DETERMINATION OF LEAF NITROGEN CONTENT USING SIMPLE AND EFFECTIVE METHODS

MSC THESIS

BY

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JIMMA, ETHIOPIA

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A Thesis

Submitted to School of Graduate Studies Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) in Partial Fulfillment of the Requirements for the Degree of Master of Science in Agronomy

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STATEMENT OF THE AUTHOR

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BIOGRAPHICAL SKETCH

The author, Misganu Etefa Hunde, was born on 24 May 1988(G.C) in Mekoworeda, Ilubabor Zone of Oromia Regional State of Ethiopia. He attended elementary school (grade 1-4) at HomaSeriti Elementary School from 1996 – 1999 (G.C) & (5-8) at Meko Elementary School from 2000-2003(G.C). After completing elementary education, he was enrolled at Bedelle Secondary School in Bedelle town, where he pursued and completed his Secondary Education (grade 9-10) from 2004 – 2005(G.C). After completing his secondary school, he attended his Preparatory school from 2006-2007 (G.C) in Beddele town. Then, he joined Mekelle University in 2008 and graduated with Bachelor of Sciences degree in Dry land crop & Horticultural in July 2010(G.C). After graduation, he was employed by Meko Agricultural & Rural Development Office and assigns him as a Crop Production expert (Agronomist). After serving the ARDO for three years, he joined the School of Graduate Studies of Jimma University in September 2013(G.C) to pursue a study leading to Master of Science degree in Agronomy.

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LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDEX

TABLE OF CONTENTS

Contents   **Page**

TABLE OF CONTENTS *(Cond't)*

LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA   Analysis of variance

CCI   Chlorophyll content index

CCM   Chlorophyll content meter

Chl   Chlorophyll

(DGCI   Dark green color index

(DMSO)   Dimethylsulphoxide

(HPLC   High performance liquid chromatography

LCC   Leaf chlorophyll concentration

LNC   Leaf nitrogen concentration

(NDVI)   Normalized Difference Vegetation Index

NIR   Near infrared

RED   Red band

SPAD   Soil-plant analysis development

ABSTRACT

Chlorophyll and Nitrogen are related to each other. Estimating one will help to predict the other. Many techniques are invented to estimate these two. In many cases evaluation of chlorophyll and nitrogen content in plants need to destructive methods, more time and organic solvents. Laboratory plant testing was usually time-consuming and high-costing. Hence, plant nutrient variability must be measured rapidly and the information made known to the farmers before the new season starts. The objectives of this paper was to evaluate the leaf chlorophyll content of different leaf colors in crop growth stage to determine leaf nitrogen content with non-destructive methods in crop leaves. SPAD-502 meter and CCM-200 meter were two reliable measurements for total chlorophyll content and chlorophyll content index respectively. Different leaf colors of three field crops (maize, wheat & barley) and one perennial crop(coffee leaves) were selected to compare the performance of a handheld SPAD Chlorophyll meter and CCM-200 chlorophyll meter reading for total leaf chlorophyll and chlorophyll content index. Leaves were sampled and Chlorophyll was quantified using these two methods. Both techniques were compared against nitrogen content of leaf analyzed using standard laboratory procedures (Kjeldahl method).Mean comparison among the treatments (leaf colors) explained highly significant difference (p<0.001) with those two parameters (SPAD, CCM&N) at both growth stage. Significant correlations (r^2=0.74, 0.88, 0.80&0.96 at vegetative and r^2=0.93, 0.91, 0.54 &0.74 at flowering) were observed between total leaf chlorophyll (SPAD-502 reading) and chlorophyll content index (CCM-200reading) as well as (r^2=0.96, 0.84, 0.99&0.99 at vegetative and r^2 =0.92, 0.86, 0.82&0.90 at flowering) for both reading with leaf N concentration (LNC) in all crops. From the result, the color of crop leaves was closely correlated with nitrogen (N) status and can be quantified easily with SPAD-502 meter and CCM-200 meter. The establishment of the relationship between SPAD-502 meter and CCM-200 meter reading and N status of leaf were important for crop monitoring and N diagnosis in the field at different crop growth stage.

Key words*:-Nitrogen, Chlorophyll, leaf color, SPAD-502chlorophyll meter, CCM-200 Chlorophyll Meter*

1. INTRODUCTION

Chlorophyll and Nitrogen are among important components found in plant leaf. Nitrogen deficiency causes lower or unhealthy crop productions (Patane and Vibhute, 2014). From all metabolic elements which plants use from soil, nitrogen is needed in the largest amounts (Tucker, 2004). Previous studies e.g. Guendouz *et al*., (2014), indicated that there are positive correlations among N uptake, leaf N concentration, leaf chlorophyll content, and grain yield .The concept of using the crop to asses crop N status indicates close link between leaf chlorophyll content and leaf N content, because majority of leaf N is contained in chlorophyll molecules (Charles *et al.,* 2013). Chlorophyll and nitrogen (N) status of leaves provide valuable information about plants' physiological conditions (Pal *et al.,* 2012). Nitrogen exists in organic and inorganic form and the largest proportions of plant nitrogen content are found in seeds, leaves, shoots and roots.

Nitrogen deficiency leads to loss of leaf green color, decrease leaf area and the associated photosynthetic capacity of the leaves (Bojovic and Markovic., *2009)*, which in turn result in yield reduction. Leaf color can be used to indicate the nitrogen status of the plants. For most leaves green- color is mainly due to chlorophyll *a* and *b*. i.e. foliar chlorophyll content is a good indicator of plant N status and photosynthetic capacity of the leaves (Chang and Robison, 2003). (Evans, 1989) reported the photosynthetic capacity of leaves is related to the nitrogen content primarily because the proteins of the Calvin cycle and thylakoids represent the majority of leaf nitrogen and thylakoid nitrogen is proportional to the chlorophyll content (50 mol thylakoid N mol ~ Chl).

Leaf color and chlorophyll content of the leaves can be measured by SPAD chlorophyll meter (*Mahdi et al., 2012).* Chlorophyll concentration is normally measured using a spectrophotometer in laboratory. In some remote places, however, it is impossible to collect leaves, preserve, and bring them to laboratory to measure their chlorophyll content (Charles *et al.,* 2013). Based on this need, measurement of chlorophyll content is observed through its color.

N is important for the synthesize of chlorophyll and the proportion of leaf N allocated to the chloroplast amountsapproximately 75 %(Ming Wu *et al.*, 2013)**.** A positive correlation between leaf N or N fertilization rate and chlorophyll content is well documented for a large number of plant species and has been investigated for rapid N status determination using chlorophyll meters in most major crops (Guendouz *et al*., 2014).

Chlorophyll content is linked directly to photosynthetic potential and primary production energy (Steele et al, 2008). In other word, leaf chlorophyll concentration is an important parameter that is frequently measured as an indicator of chloroplast development and general plant health (Giovanni*et al.,* 2011). Leaf chlorophyll content can be analyzed from destructive and non-destructive measurements. Destructive measurement destroys the sample; require complicated and time consuming experiments (Lee and Yaw Chung, 2001). Non-destructive estimation using chlorophyll meters is, however, much rapid and is reliable than the destructive approach and is potentially used in forest and crop management (Berg and Perkins 2004).The ability of such meters to assess both chlorophyll and nitrogen content has also been well established for many agricultural species such as cabbage, cotton, pea, muskmelon, corn, wheat, several fruit tree species and grasses (Unagaria *et al.,* 2015).

The chlorophyll meter Soil Plant Analysis Development (SPAD-502) and Chlorophyll content meter (CCM-200 plus) are among the non- destructive chlorophyll meters that are used in these wider applications. SPAD-502 meter is a hand-held device that is widely used for the rapid, accurate and non-destructive measurement of leaf chlorophyll concentrations (Qihua Ling et al., 2000). The meter assesses plant N status, greenness or the relative chlorophyll concentration of leaves (Kariya *et al.,* 1982; Torres-Netto*et al*., 2005) by measuring the transmittance radiation through a leaf at two wavelengths centered near 650 nm and 940 nm (Pinkard*et al.,* 2006). It has been employed extensively in both research and agricultural applications, with a range of different plant species. The use of this meter (non-destructive measure) permits repeated measurement throughout the growing season. Uchino *et al*, (2013) has reported that there is a strong correlation between SPAD reading and leaf nitrogen concentration (LNC) in various plant species including rice, maize and woody plants.

The Chlorophyll Content Meter (CCM-200 plus) is a handheld, battery operated instrument designed for the rapid, nondestructive, determination of chlorophyll content in intact leaf of samples (www.apogeeinstruments.comtechsupport@apogeeinstruments.com). It is far less time consuming and allows samples to be monitored multiple times over various stages of an entire growth cycle. CCM-200 calculates the chlorophyll content index (CCI), which is defined as the ratio of percentage of transmission at 935 nm to 635 nm through leaf tissues (Richardson *et al.,* 2002). Both instruments are reliable and determine the leaf chlorophyll and leaf nitrogen concentration at different phonological stage of crop growth as well as from different leaf color to give immediate solution for foliar application or fustigation and side dress of nitrogen fertilizer.

1.2 Objectives of the Study

The objectives of this work were to

1. Evaluate chlorophyll contents of different leaf colors by using SPAD-502 and CCM-200

2. Explain the significance of non-destructive measurements of leaf chlorophyll concentration over destructive methods.

3. Identify the effect of leaf chlorophyll and leaf nitrogen determination of SPAD-502 and CCM-200 Meter at different crop growth stage

2. LITERATURE REVIEW

2.1 Chlorophyll

Chlorophyll is the green photosynthetic pigment in the cells of plants. It is the pigment that allows plants (including algae) to use sunlight to convert simple molecules into organic compounds via the process of photosynthesis. Chlorophyll is a driver of the physiological function of leaves. Quantifying chlorophyll content can provide information regarding the physiological state of leaves. Chlorophyll is a green photosynthetic pigment which helps plants to get energy from light. According to Pal *et al.,* (2012) definition, Chlorophyll (Chl), the essential green pigment of plants, harvests solar energy and converts it into chemical energy through the process of photosynthesis. It is found in crop/plant leaf and recognized by leaf color. So that leaf color is usually used as a guide for assessments of nutrient status and plant health. The plants use the energy to combine carbon dioxide and water into carbohydrate to sustain their life process (Abdul Hakim *et al*, 2013). Leaf chlorophyll concentration is an important parameter that is frequently measured as an indicator of chloroplast development, photosynthetic capacity, leaf nitrogen content, or general plant health (Ling *et al.*, 2000).

Chlorophyll Determination

Determination of the leaf chlorophyll content is a common procedure for plant scientists. Destructive techniques have been traditionally used for the determination of chlorophyll content in stands of vegetation (Giovanni*et al.,* 2011). Chlorophyll analysis has been conducted in numerous studies due to the importance of this pigment in the physiology of plants (Xueyun*et al.,* 2013). Determination of chlorophyll content as an indirect method of estimating the productivity of vegetation represents a good way to gain an understanding of the photosynthetic regime of plants (Biljana and Stojanovi, 2005). Most of traditional methods of pigment analysis (e.g. high performance liquid chromatography (HPLC)) require destruction of the measured leaves which are not ideal to obtain long term data (Sims and Gamon, 2002). It is also time consuming and expensive to process the samples. Direct and indirect methods can be used to investigate primary organic production. Indirect methods are often used in practice for an approximate estimate of the value of organic production because it is fairly difficult to employ direct methods in plant communities.

As indirect methods, it is possible to monitor and measure all phenomena and processes correlated with productivity (Aranaya *et al.,* 2003). Analytical techniques used to extract chlorophyll from plant leaves are destructive and based on the use of organic solvents. Measurement of chlorophyll content with portable meters is an easy way to quantify crop nutrient status. In the laboratory, it is commonly determined photometrically following extraction of the pigments using an organic solvent, such as acetone or dimethyl formamide (Porra*et al.,* 1989). While this method is well-established and accurate, it is time consuming, destructive (the leaf material must be excised from the plant, and is lost), and necessitates the use of toxic or flammable chemicals.

2.2 Nitrogen

Among agricultural chemicals, nitrogen (N) based chemicals are the most important materials for crop growing and on the other hand, the most concerning nutrient element for maintaining clean environment (Malek*et al*,2012). Nitrogen (N) is an essential element for crop growth and its accurate assessment in plants is a key to nutrient management. (Xiong*et al.*, 2015) report, when excessive N fertilizer is introduced to an agricultural system, crops are more susceptible to environmental stress and pest pressure, but with insufficient N fertilizer input, crop health and productivity suffer Nitrogen is an important and costly input for non-leguminous grain crops and producers are applying N fertilizer in large amounts to ensure high yields over a range of environmental conditions (Kyveryga*et al.,* 2007).

Excessive N fertilization may lead to runoff, leaching, and nitrate pollution. A delayed N application and the use of remote sensing tools might allow a producer to apply a more economically beneficial N rate to their fields. It is the main plant mineral nutrient needed for chlorophyll production and other plant cell components (proteins, nucleic acids, amino acids) (Rafael*et al.*, 2013**).** They argued that**,** crop yield is affected by plant N status. Thus, the optimization of nitrogen fertilization has become the object of intense research due to its environmental and economic impact. Nitrogen is one of the major nutritional elements that limit crop yields. Farmers in many parts of the world likely to apply this element in excess amount for achieving high yield.

Excessive N application Nitrogen is a key component of chlorophyll and, as such, divergent levels of nitrogen in any given plant will generally be reflected in the concentration of chlorophyll in plant leaves (Donahue *et al.,* 1983),decreased grain yield and increased N loss in a wheat-soil system (Wang *et al.,* 2011).

2.2.1 Nitrogen Determination

The accurate assessment of nitrogen content in plants and soil is a key point to nutrient management. Efficient use of N fertilizer has become crucial due to fertilizer costs and the impact of excessive N on the environment and diagnostic tools for estimating plant N status have an important role in reducing N inputs while maintaining yield (Robert et al., 2011).Nitrogen content in the leaf was in relation with colour of leaf (Cabrera, 2004). Nitrogen is a key component of chlorophyll and, as such, divergent levels of nitrogen in any given plant will generally be reacted in the concentration of chlorophyll in plant leaves (Donahue *et al.,* 1983). The most abundant N-bearing compound in green leaves accounts for 30 to 50 percent of the total N, and it has absorption features at 1500, 1680, 1740, 1940, 2050, 2170, 2290 and 2470 nm (*Elvidge, 1990*).

According to Westerveld *et al.,* (2007) Nitrogen deficiency results in chlorosis (yellowing) of leaves due to a drop in chlorophyll content. Research investigated by (Benincasa and Guidici,2002; Richardson *et al,*2009) characterize most plants as the nitrogen deficiency initiates senescence on the lower, older leaves while the metabolites from the breakdown of their proteins and chlorophyll are transported to the upper, younger leaves. Adequate nitrogen also produces thinner cell walls in plant leaves; resulting in tender, more succulent plants (Donahue *et al.* 1983).Chlorophyll content is approximately proportional to leaf nitrogen content. Nitrogen is necessary for the production of protein and chlorophyll and these are essential for plant development, yield, post-grazing re-growth and reproduction (Vickery, 1981).
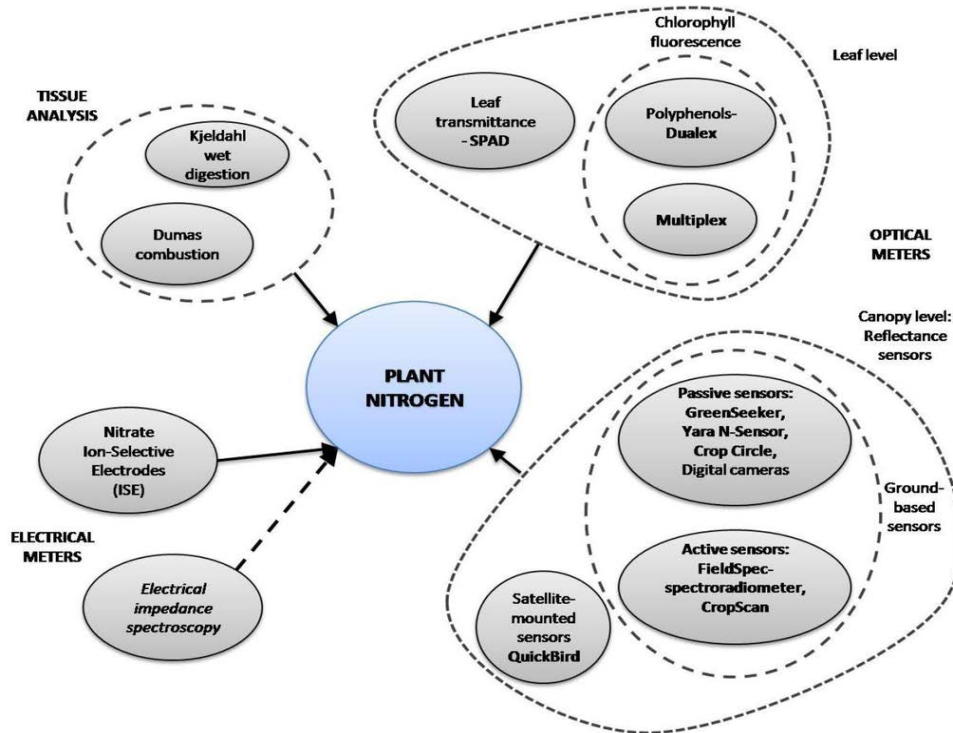
Fig.1 Methods for plant nitrogen sensing (Accepted from Rafael *et al*., 2013)

Methods based on tissue analysis, such as Kjeldahl-digestion and Dumas-combustion, have been widely applied to plants due to their reliability in organic nitrogen determination, but they are time-consuming and destructive (Rafael *et al*, 2013).

Several studies have reported the fastest and non-destructive new tools designed for plant N status estimation. Optical properties of some leaf pigments, such as chlorophyll and polyphenols, have been used as plant N status indicators (Mainard*et al*., 2008). Some of these tools measure leaf chlorophyll content, which is highly correlated to plant N status (*i.e.*, SPAD-502, Dualex, Chlorophyll fluorescence) (Rafael *et al.,* 2013). (Unagaria *et al.,*2015) applied calibration of widely used TYS-A (SPAD meter) and CCM-200 (Chlorophyll content index (CCI) meter) chlorophyll meters was attempted for chlorophyll estimation in bread wheat (Triticumaestivum L.) leaves .Accordingly, the chlorophyll values for the wheat leaves were measured with chlorophyll meters and same samples were used in standard photometric measurements in laboratory and significant linear relationship with chlorophyll content (R^2=0 .92).

With functional relationship represented with regression models used to convert the SPAD value to actual chlorophyll content for wheat.A determination of the chlorophyll content thus allows the N nutrition level of the plant, and indirectly the N supplying capacity of the soil, to be estimated (Adrijana *et al.,* 2008). As chlorophyll, nitrogen determination is also similar techniques.

2.3 Methods of Chlorophyll and Nitrogen Determination

2.3.1 Destructive methods of leaf chlorophyll and leaf nitrogen determination

Analytical techniques used to extract chlorophyll from plant leaves are destructive and based on the use of organic solvents. In many cases evaluation of chlorophyll and nitrogen content in plants need sample destructions, more time and organic solvents. The conventional techniques for assessing Chl and N status in plants cannot satisfy the requirements of precision farming (Pal *et al.,* 2012). Techniques utilized to extract photosynthesis pigments from plant materials are based on methods that use organic solvent such as acetone, dimethylsulphoxide (DMSO), methanol, petroleum ether and others (Inskeep and Bloom 1985). Acetone 80 % is the most used of these solvents. However, these methods require sample destruction. There can be significant losses of these pigments during the extraction process and extract dilution that can cause high variability in the results. Using DMSO modified the extraction methodology to eliminate the squashing and centrifuge stages. The storage period of these pigments is increased by the use of this solvent so that the spectrophotometer analyses did not have to be carried out immediately after extraction. In general, traditional methods for chlorophyll and N determination include soil testing, plant tissue analysis, and long-term field trials. Although these methods are accurate, they are destructive, time-consuming, and expensive. Some destructive methods of chlorophyll extraction are listed below.

2.3.2 Nondestructive methods of leaf chlorophyll and leaf nitrogen determination

Non-destructive optical techniques based on leaf absorbance and reflectance of light by leaves has been proven as alternative time-saving and simple techniques to quantify Chl in a number of agricultural species. Portable nondestructive meters have been successfully used to determine foliar chlorophyll or N of many plants. Chlorophyll meters (e.g., SPAD-502, Minolta, and Osaka, Japan) have been used to assess plant N status by measuring transmittance radiation through a leaf at two wavelengths centered near 650 nm and 940 nm (Pinkard *et al.,* 2006). The chlorophyll

meter readings have been positively correlated with destructive chlorophyll measurements in many crop species (Zhu *et al.,* 2012) and considered as a useful indicator of the need of N top-dressing during the crop growth (Naderi *et al*. 2012).(Nyl *et al.,*2012) conducted to determine the relationships between a portable chlorophyll meter(SPAD) reading and photosynthetic pigments, leaf nitrogen status and chlorophyll fluorescence variables in leaves of jatropha (Jatrophacurcas L.) to detect leaf greenness, chlorophyll content, nitrogen content and photosynthesis performance respectively and they found good correlations between SPAD reading and total N concentration (R^2=0.99), as well as with content of chlorophyll a (R^2=0.97), chlorophyll b (R^2=0.96) and carotenoid (R^2=0.75)

Leaf Color Chart

One of the recently introduced nitrogen management approach was estimating the leaf nitrogen concentration by the measurement of leaf greenness. Yosef (2013) reported that leaf color chart (LCC) is an easy-to-use and inexpensive diagnostic tool for monitoring the relative greenness of a rice leaf as an indicator of the plant N status. According to this author, Inexpensive leaf color chart (LCC) has been proved as a quick and reliable tool to decide the time when fertilizer N is needed to be applied for the crop growth. The use of the LCC, farmers can apply N at the right time, thereby increasing the productivity and profitability of direct rice and reduction of use of nitrogen fertilizer. It is a simple color chart, consists of many shades of leaf color from light green to dark green. It is used mainly for Nitrogen estimation. For N estimation, Leaf is cut and it is compared with color in the chart. Different species crop uses different chart. This is the easiest but also the least accurate method and is suitable for medium area (Patane and Vibhute, 2014).

Estimating by image processing

This method is used by capturing leaf image by portable camera and relationships between nitrogen content and leaf colors in red (R), green (G), blue (B) and near infrared (IR) are examined. Auearunyawat *et al* (2012) states that, leaf color image and leaf IR image have relationships with nitrogen content of sugarcane. This relationship showed the potential value of leaf color indices and IR index to estimate nitrogen concentration in two months old sugarcane.Yuzhu*et al* (2011) took color image with a digital camera for nitrogen determination in pepper.

According to these authors, color images were processed in order to determine the averages of the red (R), green (G) and blue (B) colors. There were significant negative relations between *G/(R+G+B)* ratio of coverage image and the indexes of pepper nitrogen status, such as inorganic nitrogen in soil, total nitrogen of plant, nitrate concentration of leafstalk and SPAD reading at flowering and fruiting stages.Luna *et al*(2010) reported that, color image analysis provides an accurate and quick way for nitrogen estimation and can contribute for early detection of nitrogen deficiency in tomato seedlings. But their results showed that color image analysis correlated better with the status of plant nitrogen than the SPAD.

Chlorophyll meter

Application of chlorophyll meters save time and resources. They quickly estimate the chlorophyll content of leaves at leaf absorbance of two different wavelength regions using two light emitting diodes (LEDs)(Giovani*et al.*,2011).Chlorophyll meter can conveniently estimate foliar chlorophyll and nitrogen (N) contents in many species. There are many types of chlorophyll meters which determine chlorophyll concentration of leaves and indirectly nitrogen contents of that leaves. Among these Minolta sapd-502 and CCM-200 Opti-Science which are used in this study are the most important. Chlorophyll meters are extensively used in agriculture; they quickly estimate the chlorophyll content of leaves with a hand-held device that measures the leaf absorbance in two different wavelength regions using two light emitting diodes (LEDs). Islam *et al.*(2009) reported that, the relationship between SPAD value and chlorophyll content was very close (R^2 =0.80) at panicle initiation and flower initiation stages for rice crops.

SPAD-502 Chlorophyll Meter

The SPAD 502 plus Chlorophyll Meter instantly measures chlorophyll content or "greenness" of your plants to reduce the risk of yield-limiting deficiencies or costly over fertilizing (Tevis*et al.,* 2014). SPAD readings are calculated based on two transmission values: the transmission of red light at 650nm, which is absorbed by chlorophyll, and the transmission of infrared light at 940 nm, at which no chlorophyll absorption occurs (Xiong *et al.,* 2015).they also reported, a close relationship between SPAD value and chlorophyll content per leaf area in both the monocot and dicot groups. Variable rate technology for fertilizer application has been developed to reduce environmental risks and increase fertilizer use efficiency. For precise application of this

technology, it requires an intact determination of plant nutrient status in the field. (Jifon *et al.,*2005) evaluated the utility of two handheld transmittance-based Chl content meters (SPAD-502, Minolta Corp) one reflectance-based meter (Observer, Spectrum Technologies), in estimating Chl and N concentrations in intact leaves of several citrus cultivars and they reported that, total Chl determined analytically, correlated well with nondestructive Chl meter readings (r^2: 0.72 to 0.97; $P<$ 0.0001), but regression models differed among cultivars using the same meter and also among meters for a given cultivar.

Sabrina *et al* .(2009) estimate foliar chlorophyll content of 13 tree species from the tropical rain forest in French Guiana and calibrate between SPAD units and extracted chlorophyll values. Their homographic model best accurately predicted total chlorophyll content (μg cm^{-2}) from SPAD units ($R^2 = 0.89$). Steele *et al.,* (2008) compared the performance of a handheld SPAD Chlorophyll meter and a recently developed Red Edge Chlorophyll Index (CIred edge) for Chlorophyll estimation in grapevine (*Vitis*spp.) leaves. Liu *et al.,* (2012) evaluated the utility of SPAD-502 chlorophyll meter measuring in Chlorophyll < 300 mg/m^2 to inform nitrogen fertilization rates of tea (*Camellia sinensis* L.) at different places and times. They reported that SPAD readings could estimate the chlorophyll content of tea leaves regardless of temporal and spatial considerations.SPAD reading also indicate N nutritional status, assisting in the adjustment of N fertilization during the potato plant cycle (Fabrício *et al.*, 2012). Scientists also investigate SPAD values were well-correlated with both total chlorophyll and total N leaf concentration, and the regression coefficients were higher when relationships were calculated on a leaf-area basis of tobacco (Fabio and Renato, 2009). For relationships, SPAD-total chlorophyll and SPAD-total N, the best fittings were obtained with quadratic equations.Earl and Tollenaar (1997) found a close correlation ($R^2 = 0.98$) between SPAD readings and maize leaf absorptance in the field, and Dana Martinez et al., (2004) compared SPAD readings with "greenness" measurements taken with a digital camera during wheat senescence ($R^2= 0.91$). Generally, there is a close correlation between SPAD readings and leaf Chl or N content.
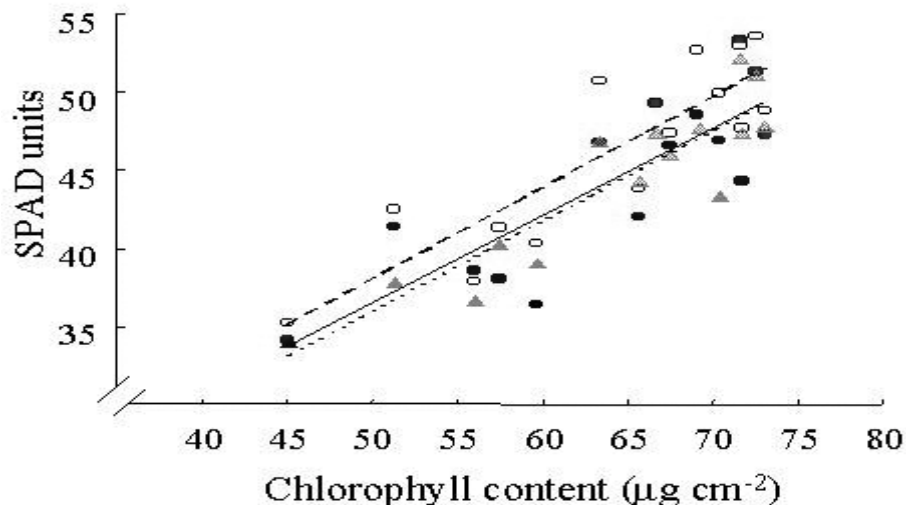
Fig.2 The Relationship between Extractable Chlorophyll Content and SPAD Readings in Wheat
Leaves (accepted from Martínez and Guiamet, 2004)

CCM-200 chlorophyll meter

The Opti-Sciences CCM-200 Chlorophyll Content Meter is marketed as an instrument that provides fast, accurate chlorophyll readings on the intact leaves of plants and crops, without the need for grinding or destructive chlorophyll assays. CCM-200 calculates the chlorophyll content index (CCI), which is defined as the ratio of percentage of transmission at 935 nm to 635 nm through leaf tissues (Richardson *et al.,* 2002) and instance CCI is commonly promoted to measure leaf nitrogen content as an indicator of plant nutrient status (Biber *et al.,* 2007) The CCM is useful for improving nitrogen and fertilizer management, and is ideal for crop stress, leaf senescence, plant breeding, health determination, and other studies (Biber *et al.,* 2007). (Pal*et al.,* 2012) explained there is a pressing need for non-destructive techniques to estimate Chland N content in the leaf of the Damask rose. The regression models were developed with destructively measured parameters (total Chl and N) as the dependent variable and a parameter derived from CCM-200 as the independent variable (CCI). Ghasemi *et al*. (2011) conducted research work on eight years old Asian pear trees during June 2008 in Tehran, Iran. They reported that, there was positive and linear correlation between CCM-200 data and chlorophyll *a* (R^2=0.72), chlorophyll *b* (R^2=0.85), total chlorophyll (R^2=0.90), and total nitrogen content (R^2=0.76) in Asian pear leaves. Thus, it can be concluded that, CCM-200 can be used in order to predict both chlorophyll and nitrogen content in Asian pear leaves. Mustafa *et al* (2011)

estimated chlorophyll and nitrogen content in Asian pear leaves using chlorophyll content meter (CCM-200). They develop regression model, and the results showed that, there was positive and linear correlation between CCM-200 data and chlorophyll *a* ($R^2=0.72$), chlorophyll *b* ($R^2=0.85$), total chlorophyll ($R^2=0.90$), and total nitrogen content ($R^2=0.76$) in Asian pear leaves.

2.4 Types of Crops Used In Research Works

2.4.1 Wheat

Modern technology of wheat production mainly based on numerous scientific farming measures as well as application of mineral fertilizers. According to Bojović and Marković(2009)**,** mainly one third of applied nutrient wheat plants are able use during vegetative period. In the field practices is very important optimize quantity of fertilizers, decrease expenses of production and improve efficiency of wheat plant of nitrogen absorption, accumulation and reutilization. Nitrogen content in the leaf was in relation with color of wheat leaf. Deficiency of nitrogen leads to loss green color in the leaves, decrease leaf area and intensity of photosynthesis. Understanding the processes that govern N uptake and distribution in crops is of major importance with respect to both environmental concerns and the quality crop products (Martínez and Guiamet, 2003).The chlorophyll index values of wheat estimated by portable chlorophyll meters (TYS-A and CCM-200) have statistically significant functional relationship with actual chlorophyll content(LUNAGARIA *et al.*,2015.

2.4.2 Maize

Maize (*Zea mays* L.) is one of the most important constitutes one of the major yield limiting factors for cereal crops of the world extensively grown in irrigated cereal production. Testing of corn leaf tissue to compare leaf nitrogen concentration with critical levels is a well-established procedure to document a crop nitrogen deficiency (Schepers *et al.,* 1992). Sawyer *et al.* (2004) reported monitoring the corn plant as a means to determine N status and seasonal availability has advantages in that the plant integrates N supply over a period of time, and hence can reflect N supply as affected by weather, soil processing, and fertilization. In irrigated corn, there are repeated opportunities to apply needed N during the growing season with irrigation water and Minolta SPAD-502chlorophyll meters were used to take the readings (Schar*et al.,* 2006).

Robert *et al.*(2011) quantify corn (*Zea mays* L.) leaf greenness with a digital camera and image-analysis software and establish the relationship with yield, leaf N concentration, and chlorophyll meter (or SPAD, soil plant analysis development) values. They conducted at five sites with N treatments ranging from 0 to 336 kg N ha^{-1}. At tasseling, the ear leaf was sampled for color analysis and SPAD measurements, and then analyzed for total N. There was a close relationship (typically $r2 \geq 0.70$) of SPAD and DGCI with leaf N concentration. Within a location, yield increased linearly in most cases with both SPAD (average $r^2 = 0.79$) and DGCI (average $r^2 = 0.78$). Digital-image analysis was a simple method of determining corn N status that has potential as a diagnostic tool for determining crop N needs.
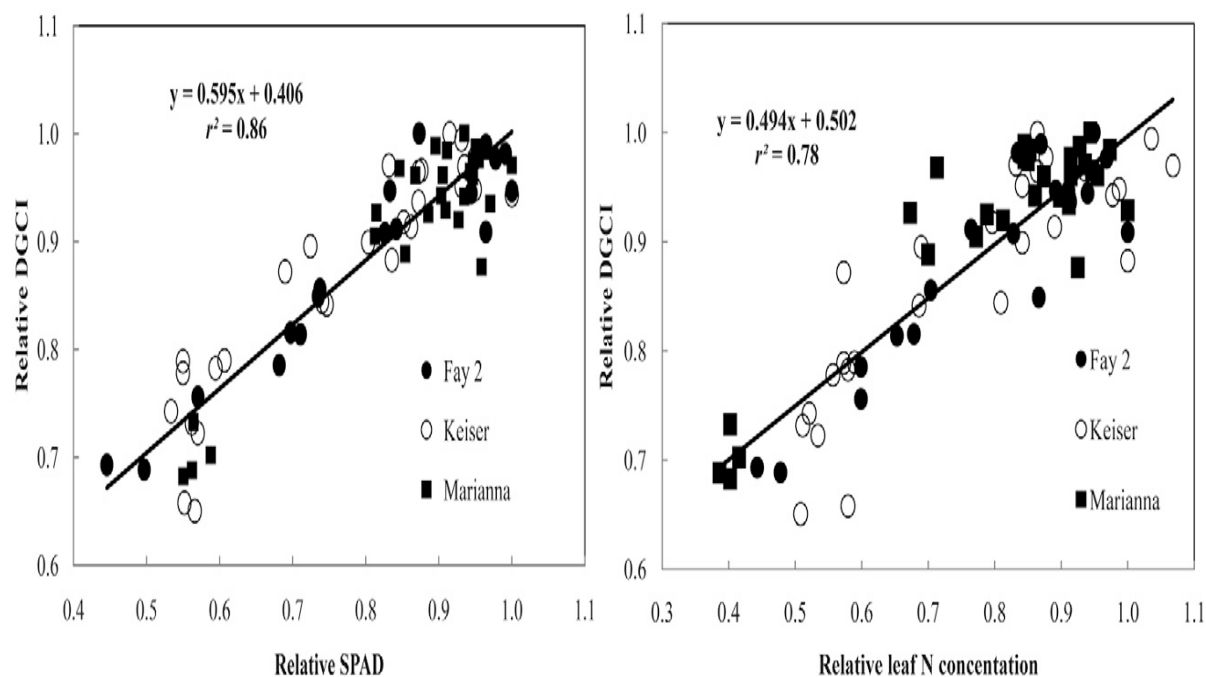
Fig.3 Relationship in corn between relative dark green color index (DGCI) and relative chlorophyll meter (SPAD),*soil plant analysis development)right and the relationship in corn between relative dark green color index (DGCI) and relative leaf N concentration for(left)(accepted from* Robert *et al.*,2011).

2.4.3 Coffee

It is the third important cash crop that targeted for this study. Nitrogen is the key element in the realization of the yield potential of both traditional and modern day coffee varieties along with mixed crops (Anand and Pereira, 2006). Research conducted in 2003 by Alena *et al.* (2004) established correlation between the photosynthetic pigments content extracted in DMSO, the total nitrogen content and the chlorophyll a fluorescence variables with the SPAD-502 readings in *Coffee canephora Pierre leaves*. Their result showed that SPAD-502 has been a good tool to diagnose the integrity of the photosynthetic system in coffee leaves and can thus help in the advanced interpretations of the photochemical process of these plants. In addition to this, Rodriguez et al, (2009) established correlations among the photosynthetic pigment content, total N, and the photosynthetic variables with the SPAD-502 readings in *Coffeea arabica* leaves. The results showed that all variables decreased with time. However, correlation increased linearly with N doses.

2.4.4 Barley

The fourth important crops used in this study collection for chlorophyll reading and nitrogen determination for the study Barley (Hordeumvulgare L.) is a plant widely distributed and cultivated in eastern Asia and used as food stuffs such as bread and cakes (Ikeguchi *et al.,* 2014) and also it is one of the major cereal crops that are largely produced in the central and south east mid and high altitude areas of Ethiopia with ranked fifth important cereal crop after Teff, Maize, Wheat and Sorghum (Kulumsa ARC, 2014). The nutrient profiles of green cereal plants change quickly (especially barley and wheat) as they grow, the plant grows, the chlorophyll, protein and vitamin content of cereal grasses declines sharply and the level of cellulose (indigestible fiber) increases (Perricone,2005).

Barley also used in experiment as target crops to study CCM and SPAD reading at different growth stage by using different leaf colors. Lucie *et al* .(2013) works on qualitative and quantitative evaluation of chlorophyll content in food supplement using HPLC and HPTLC in barley crop.

3. MATERIALS AND METHODS

3.1 Description of the Study Area

This study was conducted in three districts of Jimma Zone, South West Ethiopia. The first site was Manna Woreda (somodo), where data on wheat and barley were collected. The second one was JUCAVM horticultural farm where data on coffee were collected. The third study site was Kersa district, birbirsa irrigation site where data on maize were collected. Manna is approximately 20 kms from Jimma town while kersa is 28 km away from Jimma.

Table-1 Specific Information of the Study Area

	PLACE (WOREDAS)		
	Manna	Kersa	Jimma
Agro-ecological zone	Dega (12%), Woinadega (63%) and kola (25%)	Dega (14%), Woinadega (66%) and kola (20%)	Dega (12%), Woinadega (63%) and kola (25%)
Temperature	18.9 °C	$20c^o$	$22.83\ c^0$
Altitude	2040 m	2200 m	1,780 m
latitude	7°46.5 and 7°51.5	7°39′N 36°50′E	7°40′N 36°50′E
longitude	36°40 and 36°42 in East	7°39′N 36°50′E	7°40′N 36°50′E
Average Rainfall	1,467 mm	1140 mm.	1624 mm.
Major crops	Coffee, cereal, vegetables	37.5% under annual crops, coffee	Coffee, cereal, vegetables

Sources : CSA (2005)

3.2 Data Collection

3.2.1. Experimental Design and Field Management

The experiment was set up in a randomized complete block design with three treatments of leaf colors (deep green, dark green and yellow color) by replicating leaf number in each crop at two different growth stage (flowering and vegetative; see table 2 below). Five plants were selected for each leaf color and total fifteen plants(fifteen leaves) for all treatments (deep green, dark green and yellow green leaves) were chosen from these trees in each block randomly. Ten different SPAD and CMM readings were taken from the selected leaves (ten reading points for

one specific leaf; see table 3 below) and total fifty readings for one treatment and one hundred fifty readings for all treatments and 300 readings by both SPAD and CCM. Reading of specific leaf was taken averegically. Five leaves from each tree were then detached and taken to the laboratory for leaf nitrogen analysis. The measurements were conducted at two different phonological stages: vegetative and flowering stages.

Table .2 Treatment Combination of Different Leaf Colors In Different In Different Crops at Different Growth Stage

Growth stage	Crop type	No. Of Leaves	Leaf Colors		
			Yellow green	Dark green	Deep green
Vegetative stage	Wheat	5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5
	Maize	3	1,2,3	1,2,3	1,2,3,
	Barley	5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5
	Coffee	5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5
Flowering stage	Wheat	5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5
	Maize	3	1,2,3	1,2,3	1,2,3
	Barley	5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5
	Coffee	5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5

A. Deep Green   B. Dark Green   C. Yellow Green

Fig 3 . Types of Leaf Colors (Deep Green, Dark Green, Yellow Green)

Experimental Procedures

Sample of different leaf colors of different crops were collected at different growth stage. For one treatment (one leaf color), sample of five leaves were randomly taken from five individual crops. The SPAD and CCM reading were taken from ten places of reading points on one specific leaf and the average was taken and parametric estimation was done as flows.

1. Minolta SPAD reading:-sample of individual leaf color was taken and recorded the SPAD values from different ten points of the leaf and averaged. This repeated for other leaves and other leaf color or treatments.

2. Opt-science CCM-200 reading:-As SPAD reading, ten points of reading was taken from individual leaf and averaged. There was also repetition for others

Table 3 Number of SPAD-502(CC) Reading and CCM-200(CCI) Reading On Single Leaf

number of leaf	Leaf color (treatments)		
	Yellow green	Dark green	Deep green
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
1	10x	10x	10x
	Average	Average	Average

Fig4. SPAD and CCM-200 reading

Nitrogen analysis by Kjeldahl methods

The collected leaves of all crops or all treatment (deep green, dark green and yellow green) were analyzed in laboratory by Kjeldahl methods. The collected samples of different leaf colors oven dry at 70 c0for 48 hours. The oven dried leaf powdered prepared for Kjeldahl procedures. The sample were taken the procedures and take places in three steps for nitrogen analysis. These steps were digestion processes of the sample, distillation and titration with different chemicals. Digestion consumed 6ml of concentrated sulfuric acid and powder of copper sulfate and at 120c0 and stayed for 3 hours until the sample digested.

After digestion, distillation was take place with normal sodium hydroxides and 4% of boric acid and continuously titration with 0.1 normality of hydrochloric acid and color of the distilled sample changed from green to pink by consuming some amount of Hcl. Finally the consumed of ml of normal hydrochloric acid calculated by the following procedures and the content of nitrogen observed.

The collected leaves were taken to the laboratory and their nitrogen content was analyzed following Kjeldahl procedure. % nitrogen in each leaf per unit of dry matter was then calculated using eqn 1

%Nitrogen= [(ml standard acid x N of acid) - (ml blank x N of base)] - (ml std base x N of base) x 1.4007 weight of sample in grams …………………………………………… (eqn. 1)

3.3 Data Analysis

The collected data were analyzed following glm for ANOVA using SAS vr. 9.2. Means of significant treatment effects were compare using the Least Significant Difference (LSD) test at 5% probability levels (Gomez and Gomez, 1984). Regression analysis was done to develop relationships between leaf nitrogen concentrations, total chlorophyll in leaf (SPAD reading) and chlorophyll content index (CCM readings). Pearson correlation analysis was carried out using the same software to investigate associations among grain yield and yield traits of the crops.

4. RESULT AND DISCUSSION

4.1 Comparisons of Different Leaf Colours in Vegetative Crop Growth Stage

Table 4 Treatment comparison of CCM-200 and SPAD-502 in different leaf colors

Crop type	Treatment	CCM-200 Reading(CCI)	SPAD-502 Reading(CC)
Wheat	Deep green	462.1a	229.36a
	Dark green	408.98a	178.54a
	Yellow green	255.92b	96.42b
	LSD (5%)	73.993	50.89
	CV (%)	13.51	20.76
Maize	Deep green	417.0a	577.3a
	Dark green	284.0b	527.3a
	Yellow green	65.67c	208.3b
	LSD (5%)	58.87	51.68
	CV (%)	44.68	52.28
Coffee	Deep green	259.62a	434.24a
	Dark green	166.40b	403.42a
	Yellow green	78.74c	252.58b
	LSD (5%)	65.033	58.65
	CV (%)	26.50	11.06
Barley	Deep green	1222.48a	736.42a
	Dark green	483.24b	517.52b
	Yellow green	161.58c	335.70c
	LSD (5%)	153.15	51.75
	CV (%)	21.72	6.69

From the table above, there were significant differences between different leaf colors of the whole crops but in only numerical difference of deep green and dark green treatments of some crops and this because the leaf reflectance. In other case, there were also horizontal relationships of both reading within treatments. This result shows that, the purpose of SPAD-502 and CCM-200 measurement for total chlorophyll content and chlorophyll content index respectively at vegetative stage would be effective by nondestructive measurement. This is similar with (Walter

Riedell, 1999) the SPAD Chlorophyll Meter sensor clamps on intact leaves and instantly measures leaf chlorophyll "greenness" of corn and there is a close relationship between chlorophyll level and leaf N, the Chlorophyll Meter readings are an indicator of leaf N level. Similar argument with(van den Berg and Perkins, 2004) the CCM is an effective tool for rapid and nondestructive estimation of relative chlorophyll and nitrogen content in sugar maple leaves during the growing season. (Biber*et al.*, 2007) the CCM is useful for improving nitrogen and fertilizer management, and is ideal for crop stress, leaf senescence, plant breeding, health determination, and other studies. (Fabrício*et al.*, 2012) report SPAD reading indicate N nutritional status, assisting in the adjustment of N fertilization during the potato plant cycle.

Comparison of different type of leaf color were also explained the relationship of SPAD (total chlorophyll content) and CCM (chlorophyll content index as measured from range of deep green color to yellow green color differentiating into three groups in all three parameters measurements as(a-b-c) unless in some extent of crop leaf was close gap to identify leaf color during data recording. The data in this study indicate that the SPAD and CCM was an effective tool for rapid and nondestructive estimation of relative chlorophyll content and chlorophyll content index in maize, wheat, barley and coffee leaves during the growing season. The estimation of the accuracy of the chlorophyll content index(CCM) was 86.5% 65.4%, 73.5%, 77.3%, and that of total chlorophyll (SPAD) was80%48%, 89% and 93.4% with similar range with (Zoran *et al*, 2012) 84% for Chlorophyll concentration for SPAD and CCM in their work. This differential statement of treatment comparison in leaf color affect photosynthetic activity as (Nyl*et al.,* 2012) said that Photosynthesis yield showed a slight increase when the SPAD value was increased; however, when the SPAD values were around20 or 60, the photosynthesis yield was lower than 0.7 and(Alena *et al.,* 2005) also found the relative SPAD-502 reading has been shown a good tool to diagnose the integrity of the photosynthetic system in coffee leaves, and can thus help in the advanced interpretations of the photochemical process of these Plants. Thus the portable chlorophyll meter SPAD-502 can help in the advanced interpretations of the photochemical process in plants of the species under study. (Amer, 2005) strong positive correlations between Chlorophyll meter readings and measured Chlorophyll were found for cultivars (P< 0.0001) in Citrus sp. Leaves. These data provide evidence that the chlorophyll meter can be used to determine top dress N needs at pre-panicle initiation and panicle differentiation. This yield might

be the result of a reduction in the total amount of photosynthetic pigment content in leaf samples at the lower SPAD values and photorespiration at the high SPAD values. There were also(Marcos *et al.,* 2014) the CCM 200 plus meter provided good estimates of chlorophyll content for all leaves in Origanumvulgare ssp. hirtum, independent of leaf age, position within the plant and phonological stage. Nevertheless, the meter loses efficiency with increasing chlorophyll con-centration and/or increased leaf thickness. As the leaves develop mesophyll thickness and chlorophyll content increases. The increased thickness given by greater development of spongy parenchyma leads to higher variability in the readings and linear relationship distortion. So that, the SPAD-502 and CCM 200 plus portable meter was a very useful tool to estimate chlorophyll content, aiming to relate these values with different situations of environmental stress, nutritional management programs.

4.2 Comparison of Different Leaf Colors at flowering stage

Table 5. Treatment comparison of CCM-200, SPAD-502in different leaf colors

Crop type	Treatment	CCM-200 Reading(CCI)	SPAD-502 reading
Wheat	Deep green	215.72a	444.04a
	Dark green	170.52b	399.44b
	Yellow green	75.20c	218.16c
	LSD (5%)	42.196	42.79
	CV (%)	18.81	8.29
Maize	Deep green	455.23a	550.4a
	Dark green	150.47b	339.73b
	Yellow green	100.43c	289.03b
	LSD (5%)	33.13	77.721
	CV (%)	6.21	8.722549
Coffee	Deep green	1099.1a	704.42a
	Dark green	418.0b	490.72b
	Yellow green	72.5c	240.12c
	LSD (5%)	72.97	89.454
	CV (%)	35.32	12.82042
Barley	Deep green	630.6a	703.80a
	Dark green	417.6b	486.40b
	Yellow green	62.0c	236.20c
	LSD (5%)	54.06	89.404
	CV (%)	65.59	12.89

Different leaf colors of the whole crops of the work under SPAD and CMM reading as well as consecutive concentration of nitrogen content in each leaf colors express the difference within a

leaf color (a-b-c) and similar (aa-bb-cc) category among parameters (SAPD, CCM and Nitrogen) in only similar group of deep green and dark green in maize under SPAD reading and this is may cause of environmental effect or very close range of the leaf color.

4.3 Regression and Correlation Model

Regression analysis is a statistical tool for the investigation of relationships between variables. This graph show the relationship between SPAD, CCM and Nitrogen concentration at different growth stage in different crop types with their correlation coefficient. Chlorophyll Content Index as measured by the Opti-Sciences CCM-200 and leaf nitrogen concentration measured by SPAD-502 were found to correlate positively with Nitrogen concentration in all crop types(wheat, maize, barley and coffee)at both growth stage.

4.3.1 Relationship between SPAD (leaf chlorophyll content) and (CCI) CCM readings
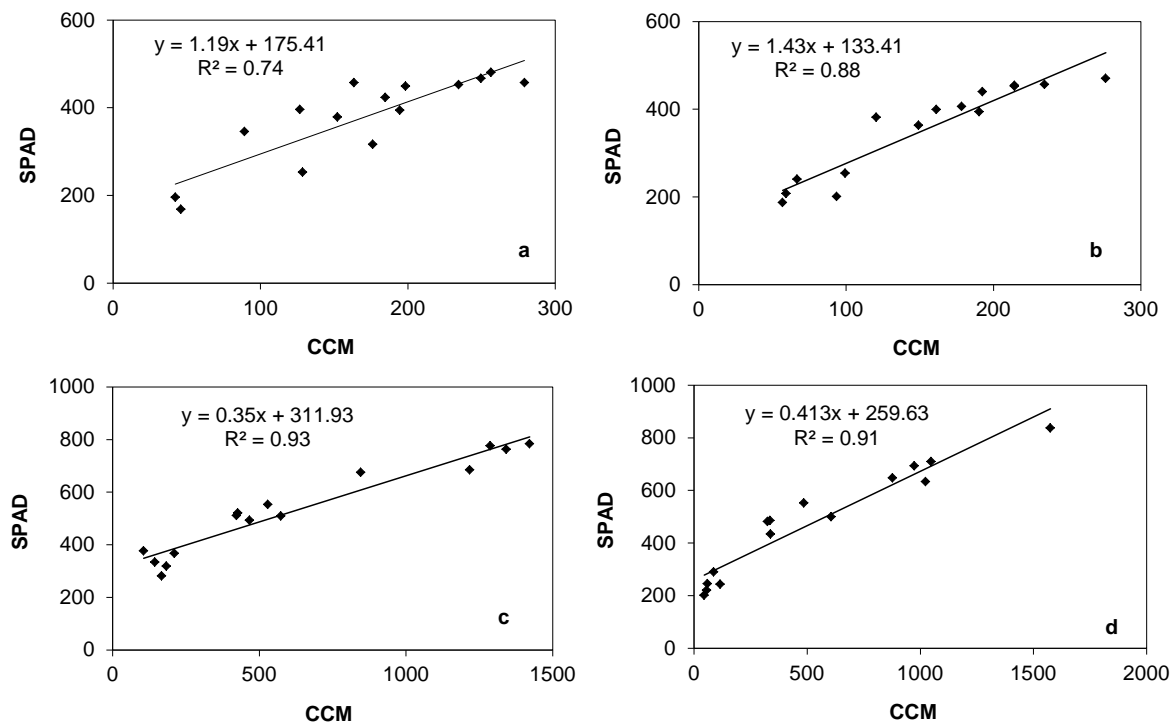
Figures 5. Correlations between leaf SPAD and CCM readings of wheat (a and c) and coffee (b and d). Panels 'a' and 'b' represents readings taken at vegetative stage while panels 'c' and 'd' represents readings taken at flowering stages for both wheat and coffee.

Significant positive linear relationships were observed between SPAD and CCM readings measured for all crops considered in the study (Figs. 5a-d, 6a-d). The correlation increased with

crop developmental stages. The regression coefficient, R^2 between SPAD and CCM for each crop and developmental stage ranged from 0.54 - 0.96 indicating strong relationship between the two readings. The correlations were, however, stronger with developmental stage and were stronger at flowering than at vegetative stage (Figs. 5a and b). The results were in agreement with findings reported by Wang *et al*. (2014). The authors also found lower R^2 values in the vegetative stage of rice crops compared to the coefficient observed at tillering stages. The slopes of the regression lines except for barely (Figs. 2b and d), on the other hand, decreased with increasing developmental stages.

For example, for wheat the slope decreased from 1.19 to 0.35 and for coffee the values decreased from 1.43 to 0.41 while the crops advanced from vegetative to flowering stages (Figs. 5a – d). SPAD quantifies the relative amount of chlorophyll present in leaf by measuring the leaf transmittance in two wave bands (400 – 500 nm and 600 – 700 nm) and reports the readings in arbitrary unit (Sim*et al*., 2015). These readings are proportional to the leaf chlorophyll concentrations which on the other hand are correlated with photosynthetic activities and leaf nitrogen status (Evans, 1983; Seemann*et al*., 1987).

The strong correlation observed between SPAD and CCM readings enable the usability of both equipments in predicting the chlorophyll contents in crops and ease their managements. Correlation between the SPAD and CCM readings were observed to be crop species specific. These crop species specific readings were probably related to the differences in the leaf reflectance distributions of each crop (Shibayama and Watanabe, 2007 and Homem*et al.,* 2001). Differences in leaf anatomical characteristics such as leaf thickness, leaf water contents and cuticle reflectance affect leaf reflectance distributions (Sim*et al*., 2015).

Crop developmental stages and environmental conditions (Peng*et al,* 1993; Shukla*et al,* 2004) also affect leaf reflectance distributions. For establishing individual regression models, it requires calibration of each of the equipment for each crop species (Marquard and Tipton, 1987).
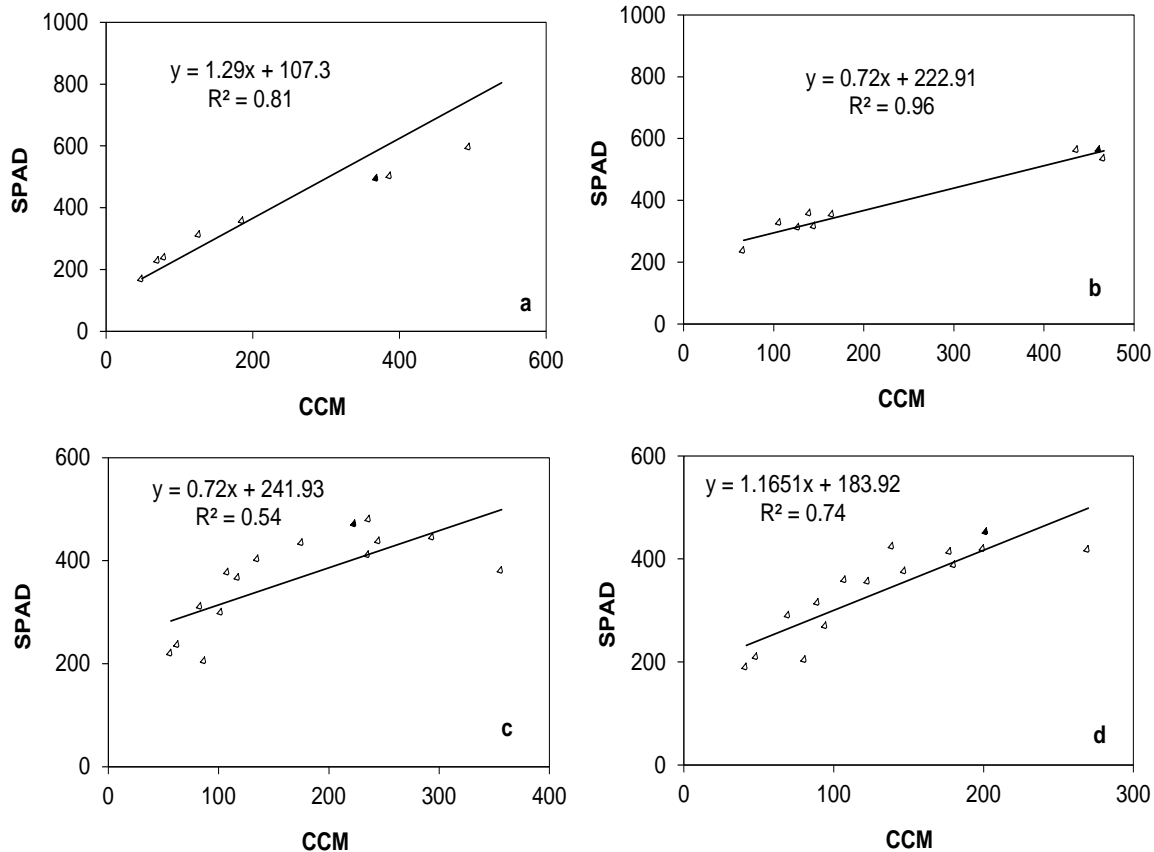
Figure 6. Correlations between leaf SPAD and CCM readings of maize (a and c) and barley (b and d). *Panels 'a' and 'b' represent readings taken at vegetative stage while panels 'c' and 'd' represent readings taken at flowering stages for both maize and barley.*

4.3.2 Relationship between leaf N concentration and SPAD/CCM readings

Leaf nitrogen concentration of each crop increased with increasing SPAD and also CCM readings (Figs. 7 and 8). Though, the correlation between the readings and leaf nitrogen concentrations were significantly different among the different crop species, the relationships between the readings and leaf nitrogen concentration in all crops followed a logarithmic model. In addition, relationships between the readings and leaf nitrogen concentration strongly differ with the developmental stages of the crops.

Mustafa et al. (2011) also said statistical analysis showed linear correlation between CCM readings and leaf chlorophyll content and also leaf nitrogen content.

The regression coefficient, R^2 between total leaf chlorophyll (SPAD reading) and N concentration varied from 0.76 – 0.98. The coefficient between chlorophyll content index (CCM reading) and N concentration, on the other hand, ranged between 0.17 – 0.96 (Figs. 7 and 8). From the Boussadia *et al.* (2011) study on olive tree leaves, maximum net photosynthetic assimilation rates, chlorophyll fluorescence parameters and the SPAD Chlorophyll index were therefore measured simultaneously and the Chlorophyll and nitrogen content of the leaves were analyzed and significant correlations were established in the olive tree leaves between SPAD-502 readings on the one hand and, nitrogen content, photosynthetic assimilation rate, and Chlorophyll fluorescence parameters. (Pal*et al.*, 2012) explained there is a pressing need for non-destructive techniques to estimate Chl and N content in the leaf of the Damask rose. The regression models were developed with destructively measured parameters (total Chl and N) as the dependent variable and a parameter derived from CCM-200 as the independent variable (CCI).They found that polynomial regression models are suitable for non-destructive estimation of total Chl, and the model predicted values were very close to traditionally measured values with a root mean square prediction error (RMSE*p*) less than $0.20 mg \; g^{-1}$ of Chl. In the case of N estimation, a power regression model was appropriate. Ghasemi et al. (2011) estimate positive and linear correlation between CCM-200 data and chlorophyll *a* ($R^2=0.72$), chlorophyll *b* ($R^2=0.85$), total chlorophyll ($R^2=0.90$), and total nitrogen content ($R^2=0.76$) in Asian pear, Mustafa *et al* .(2011) estimated chlorophyll and nitrogen content in Asian pear leaves,), Earl and Tollenaar (1997) found a close correlation ($R^2 = 0.98$) between SPAD readings and maize leaf absorptance in the field.
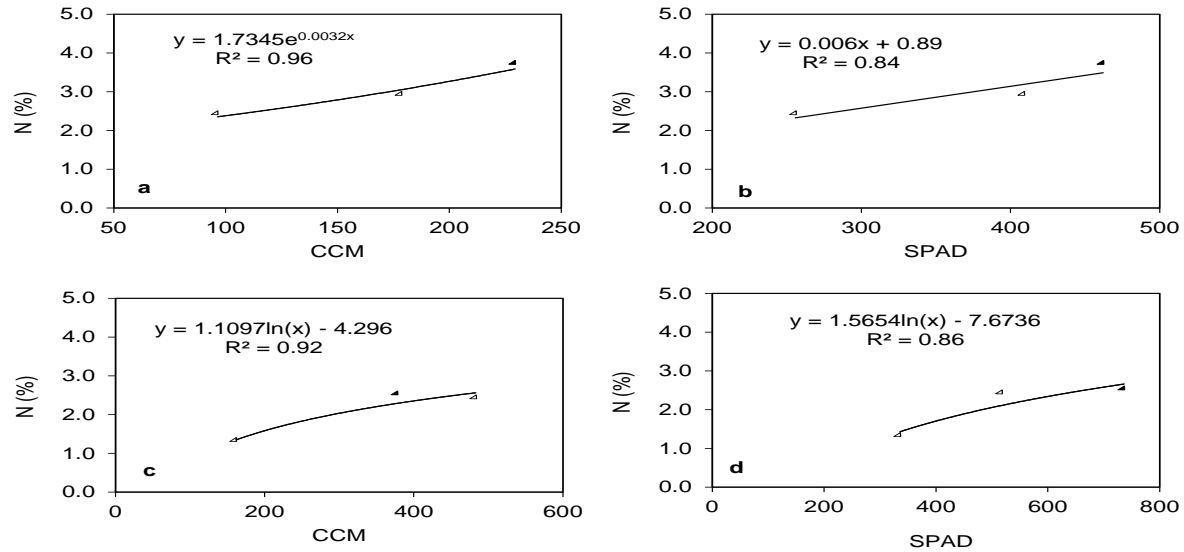
Figure7. Correlations between leaf CCM and leaf SPAD readings of wheat (a and c) and coffee (b and d) and their leaf nitrogen concentration. *Panels 'a' and 'b' represent readings taken at vegetative stage while panels 'c' and 'd' represent readings taken at flowering stages for both crops.*

The strong correlation between SPAD reading and leaf nitrogen concentration were also reported in some other crops (e.g. oil palm) (Law *et al.,* 2014) and rice (Wang *et al.*, 2014). In these studies, it was indicated that leaf nitrogen concentration was also positively and strongly correlated with leaf photosynthetic activities. (AMER, 2005) total Chlorophyll determined analytically, correlated well with nondestructive Chlorophyll meter readings (r^2: 0.72 to 0.97; P< 0.0001) was observed in Citrus sp. Leaves.That is leaf photosynthetic capacity of the leaves is also strongly correlated with SPAD readings indicating the robustness of SPAD and CCM meters for non-destructive and rapid determination of the photosynthetic rate of leaves in the field.

The same logarithmic relationship of leaf nitrogen concentration and CCM-200 was studied by (SILLA *et al,* 2009) and their result show that the CCM-200 provides good estimates of chlorophyll content in the Quercus species studied by randomly sampled 30 leaves from 8–12 trees per species and leaf stage. The best results were obtained with Q. *faginea,* with high coefficients of determination on all sampling dates. The CCM-200 worked satisfactory in the other three species, but performance was more variable, depending on the leaf stage. The relationship between CCI values and chlorophyll values was clearly non-linear, and it was best

explained by the log-log model, as chlorophyll contents showed a tendency to stabilize with high CCM-200 readings.(João Paulo *et al,* 2012) generate mathematical models that are able to report the contents of chlorophyll and carotenoids in the leaves of the castor oil plant and the results indicated that, with the exception of chlorophyll *b*, the ClorofiLOG® 1030 portable chlorophyll meter estimated the concentration of photosynthetic pigments with high precision, thus saving time and the chemical reagents that are typically used in conventional procedures.As they reported, there was a significant relationship between the portable chlorophyll meter measurements and the total chlorophyll that was determined in the laboratory (the classical method), and the model that best expressed this relationship was a linear model, with a coefficient of determination of 0.84 as the result in this work also express.

Panel a: y = 0.5842ln(x) - 1.1657, $R^2 = 0.99$ (axes: N (%) vs CCM)

Panel b: y = 1.3618ln(x) - 6.1823, $R^2 = 0.99$ (axes: N (%) vs SPAD)

Panel c: $y = 1.5451e^{0.0018x}$, $R^2 = 0.82$ (axes: N (%) vs SPAD)

Panel d: $y = 1.9118e^{0.0031x}$, $R^2 = 0.90$ (axes: N (%) vs CCM)
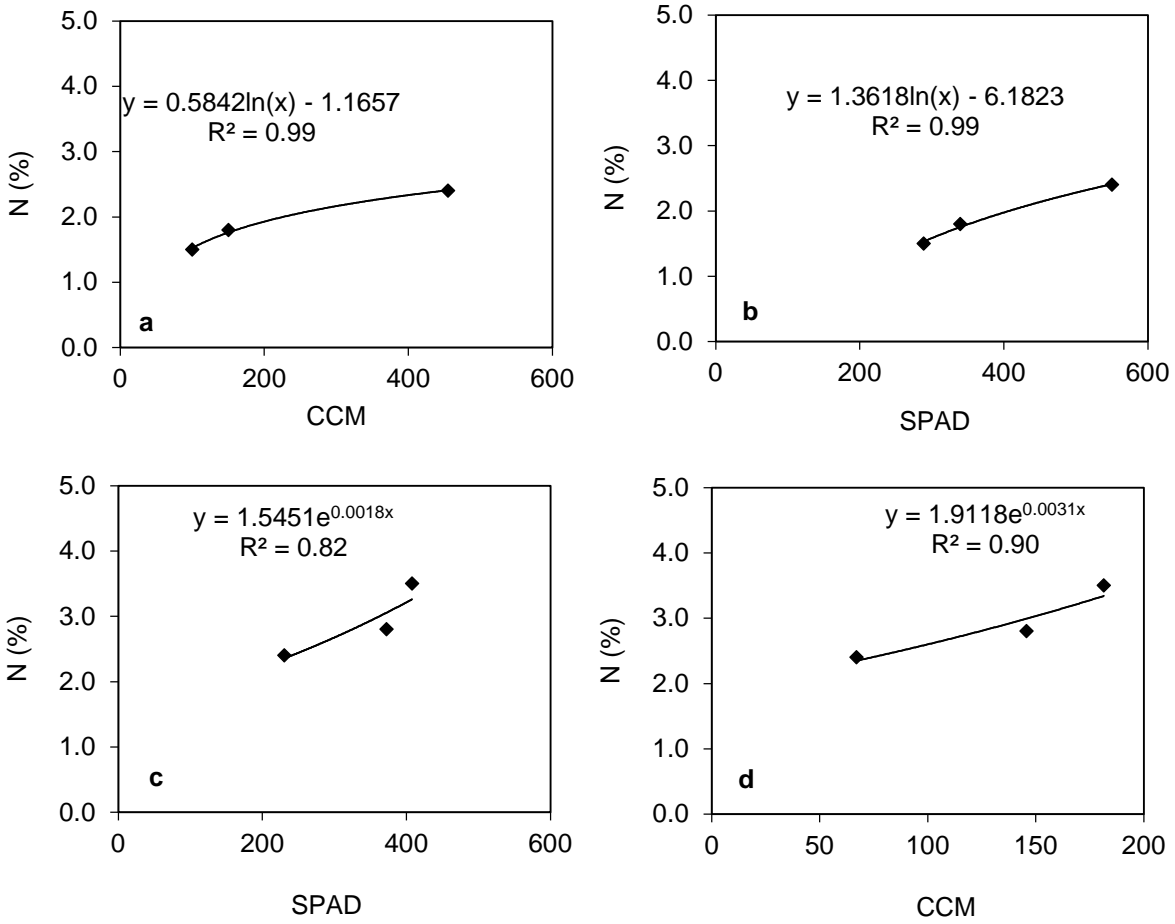
Figure 8. Correlations between leaf CCM and leaf SPAD readings of maize (a and c) and barley (b and d) and their leaf nitrogen concentration*. Panels 'a' and 'b' represent readings taken at vegetative stage while panels 'c' and 'd' represent readings taken at flowering stages for both crops.*

5. SUMMARY & CONLUSION

Chlorophyll content meters (SPAD-502 and CMM-200), despite their inherent limitations, have the potential to enable rapid assessment of the relative condition of leaf chlorophyll content and leaf chlorophyll index in a variety of crop species, both for agricultural and ecological studies. The CCM 200 plus meter and SPAD-502 provided good estimates of chlorophyll index and chlorophyll content for all leaves, dependent of leaf colors within plant phonological stage. The whole result of the research (analysis of variance, mean comparison, means of significant treatment effect at 5% probability level, relationship of correlation coefficient among parameters) were explained the efficiency use of SPA-502 and CMM-200 to determine leaf chlorophyll concentration depending on leaf colors and deep green and yellow green were successful to determine rather than use dark green (medium leaf color).

According to this result, chlorophyll index of CCM reading & total chlorophyll measurement of SPAD readings were closely related to leaf N content in different leaf color at different growth stage. They can be used to monitor the N status of coffee, maize, wheat & barley in this research work and thereby to adjust the rate of N fertilization in order to increase N use efficiency and solve timely adjustment of crop nitrogen status even by side dress or foliar application by leaf diagnosis of SPAD and CCM reading. This indicated that, the purpose of CCM &SPAD in determination of leaf nitrogen content in crop leaf at different growth stage solve the problem of expensive, time consumption , use of chemicals in involved destructive methods of leaf nitrogen determination.

A further benefit of these optical instruments is that they avoid the need to use hazardous solvents, reducing environmental contamination and human health hazards.

Even though the maximization of plant nitrogen-use-efficiency (NUE) has direct impact on increasing crop production, due to the increase of agronomic value of the fertilizer, optimizing the use of nitrogen will reduce the adverse effect on the environment that can be caused by nitrate leaching and nitrous oxide production.

 Accordingly, NUE can be achieved through measurement of the foliar N content of crops during growth. Chlorophyll meter techniques allow fine-tuning N management to field conditions for

the whole field or for management zones established in the field to address spatial variability concerns. This reduces the risk of having yield-limiting N deficiencies and reduces the potential for over-fertilization and possible no contamination of ground and surface water.

In conclusion, a chlorophyll meter can conveniently estimate foliar chlorophyll and nitrogen (N) contents in many species. Farmers and researchers need to use SPAD and also CCM to best estimate the nitrogen content of their crops specially at flowering stage. This will help them to minimize cost and optimize crop production. However, leaf color differences must be considered when using the equipments to directly estimate foliar N contents of the crops from the corresponding SPAD/CCM readings. Further research is required to explore the relationship between foliar N contents and SPAD/CCM readings for other crops.Improving the accuracy of non-destructive N estimations of crops also need to consider the phonological stage of the crops.

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APPENDEX

Table1. Sample of chlorophyll reading for specific leaf color for each crop at different growth stage

	crop type		**maize**		crop type		**wheat**
	growth stage		vegetative		growth stage		vegetative
	chlorophyll reading				chlorophyll reading		
	leaf color	CCM-200	SPAD-502		leaf color	CCM-200	SPAD-502
	DPG				DPG		
1	"	44.1	50.8	1	"	38.2	54.8
2	"	45.7	**55**	2	"	34.8	67.9
3	"	45.8	55.3	3	"	45.7	56.8
4	"	46.6	50.5	4	"	34.7	52.4
5	"	**48.3**	50.4	5	"	39.7	48.9
6	"	42.7	51	6	"	42.3	44.7
7	"	41.9	52.3	7	"	44.2	57.9
8		47.4	118.5	8	"	43.1	58.6
9	"	45.6	57	9	"	32.2	57.4
10	"	40.2	52	10	"	38.7	58.5
average		**44.83**	**59.28**	Average		**39.36**	**55.79**
total		448.3	592.8	Total		393.6	557.9
	DRG	CCM-200	SPAD-502		DRG	CCM-200	SPAD-502
1	"	22.1	38.7	1	"	26.8	56.9
2	"	22.4	49.3	2	"	29.8	47.8
3	"	21.6	44.5	3	"	29.6	49
4	"	21.7	42.5	4	"	26.5	44.6
5	'	19.5	44.9	5	"	22.5	43.5
6	"	19.8	35	6	"	19.6	41.2
7	"	17.8	35.5	7		23.4	38.8
8	"	29.2	39.9	8	"	23.4	36.8
9	"	27.4	39.2	9	"	25.7	34.7
10	"	24.2	37.7	10	"	21.1	29.6
average		**22.57**	**40.72**	Average		**24.84**	**42.29**
total		248.27	407.2	Total		248.4	422.9
	YG	CCM-200	SPAD-502		YG	CCM-200	SPAD-502
1	"	6.4	23.1	1	"	7.8	41.6
2	"	6.3	21	2	"	16.8	40.6

3	"	7.2	22	3		14.7	37.9
4	"	8.2	20.9	4	"	12.6	31.4
5	"	8.7	21.7	5	"	13.6	22.9
6		8.4	23.8	6	"	12.1	12.8
7	"	7.2	24.1	7	"	5.6	10.6
8	"	6.3	22.1	8	"	5.1	11.4
9	"	5.9	20.7	9	"	2.3	9.4
10	"	6.2	25.7	10	"	2.5	7.8
average		**7.08**	**22.51**	**Average**		9.31	22.64

	crop type		**coffee**		crop type		**barley**
	growth stage		**bean b.**		growth stage		**vegetative**
		chlorophyll reading				chlorophyll reading	
	leaf color	CCM-200	SPAD-502		leaf color	CCM-200	SPAD-502
p.r	DPG			P.r	DPG		
1	"	155.6	77.6	1	"	18.7	52.7
2	"	119.6	76.5	2	"	25.8	43.2
3		128.5	73.2	3	"	34.7	46.3
4	"	109.1	75.2	4	"	32.6	51.4
5	"	134.6	78.5	5	"	44.1	52.7
6	"	138.5	77.1	6	"	33.8	55.6
7	"	129.8	78	7	"	34.9	53.4
8	"	138.8	75.1	8	"	19.5	54.6
9	"	127.5	74.3	9	"	12.8	59.7
10	"	159.3	77.6	10	"	8.6	56.8
average		**134.13**	**76.31**	**AVERAGE**		**26.55**	**52.64**
total		1341.3	763.1	TOTAL		265.5	526.4
		CCM-200	SPAD-502			CCM-200	SPAD-502
	DRG				DRG		
1	"	46.8	54.8	1	"	19.3	45
2	"	47.4	56.3	2	**"**	19.9	43.9
3		55.2	53.8	3	"	32.3	52.8
4	"	56.2	54.3	4	"	31.2	51.2
5	"	61.1	58.1	5	"	31.9	56.7
6	"	54.6	52.3	6	**"**	12.7	47.9
7	"	55	53.6	7	"	16.7	49.5
8	"	46.5	54.5	8	"	12	45.6

9	"	52.5	57.4	10	"	7.8	43.2
10	"	52.8	58.3				
average		**52.81**	**55.34**	**AVERAGE**		**20.42222**	**48.42222**
total		528.1	553.4	TOTAL		183.8	435.8
	YN	CCM-200	SPAD-502		YG	CCM-200	SPAD-502
1	"	26.3	31.8	1	"	6.6	27.9
2	"	23.3	27.4	2	"	7.3	28.9
3	"	24	28.7	3	"	10	27.8
4	"	25.6	29.3	4	"	11.2	31.3
5	"	24.8	34.2	5	"	12.8	30.3
6	"	24.9	41.1	6	"	7.5	19.6
7	"	24.3	42.6	7	"	4.6	15.4
8	"	13.3	44.6	8	"	2.5	21.4
9	"	10.4	43.5	9	"	3.2	23.1
10	"	10.9	44.5	10	"	4.2	12.3
average		**20.78**	**36.77**	**AVERAGE**		**6.99**	**23.8**

DPG=Deep green,DRG=Dark Green,YG=Yellogreen,YN=Yello normal

Table 2. The Average Reading Of Ccm-200 And Spad-502 With Nitrogen Analysis By Kjedhal Methods (Summurized Raw Data)

wheat	trtmt	leaf color	CCM-200	SPAD-502	N. content
	T1	deep gr	229.36	462.1	**3.7ml/gm**
	T2	dark gr	178.54	408.98	2.9ml/gm
(veg.st)	T3	yellow gr	96.42	255.92	2.4ml/gm
	T total		**504.32**	**1127**	**9ml/gm**
	T1	deep gr	215.72	444.04	3.3ml/gm
	T2	dark gr	170.92	399.4	2.4ml/gm
(Flow.)	T3	yellow gr	75.2	218.16	1.6ml/gm
	T total		461.84	1061.6	7.3ml/gm
	T1	deep gr	297.44	480.7	2.9ml/gm
	T2	dark gr	195.2	435.6	2.7ml/gm
(maturity)	T3	yellow gr	96.42	230.12	1.7ml/gm
	T total		**589.06**	**1146.42**	**7.3ml/gm**
Coffee	trtmnt	leaf color	CCM-200	SPAD-502	N.cont.
	T1	deep gr.	3781	736.42	2.4ml/gm
	T2	dark gr.	483.24	517.52	2.5ml/gm
	T3	yelloe nr.	161.58	335.7	1.3ml/gm
(b.b stag)	T4	deep yel.	73.86	223.26	1.3ml/gm
	T total		**4499.68**	**1812.9**	**7.5ml/gm**
	T1	deep gr.	1099.06	704.42	2.1ml/gm
	T2	dark gr.	418	490.72	1.9ml/gm
(flow.stg	T3	Yellow nr.	72.46	240.12	1.5ml/gm
	T4	deep yel.	25.6	108.34	1.5ml/gm
	T total		**1615.12**	**1543.6**	**7ml/gm**
maize	trtmt	leaf color	CCM-200	SPAD-502	N.cont.
	T1	deep gr.	417.2	527.7667	4.3ml/gm
	T2	dark gr.	226	407.5	1.7ml/gm
(veg.stg)	T3	yellow	66.43333	208.8333	0.9ml/gm

		gr.			
	trt total		**709.6333**	**1144.1**	**6.9ml/gm**
	T1	deep gr.	455.2333	550.4	2.4ml/gm
	T2	dark gr.	150.4667	339.7333	1.8ml/gm
(flow.st)	T3	yellow gr.	100.4333	289.0333	1.5ml/gm
	trt total		706.1333	1179.167	5.7ml/gm
barley	trtmt	Leaf color	CCM-200	SPAD-502	N.cont.
	T1	deep gr.	259.62	434.24	9.6ml/gm
	T2	dark gr.	166.4	403.42	3.8ml/gm
(veg.stg)	T3	yello gr	78.74	252.58	2.6ml/gm
	trmt total		**504.76**	**1090.24**	**16.1ml/gm**
	T1	deep gr.	181.58	408.06	3.5ml/gm
(Flow.)	T2	dark gr.	145.84	372.56	2.8ml/gm
	T3	yello gr	67.12	230.82	2.4ml/gm
	trmt total		**394.54**	**1011.44**	**8.7ml/gm**

Table 3. ANOVA (Analysis Of Variance) for CCM-200& SPAD-502 different crops *STAGE I (vegetative/)*

CROP TYPE	CCM-200 reading(CCI)		SPAD-502 reading(LCC)	
	Mean values	Pr> F	Mean values	Pr> F
Wheat	57299.408**	<0.0001	22499.50867**	0.0010
Maize	94396.7778*	0.0469	120211.0000**	0.2167
Coffee	40909.84867*	0.0007	47252.4446**	0.0002
Barley	1479541.453**	<.0001	201293.5340***	<.0001

STAGE II (Flowering)

Table 4. ANOVA (Analysis Of Variance) for CCM-200& SPAD-502

Crop type	CCM-200 reading(CCI)		SPAD-502 reading(LCC)	
	Mean values	Pr> F	Meanvalues	Pr> F
Wheat	25729.01067**	<0.0001	71561.1440**	<.0001
Maize	110634.5478**	<.0001	57631.7344*	0.0015
Coffee	1364290.146**	<.0001	270035.4500**	<.0001
Barley	412605.2667*	0.0175	273760.4667**	<.0001

**= highly significant difference,*significance difference