Addis Ababa University

Studies on the Economic Benefits and Extent of Drought Adaptation of Apple (*Malus domestica* Borkh.) Genotypes Introduced to Ethiopia

By Abayneh Melke Woldegebriel

A Dissertation Submitted to the Department of Plant Biology and Biodiversity Management in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology (Plant Physiology)

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A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Plant Biology & Biodiversity Management

Approved by Examining Board:

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A B S T R A C T

The present study was aimed at evaluating the economic benefits and adaptability of apple (Malus domestica Borkh.) genotypes domesticated, propagated, and cultivated in five different locations of Ethiopia's highlands. In addition to in depth studies on the economic benefits of this useful fruit crop, the present thesis also focused on identifying early maturing, drought tolerant, as well as diseases and pest tolerant genotypes with desirable fruit yield and quality characteristics preferred by both the farmers and consumers. To this end, major apple genotypes introduced to Ethiopia during the last century were evaluated at five distinct geographical locations and under controlled glasshouse conditions. Eco-geographic characterization in five selected locations (namely: Holetta, Debrebirhan, Degem, Hidabu-abote and Agena) on eight apple genotypes (Anna, Dorsette golden, Princesa, Granny smith, Crispin, Gala, Golden delicious and Red delicious) identified specificity in the time of flowering, fruit setting and maturity, as well as adaptability of genotypes to the environments with respect to their chilling requirements for flowering and fruit setting. Results from field study conducted at these locations revealed that cultivar evaluation and selection will depend on growers' preferred attributes such as maturity status of the genotypes (early, medium or late), fruit yield per tree, fruit weight, size and color, the type of rootstock used and branching habit of the scion. The present thesis found that genotypes Anna, Dorsette golden and Princesa consistently showed early maturity and high fruit yields at all the tested sites. Field studies conducted at Debrebirhan addressed physiological response of the eight genotypes to drought stress, by considering different physiological traits, including plant water relations (RWC), leaf water potential (ψw), net photosynthesis (Pn), stomatal conductance (Gs), transpiration rate (E), as well as water use efficiency (WUE). Total chlorophyll (Chl) content; growth performaces such as root dry mass (RDM), total biomass (BM), total leaf area (TLA), specific leaf area (SLA) and leaf area ratio (LAR) were determined and compared among the studied genotypes. Highly strong positive relationships were obtain between biomass and water use efficiency (r^2 = 0.92); and between biomass and root dry mass (r^2 =0.70). Drought susceptibility index identified that Anna, Dorsette golden and Granny smith were drought tolerant genotypes. Throughout the study period, these genotypes maintained higher RWC, ψw, WUE, Pn, RDM and low rate of Gs and E, compared to Golden delicious, Red delicious and Royal gala.

Conversely, genotypes Golden delicious, Red delicious and Royal gala showed higher rates of Gs and E, hence their classification as drought susceptible genotypes. The aforementioned genotypes were also evaluated for drought tolerance in a glasshouse to further characterize their adaptability for drought prone areas. In addition to repeating measurements on the physiological parameters considered for the field studies, biochemical determinations on chlorophylls 'a' and 'b', proline, soluble sugar, lipid peroxidation expressed as malondialdehyde (MDA) content, drought induced soluble proteins (dehydrins) and antioxidant enzyme (AOX) activities, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) were measured in leaves of apple genotypes maintained in a glasshouse and subjected to induced soil water potential (Ψmd) of ~ −2.75 MPa). Induced drought stress resulted in reduced RWC, leaf water potential (LWP), Gs, E and Pn in all the genotypes studied. Under drought stress, apple genotypes Anna, Dorsette golden and Granny smith maintained higher RWC, higher LWP and lower SLA, compared to Golden delicious, Red delicious and Royal gala. Clearly, the glasshouse studies confirmed the field studies that the former group of genotypes (Anna, Dorsette golden and Granny smith) are considered as drought tolerant, compared to Golden delicious, Red delicious and Royal gala. The overall results from the glasshouse and field experiments showed that plant water relations (RWC, LWP) and gas exchange measurements (Gs, E and Pn) appeared as a greater index of genotype's tolerance or susceptibility to drought stress, followed by the elevated activities of antioxidant enzymes.

Key words/Phrases: Apple phenology, tropical highlands, drought stress, drought susceptibility index, biomass, water use efficiency, leaf gas exchange, proline,

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ACRONYMES

ABA - Abscisic acid
ALDH - Aldehyde dehydrogenases
AOX - Antioxidant enzyme
APX - Ascorbate peroxidase
AsA - Reduced ascorbate
ASC - ascorbate
ATP - adenosine triphosphate
BM - Biomass
bp - base pair
BSA - bovine serum albumin
CAT - Catalase
Chl - Chlorophyll
DEPs - Differentially expressed proteins
DHA - Dehydroascorbate
DHAR - Dehydroascorbate reductase
E – Rate of Transpiration
ETC - Electron transport chain
GLM - General Linear Model
GPX - Glutathione peroxidase
GR - Glutathione reductase
Gs - Stomatal conductance
GSH- Reduced glutathione
GST- Glutathione-S-transferase
HSPs - Heat shock proteins
LAR - Leaf area ratio
ROOH- Organic peroxide
ROS - Reactive oxygen species
RWC- Relative water content
SLA - Specific leaf area
SOD - Superoxide dismutase
TBA – Thiobarbituric acid
TDR - Time domain Reflectometer
LA - Leaf area
WUE- Water use efficiency

CHAPTER ONE: Background of the study

1.1. Introduction

Fruit crops play important roles in food security and as a source of income around the world. They are delicious and highly nutritious, rich vitamins and minerals that the cereal based diets are unable to supply in our daily food intake. Furthermore, fruits supply raw materials for ago-industries and could be sources of foreign currency (Joosten, 2007). In countries such as Ethiopia, the development of fruit industry will create employment opportunities, particularly for farming communities (Kahsay *et al.,* 2008; Keyzer *et al.,* 2000). For small-scale growers fruits contribute for improved nutritional benefits that cannot be compensated by cereal based dietery system and income generation when the household, are sold directly (Girmay *et al.,* 2014). Accordingly, marketing fresh and processed fruit products generates income which can act as an economic buffer and seasonal safety net for rural farm households in many developing countries (Girmay *et al.,* 2014; Haji, 2007). Diversification into fruit production can generate employment and enable small-scale farmers to embark on a range of production, processing and marketing activities to complement existing income-generating activities (Honja, 2014; Rolien and Andre, 2007). Apple is among the fruit crops grown for several benefits it provides to growers besides serving as an income source; it has high nutritional values including essential vitamins and minerals and/or micronutrients that rarely found in daily staple in, many developing nations (Fetena *et al.,* 2014; Getachew *et al.,* 2012).

In Ethiopia, apple was first brought to Chencha by protestant Chiristian missionary named Mr. Ralph about 60 years ago and was established in the garden of Chencha Kale-Hiwot Church in the south of the country (Ashebir *et al.,* 2010). The climatic and soil conditopns of the area are conducive for diverse varieties of apple. Further, Chncha has been serving as a resource base for the rest of the country. As a result, apple grafts have been distributed to the rest of areas in the country from Chencha (Fetena *et al.,* 2014). Currently, Chencha has a potential for producing about 15-20 metric-tons of apple fruits per year while the overall country production is estimated to be 50 metric-tons (Girmay *et al.,* 2014). Since the total production does not meet Ethiopia's demand, the nation imports about 350 metric-tons of apple fruits mainly from South Africa, New Zealand, Italy, France, Chile, USA Iran,

Turkey, China and Israel, and this showing an unmet market demand for apple fruits (EHDA, 2012).

Ethiopia is one of the county's in the tropics with highlands more than 47% of its total area and has had a sufficient seasonal low temperature during winter months that create suitable agro-ecological conditions for the production of temperate fruits (Amede *et al.*, 2004). Furthermore, (German *et al.*, 2006) confirmed that the diverse agro-ecology in Ethiopia with diverse soil types and water resources favor the cultivation of temperate fruit trees at large in its highlands. Also in Ethiopia, areas with average minimum and maximum temperature of 4º C and 18º C respectively during winter months favoured successful flowring and fruit setting that resulted in good fruit yields (Ashebir *et al.*, 2010). However, with environments shifting dramatically in climatic conditions in the last decades, drought has become a serious constraint on temperate fruit production and other field crops grown in these highland areas and this requires serious consideration for safeguarding the production of fruit trees and other food crops to secure food insecurity, producers' incomes and nutrition. Detailed studies on fruit trees have been conducted under temperate climate conditions, to quantify the negative impact of droughts and other abiotic constraints in comparison with tropical highlands where little has been done against abiotic constraints including drought and inadequate chilling conditions (Melke, 2015). The possible solutions have been suggested by many researchers, including selection of some adaptable genotypes to the local conditions in respect of genotypes chilling requirement, selection of adaptable rootstocks for drought tolerance and alternatively, breeding new varieties that will cope with drought conditions (Forsline *et al.*, 2003; Liebhard *et al.*, 2003b).

Drought problem in apple growing areas of Ethiopia is compounded with erratic, unpredictable rainfall, high temperatures and high levels of solar radiation during dry season (mainly from October to February) (FAO, 1984). Considering all of these factors, drought stress is still considered as the most limiting factor for achieving sustainable fruit production. Furthermore, the highland areas are characterized by mountainous and dissected topography that lead to diversity in rain fall pattern, microclimate, soil types and other biophysical features that are all contributed to unpredictable changes in climatic conditions that makes the environment vulnerable to drought stress and require physically and culturally appropriate adaptation strategies (Ashebir *et al.*, 2010; Bishaw and Abdelkadir, 2003; Ayele and Tefera, 1999, Unpublished). In this context, developing crop plants with inbuilt drought tolerance

mechanisms is considered a promising strategy for sustainable economic production of fruits in drought-prone environments and is therefore an important goal for plant physiologists and breeders in targeting these areas. Until now, limited or no research has been done regarding abiotic and biotic stresses affecting apple production at Ethiopian highland conditions. This study sought to identify drought tolerant genotypes with desirable fruit yield and quality attributes that contributing to food security and consequently, economic growth. Interest in drought adaptation, in its basic or applied aspects, has been growing recently; because concerns about climate change and water scarcity in agriculture are important reasons for expanding this research in the global context (Blum, 2011; Volk *et al.,* 2009; Yanbao *et al.,* 2006; Yang *et al*., 1996). The study also explored different assessing methods based on the formulated hypothesis and research questions and objectives to assess socioeconomic benefits of apple production and identify drought tolerant genotypes from introduced apple genotypes. This is the first systematic research on apple genotypes introduced to Ethiopia and it will potentially promote apple cultivation with utilization of these genotypes screened based on the present study in accorrance with their yield performance, tolerance to drought and other abiotic and biotic factors that constrained production. Currently, apple production in Ethiopian highlands is increasing rapidly and more new commericial farms being developed in areas where none previously existed. Therefore, characterizing cultivars against abiotic stress tolerance such as drought, inadequate chilling temperature stress, salinity others that negatively contribute to sustain the emerging fruit industry.

1.2 Apple (*Malus domestica* Borkh.): Taxonomic position, origin and distribution

Cultivated apple is a result of natural hybridization of various species of the genus *Malus* Mill., a member of the *Rosaceae* Juss., subfamily *Pomoideae* (pome fruits) (Hummer and Janick, 2009; Webster 2005a; Jackson 2003). Over hundred botanical names have been published for the cultivated apple (Qian *et al.,* 2010). However, *Malus domestica* Borkh. is the most commonly used scientific name, especially in the horticultural sciences. Some morphological characteristics shared by apple cultivars in the world are: woolly pubescence on young stems and on the abaxial surface of the leaves, dull green leaves, elliptic-ovate shape, with irregularly saw-toothed margins, woolly pubescence on flower stalks and calyx, and pome fruits indented at the base with persistent calyx (Hummer and Janick, 2009;Harris *et al.,* 2002). *M. domestica* is thought to have originated in Central Asia; between western to North western China, Uzbekistan and Kazakistan (Hancock *et al.,* 2008), where its primary

ancestor, *Malus sieversi* was found a widespread forest tree in these regions Also, *Malus sieversii* is widespread in the Tien Shan Mountain of China (Velasco *et al.*, 2010) and, is the only wild species that shares all the characteristics of *M. domestica*, in terms of fruit and tree morphology (Fig. 1). Its fruits are highly variable and display the full range of colours, forms and tastes found in cultivated apples across the world.

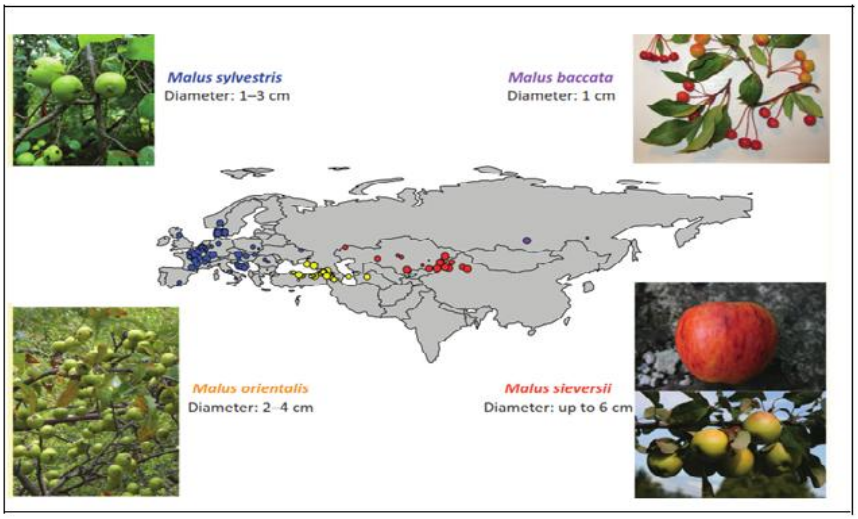

Figure 1.1. Distribution of wild apple as inferred from their geographic origin: *Malus sylvestris* (blue), *Malus orientalis* (yellow), *Malus sieversii* (red) and *Malus baccata* (purple) (Velasco *et al.*, (2010).

Ancient trade routes that linked China to the Middle East and Europe are thought to have facilitated the repeated short and long distance dispersal of *Malus sieversii* to the east and west from its area of origin in Central Asia (Ignatov and Bodishevskaya, 2011). As a result of this movement, hybrids could have occurred to the east with species native to China (e.g., *Malus baccata, Malus mandshurica,* and *Malus prunifolia*) and to the west with European species (e.g., *M. orientalis* and *M.sylvestris*) (Belfanti *et al.*, 2004); but, the native range of *Malus domestica* is difficult to determine, as the species is a product of domestication and multiple hybridizations across the world over thousands of years (Robinson *et al.*, 2001). Its primary ancestor is native to the foothills between western China, Kazakhstan, Kyrgystan, Tajikistan, Turkmenistan and Uzbekistan (Way *et al.*, 1989).

1.3. Genetic diversity of apple

The presence of high genetic diversity of wild apple in central Asia near the Tian Shan mountain range (between north western China, Tajikistan and Uzbekistan) indicates that the progenitor species for domesticated apple includes *Malus sieversii* Roem., *Malus orientalis* Uglitzk., and *Malus sylvestris* L. (Mill.), were originated in these areas (Velasco *et al.,* 2010; Harris *et al.,* 2002). Of these, *Malus sieversii* is native to central Asia where the cultivated apple (*M. domestica* Bork.) originated, and these wild relatives significantly contributed for the present apple genomic study (Cornille *et al.,* 2012). Thus, apple has both a long history as a perennial fruit crop and a continued history of crop improvement up to the present day (van Treuren *et al*., 2010; Yan *et al.,* 2008).

Most species in the genus *Malus* can be readily hybridized (Hancock *et al.,* 2008), and the capacity for inter-species hybridization within the genus *Malus* is evident by the numerous named hybrids among *Malus* species (Korban, 1986). The majority of *Malus* species are diploid and inter-fertile, as there are no apparent physiological or genetic barriers (Conner *et al.,* 1997). Korban *et al.,* (1992) reported that many hybrid species of the genus *Malus* are derived naturally or artificially from inter-species hybridization are recognized as diploid (2n= 2x = 34) and several cultivated types are triploid (Hancock *et al.,* 2008). A study conducted by Kron and Husband (2009) in southern Ontario examined populations of the introduceddiploid *M.domestica* and the native tetraploid crabapple *Malus coronaria,* and found that their geographic ranges and flowering times overlapped sufficiently for cross-pollination to occur. This study also indicates that 27.7% of seed from open-pollinated fruit was found to be of hybrid origin, and suggests that inter-crossing does happen in natural populations that contributed for the potential gene flow from domestic apples into native crabapple populations, but, occurs at low frequencies. Coart *et al.,* (2006) also evaluated hybridization between *M.domestica* and the European wild crabapple *M. sylvestris* by looking at nuclear microsatellites from a large sample of trees, the majority from Belgian forests, and found that 11% of the sampled *M. Sylvestris* trees were of hybrid origin.

1.4. Production trends and some popular apple cultivars of the world

Apple is one of the most widely cultivated perennial fruit crops ranked fourth in the world, following citrus, grape and banana (Hummer and Janick, 2009). When considering production volume in terms of tonnage (FAO, 2014), apple is now one of the top 20 most productive crops in the world. There are more than 10,000 documented cultivars of apples, resulting in a range of desired characteristics, but only a few major cultivars now dominate the world fruit production (Janick *et al.*, 1996), These elite cultivars include McIntosh, (1811s, Ontario, Canada), Jonathan (1820s, New York, USA), Cox's Orange Pippin (1830s, England), Granny Smith (1860s, Australia), Red Delicious (1870s, Iowa, USA), Golden Delicious (1890s, West Virginia, USA), Braeburn (1950s, NewZealand), Gala (1940s, NewZealand) and Fuji (1930s, Japan). The majority of cultivars are diploid (e.g., Fuji, Red Delicious, Golden Delicious, Gala, Granny Smith, Jonathan, McIntosh), while some are triploid (e.g., Jonagold) (Hampson and Kemp 2003; Westwood 1993) and a few are tetraploids (e.g., Antonovka Ploskaya, Wealthy Tetraploidnyi, Papirovka Tetraploidnaya, McIntosh Tetraploidnyi) (Sedov and Makarkina 2008).

1.5. Requisite production conditions

In general, *M. domestica* is considered best adapted to the cool-temperate zone between about 35-50° latitude, in areas with high light intensity, warm days, and cool nights (Rieger 2006; Webster 2005a&b). It has a more northern range than many other fruit crops due to its relatively late blooming and cold hardiness (Rieger 2006; Foster *et al.*, 2003). It is also grown in semi-arid, subtropical and tropical areas, where irrigation, altitude, and various cultural strategies are used to overcome climatic limitations (Hänninen and Tanino, 2011; Hampson and Kemp 2003; Westwood 1993). The main challenge for producing temperate fruit crops in tropical areas is lack of effective accumulated chilling during winter months (Melke, 2015; Lopes *et al.*, 2013; Ashebir *et al.*, 2010), and this requires selection of varieties with low to medium chilling requirements according to the exisiting chilling condition of the location (Tromp *et al.*, 2005; Tustim, 1990).

1.6. Pollination and fruit setting in apple

Flowering occurs in early spring when white to deep pink flowers develop in a cyme-like inflorescence of 4-6 flowers. The centre flower which opens first is often referred to as the "king bloom" (Westwood, 1993), and most flowers are borne terminally on spurs and less

frequently, laterally on long shoots. Flowers borne on short spurs begin the transformation from vegetative buds to flower buds 4-6 weeks before lateral buds (Jackson 2003). The flowers are hermaphroditic with the ovary embedded in the floral cup and flower parts located above the ovary. A normal flower contains five carpels each with two ovules, five sepals, petals and styles and usually 20 stamens (Rieger 2006; Dennis 2003). Flowering can be affected by many biotic (endogenous phytohormones, previous year's crop load, pathogens and pests) and abiotic (light, water stress, nutrients, temperature and exogenously applied chemicals) factors as well as cultural practices including: grafting, pruning, scoring and/or ringing the base of the tree (Jackson 2003).

Most genotypes of of *M. domestica* rely on pollination to produce fertilized seeds but some genotypes can produce unfertilized (apomictic) seeds (Wertheim and Schmidt, 2005; Westwood 1993). The majority of cultivars cannot self-pollinate due to a multi-allelic S-locus (S-RNase) - mediated gametophytic self-incompatibility mechanism (Sassa *et al.,* 1994). Thus, the majority of cultivars display high levels of allelic heterozygosity and thus when propagated from seed, is not true-to-type, in that they are extremely variable and generally bear fruits of poor size, appearance and quality (Webster and Wertheim 2003). Incompatibility genes are sufficiently disparate between cultivars that almost all cultivars are cross-fertile. Much study has gone into this area of research and compatibility lists are generally available (Kemp 1996). For consistent cropping, it is recommended that about 10% of an orchard area be devoted to pollinizer (pollen donor) cultivars. These pollinizer cultivars can be either another compatible apple cultivar or a specialized crabapple pollinizer cultivar (Westwood 1993). Dennis (2003) reported that Crabapples are commonly used as an alternative or additive source of pollen because they are heavy bloomers and provide a large source of compatible pollen. *M. domestica* flowers are predominantly insect-pollinated, mainly by bees when grown as a commercial crop. Mature pollen grains have 3 germinal furrows and are rugulate, having folds or wrinkles (Pratt, 1988). Pollen grains are large and heavy resulting in very little wind pollination. (Jackson, 2003) Pollen fertility of most apple cultivars is close to 100% but is reduced in some cultivars, such as McIntosh, by unknown factors and in others, such as Jonagold, by triploidy. The period of flowering during which viable pollen is produced varies depending on weather conditions and generally lasts from 7 to 30 days. The effective pollination time, the period during which the ovule is capable of being fertilized and the time required for the pollen tube to grow from the stigma to the ovule, varies from 2 to 9 days (Pratt, 1988).

Fruit reach maturity 120-150 days after bloom for most cultivars and weigh about 150-350 grams (Rieger, 2006; Westwood, 1993). Fruit development can be divided into three stages: (i) the first 25 days when petals fall, fruit growth is rapid, embryo in the seed develop slowly and growth is predominated by cell division, (ii) the next 50 days (up to 75 days after petal fall) when the embryo develops rapidly and the fruit approaches its final size with growth predominated by cell enlargement, and (iii) the last roughly 14 days (up to 90 days after petal fall) during which the seed testa turns brown and the fruit enlarges slightly, ripens and, in some cultivars, falls (Pratt, 1988). In cultivation, about 1 to 5% of apple flowers develop into mature fruit. The others fail because of lack of pollination, competition between fruits or cultural practices (i.e., thinning to promote fruit size and quality and discourage biennial bearing). Biennial bearing results from heavy cropping in one year which acts to inhibit flower bud initiation and reduce flowering in the second year (Jackson 2003).

1.7. Drought stress in apple: Physiological and biochemical perspectives

1.7.1. Physiological consequences of drought stress

The primary physiological consequence of drought stress is inhibition of photosynthesis under conditions of stomatal closure (Jimenez *et al.,* 2013), that consequently affect plant growth and development. When drought becomes more severe, the greatest impact is due to photosystem damage from excess light or impairments to photophosphorylation, Rubisco activity, and the regeneration of ribulose-bisphosphate (Cao *et al.,* 2004). During drought periods, the utilization and consumption of absorbed light energy is out of balance and the rate of photosynthesis decreases; because excess excited energy cannot be consumed via CO_2 assimilation (Farooq *et al.,* 2009; Reddy *et al.,* 2004 a&b). Although such excess energy can be partially dissipated through non-photochemical quenching (NPQ), photorespiration, the Mehler reaction, and other processes, plant leaves often undergo photo-oxidative stress that results in an accumulation of reactive oxygen species (ROS) (Flexas and Medrano 2002; Yordanov *et al.,* 2000). If these accumulated ROS cannot be detoxified quickly by the antioxidant systems, photo-oxidative damage can occur (Li *et al.,* 2011). Under such conditions, plants protect the photosynthetic apparatus through various mechanisms, e.g., conversion of the state in the xanthophyll-cycle pigments to dissipate excess light, a means that is considered the most efficient (Sircelj *et al.,* 2007; Yang *et al.,* 1996). Other mechanisms might include enhanced activity by antioxidative systems

(Pastori and Foyer, 2002), and these photosynthesis and photo-protective mechanisms differ among plant species and according to the severity of drought (Bassett *et al.,* 2011; Bartels, 2001). Various biochemical and physiological pathways in plants including sugar and amino acid metabolism, synthesis of different protective proteins, generation of reactive oxygen species (ROS), induction of antioxidant defence system, stomatal closure and suppression of photosynthesis are all triggered by drought stress (Fulda *et al.,* 2011; Razavi *et al.,* 2011; Bartels and Sunkar 2005).

1.7.2. Physiological responses of apple fruit trees to drought stress

Plants adapt to drought conditions through three major mechanisms: drought escape, drought avoidance or dehydration postponement and drought tolerance or dehydration tolerance (Farooq *et al.,* 2009, Blum, 2005). Prior understanding of each mechanism in which plants respond to drought would significantly help plant physiologists to determine the relevant physiological traits for drought adaptation.

Drought escape

Drought escape is expressed as the ability of a plant to complete its life cycle before the development of plant water deficits. It is expressed via phenology; rapid plant development or by adapting the length of the developmental phases to the climate and soil conditions for optimizing the water resources (Jimenez et *al.,* 2013). Furthermore, it drought escape is observed in early- maturing genotypes in agro-ecological regions where the limitations of water occur late in the growing season or in late-maturing genotypes grown in regions where drought occurs early in the season.

Dehydration avoidance (high plant water potential)

High plant water potential revealed the ability of a plant to sustain a high water status or a relatively higher level of hydration under conditions of soil or atmospheric water stress, seemingly unaffected by the water limitation (Šircelj *et al.,* 2007). Accordingly, the different genotypic responses to high rates of evapotranspiration are: (i) reduction of water loss by an increase in stomatal and cutilar resistance, absorbed radiation, or a reduction in exposed leaf area; (ii) ability to access and maintain water uptake either by increased root density and

depth, or liquid phase conductance varied with genotypes. Thus, water savers have low stomatal conductance, and reduced radiation absorption, which contribute to water maintenance in the plant tissues (Nemeskéri *et al.,* 2009), while water spenders develop an efficient root system which taps water from deep layers of the soil (Eghball, and Maranville, 1991)

Drought tolerance

Drought or dehydration tolerance is described as the relative capacity to sustain or conserve plant function in dehydrated state (Blum, 2005; Green *et al.,* 2003). Dehydration tolerance involves cellular activities, such as accumulation of metabolites for protection of cell membranes (osmoprotectants), osmotic adjustments to increase the ability for cells to take up water and enhance cellular activities to ensure maintenance of stomatal conductance and photosynthesis under extreme moisture stress conditions (Manavalan *et al.,* 2009). Dehydration tolerance is a second line of defence, when plants experience prolonged periods of stress. During this occasin, the ability of a plant to endure periods with low-tissue water status (Levitt, 1980), or to postpone dehydration (Jaleel *et al.,* 2009) is exerted via maintenance of turgor through osmotic adjustment, increase in cell elasticity or a decrease in size.

1.7.3. Physiological characterization of drought responses in apple genotypes

Effect of drought stress on apple tree water relations

Apple tree water use has a good correlation to leaf area (Angelocci and Valancogen, 1993). In apple trees in the field, it appears that stomata are well coupled with photosynthesis; usually not opening more than needed to maintain a constant internal CO_2 concentration (Lakso, 1994). This means factors affecting photosynthesis will also affect water loss. According to Lasko (1994), fruits of apples have a very high content of solutes and show increasingly negative osmotic potential as the season progresses. Thus, fruit ψ_w is much higher than leaf ψ_w due to which apple fruits shrink (loss water) during the day as leaf ψ_w becomes more negative and then expands again as leaf ψw begins to increase (Volz *et al.,* 1994). Lang and Volz, (1998) indicated that the two processes in the soil-plant-atmosphere continuum, viz. water up-take by roots and water loss via stomata of the apple trees greatly influence tree water relation and hence orchard water use.

The two key characteristics of apple tree root system of relevance to water relation are the extremely low root length density (RLD) in soil and a very non-uniform root distribution (Lakso, 1994). The implications of these low RLD and erratic rooting are: (i) reduced effective soil volume explored for water and nutrients (specially that of non-mobile nutrients), (ii) less competition (e.g., apple root s are very poor competitors compared to weeds or cover crops) (Merwin and Ray , 1997), (iii) root distribution may concentrate in the wetted zone, especially if nutrients are supplied by fertigation (Neilsen *et al.,* 2000) and, (iv) localized drying in the rhizosphere (interface between roots and soil) during times of high transpiration at midday.

Naturally, apple rootstock root systems have non-uniform distributions and differ in their responses to soil structure and soil moisture deficit (Fernandez *et al.,* 1995), which indicates that apple rootstocks vary greatly in their level of tolerance to water stress (Sircelj *et al.,* 2007). Liu *et al.,* (2012) also indicates that Gala apple scions grafted onto drought resistance rootstocks of *M. sieversii* or *M. hupehensis* roots showed different responses as determined by a number of physiological and morphological traits. This was manifested by a reductions in biomass, growth rate and leaf area under drought conditions, but, *M. sieversii* showed smaller reductions in these traits during drought treatment than *M. hupehensis*. Furthermore, a larger increase in whole plant water use efficiency (WUE) was measured in grafts on *M. sieversii* rootstocks compared to *M hupehensis*. Bassett *et al.,* (2011) also reported that Leaf size and number have been shown to respond negatively to drought, resulting in longer intervals between newly initiated leaves and smaller sizes, all features designed to reduce transpiration to conserve water in *M. sieversii* rootstocks (Richards *et al.,* 2009; Volk *et al.,* 2005).

Stomata may not open at all to prevent water loss from the leaves to maintain turgor and carry on photosynthesis longer into a mature apple leaves to adjust osmotically by as much as -2 MPa or more over time as drought stress develops (Lakso *et al.,* 1984). The osmotic adjustment is due to the accumulation of monosaccharides, especially sorbitol (Lakso, 2004). Water stress could, therefore, impose stomatal and/or non-stomatal limitations of photosynthesis, and often thought to be the first line of defense against water stress (Mpelasoka *et al.,* 2000). Stomatal behavior is also associated with water use efficiency (WUE), defined as the amount of biomass accumulated per unit of water transpired (Bassett *et al.,* 2011). The WUE of seedling apple rootstocks has increased for trees exposed to a water stress treatment (Liu *et al.,* 2012a, 2012b; Ma *et al.,* 2010); when vegetative tissue was evaluated in a field experiment, WUE was 17% higher for the semi-dwarfing genotype

Malling-Merton 106 (MM.106) compared to the dwarfing M.9, and water stress increased the WUE of both genotypes (Liu *et al.,* 2012). Also, Mohammad Reza *et al.,* (2012) reported that in a controlled environment experiment, the more vigorous MM.111 was found to have a higher WUE compared to the dwarfing rootstock B.9.

Shoot-root ratio

Reduced shoot/root ratio is associated with the adaptation mechanism of plants through an extension of their root system to capture more water and at the same time reduce canopy structures including leaves, in order to minimise water loss (Dichio *et al.,* 2002). In apples and related *Malus* species, the ratio of shoots and roots in water stressed environments has been used as an indicator of drought response (Ma *et al.,* 2010). In these species a change in ratio signifies that drought tolerant genotypes partition more dry matter to roots than shoots as an adaptation mechanism during drought stress (Cao *et al.,* 2004). Thus, shoot/root ratio has been recommended as a good characteristic for selecting drought tolerant genotypes, but, it requires a great emphasis for deep rooted rootstocks that an extended root system increased dry matter in roots at the expense of an accumulation of dry matter in harvestable organ (Glenn, 2010).

Drought stress effects on apples fruit growth and development

Naturally, reproductive growth is usually more sensitive to water stress than vegetative growth in many fruit tree crops (Lakso, 2004). The most common fruit response to water stress is the reduction of fruit growth (Ebel *et al.,* 1993) in response to water stress during the early cell-division period and, reduce the potential for good fruit size at harvest. Fruit firmness may increase due to reduction of fruit size by water stress (Mpelasoka *et al.,* 2000), and resulted in increased dry matter, percent soluble solids, delays starch degradation, and lead to earlier ethylene production (Mpelasoka *et al.,* 2000; Ebel *et al.,* 1993) in apples which resulted in low quality fruit. Most calcium uptake into the fruit takes place during the first several weeks of the growing season, and calcium uptake by roots during dry seasons are often low, and calcium related disorders such as cork spot, bitter pit, and internal breakdown in storage resulted in loss of quantity and quality of harvestable fruits (Bohnert *et al.,* 1995).

1.7.4. Physiological parameters affected by drought stress

Stomatal conductance and rate of transpiration

Stomatal conductance is a function of density, size and opening of stomata and it acts as a plant's primary defence mechanism when exposed to drought conditions (Chaves *et al.,* 2003). Drought tolerant genotypes ensure that water loss is reduced through minimal stomatal opening and at the same time allowing carbon dioxide in for photosynthesis (Cruz de Carvalho *et al.,* 1998). Due to its critical role in regulating water and gas, stomatal conductance has been recommended as a reliable parameter in screening for drought tolerance. In apples, significant genotypic variations were observed in stomatal conductance when exposed to drought conditions, providing room for the selection of genotypes adapted to drought conditions (Atkinson *et al.,* 2000 a&b).

Transpiration is directly proportional to the vapour-pressure gradient from the leaf to the air, and inversely proportional to the total resistance to water vapour transport of the air boundary layer and the leaf (Hsiao, 2000). In terms of drought adaptation, genotypes which reduce transpiration, when exposed to drought conditions, show their ability to tolerate drought (Ma *et al.,* 2010). This reduction in transpiration results from reduced leaf area, low stomatal frequency and orientation of leaves, to ensure low radiation loading and evaporative water loss to the environment (Farooq *et al.,* 2009). However, reduction in transpiration, due to reduced stomatal conductance may reflect limited photosynthetic capacity, resulting in reduced carbon assimilation (Cao *et al.,* 2004).

Photosynthesis

Genotypes which maintain high net photosynthesis under water stress conditions generally indicate an ability to tolerate drought conditions (Farooq *et al.,* 2009). High net photosynthesis is also associated with high chlorophyll maintenance under water stress conditions (Bertolli *et al.,* 2012). Therefore, the selection of genotypes with high net photosynthesis due to high chlorophyll concentration and low stomatal conductance may contribute to an improvement in the yield performance of apples under water stress conditions (Caspari *et al.,* 2004). It appears that the impairment of the photosynthetic apparatus occurs at a higher water stress than that which results in stomatal closure (Sinclair *et al.,* 1984). Furthermore, drought stress resulted in a reduction in the activity of RuBP carboxylase, PEP carboxylase, electron transport, photophosphorylation, chlorophyll and protein synthesis (Guo *et al.,* 2006).

Leaf chlorophyll content

Chlorophyll content is positively associated with photosynthetic rate which increases biomass production, and a significant relationship was observed between chlorophyll content, yield and yield components (Farooq *et al.*, 2009). Photosystem II (PSII) is highly sensitive to environmental inhibiting factors and water stress will damage its reaction centers severely. The chemical reaction of PSII is also affected strictly by water stress (Paknejad *et al.,* 2009). Chlorophyll concentration has been known as an index for evaluation of source, therefore decrease of this can be consideration as a non stomata limiting factor in the drought stress conditions. Several studies demonstrated that chlorophyll content is positively correlated with photosynthetic rate (Wang *et al.,* 2008). Increasing the chlorophyll content in crops may be an effective way to increase biomass production and grain yield in cereals Habibi *et al.,* 2011).

Relative water content

Leaf relative water content (RWC) is the amount of water in leaf tissues expressed as a ratio in relation to the maximum amount of water the leaf can hold at the point of saturation (Suriya-arunroj *et al.,* 2004). High RWC indicates the ability of genotypes to retain plant tissue water under drought stress; as in apples (Fernandez *et al.,* 1997), and peach (Bianco, *et al.,* 2000), which indicates wide variation in RWC among genotypes. Apple genotypes showed wide variation in RWC during drought stress that indicated different ability of genotypes in response to drought. Bayoumi *et al.,* (2008) also found a strong positive correlation (0.84) between RWC and yield under water stress in wheat. Considering that RWC strongly correlated with yield in wheat, it can be used as a trait for germplasm selection under drought conditions.

Water potential (Ψ)

In apple fruit trees, water potential revealed the sum of the component potential arising from the effect of pressure (pressure, or (Ψp), solutes (osmotic potential, or (Ψs), and matrix (matrix potential, or (Ψm) (Sircelj *et al.,* 2007). Water potential (Ψ) is referred as a fundamental measure of plant water stress. Jones and Higgs, (1982) indicates that water potential can be described best as the chemical potential of water, as related to the change in Gibbs free energy, as water is added or removed from a system while other environmental conditions remain constant. They further reported that leaf water potentials (Ψ_L) are measured by excision of a leaf followed by its insertion into a pressure chamber. Pressure around the leaf is then increased until xylem sap is exuded from the petiole, and it is at this point where Ψ_L is equal to the negative value of the pressure applied, measured in units of MPa (DaMatta *et al.,* 2003). Accordingly, Midday leaf water potential (Ψ_{MD}), predawn leaf water potential (Ψ_{PD}), and midday stem water potential (Ψ_S) are three of the most common indicators for plant water status.

Numerous studies have compared the three sampling methods. Overall, Ψ_S is more representative of actual plant water status for fruit trees subjected to irrigation compared to Ψ_{MD} or Ψ_{PD}. Midday stem water potential is also a more stable measurement than Ψ_{MD} and Ψ_{PD} because ΨS is influenced by all the tissues the branch, from which the leaf originated, supports (Shackel *et al.,* 1997). As well the leaves selected for Ψ_S are not subjected to the same rapid changes in radiation and transpiration demand that occurs in leaves sampled from the outer canopy for Ψ_{MD} (Mccutchan and Shackel, 1992). Under irrigated conditions Ψ_{PD} corresponds to the wettest region of the soil in contact with the roots, and it does not represent the stress experienced by above ground tissues at midday when demand for water is the greatest (Williams and Araujo, 2002). Shackel *et al.,* (1997) indicates that ΨS is particularly sensitive for evaluating the differences between the water status of fruit trees exposed to non-deficit and deficit irrigation treatments. Midday stem water potential has been positively correlated with irrigation additions in peach, and positively correlated with total yield in apple production (Naor *et al.,* 1999).A comprehensive review by Behboudian *et al.,* (1994) suggested that Ψ_S is currently the best method to measure water status of irrigated fruit trees.

Osmotic adjustment (OA)

Osmotic adjustment refers to the lowering of osmotic potential arising from the net accumulation of solutes in response to water deficits (Turner and Jones 1980). High concentrations of compatible solutes such as, sugar alcohols, quaternary ammonia compounds, proline and tertiary sulfonic compounds in plant metabolic system cause loss of either enzyme activity of membrane integrity that occurs when water is limiting (Schwab and Gaff 1986, 1990). Osmotic adjustment facilitates critical growth functions (root growth, meiosis and pollen development) and metabolic operations (when they require fluxes of water or solutes between cells and organelles). Degrees of adjustment vary with species, cultivars, and different organs of the same plant at the leaves, roots, and reproductive organs (Morgan, 1980 a&b). The degree of osmotic adjustment is influenced by factors such as the rate of development of water deficit, the degree of water deficit per se, genotype per se and other environmental conditions (temperature and light) (Turner and Jones 1980).

1.8. Biochemical, physiological and molecular responses to drought stress

Depending on the severity and duration, drought stress enforces an osmotic stress that leads to turgor loss, disorganization in membrane integrity, denaturing or deactivation of proteins, generation of reactive oxygen species (ROS) and oxidative damage. (Taiz and Zeiger 2006; Bartels and Sunkar 2005; Larcher 2003). These effects consequently result in repression of photosynthesis, metabolic disorders, damage in cellular structure and disruption in growth and development (Krasensky and Jonak 2012; Valliyodan and Nguyen 2006; Reddy *et al.*, 2004). Plants have several strategies to face drought stress (Fig. 2): adaptation mechanisms by which plants can survive under water deficit or avoidance mechanisms by which plants get the specific growth habit to avoid the water shortage (Levitt 1980).

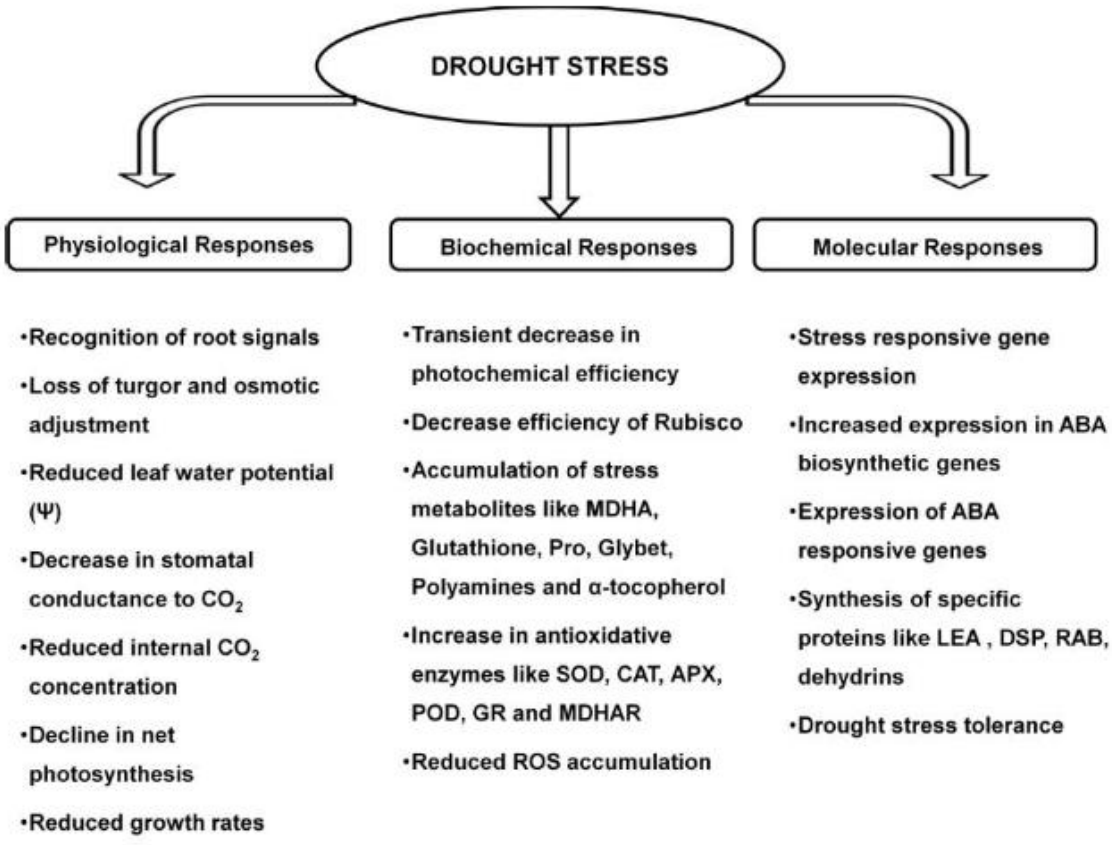

Figure 1.2. Physiological, biochemical and molecular responses of higher plants to drought stress (Adapted from Reddy *et al.*, 2004).

1.8.1. Antioxidant Defense Systems

Super oxide dismutase (SOD, EC 1.15. 1.1) is the metalloenzyme which converts O−• to H_2O_2 and was first demonstrated in maize which contain six genetically and biochemically distinct isozymes (Scandalios, 1993). The upregulation of SODs is implicated in combating oxidative stress caused due to abiotic stress and have a critical role in the survival of plants (Tuna *et al.,* 2008). On the basis of metal ion in its active site, SOD is classified as copper and zinc (Cu/Zn SOD), manganese (MnSOD) or iron (FeSOD) containing SODs. Cu/ZnSOD is located in the cytosol and chloroplast of the plant cell, MnSOD is in the matrix of the mitochondria and peroxisomes (Arbona *et al.*, 2008).

Catalases (CAT, EC 1.11.1.6) mainly localized in the peroxisomes are tetrameric heme containing enzymes which convert $2H_2O_2$ to O2 + $2H_2O$ (Ben Amor *et al.,* 2005; Srivalli *et al.*, 2003). Many plants contain multiple catalase isozyme forms, two in castor bean, six in Arabidopsis (Frugoli *et al.*, 1996), and they can directly dismutate H_2O_2 or

oxidise substrates, such as methanol, ethanol, formaldehyde, and formic acid. Plant catalases can be classified into three classes: class I catalases are most prominent in photosynthetic tissues, and are involved in the removal .of H_2O_2 produced during photorespiration; class II are highly produced in vascular tissues and may play a role in lignification, their exact biological role remaining unknown; class III are highly abundant in seeds and young plants and their activity is linked with the removal of excessive H_2O_2 produced during fatty acid degradation in the glyoxylate cycle in glyoxisomes (Willekens *et al.,* 1994). Catalases are the principal scavenging enzymes which can directly dismutate H_2O_2 and is indispensable for ROS detoxification during stress (Van Breusegem *et al.,* 2001).

Glutathione peroxidase (GPX, EC 1.11.1.9) are the family of multiple isozymes which catalyze the reduction of H_2O_2 and cytotoxic hydroperoxides to alcohols (Dixon *et al.,* 1998). Thus, besides scavenging of H_2O_2, GPxs also serve to detoxify products of lipid peroxidation formed due to activity of reactive oxygen species (ROS). GPxs in plants are classified into three types: selenium-dependent (GPx, EC 1.11.1.19), the nonselenium-dependent phospholipids hydroperoxide GPx (PHGPX), and glutathione transferases (GST, EC 2.5.1.18) showing GPx activity (GST-GPx). These two enzymes GPx and GST differ in their subunits, the bonding nature of selenium at the active centre and their catalytic mechanisms. The substrate for catalytic reaction of GPx is H_2O_2 or organic peroxide ROOH. GPx decomposes peroxides to water (or alcohol) while simultaneously oxidizing GSH.

Ascorbate peroxidase (APX, EC 1.11.1.1) is involved in the scavenging of H_2O_2 in water–water and ascorbate–glutathione cycles and utilizes AsA as the electron donor. APXs (ascorbate peroxidases) reduce H_2O_2 to water and play an important role in the antioxidant system of plants (Kangasjärvi *et al.,* 2008). The APX family consists of at least five different isoforms including thylakoid and microsomal membrane bound forms, as well as soluble stromal, cytosolic and apoplastic enzymes (Noctor and Foyer, 1998). The chloroplastic isoform of APX is very labile and the half life is less than 30 sec in the absence of AsA, whereas that of the cytosolic form is 40–60 min (Miyake and Asada, 1992). APX has a higher affinity for H_2O_2 than CAT and POD and it may have a more crucial role in the management of ROS stress or may be responsible for the fine modulation of ROS signaling (Davletova *et al.,* 2005).

1.8.2. Proline

Proline is a multifunctional metabolite and functions as an osmolyte in osmotic adjustment, a stabilizer of sub-cellular structures (protection of plasma membrane integrity,) and scavenger of free radicals (hydroxyl radical scavenger), it acts as an energy sink, a source for carbon and nitrogen and a stress- related signal (Bartels and Sunkar 2005; Nanjo *et al.*, 1999). Intracellular proline accumulation is a common metabolic response to osmotic stress and P5CS is the key enzyme in this pathway (Perez- Arellano *et al.*, 2010). Proline content under stress is regulated greatly by P5CS, ProDH and P5CDH (A'-pyrroline-5-carboxylate dehydrogenase) which catabolizes A'-pyrroline-5-carboxylate (P5C) under stress (Seki *et al.*, 2007; Yoshiba *et al.*, 1997). The accumulation of proline under drought stress can occur via the glutamate-dependent pathway (Delauney and Verma 1993). L-proline is synthesized from L-glutamic acid via A1 -pyrrolinc-5-carboxylatc (P5C) that is catalyzed by two enzymes P5C synthase (P5CS) and P5C reductase (P5CR). The oxidation of L- proline is another main pathway which controls the proline level and is catalyzed by proline dehydrogenase (ProDH) that converts L-proline to P5C which is converted to L-glutamic acid by P5C dehydrogenase (P5CDH).

1.8.3. Lipid peroxidation (LPO)

The final product of lipid peroxidation expressed as Malondialdehyde (MDA) content has been considered an indicator of oxidative damage (Ge *et al.*, 2006) and it is commonly considered as one of the best physiological components of drought tolerance in plants (Meloni *et al.*, 2003). Low MDA content was always associated with drought stress resistance (Turkan *et al.*, 2005; Bacelar *et al.*, 2007) and also other environmental stresses (Sairam and Saxena, 2000). LPO, products are formed from polyunsaturated precursors that include small hydrocarbon fragments of low molecular weight such as ketones, MDA, etc and these compounds react with thiobarbituric acid (TBA) to form coloured products called thiobarbituric acid reactive substances (TBARS) (Esfandiari*et al.*, 2007). The overall effects of LPO are to decrease membrane fluidity; make it easier for phospholipids to exchange between the two halves of the bilayer; increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels and damage membrane proteins, inactivating receptors, enzymes, and ion channels (Sanchez-Rodriguez *et al.*, 2010).

1.8.4. Proteins

These studies suggest that plants acclimation to water deficit is based on a wide range of metabolic pathways and this adaptation is not limited to a single mechanism (Fig. 3). Drought accumulated products are grouped in two main categories: functional proteins that presumably function in drought tolerance, and regulatory proteins (Reddy *et al.*, 2004; Bartels and Sunkar 2005; Shinozaki and Yamaguchi-Sinozaki 2007).

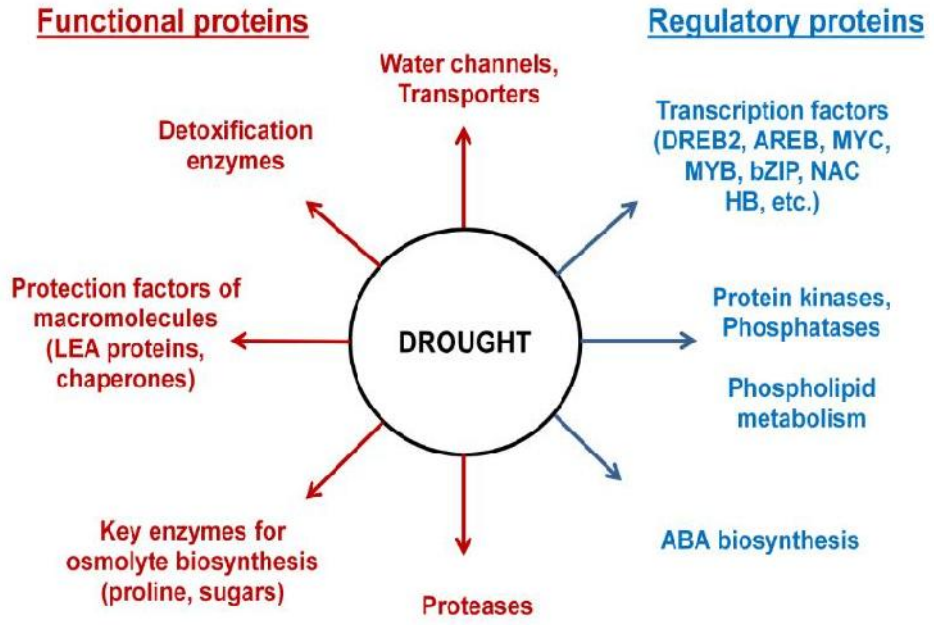

Figure 1.3. Function of drought stress inducible genes in stress tolerance and response (Shinozaki and Yamaguchi-Sinozaki 2007)

Tissues subjected to water deficit generally show a reduction in protein synthesis as measured by amino acid incorporation (Verslues *et al.*, 2006) or by polyribosome analysis (Volaire *et al.*, 1998). Aside the quantitative effect, water stress usually causes qualitative changes in protein patterns (Hajheidari *et al.*, 2005), resulting in the synthesis of stress-polypeptides (stress proteins) as indicated by Vardhini (2014). Drought-inducible proteins are divided into two main groups: abiotic stress tolerance proteins including chaperones, detoxification enzymes, and mRNA-binding proteins; and regulatory proteins such as protein kinases, protein phosphatases, or other signal-related proteins (Wechsberg *et al.*, 1994). Different plant organs (e.g., root, stem, and leaf) contain different drought-inducible proteins and show distinct responses to drought (Navari-Izzo *et al.*, 1990).

2. Hypothesis, research questions and objectives

2.1. Research Hypothesis

Hypothesis 1: Drought tolerant genotypes interact with the environment in terms of phenological, fruit yield and desirable fruit characteristics that may meet the criteria for selection of genotypes

Hypothesis 2: Physiological and biochemical responses of different apple genotypes under field conditions and controlled or glasshouse conditions vary accordingly with many attributes or traits Following these hypothesis, the following questions were formulated:

2.2. Research questions

i. What are the major environmental conditions considered for apple production in Ethiopia? And, which environmental factor affects the phenological and yield behavior of apple?
ii. Which physiological traits involved as an early indicator of drought tolerance in apple?
iii. What are the important criteria for selection of genotypes when considering farmers preferences? And both researchers and farmers evaluation? Also, how do these criteria should meet the adaptability and fruit yield characteristics apple genotypes?
iv. Do variations exist among apple genotypes introduced to Ethiopia in terms of drought tolerance behavior? If variations exist, which physiological or biochemical traits are involved in indicating drought tolerance?

2.3. Objectives

General objective

The general objective of the present study was to evaluate the economic benefits and livelihood impacts, as well as assessing the eco-physiological adaptability and fruit yield performances of eight apple genotypes in five selected highland regions of Ethiopia.

Specific objectives

The specific objectives of the present thesis were to:

1. conduct studies on field performances and socioeconomic benefits of apple genotypes grown in Ethiopia's five distinct geographical locations;
2. examine responses of apple genotypes to drought stress grown under field conditions in Ethiopia's typical central highlands;
3. assess physiological attributes of the studied apple genotypes for drought adaptation by profiling the associated biochemical markers; and, conduct studies on how drought tolerance in apple is associated with antioxidants, lipid peroxidation, protein accumulation and stomatal control.

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CHAPTER TWO

Studies on field performances and socioeconomic benefits of apple (*Malus domestica* Borkh.) genotypes grown in Ethiopia

Abstract

Eight apple genotypes (Anna, Dorsette golden, Princesa, Granny smith, Crispin, Gala, Golden delicious and Red delicious) were selected and evaluated for adaptability, yield performances and impacts on the livelihoods of the apple growers' at five major apple growing districts of Holetta, Debrebirhan, Degem, Hidabu–Abote and Agena. Farmers' preferences to apple cultivars were evaluated through focus group discussions (FGDs) and participatory variety evaluation and selection during the 2017/18 cropping seasons. Both qualitative and quantitative approaches were employed in data collection through use of questionnaire, interview, FGDs and field observations from various institutions and organizations these involved in the research, production, processing and marketing of fruits and vegetables, as well as companies involved in apple fruit imports. The primary data collected from key informants and field studies were collated against the secondary data obtained from the aforementioned institutions and were analyzed using appropriate analytical tools. Phenological data such as number of days from leaf shed to the onset of bud break, number of days to first flowering, number of days to 50% flowering, number of days to first fruit setting; number of days to 50% fruit setting, number of days to first mature fruit, number of mature fruits per tree, average fruit weight (g) per tree were collected and evaluated.

Also, studies on growers-oriented participatory variety evaluation were conducted so as to identify apple genotypes with farmers' preferred attributes such as maturity periods of the genotypes, fruit weight, fruit size and fruit yield (marketable and unmarketable) per tree, fruit color, postharvest quality, number of new spur shoot growth, rootstock preference, as well as tolerance to diseases, pests, and drought. Farmers identified nine desirable attributes, namely, early leaf shedding followed by early bud-break, early flowering and fruit setting, high fruit yields, large fruit size, adequate spur shoot development, good branching, early fruit maturity, resistance to pests and diseases, and drought tolerance at a time of terminal moisture stress. Field studies revealed that the early maturing genotypes Anna, Dorsette golden, and Princesa consistently yielded more fruits at all the tested sites, compared to the

medium maturing Granny smith and Crispin; and the late maturing Gala, Golden delicious and Red delicious. The result of the study also revealed that apple production in the studied areas has a multifaceted contribution to improving farmers' livelihoods, including the construction of better homes, ability to send children to school, changes in dressing and feeding habits, able to lead urban lifestyles in rural settings, as well as improving family income and food security. The present study, however; identified several constraints for local apple production, including land fragmentation and scarcity of agricultural inputs, as well as lack of relevant knowledge on techniques for fruit crop management.

Key words/Phrases: Apple phenology, Chencha, Debrebirhan, Dorsette golden, Kiremit, tropical highlands

2. Introduction

2.1. Suitability of Ethiopia's highlands for apple production

The highlands of Ethiopia are characterized by long growing season, presence of cold temperature during winter months, and availability of fresh water for the production of many temperate fruits and vegetables (EHDA, 2012; Alemayehu *et al.,* 2010; Agonafir, 1991). Among the major fruits grown in highland areas are apple, pear, peach and plum. These are the most common temperate fruits cultivated mainly in small patches in home gardens and in a few private commercial farms (Central Statistical Authority, CSA 2013; Ashebir *et al.,* 2010).

Promoting temperate fruits in highland areas play an important role for income generation and for avoiding nutritional imbalance of the cereal-based dietary system of the population. Fruits supply essential vitamins and minerals that cereal diets are unable to provide as a staple food (Demissie *et al.,* 2009). Also, fruits supply raw materials for local food and juice processing industries, with a potential to be sources of foreign currency (Rolien and Andre, 2009). The development of fruit industry can create employment opportunities, particularly for farming communities (Berhanu *et al.,* 2002). Fruits are versatile products that, depending on need, can be consumed within the household or are sold. Marketing fresh and processed fruit products generates income which can act as an economic buffer for farm households, can generate employment and enable small-scale farmers to embark on a range of production, processing and marketing activities to complement existing income-generating activities (Keyzer, *et al.,* 2000). Girmay *et al.,* (2014) reported the contribution of apple to income and food security status of producer farmers around Chencha district in southern Ethiopia, and observed uneven distribution in the production of apple in the area. The same study indicated that farmers' food insecurity in the district is associated with limited diversification of agricultural products by the farmers.

Production of fruits and vegetables in Ethiopia is scattered throughout the country on patches of land in peasant small holders (Storck *et al.,* 1991). In contrast, the large-scale production and processing of fruits and vegetables is carried out by state organizations, predominantly by state farms (Honja, 2014) and now a days most of these farms are transferred to private sector to improve farm productivity and status of production. These state farms have been carrying out production, marketing and development activities since 1980 (Haji, 2007). Zelleke and Gebremariam (1991) indicated that fruits and vegetable

production are the most important economic activity in Ethiopia, ranging from smallholder farming to large scale commercial farms. Smallholders usually use the largest part of their vegetable produce for home consumption and for sale, while the commercial farms, both state and privately owned, produce solely for the market. According to CSA (2012), about 2,710 million tons of vegetables, root and tubers were produced in the 2010/2011 cropping seasons on 541,000 ha, creating means of livelihood for more than 1 million households. The cultivated crop production area increased by 26%, while the production volume increased by 73% between 2011 and 2013 (CSA, 2013).

A research report from northern highland areas of Ethiopia indicated that growers have benefited from apple cultivation as income source, dietary diversification and, in some hilly areas, for soil conservation due to intensive cultivation of fruit trees (Getachew *et al.,* 2012; Bishaw and Abdelkadir, 2003). However, most of the commercial apple varieties grown in highlands areas are challenged by the lack of adequate low temperatures during the winter months to satisfy their chilling temperature requirements for bud break and flowering (Fetena *et al.,* 2014; Rice and Becker, 1990). Research experiences of the national agricultural research indicates that most temperate fruit cultivars may not undergo complete dormancy due to temperature fluctuations; they experience partial dormancy, unlike the low temperature signal followed by deep dormancy similar to temperate regions (Melke, 2015; Atkinson *et al.,* 2013; Ashebir *et al.,* 2010). Thus, the national agricultural research institute recommended low chill apple cultivars such as Anna and its pollinizer Enshimer for quality fruit production; because these cultivars are easily satisfied by the existing low temperature for bud break and flowering, and able to tolerate temperature fluctuation during the winter months under tropical highland conditions (Foster *et al.,* 2003). As indicated by several research reports and technical working papers (Godfrey-Sam-Aggrey and Bereke Tsehei Tuku, 1986; FAO, 1984), temperate fruits can successfully be produced in highland areas of Ethiopia where there is sufficiently low temperature to break dormancy and resume growth. This low temperature requirement ranges between 0 and 7° C for high chill requiring apple cultivars and between 3 and 8° C for low chill requiring cultivars. However, most of the cultivar introduction focused on low chill requiring cultivars to overcome the high temperature fluctuation during winter months (Njuguna *et al.,* 2004; Godfrey-Sam-Aggrey and Bereke Tsehei Tuku, 1986). Some temperate cultivars can break dormancy at temperatures between 10 to 12° C, but the duration of exposure to such temperatures also varies depending on the species and cultivars of fruit trees and ranges from about 100 hours

to about 1200 hours, where a one-hour exposure is termed as one chill unit (1CU) (Stanley *et al.*, 2000; Cannell and Smith, 1983).

Lopes *et al.,* (2013) and Ashebir *et al.,* (2010) indicated that a delay in chilling temperature requirements due to temperature fluctuation in most of the highland tropics may cause a delay in bud break, blooming and successful pollination, which directly influence fruit setting and fruit development. Thus, selection of low chill requiring varieties such as Anna, Dorsette golden, Princesa and others, that are easily satisfied by the existing low temperatures were given priority for successful fruit production in highland tropics (Lakso, 1996). Similarly, Fetena *et al.,* (2014) made a survey on identification of apple varieties in Chencha, and reported that out of the 60 varieties identified, only seven (Jonagored, Crispin, Gala, BR-64, Granny smith, Red delicious and Golden delicious) are dominantly cultivated in Chencha in respect of their chilling requirements, and these require more chilling units than the low chill requiring varieties. Therefore, the sensitivity of apples to environmental conditions compels researchers to test the performance of genotypes with desirable attributes such as low, medium or high chilling requirements depending on the environmental conditions (tropical or temperate), early leaf shedding, early flowering, less premature fruit drop, early and uniform fruit maturity, tolerant to diseases and insect pests and tolerant to drought under field conditions (Cook and Jacobs, 2000; Jacobs *et al.*, 1981). In selecting desirable attributes in the new environments for successful fruit production, the involvement of farmers in variety selection can influence the adoption rate of new varieties especially by small scale farmers (Marmillod, 1987). Non- or limited involvement of farmers in the cultivar selection may continue to frustrate researchers' efforts, due to an inability to address farmers' needs (Wale and Yalew, 2007). Breeders usually place great emphasis on yield and other agronomic parameters, as opposed to other equally important traits needed or preferred by farmers (Sperling *et al.*, 1993). Generally, farmers would select varieties with desirable utility and adaptation benefits that address their immediate and long-term needs.

In recent years, awareness on the nutritional and health benefits of fruits and vegetables in Ethiopia has been increasing due to public health advocacy on the role of fruits in human nutrition and health through the provision of essential vitamins such as vitamin A, C and E that are important in fighting hidden hunger (i.e., micronutrient deficiency) (UN-OCHA, 2012). Fruit consumption in daily course can protect the health problem caused by a lack of essential vitamins and minerals such as vitamin A, zinc, iron, and iodine in the diet, that the usual cereals-based diet was unable to supply these essential nutrients (Adish, 2012;

Tabor and Yesuf, 2012; Demissie *et al.,* 2009; Abdullah and Ali, 2002; Aklilu, 2000). This partially affirms government's policy of increasing productivity of high value crops with the aim of increasing household income and improving nutrition (Jaleta *et al.,* 2009). The situation also triggered commercial production and boosted private investment in the sector by both national and international entrepreneurs (EHDA, 2010). Moreover, the horticulture industry is labor intensive, could create more rural employment opportunity than other industries (Desalegn *et al.,* 2012). Furthermore, in highland areas with increasing population and declining land size, a better understanding of the production system, marketing channels and endowed opportunities for growth will go a long way to contribute to improve return on investment for value chain actors in fruits sub-sector (AgMRC, 2015; Ayele and Tefera, 1999). The present study was designed to address the following specific objectives: (i) To evaluate some adaptable apple genotypes with desirable characteristics selected by both growers and researchers; (ii) To identify fruit production and marketing constraints and opportunities, and suggest entry points for present and future interventions and investment in the sector; and, (iii) To review the present production experiences and cultivation practices from these apple growing areas and to suggest necessary measures that need to be taken so as to improve the production status of apple in Ethiopia.

2.2. Significance of the study

In many developing countries like Ethiopia where agriculture is dominant, fruit and vegetable culture can play a significant role in improving livelihoods of the society by improving nutritional status and income earning, as well as export diversification. The rationale for the present study was premised on: (i) possibilities for diversifying the cereal crops dominated agriculture in highland areas through integrating fruit culture as alternative sources of foods and income mainly for small-holder farming; (ii) potentials for producing various horticultural crops for local and export markets; (iii) the perennial nature of fruit trees for helping control the chronic problems of land degradation in the extensive highlands of Ethiopia; and, (iv) the potential for creating opportunities for investment in fruit production export purposes.

2.3. Scope of the study

Several research reports indicate that the potential and opportunities for apple fruit production in Ethiopia's highland areas are not well explored, and the benefits derived from the production of the fruit are far from satisfactory (ATA, 2016; Girmay *et al.,* 2014; Lemito and Firdissa, 2012; EHDA, 2012; Demissie *et al.,* 2009; Rolien and Andre, 2007). The current development efforts geared towards horticulture are not well supported by research and extension activities. The present study is designed to help bridge the knowledge gap on cultivar choice and introduction, management of orchards and nurseries, environmental factors that determine fruit crop productivity, as well as assessments of constraints for marketing opportunities.

2.4. Materials and methods

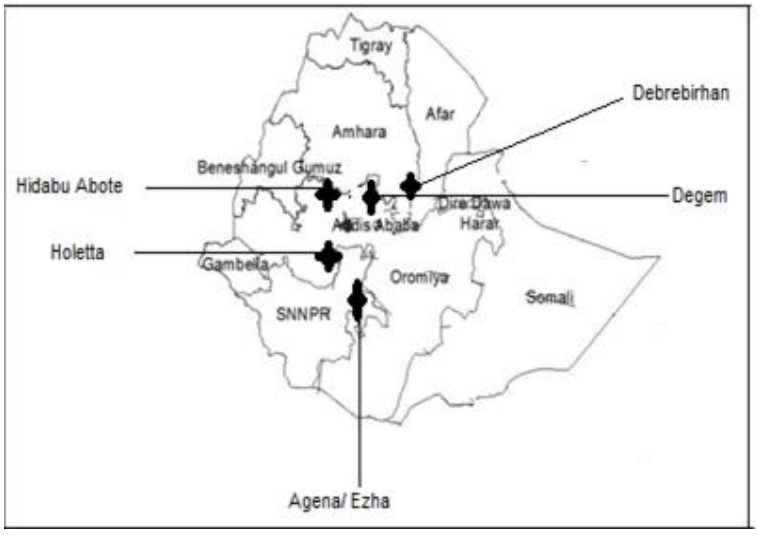
2.4.1. Site characteristics

The present study was conducted at five sites (Holleta, Degem, Hidabu–Abote, Debrebirhan and Agena/Ezha during the 2017/2018 cropping season (Fig. 1.1). The sites were selected based on the introduction of apple tree genotypes in these locations for more than ten years, expansion of production, growers' experience in orchard management and nursery activities, as well as cultivar adaptations to these sites. Detail description on site characteristics such as regional location, altitude, average annual rainfall, annual average temperature (maximum and minimum in °C) on daily basis were shown in (Table 1.1).

Table 2.1: Locations and weather records of the studied sites (2000 – 2015) average

Region	Zone	District (Woreda)	Altitude (m.a.s.l.)	Mean ann. rainfall (mm)	Mean. min. T (°C)	Mean. max. T (°C)
Oromia	West Shewa	Holetta	2400	1100	6	20
	North Shewa	Degem	2600	850	5	21
		Hidabu–Abote	2350	700	8	23
Amhara	North Shewa	Debrebirhan	2800	900	4	19
SNNPR	Gurage	Ezha/Agena	2250	650	10	26

Source: National Meteorology Agency of Ethiopia, 2015



Source: Adapted by the authors (2017)

Figure 2.1: Figure showing the location of study Wereda/sites (Debrebirihan, Holetta, Degem, Hidabu–Abote and Agena/ Ezha).

2.4.2. Informant selection procedures

Hundred home garden apple growers (20 from each Woreda) were selected through discussion with the districts' extension workers and horticultural experts. The apple growers were selected based on: (1) the number of trees they possess (15 trees and above); (2) their experience of apple cultivation for over ten years so as to obtain detailed information about the characteristics of their cultivars, the rootstocks used and other related annual crops cultivated for home consumptions and markets. Fruit growers were briefed about the objectives of the study, and were assured that this documentation would help them improve their present and future productivity. The study involved 93 men and 7 women apple growers. Unfortunately apple growing female farmers were very few in number in all the studied Weredas/Sites), hence the small sample size was used.

2.4.3. Apple genotypes used, graft production and phenology of composite plants

Apple graft production

The apple growers were provided with the genotypes which were propagated by graftage in the fruit nursery sites owned by either the government institution like MoARD (Ministry of Agriculture and Rural Development), NGOs (Non-Governmental Organizations) or private ltd. companies engaged in graft production (e.g., Faji Farm at Debrebirhan). The grafted materials (composite plants) were tagged with cultivar information (i.e., the scions and rootstocks used), and were allowed to grow for one year at the respective nursery sites with the provision of the needed care. The grafts were finally distributed to the growers under the supervision of the experts assigned from the respective district offices until the completion of the planting operation in the respective sites (MoARD, 2005). The apple growers were first trained about the grafts (i.e., the type of rootstocks and the scions used), and about the production packages regarding orchard management practices.

Genotypes of-scion and rootstock used

Genotypes of scions and rootstocks used were selected based on their history of production and adaptability at the tested sites. The survey made in each study site indicated that, of the many introduced apple genotypes to the localities, only few performed well and, consequently, the study focused on these genotypes according to the information gathered from the informants. At present, eight apple genotypes (Anna, Dorsette golden, Granny smith, Princesa, Gala, Golden delicious, Red delicious, and Crispin) were selected based on the growers' preferences. The growers preferred MM-106 rootstock in comparison with other rootstocks such as MM-111, M-7, and M-26; because, the growers believe that MM-106 contributes to better branching habits of the tree, better yield of the composite plant (i.e., scion grafted on MM-106), produce medium tree height, are tolerant to drought (requiring little or no irrigation during dry spells), tolerate apple scab disease (which attack the fruits), and woolly aphid insect pest (which attack all the aerial parts of the tree). Other rootstocks lack all the characteristics possessed by MM-106 as mentioned by the informants. It was also confirmed from the interview sessions that, at present graft productions were widely performed using MM-106 apple rootstock.

Phenological data collection

Phenological information such as leaf shade, blooming period, bloom shade, fruit set and maturity at the peak of each season were recorded following Westwood (1993). For each genotype, a total of twelve individuals of similar age, height, rootstock and similar management at site level were selected and the following pieces of phonological information were recorded: dormancy onset, which was manifested by leaf shedding during Ethiopia's rainy and cold season (Kiremt), from (June-August); chilling requirement (cold temperature requirement starting from Meher) that was between (September-November); dormancy breakage during the dry season (Bega), (December to February) and develop full bloom; new plant growth and leaf development during (Tsedey), (March and April). The new shoot growth, which will bear next year's crop above the pruned scar or above the dormant bud of the selected cultivars were counted and recorded, while water shoots/long shoots were excluded (Hessayon, 2000). Furthermore, the number of mature fruits per apple tree was counted, and average yield was computed following Elfving and Schechter (1993) and Westwood (1993).

2.4.4. Study design and approaches

The primary data were collated against the secondary data collected from the concerned district and zonal agricultural offices, research institutions, agencies, authorities, exporters and importers of fruits and fruit products, and domestic fruit processing companies.

Apple genotypes performance evaluation across locations

Locations of the experimental sites are assigned as replications and genotypes as treatments, replicated three times per site. Each genotype was represented by five trees per plot and fifteen trees per site for individual genotype. A total of 120 trees per site were considered and 600 trees were measured for all the eight genotypes evaluated at each location. The experimental design used was a randomized complete block design (RCBD), and the experimental plants considered were individuals of similar age, height, rootstock and similar management (farmers' management) at site level.

The following traits were measured on a plot basis: number of days from leaf shade (dormancy) to the onset of bud break; number of days to first flowering; number of days to 50% flowering; number of days to first fruit setting; number of days to 50% fruit setting; and

number of days to first mature fruit (Qukabli *et al.,* 2003). The number of days was counted from the start of floral bud break. To evaluate fruit yield per trees, a sample of twelve plants were randomly selected from each genotype per replication at each site was used to measure total fruit set (number of mature fruits) per tree, average fruit weight (g) per tree from ten mature fruits (Ferree, 2000; Westwood, 1993).

2.4.5. Growers oriented participatory variety evaluation of apple genotypes

Focus group discussions (FGDs)

Focus group discussions were used as a tool for participatory rural appraisal (PRA) (King, 2000), to identify key production challenges and desirable traits of apple varieties. Eight to ten farmers at each site were selected for participation in the focus group discussions (FGDs). The selection of these farmers was based on their knowledge of apple production. Farmers were briefed by the agricultural extension staff, in order to set a common understanding among members of the group. After this briefing, a research scientist led the farmers' discussion, by probing farmers on key challenges affecting the production of apples and the desirable attributes of apple genotypes, which could be considered for expansion and up-scaling of apple cultivation. The listed attributes were then scored by each farmer on a scale of 1-5, where 1 represented the most important and 5 the least important attribute.

Participatory variety evaluation

For participatory variety evaluation, farmers were not informed about genotypes with better performance in that location to avoid biased scoring (Byerlee *et al.,* 1981). The farmers involved in the FGDs were also involved in scoring the genotypes for the performance evaluation. A scale of 1-5 was used to score all genotypes for bud break, flowering, fruit setting, maturity, fruit weight, fruit size, color, new shoot growth, tolerance to diseases and pest incidence and tolerance to drought at each location (Madail *et al.,* 2010; Luckwill, 1970). Selection of the attributes was based on farmers' preferences and also on the premise that farmers tend to pay more attention to preference and quality related traits (Wale and Yalew, 2007; Hempson and Kemp, 2003; Sperling *et al.,* 2001; Etissa, 1997). The scoring considers both the growing season and at maturity, that was aimed at identifying genotypes with early

maturity, late maturity, fruit weight, fruit size and fruit yield (marketable and unmarketable) per tree and number of new shoot growth are desirable characteristics preferred by growers (Collins, 2003).

2.4.6. Socioeconomic information

Due to the homogenous nature of population in all the studied sites, the household data collected through interview and FGD informants were collated with the secondary date collected from various institutions (government and private) involved in fruit and vegetable production and marketing.

2.4.7. Statistical analysis

All the measured characters were subjected to an analysis of variance (ANOVA) using the General Linear Model (GLM) procedure in the SAS 9.3 statistical package (SAS Inc. North Carolina, USA). Genotypes were considered fixed effects while sites were considered random effects. Means for genotypes, sites and interaction between genotypes and sites were compared using the Least Significant Differences at 5% alpha level ($LSD_{Q.OS}$)- Pearson's correlation coefficients were calculated to understand the relationship between different variables.

For participatory genotypes evaluation, the data were subjected to the nonparametric Kruskal-Wallis test, also known as rank transformation test (Montgomery, 2013) to compare differences among genotypes. The Kruskal - Wallis test corresponds to a one way ANOVA of parametric data (Corder & Foreman, 2014; Steel *et al.,* 1997). The Kruskal-Wallis test was followed by stepwise step- down multiple comparisons of mean ranks at each site at the 5% alpha level (Campbell & Skillings, 1985). The stepwise step-down multiple comparison returns a sequence of subsets of groups with homogenous characteristics. However, considering that Kruskal-Wallis is a rank based analysis and only analyses ranks for different groups, Montgomery (2013) recommended comparing results from the Kruskal-Wallis test and means of scores from a standard ANOVA; if similar results are obtained then a standard ANOVA is satisfactory. Consequently, the results in this study were analyzed using one way ANOVA in SAS 9.4 (SAS Institute, 2013) and the results from the Kruskal-Wallis analysis are presented in Appendices II - V for comparison. The one way ANOVA used Least Significant Difference at 5% alpha level (LSD 0.05) to compare scores for the eight genotypes. Pattern of selection of genotypes across sites was analyzed using Spearman's rank correlation and interpreted according to Taylor (1990) who classified correlation coefficients into three major groups of low or weak association ($r<0.35$), modest or moderate (0.36 - 0.67) and strong or high (0.67 - 1).

2.5. RESULTS

2.5.1. A Study on phenological characters

Considerable variations were observed among the eight apple genotypes for all the measured characters across locations. Table 2.2. shows mean minimum and maximum values, coefficient of variation (CV) and standard deviations (SD) of all the characters measured. Accordingly, higher coefficient of variations were recorded for the following characters: days from leaf shed to bud break, days to flowering, average fruit weight and average fruit diameter (Table 2.2) across sites. Also, significant variations were observed for days to full fruit setting, days to fruit maturity and number of fruits per tree, while narrow variations were observed for days for bloom shed, days to first fruit setting and new shoot growth (Table 2.2).

Table 2. 2: Mean, minimum (min), maximum (max) values, and coefficient of variations (CV) and standard deviation (SD) for the measured characters at five locations.

Variable	N	Mean	Min	Max	CV	SD
Days from leaf shed (dormancy) to bud break (DLB)	40	56.7	45.7	72.4	20.2	11.4
Days to flowering (~ > 50% flowering) (DF)	40	61.5	42.3	85.6	23.6	14.5
Days for bloom shed (BS)	40	16.3	12.3	25.3	16.0	2.6
Days to first fruit setting (DFS)	40	14.1	10.7	17.7	12.8	1.8
Days to full fruit setting (DFFS)	40	30.1	21.7	35.3	18.7	5.6
Days to ~ > 50% fruit maturity (DFM)	40	95.6	81.4	125.6	19.4	18.5
New shoot growth for the next year's	40	8.9	5.8	15.3	1.4	0.12

Pearson correlation coefficients show a significant relationships ($r > 0.80$) among phenological characters such as days from leaf shed (dormancy) to bud break, days to flowering (> 50% flowering), days for bloom shed, days to first fruit setting and days to full fruit setting (Table 2.3). Similarly, a strong and significant correlation was recorded between yield related characters such as fruit weight and days to > 50% fruit maturity, between fruit

diameter and fruit maturity as well as fruit diameter and fruit weight (Table 2.3). New shoot growth showed weak positive relationships with days to leaf shed, bud break, days to flowering (> 50% flowering), and days to bloom shed. Fruit yield characterstics showed weak negative correlations with other phenological characterstics, because, during fruit development, more of the photo-assimilate is diverted towards fruit growth and development than plant dry matter development (Table 2.3).

Table 2.3: Pearson's Correlation Coefficients of the nine measured characters of the eight apple genotypes studied.

	DLB	**DF**	**BS**	**DFS**	**DFFS**	**DFM**	**FPT**	**FW**	**FD**
DF	0.86**								
BS	0.82**	0.81**							
DFS	0.85**	0.80**	0.82**						
DFFS	0.83**	0.82**	0.91**	0.74**					
DFM	-0.36*	-0.30	-0.38*	-0.26	-0.39*				
FPT	-0.19	-0.17	-0.16	-0.13	-0.19	0.02			
FW	-0.38*	-0.29	-0.37*	-0.25	-0.37*	0.91**	0.13		
FD	-0.35	-0.30	-0.38*	-0.26	-0.38*	0.90**	0.12	0.85**	
NSG	0.53*	0.50*	0.51*	0.-16	0.-11	-0.13	-0.25	-0.12	-0.14

N= 40; **, *: Correlation is significant at the level of 0.01 and 0.05, respectively. **N.B.** Days to (leaf shed (dormancy) to bud break (DLB), days to flowering (~ > 50% flowering) (DF), days for bloom shed (BS), days to fruit setting (DFS), days to full fruit setting (DFFS), days to ~ >50% fruit maturity (DFM), ~ number of fruits per tree (FPT), average fruit weight (g) from ten mature fruits (FW), average fruit diameter (for standard fruit size) (FD), new shoot growth which bear next year's crop (NSG).

2.5.2. Effect of sites on flowering and fruit maturity of genotypes

Days to flowering differed significantly among genotypes and sites (Table 2.4). The results also showed three categories of genotypes for flowering pattern. Anna, Dorsette golden and Princesa took shorter periods (~ 45 days) for > 50% flowering, and ~ 65 days for

> 50% fruit maturity, totaling ~ 110 days from budbreak to fruit maturity across the sites (Table 2.4), while genotypes Granny smith and Crispin showed medium maturity time ranging between ~ 65 for > 50% flowering, and ~ 85 days for > 50% fruit maturity, and took ~150 days from budbreak to fruit maturity (Table 2.4). In contrast, genotypes Gala, Golden delicious and Red delicious took more days, ~ 80 days for > 50% flowering and following ~ 95 days for > 50% fruit maturity, showing late maturity in comparison with other genotypes and took ~ 175 days from budbreak to maturity (Table 2.4).

The result indicates that early maturing genotypes Anna, Dorsette golden and Princesa significantly took similar duration to flower at all the tested sites, while the medium to late maturing type genotypes took different range of days across locations (Table 2.4). The early flowering genotypes did not show significant variations within each site, but there was significant variation between medium and late maturing type genotypes (Table 2.4).

Table 2.4: Mean of main effects (genotype and site) on flowering and maturity periods.

Factor	Days to flowering (> 50% flowering)	Days to> 50% fruit maturity
Genotypes (n=15)		
Anna	44.9	62.5
Dorsette golden	45.3	64.1
Princesa	44.5	62.3
Granny smith	67.3	87.4
Gala	79.9	95.6
Crispin	62.3	86.8
Golden delicious	76.5	93.7
Red delicious	81.3	90.5
P- Value	<0.0001	<0.0001
LSD 0.05	3.9	5.1
CV	11.6	14.7
Site (n = 24)		
Holetta	46.7	58.8
Debrebirhan	51.9	62.5
Degem	49.4	65.3
Hidabu–Abote	53.2	73.8
Agena/ Ezha	58.6	65.0
P- **Value**	<0.0001	<0.0001
LSD 0.05	3.1	3.3
CV	5.6	7.5

The result also indicates that days to maturity showed significant variations among genotypes and sites (Table 2.4). As with the flowering pattern, the maturity period showed the same pattern for early maturing genotypes that genotypes Anna, Dorsette golden and Princesa matured earlier (Table 2.4.), while those genotypes with intermediate to late maturity periods showed variable patterns in maturity periods (Table 2.4).

2.5.3. New shoot growth

New shoot growth of each genotype among locations were studied during Tsedey season (March – April), and showed significant variations among locations and genotypes (Table 2.5). The water shoot, which are rapid growing soft shoot, were excluded because these shoots are un-branched and unfruitful, hence are removed by cutting. Thus, the new wood that develops from the tip of dormant bud that can easily be identified by its greenish color and small flat growth bud, which produces a shoot, was measured.

Table 2.5: Rank correlation coefficients for new shoot growth on MM-106 rootstock of apple genotypes across location.

	Location ranking				
Genotype	Debre birhan	Holetta	Degem	H. Abote	Agena
Anna	0.61**	0.54*	0.71**	0.63**	0.52*
Dorsette golden	0.72**	0.63**	0.53*	0.58*	0.50*
Princesa	0.49*	0.47*	0.53*	0.43*	0.37*
Granny smith	0.25	0.26	0.15	0.21	0.12
Crispin	0.37*	0.22	0.17	0.21	0.15
Royal gala	0.21	0.24	0.15	0.24	0.13
Golden delicious	0.20	0.11	0.23	0.19	0.13
Red delicious	0.17	0.17	0.12	0.17	0.12

Rank correlation coefficient (Table 2.5) showed that, there is a significant positive correlation between the rankings. The new shoot growth rankings are related with the site in which the cultivar grows. For example, Anna, Dorsette golden and Princesa performed better shoot growth (Table 2.5) across locations and ranked high, as compared to Granny smith, Crispin, Gala, Golden delicious and Red delicious which showed weak correlation among locations.

2.5.4. Effects of genotype and site on fruit yield characteristics of apples

Number of fruits and fruit yield varied significantly among genotypes and sites (Table 2.6). Genotypes Anna, Dorsette golden and princesa showed a high number of fruits and fruit yield per tree at all sites, except for the genotype Red delicious which yielded lower number of fruits across locations (Table 2.6). The result also indicates that those genotypes with medium to late maturing types such as Granny smith, Crispin, Gala and Golden delicious produced less number of fruits per tree as compared with the early maturing genotypes (Table 2.6).

Table 2.6: Means of main effects (genotype and site) on number of fruits per tree, fruit yield (kg) per tree and average fruit weight of the individual genotype.

Factor	Mean no. of fruits/ tree	Mean fruit yield (kg)/tree	Mean fruit weight (g)
Genotype (n=15)			
Anna	250.6	27.8	110.07
Dorsette golden	374.8	27.92	74.5
Princesa	115.9	13.6	117.3
Granny smith	103.8	10.12	97.5
Gala	84.5	17.3	85.1
Crispin	185.2	7.19	97.8
Golden delicious	76.5	18.11	80.05
Red delicious	60.1	4.18	76.3
P- Value	<0.0001	<0.0001	<0.0001
LSD 0.05	28.5	2.14	5.85
CV	47.3	13.5	17.8
Site (n=24)			
Holetta	346.5	37.6	108.5
Debrebirhan	233.8	24.22	103.6
Degem	270.6	29.82	110.2
Hidabu–Abote	185.4	17.72	95.6
Agena/ Ezha	156. 9	14.17	90.3
P-Value	<0.0001	<0.0001	<0.0001
LSD 0.05	23.5	2.3	1.2
CV	37.4	11.2	7.4

In terms of site effects on fruit yield, Holetta gave the highest average yield (346.5 fruits/tree), followed by Degem (mean of 270.6 fruits/tree), Debrebirhan (233.8 fruits), and Hidabu-Abote (185.4 fruits); Agena/ Ezha yielded the lowest average yield of 156.9 fruits (Table 2.6). Significantly higher fruit yields per tree were observed for genotypes Dorsette golden (27.92), followed by Anna (27.8), and Crispin (18.11). Fruit weight also varied significantly among genotypes and sites (Table 2.6).

On average, the largest fruit weight was recorded for genotype Princesa (117.3), followed by genotypes Anna (110.07), Granny smith (97.5), Crispin (97.8.) and Gala (85.1) respectively (Table 2.6).

2.5.5. Evaluation of farmers' desirable attributes for apple genotypes based on focus group discussion (FGDs)

Farmers initially identified six attributes of apple genotypes, which influence their choice of varieties to grow (Table 2.7). They favored early maturing and high yielding genotypes, having the capacity to produce heavy and large-sized fruits. Farmers also favored MM-106 since this rootstock fulfills the set criteria, in addition to its ability to tolerate drought and diseases (Table 2.7). However, maturity was split into early and late maturity, while fruit weight was also split into large and small sized fruit, as well as all other characters are evaluated independently with a chosen desirable characteristics. The result inducates that early maturity, high fruit yield, large sized fruit, swetness and MM-106 rootstock were given top priority in all the tested locations.

Table 2.7: Farmers' criteria for selecting desirable attributes of apple genotypes

Genotypes character	Desirable attribute	Frequency	Percent
Time of Maturity	Earliness	65	21.82
Fruit yield	High yield	46	15.44
Fruit weight and size	Large sized fruit	52	17.45
Fruit color	Color	25	8.39
Sweetness content	TSS	55	18.46
Rootstock	MM-106 Rootstock	50	16.78
Missing	-	5	1.68
Total	-	298	100.00

Farmers priority score (Table 2.8) indicates that, all the desirable attributes listed on priority scores in order of top to low priority. Thus, tolerance to post harvest losses and resistance to field pests and diseases are secondly ranked as compared with the top priority criterias, but, varies with location (Table 2.8).

Table 2.8: Farmers' priority scores of desirable traits using a scale of 1-5 during Focus Group Discussion (FGD) by 20 farmers per site.

Trait	Locations					Mean
	Holetta	Debrebirhan	Degem	Hidabu–Abote	Agena	
Early maturity	1.00	1.00	1.00	1.00	1.00	1.00
Fruit yield	2.00	1.00	2.05	1.08	1.00	1.43
Fruit weight	1.00	1.00	1.00	1.00	1.00	1.00
Fruit size	1.00	1.00	1.00	1.00	1.00	1.00
Fruit color	1.00	1.00	2.08	2.00	4.00	2.16
Rootstock preference	1.00	2.00	1.00	1.00	2.00	1.40
Branching habit	1.00	3.00	2.00	2.00	2.00	2.00
Drought tolerance	2.00	1.00	3.00	2.00	3.00	2.20
Postharvest loss	3.00	2.00	3.00	4.00	4.00	3.20
Pest resistance	2.00	3.00	2.05	2.00	2.00	2.21

1 represents high priority and 5 represents low priority

1.5.6. Farmers' prioritized apple traits explored

Farmers recognized for the top four prioritized characters of the eight genotypes namely, early fruit maturity, fruit weight, fruit size and rootstock preference among the eight genotypes (Appendix II – V; Tables 2.8 and 2.9). Also, farmers categorized the eight apple genotypes into three categories, based on the time of maturity i.e. early, medium and late maturing types. Farmers showed a high preference for genotypes with early maturity and large fruit size with the assumption that these genotypes can produce fruit at earlier time to access early market in off season (Table 2.8) Comparison of mean scores for genotypes across sites showed that genotypes Anna, Dorsette golden and Princesa were the early maturing genotypes (Appendix II; Table 2.9). Genotypes granny smith and Crispin were medium maturing types as compared to early maturing genotypes, while genotypes Gala,

Golden delicious and Red delicious were fall into late maturing categories in all the studied locations (Table 2.9).

Table 2.9: Mean scores for eight apple genotypes for maturity, fruit yield, fruit weight, and Rootstock on a scale of (1 – 5) as 1 indicates the highest priority and 5 for the lowest priority.

Genotype	Maturity	Fruit yield	Fruit weight	Rootstock
Anna	1.00±0.02	1.74±0.06	1.56±0.04	1.00±0.03
D. golden	1.00±0.01	1.81±0.06	1.78±0.03	1.00±0.01
Princesa	1.77±0.06	1.52±0.05	2.77±0.09	2.17±0.00
G. smith	2.24±0.08	2.35±0.08	2.21±0.08	2.81±0.08
Crispin	2.36±0.05	3.31±0.07	3.85±0.12	2.00±0.01
R. gala	4.86±0.03	4.82±0.03	2.92±0.10	3.00±0.02
G. delicious	4.84±0.03	4.79±0.03	3.04±0.10	3.00±0.02
R. delicious	4.77±0.03	4.23±0.06	2.54±0.11l	3.45±0.01
Mean	2.30±0.03	2.60±0.02	2.36±0.02	1.90±0.03
LSD 0.05	1.35	5.07	3.23	2.45
CV	8	14	11	9.7

2.5.7. Variation among genotypes based on farmers' preference for the desirable traits

Rank correlations

Spearman's rank correlations of farmers' preferences were significant among all sites for early maturity, fruit weight, fruit size and rootstock preferences that were given high priority (Appendix II – V; Table 2.10), followed by fruit yield per tree and diseases and insect pest tolerance; while postharvest losses and tree branching habits were given low priority (Table 2.10). According to these categories, the measured characters such as early maturity, fruit weight, fruit size and rootstock preferences with correlation coefficients between 0.705 and 0.892, showed strong and significant correlation among all the sites, while fruit yield per tree and diseases and insect pest tolerance showed a significant correlations among all sites, except for Holetta and Debrebirhan, which resulted in a strong correlation ($r = 0.792$, $P=0.05$), followed by Agena and Degem ($r = 0.723$, $P=0.05$) .

Similarly, preferences for postharvest losses and tree branching habit showed moderately significant, but, weak correlations among all sites (Table 2. 10), which indicates less awareness of growers for postharvest management. Both moderate and strong Spearman's correlations indicate that the farmers' selection of genotypes across the sites was statistically consistent, while weak and non-significant correlations pointed towards differences in preferences for genotypes across the sites. Thus, in order of priority of characters, early maturity, fruit weight, fruit size, and rootstock preferences were given high priority, followed by Fruit yield per tree and diseases and insect pest tolerance; while Postharvest losses and branching habits were given low priority (Table 2. 10).

Table 2.10: Spearman's rank correlation coefficients between locations in order of priority of characters

	Holetta	Priority characters	Debrebirhan	Degem	H-Abote
		Early maturity			
Debrebirhan	0.854**				
Degem	0.813**		0.807**		
H. Abote	0.867**		0.705**	0.798**	
Agena	0.754**		0.768**	0.820**	0.735**
		Fruit weight			
Debrebirhan	0.810**				
Degem	0.892**		0.794**		
H-Abote	0.770**		0.861**	0.856**	
Agena	0.824**		0.746**	0.810**	0.751**
		Fruit size			
Debrebirhan	0.895**				
Degem	0.786**		0.864**		
H. Abote	0.805**		0.705**	0.716**	
Agena	0.738**		0.769**	0.765**	0.703**
		Rootstock			
Debrebirhan	0.838**				
Degem	0.801**		0.855**		
H. Abote	0.817**		0.845**	0.832**	
Agena	0.786**		0.823**	0.793**	0.726**
		Yield per tree			
Debrebirhan	0.792**				
Degem	0.669*		0.670*		
H. Abote	0.697*		0.672*	0.582*	
Agena	0.623*		0.561*	0.723**	0.648*
		Disease and pest			
Debrebirhan	0.601*				
Degem	0.632*		0.632*		
H. Abote	0.582*		0.591*	0.501*	
Agena	0.543*		0.511*	0.571*	0.481*
		Postharvest losses			
Debrebirhan	0.345*				
Degem	0.315 *		0.323*		
H. Abote	0.313*		0.291 ns	0.398*	
Agena	0.141 ns		0.285 ns	0.152 ns	0.131 ns
		Branching habit			
Debrebirhan	0.431*				
Degem	0.489*		0.356*		
H. Abote	0.256 ns		0.137 ns	0.121 ns	
Agena	0.421*		0.372*	0.191 ns	0.139 ns

1.5.8. Challenges faced by apple growing communities

FGD informants witnessed that there has been a huge land fragmentation across the studied locations due to the rain-fed dependent traditional mono cropping of cereals and mainly the present booming population pressure (Fig. 2.2), thus, the average household size of the sampled locations falls at 2.34 which is a huge population growth that never seen before. The average national household sizes of Ethiopia is, however, 4.7 and 4.8 respectively (CSA, 2014), which confirms the fast increament of population growth at the studied locations. Therefore, this clearly indicates that there is a high population pressure over a fixed agricultural land in the study area; and it negatively affects agricultural productivity and households' food security.The introduction of apple, therefore, has raised farmers' confidence due to the fact that it is more marketable and secures family income than the conventionally cultivated cereal crops.

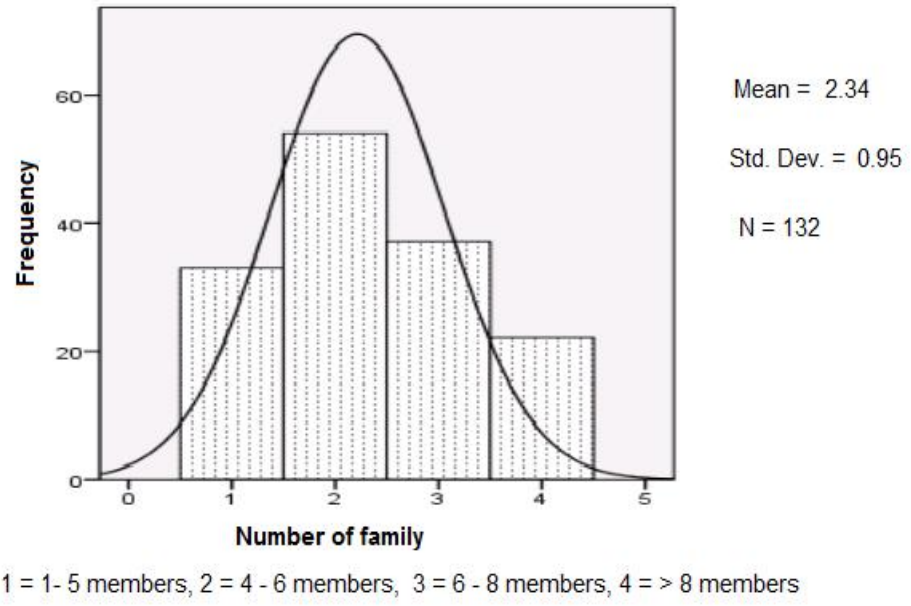


Figure 2.2: Average household size in the studied districts

2.5.9. Apple marketing in the study areas

Interviews with FGD and key informants endorsed that until recently there are three ways of marketing apple in the study areas. These include: through individual farmer households' presentation to the local market; through cooperatives and; through the brokers (Table 2.11). Accordingly, the major modes of marketing in the studied areas are through marketing cooperatives (associations) (56%); and retailing in the market (34%).

Table 2.11: Mode of marketing apple fruit in the study areas.

Mode of marketing	Frequency	Percent
Retailing in the market	45	34.09
Wholesaling from home	5	3.78
Sale through cooperatives	75	56.82
Sale through brokers	5	3.78
Missing	2	1.53
Total	132	100.0

2.6. Discussion

Based on the correlation coefficient values, phenological processes in apple showed a sequential pattern of growth. On the other hand, yield related characters such as fruit maturity, fruits per tree, fruit diameter and fruit weight showed a negative and weak correlation with other developmental processes such as days from leaf shedding to budbreak, days to flowering, days for bloom shed and days to fruit setting . This is because after fruit setting, these developmental processes are dependent on environmental variables such as temperature and photoperiod that further promotes resource competition (assimilation/sink-source) demand of the tree and followed by new shoot growth. The differences in flowering and maturity characteristics among genotypes explain that flowering, fruit setting and maturity times were varied among locations and between genotypes as some of them is early maturing, medium and late maturing respectively. This was mainly associated with the availability of chilling temperature in the locations that was adequate for breaking dormancy, followed by bud break and flowering according to the chilling temperature requirement of the genotypes (Tromp, 1980). Physiologically this was described as the effect of chilling temperature on flowering is proportional to temperature which consequently affects the rate of leaf production and initiation of flower bearing nodes in the season (Tromp, 2005).

Under mild winter tropical conditions, (Cesaraccio *et al.*, 2004) reported that the more the temperature is cooler, the higher the possibility for apple genotypes for ease of entering into dormancy followed by leaf shedding and subsequent chilling temperature requirement necessary for flower bud development and floral initiation; which may have triggered the production of nodes which contributed significantly to first flower buds; while the higher temperatures had the opposite effect, leading to prolonged periods to first flower buds and consequential late maturity (Tromp, 1997). As long as there have been enough chilling accumulates, the flower and leaf buds develop normally, otherwise trees will develop one or more of the physiological symptoms associated with insufficient chilling include delayed foliation, reduced fruit set and increased buttoning, and reduced fruit quality (Petri and Leite, 2004). Webster, (2005) indicates that lack of effective winter chilling is one of the major problems in tropical areas when growing temperate fruits. Similarly Cook and Jacobs (2000), described that warm winters in tropical highlands result in prolonged dormancy leading to poor flowering, very strong apical dominance, unsynchronized growth patterns and, consequently low fruit yield .One of the possible solutions to avoid such problems is using

low chilling requirement cultivars such as Anna (Njuguna *et al.,* 2004; Webster, 2005). Other solution which is beyond the scope of this study is the use of chemicals that show rest-breaking properties on buds (Erez, 2000; Tromp, 2005) that substitute the accumulation of chilling temperature for bud break. Effects of chemicals such as hydrogen cyanamide, (CH_2N), potassium nitrate and winter oil on the bud break of apple trees have been evaluated in Kenya, Morocco and Zimbabwe showed positive responses in effective breaking of dormancy and bud break (Mahhou *et al.,* 2003; Jackson and Bepete, 1995).

Apple genotypes are universally classified based on chilling requirements as low, medium and high chill requiring types. The present study confirmed that these early maturing genotypes such as Anna, Princesa and Dorsette golden are considered as low chill requiring genotypes, because, they can easily satisfied by the existing low temperature of the location during winter months. Conversely, genotypes Granny smith and Crispin showed relatively longer time for leaf fall, entering into dormancy, bud break, flowering and fruit setting when compared to the early maturing genotypes and are considered as medium chill requiring types, which require more chilling temperature; while the other genotypes, Gala, Golden delicious and Red delicious took some longer time than the early and medium chill requiring genotypes to satisfy the chilling requirement for bud break and flowering, and categorized as late maturing genotypes. This may indicates that cultivars are stressed or injured by insufficient cold kiremt season so that they do not fully shed their leaves. In agreement with this Rice *et al.,* (1990) reported that where cold is lacking the apple trees will suffer from a physiological disorder called delayed defoliation. Similarly Duane (1996) reported that inadequate coldness delay the defoliation of apple tree leaves, causing a delay in blooming and leafing in spring. As reviewed by Labuschagne *et al.,* (2002) the chilling requirement of different varieties varies from 200 to 1100 hours, and can be higher according to cultivar requirement and influenced by genetic variation. Similarly, Bernardi (1988) categorized the chilling requirements of the cultivars Granny Smith as intermediate, Gala, Golden Delicious, Fuji and Jonagold as high, however, this varies with locations. Legave *et al.,* (2008) reported that global warming (in France, 1976–2002) resulted in longer mean duration (3–5 days) needed to satisfy the chilling requirement of apple cultivars. The present study also indicates that the apple phenological stages observed in sequence under the condition of the study area were leaf fall in Kiremt, bud break and flowering in Tsedey, fruit growth and maturation in Bega and Belg respectively. In all the studied areas, rainfall is bimodal (National meteorology agency, 2015), in most cases from March to May the area obtains Belg rain and

the temperature becomes low. During this time most of the apple cultivars in the study areas were harvested; and following this, most apple trees shed their leaves and enter into dormancy from June- August. Westwood (1993) described that, after the period of harvesting, apple trees quickly showed yellowing, senescent leaves and probably causes sharp build up of ethylene and absicic acid (ABA) in leaves and buds. During this time of the year (June – August), the mean temperature in all the studied locations were between 1° C to 15° C, and this low temperature contribute to more effective chilling requirement of the genotypes. The sequence of phenological processes indicates that the major period of vegetative growth, blooming and flower ending is in Tsedey, fruit set and maturity in Bega and Belg which coincides with long dry seasons. According to field survey and interviewees response leaf shedding of the apple tree genotypes mostly begin from June or mid of June when the low temperature become 10° C to 11° C or lower and ends in about 60 to 75 days. Thus the chilling requirements of most genotypes seem to be about 400- 600 hrs for low chill requiring genotypes and > 600 hrs until 1800 hrs for medium to high chill requiring genotypes respectively. In agreement with this, Faust (1989) indicates that in the area with above 7°C during winter, apple tree chilling requirement is about 200 to 2000 hrs. depending on the genetic variation among genotypes and the location that the cultivar was placed.

According to the result of this study among the eight apple tree genotypes, Anna, Dorsette golden and Princesa shed its leaves fully in June as the FGD respondent witnessed. It means that the phenology of these genotypes fully coincides with the amount of accumulated chilling present in the locations. The high growth and yield of these genotypes may indicate adequacy of cold kiremt temperature. In agreement with this, Blankenship (1987) earlier described that Anna as Israeli originated cultivar with lower chilling requirement has a better performance in mild winter tropical climate. On the other hand, genotypes Granny smith, Crispin shed leaves from mid June to July, and genotypes Gala, Golden delicious and Red delicious shed leaves from July to August are subjected to delayed defoliation and may indicates that these genotypes are under stress condition due to inadequacy of cold kiremt temperature for these high chill requiring genotypes. This may indicates that insufficient cold kiremt season cause a delay in leaf shedding of genotypes. In agreement with this Rice *et al.,* (1990) reported that where cold is lacking the apple trees will suffer from a physiological disorder called delayed defoliation. Similarly Duane (1996) revealed that inadequate coldness delay the defoliation of apple tree leaves, causing a delay in blooming and leafing in spring.

As observed in the field in this study, sometimes some branches in those late maturing genotypes produce scattered flowers and set only a few fruits and later on some of the fruit dropped prematurely, and that there is little or no yield in the season. In addition to in adequate chilling, these late maturing genotypes were constrained by untimely rainfall which encourage vegetative growth.

Several research reports indicate that apple trees need to be exposed to chilling temperature during cold months of the year. Accordingly, in Ethiopia the main rainy season (Kiremt) months (June – August) and (Meher) (September to November) are the seasons where apple and other temperate fruit trees are exposed to low temperature (Godfrey-Sam-Aggrey and Bereke Tsehei Tuku, 1986) in all the highland locations that satisfy their rest requirement and be able to resume growth the following Spring (Tsedey), i.e. March to April. Thus, apple trees that obtained adequate chilling shed their leaves in Kiremt. Defoliation is therefore, depends on the cold temperature condition of the altitude in which the cultivars were grown.

This indicates that the cultivars flower initiation and bud break depends on the adequacy of cold temperature in which the cultivar grows, and flower initiation started just after shoot growth ceases and when leaves are dropped. The study also confirmed that floral initiation and development of flower buds occur during September of the season and the flowering process occurring in early spring (Tsedey) of the next season in cultivars that grow normally when received adequate chilling. Because, in Tsedey when temperature rises, growth and development of the flowers, took place and immediately followed by foliage development. For example, Genotypes Anna, Dorsette golden and Princesa bloom earlier in September in all the tested locations than other apple genotypes those bloom late in the season mainly between ends of September to November. Therefore, the development of blooming earlier in the season may indicate that, these genotypes receive sufficient chilling, while delayed blooming seems to be inadequacy of chilling and the late blooming genotypes need more chilling temperature than early blooming ones. Thus, delayed defoliation and bud break indicates that the genotype was not sufficiently chilled and manifested by continuous vegetative growth, showing that the cultivar may not be suited to climatic condition of the area.

Depending on Leaf shedding and blooming month, the genotypes are grouped into three categories in such a way that those that bloom in September are considered as early bloom, these which shed bloom in October are the mid season bloom, and November bloomers are considered the late bloom for the studied locations. Similarly, the duration of flowering varies across locations, i.e. two weeks or less for early maturing genotypes, and 2 – 4 weeks for medium to late maturing genotypes. This was accompanied by fruit setting that the fruit set and development determine how well that potential of the genotype is realized, with pollination complete. The informants from all the studied locations indicated that during cold and less frost weather condition, blooming and fruit setting as well as quality of the fruit is high; whereas, at less cold Kiremt and high cold frost Tsedey season, fruit set is very low, this may be due to frost injury or may be due to the restriction of bees (common pollinator) movement due to cold weather condition. The field study realizes that flowering time varies across locations for some medium to late maturing genotypes due to the availability of cold temperature that needed by the genotype for adequacy of chilling, the nature of the chilling requirement of the genotype, and the demand of the genotype for pollen parents; because most of the commercial cultivars of apples need pollen parents for successful pollination and fruit setting, and the climatic condition of the area affect this processes that intern affect the synchrony in time of pollination.

In apple fruit trees, earliness in blooming and maturation is related with adequate chilling while delay or late in blooming and maturation is due to insufficient or stress of cold temperature requirement (Westwood, 1993; Rice *et al.*, 1990). This agrees with the present study in such a way that early maturing genotypes received adequate chilling temperature and showed dense blooming and fruit setting; while a delay in blooming and fruit setting as well as fruit maturing was observed in medium to late maturing apple genotypes. For example, genotypes Anna, Dorsette golden and Princesa took ~120 – 150 days from blooming to harvesting across locations and this showed that there was adequate chilling temperature for these genotypes. The study also indicates that in all locations, these genotypes with medium chill requirement (Crispin and Granny smith) took some more length of days (~150 – 180 days), and the late maturing genotypes also took > 180 days (~ 180 – 210 days) from bloom to harvest. This delay in time of flowering and fruiting in medium and late maturing genotypes indicates that they are stressed by lack of adequate chilling temperature.

Under these conditions, fruit tree cultivars may not undergo their complete chilling requirement, and they rather undergo partial dormancy unlike the low temperature signal followed by deep dormancy (rest) they usually experience in the temperate region (Cook and Jacobs, 1999).

Jackson *et al.,* (1983) described that responses of apple fruit to different environmental conditions (temperature, rainfall, relative humidity of the atmosphere and various soil types) was given prior consideration before starting fruit culture in some location. Similarly, Ashebir *et al.,* (2010) indicates that in most of the tropical highland conditions where apple is growing, introducing low-chill cultivar is recommended for quality fruit production because these cultivars are easily satisfied by the existing low temperature and able to tolerate temperature fluctuation during mild winter conditions. Alternatively, medium chill-requiring cultivars were supposed to grow when supported by hand defoliation followed by dormancy breaking chemicals for better yield and fruit quality (Buban and Faust, 1982). Thus, the variations in ranges for maturity period among sites confirmed that the maturity periods of the genotypes depended on environment (growing location).

The fact that Ethiopia is in the tropics without real winter season makes it difficult to produce some high chill requiring cultivars of apples, thus, cultivars with low to medium chilling requirements are recommended for production, but with some exceptions (~ > 3200 m. a. s. l) i.e. for extreme highlands (Godfrey-Sam-Aggrey and Bereke Tsehei Tuku, 1986). Accordingly, the other problems observed in apples cultivation are incorrect introduction of germplasms by trial and error, as well as placement of cultivars without considering their true ecological niches as identified, based on their chilling temperature requirements (low, medium or high). This calls for characterization of the growing regimes for effective chilling temperature and selection of cultivars adaptable to the specific ecological areas. The study reported that the blossom denseness of genotypes increases when the chilling temperature is adequate for bud break and flowering as in the case of low chill requiring and early maturing genotypes, while blossoming performance of medium to late maturing cultivar is delayed, low in denseness and also a dropped flower buds were commonly observed in the studied locations. This agrees with study of Westwood (1993) who reported that inadequate winter chilling causes the flower buds simply to drop from the tree and have scattered less blossoming, fruiting and leafing. Similarly, Rice *et al.,* (1990) earlier recorded that when there has not been enough cold, blooming of apple trees and leaf production will be sporadic.

Studies conducted elsewhere also indicates that apple tree cultivars that obtain adequate chilling shed their flower after full blooming within two weeks whereas, those stressed by insufficient cold temperature showed a delay in blossom shedding from 3 to 4 weeks (Westwood, 1993; Rice *et al.,* 1990; Ryugo, 1988). In agreement with this, the present study shows that, medium to late maturing genotypes (Crispin, Gala, Granny smith, Golden delicious and Red delicious are stressed by inadequate kiremt cold and resulted in a delayed leaf shedding and blooming, hence, bloom shedding ends within 3 to 4 weeks. This indicates that the growing location is not matched with these genotypes and showing weak growth performance as compared to the early maturing genotypes (Anna, Dorsette golden and Princesa).

The significant correlations among genotypes and between sites for genotypes Anna, Dorsette golden and Princesa suggest the adequacy of chilling requirement of the site favors new shoot growth and these genotypes are better adapted to the studied sites. Genotypes Granny smith, Crispin, Gala and Golden delicious showed low ranking in their shoot growth performance among sites, indicating that these genotypes are poorly adaptable to the studied sites; following the genotype Red delicious that recorded the least ranking across sites as observed in this study. Thus, high ranking is mostly associated with the better adaptability of genotypes for the sites. The number of new shoot growth (this year's growth) which will bear next year’s crop after the cultivars resume growth depends on the adequacy of cold temperature of the area (David, 1983). The present study indicates that growers preferred the cultivars having the ability of early shoot growth in February that helps for early harvesting before the start of Belg rain in April of the next season for better fruit quality at each location. Accordingly, late maturing varieties are less preferred by the growers for quality fruit production, because the trees of these late maturing cultivars are exposed to Belg rain, at the middle of fruit ripening season in April and this situation encouraged the trees for untimely growth of new shoots, which comes against fruit development because of the strong competition for resources between fruit development and new shoot growth (Westwood, 1993; Quinlan, and Preston, 1971). Regarding the emerging of new shoot growth during Tsedey, two types of shoot growth observed, i.e. the water shoot (un branched and unfruitful shoots), which are rapid growing soft shoots, need to be necessarily removed by cutting; and the next new wood that develop from the tip of dormant bud that can easily be identified by its greenish color and small flat growth bud on its surface is considered a bearing shoot

needed to be identified to record the performance (fruiting potential) of each individual genotypes (Hessyon, 2000; Stucky, 1997; Robinson *et al.*, 1983).

Rank correlation showed that the new shoot growth of the eight apple tree genotypes was significant, indicating the new shoot growth was dependant on the prevailing climatic conditions that provide adequate chilling for the genotype as far as its chilling requirement is satisfied. The growth of new shoots varied between sites and among genotypes. Furthermore, genotypes Anna, Dorsette golden and Princesa showed high performance in new shoot growth. This indicates that the cultivars obtain relatively adequate chilling during the kiremt season so that they resume growth in tsedey and perform high new shoot growth; while genotypes Granny smith and Crispin showed less in new shoot growth performance, because the warmer and sunny climatic condition disfavor new shoot growth of these genotypes. As observed in the field, the new shoot growth of genotypes Anna, Princesa and Dorsette golden is long with healthy dark green leaves that coming into spur shoots (bearing shoots), and indicates that cultivars obtain adequate chilling and perform better growth at all the studied sites. Also, the spur with healthy blossom and developing fruit was observed during the field survey of this study for these genotypes.

Fear and Domoto, (1998) reported similar findings that, apple genotypes Golden delicious showed vigorous and good new shoot growth at the higher elevation (3200 m. a. s. l.) which might be due adequate chilling condition at higher altitude. Genotypes Gala and Golden delicious recorded low, and more or less similar new shoot growth, delay in defoliation (delayed until August) indicates that cultivars are stressed by insufficient kiremt cold of the locations during the time of leaf shedding, and showed poor adaptability. Tustin (1990) indicates that apple genotype Gala showed low shoot growth performance when the winter temperature rises, that the cultivars may be injured by inadequate cold temperature which might not be suited for growth. Though the genotype Red delicious is highly stressed by lack of enough kiremt cold, the least new shoot growth was recorded as compared with other genotypes in all the tested sites. This inadequacy can be recognized by the failure of the genotype to produce sufficient new growth in tsedey as the genotype is not suited for these locations.

Farmers at all the studied locations prioritized early maturity, high fruit yield, large sized fruit and the drought tolerant rootstock. However, in Faji commercial farm at Debrebirhan and some model farmers at Holetta and Degem indicate that, besides the mentioned priority, an equal attention was given for the control of postharvest losses and control of diseases and insect pests. The present study indicates that three early maturing apple genotypes (Anna, Dorsette golden and Princesa) have been preferred by the growers. Furthermore, the growing conditions suits for these genotypes for quality fruit production. Because, growing these early maturing genotypes is an adaptation strategy in areas with low chill requiring genotypes, and ensure good fruit yield mainly for market and that secure family income (Salam *et al.,* 2000; Immink and Alarcon, 1993). Thus, the preference criteria identified by the farmers in this study was mainly related with marketability of fruits than family consumption. The study also confirmed that, apple cultivation in these areas can serve as a source of cash alternative to cash making from livestock and its products, as well as from cereals.

The present studies agrees with the study conducted by Jaletta *et al.,* (2009) in central and eastern Ethiopia with regard to vegetable marketing, that vegetable quality characteristics, such as freshness, postharvest quality and early transport to final destination were given high priority, due to high market premiums. Intercropping of annual vegetables (data not shown) with fruits in an area for market and family consumption is a regular practices exercised by growers mainly to reduce land degradation (a decrease in the quality and quantity of nutrients from the soil), and this serves as a better option to improve land productivity through intensive farming; as well as contributed to soil conservation of the farm land (Lipecki and Berbec, 1997).

Differences in farmer scores among sites were observed in all genotypes, and mainly depend on the variation in time of maturity. Genotypes Anna, Dorsette golden and Princesa showed better performance across sites in their fruit yield and yield related quality characteristics such as fruit weight, fruit size; while the medium maturing genotypes (Granny smith and Crispin), and the late maturing genotypes (Gala, Golden delicious and Red delicious) showed significant variation among sites for all the studied characters including fruit yield. The differences in scores among sites for particular genotypes may be explained by the variation in chilling requirement of genotypes (Aggelopoulou *et al.,* 2010; Marguery and Sangwan, 1993).

Yield differences of the cultivars along locations showed that, Anna, Dorsette golden and Princesa have a better yield performance across the sites. Comparison of yields for the early maturing genotypes (Anna, Dorsette golden and Princesa) showed that the chilling condition of the studied areas is adequate for these genotypes with little influence of high night temperature that causes chilling reversal. The low yield performance of the medium and late maturing genotypes indicates that the chilling condition is not sufficient and they are stressed by lack of inadequate chilling temperature. Physiologically, high night temperatures during cold kiremt may cause chilling reversal in these genotypes, leading to a delayed defoliation followed by a non uniform bud break that resulted in a few flowering and poor fruit setting, and /or premature fruit drop later in the season (Oukabli *et al.,* 2003). Furthermore, cultivars unsatisfactory development is attributed to the lack of climatic adaptations, which causes some abnormalities in flower bud differentiation. In a study by (McArtney, *et al.,* 2001), an increase in night air temperature increased flower abortion in a medium to late maturing apple genotypes, while early maturing genotypes maintained significantly higher numbers of flowers under the same conditions.

The low yields of genotypes at Agena/Ezha and Hidabu-Abote, and the high yield at Holetta, Degem and Debrebirhan showed the variation in negative effect of high minimum night temperatures (> 10° C) during cold kiremt, for those medium to late maturing genotypes; as well as the soil conditions and tree canopy management such as pruning and training may influence the yielding potential of genotypes. Petri and Leite, (2004), indicates that if the buds do not receive sufficient chilling temperatures during winter to completely release dormancy, trees will develop one or more of the physiological symptoms associated with insufficient chilling include delayed foliation, reduced fruit set and increased buttoning, reduced fruit yield and quality. While crop load and the genetic biological carrying capacity (source-sink relationships) determine the potential for fruit size development in apples, the environment within which the fruit grows attenuates this potential (Garriz *et al.,* 2000).

This study showing various aspects of intervention needs in the production of apple, such as control of diseases and insects pests, orchard management practices (plant nutrition, irrigation, pruning, training etc.) have remained unsolved and still demands for more advanced research in apple and other temperate fruits. Also the prevailing planting material production exercised by different stakeholders does not meet the required standard for commercial orchard establishment to satisfy the production continuum.

This calls for optimization of propagation techniques, certification of produced materials and enhancing the capacity of planting material producers; as well as socio economic studies such as identification of producer's needs, production constraints, cost benefit analysis and adoption of technologies need to be simultaneously conducted to respond to the needs of growers in various agro-ecologies, commercial farms, and possibly agro-industries in Ethiopia (ATA, 2016; DCG, 2007).

2.7. Conclusion and recommendations

The eight apple genotypes showed significant variations in phenological characters tested at the five sites, which indicate the potential availability of adaptable genotypes with desirable attributes. Genotypes Anna, Dorsette golden and Princesa flowered and matured earlier, compared to genotypes Granny smith and Crispin that showed intermediate responses. In contrast, the late maturing genotypes Gala, Golden delicious and Red delicious showed poor adaptability to the tested locations. Clearly, the growing locations are suitable for early maturing genotypes because these genotypes got enough chilling temperatures for dormancy release, bud-break and flowering, hence resulting in better yield performances. Conversely, the medium and late maturing genotypes were stressed by inadequate chilling temperatures to accomplish their phenological processes. It is concluded that, the tested locations favor low chill requiring and early maturing genotypes than the medium to high chill requiring genotypes. Therefore, the latter genotypes have to be relocated to higher altitudes (i.e., ~ > 2700 m. a. s. l.) for satisfying their chilling requirements.Regarding growers' preferences, the interviewees and FGD participant have identified key challenges associated with the production of apple in the studied areas. The identified challenges require a holistic approach, to cater for all stakeholders involved in the value chain (production to consumption). The improved production of apple should call for creating a policy environment conducive to strengthening research, extension services, marketing, processing and utilization of apple. The farmers have identified desirable attributes, such as early maturity, high fruit yield with good weight and size (standard fruit volume), warm fruit color with longer shelf life, genotype that is tolerant to diseases, insect pests and drought, as well as rootstock which best suits the location. Considering the complexities associated with integrating these desirable attributes into a single variety, streamlined selection of genotypes to particular environmental conditions (chilling conditions) is crucial for better sustainability in production and economic profitability. The present study confirmed that the early maturing genotypes Anna, Dorsette golden and Princesa are better adapted to the tested locations and showed most of the desirable attributes identified by the growers. The low ranked medium and late maturing genotypes need to be relocated to the other higher altitudes where more chilling conditions exist to exploit the full potential of these genotypes.

The majority of sampled households (65%) in all the tested sites confirmed that they produce multiple crops in their agricultural fields. This is a highly recommendable practice which would boost households' income and ensure their food security. Regardless of the challenges faced by the framers, which ranged from apple production (i.e., field preparation, graft production and tree management) to marketing, apple has become a good source of cash to the growers in recent years. Most of the informants in the studied areas have also witnessed the multifaceted importance of apple, including improving livelihoods and securing better income status through sales of fruits and graft planting products. Also, growers acknowledged that apple helped them experience urban life in rural settings (i.e., positive changes in their nutrition and dressing styles, as well as being able to afford for some 'luxury' household goods).

In spite of the overall benefits accrued through growing apple, the present study also found out that there are multifaceted challenges which would reduce farmers' productivity. Some of these include: lack of important agricultural tools such as grafting tools, facilities for nursery operation, limited knowhow of farmers' for graft production and orchard management, land shortage and fragmentation for extensive production due to population pressure, lack of cold rooms for storing harvests, scarcity of boxes for fruit storage, as well lack of coordinated and efficient schemes for apple marketing. Producers and local traders lack reliable market information and support systems. Brokers and wholesalers in the terminal market determine prices and even sometimes refuse buying harvested produce. Due to the perishable nature of produce, producers are forced to become price takers and accept low prices offered by brokers and wholesalers and even in some cases abandon their produce in market places. The largest proportion of respondents (65%) indicated that apple growers suffer from lack of market access and/or poor prices received at farm gate. Because of cereals dominated dietary culture of Ethiopia, local demand for horticultural produce is minimal. Also, the average consumption of fruit amounts to only 1.3 kg/person/year (CSA, 2014), which is far beyond the WHO-recommendation, that is 400g/person/day, or 144kg/person/year for healthy consumption.

Based on the findings of the study, the following recommendations are suggested:

- Improve the technical knowledge and skill of farmers and development agents in apple grafts, production, field management operations such as pruninig and tree traing and crop protection;
- Strengthen research-farmer-extension linkages to select improved varieties adapted to diverse biotic and abiotic stresses and improved management practices so as to overcome the current production pitfalls to address the widely observed yield gaps;
- Develop improved and affordable postharvest handling and storage structures to prolong the shelf life of fruits and to minimize postharvest losses;

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Appendix I: Questionnaire design for data generation on the history of apple production

1. Informant identification:

Name of the informant ---------- Age --------; Sex --------; Occupation --------

Educational status: Illiterate ---------; Primary----------Secondary---; Diploma and above -----

2. Study location:

Region ----------; Zone----------; Wereda (District) ----------

3. When shall apple introduced to your area? ----------

4. How quickly was apple accepted by farmers and consumers? (1= highly accepted; 5 = less acceptance)

4.1. If your answer in # 4 is 1, justify your reason ----------

5. What is the status of your production? Home Gardner ---------- Commercial famer ---------

Private owned enterprise/agro industry ----------

6. Which diseases of apple are common in your area? Please mention ----------;

At which season? ----------

What control measures do you take? ----------

7. Which insect pests of apple are common in your area? ----------

How they are controlled? ----------

8. Do you perform pruning of apple? Yes ---------- No ---------- If yes, please specify the season : Kiremt ---------- Tsedey Bega ---------- Belg ----------

If your answer is no, what is your reason ? 1. I don't know its use ----------2. I don't know the method ---------- 3. Others ----------

9. For what purpose do you grow apple? 1. Home consumption --------- 2. Income source --

10. On phenology of apple:

Genotype	Months		
	June	July	August
Anna			
Dorsette golden			
Granny smith			
Gala			
Crispin			
Golden delicious			
Red delicious			
Princesa			

10.2. Is there any apple genotypes that doesn't shed leaves in *kiremit ?*

Yes ….; No … If yes, specify the genotype and when shall it shed leaves (Table 12.1)

10.3. Enumerate the months of blooming of apples in your garden based on the table 12.1.

10.4. Would you please rate in ranking order the apple trees based on dense blooming?

(1= dense blooming; 5 = few blooming), use table 12.1.

10.5. Rank the apple trees based on their yield performance. (1= high yielding; 5 = low yielding); refer table 12.1. for list of genotypes

11. Please specify apple yield kg/tree and the type of rootstock used?

Genotype	Average yield (kg/tree) from 9 trees per genotype	
	Rootstocks used	Yield (Kg) per tree
Anna		
Dorsette golden		
Granny smith		
Gala		
Crispin		
Golden delicious		
Red delicious		
Princesa		

12. Do you know the type of rootstock you used in your garden? Yes------ No----------

If yes, which rootstock? ---------- Why? ----------

12.1. What/when do you learn about this rootstock? ----------

13. On sustainability and future prospects for the development of apple industry in Ethiopia, please suggest the current and future benefits of apple cultivation in terms of: a) land use; b) farm profitability; c) livelihood assistance; d) crop diversification; e) soil fertility maintenance; and others ----------

Appendix II: Kruskal-Wallis mean ranks of eight apple genotypes for maturity scores at each site and across five sites.

Genotype	site										All sites	
	Holetta		Debrebirhan		Degem		H. Abote		Agena			
Anna	130.5	1	73.5	1	103.0	1	72.5	1	87.3	1	466.8	1
Dorsette golden	130.5	1	73.5	1	103.0	1	72.5	1	87.3	1	466.8	1
Princesa	130.5	1	172.2	2	181.3	2	219.3	2	271.5	2	974.8	1, 2
Granny smith	146.3	2	286.3	4	200.6	3	232.0	3	301.2	3	1166.4	3
Crispin	183.3	3	374.3	5	276.6	5	224.7	2	346.5	4	1405.4	4
Royal gala	199.2	3	210.5	3	236.2	4	462.8	5	417.8	5	1526.5	5
G delicious	220.3	4	361.1	5	200.6	3	403.5	5	413.2	5	1598.7	5
R. delicious	323.9	5	338.3	5	282.5	5	311.9	4	378.3	4	1634.9	5
Kruskal-Wallis H Test	279.4		283.3		270.9		259.9		267.2		965.6	
df	7		7		7		7		7		7	
Sig.	**		**		**		**		**		**	

*Superscripts represent homogenous rank groups at 5% alpha level

Appendix III: Kruskal-Wallis mean ranks of eight apple genotypes for fruit yield scores at each site and across five sites

Genotype	site										All sites	
	Holetta		D.birhan		Degem		H. Abote		Agena			
Anna	173.9	1	136.4	1	114.9	1	189.2	1	178.4	1	792.8	1
D. golden	167.7	1	121.5	1	94.2	1	95.5	1	156.2	1	635.1	1
Princesa	186.4	1	117.8	1	118.4	1	228.2	2	192.6	1	843.4	1
G. smith	183.4	1	297.4	2	276.2	2	336.1	3	312.4	3	1405.5	2,3
Crispin	211.3	2	338.6	3	267.5	2	447.5	4	374.2	3	1639.1	2,3
Royal gala	205.7	2	354.6	3	407.1	4	470.3	4	416.5	4	1854.2	3,4
G. delicious	487.6	4	390.1	4	347.4	3	430.7	4	456.4	4	2112.2	5
R. delicious	510.4	5	625.1	5	578.8	5	529.6	5	611.4	5	2855.3	5
K-Wallis H Test	229.3		242.9		267.9		200.6		235.3		781.2	
df	7		7		7		7		7		7	
Sig.	**		**		**		**		**		**	

*Superscripts represent homogenous rank groups at 5% alpha level

Appendix IV: Kruskal-Wallis mean ranks of eight apple genotypes for fruit weight scores at each site and across four sites.

Genotype	site										All sites	
	Holetta		D.birhan		Degem		H. Abote		Agena			
Anna	133.5	1	130.0	1	128.5	1	124.5	1	142.5	1	659	1
D. golden	133.5	1	130.0	1	128.5	1	124.5	1	142.5	1	659	1
Princesa	172.8	1	137.4	1	243.5	2	218.7	2	256.3	2	1028.7	2
G. smith	220.3	2	247.1	2	326.6	3	361.3	3	315.4	3	1470.7	3
Crispin	368.3	3	379.6	3	385.6	3	347.7	4	392. 3	4	1481.2	3
Royal gala	421.0	4	419.5	4	411.5	4	420.5	4	473.2	4	2145.7	4
G delicious	580.2	5	551.0	5	569.0	5	539.7	5	580.4	5	2820.3	5
R. delicious	555.7	5	569.8	5	556.4	5	577.3	5	516.7	5	2775.9	5
K-W H Test	260.5		259.2		262.8		264.9		271.8		1046.5	
df	7		7		7		7		7		7	
Sig.	**		**		**		**		**		**	

*Superscripts represent homogenous rank groups at 5% alpha level

Appendix V: Kruskal-Wallis mean ranks of eight apple genotypes for rootstock (MM-106) scores at each site and across five sites.

Genotype	site										All sites	
	Holetta		D.birhan		Degem		H. Abote		Agena			
Anna	92.0	1	63.0	1	74.5	1	102.9	1	165.3	1, 2	497.7	1
D. golden	92.0	1	63.0	1	74.5	1	173.5	1, 2	140.7	1	543.7	1
Princesa	203.9	2	211.2	2	125.8	1	163.7	1, 2	178.2	1, 2	882.8	1, 2
G. smith	269.0	2	221.8	2	420.2	4	351.5	3	272.5	2	1635.2	2
Crispin	302.3	3	393.8	3	292.6	2	369.4	3	310.8	3	1668.9	2
Royal gala	448. 8	4	314.1	3	446.9	4	463.8	4	456.7	4	1581.5	2
G.delicious	531.7	5	416.8	4	570.5	5	551.5	5	523.4	5	2593.9	3
R. delicious	547.0	5	494.2	4	571.4	5	515.4	5	511.2	5	2639.2	3
K-W H Test	239.8		262.6		291.8		212.1		251.4		799.8	
df	7		7		7		7		7		7	
Sig.	**		**		**		**		**		**	

*Superscripts represent homogenous rank groups at 5% alpha level

CHAPTER THREE

Responses to drought stress in apple (*Malus domestica* Borkh.) genotypes grown under field conditions at Debrebirhan in central highlands of Ethiopia

Abstract

Six apple genotypes were evaluated for drought tolerance under field conditions at Debrebirhan located in central highlands of Ethiopia at (2800) meter above sea level. The objective of the study was to evaluate performances of apple genotypes for drought tolerance by measuring changes in morphological and physiological responses to induced drought conditions. A randomized complete block design replicated four times with application of water at two levels (control and induced drought conditions) was used. Data on relative water content (RWC), Leaf water potential (ψw), net photosynthesis (Pn), stomatal conductance (Gs) and transpiration (E were collected during (2015 – 2016) growing season where drought spell frequently occurs from October – February. All the measurements were taken, starting from the first week of the drought stress treatment until the end of the experiment. There were significant differences among the apple genotypes studied, as regards the drought treatments for these morphological and physiological parameters. Drought stress significantly decreased growth and development of apple genotypes in all the tested parameters. In particular, strong positive relationship was observed between biomass and water use efficiency ($r^2 = 0.92$), and between biomass and root dry mass ($r^2 = 0.703$). Computed results for drought susceptibility index revealed that, Anna, Dorsette golden and Granny smith were drought tolerant genotypes. These genotypes maintained relatively high Pn, %RWC, leaf water potential (ψw), water use efficiency (WUE), and root dry mass. They were also characterized by low rate of stomatal conductance and transpiration rate. Conversely, genotypes Golden delicious, Red delicious and Royal gala were characterized by high rate of stomatal conductance (Gs) and transpiration (E), and recorded lower values for the other morphological and physiological parameters under conditions of drought stress, and were thus, found to be susceptible genotypes. Based on the correlation analysis, which revealed negative relationships between the tested parameters in comparison with drought susceptibility index; it can be concluded that these parameters can be used for selecting genotypes that are tolerant to drought stress.

Keywords/Phrases: Biomass, Debrebirhan, drought tolerant genotypes, drought susceptibility index, morphological and physiological parameters, and water use efficiency.

3.1. Introduction

Apple (*Malus domestica* Borkh.) is one of the oldest and most important fruit crops cultivated in the world (Jackson 2003; Laurens 1999). Apple cultivation is more extensive in the northern hemisphere, but now a days it has become one the most widespread fruit trees cultivated in mild winter tropical areas (Liu *et al.,* 2012b). According to the Food and Agriculture Organization of the United Nations (FAO, 2014), apple production worldwide has increased considerably in the last twenty years; the amount of apples produced globally is estimated at approximately 50 million tons in 1992 to 80 million tons in 2012 (FAO 2014). Currently, the leading apple producing countries in the world include: China, United States of America (USA), Turkey, Poland and India (FAO 2014). In Ethiopia, the overall country production is estimated to be 50 metric-tons (Girmay *et al.,* 2014). Since the total production does not meet Ethiopia's demand, the nation imports about 350 metric-tons of apple fruits mainly from South Africa, New Zealand, Italy, France, Chile, USA Iran, Turkey, China and Israel, and this showing an unmet market demand for apple fruits (EHDA, 2012). Regarding commercial use, apples are used for fresh consumption and processing, but, a high proportion of the apple globally is used for the fresh market. The robustness of the fruits provides short and long term storage, and makes them suitable for long distance transportation (Webster 2005). In addition, a variety of processed products are made out of apples, e.g. sauces, pastry and cakes, non-alcoholic juices, alcoholic ciders and even apple chips (Jackson 2003).

Apple trees can respond and adapt to drought stress by altering their physiological processes through various defense mechanisms (Massonnet *et al.,* 2007). Drought stress in apples can cause a reduction in plant metabolic activity; thereby resulting a decrease in net photosynthesis by decreasing stomatal conductance and/or non-stomatal limitations, as well as a decrease in carbon fixation and ultimately growth (Lawlor and Comic, 2002; Kramer and Boyer, 1995; Bethke and Drew 1992). Since stomata influence the influx of CO_2 into the leaves, a reduction in stomatal conductance to conserve water inevitably leads to a decrease in the CO_2 concentration in the intercellular spaces. This limitation in supply of CO_2 as a result of drought stress restricts the carboxylation reaction of the enzyme ribulose 1,5-biphosphate carboxylase (RuBisco) during photosynthesis (Medrano *et al.,* 2002; Farquhar and Sharkey 1982).

On the other hand, Hendrickson *et al.,* (2003) indicates that plants may also experience an inactivation of photosynthetic reactions due to biochemical limitations;

specifically plants may experience excess light energy that cannot be used in photochemistry; due either directly to an increase in irradiance or to a decrease in photosynthesis at a constant irradiance during drought **s**tress. This may result in photoinhibition (Osmond 1994) that the maximal leaf photochemical efficiency, which is characterized by sustained depression of the photosystem II (PS II) quantum yield. Under these circumstances, regulation of light harvesting is necessary to balance the absorption and utilization of light energy, thereby minimizing the potential for photoxidative or photoinhibitory damage (Adams *et al.,* 2009).

Genotypic differences in apples have been identified for a range of morphological, physiological and biochemical characteristics (Sircelj *et al.,* 2007); however, the ability of apple genotypes to perform well in variable rainfall and water-stressed environments was highly variable and mainly depends on the response of apple rootstock-scion interactions. Thus, the performances of apple fruit trees, i.e. vegetative growth, flowering, fruit yield, fruit size, fruit quality, storability, and long-term productivity of the tree, are highly dependent on crop water requirements. Most of the studies on the responses of apple tree to drought stress in tropical and sub-tropical mild winter climates were conducted on young trees grown in a container and/or in a greenhouse; but, little is known about the response apple trees to soil water depletion under field conditions. Therefore, this study investigates: (i) the influence of drought stress on morphophysiological parameters of apple genotypes under field conditions; (ii) to compare some important physiological traits, such as plant water relations, gas exchange parameters, biomass and water use efficiency that needed to be examined as an important indicator (s) for selecting drought tolerant genotypes under field conditions; that may be helpful in identifying drought tolerant genotypes for drought prone apple growing areas of mild tropical and semi-tropical environments ; and (iii) assessing the degree of genotypic differences in drought tolerance among the tested genotypes.

3.2. Materials and Methods

3.2.1. Study site and plant materials

The experimental site is located at Debrebirhan in central highland of Ethiopia at (2800) meter above sea level and receives annual average rainfall of (900 mm) during the main rain season (between June and September) (National Meteorology Agency, 2014). The mean daily minimum and maximum temperatures of the location range from (4-10^{o} C) during the rainy season in winter and (17-19^{o} C) during dry season respectively. The soil is classified as a vertisols with (pH 6.5) and its texture is clay loam under the USDA soil taxonomy classification system (Boul and Mccracken 1980) with soil profile deeper than 1 m. The experiment was conducted between July (2014) and February (2016) on 1-year-old seedlings of six apple genotypes (Anna, Dorsette golden, Granny Smith, Royal gala, Golden delicious and Red delicious) grafted on MM.106 rootstock. The grafted plants were established in Faji Temperate Fruits and Related Product Development Farm at Debrebirhan (Table 2.1). The genotypes selected are clonally propagated through stool bed sucker multiplication of the rootstock materials and transplanting of the rooted sucker to the propagation nursery for grafting of scion materials. The grafted seedlings were kept for a year until it was reached to the standards for orchard plantation under open field nursery located at Chencha (2800 m. a. s. l. @ 37 0 28'00" to 37 0 40'00"East and 6 8'55" to 6 0 25'30" North, with annual rainfall of 1150 mm), one of the popular apple growing areas in southern Ethiopia. These were then transferred to the experimental field in July 2014 for drought stress treatments.

Table 3. 1: Description of apple genotypes evaluated, including their origin, parental cross, tree characters and chilling behavior of tree (Westwood, 1993)

Genotype	Parentage/Pedigree	Tree character	Chilling behavior	Origin	Year developed
Anna	A cross between the local cultivars Red Hadassiya (a plum-sized apple) and Golden Delicious.	Moderately vigorous, precocious, goodcropper. Prefers hot summers, tolerates mild winters and considered the standard in low-chilling apples	Low chill	Israel	1965
Dorsett Golden	Grown from chance seedling of Golden Delicious. One of the most southerly apples grown in North America.	Moderately vigorous, precocious, good cropper, fruits in the tropics where no winter chilling occurs. Prefers hot summers, tolerates mild winters	Low chill	Bahamas	1964
Granny smith	A hybrid of Malus sylvestris, the European Wild Apple, with the domestic apple M. domestica as the pollinizer	Moderately vigorous, precocious, biennial cropping, heavy cropping and Prefers hot summers.	Medium chill	Australia	1968
Royal Gala	Cross of three of the world's best known apples: Kidd's Orange Red (a cross of Red Delicious and Cox's Orange Pippin) × Golden Delicious. One of the most widely available commercial fruit	Moderately vigorous, biennial cropping, good cropper	High chill	New Zealand	1940
Golden delicious	Seedling of Grimes Golden. The original tree was found on the Mullins' family farm in Clay County, West Virginia, United States and was locally known as Mullin's Yellow Seedling and Annit apple.	Moderately vigorous, spreading, biennial, heavy cropping and tolerant of late spring frosts.	High chill	USA.(Virginia)	1914
Red delicious	Original seedling known as "Hawkeye." (Bisbee Red Delicious), and first marketed as "Delicious" in1880	Moderately vigorous, spur bearer and heavy cropping.	High chill	USA (Iowa)	1880

3.2.2. Drought stress treatments

The grafts were planted (3m x 4m) spacing, trained in a central leader training method and received routine horticultural care until establishment. The trees were uniformly irrigated three times a week at peak dry season from October to February, and once a week from March to May when rainfall is moderate during the year of establishment in 2014. At each irrigation session, watering was stopped when the top soil near to the base of stem was wetted at ~ 5 cm depth on average at a time that enables normal seedlings growth. In 2015 cultivars were evaluated under two levels of irrigation; including a full (normal environment) and a deficit irrigation (intense stress environment). During (2015 – 2016), the normal irrigation treatment was continued with no limiting and was done when 20 % of the total available moisture was depleted from the root zone, while in intense stress irrigation 50 % of the total available moisture was depleted from the root zone (Allen *et al.,* 1998). Soil moisture was measured every week to compensate depleted water from stressed plants to keep the moisture level at ~ 50%. The moisture level for both control and stressed treatments was detected using the standard gravimetric methods (Clarke *et al.*, 2008) at depths of 0–20 cm. The moisture level was calculated as the difference between the weight of wet soil after full irrigation and the weight of the dry soil; i.e. 100 g of soil, that was taken from the middle of the soil near to tree trunk without disturbing the roots and the soil was oven dried at 105° C for 48 h. Thus, soil water content (%) = [(FW – DW)/DW] x 100]. Drought stress treatment under field condition was applied when there was no or sometimes little rain at the experimental location during drought seasons; from the mid October to the end of February at each year.

3.2.3. Data collection

Leaf relative water content

Midday leaf relative water content (RWC) was measured at a week interval throughout the experimental period. Six fully expanded leaves were collected at the mid-canopy position from two trees of each genotype and treatment group, and then placed in dishes containing filter paper wetted with 10 ml of deionized water; then weighed immediately for their fresh weight (FW). Turgid weight (TW) was determined from leaves floated for 24 h in distilled water in a closed container at 4^{O} C under darkness. Dry weight (DW) was determined for the same leaves after oven-drying for 48 h at 70^{O} C. RWC was calculated as: RWC (%) = [(FW-DW)/ (TW-DW)] x 100] Cook *et al.,* (2009).

Water-holding capacity (WHC) was also determined as the amount of water removed during the drying process divided by the final DW as: WHC (%) = (TW-DW)/DW x 100.

Leaf water potential (Ψw)

Leaf water potential (Ψmd) was estimated using a pressure chamber (Soil Moisture Equipment Corp., Model 3005, Santa Barbara, CA, USA). On each sampling date, Ψ measurements were taken from the terminal twigs having fully mature sun-exposed leaves monitored at midday (Ψmd; 12:00 to 13:00 hrs.), following the recommendations of Hsiao and Xu (2000) to prevent leaf water loss during measurements, that twigs were placed in a small black plastic bag covered with aluminum foil before measurement with a pressure chamber within less than 1 hr after collection. Twigs were taken from two selected plants per plot and treatments instead of leaves, because leaf petioles of apples are highly delicate and did not protrude beyond the rubber seal of the pressure bomb. Therefore, in order to standardize the measurements, shoots bearing three leaves were used in all genotypes instead of single leaves. Pressure was applied to the chamber at a rate of 0 .05 MPa s^{-1} (Turner 1981).

Leaf Chlorophyll content (SPAD) measurements

The leaf greenness or the relative chlorophyll content was measured by using chlorophyll meter (SPAD meter; Model SPAD 502, Minolta, Japan). The meter makes instantaneous and non-destructive readings on a plant based on the quantification of light intensity (peak wavelength: approximately 650 nm: red LED) absorbed by the tissue sample. A second peak (peak wavelength: approximately 940 nm: infrared LED) is emitted simultaneous with red LED for to compensate the thickness leaf. Chlorophyll relative content (SPAD unit) was determined in the flag leaf, and eight readings were taken per plant. Then, total chlorophyll content (µg ml^{-1}), obtained through SPAD units.

Gas exchange measurements

Net photosynthetic rate (Pn), transpiration rate (E), and stomatal conductance (Gs) were measured periodically throughout the study on fully expanded leaves (four leaves per plant on the third or fourth pair of leaves from the tip of a shoot) with the LC-pro- Leaf Chamber Analysis System (Li-Cor, Lincoln, England). Measurements were taken on clear days between 11:00 and 13:00 hrs. The daily course of gas exchange was taken on the same leaf during the day. Instantaneous water use efficiency was calculated by the ratio A/E.

Growth related physpological parameters

Leaf area was determined using a Portable leaf area meter model 11-3000 (LI-COR, Lincoln, Nebraska, USA). Different leaf characteristics were measured during and at the end of the experiment. Total leaf area (TLA) per plant was estimated as the sum of the leaf area of a plant measured with a leaf area meter. Leaf dry weight was determined after drying for 24 hours at 80^{o} C (Turhan *et al.*, 2005; Pirlak *et* al., 2004) to determine specific leaf area (SLA), which was calculated as (TLA/ TLDW). Growth and biomass were measured at the end of the experiment for both control and stressed plants. Plant height was measured from 5 cm above the soil surface near to the trunk to the highest active bud for growth, and biomass measurement was taken for different parts of the plant (above and below ground) in a fresh and dry forms. Roots were well washed using a micro-mesh sift and water, and roots, shoots and stem were oven-dried (48 h @ 75^{o} C) and dry weight was determined for three grafts per treatment. The number of branches was recorded for each genotype once a week after imposing drought stress. Then the average number was determined for each apple genotypes. The stem diameter of each genotype was measured with a digital caliper at 15 cm above soil surface. Measurement was taken at each sampling date; the diameter of the plants of each genotype was calculated.

Leaf senescence

Plants were scored for leaf senescence on a scale of 0 - 10, dividing the percentage of estimated total leaf area that is dead by 10 as described by Bänziger *et al.*, (2000), as follows:

1 = 10 % dead leaf area	6 = 60 % dead leaf area
2 = 20 % dead leaf area	7 = 70 % dead leaf area
3 = 30 % dead leaf area	8 = 80 % dead leaf area
4 = 40 % dead leaf area	9 = 90 % dead leaf area
5 = 50 % dead leaf area	10 = 100 % dead leaf area

The first scoring was done at second weeks of stress treatment when some of the leaves had initiated senescing till end of the experiment. This date was also appropriate for taking leaf senescence scores because an array of different leaf senescing levels could be observed. Scoring was done at five days interval on sunny days between 13:00 and 14:00 hrs. on three occasions. The scoring was done only on drought stressed apple plants, because control plants are provided with full irrigation and under normal growth condition.

Water Use Efficiency (WUE)

Prior to every irrigation session, each pot was weighed and the weight differences (kg) were converted to volume (ml). The values obtained for each pot represented the volume of water applied to that particular pot at that period. The average volume of the water used rate was determined for each genotype. The water use efficiency based on biomass was calculated according to Larcher (2003) as follow:

$$\text{WUE (g/kg)} = \frac{\text{Biomass (g/plant)}}{\text{Water used rate (kg/plant}}$$

Drought susceptibility index (S)

Drought intensity based on biomass, water use efficiency, relative water content, plant height, number of leaves per plant, stem diameter and root dry mass was first determined using both water stressed and well-watered conditions. This was done for the six apple genotypes separately. Subsequently, drought susceptibility index (S) based on relative biomass; water use efficiency (WUE) and relative water content (RWC) of drought stressed to well-watered conditions were estimated as described by Fischer and Maurer (1978).

$$D = 1 - X_s/Xw \qquad \text{[Equation 2.1]}$$

Then the drought susceptibility index (S) of individual varieties was

calculated: $Y_s = Y_w (1 - SD)$ [Equation 2.2]

$$S = \frac{(Y_w - Y_s) X_w}{(X_w - X_s) Y_w} \qquad \text{[Equation 2.3]}$$

Where;

D = Drought intensity

X_s = Average dry matter yield under water stress condition

X_w = Average dry matter yield under well-watered condition

Y_s = Individual dry matter yield under water stress condition

Y_w = Individual dry matter yield under well-watered condition

Genotypes with average susceptibility to drought have an S value of 1.0. Values of S less than 1.0 indicate less susceptibility and greater tolerance to drought, with a value of S = 0.0 indicating maximum possible drought tolerance (minimal drought effect on vegetative growth and development).

3.2.4. Statistical analysis

Six apple genotypes were evaluated under two levels of watering regime including a full irrigation (normal condition) and deficit irrigation (stress condition) using a randomized complete block design (RCBD) with four replications. Each plot contained six plants (three plants for each treatment group per plot). The data were subjected to analysis of variance (ANOVA) procedure for a randomized complete block design, followed by post hoc multiple comparisons using the Tukey test and LSD at $P \leq 0.05$; or $P \leq 0.01$ was used to identify significant differences among genotypes. Values were expressed as mean ± SD of the four replications. Individual means of water-stressed genotypes were compared to their corresponding non-stressed in a pairwise comparison analyses (t-test) and LSD @ $p \leq 0.05$ was used to determine differences in treatment means. Data analysis was performed with SAS statistical package (SAS Statistical Institute, Cary, NC, version 9.4).

3.3. Results

3.3.1. Plant water relations

Relative water content (RWC) showed highly significant variations among genotypes after the second and third weeks of stress (Table 3.2). At the second weeks of stress, the values for RWC ranged between (0.71.8±4.5) for genotype Red delicious and (0. 84.3±1.84) for genotype Dorsette golden; while at the third weeks of stress, the values for RWC ranged between (0. 41.1±2.4) for genotype Red delicious and (0.81.5±2.0) for genotype Anna ($P \leq 0.05$). After the third week of drought stress higher relative water content of 82% was recorded by genotype Anna, followed by Dorsette golden which recorded (74%). Genotype Granny smith showed better performance in RWC (66%) in comparison with other genotypes having lower values for RWC including Genotypes Royal gal (54%), Golden delicious (48%) and Red delicious (41%) respectively (Table 3.2). Furthermore, no significant differences were observed from the first to third weeks of experimental period in well watered plants for RWC (Table 3.2).

Plants kept in intense stress level (50% moisture depletion) showed a significant decrease in leaf water potential continuously, which after the first week of stress until third weeks and reaching minimum values, for genotypes: Anna, at (-2.17±0.11), Dorsette golden (-2.35±0.05), Granny smith (-2.45±0.20), Royal gala (-2.35±0.05), Golden delicious (-2.56. ±0.16) and Red delicious (-2.64±0.19) respectively (Table 3.2). The control treatment did not show significant changes in leaf water potential with the period of drought stress (Table 3.2).

Table 3.2: Effect of drought stress under well-watered and intense drought conditions on RWC and Ψw under field conditions.

Relative water content (RWC, %)

	Week (1)		Week (2)		Week (3)	
	Well watered	**Stressed**	**Well watered**	**Stressed**	**Well watered**	**Stressed**
Genotype						
Anna	82.4±7.7	80.7±2.4a	87.4±1.0	81.0±1.8 a	91.2±1.7	81.5±2.0 a
D. golden	85.8±5.1	84.8±2.7a	88.9±1.7	84.3±1.3 a	86.6±1.4	73.9±1.7 b
G. smith	89.2±1.8	87.2±1.8a	85.5±1.7	83.2±1.6 a	91.0±1.4	65.7±9.2c
Royal gala	87.2±3.2	84.7±2.0a	86.6±1.5	76.3±6.0 b	87.6±2.7	53.9±1.5ab
G. delicious	86.5±1.1	85.3±1.5a	84.8±1.8	74.4±0.6 b	85.2±0.6	48.3±7.abc
R. delicious	89.7±1.2	87.6±2.1a	85.2±1.5	71.8±4.5 b	86.7±0.1	41.1±2.abcd
P	ns	ns	ns	**	ns	**

Table. 2.2……..

Midday leaf water potential (MPa)

	Week (1)		Week (2)		Week (3)	
	Well watered	**Stressed**	**Well watered**	**Stressed**	**Well watered**	**Stressed**
Genotype						
Anna	-1.08±0.04 a	-1.21±0.06ab	-0.91±0.11abc	-1.65±0.04d	-0.82±0.15a	-2.17±0.11a
D. golden	-1.48±0.08ab	-1.37±0.0bc	-1.16±0.19cd	-1.72±0.18de	-0.91±0.14a	-2.35±0.05b
G. smith	-1.39±0.04b	-1.52±0.15abc	-1.17±0.14cd	-1.68±0.10 def	-1.02±0.19ab	-2.45±0.20c
Royal gala	-1.36±0.04 b	-1.28±0.01bc	-1.32±0.11d	-1.71±0.12 de	-1.29±0.03c	-2.35±0.05abc
G. delicious	-1.14±0.18abc	-1.32±0.04bcd	-1.37±0.08d	-1.61±0.12efg	-1.63±0.02abc	-2.56±0.16abcd
R. delicious	-1.54±0.11d	-1.31±0.08abc	-1.14±0.45cd	-1.31±0.08g defgh	-1.32±0.03cd	-2.64±0.19 e
P	**	**	**	**	**	**

Values are means ± SE of four replications, and different letters represent significant differences. Level of significance: (** = significant @ $P < 0.05$).

3.3.2. Gas exchange measurements

Drought stress resulted in a significant variation ($P< 0.05$ and $P\leq 0.01$) in net photosynthesis (Pn), stomatal conductance (Gs) and transpiration (E) among the tested apple genotypes under field conditions after the second weeks of drought stress for all the studied parameters (Table 3.3). Among genotypes, the highest values for Pn were recorded for Dorsette golden, followed by Anna and Granny smith under conditions of drought stress (Table 3.3), while the lower values for Pn were recorded for genotypes Red delicious, Golden delicious and Royal gala in stressed plants.

The differences observed after the second week of drought stress treatment indicates that the decrease in net assimilation rate remained significant for the entire experimental period for stressed genotypes of Royal gala, Golden delicious and Red delicious. Also, the rate of transpiration and stomatal conductance were high and showed similar trends in these genotypes (Royal gala, Golden delicious, Red delicious) which recorded lower rate of Pn at drought stressed conditions (Table 3.3). Drought stress induced a progressive decrease in net photosynthesis at lower rate until the third weeks in genotypes Anna, Dorsette golden and Granny smith; while it showed a sharp decrease in genotypes Golden delicious, Red delicious and Royal gala. This drastic reduction in Pn in genotypes Red delicious (4.51 µmol CO_2 $m^{-2}s^{-1}$), Royal gala (5.74 µmol CO_2 $m^{-2}s^{-1}$) and Golden delicious (6.97 µmol CO_2 $m^{-2}s^{-1}$) is accompanied by high rate of transpiration and stomatal conductance; while the reverse happened in genotypes with high assimilation rate as in Granny smith (16.21 µmol CO_2 $m^{-2}s^{-1}$), Dorsette golden (15.14 µmol CO_2 $m^{-2}s^{-1}$) and Anna (12.3 µmol CO_2 $m^{-2}s^{-1}$) under drought stress.

Table 3.3: Effects of drought stress on net photosynthesis (Pn) µmol CO2 m-2s-1, stomatal conductance (Gs) mmol m−2 s−1 , and transpiration rate (E) mmol m−2 s−1 of apple genotypes

		Week (1)		Week (2)		Week (3)	
Genotype	**Parameters**	**WW**	**S**	**WW**	**S**	**WW**	**S**
Anna	**Gs**	0.79	0.73	0.80	0.22	0.82	0.26
Dorsette golden		0.98	0.27	0.97	0.20	0.84	0.21
Granny smith		0.72	0.29	0.86	0.27	0.80	0.27
Golden delicious		0.65	0.39	0.81	0.39	1.00	0.46
Royal gala		0.76	0.53	0.70	0.47	0.78	0.54
Red delicious		0.91	0.54	1.08	0.67	1.01	0.58
LSD			7.45		16.35		3.86
CV (%)			11.6		10.82		6.25
Anna	**E**	9.01	7.59	10.74	5.00	6.02	4.49
Dorsette golden		8.75	5.86	9.58	4.96	8.37	3.39
Granny smith		10.41	6.34	10.75	6.86	8.58	4.96
Golden delicious		12.19	10.73	11.09	10.16	11.14	8.47
Royal gala		11.67	10.49	11.88	10.79	13.46	10.88
Red delicious		11.72	10.45	13.06	9.84	11.87	10.82
LSD			0.85		0.56		0.23
CV (%)			25.61		15.31		12.05
Anna	**Pn**	17.83	14.73	18.67	12.49	18.62	12.3
Dorsette golden		16.32	15.78	15.47	12.21	15.42	13.14
Granny smith		14.53	11.16	16.32	12.78	16.47	10.21
Golden delicious		17.41	11.01	16.47	8.31	17.70	5.97
Royal gala		17.27	11.18	17.74	7.74	19.90	5.74
Red delicious		19.05	11.43	15.19	5.74	15.47	4.51
LSD			2.45		1.86		0.78
CV (%)			7.09		7.39		10.97

Two-way *ANOVA* (F value)

	E	**Pn**	**Gs**	**Week 1**	**Week 2**	**Week 3**
Genotype (G)	6.87**	0.78	5.98*	1.56*	1.38*	1.17*
Watering (W)	78.35**	25.64**	75.68**	58.06**	175.62**	116.08**
G × W	0.01	8.92	0.25	48.38**	16.20**	22.18**

Values represent mean ± SE of four replications. Level of significance: *P<0.05, **P<0.01. n = 4.

3.3.3. Leaf chlorophyll measurements

Apple genotypes showed different responses to drought stresses for leaf chlorophyll content from the first week to the third week of stress treatment. Total chlorophyll content decreased in all the tested genotypes, while it was unaffected in genotype Granny smith until third weeks of drought stress, followed by genotypes Anna and Golden delicious that showed a little change in Chl. Content for the same period. (Fig. 3.1). The study indicates that the total chlorophyll content of leaves continuously shows a declining trend in chlorophyll content as the drought stress progresses.

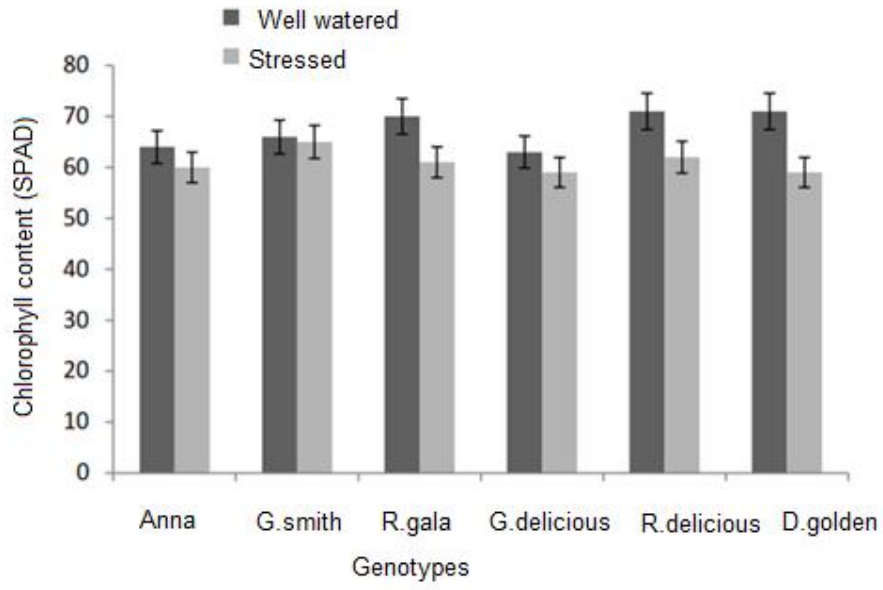

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Figure 3. 1: Effect of drought stress on leaf chlorophyll content of the six apple genotypes. Values represent Mean ±SE (n= 4). Level of significance: $P<0.05$

3.3.4. Leaf area measurements

Leaf area measurement showed that all genotypes showed significant responses under drought stressed condition as high as 1285.67 cm^2 for Golden delicious; while Granny smith recorded the lower value 802.49 cm^2 (Table 3.4). In reference to SLA, significant differences were observed under well watered and stressed conditions for genotypes Doresette golden and granny smith (Table 3.4). Under controlled conditions, Dorsette golden recorded 117.91 cm^2 and for the same period 88.88 cm^2 was recorded for stressed plants, while genotype Granny smith recorded 109.59 cm^2 and 97.08 cm^2 under well watered and stressed conditions respectively (Table 3.4). Conversely, other genotypes: Anna, Golden delicious, Red delicious and Royal gala showed no significant differences for SLA both at the control and stressed conditions (Table 3.4). Also, no significant differences were observed for leaf area ratio both under control and stressed treatment (Table 3.4).

Table 3.4: Total leaf area (TLA), specific leaf area (SLA) and leaf area ratio (LAR) of six apple genotypes under well-watered and drought stressed conditions.

Genotype	**TLA (cm^2)**		**SLA ($cm^2\ g^{-1}$)**		**LAR ($cm^2\ g^{-1}$)**	
	WW	**S**	**WW**	**S**	**WW**	**S**
Anna	1694.86a	957.13b	97.63a	91.10a	21.02a	18.61a
Dorsette golden	1884.25a	950.69b	117.91b	88.88a	27.37a	18.73a
Granny smith	1537.96a	802.49b	109.59b	97.08a	21.89a	17.08a
Golden delicious	1819.27a	1285.67ab	98.71a	102.69a	24.53a	21.90 a
Red delicious	1088.99b	901.07ab	105.1a	93.22a	20.72a	14.69b
Royal gala	1642.57a	1101.10abc	96.64a	95.37a	26.94a	22.62a
LSD (0.05)	5.7		3.48		2.43	
CV (%)	46.5		21.3		5.2	

Values with different letters are statistically different @ 0.05 by Fisher's least significant (LSD) test. Level of significance: * $P \leq 0.05$, ** $P \leq 0.01$. WW = Well watered, S = Stressed

3.3.5. Total dry biomass and water use efficiency (WUE) as affected by drought stress

At the field experiment, significant differences ($p < 0.05$) were observed for mean dry biomass of the six apple genotypes subjected to drought stress (Fig. 3.2). Among the genotypes, average dry biomass was recorded as high as 9.99 g for genotype Dorsette golden, and as low as 3.21 g for genotype Granny smith under well watered condition; while stressed plants recorded the maximm value of 2.20 g for genotype Dorsette golden and the lowest value of 0.57 g for genotype Red delicious (Fig. 3.2). Among genotypes considered, Dorsette golden, Granny smith and Anna recorded the highest values of mean dry biomass under stressed condition, followed by Royal gala and Golden delicious; while Red delicious recorded the least of all the tested genotypes (Fig. 3.2).

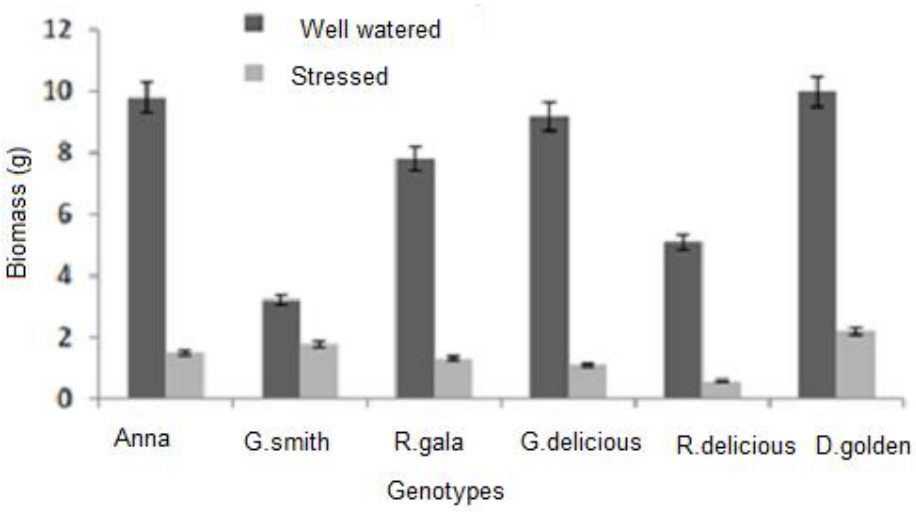

Figure 3.2: Effect of drought stress on total dry biomass of the six apple genotypes. Values represent Mean± SE (n= 4) of the four replications. Level of significance: ($p \leq 0.05$).

Highly significant differences ($p < 0.001$) were observed for WUE. The average water use efficiency ranged from 37 g/kg for genotype Golden delicious) to 13 g/kg for genotype Granny smithunder well watered condition; while stressed plants recorded as high as 4 g/kg for genotype Granny smith, and the lowest value of 1 g/kg was recorded by genotype Red delicious (Fig. 3.3). The result indicates that genotype Dorsette golden relatively took the highest value of WUE under drought stressed condition, while Golden delicious had the highest value for the control. Genotype Red delicious consistently showed relatively least value of water use efficiency under drought stressed condition (Fig. 3.3).

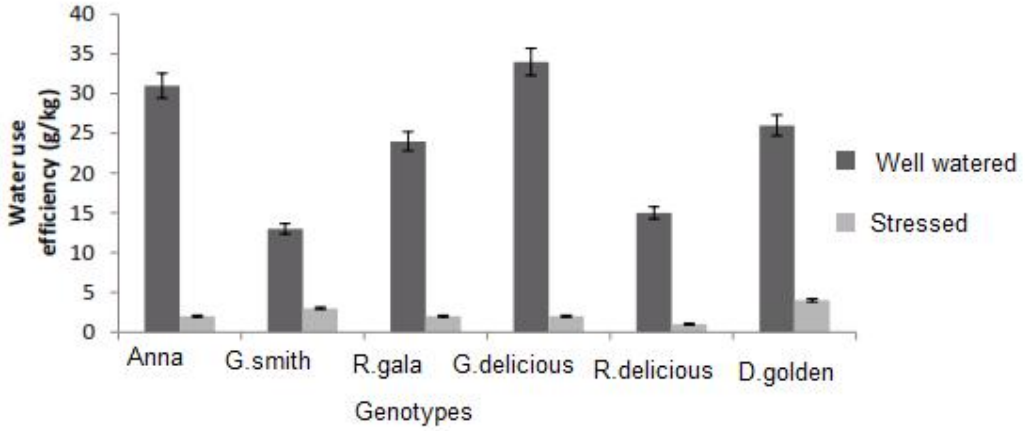

Figure 3.3: Effect of drought stress on water use efficiency of the six apple genotypes. Values represent Mean± SE (n= 4) of the four replications. Level of significance: ($p \leq 0.05$).

3.3.6. Effect of drought stress on plant height and stem diameter

Average plant height ranged as high as 115 cm for genotype Dorsette golden, and as low as 75 cm for genotype Red delicious under the control condition. For stressed treatments, the maximum height of 108 cm was recorded for genotype Dorsette golden and the lowest height of 58 cm was recorded by genotype Golden delicious (Fig 3.4). The results also showed that there were no differences in plant height for genotypes Anna, Granny smith and Red delicious under both conditions, followed by genotype Dorsette golden which showed a slight change under control and stress condition in plant height. (Fig 3.4)

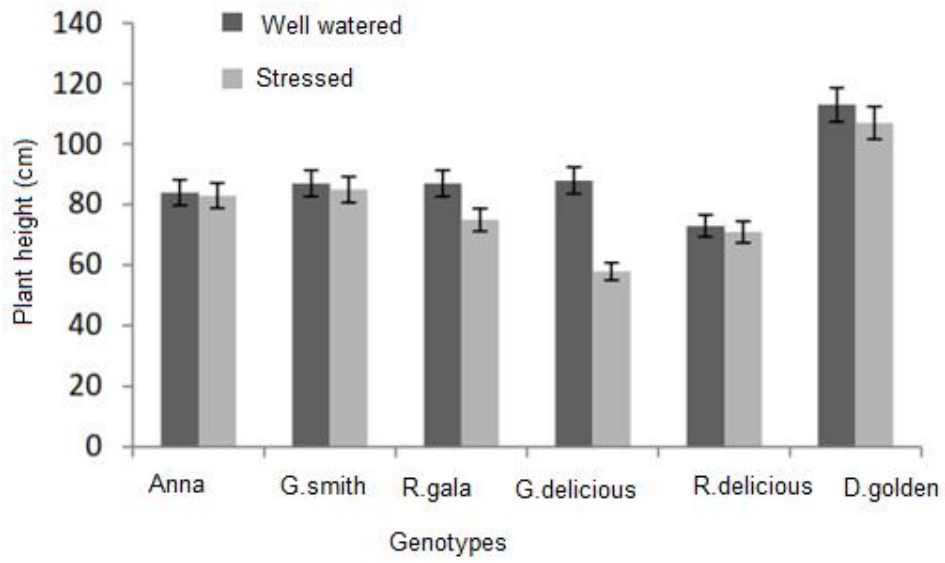

Figure 3.4: Effect of drought stress on plant height of the six apple genotypes. Values represent Mean± SE (n= 4) of the four replications. Level of significance: ($p \leq 0.05$).

The mean maximum stem diameter was recorded for the genotype Dorsette golden, which is about 0.65 cm, and the lowest value of 0.45 cm was recorded for genotypes Granny smith under well watered condition; while under stressed condition, genotype Dorsette golden relatively recorded the highest value of stem diameter (0.42 cm), followed by Granny smith (0.40 cm) Fig. 3.5. Also, genotypes Anna, Golden delicious and Red delicious) showed similar responses for stem diameter ~0.30 cm under stressed conditions, which is relatively lower than the value recorded for the other tested genotypes (Fig. 3.5).

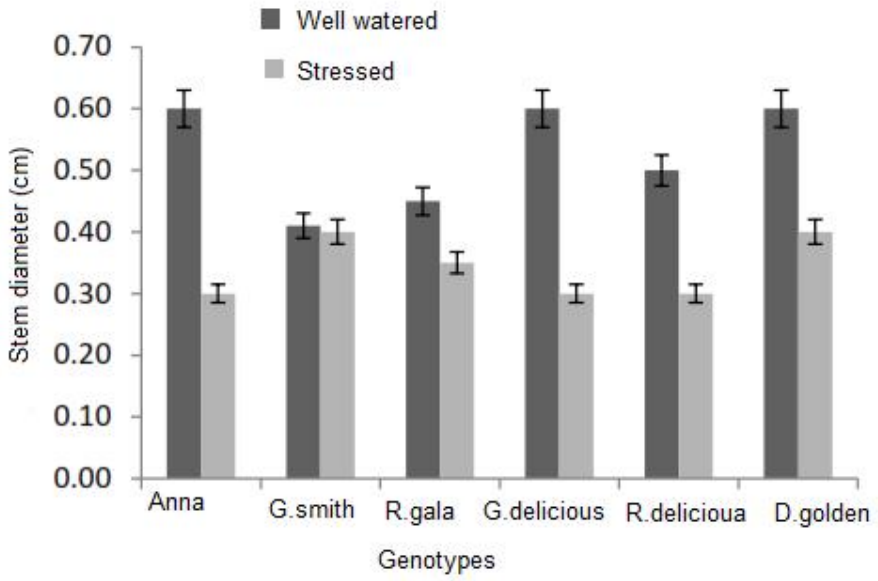

Figure 3.5: Effect of drought stress on stem diameter per plant of the six apple genotypes Values represents Mean± SE (n= 4) of the four replications. Level of significance: ($p \leq 0.05$).

The highest number of branches per plant recorded was 12 for genotype Dorsette golden, and the lowest were 9 for genotype Red delicious for the control plants. Under stressed condition, the highest number of branches was recorded by the genotype Dorsette golden (6), followed by genotypes Granny smith (3.5), while the rest of genotypes showed little variation in number of branches under stressed condition (Fig. 3.6). Exceptionally, genotype Granny smith showed no differences in number of branches under both control and stressed conditions (Fig. 3.6).

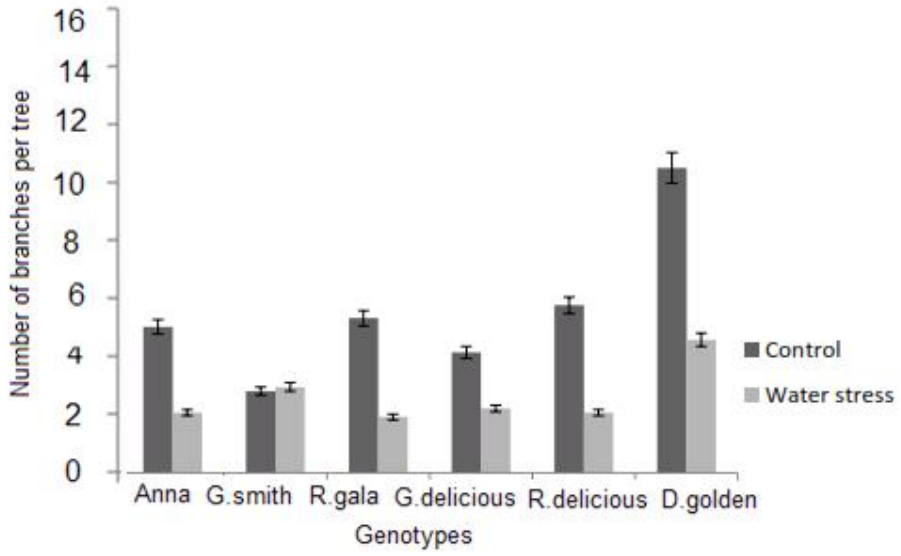

Figure 3.6: Effect of drought stress on number of branches per plant of the six apple genotypes. Values represent Mean± SE (n= 4) of the four replications. Level of significance: ($p \leq 0.05$).

3.3.7. Effect of water stress on root dry mass

The mean values for root dry mass ranged from 2.1 g for genotype Dorsette golden to the lowest value of 0.52 g for genotype Red delicious under well watered condition, and from 0.59 g for genotypes Dorsette golden to 0.22 g for genotype Golden delicious for stressed plants (Fig. 3.7). Similarly, genotypes Red delicious, Royal gala and Anna recorded lower values for root dry mass under conditions of drought stress (Fig. 3.7).

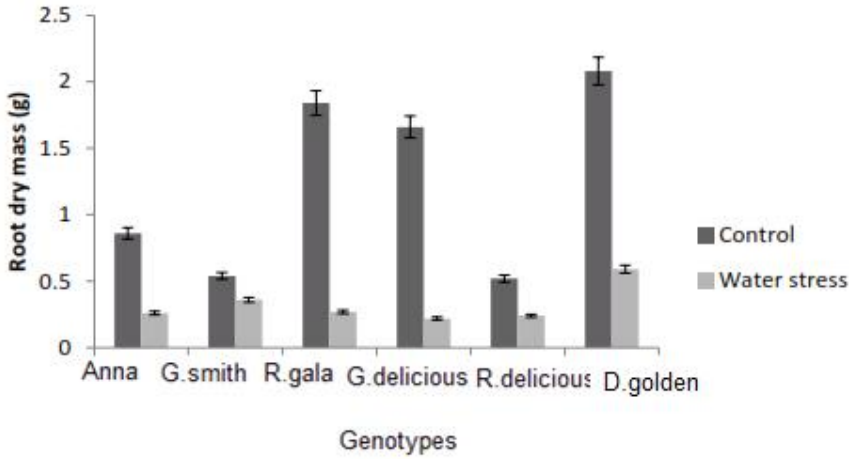

Figure 3.7: Effect of drought stress on root dry mass of the six apple genotypes. Values represent Mean± SE (n= 4) of the four replications. Level of significance: ($p \leq 0.05$).

3.3.8. Leaf senescence of drought stressed apple genotypes

Highly significant differences ($p < 0.01$) exist among the tested genotypes for leaf senescence. Mean leaf senescence ranged from 6 % to 2 %. The highest value of 6 % was recorded for Red delicious, followed by Granny smith and Dorsette golden (~4.5%) each, while the least value of 2 % was recorded equally for genotypes Anna and Royal gala as compared to the control plants (Fig. 3.8).

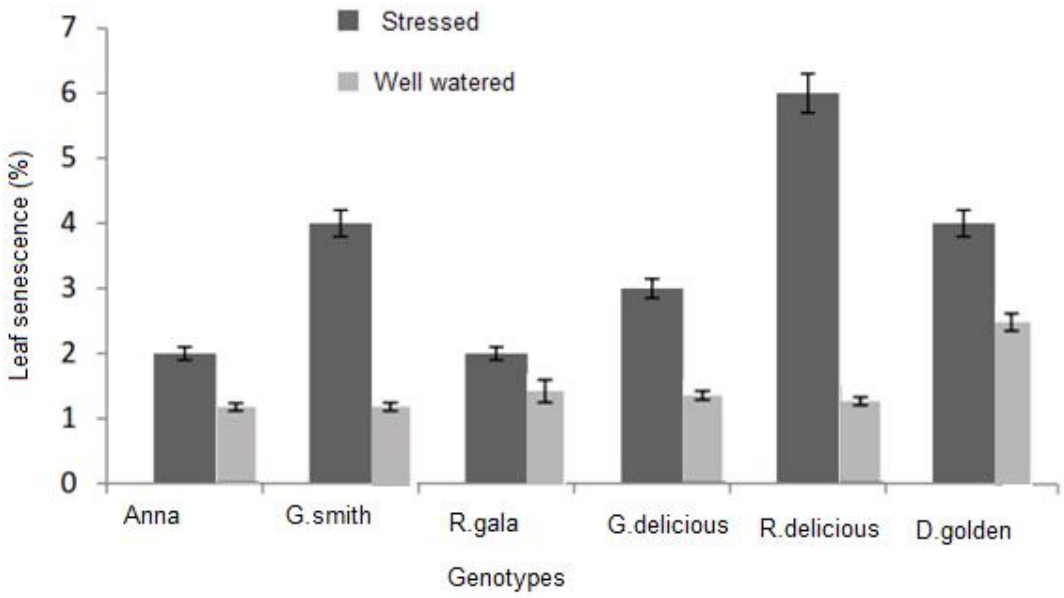

Figure 3.8: Leaf senescence of drought stressed apple genotypes. Values represent Mean± SE (n= 4) of the four replications. Level of significance: ($p \leq 0.05$).

3.3.9. Pair-wise comparison of means for the studied characters.

At the end of experiment, the tested genotypes (Anna, Dorsette golden, Granny smith, Golden delicious, Red delicious and Royal gala) showed highly significant differences ($p < 0.01$) in biomass production under stressed condition (Table 3.5) compared to the control treatment. Under drought stress, genotypes Dorsette golden and Granny smith recorded the highest values of 2.20 g and 1.78 g respectively, while the least value of 0.57 g was recorded by genotype Red delicious (Table 3.5).

The result indicated that there were also significant differences ($p < 0.01$) among genotypes for water use efficiency. Under drought stressed condition, Dorsette golden and Granny smith recorded the highest values of 4 g/kg and 3 g/kg, respectively. The least value of 1 g/kg for water use efficiency was recorded by the genotype Red delicious (Table 3.5). With reference to relative water content, it was observed that under drought stressed condition; only the genotype Granny smith did not show any significant difference from its control. Genotypes Dorsette golden and Red delicious recorded equally the same value of 71 % which was the highest under the control conditions (Table 3.5), while under drought stress, Dorsette golden and Golden delicious equally recorded lower values of 59 % after third weeks of drought stress. Genotypes Anna, Red delicious and Royal gala did not show any significant differences compared with the control (Table 3.5). Drought stress affected plant height significantly in all the tested genotypes. Accordingly, Dorsette golden recorded the highest value of 113 cm, followed by Granny smith (85 cm), Anna (83 cm), Royal gala (75 cm), Red delicious (71 cm) and Golden delicious (58 cm) respectively under drought stressed condition (Table 3.5).

Significant differences exist among apple genotypes for number of branches under the control and stressed conditions (Table 3.5). Genotype Granny smith did not show any significant variation from its control after third weeks of drought stress; while the genotype Dorsette golden recorded more number of branches (7), next to the genotypes Granny smith. However, under well watered conditions, significant differences were observed among genotypes for the number of branches except for genotypes Anna and Golden delicious which showed similar trends (Table 3.5). The effect of drought stress on stem diameter showed that genotypes Granny smith and Dorsette golden recorded equally the highest value of stem diameter (0.40 cm) followed by Royal gala (35 cm), Anna and Golden delicious which equally recorded (0.30 cm) respectively under stressed condition (Table 3.5). Under controlled condition, Anna and Dorsette golden recorded the highest value of 0.60 cm each for stem diameter, followed by Red delicious (0.50 cm), Royal gala (0.45 cm) and Granny smith (0.40 cm) respectively (Table 3.5).

With respect to the root dry mass (Table 3.5), all genotypes tested showed significant variation under the control compared to their corresponding treatment under drought stress condition. Genotypes Dorsette golden recorded the highest value of 0.59 g, which was significantly different from Granny smith (0.36 g), and also different from Anna (0.26 g), Royal gala (0.27 g), Red delicious (0.24 g) and Golden delicious (0.22 g) under stressed condition, while the same genotype Dorsette golden recorded the highest value of 2.08 g which was significantly different from the other counterparts under the control condition (Table 3.5).

Table 3.5: Pair-wise comparison of means for the parameters studied. Differences in treatment means were compared by t-test

	BM (g)		WUE (g/kg)		RWC (%)		Pht (cm)	
	Ww	**S**	**Ww**	**S**	**Ww**	**S**	**Ww**	**S**
Genotype								
Anna	9.80±5.0	1.50±0.01	31.00±16	2.00±0.03	64.00±2.0	60.00±2.0	84.00±2.0	83.00±1.0
G. smith	3.21±1.0	1.78±0.03	13.00±5	3.00±0.04	66.00±1.0	65.00±0.5	87.00±2.0	85.00±2.0
R. gala	7.81±4.0	1.31±0.01	24.00±12	2.00±0.02	70.00±5.0	61.00±0.3	87.00±7.0	75.00±3.0
G. delicious	9.18±4.0	1.10±0.01	34.00±18	2.00±0.01	63.00±3.0	59.00±0.0	88.00±17	58.00±1.0
R. delicious	5.09±3.0	0.57±0.01	15.00±8	1.00±0.04	71.00±5.0	62.00±1.0	73.00±1.0	71.00±2.0
D. golden	9.99±4.0	2.20±0.01	26.00±12	4.00±0.06	71.00±6.0	59.00±2.0	113.00±4.0	107.0±1.0
LSD (0.05)	0.07		1.94		2.07		2.84	
CV (%)	16		14		21		19	

BM = biomass; WUE = water use efficiency; RWC = relative water content; Pht. Plant height CV= co-efficient of variation; ± standard deviation; Ww= well watered; S= water stress

Table 3.5. Conti……

	NB (No.)		SD(cm)		RDM (g)		LS (%)
	Ww	**S**	**Ww**	**S**	**Ww**	**S**	**S**
Genotype							
Anna	7.00±11	4.00±1.0	3.00±0.2	0.30±0.03	0.86±0.3	0.26±0.01	20.00±1.0
G. smith	4.00±1.0	4.00±2.0	0.41±0.02	0.40±0.02	0.54±0.1	0.36±0.02	40.00±2.0
R. gala	8.00±13	3.00±1.0	0.45±0.05	0.35±0.02	1.84±1.0	0.27±0.04	20.00±1.0
G. delicious	7.00±1.0	4.00±2.0	0.60±0.2	0.30±0.02	1.66±1.0	0.22±0.01	30.00±0.0
R. delicious	9.00±14	3.00±1.0	0.50±0.1	0.30±0.0	0.52±0.2	0.24±0.02	60.00±1.0
D. golden	12.00±12	7.00±0.1	0.60±0.1	0.40±0.01	2.08±1.0	0.59±0.02	40.00±0.0
LSD (0.05)	2.43		0.03		0.07		0.18
CV (%)	5.2		7.50		4.90		2.9

NB = number of branches; SD = stem diameter; RDM = root dry mass; LS = leaf senescence , CV= co-efficient of variation; ± standard deviation; Ww= well watered; S= water stress

3.3.10. Relationship between biomass and water use efficiency

The present study indicates that there was highly significant ($p < 0.01$) association between biomass and water use efficiency (Fig. 3.9). A co-efficient of determination (r^2) of 0.92 was observed among genotypes implying that about 92 % of the variation in biomass was explained by its association with water use efficiency (Fig. 3.9).

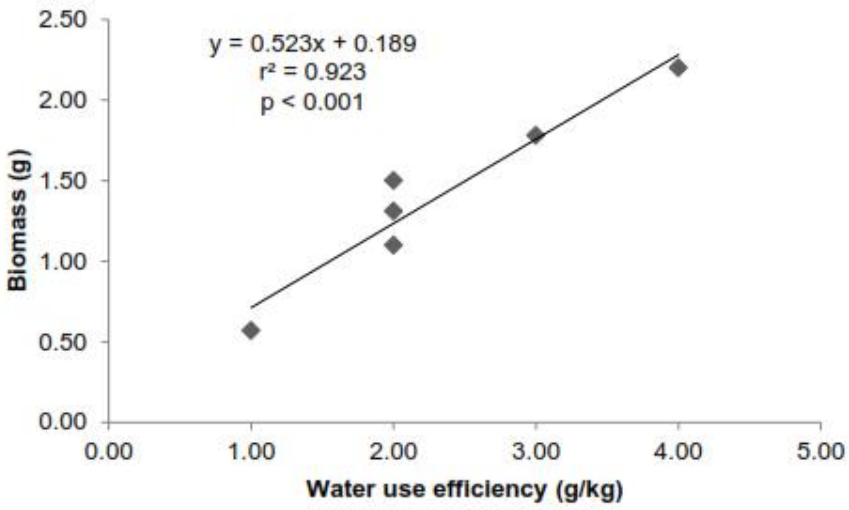

Figure. 3.9. Relationship between biomass and water use efficiency of drought stressed apple genotypes.

3.3.11. Relationship between biomass and root dry mass

A highly significant relationship ($p < 0.01$) exist between between biomass and root dry mass (Fig. 3.10). A co-efficient of determination (r^2) of 0.703 was obtained for genotypes under drought stress, and this variation in biomass could be attributed to root dry mass.

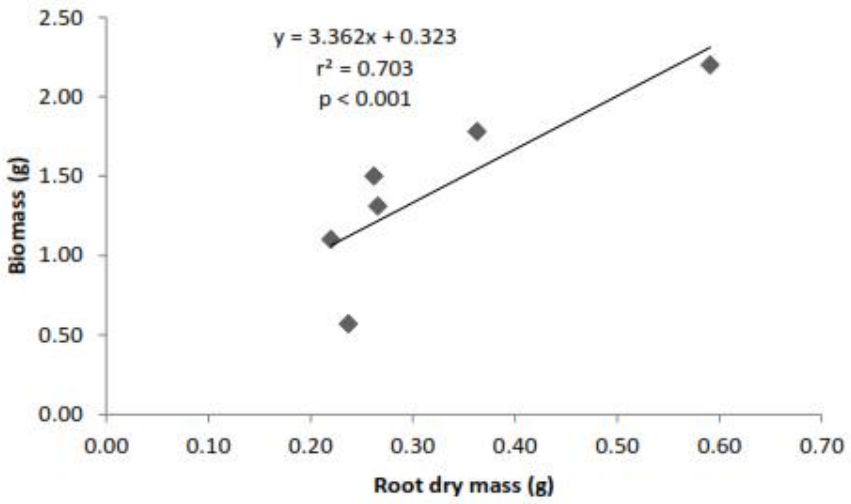

Figure 3.10. Relationship between biomass and root dry mass of water stressed apple genotypes.

3.3.12. Drought intensity and drought susceptibility index (S)

The drought intensity (D) was calculated for biomass, water use efficiency, relative water content, plant height, number of branches per plant, stem diameter and root dry mass (Table 3.6). Then the drought intensities for these indicators were subsequently used for the calculation of drought susceptibility index (S) (equation 2.2 and 2.3) for the six apple genotypes evaluated for their drought tolerance under field conditions.

Table 3.6. Drought intensity of the six apple genotypes used for this study

Drought intensity							
Traits	**BM**	**WUE**	**RWC**	**NB**	**PHT**	**SD**	**RDM**
D	0.80	0.90	0.10	0.50	0.10	0.30	0.70
Drought susceptibility index for the tested apple genotypes							
Genotype	**BM**	**WUE**	**RWC**	**NB**	**PHT**	**SD**	**RDM**
Anna	1.04	1.04	0.79	1.11	0.12	1.43	0.94
G. smith	0.55	0.85	0.19	0.10	0.24	0.10	0.45
R. gala	1.02	1.02	1.63	1.21	1.44	0.64	1.15
G. elicious	1.08	1.04	0.80	0.88	3.56	1.43	1.17
R. delicious	1.09	1.03	1.60	1.21	0.29	1.15	0.73
D. golden	0.96	0.94	2.14	1.07	0.55	0.95	0.97

D: drought intensity, BM: biomass, WUE: water use efficiency, RWC: relative water content, PHT: plant height, NB: number of branches, SD: stem diameter, RDM: root dry mass.

Among the genotypes, water use efficiency followed by biomass and root dry mass recorded higher values of drought intensity, while relative water content and plant height recorded the lowest value (Table 3.6). The drought susceptibility index of these indicators were used for the selection and ranking the apple genotypes for their drought tolerance. The drought susceptibility index based on these parameters ranged from 0.10 (both NB and SD for genotype Granny smith), to 1.63 (RWC for genotype Royal gala) (Table 3.6).

Selection and ranking of the six apple genotypes were done based on a relation between their relative performance with respect to these parameters (biomass, water use efficiency, relative water content, plant height, number of branches, stem diameter and root dry mass) under drought stressed condition and their respective drought indices obtained (Table 3.7).

The six apple genotypes were ranked according to their tolerance level to drought stress (Table 3.7). The scoring was done in such a way that genotype with lowest value of drought susceptibility index was scored number six (6), and the highest index was scored 1. Accordingly, any genotype that recorded drought index value from 42 to 35 may be considered to be tolerant to drought, from 35 to 25 may be considered moderately tolerant and lastly, less than 25 may be considered susceptible to drought stress (Table 3.7).The following ranking was therefore obtained for the six apple genotypes in decreasing order of drought tolerance; Granny smith > Anna = Dorsette golden > Royal gala = Red delicious > Golden delicious. Granny smith was relativelytolerant variety, while Anna and Dorsette golden showed moderate drought tolerance. The rest of genotypes, Red delicious and golden delicious showed higher susceptibility to drought. Therefore, the results of the present field experiment indicate that, apple genotypes Granny smith, Anna and Dorsette golden are considered relatively drought tolerant.

Table 3.7: Scoring and ranking of apple genotypes based on drought susceptibility indices of the seven morpho-physiological parameters under drought stressed condition.

Genotype	BM	WUE	RWC	PHT	NB	SD	RDM	Total score	Ranking
Anna	3	2	5	3	6	2	4	25	2
G. smith	6	6	6	6	5	6	6	41	1
R. gala	4	4	2	2	2	5	2	21	4
G. delicious	2	2	4	5	1	2	1	17	6
R. delicious	1	3	3	2	4	3	5	21	4
D. golden	5	5	1	4	3	4	3	25	2

BM: biomass, WUE: water use efficiency, RWC: relative water content, PHT: plant height, NB: number of leaves, SD: stem diameter, RDM: root dry mass.

The scale made was as follows: 42 - 35: drought tolerance; 35 – 25: Moderately tolerant; < 25: Susceptible.

3.4. Discussion

Relative water content showed significant variations ($p < 0.05$) among apple genotypes. The result indicates that genotypes Anna, Dorsette golden and Granny smith conserved much more water in their leaves under conditions of drought stress. High percentages of RWC recorded by these three genotypes under water stress give an indication that they were relatively able to maintain better plant water status within the water deficit period (osmotic adjustment), to extract deep soil moisture (root capacity) and to reduce transpiration via stomatal closure, as a water-saving mechanism. The result agrees with the findings of Choné *et al.,* (2001) that in drought tolerant grapes, the responses in stomatal conductance (Gs) and plant water status appeared to be related with the midday leaf water potentntial (ψ_w), and is highly correlated to the capacity of the grapevine to conduct water from the soil to the atmosphere. Liu *et al.,* (2012 a and b) indicates that the young Gala apple exposed to drought stress at early stage of growth showed low leaf water potential and relative water content, which resulted in lower value for net photosynthesis. The present study also indicates that differences detected in plant water status among genotypes were highly reflected in differences in net photosynthesis (Pn), based on the performance of genotypes whether they are drought tolerant or susceptible. The values for leaf water potential (Ψw) is dependent mainly on soil moisture condition of the experimental site , and showed a progressive decrease with time in stressed plants. Studies on grape vines indicates that reduced leaf water potential under drought stress resulted in loss of turgor due to the inability of the cell to obtain sufficient water to counterbalance the loss by transpiration or translocation and decrease of canopy size (Rivero *et al.,* 2007).

Castrilo and Trujillo (1994) indicates that during the period of drought stress, water potential and relative water content (RWC) decreased with an associated decrease of Rubisco activity, chlorophylls, protein content, and also found that there was a significant correlation among the components of leaf water status and photosynthetic parameters. Cifre *et al.,* (2005) indicates that differences in leaf water potential between grape cultivars under drought stress involve differences in root morphology (xylem vessels size and density), growth and distribution (generation of new fine roots) and activity which influence hydraulic conductance of the vines. The present result indicates that the leaf water potential (Ψw) data from the first week of drought stress shows that genotypes Anna, Dorsette golden and Granny smith tolerate drought stress immediately after water deficit was imposed and kept its leaves at higher RWC during progressive drought stress.

In these genotypes plants actively increased Ψw using osmotic adjustment; or, reduction in leaf RWC might activate stomatal resistance mechanisms. The data also suggested that when water constraint occurred early and decreased continuously, the shift in plant water status indicates minimum threshold water potential (ψ_w) values for individual genotypes after the third weeks of drought stress in stressed plants.

Gas exchange parameters (Pn, Gs, and E), values declined, when apple trees were exposed to drought. However, the degree of reduction differed among genotypes and stress periods. Genotypes Anna, Dorsette golden and Granny smith showed lower rate of stomatal conductance (Gs) and transpiration (E) throughout the experimental periods; indicating that these genotypes maintained high net photosynthesis. The result agreed with the findings of Silim *et al.,* (2009) that in hybrid poplar clones, drought tolerant genotypes showed lower rate of Gs and E. In similar study, Ghaderi and Siosemardeh (2011) reported that drought-tolerant strawberry cultivars subjected to drought stress had lower Gs, and E than sensitive ones. In the present study apple genotypes Golden delicious, Red delicious and Royal gala showed higher rate of Gs and E, which resulted in low CO_2 assimilation and hence low Pn. The result indicates that net CO_2 fixation rate decreased in these genotypes as drought stress developed, but, not at the same extent due to decreasing rate of stomatal conductance and transpiration. This decrease in Pn caused by drought stress has been attributed to both stomatal (restricted CO_2 availability) and non-stomatal limitations (Shangguan, 1999; Srivastava and Strasser, 1997).

The reduction in Pn under drought stress also indicates that these genotypes showing higher rate of Gs and E which indicates susceptibility to drought. The result also revealed that a smaller concentration of CO_2 may inhibit carbon uptake and ultimately growth. In addition, the result supported the findings of Socias *et al.,* (1997) that the decline in the rate of photosynthetic CO_2 uptake in the first week of drought was less pronounced compared with stomatal conductance, thus increasing WUE. The present study found that assimilation rate (Pn) was directly related to an increase or decrease in Gs and E, and also associated with WUE. It also indicates that WUE increased when less water was available. In fact, the plant gas exchange and growth can be determined within and between species, and in general, higher stomatal conductance (Gs) permits faster growth (Atkinson *et al.,* 2000; Li *et al.,* 2002).

The study presented that the rate of leaf area expansion of all genotypes declined substantially owing to drought stress in comparison with well-watered plants; but, the tolerant genotypes maintained a greater leaf area expansion rate than the susceptible genotypes. The result also indicated that tolerant genotypes could take up or retain more water than the susceptible genotype during drought stress. Guo *et al.,* (2010) reported that drought-tolerant poplar clones showed better growth under water stress. Lei *et al.,* (2006) reported that drought stress successively induced cessation of leaf and shoot growth. Zhang *et al.,* (1997) postulated that leaf expansion and stomatal behavior respond directly to soil drying before the occurrence of any detectable shoot water deficit. In the present study, differences in total leaf area (TLA) were the result of reduced leaf area production (leaf size) during the drought period. Ruiz-Sanchez *et al.,* (1997) confirmed that tolerant to drought stress includes stomatal closure and changes in leaf area and orientation, among other factors. In the present research, specific leaf area (SLA) is generally lower in genotypes (Anna, Dorsette golden and Granny smith, which are tolerant to drought stress. This could be due to an increase in Leaf water potential and relative water contents in these genotypes. This revealed that specific leaf area (SLA) is determined jointly by leaf tissue density and leaf thickness; thus, leaf tissue density decreases as leaf water content increases, and leaf water content is the most important determinant of SLA (Rieger *et al.*, 2003; Meziane and Shipley, 2001).

The present study showed that the Chl content of the drought-tolerant genotypes remained almost unchanged, while drought-susceptible genotype was substantially reduced as drought stress progressed. It indicates that drought tolerant genotypes had higher Chl content in leaves and this led to higher photosynthetic rates during drought stress than those in the susceptible genotype. Guo *et al.,* (2010) reported that drought-tolerant poplar clones maintained higher Chl during drought stress. Li *et al.,* (2006) suggested that the decrease in Chl of a drought-tolerant genotype was much lower than that of drought-susceptible barley genotypes. The present study indicates that drought stress caused a temporary increase in leaf greenness in the first week immediately after stress imposition in genotypes Anna, Dorsette golden and Granny smith; and this was mainly related to a reduction in RWC, so it may lead to an increase in pigment concentration. However in the third week, genotypes Granny smith did not show significant changes; while Anna and Dorsette golden showed some little changes in leaf greenness, indicating the drought tolerant ability of these genotypes .

Genotypes Golden delicious, Red delicious and Royal gala showed lower leaf greenness especially at the third weeks of stress, which indicates a reduced leaf greenness in drought susceptible genotypes. Flexas and Medrano (2002) reported that drought stress always reduces leaf greenness in C_3 plants leaves because of chlorophyll degradation.

Drought stress resulted in a significant decrease in growth rate, mainly because of their lower total leaf area and leaf net photosynthesis. As observed in present study, drought stress induced marked drops in leaf water-holding capacity (WHC) because of the decline in SLA, leaf area ratio (LAR), and RWC, which totally influence plant growth. As a result, drought-stressed trees had significantly smaller tree heights (TH), trunk diameters (TD), total fresh biomass (TB), total dry biomass (TDB), and TLA, although these varied by genotypes. Liu *et al.,* (2012 a and b) reported that changes in growth characteristics and biomass accumulation for apple genotypes were found in alterations to the standard irrigation protocol led to reduced growth, as manifested by smaller values for TH, TD, TB, TDB, TLA, RGR, LAR, and RWC. The result in the present study agrees with the findings of Bacelar *et al.,* (2007) who reported that the highest mean plant height (117 cm) was observed in one year old olive cultivars that received 500 ml of water treatment, which was significantly different from the 47 cm; mean plant height observed from plants grown under rainfed condition. Liu *et al.,* (2012b) indicates that in drought tolerant Honeycrisp apple genotype, growth rate was slow under drought stress conditions mainly due to the greatly reduced TLA and SLA. Stem diameter significantly decreased in all genotypes under stressed conditions. Cao (*et al.,* 2004) reported that drought stress during vegetative stage provides diminution of the growth in *Malus* seedlings leaves and stems. In terms of adaptation for apple genotypes to drought stress, Atkinson, *et al.,* (2000) reported that genotypes changed their morphological features to adapt to the extreme drought by decreasing leaf areas, turning leaf color to deep green (increasing pigment concentration) and increasing root dry mass.

Drought stress significantly decreased number of branches per plant. Except for the genotypes Dorsette golden, Granny smith and Anna, all other genotypes showed relatively equal number of branches ~ 3 under drought stress conditions. Compared to non-stressed plants, branch number reduced from nill (Granny smith) to up to 66.7% (red delicious) under drought stress conditions, indicating that apple genotypes greatly vary in their response to drought stress in this parameter.

These present results are consistent with previous study on apples Atkinson *et al.,* (1999) that water deficit reduced significantly the total leaf area and total dry matter which in turn influences the development of new branches and shoots. Many aspects of growth of apple genotypes are affected by drought stress (Jie *et al.,* 2001), including root development, stem growth, branch development, shoot emergence and leaf expansion, which is reduced due the sensitivity of cell growth to water stress. Water stress also reduces leaf production and promotes senescence and abscission in apple (Massonnet *et al.,* 2007), resulting in decreased total leaf area per plant. Reduction in leaf area reduces crop growth and thus biomass production.

Significant variations were observed among apple genotypes for root dry mass. Variation among apple genotypes for root dry mass subjected to drought stress may be attributed to the differences in root morphology and growth, and also associated with rootstock-scion interaction. In comparison to other *Malus* species, it seems that apples have different mechanisms for water stress resistance. Ma XW *et al.,* (2010) reported that water stress causes greater difference in root dry weight than shoot dry weight in all cultivated apple genotypes. Nevertheless, it has been reported that root system characters alone were less closely associated with drought resistance in some *Malus* species (Atkinson *et al.,* 2000b). From the genotypes studied here, Dorsette golden Granny smith and Anna showed relatively higher dry root mass, and it seems that those genotypes could assimilate more nutrients in roots in response to drought stress. It indicates that the fraction of root biomass in fiberous root which presumably has the greatest absorptive capacity in the root system was only increased in these genotypes.

There was significant variation among apple genotypes in the leaf duration under drought stressed condition. This variation among apple genotypes may be probably due to the ability to maintain green leaf duration and high relative water content in water-limited condition as seen in genotypes Anna, Dorsette golden and Granny smith as compared to these sensitive genotypes Golden delicious, Royal gala and Red delicious, which showed greater score for leaf senescence. This was probably due to the better osmotic adjustment by accumulation of solutes such as sugars, or by a good regulation of the stomatal conductance in these tolerant apple genotypes as indicated in the present study.

The study presented that drought stress significantly reduced above ground biomass resulting in low biomass in severe water-stressed apple genotypes after third weeks

of drought treatment. This was in agreement with the findings of Prabhu and Shivaji (2000) that indicates the main effect of drought in the vegetative period was to reduce leaf, so that the crop intercepts less sunlight. Dichio *et al.,* (2002) reported that drought stress during the vegetative stage caused a reduced growth in young olive trees. The result confirms the findings of Lu *et al.,* (1999) that identifying drought tolerant physiological mechanisms for barely cv. Mona at the soil moisture level of -0.4 MPa showed 85.2 % decrease in shoot growth on dry weight basis as compares with the control plants. Among apple genotypes studied at present, the effect of drought was severe to reduce leaf area and stem growth which resulted in reducing ability of the plants to intercept solar radiation. In general, under water-stressed condition, biomass, water use efficiency, number of leaves per plant and root dry mass were indeed closely related with highly significant positive relationship. The interpretation is that greater biomass was relatively associated with low water use, and greater water use efficiency in one side and in the other side water deficit contributes to a significant reduction in leaf area, so as to reduce water loss through transpiration with immediate consequence of decreasing in photosynthesis.

Also, the crop plant under water-limited condition tends to divert assimilates to root growth in order to capture deep soil moisture. For example, reduced plant size, leaf area, and leaf area index (LAI) are a major mechanism for moderating water use and reducing injury under drought stress (Mitchell *et al.,* 1998; Turner, 1981). Liu *et al.,* (2012a) reported that under drought stressed condition, long term water use efficiency (WUE_L) in apple genotypes were significantly correlated with total biomass (TB) and total dry biomass (TDB). Blum (2005) observed that for conditions where high WUE is an advantage because it is a marker for low water use, selection for the preferred plant type can be done by directly selecting for small plant size, small leaf area, or reduced growth duration rather than by using the more expensive selection criterion of WUE by way of carbon isotope discrimination.

The present result suggests that greater biomass production under water stress was associated with relatively low water use and greater water use efficiency as seen in apple genotypes Dorsette golden and Anna. This observation agrees with the findings of Cordon *et al.,* (2002), who compared the yield performance of two wheat genotypes differing in water use efficiency as defined by $\Delta^{13}C$, at two sites in Eastern Australia, differing in rainfall frequency. In similar way, Munoz *et al.,* (1998) reported that high yield potential and high yield under water-limited conditions are generally associated with reduced water use efficiency mainly because of high water use. The ability for crop plants to limit water use and transport, may be probably due to their osmotic adjustment within roots, because as soil water declines, it may provide an adaptive response to sustain root water uptake potentials to such an extent that the hydraulic driving force for water uptake and transport through the plant can be maintained (Turner and Jones, 1980).

3.5. Conclusion

The three apple genotypes Anna, Dorsette golden and Granny smith are recommended as moderately drought stress tolerant genotypes based on their field performance. These genotypes with moderate tolerance to drought are selected for delayed leaf senescence during progressive drought period and can enhance drought adaptation of apple genotypes; enable to produce a greater spur shoots and branches by avoiding drought stress at early stage of drought development, and are good enough in net photosynthesis (Pn) maintenance and osmotic adjustment for conferring drought tress tolerance. Biomass, water use efficiency, relative water content and root dry mass are useful, reliable and cheaper indicators t o identify and select drought tolerant apple genotypes using drought intensity and susceptibility index. Further work needs to be conducted, in order to explore the physiological mechanisms controlling canopy maintenance of the tolerant genotypes that helps in understanding of the genotype ability to produce more productive branches (spur shoots) by avoiding the severity of drought stress.

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CHAPTER FOUR

Physiological attributes for drought adaptation in apple (*Malus domestica* Borkh.): Profiling associated biochemical markers in six genotypes grown under glasshouse conditions

Abstract

The study profiled some selected physiological and biochemical parameters so as to identify drought tolerant genotype(s) from amongst six apple (*Malus domestica* Borkh.) genotypes (commonly known as Anna, Dorsette golden, Granny smith, Golden delicious, Red delicious and Royal gala) based on their sensitivity to water stress.The experiment was conducted in a glasshouse under well-watered and drought stressed conditions. Physiological parameters such as leaf relative water content (RWC), midday leaf water potential (LWP), net photosynthesis (Pn), stomatal conductance (Gs), transpiration (E), water use efficiency (A/Gs), specific leaf area (SLA), chlorophylls a and b contents, proline and soluble sugar contents were determined in leaves of the studied apple genotypes subjected to drought stress and control conditions. Sampling and measurements were performed under conditions of drought stress when midday leaf water potential (Ψmd) was approximately −2.75 MPa**.** Gs, E and Pn were determined after the first and third weeks of stress, while SLA, RWC and LWP were measured weekly throughout the stress period. Drought stress reduced RWC, LWP, Gs, E and Pn but increased SLA in the six apple genotypes studied. Under drought stress, genotypes Anna, Dorsette Golden, and Granny Smith stood first, second, and third, respectively in terms of maintaining higher RWC and LWP, but lower SLA. These genotypes were categorized as drought tolerant genotypes. In contrast, genotypes Golden Delicious, Red Delicious and Royal Gala showed higher Gs, E, but lower Pn throughout the stress period, and were thus categorized as susceptible genotypes. Genotypes Anna and Dorsette Golden exhibited better performances in almost all the physiological parameters determined in this study, and seemed to be less sensitive to drought stress. Hence, drought tolerance attributes in these genotypes could be linked to a more effective control of osmotic adjustments, as a consequence of rapid accumulation of proline and soluble sugars. Other observed attributes included increased total biomass, root-to-shoot ratio and water use efficiency.

However, further detailed studies at biochemical and molecular levels are required for more coherent and/or conclusive recommendations.

Keywords/Phrases: Chlorophylls, drought stress, leaf gas exchange, leaf water potential, relative water content, soluble sugar, proline, water use efficiency.

4.1. Introduction

Several studies conducted on apple (*Malus domestica* Borkh.) tree responses to drought stress have revealed responses similar to other trees, but much of these responses are not directly applicable across different climatic regions, especially across those apple-growing mild winter tropical areas (Fallahi *et al.,* 2011; Girona *et al.,* 2011; Farooq *et al.,* 2009; De Swaef *et al.,* 2009; Dragoni *et al.,* 2005; Lakso 2004; Marsal *et al.,* 2003; Ferree and Warrington 2003). Also, many other studies conducted in temperate climates showed variable responses of apple trees when these were exposed to drought stress in a season, which highly influenced development from vegetative to reproductive phases (Monclus *et al.,* 2009; Vitasse 2009; Naor *et al.,* 2008; Medrano *et al.,* 2003; Ebel *et al.,* 1993).

In the event of early termination of irrigation in tropical apple-growing areas, drought stress is a serious problem during the season of reproductive phases when the cell enlargement stage of fruit growth begins (Monclus *et al.,* 2006; Romero *et al.,* 2006; Marsal *et al.,* 2002; Torrecillas *et al.,* 2000). Many drought-stress studies have been conducted on potted trees under controlled conditions, and have provided useful information for field-grown trees (Tomás *et al.,* 2012). Studies on trees grown under controlled environment provide valuable information on water requirements of individual genotypes at different stages of growth and development in response to drought stress (Klamkowski and Treder, 2008; Marsal *et al.,* 2000). Such studies may be applicable for field-grown trees in order to optimize their water demand and for proper scheduling of irrigation (Mills *et al.,* 1996; Lötter *et al.,* 1985). Also, this would help the growers as an advance-warning system in their phenological calendar to adjust cultural practices in the case of a severe water shortage and to preserve the trees for long-term productivity (Bassett *et al.,* 2011; Cohen *et al.,* 1997; Bookeri, 1996).

Drought stress may induce changes in plant functions, which in turn result in stomatal restrictions on the supply of carbon dioxide. Also, drought impacts on non-stomatal components via a direct effect on ATP synthase, thus leading to a restricted ATP supply (Massonnet *et al.,* 2007; Lawlor and Comic, 2002). Drought stress can also cause damage to the reaction centers of photosystem I (PSI) and photosystem II (PSII) (Marsal and Girona. 1997). The accumulation of carbohydrates often observed under drought conditions could also limit photosynthesis through a diminished supply of inorganic phosphate to the Calvin cycle (Chaves *et al.,* 2009).

Stomatal limitation to photosynthesis is associated with a decrease in the CO_2 concentration in the cellular spaces of the leaf intracellular carbon dioxide concentration (Ci), which has been linked to impaired metabolism, e.g., inhibition of nitrate reductase and sucrose-phosphate synthase (SPS) (Lima *et al.,* 2002). Sircelj, *et al* (2005) reported that in apple genotypes the net photosynthesis and rates of transpiration decreased significantly during drought stress under field conditions; and instantaneous water use efficiency (WUEi) reflects the ability of plants to produce biomass per unit of water transpired.

Long term water use efficiency (WUE_L) can be considered as an adaptive indicator of soil drying conditions; it indicates that decreasing water content is accompanied by loss of turgor and wilting, cessation of cell enlargement, closure of stomata, reduction in photosynthesis, and interference with many other basic metabolic processes (Lei *et al.*, 2006; Jie *et al.,* 2001). Rouhi *et al.,* (2007) indicated that different almond genotypes showed varied responses to drought stress with respect to root and shoot biomass, root and shoot dry weight, total leaf area, specific leaf area and stomatal size. The authors reported that in all the tested almond genotypes, drought tolerance was significantly associated with root dry weight, leaf area, lower stomatal size, and lower specific leaf area (SLA). Liu *et al.*, (2012) indicated that different apple genotypes showed significant declines in tree height, trunk diameter, biomass production, and total leaf area in response to drought stress. Photosynthetic pigments such as chlorophylls '**a'** and '**b**' are important for plants mainly for harvesting light and production of energy and reducing power such as ATP and NADPH, respectively are prone to soil drying damages (Liu *et al.,* 2011). Organic solutes such as proline (Trovato *et al.,* 2008; Ismail *et al.,* 1994; Delauney and Verma, 1993) and soluble sugars (Wang *et al.,* 1995; Wang and Stutte, 1992) are associated with drought tolerance in several plant species; and play a central role in osmotic adjustments by preventing or reducing the loss of turgor. In the present study, drought adaptability was estimated based on physiological traits; including relative water content, leaf water potential, gas exchange parameters, chlorophylls 'a' and 'b' content, proline and soluble sugar content. The major objective here was to test the hypothesis that drought-tolerant cultivars differ from drought-sensitive ones at physiological and biochemical levels.

4.2. Materials and Methods

4.2.1. Experimental site

The study was conducted at the National Institute for Biotechnology Research, based at Holetta Agricultural Research Centre and hosted by the Ethiopian Institute of Agricultural Research. The site is found at 2390 m .a.s.l. and, is a typical representative of central Ethiopian highlands. The daily maximum and minimum temperatures of the area are 22° C and 6.3° C, respectively. The average rainfall is 1100 mm per year [Ethiopian Institute of Agricultural Research (EIAR) annual report, 2012]. The major soil type in the area is Nitosol.

4.2.2. Plant materials, experimental design, growth conditions and drought stress treatments

Six apple (*Malus domestica* Borkh.) genotypes (commonly known as Anna, Dorsette golden, Granny smith, Golden delicious, Red delicious and Royal gala), grafted on MM-106 semi-dwarfing type rootstocks, eighteen grafts from each genotypes, and a total of 108 grafs were considered for this study. The experiment was conducted between June, 2014 and May, 2015. One-year–old, uniformly grown grafts from open nursery, ranging in height between 50 to 60 cm were transferred to 50 l HDP (high density plastic) sacks filled with a mixture of top soil (collected from 0 to 20 cm deep), rotten manure and sand in a ratio of 5:1:1, respectively. Potted seedlings were irrigated three times a week so as to maintain the filled capacity (FC) ~ 80% until the beginning of drout stress treatments for normal growth of grafts, and were allowed to grow in a screen-house until September 2014. The screen-house provided better acclimation for the young grafts by allowing for free exchange of air with the external environment. Also, the grafts were trained in a central leader tree training system that encouraged the growth of lateral branches and allowed all the branches to be equally exposed to the growing conditions inside the glass house. The seedlings were then transferred to a glasshouse in October 2014 so as to apply drought stress treatments. Before transferring plants to the controlled glasshouse, the day/night temperatures was adjusted to 28^{o} C ± 2^{o} C, 18/15 h, and the relative humidity was 60–70%.

A drought treatment was started one month after transferring seedlings to the glasshouse. Drought stress treatment was applied by withholding watering for 28 days until soil water potential (Ψ_S) at predawn reached about –2.50 to –3.0 MPa. Thereafter, stressed plants were kept at 50% FC and control plants were kept at 80% FC, throughout the

experimental period. Soil moisture content for each pot was measured twice a week for the whole period of water stress using 20 cm long probes of Time-Domain Reflectometer (TDR model 1502C, Tectronix Inc. Beaverton, OR, USA) (Cerny, 2009; Topp and Davis, 1985). Plants were arranged in completely randomized design with three replications; by considering two watering regimes and six apple genotypes. Each treatment combination consisted of six plants: 3 controls at 80% FC and 3 stressed at 50% FC on plot basis.

4.2.3. Leaf gas exchange and relative water content measurements

Gas exchange measurements were conducted between 10:00 and 13:00 h, on days 7, 14, 21and 28, of the experimental period using a portable photosynthesis system (Li-6400; LI-COR Inc., Lincoln, Nebraska, USA). The youngest fully expanded and undamaged leaf, ~ eight leaves per sampled plants from branches located at the middle position was placed separately in the chamber. The leaf temperature, ambient water vapor pressure, and CO_2 concentration were maintained at 28.7 ± 1.0°C, 1.30 ± 0.15 kPa, and 480 CO_2 µmol m^{-2} s^{-1}, respectively. The net photosynthetic rate (Pn), transpiration rate (E), and stomatal conductance (Gs), was obtained from four plants per treatment. Measurement was made for 20s immediately after a stable decrease in CO_2 concentration inside the chamber was achieved. Following Mediavilla *et al.*, (2002), A/Gs ratio was taken as an estimate of intrinsic water use efficiency (WUEi), as well as the ratio of dry biomass (g) per plant to the rate of water used (kg/plant) was used to estimate long term water use efficiency (WUE_L) during progressive drought stress. The leaves used for Gs, E, and Pn were detached from the plant for RWC measurements. Immediately after cutting at the base of the lamina, the leaves were weighed to obtain the fresh weight (FW). Turgid weight (TW) was determined from leaves floated for 24 h in distilled water in a closed container at 4° C under darkness. Dry weight (DW) was determined for the same leaves after oven-drying for 48 h at 70° C. RWC was calculated as: RWC (%) = [(FW-DW)/(TW-DW)] x 100. Water-holding capacity (WHC) was the amount of water removed during the drying process divided by the final DW; it was calculated according to the modified method of Cook *et al.* (2009). Here, WHC (%) = (TW-DW)/DW x 100. The remaining fresh leaves per pot were plucked from the same plants for leaf area measurement using a LI- COR Portable Leaf Area meter (Model LI 3000, USA), and oven dried at 70° C for 72 hrs. and weighed for Specific Leaf Area (SLA) measurement. SLA was calculated as the ratio between leaf area (cm) and leaf dry matter (g).

4.2.4. Leaf water potential (leaf ψw)

Leaf water potential was measured every week using a pressure chamber (Soil moisture Equipment Corp., Santa Barbara, USA) until the end of the experiment. The leaf blade was inserted in the pressure chamber and sealed, leaving 5 cm of the petiole outside the chamber. Air pressure was then slowly increased until the xylem sap was visible at the end of the petiol at which time the pressure reading was recorded as the water potential of the leaf.

4.2.5. Chlorophylls 'a' and 'b' content Analysis (mg^{-1}g fresh weight)

Chlorophyll pigments, chlorophyll 'a' (Chl a) and chlorophyll 'b' (Chl b), were extracted from frozen fully expanded leaf samples (~0.2 g) using 80% acetone. Leaves were collected and dried with liquid nitrogen and ground into fine powder with pestle and mortar. Total pigments were extracted from the ground leaves with 5 ml of 80% acetone. The crude e xtract was then centrifuged at 1500 g for 5min, the supernatant collected, and Chls 'a' (@ ~ 430 nm), 'b' (@ ~ 470 nm) (Taiz and Zeiger, 2010), were determined using a spectrophotometer. Calculations of the chlorophylls were done using the formula of Porra *et al.,* (1989): Chl a = (12.25 x Abs $_{663:6}$) – (2.55 x Abs $_{646:6}$) (µmg/ml); Chl b = (20.31 x Abs $_{646:6}$) – (4.91 x Abs $_{663:6}$) (µmg/ml).

4.2.6. Proline and soluble sugar analyses

Free proline in fresh leaf material was determined according to Ahmad, *et al.,* (2009). Samples were taken from fully expanded and mature leaves after the third weeks of drought stress treatment for each genotype, watering treatment and replication. Fresh leaf sample of 1 g was homogenized in freshly prepared 5 ml of 3% aqueous sulfosalicylic acid. The homogenate was filtered through Whatman No. 1 filter paper and centrifuged at 14000 g for 15 min. A 2 ml aliquot of the supernatant was mixed with an equal volume of glacial acetic acid and acid ninhydrin and incubated in a water bath for1h at 100° C. The reaction was stopped in ice bath and the mixture was extracted with 4 ml of toluene and vortexed for 20-30 seconds. Subsequently, the toluene layer was separated and the final mixture was again warmed to room temperature. The upper phase with slightly red colour was taken in a separate test tube for measuring the absorbance at 525 nm, using toluene for a blank and L-proline as the standard. A standard curve was prepared using 0.1, 0.2, 0.3, 0.4 and 0.5 µmol of pure proline and used for conversion of absorbance values into proline contents.

The Proline concentration was determined from the calibration curve and was expressed as µlmol proline g^{-1} dw (Irigoyen *et al.,* 1992). For the determination of total soluble sugars, 0.5 g of the fresh leaf was crushed in a mortar and 5 ml of 80% ethanol was added to it, based on the Anthrone method (Irigoyen *et al.,* 1992). The mixture was centrifuged at 9000 g for 15 min and the supernatant was separated and further extracted with 12.5 ml of 80% ethanol. One ml of the supernatant and 1 ml of 0.2% Anthrone were mixed in a separate test tube and heated in a water bath at 100°C for 10 min. The reaction was stopped on ice for 5 min. Total soluble sugar content was determined using a spectrophotometer at 620 nm. Contents of soluble sugar were determined using a standard curve (glucose standard) and expressed as µg/g fresh weight.

4.2.7. Measurements of growth related physiological parameters

At the end of the experiment, potted plants were harvested and leaf area was determined, using AM-100 Area Meter (Analytical Development Company, Hertsford- shire, UK). Leaf dry mass was determined after oven-drying for 48 h at 70° C. Also, Plants were divided into root and shoot portions, and weighed, and oven-dried at 85^{o} C to a constant weight before being weighed again to determine total fresh biomass (TB) and total dry biomass (TDB); which were defined as the sum of the values for root, shoot, and leaf fresh and dry masses, respectively. The root: shoot ratio (RSR) was calculated as root mass divided by shoot mass. The leaf area ratio (LAR) equaled TLA divided by TDB per plant; while specific leaf area (SLA) was determined as leaf area (TLA) divided by total dry mass of the leaves. The relative growth rate (RGR) was calculated by the standard formula of RGR = (lnW2 - lnW1)/ (t2 - t1), where W1 and W2 were the initial and final dry biomass, respectively, and (t2 - t1) was the time interval (Wildy *et al.,* 2004). Roots and shoots having less than 15cm were not considered for analysis as a representative sample.

4.2.8. Statistical analyses

Data from parameters measured were subjected to normality tests, prior to an analysis of variance using the General Linear Model (GLM) in the SAS 9.3 program (SAS Inc., North Carolina, and USA). Means were compared using the Least Significant Difference test at $P \leq 0.05\%$. Analysis of variance (two-way ANOVA) was made for all the parameters measured and differences among genotypes were explored. Interactions between genotypes, drought stress and time were evaluated using appropriate statistical tools. Significant correlations among physiological parameters were determined with a Pearson's correlations coefficient test at $P \leq 0.05$.

4.3. Results

4.3.1. Plant water status

Determinations on relative water content (RWC) on the six apple genotypes are provided in (Table 4.1). None of the well-watered (WW) genotypes showed significant ($P < 0.05$) differences in their cellular RWC throughout the four-week study period. However, RWCs of all the genotypes decreased with increasing period of drought stress. Significant differences in RWCs were evident among the genotypes already during week one of the drought stress treatment, with Anna, Dorsette golden and Granny smith being characterized by significantly higher RWCs, compared to Royal gala, Golden delicious, and Red delicious. Interestingly, these differences persisted throughout the study period. These results and the consequent interactions have been verified by both one-way and two way analyses of variance (Table 4.1). However, no differences were observed under well-watered conditions until the fourth week of stress. Under drought stress conditions, genotypes Anna and Dorsette golden consistently exhibited significantly higher RWC, followed by the genotype Granny smith; while genotypes Goden delicious, Red delicious and Royal gala showed low values of RWC throughout the study period (Table 4.1).

Table 4.1: Variations among genotypes for relative water content (RWC) measured at a weekly interval after the onset of drought stress

Relative water content (RWC)								
	Week 1		**Week 2**		**Week 3**		**Week 4**	
	WW	**S**	**WW**	**S**	**WW**	**S**	**WW**	**S**
Genotype								
Anna	0.86	0.85	0.87	0.78	0.88	0.73	0.95	0.54
D. golden	0.88	0.85	0.86	0.75	0.90	0.67	0.92	0.53
G. smith	0.92	0.84	0.88	0.71	0.91	0.61	0.93	0.41
Royal gala	0.85	0.71	0.87	0.65	0.87	0.54	0.87	0.25
G. delicious	0.84	0.72	0.85	0.61	0.86	0.47	0.91	0.22
R. delicious	0.87	0.71	0.88	0.56	0.89	0.36	0.94	0.21
Mean	0.87	0.78	0.87	0.68	0.89	0.56	0.92	0.36
LSD 0.05	0.76		0.71		0.57		0.25	
CV (%)	7.09		7.39		17.23		21.73	
Two-way *ANOVA* F- values….								
Genotype (G)	5.30**		4.94**		3.97**		1.63**	
Watering (W)	76.35**		130.51**		141.28**		145.18**	
Interaction (GxW)	1.97*		5.03**		2.91**		3.87**	

Ww = Well-watered, S= Drought stressed, ns = non-significant difference; Different letters within same column shows significant differences. Levelofsignificance: (*P<0.05, **P<0.01)

4.3.2. Leaf water potential

The midday leaf water potential values of apple leaves are presented in (Table 4.2). The values decreased with the time course of drought stress treatment and the decrease was significantly different from that of the control. Leaf water potentials (LWPs) were -1.0 and -2.73 MPa one and three weeks, respectively, post stress treatments (Table 4.2). However, the rate of decline in LWP was much higher and faster at later stages (after second weeks) than at the early stages of stress development. After first week of stress treatments, apple genotypes Anna, Dorsette golden and Granny smith showed slow declines in their LWPs. Conversely, cultivars Royal gala, Golden delicious and Red delicious showed faster declines in their LWPs (Table 4.2). In general, LWPs decreased continuously up until the end of the stress period. (Table 4.2).

Table 4.2: Midday leaf water potential (LWP) of the six apple genotypes as affected by drought stress.

	Midday leaf water potential (MPa)					
	Week (1)		Week (2)		Week (3)	
	Well watered	**Stressed**	**Well watered**	**Stressed**	**Well watered**	**Stressed**
Genotype						
Anna	–0.17± 0.05b	–0.07±0. 03a	–0.93± 0.13 b	–0.68± 0.28 b	–1.27 ± 0.34a	–1.82± 0.65c
Dorsette golden	–0.10 ± 0.03a	–0.03± 0.37 a	–0.75± 0.11a	–0.61 ± 0.09a	–1.14± 0. 32a	–0.97 ± 0.42b
Granny smith	–0.61± 0.25c	–0.61± 0.21 b	–1.45± 0.10 b	–1.38 ± 0.31c	–1.53± 25 b	–0.85± 0.56a
Royal gala	–0.23± 0. 052b	–0.08± 0. 018a	–0.97± 0. 17a	–1.12± 0.62b	–1.08± 0.54 a	–2.73 ± 0. 50c
Golden delicious	–0.15± 0.05a	–0.09±0. 026 a	–0.86± 0. 32a	–1.13± 0.81 b	–1.51± 0.62 c	–2.38± 0. 64 b
Red delicious	–0.42± 0.26 c	–0.47±0.034 b	–1.18± 0. 25 c	–1.65 ± 0.54c	–1.27 ± 0.28b	–2.41 ± 0. 042b
P	**	**	**	**	**	**

Data represent mean ± SE (n = 3), Level of significance: ($P<0.05$)

4.3.3. Stomatal conductance

Two groups of apple genotypes were broadly differerentiated in terms of their stomatal conductances one and three weeks post drought treatments (Table 4.3). The first group consisted of Anna, Anna and Dorsette golden and Grany smith (with conductances ranging from 239-283 mmol $m^{-2}s^{-1}$), while the second group included Royal gala, Golden delicious and Red delicious (with conductances ranging from 495-558 mmol $m^{-2}s^{-1}$). Interestingly, reductions in stomata conductances were proportional to drought stress treatments, i.e., the first group of apple genotypes which had relatively lower stomatal conductaces under well-watered (Ww) conditions also conducted less under drought stress (S) conditions. Similarly, the second group of apple genotypes which had relatively higher stomatal conductaces under WW conditions also conducted more under S, with the exception of Red delicious which showed post stress treatment stomatal conductace of 112.75 mmol $m^{-2}s^{-1}$. There were significant ($p<0.05$) interactions among genotypes and drought stress conditions (Table 4.3).

Table 4.3: Variation among genotypes in stomatal conductance 1 and 3 weeks post drought stress

Stomatal conductance				
	Week 1		**Week 3**	
Genotype	**Ww**	**S**	**Ww**	**S**
Anna	239.00	134.50	375.50	34.25
D. golden	268.75	165.75	412.25	38.75
Granny smith	283.25	178.75	437.25	39.50
Royal gala	495.00	354.75	502.75	52.50
G. delicious	557.00	386.25	556.50	71.25
R. delicious	558.75	388.25	595.50	112.75
Mean	400.29	268.05	479.92	58.17
LSD 0.05	46.95		35.45	
CV (%)	21.31		14.25	
Two-way *ANOVA* F- values				
Genotype	1.39*		1.98*	
Watering	230.09**		161**	
Interaction	36.15**		2.69**	

Ww = Well watered, S= Drought stressed, Different letters within same column show significant differences. Level of significance: Levelofsignificance: (*P<0.05, **P<0.01)

4.3.4. Transpiration rate

Significant differences in transpiration rates (E) were observed among genotypes one week after drought stress had been imposed (Table 4.4). One way ANOVA showed highly significant variations in E in both the well-watered and stressed genotypes (Table 4.4). Genotypes Red delicious and Golden delicious showed higher Es, compared to the rest genotypes Anna, Dorsette golden and Granny smith which showed lower values of E under both well-watered and stressed conditions (Table 4.4). Two-way ANOVA indicated that there were significant interactions among genotypes and drought stress, three weeks after treatment (Table 4.4).

Table 4.4: Variation among genotypes in transpiration rate at the first and third weeks of drought

Transpiration rate				
	Week 1		**Week 3**	
Genotype	**WW**	**S**	**WW**	**S**
Anna	3.38	1.56	3.28	0.83
Dorsette golden	3.43	1.51	3.33	0.84
Granny smith	3.51	1.97	3.78	0.91
Royal gala	4.45	3.65	4.53	1.76
Golden delicious	5.45	4.05	5.27	1.39
Red delicious	5.48	4.07	5.11	1.85
Mean	4.28	2.81	4.22	1.10
LSD 0.05	0.56		0.11	
CV (%)	3.98		1.49	
Two-way *ANOVA* F- values				
Genotype	1.98*		1.13	
Watering	161.83**		87.94**	
Interaction	1.02		4.07**	

Ww = Well watered, S= Drought stressed, Ns = non-significant difference; different letters within same column shows significant differences. Levelofsignificance: (*P<0.05, **P<0.01)

4.3.5. Net photosynthesis

Net photosynthesis (Pn) differed significantly among the majority of genotypes after both the first and third week of drought stress (Table 4.5). After the third week of drought stress, all the genotypes showed lower net photosynthesis (Pn), compared to the well-watered plants of the respective genotypes. Interestingly, Anna achieved the highest Pn during both the first and third week of stress with values of 13.95 and 9.20 µmol CO_2 $m^{-2}s^{-1}$, respectively. Anna was followed by Dorsette golden (13.45 and 5.58, µmol CO_2 m^{-2} s^{-1}) and Granny smith (10.63 and 5.13 µmol CO_2 $m^{-2}s^{-1}$) in Pn during the first and third week of stress, respectively. Genotypes Royal gala, Golden delicious and Red delicious showed the lowest Pn values (Table 4.5).

Table 4.5: Variation in net photosynthesis among genotypes at the first and third weekof drought

Net photosynthesis				
	Week 1		**Week 3**	
Genotype	**WW**	**S**	**WW**	**S**
Anna	18.0	13.95	17.63	9.20
Dorsette golden	17.56	13.45	17.75	5.58
Granny smith	15.39	10.63	15.03	5.13
Royal gala	16.20	7.13	16.33	3.20
Golden delicious	16.45	7.23	15.65	3.43
Red delicious	15.15	6.75	15.31	2.48
Mean	16.46	9.86	16.28	4.84
LSD 0.05	1.43		1.02	
CV (%)	8.5		2.99	
Two-way *ANOVA* F- values				
Genotype	1.44**		1.02	
Watering	20.72**		1.24**	
Interaction	12.87**		3.57**	

Ww = Well-watered, S= Drought stressed, ns = non-significant difference; different letters within same column shows significant differences. Levelofsignificance: (*P<0.05, **P<0.01)

4.3.6. Specific leaf area

For Specific Leaf Area (SLA), drought stress treatments showed significant differences between the first and fourth weeks of stress, but, there were no differences among genotypes for SLA (Table 4.6). However, the interaction between genotype and drought stress was significant for SLA, after the second and third week of stress (Table 4.6), and showing that drought stress caused a significant increase in SLA among the tested genotypes.

Table 4.6: Variation between genotypes for specific leaf area **(**cm^2g^{-1}**)** measured at a week interval as drought stress progresses.

Specific leaf area								
Genotype	**Week 1**		**Week 2**		**Week 3**		**Week 4**	
	Ww	**S**	Ww	S	Ww	S	Ww	S
Anna	173.76	181.09	172.21	186.39	183.40	245.07	243.97	348.56
D. golden	177.19	184.53	166.31	198.41	191.12	253.45	245.97	366.03
G. smith	178.42	189.55	173.76	207.78	195.66	256.98	253.18	371.34
Royal gala	180.65	192.89	175.10	212.08	198.05	252.91	255.67	375.06
G. delicious	180.65	214.27	187.23	216.51	203.88	332.64	301.51	395.55
R. delicious	182.21	198.11	189.21	215.39	201.74	261.30	254.40	376.06
Mean	180.59	193.40	177.29	211.09	195.65	263.72	259.12	380.43
LSD 0.05	21.73		23.80		24.45		19.96	
CV (%)	34.31		30.88		32.54		22.04	
Two-way *ANOVA* F- values								
Genotype	4.67*		1.94*		2.18*		1.12	
watering	5.67**		22.11**		21.98**		4.57**	
Interaction	4.57**		4.12**		3.64**		1.24*	

Ww = well watered, S= Drought stressed, ns = non-significant difference; different letters within same column shows significant differences. Levelofsignificance: (*P<0.05, **P<0.01)

4.3.7. Chlorophyll pigment contents

Drought stress reduced the content of both chlorophyll ‘a’ and ‘b’ in apple genotypes to varying degrees, but, did not affect the chlorophyll a/b ratio (Fig 4.1). Among the tested genotypes Anna and Dorsette golden showed a non-significant change for chlorophyll ‘a’, chlorophyll ‘b’ and total chlorophyll content (Fig 4.1), while Granny smith showed little changes. Drought stress significantly affect chlorophyll ‘a’, ‘b’ and total chlorophyll content in genotypes Red delicious, Golden delicious and Royal gala (Fig 4.1). Accordingly, genotype Red delicious drastically reduced chlorophyll contents by about 50%, after third weeks of drought treatment, followed by Royal gala and Golden delicious by about 46% and 45% respectively (Fig 4.1). Despite these differences, among genotypes, the chlorophyll a/b ratio was not significantly different for all the tested genotypes (Fig 4.1).

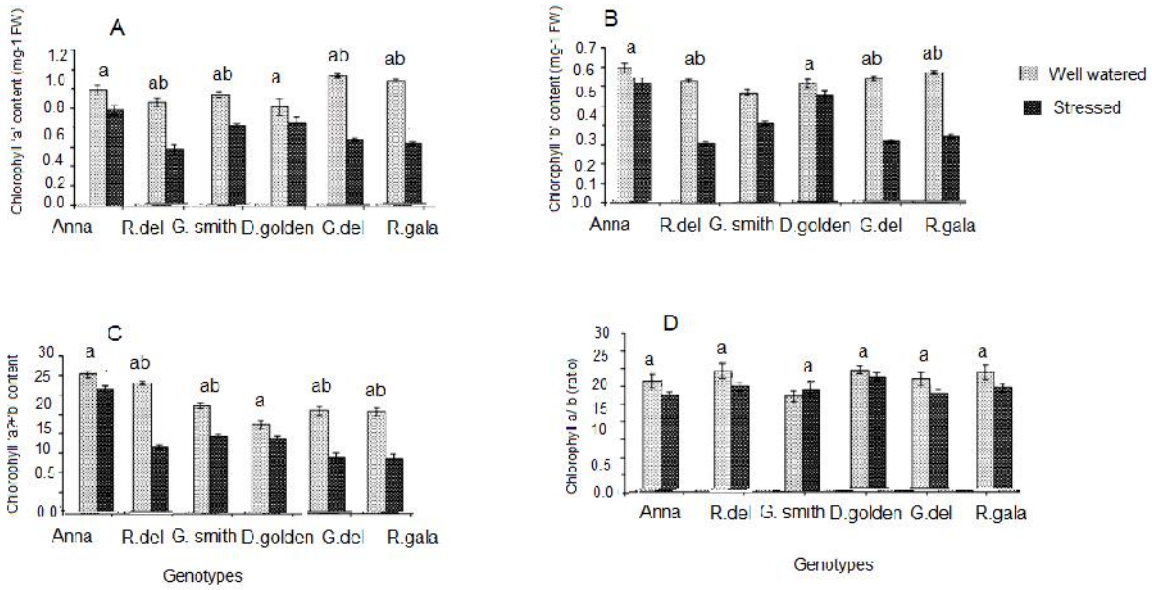

Figure 4.1. Effect of drought stress on chlorophyll content of six apple cultivars; (A): chlorophyll ‘a’, (B): chlorophyll ‘b’, (C): chlorophyll a+b contents, and (D): chlorophyll a/b ratio of each apple genotypes. Values are means ± SD (n = 3). ‘ab’ represents significant differences among treatments; while ‘a’ represent non- significant difference as calculated by t test ($p \leq 0.05$)

4.3.8. Proline content

Proline content increased under drought stress in all the tested genotypes (Fig. 4.2). Proline content of the genotypes Granny smith showed a drastic increase after the third weeks of drought stress; being approximately 50% higher than that of the control (Fig. 4.2), followed by Anna and Dorsette golden which showed ~ 45% and ~ 40% increament respectively for the same period, while Royal gala recorded (~20%) for proline content (Fig. 4.2). Genotypes Red delicious and Golden delicious recorded the least values for proline content (< ~ 15%) each after three weeks of drought stress, while (Fig. 4.2).

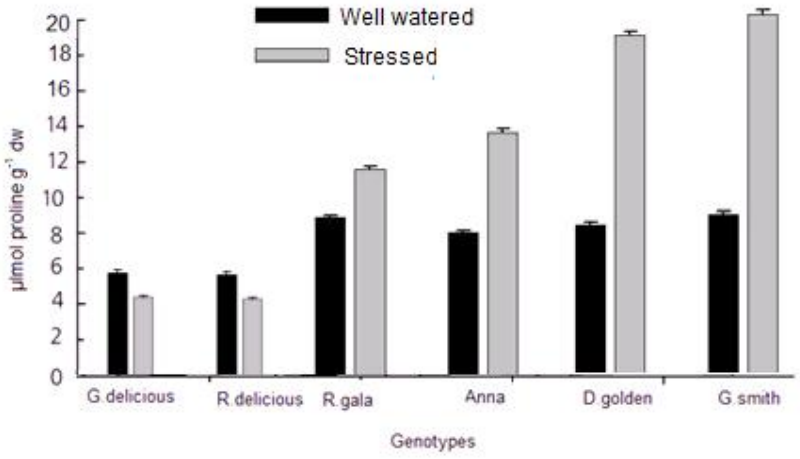

Figure 4.2: Effects of drought stress on proline content of apple genotypes after three weeks of stress treatment. Values are means ± SD (n = 3). Level of significance: ($p < 0.05$).

4.3.9. Total soluble sugar contents

Total soluble sugar concentration in leaves of apple genotypes under drought stressed condition showed random variations over time (Fig. 4.3). However, the level of soluble sugar in leaves of drought-stressed plants showed a trend toward increasing concentrations (Fig. 4.3). For example, in genotypes Anna, Granny smith, Dorsette golden and Royal gala, soluble sugar concentration increased slightly as stress treatment progresses from the first week to third weeks (Fig. 4.3). Differently in genotypes Red deliciousand, Golden delicious and Royal gala, the leaf soluble sugar content showed little or no variation between the control and stressed plants. However, the general trend in total soluble sugar concentration in response to drought stress showed increasing trends that was useful in osmotic adjustment of the plants (data not shown).

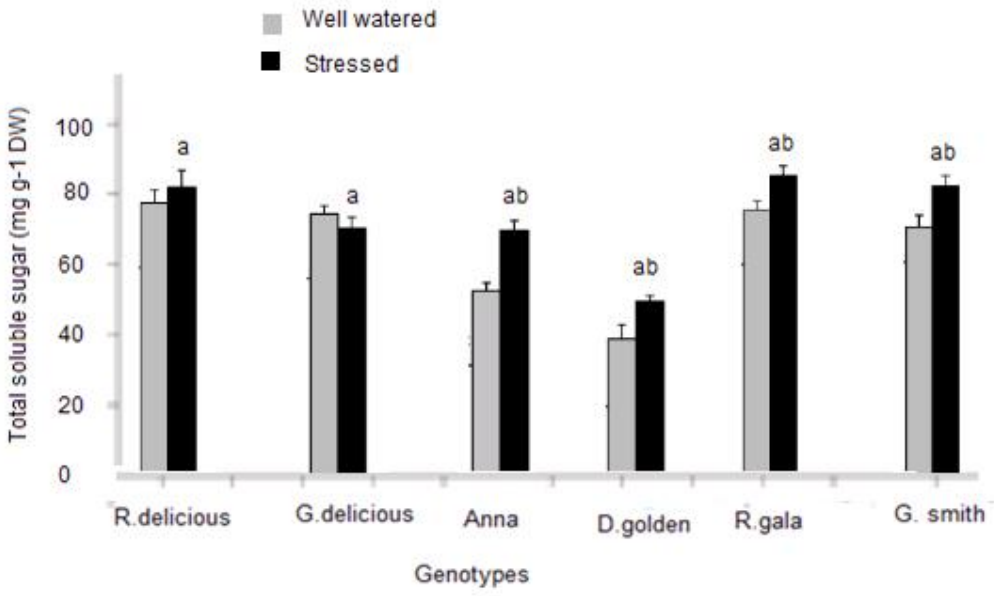

Figure 4.3: Effects of drought stress on soluble sugar content in leaves of apple genotypes. Data showed Means ± SE (n= 3). Differences were calculated by t test, and indicated by 'ab' ($p \leq 0.05$), and similar letters indicates no differences.

4.3.10. Association among growth related physiological parameters

At the end of experiment, all the genotypes tested were harvested and evaluated for some selected physiological parameters with respect to drought stress. Drought stress affected tree height (TH), trunk diameter (TD), total fresh biomass (TB), total dry biomass (TDB), and total leaf area (TLA). Both TB and TDB were positively correlated with TLA ($r = 0.57$ and 0.56, $P \leq 0.05$), but variations existed among genotypes (Table 4.7). Also, SLA was positively correlated with WHC (well-watered: $r = 0.68$; drought-stressed: $r = 0.71$, $P \leq 0.05$), but was not correlated with RWC (Table 4.7). Instantaneous water use efficiency (WUE_i) was positively correlated with stomatal conductance in well watered plants ($r = 0.65$, $P \leq 0.05$) (Table 4.7). Long-term water use efficiency (WUE_L) was significantly and positively correlated with TB and TDB (well-watered: $r = 0.83$ and 0.90; drought-stressed: $r = 0.61$ and 0.58, $P \leq 0.05$) (Table 4.7). Also, WUE_L was only weakly correlated with TLA and RSR under well-watered conditions ($r = 0.47$ and 0.36, respectively, $P \leq 0.05$) (Table 4.7), and similarly, a weak correlations existed among them under drought stress. In addition, WUE_L and SLA showed a significant negative correlation under both watering regimes (well-watered: $r = -0.82$; drought-stressed: $r = -0.78$, $P \leq 0.05$) (Table 4.7). On the other hand, a strong positive correlation was found between WUE_L and RGR for both control and stressed treatments (well-watered: $r = 0.92$; drought-stressed: $r = 0.77$, $P \leq 0.05$) (Table 4.7).

Table 4.7: Pearson's correlations coefficients among total fresh biomass (TB), total dry biomass (TDB), root: shoot ratio (RSR), relative growth rate (RGR), total leaf area (TLA), specific leaf area (SLA), relative water content (RWC), water- holding capacity (WHC), net photosynthesis (Pn), stomatal conductance (G_S), long-term water use efficiency (WUE_L) and instantaneous water use efficiency (WUE_I) of six apple genotypes under well-watered and stressed conditions.

Treat.	Parameters	TB	TDB	RSR	RGR	TLA	SLA	A	G_S
Ww	**TLA**	0.57*	0.56*						
	WHC						0.68*		
	RWC						−0.16		
	Gs							0.21	
	WUE_i								0.65*
	WUE_L	0.83*	0.90*	0.36	0.92*	0.47	−0.82*		
Stressed	**TLA**	0.36	0.18						
	WHC						0.71*		
	RWC						−0.08		
	Gs							−0.07	
	WUE_I							0.28	
	WUE_L	0.61*	0.58*	0.17	0.77*	0.38	−0.78*		

Level of significance: * $P \leq 0.05$, $n = 3$; Ww= well watered

4.4. Discussion

It has been shown that drought stress reduces relative water content and that genotypes with RW 0.50 have a high chance of recovery, but below this level may cause physiological injury to the plant (Taiz and Zeiger, 2010). The presnt study confirmed previous results of Sircelj *et al.,* (2007) that apple genotypes tolerant to drought stress maintain high RWC under stress conditions. The different responses in RWC under drought stress in apple genotypes have been attributed to water balance (Egert and Tevini, 2002), in addition to biochemical adjustments in plant cells (Chaves *et al.,* 2003). The variations among genotypes in this study could be attributed to changes in physiological processes, including water balance and photosynthesis. It can therefore be concluded that genotypes that show relatively high RWC have desirable traits for drought tolerance (Fernandez *et al.,* 1997). In the present study, the leaf water potential (LWP) declined substantially after third weeks of drought stress in all genotypes. This result was in agreement with the findings of Lima *et al.,* (2002) in pot-grown drought-sensitive and drought-tolerant Robusta coffee clones, where LWP has decreased to –3.0 MPa after six days of withholding irrigation. Such decreases in LWP resulted in a reduction of Pn in drought susceptible coffee clones. Similar studies indicates that reduction in LWP significantly reduce net photosynthesis in drought-stressed plants of banana (Adam and Barakbah, 1990), and grapevines (de Souza *et al.,* 2003, dos Santos *et al.,* 2003). Consistent with these studies, the present finding indicates that the rate of decline in LWP and net Pn during drought stress period greatly varied among genotypes (whether drought-sensitive or tolerant) and the time course of drought stress.

Under drought stress, the rate of Gs declines faster during the first week of stress for all the tested genotypes, and then gradually decreased for genotypes Anna, Dorsette golden and Granny smith in the following weeks; while it declines as a faster rate for genotypes Royal gala, Golden Delicious and Red delicious. This indicates that genotypic differences in stomatal conductance were observed under both stress and non-stressed conditions. Genotypes Anna, Dorsette golden and Granny smith responded to water stress by quickly closing their stomata (low stomatal conductance), for the maintenance of plant tissue water. Stomata closure during water stress results in improved water use efficiency in drought tolerant clonal *Malus* (Ma X.W *et al.,* 2010; Cao *et al.,* 2004; Atkinson *et al.,* 2000), which may explain the response of genotypes to water stress in the present study. The low stomatal conductance in Anna and Dorsette golden indicates either a low rate of transpiration from the leaves, or a high assimilation of carbon dioxide for increased photosynthesis, while in

genotypes Royal gala, Golden delicious and Red delicious, the high stomatal conductance contributes to increased water loss, which may be manifested by low RWC. Fernandez *et al.*, (1997) identified a positive association between stomatal conductance and dehydration in young apple seedlings grafted on three different rootstocks as the seedlings exposed to progressive drought. Similarly, Bacelar *et al.*, (2007) indicates that in three olive cultivars under contrasting water availability regimes, low stomatal conductance in drought tolerant genotypes is beneficial through continued carbon assimilation for high net photosynthesis, while the susceptible olive genotypes showed high stomatal conductance that contributes to a high transpiration rate, which consequently accelerates dehydration.

Drought stress significantly reduced photosynthesis and there were significant interactions between genotype and drought stress that indicates the existence of genotypic differences in response to reduced water availability. The differences observed among genotypes in drought stressed conditions after the third week of stress categorized the genotypes into two groups: a) genotypes Anna, Dorsette golden and Granny smith with high photosynthetic capacity; b) genotypes Golden delicious, Red delicious and Royal gala, with the lower photosynthetic capacity. The high photosynthetic capacity for genotypes Anna, Dorsette golden and Granny smith could be attributed to low stomatal conductance and lower rate of transpiration, which enabled continued assimilation of carbon dioxide. By contrast, genotypes Royal gala, Golden delicious and Red delicious maintained lower values of stomatal conductance and consequently low net photosynthesis under drought stress.

Reddy *et al.*, (2004a&b) reported that genotypes with low and extremely low net photosynthesis under drought stress are associated with a high metabolic impairment, which leads to disruption of cellular activities. The present study confirmed that the lowest net photosynthesis values for genotype Red delicious may be due to an early disruption of cellular processes, resulting from continued water loss from the onset of drought stress resulting in loss of turgor and low relative water content. Massonnet *et al.*, (2007) studied the stomatal regulation of photosynthesis in apple leaves under drought stress conditions and indicate that genotypes that maintained an active leaf canopy under drought stress showed high net photosynthesis, but, variation among genotypes existed in their response to drought. Similarly, Gomes *et al.*, (2004) in the study of orange trees submitted to drought stress reported that genotypes with drought tolerance ability exhibit high net photosynthesis due to continued carbon dioxide assimilation. Similar investigation was reported by Jie *et al.*, (2001) in apple that genotypes with relatively lower values of net photosynthesis might be

susceptible to drought; while genotypes with higher values may have a true drought tolerance mechanism. The present study also confirmed that three genotypes Anna, Dorsette golden and Granny smith have maintained low stomatal conductance and high net photosynthesis, which indicates its ability to assimilate carbon dioxide for photosynthesis under conditions of drought stress.

Drought stress significantly increased specific leaf area in all the genotypes. However, genotypes with better drought tolerant ability, such as Anna, Dorsette golden and Granny smith showed low SLA under drought conditions in the present study. The result was in conformity with (Girdthai *et al.,* 2012; Liu and Stutzel, 2004) that drought tolerant genotypes, which maintain low SLA under drought conditions, are associated with high water use efficiency. Furthermore, genotypes with small SLA under drought stress maintained an active canopy that would result in high net photosynthesis which further supports the importance of low SLA in selecting genotypes for drought tolerance (Liu and Stutzel, 2004). Perez-Harguindeguy *et al.,* (2013) also reported that SLA should be calculated from the fully expanded young and photosynthetically active leaves, based on the rule of thumb indicates that a reduction in SLA was strongly associated with drought tolerance for many different species. The differences in SLA between the current and other studies could be attributed mainly to changes in leaf dry mass, rather than changes in leaf area (Bogale *et al.,* 2011). Thus, drought tolerant genotypes may have the capacity to photosynthesize and derive less assimilates from the old leaves, maintaining high biomass production due to active photosynthesis, and hence maintaining relatively high leaf dry matter and reduced SLA. In the present study, drought susceptible apple genotypes (Red delicious, Golden delicious and Royal gala), instead of photosynthesizing more, may completely rely on the available assimilates from the old leaves, and this led to a large increase in SLA and less photosynthetic capacity. Also in the present study, three apple genotypes, Anna, Dorsetted golden and Granny smith continued photosynthesizing under drought stress and hence reduced SLA, as a result of increased leaf dry matter. Also, values for leaf area ratio (LAR) were lower because relatively less biomass was being allocated to the leaves and plants were also producing leaves with higher tissue density that decreased the leaf area per unit leaf mass. This process provided less surface area and, therefore, a lower light harvesting capability per unit of investment in leaf mass (Nair *et al.,* 2013; Hessini *et al.,* 2008). A smaller specific leaf area (SLA) in drought-stressed plants is mostly caused by a significant reduction in individual leaf area (Li *et al.,* 2009).

Additionally, when water is limiting, leaf tissues become thicker and denser (Castro-Diez *et al.,* 2000) due to increased stomatal resistance against transpiration.

Chlorophyll contents were significantly reduced by drought stress in the present study. Similar findings were presented by (Lei *et al.*, 2006; Pagter *et al.*, 2005; Cui *et al.*, 2004), that plants had reduced Chl contents under drought stress. Mihailović *et al.,* (1997) indicates that the decrease in Chl content during drought stress was due to the slow synthesis or fast breakdown of chlorophyll as a consequence of production of reactive oxygen species (ROS) in chloroplast; because an excess of excitation energy is not dissipated by the protective mechanisms under conditions of drought stress. Caemmeter and Farquhar (1981) state that drought stress cause stomatal closure and decline in CO_2 fixation; may cause production of ROS in chloroplast that causes a decline in leaf chlorophyll content. In the present study, Chl content was generally higher in genotypes Anna, Dorsette golden and Granny smith, and these genotypes were able to maintain a higher photosynthetic rate with less water transpired, had higher RWC, and growth potential than the other tested genotypes.

Proline concentration increased as a result of drought stress and the rate of increase differed between genotypes. In the present study, genotypes Anna, Dorsette golden and Granny smith exhibited a more rapid and much higher accumulation of proline compared with other apple genotypes after the first week of drought treatment and throughout the stress period. The increase in proline accumulation in stressed leaves of these genotypes was associated with a decrease in LWP and maintenance of osmotic adjustment in cells to maintain turgidity against the stress. Therefore, differences between apple genotypes for the level of proline accumulation with increasing time of drought stress might be associated with variations for osmotic adjustment to overcome drought stress (Siamak *et al.,* 2012). Thus, the rapidity of its accumulation in leaf tissue following the onset of dehydration could be regarded as one of the indicatives of osmotic regulation in drought-tolerant genotypes (Ismail *et al.,* 1994). Maestri *et al.,* (1995) indicates that a good correlation exist between the concentrations of proline and osmotic potential in both Arabica and Robusta coffee genotypes exposed to progressive drought.

Several researchers stated that the increase in proline concentration during drought stress was correlated with drought tolerance; as proline is believed to be one of the most important compatible osmolyte that play a vital role in osmotic adjustment in crops subjected

to water deficit and salinity stresses (Ahmad *et al.,* 2009; Ahmad *et al.,* 2008). Hence, the rate of accumulation of proline has been regarded as a tolerance mechanism and adaptation of genotypes to drought stress through osmoregulation in a number of crops, e.g. in sorghum (Volkmar and Woodbury, 1995) and banana (Siamak *et al.,* 2012). Proline frequently accumulates in large quantities in stressed plants as an osmotic regulator, and it serves as an osmotic protectant for many cellular structures during abiotic stress (Wahid *et al.,* 2007; Kishor *et al.,* 2005). Thus, the accumulation of proline may buffer the cellular redox potential under drought and other environmental stresses and thereby enhance the stress tolerance of plants (Machado and Paulsen, 2001). The present results revealed that increased proline accumulation was observed in apple genotypes Anna, Dorsette golden and Granny smith suggested that proline was biosynthesized as a means of regulating cellular osmosis and metabolism and conferring enhanced tolerance to severe drought stress conditions that minimized cellular damage. This higher accumulation of free proline in these genotypes suggesting that these genotypes respond quickly to drought stress by maintaining high cell turgidity due to high RWC and LWP, which enhances faster accumulation of proline as the drought stress progresses with time; and conferring enhanced tolerance to severe drought stress. Studies by (Sircelj *et al.,* 2007; Lei *et al.,* 2006) proved that free proline could act as an osmotic agent or an antioxidant in plants, decreasing the damage caused by water deficiency.

With respect to the relationships among physiological parameters in response to drought stress, two patterns were observed among the six apple genotypes tested. The first mechanism was the reduction of stomatal conductance and transpiration rate after the first week of drought stress was shown by genotypes Anna, Dorsette golden and Granny smith that led to the most efficient in gas exchange and water use; because of the high CO_2 assimilation with low water transpired. The second response type was the maintenance of high stomatal conductance and high transpiration for a prolonged drought stress, as exhibited by genotypes Royal gala, Golden delicious and Red delicious. The response of second type indicates continued loss of water through its high stomatal conductance due to the severe drought stress conditions. This continued water loss is supported by the consistently low RWC observed during the drought stress period. Kozlowski and Pallardy (2002) indicate that stomatal response is vital in maintaining water status of woody plants. Several reports indicates that an increase in resistance of the stomata increases the resistance to water movement relatively more to CO_2 movement, which leads to more reduction in transpiration rate, than in net photosynthesis, resulting in increased WUE in parallel with declining soil

water content (Lei *et al.,* 2006; Liang *et al.,* 2006; Monclus *et al.,* 2006; Yin *et al.,* 2005b; Yin *et al.,* 2004; Zhang *et al.,* 2004). Therefore, based on the stomatal conductance and transpiration rate, the six genotypes can be categorized into two: (i) drought tolerance (Anna, Dorsette golden and Granny smith), and (ii) drought susceptible (Royal gala, Golden delicious and Red delicious).

Stomatal conductance has a more direct association with transpiration than it does with net photosynthesis. The study also indicates that drought susceptible genotypes, such as Golden delicious, Red delicious and Royal gala exhibited high stomatal conductance, which contributed to high transpiration rate. On the other hand, the limited association of stomatal conductance with net photosynthesis in drought tolerant genotypes (Anna Dorestte golden and Granny smith) suggests the presence of additional non-stomata mechanisms controlling photosynthesis. Singh and Raja, (2011) reported strong relationship between stomatal conductance and transpiration in cowpea that stomatal conductance controls transpiration rate more than net photosynthesis.

Stomatal closure in response to drought stress has also been reported for grapevine (Stoll *et al.,* 2000) and tomatoes (Mingo *et al.,* 2004). In most cases, decline in turgor pressure and, thus Gs and Pn of plants subjected to a drought stress is attributed to decrease in plant water status; indicating stomatal regulation by hydraulic signals, especially in drought-tolerant genotypes (Auge and Moore, 2002; Loewenstein and Pallardy, 1998 a&b; Volkmar and Woodbury, 1995). As observed in this study and several earlier studies, stomatal response to drought stress could occur during the early stage, and may result in the reduced rate of Pn in drought-sensitive genotypes (Loewenstein and Pallardy, 1998 a&b; Saliendra *et al.,* 1995), probably as a result of chemical signals produced by the roots and transported in the xylem sap to the leaves of drought-stressed plants (Bacon *et al.,* 1998; Thompson *et al.,* 1997). In the present study, drought tolerant genotypes show the importance of water potential and cell turgor in regulating photosynthesis when drought stresses progresses. These can be ensured by stomatal and non-stomata mechanisms. The non-stomatal mechanisms, which control net photosynthesis and RWC in drought tolerant genotypes, include cell membrane stability, high proline accumulation and high soluble carbohydrates (Khan *et al.,* 2007); that may explain why drought tolerant genotypes were able to maintain high RWC and continue photosynthesising even under very low moisture content.

Under drought stress conditions, plants modify their pattern for allocation of assimilates, either by decreasing their investment to leaves relative to other organs or by altering their relative amounts of photosynthetic and non-photosynthetic tissues (Maroco *et al.,* 2000). This includes reallocation of biomass from shoots to roots, possibly improving water uptake from dry soil so that their root: shoot ratio were higher (Li *et al.,* 2009; Liu and Stutzel 2004; Kozlowski and Pallardy 2002). Increased root-shoot ratio (RSR) under drought conditions has been observed in many plant species (Dias *et al.,* 2007; Monclus *et al.,* 2006; Yin *et al.,* 2004), that all of these findings are consistent with the theory of functional balance, which states that plants will react to water deficits with a relative increase in the flow of assimilates to their roots, leading to an increased root mass ratio.

Water use efficiency (WUE) is defined as the amount of water used for growth and biomass production (Monclus *et al.,* 2006; Liu and Stutzel 2004). In many plant species, this association can be described according to Pearson's correlations (Zhang *et al.,* 2008). Although leaf area is sensitive to biomass production, and it is a direct factor modulating WUE, the RSR strongly influences efficiency because water uptake is primarily determined by the quantity of water absorbed, i.e., a function of the root component (Bauerle *et al.,* 2011; Wu *et al.,* 2008; Atkinson *et al.,* 2003). Studies have also compared WUE in woody plants, e.g., oaks (Ponton *et al.,* 2002), poplars (Monclus *et al.,* 2009) and Grapes (Chaves *et al.,* (2007; Blum, 2005) showed substantial variations in WUE.

3.5. Conclusion

Apple genotypes studied were broadly differerentiated in to two groups in their response to drought stress. The first group includes genotypes Anna, Dorsette golden and Granny smith that show low stomatal conductance, low rate of transpiration and high net photosynthesis under drought stress. These genotypes also processed high RWC, low SLA and slow decline in LWP under drought stress. The second group constitutes genotypes Golden delicious, Red delicious and Royal gala that possesses drought susceptible characteristics, due to high stomatal conductance, high transpiration rate and low net photosynthesis under drought stress. They also experience increased SLA, low RWC and faster decline in LWP during drought stress. Based on these physiological responses, genotypes Anna Dorsette golden and Granny smith are being considered as a drought tolerant and recommended to grow in a drought prone apple growing environments; provided that the existing chilling conditions of the area allows these genotypes. Therefore, it appears that both physiological and biochemical as well as morphological parameters are equally important during the screening of apple genotypes for their drought tolerance.

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CHAPTER FIVE

Improved drought tolerance is associated with enzymatic antioxidants, lipid peroxidation, protein accumulation and stomatal control in apple (*Malus domestica* Borkh.) genotypes

Abstract

A set of biochemical and physiological parameters were compared as stress indicators in order to select the best stress marker for drought stress in apple genotypes. Physiological parameters like relative water content, net photosynthetic rate, stomatal conductance, rate of transpiration, and biochemical measurements; malondialdehyde (MDA), soluble protein and antioxidant enzyme activities (superoxide dismutase, catalase, peroxidase and ascorbate) were measured in leaves of apple trees subjected to well watered and drought stress under controlled greenhouse conditions. A potted experiment consist of six apple genotypes (Anna, Dorsette golden, Granny smith, Golden delicious, Red delicious and Royal gala) were evaluated for drought tolerance ability, using these parameters. Results showed that RWC and gas exchanges appeared as a greater index of genotype's tolerance or susceptibility to drought stress, followed by activities of antioxidant enzymes. MDA and soluble protein contents appeared as reliable indicators of moderate drought stress. Genotypes showed positive and negative significant correlations between physiological and biochemical parameters in response to drought stress. The result also revealed that relative water content, gas exchanges (Pn, Gs and E) and antioxidant enzymes (SOD, GPX and CAT) are the better biomarkers for drought stress; while MDA and soluble protein contents respond only moderately to drought stress. In conclusion, physiological and biochemical markers appeared to be a better tool in determination of drought stress in apples.

Keywords/Phrases: Antioxidant enzymes, dehydrines, drought stress, gas exchange, relative water content, lipid peroxidation, soluble protein

5.1. Introduction

Plants exposure to drought stress causes many physiological and biochemical changes within plant cells including hormonal metabolism, gene regulation and changes in proteins (Doupis *et al.,* 2013; Osakabe *et al.,* 2013; Krasensky and Jonak, 2012). Studies indicates that drought stress is usually monitored on the level of selected morphological and physiological parameters as an indicators; such as reduction of plant growth, reduced leaf area expansion, reduction in photosynthetic efficiency, carbon allocation and utilization (Jaleel *et al.,* 2008; Yildirim *et al.,* 2006; Azooz *et al.,* 2004; Taylor *et al.,* 2004; Ismail, 2003; Sultana *et al.,* 2002). Recent studies conducted at cellular level using biochemical markers to detect drought stress in fruit tree species revealed that antioxidants, free amino acids (protein accumulation), lipid peroxidation, and level of proline have been proven as an early indicator of stress situation and considered as a better stress markers than the previously known growth related physiological parameters (Yanbao *et al.,* 2006; Munne´-Bosch and Penuelas, 2004; Tausz *et al.,* 2003, 2001; Navari-Izzo *et al.,* 1990). The cells are normally protected against reactive oxygen species (ROS) by the operation of an antioxidant defense system, comprised of enzymatic (superoxide dismutase (SOD), catalase (CAT) glutathione reductase (GR), ascorbate peroxidase(APX) and proxidase (POD), that directly react with and scavenge active oxygen species (AOS) such as superoxide radicals (O_2^-), singlet oxygen (O^-_1), hydroxyl radicals (OH^-) and concomitantly H_2O_2 (Athar *et al.,* 2008; Koca *et al.,* 2007; Azevedo Neto *et al.,* 2006). Accordingly, superoxide dismutase (SOD; EC 1.15.1.1) is located in various cell compartments and a major scavenger of superoxide radical (O_2^-). This enzyme converts O_2^- to H_2O_2, which is eliminated by ascorbate peroxidase (POD; EC 1.11.1.7) at the expense of oxidizing ascorbate to mono hydro ascorbate (Masood *et al.,* 2006; Lee *et al.,* 2001). Hydrogen peroxide is also scavenged by catalase (CAT; EC 1.11.1.6) and peroxidase (POD) and converted into water and oxygen (Chaparzadeh *et al.,* 2004; Mittler 2002).

The non enzymatic antioxidants (ascorbate, α- tocopherol, carotenoids and glutathione) components that can regenerate oxidized antioxidants (Misra *et al.,* 2006; Shigeoka *et al.,* 2002; Smirnoff, 1993). However, several prior studies indicates that the levels of non enzymatic antioxidants have shown increases, decreases, or no effect, depending on the species, duration of drought stress (Munne-Bosch and Penuelas, 2004; Boo and Jung, 1999; Zagdanska and Wisniewski, 1996), and cannot be equally competent with enzymatic antioxidants.

Production of ROS and as a result antioxidant enzymes activities is further enhanced when plants exposed to various abiotic stresses, such as drought (Sharma *et al.,* 2012) salinity (Weisany *et al.,* 2012), and low and high temperature (Pastori and Foyer, 2002).

Total free amino acids are accumulated during drought stress and appear most likely in osmotic adjustment (Pinheiro *et al.,* 2004; Navari- Izzo *et al.,* 1990; Hanson and Hitz, 1982). Dehydrins (dehydration induced proteins) are synthesized in response to drought stress that belong to the group II late embryogenesis abundant (LEA) proteins (Close *et al.,* 1993, 1990, 1989); and are involved in various metabolic pathways as glycolysis, the Krebs cycle, and lignin synthesis (Riccardi *et al.,* 1998). Dehydrins have been characterized as hydrophilic, heat-stable, free of cysteine and tryptophan, responsive to ABA signaling, and rich in lysine (Vardhini, 2014; Close *et al.,* 1996), and play an important role in membrane protein stability and osmotic adjustment (Chen*et al.,* 2012b). Also, a dehydrin-like proteins induced during drought stress have similar function with dehydrins that it has been protect the cells from dehydration (Close *et al.,* 1990 , Dure *et al.,* 1989), and also have a function similar to compatible solutes (such as proline, sucrose, and glycine betaine) in osmotic adjustment. Thus, drought stress responsive proteins are indeed a group of proteins that are responsible to control solute concentration in the cytoplasm (Dure *et al.,* 1993b), have a cryoprotective role in macromolecular stabilization by binding water molecules to their hydrophilic surfaces, which prevents further denaturation of cellular proteins (Close *et al.,* 1996; Wechsberg *et al.,* 1994; Dure *et al.,* 1989).

The level of lipid peroxidation measured as malondialdehyde (MDA) content found to increase with increase of drought stress (Arora *et al.,* 2008; Azevedo Neto *et al.,* 2006). Peroxidation results in the breakdown of lipids and membrane function by causing loss of fluidity, lipid cross linking, and inactivation of membrane enzymes (Girotti, 1990). Malondialdehyde (MDA) content, which is a secondary breakdown product of lipid peroxidation (Halliwell and Gutteridge, 1989b) is commonly used for assessing lipid peroxidation and oxidative damage in both leaves and roots (Zhou and Zhao, 2004; Queiroz *et al.,* 1998), and its maintenance of low levels has been associated with increased drought stress resistance in many plant species (Lima *et al.,* 2002; Moran *et al.,* 1994; Sairam *et al.,* 1998; Zhang and Kirkham, 1994).

In recent years, studies on biochemical responses of apple trees to drought stress have been limited mostly to carbohydrates (Wang and Stutte, 1992), abscisic acid (Fernandez *et al.*, 1997), osmotic adjustment (Pretorius and Wand, 2003; Atkinson *et al.*, 2000), leaf emission of volatile compounds (Ebel *et al.*, 1995). Studies conducted by Jia *et al.*, (2003) and Sircelj *et al.*, (2005, 1999) to evaluate apple trees response to drought stress indicates that antioxidant responses to drought stress are quite variable, and this variability is due to diversity of apple cultivars, environmental conditions and intensity of drought stress. Also, by expanding the earlier studies quoted above which explored drought tolerance at a biochemical level, this research further improves an understanding of the detail biochemical and physiological basis of drought tolerance in apple trees. This would provide greater opportunities for intensifying selection of promising cultivars for drought-prone regions of tropical and sub tropical apple growing areas. Furthermore, such markers would be invaluable in assisting the development of highly targeted irrigation schedules. The objectives were: (i) to identify the extent and mechanisms of intra-specific variation of water use among apple genotypes by examining antioxidant defence and related biochemical mechanisms in relation to stomatal behavior during drought stress, and (ii) to assess the relationship between biochemical and physiological parameters in reference to drought tolerance in apple fruit trees.

5.2. Materials and Methods

5.2.1. Experimental site, plant materials, and growth conditions

The experiment was conducted at the National Institute for Biotechnology Research, based at Holetta Agricultural Research Centre found at an altitude of 2390 m.a.s.l., and hosted by the Ethiopian Institute of Agricultural Research (EIAR). The daily maximum and minimum temperature of the area are (Max: 22° C and Min: 6.3° C) respectively, has an average rainfall of 1100 mm per year and dominated by nitosols (Ethiopian Institute of Agricultural Research (EIAR) annual report 2012). A one year old six apple genotypes grafted on MM-106 semi dwarfing rootstock (commonly known as Anna, Dorsette golden, Royal gala, Granny smith, Golden delicious and Red delicious) were considered in this study. Trees were transferred from open nursery to 50 ml pot filled with substrates including top soil (1- 20 cm depth), well rotted hardened manure and little course sand in the ratio 5:2:1 (v/v/v). Before transferring plants to the greenhouse, the green house was adjusted under day/night temperatures of 28° C/18° C (±2° C) respectively and relative humidity @ 60 – 70%).

5.2.2. Drought treatments and experimental design

Potted trees inside the greenhouse were equally irrigated three times a week to maintain the filled capacity (FC) favorable for normal growth and arranged in completely randomized design (CRD) replicated three times. Similar irrigation management was continued for the first growing season (June to September during 2014) for better establishment and acclimation of the grafts prior to the start of drought treatment. A total of 108 trees, eighteen from each of the six genotypes were considered. Each plot constitutes six trees (3 control and 3 stressed) from each genotypes and 36 trees per replication. Drought treatment was started by withholding the water until (Ψx) at predawn (Ψ_{Pd}) reached about –2.75 MPa. Afterwards, stressed and controlled plants were kept at 50% FC and 80% FC respectively, throughout the experimental period, from October to February of the years (2014/2015). Soil moisture content for each pot was monitored twice a week for the whole period of water stress using 20cm long probes of Time-Domain Reflectometer (TDR model 1502C, Tectronix Inc. Beaverton, OR, USA) (Cerny, 2009; Topp and Davis, 1985) .

5.2.3. Data collection

Leaf relative water content (RWC)

Leaf relative water content (RWC) was determined weekly using 10 to 15 fully expanded leaves per treatment. Leaf samples were detached from the plants and immediately weighed to determine fresh weight (FW). The same samples were placed into covered petri dishes filled with distilled water for leaves to reach full hydration for about18 h at 4° C; and weighed immediately to determine turgid weight (TW). Afterwards, leaf samples were dried in an oven at 75° C for 48 hrs. to determine dry weight (DW). Leaf RWC was calculated as: (FW – DW)/ (TW – DW) x 100.

Gas Exchange Measurements

Gas exchange measurements were taken weekly throughout the experimental period, and was measured between 10:00 am and 13:00 pm. Net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (E) were measured on mature and fully expanded leaves using a CO_2 /H_2O IRGA (LCi, ADC Bioscientific Ltd., Hoddesdon, UK). All the photosynthesis measurements were performed on the outer fully expanded leaves sampled on branches located in the middle position.

Assays of Antioxidant Enzyme Activities

For determination of antioxidant enzyme activities, 0.5 g leaf sample from control and stressed plants were frozen in liquid nitrogen and finely ground by pestle in a chilled mortar, and the frozen powder was extracted with 10 ml of extraction buffer (50 mM potassium phosphate buffer (KH_2PO4, pH 7.0) containing 1 mM ethylene diamine tetra acetic acid [EDTA], 1% polyvinylpyrrolidone [PVP], 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl [PMSF], pH 7.8). The extractions were centrifuged at 15000 g for 20 min at 4° C (Bian and Jiang, 2009; He *et al.*, 2001; Mukherjee and Choudhuri, 1983). The supernatant was collected and stored at −80° C for further analysis of SOD, CAT, APX and GPX assays; and all the enzyme activity measurements were recorded using spectrophotometer, and expressed as $min^{-1}mg^{-1}$protein.

Catalase (CAT; EC 1.11.1.6) was measured according to Aebi (1984); briefly, 10– 40 µl extract was added to 810–840 µl potassium phosphate buffer (50 mM, pH 7). The reaction was started by the addition of 150 µl of H_2O_2 solution in phosphate buffer and followed by

monitoring the decrease in absorbance at 240 nm at 20° C for 1–2 min. Accordingly, a change of 0.01 units per minute in absorbance was considered to be equal to one unit CAT activity expressed as µmol min^{-1} mg^{-1} protein. Samples without H_2O_2 were used as blank.

The activity of super oxide dismutase (SOD; EC 1.15.1.1) was assayed according to the method of Giannopolitis and Ries (1977). The reaction solution (3ml) contained 50 mM potassium phosphate buffer (pH 7.8), 60 mM riboflavin (7,8-dimethyl-10-ribitylisoalloxazine), 195 mM methionine [2-amino-4-(methyl-thio)-butyric acid], 3 mM EDTA, 1.125 mM nitro blue tetrazolium [NBT; 2,2'-di-p-nitrophenyl-5,5'-diphenyl-(3,3'-dimethoxy-4,4'- diphenylene) ditetrazolium chloride], and 100 ml of extracted solution. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. A test tubes containing the reaction mixture were irradiated under fluorescent lights at 100 µmol m^{-2} s^{-1} for 20 min and then transferred into the dark for 10 min; afterwards, the absorbance of each solution was measured at 560 nm, using spectrophotometer. A non-irradiated complete reaction mixture served as a blank. One unit of enzyme activity was defined as the amount of enzyme (µmol min^{-1} mg^{-1} protein) that would inhibit 50% of NBT photo reduction.

Glutathione peroxidase (GPX; EC 1.11.1.7) activity was measured using the method described by (Ruley *et al.*, 2004; Tuna *et al.*, 2008). A 100µl of the plant extract was added to 3ml of assay solution consisting of 3ml of reaction mixture containing 13 mmol L^{-1} guaiacol, 5 mmol L^{-1} H_2O_2, and 50 mmol L^{-1} K-phosphate buffer (pH 6.5). The presence of H_2O_2, GPX catalyzes the transformation of guaiacol to tetra guaiacol (brown product). An increase in absorbance at 470 nm for 3min at 25° C was recorded. Samples without extract were used as blank. The quantity required to degrade 0.01 mol of guaiacol per min per mg leaf tissue (FW) was expressed as one unit of GPX activity, which was expressed as µmol min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined based on the oxidation of ascorbate using the modified method of Nakano and Assada (1981). The reaction solution (3ml) contained 100 mM sodium acetate buffer (pH 5.8), 3 mM ethylenediaminetetraacetic acid (EDTA), 5 mM H_2O_2, and 100 ml of extracted solution. The reaction was initiated by adding the enzyme extract. Changes in absorbance at 290 nm were read every 10 s for 60 s. One unit of APX activity was defined as an absorbance change of 0.01 per min.

Total soluble protein concentration assay

Protein concentration was determined according to Bradford (1976) with bovine serum albumin (BSA) as the standard. Bradford reagent was made by dissolving 100 mg Coomassie Blue G-250 in 50 ml 95% ethanol, adding 100 ml 85% (w/v) phosphoric acid to this solution and diluting the mixture to 1 liter with distilled water. Briefly, 200 mg fresh leaf material with 1600 µl of ice-cold extraction buffer (50mM potassium phosphate buffer, 1mM Na-EDTA, 1 mM L-ascorbic acid, 0.02M sodium bisulphite, 20% sorbitol and 2% PVPP, (pH 7.8). In a 96-well plate, 300 µl Bradford reagent was added to 10 µl aliquot of the sample protein extract or to the protein standard and the absorbance was measured at λ = 595nm (Infinite M200 TECAN Group Ltd., Switzerland). Protein standards should be prepared in the same buffer as the samples were assayed. A convenient standard curve was made using bovine serum albumin with concentrations of 0, 10, 20, 30, 40, 50 µg/ml for the micro assay (extinction coefficient of BSA is 0.667). The standard curve was obtained by plotting the absorbance at 595 nm against µg of protein in BSA standard samples. Thus, the amount of protein calculated in a given sample indicates the absorbance values for the different samples tested.

Lipid Peroxidation (MDA) content assay

Lipid peroxidation was determined by measuring the amount of Malondialdehyde (MDA) content produced by the thiobarbituric acid (TBA) reaction as described by (Dhindsa *et al.,* 1981; Heath and Packer, 1968). A fresh leaf sample (0.5 g) was homogenized in 10 ml of 5% trichloroacetic acid (TCA). 1ml aliquot of the supernatant was added to 2 ml of a reaction solution containing 20% (v/v) tri-chloro acetic acid and 0.5% (v/v) thiobarbituric acid. The solution was placed in a water bath at 95° C for 30 min and then transferred to an ice bath to stop the reaction. Afterwards, the solution was centrifuged at 10,000 g for 10 min, the absorbance of the supernatant was read at 532 and 600 nm. Nonspecific absorbance at 600 nm was subtracted from that at 532 nm, and MDA content was calculated using this adjusted absorbance and the extinction coefficient of 155 mm^{-1} cm^{-1} (Heath and Packer, 1968).

5.2.4. Statistical Analysis and experimental design

The experimental plants were arranged in completely randomized design (CRD) with three replications in a controlled glasshouse. The data were analyzed with one-way analysis of variance (ANOVA), using watering regimes as main effect, and with two-way ANOVA (repeated-measure analysis of variance) using watering regimes and genotypes as main effects for all the parameters; to evaluate the interactions among genotypes and watering regimes. The values of biochemical variables were tested for homogeneity of variance and transformations were carried out, if necessary, to meet the statistical assumptions of ANOVA (Sokal and Rohlf, 1995). All statistical tests were considered significant at ($P < 0.05$), and means were compared with Turkey's multiple range tests. Statistical software SAS (version 9.4, SAS Institute, Cary, N.C.) was used according to the general linear model (GLM) procedure of SAS in data analysis.

5.3. Results

5.3.1. Relative water content (RWC)

Drought stress significantly affect relative water content (RWC) leading to lower plant water status in stressed plants as compared to the control (Table 5.1). Under stressed conditions, genotypes Anna, Dorsette golden and Granny smith maintained higher RWC, as compared to genotypes Golden delicious, Red delicious and Royal gala, which showed lower values for RWC during progressive drought stress (Table 5.1). The interaction among watering regime, genotypes and time was highly significant for all the tested genotypes after days 14 to 21 (Table 5.1). Trends in drought stress treatment showed that genotypes had varied responses to drought stress (Table 5.1) during the time course of the experiment.

Table 5.1. Effects of drought stress treatments on relative water content of the six apple genotypes during progressive drought

% Relative water contents (RWC						
	7 days		**14 days**		**21 days**	
Genotypes	**WW**	**S**	**WW**	**S**	**WW**	**S**
Anna	0.89	0.80	0.90	0.73	0.87	0.58
D. golden	0.87	0.73	0.88	0.66	0.88	0.53
G.smith	0.86	0.73	0.86	0.64	0.90	0.51
G. delicious	0.89	0.58	0.90	0.52	0.94	0.32
R. delicious	0.88	0.55	0.89	0.37	0.93	0.20
Royal gala	0.86	0.52	0.89	0.46	0.94	0.27
LSD P < 0.05	0.57		0.21		0.26	
CV (%)	15.31		10.97		16.10	
Two-way *ANOVA* F-Values						
Genotype	3.75**		2.54**		1.78**	
Treatment	125.63**		146.28**		168.43**	
Interaction	2.15**		3.72**		3.92**	

Ww = well watered, WS= water stressed, ns = non-significant difference; Different letters within same column shows significant differences. Level of significance: (*P<0.05, **P<0.01)

5.3.2. Gas Exchange Analyses

Stomatal conductance (Gs), transpiration rate (E) and net photosynthesis (Pn) of drought stressed and control seedlings were presented in (Table 5.2). Genotypes Anna, Dorsette golden and Granny smith showed a slow decline in Pn during the time course of stress. Accordingly, the decline in net photosynthesis in genotype Anna was recorded 12.49 (µmol CO_2 m^2 s^{-1}) after 7days to 10.10 (µmol CO_2 m^2 s^{-1}) after 21days, followed by Dorsette golden 14.01 (µmol CO_2 m^2 s^{-1}) to 10.75 (µmol CO_2 m^2 s^{-1}), and in Granny smith 14.71 (µmol CO_2 m^2 s^{-1}) to 9.31 (µmol CO_2 m^2 s^{-1}) for the same dates as compared with the control plants (Table 5. 2). Other studied parameters (Gs and E) in these genotypes, showed lower values as opposed to Pn when the stress period progresses from 7 to 21 days (Table 5. 2). Conversely, in genotypes Golden delicious, Red delicious and Royal gala, the decrease of net photosynthesis (Pn) was significant soon after the first week of stress period (Table 5.2); For example, the decrease in net photosynthesis after 7 days of drought stress in genotype Golden delicious showed 10.54 (µmol CO_2 m^2 s^{-1}); while after 21 days, it decreased to 6.51 (µmol CO_2 m^2 s^{-1}) (Table 4.2). For the same dates, the decrease in net photosynthesis for Red delicious was 10.65 (µmol CO_2 m^2 s^{-1}) to 6.74 (µmol CO_2 m^2 s^{-1}), and in Royal gala 12.41 (µmol CO_2 m^2 s^{-1}) to 5.89 (µmol CO_2 m^2 s^{-1}) respectively as compared to the control plants. The values for Gs and E for these genotypes increased against Pn for stressed plants during the entire stress period (Table 5. 2).

Table 5.2. Effects of drought stress treatments on net photosynthesis (Pn), stomatal conductance (Gs) and transpiration rate (E) in six apple genotypes under progressive drought.

	7 days after stress treatment						14 days after stress treatment			
	Pn		Gs		E		Pn		Gs	
	Ww	S	Ww	S	Ww	S	Ww	S	Ww	S
Genotype										
Anna	18.05±0.02	12.49±0.05	268±0.72	105±0.19	6.73±0.13	5.72±0.08	17.97±0.13	10.14±0. 07	275±0.34	57±0.12
D. golden	17.41±0.01	14.01±0.04	273±0.50	138±0.56	6.84±0.18	6.43±0.06	16.43±0.11	12.65±0.10	245±0. 25	62±0.14
G. smith	16.91±0.02	14.71±0.03	285±0.52	153±0.12	7.04±0.05	6.66±0.06	16.67±0.15	11.49±0.09	279±0.36	71±0.28
G. delic	13.99±0.07	10.54±0.02	271±0.62	210±0.41	9.42±0.34	8.16±0.0 7	13.41±0.22	9.42±0.05	361±0. 32	102±0.37
R. delic	14.78±0.02	10.65±0.10	268±0.21	229±0.48	7.82±0.31	7.79±0.06	13.45±0. 17	9.34±0.11	386±0.58	98±0.65
Royal gala	13.97±0.01	12.41±0.04	295±0.81	215±0.54	8.34±0.18	7.86±0. 06	12.34±0. 12	8.21±0.02	394±0.74	135±0.73
P	Ns	**	Ns	**	Ns	**	Ns	**	Ns	**
LSD (0.05)	1.27		1.95		0.08		1.02		0.05	
CV	18		11		9.60		14		6.52	

Values represent mean ± SE (n=3) of three replications. Ns= not significant; Level of significance **($P < 0.05$)

Table 5.2. Conti……

	14 days…..		21 days after stress treatment					
	E		Pn		Gs		E	
	Ww	S	Ww	S	Ww	S	Ww	S
Genotype								
Anna	5.72±0.05	3.45±0.01	17.27±0. 03	10.10±0. 015	263±0.56	17±0. 021	4.85±0. 052	2.64±0. 018
D. golden	5.19±0.04	4.73±0.03	16.26±0. 011	10.75±0. 021	267±0.42	13±0. 034	4.23±0.0 44	2.78±0. 010
G. smith	6.79±0.03	4.43±0.02	16.47±0. 04	9.31±0. 018	279±0. 72	21±0. 030	5.17±0. 049	4.46±0. 027
G. delicious	6.81±0.25	5.54±0.04	15.47±0. 032	6.51±0. 026	315±0. 65	52±0.0 65	7.52±0. 042	5.27±0.0 38
R. delicious	7.19±0.05	6.66±0.07	17.74±0. 052	6.74±0. 026	338±0. 64	57±0. 037	7.12±0.053	6.05±0. 052
Royal gala	7.14±0.0 6	6.47±0. 072	16.27±0. 05	5.89±0. 035	357±0. 50	56±0.0 33	7.48±0. 066	7.16±0.047
P	Ns	**	Ns	**	**	**	Ns	**
LSD (0.05)	0.17		0.94		0.06		0.05	
CV	3.81		7.50		3.72		1.97	

Values represent mean ± SE (n=3) of three replications. ns= not significant; Level of significance **($P < 0.05$)

5.3.3. Activity of Antioxidant Enzymes

Antioxidant enzymes; Super oxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), and Ascorbate peroxidase (APX) showed a highly variable pattern of enzymatic activity among genotypes (Fig. 5.1, A to D).According to analysis of variances (Table 5.3), irrigation levels and irrigation × genotypes were found significantly different for the elevated activity of antioxidant enzymes (Table 5.3).

SOD activity increased remarkably in genotypes Dorsette golden (75%), followed by Anna (65%) and Granny smith (55%), and also showed low values for genotypes Red delicious (25%), Golden delicious and Royal gala (20% each) during the entire stress period (Fig. 5.1A). These results indicates that the increament in activity of SOD enzyme per minute showed as high as 905.336 µ/g protein/min, in genotype Dorsette golden, 875.321 µ/g protein/min in Anna and 856.321 µ/g protein/min in granny smith during drought stress (Table 5.4). Other genotypes showed low values of 116.298 µ/g protein/min in Red delicious, 92.452 µ/g protein/min in Golden delicious and 86.354 µ/g protein/min in Royal gala for SOD activity (Table 5.4).

Increased Catalase (CAT) activity was recorded for the genotype Granny smith (75%) followed by genotype Anna (55) and Dorsette golden (50%), but, the lowest value for CAT activity was recorded by genotype Royal gala (10%) (Fig. 5.1B). The increament in activity of CAT per minute for these genotypes showed 95.556 µ/g protein/min in Anna, 84.093 µ/g protein/min in Dorsette golden, 81.029 µ/g protein/min in Granny smith, and the lower value of 38.938 µ/g protein/min in Royal gala (Table 5.4).Conversely, in genotypes Red delicious and Golden delicious, CAT activity remains unchanged for the control and stressed treatments as indicated in the present study (Fig. 5.1.B; Table 5.4).

Glutathione peroxidase (GPX) activity showed highly increased responses to drought stress in genotypes Dorsette golden (85%), followed by Granny smith (75%) and Anna (70%) during the period of drought development from days 7 to 21 (Fig 5.1C). On the other hand, under condition of drought stress, GPX activity first increased during day 7 for genotypes Royal gala and Red delicious (~ 20% each), and sharply decreased afterwards until day 21 (Fig. 5.1C). This indicates the decrease in protein concentration per minute as the drought stress progresses in these genotypes showed a decrease in sensitivity of GPX enzyme against drought stress (Table

5.4). The increase in µ/g protein/min for GPX activity was higher in genotypes Anna (73.975 µ/g protein/min), Dorsette golden (65.431 µ/g protein/min) and Granny smith (60.979 µ/g protein/min); while the lowest value was recorded for genotype Golden delicious (6.359 µ/g protein/min) (Table 5.4). Thus, genotype Golden delicious recorded the least value of (10%) for GPX activity for the entire period of drought stress treatment (Fig. 5.1C).

Ascorbate peroxidase (APX) activity also showed significant changes for genotypes studied in response to drought stress (Fig. 5.1D). APX activity remained similar for the genotype Red delicious during the entire period of stress; while showed increament in all other genotypes. (Fig. 5.1D). The maximum value for APX activity was recorded by the genotype Granny smith (75%), followed by the genotype Dorsette golden (60%) and Anna (50%), as compared to the genotypes Royal gala and Golden delicious which recorded the lowest value of (15%) and (10%) respectively (Fig. 5.1D). Increament in activity of APX in µ/g protein/min indicates that genotype Granny smith took the highest value of (61.870 µ/g protein/min), followed by Dorsette golden (53.904 µ/g protein/min) and the lower values for Anna (23.934 µ/g protein/min) and the least value of (1.685 µ/g protein/min) in Royal gala (Table 5.4). Genotypes Golden delicious and Red delicious remain similar i.e. 0.655 µ/g protein/min and 0.607 µ/g protein/min respectively.

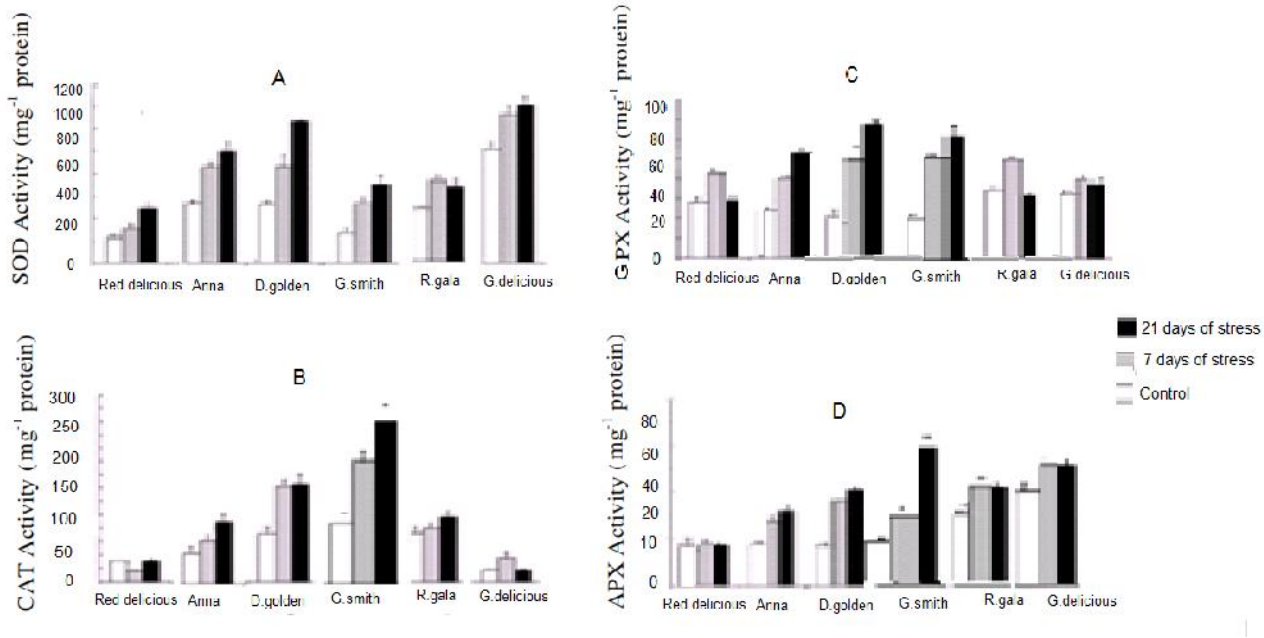

Figure 5.1 (A –D): Activity of antioxidant enzymes: (A) Super oxide dismutase (SOD), (B) Catalase (CAT); (C) Glutathione peroxidase (GPX), and (D) Ascorbate peroxidase (APX) in leaves (mg^{-1}dw protein) of the six apple genotypes as affected by drought stress. Values are mean ± SE, (n= 3). Level of significance ($P < 0.05$)

Table 5.3: Analysis of variance for Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX), Catalase (CAT) and ascorbate peroxidase (APX) enzymes activity under drought stress for apple genotypes

		SOD (µ/g protein)		CAT(µ/g protein)		GPX(µ/gprotein)		APX(µm/g protein)	
	df	**Ms**	**P.**	**Ms**	**P.**	**Ms**	**P.**	**Ms**	**P.**
Replication	2	20572.63	ns	927.114	ns	1.697	ns	0.25	ns
Watering (W)	1	1572516.0	**	17336.79	**	110.61	**	3.521	**
Error	2	40261.30		158.989		1.071		0.384	
Genotypes (G)	5	12040.146	ns	9.814	ns	1.648	ns	0.016	ns
W×G	5	336933.063	**	24.235	*	356.973	**	1.469	*
Error	5	5354.431		79.9		0.490		0.009	

ns, non significant; *, Significant @ p≤ 0.05; **, Significant @ p≤ 0.01

Table 5.4: Mean comparison of Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX), and Catalase (CAT) and Ascorbate Peroxidase (APX) enzymes activity (per minute/µ/g protein) under drought stress for apple genotypes.

Treatments	SOD (µ/g protein)	CAT (µ/g protein)	GPX (µ/g protein)	APX (µm/g protein)
Control	819.917a	68.732a	17.048a	11.735a
Drought Stress	1037.917b	126.871b	43.532b	31.173b
Anna	905.336a	95.556a	73.975c	23.934c
Dorsette golden	875.351a	84.093a	65.431c	53.904d
Granny smith	856.321a	81.029a	60.997c	61.870d
Royal gala	86.354c	38.938c	17.915a	1.685e
Golden delicious	92.452c	21.433c	6.359d	0.655f
Red delicious	116.298c	21.243c	7.056d	0.607f

Means in a column followed by the same letter are not significantly different @ p ≤ 0.01

4.3.4. Total soluble protein

The leaf soluble protein content of the apple genotypes was affected by drought stress, but the degree differed between genotypes (Fig. 5.2). Soluble protein content of genotypes Anna, Dorsett golden and Granny smith remained stable as the drought stress progresses from the first to third week, and no significant difference was observed between drought stress treatment and the control. Conversely, the soluble protein content of genotypes Red delicious, Royal gala and Golden delicious were decreased under drought stress treatment. Among them, the extent of the change in Red delicious and Royal gala were marked as nearly (20% each), followed by Golden delicious (15%) respectively.

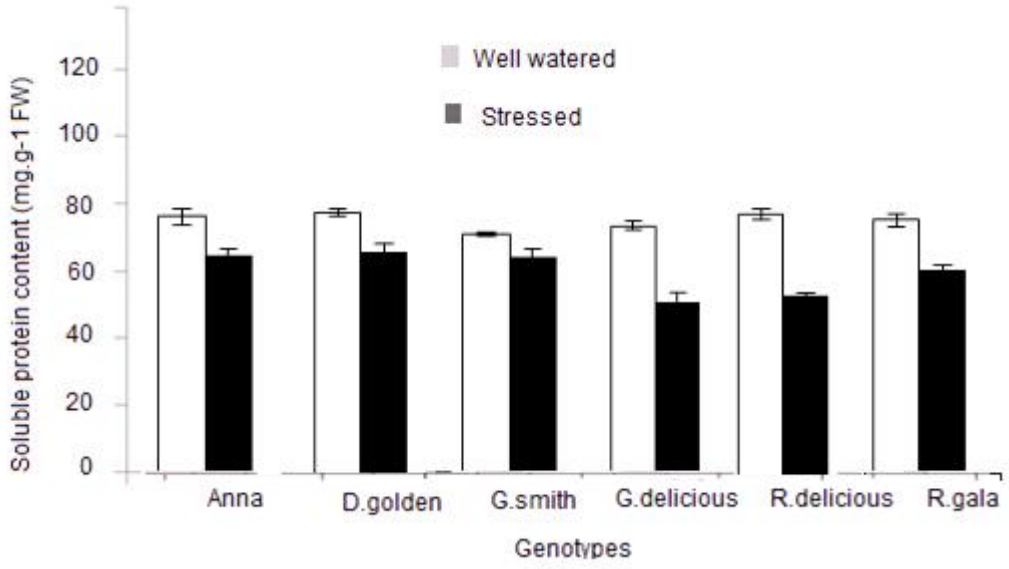

Figure 5.2: Effect of drought stress on soluble protein contents of apple genotypes. Values are means ± SD (n = 4). Level of significance: ($p < 0.05$).

5.3.5. Malondialdehyde (MDA) content

Under drought stress treatment, the MDA content raised in all the tested apple genotypes at varied concentration. (Fig.5.3). The MDA content in drought stressed Red delicious increased (~50%), followed by genotypes Golden delicious (~35%), and Royal gala (~20%) (Fig.5.3). Other genotypes recorded lower values for MDA content for stressed plants as in Dorsette golden (~5%), Anna (~10%) and (~8%) in Granny smith (Fig. 5.3).

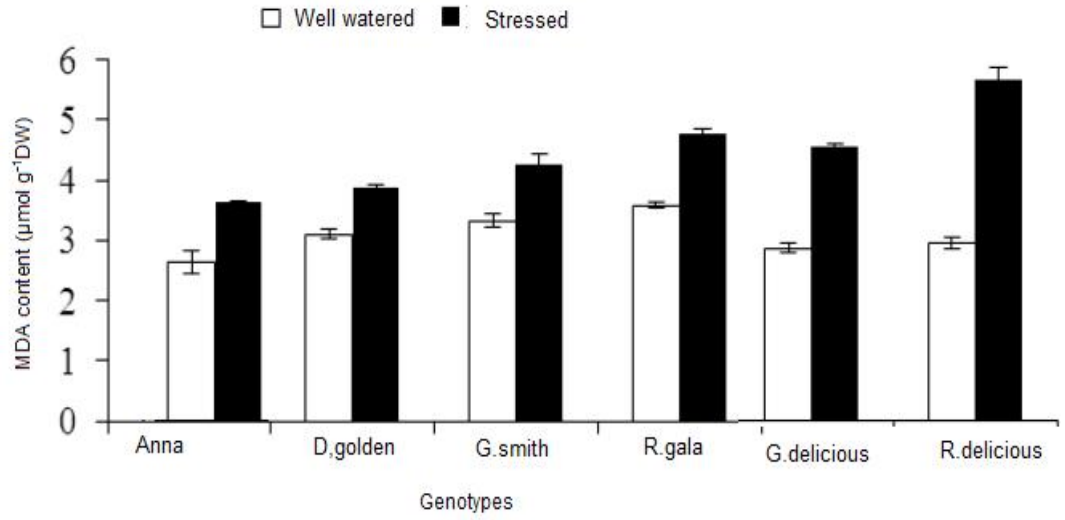

Figure 5.3: Effect of drought stress on malondialdehyde (MDA) contents of apple genotypes. Values are means ± SD (n = 4), Level of significance (P< 0.05)

5.3.6. Correlations between parameters

Pearson's correlation coefficient analysis among the physiological and biochemical parameters in apple genotypes was presented in (Table 5.5). Under well watered and stressed conditions, a significant positive and negative association ($P<0.05$; $P<0.01$) was recorded for the studied parameters. In well watered conditions, negative and non-significant correlations were observed for activities of antioxidant enzymes (Table 5.5); while in similar conditions, a strong positive correlation were established between relative water content and gas exchanges ($P \leq 0.05$) Table 5.5. Among the physiological parameters, a relative water content is significantly correlated with stomatal conductance ($r = 0.811$), rate of transpiration ($r = -0.836$), and net photosynthesis ($r= 0.836$) respectively (Table 5.5).

Regarding association among physiological and biochemical parameters, a non-significant negative correlations were exist between soluble protein content and these parameters; relative water content ($r = -0.396$); stomatal conductance ($r = -0.221$), and net photosynthesis ($r = -0.437$) for stressed plants (Table 5.5). On the other hand, a significant negative correlations were recorded for MDA contents and physiological parameters; relative water contents ($r = –0.690$), stomatal conductance ($r = -0.500$) and net photosynthesis ($r = -0.543$) Table 5.5. Antioxidant enzymes (SOD, GPX, CAT and APX) activities showed variable responses in correlations with physiological parameters (Table 5.5). Under drought stress conditions SOD activity showed significant correlation with relative water content ($r = 0.573$) and net photosynthesis ($r = -0.567$), and similarly GPX activity showed a significant negative correlation with relative water content ($r = -0.575$) and net photosynthesis ($r = -0.510$). CAT activity was also significantly correlated with relative water content ($r = 0.521$), stomatal conductance (–0.515) and net photosynthesis (–0.531). APX activity showed a negative significant correlation with relative water content ($= -0.515$), stomatal conductance ($r = -0.520$), and net photosynthesis ($r = -0.528$) (Table 5.5). Among biochemical tests, soluble protein and MDA contents showed a weak and non-significant negative correlation with all the antioxidant enzymes (Table 5.5).

Table 5.5: Pearson's correlation coefficients (r) between evaluated parameters of apple genotypes under contrasting water regimes.

Treatments	Parameters	RWC	Gs	E	Pn	Protein	MDA	SOD	GPX	CAT	APX
Control	RWC		0.845**	0.376*	0.874**	−0.343	−0.328	−0.067	–0.063	−0.014	−0.027
	Gs			0.363*	0.773**	0.211	0.043	−0.013	–0.012	−0.016	−0.021
	E				0.474*	−0.070	−0.107	−0.042	−0.047	−0.057	−0.062
	Pn					0.392	0.020	−0.017	–0.025	−0.015	−0.121
	Protein						−0.101	−0.011	−0.038	−0.015	−0.017
	MDA							−0.046	−0.053	−0.017	−0.051
	SOD								−0.031	−0.012	−0.112
	GPX									–0.010	−0.037
	CAT										−0.025
Stressed	RWC		0.811**	–0.567*	0.836**	−0.396	–0.690*	0.573*	0.575*	0.521*	−0.515*
	Gs			–0.645**	0.812**	−0.221	−0.500*	−0.161	−0.145	**−0.515***	**−0.520***
	E				0.711**	−0.164	−0.021	−0.064	–0.035	–0.281	−0.012
	Pn					−0.437	−0.543*	−0.567*	–0.512*	–0.531*	−0.528*
	Protein						−0.338	−0.363	−0.310	−0.288	−0.017
	MDA							−0.183	−0.043	−0.158	−0.026
	SOD								–0.017	−0.015	−0.051
	GPX									−0.027	−0.032
	CAT										−0.054

Level of significance: *P ≤ 0.05; **P ≤ 0.01

5.4. Discussion

Relative water content is considered a measure of plant water status, reflecting the metabolic activity in tissues and used as one of the indicator of dehydration tolerance. The present study indicates that RWC was decreased with the increasing levels of drought stress for apple genotypes. The result is in conformity with findings of Sharma and Sharma (2008) on various water stress levels in Flemish Beauty pear on *Pyrus* seedling and clonal quince rootstocks. Such a decrease in leaf RWC could be attributed to the unavailability of water in the soil (Shalhevet, 1993) or root systems (Gadallah, 2000). Wang *et al.,* (2012) also reported that RWC reduced on drought-tolerant and drought-sensitive apple rootstock genotypes; which reflects the reduced metabolic activity in tissues and is generally declined after water stress.

Drought stress severely hampered the gas exchange parameters of crop plants and this could be due to decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence, oxidation of chloroplast lipids and changes in structure of pigments and proteins (Menconi *et al.,* 1995). It is well known that leaf water status always interacts with stomatal conductance and a good correlation between leaf water potential and stomatal conductance always exist at both favorable and stressed environments. In the present study, drought stress caused a decrease in net photosynthesis to avarying degrees among genotypes; for example, the reduction in net photosynthesis in genotypes Golden delicious, Red delicious and Royal gala was related to higher rates of stomatal conductance and transpiration, which might have accompanied by low intracellular CO_2 concentration. Similar findings were also reported by Jimenez *et al.,* (2013) on Prunus rootstock, Gholami *et al.,* (2012) on figs, Alizadeh *et al.,* (2011) on apple rootstocks and by Haifeng *et al.,* (2011) on citrus rootstocks. Drought stress tolerance should involve the maintenance of cell membrane integrity; For example, Wang *et al.,* (2012) compared two apple rootstocks; *Malus prunifolia* (drought tolerant) and *Malus hupehensis* (drought-sensitive) for water stress. In these genotypes, withholding irrigation for 12 days led to considerable ultrastructural alterations in organelles in which *M. prunifolia* maintained their structural cell integrity longer than did *M. hupehensis*.

On the other hand, the study indicates that genotypes Anna, Dorsette golden and Granny smith were found drought tolerant, that maintained higher water status in leaves during drought stress and showed higher rate of net photosynthesis than that of the

susceptible genotypes. The result also indicates that tolerant genotypes could take up as well as retain more water than the susceptible genotype and thus maintain better growth during water restriction. Similar finding was reported by Guo *et al.,* (2010) on poplar clones. Several reports indicate that drought-tolerant genotypes cope with water stress better than drought-susceptible genotypes by regulating stomatal openings in response to chemical signals from roots, maintaining higher %RWC in leaves, have had a better root-shoot ratio probably by increasing their root length, hence, maintaining higher net photosynthesis.

The results of the present study showed significantly enhanced antioxidant enzymes activity in apple genotypes exposed to drought stress. According to this fact, activities of SOD, CAT, GPX and APX were increased by drought stress from day 7 to day 21, which would be beneficial to improve the cell defense system against the damage caused by reactive oxygen species (ROS) and super oxide radicals. Among the genotypes tested, Anna, Dorsette golden and Granny smith exhibited higher activities of SOD, CAT, GPX and APX, while genotypes Royal gala, Golden delicious and Red delicious showed lower response for these enzymes activity during progressive drought development. It is reported that tolerant plants had comparatively higher activity of antioxidant enzymes than that in the sensitive ones (Turkan *et al.,* 2005; Shalata *et al.,* 2001), thus, higher activity of antioxidant enzymes means higher ability to scavenge ROS in plants. The present results are in agreement with the findings of Xiao *et al.,* (2008) in poplar clones, Gunes *et al.,* (2008) in sunflower and Manivannan *et al.,* (2007) in cow pea; that antioxidant enzyme activity increased with the increase in the duration of drought stress and might be considered as a key for the decomposition of H_2O_2 and other toxic radicals that can cause cell damage. Chakraborty *et al.,* (2002) reported in the study on tea genotypes exposed to drought stress indicates that a greater increase in CAT activity was recorded with severity of stress as the plant exposed to long term drought under field condition. The present result is also in conformity with the findings of Abedi and Pakniyat (2010) on cultivars of oil seed rape (*Brassica napus* L.) that indicates increased CAT activity during moderate and severe drought stress in tolerant cultivars.

The low antioxidant enzymes activity in some apple genotypes Golden delicious, Red delicious and Royal gala in the present study indicates that these genotypes are susceptible to drought stress, and supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunit under stress conditions.

Liu *et al.,* (2008) reported that the reduction in activities of antioxidant enzymes in poplar plants exposed to drought stress may be associated with degradation caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme. Furtheremore, the expression of these antioxidant enzymes (SOD, CAT, GPX and APX) if accompanied by enhanced H_2O_2 scavenging mechanisms has been considered as an important anti-drought mechanism to cope with oxidative stress during water deficit conditions.

The degree of lipid peroxidation measured in terms of MDA content is one of the determinants which indicate the severity of stress experienced by any plant (Xu and Zhou 2006).The lipid peroxidation of cell membranes, which is usually caused by free radicals, occurs under stress conditions (Xu *et al.,* 2006). The accumulation of MDA, a product of peroxidation of unsaturated fatty acids in phospholipids, is widely used as an indicator of the degree of cellular membrane lipid peroxidation (Wang *et al.,* 2009). In the present study, the larger increase in MDA content was recorded by genotypes Golden delicious, Red delicious and Royal gala which indicates its probability of greater sensitivity to drought stress. Conversely, the lower accumulation of MDA content was observed in genotypes Anna, Dorsette golden and Granny smith that suggest an efficient scavenging of ROS. The result indicates that higher MDA content was related with drought susceptibility and the lower values for MDA content was associated with drought tolerant behavior as observed in the present study. Türkan *et al.,* (2005) found that MDA content was lower in the leaves of drought-tolerant *Phaseolus acutifolius* (Gray.) than that in drought-sensitive *P. vulgaris* L. Also, Sairam and Srivastava (2001) reported that drought-tolerant genotypes of wheat showed lower lipid peroxidation level and higher membrane stability index (MSI) value than the susceptible ones. Thus, in drought tolerant species, the intensity of drought stress and the rate of MDA formation with the intensity of water deficit regulate the peroxidation process. Therefore, MDA is often used as suitable biomarkers of membrane peroxidation damage in many plant species.

The present study showed a decrease in soluble protein content during drought stress in apple genotypes Golden delicious, Red delicious and Royal gala. These changes may be related to decreased photosynthetic capacity and increased proteolysis or inhibited protein synthesis, possibly leading to drought susceptibility (Tang *et al.,* 2008). On the other hand, in genotypes Anna, Dorsette golden and Granny smith, soluble protein content remained relatively stable and may be associated to drought tolerance. The present study suggested that genotypes with stable protein content are more tolerant to drought stress than these genotypes

that showed decreased level in soluble protein content Because of the weak response to drought stress, soluble proteins are not very good indicator of drought stress in apple trees. This was corroborated by the study of Navari-Izzo *et al.*, (1990) in leaves of stressed apple genotypes (Elstar and Jonagold) in potted experiment and found that no consistent pattern of changes in those individual free amino acids were observed in response to drought stress. Similar findings were reported by Pinheiro *et al.,* (2004) in coffee clones, and Yanbao *et al.,* (2006) in poplar clones. In conformity with these reports, the present study revealed that soluble proteins, content showed weak response to drought stress as compared to other biochemical parameters.

5.5. Conclusion

The results of the present study confirmed that physiological and biochemical parameters carried out on selected apple genotypes from varying areas of origin revealed contrasting behaviors towards drought stress. Three conclusions may be drawn: (i) the response of photosynthesis to drought stress is strongly influenced by physiological factors, indicating that relative water content and gas exchange (Gs, Pn, E) can be related to tolerant and sensitivity of genotypes to drought stress. Lower Pn values for genotypes indicate greater sensitivity to drought stress. (ii) different apple genotypes clearly responded differently to soil water deficit in terms of the activities of SOD, CAT, GPX and APX content. These results can be used as practical biochemical markers for selection of drought tolerant apple genotypes for better protection mechanisms against oxidative damage in apple genotypes. (iii) MDA status appear crucial in preventing cell oxidative damage during drought stress, while soluble protein content showed weak response to drought stress and are not a good indicator of drought stress as compared to other biochemical markers in apples. Taking the present work as a reference, it is now possible to investigate and screen the apple genotypes for their performance under mild winter areas of the tropical environments; and also the results showed different drought tolerance capabilities that could be exploited and applied for diverse apple genotypes out of these studied in the present research.

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CHAPTER SIX: General discussion and conclusions

6.1. Opportunities for apple production and research in Ethiopia

Several factors contribute to the low production of apples in Ethiopia; yields can be as low as 11.5g/tree in genotype Red delicious and as high as 45.4kg/tree in genotype Anna (Chapter 1) in comparing with the other tested genotypes across locations. However, the (EIAR, 2009) indicates that apple trees under well managed research field can yield ~ 80kg - 150kg/tree depending on the adaptability and yielding potential of the genotype. The present study also indicates that research recommendations and packages are little addressed by the growers and further efforts needed to be required from the research side to address the development needs of the country. Furthermore, the narrow genetic basis of the introduced genotypes (few in number) complicates the selection of adaptable genotypes based on their chilling requirements. It's also evident that little studies has been done in categorizing the growing locations based on the amount of chilling was the major factor that contribute for adaptability and yielding performance of genotypes (Melke, 2015; Getachew *et al.,* 2012; Ashebir *et al.,* 2010). The study indicates that three apple genotypes (Anna, Dorsette golden and Princesa) have been popularly growing at the five locations studied and accepted by the farming community due to their low chill requirement, early maturity and better yielding potential as compared with the medium and late maturing genotypes (Granny smith, Crispin, Gala, Golden delicious and Red Delicious) respectively (Chapter 1). The low adoption rate of these medium to late maturing genotypes suggests low farmer preference compared to these early maturing ones. Currently, the occurrence of frequent and intense droughts, partly due to climate change, has aggravated the problem of low production of apples, when coupled with a lack of adequate number of genotypes for selection of adaptable and better yielding genotypes to cope with the present scenarios. This study was formulated as a first step towards the selection of drought tolerant genotypes together with their desirable yield attributes based on the grower's interest. A systematic approach was utilized starting with an understanding of the chilling requirement of genotypes at different geographic locations, which has pointed towards the identification of genotypes that is adapted to low chilling temperature conditions (Chapter 1).

Also, genotypes were screened for drought tolerance in a glasshouse and field conditions (Chapter 2, 3, & 4). Accordingly, the drought tolerant genotypes maintained active canopy under both glasshouse and field conditions for all the tested physiological and biochemical

parameters, whereas susceptible genotypes showed low performance in their canopy development and branching. Those genotypes which maintained an active canopy under drought and those with low performance were further evaluated for yield performance based on the farmers' priority for selection of genotypes under field conditions to identify the preferred genotypes. Furthermore, the experiments investigating canopy maintenance and physiological mechanisms governing drought responses were conducted at Ethiopian Institute of Agricultural Research,(EIAR), at the national biotechnology research center at Holetta under controlled glasshouse conditions, and a replica of this experiment was conducted at Debrebirhan, one of the representative highland location in central Ethiopia. Also, the systematic study on farmers' preference of genotypes was implemented at five selected locations for identification of production gaps and potential on- farm trial, using participatory on-farm evaluation of genotypes in the context of researchers and researchers-farmers evaluation for desirable fruit yield and yield characteristics of genotypes.

The identification of genotypes with desirable attributes, such as low chill requirement, early flowering and fruit setting, early fruit maturity, good branching habit, large fruit size and attractive color (mostly red and variegated color) will enhance the production of apple; and will reduce complexities associated with the scoring system for these desirable characteristics, as well as tolerant to abiotic and biotic stresses as indicated in this study (Chapter 1). In addition, the presence of genotypes with large fruits, such as genotypes Anna and Princesa is an indication of possibility for developing apple genotypes for commercial production under Ethiopian highland conditions. It has also opened up new research dimensions for apple, which would enhance its production in Ethiopia.

6.2. Application of key findings

The key findings are chapter specific (Chapters 2, 3, 4&5) and detailed discussions are provided within the respective chapters. However, this section provides an insight into the empirical findings in relation to the research questions and objectives of the study. More importantly, this section focuses on the technical application of the results for selection of genotypes for abiotic and biotic stresses tolerance from researchers and farmers perspectives and it also proposes the next course of action, which will lead to the improvement of apple production in Ethiopia. This study has also demonstrated the importance of testing the introduced apple genotypes at a national level that contributes to fully utilize the selected genotypes for country's apple production.

Ecological characterization based on the time of maturity (early, medium or late maturity) can assist in focusing the search for genotypes with the required chilling temperature requirements (low, medium and high chilling), abiotic and biotic stress tolerance, as well as placement of genotypes based on their chilling requirements by characterizing the environments on the amount of chilling exist in that locations. In addition, geographic patterns showed variation in adaptability of all the tested genotypes, which indicated that apple genotypes adapted to specific environmental conditions, depending on the nature of cultivars chilling requirement (Atkinson *et al.,* 2013; Alburquerque *et al.,* 2008; Ferree and Warrington, 2003). The failure of the ecological characterization would results in the poor morphological and physiological adaptation of apple genotypes that may be due to the rise in daily temperature of an area that can expose the plants to inadequate chilling, or it can cause the reversal of accumulated chilling Lakso, 1996). Comparison of results of the present study suggest that the cooler the environment during winter months, the better the performance of adaptable genotypes in both vegetative and reproductive growth and vice-versa. This however, varied with the temperature and rainfall patterns of the location. Also, ecological characterization in respect of chilling requirements has identified geographic gaps in apple genotypes (places with adequate or inadequate chilling conditions) will indicate that the environment need to be characterized in its patterns of rainfall distribution and temperature that determines type of cultivars introduced to the location Chmielewski *et al.,* 2004; Chmielewski and Rotzer, 2002; Cook and Jacobs, 2000).

6.3. Significance of drought tolerance and early maturity

Out of six apple genotypes tested, for drought tolerance under glasshouse and field conditions, Anna, Dorsette golden and Granny smith maintained an active canopy under severe moisture stress (Chapters 3, 4&5). The maintenance of an active canopy under low moisture indicated that these genotypes are adaptable of drought tolerance and maintained a high photosynthetic capacity during the period of water stress, while these susceptible genotypes, Golden delicious, Red delicious and Royal gala showed a low photosynthetic capacity at progressive drought.

The difference between drought tolerant and susceptible genotypes indicates the previously defined categories of drought responses in plants. Plants adapt to drought conditions through drought escape, drought avoidance and drought tolerance (Farooq *et al.,* 2009). Drought escape is associated with early flowering and maturity. Drought avoidance is

associated with reduced stomatal conductance, which further reduces net photosynthesis at an early stage of drought stress, as a water conservation mechanism, as in the case of genotypes with medium maturity such as Crispin and Gala; while these genotypes used drought tolerance mechanisms under conditions of high moisture stress (e.g Anna and Dorsette golden) ensures the maintenance of low stomatal conductance and high net photosynthesis, due to the adjustment of cellular activities during progressive and severe moisture stress.

Results from the field experiments conducted at Debrebirhan confirmed different drought tolerance levels in the three drought tolerant genotypes (Anna, Dorsette golden and Granny smith) (Chapter 3). Also field study carried out in five apple growing locations in some selected highland areas (Debrebirhan, Holetta, Degem, Hidabu-Abote and Agena) showed that drought tolerant genotypes such as Anna, Dorsette golden and Princesa had yields greater than the susceptible genotype such as Royal gala, Golden delicious and Red delicious (Chapter 3) showing poor performance in both vegetative and reproductive growth under high moisture stress condition. This study also indicates that genotypes with both early maturity and drought tolerance have the advantage of giving substantial yields even under intense drought conditions (Chapter 3). Furthermore, genotypes Anna, Dorsette golden and Princesa showed early maturity (Chapter 2), and drought tolerance (Chapters 3,4 &5). This dual benefits (early maturity and drought tolerance) have been identified in these early maturing genotypes have had an added advantage for promotion and commercialization of these genotypes in Ethiopian. Furthermore, these genotypes being proposed as promising genotypes for further promotion, and need to be further tested in multi locations for wider adaptation, in order to determine farmers' preference and yield performance across locations.

6.4. Policy considerations for introduction of new genotypes

This research has identified some serious problems related to variety introduction of perennial fruit trees. For example, Tsedey commercial apple farm located at Menagesha, 25 km to the west of Addis Aababa was devastated by crown goal (apple root canker disease) can be a good lesson for this disease was introduced with genetic materials (grafts) introduced from Israeli (EIAR, 2009). This however, urges that breeders, goodwill donors and the private sector, who are interested in introduction and commercialization of apples should take full cognizance of policy regimes governing the utilization of plant genetic resources for food and agriculture that checked by the country's quarantine regulation for new introduction.

Ethiopia, as part of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA), is governing the conservation, mobilization and utilization of plant genetic resources free of pathogens and insect pests. This calls for the use of these resources in harmony with the convention on biological diversity (CBD) (FAO, 2014) by sharing of genetic resources for non-commercial use. Therefore, any introduction of varieties passed through this regulation to ensure that the genetic materials are free of pathogens and pests that can also ensure that farmers gain appropriate benefits from the new introduction. Such benefits would be facilitated by signing material transfer agreements for easy tracking as provided for in the ITPGRFA.

6.5. Limitations of the study

Although this research has brought into light some significant results, the interpretation and application of these results should take full cognizance of some potential shortfalls. The composite plants (grafts) are raised on only MM-106 apple rootstock, and without examining the response of genotypes on other rootstocks of MM-series (MM-111, M-26, M-7, M-9 ….) and others. Because the root characteristics play a critical role in defining drought tolerance in any crop plants. Furthermore, the identification of root characteristics would strengthen the validity of tolerance levels of the drought tolerant apple genotypes. Results from the field experiments conducted over two seasons provide insights into the existence of high yielding and well-adapted genotypes. However, repeated experiments over several seasons and sites would strengthen the validity of the results, more especially on fruit yield related characteristics, which are easily affected by the environment. Farmers' selection of apple genotypes was applicable to the characteristics used in this study only. Other important attributes, such as juice and jams, apple cider, apple flavored soft drinks and other characteristics, which were not considered in this study, also play a pivotal role in determining preferred varieties. Consequently, more work needs to be undertaken with farmers in respect of this and not only with dessert apple. Disease and pests resistance of the genotypes with desirable attributes is not well understood. Therefore, more research is required to comprehend the pests and disease resistance of these genotypes. In the absence of knowledge on pests and disease resistance of these genotypes, their production should be accompanied with appropriate plant protection practices that target the most important pests and diseases.

The assessment of drought tolerance at vegetative stage only in this study is a great drawback to application of results for future apple improvement. Therefore, assessment of drought tolerance at vegetative stage only rather than reproductive stage (flowering and fruit set) provided limited understanding of effect of drought on yield of the evaluated genotype. Apple genotype which showed drought tolerance at vegetative stage in this study should be further tested at both reproductive for the identification of the yielding potential of genotypes under drought conditions.

6.6. Conclusion

The goal of this study was to explore the presence of apple genotypes with drought tolerance, high fruit yield and yield related desirable attributes when evaluated by researchers and researcher-farmers' evaluation under Ethiopian highland conditions. One of the significant results emerging from the study is the identification of genotypes with different responses to drought; drought tolerance (Anna, Dorsette golden, Princesa and Granny smith) and drought susceptible (Golden delicious, Royal gala Red delicious, and Crispin). The second major finding is the identification of drought tolerant and early maturing genotypes (Anna, Dorsette golden and Princesa) with desirable fruit yield and yield attributes scored by farmers and also researchers- farmers evaluation for commercial cultivation of these selected genotypes. Therefore, the four apple genotypes Anna, Dorsette golden, and Princesa are recommended as drought tolerant genotypes with early maturity. Other genotype, Granny smith is drought tolerant, but, because of its medium maturity, its yield potential is not comparable with other drought tolerant genotypes, so that farmers showed some reservations in selecting this genotype. Biomass, water use efficiency, relative water content, plant height, number of branches, stem diameter and root dry mass are useful, reliable, cheaper and rapid indicators to identify and select drought tolerant apple genotypes using drought intensity and susceptibility index. Physiological parameters studied indicates that the response of photosynthesis to drought stress is strongly influenced by relative water content and gas exchanges (stomatal conductance, net photosynthesis and rate of transpiration) are the most reliable indicator of drought tolerance. Biochemical parameters tested for selection of drought tolerant apple genotypes, using the activity of antioxidant enzymes (super oxide dismutase, catalase, and guacol peroxidase) showing increased activity of these enzymes for better protection mechanisms against oxidative damage in apples. Proline, total soluble sugar and lipid peroxidation (MDA content) status appear crucial in preventing cell oxidative damage during drought stress, while soluble protein content showed weak response to drought stress and are not a good indicator of drought stress as compared to other biomarkers in apples. Therefore, physiological and biochemical parameters studied on selected apple genotypes from varying areas of geographic origin revealed these parameters are crucial in indicating drought tolerance ability of the tested apple genotypes.

Future research direction

The results from this study, including the identification of some limitations, have opened up a new research agenda with the potential to significantly improve production of apples in Ethiopia. For example, some drought tolerant genotypes such as granny smith may be challenged by small growers due to its medium maturity time and tip bearing behavior. Future promotion of medium to late maturing genotypes will be required by shifting them to localities at ~ > 2700 m. a. s l. for further testing of these genotypes at the chilling conditions greater than the present testing sites to evaluate fruit yield performance. Taking advantage of the drought tolerance of apple genotypes and the presence of genotypes with promising fruit yield, further research and experimentation on apple is required across location for ensuring sustainable production. Diseases and pests cause significant reduction of yields in apple. Testing genotypes with disease and pest resistance or designing appropriate strategy for control of diseases such as apple scab and powdery mildew, and insect pest such as wooly aphid these which are frequently occur in apple growing areas should be a priority. Apple production and promotion will not expand without an appropriate policy environment. The lack of a well- structured market is impeding the production and promotion of apple in Ethiopia; and, a detailed market research project would help provide policy direction for the commercialization of apple in Ethiopia.

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