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A COMPARATIVE ASSESSMENT OF NICOTINE AND REDUCING SUGAR LEVELS IN FLUE CURED TOBACCO VARIETIES IN THE SMALL SCALE SECTOR OF MUTARE DISTRICT

BY

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Abstract

Tobacco is the most widely grown and cured non-food crop worldwide. Nicotine content and reducing sugar levels are properties that determine quality in tobacco leaves. The study aimed at determining nicotine and reducing sugar levels in leaves of varieties KRK 26R and T 76 at three leaf ripeness stages of unripe, ripe and overripe. Each variety was grown by three small scale growers and leaves harvested at the unripe, ripe and overripe stages were analysed for nicotine and reducing sugar levels. Nicotine and reducing sugar levels were determined at the Tobacco Research Board laboratory. The data was analysed using Minitab version13 statistical package. The means of nicotine content and reducing sugar levels were compared using the paired T tests for each stage of ripeness within each variety and between the two varieties. The means of nicotine content levels in variety KRK 26R were as follows; 2.633% unripe leaves, 3.87% ripe leaves and 4.477% overripe leaves. The reducing sugar levels in variety KRK 26 R were as follows; 13.39% on unripe leaves, 14.567% on ripe leaves and 7.57% on overripe leaves. The means of nicotine levels on variety T76 were as follows; 2.78% on unripe leaves, 3.223% ripe leaves and 4.273% overripe leaves. The reducing sugar levels in variety T 76 were as follows; 12.84% unripe leaves, 17.103% ripe leaves and 8.893% unripe leaves. There were significant differences ($P<0.05$) in the nicotine content in KRK 26 R between the unripe and ripe leaves, and between the unripe and overripe leaves, but there were no significant differences ($P>0.05$) in nicotine content between the ripe and overripe leaves. There were no significant differences ($P>0.05$) in the reducing sugar levels of KRK 26 R between the unripe and ripe leaves, but there were significant difference ($P<0.05$) in the reducing sugar levels between the ripe and overripe leaves. On variety T 76 there were no significant differences ($P>0.05$) in nicotine content between the unripe and ripe leaves, but there were significant differences ($P<0.05$) in nicotine content between the unripe and overripe leaves, and between the ripe and overripe leaves. On the reducing sugars levels of variety T76 there were no significant differences ($P>0.05$) between the unripe and ripe leaves but there were significant differences ($P<0.05$) between the unripe and overripe leaves and the ripe and overripe leaves. In conclusion, the study showed that nicotine levels in both varieties were within the accepted range of 2–5 % and did not seem to be affected by the stage of leaf ripeness. The reducing sugar levels of both varieties on unripe and overripe leaves were far less than the accepted range of 15-30% and only the ripe stage levels were within the accepted range of 15-30%. It is therefore important that growers reap leaves at the ripe stage only in order to attain the acceptable reducing sugar levels in their tobacco.

Key words: Tobacco, unripe, ripe, overripe, nicotine, reducing sugar levels

Declaration Page

I declare that this dissertation is my original work except where sources have been cited and acknowledged. The work has never been submitted, nor will it ever been submitted to another university for the award of a degree.

_____	_____
Student's Full Name	Student's Signature (Date
_____	_____
Main Supervisor's Full Name	Main supervisor's Signature (Date)

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Dedication

To my late father and my late brother, Mr. Bruce Arthur Mazhangara and Reason Mazhangara.

Daddy you always challenged me to be a great man.

Brother you always wanted me to go high.

So I wish you could see the man I've grown to be

List of Acronyms and Abbreviations

AO	Asp oxidase
DAO	Diamine oxidase
FAAS	Flame atomic absorption spectrometer

FAO	Food Agriculture Organisation of the United Nations
MAO	Monoamine oxidases
MATE	Multidrug and toxic compound extrusion
MPO	N-methyl transferase
NUP	Nicotine uptake permease
ODC	Orn decarboxylase
PMT	Putrescent N-methyl transferase
TIMB	Tobacco Industry and Marketing Board
TIPS	Tobacco Improved Productivity Scheme
TRB	Tobacco Research Board
WHO	World Health Organisation of the United Nations

Definition of Key Terms

Nicotine	An alkaloid that is naturally produced by plants in the family solanaceaes. It is a pale yellow to dark brown oily liquid with unpleasant pungent odour, sharp persistent bitter taste and soluble in alcohol, chloroform and ether

Reducing sugar	Any sugar that is capable of acting as a reducing agent because it has a free aldehyde group or a free ketone group and can donate electrons to another molecule. All monosaccharides are reducing sugars, along with some disaccharides, oligosaccharides, and polysaccharides.
Reaping	Harvesting of tobacco leaves
Ripening	Changes that happen naturally which occur in the time without reference to death as a consequence
Unripe	Premature leaves
Ripe	Mature leaves
Overripe	Over mature leaves

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CHAPTER 1 INTRODUCTION

1.1 Introduction

Flue-cured tobacco (*Nicotiana tabacum*) is harvested during a period of four to eight weeks as the leaves ripen on the stalk. Growers harvest the leaves from the bottom of the stalk towards the top as they ripen. Cultivars differ in the rate at which they ripen on the stalk and growers can capitalize on these differences to maximize use of their labour and curing facilities.

Nicotine belongs to a family of compounds called alkaloids and occurs naturally in several varieties of plant – including tomatoes, aubergines and potatoes – but is found at its highest levels in the tobacco plant. We don't add nicotine to our conventional cigarettes and the nicotine in our e-liquids for our vapour products is extracted from tobacco leaf.

Saccharides, particularly sugars, are natural tobacco constituents, and also are frequently added to tobacco during the manufacturing processes. Sugars in tobacco are formed via enzymatic hydrolysis of starch that begins in the early stage of the curing process. Tobacco contains a variety of carbohydrates which can account for more than 30% of tobacco weight in the blended cigarettes. Carbohydrates exist in tobacco mainly in the forms of polysaccharides such as cellulose (10%), pectin (6-12%), and starch as well as mono- or disaccharides (soluble sugars) such as fructose, glucose, and sucrose

1.2 Background to the Study

Tobacco (*Nicotiana tabacum* L.) it is an annual plant that can grow up to 3 meters tall but commonly shorter in other areas of its natural range (Tamagnone, Merida, Stacey, Plaskitt, Parr, Chang & Martin, 1998). Leaves are alternating entire and ovate to lanceolate and can reach 1.2 m long (Du Preez, 2017). Tobacco belongs to the Solanaceae which includes potatoes, eggplant, paprika and pepper (Brady, 2002). Tobacco is a plant that yields very high nicotine rich leaves and the nicotine rich leaves are controversial in terms of their usage (Tamagnone *et al*., 1998).

Tobacco is the largest provider of foreign currency to Zimbabwe and any limitations or prohibition on its production will have serious repercussions on the economy of Zimbabwe (TIMB, 2016). In 2015, tobacco worth $855 million was exported from Zimbabwe with China receiving close to 41% of the total exports (TIMB, 2016) In 2015 at a consultative workshop on the WHO Framework Convention workshop on tobacco, the Tobacco Industry Marketing Board (TIMB) defended against the ban of tobacco by pointing out the households of over three million people in Zimbabwe depend on tobacco (TIMB, 2016). Any form of restrictions and bans on tobacco have serious implications on livelihoods of farmers and will definitely and significantly impact economy of the countries such as Zimbabwe, China, Malawi and others.
Zimbabwe produces close to 20% of the world's virginia tobacco and by virtue of being a leading producer of the best leaves (ZTA, 2015).

Annual tobacco production worldwide stands at approximately 7.5 million tonnes (Assunta, 2012). The top ten producers of tobacco across the world in 2018 were China, Brazil, India, USA, Indonesia, Argentina, Zimbabwe, Turkey, Malawi and Pakistan and that puts Zimbabwe as the number one producer in Africa (Davis, Wakefield, Amos & Gupta, 2007). This fact is supported by statistics from the Tobacco Industry and Marketing Board (TIMB) which show that the 2018 product increased to 240 million kg setting a new record from a figure of 189 million kg in 2017. Tobacco has proved to be a good cash cow especially for small holder growers in traditional tobacco producing areas, and that has made tobacco to be grown out to non-traditional growing areas. Manicaland as a province produced 27 813 933 kg in 2017 from a total number of growers of 12 959 with average price of $2.91 and average yield of 2115 kg per hectare (TIMB, 2017). Mutare as a district has a registered total number of 3531 growers and has produced 7 056 624 kg at an average price $2.81 and yield of 2 229 kg per hectare (TIMB, 2017).

In Zimbabwe there were over 70 000 registered farmers growing tobacco (TIMB, 2017). Over 46 000 growers were under the contract growing and marketing scheme. The industry has also spawned several cigarette manufacturers and currently there are seven cigarette manufacturers who export over 80% of their produce (ZTA, 2015).

Tobacco smoke contains over 7 000 chemicals (Leffingwell, 1999). The majority of them are found in the tar produced by smoking cigarettes. Two hundred and fifty of those chemicals including carbon monoxide, ammonia, and hydrogen cyanide are known to be harmful to smokers and people exposed to secondary smoke. Of those,

69 are known to cause cancer (Jha, Ramasundarahettige, Landsman, Rostron, Thun, Anderson & Peto, 2013).

Nicotine is the primary substance in cigarettes that causes addiction, but most experts agree that it does not directly cause cancer (Jha *et al*., 2013). It is one of thousands of chemicals in tobacco. Dozens of them, particularly tar, which gives cigarettes and chewing tobacco their flavour, are known carcinogens (WHO, 2003). Nicotine is an addictive substance and it produces a pleasurable, relaxed feeling when inhaled in smoke or vapour or when ingested from chewing tobacco (WHO, 2008). It is the component of the liquid in e-cigarettes (Jenkins, Tomkins & Guerin, 2000). The nicotine in e-cigarette liquid is extracted from tobacco and mixed with a liquid base so that it can be vaporized when heated. Because e-cigarettes deliver nicotine without the tar and many of the other cancer-linked chemicals found in tobacco, these are thought to pose less of a cancer risk than traditional cigarettes (Jha *et al*., 2013). Most research points to cigarette smoke and not nicotine, as being the primary contributor to cancer among smokers and numerous experts agree that nicotine does not directly cause cancer (Jha *et al*., 2013).

The decapitation or topping which is the removal of the terminal bud with or without the small leaves at onset of flowering is standard practice in production of tobacco. The procedure of topping has the object to hinder the translocation of photosynthetic products and the other elements of the plant from leaves to the floret bud, which in turn increases size and weight of leaves, thus subsequent in increasing the yield and improving the quality of tobacco (Dawson & Solt, 1959). The period after topping is called the maturity stage of the leaves, being biologically similar to senescence. It has been known that the starch and sugars, which are presumed to be some of the

important constituents in issue of the quality of tobacco, are accumulated during the maturity stage, together with nicotine (Huang, Wang, Wang & Peng, 2000).

In flue- cured tobacco a leaf is considered ready for harvest when it is ripe and the term reaping is used Zimbabwe when they reach a stage of senescence (Akehurst, 1981). The uniform reaping depends on earlier operations were seedlings were uniform, the land preparation was uniform and the same plough depth and same soil texture and structure, fertilizer application was done timely and weeding was done well in time.

According to the Tobacco Research Board (TRB, 2010), one of the most confusing aspects to small scale tobacco growers is the stage to reap tobacco. Reaping tobacco leaves by number may be carried out, usually two leaves per week. Sumner & Moore (2009), noted that uniform ripe tobacco is paramount for selling top quality leaf under normal conditions as flue- cured tobacco ripens two to four leaves per week. The statement by Sumner & Moore (2009) was regarded as baboon reaping by the Tobacco Research Board (TRB, 2011), and it is the practice being followed by small scale tobacco growers.

In order for the growers to continue producing high quality leaves, it is prudent that the fragile tobacco leaves are handled methodically at each stage of production of which the reaping stage is no exception and must not be overlooked as it is very vital (TRB, 2011). For most small scale growers their tobacco may appear well grown in the land, but if it is not reaped at the optimum degree of ripeness, then an array of challenges arise. When the tobacco selling season starts tobacco growers get the auction floor impulse and may end up harvesting unripe leaves or overripe leaves and this can lead to losses in leaf quality. The poor leaf quality is reflected in among

other parameters, the nicotine content and the reducing sugar levels which may be above or below the accepted range. This study therefore focuses on nicotine and reducing sugar levels from unripe, ripe and overripe leaves so as to generate data that can be used to determine the effects of different levels of leaf ripeness on tobacco quality.

1.3 Statement of the problem

Nicotine and reducing sugar levels in tobacco are influenced by varietal characteristics through plant breeding. It has, however, been noted that the handling of tobacco leaves at the pre and post-harvest stages does also contribute to different levels of nicotine and reducing sugar in tobacco. The stages at which tobacco leaves are harvested is known to influence the nicotine and reducing sugar levels in tobacco leaves.

Small scale tobacco growers usually encounter the challenge of determining exactly when to reap tobacco leaves for curing. In some cases they can reap unripe leaves and in some cases they reap both ripe and overripe leaves. It is in light of this challenge that the quality of the tobacco from small scale growers usually has varied levels of nicotine and reducing sugar levels thus compromising the revenue earned by the farmers (TIMB, 2017). It is therefore imperative that the levels of nicotine and reducing sugar be determined at different leaf ripeness levels of unripe, ripe and overripe so that farmers can be informed accordingly.

1.4 Research Objectives

1.4.1 Major Objective

Determine nicotine and reducing sugar levels from unripe, ripe and overripe leaves of two flue cured tobacco varieties KRK 26 R and T 76 grown by small scale growers in Mutare district.

1.4.2 Specific Objectives

1. Determine the nicotine levels from unripe, ripe and overripe tobacco leaves in varieties KRK26 R and T 76.

2. Determine the reducing sugar levels from unripe, ripe and overripe tobacco leaves in varieties KRK 26 R and T 76.

1.5 Research Questions

1. What are the nicotine levels from unripe, ripe and overripe tobacco leaves in varieties KRK26 R and T 76

2. What are the reducing sugar levels from unripe, ripe and overripe tobacco leaves in varieties KRK 26 R and T 76

1.6 Assumptions/ Hypotheses

Hypotheses set 1

H_0 Nicotine levels are not different at the unripe, ripe and overripe leaf stages.

H_1 Nicotine levels are different at the unripe, ripe and overripe leaf stages.

Hypotheses set 2

H_0 Reducing sugar levels are not different at the unripe, ripe and overripe leaf stages.

H_1 Reducing sugar levels are different at the unripe, ripe and overripe leaf stages.

1.7 Significance of the study

The determination of nicotine and reducing sugar levels in tobacco is crucial to control, or maintain the levels below the addictive as well as harmful thresholds. Knowledge of nicotine levels in tobacco is especially important to the tobacco industry and in the area of toxicology to control its harmful effect on health. The information is necessary to maintain the quality within those levels that are internationally accepted and hence it no necessary for the calls for the total banning of tobacco due to harmful health effects.

One way in which small scale farmers can control the levels of nicotine and reducing sugar in the harvested tobacco is to ensure that the crop is harvested at the correct stage or ripeness. The study was therefore conducted to see if the two varieties KRK 26 R and T 76 which are widely grown by small scale growers have different levels of nicotine and reducing sugar under their production conditions. The study also aimed at determining the impact of poor timing of ripeness (management practices) on nicotine and reducing sugar levels on tobacco produced by different growers.

The study aimed at determining the impact of stage of leaf ripeness on the nicotine and reducing sugar levels in the tobacco varieties KRK26 R and T 76. The results will be useful in training small scale farmers on the effects of harvesting leaves at the incorrect stages of ripeness.

1.8 Delimitation of the Study

The research was conducted at six farms in Odzi, Mutare district. The farms are in the same ecological and agronomic region 3. These received the same amount of rainfall of about 700mm. The altitude for the area is about 1017m. The soil types are the Ferralsols and the researcher drew leaf samples from three growers with the variety KRK 26 R and three growers with the variety T 76.

1.9 Limitation of the Study

There was a relatively dry spell that affected the early stage of growth and this had a retarding effect on the overall crop growth.

CHAPTER 2 REVIEW OF RELATED LITERTURE

2.1 Introduction

It is known that Virginia type of tobacco accounts for 60 % of the global tobacco production; while Burley type of tobacco accounts for 13.6 %, dark coloured and cigar tobaccos constitute 11.3 %and Oriental type of tobacco accounts for 10 % of total global production. It is also known that 40 % of Oriental type of tobacco production is occurred in Turkey and Virginia type in Africa Zimbabwe is number one producer and with the best flavour. In Zimbabwe tobacco is the second large foreign currency earner after gold. It has different uses such as religion, industrial, medicinal and economic. Chemical changes mediated by enzymatic activity during the yellowing stage lead to the formation of desired compounds in the cured tobacco. Starch is converted into reducing sugars during yellowing and early leaf drying. As starch degrades, reducing sugar concentration increases and reaches its peak by the end of the yellowing stage. It then declines due to respiration, which oxidizes reducing sugar into carbon dioxide and water.

2.2 Origin of tobacco

Tobacco originated from South America, but is also thought to be endemic to Australia and Africa (Winter, 2000). Names such as Sacra and herba from Peru point to its native origins in South America (Lugon-Moulin, Martin, Krauss, Ramey & Rossi, 2006). In Zimbabwe, it is known that the Nyoka tobacco is native to the region (ZTA, 2015). Hanafin, & Clancy, (2015) noted that in early history, small quantities of *Nicotiana* were found in Rome and old world plants including belladonna and *nicotiana* have been found in human ruins and pipes in the near East and Africa. Columbus first observed Arawak's Indians in 1492 to be smoking brown leaves of a peculiar tobacco and seven years later Amerigos Vespucci visited an island near Venezuela and noticed that the inhabitants were already chewing dried leaves (Gately, 2007). Cultivated *Nicotiana tabaccum* originated from a natural cross between *Nicotiana sylvesteris* and *Nicotiana otophora* (Crocq, 2007). The sterile hybrid of chromosomes duplicated and gave rise to the cultivated crop, although *Nicotiana tomentosiformis* has also been suggested as one of the progenitors (Lugon-Moulin et al, 2006). Tobacco has 90 genres and between 3000 and 4000 classes with great disparity in habit and distribution on all continents expect Antarctica (Crocq, 2007). The species name *Tabaccum* derives from the American name for the plant and De Candolle (1885) noted that the vernacular names in introduction regions including China, Japan, the Philippines, Java, India and Iran are mostly variations of American names confirming the Neotropical origin of the species.

2.3 Economic importance of tobacco in Zimbabwe

The total of recorded growers improved from 81 801 in 2016 to 98 927 in 2017. Tobacco farmers who grew tobacco in 2017 increased by 32% from 73 437 growers in 2016 to 97 066 in 2017 (TIMB, 2017). The 2017 marketing period concluded in 130 days which was 15 days longer than the total of selling days in the prior year.

The 2017, volume decreased by 7% to 188.9 million kg compared to the 2016 harvest (TIMB, 2017). The income realised was $559.1 million and this fell by 6% from the 2016 levels. The average price per kg slightly rose from $2.95 in 2016 to $2.96 in 2017.

Tobacco exports in 2017 rose to 182.4 million kg from 164.5 million kg achieved in 2016. Conversely, the Chinese tobacco market share fell from 42% to 33% for 2017 exports. Total 2017 export incomes amounted to $904.4 million averaging $4.96/kg matched to 2016's tobacco exports, which amounted to $933.7 million at $5.67/kg. The Far East dominated the 2017 exports with a share of 48% followed by Africa with 21% and the European Union with 17% (TIMB, 2017). Tobacco imports increased, recording a total of 6.9 million kg in 2017 compared to 4.9 million kg in 2016 and Zambia contributed the bulk of tobacco exports to Zimbabwe.

Worldwide, over US$680 billion revenue is generated from the sale of orthodox cigarettes, with some 5 500 billion cigarettes being used up per year. The tobacco industry remains a significant source of livelihoods to the economies of numerous nations and the livelihoods of millions of people worldwide including the growers, merchants and those working in the tobacco supply chain.

Universal tobacco manufacturing capacity is anticipated to have fallen in 2017 and limitations on the manufacturing, sale, marketing and packaging of tobacco products are in place in several countries and markets.

2.3.1 Tobacco uses in a cultural context

Ritual tobacco use in shamanism is probably as old as the beginning of horticulture, some 8000 years ago (Pammel, 1911). Shamans used large amounts of nicotine to

induce acute nicotine intoxication, resulting in catatonic states representing symbolic death (Hurt & Robertson, 1998; Davis & Morris, 1991). Epidemiological studies have shown that smoking heals Parkinson's disease, with an odds ratio of about 0.5 for smokers compared to non-smokers (Le Houezec, Halliday, Benowitz, Callaway, Naylor & Herzig, 1994).

Gilles de la Tourette's syndrome is a genetic disorder resulting from basal ganglia abnormality that is typically treated with dopaminergic antagonists, such as the antipsychotic drug haloperidol. Animal studies have suggested that the use of nicotine could have beneficial effects in patients with Tourette's syndrome (Stewart, 1997).

Indians used a dressing of tobacco leaves to put on skin inflammations, toothaches to help soothe and relieve pain and help draw out the poison and heal snake wounds. Although people with mental illness are twice as likely to smoke, there may be some benefits to their habit (Szasz, 1963). It is also believed that tobacco helps people with asthma and tuberculosis to breathe better (Otanez & Glantz, 2011).

Indians would blend tobacco and leaves from the Desert Sage plant, or the root of Indian Balsam or cough root (Kalant, 2001). They believed this would similarly benefit with asthma and tuberculosis. They characteristically smoked the leaves to clear out nasal passages.

The psycho-stimulant properties of nicotine might help schizophrenia patients recompense for their reasoning shortages (Hurt, Offord, Croghan, Gomez-Dahl, Kottke, Morse & Melton, 1996).

2.3.2 Industrial uses of tobacco

American Indians used tobacco nicotine as an insecticide for seed protection, and as a vermin fumigant. Tobacco is a great insect repellent for gardens. By soaking as little as a cigarette amount of tobacco in about a litre of water, and allowing it to soak overnight, the nicotine released in the water will create an all-purpose insect repellent (Stewart, 1999). The repellent can also control mosquitoes and bed bugs. In India, crushed tobacco is scrubbed on the teeth for cleaning. This method is still used in India and marketed in stores around the country. Smidgeon tobacco dusts around peach trees willpower dissuade the feared peach tree borer from pervading your tree (Kalant, 2001). A mixture of tobacco powder, pyrethrum powder dried pyrethrum flowers crumbled into a powder is be used to control leaf rollers. Aphids are actual nuisance if allowed to thrive on garden fields and a blend of powdered garlic, compost and tobacco control aphids (Kalant, 2001).

2.4 Chemical composition of tobacco

The chemical composition of the leaf and smoke are significant to the customer. Tobacco smoke contains several chemical compounds and their presence and relative concentrations depend upon the genre of the tobacco leaf, additives and industrial processes (Hoffmann, & Wynder, 1986). The most important factor is the tobacco leaf itself. Although flavouring and other additives are applied in varying amounts by the manufacturer, the tobacco gives the favour, aroma, and chemical elements that make the smoke desirable and pleasing to the consumer (Johnstone, & Plimmer, 1959).

Chemical constituents in the tobacco leaf are influenced by many factors as the tobacco plant develops from seed to the cured leaf (Leffingwell, 2001). These factors include genetic potential, environmental conditions, cultural practices and curing

methods. Interactions among the factors also influence the chemical composition and the position of the leaf on the plant as in the case of primings, lugs, leaves and tops. The genetic makeup of the plant provides the potential to produce or not produce certain compounds and the realization of these potentials depends on variables such as cultural, curing, and processing conditions (Leffingwell, 1999).

Much of the early breeding work in tobacco was directed toward disease resistance, yield, and other agronomic characters. Not until recently have geneticists begun to explore the possibility of altering certain physical and chemical characteristics of the tobacco leaf by breeding. Different tobaccos have been developed for each of these uses and are rarely interchangeable. Considerable differences in the chemical composition of the cured leaf of these classes are due to variations in heredity, environment, cultural practices and curing methods (Leffingwell, 1999). Chemically, burley tobacco is characterized by low reducing sugar levels, high nicotine, and high nitrate nitrogen levels, whereas flue-cured tobacco is characterized by high reducing sugar levels and moderate nicotine levels, besides certain flavour and aroma characteristics that are considered desirable in cigarette production (Jenkins, Tomkins & Guerin, 2000). Some investigations have been conducted to determine if the differences in chemical constituents among these tobaccos are due to the genetic makeup of the cultivars or to the environmental variations which they are subjected to (Bacsik, McGregor & Mink, 2007). Although some researchers have studied the mode of inheritance of certain chemical compounds, except for nicotine, very few have been reported to date. Variations in other chemical elements among cultivars and breeding lines have been observed and reported. When environmental and cultural conditions are constant and strain differences are found for a constituent, one can assume that these differences are genetic (Stedman, 1968).

Nicotine is principal tobacco alkaloid comprising about 95 % of total alkaloid content (Bierut, Stitzel, Wang, Hinrichs, Grucza, Xuei, & Horton, 2008). Nicotine is pale yellow to dark brown oily liquid with unpleasant pungent odour, sharp persistent bitter taste and soluble in alcohol, chloroform and among other solvents (Blakely & Bake, 1998).

Nicotine is recognized as an essential component of tobacco cultivation since it determines the quality of tobacco in the tobacco market. In small doses nicotine has a stimulating effect, increasing activity, alertness and memory. But repeat users report only relief from the symptoms of nicotine withdrawal. It also increases the heart rate and blood pressure and reduces appetite (Sanberg, Silver, Shytle, Philipp, Cahill, Fogelson, & Mc Conville, 1997). In large doses nicotine may cause vomiting and nausea and large doses are poisonous to most animals and humans. An aliquot of 40–60 mg can be a lethal dosage for adult human beings. This makes it an extremely deadly poison (Tonstad, Heggen, Giljam, Lagerbäck, Tønnesen, Wikingsson & Fagerström, 2013). It is more toxic than many other alkaloids such as cocaine with lethal dose of 1000 mg (Maaniity, 2018). The nicotine level of tobacco should be quantitatively determined before their distribution in the market to minimize its risk (Sorensen, 2012).

Determination of nicotine levels in tobacco is crucial to control, maintain or reduce nicotine levels below the addictive as well as harmful threshold. Consequently, the knowledge of nicotine levels in tobacco is especially important to tobacco industry and in the area of toxicology to control its harmful effect on health (Thomas & Stoddart, 1975).

2.5 Nicotine content in tobacco

Nicotine is the primary substance in cigarettes that causes addiction, but most experts agree that it does not directly cause cancer. Most research points to cigarette smoke, not nicotine, as being the primary contributor to among cancer smokers. Although most experts agree that nicotine does not directly cause cancer, it is believed that nicotine may lead to a type of DNA damage that increases the risk of cancer (Lacave-Garcia, Rey-Pino, Gallopel-Morvan, Moodie, Fernández & Nerín, 2018). Research into the role of nicotine in cancer is on-going. (Lian, Wang, Qiu, Zhang, Cao, Ning & Niu, 2008). Many studies, however, do not differentiate between nicotine, tobacco, or smoking when they discuss cancer risk. This makes it difficult to determine which of them causes cancer. Even if nicotine does cause or lead to cancer, the risks of developing cancer through the use of nicotine only products are much lower than the risks from smoking (Nagata, Mizoue, Tanaka, Tsuji, Wakai, Inoue & Tsugane, 2006).

Cigarette tar is a term used to describe the toxic chemical particles left behind by burning tobacco. This substance forms a tacky brown or yellow residue (WHO, 2003). Tobacco smoke contains over 7,000 substances (Leffingwell, 1999). The common ones originate in the tar produced by smoking cigarettes. Two hundred and fifty of those chemicals including carbon monoxide, ammonia, and hydrogen cyanide are acknowledged to be detrimental to smokers and people unprotected to second-hand smoke. Of those, 69 are known to cause cancer.

Nicotine is one of thousands of chemicals in tobacco. Tar which contributes more on cigarettes and chewing tobacco their flavour, are known carcinogens (Tiffany, Warthen, & Goedeker, 2008). The nicotine in e-cigarette liquid is removed from tobacco and mixed with a liquid base so it can be vaporized when heated. Since e-

cigarettes deliver nicotine without the tar and many of the other cancer-linked chemicals found in tobacco, they are thought to pose less of a cancer risk than cigarettes (Lacave-Garcia, *et al*., 2018).

2.6 Nicotine synthesis pathway

In plants, large quantities of anatomically diverse specialized metabolites are produced through long, multistep, and often branched pathways. The proper functioning of such pathways, allowing massive metabolic flows leading to complex products from simple precursors, largely relies on the expression of a large set of metabolic and transport genes, or structural genes, in different evolving and environmental contexts (Cárdenas, Sonawane, Pollier, Bossche, Dewangan, Weithorn & Giri, 2016). The transcription factors adaptable these pathways play a critical role in such coordination, which often occurs at the transcription level (Kajikawa, Sierro, Kawaguchi, Bakaher, Ivanov, Hashimoto, & Shoji, 2017). The regulatory transcription factors and downstream structural genes, which form regulatory networks of multiple genes, or regulons, have begun to be explored intensively through molecular and genomics studies (De Sutter, Vanderhaeghen, Tilleman, Lammertyn, Vanhoutte, Karimi & Hilson, 2005).

In tobacco, nicotine is an abundant major alkaloid produced in the roots and gathering mainly in the leaves. As a defence toxin, nicotine creation is radically increased in return to damage caused by grazing herbivores and jasmonates play a central signalling role in the damage induced nicotine biosynthesis (Meldau, Erb, & Baldwin, 2012).

Nicotine has heterocyclic pyridine and pyrrolidine rings the pyrrolidine ring is formed through consecutive reactions catalysed by Orn decarboxylase (ODC), putrescine N-methyltransferase (PMT) and N-methylputrescine oxidase (MPO) whereas enzymes involved in early steps of NAD synthesis, Asp oxidase (AO), quinolinate synthase (QS), and quinolinate phosphoribosyl transferase (QPT) are responsible for the formation of the pyridine ring (De Geyter, Gholami, Goormachtig, & Goossens, 2012). It has been proposed that PMT and MPO have evolved from spermidine synthase (SPDS) and diamine oxidase (DAO), two homologous enzymes with different catalytic activities, both of which accept putrescine as a substrate and thus are involved in polyamine metabolism (Penn & Weybrew, 1958).

In tobacco roots, a pair of tonoplast-localized multidrug and toxic compound extrusion (MATE) family transporters, MATE1 and MATE2, mediate vacuolar sequestration of nicotine, whereas nicotine and vitamin B6, both with a pyridine ring, are imported into the cells by a purine permease-like transporter, nicotine uptake permease 1 (NUP1), localized at plasma membranes (De Boer, Tilleman, Pauwels, Vanden Bossche, De Sutter *et al*., 2011).

2.7 Reducing sugar levels in tobacco

The level of reducing sugars is very important in determining the quality of flue-cured tobacco. A certain level is required for the tobacco to be acceptable and usable in cigarette blends. Very little reducing sugar are in the cured leaf of any of the air-cured types. The difference in sugar content between burley and flue-cured tobacco is due to cultural and growing conditions (Wingler, & Roitsch, 2008). Genetic factors also contribute to observed differences. Flue-cured cultivars vary in reducing sugar

levels and these range from 11.76 % to 16.67 % in many flue-cured cultivars (Van, Toubart, Cousson, Darvill, Gollin, Chelf & Albersheim, 1985). In a study of eight-cultivars of flue cured tobacco there was genetic variation for levels of reducing sugar existed among other cultivar of flue-cured tobacco and that the genetic variance resulted from additive gene action (Sheen, Zhou & Jang 1999).

The conversion of starch to sugar during curing is of major importance in tobacco production (Finch, 2018). The reaction is mainly catalysed by the enzyme amylase which functions optimally from 49 to 60^{o}C, but may remain active up to 70^{o}C (Smeekens, 1998). Most of the starch in the leaf is hydrolysed in the first 20 to 60 hours of curing. In the same period reducing sugars may increase by 60 -130 per cent (Smeekens & Rook, 1997). The sugar to starch ratio increases throughout curing and it is shown to increase more rapidly during the drying phase because the reduction in respiration has less demand on total sugar along with continued starch hydrolysis. Most cured leaves have reducing sugar concentrations of up to 10- 20 per cent or less of leaf dry matter (Sheen *et al*., 1999).

Sucrose has been found to increase during curing but the rate is much slower than for reducing sugar. The sucrose which is s disaccharide apparently results from fructose which arises through starch hydrolysis (Pego, Kortstee, Huijser, & Smeekens, 2000). It was reported that levels of between 4 and 8 per cent in the cured leaf. The conversion of starch to sugar is complete when chlorophyll degradation is finished. Reducing sugar in the lamina increase during the early hours of yellowing and declines during the late hours of yellowing and during drying (Herbers, Takahata, Melzer, Mock, Hajirezaei & Sonnewald, 2000). A marked decline in sugar occurs if the yellowing period is extended well beyond full colour. One of the causes of a

decrease in yield of tobacco is when not dried at the correct developmental stage (Bürkle, Hibberd, Quick, Kühn, Hirner & Frommer, 1998).

2.8 Ripeness in tobacco

Senescence may be defined as those changes which sooner or later lead to the death of an organism or part of it (Nooden, 1984). Ageing, by contrast refers basically to the changes that happen naturally which occur with time, without reference to death as a consequence and its use need not be confined to living organisms. The ripening period can vary from about 6 to 20 weeks depending of growing region, climate, rainfall, soil texture, soil structure, management on fertilizers, weeding, topping height and variety. Ethylene production in seedlings is a possible way of differentiating the rate of ripening of cultivars (Cavlek & Grsic, 2008).

Ripening is slower under high nitrogen fertilization and moisture stress conditions and high nitrogen has a negative effect on curing (Dhindsa, Plumb-Dhindsa & Thorpe, 1981). A leaf is considered to be ripe when its colour changes from dark green to pale yellow- green (Finch, 2018). The colour change is very subjective and addicting to confusion within the farmers and growers have to learn by experience (Suggs, 1986). The rate of ripening is also, to a large extent, influenced the growing region with some parts of Zimbabwe being classified as fast growing, others being medium growing and others being slow growing regions as shown in Figure 1.

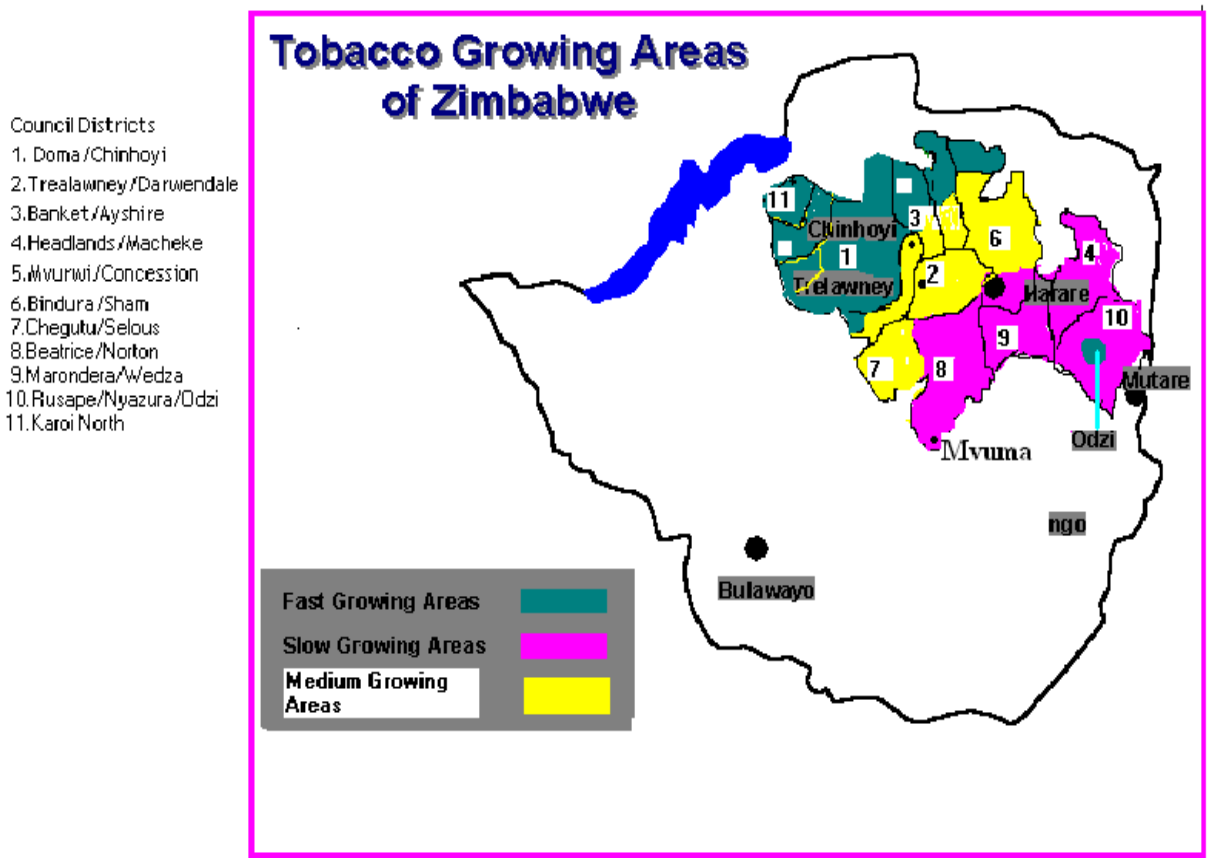

Figure 1. Tobacco growing areas of Zimbabwe

2.9 Characteristics of Kutsaga Root Knot 26 R (KRK 26 R) variety

KRK 26 R has a wider top leaf than the other K326 hybrid and K40. It has fairly short internodes and paler and less vigorous in appearance than KRK 6. The variety can be grown without fumigation or with reduced rate of nematicides. In the second and third years of growing on the same piece of land, it is greatly affected by nematodes and hence will require fumigation. The ripening rate of this variety is medium in slow growing areas and is similar to KM 10. It seems to have a consistent ripening rate which does not vary much from season to season. KRK 26 R is not as slow-ripening as K35 (TRB, 2010).

The variety is easy to reap and cure and can hang on to some extent when ripe. Guinea fowl spotting can be minimised by colouring at a higher temperature and high humidity to avoid setting green. It is prone to guinea fowl spot in the lower reaping especially if the leaf is thin. Excessive barn spot, caused by incipient frog eye can be minimised by the same curing regime. It is resistant to White mould, Wildfire, Angular race 1, Alternaria, Granville wilt and Black shank and susceptible to Wild fire race 2, Angular race 2 and Frogeye (TRB, 2010).

2.10 Characteristics of T 76 variety

The variety T76 is a tall plant with very close internodes giving a compact appearance. The leaves are very long, wide and light green in colour giving it a rather pale looking appearance even when well fertilized. On disease resistance it has a wide range such as Wild fire 0 and 1, White mould, Black shank, Alternaria, Tobacco mosaic virus, Angular leaf spot and has a medium resistance to root knot nematodes which is similar to that of RKR 26 R. It is a medium ripening variety arising from its very high resistance to Alternaria leaf spot. It has moderate holding capacity and will allow for increased body of top leaves. The pale colour of leaves can be misjudged for ripening especially under situations of under fertilization. Due to its pale colour it may be tempting to reap earlier than necessary. T 76 gives predominantly lemon cures and may produce deep lemon styles at the top if over fertilized and topped lower (TRB, not dated).

2.11 Differences between the two varieties

Variety selection is an imperative judgment for high production of flue cured tobacco. A high potential yield is probably more important than ever before due to

reduced operating margins. Nevertheless, ease of curing and specific characteristics of the cured leaf would likewise be important. Varieties will vary in cured leaf colour and other physical features wanted by buyers but these factors are also prejudiced by growing environments and curing practices. Growers ought carefully to consider any affected change in varieties grown without first trying a different variety on a limited acreage. T 76 is a new variety that is one of the best on the market which is giving the best and largest leaf in Zimbabwe. KRK 26 R is the most popular variety out of all and it gives the less challenges especially to learners. Its best merit is to be used as the first crop during the season and followed by slow growing and slow ripening varieties.

The disease resistance of varieties varies greatly and is critical to profitable production. Tobacco breeders have made tremendous progress in recent years developing resistance to the major diseases of flue-cured tobacco. It is especially important that growers have a correct identification of any diseases that may be causing field losses. Black shank, Granville wilt, and Pythium stalk rot may be confused and the presence of nematodes can make these and other root diseases more severe than expected or symptoms may not appear as expected. To further complicate matters, there have been isolated cases of less common root diseases that are not typically evaluated for in typical variety tests. If past performance of a disease resistant variety has been less than anticipated, growers are encouraged to contact local agriculture extension agent to investigate possible explanations and evaluate options. Proper identification of disease losses is essential to making the proper variety decision for the following season (TRB, not dated).

2.12 Maturity indices

The first prime characteristic of maturity indices is days to maturity and the period differs according to growing area (Gao, Deng, Zhou, Zhou, & Zeng, 2008). The other contributing factor is the ripening rate.

2.12.1 List of indices

The following are some of the tobacco maturity indices that separates a mature leaf from immature or false ripening

a) Leaf colour changes if the crop has no excess nitrogen the ripe leaf will be pale in colour compared to the one immediately above it (Weybrew, Woltz & Monroe, 1984).
b) The leaf surface shines as the leaf surface becomes dense and compacted with accumulated starch the surface will be dull.
c) Split open of the butt: if the lamina bends over away from its face the portion of the lamina near the butt will split open due to its thickness (Jian- Lin, 2010)
d) Dangle angle: with the accumulation of starch there is an increase in leaf weight and the leaf drops thereby increasing the angle of the midrib to the stalk.
e) Tip drying: generally the tip of the leaves dries off or picking up a very pale yellow (Lian, Wang, Qiu, Zhang, Cao, Ning, & Niu, 2008).
f) Midrib: the midrib changes colour to pale from dark green.
g) Hair: The hairs of the infant stage shade off or disappear.

h) Sticky ability: the leaves became so sticky when toughed that shows the accumulation of nicotine within the leaf.

i) Drawer test: i) reaping the bottom leaf of the plant.

ii) The leaf is placed in a drawer where there is no light, limited of no air movement and limited space (TRB, 2010).

iii) Then after 36-48 hours leaves should show 50% colouring if they are ready for reaping.

The maturity indices pose a challenge to farmers in the small scale tobacco production sector as many of them rely on qualitative visual experience to determine leaf ripeness rather than quantitative techniques to determine when to harvest leaves. In tobacco agronomy, as more flushes of ripening leaves come in from the fields, there is pressure on the curing barns and more leaves may become overripe in the field. Such a scenario causes challenges to small scale growers as the cured leaf quality is subsequently reduced. Several small scale growers also do not fully comprehend the consequences of harvesting either unripe or over-ripe leaves and hence this study was meant to save as a learning curve for the small scale growers to note that indeed there are penalties on tobacco quality when leaves are harvested at the inappropriate level of ripeness.

Lessons drawn from this study will go a long way in improving the quality of the crop and will be shared in meetings during the Tobacco Improved Productivity Scheme (TIPS) field discussions with farmers.

2.13 Summary

Fully mature leaves cure easily, and the quality, colour and weight are usually good. The best quality cures occur when the tobacco is allowed to mature in the field. The

stages of maturity are: premature, mature, ripe and overripe. Tobacco harvested in the ripe stage may be cured to give better colour, quality and weight than tobacco harvested in the overripe stage. Overripe tobacco does not colour, yield or sell as well as tobacco harvested and cured at proper maturation. The tobacco must mature but not become too ripe before removing leaves from the stalk.Tar is a sticky, partially combusted, particulate substance produced from the burning of tobacco during smoking. Tar is a toxic substance that damages the smoker's lungs over time through various biochemical and mechanical processes. Tar also damages the mouth by decomposing and blackening teeth, damaging the gums and desensitizing taste buds. In tobacco products, smoke tar contains most of the toxic, mutagenic and carcinogenic agents. Tar typically refers to the total aerosol residue. As it enters the lungs, tar coats the cilia and causes them to stop working and eventually die. Once gone, the cilia no longer trap toxic particles and the poisons can enter the alveoli directly, leading to lung cancer. Very less is said about tar but tar is the major culprit as far a healthy is concerned. It is nicotine that is said much of it. Nicotine is a very toxic substance, and serious or fatal poisoning may occur as a result of its ingestion, inhalation or skin absorption in small amounts.

CHAPTER 3 METHODOLOGY

3.1 Introduction

Comparison of nicotine levels from unripe, ripe and overripe and reducing sugar levels of two different tobacco flue cured varieties KRK 26 R and T 76. Ordinary nicotine methanol HPLC grade phosphoric acid and triethylamine were used. A phosphate buffer (pH) was prepared from potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate. All the samples were done in laboratory at Tobacco Research Board (TRB) Kutsaga, Harare, Zimbabwe.

3.2 Growing of the study crops

Six small scale tobacco growers in the Kubatana Community of Mutare district were selected under the TIPS training programme to participate in the study. Soil testing for the six farmers was done to determine the pH and nutrients status of the soils to ascertain the most effective fertilization program for each farmer. The soil samples were collected and sent to TRB labs for analysis. Soil samples from the six fields fell under the desired pH range for flue cured tobacco at between pH 5.5 to 6.5 and no liming was required.

For all the fields, land preparation was done in May 2018. Deep ploughing was done using a tractor drawn plough, followed by discing. Special considerations were used to guide the grower during variety selection and factors considered included; season length and estimated amount of rainfall as drought was being predicted on the first and last quarter of the summer season of 2018/2019 in Zimbabwe.

The seedlings were raised in the float beds and were sown on the same date 1 September 2018. The seed were pelleted and film coated with the aphicide silver nitrate. Clipping was done twice a week during the six weeks of seedlings growth. The ridging was done on the first week of November 2018 and basal dressing was applied differently guided by soil sample analysis reports.

Ridging was done before the first rains and basal dressing was done on the same day to insure effective fertilizer placement.

The fertilizer rates of compound C used per crop were based on the recommendations for the crop from soil analysis results and were as follows;

FARM A.: The fertilizer rates used were 30 kg N/ha, 90 kg P_2O_5/ha 52.5 kg K_2O/ha) and sulphate of potash at 10 kg ha.

Tobacco received top-dressing of 51.75 kg N/ha, at 4 weeks and at 8 weeks 28 calcium nitrate.

FARM B.: Fertilizer rates used were 30 kg N /ha, 90 kg P_2O_5/ha, 85 kg K_2O/ha and 15 kg/ha sulphate of potash.

Tobacco received top-dressing of cup 5 per plant at 4 weeks and cup 8 per plant of calcium nitrate at topping.

FARM C: The fertilizer rates used were 30 kg N/ha, 90 kg P_2O_5/ha 52.5 kg K_2O/ha sulphate of potash/10 kg ha and 43 kg of gypsum.

Tobacco received top-dressing of cup 5 per plant at 4 weeks and cup 8 per plant of calcium nitrate at topping.

FARM D: The fertilizer rates used were 25 kg N/ha, 70 kg P_2O_5/ha 52.5 kg K_2O/ha, sulphate of potash/10 kg ha and 43 kg of gypsum.

Tobacco received top-dressing of cup 5 per plant at 4 weeks and cup 8 per plant of potassium nitrate at topping.

FARM E.:

The fertilizer rates used were 50 kg N/ha, 150 kg P_2O_5/ha, 120 kg K_2O/ha).

Tobacco received top-dressing of cup 5 per plant at 4 weeks and cup 8 of calcium nitrate at topping.

FARM F.:

The fertilizer rates used were 35 kg N/ha, 110 kg P_2O_5/ha, 52.5 kg K_2O/ha and sulphate of potash at10 kg ha.

Tobacco received top-dressing of cup 5 per plant at 4 weeks and cup 8 of calcium nitrate at topping.

Transplanting was done on the 15 November after receiving the first rains. The establishment rate of transplants was satisfactory and cutworm control was done soon after transplanting. No gap filling was done and at 4 weeks after transplanting the Ammonium Nitrate and sulphate of potash was applied with and routine application of insecticides and fungicides were applied.

The topping height of 18 leaves per plant was maintained throughout. The exercise was done over the period of seven days. De suckering was done for two weeks and the use of suckercide such as N decanol and accotab. The reaping commenced at 10 weeks to pick the unripe leaves and 12 and 14 weeks for ripe and overripe respectively.

3.3. Sampling procedure

The fields were organised according to varietal selection and good management practices such as dates of transplanting and weeding and pest and disease management. The multistage sampling procedure was used to come up with the participants. From group of 28 growers only six growers were randomly selected and three fields with one particular variety KRK 26 R and the other three with a different

variety as well which was T 76. Six growers were selected basing on participation and attendance to (TIPS) Tobacco improved productivity scheme discussion.

3.4. Sample collection

Five leaves per each stage of unripe, ripe and overripe were collected from every grower same leaf position of primes were maintained and collected at the same time throughout the area of Kubatana community in Mutare district. The growers were six and the field distance was approximately 2 km from each grower for those with KRK 26 R and a distance of 600 m radius for the other three growers with T 76.

Flue cured tobacco samples were collected three times. The leave position of tobacco was priming and maintained at all three stages namely unripe leaves, ripe leaves and overripe leaves. 5 leaves were randomly picked per grower from 6 growers to give a total of 30 leaves per stage. Thus a total of 90 leaves from the six selected sample sites.

3.5 Nicotine levels determination at TRB laboratory

The nicotine in the tobacco leaves was analysed using the method reported by Saunders & Blume 1981. An isocratic mobile phase of 40 % (v/v) methanol holding 0.2 % phosphoric acid buffered to pH 7.25 with triethylamine and flow rate of 1 mL/min was used. The buffered mobile phase was primed once for the whole analysis with pronounced caution by regulating the intended pH value. Moreover the mobile phase remained used after filtration through a 0.45 μm cellulose filter and then 5 min sonication. The chamber temperature was used as a column temperature for this effort.

The UV/Vis detector was adjusted to detect nicotine at its maximum wavelength absorbance of 259 nm. Then the mobile phase was allowed to flow through the

column or the line of flow of the instrument for some minutes until stable absorbance of the mobile phase observed. After observation of stable absorbance of the mobile phase using the instrument software was set to out-zero, and the automatic injector was obeyed by the software program to inject automatically 10 µL of sample from the septum vials. As a result the nicotine peak appeared at about 7.3 min at a flow rate of 1 mL/min.

The UV spectrum of 1 nm nicotine was recorded and the spectrum showed nicotine at 236, 259 and 282 nm. To select the preferred nicotine for the analysis, 10 µL of 1 nm standard nicotine was injected to HPLC and the peak area at each of the above four wavelengths was recorded separately. The response peak area of nicotine at 259 nm was the largest. Hence 259 nm was selected for detection and quantification of nicotine. The chromatogram of standard nicotine was run at the optimum experimental conditions described above. The nicotine peak appeared at about 7.3 min.

All numerical fortitudes were complete with triplicate jabs. The extraction was done in triplicate for each leaf sample. The content of nicotine of respective sample was then quantitatively determined based on the external calibration curve Saunders & Blume 1981. The method is based on steam distillation of ground tobacco under strong basic conditions. The distillate collected is measured spectrophotometrically at three wavelengths of 236, 259 and 282 nm. Nicotine absorbs more strongly at 259 nm. Measurements at 236 and 282 nm are taken to correct for interferences.

3.6. Determination of reducing sugar levels at TRB laboratory.

The spectrophotometric determination of reducing sugar in the tobacco leaves was done by employing blue tetrazolium chloride as a colour developing reagent. The maximum absorption of formazan dye which is the product of the reaction between blue tetrazolium chloride and fructose was shown to be 530 nm. In the fructose concentration range of 0.02 mg/ml-0.14 mg/ml the calibration curve matched well to the law of Beer-Lambert. In order to take a look at the accuracy and/or recovery rate of fructose determination, the standard fructose was added to the tobacco leaves and the concentration of this standard fructose was estimated. A slightly lower concentration of the standard fructose compared with the pure one in solution was observed. However, an excellent analytical recovery was revealed under the-2% of relative error limit. When the quantitative determination of reducing sugar by the visual read-out (without using the spectrophotometer) method was done, the relative error obtained +- 10%

3.7. Analysis and Organisation of Data

The data was analysed using Minitab version 13 statistical package. Comparisons of means of nicotine and reducing sugar levels at the three stages ripeness namely; unripe, ripe and overripe were done using the paired T test to see if there were any significant differences in the levels of the nicotine and reducing sugar levels within each variety and between the two varieties.

3.8 Ethical Consideration

The ethical guidelines were followed when collecting the data and all data were treated confidentially, which was explained to the growers together with the aims of the study. Growers were free to fill in their names on the forms at the laboratory. The section revealing individuals names could be handed in separately from the document in responses to ensure anonymity. The dignity and wellbeing of growers were protected at all times as the fields, plants were also used.

3.9 Summary

The used analytical methodology for the determination of reducing sugars in extracts by UV-Visible spectrophotometry and the recovery test of the method of analysis used in this study was assessed by simultaneous extraction and determination of nicotine in the unspiked and spiked powdered tobacco leaves with 1.00 mM standard nicotine solution fulfilled the requirements to consider it validated, proving to be a specific, linear, accurate, precise and robust method against possible variations in the conditions of the method except Ph.

CHAPTER 4 DATA PRESENTATION, INTERPRETATION AND DISCUSSION

4.1 Introduction

The means of nicotine and reducing sugar levels of both varieties KRK 26 R and T 76 was obtained separately. The means nicotine and reducing sugars levels of KRK 26 R on all leaf developmental stages such as unripe, ripe and overripe were obtained. On T 76 nicotine and reducing sugar levels were obtained on unripe, ripe and unripe. The 5% level of significance was used. NS = No significance at 5% level of significance and *= significance difference at the 5% level of significance.

4.2 The means of nicotine level on both varieties

The nicotine levels of KRK 26 R and T 76 of the unripe leaves showed no significant difference. Varity KRK 26 R had a mean of 2.633 and T 76 with had a mean of 2.78 and the difference of -0.147 was not significantly different at the 5% level of significance (Table 1). There were significant differences ($P<0.05$) in nicotine levels for the ripe leaves of both varieties with a mean of 3.87 for KRK 26 R and a mean of 3.223 for T 76 (Table 1).

The leaves for overripe stage on both varieties of KRK 26 R and T 76 on nicotine levels had slightly higher means than all, with the following values of 4.477 and 4.273 respectively. With the average difference of 0.203 there was no significant difference between the means at the 5% level of significance (Table 1).

From the nicotine level perspective of three tobacco leaves developmental stages KRK 26 R had high means on ripe and overripe and T 76 had high mean on the unripe stage both at the end all means had no significant difference at the 5% level of significance (Table 1).

Table 1. Means of nicotine levels of KRK 26 R and T 76 of unripe, ripe and overripe tobacco leaves at Kubatana community Mutare

	Nicotine level %		
Variety	**unripe**	**ripe**	**overripe**
KRK 26 R	2.633	3.870b	4.477

T 76	2.78	3.223a	4.273
Diff	-0.147	0.647	0.203
value	0.657	0.04	0.203
Significance	N.S.	*	N.S.

Means followed by different letters in a column are significantly different from each other.

*= Significant difference at the 5% level of significance.

N.S. = No significant difference at the 5% level of significance.

4.3. Means of reducing sugar levels of both varieties

The reducing sugar levels of KRK 26 R and T 76 of the unripe leaves had means of 13.39% and 17.84% and a mean difference of -4.45 and these were not significantly different from each other at the 5% level of significance (Table 2).

Leaves from the ripe developmental stage of both varieties had means for KRK 26 R at 14.567% and for T 76 at 17.103% and the mean difference of -2.54 was not significantly different at the 5% level of significance (Table 2).

The overripe leaves of both varieties had a mean of 7.57% for KRK 26 R and a mean of 8.893% for T 76 and difference of -1.32 was not significantly different at the 5%

level of significance (Table 2). Even though there were no significant differences in reducing sugar levels for the two varieties at each of the three levels of leaf ripeness, the reducing sugar levels for variety T 76 were higher than the levels for variety KRK26 R (Table 2).

Table 2. Means of reducing sugar levels of KRK 26 R and T 76 of unripe, ripe and overripe tobacco leaves at Kubatana community Mutare

	Reducing sugar levels %		
Variety	**unripe**	**ripe**	**overripe**
KRK 26 R	13.39	14.567	7.57
T 76	17.84	17.103	8.893

Diff	-4.45	-2.54	-1.32
P value	0.189	0.134	0.50
Significance	N.S.	N.S.	N.S.

NS = No significant difference at the 5 % level of significance.

4.4. Nicotine levels for KRK 26 R on unripe and ripe developmental stages

The nicotine content levels for the unripe and ripe leaves for variety KRK26 were significantly different from each other at the 5% level of significance with means of 2.633% and 3.87% respectively (Table 3). The unripe and overripe nicotine content means for KRK26 R were 2.633%% and 4.477 respectively and the difference of -1.843 was significantly different at the 5% level of significance (Table 3). The ripe and overripe means for the nicotine levels were 3.87% and 4.477% with a difference of -0.607 which was not significantly different at the 5% level of significance (Table 3).

Table 3. Means of nicotine levels of KRK 26 R of unripe, ripe and overripe tobacco leaves grown at Kubatana community Mutare

	Nicotine level %				
	KRK 26 R		**KRK 26 R**		**KRK 26 R**
Unripe	2.633a	unripe	2.633a	ripe	3.870
Ripe	3.870b	overripe	4.477b	overripe	4.477

Diff	-1. 227	-1.843	-0.607
P value	0.018	0.031	0.301
Significance	*	*	N.S.

Means followed by different letters in a column are significantly different from each other
*= significant difference at the 5% level of significance.
N.S. = No significant difference at the 5 % level of significance.

4.5. Reducing sugar levels for KRK 26 R leaf ripeness stages

The unripe and ripe means for the reducing sugar levels for KRK26R were 13.39 and 14.567 respectively with a difference of -1.18 which was not significantly different at the 5% level of significance (Table 4). The unripe and overripe reducing content means for the variety were 13.39% and 7.57% respectively and difference of 5.820 was significantly different at the 5% level of significance (Table 4). The ripe and overripe reducing sugar levels for KRK26R had means of 14.567% and 7.57% respectively and the difference of 7.0 was significantly different at the 5% level of significance (Table 4).

Table 4. Means of reducing sugar level of KRK 26 R of unripe, ripe and overripe tobacco leaves grown at Kubatana community Mutare

	Reducing sugar levels %		
	KRK 26 R	**KRK 26 R**	**KRK 26 R**

Unripe	13.39	unripe	13.39b	ripe	14.567b
Ripe	14.567	overripe	7.57a	overripe	7.57a
Diff	-0.18		5.82		7.00
P value	0.495		0.002		0.033
Significance	N.S.		*		*

Means followed by different letters in a column are significantly different from each other

*= significant difference at the 5% level of significance

NS = No significant difference at the 5% level of significance

4.6. Nicotine levels for T 76 ripeness stages

The nicotine level means for the unripe and ripe leaf stages of the variety T76 were 2.78% and 3.223% respectively and the mean difference of -0.443 was not significantly different at the 5% level of significance (Table 5). The unripe and overripe nicotine content means for the variety T76 were 2.78% and 4.273% respectively and difference of -1.493 was not significantly different at the 5% level of significance (Table 5). The ripe and overripe means for the nicotine levels were 3.22% and 4.273% with a difference of -1.05 which was not significantly different at the 5% level of significance (Table 5).

Table 5. Means of nicotine levels T 76 of unripe, ripe and overripe of tobacco leaves grown at Kubatana community Mutare

	Nicotine levels %

	T 76		T 76		T 76
Unripe	2.78	unripe	2.78	ripe	3.223
Ripe	3.223	overripe	4.273	overripe	4.273
Diff	-0.443		-1.493		-1.0
P value	0.514		0.066		0.064
Significance	N.S.		N.S.		N.S.

N.S. = No significant difference at the 5% level of significance

4.7. Reducing sugar levels for T 76 leaf ripeness stages

The unripe and ripe means for the reducing sugar levels were 17.84% and 17.10% respectively and the difference of 0.73 was not significant at the 5% level of significance (Table 6). The means of reducing sugar levels for the unripe and overripe leaves on variety T 76 had values of 17.84% and 8.89% respectively and difference of 8.94 was significant at the 5% level of significance (Table 6). The means of reducing sugar levels for the ripe and overripe leaves of T76 were 17.103% and 8.893% respectively and the difference of 8.21 was significant at the 5% level of significance (Table 6).

Table 6. Means of reducing sugar levels T 76 of unripe, ripe and overripe tobacco leaves grown at Kubatana community Mutare

	Reducing sugar levels %				
	T 76		**T 76**		**T 76**
Unripe	17.84	unripe	17.84b	ripe	17.103b
Ripe	17.103	overripe	8.893a	overripe	8.893a
Diff	0.73		8.94		8.21
P value	0.605		0.022		<0.001
Significance	N.S.		*		*

Means followed by different letters in a column are significantly different from each other
*= significant difference at the 5% level of significance.
N.S. = No significant difference at the 5% level of significance.

4.8. The accepted threshold of nicotine levels in tobacco.

Nicotine constitutes approximately 0.6–3.0% of the dry weight of tobacco. Usually consistent concentrations of nicotine varying from 2–7 per cent wet weight are found in the tobacco. For both varieties the nicotine levels were within the acceptable 2-5 % range for the unripe, ripe and overripe leaves (Table 7).

Table 7. Accepted threshold of nicotine levels of KRK 26 R and T 76 of unripe, ripe and overripe tobacco leaves at Kubatana community Mutare

		Nicotine levels (%) detected		
	Accepted%	**Unripe**	**ripe**	**overripe**
KRK 26 R	2-5%	2.633	3.87	4.477
T 76	2-5%	2.78	3.223	4.273

4.9. The accepted threshold of reducing sugar levels in tobacco.

Carbohydrates are important biochemical elements of tobacco. Generally, cumulative the carbohydrate meditation does not lead to further improvements in quality unless accompanied by the harmonious interaction of other components. For example, sugars, which most sources indicate have a positive correlation with quality and taste, appear in higher concentrations in desirable leaves, but they do not imply high quality unless enough waxes and resins are present. Sugars often must be added to tobacco to balance acidic and alkaline substances, however too much sugar can lead to a biting or pungent smoke. In other words, there can be too high as well as too low a level of carbohydrates. Flue-cured cultivars vary in reducing sugar levels showed that reducing sugar levels ranged from 11.76 % to 16.67 % many flue-cured cultivars. It means both KRK 26 R and T 76 have acceptable reducing sugar levels at the ripe stage but other two stages have reducing sugar levels that are far below the acceptable reducing sugar threshold (Table 8).

Table 8. Accepted threshold of reducing sugar levels of KRK 26 R and T 76 of unripe, ripe and overripe of tobacco leaves at Kubatana community Mutare

		Reducing sugar levels (%) detected		
	Accepted%	**Unripe**	**ripe**	**overripe**
KRK 26 R	15-30%	13.39	14.567	7.57
T 76	15-30%	12.84	17.103	8.893

4.10 Discussion and Interpretation

Observations on the KRK 26 R three leaf ripeness stages have shown that the nicotine level increased by age as the unripe stage had the lowest level, followed by the ripe and the overripe with the highest content of nicotine as shown in Table 1. Despite having the lowest means of all but tracking back to the source of information the nicotine percentage differs from farmer to farmer despite having the same parent material and soil type which are ferralsols. Even on variety T 76 the nicotine content followed the pattern with the unripe leaves having the lowest means. This outcome can be attributed to the fact that the two varieties are from different parents and their genetic make-up is different as KRK 26 is a medium ripening variety and T 76 is a medium maturity to medium to slow ripening rate variety.

Looking at the means of the ripe stages of the two distinct varieties the nicotine levels were different and KRK 26 R had a high mean as compared to T 76 but on the unripe stage T 76 had the high mean. It is believed that the nicotine concentration in tobacco is closely correlated with the amount of nitrogen (N) supplied. However the high means from the KRK 26 R can be as a result of time when the variety was bred. The variety KRK 26 R is older than the variety and T 76 which is a new variety on the market and that why it is still on limited release. The older generations of breeders were looking more on attributes such as disease resistance than low nicotine content.

For the overripe leaves, the variety KRK 26 R was dominant on the nicotine content than its counterpart despite the fact that statistically there is no significant difference. This can be as a result of genetic make-up. It was recorded earlier on that nicotine is manufactured in the roots and an additional increase of the nicotine concentration is

obtained by removal of axillary buds. The wounding caused by routine leaf harvests, however, did not change the leaf nicotine concentration, and neither did reducing leaf harvest times (Leffingwell, 2001). On variety T 76 the nicotine content increased from 3.223% to 4. 273% on overripe stage despite being lower than that of variety KRK 26 R. It is thus also implied that the nicotine concentration increases with time and age as it is also manufactured in the roots.

The reducing sugars concentration on unripe stages of KRK 26 R was lower than the content in variety T 76 even though statistically there was no significant difference. This can be as a result of the contrast between new and old varieties as the new crop of breeders were concentrating more on increase of reducing sugar levels as it line with good and desirable quality. Increasing the carbohydrate concentration does not lead to further improvements in quality unless accompanied by the harmonious interaction of other components. For example, sugars, which most sources indicate have a positive correlation with quality and taste, appear in higher concentrations in desirable leaves, but they do not imply high quality unless enough waxes and resins are present (TRB, 2010).

The ripe stage from both varieties showed that the leaves had accumulated more of the reducing sugar levels that any other developmental stage. This could had be attributed to the high accumulation of carbohydrates. The soluble sugars not only serve as nutrients, but also act as signals for plant growth and development, but how sugar signals are perceived and translated into physiological responses in plants remains unclear (Akehurst, 1981). This can be as a result of the stage at which the plant requires more energy.

On the reducing sugars levels at the overripe leaf stages on all varieties, the sugars dropped drastically with close to 50% of the ripe and unripe stage. This can be as a result of the fact that plant growth is a highly energy-demanding process that requires optimal sugar balance particularly that of sucrose, between photosynthetic and non-photosynthetic cells. At this stage both senescence and ageing will be setting in. Senescence may be defined as those changes which sooner or later lead to the death of an organism or part of it (Noodén, 1984) while ageing, by contrast refers to basically to the changes that happen naturally which occur in the time without reference to death as a consequence.

4.11. Nicotine levels on leaf ripeness stages of KRK 26 R

With the same variety two leaf ripeness stages showed significant difference and this could have been due to age of leaves and translocation of photosynthesis toward the pre development of seed due to decapitation of the apical bud. All nutrients will be channeled to the leaves versus the unripe stage where assimilates will be channeled toward vegetative growth.

However, it cannot be postulated that the two leaf ripeness stages compete with each other for photosynthates as there is another stage in between. From literature, it is known that nicotine is produced in the roots and it increases with wounding exercises such as de suckering (Akehurst, 1981).

During the growth stage the major sink was the leaf and which contains half or more of the whole plant. However it appears that the effect of the ripe and the overripe on nicotine does not differ much in magnitude between the two leaf ripeness stages because of the similarity of assimilates distribution.

4.11.1 Reducing sugar levels on leaf ripeness stages of KRK 26 R

In the present study, the distribution of assimilate between the unripe and ripe showed no significant difference but the ripe stage had the higher reducing sugar levels and this is as a reason of the accumulation of more carbohydrate as the leaves grow more to the ripe stage. The unripe stage comes with very low reducing sugar levels as the leaves have more of the chlorophyll and distribute assimilates to the growing apical tip in preparation of seed development. The ripe stage comes after decapitation of the bud topping and the plant will be channeling all assimilates to leaves.

The unripe stage showed more reducing sugar levels than overripe and the was because the unripe stage building more of the energy for plant growth and the plant will be at the peak of its photosynthetic processes. The overripe stage had very low levels of the reducing sugar as shown in Table 4 and this was as a result of senescence. The results suggest that the ripe stage increased in the exportation of photosynthates to the ripe leaves and the overripe leaves came with very low of the reducing sugar and this was as a result of senescence and use of the stored energy by old leaves.

4.11.2. Nicotine levels on leaf ripeness stages of T76

The unripe leaf stage had low nicotine content than the ripe stage and this is evident from the fact that more nicotine is produced by more wounding and the first stage of wounding takes place at the removal of the apical bud. With crop ageing and when

the roots are growing, coupled with mechanical damage during operations such as re-ridging there is likely to be more the nicotine that will be produced.

The unripe leaf stage had less nicotine content than the overripe leaf stage and the two stages had some significant difference. The older leaves accumulate more nicotine as nicotine is a defense mechanism of the plant. It is believed that nicotine precursors are produced at a very tender age of the plant and the distribution of nicotine will be more from the older to tender leaves. These findings suggest that the overripe stage had more nicotine content than the ripe stage of T 76. The above mentioned reasons of wounding and defense mechanism came to the show as a results in table 5 show the trend.

4.11.3 Reducing sugar levels on leaf ripeness stages of T 76

It has been postulated that since the unripe and the ripe stages are close to each other and they hand over and take over simultaneously and these two stages might compete with each other for photosynthates in carbohydrates accumulation and hence the unripe stage has more reducing sugar than ripe.

The unripe stage has more of the reducing sugars than overripe stage and the result came with significant difference. The overripe stage came up with very low reducing sugar and this is as a result of senescence stage advancement and the leaves were yellowing. The ripe stage had more of the reducing sugar than the overripe and the difference was significant. The overripe leaves were moving to a stage when they cannot photosynthesize and there is need to use all its stored energy before necrosis.

4.12 Summary

the distribution of assimilate between the unripe and ripe showed no significant difference but the ripe stage had the higher reducing sugar levels and this is as a reason of the accumulation of more carbohydrate as the leaves grow more to the ripe stage. The unripe stage comes with very low reducing sugar levels as the leaves have more of the chlorophyll and distribute assimilates to the growing apical tip in preparation of seed development. The unripe leaf stage had low nicotine content than the ripe stage and this is evident from the fact that more nicotine is produced by more wounding and the first stage of wounding takes place at the removal of the apical bud for T 76. The unripe stage showed more reducing sugar levels than overripe and the was because the unripe stage building more of the energy for plant growth and the plant will be at the peak of its photosynthetic processes for KRK 26 R. the means of the ripe stages of the two distinct varieties the nicotine levels were different and KRK 26 R had a high mean as compared to T 76 but on the unripe stage T 76 had the high mean. It is believed that the nicotine concentration in tobacco is closely correlated with the amount of nitrogen (N) supplied. However the high means from the KRK 26 R can be as a result of time when the variety was bred. The variety KRK 26 R is older than the variety and T 76 which is a new variety on the market and that why it is still on limited release. The older generations of breeders were looking more on attributes such as disease resistance than low nicotine content.

CHAPTER 5 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 Introduction

Tobacco is a valuable cash crop. It is the most widely grown non-food crop in the world with the leaf as the most valuable part of the plant. Thousands of small scale growers in Zimbabwe depend on the tobacco production for their livelihood. However, the scientific information available with regards to the response of unripe, ripe and overripe stage on nicotine and reducing sugar is very limited. Therefore, a field experiment was conducted at Kubatana rural community in Mutare district the province of Manicaland Zimbabwe, from October-2018 to March 2019 and the crop was rain fed.

5.2 Discussion

An experiment of the comparison of nicotine levels and reducing sugar levels from unripe, ripe and overripe on two different tobacco flue cured varieties was conducted at Kubatana community and Tobacco Research Board laboratory analysed the nicotine and reducing sugar levels. The varieties established were KRK 26 R and T 76 from six small scale growers and the growers were selected from the Tobacco Research Board TIPS participants.

From the perspective of nicotine levels, the three tobacco leaf ripeness stages on KRK 26 R had high means on ripe and overripe and T 76 had high means on the unripe stage but all the means had no significant difference.

On KRK 26 R the reducing sugar decreased from unripe to overripe and also for T76 but the ripe leaves had slightly higher levels than the ripe leaves. At the end, both varieties and all three leaf ripeness stages had no significant difference.

On KRK 26R leaf ripeness stages the nicotine levels on the unripe and ripe stages and unripe and overripe stages had significant differences, while the ripe and overripe levels had no significant difference. On the same variety on reducing sugars the unripe and overripe leaves had no significant difference while the unripe and overripe leaves, and the ripe and overripe leaves had some significant differences.

With T76 on unripe and ripe on nicotine level the two stages had no significant difference and unripe and overripe and ripe and overripe there had significant differences. However, on the same variety, the unripe and ripe leaf stages had no significant difference. The unripe and overripe stages and the ripe and overripe had stages had significant differences.

Overall, the nicotine levels for the two varieties were within the accepted threshold level of 2-5 % at all leaf ripeness stages. However, the reducing sugar levels for the unripe and the overripe stages for both varieties were content were outside the accepted levels of 15-30%. It was only the reducing sugar levels for the ripe leaf stage which were within the accepted range of 15-30%.

5.3 Conclusion

The results revealed that the nicotine level from unripe to overripe stage increase by age in preparation to defend the plant and reducing sugars start very high in the leaves and decrease as the plant grow older. Therefore, taking the findings of the present study into consideration, it may be concluded that the nicotine levels fall

under the accepted threshold for all the leaf ripeness stages. The reducing sugar levels were within the accepted range only for the ripe leaf stage. It can be concluded therefore that even though leaf ripeness stages had not impact on the nicotine content, there is significant impact on the reducing sugar levels when the leaves are either unripe or overripe. However the nicotine content increases at the decapitation of the apical part and the removal of suckers. The more the tobacco plant damaged the more it increases the nicotine and the reducing sugar decreases as plant maturity and at unripe stage. The highest levels of reducing sugars are found at the ripe stage only and that resample good quality tobacco.

5.4 Implications

The results obtained from this study imply harvesting tobacco leaves at the three leaf ripeness levels of unripe, ripe and overripe has no significant impact on the nicotine levels. This could be attributed to the fact that nicotine content is not highly influenced by agronomic practices but by the inherent capacity bred into each variety. The results further showed that the reducing sugar levels are influenced by the stage of leaf ripeness with only the ripe leaves meeting the accepted threshold level of between 15% and 30%. This implies that reducing sugar levels are influenced by agronomic practices and hence the need to standardize harvesting procedures and to put more emphasis on training of small scale tobacco growers on harvesting tobacco leaves at the proper leaf ripeness stage.

5.5 Recommendations

From the current study, the following recommendations are proposed;

1. Farmers need to be trained on determination of leaf ripeness in readiness for harvesting so that they harvest leaves at the ripe stage only as it has been shown in the study that harvesting leaves at the unripe and overripe stages does affect the reducing sugar levels of the tobacco leaves.
2. It is also recommended that small scale tobacco growers need to be assisted with adequate curing facilities so as to remove the impediment of limited curing capacity which in many cases leads farmers to harvest overripe tobacco leaves.

5.6 Suggestions for further research

1. It is necessary to redo the same experiment but with the addition of more type of tobacco such as the new tobacco type in Zimbabwe which is Cigar.

2. There is need to repeat the study it under high plant population of more than 15 000 plants per hectare.

3. The study should be done for two more seasons and more sites and with more of the Kutsaga new varieties on different soil types and growing environments.

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APPENDICES

Appendix 1 T test means for nicotine levels of KRK 26 R and T 76 unripe

	N	Mean	StDev	SE Mean
C1	3	2.633	0.372	0.215
C2	3	2.780	0.767	0.443
Diff	3	-0.147	0.491	0.284

95% CI for mean difference: (-1.367, 1.074)

T-Test of mean difference = 0 (vs not = 0): T-Value = -0.52 P-Value = 0.657

Appendix 2 T test means for nicotine levels of KRK 26 R and T 76 ripe

	N	Mean	StDev	SE Mean
C1	3	3.870	0.165	0.095
C2	3	3.223	0.240	0.138

Diff	3	0.647	0.230	0.133

95% CI for mean difference: (0.075, 1.219)

T-Test of mean difference = 0 (vs not = 0): T-Value = 4.86 P-Value = 0.040

Appendix 3 T test means for nicotine levels of KRK 26 R and T 76 overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	4.477	0.703	0.406
C2	3	4.273	0.265	0.153
Diff	3	0.203	0.535	0.309

95% CI for mean difference: (-1.126, 1.532)

T-Test of mean difference = 0 (vs not = 0): T-Value = 0.66 P-Value = 0.578

Appendix 4 T test means for reducing sugar levels of KRK 26 R and T 76 unripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	13.39	1.67	0.97
C2	3	17.84	2.78	1.60
Diff	3	-4.44	3.93	2.27

95% CI for mean difference: (-14.21, 5.32)

T-Test of mean difference = 0 (vs not = 0): T-Value = -1.96 P-Value = 0.189

Appendix 5 T test means for reducing sugar levels of KRK 26 R and T 76 ripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	14.567	1.296	0.748
C2	3	17.103	1.511	0.872
Diff	3	-2.54	1.79	1.03

95% CI for mean difference: (-6.99, 1.91)

T-Test of mean difference = 0 (vs not = 0): T-Value = -2.45 P-Value = 0.134

Appendix 6 T test means for reducing sugar levels of KRK 26 R AND T 76 overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	7.570	1.332	0.769
C2	3	8.893	1.484	0.857
Diff	3	-1.32	2.81	1.62

95% CI for mean difference: (-8.30, 5.65)

T-Test of mean difference = 0 (vs not = 0): T-Value = -0.82 P-Value = 0.500

Appendix 7 T test means for nicotine levels of KRK 26 R unripe and ripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	2.633	0.372	0.215
C2	3	3.870	0.165	0.095
Diff	3	-1.237	0.290	0.168

95% CI for mean difference: (-1.958, -0.516)

T-Test of mean difference = 0 (vs not = 0): T-Value = -7.38 P-Value = 0.018

Appendix 8 T test means for nicotine levels KRK 26 R unripe and overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	2.633	0.372	0.215
C2	3	4.477	0.703	0.406
Diff	3	-1.843	0.577	0.333

95% CI for mean difference: (-3.278, -0.409)

T-Test of mean difference = 0 (vs not = 0): T-Value = -5.53 P-Value = 0.031

Appendix 9 T test means for nicotine levels of KRK 26 R ripe and overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	3.870	0.165	0.095
C2	3	4.477	0.703	0.406
Diff	3	-0.607	0.759	0.438

95% CI for mean difference: (-2.493, 1.280)

T-Test of mean difference = 0 (vs not = 0): T-Value = -1.38 P-Value = 0.301

Appendix 10 T test means for reducing sugar levels of KRK 26 R unripe and ripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	13.390	1.676	0.968
C2	3	14.567	1.296	0.748
Diff	3	-1.18	2.46	1.42

95% CI for mean difference: (-7.29, 4.94)

T-Test of mean difference = 0 (vs not = 0): T-Value = -0.83 P-Value = 0.495

Appendix 11 T test means for reducing sugar levels KRK 26 R unripe and overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	13.390	1.676	0.968
C2	3	7.570	1.332	0.769
Diff	3	5.820	0.403	0.233

95% CI for mean difference: (4.819, 6.821)

T-Test of mean difference = 0 (vs not = 0): T-Value = 25.02 P-Value = 0.002

Appendix 12 T test means for reducing sugar levels of KRK 26 R ripe and overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	14.567	1.296	0.748
C2	3	7.570	1.332	0.769
Diff	3	7.00	2.27	1.31

95% CI for mean difference: (1.37, 12.63)

T-Test of mean difference = 0 (vs not = 0): T-Value = 5.35 P-Value = 0.033

Appendix 13 T test means for nicotine levels of T 76 unripe and ripe

Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	2.780	0.767	0.443
C2	3	3.223	0.240	0.138
Diff	3	-0.443	0.977	0.564

95% CI for mean difference: (-2.869, 1.983)

T-Test of mean difference = 0 (vs not = 0): T-Value = -0.79 P-Value = 0.514

Appendix 14 T test means for nicotine levels T 76 unripe and overripe

Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	2.780	0.767	0.443
C2	3	4.273	0.265	0.153
Diff	3	-1.493	0.699	0.403

95% CI for mean difference: (-3.229, 0.242)

T-Test of mean difference = 0 (vs not = 0): T-Value = -3.70 P-Value = 0.066

Appendix 15 T test means for nicotine levels of T 76 ripe and overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	3.223	0.240	0.138
C2	3	4.273	0.265	0.153
Diff	3	-1.050	0.485	0.280

95% CI for mean difference: (-2.256, 0.156)

T-Test of mean difference = 0 (vs not = 0): T-Value = -3.75 P-Value = 0.064

Appendix 16 T test means for reducing sugar levels of T 76 unripe and ripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	17.84	2.78	1.60
C2	3	17.10	1.51	0.87
Diff	3	0.73	2.09	1.21

95% CI for mean difference: (-4.46, 5.93)

T-Test of mean difference = 0 (vs not = 0): T-Value = 0.61 P-Value = 0.605

Appendix 17 T test means for reducing sugar levels of T 76 unripe and overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	17.84	2.78	1.60
C2	3	8.89	1.48	0.86
Diff	3	8.94	2.32	1.34

95% CI for mean difference: (3.18, 14.70)

T-Test of mean difference = 0 (vs not = 0): T-Value = 6.68 P-Value = 0.022

Appendix 18 T test means for reducing sugar levels of T 76 ripe and overripe
Paired T for C1 - C2

	N	Mean	StDev	SE Mean
C1	3	17.103	1.511	0.872
C2	3	8.893	1.484	0.857
Diff	3	8.210	0.229	0.132

95% CI for mean difference: (7.642, 8.778)

T-Test of mean difference = 0 (vs not = 0): T-Value = 62.18 P-Value = 0.000