CHARACTERIZATION OF SEED OIL AND EVALUATION OF OXALATE OXIDASE ACTIVITY AMONG KENYAN SUNFLOWER VARIETIES

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Characterization of Seed Oil and Evaluation of Oxalate Oxidase Activity among Kenyan Sunflower Varieties

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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ABREVIATIONS

AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists Society
EPPO	European and Mediterranean Plant Protection Organization
EPZA	Export Processing Zone Authority
FAO	Food and Agriculture Organization
FID	Flame Ionization Detector
GLM	General Linear Model
Ha	Hectare
KAPA	Kerania Packers
MUFA	Monounsaturated Fatty Acids
ND	North Dakota
NDS	North Dakota State University
PUFA	Polyunsaturated Fatty Acids
SE	Standard error
SFA	Saturated Fatty Acids
SSR	Sclerotinia Stem Rot
USDA	United States Department of Agriculture

ABSTRACT

The growing of sunflower (*Helianthus annuus L.*) oilseed varieties in Kenya is faced with challenges of infection by disease and infestation by insect pests, which lower yield of seeds and oil. Infection of sunflower in farms by the fungus *Sclerotinia sclerotiorum* results in heavy losses of the crop. In an effort to encourage growth of suitable sunflower oilseed varieties, this study endeavored to search for varieties with superior oil yield and expressing resistance to degradation by oxalate generated by S. *sclerotiorum* during infection. Wheat oxalate oxidase has been shown to confer some resistance to *S. sclerotiorum*. This study investigated the presence of oxalate oxidase in six sunflower varieties available in Kenya, namely: Kenya Fedha, Rekord, Issanka, H8998, H4038 and H4088. A calorimetric enzyme assay was used to screen for the enzyme activity in sunflower leaf tissue. A detached leaflet assay was conducted and lesion size measured following degradation by exogenously applied oxalic acid on leaf tissue. Sunflower oil was characterized from the six varieties. Acid Value, Saponification Value, Iodine Value, Peroxide Value, Relative Density, Refractive Index and Fatty Acid composition of the oil were determined. The oil content was also determined. The relationship between the oil quality/quantity and level of oxalate oxidase activity was also investigated.

Results revealed that the selected sunflower varieties had an oil content ranging from 40.92% to 50.55% w/w. Varieties H8998 and H4088 had 50.55% (w/w) and 49.41% (w/w) oil content, respectively. The two varieties may thus be recommended for commercial oil extraction. The oils were found to be highly

unsaturated at levels of 81.93 to 89.09% of all total fatty acids, making sunflower oil superior to many edible fats and oils used commercially. Peroxide values ranged from 1.04 to 2.98 meq/kg oil while acid values ranged from 0.14 to 0.28 mg KOH/g oil. Saponification values of 162.65 to 171.78 mg KOH/g of oil were also recorded, an indication that the oils were composed of high molecular weight fatty acids. The oil was also found to be pure and light as indicated by Refractive Index and Relative Density values of 1.4709 to 1.4724 and 0.9106 to 0.9193, respectively, making it suitable for various cooking options.

All the varieties responded differently to oxalic acid degradation as characterized by differences in lesion areas per variety ($p<0.05$) at different acid concentrations. The variety H4088 showed higher oxalate oxidase activity and hence higher resistance to degradation by oxalic acid compared to other varieties and was second highest in oil content (49.40% w/w). This study, therefore, recommends that H4088 be promoted to farmers. The variety H8998 which had relatively less oxalate oxidase activity but highest oil content (50.55% w/w) may also be recommended for transformation with the resistance gene to enhance its oxalate oxidase activity. This study formed a basis for further investigation on resistance of Kenyan sunflower to *S. sclerotiorum* infection and recommends biological studies using the natural pest to be carried out. It also recommends that molecular studies be conducted to determine the genetic potential for resistance in the six varieties.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General introduction

Sunflower (*Helianthus annuus L.*) is the fourth important oil crop in the world (Cerboncini *et al.,* 2001). The growing of sunflower is faced with challenges of infestation by disease and infection by insect pests (Allison, 1999). However, plants ward off pathogen infections by eliciting an array of defense mechanisms (Kim *et al.,* 2008). Pathogenesis-related (PR) protein accumulation has been observed in many plant species, induced upon infection by pathogenic organisms such as fungi. These proteins are usually associated with acquired resistance (Stintzi *et al.,* 1993) and it has been suggested that they are involved in defense of plants against pathogens. Oxalate oxidase belongs to the germin family of proteins and catalyzes the degradation of oxalic acid to produce carbon dioxide and hydrogen peroxide (Chiriboga, 1966). The level of oxalate oxidase activity in plant tissue may directly be related to resistance of the plant against oxalic acid producing pathogens. Complete resistance in cultivated sunflower has not been reported but significant differences in the level of susceptibility have been identified in diverse germplasm (Nelson and Lamey, 2000; Rashid and Dedio, 1992). Oxalate oxidase activity in Kenyan sunflower germplasm has not been investigated. This study, therefore, intended to determine oxalate oxidase activity in some Kenyan sunflower varieties as a measure of the level of resistance to the effects of oxalic acid, the toxicity and pathogenicity factor for some fungal pathogens such as *Sclerotinia sclerotiorum* (Lib) de Bary.

The chemical composition of an oil extract gives a qualitative identification of the oil, and is a very important selective application guide in the commercialization and utility of oil products (Nwobi *et al*., 2006). The functional and nutritional values of different vegetable oils are dependent on the nature of the different fatty acids which are incorporated like building blocks into the oil (Odoemelam, 2005). Several food preparations incorporate sunflower seed oil to improve their nutritional quality. Pure sunflower oil has been known to be a superior vegetable oil that is rich in protein, many minerals, vitamins, and essential fatty acids (Penny, 1999). The extraction and characterization of oil from sunflower seeds have been carried out extensively elsewhere, but little has been reported on the oil yield and physicochemical properties of sunflower seed oil from Kenyan varieties. This study endeavored to characterize oil from six Kenyan sunflower varieties as a first line in the promotion of the use of sunflower oil in the country.

The study also sought to establish the relationship between oil quality/quantity and oxalate oxidase activity in sunflower leaf tissue upon challenge with exogenously applied oxalic acid.

1.2 PROBLEM STATEMENT

Infestation of sunflower farms by pests such as the S. *sclerotiorum* has contributed to low yields and therefore reluctance by Kenyan farmers to grow the crop. In an attempt to combat the disease, farmers have been relying heavily on the use of broad spectrum fungicides as well as sowing certified disease-free seeds. They have also been trying to plant the crop on non-infested soils. Despite this, the present production capacity remains low. As a first line in developing the oil industry, there is the need to increase sunflower yield, by way of reducing parasitic infestation like sclerotinia disease (white mold). Wheat oxalate oxidase has been shown to confer some resistance to S. *sclerotiorum* (Lu *et al.*, 2000), but the activity of this enzyme in sunflower, particularly in the Kenyan varieties, has not been investigated.

Most commercially fried foods and bakery products (cookies, doughnuts, cakes and pastries) are made with hydrogenated fats such as shortening and margarine. Saturated and trans-fatty acids from these fats tend to increase levels of "bad" cholesterol and therefore increase the risk of cardiovascular diseases. Sunflower oil is known to be superior to many such oils and fats due to its high level of polyunsaturated and monounsaturated fatty acids (Penny, 1999), which tend to lower the risk of heart disease. The nature and quality of sunflower oil from Kenyan varieties remains unknown thus necessitating investigation.

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1.3 JUSTIFICATION

Farmers have for long relied on use of fungicides to combat sclerotinia diseases implying high fungicide bills that they can hardly meet. It is therefore imperative to identify Kenyan sunflower varieties that have natural resistance to fungal infection. This study sought to determine activity of oxalate oxidase, which is associated with resistance against *S. sclerotiorum* infection. The quality and quantity of oil from the sunflower seeds was also determined. It was hoped that the most resistant and high yielding sunflower varieties would be promoted in the country in order to cut down on the farming costs and the oil/seed import bill which stands at a cost of above $140 million annually. Cultivation of resistant sunflower lines would also discourage the use of fungicides which negatively affect human and animal health and have adverse ecological implications. The results of this study would also sensitize the government on the need to develop a comprehensive national policy on growth and development of the oil industry.

1.4 HYPOTHESES

1.4.1 Null hypothesis

- Sunflower oilseed varieties in Kenya have no oxalate oxidase activity.
- Sunflower seeds from Kenyan varieties have low oil content.
- Sunflower oil from Kenyan varieties is of unacceptable quality.
- There is no relationship between the oil quality/quantity and oxalate oxidase activity.

1.4.2 Alternative hypothesis

- Some sunflower oilseed varieties in Kenya have high oxalate oxidase activity.
- Sunflower seeds from Kenyan varieties have high oil content.
- Sunflower oil from Kenyan varieties is of superior quality.
- There is a relationship between the oil quality/quantity and oxalate oxidase activity.

1.5 OBJECTIVES

1.5.1 General Objective

To identify Kenyan sunflower varieties expressing a high level of oxalate oxidase activity and with superior oil yield.

1.5.2 Specific Objectives

- To evaluate the level of oxalate oxidase activity in Kenyan sunflower varieties
- To characterize sunflower oil quality and content from Kenyan sunflower varieties
- To establish the relationship between oil quality/quantity and oxalate oxidase activity

CHAPTER TWO

2.0 LITERATURE REVIEW

Plants often face the challenge of severe environmental conditions, which include various biotic and abiotic stresses that exert adverse effects on plant growth and development. Many pathogens establish intimate relationships with their hosts in order to suppress plant defenses and promote the release of nutrients (Freeman and Beattie, 2008). Necrotrophs produce toxins or tissue-degrading enzymes that overwhelm plant defenses and promote the quick release of nutrients (Wolfgang, 1996). The plant may succumb to the infection and disease results.

2.1 Sclerotinia disease

S. sclerotiorum is a necrotrophic ascomycete fungus with a broad host range of over 400 species. It is one of the major pathogens of sunflower (*H. annuus L.)* and causes the sclerotinia disease, commonly known as 'white mold' (Boland and Hall, 1994). The pathogen can attack all parts of the plant at every stage of plant growth, predominantly the capitulum, leaf and stem (Guyla *et al.*, 1989), hence sclerotinia head and stem rot, respectively (Figure 1). It is of great economic importance as it can cause yield losses of up to 100% (Rashid, 1993).

2.2 Taxonomy: *Sclerotinia sclerotiorum* (Lib.) de Bary. Melvin *et al.*, 2006.

Kingdom: Fungi	Order: Helotiales
Phylum: Ascomycota	Family Sclerotiniaceae
Class: Discomycetes	Genus: *Sclerotinia*

2. 3 Disease symptoms

Leaves usually have water-soaked lesions that expand rapidly and move down the petiole into the stem. Infected stems of some species will first develop dark lesions whereas the initial indication in other hosts is the appearance of water-soaked stem lesions. Lesions usually develop into necrotic tissues that subsequently develop patches of fluffy white mycelium, often with sclerotia, which is the most obvious sign of plants infected with *S. sclerotiorum* (Melvin *et al.,* 2006).

2. 4 Disease cycle

There are four stages in its life cycle: sclerotia, apothecium, ascospore and mycelium (Purdy, 1979). Infection of susceptible plants can occur from mycelium that originates from eruptive germination of sclerotia in soil. Hyphal germination of sclerotia causes infection by first invading non-living organic matter and forming a mycelium, which is an intermediate necessity for mycelium infection. Apothecia can be developed from sclerotia and eject ascospores. Then ascospores may settle on nonliving plant parts, or invade healthy plant parts where they penetrate plant tissues and establish infection (Lu, 2003). Figure 1 below shows the life cycle of *S. sclerotiorum*.

Sunflower is the only crop that *Sclerotinia* consistently infects through the roots. As the fungus grows in and on the tissues, sclerotia are formed; most are produced in the decayed stem pith and on the roots as the plant dies. *Sclerotinia* survives through harsh conditions as sclerotia in the soil or in plant debris (Lu, 2003).

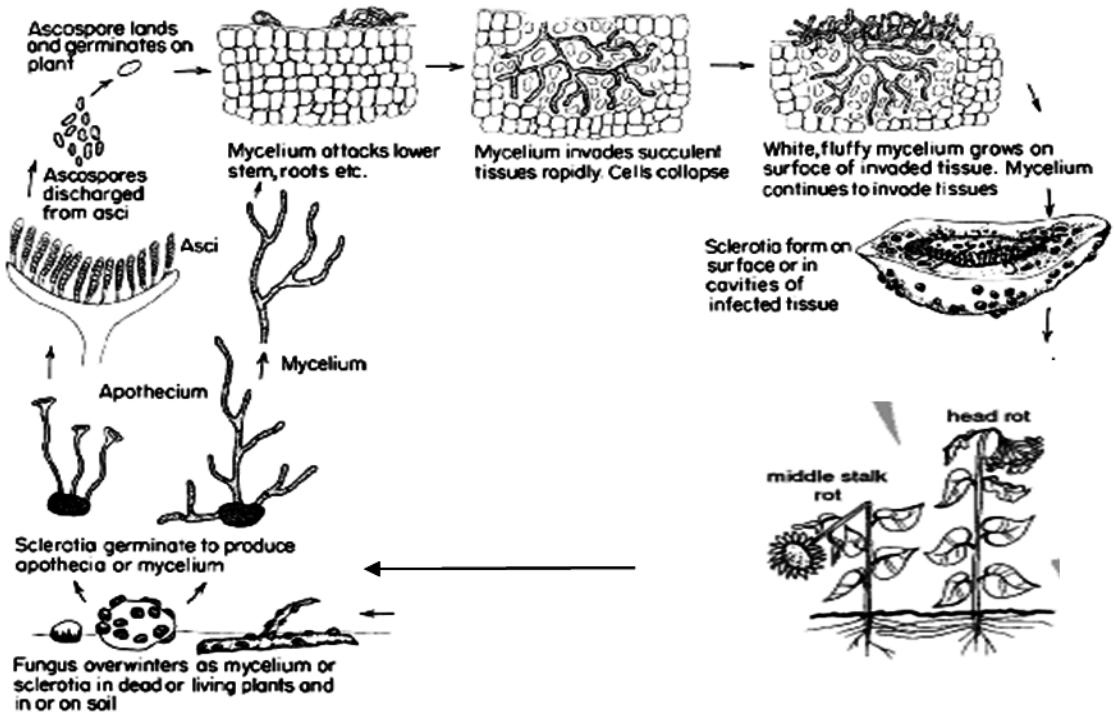

Figure 1. Disease cycle of *Sclerotinia* disease of sunflower (Reprinted - with modification - from Agrios, G. N. 1997. *Plant Pathology*. 4th ed).

2.5 Mechanism of disease

Oxalic acid is the major phytotoxic and pathogenicity factor of *S. sclerotiorum* in plants (Cessna *et al*., 2000). Mutants which are deficient in oxalate biosynthesis are less pathogenic than the wild-type fungus (Godoy *et al*., 1990). The fungus synthesizes and secretes millimolar concentrations of oxalic acid (OA) into infected host tissues (Godoy *et al*., 1990; Marciano *et al*., 1983; Maxwell and Lumsden, 1979). This acid acts as a toxin and causes wilting syndrome in sunflower (Noyes and Hancock, 1981).

Oxalic acid acts by acidifying the plant tissue. Early in pathogenesis, oxalate accumulates in the infected tissues and increases in concentration as the pathogen

colonizes the plant. The pH decreases to 4.5 or below. Since the pH optima for fungal extracellular enzymes are generally below 5.0, the lowered pH of the infected tissue has been found to enhance their activity. Such enzymes include polygalacturonase which also degrades the plant cell wall (Cessna *et al*., 2000).

Oxalate is also known to be a chelater of divalent ions. It chelates calcium ions (Ca^{2+}) from the cell wall, rendering the stressed tissue susceptible to fungal degradative enzymes. (Baterman and Beer, 1965; Lumsen, 1979). These enzymes include pectinases, β-1, 3-glucanases, glycosidases, cellulases, xylanases and cutinases (Annis and Goodwin, 1997). Chelation of Ca^{2+} has been proposed to compromise the function of Ca^{2+}-dependent defense responses.

The infected plant usually mounts a resistance response against the pathogen through the oxidative burst. This is required for all plant species to develop germplasm resistant to pathogen. Various studies have demonstrated that oxalate suppresses the oxidative burst through inhibiting the free radical-generating O-diphenol oxidase directly or by blocking a signaling step that leads to activation of the oxidase (Cessna *et al*., 2000; Ireneusz *et al*., 2006).

Oxalic acid also induces foliar wilting during fungal infection by manipulating guard cells; increasing stomatal conductance and transpiration as well as decrease in plant biomass (Noyes and Hancock, 1981). It disrupts stomatal closure at night through interfering with abscisic acid (ABA) action. It has been proved that oxalate interferes with ABA-induced stomatal closure at night by co treatment of ABA (100mM) with 1 to 10 mM oxalate. Oxalate significantly increased the

stomatal aperture compared to the buffer control at a concentration of 10mM. (Guinaraes and Stotz, 2004). It stimulates K+ uptake and starch degradation in guard cells leading to an increase in osmotically active solutes which are responsible for the oxalate-dependent stomatal opening.

Since effective pathogenicity of *S. sclerotiorum* requires secretion of oxalate, understanding its biosynthesis is important in determining the enzymes involved in pathogenicity. Although there is little information on oxalate biosynthesis in *S. sclerotiorum*, several biochemical pathways have been proposed as the potential route by which the pathogen synthesizes oxalate (Hua *et al*., 2006). The proposed mechanisms through the tricarboxylic acid (TCA) and glyoxylate cycles are shown in the figures 2 and 3 below.

The presence of malate, fumarate and succinate from the infected plants suggest an operative TCA cycle as reported from a number of fungi. It is suggested that oxalate biosynthesis is a complex biochemical process and not a simple TCA cycle, the reason why it is not understood (Hua *et al*., 2006). It is also suggested that the glyoxylate cycle can explain the variance in concentration and the presence of high oxalate concentration (Michael and Gerald, 2002).

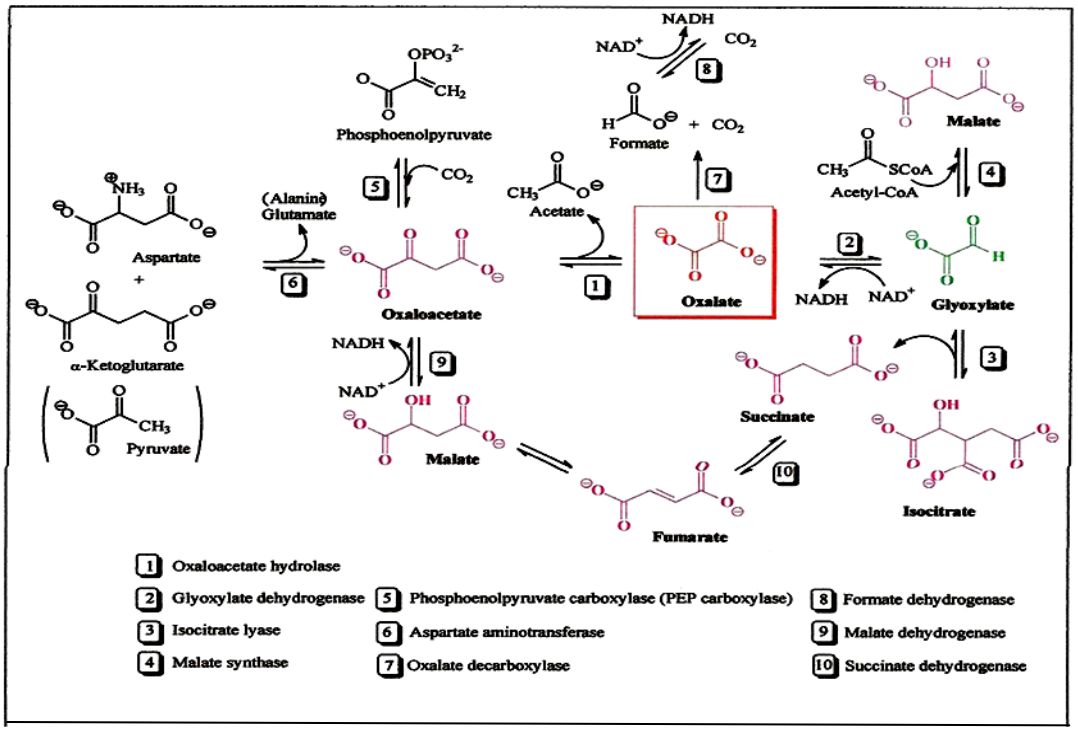

Figure 2. Proposed metabolic pathways for oxalate synthesis (Adapted from Franceschi and Nakata, 2005).

2.6 Counteracting the effects of oxalic acid

Although a particular plant species may be a susceptible host for a particular pathogen, some individuals ward off pathogen infections by eliciting an array of defense mechanisms which include reinforcement of the cell wall (Bradley *et al*., 1992), synthesis of defense-related secondary metabolites (Dixon and Lamb, 1990), activation of defense-related genes and localization of cell death or the hypersensitive response (Kim *et al*., 2008).

A common strategy for combating *Sclerotinia* pathogen is to degrade oxalate. There are three classes of known enzymes that can catabolize oxalic acid, namely oxalate oxidase (OXO) (Lane *et al*., 1991), oxalate decarboxylase (Mehta and

Datta, 1991), and oxalyl-CoA decarboxylase (Lung *et al*., 1994). The bacterial oxalyl-CoA decarboxylase gene could be used for oxalate degradation and engineering *Sclerotinia* resistance in plants. However, both fungal and bacterial oxalate decarboxylases convert oxalate into CO_2 and formic acid, which might have a toxic effect on plant cells. Therefore, scientists have been focusing on OXO.

Oxalate oxidase belongs to the germin family of proteins and catalyses the degradation of oxalic acid to carbon dioxide and hydrogen peroxide (Chiriboga, 1996; Dickman and Mitra, 1992). It is expressed during germination, where it associates with cell wall components such as glucuronogalactoarabinoxylans. Its activity increases the pH at the site of infection following contact with oxalate-secreting pathogens (Rollins, 2003).

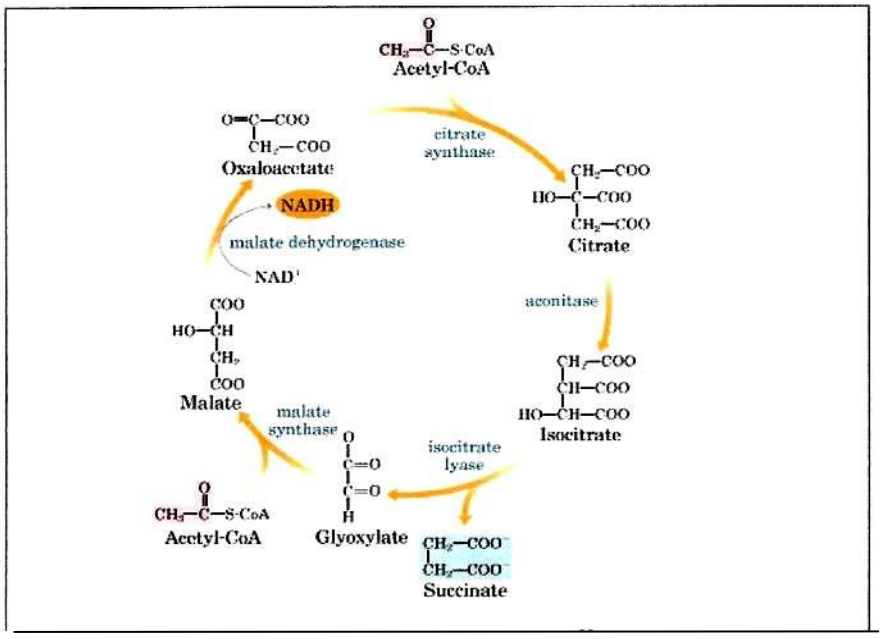

Figure 3. The TCA and Glyoxylate cycle (Adapted from Michael and Gerald, 2002).

Oxalate oxidase activity has been reported in cereals in response to pathogen attack e.g. maize, wheat, oat, rye and barley (Dunwell *et al*., 2000). Germin-like proteins have also been isolated from many higher plants. They have a high sequence identity to wheat germin. Wheat germin is found to be an apoplastic, multimeric and glycosylated enzyme with extreme resistance to heat and chemical degradation by protease or H_2O_2 (Lu *et al*., 2000).

There are a number of other enzymes which are known to cause production of hydrogen peroxide whose effect of increasing the pH renders the plant tissues resistant to fungal degradative enzymes. Sunflower carbohydrate oxidase has antifungal activity and confers resistance to fungal pathogens when expressed (Stuiver *et al*., 2000). It generates H_2O_2 which triggers increased defense-gene expression and increased sensitivity to pathogen attack. Glucose oxidase activity within the plant is also known to cause production of H_2O_2 (Ireneusz *et al*., 2006).

The oxalate oxidase gene may also play a role in cell wall defense unrelated to its enzyme activity. Schweizer *et al.* (1999) showed that the expression of a mutant wheat oxalate oxidase sequence lacking enzyme activity conferred increased resistance to penetration of plant tissue by *S. sclerotiorum* (20%–67% of control levels).

Secondary plant metabolites are also involved in plant defense and usually belong to one of three large chemical classes: terpenoids (essential oils, pyrethrins, saponins), phenolics (flavonoids, anthocyanins, phytoalexins, tannins, lignin, and furanocoumarins) and alkaloids (cocaine, morphine, and nicotine) (Freeman and

Beattie, 2008). Analysis has been done using four sunflower lines and it has been found that the amount of phenolic compounds depend on the sunflower line. Higher constitutive and induced phenolic content and phenylalanine ammonia-lyase activity are present in the most resistant line; these differences correlate with absence or presence of disease symptoms (Metraux and Raskin, 1993).

Transgenic technology has been used to successifully transfer oxalate oxidase genes from cereals to other plants including sunflower. Wheat oxalate oxidase-transgenic sunflower plants have been generated by agrobacterium-mediated transformation under a constitutive promoter SCP1 (Lu *et al*., 2000; Scleonge *et al*., 2000; Zaghmount *et al*., 1997). The Sclerotinia-induced lesions in transgenic sunflower leaves were shown to be significantly smaller than those in control leaves.

2.7 The role of fungicides

Chemical control using fungicides is often a vital part of an integrated disease management as they control many diseases satisfactorily whereas cultural practices often do not provide adequate disease control (McGrath, 2004; Mueller *et al.*, 2004a). No fungicide has been registered for control of *Sclerotinia* rot or wilt on sunflower (Lamey, 1998) though studies have shown that control of SSR with fungicides is possible, but that the degree of control is inconsistent especially when incidence of SSR is high (Bradley *et al*., 2006). To reduce the potential of resistance developing in SSR populations, it is recommended that fungicides with different chemistries, and therefore, modes of action, be alternated in a spray program (Buck *et al.*, 2003; Jeffers *et al.*, 2001).

2.8 Exploiting genetic resistance

Since there are no effective chemical controls available to apply on a large scale, the development of hybrids with adequate genetic resistance is necessary. Although complete resistance in cultivated sunflower has not been reported, significant differences in the level of susceptibility have been identified in diverse germplasm (Nelson and Lamey, 2000; Rashid and Dedio, 1992). Efforts in recent years by the USDA-Agricultural Research Service (ARS) Sunflower Research Unit in Fargo, N.D. and the NDSU Agricultural Experiment Station have brought about the introduction of some sunflower lines that show more resistance to *Sclerotinia*. USDA scientists have found sources of resistance to both head rot and stalk rot and are attempting to transfer the collection of genes responsible for resistance from wild species of sunflower into cultivated sunflower (Dale, 2007).

2.9 Sunflower oil

Due to health considerations there has been a significant shift in the sources of fats consumed in the last decades. Vegetable oils are one of the best sources of essential fatty acids in the diet. Sunflower oil, together with other vegetable oils as olive and soybean oils, are increasingly becoming important because of their high content in mono- and polyunsaturated fatty acids when compared to animal fats (Harwood *et al.,* 1994).

The sunflower seed is also rich in many minerals and vitamins. The nutrients found in sunflower seeds include protein, thiamine, Vitamin E, iron, phosphorous, potassium, calcium, and essential fatty acids like linoleic acid and oleic acid

(Putnam *et al.,* 1990). They have a high iron and potassium content, with higher calcium levels than soybeans. This great proportion of nutrients in the seeds is believed to improve cardiovascular health. Their high proportion of potassium, but low sodium content, makes the sunflower seeds act as a diuretic, which helps to lower high blood pressure (Diane, 1997). The essential linoleic acid is necessary for growth and prostaglandin production, and tests have shown it to be beneficial in reducing levels of serum cholesterollant (Penny, 1999).

Sunflower oil is also considered a premium oil because of its light color, high level of unsaturated fatty acids contributed mainly by linoleic and oleic acids, and lack of linolenic acid, brand flavor and high smoke points (Mike, 2004).

Fatty acids consist of the elements carbon (**C**), hydrogen (**H**) and oxygen (**O**) arranged as a carbon chain skeleton with a carboxyl group (**-COOH**) at one end. Mono-unsaturated fatty acids (MUFAs) such as oleic acid are distinguished from the other fatty acid classes on the basis of having only 1 double bond. In contrast, poly-unsaturated fatty acids (PUFAs) as linoleic and linolenic acids have 2 or more double bonds while saturated fatty acids (SFAs) like stearic acid have none (Pearson, 1976).

The PUFAs in vegetable oil give it its nutritional value but they also are the cause of its instability (Passmore and Eastwood, 1996). From a chemical point of view, saturated fats are more stable than unsaturated fats, which are more unstable than polyunsaturated fats (Nawar, 1996).

The double bonds in polyunsaturated fatty acids between the carbon bonds are sensitive to oxidation. Under mild conditions, molecular oxygen reacts with the double bonds following a free radical mechanism, the so-called autooxidation. The oxidation of fatty acids changes the chemical properties of the fat; it reduces the nutritional value of the fat, darkens its colour and can cause off–flavours (Staprans *et al*., 1996a; Kanazawa *et al*., 2002). According to Medical Food News Reviews, thermal stress speeds up oxidative reactions (Mike, 2004) and thus lipid oxidation is a major concern in frying operations. Bottles containing vegetable oil should be kept out of the sunlight as light can also cause vegetable oils to oxidize slowly (Kamau and Nanua, 2008).

Edible vegetable oils are relatively resistant to oxidation because of the antioxidants they have (Notee and Romito, 1971). However, once the oxidation process begins, it progresses quickly since oxidation is an autocatalytic reaction (Isabel and Mariano, 2000). To prevent or retard the oxidation, various protective measures may be used, such as gassing with inert gases, addition of antioxidants, appropriate packaging, special formulation, etc. Most of these methods can only be used for a limited range of products. Therefore, the most common procedure is the addition of antioxidants mainly of fat soluble nature (Löliger and Wille, 1993). Various chemicals have been designed to function as antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxyl toluene (BHT), and tert-butyl hydroquinone (TBHQ). The most readily acceptable oxidation inhibitors are common food ingredients, as their use is not limited by legislation (Said and Ahmed, 2005).

Many methods that have been developed to assess the extent of oxidative deterioration are related to the measurement of the concentration of primary or secondary oxidation products or of both. The most commonly used methods are peroxide value (PV) that measures volumetrically the concentration of hydroperoxides, anisidine value (AV), spectrophotometric measurement in the UV region and gas chromatographic (GC) analysis for volatile compounds (Frankel, 1998).

Oleic/Linoleic (O/L) acid ratio is considered to be a measure of oxidative oil stability. This ratio has been used to predict the shelf life of fat and oils (James and Young, 1983). Varieties with higher O/L ratios have been found to exhibit increased oil stability and increased shelf life of products. Ratios above one are generally preferred (Kratz *et al.*, 2002). The shelf life or stability of any oil is measured by the number of days before the onset of oxidative rancidity, a process that involves the whole seed, the oil or oil product by exposure to heat and air.

2.9.1 Uses of sunflower

Non-dehulled or partly dehulled sunflower seed has been substituted successfully for soybean meal in isonitrogenous (equal protein) diets for ruminant animals, as well as for swine and poultry feeding (Putnam *et al.*, 1990).

Sunflower oil has a smoothing effect on the skin and manufacturers of cosmetic producers continue to explore how best to include the oil in their formulations. Today, the oil that is extracted from sunflowers is used as a non-occlusive moisturizer for the face and body (Baumann, 2005).

Sunflower oil has been used in certain paints, varnishes and plastics because of good semidrying properties without color modification that is associated with oils high in linolenic acid (Levin, 2008).

Utilizing an emerging technology called rhizofiltration, hydroponically grown plants are grown floating over water. Possessing extensive root systems, they are able to reach deep into sources of polluted water and extract large amounts of toxic metals, including uranium. Such a process has been utilized in the former Soviet Union to decontaminate water polluted as a result of the 1986 accident at the Chernobyl nuclear power plant. The roots of floating rafts of sunflowers were able to extract 95% of the radioactivity in the water (Tome *et al*., 2008).

Scientists discovered several years ago that hydrogen could be produced from sunflower oil, offering a renewable energy source (biofuel) that was friendly to the environment. With the price of fuel escalating, the thought of using hydrogen powered engines (by way of sunflower oil) to propel our vehicles is tantalizing (Levin, 2008).

2.10 The Kenyan oil industry

A report by Export Processing Zone Authority of Kenya (2005) suggests that increased domestic production of oilseeds by local manufacturers in Kenya has been constrained by inadequate supply of raw materials, leading to efforts by the FAO to initiate development of raw material centers in the country, especially in the key growing areas of Western Kenya and the Lake Victoria basin where these

small-scale, resource-challenged farmers who are vulnerable to food and nutritional insecurity live.

A survey conducted by the Kenya Agricultural Research Institute (KARI) in Western Kenya revealed that farmers attributed the low production to poor agronomic practices, inadequate pest and disease control, lack of high yielding varieties, decline in soil fertility, shortage of good quality seeds at planting, low producer prices, shortage of sunflower seed for processing, lack of access to credit, lack of markets and market information, weak research - extension – farmer linkages and low adoption of developed technologies (Okoko *et al.*, 2008).

To address some of these constraints, KARI has been trying to scale up promising sunflower varieties, enhance value addition activities and to link farmer marketing groups to existing and new market opportunities. In January 2007, KARI released the sunflower varieties of Kenya Shaba and Kenya Fedha after trials. In 2008, several other hybrid varieties were released among them H4038 and H4088. Others include: H-008, H-893, H-894, H-8938, H-8998, H-001, H-898, Kensun 22, Kensun 33, Super 400, Rekord, Comet, Hungarian White and Kenya White (Thagana and Riungu - KARI Headquarters Library**)**. Processing companies like Bidco have also actively supported and encouraged local farming of vegetable oil crops, particularly the palm oil.

In 2006, the Agriculture Ministry announced the oil crops development policy to boost sunflower farming. The ministry expressed concerns that the country hardly meets 20 per cent of its national requirement for vegetable oil and fats. As of 2005,

domestic production of edible oils was estimated at 380,000 metric tonnes, only about one-third of its annual demand. The remainder is imported, at a cost of $140 million, making edible oil the country's second most important item after petroleum.

Currently there is a call by EPZA of Kenya for local and foreign investors to invest in the edible oil sector in areas such as oilseed production, marketing and oil processing, production of better high yielding seeds and plant materials cross breeds, provision of pre-cooling and cold storage facilities for vegetable products, agricultural extension services, farmers' training for better production methods, quality control and improvement, credit and loan facilities to farmers to boost the edible oil sector.

There are about 30 vegetable oil refineries in the country. The larger companies include Bidco Oil Refineries, KAPA Oil Refineries, Palmac Oil Refiners, Pwani Oil Refiners and Unilever.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant materials

Six sunflower seed varieties (Kenya Fedha, Issanka, Rekord, H4088, H4038 and H8998) and one variety of wheat seeds (Kwale) were obtained from KARI, Njoro and Kenya Seed Company, Nairobi and Kitale.

3.2 Chemicals

All chemicals used in various analyses were of analytical grade and were obtained from Sigma-Aldrich Chemie Ltd and Riedel-de Haen (Germany) through a local supplier – Kobian Co. Ltd. The enzyme (Horseradish peroxidase), microfuge tubes, micropipettes and micropipette tips were also sourced from Kobian Co. Ltd.

3.3 Preparation of soil medium

The soil medium was prepared by mixing loam soil, compost and sand in the ratio 3:2:1 as described by Bunt, 1976. This mix ensured the soil was well conditioned, had good drainage and had the right cation and anion capacity exchange. Nutrition was supplemented by application of foliar feed (Bayfolan, Bayer Crop Science, Germany) once a week.

3.4 Germination of seeds

3.4.1 Germination of sunflower seeds

Seventy five seeds of each of the six varieties of sunflower were planted (fifteen plants every week from which to harvest eighteen leaves) in plastic polythene bags in a greenhouse at Jomo Kenyatta University of Agriculture and Technology.

Planting was done using the completely randomized block design for five weeks per season and for two consecutive seasons. The seedlings were constantly watered to field capacity. Leaves were randomly harvested at the age of 1½ months and used in various analyses.

3.4.2 Germination of wheat seeds

This was done by planting several seeds randomly in polythene bags (in a similar manner as sunflower seeds) followed by constant watering till the seedlings were 1½ month old. Simple random sampling was applied in harvesting the wheat leaves. The planting was done for two consecutive seasons as well.

3.5 Oxalic Acid Bioassay

Leaflet assays were conducted to assess the ability of oxalate oxidase to prevent damage in response to application of oxalic acid to plant tissue. Detached sunflower leaflets were arranged on inverted weigh boats in 12-cm petri dishes containing dampened paper towels. Eighteen leaflets were used for each variety, three (replicates) for each concentration of oxalic acid in the range of 0-10mM. Each leaflet was wounded in four locations on the abaxial surface with an 18-gauge needle and 15µl oxalic acid applied to each wound. Leaflets were incubated for 48hrs at room temperature. To quantify lesion diameter, leaflets were washed with distilled water and viewed with a dissecting microscope (SZ ZT, Olympus, Japan). The controls (wheat leaves) were given the same treatment as the test samples and lesion areas compared against those of the sunflower leaflets.

3.6 Oxalate Oxidase Assay

The assays focused on the release of H_2O_2 as a measure of oxalate oxidase activity and was conducted according to the modified Sugiura *et al*. method, 1979. To determine oxalate oxidase activity in sunflower plants, leaf discs (5mm diameter) were incubated in 1.5ml microfuge tubes with 200µl of assay buffer (18mg oxalic acid in 100ml of 2.5mM succinic acid, pH 4) and reactions incubated for 15 min at 37^{o} C. After incubation, developing solution (135µl) was added to the tubes and the reactions allowed to continue at room temperature for 30 min. The developing solution consisted of 6mg of aminoantipyrene dissolved in 30µl of *N, N*-dimethylaniline, which was then added to 100ml of 0.1M sodium phosphate buffer, pH 7.0 containing 57µl of a 140mg ml^{-1} solution of horseradish peroxidase. The contents of the microfuge tubes were diluted ten times prior to measurement of absorbance at a wavelength of 550nm using a UV-Visible spectrophotometer (UV-1601 PC Shimadzu Corp, Japan). A plot of hydrogen peroxide standards in the range of 0-10mM was used to quantify the sample H_2O_2 concentrations. The positive controls (wheat leaf discs) were given the same treatment as the samples, while the blank was composed of the buffer system without leaf discs.

3.7 Oil quality and quantity analysis

3.7.1 Fatty Acid Analysis

Extraction of total lipids was done using the modified method described by Bligh and Dyer (1953).

One gram of ground sunflower seed sample was weighed into a 50ml glass-stoppered centrifuge tube and denatured over boiling water (100^{o} C) for 3 min. Two millilitres of water and 7.5ml of 2:1 v/v methanol-chloroform mixture were then added. The mixture was shaken thoroughly and left at room temperature for two hours with intermittent shaking.

After centrifugation, the supernatant was decanted into another centrifuge tube and the residue resuspended in 9.5ml of methanol-chloroform-water (2:1:0.8 v/v). The homogenate was then shaken and centrifuged (10 min). This step was repeated twice and the supernatants combined with the first extract. To the combined extracts, 7.5ml of each of chloroform and water was added, shaken and centrifuged (10 min).

The lower chloroform phase was withdrawn using a Pasteur pipette and brought to dryness using a vaccum rotary evaporator at low temperature (40^{o}C). The lipid residue in the flask was completely dried under vaccum in a dessicator over fresh KOH pellets (about 2hr) and the weight of the lipids measured. Finally the residue was reconstituted using 2ml of 2:1 methanol-chloroform mixture and stored in the freezer until required.

Extraction of fatty acid methyl esters (FAMEs) was carried out as described by Christie, 1993. Fatty acid analysis was done using Gas Chromatography ISO

5508: 1990 method (using GC 14A, Shimadzu Corp, Japan). Operating conditions were as follows:

- Detector: FID (temperature - 220^{o}C)
- Injection temperature - 220^{o} C
- Column temperature - 170^{o}C
- Flow rate - 50 ml/min.
- The carrier gas - Nitrogen
- Integrator – CR 6A, Shimadzu Corp, Japan
- Glass column measuring 3m x 3mm internal diameter and packed with 15% diethyleneglycol-succinate (DEGS) on Uniport B.

From the elution profiles, saturated, polyunsaturated, and monounsaturated fats were calculated as sums of individual free fatty acids using previously determined fatty acid standards.

3.7.2 Determination of Oil Content

Ten grams of ground sunflower seed was put in an extraction thimble and total lipids extracted by the Soxhlet Method (1879) using 150ml of petroleum spirit for 16hr at 80^{o}C. The oil was dried in an oven at 105^{o}C for one hour, cooled in a dessicator and percentage oil content calculated on weight by weight basis.

3.7.3 Determination of Acid Value (AV)

Three grams of sunflower oil sample was accurately weighed and placed in a 200ml conical flask. Forty milliliters of the solvent mixture (96% ethanol and diethyl ether, v/v, in ratio 1:1) was added with gentle warming to ensure the

samples dissolved completely. Two to three drops of 1% phenolphthalein indicator were added and the mixture titrated with carefully standardized 0.1N KOH solution. Acid value was calculated as:

$$\text{Acid Value} = \frac{56.11 \times f \times V}{W}$$

W = Weight of sample (gms)

V = Volume of 0.1N KOH used (cm^3)

f = factor for KOH

3.7.4 Determination of Saponification Value (SV)

Two grams of sunflower oil were weighed into a conical flask. Exactly 25ml of carefully standardized 0.5N alcoholic KOH was added. Twenty five millilitres of 0.5N alcoholic KOH was also pipetted as the blank and subjected to the same conditions as the samples. The mixture was boiled for 30 min under reflux. Two drops of 1% phenolphthalein indicator were then added. The mixture was titrated with 0.5N HCl until the pink colour disappeared. The saponification value of each sample was calculated using the formula:

$$SV = \frac{28.05 \times f \times (B-A)}{W}$$

W = Weight of sample (gms)

f = Factor for HCl

B= Volume required for the blank (cm^3)

A = Volume required for the sample (cm^3)

3.7.5 Determination of Peroxide Value (PV)

Two grams of sunflower oil were weighed into a glass stoppered flask. Twenty five millilitres of acetic acid- chloroform mixture (in ratio 3:2) was added and the oil dissolved. One milliliter of saturated potassium iodide (KI) solution (4 parts KI in 3 parts distilled water) was added, mixed and placed in the dark for

10 min. Thirty millilitres of distilled water was added, mixed and followed by addition of 1ml of 1% starch indicator. The mixture was titrated with 0.01N sodium thiosulphate until the blue colour disappeared. A blank test containing all the reactants other than oil was carried out at the same time. The peroxide value of each sample was calculated as follows:

$$PV = \frac{100\,(B-A)\,N}{W}$$

W = weight of sample (gms)

B= Volume required for the blank (cm^3)

A = Volume required for the sample (cm^3)

N = Normality of sodium thiosulphate

3.7.6 Determination of the Refractive Index (RI)

Sunflower oil was filtered through a filter paper to remove impurities and traces of moisture. A stream of water was circulated through the instrument (Abbe Refractometer Type 3, Atago, Japan) and the temperature of the refractometer adjusted to 25^{o}C. After ensuring that the prisms were clean and dry, a few drops of the oil sample were placed on the prism. The prisms were then closed and sample allowed to stand for 1-2 min. The instrument and lighting were adjusted to obtain the most distinct reading possible.

3.7.7 Determination of Relative Density (RD)

A dry pycnometer (25ml capacity, with a fitted thermometer) was filled with the prepared sunflower oil samples in such a manner to prevent entrapment of air bubbles after removing the cap of the side arm. The stopper was inserted and sample temperature maintained around 15.5^{o}C either by immersing in a warm water bath or in ice. Any oil that had spilled out of the capillary opening was carefully wiped and the bottle thoroughly dried. The samples were then quickly weighed on an electronic analytical balance (Type AEG-220, Shimadzu Corp,

Japan) ensuring that the temperature did not fall below 15.5^{o}C. The density of oil was determined relative to that of water measured under the same conditions.

3.7.8 Determination of Iodine Value (IV)

Zero point two grams of sunflower oil sample was accurately weighed into a clean, dry, 500ml glass-stoppered flask containing 20ml of carbon tetrachloride and 25ml of Wij's solution pipetted into the flask. The flask was swirled and allowed to stand in the dark for 1hr. Twenty millilitres of 10% potassium iodide was added followed by 100ml of freshly boiled and cooled water. Excess iodine was titrated with 0.1N sodium thiosulphate using 1% starch as indicator. A blank containing all the above reactants apart from the oil sample was analyzed at the same time and under the same conditions.

3.8 Data handling and analysis

In all experiments, data was collected on spreadsheets and statistically analyzed by subjecting it to ANOVA using general linear model SAS package (SAS Institute Inc, USA). Separation of means was done by Duncan's grouping of means at 5% significance level. Results were presented in figures as means +□ standard errors.

CHAPTER FOUR

4.0 RESULTS

4.1 Oxalic Acid Bioassay

Degradation of leaf tissue occurred following direct application of oxalic acid on the leaves. Lesions were formed on both sunflower and wheat leaves as shown in figure 4 below. Results obtained from this assay indicated a general increase in lesion size with increasing concentration of oxalic acid (Figure 5).

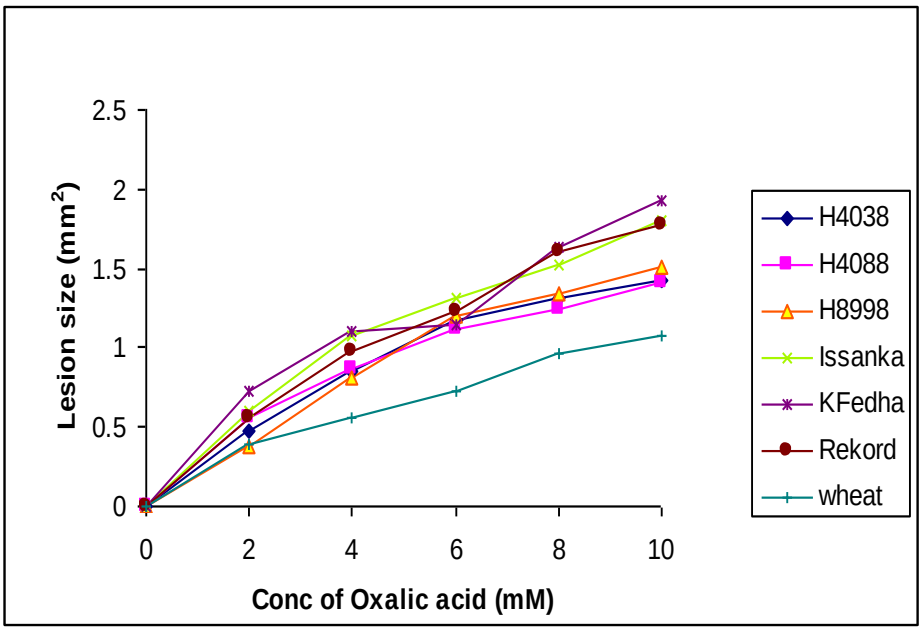

Figure 5. General increase in lesion areas following application of increasing concentrations of oxalic acid

All the varieties responded differently to the effects of oxalic acid ($p<0.05$) as indicated by the differences in mean lesion areas per concentration of oxalic acid (Table 1). With notable outcome was the high degradation of Kenya Fedha, Issanka and Rekord by oxalic acid while varieties H4038 and H4088 had relatively lower degradation. Degradation of H8998 was intermediate while

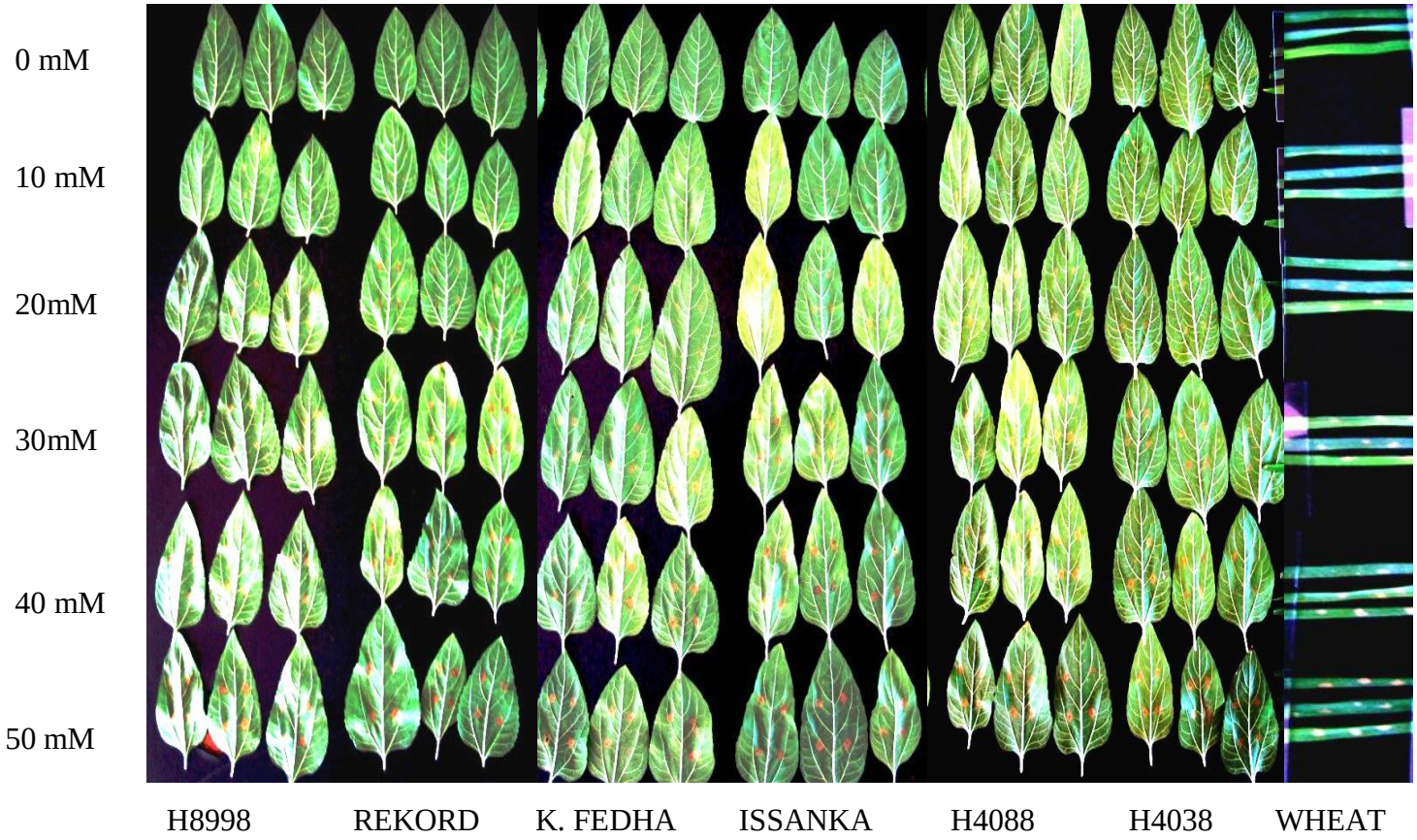

Figure 4. The tan-coloured lesions formed after incubation of leaves with increasing concentrations of oxalic acid.

wheat had the least mean degradation. All the six varieties of sunflower generally showed lower resistance to the degrading effects of oxalic acid compared to the control (Figure 5). Wheat proved to be up to 2.8 times more resistant to oxalic acid degradation than sunflower at pathophysiological concentrations.

Table 1. Mean lesion areas (mm^2) following degradation of leaf tissue by increasing concentrations of oxalic acid

Variety	Concentration of oxalic acid (mM)					
	0	2	4	6	8	10
H4038	0	0.48	0.85	1.18	1.31	1.43
H4088	0	0.56	0.87	1.12	1.24	1.41
H8998	0	0.37	0.81	1.20	1.34	1.51
Issanka	0	0.59	1.08	1.31	1.52	1.80
K. Fedha	0	0.73	1.10	1.14	1.63	1.92
Rekord	0	0.56	0.98	1.22	1.60	1.77
Wheat	0	0.39	0.56	0.73	0.97	1.07

4.2 Oxalate Oxidase Assay

The oxalate oxidase assay results showed a highly significant difference between the means for the control and sunflower varieties ($P<0.05$). The wheat (control) leaf discs produced up to 11.57mM (2.74 times more) H_2O_2 compared to the sunflower leaf discs (Figure 6B).

Among the sunflower varieties, the mean H_2O_2 production was significantly different ($p<0.05$), an indication that different varieties responded differently to

the effects of oxalic acid. The varieties H4088 and H4038 produced 6.34 and 6.35 mM concentrations of hydrogen peroxide respectively, closely followed by H8998 which produced 5.35 mM.

Figure 6A. The purple coloured product formed following incubation of leaf discs with 135 µL of reaction mixture during oxalate oxidase assay.

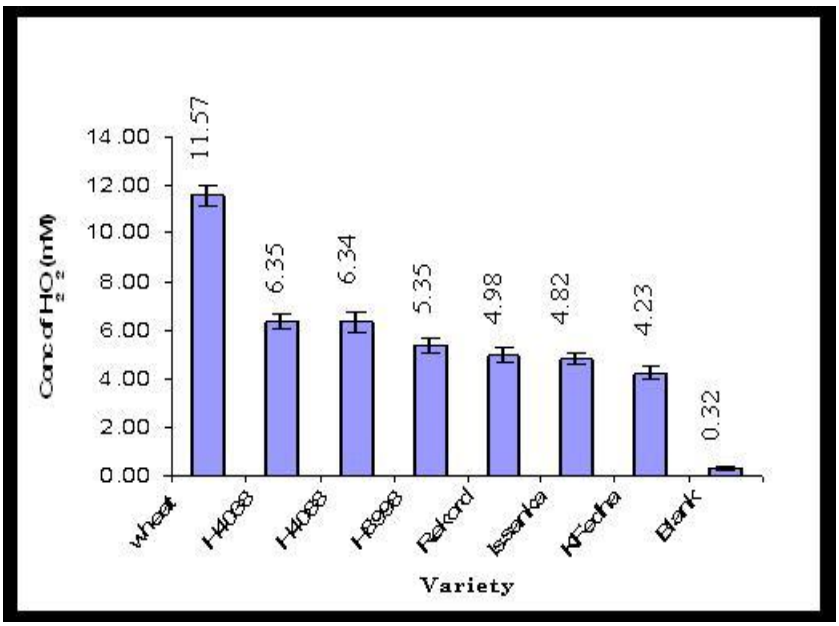

Figure 6B. Concentrations of H_2O_2 generated from leaf discs of wheat and different sunflower varieties. H_2O_2 content was determined spectrophotometrically at 550 nm

4.3 Oil analysis

The results of oil content on dry weight basis indicated a significant difference in the oil content among the six sunflower varieties ($p<0.05$). The oil content ranged between 40.92% w/w and 50.55% w/w as shown in table 2. Variety H8998, which according to Kenya Seed Company is mainly sold commercially for oil extraction, had the highest oil content of 50.55% w/w followed closely by H4088 with 49.41% w/w and Rekord, 47.66% w/w. There was no significant difference in oil content of Kenya Fedha, Issanka and H4038.

From the results, oxidation levels differed significantly ($p<0.05$) as peroxide values ranged from 1.04 to 2.98 meq/kg (Table 2). Oil from H8998 was the most highly oxidized followed by oils of Rekord and Issanka. Those of H4088 and H4038 were the least oxidized.

Iodine values ranged from 114.55 to 128.97 mg/g of oil. The difference between IVs of oils from the various varieties was not highly significant ($p=0.0035$). Oil from the variety H8998 showed the highest level of unsaturation (128.97 mg/g) followed closely by H4038 (Table 2).

Acid values ranged from 0.14 to 0.28 mg KOH/g oil. Oil from Issanka showed a marked difference by exhibiting the highest AV. There was insignificant difference between AVs of oils from all other varieties (Table 2).

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Table 2. Sunflower seed oil yield and physicochemical characteristics of the oil

Variety	% Oil content	Peroxide value	Acid value	Saponification value	Iodine value	Refractive index	Relative density
H8998	50.55 ± 0.60 ^{a}	2.98 ± 0.01 ^{a}	0.14 ± 0.00 ^{b}	162.65 ± 3.57^{a}	128.97±0.95^{a}	1.4718 $^{a\,b}$	0.9184 ^{b}
Rekord	47.66 ± 0.76 ^{b}	2.49 ± 0.00 ^{b}	0.16 ± 0.02 ^{b}	165.10 ± 3.10^{a}	119.45±1.97^{cd}	1.4717 $^{a\,b}$	0.9152 ^{c}
Issanka	41.21 ± 0.82 ^{c}	2.60 ± 0.13 ^{b}	0.28 ± 0.00 ^{a}	168.63 ± 0.28^{a}	121.88±1.10^{bc}	1.4724 ^{a}	0.9193 ^{a}
K.Fedha	41.60 ± 0.58 ^{c}	1.25 ± 0.25 ^{c}	0.14 ± 0.00 ^{b}	171.78 ± 3.38^{a}	115.44±0.98^{de}	1.4719 $^{a\,b}$	0.9182 ^{b}
H 4038	40.92 ± 0.22 ^{c}	1.04 ± 0.04 ^{d}	0.14 ± 0.00 ^{b}	165.33 ± 2.73^{a}	125.57±0.88^{ab}	1.4718 $^{a\,b}$	0.9110 ^{d}
H4088	49.41±0.25 ^{ab}	1.04 ± 0.04 ^{d}	0.14 ± 0.00 ^{b}	165.79 ± 4.35^{a}	114.55±1.09^{e}	1.4709 ^{b}	0.9106 ^{d}

* Means in the same column with similar letters indicate insignificant difference at 5% significant level.

Results on refractive index showed values ranging from 1.4709 to 1.4730. There was insignificant difference between RI values of oils from all the varieties (Table 2).

There was a significant difference in the relative densities of oil from the different varieties ($p<0.05$). Values ranged between 0.9198 and 0.9106. Issanka had the most dense oil followed by varieties H8998 and K. Fedha. Oils from H4038 and H4088 had the least density (Table 2).

The results of saponification values of oils from the different varieties showed insignificant difference ($p=0.4476$). Values ranged from 162 to 171 mg KOH/g as shown in table 2.

There was a significant difference in fatty acid composition of oil from all the six varieties ($p<0.05$) with the exception of linolenic acid and palmitoleic acid (Table 3). Sunflower oil from all the varieties was highly unsaturated. Polyunsaturation which was mainly contributed by linoleic acid ranged between 61-68% of all fatty acids, while oleic and palmitoleic acids contributed to 14-23% of monounsaturation. A saturation level of 11-17% of total fatty acids was observed and was contributed mainly by palmitic, stearic and myristic acids. Variety H4088 showed a marked difference by exhibiting nearly 1% of lauric acid compared to the other varieties which had none.

Table 3. Percentage fatty acid composition of sunflower oil from different varieties

fatty acid	Kenya Fedha	Issanka	Rekord	H 8998	H4038	H4088
Caprylic acid ($C_{8:0}$)	0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.1 ±0.00	0.1 ± 0.00
Lauric acid ($C_{12:0}$)	0.02±0.01	Not detected	Not detected	Not detected	Not detected	0.97± 0.00
Myristic acid ($C_{14:0}$)	0.20±0.06	0.15±0.03	0.06±0.03	0.21±0.1	0.11 ±0.00	0.67 ±0.02
Palmitic ($C_{16:0}$)	12.21±0.88	10.47±0.07	10.42±0.50	8.07±0.31	6.46 ±0.03	8.30 ±0.07
Palmitoleic acid ($C_{16:1}$)	0.44±0.11	0.21±0.06	0.11±0.05	0.43±0.23	0.35 ±0.01	0.08 ±0.01
Stearic ($C_{18:0}$)	4.70±0.19	5.29±0.19	4.76±0.41	3.64±0.38	4.86 ±0.02	5.19 ±0.01
Oleic ($C_{18:1}$)	13.60±0.44	15.05±0.83	15.80±1.40	21.83±0.83	19.96 ±0.16	22.96±0.04
Linoleic acid ($C_{18:2}$)	66.54±1.41	67.82±0.96	66.13±2.54	66.54±0.41	67.80 ±0.18	60.66 ±0.01
Linolenic acid ($C_{18:3}$)	0.40±0.15	0.84±0.47	0.39±0.21	0.10±0.06	0.37 ±0.03	0.60 ±0.02
Total saturated	17.14± 1.14	15.92±0.30	15.25±0.95	11.94±0.71	11.53±0.05	15.23±0.10
Total monounsaturated	14.00±0.55	15.26±0.89	15.91±1.01	21.87±1.06	20.31±0.17	23.04±0.05
Total polyunsaturated	66.94±1.56	68.66±1.43	66.52±2.75	66.64±0.47	68.17±0.21	61.26±0.03
Total unsaturated	80.94 ± 2.11	83.92 ± 2.22	82.43 ± 3.75	88.51 ± 1.53	88.48±0.38	84.30±0.08

$C_{x:0}$ – saturated $C_{x:1}$ – monounsaturated $C_{x:2}$ or $_{x:3}$ - polyunsaturated

Oleic – Linoleic acid ratios of oils from the different varieties ranged from 0.2 to 0.38. Oil from H4088 had the highest ratio (0.38) closely followed by the oils of H8998 (0.33) and H4038 with 0.29 (Figure 7).

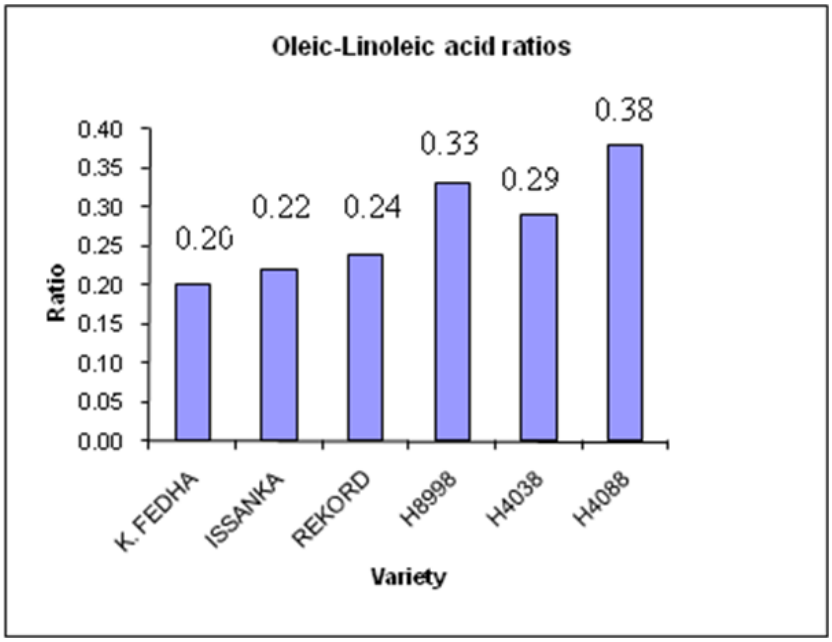

Figure 7. Oleic-Linoleic acid ratios of oils from different sunflower varieties.

No direct relationship was observed between oxalate oxidase activity and oil content of the test varieties. However, the variety H4088 proved to have the highest oxalate oxidase activity as well as a high oil content (49.41% w/w) (Figure 8). The variety H8998 which had the highest oil content (50.55% w/w) was third in oxalate oxidase activity while H4038 was low in oil (40.92% w/w) but second highest in oxalate oxidase activity.

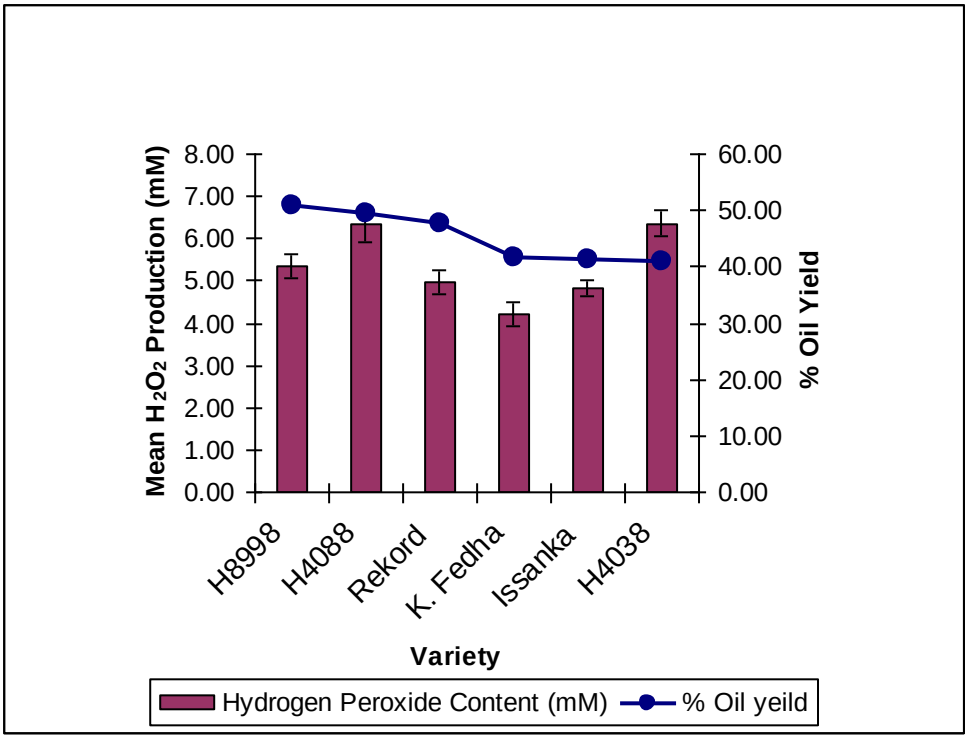

Figure 8. Relationship between oxalate oxidase activity and oil yield

CHAPTER FIVE

5.0 DISCUSSION

In this study, direct application of oxalic acid to sunflower leaf tissue caused marked tissue injury and necrosis similar to that observed during *S. sclerotiourm* infection (Livingstone *et al*., 2005).

Results of oxalic acid bioassay demonstrated differences in susceptibilities of sunflower to degradation upon challenge with exogenously applied oxalic acid. The observed significant differences may have been due to differences in parameters such as expression levels of oxalate oxidase (Livingstone *et al*., 2005) and other enzymes known to cause production of hydrogen peroxide such as sunflower carbohydrate oxidase (Stuiver *et al*., 2000), glucose oxidase (Ireneusz *et al*., 2006) among those which are known to catabolize oxalate such as oxalate decarboxylase (Mehta and Datta, 1991). Expression and activity of secondary plant metabolites involved in plant defense like phenolics has also been found to differ in different sunflower lines (Metraux and Raskin, 1993).

The protective role of oxalate oxidase was clearly demonstrated in this study. There was more resistance to degradation in wheat leaves as evidenced by relatively smaller lesions compared to sunflower (Figure 5). In the oxalate oxidase assay, wheat leaves also produced the highest amount of H_2O_2 (Figure 6b), an indication that it expresses higher levels of OXO compared to sunflower. The relatively little amount of hydrogen peroxide produced by sunflower leaves is therefore unlikely to play a protective role in sunflower defense against oxalic acid producing pathogens.

Collapse of leaf tissue and formation of lesions was observed following challenge with particularly higher concentrations of oxalic acid. The collapse may have been due to the action of oxalic acid which is said to manipulate guard cell function by inducing stomatal opening and inhibiting abscisic acid-induced stomatal closure, thus inducing foliar wilting and consequent death of leaf tissue.

The 48 hour period that was used in this study covered the critical phases when fungal ascospores or mycelia initiate their attack on leaf epidermal cells and when plant responses determine the ultimate success or failure of attempted infection (Helene *et al*., 2000).

The enzyme assay was conducted at pH 4. This is the optimum pH for the fungal polygalacturonase activity (Cessna *et al*., 2000), suggesting synergism between oxalate secretion and pectin degradation.

Though the results demonstrated oxalate oxidase to be useful in resistance of plants against oxalic acid-producing pathogens, the leaflet assays were performed under room temperature and high humidity conditions. This may not be the case in the field as variable growth and environmental conditions come into play.

The sunflower varieties evaluated proved to have as much as 50% of oil. From the results in table 3, it was evident that the oil is highly unsaturated (81% to 89% of total fatty acids) and thus a good source of the essential fatty acids in the diet, which our bodies cannot synthesize. These acids, particularly the polyunsaturated fatty acids, are associated with many health benefits such as alleviating conditions like liver degeneration, arthritis-like conditions, eczema-like skin eruptions, heart and

circulatory problems, and growth retardation, among other health conditions (Passmore and Eastwood, 1986).

In addition, the high proportion of unsaturated fatty acids in sunflower oil means that it can be heated to high temperatures without smoking, leading to faster cooking time and absorption of less oil (Miller *et al*, 1987).

The high level of unsaturation was confirmed by the determination of iodine value. Oil from the various varieties in this study had IVs of between 114 and 128 mg/g, which closely match values of between 118 to 143 mg/g for most vegetable oils (Pocklington, 1990). Oils from H8998 and H4038 were particularly highly unsaturated as confirmed by fatty acid analysis (Table 3). This suggests that they are suitable for use as edible oils and for manufacture of margarine. The IVs also indicate that sunflower oil has good semidrying qualities and therefore suitable for manufacture of paints and plastics.

Peroxide values of oils from the different sunflower varieties showed values lower than 3 meq/kg of oil. This was an indication that the oils were freshly extracted and that they are less liable to oxidative rancidity at room temperature, thus good for storage. Peroxide values above 30 meq O_2/kg of oil are an indication of deterioration of the oil (Naohiro and Shun, 2006).

Free fatty acids in the oil are also capable of being autooxidized (Berdanier *et al*., 2007) and thus can increase the PV of the oil. This explains why Issanka, which had the highest acid value (free fatty acids), also had a relatively higher peroxide value. The same relationship was observed with H4088 which had the smallest peroxide

value as well as acid value. The PV of 2.98 meq/kg observed with H8998 is a reflection of the high level of unsaturation of its oil as confirmed by its high iodine value and an indication of breakdown of the oil, which in the long run may result in oxidative rancidity.

Acid value is a barometer of rancidity in oils that measures the extent to which the constituent glycerides have been decomposed by lipase action, thus forming free fatty acids (hydrolysis). The low acid values of 0.14 to 0.28 mg KOH/g oil obtained are a general indication that the oils were freshly extracted, not rancid and thus edible (AOAC, 1980; Pearson, 1976). Acid values above 3 mg KOH/g are suggestive of degradation of the oil (Naohiro and Shun, 2006).

The very low AVs obtained also indicate the suitability of sunflower oil in industrial use with minimal refining loss. This value serves as a control measure in the production of refined oils, in the production of blown, bodied, and chemically modified oils (AOCS, 1980).

Refractive Index results showed values ranging from 1.4709 to 1.4730 which closely agree with values suggested for edible vegetable oils (Williams and Hilditch, 1964). This indicates that sunflower oil extracted from the six varieties was pure (no adulteration) and therefore minimizes purification procedures during processing such as filter pressing and/or centrifugation.

Relative Density values ranged between 0.9184 and 0.9106; this lies within the range of 0.89 – 0.92 g/ml reported for edible oils (Odufoye, 1998). The results suggest that sunflower oil is light making it desirable for many cooking purposes.

Saponification values of oil from all the six varieties were lower (162.65 to 171.78 mg KOH/g) than for most vegetable oils rich in C_{18} fatty acids (188-196 mg KOH/g), indicating that they contained mainly the long chain fatty acids (Pearson, 1976). The low saponification values also suggest that the oils can be used for candle and soap production and as chemical feedstocks for lubricants

Oleic/Linoleic acid ratios determine the oxidative stability of an oil and generally ratios above one are preferred (Kratz *et al.*, 2002). From this study, O/L ratios were quite low ranging from 0.2 to 0.37 (Figure 7). This is because the evaluated varieties were high in linoleic acid compared to oleic acid. The varieties H4088 and H8998 had ratios above 0.3 meaning they are likely to be more stable and have longer shelf lives compared to the other varieties.

No direct relationship was found between the oil yield and oxalate oxidase activity (Figure 8). The overall objective of this study was, however, met. The variety H4088 was identified as the most resistant to oxalic acid degradation and hence to *S. sclerotiorum* infection, as well as having an acceptable oil yield and quality. This important finding is expected to contribute significantly to sunflower cultivation in Kenya. This variety is relatively new in the Kenyan market and needs to be promoted widely for farming. This will in turn increase the overall sunflower yield and enhanced food security in the country.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

The selected Kenyan sunflower varieties have low resistance to the effects of oxalic acid.

The variety H4088 was the most superior among the selected varieties due to its relatively higher levels of oxalate oxidase activity and high oil content.

From the relative density and refractive index analyses done on the oil, it was deduced that oil from Kenyan sunflower varieties is light and pure making it desirable for many cooking purposes. It also has a high level of unsaturated fatty acids.

The high levels of linoleic acid give the oil good semidrying properties, meaning it may suitably be used in manufacture of paints and plastics without colour modification.

The low saponification values also suggest that the oil is good for soap and detergent production.

This study therefore recommends that

- Further investigation of resistance using the natural pest, *S. sclerotiorum*, be carried out.
- Molecular studies be conducted to determine the genetic potential for resistance in the six varieties.

- The possible contribution of other oxidases and secondary plant metabolites namely phenolics, terpenoids and alkaloids to resistance be investigated.
- Cultivation of H4088 be encouraged to produce sunflower seed oil because of its high oil yield and resistance to oxalic acid degradation.
- Variety H8998 be targeted for resistance improvement due to its high oil yield.
- Sunflower oil be promoted for domestic consumption due to its quality and potential health benefits
- The protein, mineral and vitamin content of oil from Kenyan sunflower varieties be investigated.

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APPENDICES

Appendix 1. GLM procedure for oxalic acid bioassay (0-10mM)

Dependent Variable: area

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	51	763.092392	14.962596	73.19	<.0001
Error	1283	262.297062	0.204440		
Corrected Total	1334	1025.389454			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Variety	6	99.7371547	16.6228591	81.31	<.0001
Rep	3	0.6518018	0.2172673	1.06	0.3639
Conc	5	569.5821278	113.9164256	557.21	<.0001
Variety*Conc	30	85.5579520	2.8519317	13.95	<.0001
Variety*Season	7	7.5633555	1.0804794	5.29	<.0001

Duncan Grouping	Mean	N	variety
A	1.23443	192	K Fedha
A	1.22394	188	Issanka
A	1.19184	190	Rekord
B	0.94864	191	H8998
C	0.79586	191	H4038
C	0.78719	192	H4088
D	0.44152	191	wheat

Appendix 2. GLM procedure showing the effect of different oxalic acid concentrations (0-10mM) in separate seasons

Season 1

The GLM Procedure

Dependent Variable: area

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	239.8066878	47.9613376	120.84	<.0001
Error	492	195.2794375	0.3969094		
Corrected Total	497	435.0861253			

Duncan Grouping	Mean	N	conc
A	1.93476	84	10
B	1.61138	80	8
C	1.03190	84	6
D	0.66506	79	4
E	0.23160	81	2
F	0.03456	90	0

Season 2

The GLM Procedure

Dependent Variable: area

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	383.0165259	76.6033052	148.90	<.0001
Error	612	314.8499686	0.5144607		

Duncan Grouping	Mean	N	conc
A	2.21096	104	10
B	1.80673	104	8
C	1.14369	103	6
D	0.73000	101	4
E	0.30267	101	2
F	0.00000	105	0

Appendix 3. GLM Procedure for Oxalate oxidase assay

The GLM Procedure

Dependent Variable: Conc

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	539.9531438	77.1361634	102.77	<.0001
Error	56	42.0326000	0.7505821		
Corrected Total	63	581.9857438			

Duncan Grouping		Mean	N	variety
	A	11.5675	8	wheat
	B	6.3525	8	H4038
	B	6.3400	8	H4088
	C	5.3525	8	H8998
D	C	4.9763	8	Rekord
D	C	4.8188	8	Issanka
D		4.2288	8	KFedha
	E	0.3163	8	Blank

Appendix 4. GLM Procedure for Oil Yield

The GLM Procedure

Dependent Variable: YIELD

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	310.8111611	62.1622322	56.15	<.0001
Error	12	13.2849333	1.1070778		

Duncan Grouping		Mean	N	VARIETY
	A	50.9333	3	H8998
B	A	49.4000	3	H4088
B		47.6600	3	Rekord
	C	41.6033	3	Kfedha
	C	41.2100	3	Issanka
	C	40.9300	3	H4038

Appendix 5. GLM procedure for Acid Value

Dependent Variable: AV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.04783168	0.00956634	35.21	<.0001
Error	12	0.00326012	0.00027168		
Corrected Total	17	0.05109180			

Duncan Grouping	Mean	N	VARIETY
A	0.28157	3	Issanka
B	0.16400	3	Rekord
B	0.14060	3	H4038
B	0.14057	3	H4088
B	0.14040	3	Kfedha
B	0.14033	3	H8998

Appendix 6. GLM procedure for Peroxide Value

The GLM Procedure

Dependent Variable: PV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	11.96953269	2.39390654	129.68	<.0001
Error	12	0.22152025	0.01846002		

Duncan Grouping	Mean	N	VARIETY
A	2.9775	3	H8998
B	2.5607	3	Issanka
B	2.4864	3	Rekord
C	1.1629	3	Kfedha
C	1.0318	3	H4038
C	1.0311	3	H4088

Appendix 7. GLM Procedure for Saponification Value

The GLM

Dependent Variable: SV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	150.9481137	30.1896227	1.03	0.4476
Error	11	323.1095333	29.3735939		
Corrected Total	16	474.0576471			

Duncan Grouping	Mean	N	variety
A	171.780	3	Kfedha
A	168.630	3	Issanka
A	165.787	3	H4088
A	165.333	3	H4038
A	165.100	2	Rekord
A	162.650	3	H8998

Appendix 8. GLM Procedure for Iodine Value

The GLM Procedure

Dependent Variable: IV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	322.3708500	53.7284750	16.86	0.0035
Error	5	15.9336500	3.1867300		
Corrected Total	11	338.3045000			

Duncan Grouping		Mean	N	variety
	A	128.970	2	H8998
B	A	125.570	2	H4038
B	C	121.880	2	Issanka
D	C	119.450	2	Rekord
D		116.420	1	KFedha
D		114.545	2	H4088
D		114.450	1	Kfedha

Appendix 9. GLM Procedure for Refractive Index

The GLM Procedure

Dependent Variable: RI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3.3961111E-6	6.7922222E-7	1.70	0.2084
Error	12	4.7866667E-6	3.9888889E-7		

Duncan Grouping		Mean	N	VARIETY
	A	1.4724000	3	Issanka
B	A	1.4719333	3	Kfedha
B	A	1.4718333	3	H4038
B	A	1.4717667	3	H8998
B	A	1.4717000	3	Rekord
B		1.4709333	3	H4088

Appendix 10. GLM Procedure for Relative Density

The GLM Procedure

Dependent Variable: RD

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00022268	0.00004454	404.87	<.0001
Error	12	0.00000132	0.00000011		

Duncan Grouping	Mean	N	VARIETY
A	0.9193000	3	Issanka
B	0.9184000	3	H8998
B	0.9182667	3	Kfedha
C	0.9152000	3	Rekord
D	0.9110333	3	H4038
D	0.9106333	3	H4088

Appendix 11. GLM procedures for fatty acid analysis

The GLM Procedure

Dependent Variable: caprylic

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.02221042	0.00444208	19.04	<.0001
Error	10	0.00233333	0.00023333		
Corrected Total	15	0.02454375			

Duncan Grouping	Mean	N	VARIETY
A	0.10000	2	H4038
A	0.10000	2	H4088
B	0.03000	3	H8998
B	0.01667	3	Issanka
B	0.01000	3	Rekord
B	0.00667	3	Kfedha

Dependent Variable: lauric

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.62890833	0.32578167	3759.02	<.0001
Error	10	0.00086667	0.00008667		
Corrected Total	15	1.62977500			

Duncan Grouping	Mean	N	VARIETY
A	0.970000	2	H4088
B	0.026667	3	Kfedha
C	0.000000	3	H8998
C	0.000000	3	Issanka
C	0.000000	2	H4038
C	0.000000	3	Rekord

Dependent Variable: **myristic**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.56350000	0.11270000	61.92	<.0001
Error	10	0.01820000	0.00182000		
Corrected Total	15	0.58170000			

Duncan Grouping		Mean	N	VARIETY
A		0.67000	2	H4088
B		0.15333	3	Kfedha
B		0.15333	3	Issanka
C	B	0.11000	2	H4038
C	B	0.08667	3	H8998
C		0.06000	3	Rekord

Dependent Variable: **palmitic**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	58.59500000	11.71900000	13.52	0.0004
Error	10	8.66680000	0.86668000		
Corrected Total	15	67.26180000			

Duncan Grouping	Mean	N	VARIETY
A	12.1467	3	Kfedha
A	10.4433	3	Issanka
A	10.4167	3	Rekord
B	8.3000	2	H4088
B	7.5800	3	H8998
B	6.4600	2	H4038

Dependent Variable: **palmitoleic**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.79169375	0.15833875	2.35	0.1173
Error	10	0.67390000	0.06739000		
Corrected Total	15	1.46559375			

Duncan Grouping		Mean	N	VARIETY
	A	0.6533	3	H8998
B	A	0.5867	3	Kfedha
B	A	0.3450	2	H4038
B	A	0.2700	3	Issanka
B	A	0.1067	3	Rekord
B		0.0750	2	H4088

Dependent Variable: **stearic**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	8.45086667	1.69017333	7.40	0.0038
Error	10	2.28383333	0.22838333		
Corrected Total	15	10.73470000			

Duncan Grouping		Mean	N	VARIETY
	A	5.1850	2	H4088
	A	5.1733	3	Issanka
	A	4.8550	2	H4038
	A	4.7567	3	Rekord
	A	4.3400	3	Kfedha
	B	3.1300	3	H8998

Dependent Variable: **oleic**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	198.7241667	39.7448333	21.40	<.0001
Error	10	18.5750333	1.8575033		
Corrected Total	15	217.2992000			

Duncan Grouping		Mean	N	VARIETY
	A	22.955	2	H4088
B	A	21.827	3	H8998
B		19.955	2	H4038
	C	15.797	3	Rekord
	C	15.050	3	Issanka
	C	13.600	3	Kfedha

Dependent Variable: **linoleic**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	74.53273333	14.90654667	40.50	<.0001
Error	10	3.68106667	0.36810667		
Corrected Total	15	78.21380000			

Duncan Grouping	Mean	N	VARIETY
A	67.8267	3	Issanka
A	67.8000	2	H4038
B	66.5367	3	Kfedha
B	66.2433	3	H8998
B	66.1267	3	Rekord
C	60.6600	2	H4088

Dependent Variable: **linolenic**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.38023333	0.07604667	1.68	0.2276
Error	10	0.45366667	0.04536667		
Corrected Total	15	0.83390000			

Duncan Grouping		Mean	N	VARIETY
	A	0.6000	2	H4088
B	A	0.4067	3	Kfedha
B	A	0.3867	3	Rekord
B	A	0.3700	2	H4038
B	A	0.3467	3	Issanka
B		0.0667	3	H8998

Appendix 12. Fatty acid elution profile