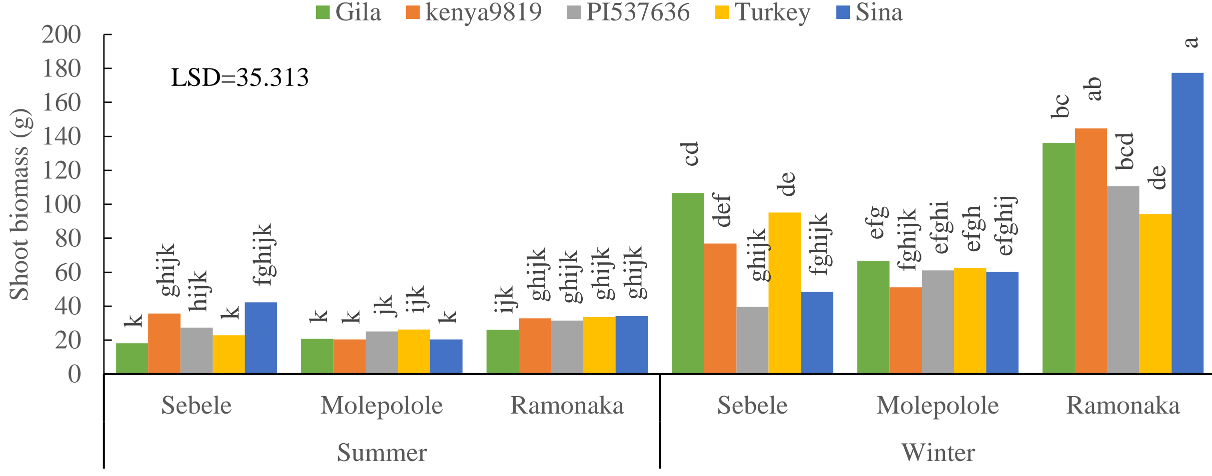

Figure 3.8. Effect of planting season, location, and genotype on the shoot biomass. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.8 Root biomass

The main effect of genotype, interactions of season × location × genotype, season × genotype, and location × genotype were not significant ($P > 0.05$) for root biomass of safflower (Appendix 2). Nevertheless, a highly significant ($P < 0.001$) interaction effect of season and location was found for safflower root biomass (Appendix 2). The results showed that planting safflower in winter substantially increased root biomass of safflower by 239.04% compared to summer planting (Figure 3.9). Safflower planted at Ramonaka in winter produced plants with higher root biomass of 13.2 g/plant than those planted in any locations and seasons (Figure 3.9). On the contrary, safflower planted at Molepolole in summer had noticeably lower root biomass of 2.0 g/plant than those planted in other locations and seasons with exception of summer planted ones in other locations (Figure 3.9). In winter, safflower planted at Molepolole and Sebele had no significant (P

> 0.05) difference in root biomass. Generally, root biomass ranged between 2.02 to 13.2 g/plant depending on location and season (Figure 3.9).

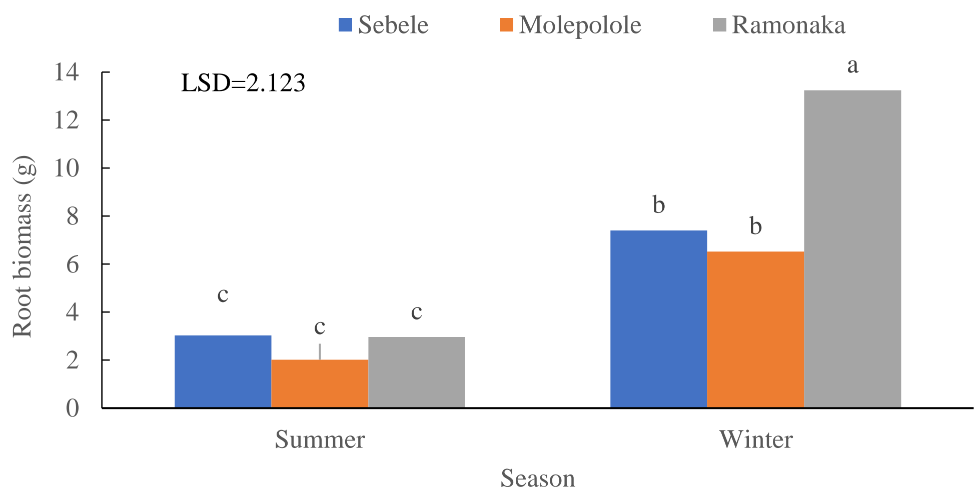

Figure 3.9. Interaction effect of planting season × location on the root biomass.
Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.9 Number of primary branches per plant

The main effect of genotype, the interactions of season × location × genotype, season × genotype, and location × genotype were not significant (P > 0.05) for number of primary branches/plant (Appendix 3). However, there was a highly significant (P < 0.001) season and location interaction for number of primary branches/plant (Appendix 3). Figure 3.10 depicts that safflower planted at Ramonaka in winter produced 13.5 primary branches/plant which was noticeably higher than primary branches/plant produced in any locations and seasons. On the contrary, the lowest number of primary branches/plant of 6.4 was produced by safflower planted at Molepolole in winter which

was different from safflower planted at Sebele in winter (Figure 3.10). In summer, a substantially high number of branches/plant was observed in Sebele and Ramonaka but their number of primary branches/plant did not significantly (P > 0.05) differ, but they were substantially lower than the number of primary branches/plant of safflower planted at Ramonaka in winter (Figure 3.10). Generally, throughout the seasons and locations, the number of primary branches/plant ranged between 6.4 and 13.52.

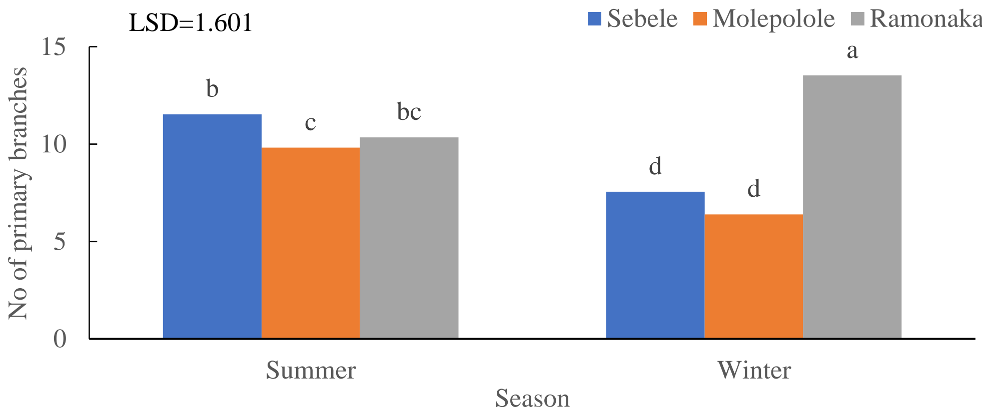

Figure 3.10. Effect of planting season × location on the number of primary branches/plant Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.10 Number of capitula/plant

The season × location interaction effect significantly (P < 0.001) influenced the number of capitula/plant (Appendix 3). The results revealed that safflower planted at Ramonaka during winter produced 46.7 capitula/plant which was statistically (P < 0.05) higher than that of other locations and seasons (Figure 3.11A). The number of capitula/plant showed no significant (P>0.05) differences across locations during summer. Likewise, in winter, the number of capitula/plant was similar across locations except in Ramonaka (Figure 3.11A).

The interactions of season × location × genotype, season × genotype, and location × genotype were not significant (P>0.05), hence the main effect of genotype for the number of capitula/plant was presented. There was significant (P<0.05) genotypic variation for the number of capitula/plant (Appendix 3). Plants of the genotype Sina had outstandingly higher number of capitula/plant (30.47) than the genotype PI537636 (Figure 3.11B). However, all genotypes produced relatively similar number of capitula/plant except for PI537636 which showed substantially lower capitula number/plant (21.37) (Figure 3.11B). In general, the number of capitula/plant ranged between 21.37 to 30.47 depending on genotype (Figure 3.11B).

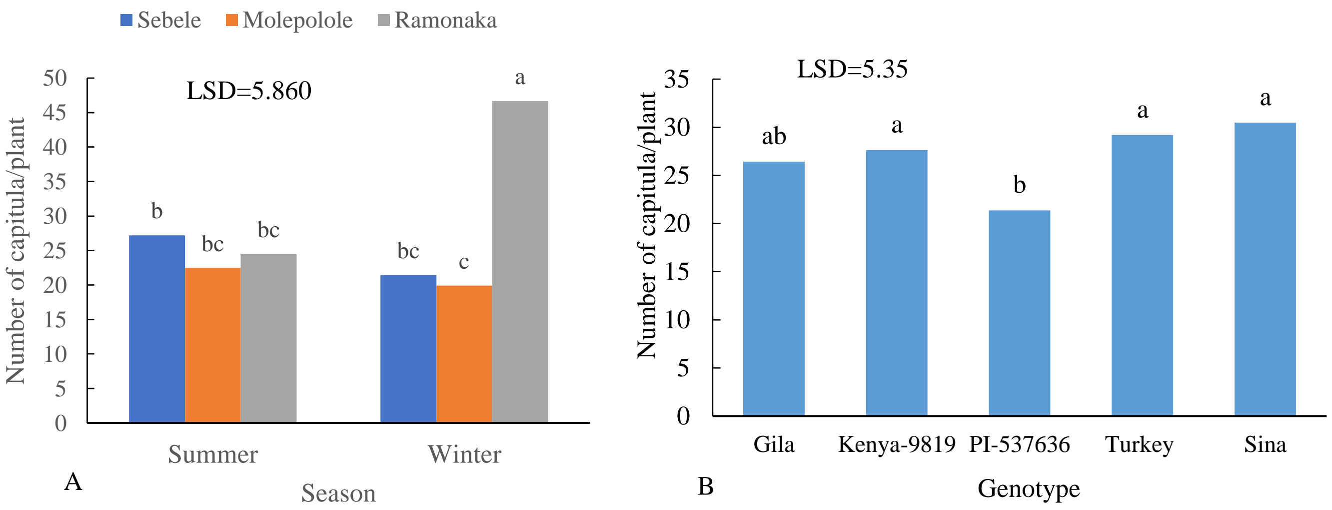

Figure 3.11. Effect of season × location (A) and genotype main effect (B) on the number of capitula/plant. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.11 Capitula weight

The interaction of season x location x genotype was not (P > 0.05) significant for capitula weight (Appendix 3). However, the season and location interaction significantly (P < 0.001) influenced the capitula weight (Appendix 3). Figure 3.12A demonstrated that safflower planted at Molepolole

in winter produced noticeably heavier capitula weight of 2.47 g than those produced by safflower plants grown in other locations and seasons with exception of safflower planted at Ramonaka in winter (Figure 3.12A). In contrast, safflower planted at Ramonaka in summer produced plants with considerably smaller capitula weight of 1.21 g than that of other locations and seasons (Figure 3.12A). Additionally, safflower planted at Molepolole and Sebele, and Sebele and Ramonaka in summer and winter, respectively had similar capitula weight (Figure 3.12A).

The season × genotype interaction effect significantly ($P < 0.05$) influenced the capitula weight (Appendix 3). Genotypes Kenya9819 and Sina consistently exhibited significantly ($P < 0.05$) higher and lower capitula weight, respectively than other genotypes independent of season (Figure 3.12B). The highest capitula weight of 3.1 g was exhibited by genotype Kenya9819 grown in winter which was noticeably higher than capitula weight than that of any genotype in all seasons (Figure 3.12B). The genotype Sina grown in summer had the lowest capitula weight of 1.43 g in comparison to other genotypes grown in various seasons except for genotypes Gila and PI537636, and Sina planted in summer and winter, respectively (Figure 3.12). Genotypes PI537636 and Turkey, and Kenya9819 planted in winter and summer, respectively had noticeably greater capitula weight than that of any genotype planted in winter and summer, except for genotype Kenya9819 planted in winter (Figure 3.12B).

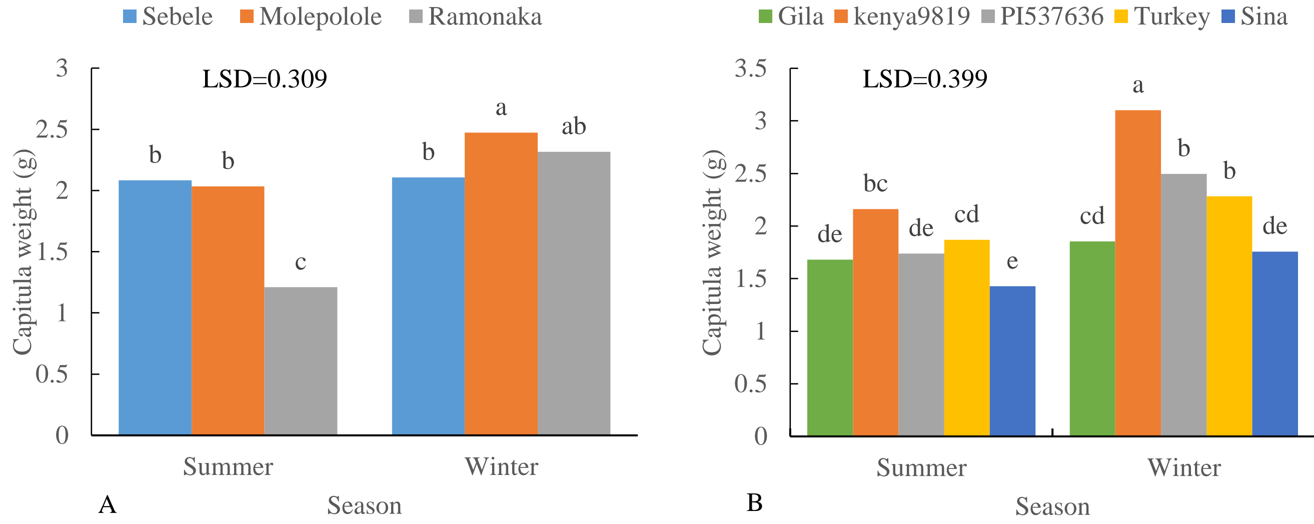

Figure 3.12. Effect season × location (A) and season × genotype (B) on the capitula weight. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.12 Capitula diameter

The main effect of location, interactions of season × location × genotype, season × genotype, and location × season were not significant (P > 0.05) for capitula diameter (Appendix 3). However, the seasonal variation significantly (P < 0.001) influenced the capitula diameter (Appendix 3). Planting in winter resulted in a substantial increase (4.69%) in the capitula diameter compared to safflower planted in summer (Figure 3.13A). Furthermore, genotype × location interacted significantly (P < 0.01) to influence capitula diameter (Appendix 3). The interaction showed that Sina produced noticeably smaller capitula diameter than any genotype in all planting locations (Figure 3.13B). The Turkey grown at Sebele attained the largest capitula diameter of 25.75 mm than the rest of genotypes planted in other locations except for genotypes Gila and Kenya9819, Gila, PI537636, and Turkey, and Gila, Kenya9819, and PI537636 planted at Sebele, Molepolole, and Ramonaka, respectively (Figure 3.13B). At Molepolole and Ramonaka, the genotypes had no marked

differences in capitula diameter excluding genotype Sina (Figure 3.13B). Generally, the capitula diameter ranged between 21.71 to 25.75 mm depending on the genotype and location (Figure 3.13B).

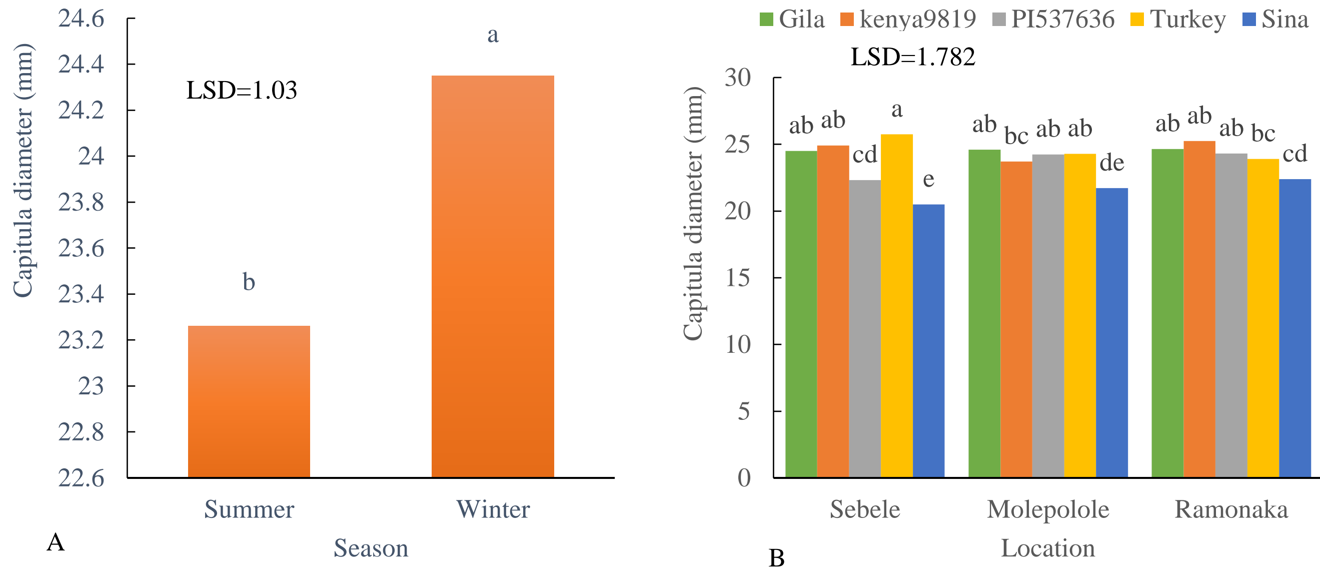

Figure 3.13. Effect of season × genotype (A) and seasonal main effect (B) on capitula diameter. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.13 Thousand seed weight

A significant ($P < 0.05$) interaction of season, location, and genotype was found for the 1000-seed weight (Appendix 3). The genotype Kenya9819 planted in winter at Ramonaka showed remarkably higher 1000-seed weight (46.0 g) than the rest of the genotypes grown at various locations in different seasons with exception of genotypes Sina and Kenya9819 planted at Ramonaka and Molepolole, and Molepolole in winter and summer, respectively (Figure 3.14). The genotype Gila consistently produced significantly ($P<0.05$) lower 1000-seed weight than the rest of genotypes planted in various locations and seasons (Figure 3.14). In general, the lowest 1000-

seed weight was observed in safflower plants grown at Ramonaka in summer compared to other locations and seasons (Figure 3.14). The 1000-seed weights ranged between 19.77 to 46.04 g depending on the season, location, and genotype (Figure 3.14).

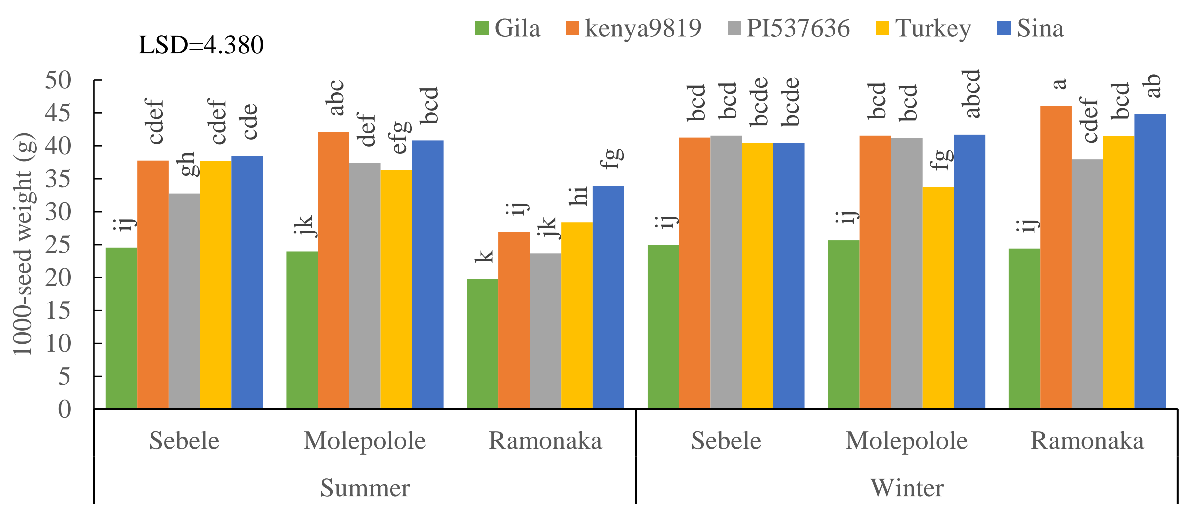

Figure 3.14. Effect of planting season × location × genotype on 1000-seed weight. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.14 Seed yield

With respect to seed yield, a significant ($P < 0.001$) interaction between season and location was found for this trait in the current study (Appendix 3). The results demonstrated that safflower grown in winter at Ramonaka produced statistically higher seed yield of 3803.13 kg/ha than that was obtained in other locations and seasons (Figure 3.15A). In contrast, safflower planted at Ramonaka accumulated a seed yield of 820.53 kg/ha in summer which was lower than what was attained in other locations and seasons with the exception for safflower planted at Molepolole in the same season (Figure 3.15A). Safflower seed yield/ha at Sebele and Molepolole was not

affected by season (Figure 3.15A). Generally, safflower planted in winter had higher seed yield by 84.4% in comparison with that planted in summer (Figure 3.15A).

The interactions of season × location × genotype, season × genotype, and location × genotype were not significant (P>0.05), hence the main effect of genotype for seed yield was presented. A marked (P < 0.05) genotypic variation was found for seed yield in the current study (Appendix 3). The genotype Kenya9819 accumulated a seed yield of 2265.22 kg/ha which was statistically greater than that of any genotype with exception for seed yield of genotypes Turkey and Sina (Figure 3.15B). The seed yield of genotypes Gila, PI537636, Turkey, and Sina did not vary (Figure 3.15B). Generally, seed yield ranged between 1490.44 and 2265.22 kg/ha depending on genotype (Figure 3.15B).

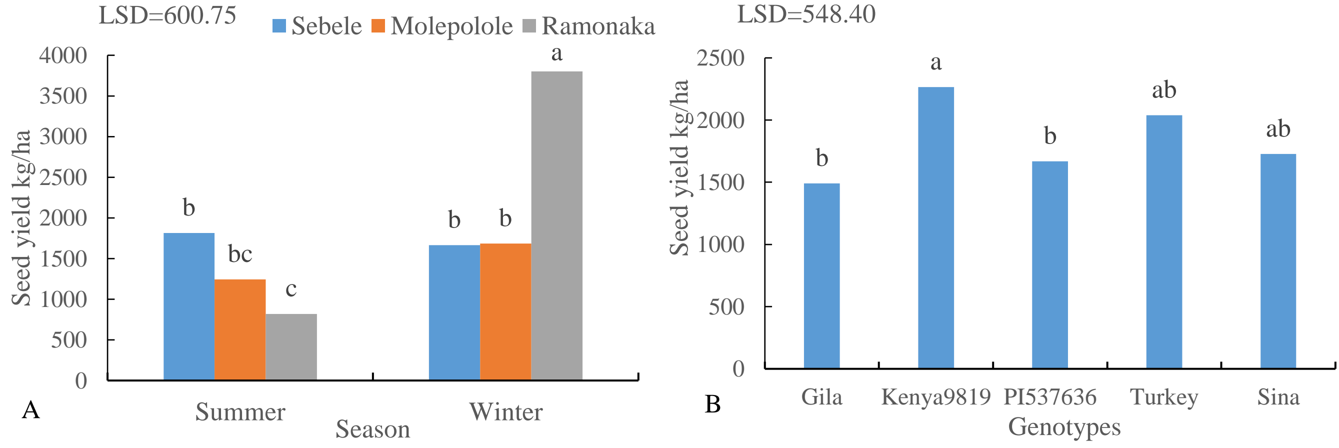

Figure 3.15. Effect of planting season × location (A) and genotypic effect (B) on the seed yield Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.15 Oil content

A significant ($P < 0.01$) interaction of year, season, and location was found for oil content (Appendix 3). Generally, oil content was higher in winter-planted safflower than in summer-planted safflower (Figure 3.16). The interaction effect demonstrated that genotype Gila consistently had the highest oil content ranging between 28.3-42.3% depending on planting season and location (Figure 3.16). The genotype Gila planted at Molepolole in winter produced seed with substantially the highest oil content of 42.27% in comparison with other genotypes sown at any location and season (Figure 3.16). In contrast, the genotype Turkey planted at Ramonaka in summer had the lowest oil content of 11.82% compared to genotypes planted at various locations and seasons (Figure 3.16). The seed oil content of the genotype Gila planted at various locations in summer and winter was not significant ($P>0.05$) but remained noticeably higher than that of other genotypes in other locations and seasons with exception of Gila planted at Molepolole in winter (Figure 3.16). The second highest oil-producing genotypes were Kenya9819 (17.0-32.2%) and PI537636 (15.7-33.4%) depending on season and location (Figure 3.16). Generally, safflower oil content ranged between 11.8-42.3% depending on the genotype, location, and season (Figure 3.16).

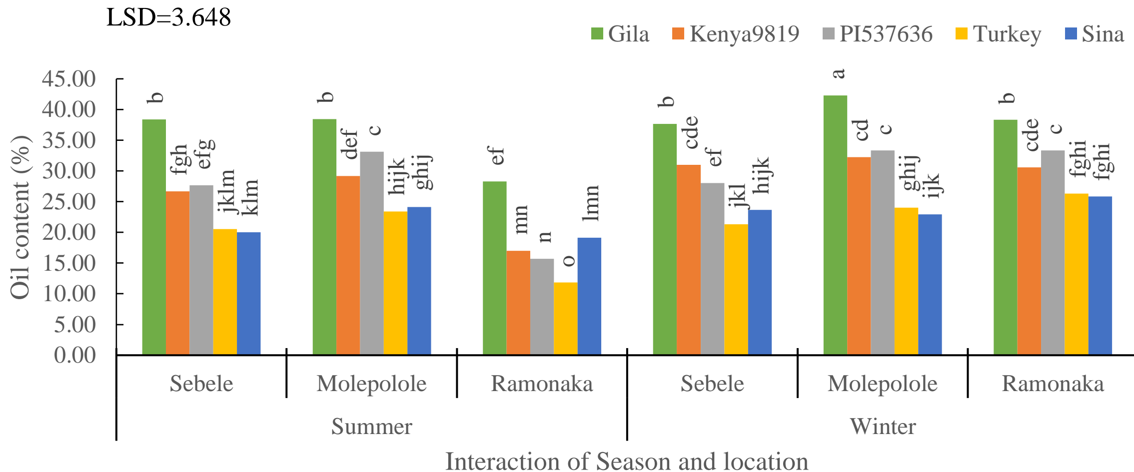

Figure 3.16. Effect of planting season, location, and genotype on oil content.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.16 Oil yield

Oil yield was statistically ($P < 0.001$) affected by the interaction of season and location (Appendix 3). The results showed that the highest oil yield of 1120.92 kg/ha was obtained in safflower plants grown at Ramonaka in winter which was markedly higher than that was produced in other locations and seasons (Figure 3.17A). On the contrary, the lowest oil yield of 147.70 kg/ha was produced by safflower plants planted in summer at Ramonaka compared to safflower plants in other locations and seasons (Figure 3.17A). Oil yield of plants grown at Sebele and Molepolole was relatively similar independent of season. In general, safflower planted in winter had oil yield increment of 114.16% in comparison with summer planted safflower (Figure 3.17A). Generally, the oil yield/ha ranged from 147.70 to 1120.92 kg/ha depending on season and location (Figure 3.17A).

There was a significant ($P < 0.05$) interaction effect of location and genotype for safflower oil yield (Appendix 3). The genotype Kenya9819 planted at Ramonaka had maximum oil yield of 752.53 kg/ha than that of the rest of the genotypes planted at various locations with exception of all genotypes planted at Ramonaka, genotype Gila planted at Molepolole, and genotypes Gila and Kenya9819 planted at Sebele (Figure 3.17B). The lowest oil yield of 247.04 kg/ha was produced by the genotype Sina planted at Molepolole although it was statistically similar ($P > 0.05$) to the oil yield produced by genotypes Kenya9819, Turkey, and PI537636 planted at Molepolole, and genotypes Sina, Turkey, and PI537636 planted at Sebele (Figure 3.17B). Generally, safflower genotypes planted at Ramonaka produced higher oil yield than other locations (Figure 3.17B).

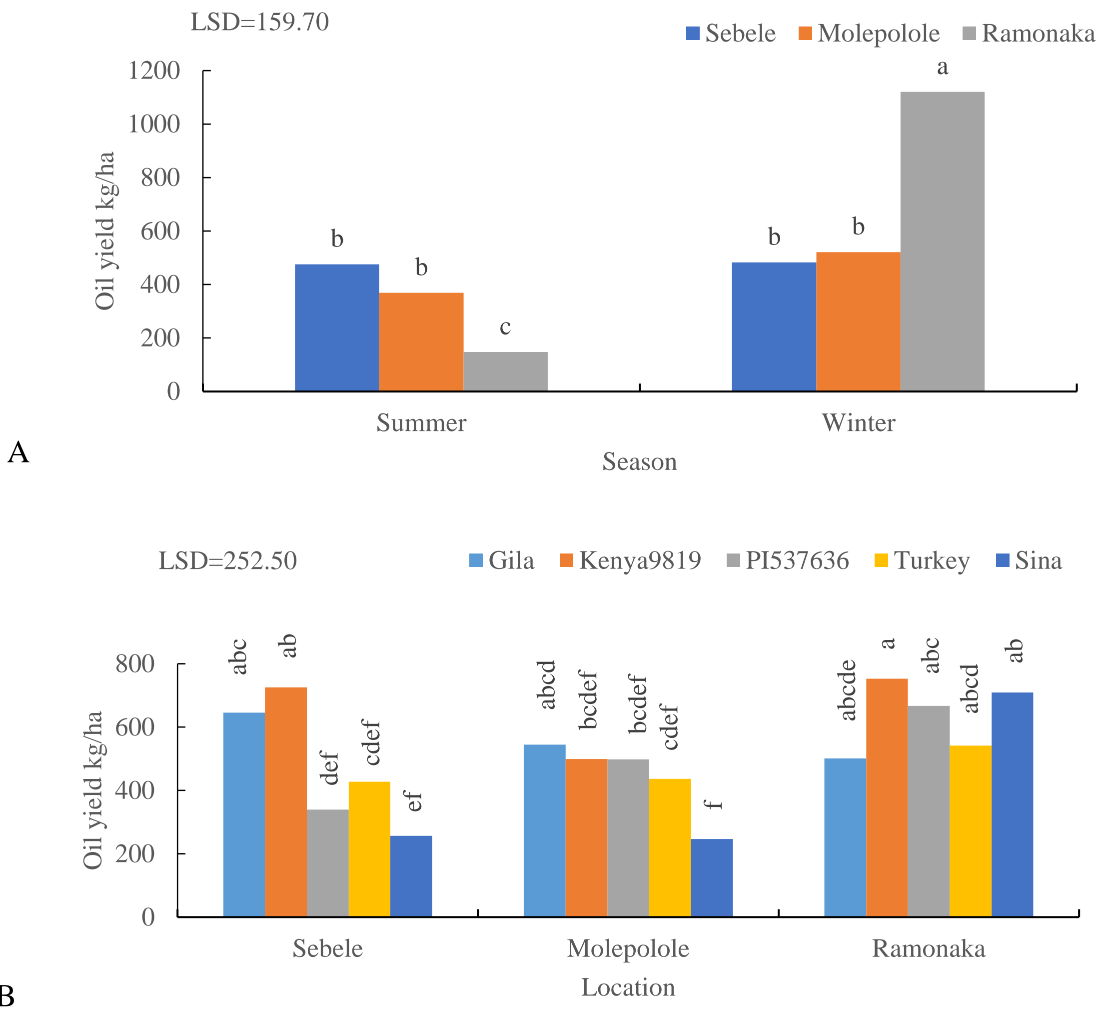

Figure 3.17. Effect of planting season and location (A) and location and genotype (B) on the oil yield. Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

3.3.17 Correlations of yield and yield components

Figure 3.18 demonstrates that there is a positive correlation between seed yield and yield components with the exception of oil content (Figure 3.18). On the other hand, oil yield revealed positive correlation with all the studied traits. 1000-seed weight showed a significant positive correlation with capitula weight and the phenological traits (Figure 3.18). Oil content exhibited a significant ($P < 0.05$) positive correlation with the phenological traits and shoot biomass while it

had no substantial correlation with plant height, number of branches, and capitula number (Figure 3.18). The number of primary branches/plant positively correlated with the growth traits and number of capitula/plant (Figure 3.18). Number of capitula/plant showed a significant ($P < 0.05$) positive correlation with the growth parameters (plant height and shoot biomass) and capitula diameter. Capitula diameter significantly ($P < 0.05$) correlated with the phenological traits, growth parameters and capitula weight. Capitula weight correlated with the growth traits and phenological traits. The growth traits correlated positively with each other and with the phenological traits (Figure 3.18). Likewise, the phenological traits significantly ($P < 0.05$) correlated positively with each other (Figure 3.18).

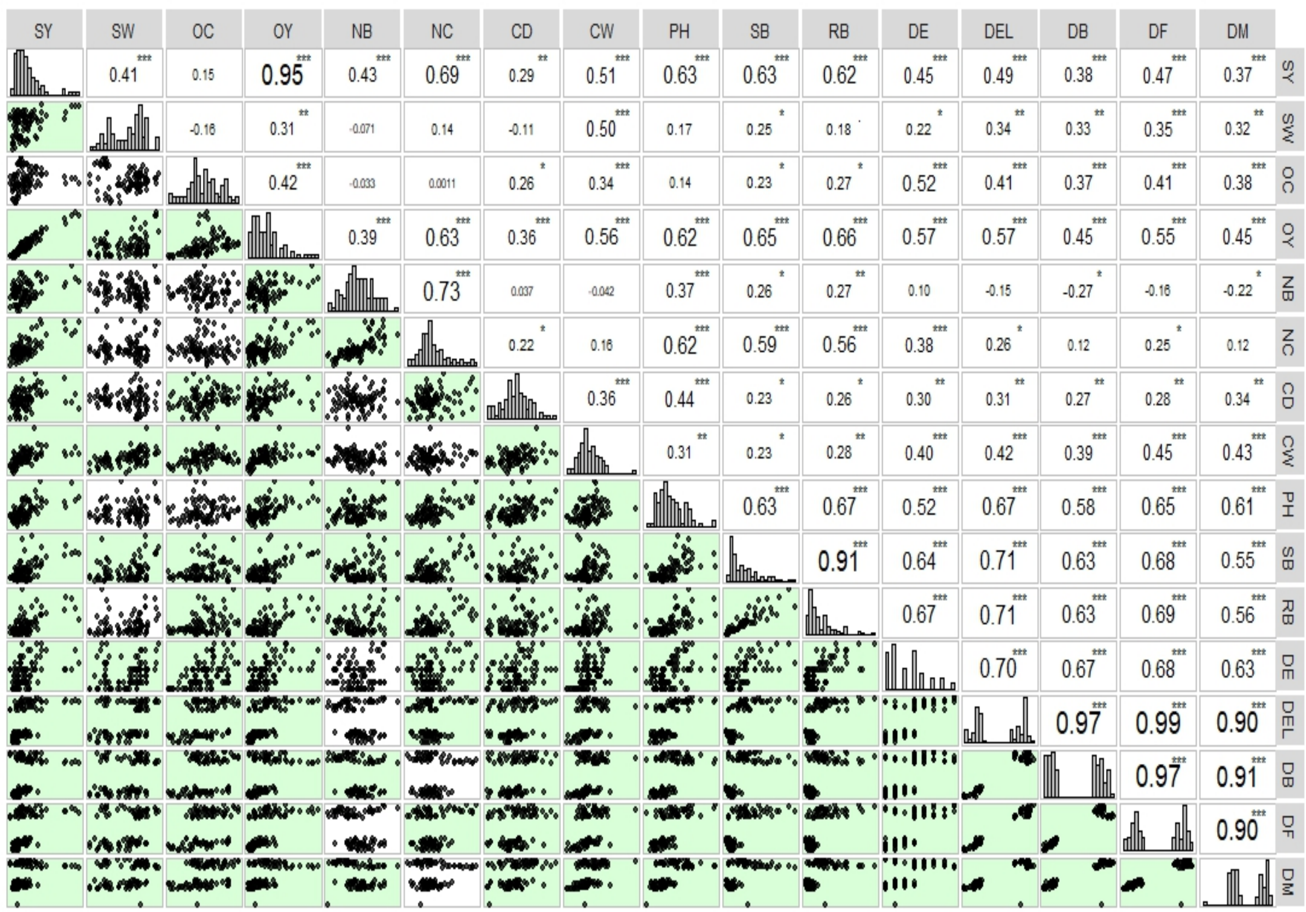

Figure 3.18. Pearson correlation matrix showing relationship among the studied traits.

*: P≤0.05; **: P≤0.01; ***: P≤0.001.

Seed yield kg/ha (SY), 1000 seed weight (SW), oil content (OC), oil yield (OY), number of primary branches/plant (NB), number of capitula/plant (NC), capitula diameter (CD), capitula weight (CW), plant height (PH), shoot biomass (SB), root biomass (RB), days to emergence (DE), days to elongation (DEL), days to branching (DB), days to flowering (DF), and days to maturity (DM).

3.3.18 Genotype × environment (G×E) interactions for seed yield using GGE biplot analysis

The G×E interactions for seed yield kg/ha were determined through the combined seasons GGE biplot analysis. In this study, a test environment refers to year and location combination. The biplots examined 94.17% of the total variation (PC1 and PC2) of the environment-centered G×E

table. The arrowed line is the average environment coordinate (AEC) abscissa it points to higher mean yield across environments. The AEC coordinate (green line without arrows) in either direction far away from the biplot origin indicates a greater G×E interaction (GEI) effect and poor stability. The genotypes located on the right of the line cutting the axis, representing the mean seed yield according to the mean center of coordinates, yielded higher seed yield than the mean. While the genotypes on the left yielded lower seed yield than the average. The results showed that Kenya9819 and Turkey were the highest-yielding genotypes while Sina, PI537636, and Gila were poor yielding genotypes (Figure 3.19a, b) as shown by their projections onto the average environment coordination (AEA) axis. The genotypes Sina and Gila had greater instability as they had the longest vector of the genotypes on either side of AEA (Figure 3.19a, b). While the genotypes Kenya9819 and Turkey had greater yield stability followed by PI537636 though low yielding (Figure 3.19a, b).

A polygon is drawn on genotypes that are farthest from the biplot origin so that all other genotypes are confined within the polygon. The equality lines divide the biplot into sectors, and the winning genotype for each sector is the one located on the respective vertex. The which-won-where polygon view revealed that the test environments (Sebele and Molepolole) fell within one sector of the four sectors (Figure 3.19b). Genotypes Gila and PI537636 each fell within a sector that did not have any environment. On the other hand, genotypes Kenya9819 and Turkey fell within a sector that had two environments (Sebele and Molepolole) suggesting that these genotypes were high yielding at those locations although Kenya9819 was the winner genotype because it fell at the vertex of that sector (Figure 3.19b). Genotype Sina fell within a sector that had Ramonaka suggesting that it was a winning genotype at Ramonaka (Figure 3.19b).

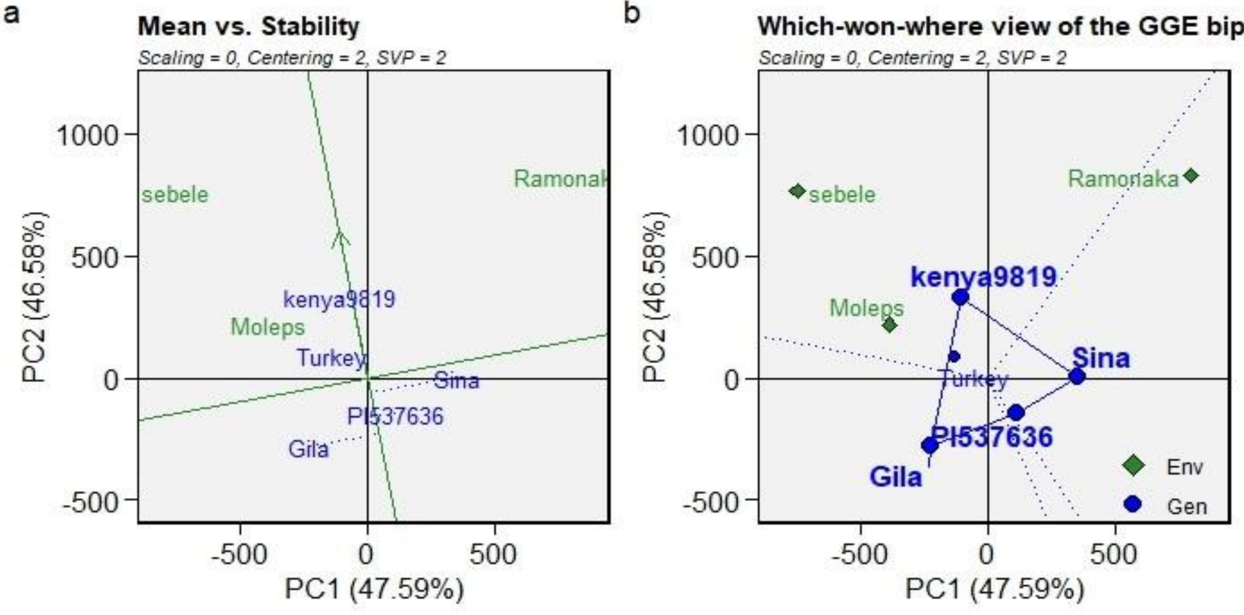

Figure 3.19. The vector view of GGE biplot showing the mean vs stability (a) and which-won-where view of the GGE biplot (b). Moleps means Molepolole

The lines that join the test environments to the biplot origin are called environment vectors. The average environment (represented by a line with the arrow) has the average coordinates of all test environments (Sebele, Molepolole, and Ramonaka), and AEA (Average-Environment Axis) is the line that passes through the average environment and the biplot origin. The length of the location (environment) vector from the biplot origin, shows the discriminative ability of the location. Figure 3.20a shows that Ramonaka and Sebele had the longest vector from the biplot origin hence the most discriminative. Molepolole had the shortest vector closer to the biplot origin hence least discriminative. The representativeness of location is determined by the closeness of its angle with AEC, with smaller angle between location vector and the AEC indicating that the test location is more representative of other test environments. The representativeness analysis revealed that Sebele had a smallest angle with the AEA, hence, it is more representative of the other test environments followed by Ramonaka while Molepolole was the least representative (Figure

6.20a). The cosine of the angle between the vectors of two environments approximates the correlation between them. Sebele and Molepolole had a strong relationship in the ranking of genotypes as indicated by an acute angle between them (Figure 3.20b). Ramonaka on the hand showed a weak relationship with both Sebele and Molepolole (Figure 3.20b).

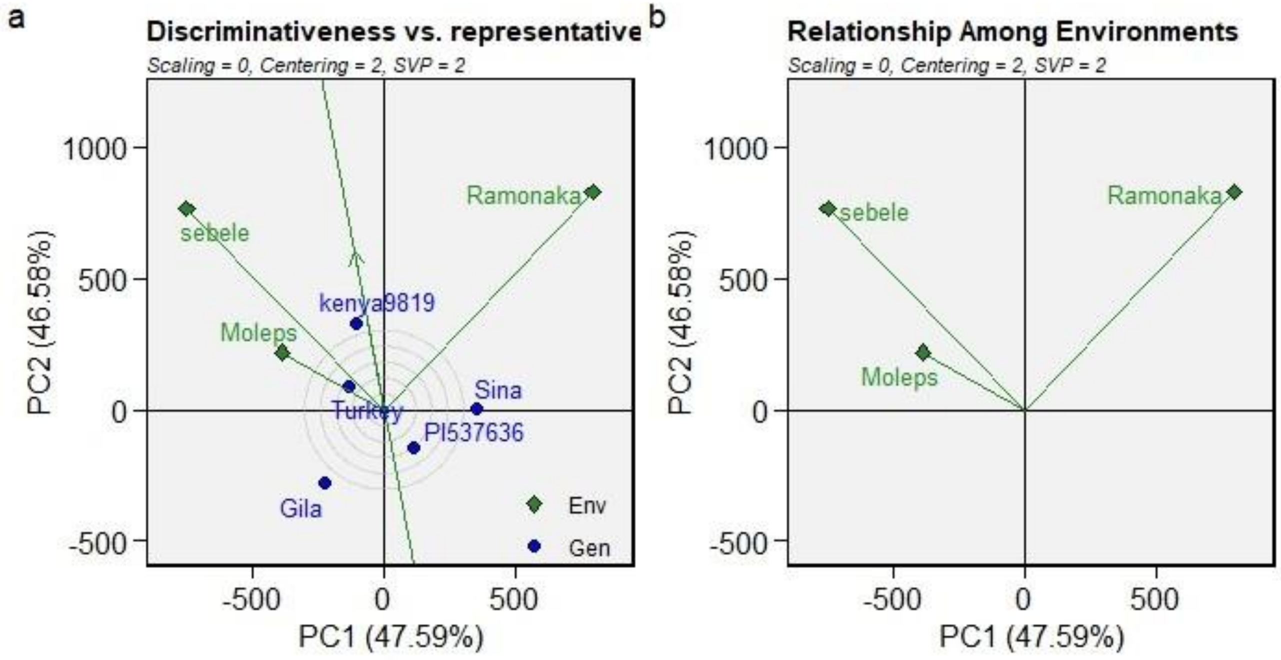

Figure 3.20. A vector view of GGE biplot showing the discriminative versus representativeness (a) and relationship among test environments (b). Moleps means Molepolole

An ideal genotype was denoted by a small circle with an arrow pointing to it and had both the highest mean yield and higher stability across the environments. When ranking genotypes, Kenya9819 was found to be the most desirable genotype (Figure 3.21a). On the other hand, Gila was the least desirable genotype (Figure 3.21a). Generally, genotypes ranked as Kenya9819 > Turkey > Sina > PI537636 > Gila. Regarding the ranking of environments, the ideal environment was used as the center of a set of concentric circles (Figure 3.21b). Environment ranking biplot

revealed that Sebele was closest to the ideal environment, hence, desirable for selecting genotypes adaptable in all the three test environments while Ramonaka was the least desirable (Figure 3.21b).

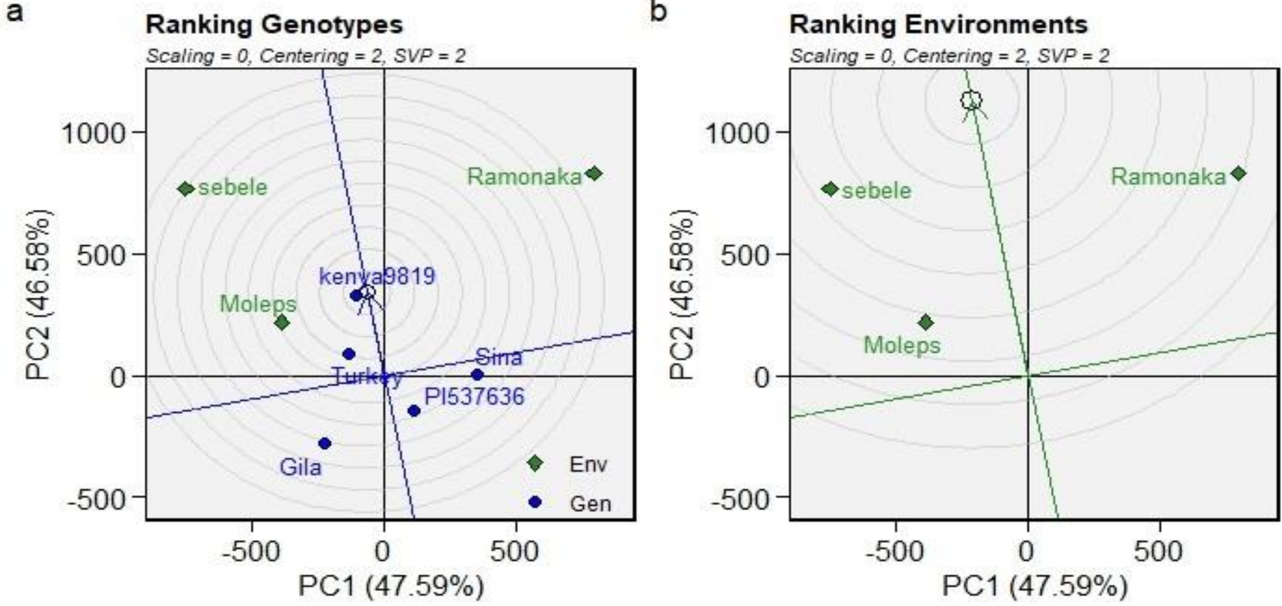

Figure 3.21. The vector view of GGE biplot ranking genotypes (a) and ranking environments (b). Moleps means Molepolole

3.3.19 Genotype by yield*trait (GYT) biplot

The yield*trait biplot was based on the fact that yield is the most important trait while all other target traits are only important when combined with high yield. In the GYT biplot below (Figure 3.22) yield was not included because it was already integrated into each of the yield-trait combinations. This biplot presents data from three locations replicated in winter and summer. The average tester coordination view of the GYT biplot was used to rank genotypes based on their overall superiority among the yield-trait combinations and to show their trait profiles following the methodology by Yan and Frégeau-Reid (2018). The cosine of the angle between the vectors of two traits approximates the Pearson correlation between them. Therefore, an angle less than 90°

denotes a positive correlation, an angle larger than 90° denotes a negative correlation, and an angle of 90° denotes zero correlation. Figure 3.22A depicts that most of the studied yield-trait combinations correlated positively with each other as shown by the acute angle between the vectors. Yield*oil content was positively correlated with yield*all phenological traits, yield*capitula diameter, yield*capitula weight, and yield*oil yield while it negatively correlated with the rest of the traits (Figure 3.22A).

Further, an acute angle denotes that the genotype is above-average for the trait while an obtuse angle denotes that the genotype is below-average for the trait; and a right angle denotes that the genotype is average for the trait. Figure 3.22A shows that genotype Kenya9819 had an acute angle with all the studied yield*traits indicating that it is above average for all traits. As for genotype Turkey an acute angle was observed in relation to all the yield*traits except for yield*oil content indicating that it is above average for most of the studied traits (Figure 3.22A). Inversely, genotypes Gila and PI537636 had an obtuse angle with all yield*trait combinations indicating that they were below-average for all traits except for yield*oil content which was above-average (Figure 3.22A). On the other hand, genotype Sina was above-average for yield*shoot biomass, yield*number of capitula/plant, yield*number of branches, yield*plant height, yield*root weight, and yield*1000-seed weight (Figure 3.22A).

An ideal genotype was denoted by a small circle with an arrow pointing to it and had both the highest mean yield*trait combination and is overall superior. Therefore, when ranking genotypes in terms of overall superiority based on the yield*trait combinations, genotype Kenya9819 was found to be an ideal genotype followed by Turkey then Sina and PI537636 while genotype Gila was the least desirable (Figure 3.22B).

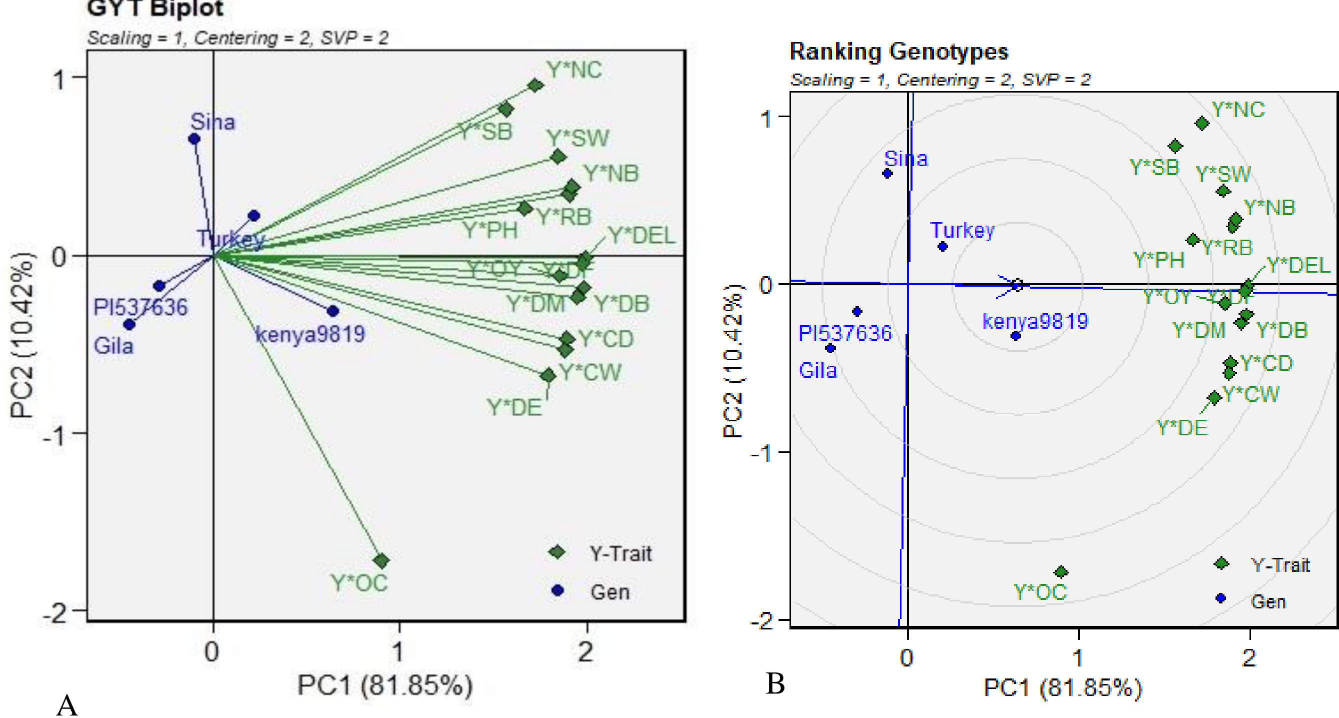

Figure 3.22. The tester coordination view of the yield*trait (GYT) biplot showing correlations among yield-trait combinations (A) and ranking of genotypes.

Seed yield kg/ha (Y), 1000-seed weight (SW), oil content (OC), oil yield (OY), number of primary branches/plant (NB), number of capitula/plant (NC), capitula diameter (CD), capitula weight (CW), plant height (PH), shoot biomass (SB), root biomass (RB), days to emergence (DE), days to elongation (DEL), days to branching (DB), days to flowering (DF), and days to maturity (DM).

3.4 Discussion

3.4.1 Number of days to emergence

Safflower phenological development was greatly affected by variations in seasons, locations, and genotypes in this study. The results revealed a significant interaction of genotype and season on number of days to emergence of safflower. For example, genotypes Kenya9819 and Gila grown in winter took substantially more days to emergence than genotypes Gila, Turkey, Sina, and PI537636 planted both in winter and summer. Additionally, the genetic and seasonal variation observed on days to emergence in the current study showed the presence of genotype and

environmental (G×E) interaction on safflower seed emergence. For instance, safflower planted in winter at Ramonaka took longer to emerge than in other locations and seasons. This was attributed to differences in soil texture across the locations. Soils at Ramonaka are clay loam, therefore, in winter these soils tend to have low temperatures and take a long time to warm up consequently delaying seedling emergence unlike sandy clay loam and sandy loam soils found at Molepolole and Sebele, respectively which had more sand particles (66-78%) (Table 5.1) that warm up quickly. According to Akter et al. (2016), clay soils have finer soil pores and tend to retain more moisture and thus making them attain lower temperature than other soil types. In summer, differences in soil texture may have contributed to variation in the timing of safflower seedling emergence across the locations. Thus, emergence was faster in Ramonaka because the soils were clay loam in texture which is better in retaining moisture that is needed for germination and seedling emergence. This is because the duration of germination and seedling emergence is determined by water availability and plant species (Haj Sghaier et al., 2022).

Genotype and environmental interaction on phenological traits of safflower have been reported to strongly vary with environmental conditions, especially temperature and rainfall (Beyyavas et al., 2011; Moghaddam et al., 2014; Kose et al., 2018; Arshad et al., 2020; Chehade et al., 2022; Malve et al., 2022; Thoday-Kennedy et al., 2023). Meticulous phenotyping of safflower is important in understanding safflower plant behaviour under varied growing environments and recognizing unique traits for crop breeding. Genotypic variation in number of days to emergence of safflower is well documented in literature (Amini et al., 2008; Golkar et al., 2011b; Moatshe, 2019; Chehade et al., 2022). Studies of Singh et al. (2008) and Golkar (2011; 2014) reported that number of days to emergence of safflower seeds was governed by additive and dominance gene effects.

Generally, genotypes sown in winter took a longer time to emerge than those sown in summer mainly because of temperature variations between the two seasons. In summer, emergence occurred in November when temperatures were warm while in winter emergence occurred in May when temperatures were cool (Appendix 15). Furthermore, Torabi et al. (2015) found a significant negative correlation between days to emergence and temperature in safflower with lower than optimum temperatures of 22°C delaying emergence. A similar interaction of season and genotype for the number of days to emergence in safflower was reported by Moatshe and Emongor (2019). Longer duration of phenological stages of safflower grown in Botswana during winter has been attributed to cooler air temperature that favours growth and development than in summer (Kedikanetswe, 2012; Emongor et al., 2015, 2017; Oarabile, 2017; Moatshe & Emongor, 2019). The best temperature for safflower seed germination and growth is 15.6°C and 20-32°C, respectively (Kaffka & Kearney, 1998; Torabi et al., 2013, 2015). In addition to this, Haj Sghaier et al. (2022) affirmed that lower temperatures caused rapeseed germinating seeds to have a slower metabolism and subsequently slower growth, while higher temperature triggered faster metabolism and consequently disintegrating the seed energy required for growth.

3.3.2 Number of days to stem elongation

A significant interaction of season × location × genotype was found for number of days to stem elongation in this study. This indicated that the ability of safflower genotypes to initiate stem elongation was influenced by an interaction of genetic and environmental factors. For instance, in winter, the genotypes Gila, Sina, Kenya9819, and Turkey at Ramonaka, Gila at Sebele and Turkey at Molepolole took a longer period to reach stem elongation than other genotypes planted in different locations and seasons. This highlighted the inconsistencies and instability in the

performance of genotypes across locations and seasons, that is, some genotypes took a longer time to start elongating at a certain location while they took less time when planted at a different location in the same season. This demonstrated that the variations in temperature, day length, and soil type observed across the locations and seasons had a large influence in moderating genotypic responses regarding days to elongation. Likewise, Koutroubas et al. (2004) reported that environmental conditions, such as solar radiation, soil moisture and water, air temperature, and soil nutrient content significantly influenced performances of safflower varieties, whereby, the same variety was able to perform differently under different climatic conditions.

Furthermore, genotypes took longer time to initiate stem elongation in winter as compared with summer because low winter temperatures prolonged the rosette stage and subsequently delaying the onset of stem elongation stage. The opposite was true during summer because high temperatures reduced the duration of the rosette stage and subsequently shortening the days to stem elongation. In the current study, safflower plants took 24.3-69.7 days to reach elongation stage depending on genotype, location, and season of planting. During the experimental period the average minimum and maximum temperatures in summer and winter ranged between 15.4-20.2°C and 29.5-30.3°C, and 2.3-8.5°C and 23.2-26.2°C, respectively. Temperature has been reported to significantly influence the length of the phenological stages of safflower (Wachsmann et al., 2010; Emongor et al., 2017; Emongor & Emongor, 2023). Safflower seedlings require low temperatures of 15–20°C after emergence for rosette development and root growth, but high temperatures of 20–30°C during stem elongation and reproduction phenological stages (Li et al., 1997; Carapetian, 2001; Emongor & Oagile, 2017; Emongor & Emongor. 2023). Similarly, Dajue and Mundel (1996) highlighted that cold temperatures lengthen the duration of rosette stage and subsequently delaying the onset of stem elongation. On average, safflower planted at Ramonaka in winter took

a long time to reach the stem elongation stage than safflower planted during winter at Molepolole and Sebele which reached the elongation stage earlier. This was because safflower planted at Ramonaka emerged late as compared with other locations and thus the onset of stem elongation was delayed. A study in Botswana showed that differences in days to elongation between summer and winter was attributed to warmer temperatures in summer (average minimum and maximum temperatures were 17-23°C and 28-36°C) than winter (average minimum and maximum temperatures were 4-16°C and 22-29°C) (Moatshe, 2019). Part of the variation in days to elongation of safflower in the different locations, seasons, and genotypes in the current study could be explained by gene expression. Many authors have reported genetic variation occurring in safflower germplasm grown in different environments (Yang et al., 1993; Mehthre et al., 1995; Alizadeh & Jirair, 2006; Golkar, 2011; 2014; Bella et al., 2019). Overall, the results demonstrated that days to stem elongation in safflower was highly influenced by genotype × environment (G x E) interaction and this corroborated those reported in literature (Cosge & Kaya, 2008; Koc, 2019; Bella et al., 2019; Moatshe & Emongor, 2019). Large genetic variation is useful in breeding, selection, and improvement of the productivity and adaptability of safflower genotypes (cultivars) in different environments.

3.4.3 Number of days to first branching

A significant interaction of season × genotype for number of days to branching was observed in this study. This suggested that genotypic environmental factors interacted with each other to influence the timing of branching. From this study, genotype Gila planted in winter took the longest time to start branching while genotype Sina planted in summer took the shortest time to start branching as compared with the duration of other genotypes planted in various seasons and

locations. Generally, the timing of branching was influenced by the timing of stem elongation, therefore, any factor which delayed stem elongation consequently delayed the timing of branching. Thereby, genotypes that took a long and short time to reach elongation stage also took a long and short time to branching stage, respectively. Moatshe and Emongor (2019) also found that the number of days to the onset of branching was influenced by environmental factors with winter season delaying the timing of branching.

The interaction of season × location statistically affected the onset of branching in the current study. This showed that the environmental conditions interacted to influence days to the onset of branching. Generally, branching started significantly earlier in Ramonaka during summer and longer in Sebele during winter than safflower planted in different locations and seasons. This was partly attributed to earlier seed emergence and reaching the elongation stage earlier in Ramonaka in summer than Sebele and Molepolole. Additionally, timing of branching was long during winter in Sebele followed by Molepolole because safflower planted at these sites experienced chilling injury in 2021/2022 season (Appendix 15) during the stem elongation stage in July (the coldest month) with the degree of injury being higher at Molepolole, hence slowing their growth. Nevertheless, safflower grown in Ramonaka during winter escaped chilling injury because they were at the rosette stage during the coldest phase. Koc (2019), stated that a longer rosette habit is advantageous in avoidance of winter chilling injury, and hence inhibiting plants from being injured by low winter temperatures. Therefore, plants with prolonged rosette period are more cold tolerant (Yasari et al., 2016). However, some of the plants from Molepolole and Sebele were able to recover and developed new leaves. According to Landry et al. (2017), if meristems survive, plants are able to recover from chilling injury and regrowth starts as new leaves replenish the injured ones. Another possible reason why plants from Sebele and Molepolole were seriously damaged by frost

injury was because the soils from these locations had a high percentage of sand (66-78%) than Ramonaka which had 39% sand. According to Flohr et al. (2016), sandy soils are susceptible to frost because of their low bulk density, low water-holding capacity, and moderately low nutritive level compared with other soil types. The findings of this study conform with those reported in literature which revealed that temperature significantly influences onset of branching in safflower (Wachsmann et al., 2010; Carapetian, 2001; El-Bassam, 2010; Bella et al., 2019; Emongor & Emongor, 2023).

3.4.4 Number of days to flowering

The seasons, locations, and genotypes interacted significantly to influence the number of days to flowering in this present study. This highlighted that the timing of flowering in this study was explained by G × E interaction. From the results of the current study, genotypes Gila planted at Ramonaka and Turkey planted at Molepolole and Ramonaka in winter took the longest time to reach the flowering stage while genotypes Sina and PI537636 grown at Ramonaka in summer took the shortest time. These genotypic variations in summer and winter for the onset of flowering stage suggested that genotypes varied in their ability to respond to changes in temperature and photoperiod across the seasons. Furthermore, genotypes Sina and PI537636 flowered earlier both in summer and winter, showing better adaptability to temperature and day length variations in different seasons and locations than other genotypes. This could also mean that genotypes Sina and PI537636 were the first ones to perceive the changes in the day length in winter (less sensitive to short day length) and hence, initiated flowering earlier than other genotypes. Studies in different countries have shown that the onset of flowering in safflower varied between 70-83 days after planting, depending on environmental conditions and genotypes (Uslu et al., 2002; Golkar et al.,

2011b; Sajid et al., 2021; Chehade et al., 2022). In the present study, the number of days to flowering ranged between 52 to 128 depending on genotype, planting location, and season. Days to flowering of safflower is known to be highly influenced by genotype and environment interaction (Cosge & Kaya, 2008; Shabana et al., 2013; Moatshe & Emongor, 2019). Genetic variation of safflower genotypes grown in different environments is well documented (Yang et al., 1993; Mehthre et al., 1995; Alizadeh & Jirair, 2006; Golkar, 2011; 2014; Golkar et al., 2017; Bella et al., 2019). Furthermore, Kotecha (1979), and Shahbazi and Seaidi (2007) reported additive gene effects in the control of days to flowering of safflower. While Gupta and Singh (1988a) reported over and partial dominance of gene action over the same trait. On the other hand, Golkar (2011) and Golkar et al. (2012) reported dominance gene effects were involved in the genetic control of days to flowering of safflower. Singh et al. (2008) reported that both additive and dominance gene effects-controlled days to flowering of safflower. The inconsistencies observed in the above explanations can be explained by variation due to genotype x environmental interactions observed in different growing conditions and sites. This corroborates with the results of Koutroubas et al. (2004), Alizadeh and Jirair (2006), and Bella et al. (2019) who revealed that variability among genotypes of safflower for the number of days to flowering was due to genotype x environment interaction.

Generally, safflower planted in winter took significantly longer time to start flowering than in summer independent of location and genotype. This was mainly attributed to variations in temperature and photoperiod across seasons. Safflower is a long day plant, therefore, in winter flowering was delayed because of decreased photoperiod while in summer flowering occurred earlier because of increased photoperiod. Furthermore, evidence revealed that low temperatures and short day length during the photoperiod-sensitive phase delayed the flowering period of

safflower (Daba et al., 2016; Torabi et al., 2020). Furthermore, it was highlighted that the progressive rise in night temperature causes flowering to occur prior to time (Parthasarathi et al., 2022), and this may explain why flowering was earlier in summer than in winter in the current study because the night temperatures were highest in summer. According to Siddi et al. (2022), the high variation of environments for days to flowering was attributed to the low night temperatures. Shabana et al. (2013) also explained the variation in days to flowering of safflower observed in their study was due to temperature variation experienced during the crop growth.

3.4.5 Number of days to maturity

Phenological traits of days to flowering and physiological maturity are important stages that affect safflower yield (Weiss, 2000; Golkar, 2014; Oarabile, 2017). The findings of this study revealed a highly significant main effect of season was found for number of days to maturity. That is, safflower planted in summer reached physiological maturity earlier than winter-planted safflower. This was ascribed to variations in temperature and photoperiod between the two seasons with winter having very low minimum temperatures than summer. It is commonly known that lower temperatures slow the phenological development of any crop while high temperatures shorten it, therefore, maturation cycle was fast in summer and slow in winter. According to Ferrante and Mariani (2018) temperature was important because it informs the plant the current season thus allowing optimization of their phenological development in relation to thermal, radiative, and rainfall. Likewise, Cosge and Kaya (2008) found that days to maturity varied significantly with season. Correlation analysis in the current study demonstrated that the number of days to maturity was directly influenced by the number of days to elongation, flowering, and branching hence, any delays in the duration of these earlier stages will delay plant maturity. Thoday-Kennedy et al.

(2023) emphasized that changes in rainfall patterns and temperature across the years significantly influenced safflower development. Moatshe et al. (2019) and Moatshe (2020a), attributed the longer physiological maturity of winter grown safflower in Botswana than summer grown safflower to lower minimum average temperatures in winter (4-16°C) than summer (17-23°C). In the current study, days to physiological maturity ranged between 93 to 173 depending on genotype, location, and season. Moatshe (2019) in Botswana, observed that days to physiological maturity of safflower were between 89 and 142 depending on genotype and season of planting (summer or winter). Seasonal variation in days to physiological maturity was explained by changes in temperature between winter and summer (Moatshe, 2019). Planting safflower in summer in Botswana enhances all the phenological stages of safflower due to the higher temperatures (Emongor et al., 2015; 2017; Moatshe, 2019). Shabana et al. (2013) observed that safflower accessions varied between 113-121 days from planting to physiological maturity. Other researchers have found that days to maturity of safflower ranged between 93-154 days depending on genotype, planting location and year (Uslu et al., 2002; Golkar et al., 2011b; GRDC, 2017; Sajid et al., 2021; Chehade et al., 2022). The impact of planting season on safflower phenological development is widely documented in literature (Wachsmann et al., 2010; Alizadeh & Jirair, 2006; Golkar et al. 2011; Kedikanetswe, 2012). Ahadi et al. (2011) highlighted that days to flowering and maturity in safflower were affected by planting time in Iran and the phenological stages were prolonged with decrease in mean air temperature. Temperature affects phenological stages of all plants from emergence to physiological maturity with significant impact experienced at the later stages (Slafer & Rawson, 1994). Goudriaan and Laar, (1994) and Ritche and Ne Smith, (1991) reported that the rate of crop development was significantly influenced by temperature. Crop developmental rate was found to be positively correlated with temperature within the physiological

range, with growth being hastened when temperature was increased (Porter & Decolle, 1988; Salisbury & Ross, 1992; Goudriaan & Laar, 1994).

In the current study locations varied enormously in their number of days to maturity with plants from Ramonaka maturing earlier than those at Sebele and Molepolole. Generally, safflower planted at Ramonaka matured early because their growth was not delayed by the devastating effect of chilling injury that was experienced by safflower planted at Molepolole and Sebele in winter in 2020/2021. Similarly, Sajid et al (2021) reported that the days to maturity in safflower varied significantly with planting location. In contrast, Hussein et al. (2018) found that number of days to maturity in safflower was not influenced by planting location.

On the other hand, a significant genotypic variation was found for number of days to maturity, with genotype Sina maturing earlier than genotypes, Gila, Turkey, and Kenya9819. The presence of genotypic variation for days to physiological maturity of safflower is well known (Amini et al., 2008; Ahadi et al., 2011; Shinwari et al., 2014; Yasari et al., 2016; Arslan & Culpan, 2018; Muhammad et al., 2020; Sajid et al., 2021). The early maturing genotypes like Sina and PI537636 may be suitable for use in earliness management. Earliness management approach is ideal for farmers because it can reduce the chances of plant growth/harvest coinciding with bad weather. Days to seed emergence and physiological maturity are critical phenological stages in plant earliness. Golkar (2011) reported additive and dominance gene effects-controlled earliness in safflower. Additive gene effects in the control of days to physiological maturity of safflower has been reported by Shahbazi and Seaidi (2007). Gupta and Singh (1988a) reported over and partial dominance of gene action over the same trait. While Golkar (2011) reported that dominance gene

effects took part in the genetic control of days to maturity of safflower. Singh et al. (2008) reported that additive and dominance gene effects-controlled days to maturity of safflower.

3.4.6 Plant height

The interaction of season and location substantially influenced the plant height of safflower. This showed that plant height was highly controlled by interaction of environmental factors. For instance, significantly taller plants were produced at Ramonaka in winter than plant heights in other locations and seasons. This was partially attributed to fertile clay loam soils of Ramonaka which promoted vegetative growth of safflower including plant height. Similarly, Emongor (2010), highlighted that safflower plant height depended on soil moisture, temperature, photoperiod, planting date and soil fertility. Furthermore, safflower planted at Molepolole in winter had the shortest plants because these plants were injured by frost which reduced stem elongation. Kolanyane (2022) reported that safflower planted in winter of 2021, the response of safflower plants to applied N and P fertilizers with regard to plant height was poor because of low temperatures of -6.3-4.2°C that happened at the elongation stage of safflower. The susceptibility and severity to chilling injury by a plant tissue is controlled by the genetic makeup of the plant, developmental stage, metabolic status of the tissue, and environmental factors like temperature, relative humidity, light, and atmospheric composition (Lim et al., 2009; Chaudhary et al., 2017; Wassan et al., 2021). Atmospheric temperature lower than -4°C during the stem elongation and branching stages of the safflower is known to cause damage to the growing point which can results in death of a plant (Wachsmann et al., 2010). Plants may recuperate from chilling injury by producing new shoots underneath damaged areas, although plant growth rate and seed yield may be negatively affected (Wachsmann et al., 2010; Kolanyane, 2022). Safflower plants in the current

study recovered from chilling injury when temperatures warmed up in September. The results of the present study agree with the studies by Emongor et al. (2015) and Emongor and Oagile (2017).

Plant height is one of the developmental traits that indicates the overall plant growth. However, it is is controlled by genetic make-up, soil nutrition and environmental conditions (Arif et al., 2012). It was evident from the present study that planting safflower in summer limited degree of stem elongation and consequently plant height. Higher and drier summer temperatures experienced in the current study contributed to the stunted growth indirectly through accelerated maturation cycle. Koutroubas et al. (2004) hypothesized that the longer growing period particularly during the stem elongation stage, contributed to taller safflower plants. In summer, no significant differences were observed among the locations for plant height. This suggested that safflower plant height was stable across the locations in summer and unstable during winter due to large diurnal temperature ranges and short-day length which slowed vegetative growth and phenological development and resulted in enhanced internode growth and stem elongation (Emongor et al., 2015; 2017; Moatshe, 2019). It is commonly known that stem elongation increases with an increase in greater variation of day and night temperatures (DIF) during elongation phase depending on the plant species (Erwin & Heins, 1995; Ohtaka et al., 2020; Wu et al., 2021). Erwin and Heins (1995) stated that plant stem elongation responses to DIF decreased as photoperiod length increased. This implies that as photoperiod length increases in summer, the plants show less sensitivity to DIF, hence short plant height. Moreover, Thingnaes et al. (2003) revealed that at low average daily temperatures stem length was found to increase rapidly with increasing DIF, whereas under high average daily temperatures stem length was estimated to decrease with DIF. Therefore, this explained why winter grown safflower plants were taller than summer safflower plants.

The season and genotype interaction significantly influenced plant height of safflower. For example, the genotype Turkey planted in winter had significantly taller plants than plant height of other genotypes in any season. In contrast, the genotype Kenya9819 planted in summer had shorter plants than the rest of the genotypes planted in any season with the exception of genotypes PI537636 and Sina planted in summer. The findings of the present study suggested that there was G x E interaction for plant height. Bey et al. (2021) suggested that variations in plant heights over the years observed in their study showed that this trait was greatly affected by environmental conditions and was characterized by a low heritability. Moreover, a significant interaction of genotype and season in the current study showed that plant height was greatly impacted by the genotype's ability to adapt to different environmental conditions experienced over seasons. The genotype Turkey was able to maintain its taller height in both seasons (winter and summer) while the height of genotypes Gila and Sina varied significantly over seasons. Similarly, Koutroubas et al. (2004) found that safflower genotypes varied in plant height due to seasons. They further explained that longer growing period provided by earlier sowing or planting date might have contributed to the taller plants. Bella et al. (2019) highlighted that plant height was a morphological trait that was under genetic control, however it is impacted by environmental factors such as altitude, temperature, solar radiation, and moisture. While Kotecha (1979), Shahbazi and Saidi (2007), and Golkar et al. (2012) all reported that plant height of safflower influenced by additive gene effects. Other studies have shown that safflower plant height is affected by several environmental and genotypic differences (Uslu et al., 2002; Beyyavas et al., 2011; Golkar et al., 2011b; El-Lattief, 2012; Shinwari et al., 2014; Aguilera-Molina et al., 2021 ; Bey et al., 2021; Sajid et al., 2021; Beyyavas & Dogan, 2022; Thoday-Kennedy et al., 2023). Other studies have

reported significant genotype and season interaction for plant height of safflower (Esendal et al., 2008; Cosge & Kaya, 2008; Moatshe et al., 2016). Naseri (2011) stated that the higher safflower height attributed to adequate soil moisture and low temperatures which contributed to better growth.

3.4.7 Shoot biomass

The interaction of location, season, and genotype substantially impacted the shoot biomass of safflower. The significant interaction of genotype, season and location on safflower shoot biomass observed in the current study indicted G x E interaction influenced shoot biomass of safflower. For example, the genotype Sina planted at Ramonaka in winter produced plants with higher shoot biomass than biomass of other genotypes grown in various locations and seasons with exception of genotype Kenya9819 planted at the same location and season. While genotype Gila planted in summer at Sebele had plants with the lowest shoot biomass compared to other genotypes planted in other locations and seasons with exception of all genotypes planted in summer at all locations and genotypes PI537636 and Sina at Sebele, and Kenya9819 planted at Molepolole in winter. The current results suggested that safflower genotypes showed different magnitudes of vegetative growth (shoot biomass) when planted in various locations and seasons. This was also attributed to the genotypes' differences in morphological features such as leaf size, plant height, and branching pattern which were influenced by the different environmental condition such as rainfall, soil types and temperature that occurred in different seasons and growing locations. Most genotypes in the current study produced maximum shoot biomass at Ramonaka when planted in winter compared to other locations and summer. This was explained by the fertile clay loam soils in Ramonaka plus the slow phenological development induced by cool winter temperatures that promoted shoot

biomass production due to excellent vegetative growth. In summer the safflower plant growth rate is high resulting in lower accumulation of dry mass and biological yield (Moatshe, 2019). GRDC (2017) emphasized that although safflower can be grown in different soil types, it thrives better in neutral to alkaline soils that are well drained and have high water holding capacity like clay loams. Moreover, planting safflower in winter favoured the production of more shoot biomass than in summer irrespective of location and genotype suggesting that genotypes express their greatest genetic potential when environments are favourable. Johnston et al. (2002) highlighted that long growing season required by safflower permits deep root growth, enabling safflower to use water and nutrients from a greater soil depth than crops with a shorter growing season. Koutroubas et al. (2004) found that safflower genotypes had varying shoot dry matter content at flowering stage and the dry matter content was variable between years suggesting G x E interaction. Moatshe et al. (2019) observed significant genotype x season x plant density variation of safflower dry matter and biological yield. They also attributed their results to variation of the variables leaf area index, chlorophyll content, net assimilation rate and crop growth rate of the different safflower genotypes as influenced by season and plant density (Moatshe et al., 2019). Gonzalez et al. (1994) highlighted that yield components of safflower are genetically controlled, however, they are also influenced by environmental factors and agronomic practices. Parthasarathi et al. (2022) revealed that exposure of safflower plants to high temperatures reduced plant height and total biomass. Moreover, the morphological traits of safflower are known to be impacted by additive and non-additive gene effects (Kotecha, 1979). In contrast, Abbadi and Gerendas (2012) found that safflower genotypes did not vary in their shoot biomass.

3.4.8 Root biomass

The season and location interacted significantly to influence the root biomass in the current study. The results revealed that planting safflower in winter substantially increased root biomass of safflower by over 50% compared to summer planting. Safflower planted at Ramonaka in winter produced plants with significantly higher root biomass than safflowers planted in other locations and seasons. While safflower planted at Molepolole in summer had lower root biomass than that of safflower planted in other locations and seasons. The significant interaction of season and location on root biomass of safflower was attributed to environmental conditions especially temperature. The cool temperatures in winter especially at night (≤ 4°C) in the growing locations (sites) increased the vegetative growth (shoot and root) of safflower. Winter grown safflower in Botswana has been reported to have significantly higher vegetative growth (leaf area index, plant height, net assimilation rate, and number of branches per plant) and biological yield (total dry matter of shoot and roots) than summer grown plants (Emongor et al., 2015; 2017; Moatshe et al., 2016; 2019; 2020a; Moatshe, 2019). This was attributed to the longer growing period in winter (135-165 days after sowing) induced by cool night temperatures (≤ 4°C) than summer (90-116 days after sowing) grown (≥ 22°C) safflower (Emongor et al., 2015; 2017; Moatshe et al., 2016; 2019; 2020a). The effect of growing season on vegetative growth of safflower is well established (Wachsmann et al., 2001; Alizadeh, 2005; Ahadi et al., 2011; Golkar et al., 2011b; Kedikanetswe, 2012; Shabana et al., 2013; Oarabile, 2017; Bey & Kurt, 2023). Cool winter temperatures during the rosette stage of safflower has been reported to slow down growth but promoted the partitioning of photoassimilates root growth (Dajue & Mundel, 1996; Emongor & Oagile, 2017). According to Reddy et al. (2017) and Walne and Reddy (2022), high biomass partitioning to the plant root system at lower temperatures was due to less demand for photosynthates from above-ground

biomass undergoing lower growth rates. Therefore, in the current study, slow growth habit of safflower in winter during the rosette stage might have contributed to the increased root biomass through biomass partitioning to the root. In the current study, the soils of Ramonaka had cation exchange capacity (CEC) of 14 meq/100g that was about quadruple the CEC of Sebele and Molepolole, suggesting that soil CEC might have been a limiting factor at those locations (Table 5.1). Therefore, the fertile clay loam soils of Ramonaka might have contributed to the increased biomass (shoot and root) production. Jafarzadeh et al. (2008), found that low soil CEC was among the soil factors that limited safflower production in Iran.

3.4.9 Number of primary branches/plant

The season and location interaction substantially affected the number of primary branches/plant in the current study. Safflower grown at Ramonaka in winter accumulated the greatest number of primary branches/plant than in any locations and seasons. This was attributed to environmental factors mainly soil fertility and temperature. The soils at Ramonaka are clay loam which are fertile that promoted vegetative growth compared to the sandy loam and sandy clay loam soils in Sebele and Molepolole, respectively. The cool winter temperatures of ≤ 4°C significantly promoted the number of primary branches/plant than summer when the average temperatures are ≥22°C. Temperature plays an essential role in the phenological development and vegetative growth of safflower (Dajue & Mündel, 1996; Weiss, 2000; Carapetian, 2001; Emongor et al., 2017; GRDC, 2017; Moatshe et al., 2020a; OECD, 2020; Kolanyane, 2022; Emongor & Emongor, 2023). Winter grown safflower in Botswana has been reported to have significantly higher branch number per plant than summer grown plants (Kedikanetswe, 2012; Emongor et al., 2015; 2017; Moatshe et al., 2020a). Sampaio et al. (2017) reported that there were a greater number of branches per plant

of safflower in winter than in summer, irrespective of plant density since low winter temperatures prolonged crop growth cycle compared to high summer temperatures. Sarkees and Tahir (2016) also found that there were significantly fewer branch numbers per plant in spring grown safflower than winter. They attributed this to shortened safflower growth cycle in summer than winter to a short duration of vegetative phase and/or branching which hindered lateral growth in spring (Sarkees & Tahir, 2016; Emongor et al., 2015; 2017; OECD, 2020; Moatshe et al., 2020a). Salisbury and Ross (1992) reported that long days have a suppressive effect on branching, this could explain why summer grown safflower plants had fewer branches in summer than winter in the present study.

In the current study, safflower planted in Ramonaka had the longest rosette stage duration as compared to other locations and these gave the plants time to produce more vegetative growth including number of primary branches/plant. The long rosette of safflower induced by low temperatures of ≤ 4°C caused the plants to grow an extremely dense clump of leaves with delayed stem elongation resulting in high number of primary branches in the winter planting (Carapetian, 2001; GRDC, 2017; Moatshe, 2019; OECD, 2020). GRDC (2017), emphasized that planting early in winter permits more time for a large rosette and an extensive branch structure to develop, establishing a high yield potential. The low number of primary branches/plant being produced in Sebele and Molepolole during winter was partly explained by the low chilling temperatures (-3-1.6°C for 10 days) that caused chilling injury which limited their degree of branching. However, Arslan (2007c) found low heritability for number of branches/plant of safflower suggesting that this trait is greatly affected by environmental factors and hence explaining the lack of genetic variations in the current results. Although Golkar et al. (2012) found that both additive and non-

additive genetic effects were vital in the inheritance of branches/plant in safflower, but there was no genotypic variability for this trait. A similarly, the absence of genotypic variation for number of primary branches/plant in safflower is reported in literature (Uslu et al., 2002; Esendal, 2006; Baloch et al., 2015). In contrast, the presence of genotypic variation for number of primary branches/plant in safflower was previously reported in literature (Amini et al., 2008; Beyyavas et al., 2011; Emongor et al., 2017; Arslan & Culpan, 2018; Muhammad et al., 2020; Bella et al., 2019). However, Hussein et al. (2018), found that the number of primary branches/plant was not affected by the interaction of genotype and location, which was also evident in the current study. Discrepancies in information concerning genotypic influence for this trait could be a result of differences in the type of genotypes used and environmental conditions in the different experimental sites or locations.

3.4.10 Number of capitula/plant

The impact of season and location interaction was substantial for the number of capitula/plant in the current study. The significant interaction of season and location was attributed to the critical role environmental attributes such as temperature, soil type and rain amount and their distribution have on the capitula number/plant. Safflower planted at Ramonaka during winter produced the highest capitula/plant compared to other locations and seasons. The soils at Ramonaka are clay loam which are more fertile than the sandy loam and sandy clay loam soils in Sebele and Molepolole, respectively. Safflower planted in Ramonaka in winter had better vegetative growth (shoot and root biomass), a greater number of primary branches/plant, and taller plants than safflower planted in Sebele and Molepolole in both summer and winter due to better soil fertility and type in Ramonaka. Also, the high primary branch number/plant in safflower plant in winter at

Ramonaka explained the high capitula number/plant because each branch ends in one or more capitula (Dajue & Mündel, 1996; Weiss, 2000; GRDC, 2017; OECD, 2020). This is further evidenced by a significant positive correlation (r = 0.73) between the number of primary branches/plant and capitula number/plant found in the current study suggesting that part of the variation in capitula number/plant was due to the number of primary branches/plant. Arslan (2007a) found low heritability for the number of capitula/plant suggesting that this trait can be influenced by environmental conditions. In contrast, Emongor et al. (2017) found that the number of capitula/plant did not vary significantly whether safflower was planted in summer or winter. There was a high positive correlation between the capitula number/plant and plant height (r=0.62), shoot biomass (r=0.59), and root biomass (r=0.56) observed in the current study suggesting that part of variation in capitula number/plant was due to these growth attributes.

The variation in capitula number/plant observed in summer was attributed to rainfall amount received during the experimental period and soil type. In summer, Sebele received 202.5 mm of rainfall which has sandy loam soils with excellent drainage while Molepolole with sandy clay loam and Ramonaka with clay loam soils received 283.6 and 189.3 mm, respectively. The heavy rainfall of 111.6 mm received in December at Molepolole caused waterlogging which reduced safflower growth slightly in summer. Safflower plants do not stand waterlogging since it predisposes the crop to root diseases (Emongor & Oagile, 2017; Bergman & Kandel, 2019; OGTR, 2019; OECD, 2020; Emongor & Emongor, 2023). The best soils for safflower growth and development should be fertile, well-drained with better water holding capacity (Bergman & Kandel, 2019; OGTR, 2019; OECD, 2020). The cool winter temperatures of ≤ 4°C also promoted vegetative growth of safflower which led to higher capitula number/plant than summer when the average temperatures

are ≥22°C. Winter grown safflower in Botswana has been shown to have a higher capitula number/plant than summer grown plants (Emongor et al., 2015; 2017; Moatshe et al., 2020b). The effects of the growing season on capitula number/plant are well known (Wachsmann et al., 2001; Alizadeh, 2005; Golkar et al., 2011b; Emongor et al., 2015; Moatshe & Emongor, 2019).

There was a significant genotypic variation for capitula number/plant in the present study. The genotypes PI537636 and Sina had remarkably the highest and lowest capitula number/plant, respectively. This genotypic variation with respect to capitula number/plant of safflower was explained by genetic variations among the studied genotypes. The significant genotypic variation observed for capitula number/plant in the current study further suggested the possibility of breeding safflower genotypes through direct selection for this trait. Bey et al. (2021) hypothesized that a high difference in capitula number/plant among safflower cultivars was a result of differences in plant genetics. Past studies also have revealed the presence of genetic variation of capitula number/plant in safflower (Amini et al., 2008; Ahadi et al., 2011; Beyyavas et al., 2011; Golkar et al., 2012; Golkar, 2014; Ahmad et al., 2016; Arslan & Culpan, 2018; Kose et al., 2018; Bella et al., 2019; Muhammad et al., 2020; Bey et al., 2021; Sajid et al., 2021; Beyyavas & Dogan, 2022; Chehade et al., 2022). Additive x additive and dominance x dominance epistasis gene effects have been reported for the genetic regulation of capitula number/plant. Other researchers have reported dominant gene effects in the regulation of capitula number/plant of safflower (Pahlavani et al., 2007; Golkar, 2014; Golkar et al., 2012; 2017). Sahu and Tewari (1993) indicated that capitula number/plant was under the regulation of additive-dominance gene effects. While Ramachandram and Goud (1981) highlighted that mean comparison of reciprocal effects

demonstrated that maternal effects contributed a crucial part in the inheritance of capitula number/plant.

3.4.11 Capitula weight

The present study revealed a season x location, and season x genotype interactions significantly influenced capitula weight of safflower. The interactions of season x location, and genotype x season suggested genotype x environment (G x E) interaction indicating that safflower genotypes under study in the current research responded differently to the environmental conditions in different locations and seasons. For example, safflower planted at Molepolole in winter produced significantly heavier capitula weight than those produced by safflower plants grown in other locations and seasons with exception of safflower planted at Ramonaka in winter. The genotype Kenya9819 planted in winter produced plants with significantly higher capitula weight than other genotypes planted either in winter or summer. On the other hand, genotype Sina planted in summer had the lowest capitula weight compared to that of other genotypes planted in different seasons. Heavy capitula is an indication of more and larger seeds in the capitula with the potential for high seed yield. The interaction of season x location in influencing capitula weight was partially ascribed to the fertile soils and better vegetative growth that was produced in winter than in summer at these locations. On the contrary, safflower planted at Ramonaka in summer produced low capitula weight because of excessive and prolonged rainfall that coincided with seed-filling and pre-harvest stage which caused poor grain filling and some capitula got soaked with water due to its upright cup like nature resulting in seed discolouration and rotting before harvest resulting in reduction in the capitula weight and consequently seed yield (Appendix 13 and 14). Zhao et al. (2022) highlighted that seasonal rainfall in the Victoria Wimmera region of Australia had a

negative effect on the agronomic traits of safflower such as capitula weight, seed number/capitulum, and seed yield. Safflower capitula has an upright cup like nature that makes it to be easily saturated by rain which may result in the staining of seed, diminishing its value and/or sprouting of seeds (GRDC, 2017).

The results further revealed the interaction of genotype x season on capitula weight of safflower agree with the findings of Singh et al. (2004), Salamati et al. (2011), Hussein et al. (2018), Chehade et al. (2022) and Zhao et al. (2022) who concluded that safflower genotypes responded to G× E interaction over environments. Genotype x E interaction is an important factor for detecting and selecting stable and productive cultivars of safflower in breeding programs. The significant season x location, and genotype x season interactions on safflower capitula weight suggested instability of this trait over different growing locations and seasons induced by environmental attributes such as rainfall, temperature and soil fertility discussed in other sections of this thesis. Generally, the climatic conditions were more favourable for safflower growth in winter than summer, explaining partly the variation in capitula weight between seasons.

3.4.12 Capitula diameter

Genotype x location interaction for capitula diameter was significant in this study. The interaction revealed that genotype Sina consistently produced substantially smaller capitula diameter than any genotypes in all planting locations. The genotype Turkey grown at Sebele had the largest capitula diameter of 25.75 mm than the rest of the genotypes planted in other locations except for genotypes Gila and Kenya9819, Gila, PI537636, and Turkey, and Gila, Kenya9819, and PI537636 planted at Sebele, Molepolole, and Ramonaka, respectively. The significant interaction of genotype x

location in the current study on safflower capitula diameter (size) suggested G x E interaction indicating that safflower genotypes under study responded differently to the environmental conditions in different locations. The results further indicated that the genetic control of safflower capitula size is also influenced by environmental factors in the different planting locations. The significant genotype x location interaction on capitula size implied that environmental factors are contribute differences in plant growth and development and must be considered in safflower breeding programs. Safflower genotypes have been reported to be substantially influenced by G× E interaction with respect to capitula size (Singh et al., 2004; Esendal, 2006; Salamati et al., 2011; Hussein et al., 2018; Culpan & Traits, 2023). Safflower capitula diameter was also reported to depend on genotype, crop management, and growing conditions (Uslu et al., 2002; GRDC, 2010; Golkar et al., 2011b; Shinwari et al., 2014; Arslan & Culpan, 2018; Kose et al., 2018). The capitula diameter ranged between 21.71 to 25.75 mm depending on genotype and growing location. In literature capitula diameter of safflower was reported to range between 5.35-51.3 mm depending on genotype, agronomic and environmental factors (Uslu et al., 2002; GRDC, 2010; Golkar et al., 2011b; Shinwari et al., 2014; Arslan & Culpan, 2018; Kose et al., 2018; Moatshe et al., 2020a). However, Golkar et al. (2012) reported greater effect of dominance gene action than additive gene action in controlling capitula diameter suggesting that direct selection of genotypes with superior capitula diameter can be achieved in safflower breeding. What was interesting about genotype Sina is that even though it had low capitula diameter and capitula weight, it also had numerous capitula/plant and higher 1000-seed weight which placed it at a perfect spot in terms of seed yield. Therefore, even though Golkar et al. (2011b) suggested that capitula diameter may be used as a selection index to increase seed yield of safflower, consideration should be made during selection to avoid exclusion of genotypes with small capitula diameter because some may have desirable

yield traits such as high 1000-seed weight and numerous capitula/plant like it was found in the current study. Thus, genotypes with small capitula diameter do not necessarily mean that they have low yield potential. Furthermore, Esendal (2006) found low heritability for capitula diameter meaning that this trait may be regulated by environmental conditions.

Also, in the current study there was significant seasonal variation for capitula diameter of safflower. Safflower planted in winter had significantly larger capitula diameter (size) than summer planted. These seasonal variations on capitula size were attributed to temperature differences between summer and winter. The cool winter temperatures of ≤ 4°C promoted vegetative growth of safflower which led to higher capitula size than summer when the average temperatures are ≥22°C. Winter grown safflower in Botswana has been earlier reported to have a larger capitula diameter than summer grown plants (Emongor et al., 2015; 2017; Oarabile, 2017; Moatshe et al., 2020a). The effects of the growing season on safflower capitula diameter have been documented in other parts of the world (Wachsmann et al., 2001; Alizadeh, 2005; Ahadi et al., 2011; Golkar et al., 2011a; Shabana et al., 2013). Safflower capitula growth seems to be reduced by high summer temperatures due to compressed phenological cycle. Moreover, literature has shown that a short maturation cycle reduces the yield and yield components of safflower (Uslu et al., 2002; Emongor & Emongor, 2023).

3.4.13 Thousand seed weight

A significant interaction of season x location x genotype was found for the 1000-seed weight in this study. Genotype Kenya9819 grown at Ramonaka in winter produced the highest 1000-seed weight which was significantly higher than that of other genotypes in different locations and

seasons except for genotypes Sina planted at Ramonaka and Molepolole in winter, and Kenya9819 planted at Molepolole in summer. On the contrary, the genotype Gila consistently produced considerably lower 1000-seed weight than other genotypes planted in different locations and seasons. The significant interaction of genotype x season x location showed G x E interaction as safflower genotypes performed differently in different locations and seasons (environments) for 1000-seed weight. This demonstrated that genotypes differed in their genetic potential for 1000-seed weight and their degree of variation depended more on the environment where they were grown. Likewise, Ali et al. (2020) and Zanetti et al. (2022) observed that safflower 1000-seed weight varied with location and season. Climatic conditions during flowering and seed development stages affects seed weight and its chemical composition in different oilseed crops (Mustafa et al., 2015, 2016). Genotypes of oil crops such sunflower and safflower behave differently under various climatic conditions (Qadir et al., 2006; Mustafa et al., 2016). Genetic x E interaction for 1000-seed of safflower is reported in literature (Cosge & Kaya, 2008; Zraibi et al., 2014; Golkar et al., 2017; Santos et al., 2017; Hussein et al., 2018; Arslan & Culpan, 2018; Bella et al., 2019; Bey et al., 2021; Ahmadvandi et al., 2022; Beyyavas & Dogan, 2022; Koç, 2021; Chehade et al., 2022; Zhao et al., 2022). Also, 1000-seed weight is reported to be under the control of additive gene effects (Mandal & Banerjee, 1997; Golkar et al., 2012). While Shahbazi and Saeidi (2007) reported that 1000-seed weight was governed by additive-dominance gene effects. The genotype Gila consistently produced significantly low 1000-seed weight independent of location and season this was attributed to the low seed hull (pericarp) content which is genetically controlled. Urie (1986) reported that partial hull was recessive to white hull. According to Ebert and Knowles (1966), striped seed and reduced pericarp (such as in the seed of Gila) are controlled by recessive genes *th* and *stp*, respectively. The low hull content in the seed of Gila might have

contributed to the low 1000-seed weight. A recent study showed that 1000-seed weight was positively correlated with the hull content (Cerrotta et al., 2020). Therefore, genotypes with a low hull content will most likely exhibit low 1000-seed weight.

In general, the lowest 1000-seed weight was registered in Ramonaka during summer in respect to other locations and seasons. This was ascribed to excessive and prolonged rainfall that occurred at Ramonaka during the seed development stage in summer which caused discoloration of seeds and capitula (Appendix 13) thus, decreasing the seed value. Prolonged phenological development and higher biomass production in safflower grown in winter partly explained the higher 1000-seed weight than in summer. Zanetti et al. (2022), highlighted that prolonged vegetative development can contribute to the remobilization of photosynthates during seed filling stage, consequently increasing 1000-seed weight remarkably.

3.4.14 Seed yield

A significant interaction between season and location was found for seed yield in the current study suggesting instability of this trait over seasons in different locations (environments). The safflower grown in winter at Ramonaka produced significantly higher seed yield than in other locations and seasons. In contrast, safflower planted at Ramonaka in summer produced lower seed yield than in other locations and seasons. The high safflower seed yield at Ramonaka planted in winter was attributed to the fertile soils and better vegetative growth. In contrast, safflower planted at Ramonaka in summer produced the lowest seed yield due to low capitula and 1000-seed weights caused by excessive and prolonged rainfall which coincided with seed-filling and pre-harvest stages which caused poor grain filling and rotting of some capitula, and seed discolouration leading

to low seed yield (Appendix 13 and 14). Excessive seasonal rainfall in the Victoria Wimmera region of Australia has been reported to negatively impact on safflower capitula weight, seed number/capitulum, and seed yield (GRDC, 2017, OECD, 2020; Zhao et al., 2020). Safflower capitula has an upright cup like nature that makes it to be easily saturated by rain which may result in the staining of seed, diminishing its value and/or sprouting of seeds (GRDC, 2017, OECD, 2020; Zhao et al., 2020). Safflower requires minimal rainfall or irrigation at physiological maturity to ripen the capitula (FAO, 2010; Emongor & Emongor, 2023). Excess rainfall at flowering stage of safflower promotes leaf and capitula diseases thus reducing seed yield (Dajue & Mündel, 1996; Emongor & Emongor, 2023). Prolonged rainfall at flowering and temperatures greater than 32°C reduces effective pollination and seed set (Mündel et al., 1992). While excessive rainfall just before harvest causes seed discolouration, rotting of seeds, and germination of seed while still attached to the capitulum since safflower seed have no dormancy (Bérvillé et al., 2005; McPherson et al., 2009; Emongor, 2010; GRDC, 2017; OECD, 2020).

Safflower planted in winter had higher seed yield by 84.4% than summer planted in the current study. This was explained by the longer growing period in winter (173 days to physiological maturity), therefore, higher leaf area index (LAI), net assimilation rate (NAR), shoot and biomass, plant height, branch number/plant, capitula number/plant, capital weight and size, and 1000-seed weight than summer (93 days to physiological maturity) grown safflower. Similarly, Moatshe (2019) reported seasonal variation in safflower on seed yield in Botswana with safflower planted in winter producing higher yield than summer. This was attributed to the longer growing period in winter (135-147 days to physiological maturity), higher LAI, NAR, biological yield, and yield components than summer (100-116 days to physiological maturity) grown safflower (Moatshe,

2019). The longer physiological maturity of winter grown safflower in Botswana than summer grown safflower was explained by lower minimum average temperatures in winter (4-16°C) than summer (17-23°C) leading to excellent vegetative growth, high yield components and yield in winter. Ozkaynak (2013) found a positive correlation between safflower seed yield and the length of vegetative period, with longer vegetative period increasing the yield. Chehade et al. (2022) reported that seed development and nutrient reserve accumulation are highly susceptible to environmental changes, which can influence the qualitative and the quantitative traits of the final yield of safflower. High summer temperatures greatly influence crops by altering root and shoot growth, photosynthetic membranes, activities of carbon metabolic enzymes, starch accumulation, and sucrose production thus subsequently resulting in a yield decrease (Parthasarathi et al., 2022). Sehgal et al. (2018) reported that heat stress hindered accumulation of starch in the seed of field crops by influencing their metabolic pathways, although the changes were crop-specific and depended upon the duration of exposure to heat stress. Weather conditions significantly affect the seed yield of safflower, despite its high rusticity and adaptability to various environments (Zanetti et al., 2022). Seasonal variation in safflower seed yield due to the differences in climatic conditions such as humidity, temperature, and sunshine between winter and summer are reported in literature (Koutroubas et al., 2004; Tayebi et al., 2012; Emongor et al., 2013; Hassan et al., 2015; Tahmasebpour et al., 2016). Safflower grown at Sebele and Molepolole in winter was low in seed yield compared to Ramonaka in the current study due to chilling injury experienced which reduced vegetative growth and crop stand at harvest.

Significant genotypic variation was found for seed yield in this study. The genotype Kenya9819 produced a seed yield which was significantly higher than that of any genotypes except for seed

yield of genotypes Turkey and Sina. The genotypic variation of safflower seed yield was ascribed to genetic differences among the genotypes. Genotype variation of safflower seed yield is known to be governed by additive gene effects (Golkar et al., 2012; 2017; Golkar, 2014; Nakhaei, 2014). Also, gene dominance in the control of safflower seed yield has been reported (Mandal & Banerjee, 1997; Deedawat et al., 2015). Genetic variation among safflower genotypes is documented in literature (Dajue & Mündel, 1996; Rahmatalla et al., 2001; Kizil et al., 2008; Killi et al., 2016; Korononeo, 2023). Korononeo (2023) reported that the Sina registered greater seed yield ranging 2862-3505 kg/ha than any genotype, but this varied with growing season. This genotype has been reported to be high yielding and adaptable in various environments producing better seed yield than other genotypes (Poordad, 2003; Kizil et al., 2008; Amoughin et al., 2012; Moatshe et al., 2016; Arslan & Culpan, 2018; Bella et al., 2019; Koc, 2021). Depending on safflower genotype Abd El-lattief (2012) and Hamza (2016) reported seed yield in the range of 512 to 2846 and 1978 to 2510 kg/ha, respectively in Egypt. At Turkey, safflower seed yields of 827-3111 kg/ha depending on genotype have been reported (Kizil et al., 2008; Killi et al., 2016). In the present study safflower seed ranged between 820-3803 kg/ha dependent on plant genotype and growing season which is within ranges reported elsewhere in literature.

The was a substantial positive correlations of plant height, number of primary branches/plant, number of capitula/plant, capitula weight, and 1000-seed weight with seed yield of safflower. This indicated that the above variables are yield components of safflower. For example, greater stem reserves of taller plants may support grain filling better and might extend this period that is crucial for yield formation (Dordas & Sioulas, 2009; Bella et al., 2019; Chehade et al., 2022). Evaluation of morphological and yield variables and their relationships is important in understanding how the

direct components of yield are related to the various morphological parameters of the crop species and vice versa. Moatshe (2019) and Bey and Kurt (2023) reported positive correlations between safflower seed yield and capitulum number/plant, seed number/capitulum, capitulum weight, 1000-seed weight, plant height, branch number per plant and biological yield. Chaundry (1990) evaluated 50 safflower genotypes and suggested that for number of seed/capitulum, number of capitula/plant and 1000-seed weight may be used as a main selection criterion for high seed yield. While Ahmadzadeh et al. (2012) observed a substantial relationship of number of seeds/capitula, 1000-seed weight, days to 50% flowering with seed yield of safflower and suggested that these parameters may be used as a main selection criterion for high yielding genotypes under irrigated and rainfed conditions. Furthermore, positive correlations of safflower yield components with seed yield have been documented in literature (Moghaddasi & Omidi, 2010; Soleymani et al., 2011; Pavithra et al., 2016; Wang et al., 2018; Moatshe et al., 2020a; Pattar & Patil, 2020; Koc, 2021; Chehade et al., 2022).

3.4.15 Oil content

The importance of safflower is mostly due to the oil produced from its seeds which also determines the success of safflower cultivation (Singh & Nimbkar, 2006; Moatshe et al., 2020b; Zemour et al., 2020; Emongor & Emongor, 2023). The significant interaction of genotype x season x location showed G x E interaction as safflower genotypes performed differently in different locations and seasons (environmental factors such as soil type and fertility, temperature, and rainfall amount) for seed oil content. This revealed that the environmental factors contributed to high genetic variability in the oil content of safflower genotypes. Therefore, for safflower breeding programs after producing elite lines or hybrid, genotypes should be tested in different environments before

recommending them. For example, the genotype Gila planted at Molepolole in winter produced seed with significantly greater oil content of 42.27% than the rest of the genotypes planted at different locations and seasons. In contrast, genotype Turkey planted at Ramonaka in summer had the smallest oil content of 11.82% compared to other genotypes planted at various locations and seasons. The oil content of the genotype Gila planted at various locations in summer and winter did not vary but remained noticeably higher than that of other genotypes in other locations and seasons indicating genotype stability in all locations and seasons with reference to seed oil content. Genetic x environment interaction for seed oil content of safflower is well known (Singh et al., 2004; Torbaghan, 2015; Ebrahimi et al., 2016; Alizadeh et al., 2017; Golkar et al., 2017, 2020; Hussein et al., 2018; Bey et al., 2021; Koç, 2021; de Oliveira Neto et al., 2021, 2022; Sajid et al., 2021; Zemour et al., 2021; Zhao et al., 2022; Culpan & Traits, 2023; Subaşı & Başalma, 2023). While Nguyen et al. (2016) highlighted that the oil content of crops was sensitive to both genetic and environmental factors such as drought stress and heat stress. Drought stress caused by high temperatures during the grain filling stage of safflower was reported to reduce oil content and modify the oil composition (Dwivedi et al., 1993; Izquierdo et al., 2006; Mosupiemang et al., 2022a; Roche et al., 2010; 2019; Whaley & Eskandari, 2019; Navas-López et al., 2020). The oil content of a crop has also been reported to vary with cultivar, genetic traits, soil characteristics, planting date, and climate (Rahamatalla et al., 2001; Shabana et al., 2013). Safflower seed oil content is a qualitative trait which is affected by genotype x environment interaction (Golkar, 2014). Additive (Golkar et al., 2011b) and dominance (Gupta & Singh, 1988a) gene effects was found in the genetic control of safflower seed oil content. However, epistatic effects are reported to have significant effect on the genetic regulation of safflower oil content (Pahlavani et al., 2007). Dominant alleles are also reported to be involved in the genetic regulation of safflower seed oil

content (Ramachandram & Goud, 1981). Genotypically, the spiny safflower cultivars are known to produce more oil content than spineless ones (Weiss, 2000). Likewise, Beyyavas et al. (2011) demonstrated that out of the 26 genotypes they studied, Gila was among the top with higher oil content irrespective of planting year. Also, Zemour et al. (2021) observed that genotypes that produces spiny leaves like Gila form seeds high in oil content. While Mosupiemang et al. (2022b) found that the genotype Gila had the highest oleosin gene content and a smaller oil body diameter which are characteristics of a high oil-producing genotype.

Safflower seed oil content is reported to vary between 15-64.6% depending on genotypes, environmental conditions, morphology, physiology, agronomic practices, growing seasons, and geographical regions (Samanci & Ozkaynak, 2003; Elfadl et al., 2005; Kizil et al., 2008; Ahmadzadeh et al., 2014; Hamza, 2015; Arslan & Culpan, 2018; Moatshe et al., 2020b; Aguilera-Molina et al., 2021; Bey et al., 2021; Chehade et al., 2022; Thoday-Kennedy et al., 2023). The oil content in the present study ranged between 11.8-42.3% depending on genotype, location, and season. On the contrary, Koc (2019) did not find any difference in safflower seed oil content over the years. He attributed this to lack of environmental effects on seed oil content.

Furthermore, the results revealed that safflower genotypes had noticeably higher seed oil content in winter than in summer irrespective of location. This suggested that the lower temperatures (4-16°C) and longer phenological stages in winter favoured seed filling and oil production in safflower. Emongor and Emongor (2023), highlighted that reduced oil content in summer-grown safflower was attributed to contracted phenological stages. High temperatures during the grain filling stage of safflower induces water stress which leads reduction in seed oil content and

modified oil composition (Izquierdo et al., 2006; Whaley & Eskandari, 2019; Roche et al., 2010; 2019; Navas-López et al., 2020). When temperature is high the extent of decrease in oil content was dependent on the stage of grain filling and/or genotype (Cantagallo et al., 2004; Rondanini et al., 2006). Among environmental variables, drought, correlated with high temperatures at the end of the growing season of safflower significantly reduced seed oil content (Quadir et al., 2006; Istanbulluoglu et al., 2009; Ashrafi & Razmjoo, 2010; Oraki et al., 2011; Fernández-Cuesta et al., 2014; Zemour et al., 2021). Drought affects the photosynthetic activity and availability of photoassimilates necessary for oil biosynthesis during grain filling (Kafi & Rostami, 2008; Zahedi et al., 2009; Mosupiemang et al., 2022a). The decrease in oil content is also a result of changes in the metabolic pathways essential in the synthesis and production of oil and by affecting the activity of enzymes responsible for oil biosynthesis (Koocheki et al., 2016; Fahad et al., 2017). Sehgal et al. (2018) reported that under drought conditions the decrease in oil content was due to a decrease in the accumulation of digestible carbohydrates and of sugars from stem to developing seeds. A study in United States on soybean oil content by Lobell and Asner (2003) found that in a growing season, every 1°C rise in temperature above the optimum results with up to 17% decrease in oil yields. Hocking and Stapper (2001) also reported similar findings when studying safflower and linseed. The decrease in oil content was influenced by high temperature due to low accumulation of intercepted solar radiation during the critical period (Aquirrezabal et al., 2003) or a shortened grain filling period (Ploschuk & Hall, 1995). Mustafa et al. (2016) reported that for most oil crops, cold temperatures, adequate moisture, long days during and after flowering increases grain and oil yield, and oil quality.

The results showed that oil content had significant positive correlations with phenological parameters such as days to elongation, branching, flowering, and maturity, and shoot biomass. The more days it took to reach the above phenological stages or the higher the shoot biomass the higher the oil content. The long safflower growth period increased the accumulation of dry matter and photoassimilates (carbohydrates) necessary for grain filling process and oil biosynthesis. Short growth period of safflower reduces seed oil content due to low accumulation of photoassimilates (Moatshe et al., 2020a; Emongor & Emongor, 2023). Early planting of safflower was reported to increase oil content in many genotypes due to accumulation of nutrient reserves and excellent seed filling processes which influenced quantitative and qualitative traits of seed yield and oil content (Yau, 2007; Mohammadi et al., 2018; Sehgal et al., 2018; Chehade et al., 2022). According to Emongor and Emongor (2023), reduction in oil content in summer-planted safflower is ascribed to contracted phenological stages.

3.4.16 Oil yield

Season and location interacted significantly affected safflower oil yield/ha in this study. The highest and lowest oil yield of 1120.92 and 147.70 kg/ha was produced by safflower plants grown at Ramonaka in winter and summer, respectively, compared to that which was produced in other locations and seasons. Also, there was significant interaction of location and genotype for safflower oil yield. The genotypes Kenya9819 and Sina planted at Ramonaka and Molepolole had highest (752.53 kg/ha) and lowest (247.04 kg/ha) oil yield, respectively. The significant interactions of season x location, and genotype x location indicated G x E interaction implying that environmental factors affected the oil yield of safflower genotypes by impacting the seed yield and oil content. Oil yield is a function of oil content and seed yield. The climatic factors discussed

in other sections of this thesis influenced seed yield and oil content. Abbadi and Gerendas (2012), highlighted that a reduction in a certain yield component due to drought stresses during growth and development of safflower reduced seed and oil yield because oil yield is a product of oil content and seed yield/ha. While Soleymani et al. (2011), Shabana et al. (2013) and Chehade et al. (2022) reported that oil yield of safflower was influenced by the environmental factors that caused variations in the oil content and seed yield. Omidi (2006) reported year x location x genotype interaction was statistically different which showed that safflower genotypes responded differently under different climatic conditions. Koç (2021) reported that because oil yield was obtained by multiplying oil content and seed yield, all the factors that influenced G x E increased seed yield and oil content also increased oil yield of safflower. Genotype x environment interaction for safflower oil yield has been reported in literature (Subaşı & Başalma, 2021). Seed yield and oil content should be taken into consideration in the genetic improvement of safflower oil yield during breeding (Baljani et al., 2015).

In this study the safflower oil yield ranged between 148-1121 kg/ha depending on genotype, location, and season. The reported safflower oil yield in literature ranges between 10.3-3597 kg/ha (El-Lattief, 2012; Killi et al., 2016; Arslan & Culpan, 2018; Bella et al., 2019; Moatshe et al., 2020b; Ahmadvandi et al., 2022; Beyyavas & Dogan, 2022; Chehade et al., 2022) depending on genotypic, agronomic practices and environmental factors. Also, oil yield exhibited a strong positive correlation with seed yield ($r = 0.95$), capitula weight ($r = 0.56$), capitula number/plant ($r = 0.63$), phenological variables and weak a positive correlation with oil content ($r = 0.42$) suggesting that high oil yield in the current study was associated with higher seed yield, yield components and oil content. The oil yield of oil crops is positively correlated with grain dry mass, grain yield and oil content (Moghaddasi & Omidi, 2010; Amoghein et al., 2012; Moatshe et al.,

2020b). Positive correlations between safflower oil yield with oil content and seed yield is well known (Yan & Kang, 2003; Sharifmoghaddassi & Omidi; 2009; Naserirad et al., 2013; Emongor et al., 2015; Bella et al., 2019; Moatshe et al., 2020b; Koç, 2021; Bey & Kurt, 2023).

3.4.17 Genotype x environment (G×E) interactions for seed yield using GGE biplot analysis

To determine high performing genotypes in multi-environment yield experiments is difficult because of high G×E interactions (Kaya et al., 2006; Subaşı & Başalma, 2021; Koç, 2021). However, determination of G×E interaction with biplot analysis is a significant component of genotype selection process in multi-environment studies (Yan et al., 2000; Kaya et al., 2006; Pourdad & Moghaddam, 2013; Subaşı & Başalma, 2021; Koç, 2021). Biplot analysis is a significant method in crop breeding and agricultural research. GGE biplot analysis gives an easy and complete solution for G×E interaction data analysis, which is a challenge for plant breeders, geneticists, and agronomists (Yan et al., 2000; Kaya et al., 2006; Pourdad & Moghaddam, 2013; Subaşı & Başalma, 2021; Koç, 2021). GGE biplot analysis makes the evaluation of genotypes efficient and provides a comprehensive understanding of the target environment and test environments (Yan et al., 2000; Kaya et al., 2006; Yan &Tinker, 2006; Alizadeh et al., 2008; Ebrahimi et al., 2016; Pourdad & Moghaddam, 2013; Subaşı & Başalma, 2021; Koç, 2021).

The G×E effect for seed yield revealed that genotypes responded differently in different environmental conditions which necessitated studying safflower genotypes at multiple locations and seasons. The results of the present study showed that the biplot examined 94.17% of the total variation of the environment-centred G×E table which implies that the biplots effectively explained the variation of GGE (Yan &Tinker, 2006; Alizadeh et al., 2008; Ebrahimi et al., 2016; Pourdad & Moghaddam, 2013; Koç, 2021; Subaşı & Başalma, 2021). The biplots identified

Kenya9819 as the highest-yielding and Gila as the poor-yielding genotypes. Furthermore, stability analysis showed that Sina and Gila had greater yield instability while Kenya9819, Turkey, and PI537636 had greater yield stability. This suggested that the relative performance of Kenya9819, Turkey, and PI537636 was consistent across the environments. The current results agree with those reported in literature (Yan & Tinker, 2006; Kaya et al., 2006; Koç, 2021; Subaşı & Başalma, 2021). In the current study, the which-won-where/ what is best for what polygon view analysis showed that the test environments fell within two sectors showing that genotypes were showing high performance at specific locations. According to Yan et al. (2007), if all environment markers fall into a single sector, it indicated that a single cultivar had the highest yield in all environments. In the current study, the genotype Kenya9819 was a winner in the test environments Sebele and Molepolole. It was concluded that the genotype Kenya9819 was adaptable to a wide range of environmental conditions such as soil type, moisture, and temperature of the study locations across the seasons. When ranking the locations, Molepolole had the shortest vector indicating that all genotypes perform equally in this environment and cannot be used in selecting superior genotypes because it gives insufficient information about genotype discrimination. Although Ramonaka was the most discriminative of all test environments it had large angle from the AEC abscissa, therefore it cannot be used in selection of superior genotypes, but it may be used for culling unstable genotypes. On the other hand, Sebele had high representativeness and discriminative ability hence considered an ideal location for selecting genotypes adapted to the whole region. Sebele and Molepolole had a strong relationship indicating that they were highly correlated in their ranking of the genotypes. The results agree with those in literature (Yan et al., 2007; Kaya et al., 2006; Koç, 2021; Subaşı & Başalma, 2021).

3.4.18 Genotype by yield*trait biplot

To evaluate the overall superiority of the genotypes for all the studied traits, the genotype x yield trait (GYT) methodology was used. This approach is based on the hypothesis that an ideal genotype should have superior levels for multiple desired traits (Yan & Frégeau-Reid, 2018). Therefore, the results of the biplot revealed that most of the studied yield-trait combinations positively correlated with each other mostly because they are yield components. This indicated that these traits can be used in selection for breeding purposes without affecting/compromising the level of another trait. Similar findings were reported in literature (Karahan & Akgün, 2020; Kendal, 2020; Gholizadeh et al., 2023). Overall, genotypes Turkey and Kenya9819 were ranked as superior because they had above-average yield*trait combinations. Thereby, these genotypes are recommended as all-purpose/traits genotypes in the southern part of Botswana independent of season and location. This is in accordance with the hypothesis by Yan and Frégeau-Reid (2018) which states that the superiority of a genotype should be judged by its levels of combining yield with other desired traits, instead of its levels in individual traits. This is because the selection of genotypes based on the seed yield only without considering other traits of interest may be misleading (Takele et al., 2022). In contrast, genotype Gila ranked last in terms of overall superiority. This means that this genotype cannot be used as multi-trait genotype rather it can be used for breeding of specific individual traits like oil content. The GYT method has not been appraised in safflower genotype selection, but it can be a novel approach for selecting genotypes not only based on one trait but a combination of yield with other traits. Thus, in selection of desirable genotypes, the combined effects of yield-trait are highly significant than the effects of individual traits (Kendal, 2020).

3.5 Conclusion and recommendations

The phenological development, growth, yield, yield components, oil yield, and oil content of safflower were significantly influenced by genotype, location, seasons, and their interactions depending on the variable indicating G x E interaction influenced safflower growth and development. The climatic conditions such as the amount and distribution of rainfall, soil types and air temperatures allowed safflower genotypes to perform differently at various locations and seasons. Growing safflower in winter prolonged phenological stages (days to emergence, elongation, branching, flowering, as well as maturity) compared to summer. This resulted in increased vegetative growth, seed and oil yield, yield components, and oil content depending on the genotype and location. The major source of variation among the seasons was temperature, rainfall, and day length. Therefore, winter planting of safflower in Botswana was preferred over summer. However, the planting date of safflower in winter needs to be scrutinized so that the reproductive stage does not coincide with low chilling temperatures that normally occurs in July and August to avoid chilling injury. The biplot analysis provided significant advantages in identifying the promising genotypes. The biplots identified Kenya9819 and Gila as the highest-yielding and poor-yielding genotypes, respectively. Stability analysis showed that Kenya9819, Turkey, and PI537636 had greater yield stability while Gila and Sina were the least stable. Sebele was the most representative and discriminative of all test environments hence, the ideal environment for selecting safflower genotypes adaptable to all three locations. Kenya9819 was a winner genotype across all locations studied and the highest seed-yielding and stable genotype. The yield by trait combination biplot successfully delineated the genotypes based on those with above-average as well as below-average yield component trait combinations. In this regard, genotypes Kenya9819 and Turkey were found to have above average values of yield*trait

combinations followed by Sina, while PI537636 and Gila had below-average values. Therefore, genotypes Kenya9819 and Turkey were recommended to be planted in the southern region of Botswana because of their overall superiority. However, genotype Gila could be used for breeding purposes to improve the seed oil content of other genotypes due to its high seed oil content. Furthermore, genotype Turkey that produces high biomass and has less spines are recommended for use as leafy vegetable.

3.6. References

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Chapter 4

Oleosin Expression Patterns and Size of Oil Bodies as a Factor in Determining Oil Content in Safflower (*Carthamus tinctorius* L.) Genotypes

Abstract

The climate crisis and the Ukraine war have shown the vulnerability of various crop commodities. One of those badly affected is cooking oil, leading to a shortage in several countries. This coupled with the need for healthier cooking oil, increases proportionally with the world population and has resulted in escalated cooking oil prices. Thus, continued evaluation of alternative oil crops that can do well in marginal lands becomes a vital practice to undertake. Safflower is one of the marginalized oil crops with high-quality oil containing essential fatty acids beneficial to human health. Screening safflower genotypes for oil content is critical for its breeding and adoption in non-native areas. Therefore, this study delineates the relationship between oleosin genes and oil bodies in regulating the oil content of safflower seeds. Oleosin genes and oil bodies from the seeds of five safflower genotypes were isolated and quantified using qPCR and fluorescence microscope respectively and evaluated against the seed oil content. The results showed an inverse relationship where smaller oil bodies were displayed by genotypes with high oil content. A high relative expression of oleosin genes was observed in genotypes with high oil content (Kenya9819 and Gila). Of the eight Ctoleosin genes that were studied, it was observed that Ctoleosin genes (1, 4, 6, 7, and 8) were highly reliable in characterizing safflower genotypes based on the oil content. Kenya9819 and Gila genotypes were found to have high oil potential, and this was confirmed by a higher accumulation of the oleosin gene. A high correlation coefficient between oleosin, oil content, and oil body was also observed in this study. The findings suggested that selected oleosin

genes and oil bodies are important traits to consider when characterizing oil seed crops for oil content.

4.1 Introduction

Seeds store lipids in the form of small spherical intracellular organelles, called oil bodies, with sizes ranging between 0.5 and 2.0 µm in diameter (Leprince et al., 1997). Song et al. (2017) suggested that some oil body diameters can be 2-3 times larger. The oil body sizes vary with species depending upon the set of surrounding proteins mainly oleosin and the nature of lipids stored (Mawlong et al., 2019). These stored lipids, mainly triacylglycerols (TAGs), provide energy for seed germination and seedling growth (Lu et al., 2018). Oil bodies accumulate in maturing seeds, and in seeds with high oil and they fill much of the cytoplasmic space by the start of dormancy Schmidt and Herman (2008) and they occupy a larger area in the physiologically mature oilseed which ensures oil body stability (Cai et al., 2018). Oleosins are the most abundant proteins associated with oil bodies (Siloto et al., 2006) covering about 80% of the oil body surface (Romero-Guzmán et al., 2020). The role of oleosin proteins is to stabilize the lipid bodies in developing seeds, and mature seeds, and act as recognition signals for lipase biding in germinating seeds (Ling, 2007; van der Schoot et al., 2011). The formation of oleosin contributes to the buildup of discrete oil bodies in plant tissues thus stabilizing the oil body surface Li and Fan (2009) and preventing coalescence of the oil bodies (Alexander et al., 2002). Oil bodies can be found in other plant tissues such as fruit but only pollen and seeds produce oleosins where the oil bodies are subjected to developmentally regulated desiccation and hydration (Schmidt & Herman, 2008). Therefore, the expression of oleosin genes is tissue-specific but appears to be universally abundant in seeds that store oil and this is not the case for oil-storing fruits (Fang et al., 2014; Capuano et al., 2007).

Previous study has shown an inverse relationship between the size of oil bodies to the amount of oleosin in maize and most importantly that oil content was highly influenced by oleosin content (Ting et al., 1996). Ho et al. (2014) found that the size of oil bodies in mesocarp tissue of high oil yielding palms were significantly smaller than the oil bodies of low oil yielding palms thereby confirming the inverse relationship between oil body size and oil yield. A similar study on rice oleosin also confirmed that seed oil content was negatively correlated with oil body size and that oleosin participates in the formation of oil bodies and enlarges oil storage capacity (Liu et al., 2018). All these studies suggest that the accumulation patterns of oleosins in oil seed crops are an important trait that may be used to screen genotypes for oil content.

Safflower (*Carthamus tinctorius* L.) is an oil seed crop with high-quality vegetable oil rich in polyunsaturated (linoleic and arachidic acid) monounsaturated (oleic and palmitoleic acid) fatty acids (Burdge & Calder., 2005; Brenna et al., 2009; Mišurcová et al., 2011; Orsavova et al., 2015; Piccinin et al., 2019; Moatshe, 2019; Moatshe et al., 2020; Emongor & Emongor, 2023). Safflower oil is better in nutrition than olive and canola oils (Bergman, 1997; Corleto et al., 1997) and sunflower oil (Dajue & Mündel, 1996) due to its lower percentage of saturated fatty acids (Moatshe et al., 2020; Emongor & Emongor, 2023). The human body cannot make polyunsaturated fatty acids (PUFAs) due to the absence of appropriate enzymes, therefore they must come from the diet (Burdge & Calder, 2005; Brenna et al., 2009; Mišurcová et al., 2011; Glick & Fischer, 2013; Emongor & Emongor, 2023). Safflower seed oil contains both ALA and LA. Safflower seed oil content ranges between 16.1–64.6% depending on cultivars, environmental conditions, cultural practices, and seasons (summer, winter and spring depending on geographical region) of growth (Samanci & Ozkaynak, 2003; Elfadl et al., 2005; Hamza, 2015; Killi et al., 2016; Emongor et al.,

2017; Moatshe et al., 2020; Chehade et al., 2022; Emongor & Emongor, 2023). Seed oil content is a very important economic trait for safflower and is considered one of the most important factors affecting the success of safflower adoption in new areas (Bassil & Kaffka, 2002; Emongor et al., 2017). Safflower oil can be used as a vegetable oil for cooking, margarine production, salad oil, infant formulations, paints, and varnishes (Singh & Nimbkar, 2006; Dunford, 2012; Liu et al., 2016). Oleosin bounded oil bodies have the potential to be incorporated in the food industry, in the preparation of cosmetic products and pharmaceuticals and this makes safflower a perfect candidate for such technology because it has quality oil that is rich in oleic and linoleic acid and low allergic reactions. Oil bodies can be used as an ingredient in dairy like food, beverages, salad dressings, sauces, edible films, coatings and hair, products (Nikiforidis et al., 2014; Cai et al., 2018). Therefore, studies on oil body size and their associated oleosins proteins are of great importance in the establishment of this promising technology. In the current study, the oil bodies were isolated from mature safflower seeds and their size was measured, oleosin genes were quantified from developing safflower seeds and the oil was extracted from safflower seeds and oil content was determined. These were done to compare oleosin gene expressions in relation to oil body size and oil content among safflower genotypes. Studies comparing the relationship between oleosin gene expression, oil body size and oil content of safflower genotypes are limited in literature. Therefore, the results of the current study may guide in the selection of high oil-yielding safflower genotypes and in breeding for high seed oil content by regulating the levels of oleosin genes surrounding the surface oil bodies.

4.2 Materials and Methods

4.2.1 Experimental site

This study was conducted at the Botswana University of Agriculture and Natural Resources (BUAN) Content Farm during winter 2020/2021 and summer 2020/2021. This site is located at the latitude of 24° 33' South and longitude of 25° 54' East in Sebele, Gaborone in the southern part of Botswana. Full description of the site is covered under section 3.2.1. The experimental sites were disc ploughed using a tractor to have a smooth planting bed. Two seeds were sown per hill at a depth of 2.5 cm and two weeks after emergence plants were thinned to one plant per hill. The spacing was 40 cm between rows (inter row spacing) and 25 cm within plants (intra row spacing) (Moatshe et al., 2016). Plants were mainly rainfed and supplementary irrigation was provided in periods of prolonged dry spells where no rainfall was received within two weeks. The experimental unit was 4 m by 5 m in size.

4.2.2 Experimental design

The experiment design was a randomized complete block with three replications. The treatments were five safflower genotypes (Gila, Sina, Turkey, PI537636, and Kenya9819). The experiments were carried out in summer (November 2020 to February 2021) and winter (May to October 2021).

4.2.3 Plant material

Five safflower genotypes used in this study were, Sina, Gila, Turkey, PI537636, and Kenya9819. The plants were grown (for two seasons winter 2020/2021 and summer 2020/2021) until they reached physiological maturity then their seeds were harvested and used for oil content determination and oil bodies isolation. For RNA isolation, the flowers containing immature seeds were harvested 20 days after flowering. The flowers for RNA were carefully cut out and

immediately frozen in liquid nitrogen and stored at -80°C until use. Before RNA extraction, the seeds were separated from the flowers and then ground in liquid nitrogen.

4.2.4 Oil content determination

Oil from the safflower seeds was quantified using the soxhlet n-hexane extraction method (Wrolstad, 2005) as described in chapter 3.2.

4.2.5 Isolation of oil bodies

Isolation and purification of oil bodies was done following the method of Tzen et al. (1997) with minor modifications. Physiological mature seeds (20 g) were used for oil body isolation whereby, the seeds were homogenized at 4°C in 50 ml grinding medium (0.6 M sucrose and 10 mM sodium phosphate buffer pH 7.5) with a blender (12 000 rpm) for 90 s. After blending, the homogenate was filtered through two layers of mutton cloth. After filtration, each 200 µl portion of the homogenate was transferred to a 1.5 ml Eppendorf tube, and 200 µl of flotation medium (grinding medium containing 0.4 M sucrose) was layered on top. The tube was centrifuged at 10,000 X g for 20 min and the supernatant was collected and resuspended in 400 µl of detergent washing solution (0.2 M sucrose, 5 mM sodium phosphate buffer pH 7.5, and 0.1% Tween 20). The resuspension was transferred to two 1.5 ml Eppendorf tubes (200 µl in each), and 200 µl of 10 mM sodium phosphate buffer (pH 7.5) was layered on top, and the tubes were centrifuged at 10,000 X g for 20 min. The supernatant was collected and resuspended in 400 µl of ionic elution buffer (grinding medium additionally containing 2 M NaCl). The resuspension was transferred to two 1.5 ml Eppendorf tubes (200 µl in each), 200 µl of floating medium (grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top, and the tubes were centrifuged at

10,000 X g for 20 min. Then, the supernatant was collected and placed in a new 1.5 ml Eppendorf tube then 200 µl of 10 mM sodium phosphate buffer pH 7.5 was layered on top and the tubes were centrifuged at 10,000 X g for 20 min. The supernatant was collected and resuspended in 200 µl of grinding medium mixed with 200 µl of n-hexane and the tube was centrifuged at 10,000 X g for 20 min. The upper hexane layer was removed. Then the oil bodies were collected and resuspended in 200 µl of grinding medium. The resuspension was transferred to a new 1.5 ml Eppendorf tube while 200 µl of flotation medium was layered on top, and the tubes were centrifuged. The supernatant was collected and resuspended in a grinding medium and stored at 4°C till use. This final medium was termed salt-washed oil bodies.

4.2.6 Imaging

Identification of oil bodies was done by staining the isolated oil bodies with a Nile red dye as a fluorophore (1 mg of Nile red/ml of acetone) in a ratio of 1:100 v/v Nile red to oil bodies and incubated for an hour at 20°C. The stained oil bodies appeared as red circles when excited with green light under a fluorescence microscope (Carl Zeiss Scope. A1, Leica Microsystems CMS GmbH, Wetzlar, Germany). Samples were placed on a microscope, covered with a cover slip, then visualized under magnification of 40x, and images were photographed using Axiocam 305 digital camera. A total of six slides were prepared per genotype (3 biological reps plus 3 technical reps). Ten oil bodies were measured per micrograph using the microscope inbuilt software (Zeiss ZEN lite software). Then the average of the 10 measured oil bodies was used as the final reading (oil body diameter).

4.2.7 RNA extraction and cDNA synthesis

Total RNA was isolated from maturing safflower seeds (20 days after flowering) as Lu et al. (2018) observed that the expression pattern of Ctoleosin genes peaks during 15-25 days post onset of flowering, and decreases thereafter. After collection, the seeds were snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. Frozen seeds were ground in liquid nitrogen, and total RNA was extracted with the Quick-RNA Kit MiniPrep (Zymo Research Corp, United States of America, Tustin, California) as per the manufacturer's instruction. The quality and quantity of the extracted RNA were checked on both the 0.8% agarose gel and Nanodrop (Thermo Fisher Scientific, USA, Wilmington, DE). Samples with traces of contamination and low concentration of RNA were discarded, and a new extraction was undertaken. Samples with good quality and quantity were subjected to cDNA synthesis. The DNA-free RNA was converted into first-strand complementary DNA (cDNA) using a ReverTra-Ace-α synthesis kit (Toyobo Co., Ltd, Osaka, Japan) with an Oligo (dT) primer (Toyobo Co.LTD, Osaka, Japan). The cDNA synthesis success was checked by amplifying the cDNA using a set of plant reference markers Elongation factor (EF1 and EIF-5A) to check the presence of the amplified band. cDNAs that showed banding were used for quantification of the gene of interest whereas cDNAs that did not show any banding after amplification were redone.

4.2.8 Gene of interest selection and design of primers

Oleosin genes from various plants' genome databases were used to blast search homologs against the safflower genome in the National Center for Biotechnology Information, U.S. National Library of Medicine (NCBI) website, and were cross-checked with Lu et al. (2018). From the identified gene homologs, the sequences were used to design the primers using the Primer3 online software

tool. Suitably designed primers for qPCR were assembled and purchased from Inqaba Biotechnical industries (Pty) LTD, South Africa.

4.2.9 Quantification of oleosin gene expression

The designed primers (Ctoleosin 1, 2, 3, 4, 5, 6, 7, and 8) were used to quantify the mRNA abundance of oleosin genes in safflower (Table 4.1). The quantification was monitored by Fluorescent, Quantitative Detection System (qPCR instrument) (Bioer, China, Zhejiang) using Light Cycler 480 SYBR Green I Master Kit (Roche, Germany, Mannheim) to amplify 50 ng cDNA using the designed pair of primers. Two reference genes of EF1 and EIF-5A were used as internal standards and their normalized values were used to calculate the relative abundance of respective oleosin mRNAs using the $2^{-\Delta\Delta Ct}$ method. The profiling of mRNA quantification was run with three technical replications. Relative gene expression (R) was calculated using Equation.

$$R = 2^{[\Delta Ct\ sample - \Delta Ct\ control]}$$

$$R = 2^{-\Delta\Delta Ct}$$

Table 4.1. Primers used for gene expression

Primer name		Sequence
Ctoleosin 1	Ctoleosin 1- F	ATTGATCGCCGTCTTCATCC
	Ctoleosin 1- R	CCGTCACGTACGAGTAGATCCA
Ctoleosin 2	Ctoleosin 2 - F	ATTTTCAGCCCCGTGTTGG
	Ctoleosin 2 - R	CAGAAGAAAACAAACACGGCG
Ctoleosin 3	Ctoleosin 3 - F	TTCAGGAAGAGCCACCAGATCA
	Ctoleosin 3 - R	TGAGCCCTCCGTTTTGCAT
Ctoleosin 4	Ctoleosin 4 - F	ATGGACAACGGCCAACTCAA
	Ctoleosin 4 - R	CCAGTGGAAACGAAAAAGACGA
Ctoleosin 5	Ctoleosin 5 - F	TTCATCCTCTTCAGCCCCATC
	Ctoleosin 5 - R	GCAGTTGACCAGGAACGACAA
Ctoleosin 6	Ctoleosin 6 - F	CAGATACCGTGGACTACGCCA
	Ctoleosin 6 - R	CGTACATGCCCATATCGTGG
Ctoleosin 7	Ctoleosin 7 - F	ATCTTCGGCCCTTTGCTGTT
	Ctoleosin 7- R	AACCCATCCCAACGTAGCAAG
Ctoleosin 8	Ctoleosin 8- F	CCTCATCTTCTTTTCGCCCATC
	Ctoleosin 8 - R	ACCCGAAGACACACAGGAATCC
EF1	EF1 F	TCTGGTGTCACTGCTGAAGG
	EF1 R	TCCTCACCGAAAAGATCCAC
EIF-5A F	EIF-5A F	TGTCCCTCATGTCAACCGTA
	EIF-5A R	GCATCATCAGTTGGGAGCTT

4.2.10 Data Analysis

One-way analysis of variance (ANOVA) was performed using Sigmaplot program version 14.0. Treatment means were compared using Fisher's least significant difference (LSD) procedure at a significance level of 5%. Pearson's correlation coefficient was used to test if there were correlations between oleosins, oil content, and oil bodies.

4.3 Results

The results showed a highly significant ($P<0.001$) genotypic variation for oil content. Genotype Gila had a remarkably high oil content of 38.02% followed by Kenya9819 and PI537636 with an oil content of 28.85% and 27.85%, respectively (Table 4.2). The rest of the genotypes had a low

oil content averaging 21.39%. In general, the oil content ranged between 20.94 to 38.02% depending on the genotype (Table 4.2).

Table 4.2. Oil body diameter and oil content of safflower seeds

Genotype	**Oil body diameter (µm)**	**Oil content (%)**
Gila	1.54±0.20b	38.02±0.76a
Kenya9819	2.83±0.20a	28.85±0.76b
PI537636	3.23±0.20a	27.85±0.76b
Sina	3.20±0.20a	21.83±0.76c
Turkey	2.76±0.20a	20.94±0.76c
LSD	0.569	2.58
f-statistic	11.81***	82.24***

Values followed by dissimilar letters in the same column in treatment are significant at P=0.05 according to Fischer LSD. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$. Values in the columns represent the means and SEM.

Safflower genotypes under study had statistically ($P<0.001$) varying sizes of seed oil body diameters (Table 4.2). Genotype Gila had a substantially smaller oil body diameter of 1.54 µm than other genotypes which had similar oil body size (Table 4.2). The oil body diameters ranged between 1.54 to 3.23 µm. The microscopy imaging showed that most of the oil bodies were very small in size, although there were some large oil bodies among safflower genotypes; Kenya9819, Turkey, Sina, and PI537636 (Figure 4.1). The observed oil bodies were spherical in shape (Figure 4.1).

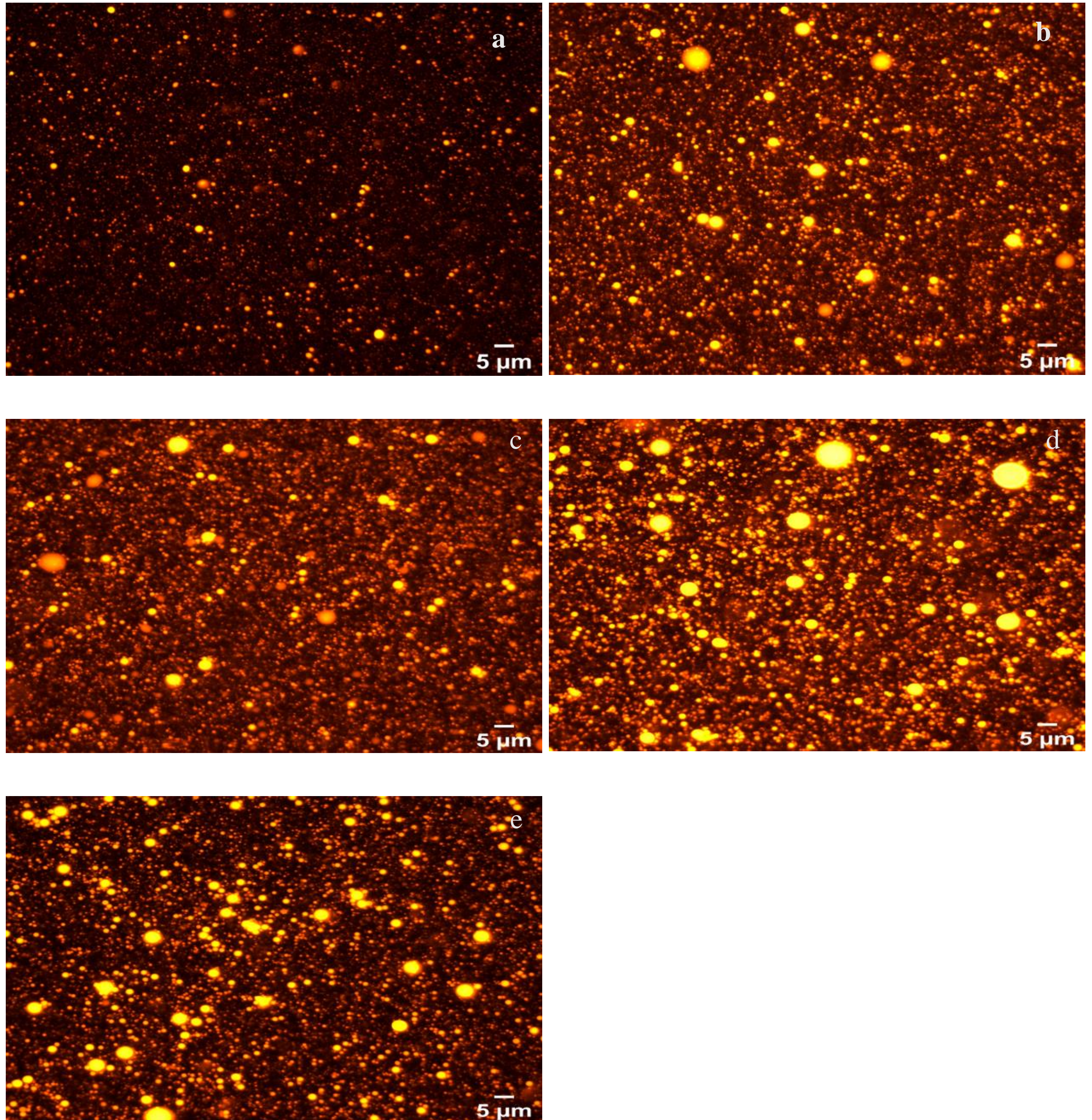

Figure 4.1. Microscopic identification of seed oil bodies using fluorescence technique for safflower genotypes a; Gila, b; Kenya-9819, c; PI-537636, d; Sina and e; Turkey.

A total of eight selected genes were used for the relative expression and of the eight, only five gave a satisfactory expression of genes and were used in the current study. The relative expression results showed that the oleosin genes were variably expressed in the seeds of safflower genotypes

(Figure 4.2 and 4.3). Genotypes Kenya9819 and Gila showed a higher level of gene expression than other genotypes under investigation. Kenya9819 exhibited the greatest expression level of Ctoleosin genes 1, 6, and 7 (Figures 4.2 a, c, d) while Gila showed the greatest expression level of Ctoleosin genes 1 and 8 (Figure 4.2a and 4.3). All the genotypes showed a considerably similar expression level of Ctoleosin 4 (Figure 4.2b). The genotypes which exhibited lower oil content (Sina and Turkey) also showed low expression levels of oleosin genes (Figures 4.2 and 4.3).

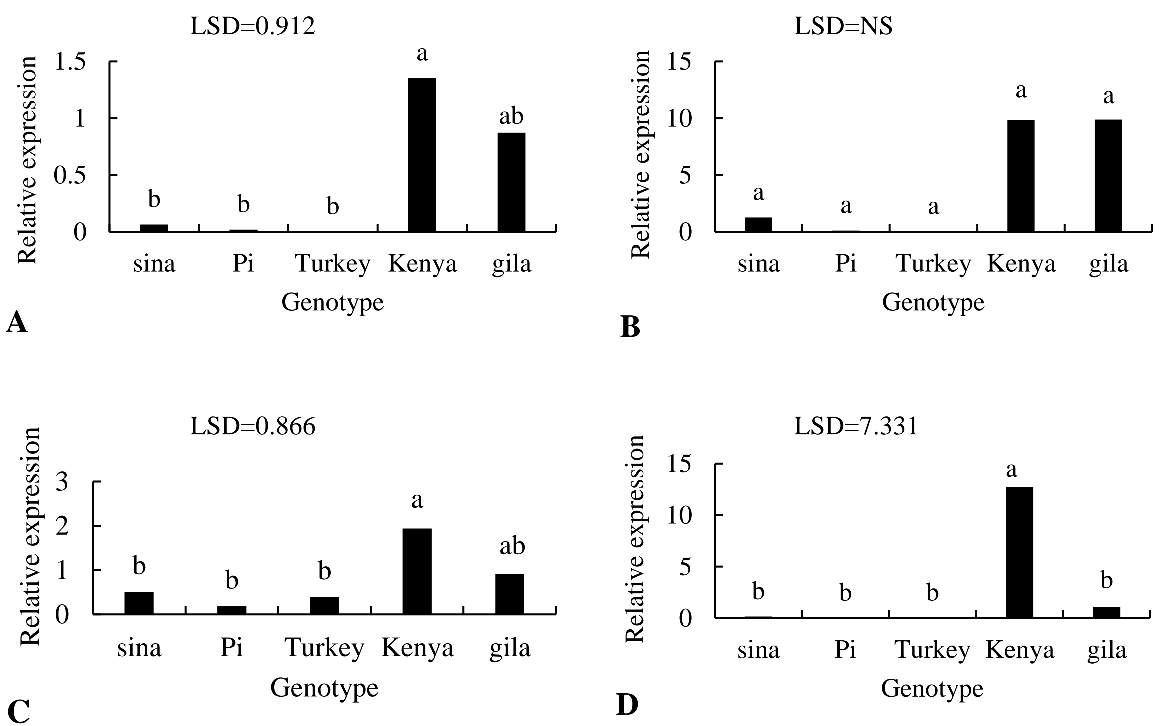

Figure 4.2. The relative expression of Ctoleosin 1 (a), 4 (b), 6 (c), and 7 (d) genes in winter planted safflower at 20 days after flowering.

Values with dissimilar letters in a treatment indicate significant differences according to Fisher's LSD.

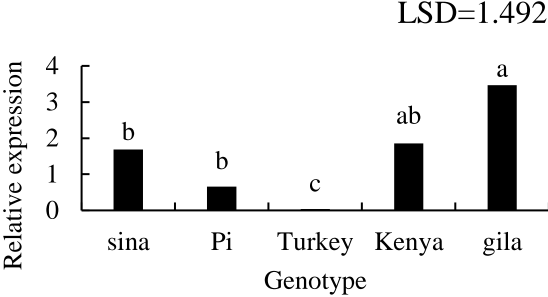

Figure 4.3. The relative expression of Ctoleosin 8 genes in winter planted safflower at 20 days after flowering.

Values with dissimilar letters in a treatment indicate significant differences according to Fisher's LSD

Oil bodies showed a negative correlation with oil content and Ctoleosin 8 (Table 4.3). On the other hand, oil content showed a positive relationship with Ctoleosin 1, 4 and 8. The results showed that 20% of the studied Ctoleosin genes had a significant correlation with oil body size while 60% of them had a significant correlation with oil content.

Table 4.3. Pearson correlation coefficient of oil body diameter, oil content, and oleosin

	Oil content	Ctoleosin 1	Ctoleosin 4	Ctoleosin 6	Ctoleosin 7	Ctoleosin 8
Oil body size	-0.53**	-0.24 ns	-0.34 ns	-0.08 ns	0.2 ns	-0.54*
Oil content	1	0.55*	0.45*	0.25 ns	0.21 ns	0.74**

Values for correlation are significant according to Pearson's correlation $P \leq 0.05$ *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$. ns=not significant

4.4 Discussion

The results of the current study indicated that a significant variation existed in oil content among safflower genotypes. This demonstrated that the genotypes had a varying genetic capacity for oil

content. The presence of genetic variation in safflower oil content is reported in literature (Beyyavas et al., 2011; El-Lattief, 2012; Zemour et al., 2021; Chehade et al., 2022). Safflower seed oil content is a qualitative trait which is reported under the control of additive (Golkar et al., 2011), dominance (Gupta & Singh, 1988a), and epistatic (Pahlavani et al., 2007) gene effects. Furthermore, Ramachandram and Goud (1981) reported that dominant alleles governed safflower seed oil content. Safflower seed oil content is reported in literature to range between 16.1 to 64.6% depending on cultivars, environmental conditions, cultural practices, and seasons (summer, winter and spring depending on geographical region) of growth (Killi et al., 2016; Arslan & Culpan, 2018; Saisanthosh et al., 2018; Bella et al., 2019; Moatshe et al., 2020b; Bey et al., 2021; Chehade et al., 2022; Emongor & Emongor, 2023; Thoday-Kennedy et al., 2023). The seed oil content of safflower genotypes in the current study ranged between 21 to 38% which is within the range reported in literature.

Lipids are one of the economic products stored in seeds in the form of triacylglycerol (Tzen et al., 1993; Zienkiewicz et al., 2010). Triacylglycerol is the major component of the oil body (Tzen et al., 1992; Siloto et al., 2006; Shimada & Hara-Nishimura, 2010). Oil bodies are vital in understanding oil content in crops (Song et al., 2017). In the current study, safflower genotypes substantially varied in their oil body size suggesting the presence of genetic variability for this trait. This was attributed to differences in oil content among genotypes. Abbas et al. (2020) and Chen et al. (2023) also reported genotypic variation of oil body size in *Arachis hypogaea* (peanuts) and *Brassica napus* (oilseed rape) seeds, respectively. Abbas et al. (2020) hypothesized that differences in oil bodies among peanut cultivars were attributed to variations in the acidic amino acid and protein content on the surface of the oil bodies. In this study, an average oilbody diameter

of safflower seeds ranged between 1.56 and 3.23 µm with an average of 2.7 µm which was higher than those reported in other studies (Chen et al., 2019; Lan et al., 2020). Chen et al. (2019) and Lan et al. (2020) reported oil body diameter ranging from 0.5 to 2.5 µm in rapeseed oil and safflower. The variation in oil body diameter in relation to what is reported in literature could be due to the presence of some overly large oil bodies that were identified in the current study or differences in crops. The size of oil bodies varies with plant or crop species depending upon the type of surrounding proteins (oleosin) and nature of lipids stored (Tzen & Huang, 1992; Mawlong et al., 2019). Murphy (2012) also reported large oil bodies in crops, and they play the same role of energy reservoir. Chen et al. (2023) reported a direct proportionality between oil content, number of oil bodies and the total area of oil bodies. Inducing a decrease in the distance between oil bodies lead to the occurrence of irregularly shaped oil bodies and oil body aggregation of *Brassica napu*s (Chen et al., 2023). Therefore, the occurrence of large oil bodies observed in the current study could have been a result of oil body aggregation. Lan et al. (2020) reported that safflower oil bodies were nearly monodisperse unlike in the present study where a presence of some overly sized oil bodies was observed which led to polydispersity in oil body size.

Oil body size of safflower was found to negatively correlated with oil content implying that smaller oil-bodied crop give a higher oil content and yield. The genotype Gila, which recorded the highest oil content had the smallest oil body diameter (size) which showed that oil body size was inversely correlated with oil content. Mawlong et al. (2019) also reported that oil body size was negatively correlated with oil content in *Brassica juncea*. Difference of opinions regarding the size of oil bodies are documented in literature (Hu et al., 2009; Mawlong et al., 2019). *Brassica napus* cultivars that have larger oil bodies also have low oil content (Mawlong et al., 2019). However,

some cultivars of *Brassica napus* do not show any relationship between oil body size and oil content (Mawlong et al., 2019). The variation in oil body size was suggested to be due to oleosin content (Hu et al., 2009). The oleosins maintain the stability of oil bodies hence preventing the organelles from coalescing by providing stearic hindrance during desiccation (Tzen & Huang 1992, Tzen et al., 1993, Ross et al., 1993, Ting et al., 1996). The present results showed a significant variation in the relative expression of different oleosin genes in safflower seeds among safflower genotypes. This was attributed to the differences in the quantity of oil content observed among the studied genotypes. For instance, the genotypes that had higher oil content (Kenya9819 and Gila) exhibited the highest oleosin gene content. This suggested that the amount of oleosin genes present in seed can be used to distinguish between high oil-yielding genotypes and low oil-yielding genotypes. Research in Arabidopsis showed that gene mutation encoding oleosin protein resulted in large size of oil bodies (Siloto et al., 2006). Furthermore, disruption of the gene encoding oleosin also led to altered aggregation of lipids and proteins which caused delay in seed germination (Siloto et al., 2008; Shimada et al., 2008). Shimada et al. (2008) in Arabidopsis reported an inverse relationship between oil body size and total oleosin levels.

The results of this study have shown that oil body size had a significant inverse correlation with the Ctoleosin genes. Likewise, Marin et al. (2020) found that the relative amount of oleosins and oil content determine the size of the oil bodies in coffee. Similarly, Siloto et al. (2006) reported that a slight reduction in OLEO1 content resulted in larger and homogeneous oil bodies and further reductions led to the production of oil bodies with diverse sizes in arabidopsis. On the contrary, Ting et al. (1996) found larger oil bodies in high oil-yielding maize and smaller and irregularly shaped oil bodies in low oil-yielding maize. Ting et al. (1996) further found that seed oleosin genes

in maize are expressed independent of the oil contents. These contradictions might be due to the variances in the plant species suggesting that expression patterns of oleosins and accumulation of oil bodies are highly influenced by the plant species studied.

4.5 Conclusion and Recommendations

The present work reported the use of oleosin genes in characterizing safflower genotypes based on oil content, where Kenya9819 and Gila genotypes were correctly characterized to have high oil potential, and this was validated by smaller oil bodies and higher oil content of the two genotypes. The inverse correlation of oil bodies and oil content was also identified as an important trait to use when characterizing oil seeds for oil content. A significant correlation between oil content, oil bodies and oleosin genes obtained from the current study suggests that breeding for high oil content in safflower can be achieved by regulating the levels of oleosin genes that are embedded on the surface of seed oil bodies and subsequently increasing the oil storage capacity of the seed. Therefore, breeding for higher seed oil yield by targeting the oleosin genes may serve as a novel way of meeting the increasing demands for seed oil. Further studies should evaluate the relationship of oil content, oil body, and oleosin genes at different seed development stages which could help in understanding the critical stage of oil formation in safflower seeds. Moreover, further studies should assess the nutritional composition of the isolated oil bodies and their possible use in the food industry as mayonnaise.

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Chapter 5

The Response of Safflower Genotypes to Drought Stress Induced at Different Phenological Stages

Abstract

Drought stress impact fuelled by climate change is becoming a global concern as it threatens food security in many arid and semi-arid lands (ASALs). However, planting drought-tolerant crops such as safflower (*Carthamus tinctorius* L.), in the ASALs can help ensure food security and sustainability. Although safflower is known to be drought tolerant, there is a need to select genotypes that would better adapt to drought-stress conditions promoted by climate change in the ASALs. Greenhouse and field experiments were conducted during the 2021/2022 planting season to evaluate the response of safflower genotypes to drought stress induced at various developmental stages. Factors under study were stress condition (stressed and control), developmental stages, and five safflower genotypes. The results revealed that drought stress reduced leaf chlorophyll content, leaf relative water content (LRWC), and plant height varying with genotype, duration of water stress, and phenological stage of safflower. On the other hand, the contents of ascorbate peroxidase (APX) and proline content increased in stressed plants varying with genotype, duration of stress and phenological stage of safflower as a physiological mechanism of overcoming the negative effects of drought stress on growth and development of safflower. There was statistical ($P \leq 0.05$) genetic variation to drought stress, duration of water stress and phenological stage of safflower as evidenced by different contents of chlorophyll content, LRWC, proline, and APX, and plant height. Biplot analysis showed that the genotype Kenya9819 was the most superior and drought tolerant. While the genotype Gila ranked poorly and most susceptible to drought. The results indicated that drought stress tolerance was very complex, and it involves several mechanisms

either working synergistically or independently based on the traits involved. Therefore, more drought stress tolerance traits need to be studied to make more informed choices during genotype selection and evaluation.

5.1 Introduction

Drought is amongst the abiotic stress factors that seriously hinders crop productivity and yield in arid and semi-arid lands (ASALs). The consequences of drought stress are anticipated to worsen in the future as a consequence of progressive global warming with one of the negative results being the increasing margins of desertification in the ASALs (Singh & Laxmi, 2015; Emongor & Emongor, 2023). Fortunately, some plants have developed acclimation and adaptation mechanisms in response to drought stress. Several traits like reduced water loss, build-up of osmotic potential, synthesis of compatible solutes, dissipation of excess energy, activation of antioxidant defence and repair systems, generation of sclerenchymatous tissue, and strengthening of the plasma membrane cell wall interaction aid in drought tolerance (Laxa et al., 2019; Mosupiemang et al., 2022a). Plant tolerance tactics can be split into resistance and avoidance mechanisms, which enable plants to survive dehydration and prevent the exposure of plants to osmotic stress through deep rooting or a short growth season (Kantar et al., 2011; Mosupiemang et al., 2022a). Interestingly, plants can recognize even the lowest environmental stress signal (Pandey et al., 2017). The plant reaction to water deficit stress is controlled by the species' intrinsic strategy along with the severity and duration of the stress period (Naderi et al., 2014).

The hostile impact of drought stress fuelled by climate change are also posing a threat to the United Nations Sustainable Development Goal number 2 of ending hunger, achieving food security and improved nutrition, and promoting sustainable agriculture by 2030. This global concern prompts the need to incorporate drought and saline-tolerant and multipurpose crops such as safflower into cropping systems, especially in the ASALs. Among oilseed crops, safflower is recognized as the most drought-tolerant and can produce a good yield in ASALs (Weiss, 2000; Emongor & Emongor, 2023). Its tolerance to low moisture conditions is mainly a results of its effective deep tap root and many fine lateral roots which can significantly tolerate periods of moisture scarcity (Emongor, 2010; Bahrami et al., 2014; Mosupiemang et al., 2022a). Safflower genotypes differ in their response to drought stress and thus genotypes that demonstrate excellent drought tolerance characteristics are suitable to be used by farmers because they can save the costs of implementing drought management practices (Mosupiemang et al., 2022a). Previous studies on drought tolerance mechanisms of safflower indicated that genotypes respond differently to drought stress (Sajedi et al., 2012; Thippeswamy et al., 2013; Hussain et al., 2016; Bortolheiro & Silva, 2017).

Chlorophyll is considered to be the most critical chloroplast component for photosynthesis, and its relative concentration has a positive correlation with the photosynthetic rate of plants (Ansari et al., 2019). Photosynthetic pigments like chlorophyll content have been reported to decrease with drought stress in many plant species including safflower (Amini et al., 2013; Mohammadi et al., 2016; Farooq et al., 2020). Plant height is one of the variables that signify the vegetative growth of plants. Plant height is known to be reduced by drought stress, especially if it occurs during the vegetative stage (Tayebi et al., 2012; Kazemeini et al., 2015; Joshan et al., 2019). Similarly, LRWC was found to decrease with drought stress (Mohammadi et al., 2016; Manvelian et al., 2021).

Plants enhance the production of osmoprotectants or osmolytes such as proline for the protection of membranes, proteins, and enzymes against various stresses (Singh et al., 2015; Mosupiemang et al., 2022a). Studies on drought tolerance mechanisms of safflower have revealed an increase in proline levels with drought stress (Sajedi et al., 2012; Aeini et al., 2018; Farooq et al., 2020; Çulha et al., 2021). Exposure of plants to unfavourable environmental conditions like drought stress also increased the production of ROS which are highly reactive and toxic, causing damage to proteins, lipids, carbohydrates, and DNA which ultimately results in cell death (Gill & Tuteja, 2010). Excessive ROS accumulation is subject to control by a complex mechanism of enzymatic and non-enzymatic antioxidant systems (Islam et al., 2022). The major ROS-scavenging antioxidant enzymes of plants include SOD, APX, CAT, POX, and PrxR, MDHAR, DHAR, and GR (Blokhina et al., 2003; Mittler et al., 2004). Ascorbate peroxidase was reported to be an efficient regulator of ROS, as it contributes maximally to hydrogen peroxide detoxification (Pandey et al., 2017). Ascorbate peroxidase has a high affinity to H_2O_2, and it is present in every cell compartment of plants, and its expression is highly regulated, making it important in the removal of H_2O_2 for signalling purposes (Mittler & Poulos, 2005). Studies on the activities of APX in safflower genotypes under drought stress are scanty. However, the levels of APX were reported to increase with drought stress in safflower (Amini et al., 2013; Çulha et al., 2021).

Different safflower genotypes have different degrees of tolerance to drought stress depending on the phenological stage and duration of stress. Moreover, studying different mechanisms that different safflower genotypes display under different environmental conditions may aid in the selection and breeding for drought tolerance. Therefore, this study seeks to evaluate the impact of

drought stress induced at different phenological stages and drought stress duration on the physiological and biochemical traits of safflower genotypes.

5.2 Materials and Methods

5.2.1 Experimental design and setup

Field and greenhouse experiments were conducted at the Botswana University of Agriculture and Natural Resources (BUAN) Content Farm in Sebele during the winter of 2021/2022 planting season (May 2021 until September 2021). This site is located at the latitude of 24° 33' South and longitude of 25° 54' East in Sebele, Gaborone in the southern part of Botswana. The experiment was arranged in a 2 x 3 x 5 split split-plot design and a 2 x 3 x 5 factorial design in the field and greenhouse, respectively. Factors under study were stress condition (stressed and non-stressed control plants), three phenological stages (rosette, branching, and flowering), and five safflower genotypes (Turkey, Kenya9819, Sina, PI537636, and Gila) making a total of 30 experimental units and were replicated three times. For the greenhouse experiment, a total of 90 pots each having 30 kg volume were used. Firstly, soil was sieved and a total of 25 kg of soil was weighed into each pot. For a field experiment, plants were planted along the drip lines at an inter- and intra-row spacing of 1 and 0.25 m, respectively. A sub-sub plot constituted of 2 x 2 m plots spaced 1 m apart within the sub-plots which were 8 x 14 m spaced 2 m apart while the main plots (stress and non-stress) were 8 x 46 m spaced by 2 m apart. The experiments were both conducted concurrently during winter which is the dry season.

5.2.2 Drought stress induction

Drought stress was imposed at the onset of the following phenological stages; rosette (two weeks after emergence), branching, and flowering stages. In the non-stressed treatment, plants were irrigated throughout the crop cycle to satisfy plant water needs. Drought stress treatment was imposed by withholding irrigation completely at each phenological stage. Two access tubes were installed in the middle of each sub-sub plot/pot at a depth of 5 and 20 cm (greenhouse experiment) and 20 and 40 cm (Field experiment) to measure soil moisture. The soil moisture was measured by inserting the moisture meter (Theta probe type ML2x, Delta-Devices, Cambridge England) into the access tubes and readings were taken at each assessment date. Data was collected every after 10, 20 and 30 days of stress imposition. For the field experiment data was collected for the rosette and branching phenological stages but not flowering stage because it started raining heavily at the start of the flowering period.

5.2.3 Determination of leaf relative water content

Sampling was done at solar noon ±2 hours as this is the most stable time of the day concerning irradiance and temperature and their effect on leaf water relations. To minimize water loss during the transfer of the leaves to the laboratory, leaves were immediately enclosed in labelled plastic ziplock bags after being picked. The topmost fully expanded healthy leaves were used for measurements. Four leaf samples were collected from different plants in each plot or pot. Each leaf was labelled and weighed to determine the fresh weight. To obtain turgid weight, leaves were soaked in distilled water for 24 hours at room temperature. Then after gently wiping the water from the leaf surface with a paper towel, turgid weight was measured. Leaf dry weight was

determined by oven drying the leaves for 48 hrs at 66°C, then weighed to get dry weight. The LRWC was calculated as per the formula:

$$RWC\ (\%) = [(FW-DW) / (TW-DW)] \times 100$$

Where FW is the initial fresh weight, TW is turgid fresh weight and DW is the dry weight.

5.2.4 Chlorophyll content

Five fully expanded fresh leaves were measured, simultaneously using a chlorophyll meter (SPAD-502 plus, Konica Minolta).

5.2.5 Plant height

Plant height was measured from the ground level to the apex of the main stem at each assessment date.

5.2.6.0 Sampling for proline and Ascorbate Peroxidase

At each assessment date, leaf samples for laboratory work were collected from intact leaves (leaves without injury) and they were quickly frozen in liquid nitrogen and stored at -80°C until further analysis. Then, the stored leaf samples were ground to a fine powder in liquid nitrogen using a pre-cooled pestle and mortar and then used for quantification of APX and proline.

5.2.6.1 Proline determination

Proline was determined as per the method of Bates et al. (1973). Approximately 0.2 g of ground leaf samples were weighed and extracted by homogenizing in 10 mL of 3% (w/v) aqueous sulfosalicylic acid using a pestle and mortar. Then, the extracted leaf samples were transferred into

15 ml tubes and kept in ice until using as crude samples. The crude samples were centrifuged at 3,000 g for 20 min at 4°C. Then 2 ml of supernatant was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in a test tube. Then samples were incubated in a boiling water bath for 60 min, followed by shocking in an ice-cold water bath for 15 mins then allowed to cool at room temperature. The reaction mixture was extracted with 4 ml toluene and mixed vigorously for 15-20 seconds. Then 300 µl of chromophore (upper part) containing toluene was transferred into a 96-well microplate. Absorbance was read at 520 nm in a spectrophotometer (SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) set to endpoint mode and using toluene as a blank. Proline standards were made from pure proline and were put through the same process as of the sample. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

5.2.6.2 Determination of Ascorbate peroxidase activity

Ascorbate peroxidase (APX) was determined as per Nakano and Asada (1981) with some minor modifications. Approximately 0.2 g of ground leaves was weighed and extracted by homogenizing in 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.8 with 0.1 mM EDTA) supplemented with 1 mM ascorbate. The samples were then centrifuged at 15,000×g for 20 min at 4°C. The supernatant was transferred to a new 2ml Eppendorf tube, and the pellet was re-suspended in 0.8 mL of the potassium phosphate buffer, and the suspension was centrifuged for 15 min at 15,000 × g at 4°C. Then supernatant pipetted out and combined with the initial supernatant in 2 ml Eppendorf tubes then stored at -80°C and used as a crude enzyme extract in the quantification of APX. The reaction mixture for APX analysis (with a total volume of 300 µl) contained 50 mM potassium phosphate buffer (at pH 7.0), 0.5 mM ascorbate, 0.25 mM EDTA, 3% H_2O_2, and 15 µL

of crude leaf extract was used to initiate the reaction. Then absorbance was recorded after every 30 sec for 3 minutes at 290 nm. The enzyme activity was calculated from the extinction coefficient (2.8 mM^{-1} cm^{-1}) for the reduced ascorbate. The APX activities were determined spectrophotometrically using SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) set to kinetic mode and expressed as micromoles of ascorbate per minute per gram of fresh weight.

5.2.9 Statistical analysis

All measured variables were analysed using one-way, analysis of variance (ANOVA) using R-Software version 4.2.2 and the agricolae package version 1.3-5. Treatment means effects were compared using Fisher's least significant difference (LSD) procedure at a significance level of 5%. The GT biplot analyses for all studied traits of genotypes were executed on R-Software version 4.2.2 using the METAN package of Olivoto and Lúcio (2020).

5.3 Results

5.3.1 Chlorophyll content

The main effect of genotype, the interactions of stress condition × stage of development × genotype, stress condition × genotype, and stage of development × genotype were not significant ($P > 0.05$) for chlorophyll content at 10 days of stress induction under greenhouse experiment (Appendix 4). However, there was a significant ($P < 0.05$) interaction of stress condition and stage of development for chlorophyll content of safflower stressed for 10 days under the greenhouse (Appendix 4). A statistically ($P > 0.05$) higher level of chlorophyll content of 56.07 SPAD reading

was observed among the control plants during the flowering stage than on other developmental stages and stress conditions, with exception of plants that were stressed at the branching stage (Figure 5.1). Generally, there were no noticeable differences in the chlorophyll content of stressed and non-stressed plants at day 10 of withholding water under the greenhouse experiment during the rosette and branching stages. On the contrary, the plants that were stressed for 10 days at the flowering stage exhibited a significantly ($P < 0.05$) lower chlorophyll content than their respective control plants (Figure 5.1).

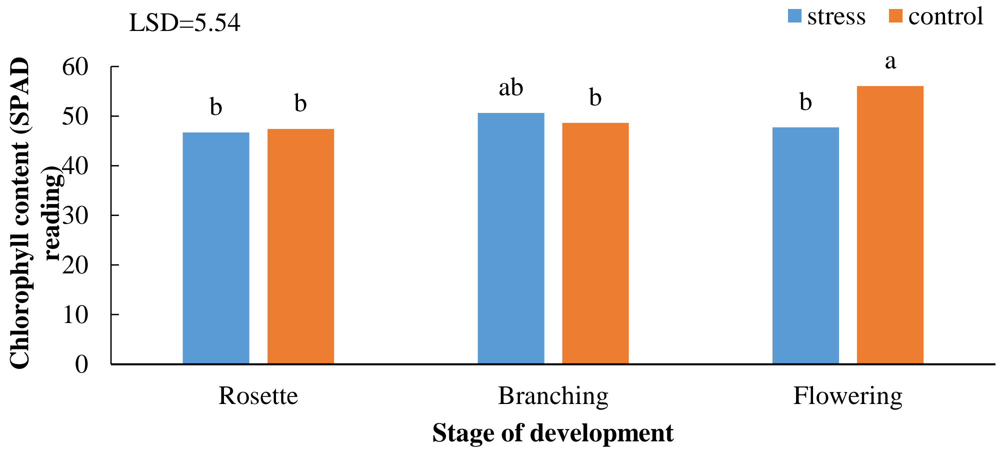

Figure 5.1. Effects of stress condition and stage of development on the chlorophyll content of safflower stressed for 10 days under the greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The main effect of stress condition, the interactions of stress condition × stage of development × genotype, stress condition × genotype, stage of development × genotype, and stress condition × stage of development were not significant ($P > 0.05$) for chlorophyll content after 10 days of stress induction under field experiment (Appendix 4). Therefore, the main effect of stage of development and genotypes are presented. Figure 5.2A depicts that the chlorophyll content was generally higher

(52.3 SPAD reading) during the branching stage than the rosette stage at 10 days of withholding water. With respect to genotypes, Turkey recorded chlorophyll content of 52.9 SPAD reading which was markedly high as compared with other genotypes with exception of genotype Gila (Figure 5.2B). The least chlorophyll content of 47.6 was observed on genotype PI537636, although it was not statistically different from genotypes Kenya9819 and Sina (Figure 5.2B).

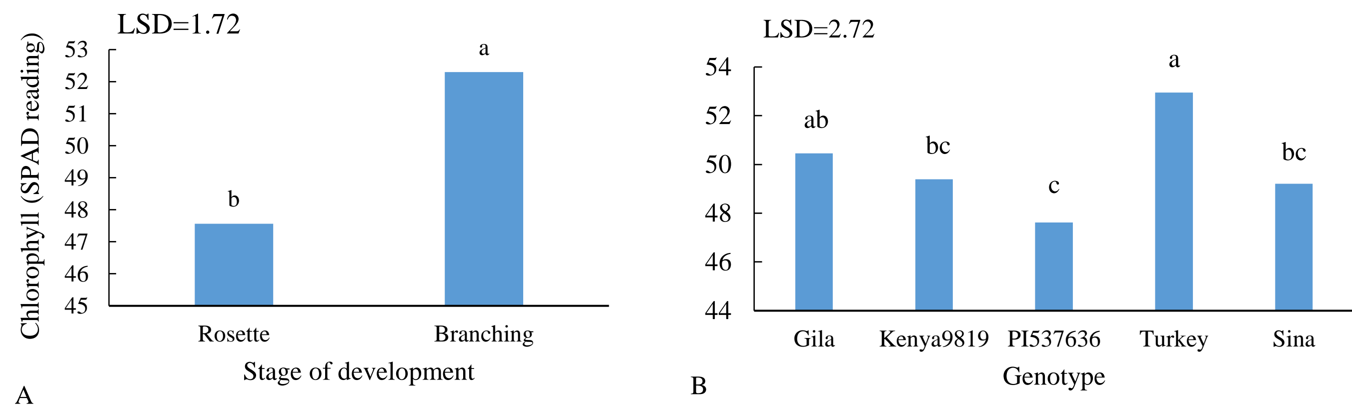

Figure 5.2. The main effect of stage of development (A) and genotype (B) on the chlorophyll content of safflower stressed for 10 days under the field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The interactions of stress condition × stage of development × genotype, stress condition × genotype, and stage of development × genotype was not significant ($P > 0.05$) for chlorophyll content after 20 days of stress induction under greenhouse experiment (Appendix 4). However, the interaction of stress condition and stage of development was significant ($P < 0.001$) at day 20 of stress imposition in the greenhouse (Appendix 4). Generally, the chlorophyll content was noticeably higher among the control plants than the stressed plants (Figure 5.3). The highest chlorophyll content of 54.8 SPAD reading was observed among the control plants during the

flowering stage this was significantly like plants that were stressed at the rosette and the control plants at the branching stage (Figure 5.3). The chlorophyll content was statistically (P > 0.05) similar among the control and stressed plants at day 20 of withholding water during the rosette stage. However, there was a noticeable variation among the stressed and control plants during the subsequent stages of development (branching and flowering stages) with the stressed plants exhibiting low leaf chlorophyll content. A remarkably low chlorophyll content of 12.1 SPAD reading was observed among the stressed plants during the flowering stage (Figure 5.3).

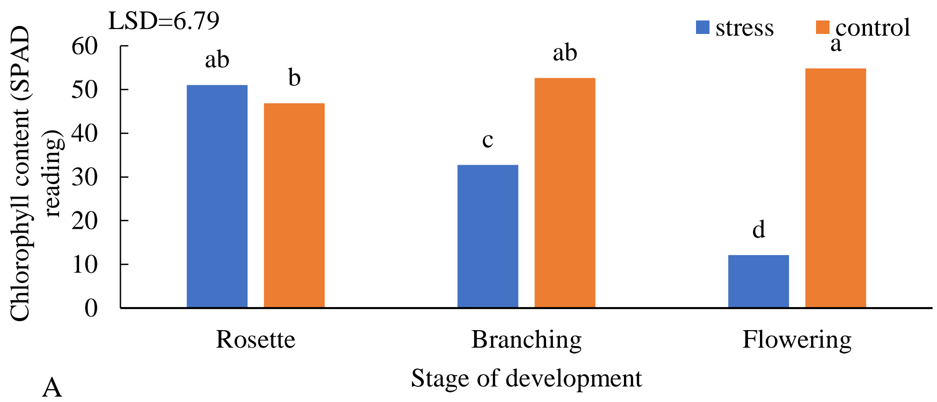

Figure 5.3. The effect of stage of development and stress condition on the chlorophyll content of safflower stressed for 20 days under the greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The main effect of stress condition, the interactions of stress condition × stage of development × genotype, stress condition × genotype, stage of development × genotype, and stress condition × stage of development were not significant (P > 0.05) for chlorophyll content after 20 days of stress induction under field experiment (Appendix 4). Therefore, the main effects of stage of development, genotype, and condition of the plants are presented. Figure 5.4A shows that at day 20 of withholding water, the chlorophyll content was high during the branching stage (54.2 SPAD

reading) as compared with the rosette stage (47.3 SPAD reading) independent of stress condition and genotype. The genotypes recorded significantly similar values of chlorophyll except for genotype PI537636 which recorded the least chlorophyll content independent of stress condition and stage of development (Figure 5.4B).

The main effect of stress condition was highly significant ($P < 0.001$) for chlorophyll content at day 20 of withholding water under the field experiment (Appendix 4). The results showed that chlorophyll content was substantially higher among the non-stress plants (52.6 SPAD reading) than on the stressed plants (48.9 SPAD reading) (Figure 5.4C).

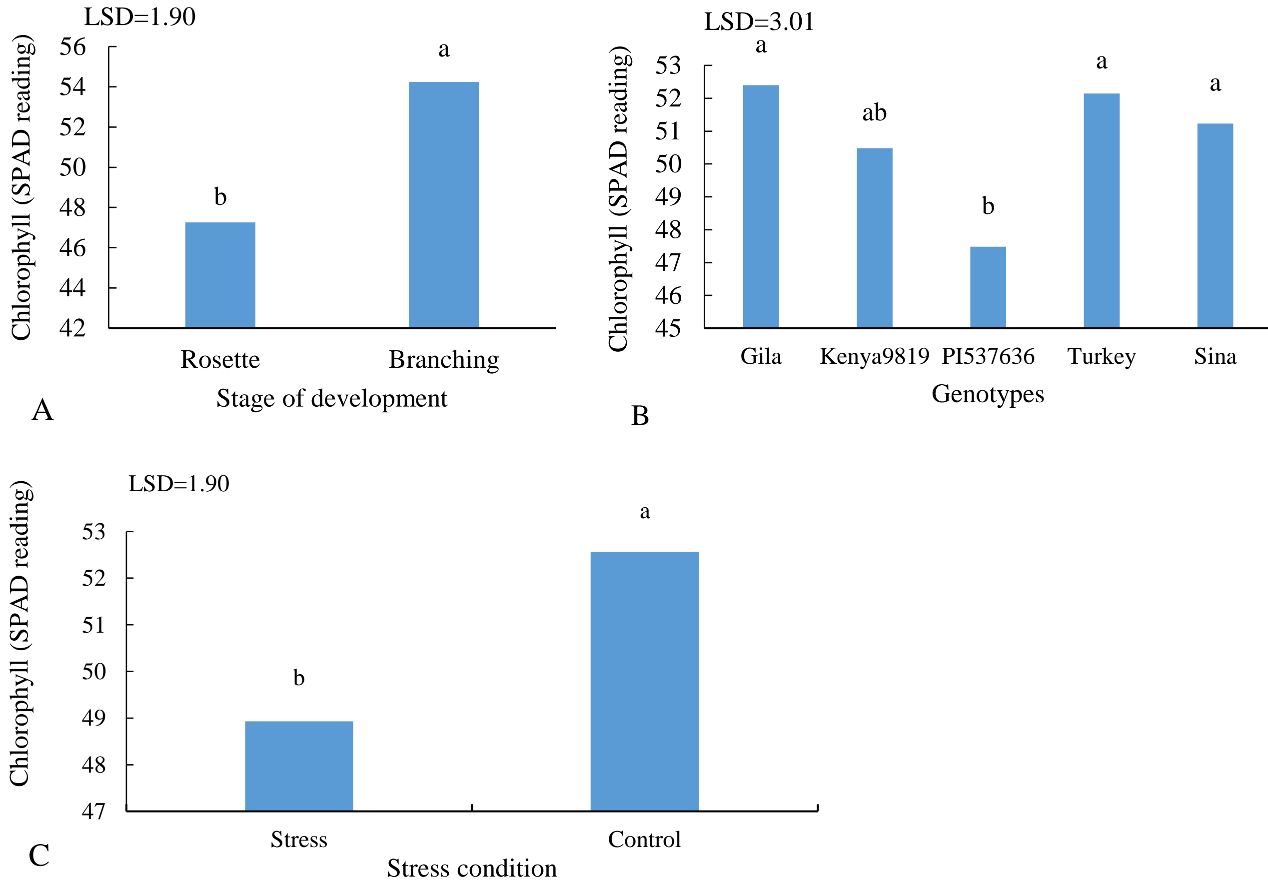

Figure 5.4. The main effect of stage of development (A) and genotype (B) on the chlorophyll content of safflower stressed for 20 days under the field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The interaction of stress condition and stage of development, condition and genotype, and stage of development and genotype significantly ($P < 0.05$) influenced the chlorophyll content of safflower plants at 30 days after imposition of stress under the greenhouse experiment (Appendix 4). A chlorophyll content of 63.1 SPAD reading was observed among the stressed plants during the rosette stage which was noticeably high as compared with other stages and stress conditions (Figure 5.5A). Generally, during the rosette stage, high chlorophyll content was found on the

stressed plants as compared with the non-stressed control plants while the opposite was true during the branching stage where stressed plants had the lowest chlorophyll content (Figure 5.5A).

There was a significant ($P < 0.05$) interaction between stress condition and genotype for chlorophyll content among plants that were stressed for 30 days under the greenhouse (Appendix 4). The results showed that the control plants had statistically higher chlorophyll content than the stressed plants independent of genotype except for genotypes PI537636 and Turkey which had similar values to the control plants (Figure 5.5B). Generally, genotypes without water stress had similar chlorophyll content (Figure 5.5B). Stressed genotypes Gila and Sina had the least chlorophyll content of 32.7 and 31.5 SPAD readings, respectively compared to other genotypes stressed or not stressed (Figure 5.5B). Among the stressed plants, genotypes Kenya9819, PI537636, and Turkey had similar chlorophyll content (Figure 5.5B).

There was a significant ($P < 0.05$) interaction between stage of development and genotype for chlorophyll content among plants that were stressed for 30 days under the greenhouse (Appendix 4). Generally, chlorophyll content was noticeably high during the rosette stage as compared with the branching stage irrespective of genotype (Figure 5.5C). The lowest chlorophyll content of 25.82 and 23.23 SPAD reading was found on genotypes Gila and Sina respectively during the branching stage. Furthermore, during the branching stage, genotypes Kenya9819, Turkey, and PI537636 had relatively similar chlorophyll content (Figure 5.5C).

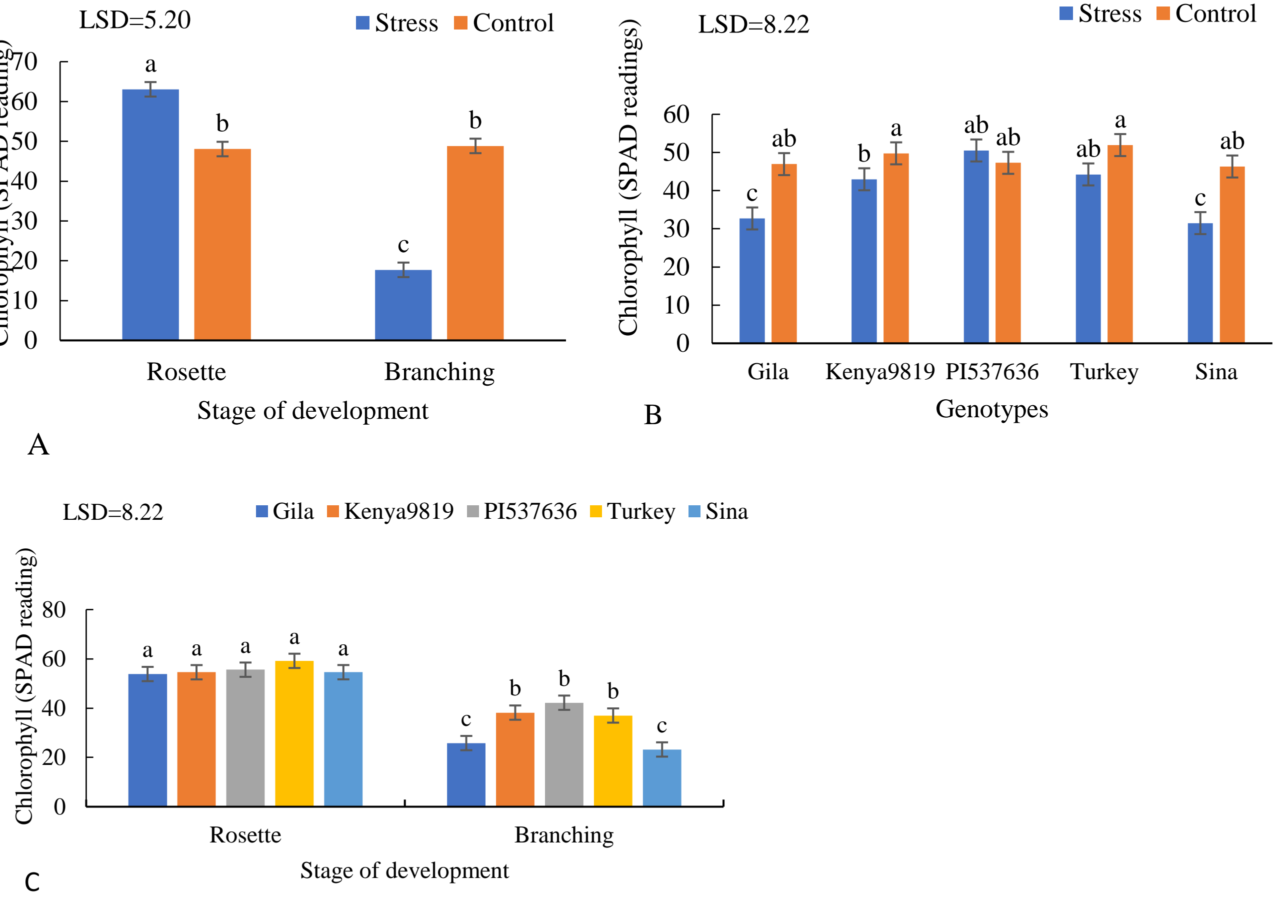

Figure 5.5. The effect of stage of development and stress condition (A), effect of stress condition and genotype (B), and effect of genotype and stage of development (C) on the chlorophyll content of safflower stressed for 30 days under the greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The main effect of stress condition, the interactions of stress condition × stage of development × genotype, stress condition × genotype, stage of development × genotype, and stress condition × stage of development were insignificant ($P > 0.05$) for chlorophyll content after 30 days of stress induction under field experiment (Appendix 4). Therefore, the main effects of stress condition and stage of development are presented. At 30 days of withholding water under the field experiment, the stressed plants had noticeably had lower chlorophyll content of 49.8 SPAD reading than the

control plants that had 53.6 SPAD reading (Figure 5.6A). Figure 5.6B shows that the chlorophyll content was markedly higher during the branching stage (55.6 SPAD reading) than at the rosette stage (47.7 SPAD reading).

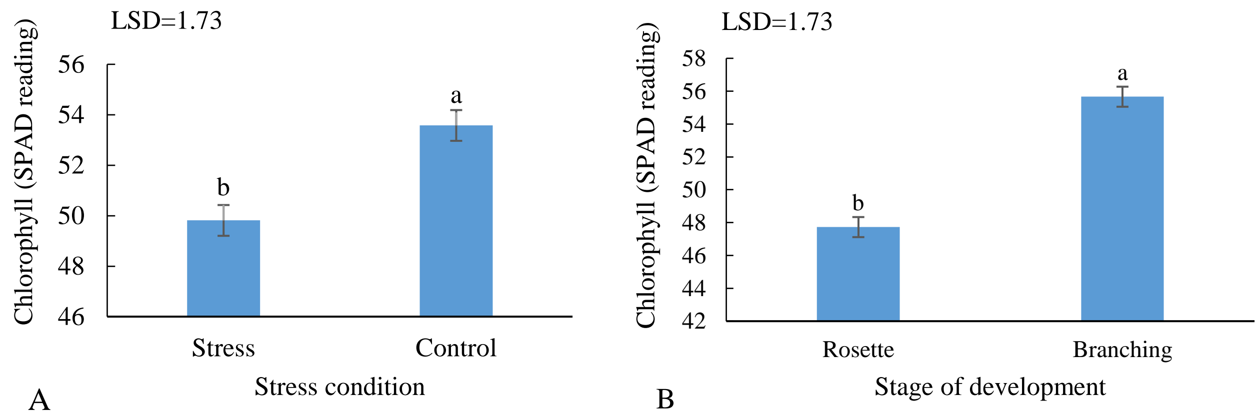

Figure 5.6. The main effect of stress condition (A) and stage of development (B) on the chlorophyll content of safflower stressed for 30 days under the field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The results showed that drought stress increased the leaf chlorophyll content of safflower at the rosette stage irrespective of stress duration under the greenhouse experiment except at day 10 where there were no marked differences between the control and stress plants (Figure 5.7A). Among the stressed plants, the highest chlorophyll content of 63.06 SPAD reading was observed at day 30 of stress induction while the lowest values (46.71 SPAD reading) were observed at day 10. Generally, drought stress increased the chlorophyll content by 4.2 and 13.5% on days 20 and 30 respectively, relative to the control at the rosette stage under the greenhouse experiment (Figure 5.7A). The leaves from stressed plants were wilting and dark green in colour while those of control plants were shiny with bright green colour during the rosette stage.

Drought stress caused no marked reduction in the chlorophyll content of safflower at the rosette stage during the initial stages of stress induction (10 days) as compared with control plants under the field experiment (Figure 5.7B). However, a substantial decrease in the chlorophyll content was found in stressed plants after 20 days of stress induction (Figure 5.7B). At day 30, a high chlorophyll content of 49.29 SPAD readings was observed in the non-stress control plants while the stress plants registered a low value of 46.16 SPAD readings. Generally, drought stress reduced chlorophyll content by 6.7% at day 30 during the rosette stage under the field experiment (Figure 5.7B).

Generally, drought stress caused a decrease in the chlorophyll content of safflower at the branching stage under the greenhouse experiment and the reduction was linear (Figure 5.7C). However, on day 10 there was no noticeable variation in the chlorophyll content among the stressed and control plants (Figure 5.7C). However, after day 10 there was a marked reduction in the chlorophyll content due to drought stress which progressed linearly 30 days of after stress induction. At days 20 and 30, drought stress reduced chlorophyll content by 37.8 and 63.7%, respectively, with the rate of decrease increasing with the duration of stress (Figure 5.7C).

Overall, drought stress caused a decrease in the amount of chlorophyll irrespective of stress duration during the branching stage under the field experiment (Figure 5.7D). However, there were no marked reductions in the chlorophyll content during the early days (10 days) of stress induction (Figure 5.7D). Moreover, as drought stress progressed to 20 and 30 days, there was a substantial decrease in the chlorophyll content of stressed plants relative to control plants under field conditions during branching stage (Figure 5.7D). In this regard, drought stress reduced chlorophyll

content by 9% and 7.6% at day 20 and 30 respectively during the branching stage under the field experiment (Figure 5.7D).

During the initial days of stress induction (day 10), the chlorophyll content was similar among the stressed and control plants during the flowering stage under the greenhouse experiment (Figure 5.7E). However, as stress duration progressed to day 20, drought stress reduced the leaf chlorophyll content of safflower by 77.9% (Figure 5.7E).

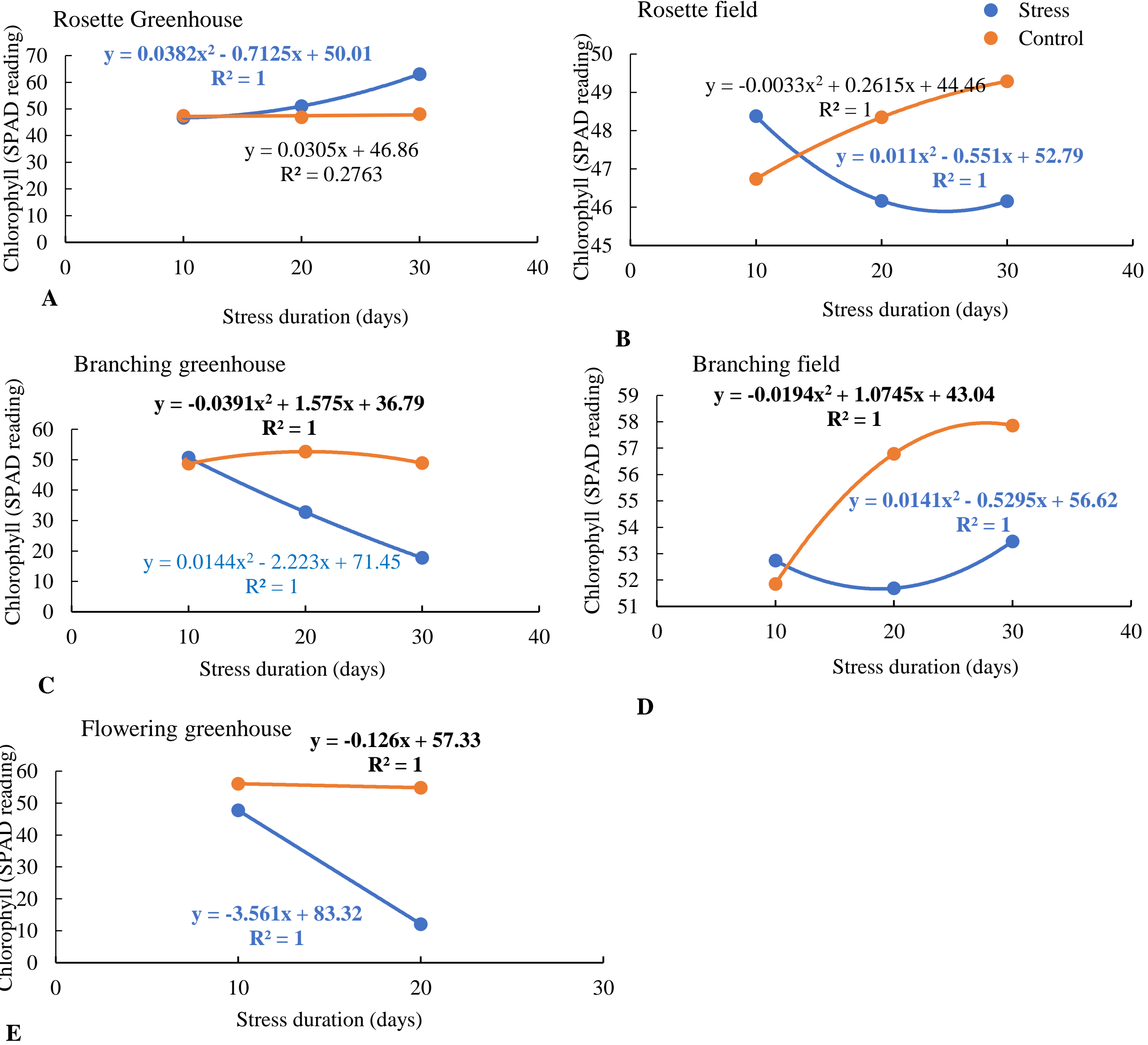

Figure 5.7. The effect of stress duration at different phenological stages (rosette greenhouse (A), rosette field (B), branching greenhouse (C), branching field (D), flowering greenhouse (E)) on the chlorophyll content of safflower stressed.

5.3.2 Plant height

There was a highly significant ($P<0.001$) interaction of stage of development and genotype for plant height at day 10 of withholding water under the greenhouse and field experiments (Appendix 5). The results showed that plant height increased as the plants developed irrespective of genotype,

with the rosette stage recording noticeably lower plant height than that of the subsequent stages (Figure 5.8A, B). Concerning the greenhouse experiment, genotype Turkey registered the highest plant height of 110.5 cm at the flowering stage than other genotypes at any phenological stage (Figure 5.8A). Generally, the plants at the rosette stage attained substantially similar plant height under the greenhouse and field experiment (Figure 5.8 A, B). Under field conditions, during the branching stage, the highest plant height of 63.93 and 57.95 cm were observed on genotypes Sina and Kenya9819, respectively while the lowest height of 44.8 cm was observed on genotype Gila (Figure 5.8B). In the greenhouse study after 10 days of drought imposition at flowering stage, safflower plant height had no noticeable variation among genotypes except for genotype Turkey which had statistically taller plants than any genotypes (Figure 5.8A). Within rosette and branching stages 10 days after water stress imposition there was no variation in plant height among genotypes except for genotypes PI537636 and Turkey which had marked differences in plant height in the greenhouse experiment (Figure 5.8A).

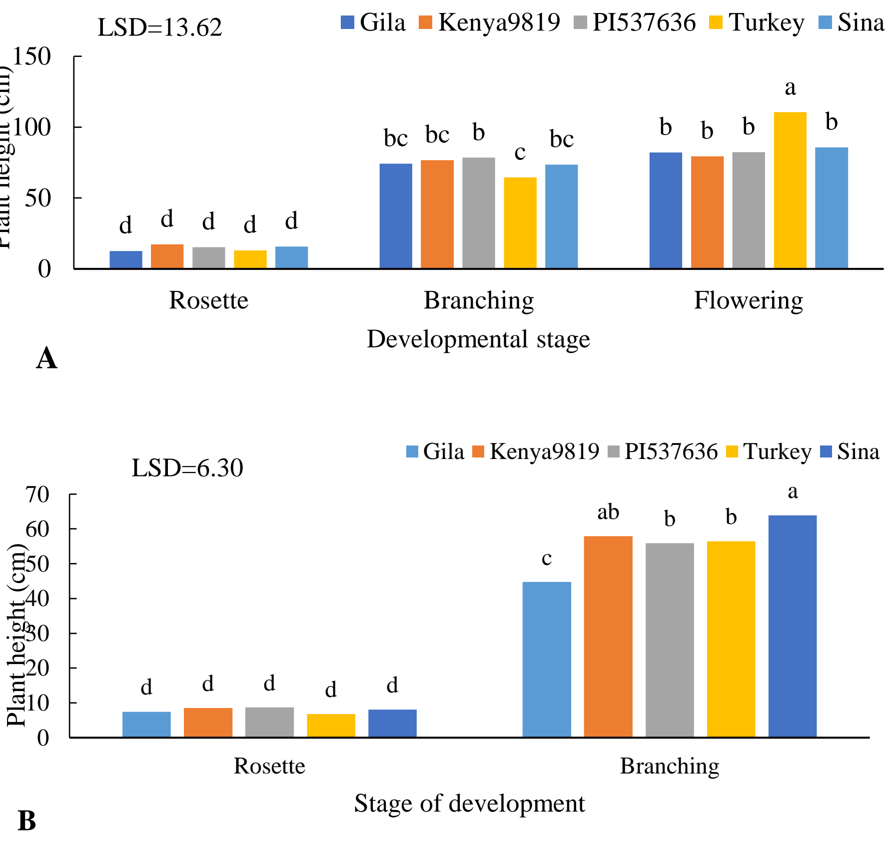

Figure 5.8. The interaction of stage of development and genotype on the plant height of safflower stressed for 10 days under greenhouse (A) and field experiment (B).

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The main effect of stress condition and genotype, the interactions of stress condition × stage of development × genotype, stress condition × genotype, stage of development × genotype, and stress condition × stage of development were not significant ($P > 0.05$) for plant height after 20 days of stress induction under greenhouse and field experiment (Appendix 5). Therefore, the main effect of the stage of development is presented. Under both the greenhouse and field experiment at day 20, plants were shorter during the rosette stage and their height increased in the subsequent developmental stages with the maximum height of 85.25 cm being recorded at the flowering stage

(Figure 5.9 A, B). In the greenhouse experiment, safflower plants at flowering stage were significantly (P<0.05) taller than those at rosette and branching stages (Figure 5.9A). Also, plants in the branching stage were significantly (P<0.05) taller than those at the rosette stage in both greenhouse and field experiments 20 days after water stress imposition (Figure 5.9 A, B).

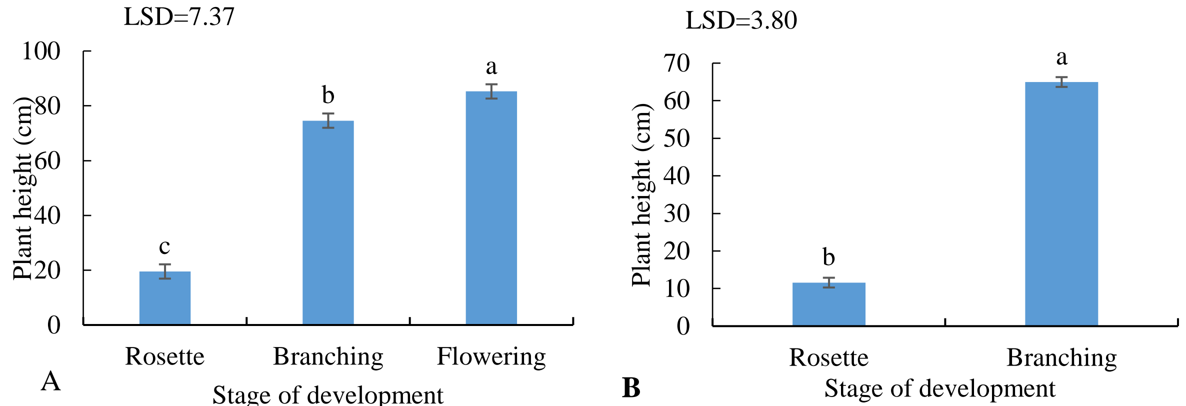

Figure 5.9. The interaction of stage of development and genotype on the plant height of safflower stressed for 20 days under greenhouse (A) and field experiment (B).

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD. Error bars represents standard error of LSMEAN.

The interactions of stress condition × stage of development × genotype, stress condition × genotype, stage of development × genotype, stress condition × stage of development, and the main effect of genotype were not significant (P > 0.05) for plant height after 30 days of stress induction under greenhouse and field experiment (Appendix 5). Therefore, the main effects of stress condition and stage of development are presented. Plant height was substantially lower among the stressed plants than the control plants under both greenhouse and field experiments (Figures 5.10 A, B). Plant height was significantly low during the rosette stage as compared to the branching stage in both greenhouse and field experiments (Figures 5.10 C, D).

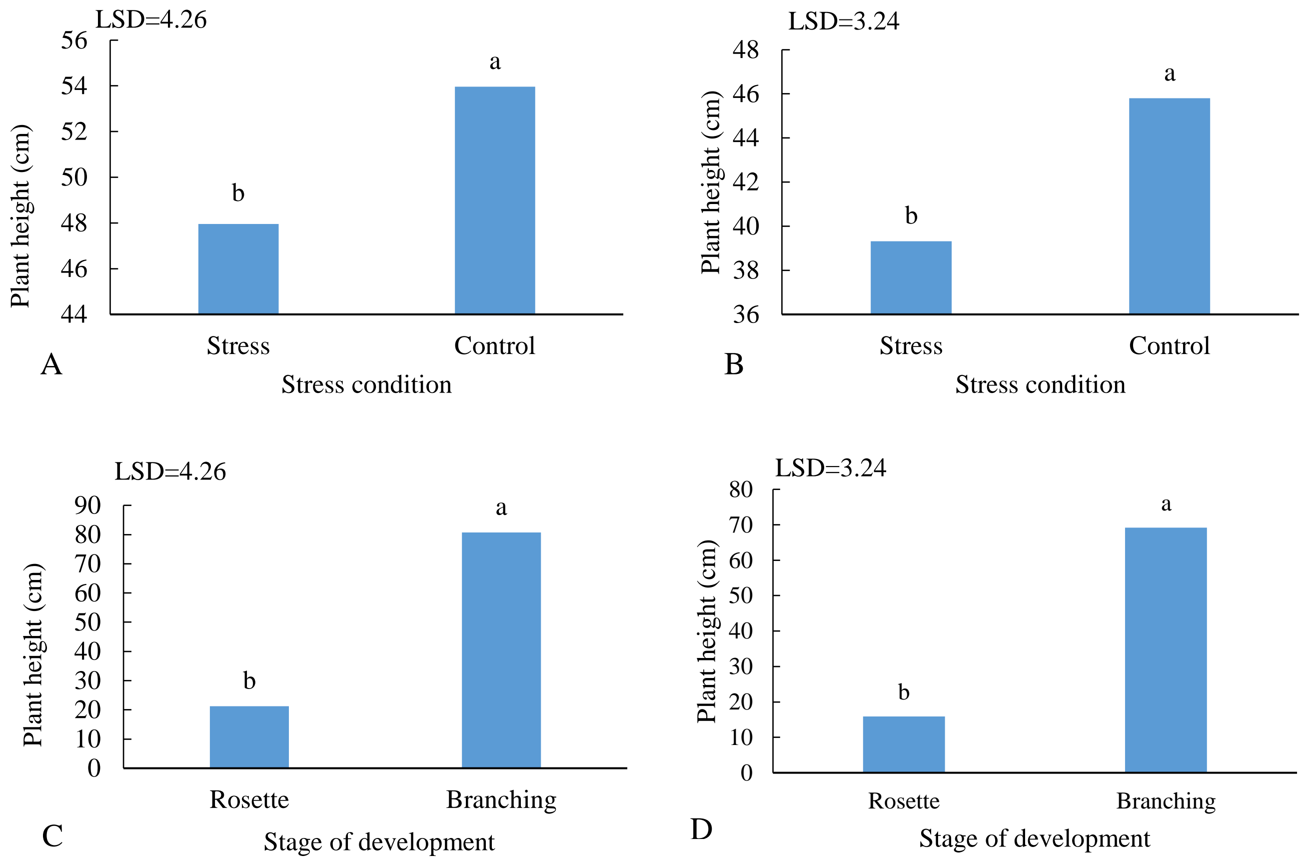

Figure 5.10. The main effect of stress condition under greenhouse (A) and field experiment (B); stage of development under greenhouse (C) and field experiment (D) on the plant height of safflower stressed for 30 days.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

During the early stages of stress induction (days 10 and 20), there were no marked differences in plant height among the stressed and control plants during the rosette stage at the greenhouse experiment (Figure 5.11A). Moreover, there was a substantial reduction in plant height as stress progressed to day 30. At day 30, the control plants attained a height of 24.27 cm which was taller than the height (18.12 cm) of the stressed plants during the rosette stage in the greenhouse (Figure 5.11A). Generally, drought stress reduced plant height by 25.3% at day 30 relative to the control

plants (Figure 5.11A). The results further showed that the stressed plants stopped growing when they were stressed beyond 20 days, unlike the control which increased growth by 19.6% (Figure 5.11A). Safflower genotypes had similar plant height at day 10 irrespective of stress conditions during the rosette stage under the greenhouse experiment (Figure 5.11A).

Drought stress decreased the plant height of stressed plants irrespective of stress duration during the rosette stage under the field experiment (Figure 5.11B). However, no marked difference in plant height was observed during the initial days (day 10) of stress induction among the stressed and control plants. However, as drought period increased plant height was significantly ($P<0.05$) reduced linearly at the rosette stage in the field experiment (Figure 5.11B). Generally, drought stress reduced plant height by 25.5 and 31.9% on days 20 and 30 respectively, relative to the control plants (Figure 5.11B). The results revealed that the stressed plants increased growth by 30.3% from day 20 to day 30 while the control increased growth by 42.5% during the rosette stage under field experiment (Figure 5.11B).

Drought stress did not cause any marked differences in the plant height of safflower plants during the branching stage under the greenhouse experiment before 20 days after imposition water stress (Figure 5.11C). However, after 20 days water stress imposition drought stress reduced safflower plant height in the greenhouse (Figure 5.11C).

Drought stress did not cause a decrease in the plant height of safflower at the initial stages of stress induction (days 10 and 20) during the branching stage under the field experiment (Figure 5.11D). However, it caused a substantial reduction in the plant height at 30 days of stress induction. Thus, drought stress reduced plant height by 9.5% relative to the control plants during the branching stage under the field experiment (Figure 5.11D). Drought stress caused a substantial decrease on

safflower plant height after 10 and 20 days of water stress imposition during flowering in the greenhouse experiment (Figure 5.11E). Plant height at the flowering stage decreased by 7.8 and 6.6% after 10 and 20 days of drought stress respectively in the greenhouse (Figure 5.11E).

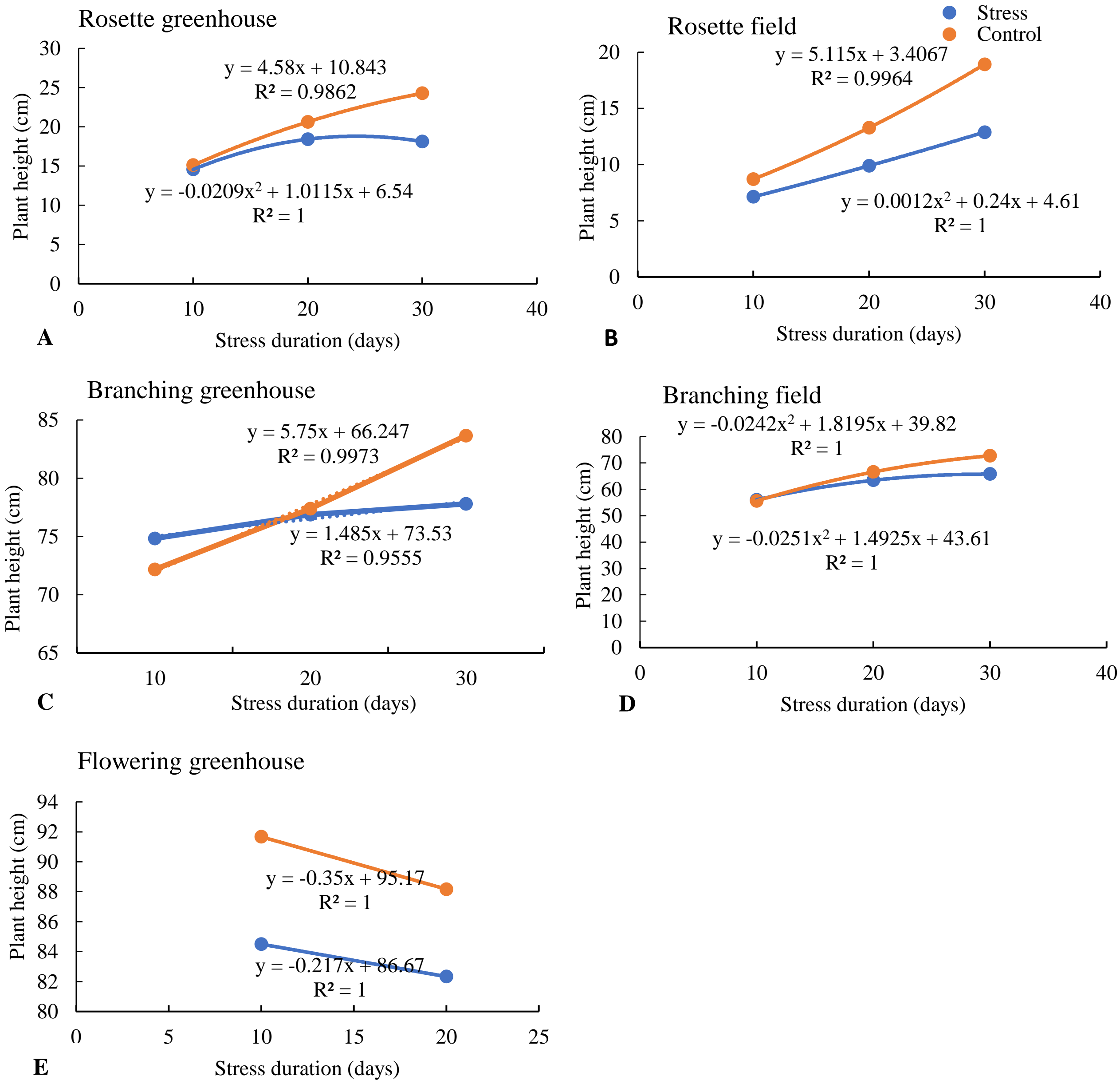

Figure 5.11. The effect of stress duration on the plant height of safflower stressed at different phenological stages (rosette greenhouse (A), rosette field (B), branching greenhouse (C), branching field (D), flowering greenhouse (E)).

5.3.3. Leaf relative water content

There was a significant (P<0.001) interaction between stress condition and stage of development for LRWC at day 10 of withholding water under the greenhouse experiment (Appendix 6). The highest LRWC (78.09%) was observed during the branching stage in the control plants at the branching stage which was noticeably higher than LRWC of plants at rosette and flowering stages both control and stressed, and stressed plants, respectively after 10 days of water stress (Figure 5.12). At day 10, there were no marked differences in LRWC among stressed and control plants within rosette and branching stages under the greenhouse experiment (Figure 5.12). In contrast, during the flowering stage, the stressed plants had the lowest LRWC of 54.51% as compared with control plants (Figure 5.12). Furthermore, there was no marked variation in LRWC of control plants in branching and flowering phenological stages at 10 days of water stress in the greenhouse (Figure 5.12)

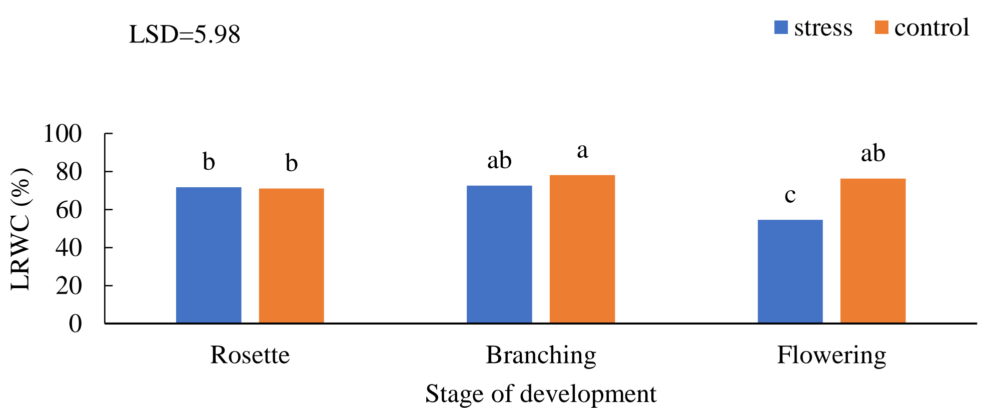

Figure 5.12. The effect of stage of development and stress condition on the LRWC of safflower stressed for 10 days under greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a significant (P<0.05) interaction of stress condition and stage of development for LRWC at day 10 of withholding water under the field experiment (Appendix 6). On day 10, LRWC was very high during at the rosette stage as compared with the branching stage in both control and stressed plants (Figure 5.13A). However, control plants at the rosette stage had LRWC of 80.4% which was substantially higher than the LRWC (70.6%) of stressed safflower plants at the flowering stage after 10 days of water stress (Figure 5.13A). During the rosette stage on day 10 of water stress, the LRWC was similar between stressed and control plants in the field experiment (Figure 5.13A). In contrast, during the branching stage, LRWC was markedly lower stressed plants than control plants after 10 days of water stress in the field study (Figure 5.13A).

There was a significant (P<0.05) genotypic variation for LRWC at day 10 of stress imposition under the field experiment (Appendix 6). Genotypes Sina (78.17%) and PI537636 (78.90%) had markedly higher LRWC than genotype Kenya9819 (74.04%) after 10 days of water stress in the field experiment (Figure 5.14B). Generally, all genotypes showed substantially, similar LRWC except for Kenya9819 which registered the lowest LRWC (Figure 5.14B).

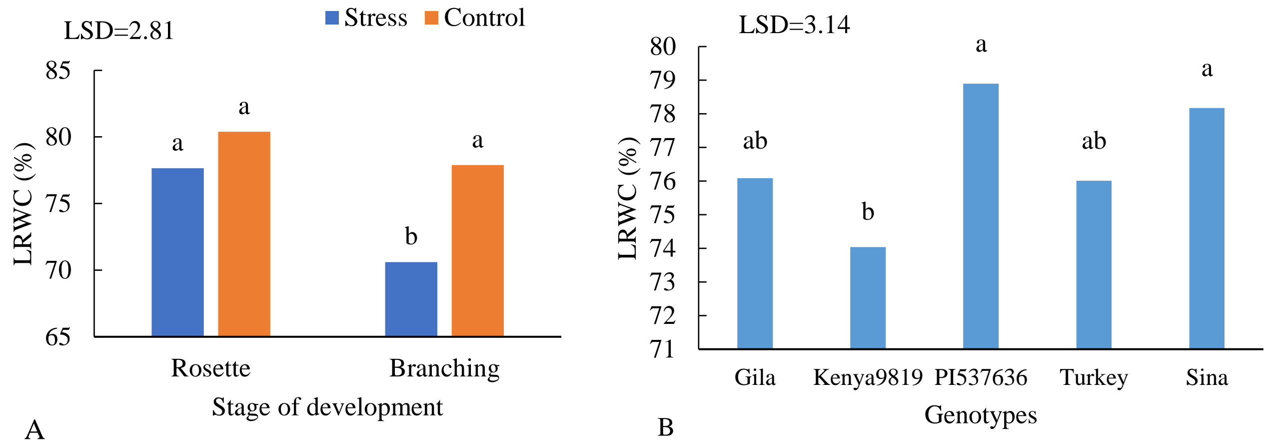

Figure 5.13. The effect of stage of development and stress condition (A) and the main effect of genotype (B) on the LRWC of safflower stressed for 10 days under field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a highly significant (P<0.001) interaction of stress condition and stage of development for LRWC at day 20 of stress induction under the greenhouse experiment (Appendix 6). The LRWC was high during the rosette stage irrespective of stress condition as compared to other developmental stages except for LRWC of control plants at branching and flowering phenological stages after 20 days of water stress in the field experiment (Figure 5.14). Water stress after 20 days remarkably reduced LRWC of safflower plants at branching and flowering phenological stages compared to control plants (Figure 5.14). At the flowering stage after 20 days of water stress, stress reduced LRWC by 71.45% (Figure 5.14). The control plants had statistically similar LRWC irrespective of stage of development in the field experiment after 20 days of water stress (Figure 5.14). The lowest LRWC of 20.7% was observed among stressed plants at the flowering stage (Figure 5.14).

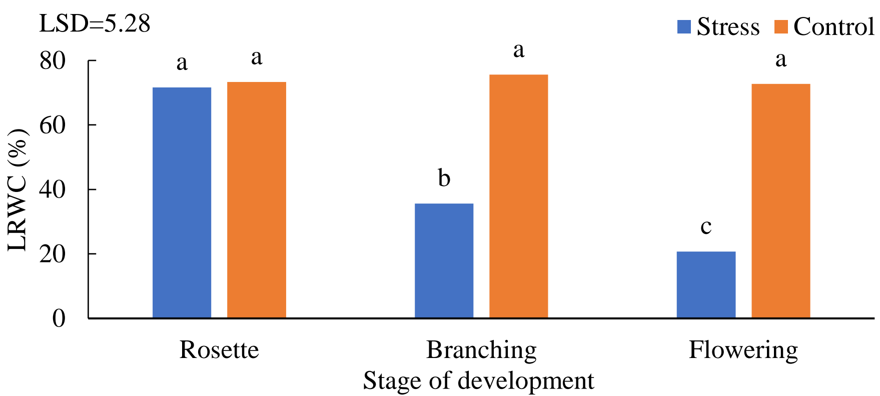

Figure 5.14. The interaction of stage of development and stress condition on the LRWC of safflower stressed for 20 days under greenhouse experiment.

Means followed by dissimilar letters are significant at P<0.05 according to Fisher LSD.

The interactions of stress condition × stage of development × genotype, stress condition × genotype, stage of development × genotype, stress condition × stage of development, and the main effect of genotype were insignificant (P > 0.05) for plant height after 20 days of stress induction under field experiment (Appendix 6). Therefore, the main effect of stress condition and stage of development is presented. Figure 5.15A showed that stressed plants had noticeably lower LRWC than the control plants independent of genotype and stage of development (Figure 5.15A). With respect to the stage of development, LRWC was higher during the branching stage than the rosette stage after 20 days of stress in the field experiment (Figure 5.15B).

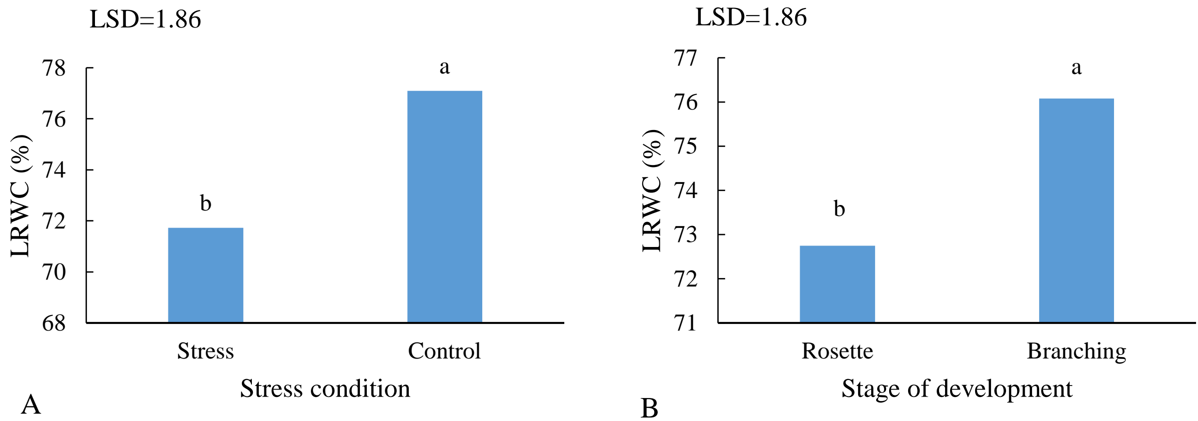

Figure 5.15. The main effect of stress condition (A) and stage of development (B) on the LRWC of safflower stressed for 20 days under field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a significant (P<0.05) interaction of stress condition × stage of development × genotype for LRWC at day 30 of stress imposition under the greenhouse experiment (Appendix 6). After 30 days of water stress, stressed plants had significantly (P<0.05) lower LRWC than control plants irrespective of genotype and stage of phenological development in the field study (Figure 5.16). The highest LRWC of 80.1% was recorded in the control plants of the genotype Turkey at branching stage of development (Figure 5.16). The lowest LRWC of 11.0% was recorded in stressed of the genotype Gila during the branching stage of safflower growth and development (Figure 5.16). Furthermore, control plants had relatively similar LRWC irrespective of stage of development and genotype (Figure 5.16). Among stressed plants the genotype Gila at branching stage had LRWC of 11.0% which was noticeably lower than LRWC than other genotypes and developmental stages except for genotypes Turkey and Sina during branching stage (Figure 5.16).

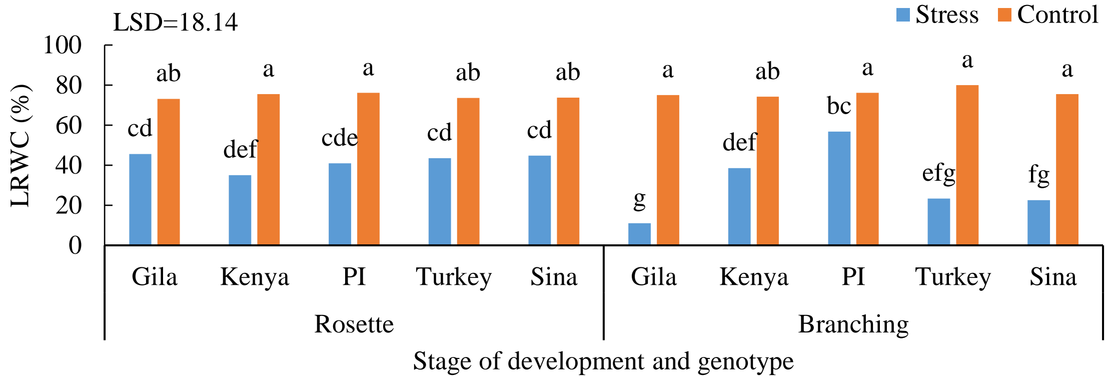

Figure 5.16. The effect of stress condition, stage of development and genotype on the LRWC of safflower stressed for 30 days under greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a significant ($P<0.05$) interaction of stress condition and stage of development for LRWC at day 30 of stress imposition under the field experiment (Appendix 6). A substantially high LRWC of 82.3% was observed during the rosette stage among the control plants which was noticeably higher than LRWC of stressed and control plants at either rosette or branching stage (Figure 5.17). On the contrary, the least LRWC of 65.7% was observed among stressed plants during the branching stage which was remarkably lower than LRWC of stressed and control plants at both rosette and branching stages of development of safflower plants in the field experiment (Figure 5.17).

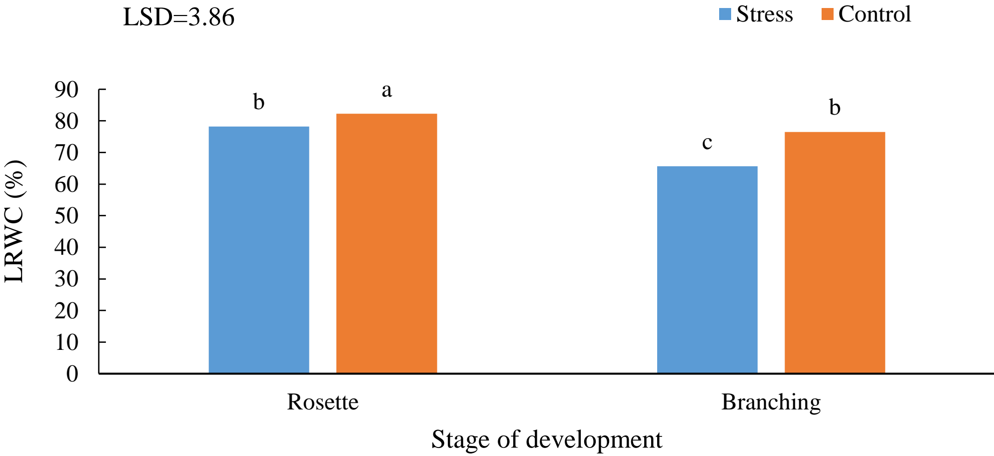

Figure 5.17. The effect of stress condition and stage of development on the LRWC of safflower stressed for 30 days under field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

Drought stress did not cause reductions in LRWC during the early stages of stress (days 10 and 20) but noticeable reductions were observed when stress was beyond 20 days and the reduction in LRWC increased linearly up to 30 days during the rosette stage under the greenhouse (Figure 5.18A). Drought stress reduced LRWC by 43.6% at day 30 compared to control plants during the rosette stage under the greenhouse (Figure 5.18A).

During the rosette stage, control plants had higher LRWC by 3.41-6.14% depending on number of days water stress was imposed in the field experiment (Figure 5.18B). The highest reduction of 6.14% in LRWC was observed on stressed plants on day 20 at rosette stage in the field experiment (Figure 5.18B).

Drought stress caused a reduction in the LRWC of safflower regardless of stress duration during the branching stage in the greenhouse experiment (Figure 5.18C). However, there were no marked

differences in the LRWC of stress and control plants during the first 10 days of stress induction (Figure 5.18C). However, substantial reduction in LRWC was observed among stressed plants in comparison to the control plants at 20 and 30 days of water stress during the branching stage in the greenhouse experiment (Figure 5.18C). Drought stress reduced LRWC by 52.9 and 60.0% at day 20 and 30 respectively, relative to the control plants during the branching stage in the greenhouse experiment (Figure 5.18C).

Drought stress reduced LRWC during the branching stage irrespective of stress duration at the branching stage in the field experiment (Figure 5.18D). Water stress decreased LRWC by 9.3, 7.6, and 14.1% on days 10, 20, and 30 respectively, with the greatest rate of decrease being recorded 30 days of stress in the field experiment (Figure 5.18D).

Regarding the flowering stage in the greenhouse experiment, drought stress reduced the LRWC by 28.5 and 71.5% at day 10 and 20, respectively in the greenhouse experiment (Figure 5.18E). The stressed plants had a very low LRWC of 20.7% at day 20 and almost all plants were wilted at this time unlike in other stages (branching and rosette) and the plants did not reach day 30 in the greenhouse experiment (Figure 5.18E).

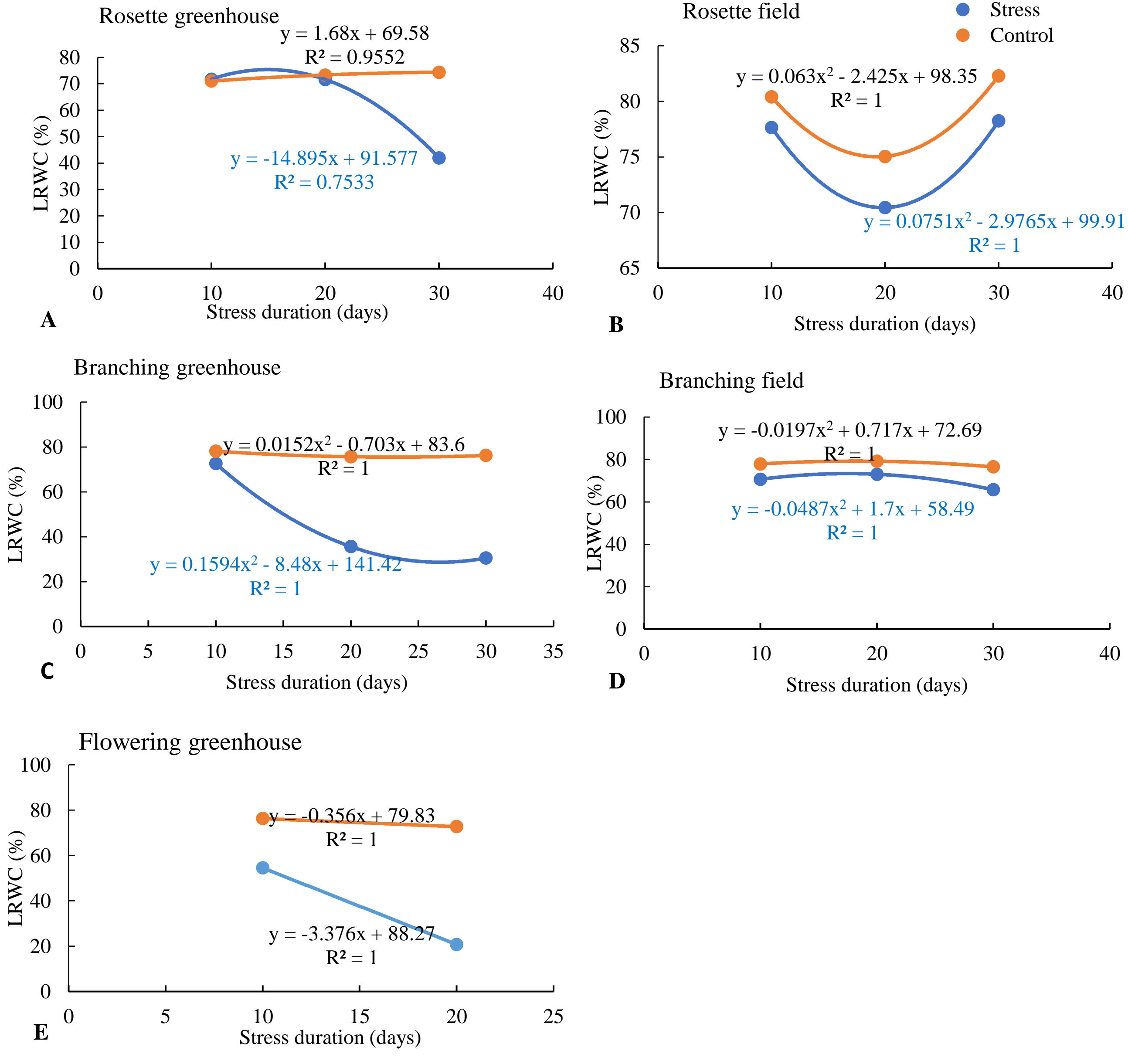

Figure 5.18. The effect of stress duration on the LRWC of safflower stressed at different phenological stages (rosette greenhouse (A), rosette field (B), branching greenhouse (C), branching field (D), flowering greenhouse (E)).

5.3.4 Proline content

There was a highly significant ($P<0.001$) interaction of stress condition, stage of development, and genotype for proline content at day 10 of stress induction under the greenhouse experiment (Appendix 7). The results showed that stressed plants of the genotype Gila at flowering stage had substantially higher level of proline content (24.22 µmoles/ g FW) than other genotypes at any developmental stage whether water stressed or not 10 days after water stress imposition (Figure 5.19). The lowest proline content of 3.97 µmoles/ g FW was produced by control plants of the genotype Kenya-9819 at the branching stage which was similar to the proline contents of control plants of other genotypes during the branching stage of safflower (Figure 5.19). In general, at day 10 of stress induction, stressed plants had higher proline content than control plants irrespective of genotype and developmental stage (Figure 5.19). Stressed plants at the flowering stage had substantially higher proline content than control and stressed plants of any genotype and developmental stage (Figure 5.19). After 10 days of water stress during the rosette stage, the control and the stressed plants had noticeably similar proline content irrespective of genotype (Figure 5.19). However, during the branching stage at day 10, stressed plants had relatively higher proline content depending on the genotype (Figure 5.19).

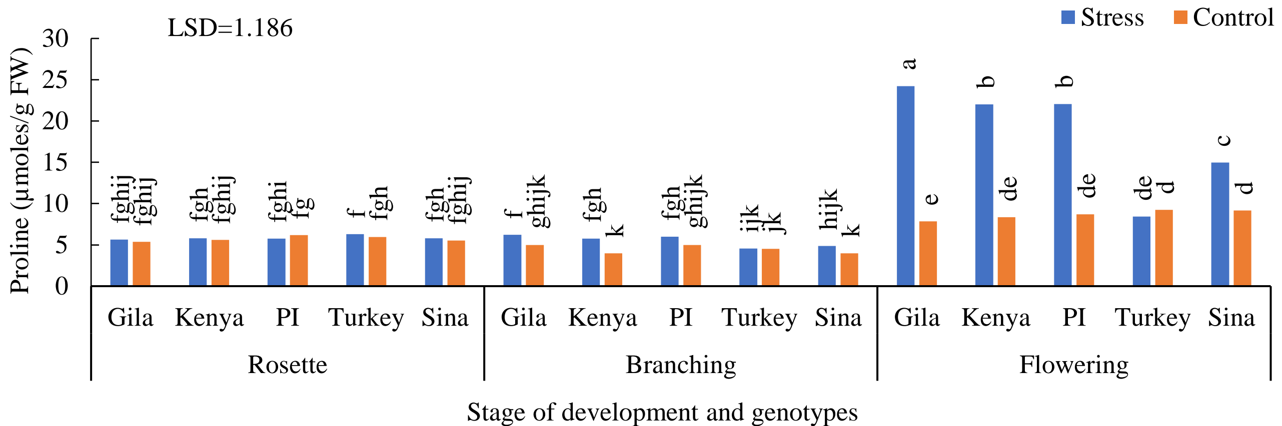

Figure 5.19. The effect of stress condition, stage of development, and genotype on the proline content of safflower stressed for 10 days under greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a highly significant (P<0.001) interaction of stress condition and stage of development for proline content at day 10 of withholding water under field experiment (Appendix 7). In the field experiment after 10 days of water stress, stressed plants at the branching stage had a significantly (P<0.05) higher proline content of 21.24 µmoles/ g FW than control and stressed plants at the rosette stage and control plants at the branching stage (Figure 5.20A). Generally, the proline content was high during the branching stage as compared with the rosette stage in the field experiment (Figure 5.20A). Control plants at the branching stage had substantially higher proline content of 16.81 µmoles/ g FW than control safflower plants at the rosette stage after 10 days of water stress in the field (Figure 5.20A). However, at the rosette stage in the field experiment 10 days after water stress there was no marked variation in proline content between control and stressed safflower plants (Figure 5.20A).

There was a significant (P<0.05) main effect of genotypes for proline content at day 10 of stress induction under the field experiment (Appendix 7). Genotype PI537636 had a substantially

(P<0.05) higher level of proline of 18.793 µmoles/ g FW than other genotypes except for Kenya9819 and Turkey (Figure 5.20B). Genotype Sina had noticeably the lowest proline content of 15.469 µmoles/ g FW than other genotypes except for genotype Gila in the field experiment (Figure 5.20B).

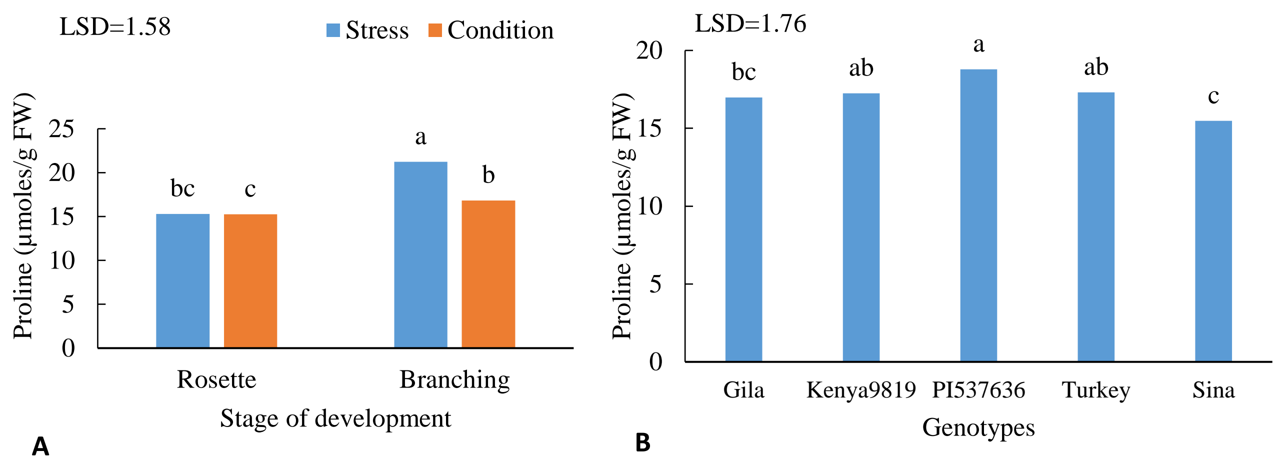

Figure 5.20. The effect of stress condition and stage of development (A) and the main effect of genotype (B) on the proline content of safflower stressed for 10 days under field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a highly significant (P<0.001) interaction of stress condition, stage of development, and genotype for proline content at day 20 of stress induction under the greenhouse and field experiments (Appendix 7). With respect to the greenhouse experiment, stressed plants of the genotype Gila during the flowering stage registered a substantially higher proline content of 274.18 µmoles/ g FW than other genotypes at any stage of development stage and stress condition after 20 days of water stress (Figure 5.21A). Generally, stressed plants had noticeably higher proline content than control plants irrespective of genotype and stage of safflower development (Figure 5.21A). Control plants had relatively similar and low contents of proline irrespective of stage of development and safflower genotype after 20 days of water stress (Figure 5.21A). In contrast, the

proline content of stressed plants varied significantly with stage of development as well as genotype (Figure 5.21A). During the rosette stage, there were insignificant ($P > 0.05$) differences between the stressed and the control plants except for genotype PI537636 (Figure 5.21A).

Under the field experiment at after day 20 of stress induction, the highest level of proline content 22.56 µmoles/ g FW was observed in stressed plants of the genotype PI537636 which was statistically greater than the proline content of any genotype, development stage, and condition (stressed or control) of the plant (Figure 5.21B). The effect of genotype and stress condition under field experiment at the rosette stage followed a similar trend with the greenhouse experiment where there were no marked differences between stressed and control plants except for genotype PI537636 (Figure 5.21B). At the branching stage, there was a substantial difference in the proline content of stressed and the control plants, with stressed plants exhibiting the highest proline contents irrespective of genotype (Figure 5.21B). Also, during the branching stage stressed plants of the genotypes Gila, Kenya9819, Turkey, and Sina had similar proline contents except for the genotype PI537636 (Figure 5.21B).

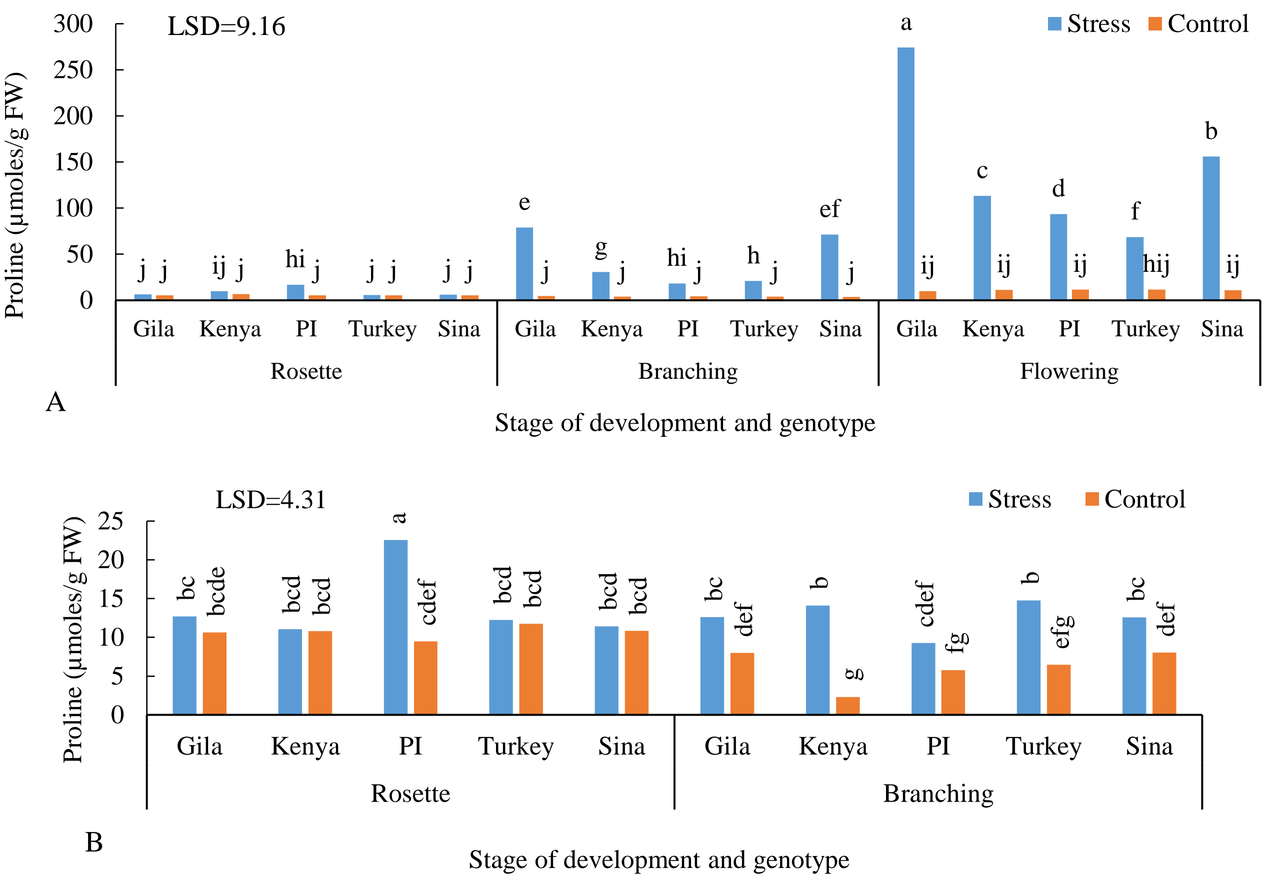

Figure 5.21. The interaction of stress condition, stage of development, and genotype on the proline content of safflower stressed for 20 days under greenhouse (A) and field experiment (B).

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a highly significant (P<0.001) interaction of stress condition, stage of development, and genotype for proline content at day 30 of stress induction under the greenhouse (Appendix 7). Plants of the genotype Sina stressed for 30 days at the flowering stage recorded the highest proline content of 228.47 µmoles/ g FW which was statistically (P<0.05) greater than proline contents of plants any genotypes at any stage of development and stress condition exception for stressed plants of the genotype Gila at the same stage (Figure 5.22). Stressed plants of all genotypes under study had substantially higher proline content than control plants of all genotypes in different developmental stages after 30 days of water stress in the greenhouse experiment (Figure 5.22).

Control plants of all genotypes in different developmental stages had relatively similar proline content after 30 days of water stress in the greenhouse experiment (Figure 5.22). Generally, after 30 days of water stress in the greenhouse, stressed plants at branching stage had markedly higher proline content than stressed plants at the rosette stage except for plants of the genotype PI537636 (Figure 5.22).

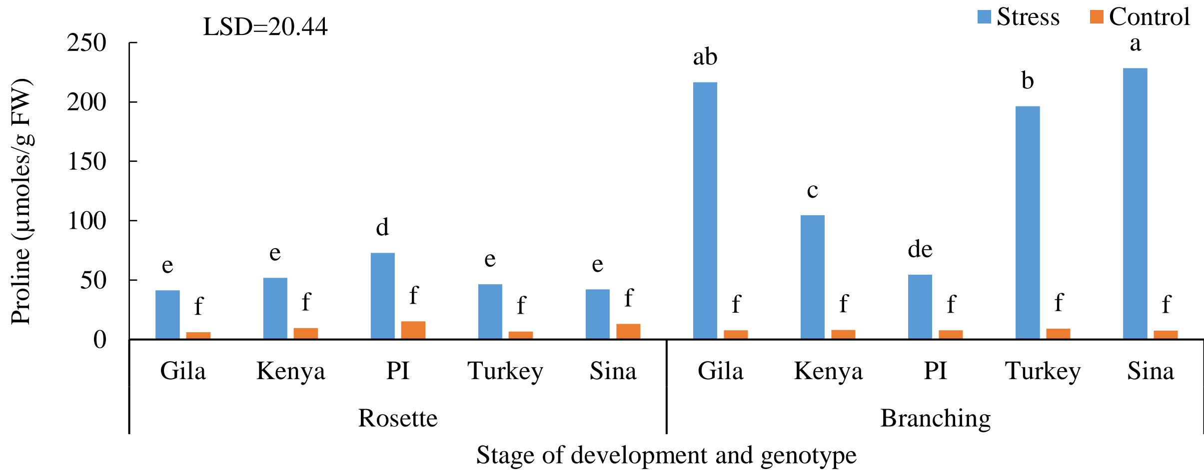

Figure 5.22. The effect of stress condition, stage of development, and genotype on the proline content of safflower stressed for 30 days under greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a significant ($P<0.05$) interaction of stage of development and genotype for proline content at day 30 of withholding water under field conditions (Appendix 7). Safflower plants of the genotype Sina at the branching stage had proline content of 18.08 µmoles/ g FW which was substantially higher than proline content of plants of the genotypes Kenya9819, and PI537636 and Turkey at branching and rosette phenological stages, respectively under field conditions (Figure 5.23). The lowest proline content of 8.76 µmoles/ g FW was observed in plants of the genotype Kenya9819 at the rosette (Figure 5.23). The proline contents of plants of genotypes Sina and Gila

did not significantly (P>0.05) vary in both rosette and branching phenological stages under field conditions (Figure 5.23).

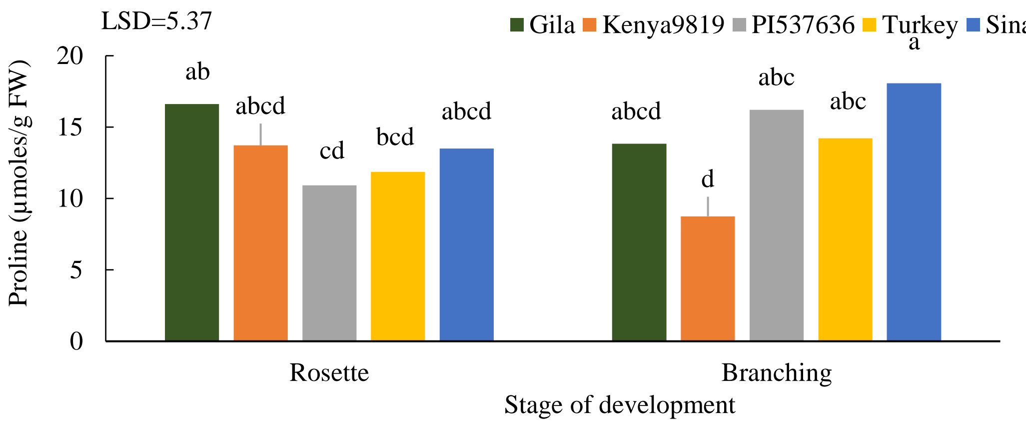

Figure 5.23. The effect of stage of development and genotype on the proline content of safflower stressed for 30 days under greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There were no significant (P>0.05) differences in proline content observed between control and stressed plants 10 days after drought stress induction at rosette stage under the greenhouse experiment (Day 10) (Figure 5.24A). However, as stress duration progressed to 20 and 30 days, significant (P<0.05) variation in proline content was observed between stressed and non-stressed control plants (Figure 5.24A). At the rosette stage, 30 days after drought stress induction, stressed safflower plants had 5x more proline content (51 µmoles/ g FW) than control plants (10.16 µmoles/ g FW) in the greenhouse (Figure 5.24A). The increase in proline content in both stressed and control plants at the rosette stage with increase in drought stress duration was quadratic (Figure 5.24). Generally, drought stress increased the proline content by 100 and 402% relative to control

plants at 20 and 30 days of water stress, respectively at the rosette stage in the greenhouse experiment (Figure 5.24A).

Proline content was outstandingly high among the stressed plants at day 20 as compared with the control during rosette stage under the field experiment (Figure 5.24B). Proline content of stressed plants was higher by 31% than control plants at the rosette stage after 20 days of water stress in the field experiment (Figure 5.24B). However, 10 and 30 days after drought stress induction the proline content of control and drought stressed plants had no marked variation on each day respectively during the rosette stage under the field experiment (Figure 5.24B).

During the branching stage an increase in duration of water stress up to 30 days substantially increased leaf proline content of drought stressed plants compared to control plants in the greenhouse study (Figure 5.24C). The response of the safflower plants to increase in drought stress was quadratic (Figure 5.24C). In the field experiment during the branching stage, increase drought stress duration substantially increased proline content of drought stressed plants compared to control plants except for 30 days after water stress (Figure 5.24D). The response of safflower plants to increase in drought stress duration was quadratic (Figure 5.24D).

Drought stress increased remarkably increased accumulation of proline in water stressed plants compared to control plants during flowering stage under greenhouse experiment (Figure 5.24E). After 30 days of flowering at the flowering stage of safflower the proline content was 11.6x higher than control plants in the greenhouse experiment (Figure 5.24E).

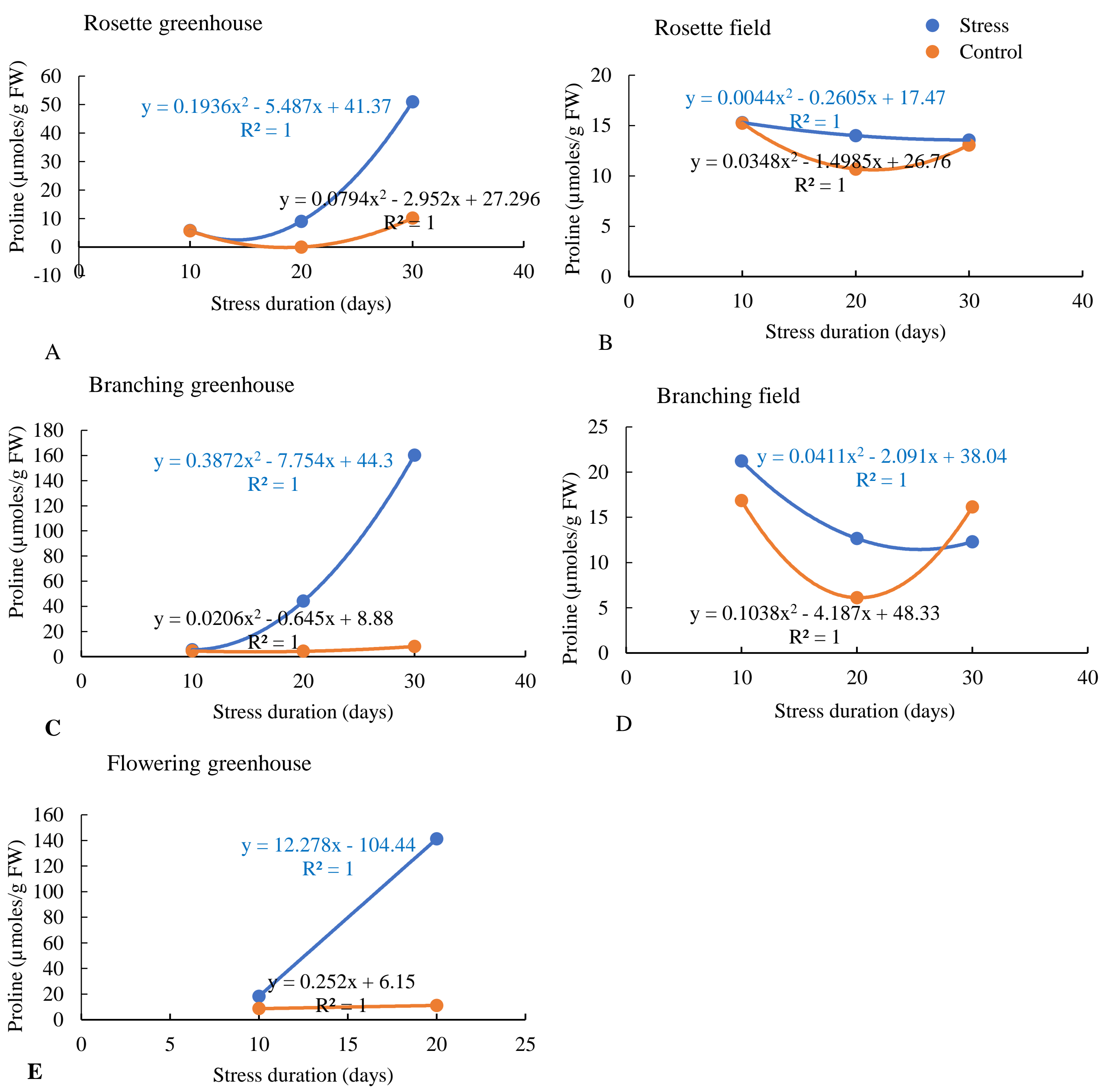

Figure 5.24. The effect of stress duration on the proline content of safflower stressed at different phenological stages (rosette greenhouse (A), rosette field (B), branching greenhouse (C), branching field (D), flowering greenhouse (E)).

5.3.5 Ascorbate peroxidase

There was a highly significant ($P<0.001$) interaction of stress condition, stage of development, and genotype for APX activities at day 10 of stress induction under the greenhouse and field

experiments (Appendix 8). With respect to the greenhouse experiment at day 10 of stress induction, water stressed plants of the genotype Kenya9819 at branching stage accumulated significantly ($P<0.05$) higher level of APX (1.67 U g^{-1} FW) than other genotypes at any stage of development and stress condition with exception of stressed plants of the genotype PI537636 at the rosette stage (Figure 5.25A). In general, water stressed plants had statistically ($P<0.05$) higher APX content than control plants at all developmental stages and in all genotypes in the greenhouse study (Figure 5.25A). During the rosette stage, stressed plants of genotypes Gila, Kenya9819, and PI537636 had substantially higher content of APX than control plants of the same genotypes (Figure 5.25A). While plants of genotypes Turkey and Sina had relatively similar levels of APX irrespective of the stress condition at the rosette stage (Figure 5.25A). At the branching stage, stressed plants of genotypes Kenya9819 and PI537636 had substantially higher content of APX than stressed or control plants of other genotypes in the greenhouse (Figure 5.25). At the flowering stage, water stressed plants of the genotypes Gila, Kenya9819, Turkey, and Sina had similar APX contents except for plants of the genotype PI537636 in the greenhouse study (Figure 5.25A).

In the field experiment at day 10 of stress induction, stressed plants of the genotype Turkey at the branching stage accumulated remarkably higher content of APX (7.99 U g^{-1} FW) than other genotypes in any stress condition and stage of development (Figure 5.25B). Generally, control plants of all genotypes had statistically ($P > 0.05$) similar APX contents in all developmental stages and genotypes (Figure 5.25B). In general, control plants of all genotypes and developmental stages had relatively low APX contents compared to stressed plants in the field experiment (Figure 5.25B).

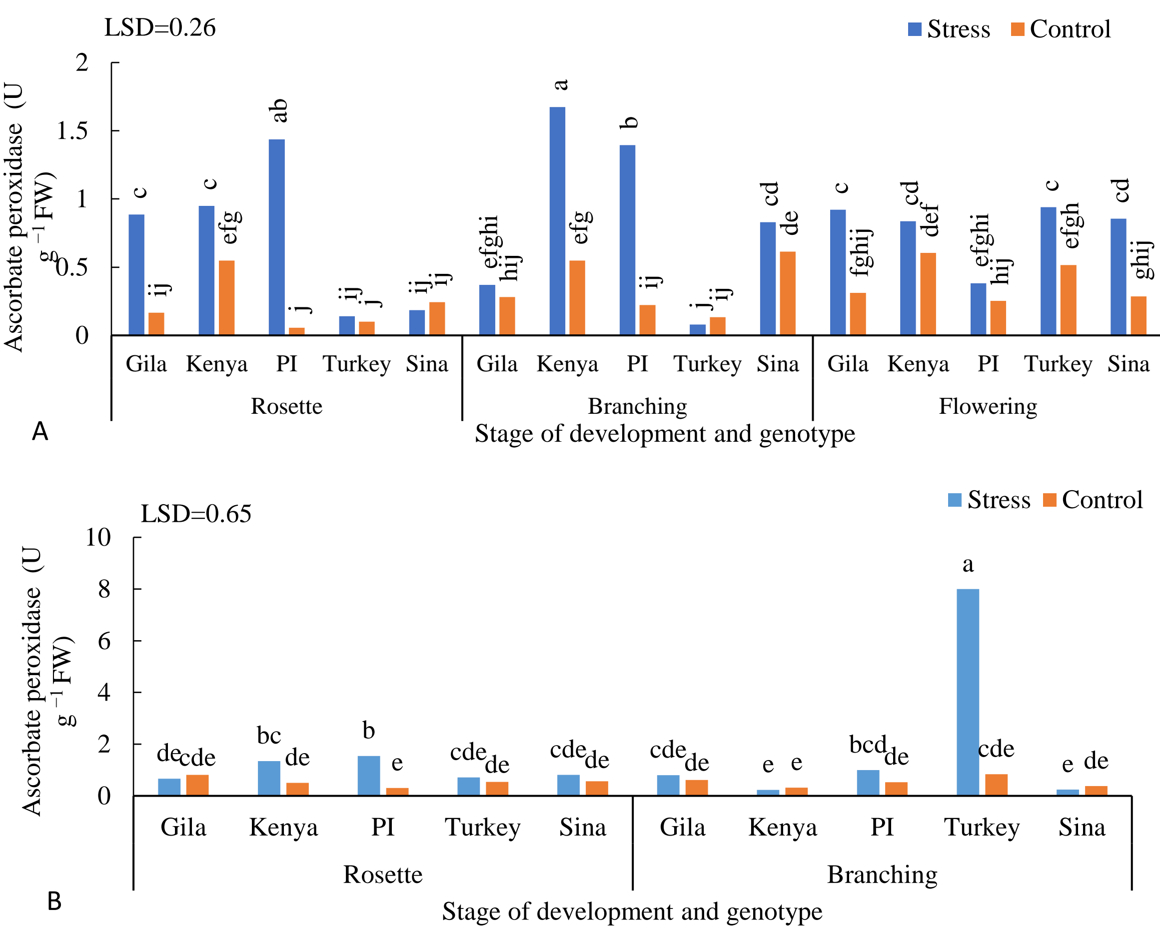

Figure 5.25. The effect of stress condition, stage of development, and genotype on the APX unit of safflower stressed for 10 days under greenhouse (A) field experiment (B).

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a highly significant ($P<0.001$) interaction of stress condition, stage of development, and genotype for APX activities at day 20 of stress induction under the greenhouse experiment (Appendix 8). Stressed plants of the genotype PI537636 at 20 days of water stress during the flowering stage had noticeably higher APX content (20.89 U g^{-1} FW) than other genotypes in any stress condition and stage of development (Figure 5.26). In general, control plants of all genotypes

and developmental stages had similar (P>0.05) activities of APX irrespective of stage of development (Figure 5.26). During rosette and branching stages at day 20 of stress induction in the greenhouse, APX content of control and stressed plants of all genotypes were similar (Figure 5.26). In contrast, during the flowering stage at 20 days of water stress there was a substantial genotypic variation with respect to APX content in the greenhouse experiment (Figure 5.26).

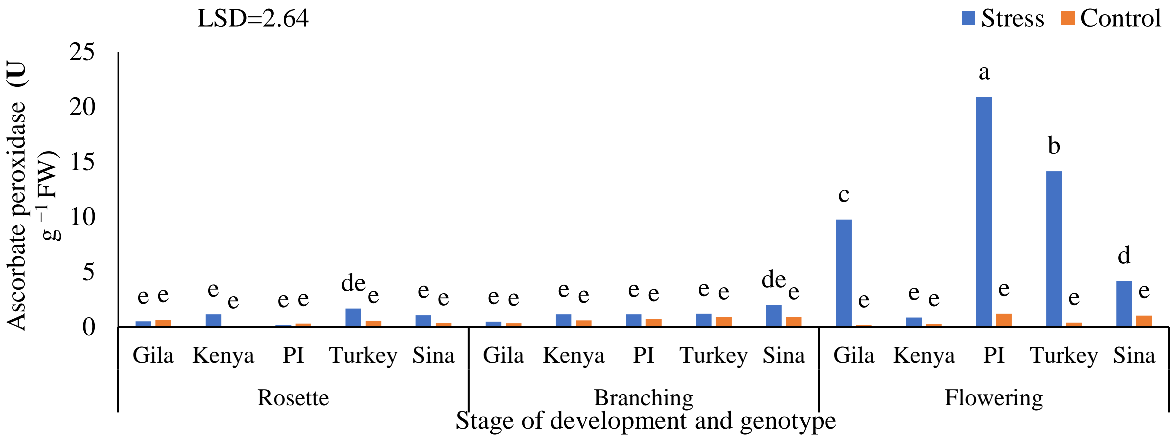

Figure 5.26. The effect of stress condition, stage of development, and genotype on the APX unit of safflower stressed for 20 days under greenhouse experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

The main effect of stage of development and genotype and the interactions of stress condition × stage of development × genotype, stress condition × genotype, stage of development × genotype, and stress condition × stage of development was not significant (P > 0.05) for APX activities at day 20 days of stress induction under field experiment (Appendix 8). However, the main effect of stress condition was highly significant (P<0.001) for APX activities (Appendix 8). Figure 5.27 depicts that the APX activity was substantially (P<0.05) high among stressed plants (1.64 U g^{-1} FW) than the control plants (0.6 U g^{-1} FW) independent of plant genotype and stage of development in the field experiment (Figure 5.27).

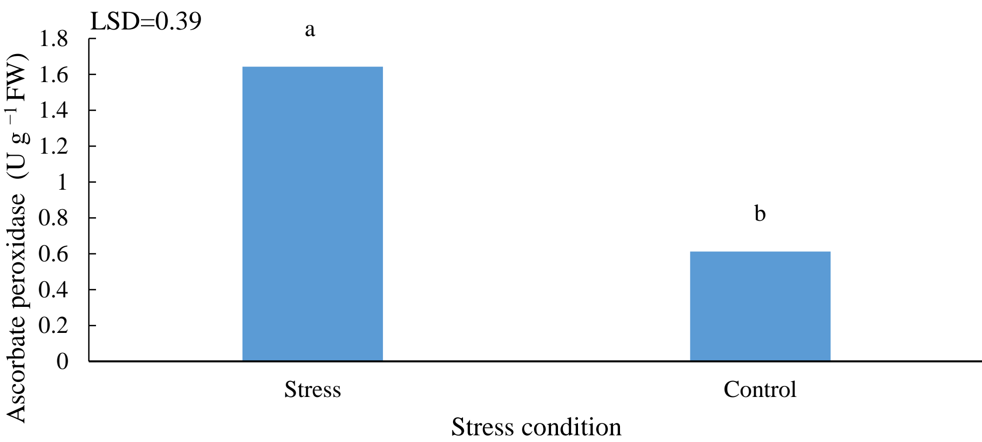

Figure 5.27. The main effect of stress condition on the APX unit of safflower stressed for 20 days under field experiment.

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

There was a highly significant (P<0.001) interaction of stress condition, stage of development, and genotype for APX activities at day 30 of stress induction under the greenhouse and field experiment (Appendix 8). With respect to the greenhouse experiment at day 30 of stress induction, stressed plants of the genotype Kenya9819 at the rosette stage had significantly (P<0.05) high content of APX (2.88 U g^{-1} FW) than other genotypes at any stage of development and condition of stress (Figure 5.28A). The second highest content of APX (1.53 U g^{-1} FW) was observed among stressed plants of the genotype Turkey at the branching stage but it was similar to stressed plants of the genotype Sina at the rosette stages (Figure 5.28A). Generally, stressed plants had greater APX content than control plants of all genotypes and developmental stages at 30 days of water stress (Figure 5.28A).

With respect to the field experiment, stressed plants of the genotype Sina at the rosette stage had significantly (P<0.05) higher content of APX (3.00 U g^{-1} FW) than other genotypes at any stress condition and stage of development (Figure 5.28B). The APX contents of stressed plants of the

genotypes PI537636 and Turkey at branching and rosette stages, respectively was markedly (P<0.05) greater than that of stressed or control plants of all genotypes and developmental stages except for stressed plants of the genotype Sina at the rosette stage (Figure 5.28B).

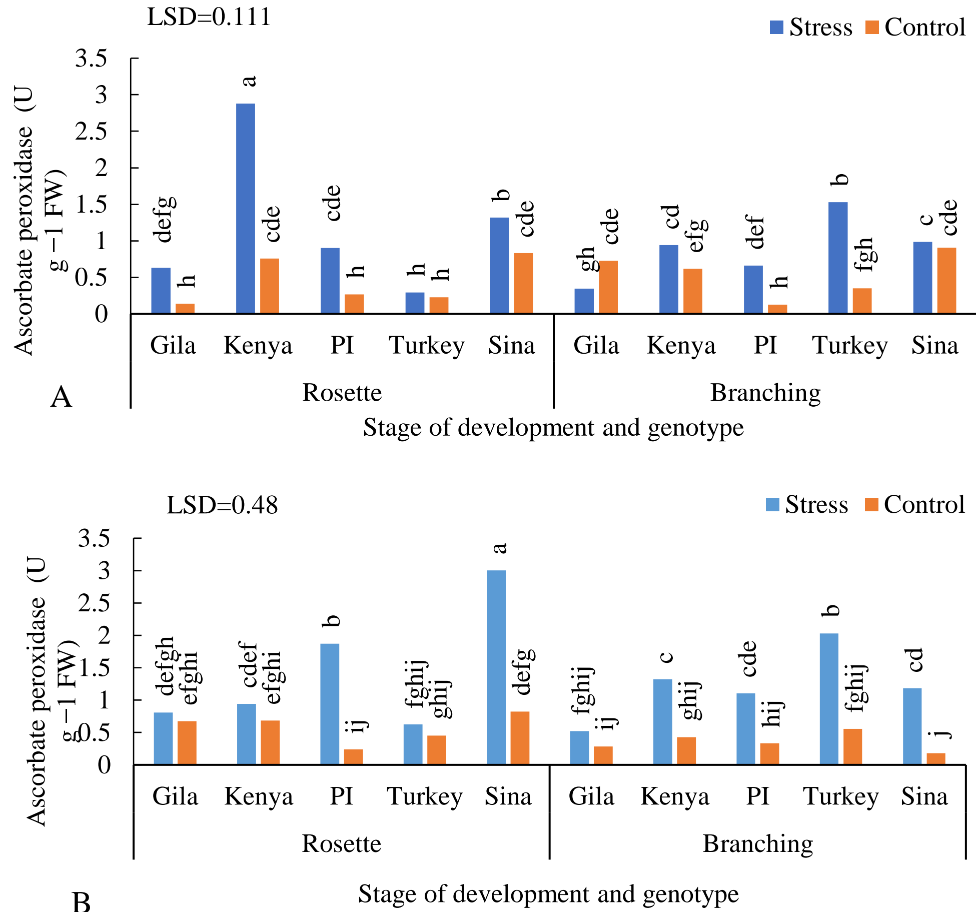

Figure 5.28. The interaction of stress condition, stage of development, and genotype on the APX unit of safflower stressed for 30 days under greenhouse (A) and field experiment (B).

Means followed by dissimilar letters are significant at P=0.05 according to Fisher LSD.

In the greenhouse experiment at the rosette stage, increase in water stress duration significantly (P<0.05) enhanced APX level of stressed plants in comparison to control plants (Figure 5.29A). The APX level increased by 227, 137, and 173% after 10, 20, and 30 days of stress imposition,

respectively compared to control plants at the rosette stage in the greenhouse experiment (Figure 5.29A).

In the field experiment at the rosette stage, increase in drought stress duration significantly ($P<0.05$) increased APX content in stressed plants compared to control plants, the increase in APX was quadratic (Figure 5.29B). Similarly, in the greenhouse experiment at the branching stage, increase in drought stress duration remarkably increased APX content in stressed plants compared to control plants, the increase in APX was quadratic (Figure 5.29C). Similar results were observed in the field experiment at the branching stage except the response to increase in drought stress duration was linear with respect to APX content (Figure 5.29D).

Drought stress increased the activities of APX regardless of stress duration during flowering stage under greenhouse experiment (Figure 5.29E). In this regard, drought stress increased the APX content 1.03 and 15.6x higher in stressed plants after 10 and 20 days of drought stress, respectively than control plants (Figure 5.29E).

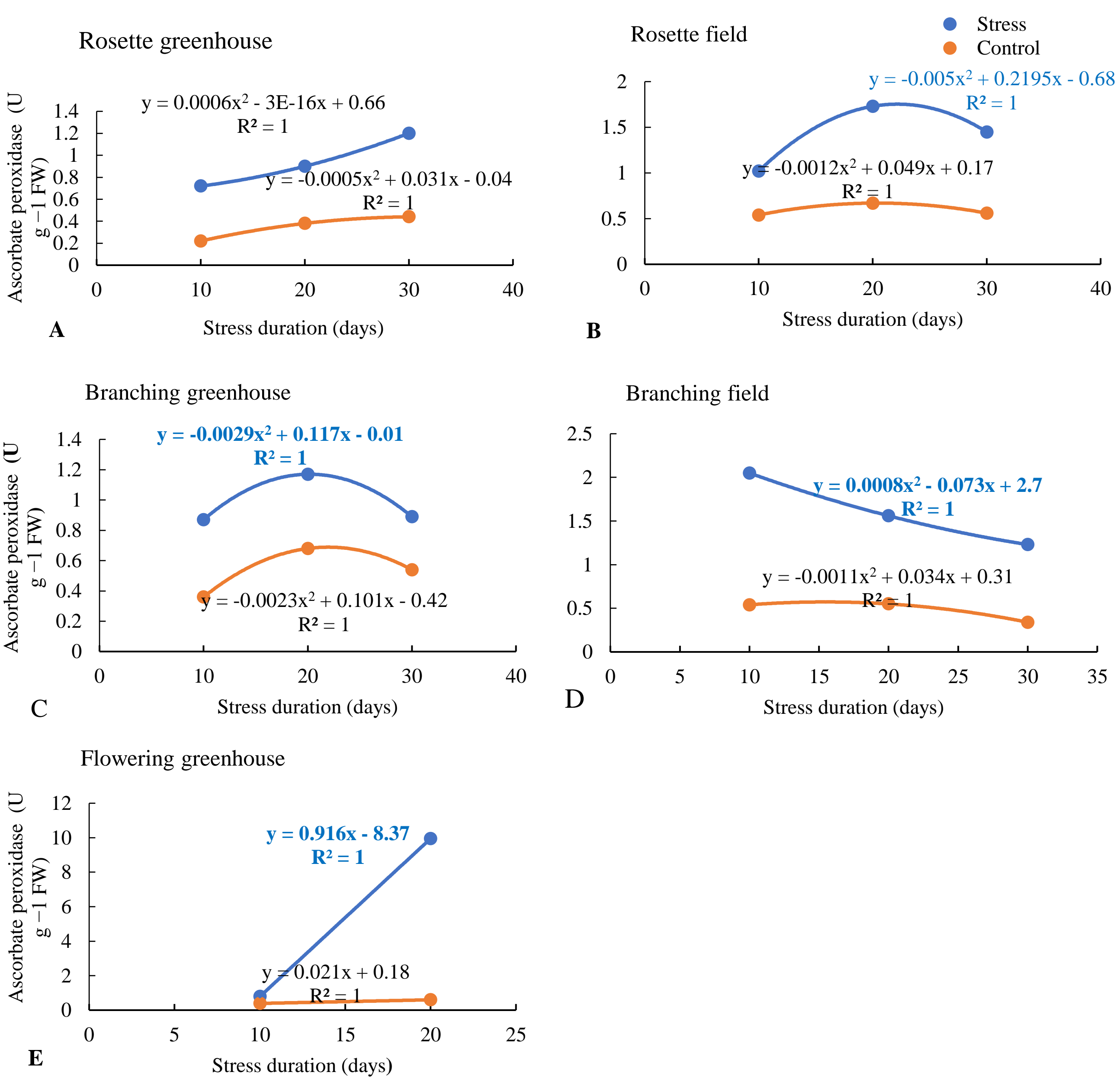

Figure 5.29. The effect of stress duration on the APX unit of safflower stressed at different phenological stages (rosette greenhouse (A), rosette field (B), branching greenhouse (C), branching field (D), flowering greenhouse (E)).

5.3.6 The associations among traits and the trait profiles of the genotypes

The genotype by trait (GT) biplots presented in figures 5.30-5.32 are trait means of five safflower genotypes drought stressed for 30 days at different phenological stages under greenhouse and field

conditions. Notably, only stressed plants were used. The Pearson correlation (r) between any two traits was estimated by the cosine of the angle between the vectors of the traits. In this regard, vectors of two traits with acute angle represent positive correlation, those of obtuse angle represent negative correlation, while those of right angle indicate the absence of correlation. The angle between a genotype and a trait designates the approximate content of the genotype for the trait. Therefore, an acute angle designates that the genotype is above average for the trait; an obtuse angle designates that the genotype is below average for the trait while a right angle designates that the genotype is average for the trait.

5.3.6.1 Rosette greenhouse

Figure 5.30A displays that proline and APX correlated positively with each other and they both correlated negatively with chlorophyll content. Therefore, genotype Kenya9819 which had above-average plant height, proline and APX, had below-average chlorophyll content and LRWC. Additionally, genotype PI537636 which accumulated above-average LRWC, proline, and APX had below-average chlorophyll content and plant height. Furthermore, chlorophyll content correlated positively with plant height. Thus, genotypes Turkey and Kenya9819 which had the tallest plant height also had above-average chlorophyll content. Moreover, LRWC correlated negatively with chlorophyll content. Hence, genotype Sina which had above-average LRWC had below-average chlorophyll content (Figure 5.30A).

6.3.6.2 Rosette field

Chlorophyll content was negatively correlated with plant height and APX (Figure 5.30B). The genotype Gila which had high chlorophyll and proline content accumulated below-average APX

and had short plants (Figure 5.30B). Analogously, genotypes Sina and PI537636 which had above-average APX content and tall plants but accumulated below-average proline and chlorophyll content (Figure 5.30B). Genotype Kenya9819 accumulated above-average proline, LRWC, and APX but it had average plant height and chlorophyll content (Figure 5.30B).

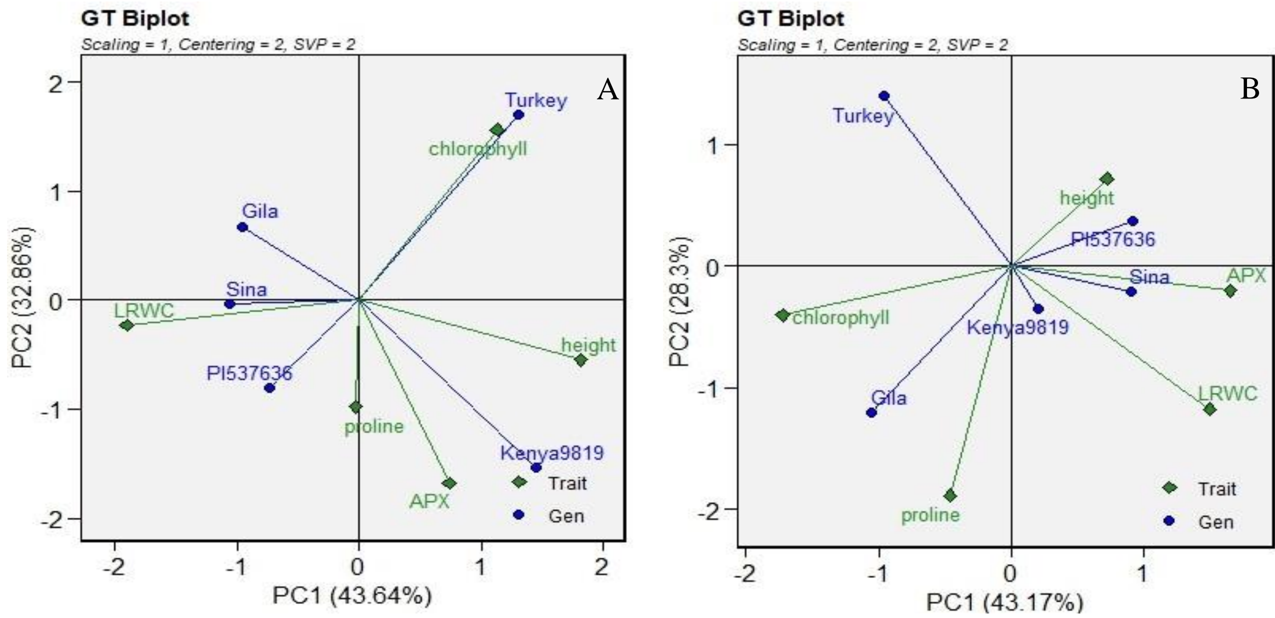

Figure 5.29. GT biplot showing associations among drought traits during the rosette stages under greenhouse (A) and field experiment (B).

5.3.6.3 Branching greenhouse experiment

Figure 5.31A depicts that proline and APX correlated positively with each other, and they both correlated negatively with plant height, chlorophyll content and LRWC. Therefore, genotypes Turkey and Kenya9819 that accumulated above-average proline and APX had below-average chlorophyll content, LRWC, and plant height (Figure 5.31A). Although genotypes Sina and Gila accumulated above-average proline content, they had below-average APX, LRWC, and

chlorophyll content. As for PI537636, it had the highest plant height, chlorophyll content, and LRWC and it accumulated below-average APX and proline (Figure 5.31A).

5.3.6.4 Branching field

Ascorbate peroxidase correlated negatively with all traits except for plant height (Figure 5.31B). Further, genotypes PI537636 and Turkey which had above-average APX, and plant height had below-average chlorophyll content, proline, and LRWC (Figure 5.31B). Genotype Kenya9819 had high LRWC, chlorophyll content, and plant height and it accumulated below-average APX and proline (Figure 5.31). On the other hand, genotype Sina had below-average plant height, chlorophyll content, and LRWC and it had average APX and above-average proline content (Figure 5.31B). Moreover, genotype Gila accumulated above-average proline and chlorophyll content and below-average plant height, and APX (Figure 5.31B).

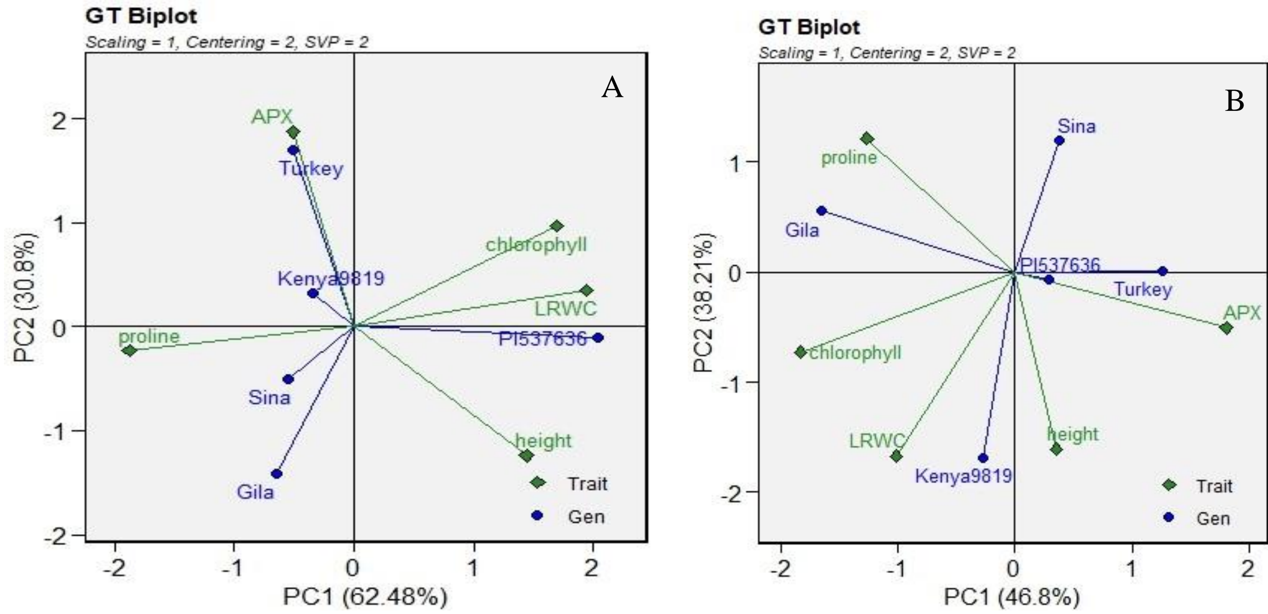

Figure 5.30. GT biplot showing associations among drought traits during the branching stages under greenhouse (A) and field experiment (B)

5.3.6.5 Flowering greenhouse

Chlorophyll content and LRWC correlated positively with each other, and they correlated negatively with other studied traits (Figure 5.32). Therefore, genotypes Sina and Kenya9819 that had above-average chlorophyll contents and LRWC had below-average proline, APX, and plant height (Figure 5.32). Furthermore, APX and proline correlated positively with each other, and they both correlated negatively with chlorophyll content and LRWC (Figure 5.32). Similarly, genotypes Gila and PI537636 accumulated above-average proline and APX and had below-average LRWC and chlorophyll content (Figure 5.32). On the other hand, genotype Turkey had above-average APX, LRWC, and plant height but accumulated below-average proline and chlorophyll content (Figure 5.32).

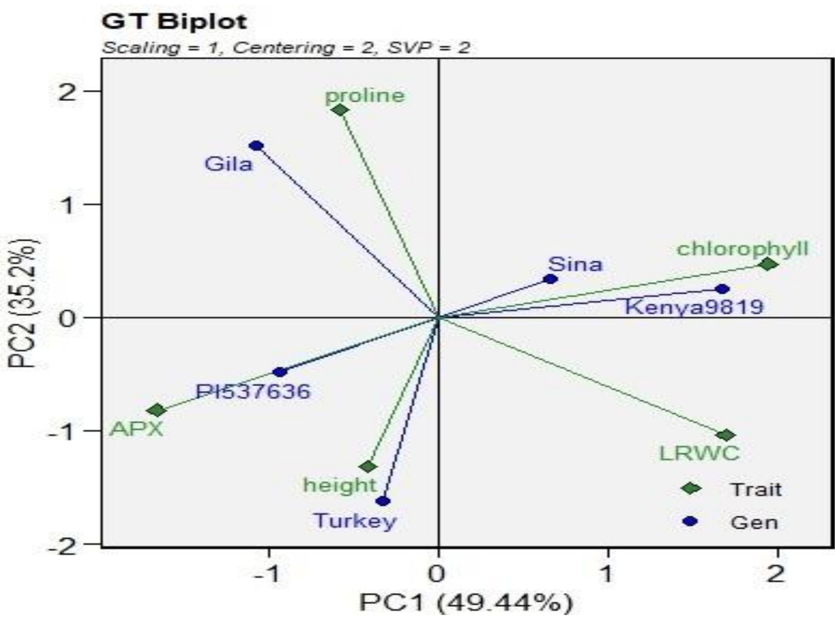

Figure 5.31. GT biplot showing associations among drought traits during the flowering stage under greenhouse.

5.3.7 Superiority rank of the genotypes based on their trait profiles

An ideal genotype is denoted by a small circle with an arrow pointing to it and has a superior trait profile overall. A superior genotype is located closest to the ideal genotype. Figure 5.33 ranks genotypes based on their overall superiority, and it shows that during the rosette at the greenhouse, genotype Kenya9819 was considered as the best genotype followed by genotype PI537636 as they were very close to the ideal genotype (5.33A). The genotypes are ranked as follows; Kenya9819 > PI537636 > Sina > Turkey > Gila (Figure 5.33A). Additionally, during the rosette stage under field conditions, genotype Kenya9819 was the best while Turkey and Gila ranked poorly (5.33B). Generally, genotypes ranked as follows Kenya9819 > Sina > PI537636 > Gila >Turkey during the rosette stage under field conditions (Figure 5.33B). During the branching stage under greenhouse experiment, genotype PI537636 ranked first followed by Kenya9819 (Figure 5.33C). Overall, genotypes ranked as follows during the branching stage under the greenhouse experiment; PI537636 > Kenya9819 > Turkey > Sina > Gila (Figure 5.33C). On the other hand, Kenya9819 showed the best performance followed by PI537636 under field experiment during the branching stage (Figure 5.33D). During the branching stage under field experiment, genotypes ranked as follows Kenya9819 > PI537636 > Gila > Turkey > Sina (Figure 5.33D). With respect to the flowering stage under the greenhouse experiment, genotype Sina outperformed other genotypes followed by Kenya9819 (Figure 5.33E). Generally, ranking of genotypes was as follows; Sina > Kenya9819 > Turkey > PI537636 > Gila during the flowering stage (Figure 5.33E). Notably, genotype Kenya9819 outperformed other genotypes when stressed at different phenological stages under greenhouse and field conditions. On the contrary, overall, genotype Gila performed poorly when compared with other genotypes irrespective of the phenological stage under greenhouse and field experiments.

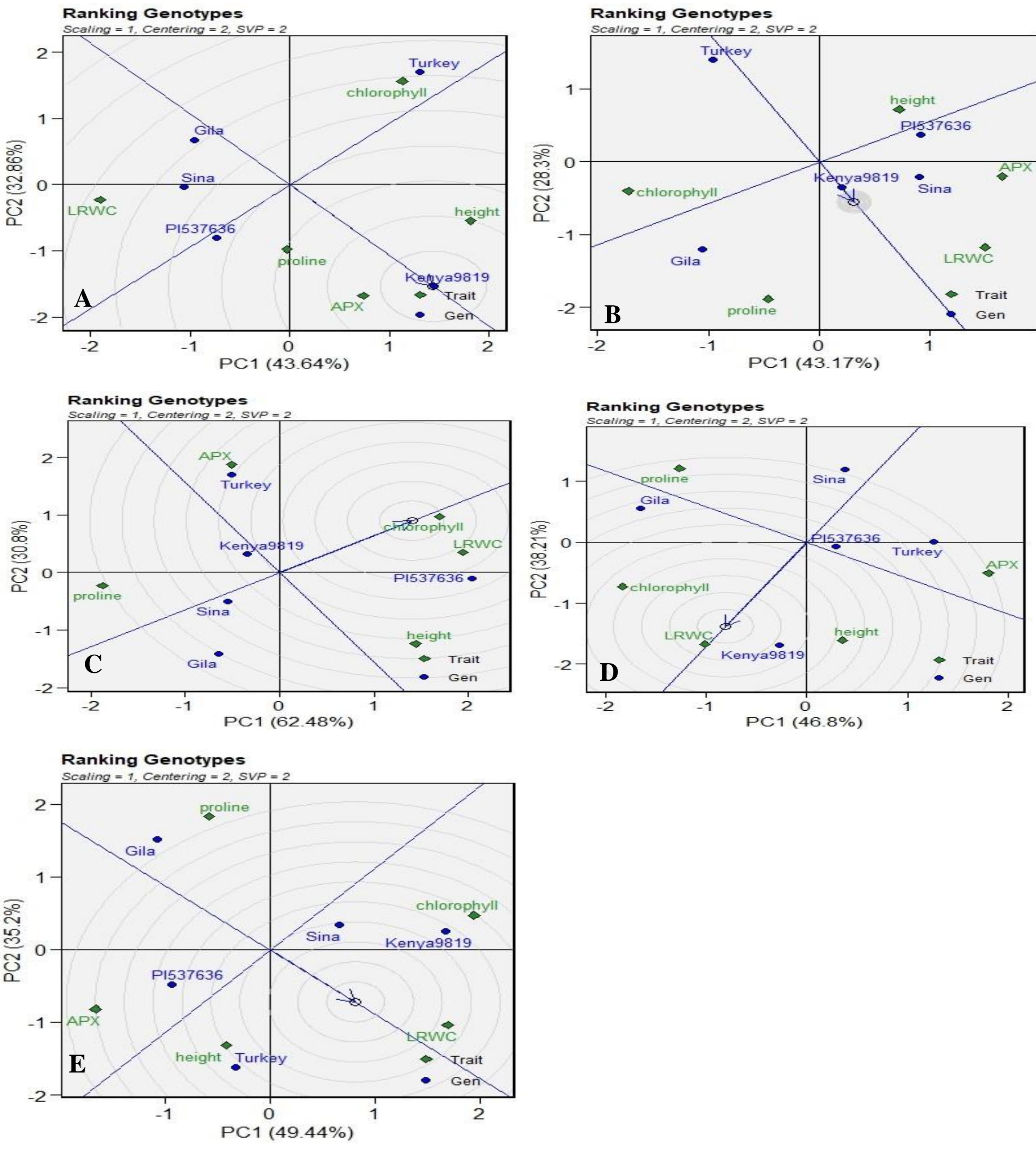

Figure 5.32. GT biplot ranking genotypes based on overall superiority.

Safflower genotypes stressed for 30 days at a) rosette greenhouse; b) rosette field; c) branching greenhouse; d) branching field; and stressed for 20 days at e) flowering greenhouse.

5.4 Discussion

The interaction of genotype and stress condition, genotype and stage of development, and stress condition and stage of development interacted significantly to influence chlorophyll content of safflower in the greenhouse experiment. While in the field study the main effects of stress condition, duration of drought stress, phenological development, and genotypes significantly influenced leaf chlorophyll content of safflower. Drought stress duration caused a significant reduction in the chlorophyll content of safflower genotypes at different stages of growth and development in the current study. As the duration of drought stress increased leaf chlorophyll content of safflower reduced. This demonstrated that the chlorophyll content of safflower was greatly influenced by genetic factors, stage of development and environmental factors. Several studies have stated that drought stress decreased leaf chlorophyll content of safflower (Thippeswamy et al., 2013; Canavar et al., 2014; Mohammadi et al., 2016; Bortolheiro & Silva 2017; Farooq et al., 2020; Chavoushi et al., 2020; Joshi et al., 2021). Amini et al. (2013) stated that drought stress diminished leaf chlorophyll content of safflower with the rate of decrease varying among the genotypes suggesting that some genotypes showed a high degree of drought tolerance. In the current study, drought stress decreased safflower leaf chlorophyll content differently among genotypes. Drought stress reduced leaf chlorophyll content from plants genotypes Kenya9819 (13.7%) and Turkey (14.8%) compared to plants of genotypes Gila (30.3%) and Sina (32.0%) 30 days after withholding water in the greenhouse. Furthermore, on day 30 of withholding water in the greenhouse experiment, water stress was not reduced by leaf chlorophyll content of plants of the genotype PI537636. Joshi et al. (2021) working with 24 genotypes of safflower reported genotypic differences in response to water stress. Similarly, Thippeswamy et al. (2013) found that the chlorophyll content of safflower cultivars decreased with increasing water

stress compared to control plants, but the percent decrease was higher in cultivar Nira as compared to A1 cultivar. Likewise, other studies found a significant genotypic variation in the chlorophyll content of drought-stressed safflower genotypes (Amini et al., 2013; Çulha Erdal et al., 2021; de Almeida Silva et al., 2023). The findings of the present study revealed that an increase in water stress duration significantly reduced leaf chlorophyll content of safflower at various stages of development. Also, there was significant genotypic variation for chlorophyll content at various stages of safflower growth. Drought tolerance is under the control of polymorphic genes and environmental factors (Pinto et al., 2010; Jegadeeswaran et al., 2021; Poodineh et al., 2021).

Stress condition and stage of development interacted to influence leaf chlorophyll content. At the flowering stage (12.1 SPAD reading) stressed plants exhibited a significantly lower chlorophyll content than corresponding plants in either rosette (51.06 SPAD reading) or branching (32.75 SPAD reading) stages after 20 days of water stress induction in the greenhouse. The same trend existed in other drought stress durations in greenhouse and field experiments. This was partially attributed to insufficient soil moisture which was needed for nutrients uptake from the soil. According to Nouri et al. (2023), drought stress decreased absorption of nutrients such as N and Fe, which performs an important function in the structure and biosynthesis of chloroplasts resulting in a decrease in leaf chlorophyll content. In safflower, the flowering stage is the most significant phenological stage (Quiroga et al., 2001; Zareie et al., 2013; Emongor & Emongor, 2023). Many studies revealed that the highest damage on grain and oil yield, and major yield components of safflower was caused when drought stress takes place at the flowering stage (Saini & Westgate, 1999; Abolhasani & Saeidi, 2006; Lovelli et al., 2007; Yau, 2007; Farooq et al., 2009;

Istanbulluoglu et al., 2009; Koutroubas et al., 2009; Abd El-Lattief, 2013; Zareie et al., 2013; Mostafaie et al., 2014; Alizadeh et al., 2020; Poodineh et al., 2021).

Significant variation in leaf chlorophyll content was observed during the branching and flowering stages with the stressed plants exhibiting low chlorophyll content. This suggested that as the plants transition from the vegetative stage to the reproductive stage they need more water to support reproductive growth, and this simultaneously hastens depletion of available water. This was more evident during the flowering stage as stressed plants senesced and died before reaching day 30 in the current study. This demonstrated that drought stress interrupted the chlorophyll synthesis of stressed plants. The destruction of chlorophyll content in drought-stressed plants was reported to be a result of overproduction of ROS which caused lipid peroxidation and consequently led to significant chlorophyll degradation (Atta et al., 2020; Amir et al., 2021). Marcińska et al. (2013) emphasized that a reduction in chlorophyll content with drought stress implies that there is minimal capacity for light harvesting. The rate of chlorophyll reduction due to drought stress was very high under greenhouse as opposed to field conditions in the current study. Safflower is known to have a deeper root system which helps to scavenge water from deeper soil layers (Singh & Nimbkar, 2006; Emongor, 2010; Bhattarai et al., 2020). Therefore, planting in pots might have restricted root growth due to shallow soil depth. Additionally, Bayati et al. (2022) emphasized that a reduction in the chlorophyll content of plants undergoing drought stress is a common sign of oxidative stress which can be caused by pigment photooxidation and chlorophyll degradation. Similarly, Hussain et al. (2019), revealed that water deficit conditions significantly reduced the chlorophyll contents of safflower because of enhanced oxidative stress and deterioration or photooxidation of the chlorophyll pigments. Yang et al. (2021) contended that drought stress

makes it hard for plants to absorb nutrient resulting in symptoms of nutrients deficiency which is also displayed as decreased chlorophyll content.

The interaction of drought stress condition and stage of development in the current study additionally showed that at day 30 of withholding water during the rosette stage, high chlorophyll content was found on the stressed plants as compared with the control. However, the opposite was true during the branching stage where stressed plants had the lowest chlorophyll content. This suggested that developmental stage influenced the adaptive ability of safflower to cope with drought stress with regards to chlorophyll synthesis. Monteoliva et al. (2021), highlighted that a higher chlorophyll content under stress in plants could be a result of mechanisms such as the initiation of the antioxidant system. Similarly, Canavar et al. (2014) revealed that during the vegetative stage, higher chlorophyll content was observed in drought-stressed safflower than in non-stressed safflower. Additionally, Bortolheiro and Silva (2017), found that safflower line IMA 14 had an increased amount of total chlorophyll under water deficit conditions during the vegetative stage, thereby being considered stress tolerant. Zhang et al. (2020), Monteoliva et al. (2021) and Yang et al. (2021), contended that not all plants under drought reduce their chlorophyll content and that their ability to maintain chlorophyll content may indicate higher drought tolerance but this varies with the plant genotype, stress duration and intensity. The increment in chlorophyll content with drought stress duration was only observed during the rosette stage which may imply that safflower was more tolerant to drought at the rosette stage as opposed to the branching and flowering stages. It is also worth noting that these leaves were rolling and were dark green in colour showing signs of stress while those of the control looked shiny and bright green in colour.

With regards to the field experiment the main effect of stage of development at day 10, 20, and 30 of withholding water influenced the chlorophyll content. This highlighted that the age of the plant influenced the chlorophyll content. For example, the results showed that chlorophyll content was higher during the branching stage than the rosette stage and this was also true at day 30 under greenhouse experiment. Literature has shown that leaf chlorophyll content changes with plant age partly due to changes in leaf N, maturity, and senescence (Li et al., 2022). Zdunek-Zastocka et al. (2021) found that chlorophyll content decreased with plant age in field potatoes.

Generally, leaf chlorophyll content is an essential factor in determining the photosynthetic rate and dry matter accumulation in plants (Chaves, 1991; Yordanov et al., 2000; Wahid & Rasul, 2005; Farooq et al., 2009; Amini et al., 2013). However, water stress is reported to cause decrease in chlorophyll content (mainly a and b) and triggering the breakdown of the photosynthetic apparatus in safflower and other plants (Chaves 1991; Yordanov et al. 2000; Farooq et al., 2009; Hussain et al., 2016; Joshi et al., 2021). Thus, other studies hypothesized that although safflower is drought tolerant, it responds very well to supplementary irrigation (Kocaman et al., 2016; Parameshnaik et al., 2022).

Plant height is one of the noticeable symptoms of water deficit during the vegetative period (Yang et al., 2021). Results revealed that there was a substantial interaction of stage of development and genotype for plant height at day 10 of withholding water under the greenhouse and field experiment. This implied that plant height of safflower genotypes varied with stage of development. The results further showed that plant height increased with increase in the phenology of safflower, with the rosette stage having significantly shorter plants than plants at branching or

flowering stages depending on genotype. For example, in the greenhouse experiment, plants of the genotype Turkey at flowering stage had significantly the tallest plants (110.5 cm) than plants of other genotypes at any phenological stage. While in the field experiment, plants of the genotypes Sina (64 cm) and Kenya9819 (58 cm) at the branching stage were taller than plants of the genotype Gila (45 cm). Other researchers have found significant genotypic variation in plant height at the vegetative stages of safflower (Eslam, 2011; Kazemeini et al., 2015; Aeini et al., 2018; Moatshe, 2019). Plant height is a morphological trait under the influence of additive genes (Shahbazi & Saeidi, 2007; Farooq et al., 2009; Golkar et al., 2012; La Bella et al., 2019; Moatshe, 2019; Kolanyane, 2022; Korononeo, 2023), but it is also influenced by environmental and cultural factors, and stage of development (Zareie et al., 2013; Killi et al., 2016; La Bella et al., 2019). Plant height is not influenced by extra-nuclear genes (Mandal & Banerjee, 1997; Golkar, 2014). Therefore, cyclic selection should be effective for improvement of safflower plant height.

The main effect of stress condition was significant for plant height under both greenhouse and field experiment at day 30 of water stress induction independent of stage of development and genotype in the current study. Drought stress after 30 days significantly reduced plant height of stressed safflower plants by 31.9 and 25.5% in the field and greenhouse experiments (rosette stage), respectively compared to control plants. Furthermore, as drought stress duration increased the degree of plant height reduction increased. This showed that even though safflower is known to be drought tolerant, exposing it to drought stress conditions for an extended period (30 days), retards its growth. Generally, growth is achieved through cell division and enlargement, and differentiation, which comprises genetic, ecological, physiological, and morphological processes and their interactions. However, cell growth is one of the most drought-sensitive physiological

processes because of the decrease in turgor pressure (Taiz & Zeiger, 2006; Farooq et al., 2009). During severe water stress, cell elongation of plants is hindered by disruption of water flow from the xylem to the elongating cells (Nonami, 1998; Taiz & Zeiger, 2006; Farooq et al., 2009). Therefore, the decline in plant height of safflower in this study could be attributed to disruption of cell division and elongation in the meristematic tissues of safflower shoots caused by water stress. Nonami (1998), Kaya et al. (2006), and Hussain et al. (2008) revealed that diminished mitosis, cell elongation, and expansion resulted in reduced plant height, leaf area and crop growth under drought stress conditions. The reduced safflower plant height because of drought stress in the current study may also be due to decreased CO_2 assimilation, tissue membrane damage, and inhibition of enzymes activity, increased leaf shedding, and alterations in plant water relations (Yang et al., 2021; Atta et al., 2022). While Manvelian et al. (2021) indicated that water stress decreased safflower plant height due to low synthesis of photoassimilates caused by limited access of plants to water, and CO_2 resulting low partitioning of photoassimilates to plants' growing parts. Several other researchers have shown that drought stress led to a decline in safflower plant height (Tayebi et al., 2012; Canavar et al., 2014; Salem et al., 2014; Kazemeini et al., 2015; Aeini et al., 2018; Joshan et al., 2019; Esmaeilzadeh et al. 2022). Likewise, Parameshnaik et al. (2022) found improvement in plant height of safflower as a result of scheduled irrigation at different phenological stages as it contributed to adequate soil moisture availability throughout the growth period.

Drought is an abiotic factor that threatens world food security. The severity of drought cannot be predicted because it is influenced on many factors such as occurrence and rainfall distribution, evaporative demands, and moisture holding capacity of soils (Wery et al., 1993; Hussain et al.,

2016). Leaf relative water content, water potential, and temperature, stomatal resistance, rate of transpiration, and canopy temperature are important variables that affect plant water relations. In the current study a significant interaction of drought stress condition and stage of development on LRWC at day 10 and 20 of withholding water under the greenhouse and at day 10 and 30 under field experiment was observed. Drought stress significantly reduced LRWC of safflower plants dependent on the stage of development and duration of drought stress. As the duration of the drought stress increased from 10-30 days in each development stage degree of reduction of LRWC increased and by day 30 in the greenhouse experiment all plants had died. The reduction in LRWC was mainly due to depletion of available water that occurred faster during branching and flowering stages as compared to the rosette stage (appendix 16). The results also showed that the flowering stage of safflower was highly sensitive to drought stress mostly because of the high-water requirement to prevent flower abortion and for effective seed filling process as compared with the earlier stages of growth. Furthermore, the results showed that most stressed plants were wilted at day 20 and they died before reaching day 30. This demonstrated how severity of drought stress affected safflower plants and that the duration of stress and soil moisture availability determines LRWC of safflower plants. Leaf relative water content in safflower decreased significantly with drought stress at the flowering stage (Eslam, 2011; Hojati et al., 2011; Bortolheiro & Silva, 2017; Ghassemi-golezani & Afkhami, 2018; Bijanzadeh et al., 2022; Esmaeilzadeh et al., 2022; de Almeida Silva et al., 2023). Mosupiemang et al. (2022a) highlighted that although LRWC of safflower was reduced by drought stress at rosette and branching stages, the effect was more noticeable at the flowering stage. Vegetative and reproductive stages of safflower are known to be sensitive to water stress (Koutroubas et al., 2009; Bijanzadeh et al., 2013; 2022). Water stress has also been revealed to reduce LRWC in other crops such as rice (Farooq et al., 2009), wheat

(Siddique et al., 2001; Abbate et al., 2004), chickpea (Nayyar et al., 2006), snap bean (Omae et al., 2007), and maize (Németh et al., 2002). Nakanwagi et al. (2020) found that crop moisture requirements increase with growth stage because the amount of water required at seedling stage was less than that of subsequent stages.

There was a significant interaction of stress condition × stage of development × genotype for LRWC at day 30 of stress imposition under the greenhouse experiment in the current study. After 30 days of water stress, drought stress significantly reduced LRWC of safflower plants depending on genotype and stage of development. The lowest and highest LRWC of 11.0 and 56.8% was recorded in stressed plants of genotypes Gila and PI537636, respectively during the branching stage of safflower growth and development. The interaction of stress condition x stage of development x genotype on LRWC suggests that genetic and environmental factors control the LRWC of safflower. This also revealed that as plants advance in age and when exposed to prolonged stress duration genetic variability for LRWC was induced hence delineating the differences in genetic adaptability and sensitivity of genotypes to drought stress. This arguably emphasizes the importance of stress duration in the selection of stress-tolerant safflower genotypes. Thus, stressed genotypes at the rosette stage and PI537636 at the branching stage showed better genetic adaptability and less sensitivity to drought stress than other genotypes. This is because drought tolerant genotypes tend to maintain higher LRWC than sensitive ones.

Also, in the current study there was significant genotypic variation for LRWC at day 10 of stress imposition under the field experiment. Genotypes Sina (78.17%) and PI537636 (78.90%) had markedly higher LRWC than genotype Kenya9819 (74.04%). Genotypic variation in the decrease LRWC of safflower due to drought stress has been stated in literature (Hojati et al., 2011; Eslam,

2011; Nikzad et al., 2013; Canavar et al., 2014; Bortolheiro & Silva, 2017; Esmaeilzadeh et al., 2022). Hojati et al. (2011) reported that LRWC of safflower varieties substantially declined in response to drought stress and the rate of decrease was high under severe drought stress conditions. While, Roudbari et al. (2012) reported that drought stress led to marked decrease in LRWC irrespective of safflower genotype. Genotypic variation in LRWC could be due to variations in the capacity of the genotypes to absorb water (Roudbari et al., 2012). The findings of Roudbari et al. (2012) further showed that safflower genotypes which had high LRWC had the lowest yield loss, longer stomata, and high leaf area index (LAI) compared with drought sensitive genotypes. During seed filling stage in five safflower genotypes, drought stress imposed catastrophic ramifications in LRWC, stomatal conductance, leaf temperature, osmotic adjustment, and leaf weight (Eslam, 2011). The results of Roudbari et al. (2012), Eslam (2011) and those of the current study suggest that LRWC can be used to distinguish between drought tolerant and sensitive genotypes of safflower. While Keyvan (2010) highlighted that variations in LRWC of plants of wheat genotypes that were under drought stress was due to variations in the ability of the genotypes to absorb water from soil or ability of stomata to reduce water loss. On the contrary, Bortolheiro and Silva (2017) found a non-significant genotypic effect for LRWC in safflower after 30 days of stress induction.

Drought comprises of various stresses that weakens the morphological, phenological, physiological, biochemical, and molecular functioning of plants and finally impact crop growth and production (Yordanov et al., 2000; Wang et al., 2003; Bartels & Sunkar, 2005; Hussain et al., 2016; Song et al., 2023). Drought escape, solute and antioxidant accumulation, photosynthesis, and changes in phytohormone composition are some of the strategies used by plants to resist water deficit conditions (Farooq et al., 2009; Hussain et al, 2016; Esmaeilzadeh et al., 2022;

Mosupiemang et al., 2022a; Song et al., 2023). Free proline content has been revealed to surge in plants including safflower as water supply decreases (Alian et al., 2000; Zhang et al. 2006; Hussain et al., 2016; Mohammadi et al., 2016; Esmaeilzadeh et al., 2022). Proline is an antioxidant that works as a molecular chaperon that helps plants to maintain protein integrity and increase the functioning of different enzymes under water deficit conditions (Rajendrakumar et al., 1997; Verbruggen & Hermans, 2008; Farooq et al., 2009; Hayat et al., 2012; Hussain et al., 2016; Mohammadi et al., 2016; Banerjee & Roychoudhury, 2018; Yang et al., 2021; Mosupiemang et al., 2022a). Several studies highlighted that proline is a ROS scavenger and singlet oxygen quencher (Smirnoff et al., 1989; Alia & Mohanty, 2001; Matysik & Cumbes, 2002; Farooq et al., 2009; Wang et al., 2009; Hussain et al., 2016; Banerjee & Roychoudhury, 2018). There was a statistical interaction of drought stress condition, stage of development, and genotype on proline content at day 10, 20, and 30 of stress induction under the greenhouse experiment and at day 20 under field experiment. This interaction of stress condition, stage of safflower growth and development, and genotypes showed the multidimensional factors involved in plants adaptation to water stress. The duration of the drought stress increased proline content in safflower plants in comparison to control plants depending on genotype and phenological stage. For example, safflower plants of the genotype Sina stressed for 30 days at the flowering stage recorded the highest proline content of 228.47 µmoles/ g FW which was significantly higher than proline contents of plants of other genotypes at any stage of development and stress condition exception for stressed plants of the genotype Gila at the same stage in the greenhouse experiment. Stressed plants of all genotypes had significantly higher proline content than control plants of all genotypes in different developmental stages after 30 days of water stress in the greenhouse experiment. The results indicated that genetic, environmental, and phenological factors take part in the biosynthesis

of proline in water stressed safflower plants. Safflower genotypes tolerant to abiotic stresses such as drought and excessive salinity have been reported to show elevated proline concentrations (Ahmad et al., 2012; Hussain et al., 2016; Chavoushi et al., 2019). Mohammadi et al. (2016) in Iran found that the highest leaf proline content of all four safflower genotypes (Faraman, Goldasht, Sina, and Soffeh) was recorded under severe drought stress conditions. The proline level of the Sina was less than that of other genotypes under all irrigation regimes (Mohammadi et al., 2016). The genotype Goldasht had the highest proline content under the least water availability, followed by Soffeh and Faraman (Mohammadi et al., 2016). Sajedi et al. (2012) reported that safflower genotypes Esfahan native and Esfahan-14 had significantly higher proline content than genotypes PI537598 (Sina) and IL111 under water deficit growing conditions, which described their drought stress tolerance. Sajedi et al. (2012) further found proline and two enzymes [P5C reductase(P5CR) and P5C synthetase (P5CS)] involved in the proline biosynthetic pathway in two safflower genotypes A1 (drought tolerant) and Nira (drought sensitive). The drought tolerant variety A1 was characterized by higher proline content and increased activity of P5CS synthetase than Nira. Implying that drought stress increases the activity of the enzyme P5CS which catalyzes the conversion of glutamatic acid to Δ'-pyrroline-5-carboxylate (P5C) an intermediate to proline biosynthesis in plant tissues (Vogel & Davis, 1952; Sekhar et al., 2007; Hayat et al., 2012). Genotypic variation in proline content of safflower is well established (Thippeswamy et al., 2010, 2013, 2021; Javed et al., 2013; Çulha et al., 2021). Studies on different plants has revealed that proline level was greater in drought-tolerant genotypes than in drought-sensitive genotypes (Ahmed et al., 2009; Roy et al., 2009; Ahmad et al. 2012; Hassan et al., 2015; Hussain et al., 2016; Mohammadi et al., 2016; Carvalho et al., 2019; Thippeswamy et al., 2013, 2021; Shin et al., 2021). On the contrary, Farooq et al. (2020) found the absence of genotypic variation for proline in

safflower genotypes under water deficit stress. They postulated that proline content had no connection with the genetic nature of the safflower.

Also, in the current study, at day 10 of stress induction, the highest proline content was recorded during the flowering stage as compared to other growth stages of safflower. This was attributed to the low LRWC that was found during the flowering as compared to other growth stages. According to Chiang and Dandekar (1995), plant tissues like rosette leaves that exhibited high water contents accumulated low levels of proline in Arabidopsis. Similarly, Zdunek-Zastocka et al. (2021) found that proline content varied widely with developmental stages and plant age. Moreover, during the flowering stage, the highest proline content was found among stressed plants with exception of genotype Turkey which was not affected by drought stress condition. This highlighted the sensitivity of the flowering stage to drought stress with effects varying with genotype. Therefore, genetic variability for proline content may form part of the basis for selecting drought tolerant genotypes. Khanna-Chopra and Selote (2007) pointed that drought tolerance of a crop is genetically determined but interaction with the environment influenced the expression of the plant traits. Servani et al. (2014) found that the increase in proline content due to drought stress was more severe at the flowering stage than at the vegetative stage of safflower. While Nakanwagi et al. (2020) reported that crop moisture requirements of crops increases with growth stage.

In the current study, drought stress significantly increased proline content of safflower plants with proline content increasing either linearly or quadratically with increase in water stress duration. This revealed the active participation of proline in guarding plant cells against osmotic stress resulting from drought stress. Surge in proline level in drought stressed safflower plants with

increase in drought duration is widely reported (Mohammadi et al., 2016; Amir et al., 2021; Esmaeilzadeh et al., 2022). Mahdavi et al. (2011), highlighted that proline is hydrophilic and eases stress damage in plant cells by reducing the water potential. Exogenous application of proline to plants has been revealed to provide osmoprotection and enhanced the growth of plants experiencing to water stress (Ali et al., 2007; Kamran et al., 2009; Hayat et al., 2012). Exogenous application of proline to maize (*Zea mays*) at the seedling and/or vegetative stage increased growth under water deficient conditions (Ali et al., 2007). While soaking wheat (*Triticum aestivum*) seeds in proline as pre-sowing treatment relieved the undesirable effects caused by drought stress resulting in increased growth and yield (Kamran et al., 2009).

Under drought stress conditions, plants produce very high concentrations of ROS (Farooq et al., 2009; Gill & Tuteja 2010; Hasanuzzaman et al., 2012; Hussain et al., 2016). ROS may react with various cellular molecules (lipids, nucleic acids, proteins, and DNA) and cause irreversible oxidative damage to cells. High concentrations of ROS such as hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals result in oxidation of lipids, proteins, damages in nucleic acids, inhibition of enzymes, and ultimately death of plant cells (Sharma & Dubey 2005; Farooq et al., 2009; Hussain et al., 2016). Survival of plants rely on the balance of ROS and antioxidative defense system (Selote & Khanna-Chopra 2006, 2010; Slabbert & Krüger, 2014). Antioxidant enzymes such as APX, CAT, SOD, GR, and GPX, and non-enzyme antioxidants such as ascorbic acid and reduced glutathione play an important role to reduce deleterious effects of ROS (Farooq et al., 2009; Hasanuzzaman et al., 2012; Hussain et al., 2016; Shin et al., 2021). Ascorbate peroxidase (APX) is widely distributed across cell organelles and it is an efficient scavenger of H_2O_2 during stress (Das & Roychoudhury, 2014). The results showed that the activities of APX

increased significantly in drought stressed safflower plants in both greenhouse and field experiments. There was a significant interaction of drought stress condition, stage of development, and genotype for APX activities at day 10, 20, and 30 of stress induction under both greenhouse and field experiments except at 20 days in the field experiment. The activity of APX in safflower genotypes varied with the stage of development, water stress condition irrespective of duration of the water stress. Additionally, the activity of APX elevated with increase in water stress duration (10, 20, and 30 days) depending on genotype, stage of safflower growth, and development. In the current study the activity of APX was also detected after 10 days of water stress induction in greenhouse and field experiments depending on genotype, stage of development and condition of water stress. This observation suggested that drought stressed safflower plants produced APX earlier to scavenge ROS even before visual symptoms of stress were observed. Slabbert and Krüger (2014) found increased activity of APX in stressed amaranthus plants 7 days earlier than the activities of SOD and GR which occurred 10-12 days later. Moreover, Zhang et al. (2020) reported that the highest level of APX in ten different ground cover seedlings for roof greening were recorded under moderate drought stress, suggesting that APX activities were first triggered under moderate drought stress to scavenge ROS. Additionally, studies on safflower also revealed an increment in APX levels with drought stress (Hojati et al., 2011; Sajedi et al., 2012; Amini et al., 2013; Khosrowshahi et al., 2018; Sadeghi et al., 2021).

In the current study, at day 10 and 30 of water stress induction in the greenhouse experiment, genotype Kenya9819 stressed during the branching stage accumulated significantly higher level of APX (1.67-2.88 U g^{-1} FW) than other genotypes at any stage of development. On the other hand, at day 10 of stress induction in the field experiment, genotype Turkey stressed at the

branching stage accumulated significantly higher level of APX (7.99 U g^{-1} FW) than other genotypes in any stress condition and stage of development. While genotype PI537636 stressed for 20 days during the flowering stage in the greenhouse had significantly higher level of APX (20.89 U g^{-1} FW) than other genotypes in any stress condition and stage of development. With respect to the field experiment at day 30, stressed genotype Sina at the rosette stage had significantly higher level of APX (3.00 U g^{-1} FW) than other genotypes at any stress condition and stage of development. This indicated that these genotypes had different activities for APX depending on the stage of development and stress duration. High levels of APX activities in drought-stressed plants suggested that these genotypes were very competitive in scavenging ROS through APX, which is a good characteristic of drought-tolerant genotypes. Moreover, higher genetic variability for APX among safflower genotypes under water stress suggested that APX can be one of the traits that can be used in selection for drought tolerant safflower genotypes. The results also indicated that safflower genotypes responded differently to oxidative injury, because of their genetic-based variances in their enzyme antioxidant systems. Several studies have revealed that an increase in the activities of APX in drought-tolerant plant genotypes than in sensitive genotypes (Khanna-Chopra & Selote, 2007; Ahmed et al., 2009; Sharma et al., 2012; Zlatev & Lidon, 2012; Naderi et al., 2014; Laxa et al., 2019; Tiwari et al., 2023). APX activities are reported to be induced by drought stress in various plants (Sofo et al., 2005; Mattos & Moretti, 2015; Dar et al., 2017; Hasanuzzaman et al., 2019). While Grant (2012) emphasized that an increase in APX level in the leaves of drought-stressed plants explained why the chloroplasts were sufficiently protected against ROS.

Drought stress has been reported to increase the levels of SOD, GPX, and CAT enzymes in four safflower genotypes (Esfahan, Esfahan 14, PI537598, and IL-111) (Sajedi et al., 2012). The genotype IL-111 had significantly lower activity of SOD, GPX and CAT enzymes than other safflower genotypes resulting low safflower grain yield (Sajedi et al., 2012). These antioxidant enzymes can be used as indices for drought tolerance in plants because they guard the plants from oxidative damage caused by ROS. It seems an increase in APX, SOD, CAT and GPX levels under conditions of water deficit stress could prevent oxidative damage; therefore, an immediate surge in the levels of these enzymes cause a decline of deleterious effects of H_2O_2 during drought stress. Pasternak et al. (2005) reported that H_2O_2 can be removed using the ascorbate-glutathione cycle in which APX and SOD are the key enzymes. Hojati et al. (2011) reported that antioxidant compounds ascorbic acid, α-tocopherol, GSH, SOD, CAT, and POX enhanced under drought stress in two safflower genotypes. While Amini et al. (2013) found positive correlations between antioxidant enzyme activities of CAT, APX, and POX, and seed yield of 64 safflower genotypes grown under drought stress conditions. A statistical genotypic (6 genotypes) variation of increased activities of CAT, APX, and glutathione reductase enzymes in safflower plants grown under water stress conditions which showed the significance of these enzymatic antioxidant mechanisms in drought tolerance of safflower (Javed et al., 2013).

Although the studied traits were all useful in discriminating genotypes for drought tolerance, no single trait was considered the best as they were all equally important. In particular, the GT biplots revealed that plants of genotype Gila under water stress had high proline content at all the phenological stages in the greenhouse and field experiments except at the rosette stage in the greenhouse. However, water stressed plants of Gila had lower levels of the other traits when

compared with other genotypes which showed that it was not competitive. Water stressed plants of the genotype Kenya9819 had low levels of chlorophyll content, plant height, and LRWC but had high levels of APX and proline which was not always the case with other genotypes. The high activity of APX and proline level in drought stressed safflower plants showed their involvement in lessening the adverse effects of drought stress. The results of the current study agree with those reported in literature (Khanna-Chopra & Selote, 2007; Ahmed et al., 2009; Roy et al., 2009; Grant, 2012; Naderi et al., 2014; Hassan et al., 2015; Laxa et al., 2019; Tiwari et al., 2023).

To evaluate the overall superiority of the genotypes for all the studied drought stress traits, the GT methodology was used. This approach is founded on the hypothesis that an ideal genotype should have superior levels for multiple desired traits (Yan & Frégeau-Reid, 2018). Generally, the GT biplots showed that genotype Kenya9819 was superior at all the studied developmental stages under both the greenhouse and field experiment except during the flowering and branching stages under the greenhouse where it was the second best after PI537636 and Sina, respectively. Hence, genotype Kenya9819 was the most drought tolerant when compared with other genotypes. On the other hand, genotype Gila was the least drought tolerant because it ranked poorly at all the studied developmental stages under greenhouse and field experiments although it was second to last at the rosette and branching stages under field conditions. Therefore, genotype Gila was considered drought-sensitive when compared with other genotypes. The GT biplots also revealed that genotypes PI537636, Sina, and Turkey ranked fairly (moderately drought tolerant) depending on the developmental stage and growing condition (greenhouse or field experiment).

5.5 Conclusion and recommendations

The present study showed the differential response of safflower genotypes to drought stress as demonstrated by their chlorophyll content, plant height, LRWC, proline content, and APX activities as stress progressed. In addition, genotypes responded variably to stress when drought stress was induced at different developmental stages. The rosette stage was the most tolerant while the flowering stage was the most drought sensitive. Therefore, to increase the productivity of safflower especially in arid and semi-arid areas supplemental irrigation should be provided and the use of tolerant genotypes should be incorporated into the cropping system to curb the effects of drought stress. The genotype, Kenya9819 was an overall superior / competitive genotype under drought stress conditions and hence, considered drought stress tolerant. On the other hand, genotype Gila ranked poorly in most of the traits and hence, is considered susceptible to drought stress. Finally, studies evaluating the response of safflower genotypes to drought stress can never be enough because of climate change. Moving forward, the selection of drought tolerant safflower genotypes should be conducted at different locations across Botswana and longer drought stress durations where stress severity is high and multiple traits should be used to make more informed choices.

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Chapter 6

General Discussion

Safflower is known to thrive in ASALs (Weiss, 2000; McPherson et al., 2004; Emongor & Emongor, 2023) due to the crop's drought, cold, heat, and salt tolerance characteristics (Bassil & Kaffka, 2002; Emongor & Oagile, 2017). However, its performance under some environments is genotype specific (Hojati et al., 2011; Bassil & Kaffka, 2002; de Oliveira Neto et al., 2022; Emongor & Emongor, 2023). Therefore, the present study evaluated the adaptability of safflower genotypes across various seasons and locations as well as under drought stress conditions. The first study (Chapter 3) was aimed at evaluating safflower genotypes in response to environmental variations (season and location). The results revealed that environment × genotype interaction significantly affected the phenological development of safflower. This demonstrated that the variations in temperature and soil type observed across the locations and seasons had a large influence in moderating genotypic responses regarding days to phenological development. The G×E on phenological traits of safflower have been reported to substantially differ with environmental conditions, especially temperature and rainfall (Arshad et al., 2020; Chehade et al., 2022; Malve et al., 2022; Thoday-Kennedy et al., 2023). Furthermore, genotypes took longer time to initiate stem elongation, branching, flowering, and to reach maturity in winter than summer because low winter temperatures prolonged the rosette stage and subsequently delayed the onset of the subsequent stages. Safflower seedlings need low temperatures for rosette formation and root growth but warm temperatures for stem elongation and flowering (El-Bassam, 2010; Afzal et al., 2022). In summer, phenological development was fast because high temperatures reduced the duration of the rosette stage and subsequently shortening phenological development. On the other

hand, phenological development was long during winter in Sebele followed by Molepolole because safflower planted at these sites experienced chilling injury in 2021/2022 season during the stem elongation stage with the degree of injury being higher at Molepolole, hence slowing their phenology. Plants at Ramonaka during winter avoided chilling injury because they were at the rosette stage during the coldest period. Some of the plants from Molepolole and Sebele were able to recover and developed new leaves. The plants from Sebele and Molepolole were seriously damaged by chilling injury because the soils from these locations had a high percentage of sand (66-78%) than Ramonaka which had 39% sand. According to Flohr et al. (2016), sandy soils are susceptible to frost because of their low bulk density, low water-holding capacity, and moderately low nutritive level compared with other soil types.

The growth, yield and yield components of safflower were found to vary widely due to G×E interaction. Most genotypes produced significantly higher, growth, yield and yield components at Ramonaka in winter than in other locations and seasons. This was mostly attributed to the longer phenological development in winter that increased the accumulation of vegetative growth resulting in high yield and yield components. Additionally, differences in soil texture and fertility contributed to variations among locations. Better yield at Ramonaka was because soils were fertile (clay loam) which promoted vegetative growth compared to the sandy loam and sandy clay loam soils in Sebele and Molepolole, respectively. This highlighted the importance of planting location in the success of safflower production despite it being known to thrive in contrasted environments. A significant genotypic effect on the phenological development, growth, yield, yield traits, and oil content was evident in the studied traits, signifying the possibility of breeding safflower genotypes through direct selection of these traits. Most of these genotypic differences observed in this study

were influenced by the environmental factors as shown by their varying response to the environmental conditions in the studied locations and seasons. This highlighted the impact of G×E in selection and breeding of stable and adaptable genotypes.

To understand the G×E for safflower, GGE biplot analysis was employed because of their effectiveness in interpreting G×E interactions (Yan & Tinker, 2006; Yan & Frégeau-Reid, 2018). According to the biplots, Kenya9819 was identified as the highest-yielding and Gila as the poor-yielding genotypes. On the other hand, stability analysis showed that Sina and Gila had greater yield instability, and this implied that their performance was environment specific while Kenya9819, Turkey, and PI537636 had greater yield stability. This suggested that the relative performance of Kenya9819, Turkey, and PI537636 was consistent across the environments studied. Moreover, the which-won-where analysis showed that the test environments fell within two sectors showing that genotypes were showing high performance at specific locations/environments. In the current study, the genotype Kenya9819 was a winner in the test environments Sebele and Molepolole. It was found that genotype Kenya9819 was stable and adaptable to a diverse environmental condition of the study locations and growing seasons (summer and winter).

To select genotype(s) that have superior levels of the studied traits, the GYT biplots were used because of their efficacy in identifying genotype/s that have overall superior levels of several desired traits (Yan & Frégeau-Reid, 2018). The results revealed that most of the studied yield-trait combinations positively correlated with each other mostly because they are yield components. This indicated that these traits can be used in selection for breeding purposes without affecting or

compromising the level of another trait. Overall, genotypes Kenya9819 and Turkey were ranked as superior because they had above-average yield*trait combinations. On the other hand, genotype Gila ranked last in terms of overall superiority.

Safflower seed oil content is an essential economic trait for safflower genotypes (cultivars) because it affects the success of safflower adoption and production (Singh & Nimbkar, 2006; Emongor & Emongor, 20233). Therefore, evaluating safflower genotypes for oil content is necessary for breeding and production purposes, especially in non-native areas. Therefore, chapter 4 (study 2) was aimed at determining the relationship between oleosin genes and oil bodies in regulating the oil content of safflower seeds. The results showed that a marked variation existed in seed oil content and oil bodies of safflower genotypes, implying that there is genetic variability for these traits. The results further showed that oil body size and oil content of safflower were negatively correlated with each other suggesting that the smaller the seed oil-body the higher the seed oil content. For instance, genotype Gila that recorded the highest oil content had the smallest oil body diameter (size) which showed that oil body size was inversely correlated with oil content. Moreover, the results showed a substantial genetic variation in the relative expression of different Ct oleosin genes in safflower seeds. This was ascribed to the variation in the quantity of oil content and oil body size observed among the studied genotypes. For example, the genotypes Kenya9819 and Gila that produced higher oil content exhibited the highest Ctoleosin genes and smaller oil body size. This showed that the amount of oleosin genes present in seed can be used to distinguish between high and low oil-yielding genotypes. This information is important for breeding purposes because of the potential to modify seed oil content by optimizing the oleosin level.

Although safflower has proven to be a potential oil crop in Botswana its productivity is prone to frequently occurring droughts enhanced by climate change leading to water stress at the critical stages of growth and development. Environmental factors influence crop growth and development which are the significant variables that can reduce crop yields (Farooq et al., 2009; Mustafa et al., 2016; Emongor & Emongor, 2023). Environmental factors during flowering and seed filling stages affect quality and productivity of oilseed crops (Farooq et al., 2009; Mustafa et al., 2016; Hussain et al., 2016; Song et al., 2023). Therefore, this necessitated the need to screen genotypes for their level of tolerance to drought stress and this was the aim of chapter 5 (third study). The greenhouse and field studies revealed that drought stress diminished chlorophyll content, plant height, and LRWC and in response to drought stress, the levels of APX and proline increased irrespective of genotype and stage of development of safflower. However, this varied across different stress durations with the effect being more severe under long stress duration (30 days). This demonstrated how the duration of drought stress affected safflower plants. Past studies revealed the malicious effects of drought stress in plants with the effects being more severe as stress duration increased (Thippeswamy et al., 2013; Bortolheiro & Silva 2017; Farooq et al., 2020; Chavoushi et al., 2020). Generally, water deficit stress reduces plants size, branch numbers (vegetative growth), seed yield, seed oil content and yield (Mustafa et al., 2016; Bortolheiro & Silva 2017; Chavoushi et al., 2020; Farooq et al., 2020; Mosupiemang et al., 2022a). Water deficit during grain filling stage spring safflower genotypes significantly decreased seed and oil yield in ASALs (Pasban, 2011; Mustafa et al., 2016).

The levels of these studied traits also varied with the stage of development among the stressed and non-stress safflower genotypes. For instance, at the flowering stage stressed safflower plants

exhibited a significantly lower chlorophyll content and LRWC and higher proline content than corresponding control plants in either rosette or branching stages after 20 days of water stress induction in the greenhouse. The same trend existed in other water stress durations in greenhouse and field experiments. This suggested that as the plants transition from the vegetative stage to the reproductive stage they need more water to support reproductive growth, and this simultaneously hastened depletion of available water. This was more evident during the flowering stage as stressed plants senesced and died before reaching day 30 in the current study.

In most of the studied traits, significant genotypic differences between the stressed and non-stressed genotypes were found when the stress severity was high (day 30). This revealed that when plants were exposed to prolonged stress duration genetic variability was induced hence delineating the differences in genetic adaptability and sensitivity of genotypes to drought stress. This arguably emphasizes the importance of stress duration in the selection of stress-tolerant safflower genotypes. Furthermore, the presence of genetic variability among the stressed plants suggested differences in the drought tolerance levels among safflower genotypes.

To determine the overall superiority of the genotypes for all the studied drought tolerance traits, the GT methodology was used. This approach is based on the hypothesis that an ideal genotype should have superior levels for multiple desired traits (Yan & Frégeau-Reid, 2018). Generally, the GT biplots showed that genotype Kenya9819 was superior at all the studied developmental stages under both the greenhouse and field experiment except during the flowering and branching stages under the greenhouse where it was the second best after PI537636 and Sina, respectively. Hence, genotype Kenya9819 showed some level of tolerance when compared with other genotypes. On

the other hand, genotype Gila was the least drought tolerant because it ranked poorly at all the studied developmental stages under greenhouse and field experiments although it was second to last at the rosette and branching stages under field conditions. Therefore, genotype Gila was considered drought-sensitive when compared with other genotypes. The GT biplots also revealed that genotypes PI537636, Sina, and Turkey ranked fairly (moderately drought tolerant) depending on the developmental stage and growing condition (greenhouse or field experiment).

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Chapter 7

General Conclusions and Recommendations

7.1 General Conclusion

The first study (chapter 3) provided insight into how season, location, genotype, and their interactions influenced the growth, phenological development, yield, and oil content of safflower. Thus, the findings revealed that the phenological development, growth, yield, yield components, oil yield, and oil content of safflower were significantly influenced by genotype, location, seasons, and their interactions. The climatic conditions such as the amount and distribution of rainfall, soil types and air temperatures allowed safflower genotypes to perform differently at various locations and seasons. Growing safflower in winter was recommended over summer because it results in prolonged phenological stages which promoted vegetative growth, seed and oil yield, yield components, and oil content. The results also revealed that although the benefits of planting safflower in winter were noticeable, correct planting date is critical so that the reproductive stages do not coincide with low chilling temperatures that normally occur in July and August to avoid chilling injury. The biplots analysis for G×E revealed that Kenya9819 and Gila as the highest-yielding and poor-yielding genotypes, respectively. Further, the biplots showed that Kenya9819, Turkey, and PI537636 had better yield stability whereas Gila and Sina were the instable. Sebele was the most representative and discriminative of all test environments hence, a suitable environment for choosing safflower genotypes that are adaptable to all the studied sites. The yield by trait combination biplot successfully ranked the genotypes based on those with above-average as well as below-average yield by trait combinations. In this regard, genotypes Kenya9819 and Turkey had above average values of yield*trait combinations followed by Sina, on the other hand Gila and PI537636 had below-average values. Therefore, genotypes Kenya9819 and Turkey were

recommended to be grown in the Greater Gaborone region of Botswana due to their overall superiority. The second study provided evidence that it is possible to use oleosin genes in characterizing safflower genotypes based on the oil content, where Kenya9819 and Gila genotypes were correctly characterized to have high oil potential as validated by their smaller oil bodies and higher oil content. A significant correlation between oil content, oil bodies, and oleosin genes found in the present study suggested that breeding for high oil content in safflower can be accomplished by regulating the quantity of oleosin genes that are embedding the surface of seed oil bodies and subsequently enhancing the oil storage capacity of the seed. The third study evaluated safflower genotypes based on their level of tolerance to drought stress. The results demonstrated a varying response of safflower genotypes to drought stress as shown by their varying levels of chlorophyll content, plant height, LRWC, proline, and APX as stress progressed. In addition, the development stage and stress duration contributed to genotypic variations for drought stress. In particular, the rosette stage was the most tolerant stage while the flowering stage was the most drought-sensitive stage. The genotype, Kenya9819 was an overall superior (competitive) genotype under drought stress conditions and hence, considered most drought stress tolerant. On the other hand, genotype Gila ranked poorly in most of the traits and hence, it was considered less tolerant to drought stress. To increase productivity of safflower in the ASALs, supplemental irrigation should be provided at flowering and grain filling stages; and the use of tolerant genotypes should be incorporated into the cropping system to curb the impact of drought stress.

7.2 Recommendations and Future research

Genotype Turkey that produced high biomass and has fewer spines was recommended for use as leafy vegetable and petal production for herbal tea, colouring foods, cosmetics and pharmaceuticals. On the other hand, genotype Gila could be used for breeding purposes to improve the seed oil content of other genotypes due to its high seed oil content. The G×E interaction for safflower in Botswana should be conducted in more locations, especially in agroecological zones that were not represented in this study. In addition, more genotypes should be explored, especially those that are known to have a higher oil yield. Breeding for greater seed oil yield by targeting the oleosin genes may aid in meeting the increasing demands for seed oil. Other studies should evaluate the relationship between oil content, oil body, and oleosin genes at different seed developmental stages which could help in understanding the critical stage of oil formation in safflower seeds. Oleosin bounded oil bodies should be explored for their potential to be incorporated in the food industry, in production of mayonnaise and this makes safflower a better option because its high-quality oil which is rich in oleic and linoleic acid and minimal allergic reactions and saturated fatty acids.

It was recommended that selection of drought tolerant safflower genotypes should be done at different environments and longer drought stress durations to increase stress severity. Multiple drought tolerance traits should be used during safflower germplasm evaluation for drought tolerance this will assist in making informed choices. Safflower is a climate-smart crop that is adapted to various abiotic conditions in ASALs such as Botswana and because of its diverse uses its production should be promoted in the country and other Southern African Development Cooperation (SADC) countries with similar climatic conditions. With successful execution of

precise support policies in provision of subsidies to promote production, pricing, and marketing of safflower by the Botswana government has potential to improve food security, incomes, and livelihoods of many Batswana, and reduce the import bill of cooking vegetable oils.

Appendices

Appendix 1. Interaction of season, location and genotype on phenological characteristics of safflower.

Treatment effects	Days to emergence	Days to Elongation	Days to branching	Days to flowering	Days to maturity
Season	347.43***	7078.88***	17509.5***	17595.9***	479.76***
Location	12.24***	2.57ns	158.69***	71.010***	5.19**
Genotype	42.51***	16.93***	16.54***	32.56***	2.53**
Season x location	66.03***	72.08***	23.82***	187.56***	2.06ns
Season x genotype	13.11***	0.86ns	7.11***	1.58ns	1.29ns
Location x genotype	1.027ns	1.32ns	0.79ns	1.92ns	1.09ns
Season x location x genotype	1.84ns	2.82**	0.81ns	2.46*	1.14ns

*: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

Appendix 2. The growth parameters of safflower

Treatment effects	Plant height	Shoot biomass	Root biomass
Season	247.26***	178.67***	108.55***
Location	32.40***	28.95***	14.19***
Genotype	46.01***	1.34ns	10.08ns
Season x location	35.51***	19.47***	10.08***
Season x genotype	9.57***	1.91ns	2.36ns
Location x genotype	1.02ns	2.01ns	0.55ns
Season x location x genotype	1.80ns	2.85**	0.69ns

*:P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

Appendix 3. Yield, yield components, and oil content of safflower.

Treatments	No of primary branches	No of capitula/ plant	Capitula diameter	Capitula weight	1000-seed weight	Seed yield/ha	Oil content	Oil yield
Season	9.24**	7.49**	11.21***	34.40***	95.31***	39.58***	119.56***	67.10***
Location	23.15***	26.65***	0.86ns	10.42***	16.99***	8.31***	48.33***	6.38**
Genotype	1.45ns	3.44*	13.57***	15.87***	105.94***	2.56*	143.25***	3.55*
Season x location	24.5***	27.24***	0.13ns	12.49***	39.02***	30.72***	60.7***	42.58***
Season x genotype	2.07ns	2.43ns	0.53ns	2.51*	4.42**	1.43ns	2.1ns	2.34ns
Location x genotype	0.21ns	1.03ns	2.18*	1.20ns	3.21**	1.86ns	3.3**	2.34*
Season x location x genotype	1.21ns	0.80ns	1.92ns	0.56ns	2.38*	1.13ns	3.0**	0.77ns

*: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

Appendix 4. Chlorophyll content of safflower genotypes stressed at different phenological stages under the greenhouse and field experiment.

	Greenhouse			**Field**		
Treatments	**Day 10**	**Day 20**	**Day 30**	**Day 10**	**Day 20**	**Day 30**
Stress condition	2.31ns	98.73***	19.612***	19.61ns	14.95***	19.27***
Phenological stage	3.01ns	21.15***	149.69***	31.06***	55.04***	85.69***
Genotype	1.09ns	1.08ns	5.36**	4.30*	3.54*	1.44ns
Stress condition× Phenological stage	3.82*	47.65***	160.41***	0.20ns	2.42ns	0.54ns
Stress condition × genotype	0.42ns	1.35ns	3.22*	0.89ns	0.36ns	2.10ns
Phenological stage × genotype	1.46ns	0.58ns	3.46*	1.18ns	0.78ns	2.29ns
Stress condition × Phenological stage × genotype	0.59ns	0.25ns	1.73ns	0.93ns	1.22ns	0.58ns

*: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

Appendix 5. ANOVA summary table for plant height of safflower genotypes stressed at different phenological stages under the greenhouse and field experiment.

	Greenhouse			Field		
Treatments	**Day 10**	**Day 20**	**Day 30**	**Day 10**	**Day 20**	**Day 30**
Stress condition	1.138ns	0.15ns	8.13***	0.17ns	3.03ns	16.38***
Phenological stage	336.24***	183.19***	798.56***	1180.97***	806.74***	1106.9***
Genotype	0.74ns	1.06ns	0.50ns	5.40**	2.18ns	2.46ns
Stress condition× Phenological stage	1.38ns	1.03ns	0.01ns	0.50ns	0.004ns	0.08ns
Stress condition × genotype	0.56ns	0.70ns	0.47ns	1.19ns	0.89ns	1.80ns
Phenological stage × genotype	3.89***	1.94ns	0.50ns	4.63**	1.07ns	1.68ns
Stress condition × Phenological stage × genotype	0.25ns	0.79ns	1.75ns	1.36ns	0.33ns	0.93ns

*: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

Appendix 6. ANOVA summary table for LRWC of safflower genotypes stressed at different phenological stages under greenhouse and field experiment.

	Greenhouse			Field		
Treatments	**Day 10**	**Day 20**	**Day 30**	**Day 10**	**Day 20**	**Day 30**
Stress condition	23.39***	420.74***	189.55***	25.95***	33.91***	30.22***
Phenological stage	10.96***	98.49***	2.93ns	23.62***	13.07***	46.51***
Genotype	2.07ns	1.52ns	1.71ns	3.09*	1.27ns	1.67ns
Stress condition× Phenological stage	14.38***	98.96***	5.46*	5.32*	0.69ns	6.25*
Stress condition × genotype	1.18ns	1.25ns	1.29ns	2.06ns	0.12ns	1.43ns
Phenological stage × genotype	0.30ns	1.92ns	2.27ns	1.89ns	0.84ns	1.84ns
Stress condition × Phenological stage × genotype	0.38ns	1.76ns	3.12*	0.98ns	1.84ns	1.11ns

*: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

Appendix 7. ANOVA summary table for proline content of safflower genotypes stressed at different phenological stages under the greenhouse and field experiment.

	Greenhouse			Field		
Treatments	**Day 10**	**Day 20**	**Day 30**	**Day 10**	**Day 20**	**Day 30**
Stress condition	524.04***	2380.3***	909.12***	16.26***	53.46***	1.99ns
Phenological stage	1276.32***	1224.8***	280.00***	46.53***	19.24***	0.57ns
Genotype	47.59***	174.9***	19.32***	3.67*	1.20ns	1.86ns
Stress condition× Phenological stage	387.32***	1011.2***	302.36***	15.61***	5.79*	3.31ns
Stress condition × genotype	50.88***	179.4***	21.79***	1.97ns	2.28ns	2.52ns
Phenological stage × genotype	34.44***	86.4***	40.53***	1.24ns	4.44**	2.94*
Stress condition × Phenological stage × genotype	43.90***	89.56***	36.42***	2.04ns	7.00***	2.0ns

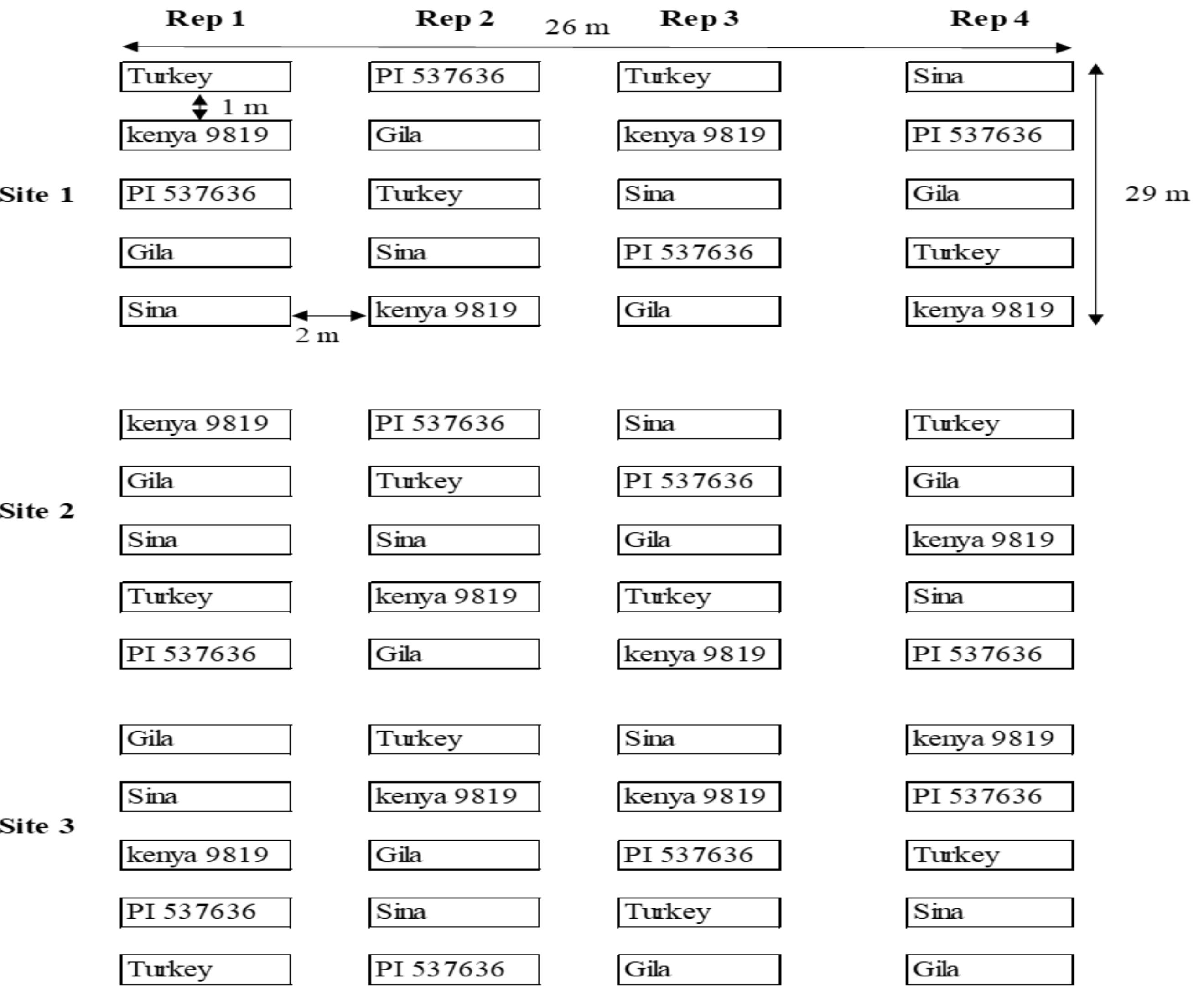
*: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

Appendix 8. Ascorbate peroxidase activities of safflower genotypes stressed at different phenological stages under greenhouse and field experiment.

	Greenhouse			Field		
Treatments	**Day 10**	**Day 20**	**Day 30**	**Day 10**	**Day 20**	**Day 30**
Stress condition	159.16***	102.70***	124.80***	95.78***	28.14***	134.59***
Phenological stage	9.63***	77.59***	4.54*	25.67***	0.58ns	8.37**
Genotype	27.45***	12.23***	44.06***	54.42***	1.28ns	9.44***
Stress condition× Phenological stage	1.03ns	74.78***	17.23***	26.37***	0.02ns	0.000ns
Stress condition × genotype	15.10***	10.17***	15.78***	45.34***	1.55ns	10.45***
Phenological stage × genotype	18.31***	13.32***	31.78***	65.86***	2.04ns	18.29***
Stress condition × Phenological stage × genotype	12.20***	12.42***	23.11***	54.50***	0.70ns	9.30***

*: P≤0.05; **: P≤0.01; ***: P≤0.001. ns=not significant. Values in the columns represent the F-values.

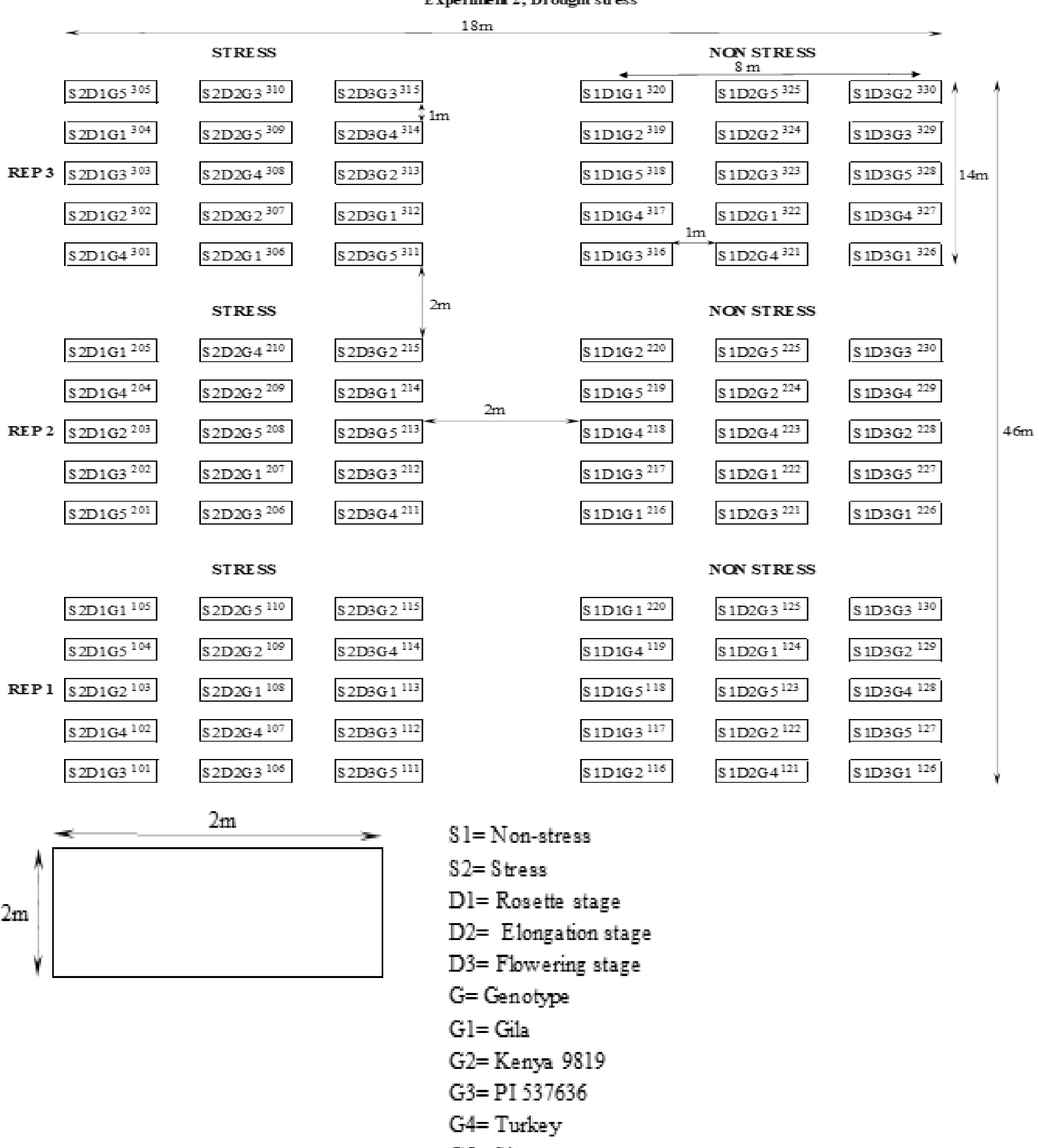
Appendix 9. Multilocation field map



Appendix 10. Greenhouse drought stress field map

stresss			non stress		
PI 537636 [101]	PI 537636[201]	Sina[301]	Kenya 9819[106]	Turkey[206]	Gila[306]
Turkey[102]	Turkey[202]	PI 537636[302]	PI 537636[107]	Kenya 9819[207]	Sina[307]
Kenya 9819[103]	Gila[203]	Gila[303]	Sina[108]	Sina[208]	Turkery[308]
Sina[104]	Kenya 9819[204]	Turkey[304]	Turkey[109]	Gila[209]	Kenya 9819[309]
Gila[105]	Sina[205]	Kenya 9819[305]	Gila[110]	PI 537636[210]	PI 537636[310]

Appendix 11. Field map of drought stress experiment



Appendix 12. Sample isolated creamy oilbodies from genotype Gila

Appendix 13. Safflower choked by watterlogging at Molepolole summer 2021/2022 (a) and safflower capitula rotten due to excess rainfall at Ramonaka during summer 2020/2021 season (b)

Appendix 14. Rainfall data for study 1 (chapter 3)

	2020/2021			2021/2022		
Month	**Sebele**	**Molepolole**	**Ramonaka**	**Sebele**	**Molepolole**	**Ramonaka**
September	47	15	0	0	0	0
October	56	70	38.5	44	0	0
November	0	37	24.5	0	29	64
December	9.5	111.6	21.8	41	36	83
January	40	5	85.5	5	17.5	5
February	50	50	19	0	0	0
March	0	0	0	24	32.5	20
April	-	-	-	18	183	24
Total for experimental period only	99.5	198.6	150.8	88	269	132
Pre sowing total	103	85	38.5	44	29	64

Appendix 15. Temperature data

Summer trial 2020/2021 temperatures (For Sebele, Molepolole, and Ramonaka)

	Nov-2020		Dec-2020		Jan-2021		Feb-2021		Mar-2021	
Day	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**
1	25.1	17.3	32.4	19.8	30.5	22.9	26.4	19.5	25.3	15.1
2	29.3	14.8	33.4	14.2	27.5	19.5	23.5	19.8	26.6	14.5
3	24.7	15.9	25.3	21	25.2	16.9	25.4	18.9	29.6	14.3
4	29.9	18	27.9	16.6	29.9	17.8	25.8	20.2	32.5	16.9
5	28.8	16.4	24	18	31.7	19.3	22.6	20.5	32.9	16.1
6	31.8	17.6	26.6	17.1	31.7	22.2	22.5	19	30.9	16.5
7	35.6	16.9	28.7	17.4	26.8	20.9	26	18.2	32.9	16.7
8	37	19.5	24.6	20.1	28.7	20.3	30.1	19	32.8	15.1
9	28.7	17.7	28.7	18.3	31.8	19.8	29	19.5	27.5	
10	23.9	18.7	32.6	18.9	32.1	21.9	28.9	18.6	34.6	16.9
11	27	17	32.8	21.1	31.3	22.9	29.6	19.7	34.2	13
12	25.1	17.9	33.8	20.7	30.5	20.8	30.6	18.6	31.3	16.3
13	28	16.3	28.7	18.4	33	19.9	31.5	18.6	30.2	17.3
14	30.9	15.9	24.3	20.1	30.8	19.3	32.2	19.7	28.7	12.1
15	32.3	16.6	29.3	20.1	31	20.2	32.6	19.9	29.5	15.2
16	33	15.9	29	18.8	31.3		28.9	20.6	30.5	14.9
17	27.6	20.2	21.9	18.8	32.2	21.1	30.6	17.6	27.3	19.3
18	34.7	15.4	24.9	19.4	31		33	17.9	29.6	17
19	34.3	19.3	25.8	19.3	29.3	20.2	34.4	19.9	32.4	14
20	30.4	20	28.4	20.8	33.8	17.6	33.4	18.3	28	15.9
21	21.8	17.6	28.8	19.1	32	21.6	32.2	18.6	25.2	17.3
22	28.9	19.1	32.2	20.9	33.6	21.1	26.9	19.6	30.3	14.1
23	32	19.3	33.9	22.4	35.3	17.3	27.4	18.9	24.8	17.2
24	35.5	19.5	34.9	21.8	32.7	22.1	24.9	19.1	21.6	18.3
25	33.6	15.3	28.5	20.9	32.3	18.9	25.4	19.9	28.3	16.3
26	30.8	20	30.4	19.1	27.3	20.2	27.3	17.6	29.1	13.2
27	31.7	20.5	33.1	19.5	28.1	20.3	25.9	14.8	31.9	11.3
28	27.4	20	34.7	21.1	28.5	20.8	26.9	16.2	30.5	13.2
29	30.3	17.5	33	23.2	25.5	20.6			28.3	15.5
30	28	19.8	35.3	20.5	28.1	19.7			28.2	12.9
31			31.9	17.9	26.3	18.7			29.4	14.2
AV	**29.9**	**17.9**	**29.7**	**19.5**	**30.3**	**20.2**	**28.4**	**18.9**	**29.5**	**15.4**

Summer trial 2021/2022 temperature (Sebele, Molepolole, and Ramonaka)

	Dec-2021		**Jan-2022**		**Feb-2022**		**Mar-2022**		**Apr-2022**	
Day	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**
1	34.4	20.5	31.6	19.2	31.5	18.1	32.1		31.7	12.7
2	28	18.7	32.7	20.2	33.8	20.4	34.8		33.2	14.7
3	34.5	19.9	32.3	18.8	34.6	21.9	33.3	19.3	34	17.1
4	32.7	22.3	31.3	19.1	32.2	19.9	26.5	20.2	30.4	18.7
5	23.5	18.7	32.1	19.9	29.8	19.7	29.6	20.5	31.2	17.5
6	31.5	17.8	31.5	20.1	29.3	19.6			33.1	17.7
7	32.1	18.7	27	19.6	30.8	17.3	29.1		33.1	19.5
8	28.7	19.2	29	19.1	33.9	16.9	25	18.4	24.6	18.9
9	29.8	18.7		18	33.6	20.8	21.1	18.7	22	16.5
10	32.6	19.7	28.3	16.4	34.3	17.5		18.2	19.7	16.1
11	32.4	20.7	26	20.1	33.3	16.9	29.1		21	16.4
12	26.9	19.1	30.7	18.5	31.6	15.1	30.7		28	14.5
13	26	19.5	30	20.4	32.3	17.8		16.7	31	15
14	30.7	17	30.8	19.9	34.1	16.6	28.2		28.5	16.5
15	33.7	18.1		20.8	32.4	20.7	28.8	17	22.5	15.9
16	24.9	17.1	28.2		32.8	18.6		15.9	27.1	19.6
17	28.6	18.6		18.5	34.5	19.6			20.6	16.1
18	20	16.9	27.3	20.3	32.6	20.3			24.1	15.8
19	23.3	14.2	32.1	17.9	30.6	20.8			22.9	14.4
20	24.8	16.2	31	19.6	35.2	19.8			23	12.6
21	26.7	14.8	32.6		36.2	18.1			20.6	15.2
22	30.1	17.2	28.5	15.7	35.7	21	27.4		25	15.5
23	31	16.8		16.3	33.8	19.3	27.7	14.3	28.4	14.4
24	32.6	13.4	31.5	15.7	34.2	18.8	29.6	15.7	30	15.5
25	32.2	16.9	34.5	17.6	33.6	19.6		19.7	30.6	16.7
26	28.8	17.8		19.4	30.4	19.3			27.6	16.1
27	23.7	17.8	31.6		34.6	19.5			22.7	17.2
28	30.2	14.2		21.1	31.5	19.6			22.5	11.5
29	34.4	16.4	25.7	19.3			28.7		26.1	7.6
30	33.8	19.2	25.4	19.3					29.6	9.6
31	34.6	19.7	28.1	19.8						
AV	**29.6**	**17.9**	**30.0**	**19.0**	**33.0**	**19.1**	**28.9**	**17.9**	**26.8**	**15.5**

Winter trial 2020/2021 (For Sebele, Molepolole, and Ramonaka)

	May-2021		Jun-2021		Jul-2021		Aug-2021		Sep-2021		Oct-2021	
Day	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**
1	23.8	8.5	20.6	5.1	26	6.5	23.7	1	31.6	5.2	24.1	17
2	21.5	14.3	21.4	1	25.7	2.8	26.8	2.3	29.4	13.9	28.7	13.6
3	22.8	8.9	21.3	5.3	26.9	1.9	23.7	7.1	32.9	13.2	29.3	11.1
4	24.1	7.8	19.7	6.3	27.9	2	24.7	7.3	31	13.1	28.5	8.4
5	26.1	7.3	18.5	1.8	25.9	3	25.3	3.5	30.3	18.5	27.2	15.3
6	26.7	8.5	19	3.3	18.3	8.1	28	4.7	30.5	17.5	31.7	16.8
7	26.5	12.3	19.1	6.3	21.6	5.1	28.8	5.1	29	12.3	28.1	17.9
8	26	12.7	19.1	5.8	22.3	3.9	28	11	20.6	16.9		
9	24.7	60	19.3	9.5	24	4.3	29.4	8.7	26.6	10.9	33.8	17.7
10	25.5	6.3	22	7.1	24.1	2.3	25.2	7.7	26	13.6	32.3	13.2
11	26.7	5.4	23.3	4	23.2	1.8	24.5	12.1	27.1	10	23.4	15
12	28	6.4	25.1	3.2	25.7	4.1	28.7	7.7	27.4	8.9	29.3	10.7
13	27.1	4.9	26.2	4.6	16.7	7.2	27.7	6.3	29.9	5	33.4	11.2
14	27.8	4.3	25.3	3.1	16.2	-0.2	17.9	11.9	33.1	10.8	36.3	12.9
15	29.1	6.1	24.9	2.4	18.2	1.2	20.2	12.3	35.3	13.5	37.2	15.3
16	26.2	6.1	24.5	3.7	18.5	3.5	23.8	7.7	34.4	11.1	36.3	17.6
17	25.5	6.8	26.1	3		1	26	9.8	28.4	11.1	30	15.8
18	25.4	3.4	25.9	3.3	23.1		30.1	8.7	22.2	14.7	28.1	14.5
19	27.5	9.2	20.7	12.5	18.9	3.4	27.5	4.6	27.5	10.9	21.2	14.2
20	25.4	6.3	20.3	9	19.7	-0.7	29.9	5.1	34	15.2	26.1	11.7
21	27.5	6.3	20.4	4.4	22.9	-0.1	28.4	12.8	31.5	15.1	28.2	14.2
22	25.5	6.3	19.9	2.7	17.4	-3	30.4	15	24	15.3	30.2	16.3
23	23.8	9.1	23	4.2	16.5	-1.8	26.1	14.9	27.4	9.4	35.2	15.2
24	18.1	2.4	24.5	3.4	17.8	0	27.2	13.6	30.8	12.1	31.6	15.8
25	23.1	5.7	26.2	3.1	17.6	1.6	27.1	14	33.9	13.2	28.5	16.3
26	23.3	3.6	26.9	3.1	18.1	-0.8	26.8	12.8	34.6	14.3	29.2	17.6
27	25	3.8	26.3	3.1	19.8	1.7	32.8	15	32.9	16.4	28.7	18.6
28	26	4	28.7	2.3	20.9	-0.6	18.5	12.4	35.9	15.7	20.3	19.3
29	27.2	4.9	28.8	5.2	24.2	1.5	21.2	-2.3	35.7	18.1	30.8	17.3
30	25	2.3	28.5	7.9	23.7	2.3	24.2	5.5	26.4	20.6	32.5	16.7
31	23.2	3.7			22.6	5.8	29	4.2			32.6	15.8
AV	**25.3**	**8.3**	**23.2**	**4.7**	**21.5**	**2.3**	**26.2**	**8.5**	**30.0**	**13.2**	**29.8**	**15.1**

Winter trial 2021/2022 (For Sebele, Molepolole, and Ramonaka)

	May-2022		Jun-2022		Jul-2022		Aug-2022		Sep-2022		Oct-2022	
Day	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**
1			20.6	-0.3	20.6	6.5	22.4	12.5	23.5	8.5	34.7	15.6
2	23.7	11.6	20.7	1.5	21	5.7	20.3	10.7	25.7	6.4	36.6	16.5
3	24.3	10.5	20.6	1.6	21.8	5	20	13.2	30.2	6.2	38.1	17.4
4	25.6	9.9	20.6	1.5	23.3	3.7	27.6	9.2	31.8	7.4	39.3	18.3
5	25.6	11.2	23.6	2.6	24.6	3.4	27.9	4.4	32.2		35.4	20.1
6	26.1	8.4	20.6	6	24.9	6.6	29.8	4.4		9.9	38.2	18.2
7	25.3	8.6	21.4	2	24.3	1.3	31.6	7.2	25.5		37.4	17.8
8	24.2	10.7	22.1	2.5		1.8	25.7	10.3	27.6	16.9	33.6	21.8
9	24.1	4.9	22.1	4.1	22.1		26.3	3.3	32	8.3	36.1	25.2
10	23.2	5.7	22.1	2.8	24.7		26	5.1	32.5	12.5	36.3	16.3
11	25.1	7	20.3	4.9	22.4	4.3	27	5	34.4	11.1	34.5	19.1
12	27	7.3	21	1.4		2.2	30.4	6.5	34.7	12.1	35.8	16
13	26.6	7.3	22.8	1.3	24.3		31.5		24.1	15.8	35.8	17.1
14	28.5	8.1	24	1.8	25	4.1	22.9	5.5	24.3	11.7	36.2	16.8
15	26.9	8.2	17.8	8.8	23.3	2.7	21.8	2.3	28.4	11	36.6	20
16	21.8	10.7	18	1.3	20.8	5.9	24.3	-0.1		8.2	28.7	19.7
17	21	9.2	20.1	1.6	20.5	2.8	30.4	2.9	32.4		20.6	17.9
18	22.4	10.5	23.5	-0.6		5.3	30.6	7	34.3		26.7	16
19	23.6	5.4	25.3	-2.5	22.1		20.6	8.2	31.6	17.7	30.6	16.1
20	24.6	8.7	23.7	8.9	21.5		23.7	2	26	11.1	32	18.1
21	11	2.8	21	10.1	24.1	10.9	23	2.4	26.6	6.4	32.9	16.2
22	17.7	4.7	18.3	8.6		8.2	23.1	5.9	27.4	12.8	33.6	19.9
23	22.2	5.2	21.9	9.2	23.9	10.8	24.3	5.1	31.3	9.3	29.9	17.4
24	25	7.5	22.3	6.7	25.7		24.5	7.5	33.9		33.8	19.3
25	26	6.2	15.7	9.6	22.3	3.9	27.4	4.3	33.7		36.3	16.2
26	26.5	5.9	19.8	6.2	22.5	8.7	29.2	4.6	35.6	15.9	34.9	16.7
27	25.1	6.2	19.1	6.4	23.3	8.1	30.9	8.1	35.6	16.7	28.5	17.1
28	23.3	9.3	16.4	2.4		6.2	31.4	7.9	32.9	18.4	32.7	16.5
29	26.2	8.4	18.2	4.1	23.9		31.4	11	30.2	16	26.4	16.2
30	22.3	4.5	19.1	0.9	26.7		30.4	10.1		13.3	29.2	18.1
31	21.4	1.2			23.2	5.4	20.6	13.4	23.5	8.5	32.4	18.8
AV	**23.9**	**7.5**	**20.9**	**3.9**	**20.6**	**6.5**	**26.4**	**6.7**	**30.3**	**11.8**	**33.3**	**17.9**

Appendix 16. Moisture content for drought experiment

				Greenhouse experiment		Field experiment	
genotype	condition	stage	day	moisture (5cm)	moisture (20 cm)	moisture (20 cm)	moisture (40cm)
gila	control	rosette	0	20.67	20.98	13.81	14.44
kenya	control	rosette	0	18.67	19.33	12.98	11.96
PI	control	rosette	0	20.67	19.83	13.67	14.56
Turkey	control	rosette	0	20.47	21.00	13.04	12.81
Sina	control	rosette	0	19.67	20.63	14.97	15.87
gila	stress	rosette	10	8.55	10.45	7.70	12.97
kenya	stress	rosette	10	8.69	9.89	10.30	3.45
PI	stress	rosette	10	8.02	9.13	5.60	4.40
Turkey	stress	rosette	10	8.46	9.80	6.97	11.60
Sina	stress	rosette	10	9.02	10.76	5.23	2.17
gila	control	rosette	10	19.60	20.45	13.27	19.25
kenya	control	rosette	10	19.37	19.60	13.70	14.33
PI	control	rosette	10	20.83	23.75	12.07	12.75
Turkey	control	rosette	10	17.98	18.90	12.63	18.00
Sina	control	rosette	10	19.67	19.50	13.77	12.10
gila	stress	rosette	20	3.43	5.50	5.67	1.93
kenya	stress	rosette	20	3.43	5.50	4.83	0.80
PI	stress	rosette	20	2.67	3.57	4.33	2.60
Turkey	stress	rosette	20	2.20	3.77	5.40	2.40
Sina	stress	rosette	20	3.50	4.30	3.50	1.07
gila	control	rosette	20	19.37	19.60	11.40	16.35
kenya	control	rosette	20	23.07	20.90	9.13	13.07
PI	control	rosette	20	17.67	17.13	9.67	12.10
Turkey	control	rosette	20	18.87	23.87	9.47	13.97
Sina	control	rosette	20	15.83	16.93	10.87	13.85
gila	stress	rosette	30	0.93	1.87	2.40	0.20
kenya	stress	rosette	30	0.50	0.70	1.30	0.00
PI	stress	rosette	30	0.47	1.10	2.70	0.27
Turkey	stress	rosette	30	0.60	1.67	2.13	0.87
Sina	stress	rosette	30	1.23	1.20	1.93	0.30
gila	control	rosette	30	20.93	20.87	10.27	16.30
kenya	control	rosette	30	26.57	26.33	8.40	11.07
PI	control	rosette	30	23.67	21.00	7.17	10.60
Turkey	control	rosette	30	27.70	27.47	10.30	10.23
Sina	control	rosette	30	22.33	21.53	6.57	13.03
gila	control	branching	0	18.93	18.30	7.30	4.20
kenya	control	branching	0	18.67	19.33	8.10	3.60
PI	control	branching	0	25.67	18.83	6.50	7.47
Turkey	control	branching	0	22.47	21.00	6.53	4.30
Sina	control	branching	0	19.67	19.60	6.77	3.40
gila	stress	branching	10	1.30	0.90	4.30	5.03
kenya	stress	branching	10	1.57	1.43	6.60	6.89
PI	stress	branching	10	3.33	3.13	5.54	6.03

Turkey	stress	branching	10	2.90	2.43	3.90	4.76
Sina	stress	branching	10	1.30	1.37	3.80	4.36
gila	control	branching	10	11.87	12.77	12.97	12.50
kenya	control	branching	10	6.70	9.60	13.23	14.45
PI	control	branching	10	9.87	12.73	11.20	12.10
Turkey	control	branching	10	15.47	10.67	11.83	12.25
Sina	control	branching	10	12.90	14.73	12.67	12.50
gila	stress	branching	20	0.00	0.00	4.20	4.99
kenya	stress	branching	20	0.03	0.00	4.17	4.89
PI	stress	branching	20	0.00	0.00	4.00	5.04
Turkey	stress	branching	20	0.33	0.03	2.90	3.67
Sina	stress	branching	20	0.00	0.00	2.00	3.05
gila	control	branching	20	9.73	10.20	11.80	12.80
kenya	control	branching	20	12.33	10.03	10.37	11.60
PI	control	branching	20	8.13	11.73	10.67	11.80
Turkey	control	branching	20	9.83	10.53	11.53	12.40
Sina	control	branching	20	11.87	10.37	10.23	13.20
gila	stress	branching	30	0.00	0.00	3.67	3.89
kenya	stress	branching	30	0.00	0.00	3.40	3.88
PI	stress	branching	30	0.00	0.00	3.02	4.06
Turkey	stress	branching	30	0.33	0.00	2.10	3.10
Sina	stress	branching	30	0.00	0.00	1.11	1.98
gila	control	branching	30	12.87	14.07	13.60	13.98
kenya	control	branching	30	7.90	9.60	14.17	14.89
PI	control	branching	30	8.83	12.03	11.97	13.06
Turkey	control	branching	30	12.77	12.57	12.53	12.78
Sina	control	branching	30	8.73	10.97	12.77	13.89
gila	control	Flowering	0	21.47	19.53		
kenya	control	Flowering	0	31.87	22.20		
PI	control	Flowering	0	23.40	23.13		
Turkey	control	Flowering	0	38.77	34.07		
Sina	control	Flowering	0	37.70	25.43		
gila	control	Flowering	0	39.67	30.43		
kenya	control	Flowering	0	24.47	18.30		
PI	control	Flowering	0	22.80	19.83		
Turkey	control	Flowering	0	29.07	21.77		
Sina	control	Flowering	0	25.90	22.73		
gila	stress	Flowering	10	1.07	0.87		
kenya	stress	Flowering	10	4.33	2.10		
PI	stress	Flowering	10	1.53	1.93		
Turkey	stress	Flowering	10	4.40	3.23		
Sina	stress	Flowering	10	2.33	1.60		
gila	control	Flowering	10	13.10	15.63		
kenya	control	Flowering	10	16.77	14.90		
PI	control	Flowering	10	13.33	14.37		
Turkey	control	Flowering	10	16.17	14.83		
Sina	control	Flowering	10	18.63	17.00		
gila	stress	Flowering	20	0.00	0.00		

kenya	stress	Flowering	20	0.00	0.00		
PI	stress	Flowering	20	0.00	0.00		
Turkey	stress	Flowering	20	0.00	0.00		
Sina	stress	Flowering	20	0.00	0.00		
gila	control	Flowering	20	11.80	13.83		
kenya	control	Flowering	20	14.27	13.07		
PI	control	Flowering	20	14.80	14.57		
Turkey	control	Flowering	20	14.33	11.37		
Sina	control	Flowering	20	16.90	13.23		