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Review

Air pollution and their mitigation measures in Brazilian poultry production

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The Brazilian broiler industry is the most prominent branch in farming, due to its high levels of productivity. However, there is a growing concern about the environmental damages caused by such an activity, in special the atmospheric air pollution, as a consequence of the high volume of residues generated and the damages made to the environment. Air pollution, despite being most of the time detected by undesirable odoriferous substances, can be caused by numerous atmospheric pollutants such as particulate matter, odorless gases, non-volatile compounds, amongst others. This negatively impacts the health of both humans and animals, as well as of the surrounding ecosystem. Broiler production can be understood as a range of steps necessary to poultry meat production. Hence, their emissions can be computed all the way from the grain production, the feed fabrication, the poultry farming to, finally, the slaughtering and recycling of animal based products. As the poultry chain is well-segmented in its functions, it is necessary to establish and quantify the real impacts caused by its sectors and define mitigation controlling measures in regards to air pollutants.

Key words: Air quality, atmospheric pollutants, control, environment, productivity.

INTRODUCTION

Poultry, in the recent Brazilian economy, had an increase of productivity that is intimately associated to the technological advance and, mostly, to the progress of the industrial sector. However, as in all agricultural activities, this sector generates a large amount of pollutants to the environment, in particular air pollutants (Oliveira and Biazoto, 2012). Poultry production systems generate harmful emissions to the atmospheric air, from food and

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supply production, such as soybean and corn, for example, to the slaughter industry, prior to the subsequent marketing of the meat. Emissions to the environment range from undesirable odors, due to the concentration of sulfuric gas, ammonia and methane, to the suspension of particulate matter and dust (Meda et al., 2011; Copeland, 2014); the discharge of high volumes of carbon monoxide and carbon dioxide produced during the periods in which heating systems are active, must be considered.

Furthermore, factors such as the improper system management and failures in computing all the balance of the released gases for the manufacturing of the supplies can contribute to the underestimation of the polluting potential of the poultry farms. Therefore, it is necessary to study the real impacts that cause poultry production on the environment. According to Valipour et al. (2012), without the exact information about quantity and quality of pollution sources, reducing or eliminating industrial pollutions are not possible.

Hence, the aim of this review article was to identify the types and means of air pollution associated to the poultry production in Brazil and to list a set of mitigation measures to reduce the impacts caused by their emissions.

AIR POLLUTION IN THE POULTRY CHAIN

Atmospheric pollution can be understood as the air contamination by insertion or temporary maintenance of substances that are normally absent in natural air composition or are present in amounts proportionally superior than the natural (Barrenetxea, 2003). The unpleasant odor is the most sensible form of pollution to humans and becomes a difficult problem to deal with when it concerns a reasonable number of people, intervening in their well-being (Licco, 2002). However, several other pollutants can contaminate the atmospheric air without our perception, as the presence of particulate matter and odorless gases in high concentrations.

Poultry production in Brazil is a well-segmented sector with clearly defined functions throughout its several processes. The impacts related to air quality must be considered in order to determine its potential as a pollutant source and waste generator. All steps that directly or indirectly contribute to the production and/or processing of any supply or natural property that will be subsequently used in the sector can be considered as part of the poultry chain. Therefore, in relation to air quality, the main stages that represent any environmental impact form are these: grain production, feed factories, growing barns, and the slaughtering industry. This article will approach air pollution sources, as well as the proposals and suggestions of mitigation measures of control to reduce the environmental damage caused by the poultry production chain.

AIR POLLUTION FROM THE GRAIN PRODUCTION TO THE FEED MANUFACTURING

Broiler food and feed production is constantly modernizing and increasing its productivity rates. However, the same time, there is a significant contribution to the generation of atmospheric pollutants in the cultivation and management of the cultures utilized in feed manufacturing. Nearly the totality of broiler concentrates in Brazil is formulated from two basic ingredients: corn, a great energy source, and soybean meal, which contributes with high quality proteins and good availability of amino acids (Opalinski et al., 2006). These two foods have high digestibility when compared to other ingredients used in the formulation of broiler feeds (Olukosi et al., 2007). Furthermore, from the nutritional point of view, they complement each other by supplying limiting amino acids, lysine, and methionine essential to growth and protein synthesis of the animal organism, increasing the productivity performance (Bertechini, 2012).

In order to grow maize and soybean, appropriate soil preparation, use of chemical fertilizers and the application of the required agrochemicals are strictly necessary. The environmental impact in due to the mixing and preparation of the soil happens is due to the emission of particulate matter, mainly silt (Huggins et al., 2007; Olson et al., 2014). Emissions of particulate soil is the second largest source of dust, behind the sea salt. When the soil is airborne into the atmosphere, it can be transported to and get in contact with human body. This may result in problems such as eye irritation, respiratory disorders, lung disease, and an increased risk of lung and skin cancers (Sing and Sing, 2010). With concern to the use of chemical fertilizers, it is important to remember that they are derived, mostly, from petroleum and that their production releases undesirable pollutants, such as nitrogen oxides and sulfur dioxides. These products, when applied to the soil in excess can cause environmental adversities, such as acid rain, also in addition to causing dependence on fossil energy mix.

In regards to the agrochemicals applied on cropping systems, there is no doubt that they can damage human health and to the local biodiversity where the farming is located (Silva et al., 2005). This is due to contamination and percolation risks of these compounds, reaching water bodies but also volatilizing to the in atmosphere. Employees working directly with the soil under these conditions are mostly susceptible to respiratory diseases (Pignati et al., 2014). There is also an adverse effect from the contamination and cross-infection in humans due to the consumption of livestock products which are fed with contaminated grains (Fiocruz, 2011). The risks and effects may be even more pronounced due to a higher dispersion of pollutants to natural biomes and also to the usage of inadequate and inappropriate techniques of applying agrochemicals. Amongst the most susceptible segments of the population are the children, who once

exposed to contamination may develop of cancer (Curvo et al., 2013).

Growing maize also leads to emission of particulate matter to the environment via pollen, which can cause allergies and predisposition to other respiratory diseases (Solé et al., 2008). It is also important to emphasize that, in air pollution, all damage caused by the emission of a particular air pollutant, be it a gas or particulate matter, depends on the concentration level and exposure time to the contaminant. Therefore, it is possible that certain cultivation techniques applied to extensive monoculture fields will result in a potentially relevant pollutant source in agriculture.

MITIGATION MEASURES OF AIR POLLUTION IN GRAIN FARMING

As controlling measures for pollutant emissions to the air, originated from farming techniques for the production of grains, one example is the appropriate application of fertilizers. With the rational use and the more widespread application of organic fertilizers, along with the adoption of techniques such as direct planting, pollutant and particulate matter emissions can be significantly reduced (Kibblewhite et al., 2008).

Another measure that has been getting more popularity, but that is still very controversial, is the use of transgenic species, which are organisms that present more resistance to plagues and that require relatively smaller amounts of agrochemicals, in addition to yield higher productivity per area (Vallero, 2014). However, there is an urgent need for developing detailed studies on the crossover effect and the permanence time of the agrochemicals in the body of the animals that will consume the processed grain in the feed. In addition, the possibility of changing gene expression regarding the physiological activities, which may be transferred to humans, is still to be evaluated. The principle of labelling transgenic food must be adopted in case of doubt or lack of scientific knowledge about its effects in human health, animal welfare and in the protection of the ecosystems (Nodari and Guerra, 2003).

AIR POLLUTION IN GRAIN FACTORIES

After being harvested, the grains proceed to beneficiation and are then sent to the feed factories. In the case of soybeans, it usually goes through a crushing process for oil extraction and then is toasted. This step is needed because raw soybean has anti-nutritional factors, such as enzymes inhibitors and allergic substances, which may result in an inappropriate use of nutrients by the animals that consume it (Xavier-Filho and Campos, 1989). Corn, in its turn, arrives at processing plant as a whole grain, passes through milling and subsequent crushing to acquire appropriate grading. These processing factors, in addition to improving the food digestibility, increase its nutritional value as well and contribute to the improvement of the homogenization of feed and of the ingredients added to the ration.

However, the processes of crushing, mixing and incorporating others ingredients, such as vitamins, minerals, amino acids, anticoccidials, and growth promoters, among others, emissions of a great amount of particulate matter is involved. During the manufacturing process, some important aspects called are checked, such as dust, the emission of gases and undesirable odors. Even though dust is one of the most present pollutants during the process, it is confined to the internal storage step of crushing and dosing of the ingredients, including the management of silos and sacks of grains and the granular ingredients.

The risks of air pollution in this kind of activity reach mainly factory employees, but also affect those who live in the surrounding area. Main pollutants involve the emissions of gases, particulate matter, and undesirable odors, as well as of course, the solid and liquid wastes, which need to have an appropriate destination in order to avoid environmental contamination. Hence, the adoption of good manufacturing practices with a set of principles, rules and procedures is crucial. These for practices may involve proper food handling, involving the process as a whole and aiming to ensure the production of food that is safe and free of contamination by pathogenic microorganisms, toxins, chemical, and physical products, in addition to reducing the environmental impacts (Pilecco, 2011).

MITIGATION MEASURES OF AIR POLLUTION IN FEED FACTORIES

In the case of the feed factories, several measures can be taken to reduce emissions of air pollutants. One of the most significant is using personal protective equipment (PPE), which is an effective way to guarantee the health and safety of workers (Pilecco et al., 2012). As indispensable examples of PPEs are the use of filter masks and goggles to avoid inhalation of undesirable particles and contact with the eye and the use of appropriate clothing, gloves and footwear to reduce the adherence of these particles onto the skin. After all, the length of the working day of an average of eight hours, and the time of exposure to the pollution generated in factories may be high, which, depending on the concentration of harmful compounds, may represent irreversible damage to health.

Thus, the production management, in this case, must propose some effective measures, with clear objectives and well-defined proposals. Subsequently, the producer will be able to determine, along with the regulatory agencies, if their environmental actions were effective and practice. As an example of measures related to the generated pollutants in animal feed production factories,

the following are listed:

i. Smoke, odors and gases: the use of air scrubbers to prevent the release of these pollutants has been suggested.
ii. Dust: can be treated through the adoption of collectors (e.g: bag filter), which work as a cyclones and are usually positioned near the largest dust sources, such as the dosing and the blending rooms. Besides, attention should be paid so that the equipment receives appropriate monitoring and constant maintenance, since it can be the cause of leaks. Another way of avoiding particulate material is the appropriate handling of the sacks, which are usually commercialized or sent to recycling. The use of indoors biofilters combined with mechanical ventilation and exhaust for the renovation and improvement in air quality is also indicated (Nicolai and Lefers, 2006).

Even though the objective is always to get the optimum profits by improving productivity, the measures for controlling and reducing pollutants emissions of whatever form or nature can present themselves as a way of adding value to the products.

AIR POLLUTION IN POULTRY BARNS

Envisioning the increase of productivity, the enforcement of sanitary control, the ease of handling, and the optimized environmental control, the poultry farming has significantly increased in intensity. However, the confined growing of animals, in spite of being very productive, presents some disadvantages in relation to the emission of pollutants which must be discussed and taken into account.

Broilers are usually reared in confined systems, on floors covered with a material of specific characteristics, commonly known as litter. The litter can be composed different materials usually easily acquired in the region, are at a low price rate, having good absorption capability and no risk to the health of the animals. The main used materials are shavings, coffee husks, peanut hulls, rice husks, dry grass, and chopped corncob, among others (Garcia et al., 2012). Due to the usually practiced high stocking densities, the broilers have limited mobility, moving about exclusively for the purpose of feeding and resting. In addition, at the end of the cycle, approximately 2.19 kg in natural matter of waste are produced per animal (Santos and Lucas Jr., 2003). From poultry waste, gases and undesirable odors are released which, when reaching high concentrations, are harmful to the animals, farm workers, and people living nearby.

ATMOSPHERIC POLLUTANTS FROM POULTRY HOUSES

Air pollutants can be classified into dust, smoke, mist, moisture, vapors and gases, and carry biological materials such as pollen, fur and microorganisms (CETESB, 2013). Besides, the exposure time and concentration of pollutants inside poultry houses may be associated with the development of respiratory diseases in birds and humans (Nääs, 2004). The particulate matter emitted by poultry houses may contain pathogenic microorganisms in suspension, such as the virus for New Castle's disease, the avian-influenza virus, *Escherichia coli*, *Salmonela* sp. and campylobacter (Cambra-López et al., 2010).

Thus, it has been demonstrated that dust and microorganisms contribute to the development of respiratory diseases, and their emission levels are mainly related to improper management. Baêta and Souza (2010), mentioned that the dust particle size inside the facilities range between 1 to 150 µm, while its production may reach up to 54 mg/bird/day. Hinz and Like (1998) claimed that the dust concentrations are larger in the winter, since the curtains are usually closed in this period to provide poultry with greater thermal comfort.

As for the gaseous pollutants that can be found inside poultry barns, they are mostly harmful, since they may cause direct harm to the health of humans and animals. When present in high concentrations, they act in the respiratory tract and promote secondary effects with systemic reactions in the organism after their absorption (Kampa and Castanas, 2008). Gases and vapors concentrate in a heterogeneous pattern inside the poultry houses. These pollutants have the tendency of moving both by diffusion and convection, precipitating according to their respective molecular weight (Baêta and Souza, 2010).

The most significant gaseous pollutants in the case of poultry houses are ammonia, carbon dioxide, carbon monoxide, methane and hydrogen sulphide (Barrasa et al., 2012). Out of these gases, ammonia is the main pollutant affecting the health of animals and workers (Menegali et al., 2012). Complications from NH_3 exposure in animal rearing facilities have long been recognized. These include respiratory disease (exposure 200 ppm, Anderson et al., 1964), eye damage (exposure 50 and 75 ppm, Miles et al., 2006), inefficient feed conversion (exposure 100 ppm, Charles and Payne, 1966), and decreased weight gain (exposure 50 and 75 ppm, Miles et al., 2004; exposure 50 ppm). More recently, environmental issues related to gaseous emissions from livestock barns are becoming a concern for the animal production industry (Moore et al., 2011) due to the possibilities of compromising terrestrial bio-diversity, inducing aquatic nutrient enrichment, and deteriorating air quality (Mukhtar et al., 2003; Miles et al., 2013).

Ammonia is water-soluble and lighter than air and, for this reason, can be absorbed by dust particles, litter fragments and through animal mucosa. Lima et al. (2011), claim that the concentrations of ammonia in poultry houses are usually around 20 ppm. The type of ventilation, whether natural or mechanical has an important influence on the dispersion of pollutant gases

and air exchange inside Brazilian poultry barns (Mendes et al., 2014). The highest concentration of this gas is in the height of 50 cm, at the level of the birds, forming an undesirable microenvironment as a product of the microbial fermentation of excreta (Ferreira, 2010). When in concentrations higher than tolerable, ammonia causes primarily irritation of the mucous membranes of eyes and respiratory system in birds and later, after falling into the bloodstream, has a toxic effect over the physiological metabolism, leading to a decrease in ration consumption and reduced weight gain, therefore interfering in the well-being and health of the broilers (Kilic and Yaslioglu, 2014). Alencar et al. (2002) proposed that tolerable exposure levels to workers are around 25 ppm for an 8 h working day, but for smaller workloads, it can be up to 35 ppm.

Jones et al. (2013) identified significant ammonia emissions upwind in poultry houses, causing damage and corrosion to the facilities close to them. In long term, emissions lead to a great amount of ammonia in the bodies of water, provoking an effect known as “blooming of the algae” (Wiegand et al., 2011). As a consequence, an excessive development of algae such as the diatomaceous and the cyanobacteria takes place, which are responsible for threats to the human health and to the surrounding environment. Examples of health threats of ingestion of diatomaceous and cyanobacteria are: damages to the neurological system and to the liver, irritation of the skin, allergies, respiratory diseases, and increased mortality of fish and other organisms (CETESB, 2013). Despite all the deleterious consequences of its excessive emissions to the environment, Brazilian legislation does not currently regulates emissions of ammonia nor incentivizes the adoption of effective measures to control its spread.

The acceptable levels for carbon monoxide for animals and humans, in turn, are in the order of 10 and 50 ppm, respectively (Wathes, 1999). These values are for 8 h working days and continuous exposition of the animals during the full production cycle. This pollutant is toxic, odorless and is present in the facilities as a product of incomplete combustion of equipment used for heating in the initial phase of bird lives and also due to improper ventilation (Nääs et al., 2007). Carbon monoxide causes, in birds, intoxication by the cells’ inability to carry oxygen, competing with carbon and causing hypoxia with the consequent death of animals that are exposed to elevated concentrations of this gas (WHO, 1999).

Carbon dioxide is naturally present in poultry barns. Its concentrations inside the facilities are affected by the metabolic aspects of the animals and to the biological processes occurring in the microenvironment (Hellickson and Walker, 1983). The combustion of fuel used to heat the air up in the initial phase is another factor that contributes to the air pollution inside the facilities. CO_2 concentrations around 600 to 4000 ppm do not threaten animal health; however, they increase of the respiratory rate, consequently increasing heat production (Alencar et al., 2002). The increased heat production caused by CO_2 accumulation will contribute to decreasing the productive performance. In case of continuous exposure, CO_2 levels of up to 3000 ppm are adopted for birds and 5000 ppm for workers with 8 h working days (Wathes, 1999).

The concentrations of methane and hydrogen sulphide inside the poultry houses are relatively low (Nääs et al., 2007). Their effect is more pronounced at the stage of disposal and improper handling of wastes. This is due the typology of the houses used in Brazil, which are usually open, favoring the air renewal and the dilution of pollutants. However, with the use of mechanically ventilated systems, such as dark houses, in order to increase the productivity, the hydrogen sulfide gas has been shown to be a serious problem. Hydrogen sulfide gas is formed by bacterial reduction of sulfate and the anaerobic decomposition of sulfur-containing organic compounds present in manure under (Arogo et al., 2000). The presence of this pollutant has been responsible for many animal and human deaths (Donham et al., 1982). Tolerable concentrations of hydrogen sulfide for broilers must be less than 2 ppm in the first week of life, and be between 3 and 6 ppm in the 4^{th} and 6^{th} weeks. High levels of this gas, may adversely affect the performance and quality of broiler meat, resulting in economic losses (Wang et al., 2011).

As an effective way to minimize the concentration, the accumulation, and the production of these gases, the company can carry on the proper treatment of residues through composting and anaerobic biodigestion (Kelleher et al., 2002).

MITIGATION MEASURES TO REDUCE AIR POLLUTION IN POULTRY HOUSES

The accumulation of gases inside poultry facilities represents the main factor of air pollution in the production cycle. The causes for decreasing air quality in these houses are not specific. Instead, the cumulative effect of some determinants such as ventilation, stocking density, age, the time of the year, adequate management of wastes and humidity excess. Besides impacting the performance of the animals and the health of workers, these factors affect the air quality of residents adjacent to the polluting sources. Therefore, they may cause discomfort and inconveniences, setting the poultry activity as a villain to the socioeconomic progress of the region where it is installed.

Since the causes of the decrease in air quality are diverse, the main proposals of mitigation measures to reduce the impact caused by its respective factors are listed as follow:

i. Stocking density: the larger the density of the poultry stocking is, the higher the dust formation and particulate material dispersion inside the facility will be. It is recommended that the density be of 12 to 14 broilers/m^2, so the animal activity will not cause excessive dust to

become airborne or waste of ration, in addition to easing the maintenance of flock uniformity. Mendes et al. (2012) has demonstrated that lower stocking densities in laying hens also allow for reduced emissions of NH_3, become with fewer birds occupying the same floor area, the manure will dry out faster, leading to reduced microbial activity responsible for ammonia emission. By using larger densities, more attention should be given to the handling and, especially to the air-conditioning equipment and the renewal of air inside the facilities.

ii. Ventilation: the ventilation, positive, negative, lateral or in tunnel, aims to dilute the pollutants inside poultry houses and drag them out of the installation, decreasing the undesirable concentrations of pollutants. It is recommended that the wind speed inside the facilities be between 2.5 to 3.0 m/s.

iii. Air and litter humidity: the air humidity must be in the optimal rate of 50 to 70% in order to ease the dispersion of pollutants and the exchanges of the animals with the environment in case of environmental stress.

iv. Curtains: attention must be given to the adequate handling of curtains, ensuring that they are lowered during the warmest hours of the day and in accordance with the time of the year. Even more attention should be paid during the winter period, where curtains are usually raised in order to favor the thermal comfort of the animals.

v. Use of natural barriers: the use of natural barriers is highly recommended, they include trees and bushes, in order to isolate the farm without impairing the natural ventilation.

vi. Farm isolation: the poultry house, if possible, must be isolated from human inhabited areas.

vii. Adequate handling of dead animals: it is estimated that mortality ranges from 3 to 5% in poultry houses. Dead animals must be handled properly. There are two carcass elimination measures that can be adopted in order to reduce air pollution: the composting of dead animals and the use of septic tanks for disposal. These two measures envision the non-emission of methane and odors. The compost of dead animals mixed with the litter material, can be used as an organic fertilizer.

viii. Control of ammonia emissions and odors: in spite of this sort of control not being currently used in Brazil, according to Mostafa and Buescher (2011), the use of dry filters attached to cyclones in closed poultry houses is a very good alternative to mitigate ammonia emissions. Another option is to treat the emissions through electro-chemical oxidation, by “disnitrifying” ammonia into gaseous dinitrogen by using hyperchlorous acid; however, the cost of this process is high (Bejan et al., 2013). In these mechanisms, the use of biofilters in closed sheds allows the processing of generating harmful gases at the end of the process, carbon dioxide, water, minerals, volatile compounds and microbial biomass (Kafle et al., 2015).

ADEQUATE HANDLING OF POULTRY WASTE AS A MEANS TO REDUCING THE PRODUCTION OF ATMOSPHERIC POLLUTANTS

At the end of the growth out cycle, the producer is left with large volumes of waste, including the litter volume used for the lodging of chicks one day of age until slaughter. The litter contains plant nutrients, such as N, P, K and trace elements, such as Cu, Zn and As, pesticide residues, pharmaceuticals such as coccidiostats, endocrine disruptors and microorganisms (Bolan et al., 2010). Moreover, it may also contain pathogenic viruses such as avian influenza (Reis et al., 2012). This material of heterogeneous composition possesses great pollution potential and produces large quantities of CH_4 and CO_2 during the decomposition process. Therefore, measures and alternatives that reduce this undesirable impact are extremely necessary. The practice of composting and anaerobic biodigestion is becoming more popular and presenting good results in the reduction of pollution impacts. Moreover, composting yields excellent organic fertilizers and useful energy that can be used in the property (Kelleher et al., 2002).

COMPOSTING AS A MITIGATION MEASURE FOR AIR POLLUTION FROM BROILERS WASTE

The composting process is an ancient technique that consists on the biological decomposition of complex organic compounds into simpler molecules, with the mineralization of elements desirable for organic agriculture. It is an aerobic process, in which straw is mixed with the waste until a ratio of carbon:nitrogen of around 25:1 is established; the biological degradation process usually lasts for approximately 90 to 120 days (Fialho et al., 2005). Aerating poultry litter compost is very important because it enables the elimination of salmonella (Bodí et al., 2013). At the end of the process, a nutrient-rich organic fertilizer will remain; which will release carbon dioxide to the environment instead of methane, which is 21 times more harmful to the atmosphere. Despite being a good alternative for the waste treatment and reduction of the pollutant potential of waste, the composting process still has a few disadvantages. A considerable amount of area is required for the disposition of the waste and for the constant inversion of the composted windrows.

ANAEROBIC BIODIGESTION AS A MITIGATION MEASURE OF AIR POLLUTION

The anaerobic biodigestion is a natural biological process in which organic matter is reduced to methane in environments free from oxygen (Chen et al., 2008). It consists of four phases or stages of bacteriological

decomposition of organic matter: hydrolytic, acidogenic, acetogenic and methanogenic. Anaerobic biodigestion provides a variety of benefits. The odors are significantly reduced or eliminated; a liquid relatively clean for washing and irrigation is produced; the pathogenic agents are substantially eliminated in the liquid and solid products; and reduced emissions of greenhouse (Burke, 2001).

The biodigestors are yet important to the rural sanitation, since the anaerobic digestion process promotes the reduction of organic load, the reduction of solids and the reduction of pathogenic microorganisms present in the effluents. Besides stimulating the recycling of the organic matter and of the nutrients, they enable the sanitizing of the facilities where animals are raised, promoting the treatment of its waste, reducing incidence of flies and unpleasant odors (Bolan et al., 2010).

AIR POLLUTION IN POULTRY SLAUGHTERHOUSES

Poultry slaughterhouses produce a considerable quantity of odors and of toxic pollutants, such as the emissions from combustion in boilers, which are harmful to the environment and to the adjacent population. This is due to cold stores having a sector that carries out the recycling of the material of animal origin attached in their working plant (Licco, 2002).

The main impacts of slaughterhouses are the residual waters and the toxic effluents generated from the humid transformation of residues. In dry transformation, there is air pollution, by non-condensable gases and vapors such as odors, derived from the recycling processes and from the transformation of matter of animal origin. Miller (1975) notes that the main impact of slaughterhouses is the unpleasant odor derived from the rendering plants. Furthermore, about 80 to 85% of the total energy required in a slaughterhouse is produced by the combustion of fuel in the boilers at the industrial unit (thermal energy – vapor and hot water). The main emitted pollutants are the sulfur oxides, nitrogen oxides and particulate matter (CETESB, 2008).

In Brazil, slaughterhouses with precarious installations and hygiene conditions that do not have a system for treating and final disposal of residues are commonly found; this makes the subject of utmost importance. In addition, the Brazilian meat is exported to various countries that usually demand, amongst other conditions, the treatment of the pollution caused by the activity. Of the total amount of broiler chicken meat produced in the country, about 30% is exported to approximately 142 different countries (ABPA, 2014).

MITIGATING MEASURES FOR THE REDUCTION OF AIR POLLUTION IN POULTRY SLAUGHTERHOUSES

The reduction of odor generating sources in slaughterhouses is reached, mainly by maintaining the hygiene level of the environment, and avoiding the accumulation of unpleasant materials. For the treatment of the odor generating sources, biofilters, air scrubbers and filtration with activated carbon can be used (Seth, 2005). In most cases, an efficient solution is obtained with the combination of various methods (Barros, 2007). Oliveira (1990) and Sinhorini (2013) indicate that the intensity of the odor in the facilities of a rendering plant is directly related to the time elapsed from the slaughter of the animal until the instant of residue processing. Therefore, it can be concluded that the shorter the residues are handled, the smaller the amount of undesirable odor emitted. Another important aspect is the careful selection of the location of the slaughterhouse. When designing the slaughterhouse, areas with superior topography must be prioritized, the dominant wind directions must be observed and attention must be dispensed to the distancing from populated areas (Dias, 1999). Regarding odor reducing strategies, the use of biofilters has shown to decrease emissions of odor caused by hydrogen sulfide by around 95% and about 80% by ammonia. However, one must be able to control humidity (30 to 70%) and temperature (38 to 58°C) of the biofiltration process (Nicolai et al., 2006).

In order to control atmospheric emissions, the following is suggested: the particulate material from the boilers that use wood as fuel must be treated through cyclones, through electrostatic precipitators, through gas scrubbers or bag filters (Ferreira et al., 2002). The boilers that run based on fossil fuel (combustible oils) must have their emissions treated by adsorption in activated carbon, through air scrubbers towers and incineration (Oliveira, 1990; Licco, 2002; FEAM, 2010). Brazilian legislation has set standards and resolutions which established maximum limit of pollutant emissions at national level.

Table 1 lists the major air pollutants emitted by poultry production industry, related to its emission source and the main mitigating control measures aiming at improving air quality and reducing threats. To evaluate the control of emissions and success of mitigating actions to control, some techniques have been used: life cycle assessment (LCA) and environmental flow diagram (EFD). The technique of life cycle assessment aims to understand and evaluate the magnitude and significance of the potential environmental impact of a production system (Goedkoop et al., 2008). The use of LCA practice in the production of broilers is a technique able to infer the polluting capacity of generation system as well as identify their sustainability (Boggia et al., 2010); and can be adopted in poultry production. Environmental flow diagram can also be adopted to assess the impacts of poultry. The EFD is based on the power reference system and process flow diagram for a particular industry sector (Valipour et al., 2013). EFD has been applied in civil and industrial construction quite successfully. Occurs through the use of software by companies in order to encode the pollutant sources in the receiving

Table 1. Main air pollutants emitted by the production of broilers, the major emission sources and mitigation measures for improving air quality and reducing environmental impacts.

Chain steps	Air pollutants	Emission sources	Damages	Mitigating measures
Grain farming	*Particulate matter *Agrochemicals in suspension *Excess pollen	*Inadequate soil management *Excessive application of pesticides and fertilizers	*Health problems *Air pollution, soil and water by pollutants *Uncomfortable	*Rational agricultural practices such as tillage *Precise use of fertilizers and pesticides in crop management
Feed factories	*Particulate matter *Smoke, odors and gases	*Inadequate management of the ingredients	*Health problems *Uncomfortable	*Manpower training *Use of PPE *Gas scrubbers and filter collectors for emissions of harmful gases and dust, respectively.
Production of broiler chickens	*Dust and microorganisms *Ammonia, carbon dioxide, carbon monoxide, methane and hydrogen sulphide *Odors	*Inadequate management of waste *Poor ventilation *Generously sized equipment	*Health problems *Uncomfortable *Decreased productivity *Development of disease *Contamination of natural resources	*Proper management of waste (composting and biodigestion) *Good ventilation management practices, humidity and density of animals *Use of biofilters in the case of closed sheds
Poultry slaughterhouse and recycling of animal products	*Dust and odors *NOx e SOx *CO	*Industrial boilers and other recycling processes *Inadequate management of waste	*Health problems *Uncomfortable *Air pollution	*Effective management of animal waste *Biofilters, cyclones, through electrostatic precipitators, through gas scrubbers or bag filters

NOx = nitrogen oxides; Sox = sulfur oxides; CO = carbon monoxide; PPE = personal protective equipment.

environment and then determine the energy optimization solutions and reduce environmental pollutants (Valipour et al., 2013). In this way, it can be used successfully in the poultry industry to mitigate the pollution generated.

FINAL CONSIDERATIONS AND CONCLUSIONS

The decision-markers of the poultry production activity must should be conscious of its pollutant potential and search for alternatives in order to minimize their impacts in the environment. On the other hand, consumer increasingly demands for products that are environmentally correct and safe. The processes of rearing broiler chickens and products of animal origin must have an adequate handling of its residues and implement production alternatives that are less aggressive to health and environment, adjusting the production to the current conditions and environmental laws.

Emissions of pollutants from the poultry industry, starting from the production of grains for the fabrication of rations until the slaughter of the animals is significant. The producer and the processing industries must receive all the support necessary in order to implement handling practices and new technologies to mitigate the impacts caused.

The mitigation of atmospheric pollutants in all the steps of the poultry industry is possible due to the existence of emission control methods that have proven to be effective. The reduction of pollutant emission must be faced as a routine procedure, as a requirement of the productive process and not as an obstacle that can be neglected by the agents of the industry.

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Conflict of Interests

The authors have not declared any conflict of interests.

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Influence of lactation stage, delivery order of calving and genetic group on milk quality

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Milk production and quality are influenced by environmental factors such as nutrition, genetic factors such as breed and physiological. The aim of the present study was to evaluate the influence of lactation stage, delivery order and genetic group on the quality of milk from crossbred cows 3/4 Gyr, 7/8 Gyr and 15/16 Gyr. Fresh milk samples were collected from crossbred cows. Fat, protein, lactose, non fat dry extract (NDE) and somatic cell count (SCC) of milk according to lactation stage, delivery order of calving and genetic groups were evaluated. The experimental design was completely randomized. Tukey test at 5% probability was used. Animals at the final third of lactation (201 to 305 days) showed higher fat, protein, NDE and SCC levels when compared to animals of other lactation stages. First delivery animals produced milk with higher content of solids compared to animals with 2 deliveries and above three deliveries. First calving the animals produced milk with higher solids content compared to animals with two calving and above three calving. Fat, protein and NDE results of fresh milk were consistent with limits established by the Brazilian legislation for milk quality, regardless of lactation stage, delivery order of calving and genetic group. Milk SCC was higher in cows in advanced lactation stage, The results were however below limits required by law. 3/4 crossbred Gyr cows showed higher SCC, with average values above limit established by current legislation.

Key words: Somatic cell count (SCC), fat, non fat dry extract (NDE), fresh milk.

INTRODUCTION

Milk production and quality are influenced by environmental factors such as nutrition, genetic factors such as breed and physiological factors such as age at first delivery and delivery order of calving (Galvão Junior et al., 2010).

Animal age has a direct influence on milk quality and production, that is, production increases from the first lactation until the cow reaches physiological maturity, slowly decreasing as the animal becomes older, and this effect is directly related to delivery order of calving (Soares et al., 2009). To Rennó et al. (2002), there are other factors influencing milk production such as season,

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Table 1. Number of animals according to lactation stage, delivery order of calving and genetic groups.

Lactation stage	n = number of animals
0 to 100 days	46
101 to 200 days	79
201 to 305 days	88
Total	213
Delivery order of calving	
One delivery First calving	124
Two deliveries Second calving	58
Above 3 deliveries Above three calving	31
Total	213
Genetic group	
3/4 Gyr	34
7/8 Gyr	38
15/16 Gyr	76
Total	148

herd, and milking, age and lactation period.

The period between birth and cow drying is called lactation period (LP), with average duration of 305 days; thus the animal has a dry period of about 60 days and has one delivery per year (Rangel et al., 2009).

In early lactation, there is an increase in somatic cell count (SCC) of milk due to the presence of immunoglobulins and consequently defense cells. At the end of lactation, there is also an increase in SCC due to increased natural desquamation of the mammary gland epithelium (Harmon and Reneau, 1993).

SCC levels above those allowed by law are indicator that the dairy farm has a large number of animals affected with subclinical mastitis, and high SCC in milk influences the quality of the final product and may be a health risk for consumers. Alternative techniques and methods have been increasingly researched to prevent microorganisms from outside, which cause subclinical mastitis in cattle.

Knowledge of the productive potential of each breed and each crossing used in climate conditions in Brazil should be studied in order to have security when indicating a particular animal for the various production systems (Rennó et al., 2002).

Knowledge of the productive potential of each breed and climate conditions in Brazil should be studied in order to have security when indicate a particular animal for the various production systems (Rennó et al., 2002).

Animals with a higher proportion of Gyr had longer durations of lactation, which exceeds the average daily milk production and the total production of animal milk with higher composition of Holstein (Manus et al., 2008).

So, the aim of the present study was to evaluate the influence of lactation stage, delivery order of calving and genetic group on the quality of milk from crossbred cows.

MATERIALS AND METHODS

The study was conducted at Fazenda Medalha located in the municipality of Rio Verde, southwestern State of Goiás in the period from July to August 2014, where fresh milk samples were collected from crossbred animals throughout the experimental period. Other breeds for variables lactation stage and order of calving were studied. While the variable genetic group were studied, only cows 3/4 Gyr, 7/8 Gyr and 15/16 Gyr data were grouped according to Table 1.

The dairy farm had a total area of 166 ha, 35 ha devoted to grazing and housing of animals for milk production, handling corrals, milking parlor with bulk tank with capacity of storing 4000 L of milk and shed for storage of inputs and shelter for agricultural machinery.

The milking parlor was 2x6 fishbone type with paved waiting corral, milk piping system, closed circuit with six sets of liners and individual milk meters.

The dairy farm had natural shaded and unpaved area are for the tract of animals, where diet composed of feed and corn silage directly produced on the farm were provided.

The milking parlor was 2x6 fishbone type with paved waiting corral, milk piping system, closed circuit with six sets of liners and individual milk meters. The dairy farm had natural shaded and unpaved area are for the tract of animals, where diet composed of feed and corn silage directly produced on the farm were provided. The cows regularly received all mandatory vaccines (Foot-and-mouth disease, Brucellosis and Carbuncle) according to recommendations from veterinarians accredited by the Regional Council of Veterinary Medicine and Animal Science of the State of Goiás and vaccine schedule requirements were stipulated by the Agricultural and Livestock Defense of the State of Goiás.

Fresh milk samples were collected at the end of the morning milking with the aid of individual collectors coupled to each set of liners, which had a valve at the bottom such that before collection of milk samples, it was set to stir for five seconds for milk homogenization, thereafter the valve was turned to emptying

Table 2. Mean values and standard deviation of fat, protein, lactose, non fat dry extract (NDE) and somatic cell count (SCC) and log SCC of crossbred cows at different lactation stages.

Parameter	Lactation stage		
	0 - 100 days (n = 46)	101 - 200 days (n = 79)	201 - 305 days (n = 88)
Fat (%)	3.60±0.52[a]	3.28±0.60[b]	3.71±0.89[a]
Protein (%)	3.15±0.25[b]	3.11±0.29[b]	3.65±0.40[a]
Lactose (%)	4.72±0.16[b]	4.81±0.25[a]	4.52±0.22[c]
NDE (%)	8.85±0.32[b]	8.92±0.48[b]	9.20±0.40[a]
SCC (CS/mL)	278.109±574.196[a]	304.848±761.966[a]	455.511±533.367[a]
Log SCC	6.10±0.49[b]	6.02±0.59[b]	6.44±0.45[a]

Different lowercase letters in line differ according to Tukey test at 5% probability.

position, transferring the milk from the meter to collection flasks. Milk samples were aseptically collected in plastic bottles containing approximately 40 mL of preservative Bronopol® for chemical composition and SCC analyses. Overall, six collections were performed throughout the experimental period at intervals of three days between collections.

Immediately after collection, milk samples were packed in isothermal boxes containing ice and transported to the Laboratory of Animal Products, Federal Institute of Education, Science and Technology of Goiás – Rio Verde Câmpus, and then sent to the Laboratory of Milk Quality (LQL), Research Center of Food of the Animal Science and Veterinary School, Federal University of Goiás, Goiânia, GO, for carrying out electronic analyses.

Fat, protein, and non fat dry extract (NDE) were evaluated through the analytical principle based on the differential absorption of infrared waves by the milk components using MilkoScan 4000 equipment (Foss Electric A / S. Hillerod Denmark). Samples were previously heated in water bath at temperature of 40ºC for 15 min to dissolve fat. Results were expressed in percentage (International Dairy Federation, 2000).

SCC analysis was performed according to the analytical principle based on flow cytometry using Fossomatic 5000 Basic equipment (Foss Electric A / S. Hillerod, Denmark). Before analysis, samples were previously heated in water bath at a temperature of 40°C for 15 min to dissolve fat. Results were expressed in SC/mL (ISO 2006).

Results were submitted to analysis of variance with factors being compared: lactation stage (early: up to 100 days of lactation; intermediate: from 101 to 200 days of lactation and late: from 201 to 305 days of lactation), delivery order (one delivery; two deliveries; above three deliveries) and genetic group (3/4 Gyr; 7/8 Gyr; 15/16 Gyr).

The experimental design was completely randomized. Tukey test at 5% probability and the SISVAR software were used (Ferreira, 2003).

RESULTS AND DISCUSSION

The mean fat, protein, NDE and SCC values of crossbred cows at different lactation stages were consistent with values established by Normative Instruction 62/2011 (Brazil, 2011), which established minimum fat, protein and NDE levels of 3.00, 2.90 and 8.40%, respectively, and maximum somatic cells count of 500 thousand SC/mL (Table 2). Brazilian milk quality law does not establish minimum or mean lactose values.

According to the Table 2, fat values showed significant differences ($p < 0.05$) in relation to different lactation stages ,and cows at intermediate stage (101 to 200 days) showed lower fat content (3.28%±0.60) compared to early (0 to 100 days) and late stages (201 to 305 days), which showed fat content of 3.60%±0.52 and 3.71%±0.89, respectively. Campos et al. (2006) evaluated milk composition in different periods of the first lactation stage in high-production cows and observed fat contents close to those of the present study, 3.62, 3.77, 3.59 and 3.55% in the second, fifth, eighth and eleventh lactation week, respectively.

Fat content is higher at the beginning and end of lactation and lower in milk production peak, considering standardized lactation of 305 days (Kolver et al., 2007). Fat is the milk component that varies the most along the lactation period of cows (Certódes et al., 2004).

At the late lactation stage, protein content was 3.65%±0.40, significantly differing ($p<0.05$) from protein contents at the early lactation stage (3.15%±0.25) and at the intermediate stage, protein content was 3.11%±0.29. Noro et al. (2006) observed protein contents below those of the present study (2.99%) at the early lactation stage and 3.32% at the late lactation stage. Campos et al. (2006) observed protein content was below that established by legislation at 150 days and 240 days of lactation, with average values of 2.84 and 2.86% respectively.

According to Costa et al. (2009), when the animal advances in its lactation stage, there is a downward trend in the daily milk production and consequently the lactose content decreases, causing an increase in fat and protein levels.

The lactose content varied during lactation stages, which was higher at the intermediate stage (101 to 200 days), significantly differing ($p<0.05$) from the early and late stages, which results were 4.81%±0.25, 4.72%±0.16 and 4.52%±0.22 lactose, respectively. Campos et al. (2006) evaluated cows up to eleven weeks of lactation, corresponding to 77 days of lactation and observed average lactose content of 4.65%, and these results are very close to those described in this study.

Table 3. Mean values and standard deviation of fat, protein, lactose, non fat dry extract (NDE) and somatic cell count (SCC) and log SCC of crossbred cows at different delivery orders calving.

Parameter	Delivery order calving		
	One delivery	Two deliveries	Above three deliveries
	First calving (n = 124)	Second calving (n = 58)	Above third calving (n = 31)
Fat (%)	3.59±0.58[a]	3.55±0.67[ab]	3.22±1.25[b]
Protein (%)	3.31±0.35[b]	3.52±0.55[a]	3.15±0.27[b]
Lactose (%)	4.78±0.21[a]	4.52±0.27[c]	4.67±0.11[b]
NDE (%)	9.08±0.41[a]	9.01±0.52[a]	8.79±0.32[b]
SCC (SC/mL)	317.306±694.238[a]	501.310±637.146[a]	275.452±270.612[a]
Log SCC	6.11±0.55[b]	6.42±0.53[a]	6.23±0.46[ab]

Different lowercase letters in line differ according to Tukey test at 5% probability.

Lactose is related to regulation of the mammary gland osmotic pressure, so that increased lactose production leads to higher milk production (Peres, 2001). According to Rangel et al. (2009b), increased SCC values cause reduction in lactose and NDE percentage.

The reduction in the lactose percentage may be explained by the loss of lactose from the mammary gland to blood due to changes in the permeability of the separation membrane. Thus, in healthy mammary gland, the more lactose is secreted, the more liters of milk are produced (Reis et al., 2012).

SCC evaluation according to count in absolute numbers showed no significant difference ($p>0.05$) in the different lactation stages; however, to reduce the variation range, the results (SC/mL) were transformed into Log SCC for statistical evaluation, revealing that there was an increase of somatic cell count at the end of lactation (201 to 305 days).

Voltolini et al. (2001) observed that in relation to the lactation stage, SCC showed a numerical increase at the beginning and end of lactation; however, these values do not show significant differences. The lactation stages could exert greater influence on SCC in herds with high counts. Moreover, the beginning and end of lactation are stressful stages for the cow, which will naturally increase SCC.

The mean values and standard deviation of fat, protein and NDE of crossbred cows at different delivery orders order calving are described in Table 3. Fat, protein and NDE values are within standards set by Normative Instruction 62 of December 29, 2011.

Higher fat contents were observed in cows of first and second deliveries calving, differing ($p<0.05$) from animals of one delivery calving and animals above three deliveries calving, with 3.55%±0.67, 3.59%±0.58 and 3.22%±1.25 fat, respectively. From the third delivery calving, there is a tendency of fat to decrease. Due to its mechanism of synthesis, fat is the milk component of greatest variation, where the delivery order of calving can influence milk fat content, among other factors (Galvão Junior et al., 2010).

Second delivery calving cows showed higher protein content compared to first delivery calving cows and those above three deliveries calving, significantly differing from each other ($p<0.05$), with 3.52%±0.55, 3.31%±0.35 and 3.15%±0.27 protein, respectively. Different results were found by Noro et al. (2006), who observed lower protein content in milk from first delivery calving cows, and Souza et al. (2010) who found no difference in milk protein content of cows up to the fifth delivery calving.

According to Santos and Fonseca (2006), first delivery calving cows are still in body growth phase and development of the mammary gland and therefore have lower milk production capacity.

Milk lactose contents varied among delivery orders of calving, and first lactation showed higher lactose percentage, followed by cows with more than three deliveries calving and cows with two deliveries calving. According to Cunha et al. (2008), lactose is the milk component with the highest osmotic capacity.

Different results were observed by Corrêa (2010), who reported that the lactose percentage in milk was higher in the first and second lactation, keeping constant from the third to the seventh lactation, and decreasing with increasing delivery orders of calving and the lowest point is in the ninth lactation.

Second delivery cows showed SCC above values established by law. According to Magalhães et al. (2006), first delivery animals primiparous cows have less contact with mastitis-causing pathogens. As lactations increase, which coincides with increasing age, animals become more susceptible and are more often exposed to infection.

Animals with higher number of lactations have higher SCC, that is, animals with SCC over 100,000 SC/mL produce less milk. SCC indicates inflammation of the mammary gland, in the majority of cases resulting from bacterial infection (Cunha et al., 2008).

Milk production losses due to increased SCC are absolute, that is, it is independent of the animal production level. They begin to occur from SCC of 17,000 SC/mL and are different for primiparous and multiparous

Table 4. Mean values and standard deviation of fat, protein, lactose, non fat dry extract (NDE) and somatic cell count (SCC) and log SCC of cows of different genetic groups.

Parameter	3/4 Gyr (n = 34)	7/8 Gyr (n = 38)	15/16 Gyr (n = 76)
Fat (%)	3.74±0.59[a]	3.99±1.09[a]	3.25±0.58[b]
Protein (%)	3.73±0.36[a]	3.58±0.50[a]	3.01±0.25[b]
Lactose (%)	4.60±0.12[b]	4.55±0.25[b]	4.71±0.21[a]
NDE (%)	9.28±0.39[a]	9.09±0.40[a]	8.71±0.38[b]
SCC (CS/mL)	508.118±432.l312[a]	358.605±598.596[a]	402.697±774.769[a]
Log SCC	6.54±0.43[a]	6.29±0.47[b]	6.28±0.49[b]

Different lowercase letters in line differ according to Tukey test at 5% probability.

(Coldebella et al., 2004). The mean values and standard deviation of fat, protein, NDE and SCC of crossbred cows of different genetic groups are presented in Table 4.

The milk fat content differed ($p<0.05$) among genetic groups, and 15/16 Gyr cows showed less fat when compared to 3/4 and 7/8 Gyr cows, which fat contents were 3.25%±0.58, 3.74%±0.59 and 3.99%±1.09, respectively.

Reis et al. (2012) evaluated the effect of breed on milk yield and composition and concluded that Holstein cows were more productive, however, milk had lower concentrations of lipids and proteins than milk from Gyr cows and crossbred animals, and the average fat values were different when compared with the present study, 3.64, 3.49 and 3.68%, respectively, for Gyr, Holstein and crossbred cows.

Protein levels were higher in 3/4 and 7/8 Gyr cows, being significant ($p<0.05$) when compared with 15/16 Gyr cows, which values were 3.73%±0.59, 3.58%±0.50 and 3.01%±0.25, respectively. The average milk protein levels of crossbred cows were higher than those of Holstein cows, averaging 3.28 and 3.22%, respectively, for animals in extensive production system.

Both are affected by the technological improvement of the production system with a decrease in milk protein (Gonzalez et al., 2003). Oliveira et al. (1999) pointed out that NDE is the cause of variation of protein content, since the main solid represented in the NDE assessment is protein.

The lactose content of milk from cows of different genetic groups showed significant difference ($p<0.05$) and for 3/4 and 7/8 Gyr cows, the lactose content was lower when compared with 15/16 Gyr cows, with lactose contents of 4.60%±0.12, 4.55%±0.25 and 4.71%±0.21 respectively. Botaro et al. (2011) evaluated the composition of milk from Holstein, Jersey and Gyr cows and observed lower average lactose contents of 4.42, 4.30 and 4.45% respectively. The reduction in the lactose content can be as a result of reduced synthesis of this component in cows with mastitis.

When actual data of milk SCC were transformed into Log CCS, significant differences ($p<0.05$) among results were observed, and 3/4 Gyr cows were more susceptible to mastitis compared with 7/8 and 15/16 Gyr cows, which results were 6.54±0.43, 6.29±0.47 and 6.28±0.49, respectively.

Reis et al. (2012) evaluated Gyr, Holstein and Crossbred cows and found values lower than those of the present study, whose parameters are within standards required by NI/62 2011 (Brazil, 2011), with average values of 395,000 SC/mL, 472,000 SC/mL and 423,000 SC/mL, respectively.

SCC quantitatively indicates the degree of infection of the mammary gland. This infection compromises milk composition, resulting in damage of the mammary gland secretory cells, leading to a reduction of casein, fat, lactose synthesis and increase in total protein concentration (Deitos et al., 2010).

Conclusion

The fat, protein and NDE results of fresh milk were consistent with limits established by the Brazilian legislation for milk quality, regardless of lactation stages, delivery order and genetic group. Milk SCC was higher in cows with advanced lactation stage; however, with results below limits required by law. 3/4 Gyr cows showed higher SCC, with average values above limits established by the Brazilian legislation.

Conflict of Interests

The author have not declared any conflict of interest

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Full Length Research Paper

Isolation and identification of some pathogenic fungi associated with cassava (*Manihot esculenta* Crantz) root rot disease in Cameroon

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Root rot diseases constitute a major constraint to cassava production in Cameroon. However, not much is known about the identity of pathogens associated with them. It is in this light that this study was realized with the aim of characterizing the various root rot diseases and identifying their associated fungal causal agents in Cameroon. Sixty four cassava stems with root rot symptoms were sampled in the Littoral, Southwest and West Regions of Cameroon. Results revealed that cassava root rot (CRR) is either wet (soft) or dry depending on the region of study. Isolation was done on PDA medium enriched with Chloramphenicol. After purifying thrice on the PDA medium, 20 isolates were collected. Identification with the help of the Barnett and Hunter key revealed the existence of seven fungi including *Colletotrichum* sp., *Fusarium* sp., *Pestalotia* sp., *Geotrichum* sp., *Sphaerostilberepens, Trichoderma viride* and *Botryodiplodia theobromae*.

Key words: Cassava root rot, pathogen, sustainable agriculture, disease control.

INTRODUCTION

The actual crop yield as percentage of potential yield is more than 60% for North America, Western and Central Europe, but is less than 50% for South America and North Africa and it is about 30% for Central America and the Caribbean, Eastern Europe and sub-Saharan Africa (FAO, 2012; Valipour, 2014; Valipour et al., 2015). One of the world's most important food crops is cassava (*Manihot esculenta* Crantz) which belongs to the family *Euphorbiaceae.* Cassava roots and leaves serve as an essential source of calories and income throughout the

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tropics. Most people in Africa, Asia and Latin America depend on the cassava crop for their food and income. A significant progress has been observed in cassava production in recent times. In 2005, cassava occupied the 5th rank in the world production of food crops after maize, rice, wheat and solanum potatoes. World production of fresh cassava tubers increased from 189,099,633 tons to 232 million tons in 2008 with Sub-Saharan Africa alone producing 118 million tons per annum constituting about half of the world's production (FAOSTAT, 2010). In Cameroon where roots and tubers account for 70% of the total cultivated area and 46% of food crop production, total cassava production was estimated at 2,882,734 tons in 2008 (Agristat, 2009). In Cameroon, 80% of urban households consume cassava products on a daily basis, and about 90% of small-scale producers market at least a small part of the cassava they produce.

Despite the relative progress observed in world annual yield of cassava, its cultivation is faced with several pests and diseases. The fungal root rot disease of cassava which affects the tubers, is a disease caused by infection of the roots by fungi found in humid or poorly drained soil (Silvestre and Arrandeau, 1983). It is characterized by browning and wilting of leaves, accompanied by loss of water which may eventually lead to the death of the plant. The other symptoms are swelling of roots and a light brown coloration observed when the roots split in the soil or when they are cut open. In Africa, CRR causes enormous yield losses. It actually hinders the synthesis and storage of nutrients in the roots. This consequently limits plant development, reduces number of roots and their ability to form tubers and become mature, hence limiting production (Msikita et al., 2000).

In Cameroon, about 36% of farmers classify CRR as the second cause of reduced yields in the cassava sector. However, proper identification of pathogenic fungi associated with this disease is yet to be done (Messiga et al., 2004). In order to address this situation, this study was realized in the Littoral, South-west and West Regions of Cameroon with the aim of identifying the pathogenic agents associated with CRR through their isolation from infected plants and morphological description of their fructifications.

MATERIALS AND METHODS

Study sites

Visits and observations were done between March and June 2010 in cassava farms in eight localities, namely Douala, Dibombari, Souza, Mondoni, Batoke, Ekona, Kumba and Dschang, which are found in the Littoral, West and Southwest Cameroun. These three main agro-ecological zones are constituted principally of the humid forest with monomodal rainfall for the Littoral and South-West, characterized by an average temperature of 25°C, 4000 mm of annual rainfall with ferralitic sandy or sandy clay soil. The western highland zone on its part has an average temperature of 20°C and about 1500-2600 mm rainfall with reddish ferralitic soil formed on basalt. Samples were collected from infected cassava plants in these regions and carried to the Crop Protection Laboratory of the Department of Plant Protection, Faculty of Agriculture, University of Dschang where fungal pathogens were isolated.

Collection of samples

Two farmers' farms per locality were surveyed for the presence of plants with external symptoms such as leaf browning and discoloration of the lower part of the stems, and generalized wilting, which are most often indicative of root infections. Of all the plants identified, four were off-rooted from each farm and observations were done on the phytosanitary status of their root system. Samples of partially rotten tubers were taken from these infected plants and carried to the laboratory where fungal isolations were done using potato dextrose agar (PDA) medium.

Isolation and purification of fungi

Fungal isolation was done on PDA enriched with chloramphenicol (150 ppm) in order to avoid bacterial growth. The culture medium was poured in Petri dishes and allowed to solidify. Tubers were washed with tap water. With the help of a sterilized scalpel, 1 cm fragments were collected from the necrotic front and disinfected with 95°C alcohol with the help of a sterile pincer. The fragments were then washed with distilled water and dried with sterile blotting-paper. This exercise was undertaken under a laminar flow hood in the presence of a Bunsen burner in order to assure aseptic conditions. These sterilized explants were cultured on PDA-chloramphenicol medium at 25°C in the dark. After ten days of incubation, fungal colonies which emerged from the explants were sub cultured individually on new PDA simple culture medium. This action was repeated thrice until pure cultures were obtained. A collection of 20 isolates was constituted. Microscopic observation of each isolate was done with an optical microscope (model Olympus BH-2) at a magnification of 400x, and fungal identification was done with reference to the key of Barnett and Hunter (1972).

RESULTS

Description of root rot symptoms

At the first site, a swelling of the tuber and browning of the bark close to the stem were noted on the roots collected from all localities. Splits of these roots showed light brown coloration which is characteristic of CRR disease. A pungent smell was also noted. It was observed that the rotting was different for samples collected from the different localities (Table 1). In fact, rotting of some roots was humid and pasty which is characteristic of soft root rot while on others it was friable, characteristic of dry rot. It has been reported that relative humidity plays a major role in cassava fungal disease development (Makambila, 1994).

Isolation and identification of pathogenic fungi

Fungal isolations realized on rotten cassava samples from the eight localities revealed a great diversity of species associated with cassava root rot. In all, 20 fungal isolates were grouped into seven genera. Figure 1 presents

Table 1. Typological characterization of cassava root rot.

Nature of infection	Infected part		Region	Ecosystem
	Aerial part	Root		
Soft (wet) rot	Wilting, leaves fall-off	Brown necrotic lesions, pasty rotting	Littoral	Humid forest zone with monomodal rainfall
Soft (wet) rot	Wilting, no falling of leaves	Brown necrotic lesions, pasty rotting	Southwest	Humid forest zone with monomodal rainfall
Dry rot	Dehydration of plant, dark brown coloration	Friable rotting	West	Upper plateaux

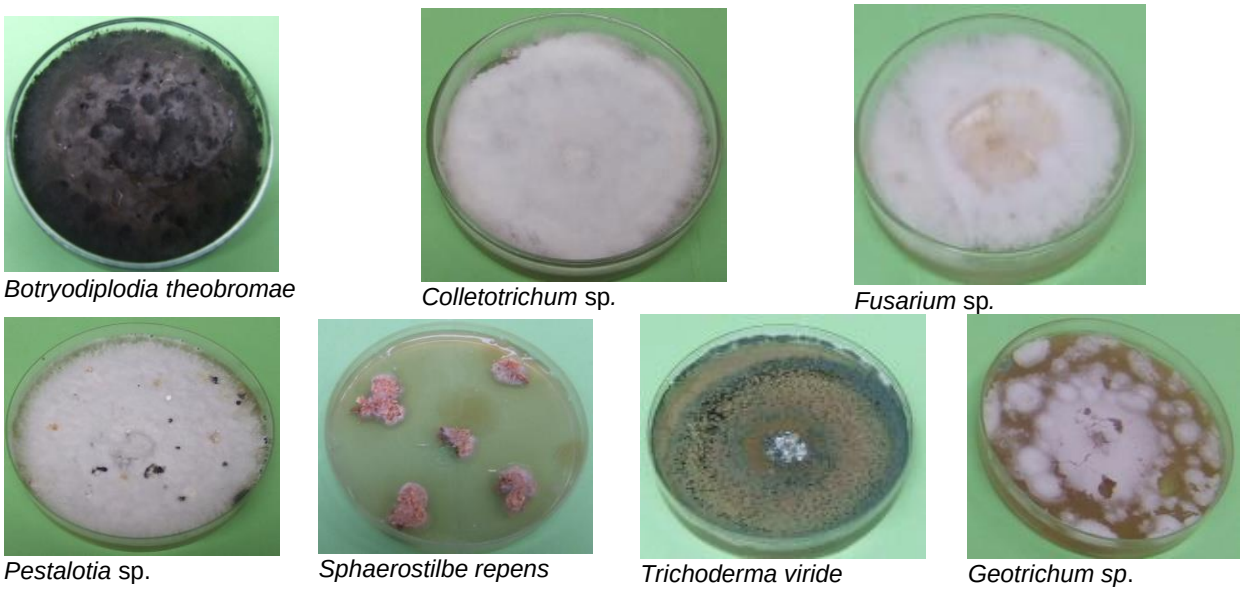

Figure 1. Diversity of fungal species associated with CRR as observed in PDA after 7 days.

presents the mycelial growth and coloration of colonies of each of these genera.

The morpho-cultural and microscopic characteristics of CRR causal agents isolated in this study are presented in Table 2.

With respect to localization, *Fusarium* sp. was the most common genus with a 50% relative prevalence, followed by *Botryodiplodia theobromae* (15%), *Colletotrichum* sp. (10%) and *Trichoderma viride* (10%) (Table 3).

DISCUSSION

Determination of infectious routes of plant pathogens and their mechanisms of infection are of great importance in any disease control program (Twumasi et al., 2014). Host-plant resistance and biological control are the cornerstones of crop protection measures against biotic stress on cassava (Herren, 1994). In fact, knowledge on the identity of the pathogen and disease infection mechanisms is of prime importance. This could be achieved by isolation and identification of disease causal agents on appropriate culture media. The choice of PDA medium for the isolation of the pathogenic fungal species in this study was based on its successful use in previous studies (Maheshwari et al., 1999; Attrassi et al., 2005), which have been mentioned as an appropriate medium for isolation of a wide range of fungi. Isolation and identification of the pathogenic fungi in this study help to show the presence of seven fungi, namely *Colletotrichum* sp., *Fusarium* sp., *Pestalotia* sp., *Geotrichum* sp., *Sphaerostilbe repens, Trichoderma viride* and *Botryodiplodia theobromae.* With a relative prevalence in terms of isolation frequency equal to 50%, fungi of the genus *Fusarium* sp. were the most encountered. They were respectively followed by *B. theobromae* (15%), *T. viride* and *Colletotrichum* sp., respectively 10%, *S. repens, Geotrichum* sp. and *Pestalotia* sp., respectively 5%. With reference to the symptoms observed on the roots/tubers of cassava, the genera *Botryodiplodia* sp. and

Table 2. Morpho-cultural and microscopic characteristics of fungi isolated in this study.

Fungi	Morpho-cultural characteristics on PDA	Microscopic characteristics
Botryodiplodia theobromae	Very rapid growth, abundant cotton-like mycelium, colorless when young and becomes deep gray or black with age	Bicellular and ovoid conidia
Colletotrichum sp.	White cotton-like mycelium which turns to gray with time forming acervuli	Abundant unicellular, fusiform conidia
Fusarium sp.	White mycelium which turns to ochre-yellow on old cultures	Spores with crescent form. Septate hypha.
Pestalotia sp.	White cotton-like mycelium-forming black acervuli	Hyphae are septate. Spores are fusiform.
Sphaerostilbe repens	Localized growth; colonies with twisted outlines, whitish when young and turning progressively red with age	They bear stilbospores. The mycelium is undifferentiated.
Trichoderma viride	Rapid growth, sparse mycelium initially colorless, but rapidly turns green with profused green conidia	Pyramidal branched conidiophores.
Geotrichum sp.	Less abundant milky white cotton-like mycelium	Hyaline Conidia. Septate hyphae with dichotomous branches.

Table 3. Relative prevalence of each pathogenic fungal species with respect to localization.

Fungi	Localization	Relative prevalence (%)
B. theobromae	Douala, Batoke, Dschang	15
Colletotrichum sp.	Batoke, Kumba, Dibombari, Dschang	10
Fusarium sp.	Mondoni, Kumba, Souza, Douala	50
Pestalotia sp.	Douala	5
Geotrichum sp.	Kumba	5
S. repens	Souza	5
Trichoderma viride	Douala, Ekona, Kumba, Souza, Dschang	10

Sphaerostilbe sp. seem to be associated with dry root rot while the genus *Fusarium* sp. is associated with wet (soft) cassava root rot (Theberge, 1985). These symptoms correspond to a particular climatic condition. For instance, the high prevalence of *Fusarium* sp. among our isolates could be due to the conditions of high rainfall, particularly common in the Littoral and Southwest regions, rendering the soils humid and favoring soft or wet rot.

Damages caused by *Colletotrichum* sp. on cassava have been signaled elsewhere. A special form of this genus, *Colletotrichum gloesporioides f. sp. Manihotis* is known to cause anthracnose diseases on cassava (Amusa, 1998; Magdalena et al., 2012) which is an important disease of cassava in tropical Africa, transmitted through breeder seeds and post-harvest debris in the field (Fokunang et al., 1997, 2001). The disease has been reported to cause total crop failure where infected propagation materials are used as seed sources (Ikotun and Hahn, 1991; Magdalena et al., 2012).

The fungi of the genus *Geotrichum* sp. probably play a role in the process of fermentation and post-harvest deterioration of tuberized roots of cassava (Noon and Booth, 1977; Raimbault et al., 1985; Oyewole and Odunfa, 1988). However, despite the fact that it contributes to crop devastation, a study revealed that *Geotrichum* sp. possibly produces dihydroisocoumarins which could be capable of inhibiting the action of *Plasmodium falciparum* (Palangpon et al., 2003).

Fungi of the genus *Trichoderma* sp. are saprophytes found in the soil. Their capacity to inhibit mycelial growth of other fungi such as *B. theobromae* and *Fusarium* sp. has been put to evidence (Manjula et al., 2005). The species *viride* though with very slow mycelial growth is seemingly capable of inhibiting the development of fungi at a distance (Cherif and Benhamou, 1990). In fact, trichodermine (an antibiotic) has been derived from *T. viride* (Dennis and Webster, 1971). The presence of *Colletotrichum* sp. and *Pestalotia* sp. at the level of cassava tuberized roots need further investigation given that Makambila (1994) highlighted the responsibility of *C. gloeosporioides* in anthracnose of cassava stem. Their specific identification would permit establishment of the

relationship between rhizospheric and aerial isolates.

The *B. theobromae* identified in this study has also been reported to cross infect other crops like cocoa, mango, banana and yam with significant tissue damage and economic losses. The cross-infectivity of this fungus on several crops calls for a review of biocontrol strategies that recommend adoption of mixed- or inter-crop systems to control fungal rot in farms (Twumasiet al., 2014).

CONCLUSION AND PERSPECTIVES

From this study, seven pathogenic fungi were revealed to be associated with cassava root rot disease namely: *Colletotrichum* sp., *Fusarium* sp., *Pestalotia* sp., *Geotrichum* sp., *S. repens, T. viride* and *B. theobromae.* Results also show a variability of types of infection with respect to locality or origin of cassava samples revealing an influence of ecological conditions on relative abundance of the pathogenic fungi. This study will help to improve cassava production; thereby contributing to poverty alleviation and food security. Given the nutritional and economic importance of cassava as well as the seriousness of the phytosanitary problems associated with the cassava sector, it will be appropriate in perspective to realize a more elaborate study to determine the prevalence of these pathogens in relation to pertinent factors. An aggressivity/pathogenicity assessment of the fungi as well as their biomolecular characterization is also imperative to confirm their identity and pathogenic importance for their possible use in the screening of cassava clones for resistance.

Conflict of Interests

The authors have not declared any conflict of interest.

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Grape pre-evaluation by berry-leaf biochemistry quantitative correlation analysis

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The quality and characteristics of grape are fundamentally determined by its biochemical components. Quantitative detection of these components in berries is a classic method to evaluate grapevine resources. However, fruits are not always available for the new generated grape plantlets due to their long juvenile stage (3 to 4 years), as well as for many other potential valuable germplasm resources, such as wild grapes. Therefore, an effective berry-independent method for grapevine evaluation should have great significance. Data were provided from both leaves and berries for 2 groups of grapevine: one group is 12 genotype different varieties or species from environmental similar collections; the other group is one variety of wine grape with 18 different treatments. After quantitative correlation tests, 9 in total 11 detected parameters in genotype different (GD) group and 5 in 9 detected parameters in treatment different (TD) group, respectively, were significantly correlated between leaf and berry, respectively were found. Higher correlation coefficients were found in GD group than in TD group. Parameters of leaf reducing sugar, total flavonoids and superoxide anion scavenging capacity were found significantly correlated to berry, in both groups. These parameters with significant correlation may potentially be used as metabolite markers to estimate the qualities and characters of some new grapevine germplasm, by using the obtained data from leaves. The prospects of this leave-dependent evaluation method have also been discussed in this report.

Key words: Leaf-dependent berry evaluation, leaf/berry quantitative correlation, parameter pair, inter-parameters pair.

INTRODUCTION

Grapevine is one of the most widely planted fruits in the world, and a large proportion is used for wine making. The pursuing for high quality, distinctive features and high adaptabilities of cultivars raises the needs of rapid development of wine grape breeding. At present, thousands of varieties have been developed and many of them broadly utilized in wine industry for their good quality or distinctive adaptation characters all over the world (Alleweldt and Possingham, 1988; This et al., 2004). New cultivars of grape are always generated from crosses using inter or intraspecific grapevine resources or domesticated from wild grapes (*Vitis* species) (Reisch

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et al., 2012). Regardless of the origins of a new variety, the systematic evaluation will be essential before it can be applied in viticulture. In order to select efficiently appropriate cross parents and screen out elite offspring, systematic evaluation of large amount of germplasm resources and cross progeny is indispensable, but a long-term and hard-task process (Alleweldt and Possingham, 1988; Nejatian, 2006). Traditionally, to evaluate potential grapevine germplasm, the candidates should be grown until they produce fruits. Adaptability and other agronomic features can be evaluated during the juvenile stage. The most important procedures are the biochemical evaluation of the berries, which have to wait for 3 to 4 years from planting the cross progeny as a result of the long juvenile stage of grapevine. Nowadays, the quality evaluation procedures is always carried by qualitative and quantitative determinations to berry composition (Guidetti et al., 2010; Shiraishi et al., 2010), and the long delay between juvenile stage to productive stage becomes a bottleneck of rapid selection. Moreover, collection of ripen grape berries from some potentially useful wild species in natural conditions also has difficulties, because of the unpredicted mature time and birds feeding. In contrast, grape leaves of any development stages is easily harvest, especially for wild resources. Therefore, if a leaf-dependent pre-evaluation method can be successfully applied in berry evaluation, time and workload in vine breeding will decrease dramatically. Sine after an earlier leafy compositional and quantitative screening, one could only focus on those most potential candidates. While most of these biochemical characters have not any detectable genetic marks for this purpose.

Moreover, if quantitative responses of certain metabolites in fruits always correlated significantly to their leaves, one may predict the effects of environmental perturbations on berry composition based on results from a leaf or tissue assays. Despite its potential importance, there are no studies assessing the quantitative correlation of biochemical traits between leaf and fruit in plants. In this research, the correlation of several important biochemical traits were tested between leaf and berry from various grape varieties, as well as one cultivar but treated differently. A leaf-dependent prediction method for berry evaluation was then proposed based on the analyzed quantitative correlations of these detected traits. Many of these detected parameters such as sugar, acidity, flavonoids, phenols contents, and anti-oxidative capacities are fundamental in grape quality and characteristic evaluation.

MATERIALS AND METHODS

Plant and experiment design

Commercially ripen fruits and full developed healthy leaves of 11 varieties of grapevines (*Vitis vinifera*) and a wild species (*Vitis heyneana*) were sampled and used in the quantitative analysis for measuring some biochemical and physiological parameters, from vineyards in Qiubei county, Yunnan province, China in 2012 as genotype different (GD) group. Grape cultivars in GD group include Yan73 (v1), Beijixing (v2), Xiahei (v3), Rose honey (v4), Crystal (v5), Cabernet Sauvignon (v6), Red rose (v7), Faguoye (v8), Zhengzhou Dawuhe (v9), America No.1 (v10), Merlot (v11), and a wild species *V. heyeara* (v12). All these field-grown grapevines in a germplasm collection were 5 to 7-year old, spur pruned, with a density of 1.6 m between rows and 1 m between plants. Vineyard management followed the local standards. Another 18 samples as treatment different (TD) group, were harvested from a wine grape cultivar cv. Rose Honey growing in a commercial vineyard (5-year old, also spur pruned, with a density of 1.2 m between rows and 0.9 m between plants) with 18 combinations of fungal regents and pesticides. Vines were separated into 2 parts and one part inoculated with 8 different strains of fungi with a non-fungus inoculation control, and followed the local management for 4 times of pesticides applying. Other parts were also inoculated with the same strains of fungi and a non-fungus inoculation control, but without any pest controlling (pesticides free). Each single treatment contains 10 grapevines. The purpose of this treatment was to create the quantitative variation of metabolites in grapevine.

Vines without obvious visible disease symptoms of each variety were sampled randomly from at least 6 plants of GD group. Samples from every 2 vines pooled as one replicate for both leaf and fruit, respectively for each variety, and preserved in an ice box, delivered to lab within 4 h for processing. For berry sampling, 2 ripen clusters for every vine were taken. For leaf samples, almost the same position (4 to 6th from the bottom of the fruit cane), similar size, full developed healthy leaves were sampled. Samples of TD group were harvested with the same method above at berry ripen stage (67 days after treatment). Six grapevines were also sampled and samples (both fruit and leaf) from every 2 grapevines were pooled as one replicate.

Determination of physio-chemical traits

Pre-treatment of leaf and berry samples

All leaf samples were cut into about 1 cm^2 pieces for each sample. Randomly selected ripen berries were picked off from clusters of each replicate sample and well mixed up. About 20 g of leaf pieces and randomly selected berries for each samples were homogenized into fine powder in liquid N_2 with a stainless grinder and transferred to a 50 ml tube, then stored at -80°C for reducing sugar, titratable acidity, total phenols, soluble protein and enzyme activity analysis; the rest of the samples were dried in wind-oven following a program of 110°C for 10 min, 80°C for 48 h (72 h for berries) and then ground into fine powder with a stainless grinder for the measurement of total flavonoids content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, and superoxide anion savaging capacities.

Determination of reducing sugar (RS) and total sugar (TS)

Fresh sample (1 g) was added with 4 ml 1 mol/L zinc acetate (containing 3% glacial acetic acid) and 4 ml 0.25 mmol/L potassium ferrocyanide, and extracted in 80°C for 10 min with 2 times of vortex. The mixture was centrifuged at 5000 rpm and the supernatants was adjusted to pH=7 by adding calcium carbonate powder. After 30 min in 60°C water bath with several times of vortex, the solution was cooled to room temperature, and metered the volume to 10 ml with distilled water. After 10 min centrifuge at5000 rpm, the supernatant was titrated with alkaline tartrate copper solution A+B (Dygert et al., 1965). The consumption of the supernatant was used to calculate the contents of RS. TS was

obtained by pre-treating the homogenate with 6 mol/L HCL and then follow the same procedure as that of RS.

Titratable acidity (TTA)

Titratable acidity was determined by sodium hydroxide direct titration. About 1 g fresh sample was weighed and extracted in a boiling water bath for 30 min, vortex several times during the bath to get all the organic acids dissolved in the solution. After cooling to room temperature, the solution was centrifuged at 5000 rpm for 10 min, the supernatants was titrated with 0.01 mol/L standard solution of sodium hydroxide. The consumption of sodium hydroxide was used for total acid calculating, and described as the content of tartaric acid (mg/g fresh weight, FW).

Total flavonoids (TF) content

Total flavonoid content of berry and leaf were determined with dried samples by using the aluminum chloride colorimetric method (Willett, 2002), with some modifications. Methanol extracts (0.5 ml), 10% aluminum chloride (0.1 ml), 1 M potassium acetate (0.1 ml) and distilled water (4.3 ml) were mixed after incubation at room temperature for 30 min. The absorbance was measured at 415 nm. Total flavonoid content was calculated by comparing the calibration with rutin trihydrate as standard substance.

Total phenols (TPh) content

Total phenols were determined according to the method of Forint phenol colorimetric. About 1 g fresh frozen sample was used to extract and determined the total phenols (Asami et al., 2003). TPh content was standardized against gallic acid and expressed as milligrams per liter of gallic acid equivalents.

Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reacting substances (TBARS), as described by Dhindsa et al. (1981). Fresh frozen tissue (0.5 g) was extracted with 10 ml trichloroacetic acid (TCA) 0.1% (w/v) for 10 min with 2 times of vortex. The mixture was centrifuged at 6000 rpm at 4°C for 10 min. 2 ml of supernatant were mixed with 2 ml 20% TCA solution (containing 0.5% (w/v) thiobarbituric acid. The mixture was heated at 95°C for 30 min, quickly cooled and centrifuged at 13,000 rpm and 4°C for 10 min. The absorbance of the supernatant was read at 532 nm with the values for non-specific absorption at 600 nm subtracted. TBARS concentration was calculated using the following formula (Heath and Packer, 1965):

TBARS concentration = [(A532 × 1000) - (A600 × 1000)] /155

Determination of total soluble protein and antioxidant enzymes

Fresh tissue (1 g) was added with 10 ml of 0.1 mol/L potassium phosphate buffer (pH 7.0), containing 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA)-Na_2, 0.5 mmol/L ascorbate and 1% polyvinyl polypyrrolidone (PVPP) and stood for 30 min with several times of vortex. The mixture was centrifuged at 13,000 rpm under 4°C for 10 min. The supernatant was used for determinations of protein content and antioxidant enzyme activity. Total soluble protein concentration(SPr) was determined as described by Bradford (1976) using bovine serum albumin as standard. Superoxide dismutase (SOD) was determined by the nitro-blue tetrazolium (NBT) method (Dhindsa et al., 1981), and guaiacol peroxidase (GPX) assay was performed using the method described by Amako et al., 1994).

Activity of phenylalanine ammonia-lyase (PAL)

The extraction and determination of PAL was performed according to the method of Carolyn et al. (1996), with some modifications as described by Xi et al. (2013). Only samples from TD group were analyzed for PAL in this research.

DPPH radical scavenging capacity

Dried sample was ground into fine powder, and about 1 g was accurately weighed into a volumetric flask. DPPH radical scavenging active substances were extracted by adding 50% of ethanol and sonicating for 30 min in an ultrasonic chamber. The mixture was filtered and the filtrate was diluted into gradient concentrations for further detection. DPPH radical scavenging capacity was measured and calculated by using the method of Li et al. (2012); absorbance was read in a spectrophotometer (S22, Biochrom Libra, England) and results were described as percentage of DPPH radical scavenged (Li et al., 2012).

Superoxide anion scavenging capacity (SA)

Preparation of gradient concentrations of sample extract is as the same process as DPPH radical scavenging capacity determination. The measurement of superoxide anion scavenging capacity was following the method of Li et al. (2012), and the SA scavenging capacity was described as percentage of superoxide anion scavenged (Li et al., 2012).

Data analysis

All data were reported as means ± standard variation values of 3 biological replicates, and analyzed by using the software of SPSS version16.0 (SPSS Inc., Chicago, IL, USA) for windows. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used for the significance determination with a significant level of 0.05. Pearson's correlation test was conducted to determine the correlations between parameters within or between leaf and berry.

RESULTS

Values of the detected parameters presented as means ± standard variation with different significances of all samples, including leaves and berries in different groups were listed and are shown in Tables 1 to 4, respectively. As genotype different resource, values of each parameter in GD group varied significantly ($P<0.05$), both in leaf and in berry (Tables 1 and 2). In TD group, the same cultivar was subjected to different treatments, and the values of every parameter also varied among treatments (Tables 3 and 4). Therefore, quantitative variations of these parameters can not only be caused by genetic factors but also by the given treatments (environment factors).

However, coefficients of variation caused by genotype are obviously higher than that of the environment factors,

Table 1. Leaf results of detected biochemical traits of genetic different (GD) group of grapevine.

Cultivar	TS mg/g (FW)	RS mg/g (FW)	TF mg/g (DW)	TTA mg/g (FW)	SPr mg/g (FW)	TPh mg/g (FW)	SOD U/g (FW)/min	GPX U/g (FW)/min	TBARS μ mol/g (FW)	DPPH (%)	SA (%)
v1	66.674±3.752^{e}	32.841±1.835i	39.485±1.562^{b}	15.5166±1.3364^{d}	3.470±0.135^{a}	2.431±0.054^{d}	40.0043±1.2896^{h}	62.0238±3.5089j	0.4663±0.0233^{a}	93.9332±2.9428^{a}	76.9021±5.9730^{ab}
v2	108.850±8.365^{a}	95.090±0.369^{a}	27.773±1.274^{fg}	9.6905±0.1922^{fg}	1.850±0.070^{f}	2.902±0.007^{b}	120.129±8.9406^{e}	161.8998±2.1628^{e}	0.0240±0.0012^{e}	68.0597±1.5056^{d}	43.7202±3.3958^{f}
v3	98.622±0.597^{bc}	84.353±0.729^{b}	63.279±0.535^{a}	10.8333±0.8036^{e}	3.273±0.032^{b}	2.749±0.017^{bc}	102.77±10.8121^{f}	85.1755±2.8866i	0.0226±0.0012^{e}	63.0239±1.9744^{e}	73.2932±5.6927^{bc}
v4	105.13±5.340^{ab}	66.649±1.389^{d}	36.855±1.740^{c}	11.4268±0.5326^{e}	1.900±0.070^{ef}	2.572±0.017^{cd}	41.5189±2.1059^{h}	97.6904±1.6192^{h}	0.0577±0.0029^{b}	51.9366±2.5669^{f}	83.1652±6.4595^{a}
v5	65.924±2.013^{e}	46.899±1.438^{g}	26.252±0.740^{g}	21.2505±0.4992^{bc}	1.150±0.101^{h}	2.565±0.016^{cd}	84.7543±1.4738^{g}	111.7384±2.0095^{f}	0.0245±0.0012^{e}	69.4929±1.8638^{d}	73.7941±5.7316^{abc}
v6	40.573±1.484^{f}	26.455±2.494j	31.800±0.699^{d}	8.8768±0.3338^{g}	2.207±0.055^{d}	1.849±0.451^{e}	155.326±1.7262^{d}	279.7306±2.5099^{b}	0.0316±0.0016^{de}	92.9329±2.9114^{a}	78.6981±6.1125^{ab}
v7	75.178±0.885^{d}	36.486±0.912^{h}	30.567±0.370^{de}	8.7334±0.1714^{g}	1.180±0.165^{h}	2.370±0.044^{d}	188.839±7.0074^{c}	203.0260±3.4238^{d}	0.0464±0.0023^{bc}	85.0873±1.7258^{b}	55.2208±4.2890^{e}
v8	65.175±2.525^{e}	51.929±1.546^{f}	29.129±1.233^{ef}	21.7514±0.3871^{b}	0.2057±0.0104^{de}	2.473±0.043^{cd}	125.064±7.2309^{e}	100.9820±1.5992^{h}	0.0225±0.0011^{e}	67.1970±1.7919^{d}	58.3772±4.5342^{de}
v9	97.762±3.788^{c}	77.894±2.318^{c}	16.307±0.329i	10.6596±0.2115^{ef}	2.440±0.085^{c}	2.584±0.034^{cd}	99.2377±2.1318^{f}	106.1515±1.9090^{g}	0.0196±0.0010^{e}	53.7264±1.3699^{f}	33.4142±2.5953^{g}
v10	78.210±3.030^{d}	62.315±1.854^{e}	23.581±0.781^{h}	20.3501±0.4037^{c}	1.573±0.055^{g}	2.392±0.098^{d}	215.656±5.6372^{b}	245.8244±4.4209^{c}	0.0382±0.0019^{cd}	79.2567±1.5431^{c}	66.7575±5.1851^{cd}
v11	45.622±1.768^{f}	32.351±1.082i	39.677±2.572^{b}	8.7215±0.1730^{f}	2.648±0.061^{c}	3.685±0.216^{a}	342.6612±6.3095^{a}	357.5628±6.4304^{a}	0.0424±0.0029^{bc}	90.0392±2.8208^{ab}	75.2043±5.8412^{ab}
v12	44.398±0.920^{f}	20.472±1.345k	24.207±1.349^{h}	24.5099±1.8114^{a}	2.841±0.252^{bc}	2.341±0.077^{d}	101.8998±4.8944^{f}	125.0646±5.9322^{ef}	0.0183±0.0038^{e}	81.8854±2.0066^{c}	38.0097±4.3428^{fg}

Values in the table are illustrated as mean ± standard variation. The same letters indicating the values are not different significantly. Otherwise, different letters indicating the values are significantly different (P<0.05) Post hoc Duncan test. TS: Total sugar content; RS: reducing sugar content; TF: total flavonoid content; TTA: total titratable acids; SPr: soluble protein content; TPh: total phenolic content. SOD: activity of superoxide dismutase; GPX: activity of Guaiacol peroxidase; TBARS: concentration of thiobarbituric acid reacting substances; DPPH: percentages of DPPH radical scavenged at the concentration of 5 mg/ml; SA: percentages of superoxide anion radical scavenged at the concentration of 10 mg/ml.

Table 2. Berry results of detected biochemical traits of genotype different (GD) group of grapevine

Cultivar	TS mg/g(FW)	RS mg/g(FW)	TF mg/g(DW)	TTA mg/g(FW)	SPr mg/g(FW)	TPh mg/g(FW)	SOD U/g(FW).min	GPX U/g(FW)·min	TBARS μ mol/g(FW)	DPPH %	SA %
v1	76.723±2.016^{g}	61.976±1.755^{e}	12.937±1.216^{a}	19.3149±0.7255^{e}	4.159±0.459^{b}	0.902±0.036^{cd}	35.2284±3.6363^{g}	92.6452±3.1893j	0.6407±0.0641^{a}	94.0354±1.5413^{a}	88.2055±4.2270^{b}
v2	177.849±5.282^{a}	162.70±6.753^{a}	3.859±0.129^{fg}	15.0122±0.5639^{f}	0.918±0.053^{f}	0.919±0.0177^{bc}	87.1901±9.5897^{a}	253.8754±2.4133^{e}	0.1509±0.0151^{d}	35.3508±0.5794^{h}	22.7818±1.7908^{e}
v3	120.32±6.605^{bc}	104.067±5.713^{bc}	10.882±0.297^{b}	25.1913±2.0731.	0.778±0.084^{f}	0.8.84±0.013^{cde}	53.088±1.3616^{de}	101.3510±2.2645^{i}	0.1404±0.0141^{de}	82.2641±1.3484^{c}	82.5355±3.9420^{bc}
v4	123.31±2.907^{bc}	98.528±5.408^{bc}	7.362±0.576^{c}	28.6425±1.0759^{c}	4.626±0.143^{a}	0.977±0.013^{ab}	23.4415±2.5245^{h}	104.1430±2.2610^{i}	0.3161±0.0316^{b}	88.3319±1.4478^{b}	94.5748±4.5522^{a}
v5	97.133±5.332^{e}	74.321±4.080^{d}	4.773±0.405^{e}	41.6238±1.5634^{a}	4.863±0.084^{a}	0.999±0.005^{a}	57.991±2.3602^{cd}	170.8425±2.2672^{f}	0.0152±0.0016^{f}	66.9622±1.0976^{d}	85.0449±4.0676^{b}
v6	48.718±2.674^{i}	34.643±1.902^{f}	8.087±0.218^{c}	21.4225±1.9315^{e}	3.090±0.113^{c}	0.679±0.097^{h}	49.028±4.5257^{ef}	336.9288±4.8217^{c}	0.2214±0.0222^{c}	90.2109±1.4786^{b}	88.0319±4.2183^{b}
v7	114.985±6.312^{c}	97.330±5.343^{c}	4.279±0.251^{ef}	11.8331±1.5713^{g}	2.980±0.128^{c}	0.772±0.013^{fg}	80.426±0.3265^{ab}	296.3325±2.5611^{d}	0.0281±0.0028^{f}	64.9301±1.0643^{e}	64.1567±3.0615^{d}
v8	128.341±7.045^{b}	106.424±5.842^{b}	11.225±0.126^{b}	31.7831±1.1938^{b}	1.301±0.210^{e}	0.825±0.022^{ef}	61.8468±1.7291^{c}	137.2536±3.1980^{h}	0.1031±0.0104^{e}	44.4341±0.7283^{g}	69.3667±3.3025^{d}
v9	106.709±3.169^{d}	97.621±4.051^{c}	3.101±0.535^{g}	16.5134±0.6203^{f}	2.101±0.102^{d}	0.727±0.016^{gh}	43.6158±2.5919^{f}	162.3003±2.1539^{g}	0.0028±0.0003^{f}	30.0247±0.4921^{i}	11.9519±1.8325^{f}
v10	83.589±2.482^{f}	76.470±3.174^{d}	5.951±0.165^{d}	31.5256±1.1842^{b}	4.995±0.380^{a}	0.845±0.033^{de}	77.6177±3.7469^{b}	375.8534±4.9878^{b}	0.1250±0.0125^{de}	52.3952±0.8588^{f}	77.8890±3.7123^{c}
v11	45.622±1.768^{i}	47.556±1.891^{ef}	11.123±1.224^{b}	13.5110±0.5074^{g}	3.270±0.184^{c}	1.064±0.061^{a}	93.6076±1.6535^{a}	546.6959±7.2550^{a}	0.2535±0.0293^{c}	84.7174±1.3886^{c}	86.4790±4.1398^{b}
v12	44.398±0.920^{i}	30.998±2.311^{f}	3.940±0.654^{g}	33.8944±1.0094^{b}	3.500±0.234^{bc}	0.570±0.45^{i}	42.0238±5.1122^{f}	188.8393±8.9200^{f}	0.1111±0.0087^{e}	63.4611±1.9452^{e}	36.9856±3.9873^{e}

Values in the table are illustrated as mean ± standard variation. The same letters indicating the values are not different significantly. Otherwise, different letters indicating the values are significantly different (P<0.05) Post hoc Duncan test. TS: Total sugar content; RS: reducing sugar content; TF: total flavonoid content; TTA: total titratable acids; SPr: soluble protein content; TPh: total phenolic content. SOD: activity of superoxide dismutase; GPX: activity of Guaiacol peroxidase; TBARS: concentration of thiobarbituric acid reacting substances; DPPH: percentages of DPPH radical scavenged at the concentration of 5 mg/ml; SA: percentages of superoxide anion radical scavenged at the concentration of 10 mg/ml.

Table 3. Leaf results of detected biochemical traits of treatment different (TD) group of grapevine

T	GPX U/g (FW)/min	SOD (U/g (FW)/min)	RS (mg/g (FW))	SPr (mg/g (FW))	TF (mg/g (DW))	TPh (mg/g (FW))	PAL (U/(g (FW)/min))	DPPH (%)	SA (%)
1	42.67±11.39^{g}	367.44±44.85^{de}	66.3±01.8^{b}	1.03±0.02^{cd}	28.4±1.13^{a}	2.17±0.06^{i}	243.06±2.86^{c}	89.13±0.24^{ab}	77.83±4.98^{a}
2	93.33±15.72^{e}	271.32±82.72^{f}	54.7±0.9^{de}	1.17±0.02^{bc}	26.4±1.31^{ab}	2.08±0.12^{i}	219.22±21.33^{de}	69.81±4.68^{ef}	70.20±1.53^{abc}
3	213.33±41.63^{cd}	494.57±29.90^{bc}	78.2±3.7^{a}	1.02±0.02^{cd}	26.9±0.64^{ab}	4.13±0.42^{b}	231.89±10.34^{de}	87.48±00.31^{ab}	76.91±0.82^{ab}
4	180±20^{cd}	514.73±11.71^{bc}	60.9±3.1^{c}	1.02±0.02^{cd}	26.3±0.39^{ab}	3.65±0.06^{de}	212.44±14.94^{e}	59.35±2.53^{h}	68.83±0.76^{b}
5	262±49.52^{bc}	368.99±25.62^{de}	61.5±3.0^{c}	1.38±0.09^{a}	26.0±0.48^{ab}	4.49±0.23^{a}	240±13.86^{c}	73.47±9.03^{de}	67.70±3.97^{bc}
6	52±3.46^{fg}	331.78±60.52^{e}	50.3±1.9^{g}	1.15±0.09^{bc}	20.6±0.38^{abc}	3.89±0.34^{d}	184.56±21.45^{hi}	29.62±1.16^{j}	63.38±1.26^{cd}
7	410.67±227.04^{a}	368.99±75.62^{de}	62.7±1.5^{c}	0.74±0.11^{e}	26.7±0.41^{ab}	4.43±0.23^{ab}	168.44±7.90^{ij}	42.45±2.02^{i}	62.10±3.23^{cd}
8	76.67±25.17^{ef}	243.41±32.67^{g}	51.0±0.3^{f}	1.06±0.10^{c}	24.6±0.44^{ab}	3.58±0.13^{e}	228.22±5.87^{d}	43.95±5.34^{i}	60.11±2.16^{d}
9	222±37.04^{cd}	527.13±20.97^{b}	57.1±3.3^{d}	1.23±0.07^{b}	20.9±0.17^{abc}	4.59±0.32^{a}	218.89±16.98^{de}	81.71±3.52^{bc}	56.30±2.25^{de}
10	68±8^{f}	578.29±18.80^{ab}	55.6±3.3^{de}	0.84±0.07^{de}	22.7±0.68^{abc}	3.79±0.06^{d}	197±14.38^{ef}	76.04±1.85^{d}	54.22±5.58^{e}
11	282.67±8.33^{bc}	590.70±20.27^{ab}	54.0±2.0^{de}	1.09±0.02^{c}	19.4±1.34^{b}	4.70±0.21^{a}	242±5.81^{bc}	64.50±0.50^{g}	50.29±1.46^{f}
12	304±141.32^{b}	601.55±11.71^{a}	46.5±1.2^{g}	0.84±0.04^{de}	27.7±0.82^{a}	4.08±0.01^{bc}	144.56±2.22^{g}	73.06±1.93^{e}	51.60±2.08^{ef}
13	303.33±49.89^{b}	488.37±58.28^{bc}	52.0±0.6^{f}	1.05±0.05^{c}	28.3±0.85^{a}	4.53±0.01^{a}	260.11±7.03^{b}	80.01±3.10^{c}	66.82±2.75^{bc}
14	240.67±133.17^{cd}	443.41±33.65^{c}	46.6±1.1^{g}	0.49±0.04^{g}	14.4±2.88^{c}	2.53±0.14^{h}	187.11±4.53^{f}	62.29±3.89^{gh}	51.82±3.11^{ef}
15	194.67±46.54^{cd}	393.80±42.20^{d}	50.4±1.9^{fg}	0.94±0.03^{d}	27.2±0.68^{ab}	2.57±0.04^{h}	300.22±15.41^{a}	82.43±0.99^{b}	65.53±1.62^{c}
16	276.33±21.01^{bc}	435.66±28.04^{cd}	45.0±1.0^{g}	0.62±0.08^{f}	24.0±1.83^{abc}	3.56±0.11^{e}	187.33±5.04^{f}	79.34±5.15^{cd}	60.07±2.37^{d}
17	154±22 ^{d}	404.65±9.30^{d}	41.3±0.9^{h}	1.09±0.08^{c}	14.2±0.96^{c}	2.95±0.04^{g}	210.78±8.70^{e}	79.9±2.13^{cd}	46.75±2.05^{f}
18	253.33±53.97^{c}	355.04±39.01^{de}	36.6±0.3^{i}	0.85±0.10^{de}	15.0±1.00^{c}	3.29±0.07^{e}	187.33±4.18^{f}	90.2±2.48l^{a}	49.88±1.87^{f}

Values in the table are illustrated as mean ± standard variation. The same letters indicating the values are not different significantly. Otherwise, different letters indicating the values are significantly different (P<0.05) Post hoc Duncan test. T: Numbers of treatments; GPX: activity of Guaiacol peroxidase; SOD: activity of superoxide dismutase; RS: reducing sugar content; SPr: soluble protein content; TF: total flavonoid content; TPh: total phenolic content. PAL: activity of phenylalanine ammonia lyase; DPPH: percentages of DPPH radical scavenged, at the concentration of 15 ug/ml; SA: percentages of superoxide anion radical scavenged at the concentration of 10 mg/ml.

Table 4. Berry results of detected biochemical traits of treatment different (TD) group of grapevine

T	GPX U/g (FW)/min	SOD (U/g (FW)/min)	RS mg/g (FW)	SPr (mg/g (FW))	TF (mg/g (DW))	TPh (mg/g (FW))	PAL (U/(g (FW)/min))	DPPH (%)	SA (%)
1	21.33±2.31^{h}	88.37±25.90^{e}	116.6±0.9^{b}	0.38±0.13^{c}	3.17±0.6^{b}	0.44±0.01^{cd}	53.44±7.94^{ab}	25.66 ±1.11^{bc}	44.52±0.43^{ab}
2	40±20.25^{fgh}	165.89±51.86^{cd}	85.3±0.2^{f}	0.43±0.03^{ab}	3.08±0.04^{b}	0.22±0.02^{f}	41.72±2.70^{cd}	23.34±1.63^{c}	41.13±1.54^{b}
3	134±6.61^{b}	106.20±90.68^{cde}	134.5±0.4^{a}	0.53±0.05^{ab}	3.68±0.07^{a}	0.41±0.02^{cd}	42.83±4.37^{c}	36.01±2.49^{a}	47.90±0.82^{a}
4	97.47±4.40^{cd}	130.23±66.23^{cde}	97.2±0.5^{d}	0.45±0.09^{ab}	3.05±0.04^{bc}	0.50±0.06^{b}	41.67±0.93^{cd}	27.92±6.05^{b}	45.11±0.59^{ab}
5	175±12.95^{a}	227.13±34.91^{bc}	103.5±1.4^{c}	0.54±0.06^{a}	3.62±0.06^{ab}	0.42±0.05^{c}	44.17±4.65^{bcd}	15.97 ±4.41^{f}	27.18±3.95^{e}
6	20.97±1.89^{h}	133.33±12.8^{d}	100.0±1.6^{cd}	0.43±0.07^{b}	3.02±0.05^{bc}	0.31±0.01^{e}	30.61±1.29^{fg}	21.79±2.3^{cd}	33.83±1.26^{d}
7	25.67±5.13^{h}	89.92±20.05^{de}	134.0±2.2^{a}	0.46±0.04^{ab}	1.76±0.04^{e}	0.42±0.03^{c}	45±1.67^{bc}	14.94±2.38^{fg}	31.13±3.23^{de}

Table 4. Contd.

8	48±18.52^{ef}	208.53±11.47^{c}	99.4±1.2^{d}	0.35±0.07^{cd}	1.36±0.06^{ef}	0.65±0.08^{a}	48.89±2.55^{b}	20.45±2.48^{d}	30.10±2.16^{de}
9	42±4.36^{f}	334.52±13.87^{a}	121.6±3.2^{b}	0.40±0.08^{b}	1.79±0.04^{e}	0.37±0.05^{cd}	45.22±5.32^{bcd}	15.77±2.15^{f}	33.09±2.25^{d}
10	60±3^{e}	302.17±13.67^{ab}	114.2±1.2^{bc}	0.51±0.02^{ab}	2.83±0.06^{c}	0.24±0.04^{f}	40.89±2.18^{cd}	18.65±0.88$^{e\ efgh}$	21.14±5.58^{f}
11	105.33±14.74^{c}	275.35±14.27^{ab}	94.4±1.8^{e}	0.38±0.06^{bc}	2.98±0.02^{c}	0.38±0.06^{cd}	49.06±1.58^{bc}	25.04±1.57^{bc}	38.30±1.46^{c}
12	20.32±3.39^{h}	188.56±50.56^{cd}	97.9±0.5^{d}	0.32±0.07^{d}	2.04±0.06^{e}	0.64±0.02^{a}	36.5±2.85^{de}	21.17±0.31^{cd}	37.11±2.08^{c}
13	99.33±0.95^{cd}	259.57±23.71^{bcd}	91.6±0.6^{f}	0.37±0.03^{c}	2.50±0.04^{d}	0.64±0.03^{a}	40.67±2.25^{cd}	17.10±1.39^{ef}	28.80±2.75^{de}
14	89.57±1.30^{d}	314.01±17.76^{ab}	96.1±0.6^{e}	0.29±0.11^{e}	1.65±0.04^{e}	0.39±0.00^{cd}	31.39±13.14^{f}	5.51±2.72^{l}	20.72±3.11^{f}
15	29.07±2.10^{g}	318.74±34.33^{ab}	111..5±0.4^{bc}	0.46±0.05^{ab}	1.43±0.05^{ef}	0.34±0.00^{d}	57.39±1.77^{a}	10.46±4.51^{j}	27.51±1.62^{e}
16	18.88±11.34^{h}	245.36±20.68^{bc}	110.5±0.7^{bc}	0.30±0.04^{e}	1.46±0.02^{ef}	0.41±0.02^{cd}	37.83±1.09^{d}	12.88±0.64^{h}	27.92±2.37^{e}
17	51.73±2.69^{ef}	271.40±7.61 ^{b}	98.7±1.2^{d}	0.53±0.05^{ab}	1.05±0.06^{f}	0.50±0.02^{b}	34.11±1.18^{e}	8.96±0.27k	29.41±2.03^{de}
18	42.9±1.97^{f}	265.88±14.27^{b}	84.2±0.5^{f}	0.38±0.02^{bc}	1.12±0.03^{f}	0.40±0.01^{cd}	25.89±1.35^{g}	18.19±1.31^{e}	27.93±1.86^{e}

Values in the table are illustrated as mean ± standard variation. The same letters indicating the values are not different significantly. Otherwise, different letters indicating the values are significantly different (P<0.05) Post hoc Duncan test. T: Numbers of treatments; GPX: activity of Guaiacol peroxidase; SOD: activity of superoxide dismutase; RS: reducing sugar content; SPr: soluble protein content; TF: total flavonoid content; TPh: total phenolic content. PAL: activity of phenylalanine ammonia lyase; DPPH: percentages of DPPH radical scavenged, at the concentration of 15 ug/ml; SA: percentages of superoxide anion radical scavenged at the concentration of 10 mg/ml.

especially on some primary metabolites, such as sugar and protein contents (Figure 1). Except few parameters, variations of different parameters between leaves and berries in GD group are very similar, however, greater variations of the detected parameters were found in berries than in leaves of the TD group (Figure 1). These variations promted us to assess the correlations of these traits between leaf and berry.

Inter-parameters' correlation within leaf or berry for both GD and TD groups were detected and the result of correlation coefficients were listed and are shown in Tables 5 and 6, respectively. Only smaller proportion (<11%) of inter-parameter pairs tested were with significant correlation within leaf or berry samples in both groups. In GD group, parameter pairs TS/RS, GPX/SOD and TF/SA showed significant correlations both in leaf and in berry. Significant correlation of inter-parameter pairs in TD group were also found, but only one inter-parameter pairs TF/SA showed significant correlation both in leaf and in berry in this group (Table 6). Correlation of different parameters within leaf (berry) implies the possible correlations of these parameters in metabolisms or functions. Interestingly enough, in coefficients of variation, there was also significant correlations between leaves and berries in both GD group (r=0.918; P<0.001) and TD group (r=0.870; P<0.05). Lower proportion of correlation as well as lower correlation coefficients of inter-parameter pairs within leaf (berry) samples ensured the effectiveness of following quantitative correlation analysis between leaf and berry.

Correlations between leaf and berry for every coordinate traits were tested for both GD and TD groups and the correlation coefficients are shown in Tables 7 and 8, respectively. Out of the 11 detected biochemical parameters, 9 showed significant correlations between leaf and berry in GD group. Amongst, parameters of TS, RS, TTA, TBARS, GPX, SOD, and SA were correlation significant at P<0.01 level, with the correlation coefficients as high as 0.84, 0.87, 0.83, 0.80, 0.98, 0.87, and 0.97, respectively. In total flavonoids (TF) and total phenols (TPh), there was also significant correlation between leaf and berry in GD group at P<0.05 level, with the correlation coefficients of about 0.68 (Table 7). Therefore, data of these correlation significant traits from leaf of GD group may be used to estimate the values of coordinating berry traits. In TD group, values of parameters RS, SPr, SA, and PAL correlate significantly between leaves and berries at P<0.01 level, with the correlation coefficients as 0.67, 0.52, 0.58, and 0.69, respectively. TF showed significant correlation at P < 0.05 level between leaf and berry with a correlation coefficient of 0.51. Parameters of TPh, SOD, and GPX showed significant correlations in GD group, while in these parameters, there was no significant correlation in the TD group (Table 8). Amongst the parameters that detected simultaneously in both GD and TD groups, RS, TF, and SA showed significant correlation between leaves and berries. Therefore, significant correlations in values of some biochemical

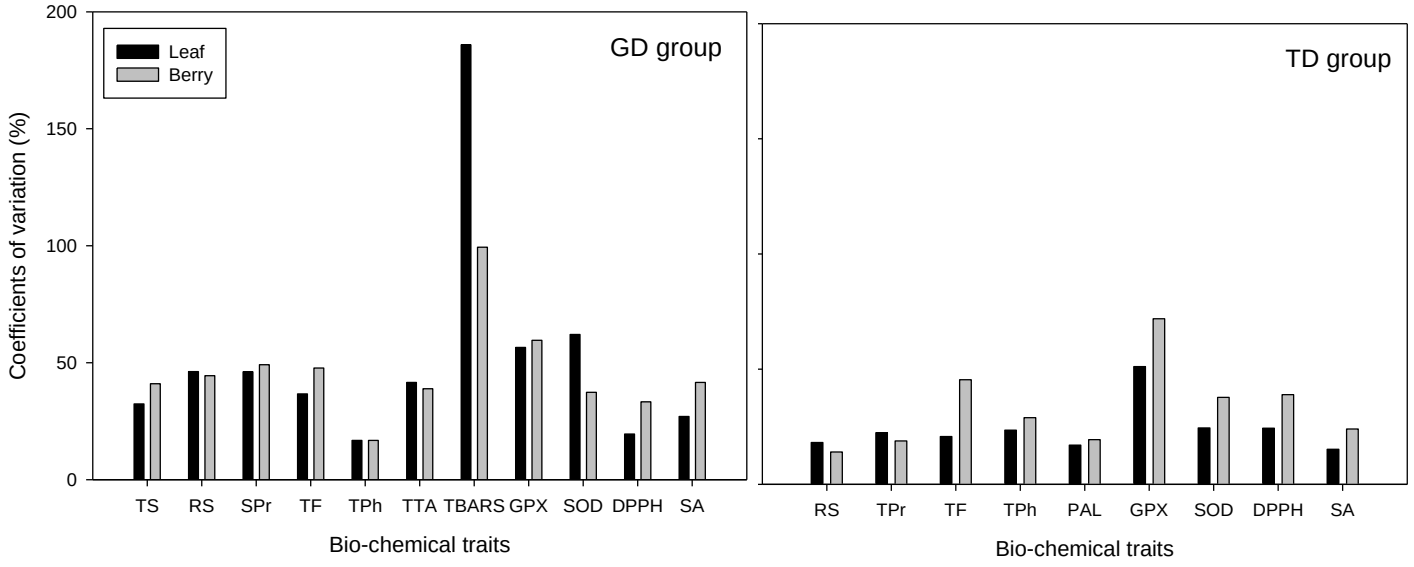

Figure 1. Comparison of coefficients of variation of detected biochemical traits within leaves and berries in both genotype different (GD) and treatment different (TD) groups of grapevine.

traits between leaves and berries of grapevines broadly existed. No doubt that the ranges of these correlated traits in berries could be primarily estimated by the detected leaf values.

Beside leaf/berry same-parameter pairs afore-mentioned, significant correlations of inter-parameter pairs between leaf and berry were also detected, such as TS.L&B/RS.L&B ("L" represents leaf and "B" represents berry), TF.B/TPr.L, TF.B/SA.L, GPX.L&B/SOD.L&B, TBARS.B/TPr.L, TF.L/DPPH.B, and SA.L/DPPH.B. Parameters RS.B and TS.B were foundto be both significantly negative correlated with DPPH.L in GD group (Table 7). Compared to GD group, more of such inter-parameter pairs between leaf and berry were found with significant correlation in the TD group. For some examples, leaf RS correlated significantly to berry TF (r=0.72), PAL (r=0.52), DPPH (r=0.65), SA (r=0.60); berry TF significantly correlated to leaf RS and SA (r=0.54); and berry PAL also significantly correlated to leaf RS, TF (r=0.62) and SA (r=0.48) at the same time (Table 8).

DISCUSSION

Correlative growth of different parts of plant has been well known, because of the continuously exchange of nutrients, metabolites, and signal molecules (Srivastava, 2002; Teale et al., 2006). Compositional correlations between different organs of plants had also evidences. Some special substances detected in certain species of plant can always be detected more or less from other parts or organs in this species of plants (Neto et al., 1992), and some of these compounds and existent patterns have been used as chemo-taxonomical parameters (Herl et al., 2008; Loreto, 2002; Figueiredo-González et al., 2012). However, the quantitative correlation of biochemical components between different parts of a plant has not been systematically studied. In some earlier studies, correlations of some nutrients, such as N, Ca, K, P, Mg, etc., between or within plant leaves and fruits has been reported (Dris et al., 1999). Correlations between metabolites in grape berries also had been studied which were focused more on correlations of inter-parameter within or between leaves and fruits, other than purposely designed to investigate the correlations of same-parameter pairs between different organs or parts of plant (Shiraishi et al., 2010). The later work has just tested the significance of correlations of the biochemical traits within grape berries. Plant cells from different parts or organs differentiated as cells with different phenotypes and functions, and will have different patterns of gene expression and the resultant metabolites. However, the high similarities of genetic background of these cells from different parts of one plant or same variety will share higher proportions of metabolic similarities compared to genetically varied cells as has proved again in this study (Figure 1). Furthermore, cells in leaves or berries of a single plant always under similar environmental conditions and may respond to these factors coordinately to produce similar defense metabolites, due to the continuously exchange of all kinds of transportable metabolites, including some defense compounds among different parts of tissues (Jørgensen et al., 2015).

Therefore, the existence of some metabolites with co-ordinating concentrations in leaves and fruits is expected. The obtained data have provided evidence and proved the existence of such kind of values' co-vibaration of some

Table 5. Correlation coefficients among parameters (inter-parameter pair)within berry (right-above) and leaf (left-below) of genotype different (GD) group of grapevine.

Parameter	TS	RS	TF	TTA	TPr	TPh	SOD	GPX	TBARS	DPPH	SA
TS	-	0.985**	-0.209	-0.118	-0.509	0.279	0.141	-0.418	-0.226	-0.509	-0.327
RS	0.916**	-	-0.211	-0.221	-0.551	0.306	0.253	-0.302	-0.234	-0.560	-0.387
TF	0.127	0.129	-	-0.062	-0.087	0.384	-0.100	-0.042	0.673*	0.600*	0.662*
TTA	-0.314	-0.269	-0.336	-	0.397	-0.036	-0.361	-0.372	-0.200	0.005	0.260
TPr	-0.125	-0.087	0.518	-0.062	-	0.159	-0.241	0.152	0.268	0.400	0.476
TPh	0.144	0.242	0.274	-0.273	0.163	-	0.284	0.144	0.282	0.249	0.472
SOD	-0.410	-0.243	-0.013	-0.251	-0.159	0.509	-	0.746 **	-0.349	-0.329	-0.129
GPX	-0.478	-0.333	-0.094	-0.350	-0.228	0.285	0.909**	-	-0.131	0.044	0.096
TBARS	-0.091	-0.270	0.201	0.022	0.493	-0.092	-0.331	-0.307	-	0.649*	0.465
DPPH	-0.773**	-0.764**	0.043	0.003	0.172	-0.103	0.455	0.548	0.422	-	0.818**
SA	-0.170	-0.215	0.578*	-0.163	0.048	0.030	0.056	0.180	0.309	0.236	-

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed). ; GPX: activity of Guaiacol peroxidase; SOD: activity of superoxide dismutase; RS: reducing sugar content; TS: total sugar; TPr: total protein content; TTA: titratable acidity TF: total flavonoid content; TPh: total phenolic content. DPPH: percentages of DPPH radical scavenged, at the concentration of 15 ug/ml; SA: percentages of superoxide anion radical scavenged at the concentration of 10 mg/ml.

Table 6. Correlation coefficients among traits in berry (right-above) and leaf (left-below) of treatment different (TD) group of grapevine.

Parameter	GPX	SOD	RS	SPr	TF	TPh	PAL	DPPH	SA
GPX	-	0.064	-0.034	0.398	0.639**	0.079	0.048	0.233	0.065
SOD	0.35	-	-0.29	-0.168	-0.27	-0.113	-0.087	-0.645**	-0.704**
RS	-0.061	0.111	-	0.35	0.178	-0.145	0.422	0.157	0.163
SPr	-0.32	-0.172	0.313	-	0.329	-0.325	0.17	0.21	0.13
TF	0.036	0.002	0.601**	0.213	-	-0.078	0.422	0.570*	0.479*
TPh	0.530*	0.484*	0.219	0.24	0.121	-	0.013	0.017	0.075
PAL	-0.202	-0.13	0.28	0.462	0.286	-0.134	-	0.196	0.278
DPPH	0.079	0.303	0.019	0.006	0.027	-0.2	0.335	-	0.844**
SA	-0.248	-0.294	0.759**	0.322	0.751**	-0.153	0.445	0.066	-

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level (2-tailed). GPX: activity of Guaiacol peroxidase; SOD: activity of superoxide dismutase; RS: reducing sugar content; SPr: soluble protein content; TF: total flavonoid content; TPh: total phenolic content. PAL: activity of phenylalanine ammonia lyase; DPPH: percentages of DPPH radical scavenged, at the concentration of 15 ug/ml; SA: percentages of superoxide anion radical scavenged at the concentration of 10 mg/ml.

parameters between leaf and berry of grapevine as indicated in Tables 7 and 8, implies the possibility to predict some berry quality-related parameter values by using values from leaves or other parts of vines. In addition, the increase of sample numbers will Increase the significance of correlation coordinately, since randomly take off of any sample datum will decrease the significance and coefficient of correlations.

Table 7. Correlation coefficients of detected physio-chemical traits between berry and leaf of the genotype different (GD) group of grapevine.

Parameter	TS.B	RS.B	TF.B	TTA.B	TPr.B	TPh.B	SOD.B	GPX.B	TBARS.B	DPPH.B	SA.B
TS.L	0.84**	0.85**	-0.25	-0.16	-0.31	0.29	-0.09	-0.45	-0.11	-0.37	-0.31
RS.L	0.82**	0.86**	-0.21	-0.10	-0.49	0.29	0.09	-0.30	-0.25	-0.50	-0.37
TF.L	0.04	0.04	0.68*	-0.12	-0.28	0.37	-0.07	-0.15	0.38	0.64*	0.55
TTA.L	-0.20	-0.27	-0.06	0.83**	0.33	-0.23	-0.20	-0.29	-0.13	-0.21	-0.00
TPr.L	-0.32	-0.29	0.58*	-0.19	-0.28	-0.12	-0.37	-0.24	0.58*	0.37	0.02
TPh.L	0.10	0.21	0.22	-0.32	-0.19	0.69*	0.51	0.40	0.04	-0.03	-0.03
SOD.L	-0.36	-0.24	0.09	-0.37	-0.00	0.18	0.80**	0.95**	-0.21	-0.00	0.11
GPX.L	-0.45	-0.35	0.00	-0.37	0.15	0.09	0.66*	0.98**	-0.10	0.15	0.18
TBARS.L	-0.17	-0.18	0.52	-0.19	0.28	0.16	-0.33	-0.29	0.87**	0.44	0.29
DPPH.L	-0.65*	-0.62*	0.31	-0.25	0.27	-0.15	0.31	0.54	0.40	0.45	0.32
SA.L	-0.24	-0.29	0.65*	0.169	0.43	0.57	-0.15	0.08	0.53	0.82**	0.97**

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level (2-tailed). Parameters with ".B" indicate the berry parameters, and ".L" indicate the leafy parameters.

Table 8. Correlation coefficients of detected physio-chemical traits between berry and leaf of the treatment different (TD) group of grapevine.

Parameter	GPX.B	SOD.B	RS.B	SPr.B	TF.B	TPh.B	PAL.B	DPPH.B	SA.B
GPX.L	0.21	0.114	0.141	-0.173	0.026	0.291	-0.097	-0.244	-0.159
SOD.L	0.228	0.286	0.158	-0.041	0.311	0.085	-0.011	0.145	0.067
RS.L	0.421	0.573	0.673**	0.455	0.723**	-0.12	.535*	0.647**	0.598**
SPr.L	0.343	-0.117	-0.096	0.517*	0.305	-0.019	0.301	0.372	0.373
TF.L	0.026	-0.515*	0.345	0.105	0.510*	0.209	0.615**	0.452	0.443
TPh.L	0.388	0.034	0.253	0.132	0.123	0.274	-0.015	0.198	-0.019
PAL.L	0.339	0.218	0.023	0.279	0.319	-0.043	0.688**	0.07	0.103
DPPH.L	0.136	0.329	0.056	0.086	0.36	-0.034	0.101	0.009	0.059
SA.L	0.2	-0.642**	0.355	0.263	0.536*	-0.073	0.479*	0.549*	0.579*

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level (2-tailed). Parameters with ".B" indicate the berry parameters, and ".L" indicate the leafy parameters.

According to the aforementioned analysis, it is impossible and unreasonable to expect all metabolites having quantitatively coordinated concentrations in different parts of plants, because of the positional and functional differences of cells.

However, it will be of great significance if some parameters or components of interest such as sugar, organic acids, flavonoids, phenols, etc., to show this kind of correlations. On the base of these correlations, it will be allowed to develop a berry-independent method for berry pre-evaluation. The values of several parameters for 2 groups in both leaf and berry samples of grapevine were measured. GD group were different varieties but shared the similar environmental

conditions, and TD group were same variety, but treated with different factors. Nine of 11 and 5 of 9 of the detected parameters in GD and TD group, respectively, showed significant correlation in values' variation between leaf and berry. Parameters of TS, TF and SA in leaves are significantly correlated to berries, both in GD and TD groups. Therefore, values of parameters TS, RS, TF, TTA TPh, GPX, SOD, and SA of leaf can be used to estimate the values in berry for the genotype differed materials but grow in similar environmental condition. Amongst sugar, acidity, and antioxidants such as total flavonoids and phenols are always important parameters for berry quality evaluation. Leaf values of parameters RS, TF, SPr, SA, and PAL can be used to estimate the corresponding values of berries for the same genotype materials but treated differently. Although, all these mentioned parameters have significant correlation in one or both groups, but correlation coefficients in GD group were obviously higher than in the TD group as indicated in Tables 7 and 8; which implies this leaf-dependent berry quality evaluation will be more reliable for those genotype varied candidates.

Theoretically, parameters which have significant correlation in values between leaf and berry, leafy values can potentially be used to estimate the ranges of the corresponding traits of berry. But how to make the estimation more accurate should have some strategies, both in experiment designing and choices of indicator leaves. Genetic differed grapevines growing at similar environment can be evaluated by just comparing the leaf values of certain parameter amongst the candidate materials, because of the higher variation coefficients among different genotypes and higher correlation coefficients of certain traits between leaf and berry (Figure 1, Tables 1 to 4, 7 and 8). More traits including some special groups of metabolites, such as organic acids, free amino acids, flavonoids, tannins, stilbenes or even anthocyanins, etc., that are not only closely related to the quality of grape but also vibrate coordinately in leaf and berry need to be developed. As for the choice of indicator leaves, almost the same physiological conditioned leaves should be chosen as indicator leaf as has been described in materials and experimental design. The fact that the existence of significant correlation of some inter-parameter pairs between leaf and berry, implies that values of some leafy parameters could also be used as indicators of some other parameters in berry, as already suggested in some similar studies (Dris et al., 1999). For examples, values of SA in leaf can be used to estimate the values of TF and DPPH in berries, and the leafy TF values can also be used to indicate the values of certain berry DPPH. As for TD group, beside the different treatments, grapevines of the same cultivar also grew under environmental conditions, the effects of different treatments on some leafy parameters could also be used to estimate the effects on corresponding traits of berries. While more parameter pairs including same- and inter-parameter pairs were found to be significantly correlated in TD group than in GD group, the relatively lower coefficients of correlation in TD group may limit the accuracy of estimation. However, as a purpose of primary estimation, it is enough for making a decision.

One might notice that this pre-evaluation method cannot evaluate the resources integrally for multiple agronomic characters. It could only be applied as one of an assistant method in early stages of screening from numerous candidates, especially for certain biochemical trait screening, for some examples, the selection of higher sugar content, special sugar/acid ratio, or higher flavonoids content materials, etc., whereas the final evaluation to resources should still be dependent upon the formal ways of evaluation, but at this time focus only on the mostly potential candidates.

Except for providing a leaf-dependent berry pre-evaluation method, the obtained results have also provided a basic relationship between results from *in vitro* and *in vivo* experiments. Nowadays, many elicitors have proved to be able to induce or modify certain kinds of secondary metabolite in grape suspension cells (Tassoni et al., 2012; Cetin et al., 2014; Cai et al., 2011; Chao et al., 2015), but if all these elicitors or factors can also cause similar responses in grapevine at plant level or fruits, is still lack the theoretic basis. In fact, some elicitors or factors did cause similar secondary metabolic responses both at plant and cell level of grapevine. For some instances, ABA can promote the synthesis of anthocyanins in certain line of grape cells (Gagné et al., 2011), in vitro cultured grape berries (Hiratsuka et al., 2001), and can also promote the coloration of fruits at plant level (Jiang and Joyce, 2003; Pirie and Mullins, 1976). The UV-B can induce the accumulation of resveratrol and other secondary metabolites responses where ever in suspension cells, *in vitro* tissues and *in vivo* of vine (Li et al., 2008; Zamboni et al., 2006). The present study has given a good explanation for these similar responses to same factor, but in different type of experimental materials. However, whether exist significant correlations in value vibration of these responding effects between *in vitro* and *in vivo* experiments needing further evidences.

Conflict of Interests

The authors have not declared any conflict of interest.

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In vitro antifungal activity of polyphenols-rich plant extracts against *Phytophthora cinnamomi* Rands

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Antifungal activity of water, ethanol, lanolin and cocoa butter plant extracts derived from seven Mexican Chihuahuan desert inhabiting plant species (*Larrea tridentata, Flourensia cernua, Agave lechuguilla, Opuntia ficus-indica, Lippia graveolens, Carya illinoensis* and *Yucca filifera*) were evaluated against *Phytophthora cinnamomi*. All plant extracts were active against *Phytophthora cinnamomi*. Two (*L. tridentata* and *F. cernua*) out of seven plant species tested had the optimal antifungal activity against this fungus specie, with minimum inhibitory concentration (MIC) values as low as 6.96 and 8.6 mg/L. Some of the plant extracts had moderate to low activity against *P. cinnamomi*, and the variations of active polyphenolic (condensed and hydrolysable tannins) compounds in the plant extracts estimated via colorimetric methods indicated that the inhibitory activity may not based on a general metabolic toxicity but perhaps the antifungal potency is conferred by group or groups of toxic metabolites. Based on the antifungal activity, crude plant extracts may be a cost effective way of protecting crops against *P. cinnamomi.* Because plant extracts contain several antifungal compounds, the development of resistant pathogens to these plant extracts may be delayed.

Key words: Antifungal activity, plant extracts, polyphenols, MIC_{50} *P. cinamomi*.

INTRODUCTION

The stramenopile *Phytophthora cinnamomi* Rands causes root rot of avocado and is one of the main limiting factors of this crop (Ceja et al., 2000; Messenger et al., 2000). In addition, this plant pathogen causes damages

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to others species as *Eucalyptus* and *Pinus* species (Linde et al., 1997), and pineapple (Allen et al., 1980). Their virulence is associated with temperature between 21 to 30°C, poorly drained soils and excessive moisture. This pathogen is diploid and heterothallic with two groups, A1 and A2 (Linde et al., 1997).

Its control is based on cultural practices, including management of soil moisture and improving ventilation by increasing drainage, and mineral nutrition care. The application of chemicals among which are the fungicides metalaxyl and fosetyl-aluminum to the soil, leaves or trunk injection (Whiley et al., 1986) and biological control agents including bacteria and fungi in soil, as *Pseudomonas* spp., *Streptomyces* spp. and *Trichoderma* spp., *Myrothecium roridum*, *Aspergillus* spp., or *Paecilomyces* spp., are other techniques useful to inhibit *P. cinnamomi* (Reeves, 1975; Gees and Coffey, 1989; Mass and Kotzé, 1990; Casale, 1990; Stirling et al., 1992; Duvenhage and Kotzé, 1993).

However, these management disease techniques present challenges and constraints in control of the disease, loss in efficiency, increased resistance to active ingredients and environmental hazards, so it is necessary to find new strategies for control, one of these strategies can be use of plant extracts as an alternative (Lira et al., 2007).

Several studies showed that secondary metabolites produced by plants have an effect on inhibiting the development of the mycelium of several pathogenic fungi (Hossein and Maldonado, 1982). Among the synthesis of secondary metabolites or phytochemicals are polyphenols which are a heterogeneous group of molecules having a structure of benzene substituted by various groups with hydroxyl functions, allowing them to be highly soluble is substances such as water.

These compounds are present in extracts of leaves, bark, wood, fruits and galls of certain ferns, gymnosperms and angiosperms (Swain, 1979). Polyphenols are important in plant physiology because they contribute to resistance to microorganisms, insects and herbivorous animals (Haslam, 1996).

Besides, these compounds help to preserve plant integrity during continuous exposure to environmental stressors, including ultraviolet radiation, high temperatures and dehydration (Lira et al., 2007). Polyphenol antioxidants are active in biological systems and probably the capacity or biological value explains its abundance in plant tissues (Meckes et al., 2004). Some plant species like *Larrea tridentata*, *Turnera diffusa*, *Flourensia cernua*, *Jatropha diocesan* among others are widely distributed in the Mexican Northern States, occupying an area of approximately 100 million hectares (González, 1975). These native plants have a high content of polyphenolic compounds (Lira et al., 2007). Plant extracts obtained with different solvents as methanol, acetone, chloroform, hexane, etc. have been reported with antimicrobial properties.

However, little attention has been given to obtaining polyphenols-rich extracts with unconventional solvents which have potential use in disease management of organic farming. The detected significant differences on the antifungal activity can be due to total polyphenols presents in the plant extracts. This is the first study on use polyphenols-rich plant extracts against *P. cinnamomi*, because there are some reports where plant extracts are used but to inhibit other *Phytophthora* species such as: *Phytophthora infestans* (Gamboa et al., 2003a, b), *Phytophthora capsici* (Galván, 2005) and *Phytophthora palmivora* (Mendoza et al., 2007) *in vitro.*

In addition, Nielsen et al. (2006), reported the effect of natural product derives from *Quillaja saponaria* which showed activity against root rot until 100% in disease control, this plant is native of desert regions and have high titers of saponins. Saponins have been reported to reduce surface tension in the nutrient solution of hydroponic systems in greenhouses and cause disintegration of the membrane of *Phytophthora* zoospores.

In this context this paper aims were to determine the *in vitro* antifungal activity of semi-desert plants extracts on inhibiting mycelial growth of *P. cinnamomi* and their MIC_{50}.

MATERIALS AND METHODS

Seven wild plant species (*L. tridentata* Sees and Moc. ex D.C. Coville, [Zygophyllaceae] *Flourensia cernua* DC [Asteraceae], *Agave lechuguilla* Torr [Agavaceae], *Opuntia ficus-indica* L. [Cactaceae], *Lippia graveolens* Kunth (Verbenaceae), *Carya illinoensis* K. Koch (Juglandaceae) and *Yucca filifera* Chabaud (Agavaceae)) were collected in the Southern region of Coahuila, (semi-desert region) during August and September, 2008. The collected plant material was transferred to the Microbiology Laboratory of The Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila, for dehydration and milling. Dehydration was carried out at room temperature for 10 days and when required in an oven for two days to have moisture content between 5 to 10%, the milling process was carried out in a miller (Thomas Wiley) 1 mm mesh. The obtained fine powder was stored in amber bottles at room temperature until extraction of polyphenolic compounds was done.

The phytochemical compounds extraction was performed by a solid-liquid procedure, using four solvents (water, ethanol, lanolin and cocoa butter). For hydrophilic solvents group Soxhlet method was used and hydrophobic solvents group infusion method was used. In firth group distilled water and ethanol (70%) were used and second group mineral oil emulsions with 10% lanolin and cocoa butter were used. Each fine powder sample was mixed in a 1:4 (w /v) ratio with the corresponding extracting agent. Soxhlet method was performed in a rotary evaporator at 60°C for 7 h while infusion method was carried out heating the solvent at 60°C, once reached this temperature; the fine powder was added and remained under these conditions during 7 h. After this, extracts were filtered and stored at 5°C in container in ramber bottles until the extracted phytochemical compounds were identified and quantified.

In this case, only condensed and hydrolysable tannins were

determined which belong to polyphenols group. Concentration of hydrolysable tannins (HT) was determined by the Folin-Ciocalteu method (Makkar, 1999). Condensed tannins (CT) were spetrophotometrically determined using the method reported by Swain and Hillis (1959). For condensed tannins determination, an aliquot of 0.5 ml of plant extract was placed in a tube, with 3 ml of HCl/butanol (1:9) and 0.1 ml of ferric reagent.

On the other hand, it was added to a tube assay series catechin (standard) in distilled water at different concentrations (0, 200, 400, 600, 800 and 1000 ppm) to determine the reference curve. Tubes were plugged tightly and were heated for 1 h in water bath at 90°C. After that, tubes were leaved to cool and absorbance was read at 460 nm. For hydrolysable tannins determination, a reference curve was done by placing 400 µl of gallic acid at different concentrations (0, 200, 400, 600, 800, and 1000 ppm) in assay tubes. Gallic acid concentrations were prepared using distilled water. Each one of the plant extract were diluted in a test tube respectively, immediately to each tube were added 400 µl of commercial Folin-Ciocalteu reagent, samples were vortexed and held for 5 min. Then 400 µl of $NaCO_3$ (0.01 M) and 2.5 ml of distilled water were added.

Finally, absorbance was read at 725 nm in UV / visible spectrophotometer. Determination of polyphenolic compounds antifungal activity from 28 plant extracts on inhibition of mycelia growth was performed through the poisoned medium technique using different concentrations (ppm) of total polyphenols (hydrolysable plus condensed tannins). The response in inhibition mycelia growth was based in Minimum Inhibitory Concentration (MIC_{50}) defined as: the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism in 50% of radial growth in contrast to control (Kumar et al., 2011). Potato dextrose agar (PDA - Bioxon) as culture medium was used, in this case, volume of each extract according to the final concentration was determined and quantified; this volume was added to a flask with the water volume and PDA and sterilized at 120°C for 15 min, then flasks were left to cool and poured in Petri dishes. Subsequently, 0.5 cm plug *P. cinnamomi* mycelia 7 days old was add and incubated at 25 ± 2°C, until the untreated control (PDA only) completely covered the Petri dish. The response variable was radial growth (cm).

This data was transformed to percent of mycelia growth inhibition by the following equation P = (CT) / C x 100, where P is inhibition percentage, C is colony diameter of the control treatment and T is the colony diameter of a specific treatment. Treatments were established under a completely randomized design with four replications.

In addition, Probit analysis by maximum likelihood method (Finney, 1971) to determine the minimum inhibitory concentrations at 50% (MIC_{50}) of each extract was used. Data were analyzed using SAS V8.1 software. The MIC_{90} and MIC_{50} values were calculated as the 90th and 50th percentile of the minimum inhibitory concentration values and their fiducials limits respective.

RESULTS

The variance analysis detected significant differences on the antifungal activity by effect of polyphenols derives Mexican plants. We observed differences in percentage of mycelia growth inhibition of *P. cinnamomi*. These percentages in mycelia growth inhibition varied from 0% (control treatment) to 100% in the highest concentration treatment where plant extracts were used. In Figure 1, is shown as totals polyphenol concentration is increase, the algae mycelia growth inhibition also increases.

The antifungal effects of plant extracts on *P. cinnamomi* were variable. Figure 1a shows that the *L. tridentata* extracts promoted the high mycelium inhibition until 100% when those was obtained with ethanol and 80% when lanolin was used, while the lowest antifungal effect was observed with cocoa butter and water solvents. It also shows that as total polyphenols concentration increase *P. cinnamomi* mycelia growth inhibition also increases.

Results obtained with *Flourensia cernua* indicated that the highest fungal inhibition effect are reached using ethanol and water as solvents, while the lowest fungal inhibition effect was observed when lanolin was used during the extraction (Figure 1b). Although the fungal inhibition effect were equal (100%) with extracts obtained using water and ethanol, the concentrations required in the later case are lower (Figure 1b).

Pecan (*C. illinoensis*) nut husk extracts showed little or no effects on *P. cinnamomi* mycelium growth inhibition, the highest inhibition effect (16%) was observed when cocoa butter extracts was used as solvent (Figure 1f). In this case, it was so that the highest (3000 ppm) concentration inhibited only 50% the mycelium growth, extractions where ethanol, water and lanolin were used as solvents no inhibitory effect were observed.

This is the first study reporting the use *Opuntia ficus-indica, Agave lechuguilla, Lippia graveolens* and *Yucca filifera* extracts against *P. cinnamomi*. The highest fungal inhibition effects (100%) using *Opuntia* extracts were observed using water as solvents and a polyphenols concentration of 4000 ppm. While little or no mycelia growth inhibition was found with the other solvents (Figure 1c).

Agave extracts showed the best fungal inhibition effect (60%) observed when water was used during extraction at polyphenol concentration of 3000 ppm (Figure 1d). Not mycelium growth inhibition effects were observed with cocoa butter and lanolin emulsions, while inhibition effect (40%) was observed when ethanol was used in the extraction in polyphenols at 40 ppm concentration. The highest mycelium growth inhibition (80%) on *P. cinnamomi* by *Lippia* extracts was observed in lanolin at 40 ppm, while no fungal inhibitory effects were observed with aqueous extracts. In general it was observed less than 50% inhibition using ethanol and cocoa butter as solvents (Figure 1e). *Yucca* extracts showed little or no effect on *P. cinnamomi* mycelium growth inhibition at the evaluated concentration (Figure 1g).

The results obtained in the present study showed that the plant species has an effect on the level of *P. cinnamomi* mycelium growth inhibition. Figure 2 shows that the highest (75.3 and 68.1%) mycelium growth inhibition was reached when *Flourensia cernua* and *L. tridentata* were used as sources of extracts. On the other hand, all other plant species showed a maximum average effect on fungal inhibition of 30%. In general, it was observed plant and solvent interaction effects on mycelium

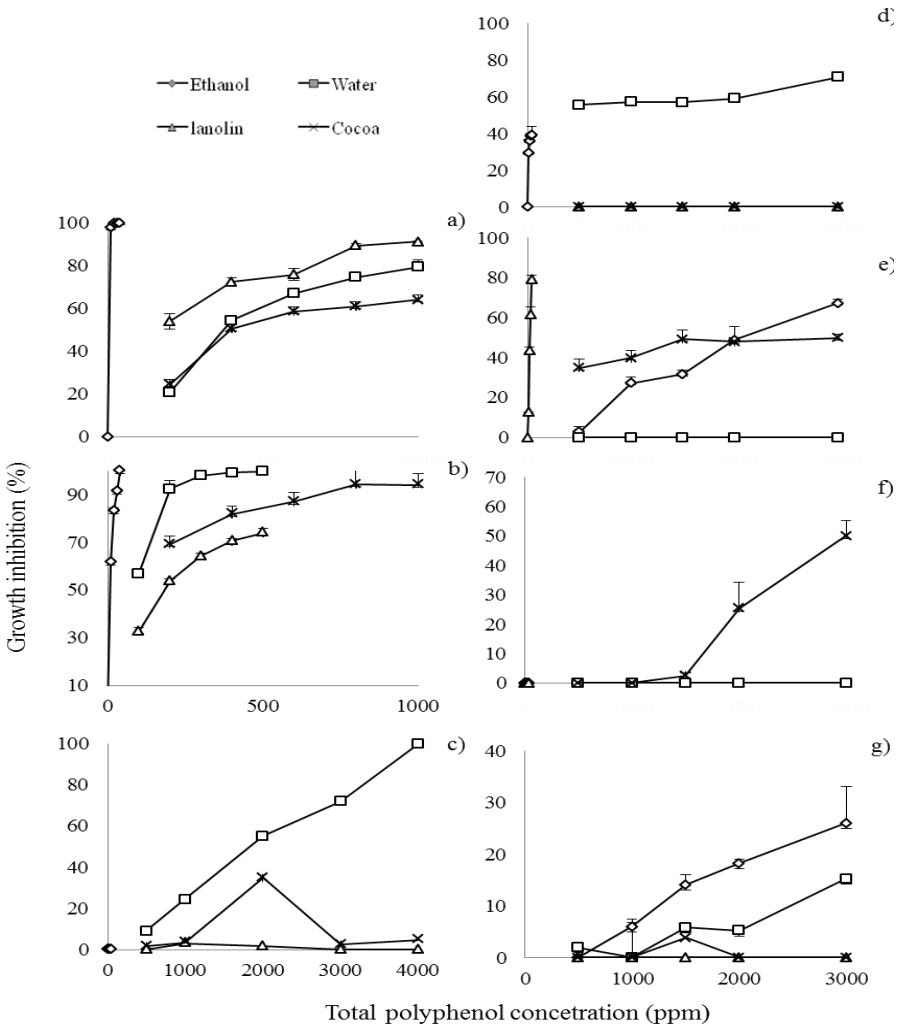

Figure 1. Inhibition response of *P. cinnamomi* at the total polyphenols concentration (PPM) obtained different solvents from (a) *L. tridentata*, (b) *F. cernua*, (c) *O. indica*, (d) *A. lechuguilla,* (e) *L. graveolens*, (f) *C. illinoensis* and (g) *Y. filifera*.

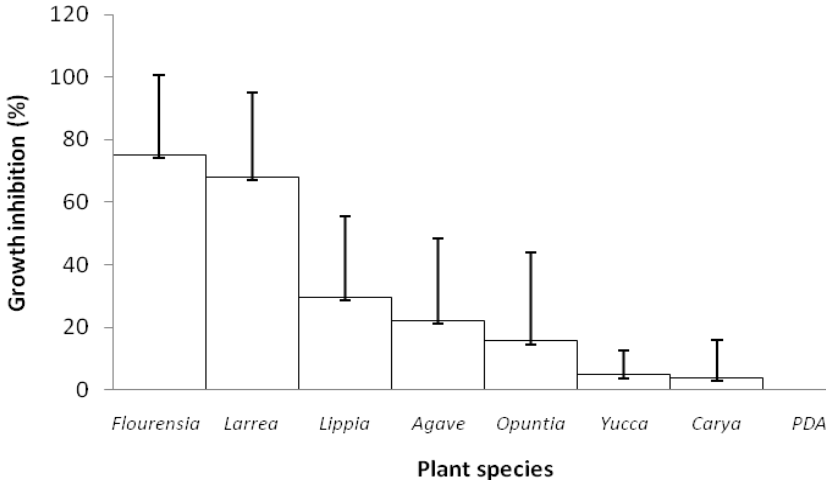

Figure 2. *In vitro* average effect on inhibition of *P. cinnamomi* with different plant extracts.

Table 1. Totals polyphenols minimum inhibitory concentrations (ppm) for inhibit mycelia of *P. cinnamomi.*

Species	Solvents	MIC_{50}	Fiducial limits 95% of MIC_{50}		MIC_{90}
			Inferior	Superior	
Larrea tridentata	Water	483.7	449.8	518.2	1431
	Lanolin	183.6	155.3	210.3	1008
	Cocoa	664	560.4	772.4	7213
	Ethanol	6.96	6.17	7.85	11.19
Flourensia cernua	Water	94.97	88.05	101.36	193.14
	Lanolin	230.12	212.83	247.65	1188
	Cocoa	112.19	62.78	157.25	619.14
	Ethanol	8.6	7.8	9.36	23.61
Opuntia ficus indica	Water	13039	5803	596284	68568
	Lanolin	5378	4524	6817	20636
	Cocoa	1867	1723	2013	3595
	Ethanol	341.95	80.82	576.41	409181
Agave lechuguilla	Water	28.87	22.39	38.05	121.7
	Lanolin	23.07	21.96	24.22	58.5
	Cocoa	252.7	209.07	298.87	326974
	Ethanol	2032	1908	2169	5952
Lippia graveolens	Water	2887	2704	3140	4825
	Lanolin	0	-	-	0
	Cocoa	0	-	-	0
	Ethanol	0	-	-	0
Yucca spp.	Water	0	-	-	0
	Lanolin	0	-	-	0
	Cocoa	0	-	-	0
	Ethanol	0	-	-	0
Carya illinoensis	Water	0	-	-	0
	Lanolin	0	-	-	0
	Cocoa	0	-	-	0
	Ethanol	0	-	-	0

Fiducial limit = confidence interval, MIC = Minimun inhibitory concetrantion in PPM.

growth inhibition of *P. cinnamomi*. The MIC_{50} of each plant extract on *P. cinnamomi*, was highly variable among solvents within each particular specie. The lowest MIC_{50} was obtained with *L. tridentata* in ethanol with 6.96 ppm, and the highest with *Opuntia* aqueous extract with 13039 ppm (Table 1). MIC_{50} analysis reveals that the lowest concentrations inhibiting 50% of mycelia growth of *P. cinnamomi* are: 6.96 of *L. tridentata* in ethanol, 8.60 of *F. cernua* in ethanol, 23.07 of *L. graveolens* in lanolin, 28.87 of *A. lechuguilla* in ethanol (Table 1).

The highest concentrations (ppm) to inhibit 50% of *P. cinnamomi* mycelia growth are: *Opuntia* aqueous extract at 13039.00, *Y. filifera* ethanol extracts with 5378.00, for *C. illinoensis* extracts using cocoa butter as solvent with 2887 and *L. graveolens* ethanolic extracts with 2032 (Table 1). The extracts that did not inhibit *P. cinnamomi* mycelia growth are: *Y. filifera* using both lanolin and cocoa butter as solvents, *O. ficus-indica* with lanolin, cocoa butter and ethanol as solvents, *A. lechuguilla* with lanolin and cocoa butter as solvent, *L. graveolens* with cocoa butter and *C. illinoensis* with water, lanolin and ethanol as solvents (Table 1).

DISCUSSION

The solvents used permitting the extraction of polyphenols from plants in this study. It was demonstrated the solvents chemical structure interaction in specific manner with the polyphenols type extracted from vegetal tissue. Because it was used two groups of solvents, one highly hydrophilic (water and ethanol) and other hydrophobic

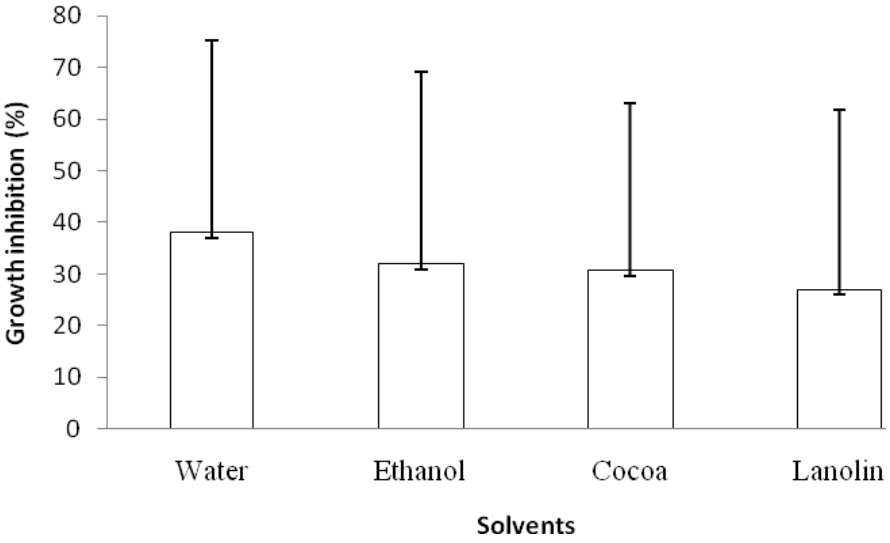

Figure 3. *In vitro* average effect on *P. cinnamomi* mycelia growth inhibition using different solvents.

(lanolin and cocoa butter) where polyphenols quantity differences obtained can be due to plant genera and solvent in this study (Figure 2). In addition, the polyphenols content in tissue is affected by season of plant tissue recollection, vegetative part, and plant growing conditions (Gamboa et al., 2003a; Hyder et al., 2005).

The differences shows on mycelia growth inhibition by polyphenols can be due to the chemical constitution of the polyphenols extracted associated with solvents (lanolin, cocoa butter, ethanol and water) may be due to the association formed between the hydrophobic region present in their structures, and the lipophilic region of the polyphenolic ester group, in comparison to the hydrophilic region of the water molecule. Lanolin is a complex mixture of esters of sterols, triterpene alcohols, esters of aliphatic alcohols and monohydroxyesters of sterols and triterpenes and aliphatic alcohols (Schlossman and McCarthy, 1978), while cocoa butter is composed by glycerides, mainly oleo-palmitostearin, oleo-distearin, oleodipalmitin, stearo-diolein, palmito-dioleintrisaturatedtriolein and triunsaturedtriolein (Beckett, 1994).

On the other hand, results of this study suggest that emulsions obtained with lanolin and ethanol inhibit better this pathogen than extracts using water or cocoa butter as solvents at low concentrations.

The antifungal effect of all extracts on *P. cinnamomi* inhibition contrast with studies shown by other authors, because research works using different plant species. Gamboa et al. (2003) reported the use *L. tridentata* extracts against *P. infestans* and shown an antifungal activity of 100% at concentrations of 4000 ppm. Our results indicated that *L. tridentata* ethanol-extracts has potential on *P. cinnamomi* control because it was observed 100% fungal growth inhibition with concentrations as low as 20 ppm (Table 1). Galván (2005) reported 100% inhibition effects on *P. capsici* using ethanolic resin at concentrations of 500 ppm derives from *F. cernua* and Gamboa et al. (2003) found mycelium growth inhibition of 67.28% to 20,000 ppm using methanolic extracts against *P.infestans*. Osorio et al. (2010) mentioned effects in inhibition (100%) on *Pythium* sp. using *C. illinoensis*.

In general, it was observed that the polyphenols obtained from plant extracts using different solvents have effects on mycelium growth inhibition of *P. cinnamomi*. Results obtained with these plant species are similar to those obtained by Gamboa et al. (2003a, b) against *Phytophthora* spp. and confirm the antifungal activity of polyphenols derives from *F. cernua* and *L. tridentata.*

From this study results, it can be inferred that solvent selection play important role on metabolites extraction. The present study showed that aqueous solvents present major antifungal response to Oomycetes (Figure 3). Ethanolic extracts is 20 times better than water and 5 times better than lanolin extracts for have higher effect on *P. cinnamomi* growth inhibition (Figure 2).

Also, lanolin can be the alternative solvent because it showed an interesting effect on polyphenol extraction from *L. graveolens* and excellent effect on antifungal response.

The MIC_{50} obtained with more effective plant extracts on mycelia growth inhibition was such as 20 ppm and derives from *L. tridentata* and *F. cernua.* These doses are lower than those needed to *in vitro* inhibit 100% of *P. cinnamomi* mycelia growth using a commercial fungicide

(Metalaxyl at 750 ppm) (Gamboa et al., 2003b).

Conclusions

It was possible *in vitro* mycelia growth total inhibition of *P. cinnamomi* using *L. tridentata* and *F. cernua* extracts obtained using ethanol and water, *L. graveolens* extracts obtained using lanolin. The best concentrations were lower than 20 ppm of total polyphenols.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Integrated management of tomato fruit borer (*Neoleucinodes elegantalis*)

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The management of *Neoleucinodes elegantalis* (Guenée) (Lepidoptera: Crambidae) with neem-based and registered insecticides most applied on tomato crops in the Agreste region on *N. elegantalis* (eggs, pre-pupae and pupae) were evaluated in this study; methomyl, chlorpyrifos, lambda-cyhalothrin, beta-cypermethrin, deltamethrin, etofenprox, chlorantraniliprole and the neem-based insecticides (azadirachtin A/B and emulsifiable neem oil) were used for studying the repellent effect on oviposition. Lufenuron, deltamethrin and indoxacarb associated with 2.5% sucrose were used as toxic baits on tomato plants. Regarding the ovicidal effect, etofenprox and methomyl had the highest egg mortality, reducing larval survival and fruit damage. Etofenprox had the highest average mortality: 50% in pupae and 38% in pre-pupae. As for repellency, all insecticides tested reduced significantly the number of eggs when compared to the control, except for azadirachtin and chlorantraniliprole. Lufenuron and deltamethrin decreased the number of eggs, showing great potential for use in toxic baits, while indoxacarb stimulated oviposition, thus not being recommended for this purpose. The appropriate use of synthetic insecticides can maximize their potential of control, as long as their use is directed to the stage of the pest that causes higher damage to the crop, or exploring their potential as oviposition deterrent, as well as their use in toxic baits.

Key words: Toxic bait, ovicidal effect, repellence, integrated management.

INTRODUCTION

The tomato crop has a great economic and social importance to Brazil. In 2011, the Brazilian production was 3,653,017 t in 57,355 ha with a productivity of 63,729 kg/ha (IBGE, 2015). This culture, however, provides a favorable habitat for several species of insects, which can achieve high rates of reproduction and survival, compromising the production (Picanço and Guedes, 1999; Picanço and Marquini, 1999). Among the insects, the tomato fruit borer (TFB) *Neoleucinodes elegantalis* (Guenée) (Lepidoptera: Crambidae) is a major pest in most tomato-growing regions. It severely infests the fruit, making them unsuitable for consumption and industrial processing (Gravena and Benvenga, 2003), which can lead to 22% production loss (Picanço et al., 2007), and even damage other solanaceous fruits, such as eggplant, scarlet eggplant, jua, jurubeba and pepper (Toledo, 1948;

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Zucchi et al., 1993).

Furthermore, control this pest is difficult due to its behavior. The female lays the eggs, preferably under the sepals of small green fruit (23 mm) and after five days, the larvae hatch, penetrating the fruit between the first and second hour of scotophase, where they remain for about 16 days (Fernández and Salas, 1985; Blackmer et al., 2001). Therefore, *N. elegantalis* control has been done almost exclusively with the use of synthetic insecticides, applied according to pre-established timetables (Lima et al., 2001).

Management practices aiming at the proper use of insecticides are necessary, thus reducing their application and maximizing effects. Among these practices, the use of repellent insecticide can be considered an important tool for deterring oviposition. Host-plant location is not only related to feeding, but also to oviposition site selection. In Lepidoptera, finding the oviposition site is mediated by the presence of one or more substances that form the specific volatiles or blends of volatiles of the host (França et al., 2009a). The alteration of the plant natural aroma by applying non-specific volatiles can promote rejection. In this sense, some synthetic chemical and botanical insecticides have shown an oviposition deterrent effect on *N. elegantalis* (Cook et al., 2007), becoming strong allies in the management of this pest.

Also, the development of tactics for pest management based on behavior manipulation has been shown as a promising alternative (Witzgall et al., 2008). Chemicals involved in behavioral management, such as feeding stimulants and semiochemicals, can become excellent tools in pest control (Foster and Harris, 1997). Likewise, attractive toxic baits containing feeding stimulants make pest management by behavioral manipulation an effective tool, helping to reduce quantity and increase efficiency of insecticides used in agricultural production systems (Potts, 1999). According to Arruda-Gatti and Ventura (2003), these baits are widely used for the integrated management of insects of agricultural importance, assisting in the decision making of whether or not to control a particular pest. However, the mortality factor is added to the toxic bait, which usually uses a synthetic insecticide. One of the advantages of toxic baits is the benefit to the environment, since they have less influence on some natural enemies, because they are not applied in the total area cultivated (Galli et al., 2004).

The objective of this study was to improve strategies that assist in the integrated management of *N. elegantalis*, directing insecticide applications at different development stages, in order to increase efficiency. Therefore, the direct effects of insecticides on eggs, pupae and pre-pupae were evaluated, as well as insecticide effects on adults (with toxic baits) and repellency/deterrence of *N. elegantalis* oviposition.

MATERIALS AND METHODS

The experiments were conducted in the Agronomy Department vegetable garden and at the Laboratory of Agricultural Entomology, Federal Rural University of Pernambuco (UFRPE).

Insect

The insects used in the experiments were obtained from a population kept under 25 ± 2°C, 65 ± 10% relative humidity and 12 h photophase, at the Laboratory of Agricultural Entomology. Breeding was adapted from the methodology developed by Prof. Marcelo Coutinho Picanço at the Laboratory of Integrated Pest Management, Federal University of Viçosa. Branches with green fruit (about 3 cm in diameter) and leaves of the tomato cultivar Yoshimatsu L-1 were put in plastic bottles with water and placed inside cages, as a site for *N. elegantalis* oviposition. The fruit were daily changed, and the eggs transferred to green fruit of organic scarlet eggplant. Each fruit was infested with about four to six eggs, according to size. The scarlet eggplant fruit were put in plastic trays lined with paper towel and kept for about 15 days, until the larvae reached the last instar, when they abandoned the fruit, spending the pupal stage on the paper towel. Pupae were transferred to wooden cages (60 × 60 × 60 cm) covered with organza until adult emergence. Adults were fed with a 10% sucrose solution.

Survey of insecticides used to control N. elegantalis

The questionnaires about insecticide use for *N. elegantalis* control in tomato crops were given to farmers from the Agreste municipalities of Camocim de São Félix and Bezerros, which are major tomato producers in the state of Pernambuco (IBGE, 2012). The survey was conducted in six properties, three for each municipality, from September 2011 to February 2012.

Ovicidal effect of synthetic and botanical insecticides

Tomato plants of the cultivar Yoshimatsu L-1, with fruit and leaves, were placed in breeding cages for *N. elegantalis* oviposition. After 48 h, 15 eggs were selected per fruit, the remaining removed and discarded with the help of a brush. Oviposition sites for the selected eggs were marked with a marker pen. The fruit were sprayed with a 5.0 ml solution of each product, with the aid of a Paasche electric micro-atomizer coupled to a compressor, calibrated with nine pounds of pressure (9 mmHg). The insecticides were applied at 10 cm from the spray table, and the control was sprayed with distilled water. The following insecticides were tested: azadirachtin A/B (Azamax®), emulsifiable neem oil (Natuneem®) (1 ml L^{-1}), chlorpyrifos (Lorsban® 480 BR) (1.5 ml L^{-1}), methomyl (Lannate® BR) (1 ml L^{-1}), beta-cypermethrin (Akito®) (0.4 ml L^{-1}), fenpropathrin (Danimen® 300 EC) (1.5 ml L^{-1}) and etofenprox (Safety®) (0.5 ml L^{-1}). The number of hatched larvae and the average number of entrance holes in the fruit were counted six days after spraying; the effect of insecticides on newly-hatched larvae (Shock Effect) was also observed, because the pest has direct contact with the dry residues. The assessment of average number of holes per fruit was carried out 21 days after application. To calculate the efficiency of the treatments on egg mortality (Ovicidal Efficiency), on preventing fruit damage by newly-hatched larvae (Larvicidal Efficiency: Shock Action) and on the full development of the pest inside the fruit (Larvicidal Efficiency: Physiological Action), the formula proposed by Abbott and adapted by Benvenga was used for comparing the treatments to the control (Abbott, 1925; Benvenga, 2009). A completely randomized design with eight treatments and five replicates, each consisting of 15 eggs was carried out; results were

Table 1. Formulas used to calculate the efficiency of chemical treatments to control *N. elegantalis* in tomato fruit, in the laboratory (Benvenga, 2009).

Ovicidal efficiency	**Formulae**
Reduction in larvae hatching compared to the control (%)	R (%) = [(NLTS – NLTR) / (NLTS)] x 100
Larvicidal Efficiency (Shock action)	
Reduction in entrance holes compared to the control (%)	R (%) = [(NOeTS – NOeTR) / (NOeTS)] x 100
Larvicidal Efficiency (Physiological action)	
Reduction in exit holes compared to the control (%)	R (%) = [(NOsTS – NOsTR) / (NOsTS)] x 100
Biological Efficiency (Ovicidal Action + Larvicidal Action)	
Reduction in hatching and exit holes compared to the control (%)	R (%) = [(NOsTS/NOTS) – (NOsTR/NOTR) / (NOsTS/NOTS)] x 100

Control	**Treatment**
NLTS = Average number of hatched larvae	NLTR = Average number of hatched larvae
NOTS = Average number of eggs	NOTR = Average number of eggs
NOeTS = Average number of entrance holes	NOeTR = Average number of entrance holes
NOsTS = Average number of exit holes	NOsTR = Average number of exit holes

submitted to analysis of variance and means were compared by the Tukey's test (P ≤ 0.05).

In the formula of biological efficiency, indices were established for the control and treatment, with an initial reference on number of eggs in relation to the number of exit holes in fruit at the end of the study (Table 1).

Effect of synthetic and botanical insecticides on pre-pupae and pupae

Pre-pupae and pupae were taken from the *N. elegantalis* breeding collection and packed in plastic pots containing tomato leaves; they were then sprayed with the same insecticides, following application techniques used in the previous experiment. Development time, mortality and possible morphological changes were observed.

Mortality values were corrected using the Abbot's formula (Abbott, 1925). A completely randomized design with six treatments and five replications, each consisting of 20 pupae or pre-pupae was carried out; results were submitted to analysis of variance and means were compared by the Tukey's test (P ≤ 0.05).

Effects of synthetic and botanical insecticides on oviposition

Tomato branches approximately 30 cm long, containing two leaves and two to three fruits (2 to 3 cm diameter), placed in plastic bottles with water, were sprayed with a 5.0 mL solution of each insecticide by a Paasche electric spray gun coupled to a compressor, calibrated with 12 mmHg. After drying, they were placed inside wooden cages covered with organza (1 × 1 × 1 m), in a hexagonal arrangement with six plants treated or untreated. The following insecticides were tested: methomyl (Lannate®) (1 ml L^{-1}), chlorpyrifos (Lorsban®) (1.5 ml L^{-1}), lambda-cyhalothrin (Karate Zeon®) (0.4 ml L^{-1}), beta-cypermethrin (Akito®) (0.4 mL L^{-1}), deltamethrin (Decis®) (0.4 ml L-1) etofenprox (Safety ®) (0.5 ml L^{-1}) chlorantraniliprole (Premio®) (0.02 ml L^{-1}), all registered for the control of *N. elegantalis.* The following neem-based botanical insecticides were used at a concentration of 1%: azadirachtin A/B (Azamax® CE - 12000 mg kg^{-1} of azadirachtin A and B) and emulsifiable neem oil (Natuneem® 1500 mg kg^{-1}). Botanical and synthetic insecticides, as well as the control were tested separately in a randomized complete block design with eight replications. Inside each cage, 30 mated females (three to four days old) were released. A 10% honey solution was used as food source in the cage. The evaluations were made 48 h after infestation by counting the number of eggs per fruit. The mean percentage of egg reduction for each insecticide was calculated, according to a formula:

PR = [(NC – NT) / (NC + NT) x 100]

where PR = mean percentage of egg reduction, NC = mean number of eggs in the control and NT = mean number of eggs in the treatment (Obeng-Ofori, 1995).

The results were analyzed by the Student's t-test at 5% probability, after homogeneity and normality tests; for lambda-cyhalothrin, chlorpyrifos and beta-cypermethrin, the data were transformed into arcsine √ (x / 100) to meet the assumptions of ANOVA, using the SAS version 8.02 program (SAS Institute, 1999-2001).

Use of toxic baits and oviposition-repellent insecticides

Tomato plants were grown in the Agronomy Department vegetable garden. When fruit reached 2 to 3 cm in diameter, four cages were assembled using PVC pipes (25 mm), with the following dimensions: 1.20 m length × 1.00 m width × 1.30 height m, covered by a voile-type cloth and having side vents with a Velcro closure system. The cages were fixed to the beds with the aid of iron clamps, to provide greater stability. Four plants with about 10 to 15 fruits were placed in each cage. Plants were sprayed with the insecticides lufenuron (Match® EC), deltamethrin (Decis®) and indoxacarb (Rumo®) at commercial concentrations, associated with

Table 2. Main insecticides used to control *N. elegantalis* in tomato crops in the Agreste region of Pernambuco, according to questionnaires data.

Commercial Name	Active ingredient	Chemical Group	Action Mode
Lorsban® 480 BR	Chlorpyrifos	Organophosphate	Acetylcholinesterase inhibitor (A)
Vexter®	Chlorpyrifos	Organophosphate	
Nufos® 480 EC	Chlorpyrifos	Organophosphate	
Malathion®1000 EC Cheminova®	Malathion	Organophosphate	
Cyptrin® 250 CE	Cypermethrin	Pyrethroid	Sodium channel modulator (B)
Decis® 25 CE	Deltamethrin	Pyrethroid	
Sumidan® 150 SC	Esfenvalerate	Pyrethroid	
Talcord® 250	Permethrin	Pyrethroid	
Akito®	Beta-cypermethrin	Pyrethroid	
Danimen® 300 EC	Fenpropathrin	Pyrethroid	
Fastac® 100	Alpha-cypermethrin	Pyrethroid	
Safety®	Etofenprox	Diphenyl ether	Sodium channel modulator
Bac-control WP®	*Bacillus thuringiensis*, kurstaki		Microbial disruptors of the mid-gut membrane
Premio®	Chlorantraniliprole	Anthranilamide	Feeding inhibition and muscular paralysis
Polytrin®	Cypermethrin + profenofos	Pyrethroid + Organophosphate	A + B
Lannate® BR	Methomyl	Oxime Methylcarbamate	Acetylcholinesterase inhibitor

2.5% sucrose (França et al., 2009b). The application was carried out with a manual backpack sprayer with a light-jet cone nozzle. After spraying, 45 female *N. elegantalis* (aged 48 to 72 h) were released per cage. At 48 h of spraying, the insects were collected and the dead females were counted. Then, bunches were removed and taken to the laboratory of Agricultural Entomology for egg quantification. Each insecticide and the control were tested separately in a completely randomized design with eight replications.

Square root data transformation (x + 0.5) was carried out when necessary to meet ANOVA prerequisites. The results were submitted to analysis of variance, and the means were compared by the t-test using the SAS version 8.02 program (SAS Institute, 1999-2001).

RESULTS

Survey of insecticides used to control *N. elegantalis*

After analyzing the questionnaires, it has been found that the main insecticides used by tomato growers in the Agreste region of Pernambuco are pyrethroids and organophosphates (Table 2); they act as sodium channel modulators and acetylcholinesterase inhibitors in nerve synapses, respectively, causing the continuous passage of nerve impulses, leading the insect to fatigue and, consequently, death (IRAC-BR, 2012).

Ovicidal effect of synthetic and botanical insecticides

The etofenprox, methomyl and fenpropathrin were primarily responsible for inviability of *N. elegantalis* eggs, with percentages of 98.6, 91.9 and 45.3%, respectively, differing from the control; etofenprox and methomyl markedly reduced larvae survival and fruit damage. Although fenpropathrin has not been as effective in reducing larval survival, it greatly decreased their entrance into the fruit, thus avoiding possible damage. The other insecticides were not considered effective. Regarding larvicidal efficiency, etofenprox, methomyl, fenpropathrin and azadirachtin produced better results, showing a reduction of larvae holes of 98, 90.63, 85.94 and 64.06%, respectively, when compared to the control. The insecticides tested showed similar performance to the

Table 3. Effect of synthetic insecticides and emulsifiable neem oil on *N. elegantalis* eggs at 27 ± 3 °C, 69 ± 5% relative humidity and 12 h photophase.

Treatment	Egg inviability (%)[a]	Larvae survival (%)[a]	Entrance holes[a,b]	Exit holes[a,c]
Azadirachtin	21.3 ± 7.71[bc]	78.6 ± 7.71[ab]	4.6 ±1.66[bc]	0.3 ± 0.20[b]
Beta-cypermethrin	27.9 ± 6.79[bc]	71.9 ± 6.79[ab]	5.4 ± 2.03[bc]	2.2 ± 0.58[a]
Chlorpyrifos	7.9 ± 3.26[c]	91.9 ± 3.26[a]	11.0 ± 1.14[ab]	0.20 ± 0.20[b]
Etofenprox	98.6 ± 1.33[a]	1.3 ± 1.33[c]	0.2 ± 0.20[c]	0.2 ± 0.20[b]
Fenpropathrin	45.3 ± 13.88[b]	54.6 ± 13.88[b]	1.8 ± 0.40[c]	1.0 ± 0.44[ab]
Methomyl	91.9 ± 3.2 6[a]	7.9 ± 3.26 [c]	1.2 ± 0.48[c]	0.0 ± 0.00[b]
Emulsifiable neem oil	14.6 ± 5.73[bc]	85.3 ± 5.73[ab]	6.0 ± 2.69[abc]	0.4 ± 0.19[b]
Control	5.3 ± 2.49[c]	94.6 ± 2.49[a]	12.8 ± 1.49[a]	1.4 ± 0.67[ab]

[a] Means (± SE) followed by the same letter in the column do not differ by the Tukey's test (P> 0.05). [b] Larvae entrance hole in tomato fruit. [c] Pre-pupae exit hole in tomato fruit.

Table 4. Effect of synthetic and neem-based botanical insecticides on the development period (days), mortality (%) and morphological changes (%) of *N. elegantalis* pre-pupae at 27 ± 3°C, 69 ± 5% relative humidity and 12 h photophase.

Treatment	Development period (%)[a]	Mortality[a] (%)	Morphological chages (%)[a]
Azadirachtin	3.2 ± 0.37[b]	15.2 ± 0.86[a]	1.2 ± 0.40[a]
Beta-cypermethrin	3.4 ± 0.24[b]	19.0 ± 1.85[a]	5.0 ± 0.31[a]
Chlorpyrifos	3.6 ± 0.24[b]	18.0 ± 0.67[a]	7.0 ± 0.24[a]
Etofenprox	3.8 ± 0.20[b]	38.0 ± 0.40[a]	8.0 ± 0.40[a]
Fenpropathrin	3.6 ± 0.24[b]	29.0 ± 1.46[a]	2.0 ± 0.40[a]
Methomyl	5.0 ± 0.31[a]	19.0 ± 0.73[a]	5.0 ± 0.00[a]
Emulsifiable neem oil	3.0 ± 0.31[b]	14.0 ± 0.37[a]	0.6 ± 0.73[a]
Control	3.8 ± 0.20[b]	17.0 ± 0.97[a]	3.0 ± 0.40[a]

[a]Means (± SE) followed by the same letter in the column do not differ by the Tukey's test (P> 0.05).

control, in relation to larvae holes in tomato fruit (Table 3).

Effect of synthetic and botanical insecticides on pre-pupae and pupae

The insecticides did not affect the pre-pupal period in relation to the control, except that methomyl lengthened this period, but with no significant morphological changes (Table 4). The insecticides tested did not affect pupal development, when compared to the control, although, among the insecticides, Fenpropathrin presented a higher pupal development period (Table 5). Etofenprox had an upper percentage of morphological changes in pre-pupae, but none of the insecticides caused significant changes (Table 4). Insecticides caused varied morphological changes in pupae, such as deformed abdomens and two-headed individuals, incomplete biological cycle and adults with atrophied wings (Figure 3). However, none of the insecticides provided a number of significant changes in pupae morphology, compared to the control, with methomyl, azadirachtin and neem emulsifiable oil causing the fewest alterations (Table 5). Etofenprox provided greater corrected mortality in pupae and pre-pupae, 35.89 and 25.30% respectively (Tables 4 and 5).

Effect of synthetic insecticides on oviposition

The following insecticides reduced significantly the number of eggs, when compared to the control: chlorpyrifos ($t = -10.39$; $P < 0.0001$; GL = 17.8), lambda-cyhalothrin ($t = -10.63$; $P < 0.0001$; GL = 20.4), beta-cypermethrin ($t = -7.95$; $P < 0.0001$; GL = 23.8), deltamethrin ($t = -10.54$; $P < 0.0001$; GL = 34), azadirachtin ($t = -5.54$; $P < 0.0001$; GL = 34), methomyl ($t= -3.72$; $P = 0.0007$; GL = 34) and etofenprox ($t = -3.91$; $P = 0.0004$; GL = 34); reduction percentages were 89.51, 74.34, 45.79, 45.16, 36.86, 25.27 and 23.37%, respectively. Chlorantraniliprole ($t = -1.60$; $P = 0.11$; GL = 34) reduced the number of eggs in just 10.30%, and neem emulsifiable oil ($t = -1.94$; $P = 0.06$; GL = 18.9) achieved a 15% reduction, thus not differing from the control (Figure 1).

Table 5. Effect of synthetic insecticides and emulsifiable neem oil on the development period (days), mortality (%) and morphological changes (%) of *N. elegantalis* pupae at 27 ± 3°C, 69 ± 5% relative humidity and 12 h photophase.

Treatment	Development period (days)[a]	Mortality (%)[a]	Morphological changes (%)[a]
Azadirachtin	8.2 ± 0.20[a]	19.0 ± 1.48[b]	1.8 ± 0.37[a]
Beta-cypermethrin	8.2 ± 0.80[a]	26.0 ± 0.66[ab]	9.0 ± 0.37[b]
Chlorpyrifos	8.2 ± 0.20[a]	37.0 ± 0.81[ab]	16.0 ± 0.58[b]
Etofenprox	8.4 ± 0.81[a]	50.0 ± 1.30[a]	6.0 ± 0.20[b]
Fenpropathrin	9.0 ± 0.31[a]	37.0 ± 1.12[ab]	6.0 ± 0.37[b]
Methomyl	8.4 ± 0.50[a]	23.0 ± 1.63[b]	2.0 ± 0.40[a]
Emulsifiable neem oil	6.4 ± 0.87[a]	10.0 ± 2.35[b]	0.8 ± 0.40[a]
Control	8.2 ± 0.48[a]	22.0 ± 1.02[b]	8.00±0.40[b]

[a]Means (± SE) followed by the same letter in the column do not differ by the Tukey's test (P>0.05).

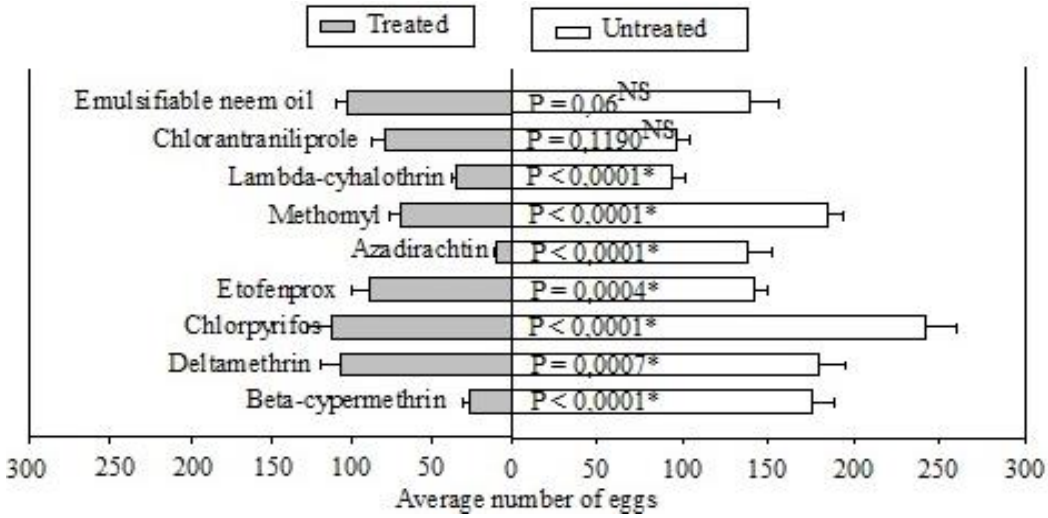

Figure 1. Number of *N. elegantalis* eggs in tomato fruit treated and not treated with synthetic and neem-based botanical insecticides at 25.42 ± 1.66°C, 69.85 ± 6.46% relative humidity and 12 h photophase. NS = non-significant, * significant at 5% probability.

Use of toxic baits and oviposition-repellent insecticides

Deltamethrin (t = -6.41, P < 0.0001; GL = 14) and lufenuron (t = -3.49; P = 0.007; GL = 8.19) reduced significantly the number of eggs when compared to the control, but female mortality was not significant (P> 0.05). Indoxacarb (t = 2.64; P = 0.02; GL = 9.44) caused a significant increase in the number of eggs compared to the control (Figure 2).

DISCUSSION

Pyrethroid insecticides are the most used by tomato growers, due to their low mammalian toxicity, environmental impact and quantity/ha. Organophosphates have a high biological activity, relative instability and a half-life in plants from two to 10 days, but in general, they are very toxic to vertebrates (Santos et al., 2007a; Santos et al., 2007b). However, despite the growth in insecticide use over the years, according to reports from producers, the damage caused by pests has also increased, probably due to selection of resistant insect populations. Thus, insecticide resistance management is an important tool in integrated pest management programs and requires the controlled use of chemicals by varying the concentration and frequency of applications, using insecticides in a rotation system or in a mixture, or applying sequence of chemicals that have different modes of action, for example (Gullan and Cranston, 2007).

The effects of chemical insecticides have been investigated more frequently to optimize their application, directing them to the most susceptible stage of the target-pest. In relation to ovicidal effect, etofenprox, fenpropathrin

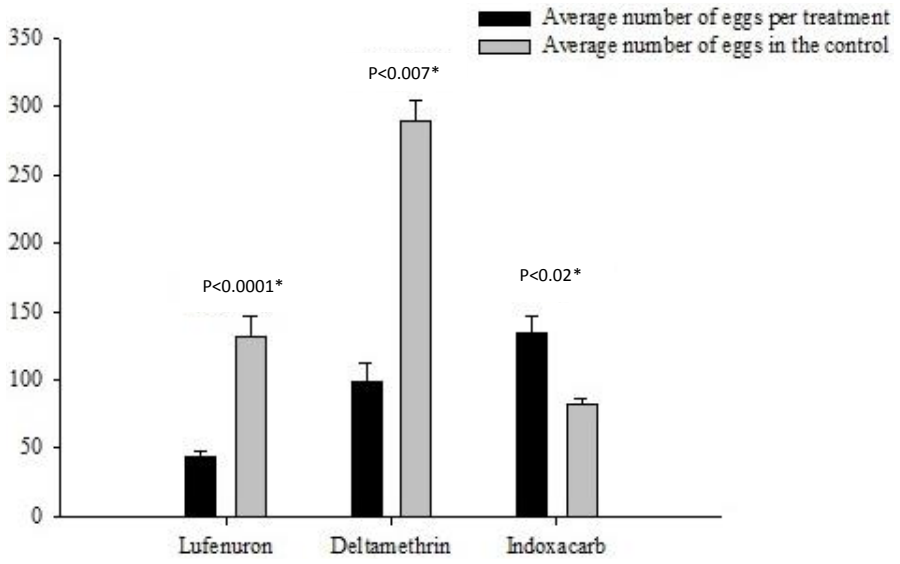

Figure 2. Number of *N. elegantalis* eggs in tomato fruit treated and not treated with synthetic insecticides, such as toxic baits associated with 2.5% sucrose.
* Significant at 5% probability.

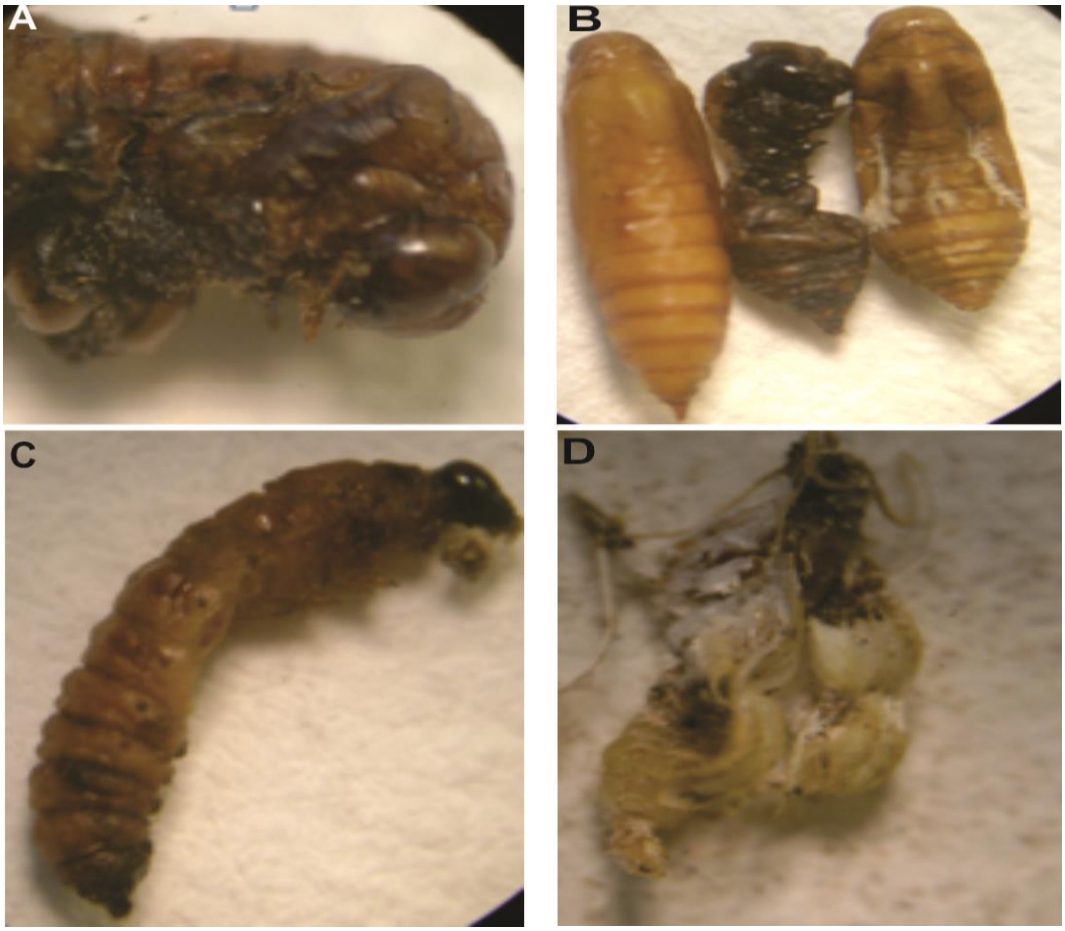

Figure 3. Pupae with two heads (A). Healthy pupae next to pupae with morphological changes (B). Deformed pre-pupae (C). Moth with deformed and stunted wings (D).

and methomyl proved to be a promising alternative for controlling *N. elegantalis* larvae before they could penetrate the fruit. Azadirachtin (NeemPro) had the highest slope of a curve dosage-mortality of *N. elegantalis* eggs compared to other insecticides, meaning that a small increase in the concentration causes significant increases in mortality; however, deltamethrin (Decis® 250 CE) was 144.58 times more toxic than the neem-based insecticides tested (França et al., 2009a).

The insect development stage in which insecticides are applied can influence their effectiveness, hence the importance of applying them at the right time (Neto e Silva et al., 2011). The insecticide lufenuron (Match® EC) was more effective on *Lobesia botrana* Den & Schiff eggs at 0 to 24 h from oviposition (Sáenz-De-Cabezón et al., 2006). However, considering insect development stages, eggs are more tolerant to chemical insecticides. The development of *Phthorimaea operculella* Zeller eggs became unaffected when exposed to various concentrations of thiacloprid (Calypso 480 SC), because larvae hatching was not affected; however, larval survival and adult emergence were reduced (Saour, 2008). 12 insecticides at different concentrations on eggs and larvae of *Grapholita lobarzewskii* Nowicki, had a larvicidal effect greater than the ovicidal (Charmillot et al., 2007).

The effects of botanical and synthetic insecticides on oviposition of some Lepidoptera species have been tested. The emulsifiable neem oil (Natuneem), azadirachtin (NeemPro), deltamethrin (Decis®) and lambda-cyhalothrin (Karate Zeon®) reduced the number of *N. elegantalis* eggs, when compared to the control, in tests of oviposition preference.[15] Aqueous extracts of cinnamon leaves and branches (*Melia azedarach* L.), powdered tobacco (*Nicotiana tabacum* L.) and the commercial product Dalneem® (3000 ppm azadirachtin) in the form of emulsifiable oil at 10% concentration caused a reduction in oviposition of *Plutella xylostela* L. (Dequech et al., 2008). Methanol extracts from neem seeds and cinnamon at 2, 4, 6, 8 and 10%, reduced oviposition of *Earias vittella* Fabricius (Gajmer et al., 2002). Aqueous solutions of emulsifiable neem oil reduced the number of eggs laid by *Leucoptera coffeella* on coffee leaves (Martinez and Martinez, 2003). However, further studies should be conducted in order to explore the deterrent or repellent effects of insecticides on *N. elegantalis* oviposition.

In this study, high natural mortality of pre-pupae and pupae was found in the control treatment. However, the soil is the preferred site for *N. elegantalis* pupation, which may have influenced their development, because during the breeding of the pest in the laboratory, this problem is commonly observed (Salas et al., 1991).

The use of toxic baits has been studied for the control of several urban pests and insect vectors of pathogens (Sackmann and Corley, 2007; Müller and Schlein, 2008); however, studies with lepidopteran pests are incipient. Research conducted with adult *N. elegantalis* found that mortality caused by lufenuron and deltamethrin associated with a 10% honey solution increased directly with the evaluation periods (0, 0.5, 1, 2, 12 and 24 h after exposure) for males, females and adults (male and females), reaching 100% mortality at 2 h of observation (França et al., 2009b).

The deterrent effect of insecticides on oviposition can be an important tool to prevent the establishment of pest insect infestations, as well as the use of toxic baits can increase the efficiency of *N. elegantalis* control.

Chemical control is the most used in *N. elegantalis* management, but its effectiveness is limited, particularly due to the pest behavior. The neonate larvae penetrate the fruit quickly, thus protecting themselves from insecticides (Eiras and Blackmer, 2003). Hence, the results obtained in this study will bring new perspectives to the management of *N. elegantalis* in tomato crops in the Agreste region of Pernambuco, by using etofenprox, fenpropathrin and methomyl as ovicidal agents, chlorpyrifos and lambda-cyhalothrin as oviposition deterrents, and lufenuron and deltamethrin in toxic baits. A significant reduction in tomato production losses is expected, and consequently, economic and social benefits for the producers, as well as a better quality product for the consumers.

Conflict of Interests

The authors have not declared any conflict of interests.

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Effect of traditional kerosene smoking and ethrel on ripening, shelf life and quality of Cavendish banana (*Musa* sp.)

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The effect of banana treatment with traditional kerosene smoking and ethrel released ethylene were investigated to determine their efficacy on ripening, shelf life and physicochemical quality attributes. Fruits at full maturity stage that are light green and three quarter full were used. The study was consisted of three factors namely ripening techniques (conventional kerosene smoking and Ethrel), exposure times (that is, 18, 24 and 30 h), and cultivars (Williams I, Poyo and Giant Cavendish). Fruits were conventionally treated with kerosene smoke released from kerosene burners and ethylene released from 10 ml of ethrel solution (2-chloro ethyl phosphonic acid). They were equally treated under airtight conditions over three sets of exposure times inside locally standard 3 m × 2 m × 3 m sized six separate commercial banana ripening chambers. Fruits were then sequentially withdrawn from the chambers on the basis of their respective exposure times and studied under ambient conditions (23±1°C and 73±1% RH). All parameters tested were invariably and progressively affected by treatment combinations over the experimental period. Significant differences ($p \leq 0.05$) in mean values were also recorded in all parameters at different stages of the ripening period. A three way significant ($p \leq 0.05$) interaction effect of the three factors was revealed on the 7th day of the ripening period on the major quality parameters, starch, TSS, and TSS/TA. Sensory quality evaluation results conducted on the 7th day of the ripening period also showed a similarly highly significant interaction effect among the treatment factors on all quality attributes tested. Ethrel treated fruits demonstrated higher sensory quality mean score values in color (3.85), flavor (3.89), taste (3.80), aroma (3.66) and total acceptability (3.67), other than mouth- feel (3.37) and degree of ripening (3.49). Fruits treated with all treatment combinations of the kerosene smoking system equally completed their maximum ripening stage on the 7th day of the ripening period. However, at this stage, fruits were found developing some off ripening effect black scars on the peel in addition to the relatively low quality attributes recorded upon them through the sensory evaluation panel. Fruits treated with ethrel completed their ripening stage on the 7th day only at the exposure time of 30 h. Those exposed to 18 and 24 h exposure times took more time and extended their ripening stage to up to the 11th day. Thus, in terms of ripening efficiency, the kerosene smoking system can be used at the lowest exposure time of 18 h under the conditions tested. The ethrel-based ripening system can similarly be used for equal ripening efficiency and better sensory quality attributes but only at the highest exposure time of 30 h.

Key words: Banana, ripening, shelf life, physicochemical quality, kerosene smoking, ethrel.

INTRODUCTION

Dessert banana and plantain (*Musa* sp.) are the fourth most important staple food crops in the world after rice, wheat and maize (Salvador et al., 2007a). They are also important sources of income for many smallholder Sub-Saharan Africa farmers (FAOSTAT, 2012).

In Ethiopia, dessert banana is the most important fruit crop, which grows in several parts of the country where the growing conditions are conducive. It is predominantly grown by smallholder farmers and is of great socioeconomic importance, especially in the south and southwestern parts of the country. In 2011, the area cultivated with banana in Ethiopia was 24,212 ha with a total production of 235,824 tons (FAOSTAT, 2012). However, although the demand for banana is steadily increasing in Ethiopia, it is so far grown only on less than one percent of the gross cultivated area, contributing only less than 0.8% of the gross value of agricultural outputs and quite negligible in export earnings (CSA, 2009). Besides, although some studies have been sporadically reported, no significant information is so far available regarding the marketing and post-harvest handling systems of banana including ripening.

The dominant commercial cultivars currently cultivated in Ethiopia are Dwarf Cavendish, Giant Cavendish, and Poyo. Cultivars like Williams I, Williams II and Grand Naine are also recently becoming into picture particularly in the Arba-Minch area where around 90% of the banana marketed in Ethiopia comes from (CFC, 2004). Others like Robusta and Butuzua are also among the recently released Cavendish banana cultivars in Ethiopia but their production is limited to certain commercial farms. The rest are less popular land races grown to a very limited extent in certain localities across the country.

Natural ripening of mature banana may result in softening with non-uniform, dull, pale yellow and unattractive color (Eduardo, 2012). In order for the fruit to attain a bright yellow peel color, a firm pulp texture, and good flavor, bananas are commercially ripened by releasing ethylene into a sealed chamber under controlled temperature and relative humidity. In doing so automated ethylene gas generators are used with calculated ethylene concentrations and exposure time. One main reason for such controlled ripening is to provide retailers and wholesalers with fruits at a stage of ripeness desired by consumers (Eduardo, 2012).

Conversely, in Ethiopia, different traditional ripening techniques are employed; which their effectiveness and effect on shelf-life and the subsequent physicochemical quality attributes are not yet studied and quantified. The most common traditional commercial banana ripening system in Ethiopia where different layers of bunches, in some cases hands, are treated involves the use of what is locally alternatively called “Chella” or “Muket” houses. Such houses, often owned by banana wholesalers, are basically airtight rooms inside which people apply kerosene smoking using kerosene burners. This traditional practice is generally known to accelerate the banana ripening process due to the presence of both acetylene (C_2H_2) and ethylene (C_2H_4) in the smoke (Sarananda, 1990). However, as stated by the same author and other previous reports, the system is often liable for lower consumer attraction, and so is being disregarded in many countries, owing to the resultant burnt scars, bruises, microbial infections, poor appearances on the peel, and contamination of the natural aroma of the fruits with the smoke. As an alternative and relatively improved ripening technique, ethylene generated from 10 ml of ethrel solution (ethephon or 2-chloro ethyl phosphonic acid), is also practiced in some developing countries for ripening of banana fruits often at semi-commercial levels for exposure time of 12 to 24 h (ICAR, 2009).

The aim of this study was thus to compare the efficacy of the traditional commercial kerosene smoking system in Ethiopia with the ethrel released ethylene system used elsewhere in the world in terms of ripening, shelf life and physicochemical quality attributes.

MATERIALS AND METHODS

Description of the study area

The experiment was carried out at Jimma University College of Agriculture and Veterinary Medicine (JUCAVM), under the laboratory of the Department of Postharvest Management (PHM). JUCAVM is found in Jimma town, which is 355 km southwest of Addis Ababa laying at about 7040’N latitude and 360 50’ E longitudes with an altitude of 1780 masl. The mean maximum and minimum temperatures were 26.8 and 11.4°C respectively, and the mean maximum and minimum relative humidity were 91.40% and 39.92% respectively.

Experimental design and treatments

The experiment was laid out in a Randomized Complete Block Design (RCBD) with a 2*3*3 factorial arrangement in three replications. The factors consisted of (1) Factor A: Ripening Techniques in two levels (i.e. kerosene smoking with kerosene burners (K) and ethrel generated ethylene (E); (2) Factor B: Exposure-Time in three levels (that is, 18 h, 24 h = local standard, and 30 h); and (3) Factor C: Cultivars in three levels (that is, Williams I = C1, Poyo = C2 and Giant Cavendish = C3). In total, there were 18 treatment combinations and 54 experimental units.

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Experimental procedures

Harvesting and transportation of banana bunches

Banana bunches of three selected Cavendish cultivars; Williams I (C1), Poyo (C2) and Giant Cavendish (C3) were obtained from Tepi Agricultural Research Center, which is located about 274 km southwest of Jimma town or 629 km southwest of Addis Ababa. Fifteen matured bunches of light-green color and about three quarter full fingers, were selected from the "Banana Variety Maintenance Trial" field. They were selected from prior selected and tagged plants that were healthy, robust and relatively uniform. Fruit bunches were carefully harvested late in the afternoon (4:00 to 5:00 p.m.) and carried on a shoulder pad of the harvesters and kept under natural tree shade for three hours in order to pre-cool the bunches, remove the field heat and slow down metabolism. Initial temperature of each bunch was recorded using a Thermo-Hygrometer (model: PWT-101, Mauritius). The bunches were then night transported to the traditional commercial banana ripening houses in Jimma town by ISUZU truck cushioned with banana leaves from all sides except from the top.

Preparation of banana hands for ripening treatment

Upon arrival at the commercial ripening houses in Jimma town, bunches were carefully unloaded and stabilized for two hours inside a ventilated shade house. Once again, the temperature of each bunch was recorded immediately after unloading using the same Thermo-Hygrometer. Bunches were then carefully de-handed in a cluster of five fingers per crown with a very sharp curved knife in order to remove most of the rachis. De-handing was performed by hanging bunches down wards from a hook and the process proceeded from the apex to the basal end in order to avoid damages to individual fingers.

In order to maintain the uniformity of the experimental materials, only uniform (in terms of overall appearance, size and stage of maturity), clean and undamaged hands were de-handed from the central part of each bunch by avoiding those in the basal and apical rows. Care was also taken to prevent the hands from bumping against each other during de-handing and washing. Immediately after de-handing, but before treatment, hands were carefully placed into the washing tank or tub and washed using 2% normal "Barakina" (Sodium hypochloride) for 3 min in clean tap water. This was done for surface disinfection and removal of the latex and any other dirt. The hands were inspected one by one for any remaining dirt, which was gently removed using wet sponge. In order to completely remove the "Barakina" from the hands, hands were sequentially transferred into the second and third tanks and rinsed with clean tap water. In the end, the hands were drained off (air dried) on a wire-meshed platform with newspaper bedding. The crowns of the hands were also brushed with Aluminum Sulphate to avoid latent infection. This process of washing and draining off the hands was carried out under shade house and took around two hours.

Preparation and treatment of banana cultivars using kerosene smoking

Three traditional commercial banana ripening chambers, locally called "Chella" or "Muket" houses, with an area of 3 m * 2 m * 3 m = 18 m^3 were used from the commercial banana ripeners in Jimma town. The washed and air-dried banana hands of the three banana cultivars were put separately inside perforated plastic boxes and placed on top of weaved wooden shelves. The hands were then conventionally treated with smoke generated from two kerosene burners per room, each filled with 2 L of kerosene, for the respective durations stated above (that is, 18, 24 and 30 h). The ripening treatment process was started simultaneously at 12:00 a.m. in all ripening rooms. As conventionally practiced by the "Chella" house operators in Jimma town and elsewhere in Ethiopia, the ripening rooms were closed and air tightly sealed up until the end of their respective exposure times. The pulp temperature and relative humidity of the respective ripening rooms were recorded at the beginning and end of the ripening process.

Preparation and treatment of banana cultivars with ethrel

Ethylene, in the presence of alkaline medium such as sodium hydroxide (NaOH), evolves from Ethrel to treat climacteric fruits such as banana and promote ripening (Mohamed and Abu-Goukh, 2003; ICAR, 2009). Using the same procedure, a 10 ml of Ethrel solution and 2 g of NaOH pellets were mixed in 5 L of water in a wide mouthed plastic buckets inside the respective ripening chambers of the three exposure times (that is, 18, 24 and 30 h). This was presumed to release 100 to 150 µL L^{-1}ethylene, which is the common concentration level used for banana ripening at destination markets (Mohamed and Abu-Goukh, 2003; ICAR, 2009). This, in place of the smoke released through kerosene burners, was used to treat fruits of the three banana cultivars through the liberated ethylene inside the respective air tight ripening chambers for the specified exposure times. A small portable electric operated fan (Model: REE-NOVA FH-04: 220-240V/50~60Hz, China) was placed near the buckets in each of the respective ethrel treatment chambers to facilitate the release and uniform circulation of the ethylene gas from the solution. The chambers were then sealed air-tight immediately after recording the prevailing temperature and relative humidity.

Termination of the ripening process and subsequent handling of treated fruits for analysis

At the end of the respective treatment periods, the temperature and relative humidity of the rooms were recorded and the treated fruits were withdrawn from the ripening rooms in order to terminate the process and keep them under ambient conditions inside the laboratory of the Department of Postharvest Management at JUCAVM. Then, fruits were assigned into meshed plastic trays (35 fingers per tray), which were randomly laid out as per the design of the experiment across the laboratory benches. Finally, samples of fruits were periodically taken for physicochemical quality analysis up until the end of their marketable life. In addition to the physicochemical measurements taken immediately after harvest (that is, before the ripening treatment), which is considered as a Day Zero measurement, random samples of 3 to 5 fruits were used for subsequent analysis at three days interval. The temperature and relative humidity of the display room were recorded three times a day (that is, morning, mid-day and late afternoon) throughout the experimental period. Ripening fruits were also inspected and records were taken everyday for problems like disease incidence and physiological deterioration.

Data collected

Data collection was started right after the arrangement of the hands in the laboratory to the respective treatments and before the application of the ripening treatments. In addition to observations taken on Day one after treatment, data collection was then continued at three days interval up until the fruits became unmarketable. Data were collected upon the following physical and chemical properties of the experimental fruits.

Physical parameters

Physiological weight loss of fruits (PWL %)

Physiological weight loss of fruits was recorded through periodical weighing of five sample fingers from each experimental unit throughout the experimental period using a precision scale (model: LS200 Sartorius GMBH Gottingen, Germany). Physiological Weight Loss of fruits in the respective treatments was calculated and expressed as a percentage of the initial weight using the following equation.

$$\text{Weight loss (\%)} = \frac{\text{Initial Fruit Weight (g)} - \text{Final Fruit Weight (g)}}{\text{Initial Weight (g)}} \times 100 \quad (1)$$

Fruit firmness (N)

Fruit firmness was measured using a Texture Analyzer (Model: TA-XT Plus, UK) and calculated by employing the method used by Fan et al. (1999). Two whole unpeeled fingers were used periodically from each treatment to measure the penetration force required to penetrate the fingers by the stainless plunger and the force was automatically recorded and expressed in Newton (N). The mean value of measurements taken from two fingers of the respective treatments was recorded as the mean firmness.

Fruit peel color

Peel color was measured using the Tri-Stimulus Colorimeter (AccuProbe HH06, USA), which was regularly calibrated before measurement with a Minolta standard white tile to *L* = 83.14, *a* = -3.67 and *b* = 10.79. Non-destructive measurements were taken periodically starting from Day 0 (Day Zero) at three days interval to the end of the experimental period and values were recorded as *a, b* and *L*. Multiple measurements were taken from two sample fruits on two opposite sides and mid- point to the two ends of each fruit. Color values for each sample were calculated as the means of the four measurements taken. Total colour change of samples was determined using the CIE (Commission Internationale de L'Eclairage) 1976 *L*a*b color scale* system, where *L**scale measures lightness ('light vs. dark') and varies from 100 for perfect white to zero for perfect black (that is, 0 to 50 indicates dark while 51 to 100 indicates light); *a** measures redness ('red vs. green') (positive when red and green when negative); and *b** measures yellowness ('yellow vs. blue') yellow when positive and blue when negative). The total color change (ΔE) was calculated by employing the following formula:

$$\Delta E = \sqrt{(L^*_i - L_f)^2 + (a^*_i - a_f)^2 + (b^*_i - b_f)^2} \quad (2)$$

Where I = initial, and f = final.

Chemical parameters

Pulp starch content

Starch staining was carried out by a 3 to 5 min dipping of a cross-sectional cut of the unpeeled sample fruits in a starch-iodine staining solution with10 g potassium iodide (KI) and 2.5 g iodine (I_2) in distilled water. It was carried out as per the rapid starch staining method and chart developed by Sylvia et al. (1993). The stained part of the sample fruit slices were then turned up and put in an upright position for about 2 min in order to allow the excess solution to be drained off. A transparent plastic sheet was then traced on the patterns of the stained areas and estimated in relation to the whole surface area of the cut dipped portion of the fruits. The sample fruits were then rated based on the chart developed previously into 10 distinguishable stages with rating numbers ranging from 1 to 10 based on the percentage of unstained area, that is, 1 means <5% unstained area, 2 (5%), 3 (10%), 4 (15%), 5 (25%), 6 (35%), 7 (45%), 8 (55%), 9 (65%) and 10 (>65%). The degree of ripening as well as the corresponding starch contents of the sample fruits was determined accordingly. A trend of increase in the rating number, from 1 to 10, was expressed as a characteristic pattern of starch loss during ripening.

Total soluble solids-TSS (°Brix)

Sample fruits were peeled and blended using a juice blender and TSS of the pulp juice was measured by the refractive index, expressed as °Brix, by a portable hand Refractometer (Model: SN-003007). The macerated samples were homogenized by adding about 40 ml of distilled water and filtered with cheese cloth. One to two drops of the filtrate was then placed on to the glass prism of the refractometer for reading within the scale. The glass prism was rinsed with distilled water and cleaned with cheese cloth in between the measurement of each of the samples drawn from the experimental units.

Titratable acidity (TA)

Titratable acidity was measured for samples of each experimental unit by titrating the pulp filtrates with 0.1N NaOH solution with 2 to 5 drops of phenolphthalein up until the indicator light pink color appeared. The volume of NaOH used up until the indicator reach the end point color was then recorded and TA was expressed as percentage of malic acid in the pulp weight of the titrate as calculated using the following formula:

$$\text{Titratable Acidity (\%)} = \frac{V_1 * N * E}{V_2} \times 100 \quad (3)$$

Where, N = Normality of NaOH, V_1 = volume of NaOH used, E = equivalent weight of acid, and V_2 = volume of sample taken for estimation.

TSS to TA ratio

The ratio between total soluble solids and titratable acidity was determined by dividing the values for TSS to the values of TA of the same sample fruit.

Pulp pH

The pH of the sample fruit juice was measured using a bench top digital pH meter (model: CP-505, Poland). The pH meter was periodically calibrated with buffer at pH 4.0 and 7.0 before taking the measurements. pH was expressed as the equilibrium measure of hydrogen ion concentration in the sample fruit juice.

Sensory quality

The organoleptic or sensory quality of sample fruits from each treatment was determined using 5-point hedonic scale (where 1 = dislike, 2 = slightly, 3 = neither dislike nor like, 4 = slightly like, and 5 = like). A panel of 18 pre-oriented under and post graduate

Table 1. Interaction effect of ripening techniques and cultivars on PWL (%) of banana fruits.

Cultivar	Day 7	
	Kerosene smoking	Ethrel
Williams I	8.10^{ab}	7.17^{bc}
Poyo	8.12^{ab}	6.43^{c}
Giant Cavendish	7.68^{ab}	8.35^{a}
SE ±	1.2	
CV (%)	15.73	
LSD (5%)	1.09	

* In each column, means followed by the same letter (s) are not significantly different (p ≤ 0.05).

Table 2. Effect of cultivars on PWL (%) of banana fruits treated with ethrel alone.

Cultivar	Day 9
Williams I	8.95^{ab}
Poyo	7.84^{b}
Giant Cavendish	10.36^{a}
SE ±	1.61
CV (%)	17.8
LSD (5%)	1.61

* In each column, means followed by the same letter (s) are not significantly different (p ≤ 0.05); Fruits treated with the kerosene smoking completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for the 9th are only for fruits treated with ethrel alone.

students from the departments of Horticulture and Post-Harvest Management were considered for the evaluation. The evaluation was carried out on the 7th day of the ripening period when most of the sample fruits attained the 6th color chart scale according to methods reported by Larmond (1987).The evaluation was made for such quality attributes like pulp color, flavor, taste, aroma, mouth-feel, level of ripening and overall acceptability. The sample fruits were peeled, hand sliced into smaller equal pieces and served in a random order across the laboratory benches of the Department of Post-Harvest Management in 3-digit coded flat glass trays. Distilled water was provided for the evaluators to cleanse their palates between each test sample. The panel test was carried out from 10:00 to 11:30 a.m. in the morning. The evaluation results were statistically analyzed to determine whether or not there were statistically significant differences in consumer preferences.

Statistical analysis

The experimental data were analyzed through the analysis of variance (ANOVA) by employing SAS software version 9.2. The least significant differences (LSD %) test was used to determine the level of significance at 5% (P < 0.05) and for mean separation and comparison of their differences among the treatment means. Data on main factor effects and their corresponding interaction effects are presented as results of the experiment only when they showed statistically significant differences.

RESULTS AND DISCUSSION

Physical characteristics

Physiological weight loss (PWL %)

Physiological weight loss increased consistently as the ripening period prolonged in all treatments. The increase in weight loss with the application of the kerosene smoking and ethrel-based ripening systems was probably due to the upsurge in respiration rate of the sample fruits over the ripening period. Dhall and Singh (2013) also reported a similar increase in PWL (2.23 to 8.60%) during tomato ripening with ethrel and ethylene which was attributed to the increased rate of respiration as well as transpiration from the peel surface with the advance of ripening. The two-way interaction effect of ripening techniques and cultivars showed a significance difference ($p<0.05$) among cultivars treated with the kerosene smoking and ethrel-based ripening system on the 7th day of the ripening period (Table 1). However, while all cultivars recorded the highest weight loss (7.68 to 8.35%) on the 7th ripening day with the kerosene smoking-based system, only Giant Cavendish recorded a similar loss under both ripening systems on the same day. All cultivars showed similar levels of weight loss with the ethrel-based ripening system after the 9th day of ripening (Table 2). Thus, the kerosene smoking-based system significantly increased the weight loss invariably much earlier than the ethrel-based system signified that it was more efficient in the acceleration of the ripening process than the later.

Firmness (N)

The data on banana fruit firmness revealed an increasing trend during the ripening period for all treatments. Significant differences ($p\leq0.05$) among mean values of firmness were recorded at different stages of the ripening period. The firmness of fruits averagely decreased significantly from 21.87 N on Day 0 to 4.36 N on the 7th day of the ripening period. While fruits treated with the kerosene smoking system attained the above stated minimum firmness level on the 7th day of ripening with all exposure times (Tables 3 and 4), those treated with ethrel attained a similar level of firmness only at the 11th day of the ripening period with the exposure time of 30 h (Table 5). With the exposure times of 18 and 24 h, varieties treated with ethrel showed differences with only Williams I and Poyo attaining the stated level of firmness at the 11th day of the ripening period. Venkata et al. (2012) also reported a similar finding on Grande Naine banana fruits, treated with smoking and ethrel, declined in firmness to 4.83 on the 8th day of the ripening period. The decline in firmness was so drastic between the initial two days and thereafter decreased gradually throughout the ripening period. This consistent softening or decline in

Table 3. Interaction effect of ripening techniques and exposure times on firmness (N) of banana fruits.

Exposure time (h)	Day 7	
	Kerosene smoking	Ethrel
18	4.59[c]	12.53[a]
24	4.30[c]	11.93[a]
30	4.20[c]	7.90[b]
SE ±	1.48	
CV (%)	19.48	
LSD (5%)	1.41	

* In each column, means followed by the same letter (s) are not significantly different ($p \leq 0.05$).

Table 4. Interaction effect of ripening techniques and cultivars on firmness (N) of banana fruits.

Cultivar	Day 7	
	Kerosene smoking	Ethrel
Williams I	4.49[c]	8.90[b]
Poyo	4.31[c]	10.01[b]
Giant Cavendish	4.29[c]	13.46[a]
SE ±	1.48	
CV (%)	19.48	
LSD (5%)	1.41	

* In each column, means followed by the same letter (s) are not significantly different ($p \leq 0.05$).

firmness corresponds to an inter conversion of insoluble pectic substances into soluble pectin (that is, by cellular disintegration leading to membrane permeability) over the ripening period (Venkata et al., 2012). Tapre and Jain (2012) also reported that the softening of banana fruits during ripening treatment is associated with the conversion of starch to sugar, the breakdown of pectin substances and the movement of water from the rind of the banana to pulp during ripening. As stated by Sandipkumar and Shanmugasundaram (2015), changes in biochemical and other properties such as peel thickness, TSS and moisture or pulp dry matter content are the main cause of alteration in mechanical properties such as firmness in banana. The result in the present study is further consolidated by the fact that the correlations between firmness and TSS as well as firmness and pulp dry matter content were found to be strongly negative (R = -0. 86) and moderately positive (R = 0.58) respectively (Annex 1).

Peel color

Significant differences ($p \leq 0.05$) in mean values of total peel color change were found at different stages of the ripening period. The obvious manifestations of banana color change during ripening such as the disappearance of the green peel color and the corresponding yellowing were clearly observed in the present experiment irrespective of variations in treatments. The results were in agreement with the reports of Tourky et al. (2014) and Salvador et al. (2007b) in that the progressive loss of the green color in the peel was due to the continuing degradation of the chlorophyll structure during ripening.

The highest total peel color change was recorded on the 7th day of the ripening period for fruits treated with kerosene smoking across all exposure times (Table 6 and 7). On the other hand, fruits treated with ethrel extended the trend till the 9th day and then declined on the 11th day (Table 8). This could be attributed to the much accelerated ripening effect of the kerosene smoking ripening system, which triggered all cultivars to complete their ripening period much earlier than the ethrel-based system. Similar results were also found by Ding et al. (2006) who reported that the color change of the peel implied the ripeness of Berangan banana and William Cavendish in that as the fruits ripen, they progressively developed a bright yellow color as chlorophyll gets degraded and carotenoids become visible. Thereafter the peel color declined as brown spots (senescent) appeared on the skin and the fruits became overripe.

On the other hand, it was observed that fruits treated with the ethrel-based ripening system developed uniform yellow peel color; while those treated with kerosene smoking exhibited deep yellow color with black spots on the peel surface leading to over-softening of the fruits at the end of the ripening period. A similar result was earlier reported by Sarananda (1990) that although the traditional banana ripening practice by smoking and subsequently storing at room temperature accelerates the ripening process, the resultant burnt scars, bruises, microbial infections and poor appearance of smoked ripe fruits lower consumer attraction.

Chemical characteristics

Starch

The starch stained a blue-black color, while areas that have lost starch remained white. Significant differences ($p \leq 0.05$) in starch un-staining were found at different stages of the ripening period. The proportion of unstained area increased faster from 1(<5% unstained) at the beginning to 10 (>65% unstained) on Day 7 of the ripening period with the kerosene smoking system for all three exposure times and cultivars (Table 9). Under the ethrel-based ripening system, cultivars showed a similar extent of starch un-staining on Day 7 of the ripening period only at the exposure time of 30 h (Table 9). With the 18 and 24 h exposure times, cultivars exhibited similar level of starch un-staining only on the 11th day of the ripening period (Table 10). This significant disappearance

Table 5. Interaction effect of exposures times and cultivars on firmness (N) of banana fruits treated with ethrel alone.

Cultivar	Day 9			Day 11		
	18 h	24 h	30 h	18 h	24 h	30 h
Williams I	6.87^{cbd}	6.07^{cd}	5.97^{cd}	4.67^{b}	4.00^{b}	3.87^{b}
Poyo	9.83^{b}	8.77^{cb}	6.20^{cd}	5.83^{b}	5.30^{b}	4.77^{b}
Giant Cavendish	15.43^{a}	15.60^{a}	5.67^{d}	13.73^{a}	13.23^{a}	4.30^{b}
SE ±		1.72			1.14	
CV (%)		19.2			17.21	
LSD (5%)		2.97			1.98	

* In each column, means followed by the same letter (s) are not significantly different ($p \leq 0.05$); Fruits treated with the kerosene smoking completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for the 9th & 11th are only for fruits treated with ethrel alone.

Table 6. Interaction effect of ripening techniques and cultivars on total color change of banana fruits.

Cultivar	Day 7	
	Kerosene smoking	Ethrel
Williams I	23.07^{c}	33.39^{a}
Poyo	26.24^{bc}	31.41^{ab}
Giant Cavendish	27.66^{abc}	26.63^{ab}
SE ±	6.57	
CV (%)	23.4	
LSD (5%)	6.29	

* In each column, means followed by the same letter (s) are not significantly different ($p \leq 0.05$).

Table 7. Effect of exposure times on total color change of banana fruits.

Exposure times (h)	Day 7
18	23.37^{a}
24	28.85^{a}
30	31.98^{a}
SE ±	6.57
CV (%)	23.4
LSD (5%)	4.45

* In each column, means followed by the same letter (s) are not significantly different ($p \leq 0.05$).

Table 8. Effect of exposure times on total color change of banana fruits treated with ethrel alone.

Exposure times (h)	Day 9	Day 11
18	29.61^{b}	28.48^{b}
24	34.88^{ab}	32.88^{b}
30	40.03^{a}	40.56^{a}
SE ±	7.16	6.94
CV (%)	20.56	20.42
LSD (5%)	7.16	6.93

* In each column, means followed by the same letter (s) are not significantly different ($p \leq 0.05$); Fruits treated with the kerosene smoking completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for the 9th & 11th are only for fruits treated with ethrel alone.

Table 9. Interaction effect of ripening techniques, exposure times and cultivars on pulp starch content of banana fruits.

Cultivar	Day 7					
	Kerosene smoking			Ethrel		
	18 h	24 h	30 h	18 h	24 h	30 h
Williams I	10.00[a]	10.00[a]	10.00[a]	7.67[d]	8.00[c]	10.00[a]
Poyo	10.00[a]	10.00[a]	10.00[a]	7.00[e]	7.00[e]	10.00[a]
Giant Cavendish	10.00[a]	10.00[a]	10.00[a]	6.00[f]	6.00[f]	9.00[b]
SE	0.14	CV (%)	1.52	LSD (5%)	0.23	

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$).

Table 10. Interaction effect of exposure times and cultivars on pulp starch content of banana fruits treated with ethrel alone.

Cultivar	Day 9			Day 11		
	18 h	24 h	30 h	18 h	24 h	30 h
Williams I	9.00[b]	9.00[b]	10.00[a]	10.00[a]	10.00[a]	10.00[a]
Poyo	8.00[c]	8.00[c]	10.00[a]	9.00[b]	9.00[b]	10.00[a]
G.Cavendish	7.00[d]	7.00[d]	10.00[a]	8.00[c]	8.00[c]	10.00[a]
SE ±		0.37			0.26	
CV (%)		4.85			3.19	
LSD (5%)		0.43			0.42	

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$); Fruits treated with the kerosene smoking-based ripening system completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for days 9 and 11 are only for fruits treated with ethrel).

of starch on Day 7 of the ripening period of the cultivars showed a highly significant correlation with peel color (R = 0.82), TSS (R = 0.96), and p^H (R = -0.80) (Annex Table 1). The results are in agreement with previous findings by Tourky et al. (2014) in that starch hydrolysis is the most important post-harvest biochemical change which occurs during the post-harvest ripening of banana and causes the accumulation of sugar that are responsible for the increase in pulp TSS content and sweetening of the fruit.

TSS (^{0}Brix)

A significant difference ($p \leq 0.05$) was generally observed in respect of the total soluble solids (TSS) of banana fruits among the treatments at different stages of the ripening period. TSS content increased consistently throughout the ripening period irrespective of treatments. Results also indicate a progressive increase in TSS and decrease in starch content with the maximum TSS (25.21°Brix) attained at the end or 7th day of the ripening period under the kerosene smoking system followed by the 9th and 11th day under the ethrel-based system (Tables 11 and 12). These results are almost in consistency with results (23.07 °Brix) obtained by Tapre and Jain (2012) at stage 7 of the ripening period of the banana fruits. Sandipkumar and Shanmugasundaram (2015) also reported an increase in TSS from 3 to 22.24°Brix during nine days of banana storage. In addition, Kulkarni et al. (2004) reported an increase in TSS and sugars in mango fruits treated with ethrel with progress in storage time. The increase in TSS during ripening may result from an increase in concentration of organic solutes as a consequence of water loss and hydrolysis of starch into soluble sugars such as sucrose, glucose and fructose (Tapre and Jain, 2012). Tourky et al. (2014) reported that banana and some other fruits contain many water soluble compounds (sugars, acids, vitamin C, amino acids and some pectin) that form the TSS. Total soluble solids, having sugar as their main component are known to be important postharvest quality attributes of the banana fruits that serve as the most useful index of ripeness.

Titratable acidity (TA %)

Although the percentage values were recorded in the narrow range, a progressive increase in TA was found with advances in the ripening period in all the treatments. The highest percentage of TA (0.34-0.36%) was recorded on the 7th day of the ripening period similarly under the kerosene smoking system equally with all the cultivars

Table 11. Interaction effect of ripening techniques, exposure times and cultivars on TSS (^{0}Brix) of banana fruits.

Cultivar	Day 7					
	Kerosene smoking			Ethrel		
	18 h	24 h	30 h	18 h	24 h	30 h
Williams I	25.33^{a}	25.13^{a}	25.40^{a}	19.20^{c}	20.44^{c}	23.67^{ab}
Poyo	24.53^{a}	24.77^{a}	26.00^{a}	19.00^{c}	21.27^{bc}	23.78^{ab}
Giant Cavendish	25.07^{a}	25.51^{a}	25.13^{a}	7.05^{d}	19.40^{c}	24.23^{ab}
SE	1.84	CV (%)	8.2	LSD (5%)	3.06	

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$).

Table 12. Interaction effect of exposure times and cultivars on on TSS (^{0}Brix) of banana fruits treated with ethrel alone.

Cultivar	Day 9			Day 11		
	18 h	24 h	30 h	18 h	24 h	30 h
Williams I	23.13^{ab}	23.41^{ab}	25.20^{a}	23.58^{ab}	24.b^{a}	25.87^{a}
Poyo	21.60^{a}	23.58^{ab}	24.07^{ab}	23.09^{b}	25.00^{ab}	25.87^{a}
G. Cavendish	11.74^{c}	23.73^{ab}	25.13^{a}	19.40^{c}	25.57^{ab}	25.87^{a}
SE $\pm$		1.73			1.44	
CV (%)		7.72			5.93	
LSD (5%)		2.99			2.5	

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$); Fruits treated with the kerosene smoking-based ripening system completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for days 9 and 11 are only for fruits treated with ethrel).

Table 13. Interaction effect of ripening techniques and exposure times on titratable acidity (%) of banana fruits.

Exposure time (h)	Day 7	
	Kerosene smoking	Ethrel
18	0.34^{a}	0.24^{b}
24	0.35^{a}	0.27^{b}
30	0.36^{a}	0.34^{a}
SE $\pm$	0.03	
CV (%)	10.14	
LSD (5%)	0.03	

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$).

and exposure times. Although a slight decline in TA (0.28%) was observed with the kerosene smoking treatment after the 7th day of the ripening period, the fruits were by then became unmarketable and discarded (Table 13). Fruits treated with the ethrel-based system attained similar level of TA percentage at the 7th day of the ripening period only at the exposure time of 30 h (Table 13). There was also a two way interaction effect of exposure times and cultivars on the 9th day of the ripening period where fruits attained similar level of titratable acidity (0.34 to 0.35%) at 30 h of exposure time (Table 14). Those treated with the exposure times of 18 and 24 h extended the stage to the 11th day of ripening (Table 15). Although results were not statistically significant, similar results were reported by Tapre and Jain (2012) with values ranging narrowly from 0.3 to 0.45% among treatments at the end of the banana ripening period. The result is as well concurring with the studies made by Zeweter (2008), and Siriboon et al. (2004) who reported that TA increased to its peak as ripening progressed, which coincided with the accumulation of ethylene and ripening, and then started declining afterwards. Thus, as stated by Dadzie and Orchard (1997), the increase in TA over the ripening

Table 14. Interaction effect of exposure times and cultivars on titratable acidity (%) of banana fruits treated with ethrel alone.

Cultivar	Day 9		
	18 h	24 h	30 h
Williams I	0.28^{e}	0.30^{de}	0.34^{abc}
Poyo	0.30^{de}	0.33^{cde}	0.38^{a}
G. Cavendish	0.22^{f}	0.30^{cde}	0.35^{ab}
SE ±		0.02	
CV (%)		7.75	
LSD (5%)		0.04	

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$); Fruits treated with the kerosene smoking-based ripening system completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for on the 9th day are only for fruits treated with ethrel

Table 15. Effect of of exposure times on titratable acidity (%) of banana fruits treated with ethrel alone.

Exposure time (h)	Day 11
18	0.33^{b}
24	0.36^{ab}
30	0.39^{a}
SE	0.04
CV (%)	10.91
LSD 5%)	0.04

Fruits treated with the kerosene smoking-based ripening system completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown on the 11th are only for fruits treated with ethrel.

period could also be regarded as a useful index of banana fruit.

TSS/TA ratio

The TSS/TA ratio, which is also taken as a ripening index (RI), generally increased as the ripening period progressed in all treatments. The maximum TSS/TA ratio (67 to 81) was recorded on the 7th day of the ripening period across all the treatments under the traditional kerosene smoking ripening system (Table 16). Cultivars treated with the ethrel-based system also showed a similar ratio in TSS/TA on the 7th day under the exposure times of 24 and 30 h (Table 16). Cultivars treated for 18 h with the ethrel-based system attained a similar TSS/TA ratio as of the 9th day of the ripening period (Table 17). This increase in TSS/TA ratio was attributed to the increase in the amount of sugars and the parallel decline in organic acids (Dadzie and Orchard, 1997). The proportional organic acids increase, which are important in giving a desired sugar-to-acid balance and pleasing fruit taste, usually decline during fruit ripening as they are respired and converted into sugar (Tourky et al., 2014).

pH

pH was expressed as the equilibrium measure of hydrogen ion concentration in the sample fruit juice. The pH value gives a measure of the alkalinity of the sample fruit juice. The pH of the pulp steadily decreased over the ripening period irrespective of treatments. A two way interaction effect on pulp pH was obtained between exposure times and cultivars on the 7thday of the ripening period (Table 18). Significantly ($p \leq 0.05$) low pH was recorded through the kerosene smoking system on the same date (Table 19). On the other hand fruits treated with the ethrel-based system showed similar level of pH only on the 11th day of the ripening time at the exposure times of 24 and 30 h (Table 20). The decline in pH as ripening progresses is in agreement with the findings of Zeweter (2008) and Hernandez et al. (2006). As ripening advances, titratable acidity increases which results in a decrease of pulp pH (Dadzie and Orchard, 1997). Pulp pH and total titratable acidity are thus important post-harvest quality attributes in the assessment of fruit ripening and quality.

Sensory quality

The data in Table 21 illustrates highly significant differences ($p \leq 0.05$) in mean values among the treatments across all the tested sensory quality parameters. Results also clearly indicate that ethrel treated fruits demonstrated higher mean score values for sensory quality attributes of color (3.85), flavor (3.89), taste (3.80), aroma (3.66) and total acceptability (3.67) other than mouth-feel (3.37) and degree of ripening (3.49). A similar result was reported by Kulkarni et al. (2010) that ethrel at 500 ppm induced uniform ripening without impairing taste and flavour of banana. All the same, Lakshmana et al. (2010) reported parallel results that the organoleptic quality of banana ripening treated with 2500 ppm ethrel dip (5 min) at 18 to 26°C and 60 to

Table 16. Interaction effect of ripening techniques, exposure times and cultivars on TSS/TA ratio of banana fruits.

Cultivar	Day 7					
	Kerosene smoking			Ethrel		
	18 h	24 h	30 h	18 h	24 h	30 h
Williams I	74.30^{bcd}	81.29^{abcd}	85.10^{abc}	58.41^{ef}	81.34^{abcd}	93.00^{a}
Poyo	67.95^{de}	75.76^{bcd}	79.97^{abcd}	50.66^{f}	74.67^{bcd}	87.30^{ab}
Giant Cavendish	71.41cde	78.68^{abcd}	81.19^{abcd}	22.15^{g}	85.64^{abc}	87.78a^{b}
SE			9.25			
CV (%)			12.46			
LSD (5%)			15.35			

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$).

Table 17. Effect of exposure times on TSS/TA ratio of banana fruits treated with ethrel alone.

Exposure time (h)	Day 9	Day 11
18	67.28^{b}	74.17^{b}
24	69.69^{b}	86.38^{a}
30	86.56^{a}	90.13^{a}
SE	6.71	8.78
CV (%)	9.01	10.51
LSD 5%)	6.71	8.77

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$); Fruits treated with the kerosene smoking-based ripening system completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for on the 9th and 11th day are only for fruits treated with ethrel.

Table 18. Interaction effect of exposure times and cultivars on pulp p^{H} of banana fruits.

Cultivar	Day 7		
	18 h	24 h	30 h
Williams I	4.98^{b}	4.89^{cd}	4.86^{de}
Poyo	4.94^{cb}	4.87^{cde}	4.86^{cde}
G. Cavendish	5.20^{a}	4.81^{de}	4.80^{e}
SE $\pm$	0.07		
CV (%)	1.35		
LSD (5%)	0.08		

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$).

Table 19. Effect of ripening techniques on pulp p^{H} of banana fruits.

Technique	Day 7
Kerosene smoking	4.44^{b}
Ethrel	5.39^{a}
SE $\pm$	0.07
CV (%)	1.35
LSD (5%)	0.04

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$).

Table 20. Effect of exposure times on p^H of banana fruits treated with ethrel alone.

Exposure time (h)	Day 11
18	5.03[a]
24	4.62[b]
30	4.47[b]
SE	0.23
CV (%)	4.9
LSD 5%)	0.23

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$); Fruits treated with the kerosene smoking-based ripening system completed their marketable shelf life and discarded on the 7th day of the ripening period. Thus mean values shown for on the 11th day are only for fruits treated with ethrel.

Table 21. Interaction effect of kerosene smoking and ethrel generated ethylene ripening on sensory quality attributes characteristics of banana fruits.

Treatment	Day 7						
	Means						
A*B*C	Color	Flavor	Taste	Aroma	Mouth feel	Acceptability	Level of ripening
E 18C1	4.37[a]	4.00[bc]	4.30[a]	3.67[bcd]	3.83[b]	4.27[a]	3.33[a]
E 18C2	4.23[ab]	3.70[de]	3.80[c]	3.40[gh]	3.33[cde]	3.70[def]	3.47[efg]
E 18C3	3.27[g]	3.70[de]	3.47[ef]	3.47[fg]	3.47[c]	3.47[g]	3.10[g]
E 24C1	3.77[c]	3.83[cd]	3.80[c]	3.80[b]	3.73[b]	3.67[ef]	3.63[b]
E 24C2	4.17[b]	4.27[a]	4.20[ab]	4.10[a]	3.50[c]	4.20[ab]	3.57[a]
E 24C3	3.47[ef]	3.70[de]	3.73[cd]	3.67[bcd]	3.27[def]	3.60[f]	3.43[b]
E 30C1	4.23[ab]	4.00[bc]	3.80[c]	3.47[fg]	3.17[ef]	3.30[h]	3.70[cd]
E 30C2	3.63[cde]	4.10[a]	3.33[fg]	3.70[bcd]	2.80[g]	3.17[i]	3.70[efg]
E 30C3	3.53[de]	3.67[de]	3.77[c]	3.63[cde]	3.27[def]	3.63[ef]	3.47[cd]
K 18C1	4.20[ab]	3.63[e]	4.10[b]	3.57[def]	3.77[b]	3.90[c]	3.80[bc]
K 18C2	4.23[ab]	3.60[ef]	3.60[d]	3.50[efg]	3.33[cde]	3.67[ef]	3.80[bc]
K 18C3	2.83[ch]	3.13[h]	3.10[h]	2.87[i]	2.67[g]	2.73[k]	3.13[h]
K 24C1	3.73[c]	3.63[e]	3.83[c]	3.70[cbd]	3.90[b]	3.87[c]	3.60[cd]
K 24C2	3.27[g]	3.43[fg]	3.30[g]	3.37[gh]	3.10[f]	2.93[j]	3.77[h]
K 24C3	3.30[fg]	3.60[ef]	3.73[cd]	3.57[def]	3.40[cd]	3.80[cd]	3.70[cd]
K 30C1	4.27[ab]	3.83[cd]	3.73[cd]	3.73[bc]	4.13[a]	4.10[b]	4.10[a]
K 30C2	3.63[cde]	3.60[ef]	3.80[c]	3.47[fg]	3.50[c]	3.73[de]	4.23[def]
K 30C3	3.67[cd]	3.37[g]	3.77[c]	3.30[h]	3.17[ef]	3.33[h]	3.83[fg]
SE (±)	0.12	0.10	0.10	0.09	0.10	0.06	0.08
CV (%)	3.13	2.77	2.65	2.48	2.98	1.69	2.20
LSD (5%)	0.20	0.17	0.16	0.15	0.17	0.10	0.13
Mean of E:	3.85	3.89	3.80	3.66	3.37	3.67	3.49
Mean of K:	3.68	3.54	3.66	3.45	3.44	3.56	3.77

In each column, means followed by the same letter (s) are not significantly different at ($p \leq 0.05$); A= Ripening Techniques (K=Kerosene; E=Ethrel); B= Exposure Times (18, 24 &30 hrs); C= Cultivars (C1, C2 &C3); *= Interaction; ns=Non significant.

70% RH was found optimal for the conversion of starch and acids and possessed better quality attributes in terms of pulp color, taste, aroma, texture, firmness and overall appearance compared to hot water dip (55°C, 5 min) and including smoking (24 h).

Slightly higher mean score value with respect to mouth-feel (3.44) was recorded for the kerosene smoking-based ripening system, which could be related to the similar higher value accounted to it in terms of degree of ripening (3.77). The relatively low scores observed in Giant Cavendish fruits under the ethrel-based ripening system, invariable across all the sensory quality attributes, could

be attributed to its inability to reach the same ripening stage on the 7th day as the other two cultivars. This situation with Giant Cavendish was likewise manifested through the mean values of most of the other physicochemical parameters tested including total peel color. In addition, the relatively low mean score values accounted to the kerosene smoking-based ripening particularly in terms of color, flavor and aroma, could be attributed to the poor performance of the system in appropriately expressing the natural traits of the fruits. Sarananda (1990) and similar other studies reported that although such traditional smoking techniques of banana using kerosene smoking generally accelerate the ripening process, primarily due to the presence of acetylene (C_2H_2) and ethylene (C_2H_4) in the smoke, they usually render the fruits into lower consumer attractions. This, among other reasons, was similarly attributed to the masked or displaced natural aroma of the fruits by the smoke, poor appearance of smoked fruits, burnt scars and bruises on the peel surface, and increasing the liability of the fruits for microbial infections.

SUMMARY AND CONCLUSIONS

Ripening of banana fruits using the traditional kerosene smoking ensures faster and uniform ripening in variably with all treatment combinations in just seven days of the ripening period. However, at this stage, fruits were found developing some off ripening effect black scars on the peel in addition to the relatively low quality attributes recorded upon them through the sensory evaluation panel. On the other hand, banana fruits treated with ethrel generated ethylene were able to complete their ripening stage on the 7th day of the ripening period only at the exposure time of 30 hours. Those treated for 18 and 24 h extended their ripening stage to the 9th and 11th day of the ripening period. However, ethrel generated ethylene treated fruits demonstrated higher mean score values in all sensory quality attributes tested other than mouth-feel, which could also be attributed to the slight delay in their ripening stage. Thus, in terms of ripening efficiency, the kerosene smoking system can be used at the lowest exposure time of 18 h under Jimma (Ethiopia) conditions. The ethrel-based ripening system can similarly be used for equal ripening efficiency and better sensory quality attributes but only at the highest exposure time of 30 h. In addition, further study is recommended for the ethrel-based ripening system with more concentrations in order to attain the more efficient 18 h exposure time recommended for the kerosene smoking system under similar conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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ANNEX

Annex Table 1. Correlation among different physicochemical parameters of banana cultivars subjected to different ripening treatments.

	Firmness	TSS	P^H	TA	TSS:TA	Starch (un-staining)	PWL	Peel Color
Firmness	1							
TSS	-.862**	1						
P^H	.803**	-.796**	1					
TA	-.819**	.755**	-.734**	1				
TSS:TA	-.819**	.955**	-.686**	.635**	1			
Starch (un-staining)	-.913**	.904**	-.800**	.842**	.868**	1		
PWL	-.788**	.840**	-.709**	.812**	.792**	.892**	1	
Peel Color	-.771**	.765**	-.600**	.769**	.741**	.820**	.782**	1

** Correlation significant at P< 0.01 level (2-tailed). *Correlation significant at P< 0.05 level (2-tailed).Starch content was indirectly measured through the iodine-staining method and expressed in terms of the unstained area of the pulp (refer starch data collection procedure under Materials & Methods).

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Effect of stocking rate on biomass variation and lamb performances for barley stubble in Tunisian semi arid region and under conservation agriculture conditions

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The integration of livestock in the practices of conservation agriculture (CA) was assessed in Tunisian semi-arid conditions. Forty five Barbarine lambs (aged 220 ± 10 days, average body weight 20 ± 2.5 kg) were used in a performance trial, carried out in the experimental station of INRAT. During the experiment, lambs were grazing on a plot of barley stubble cultivated according to CA was divided into 6 fenced equal subplots and to each subplot was assigned a stocking rate of animals (15 and 30 lambs per hectare, SR15 and SR30 respectively). The biomass of stubble and its botanical composition were estimated 2 times, using quadrats sampling technique. Live weight was determined 3 times after the start of the experiment (three 15-days successive periods) to calculate live weight gain (LWG) and daily live weight gain (DLWG). The amount of biomass varied (P <0.05) from 2204 to 2067 kg DM / ha for SR15 plots and from 2404 to 1826.5 kg DM/ha for SR30 ones. This decrease was higher with SR30 (P<0.05). Heads proportion decreased first, then leaves and finally stems. Biomass chemical composition declined with sampling period. During the first grazing period, lambs assigned to both treatments lost LW (P<0.001), mainly SR30 lambs as compared to SR15 (P<0.05, -610 and -110 g, respectively). The same trend was observed in DLWG (P<0.05). In the second period, the two groups exhibited similar LWG (about 2 kg) and DLWG (about 171 g/d). In the third period, SR15 lambs maintained their body weight, while SR30 group lost (P<0.001) about 400 g comparatively to the second period. It was concluded that under the studied feeding system, stubble grazing without supplementation allowed Barbarine sheep to maintain body conditions.

Key words: Lambs, barley stubble, stocking rate, conservation agriculture.

INTRODUCTION

In the Mediterranean Basin, livestock production and crop farming have always co-existed. Cereal stubble and straw are important feed resources mainly during summer season. A major concern for the crop/livestock

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systems in the arid or semi-arid lands is the competition for natural resources, especially for crop and other biological residues. In Tunisia, sheep husbandry (about 3.84 million ewes; OEP, 2013) is still playing an important role in rural population. Local sheep breeds are often assigned to extensive management system (OEP, 2013) including local feed resources such as crop residues (e.g. cereal straws and stubbles: about 1.5 million and 500 000 tones/year). These practices might not be independent of farming system evolution and development. In this connection, conservation agriculture (CA) based mainly on zero tillage is increasingly developed in the world (Valipour, 2014) and more and more adopted in Tunisia as it improves profitability of cereal and forage cropping. The total area cropped under CA context, mainly no-tillage increased from 27 ha 1999 to nearly 12 000 ha (INGC, 2014). The benefits from CA include social and economic advantages and combine production and environment protection. It promotes minimal disturbance of the soil (zero tillage), balanced application of chemical inputs and careful management of crop residues (Dumanski et al., 2006). In its compilation of definitions on sustainable agriculture, Gold (2007) reported that CA practices leave residue cover on the soil surface, substantially reducing the effects of soil erosion from wind and water. They also minimize nutrient loss, decreased water storage capacity, crop damage, and decreased farmability. The soil is left undisturbed from harvest to planting except for nutrient amendment. The same author reported that weed control is accomplished primarily with herbicides, limited cultivation, and with cover crops. The concept of CA is somewhat in line with other sustainable practices such as the low-input sustainable agriculture (LISA). Indeed, according to Parr et al. (1990), LISA are systems how “seek to optimize the management and use of internal production inputs (that is, on-farm resources) and to minimize the use of production inputs (that is, off-farm resources), such as purchased fertilizers and pesticides, wherever and whenever feasible and practicable, to lower production costs, to avoid pollution of surface and groundwater, to reduce pesticide residues in food, to reduce a farmer's overall risk, and to increase both short- and long-term farm profitability”. This suggest that LISA will have a physical productivity limited by the maximum on-farm resources that can be mobilized and that LISA can then be associated with lower output (Poux, 2008).

By the other hand, the integration of crop-livestock under CA holds promise to improve the efficiency and sustainability of production systems, but this is conditioned by good understanding of CA principals and appropriate use of corresponding packages. The farmer can introduce forage crops into the crop rotation, thus extending it and reducing pest problems. Forage species could be used as dual-purpose crops for fodder and soil cover. However, conflicts between the use of stubbles in livestock feeding or to cover the soil have to be resolved, particularly in drylands where fodder potential is low (FAO, 2006). This concept of CA seemed to be apparently incompatible with livestock extensive system and if it is adopted, competition with livestock feeding needs to be optimized.

The current study is part of a research program on livestock management under the context of CA which is not yet documented in the literature. Therefore, this experiment was designed to study the effect of stocking rate on stubble biomass variation and lamb growth.

MATERIALS AND METHODS

Study location

The trial was carried out in the experimental station of INRAT (24 km from Tunis, semi-arid: 350 mm of annual rainfall). Barley (variety Manel) was cropped using CA package. Indeed, barley was drilled (no tillage) in the 26th of December 2012 at a seeding rate of 100 kg/ha. A treatment by herbicide (glyphosate 100 g L^{-1}/ha) was applied in absence of later weeding. The plot was fertilized using ammonitrate (150 kg/ha). The harvest was made on the 24th of June 2013, at a cutting height of about 25 cm above ground. The registered grain yield was 1.4 t/ha.

Animals

Forty five 7-month-old Barbarine lambs (initial average weight 20 ± 2.3 kg) raised in the experimental station of Oueslatia (INRAT) were used for the experiment. They received an antiparasitic treatment and were vaccinated against enterotoxaemia. They were housed in collective boxes in a covered barn and marked on, using different colors of painting to be easily identified and separated per treatment and plot, before leaving for grazing. They had free access to clean water 3 times a day.

Experimental design, sampling and measurements

The experimental plot of 22500 m^2 was divided into 6 subplots of 3750 m^2 each, assigned randomly to two stocking rates (15 and 30 lambs per hectare, respectively for SR15 and SR30). Each treatment was triplicated using the six fenced subplots. Along with the 46 day-trial, lambs grazed twice a day (from 5.00 h to 8.00 h a.m. and from 16.00 h to 18.00 h or 17.00 h to 19.00 h) with a total grazing duration of 5 h per day. The grazing practice covered the period 19th July-3rd September 2013, with a total duration of about 46 days.

The biomass of stubble and the removal of particular fraction were estimated using quadrats sampling technique (0.25 m^2, 5 quadrats per subplot placed in zigzag to have representative samples) at the beginning of the trial and 14 days after. The cutting height was at about 4 cm from the soil. It was not possible to take samples at the end of the experiment because of the rain occurring towards the end of August and the beginning of September and the emergency of vegetation. Samples were immediately weighed and transported to the laboratory for immediate dry matter (DM) determination. Proportions of heads, leaves, stems and other vegetation were evaluated and samples were stored for chemical analysis.

In order to control lamb growing, the animals were weighed early in the morning (5.00 h am) before the start of grazing. Live weight was determined 3 times after the start of the experiment (each 15

Table 1. Variation of DM biomass with stocking rate and sampling period.

Variation		S 0	S 14	SEM	Significance
SR15	Biomass (kg DM/ha)	2204^{aA}	2067^{bB}	128.12	*
	Vegetation (%)	6.7 ^{aA}	3.6 ^{bA}	0.9	NS
	Heads (%)	33.6 ^{aA}	35.2 ^{aA}	1.84	NS
	Stems (%)	33.3 ^{aA}	36.1 ^{bA}	1.32	NS
	Leaves (%)	26.8 ^{aA}	25.1 ^{bA}	1	NS
SR30	Biomass (kg DM/ha)	2404 ^{aA}	1826.5^{aB}	132.7	*
	Vegetation (%)	5.7 ^{aA}	8.4 ^{aA}	0.99	NS
	Heads (%)	34.2 ^{aA}	26.6 ^{aB}	2.26	*
	stems (%)	31.9 ^{aB}	39.7^{abA}	1.71	*
	Leaves (%)	28.1 ^{aA}	25.2 ^{bA}	0.92	NS
SEM	Biomass (kg DM/ha)	131.05	111		
	Vegetation (%)	0.93	0.89		
	Heads (%)	2.19	2.03		
	Stems (%)	1.41	1.4		
	Leaves (%)	1.11	0.89		
Significance	Biomass (kg DM/ha)	NS	**		
	Vegetation (%)	NS	*		
	Heads (%)	NS	**		
	Stems (%)	NS	*		
	Leaves (%)	NS	**		

S0: Sampling at 0 days, S 14 : sampling after 14 days, a, b, c: Different letters in the same column mean different values; A, B, C: Different letters in the same line mean different values; SEM: Standard error of the mean; *: P<0.05; **: P<0.01.

days) and then lamb growth was followed for 3 successive periods of grazing. Live weight gain (LWG) and daily live weight gain (DLWG) were calculated.

Chemical analysis

Biomass samples were dried at 50°C ground to pass through a 1 mm-screen then were analyzed for ash and crude protein (Association of Official Analytical Chemists, 1984) and for lignocellulose fraction ADF (Van Soset et al., 1991).

Statistical analysis

Data were subject to analysis of variance using GLM procedure, (Statistical Analysis System software; SAS, 2002). The model included stocking rate (S) and period (P) effects and the interaction (S x P). SNK test was used to compare treatment effects. When P-value is below 5%, the treatment effect was considered significant.

RESULTS AND DISCUSSION

Stubble biomass

Estimated biomass yields are reported in Table 1. At the beginning of the experiment (S0), no difference was observed in biomass between the plots reserved to the 2 stocking rates (averaged 2304 kg DM/ha). Subplots assigned to the two stocking rates exhibited substantial decrease of biomass yield (P<0.05) in the second sampling time (S14: -137 and -577.5 kg DM/ha respectively with SR15 and SR30). Proportions of biomass components indicated in Table 1 showed that the proportions of heads decreased (P<0.05) in SR30 plots, but was maintained in SR15 ones. This decrease could be due to the sorting exerted by lambs which preferred heads while grazing (Brand et al., 1993). The same trend was observed by Yiakoulaki and Papanastasis (2005) who mentioned that sheep grazing on cereal stubble tend to consume heads first. Heads are selected first by sheep because they are higher in energy than the other parts of the stubbles (Houmani, 2002). Leaves proportions were not affected after 14 days of grazing in both SR15 and SR30 plots, while stems proportions increased (P<0.05) in SR30.

The observed values of stubble biomass are in the same range of that reported by Valderrabano (1991) and Cabello et al. (1992). It worthy to note that wide variation in stubble biomass amounts were observed in the literature and that several factors could affect the precision of measurements, mainly the size of quadrat.

Table 2. Chemical composition of biomass according to stocking rate and sampling period (% DM).

Variation		S0	S14	SEM	Significance
SR15					
	DM(%)	91.4^{aA}	92.1^{aA}	1.9	NS
	Ash	7.7^{aA}	7.4 ^{aA}	0.16	NS
	CP	4.6 ^{aA}	4.5 ^{aA}	0.12	NS
	ADF	45.3 ^{aA}	47.9 ^{aB}	0.81	*
SR30					
	DM(%)	92.7^{aA}	93.6^{aA}	2.1	NS
	Ash	8.1^{bA}	7.5 ^{aA}	0.27	NS
	CP	5.3 ^{bA}	4.3^{aB}	0.17	*
	ADF	46.6 ^{bA}	48.7^{aB}	0.97	*
SEM					
	DM	1.95	2.4		
	Ash	0.25	0.15		
	CP	0.17	0.10		
	ADF	0.93	0.98		
Significance					
	DM	NS	NS		
	Ash	*	NS		
	CP	*	NS		
	ADF	*	*		

S0: Sampling at 0 days, S 14: sampling after 14 days, a, b, c: Different letters in the same column mean different values; A, B, C: Different letters in the same line mean different values; SEM: Standard error of the mean; *: P<0.05.

Indeed, according to Treacher et al. (1996), a comparison of samples cut, using quadrats of 1.0 x 1.0 m (S) and 4.25 x 0.47 m (R), showed a large reduction in the coefficient of variation from 23% with S to 9% with R. The absence of later sampling times in our study did not allow to better understanding grazing evolution. Treacher et al. (1996) conducted a similar experiment on stubble grazing ewes. They noted that heads were selected first and disappeared after 4 to 8 days of grazing at stocking rates of 20 to 60 sheep/ha. They also recorded an increase of stems intake when most of the leaf had been removed. Houmani (2002) mentioned that the consumption of high-energy diets encouraged sheep to consume more stems. When the stems become very hard, sheep then tend to remove leaves.

Chemical composition variation

Nutrient contents of stubble are presented in Table 2. Dry matter proportion of stubble was similar among sampling times and stocking rates. Ash content did not change between S0 and S14 in both SR15 and SR30. However, it decreased (P<0.05) with the increase of the stocking rate in S0. Similar trends of contents were reported by Ben Said et al. (2011) in semi-arid regions from Tunisia. CP contents are relatively high in the beginning of the experiment in all the plots comparatively to literature. Indeed, the average content of this nutrient (4.9% DM at S0) is higher than which found by Avondo et al. (2000) for barely stubble (3.4% DM) and values relative to cereal straws (Houmani and Tisserand, 1999). This may be related to the richness of biomass in heads and thereby grains. The CP content was maintained in SR15 treatment, but decreased (P<0.05) by about one percentage unit 1% in SR30, 14 days after the beginning of the grazing period. Houmani (2002) conducted a similar experiment on ewes and concluded that the content of CP decreased with grazing frequency by the animals (-1.3 percentage unit), 16 days after the beginning of the experiment. This variation of the CP content of stubble is likely due to its level in grains, which decreases with the grazing duration. Also,the relatively high content of CP, even in the second sampling time, may indicate once more that the studied stocking rates were not very high. Rihani et al. (1991) suggested that the lower is the stocking rate, the higher is the digestibility and the CP content of the stubble.

The content of ADF seems relatively low (averaged 45.9% DM in S0) when compared to that of fibrous

Table 3. Variation in LW according to stocking rate and period (kg).

Variation	Period 1	Period 2	Period 3	SEM	Significance
SR15	-0.61^{bC}	2.05 ^{aA}	0.03 ^{aB}	0.51	***
SR30	-0.11^{aB}	2.07 ^{aA}	-0.4 ^{bC}	0.79	***
SEM	0.22	0.161	0.166		
Significance	*	NS	**		

a, b, c: Different letters in the same column mean different values; A, B, C: Different letters in the same line mean different values; SEM: Standard error of the mean; *: P<0.05; **: P<0.01; ***: P<0.001.

feedstuffs including cereal straws in Mediterranean regions (generally ADF content is above 50%, Susmel et al., 1994). This observation is in line with the relatively high content of CP and confirms the negative correlation between CP and ADF shown by Avondo et al. (2010). The ADF contents of stubble in the 3 plots in the first sampling time are similar (Table 2). In both treatments, ADF content increased by 2.6 and 2.1% units (P<0.05) for SR15 and SR30 plots respectively. This result is related to the changes in botanical composition between the 2 sampling time, exhibiting decreasing trends of head and leave proportions and a decrease in steam ones, mainly in SR30. The higher lignocellulosic fraction in steams comparatively to heads and leaves may be reflected in which of biomass. Our results confirmed the cell wall variation trends observed in Tunisia by Ben Said et al. (2011), for cereal stubble produced in CA condition. Controversial literature data on the nutritive value of stubble are reported. Some differences are noted comparatively with results found by Ben Said et al. (2011) for barley stubble variation between June and September in Tunisian semi-arid regions and by Avondo et al. (2000) for the same speciesin southern Italy. Chemical composition of cereal stubbles is related to different factors such as region, cereal species and varieties and climate (Rao and Dao, 1994). The compilation of chemical composition results, mainly the relatively high content of CP and the low content of ADF are in line with morphological composition of stubbles which were especially high in heads and leaves as compared to data reported in Cobarellero et al. (1992) and Ben Said et al. (2011). Differences in head proportions are mainly related to the control level of the harvesting process and used machines. Variation in chemical composition between the two sampling times especially noted in SR30 may be due to selective behavior of lambs during grazing, which induced changes in morphological composition of stubbles and thereby in chemical composition (Ben Said et al., 2011). Indeed, animals start the grazing period by sorting heads and then leaves.

Lamb performances

LWG and DLWG according to stocking rate and period are presented in Tables 3 and 4 respectively. During the first grazing period, lambs from the both treatments lost Live weight (P<0.001), but this loss was higher (P<0.05) for SR15 than SR30 group (-610 and -110 g, respectively). The same trend was observed in DLWG (-23.3 and -4.1 g/d, respectively for SR15 and SR30, P<0.05). This result couldn't be ascribed to the nutritive value of stubbles but likely to the initial body conditions of lambs and the first period represents, actually, an adaptation period for experimental conditions. Also, it is not excluded that the sorting by lambs of high amounts of heads and grains in stubble biomass could have induced some digestive disturbances in this first period particularly rich in grains. These hypotheses are confirmed in the second period, since the two groups performed similarly as reflected by the LW (around 2 kg, Table 3) and the DLWG (171 g/d, Table 4). The last finding suggests that the biomass in the plots was not limiting and was sufficient for animals assigned to the two treatments. As expected, in the third period SR15 group conserved their body weights, while SR30 group lost (P<0.001) about 400 g comparatively with the second period (Tables 3 and 4).

Biomass estimation seemed to be in line with sheep performances. In addition, data presented in Table 5 indicated that generally in this feeding system, body state of animals was preserved. Furthermore, animals in both treatments registered a similar total LWG (around 1.5 kg). Thus, stubbles have contributed to safeguard livestock even in absence of supplementation. Similar findings were reported by Treacher et al. (1996) on ewes grazing barley stubbles at different stocking rates.

These results obtained under CA conditions, should be translated in terms of stubble management strategies to comply with CA principles and objectives, including soil cover (Abbas and Zitouni, 2010). Indeed, the effect of livestock is closely related to the rate of vegetation cover before grazing (Masmoudi, 2012), the stocking rate and the duration of grazing. Köller (2003) claimed that livestock could be fully integrated into conservation agriculture, when more than 30% of the residues from the previous cropare left on the ground as mulch. Also, the study of Masmoudi (2012) showed that the integration of livestock at different levels of stocking rates requires a rate of biomass cover higher than 78% before grazing.

Table 4. Variation in DLWG according to stoking rate and period (g/d).

Variation	Period 1	Period 2	Period 3	SEM	Significance
SR15	-23.3 ^{aB}	171.1 ^{aA}	4.7 ^{aB}	43.21	***
SR30	-4.1 ^{bB}	172.8 ^{aA}	-69.05^{bC}	71.22	***
SEM	8.45	13.42	23.77		
Significance	*	NS	**		

a, b, c: Different letters in the same column mean different values; A, B, C: Different letters in the same line mean different values; SEM: Standard error of the mean; *: P<0.05; **: P<0.01; ***: P<0.001.

Table 5. Effect of stocking rate on total TLWG and DLWG.

Variation	TLWG(kg)	DLWG (g/d)
SR15	1.479	32
SR30	1.483	32
SEM	0.61	10

SEM: Standard error of the mean.

Further studies are needed in different conditions and with different crops and animal species before claiming suitable residues amounts, as related to both animal and CA requests.

Conclusions

Irrespective of the stocking rate, lambs grazing barley stubbles for one month and half after harvest were able to meet their maintenance requirements and even to grow at a rate of 30 g/day. Under the experimental conditions of the current work, the two stocking rates resulted in similar performances of Barbarine lambs. This suggests that the available biomass could support higher stocking rates.

Conflict of Interests

The authors have not declaredanyconflict of interests.

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Trichoderma: A magical weapon against soil borne pathogens

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***Trichoderma* species occur worldwide. The members of the genus *Trichoderma* are widely used as bioagent for the control of phytopathogenic fungi in agriculture sector. *Trichoderma* not only parasites pathogen but it also enhance plant plant growth, help in the bioremediation of soil etc.Biocontrol activity of *Trichoderma* is known since 1930s.The effect of *Trichoderma* on soil borne pathogens is higher as compared to chemical fertilizers and it persists in soil for longer period after application. The members of this genus are reproducing asexually by the formation of cyanide and chlamydospore, in wild habitats they reproduce by formation of ascospores. *Trichoderma* species are well known for the production of cell wall degrading enzymes. These cell wall-degrading enzymes (CWDEs) play a major role in biocontrol mechanism. They are also widely exploited in industries as sources of enzymes.Use of biocontrol agents for reducing disease incidences provides an alternative for the chemical pesticides. *Trichoderma* species are among the most studied biocontrol agents. It has been found that there are many genes which are responsible for biocontrol activity these genes are called biocontrol genes. The sequencing of these genes should be done in order to produce miccroorganisms with superior biocontrol ability and also for the production of transgenic plant, that are resistant against plant pathogens.**

Key words:*Trichoderma,* phytopathogens, lytic enzymes, biocontrol genes.

INTRODUCTION

Use of biological pesticides is continuously increasing due to public concerns about environmental pollution, human health and soil fertility. Biological pesticides are served as an alternative to chemical pesticides. Farmers generally use chemical pesticides to control plant diseases, these chemical pesticides imparts a bad impact on environment. In Europe around 250k tones of biopesticides is consumed annually. There are two types of biocontrol agents generalists (These biocntrol agents are capable of controlling a large number of taxonomically different pathogens e.g. *Bacillus, Pseudomonas, Trichoderma*, yeast etc) and Specialist (These biocontrol agents are capable of controlling only targeted species e.g. *Agrobacterium, Aspergillus*etc).

Trichoderma species are the most commonly used biocontrol agents. They are commercially marketed as

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biopesticides, biofertilizers and growth enhancers. The various mycoparasitism mechanism employed by *Trichoderma* are, competition for food and space, secretion of cell wall degrading enzymes, secondary metabolite production, host immune response induction and plant growth promotion. *Trichoderma* based bioformulation are used in greenhouse, nursery, field, orchards and hydrophonics.*Trichoderma* based bioformulations are used for crop protection in whole world. *Trichoderma* species that are most commonly used as biocontrol agents are: *T.harzianum, T. atroviride, T. asperellum, T. polysporum, T. viride.*

Trichoderma species are also known for their biodegradation capability. *Trichoderma* species have the capability of degrading toxic compounds.

Trichoderma is highly effective on root rot, foot rot, collar rot, stem rot, damping off, wilt, blight leaf spot of crops like pulses oil seeds, *cucurbitaceous* crops (cucumber, bottle gourds, ridge gourd) *solanacceuos* crops like tomato, brinjal, chilli, capsicum etc. *Trichoderma* are also effective against sheath rot, sheath blight and bacterial leaf blight of rice.

The positive effects of *Trichoderma* on agriculture crop protection have been recognized in the whole world. Our understanding of the mechanisms of biological control employed by *Trichoderma* is continuously expanding. The molecular mechanisms of the interaction of this fungus with phytopathogen can be understood through the modern molecular techniques. Samuels (1996) provides a comprehensive review of *Trichoderma* species for enzyme production and biological control mechanisms. In *Trichoderma* species sexual reproduction is not present and is believed to be mitotic and clonal. The main problem with the nomenclature of *Trichoderma* is pleomorphisim present within the genus. In *Trichoderma* there are two stages, the sexual stage is called *Hypocrea* (telomorphic) and asexual stage is called *Trichoderma* (anamorphic). The genus is called as *Hypocrea/Trichoderma* (Druzhinina et al., 2011).The genetic diversity within the genus *Trichoderma* is very high, thus there must be some technique through which we can identify the *Trichoderma* species. The necessary characters which should be present in an effective biococntrol agent are: Good lytic enzyme producer, increased plant systemic resistance, plant growth enhancer, good secondary metabolite production, pollutant degradation, good CFU maintenance in formulation. By gaining the knowledge of desirable characters new strains can be designed and developed. In case of *Trichoderma*protoplast fusion is the technique through which we can develop hybrid strains.

Trichoderma species are excellent producers of lytic enzymes like chitinase, glucanase, cellulases and xylanase, which lyase the fungal cell wall. Besides being attacking directly it promotes plant growth and induce plant resistance. *Trichoderma* species produce a variety of secondary metabolites. The *Trichoderma*species release antibiotics and other chemicals that are harmful to pathogens and inhibit growth (antibiosis).The potential use of the *Trichoderma* species (Table 1) as a biocontrol agent was suggested more than 70 years ago by Weindling (1932) who was first to demonstrate the parasitic activity of a member of this genus against soil borne fungal or bacterial pathogens.There are many other crops for which *Trichoderma* is used.

TAXONOMIC HISTORY

Although the genus *Trichoderma* has been known since the 19th century. Its association with *Hypocrea*Fr. was discovered by the Tulasne brothers in 1865; its taxonomy has remained obscure until recent decades. Bisby (1939) thought that the morphological variation could be ascribed to a single species, *T. viride*. The first serious attempt to morphologically distinguish species, or rather “species aggregate”, was made by Rifai (1969). Some new species were subsequently described by were keyed out by Domsch et al.(1980). Teleomorph connections were established by means of ascospore isolates by Dingley (1957), and by Webster and coworkers Webster andRifai (1969). In Japan, Doi (1969; 1972), studied a number of telemorphs and described them with cultural and anamorph characters, but no cultures were preserved from that study. After this no morphological differentiation was given by Doi(1979; 1986). Bissett (1984; 1991a, b, c; 1992) gave a detailed description of the morphological studies, who distinguished about 21 taxa. These studies have shown the delimitation of biological species is extremely difficult in this genus on morphological grounds alone.

Apart from morphological studies there are many other methods that are used in the taxonomy, such as study of secondary metabolites, this has shown a great diversity in this genus (Okuda et al., 1982). Physiological features that are detected by the microtiter plate assay are the useful tools that are used for identification. Isoenzyme profiles are also used for taxonomic classification (Leuchtmann et al., 1996; Samuels et al., 1994). In modern era molecular techniques such as sequences of its region of ribosomal DNA and fingerprinting techniques provides the finest resolution of taxonomic entities(Fujimori and Okuda, 1994; Meyer et al., 1992; Muthumeenakshi et al., 1994).

BIOCONTROL GENES OF *TRICHODERMA*

Trichoderma is widely used for the control of many soil borne plant pathogens (Table 2). *Trichoderma* species are the efficient producer of cell wall degrading enzymes (Srivastava et al., 2014b), some of these enzymes are of commercial importance. Many research workers have proved that *Trichoderma* species possess some

Table 1. *Trichoderma* species and its uses against different plant pathogens.

Plant	Causative agent	*Trichoderma* species used
Vignamungo (Black gram) (Raghuchander et al., 1997; Dubey et al., 2001; Mishra et al., 2011)	Macrophominaphaseolina, alternate Alternaria	*T. viride, T. harzianum*
Solanummelongena L.(Brinjal) (Jadon, 2009, Balaji and Ahir, 2011)	*Fusariumsolani F.oxysporum f. sp*	*T. viride, T. harzianum*
Cicerarietinum (Chickpea) (Mukherjee et al., 1997; Haware et al., 1999; Pandey, 2003; Poddar et al., 2004)	*F. oxysporum, R. solani, A. niger* *Chaetomium* sp, *S. rolfsii, Penicillum* spp. *M. phaseolina*	*T. harzianum, T. viride*
Capsicum annum L (Chilli), (Rini and Sulochana, 2006, Kapoor, 2008, Vasanthakumari and Shivanna 2013)	*S. rolfsii, F. oxysporum, Pythiumspp, R. solanipseudokoningii 2013*	*T. viride, T. harzianum*
Cocosnucifera L (Coconut) (Karthikeyan et al., 2006)	*Ganodermalucidum*	*T. harzianum, T. viride*
Coffeaarabica L. (Coffee); (Deb and Dutta,1999)	*Phomopsisthaeae, Glomerellacingulata*	*T. harzianum*
Vignasinensis (Cowpea) (Pan and Das, 2011)	*R. solani*	*T. harzianum*
Arachishypogaea L.(Groundnut) (Biswasand Sen, 2000; Kishore et al., 2001; Rakholiya et al., 2010; Bagwan, 2011; Sreedevi et al., 2011; 2012)	*Thievaliopsisbasicola, S. rolfsii Sacc, A. niger, R. solani, P aphanidermatum, M. phaseolina*	*T. harzianum T. viride T longibrachiatum*
Agaricusbisporus (Mushroom) (Rawal et al., 2013)	*Rhizopusstolonifer, Coprinopsiskimurae, P. glabrum, F. oxysporum*	*T. viride*
Cajanuscajan (Pigeon pea) (Hukma and Pandey, 2011)	*F. udum*	*T. viride T. harzianum*
Solanumlycopersicum (Tomato) (Sreenivasaprasad, 1999; Dutta and Das, 2002; Jayaraj, 2006,)	*F. oxysporum f. sp. lycopersici, P. aphanidermatum, R. solani, S. rolfsii*	*T. harzianum T. viride T longibrachiatum, T. virens*
Capsicum annum L (Capsicum) (Kapoor, 2008)	Alternariaalternata	*T. viride T. harzianum*
Brassica oleracea (Cauliflowe) (Sharma et al., 2004; 2005; Ahuja et al., 2012)	*R. solani, P. aphanidermatum*	*T. viride T. harzianum*
Citrus (Kalita et al., 1996; Singh et al., 2000	*F. solani*	*T. viride T. harzianum*
Gossypiumhirsutum (Cotton) (Sreenivasaprasad, 1990; Gaur et al., 2005)	*R. solani, S. rolfsii, P. aphanidermatum*	*T. viride T. harzianum*
Zingiberofficinale (Ginger)(Gupta et al., 2010)	*P. aphanidermatum*	*T. harzianum*
Sesamumindicum L (Sesame) (Tamimi and Hadvan, 1985; Sankar and Jeyarajan, 1996a,b; Jeyalakshmi, 2013)	*A. flavus, Curvularialunata, P. notatum, P. chrysogenum, F. moniliforme, F. oxysporium , R. nigricans, M. phaseolina*	*T. viride T. harzianum*

Table 2. Some biocontrol genes of *Trichoderma* and their function.

Gene	Source organism	Function
Tvsp1	*T. virens*	This gene encodes for serine protease. *Rhizoctonia solani which* affects the cotton seedlings has been controlled biologically by serine protease.
*tri*5	*T. harzianum*	This gene is responsible for the synthesis of trichothecene which inhibits the protein and DNA synthesis in the cells of the pathogens and inhibits their growth.
*TgaA*and *TgaB*	*T. virens*,	This gene exhibits antagonist activity against *R. solani*and *Sclerotiumrolfsii*
ThPG1	*T. harzianum*	This gene encodes for endopoly-galacturonase. This enzyme is involved in the cell wall degradation of the pathogens like *R. solani*and *P. ultimum*
Th-Chit	*T. harzianum*	This gene is responsible for the antifungal activity in transgenic tobacco plant.
tri5	*T. brevicompactum*	This gene helps in the production of *Trichoderm* in which shows antifungal activity against *S. cerevisiae, Kluyveromycesmarxianus, Candida albicans, C. glabrata, C. tropicalis*and *Aspergillus fumigates*
erg1	*T. harzianum*	This gene encodes an enzyme named squalene peroxidase, which helps in the synthesis of ergosterol and silencing of this gene provides resistance to terbinafine, an antifungal compound
TvGST	*T. virens*	This gene is responsible for cadmium tolerance
Thkel1	*T. harzianum*	This gene codes for putative kelch-repeat protein which helps in regulating the glucosidase activity and enhances tolerance to salt and osmotic stresses in *Arabidopsis thaliana* plants
egl1.	*T. longibrachiatum*	This gene showed biocontrol activity against *P. ultimum*in damping-off of cucumber
qid74	*T. harzianum*CECT 2413	This gene plays a significant role in cell protection and provide adherence to hydrophobic surfaces that helps the fungus in mycoparasitic activity against *R. solani*pathogen
Taabc2	*T. atroviride*	This gene has a significant role in ATP binding cassette (ABC) transporter in cell membrane pump that helps in the mycoparasitic activity
tac1	*T. virens*	This gene has its role in mycoparasitic activity against *R. solani*and *P. ultimum*
TrCCD1	*T. reesei*	This gene is involved in carotenoid metabolism that helps in the development of conidiospores and hyphal growth in *T. reesei*

Source:Srivastava et al. (2014b).

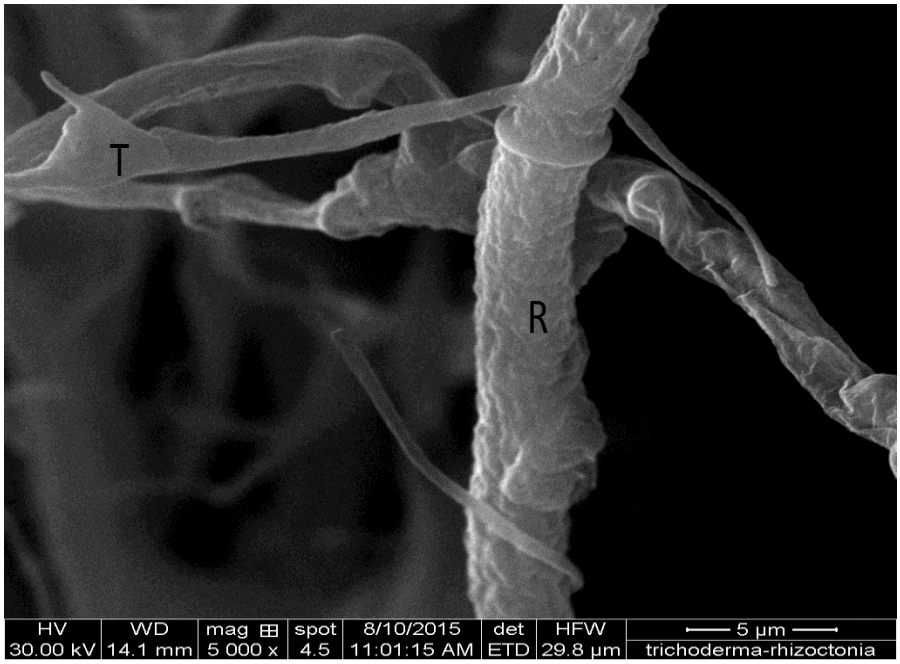

Figure 1. SEM analysis of coiling and hyphal growth depression of *Rhizoctonia bataticola* (R) *by T. harzianum* (T).

biocontrol genes that can be isolated and cloned for commercial large scale production (Massart and Jijakli, 2007).

Kuc (2001) has proven that some genes, providing resistance to abiotic and biotic stress are present in *Trichoderma*. Mycoparasistism, antibiosis and competition for the nutrients are the main strategies employed by *Trichoderma* for the phytopathogenic action (Janisiewicz and Korsten, 2002). Among the different species tested *T. herzianum* was found to be the most promising strain (Gao et al., 2002).

GENOMICS OF TRICHODERMA

The genome size of filamentous fungi is very small that is about 25 to 50 Mb. With the advancement of pulse field gel electrophoresis karyotyping of the filamentous fungi is possible. Karyotyping is helpful in the detection and translocations and variations in chromosome numbers. Through PFGE we separated chromosomal DNA from *Trichoderma* (Gilly and Sands, 1991; Hayes et al., 1993; Herrera-Estrella et al., 1993; Mäntylä et al., 1992). The expected genome size and chromosome number of *Trichoderma* ranges from 31 to 39 MB and from 3 to 7, respectively. From the data obtained through DNA homology it was found that *T. herzianum*and *T. veins* are closely related, and it was concluded that they may have the same phylogenetic origin (Herrera-Estrella et al., 1993). Mäntylä et al., (1992) determined molecular karyotypes of strains of *T. reesei* that had undergone mutagenesis and screening for the hyperproduction ofcellulase enzyme. The authors found that extensive alteration in the genome organization of these strains occurred.

The first member of the genus sequenced was *T. reesei*. *T. reesei* is the first choice, because the genome size of this organism is very small (33Mb) and it has seven chromosomes. Fungal genomics laboratory of NCSU has EST, cDNA collection and BAC libraries for academic researchers. DNA mediated transformations and gene protocols have been developed for the genomic study of *Trichoderma.*

Trichoderma and phytopathogen control

The use of *Trichoderma* as a biocontrol agent for the control of phytopathogen is an environmental friendly process. However, more detailed information about the mechanism of action of these biological agents is needed (Cortes, 1998). When *Rhizoctonia* comes in contact with *Trichoderma herzianum*, hyphae of the *T. herzianum* start to coil around *Rhizoctonia* (Chet, 1987).). It has been found that there are some lectins, secreted by *Rhizocotonia, Sclerotium* and other phytopathogenic fungi which stimulate *Trichoderma* to coil around pathogen hyphae (Figure 1). *Trichoderma* species secrete extracellular enzymes that aid in the phytopathogenic activity. Weindling (1934) reported that a strain of *Trichoderma*, *T. lignorum* secretes gliotoxin that is harmful for both *R. soleni* and *Sclerotium* americana

(Chakravarthy et al., 2011). It has been found that chitinolytic system of *Trichoderma* has six different enzymes two of which are classified as acetyl glucosamine and the rest four as endochitinase (Haran et al., 1996).

Mycoparasitism can be defined as the direct attack of one fungi over other (Fand et al.,2013). The process of mycoparasitic action involves recognition, attachment and finally killing of the fungi. CWDEs play a major role in the mycoparasitism action. Mycoparasitism action begins with the secretion of chitinase enzyme, which degrades the cell wall of the fungus. As soon as cell wall of the phytopathogen degrades it enters into the lumen of the pathogen and kills that pathogen. In *Trichoderma* there is an evidence for the participation of G α unit in coiling.

The heterometric G protein signaling is basically comprise of three parts, a G protein-coupled receptor(GPCR), a heterotrimeric G protein (α, β, γ subunits), and an effector (Neer, 1995). Various fungal genomes are available nowadays and comparative genomics pointed out that receptors can be classified into nine groups (Lafon et al., 2006). Preliminary investigations of the *Trichoderma reesei* (http:// genome.jgi-psf.org/Trire2/Trire2.home.html) and *T. atroviride* genomes revealed 16 putative proteins with 7-transmembrane domains, well distributed over all nine receptor classes. Highly conserved heterotrimeric G-proteins act as signal transducers that couple cell surface receptors to cytoplasmic effector proteins. G-protein α subunits can be classified into three major subgroups (Bölker, 1998). Further characterization of the tga1 mutant showed that this G-protein α subunit affects processes like vegetative growth, production of antifungal metabolites, and chitinase formation (Reithner et al., 2005), which are at least partially involved in *Trichoderma*biocontrol. In liquid culture the tga1 mutant produced strongly decreased chitinase activities and showed a reduced transcription of the nag1 (N-acetyl-glucosaminidase encoding) and ech42 (endochitinase 42-encoding) genes (Reithner et al., 2005). In antagonistic assays, the tga1 mutant was unable to overgrow and lyse host fungi such as *R. solani,B. cinerea*, and *S. sclerotiorum*, although infection structure formation was unaffected; nevertheless, it displayed an enhanced growth inhibition of the host fungi by over-producing and secreting low molecular weight metabolites. In contrast to the role of Tga1 in influencing growth and conidiation in *T. atroviride*, its homologue TgaA did not affect these properties in *Trichoderma virens*. tgaA mutants grew normally and sporulated like the wild type, but had a reduced ability to colonize *S. rolfsii*sclerotia, whereas they were fully pathogenic against *R*. *solani* (Mukherjee et al., 2004). No such host specificity could be observed in the *T. atroviride* tga1 mutant. Mutants of *T. virens* lacking the TgaB protein(belonging to subgroup II Gα subunits) did not show major phenotypic defects: They grew and sporulate like the wild type and biocontrol against *R. solani* and sclerotia of *S. sclerotiorum* was unaffected (Mukherjee et al., 2004).

MECHANISMS OF PLANT-DISEASE CONTROL BY *TRICHODERMA*

When *Trichoderma* spores are added into the soil they colonize the root surface and form a zone of interaction into which *Trichoderma* species release bioactive compounds. These bioactive compounds enhance plant resistance.The *Trichoderma* species produce CWDEs that degrade the cell walls of pathogen, it also produces antibiotics, it starts coiling around the pathogen hyphae.

In recent times excessive use of chemical pesticides has pose a threat on the environment.*Trichoderma* based biocontrol agents have better ability to promote plant defense response, promote plant growth and soil remediation etc. *Trichoderma* species have gained wide acceptance as effective biocontrol agents against several commercial phytopathogens (Sitansu and Amrita 2011). Micropropagules of *Trichoderma* spp. in the form of conidia are preferred over chlamydospores and mycelia biomass because of the viability and stability in field application (Rosaneet al., 2008).

BENEFITS OF TRICHODERMA

1. *Trichoderma* is extensively used for post-harvest disease control. It has been found effective against Fusarium*, Phytopthara, Scelerot* etc.
2. *Trichoderma* strains have the ability to solubilize phosphates, thus they act as plant growth promoting Rhizobacteria.
3. There are many biocontrol genes are present in *Trichoderma*. Introduction of endochitinase gene from *Trichoderma* into plants such as tobacco and potato plants have increased their resistance to fungal growth.
4. *Trichoderma* strains also play an important role in the bioremediation of soil thatare contaminated with pesticides and herbicides. They have the ability to degrade a wide range of insecticides, organochlorines, organophosphates and carbonates.
5. *Trichoderma* is environmentally friendly.
6.*Trichoderma*species are efficient producer of cellulase and other enzymes that degrade complex polysacchharides. Cellulase from *Trichoderma* are used for the biostoning of denims, to give it a stone washed appearance.
7.The extracellular enzymes obtained from *Trichoderma* are also used to increase the digestibility hemicelluloses used in poultry feed.
8. *Trichoderma* is compatible with organic manures and with other biofertilizers like *Rhizobium,Azospirilium*, *Bacillus subtilis* etc. It can be easily applied to seeds

treated with metalaxyl and thiram but not with mercurials (Gangwar and Sharma, 2013).

LIMITATIONS

1. We should not apply chemical fungicides after application of*Trichoderma* for 4 to 5 days.
2. Trichoderma should not be used in dry fields, as it requires moisture for its survival.
3. Seeds treated with *Trichoderma* should not be dry in direct sunlight**.**

CONCLUSION AND FUTURE RESEARCH

Trichoderma is a promising candidate for the biological control of plant pathogenic fungi.Data obtained from the past studies have provided many clues for future studies. Data obtained by researchers clearly shows that this fungus can be efficiently used as biocontrol agent. The genes present in the fungi have the ability to enhance host plant's resistance against phytopathogenic fungi.

Conflict of Interests

The authors have not declared any conflict of interests.

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Cultivation of golden flax with application of nitrogen and irrigation

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Flaxseed is the seed of the linen (*Linum usitatissimum* L.) and the most produced varieties are brown and golden flaxseed. The objective of this study was to investigate the influence of irrigation and nitrogen application in cultivation of the golden flax cultivation. The irrigation it was made by dripping, daily, except for days of rain occurrences, according to evapotranspiration. The treatments were separated by 0, 50, 100, 150, 200 and 250% of the daily irrigation, and the control value of 100% of the amount of evaporation by area. The seeds were planted on April 22, 2013. After flowering, data were analyzed as: Plant height, number of branches and siliques and the fresh and dry mass of silica and twigs on August 8, 2013. The crop occurred on October 10, 2013 and they were analyzed: amount of silica, number of seeds, total weight of seeds per plant, weight of 100 seeds, the production per meter and the proportion by hectare. Urea application resulted in an improvement in the production of two varieties. The combined irrigation also increases the production; however, in quantities exceeding 150% of irrigation, there was a decrease.

Key words: *Linum Usitatissimum* L., evapotranspiration, production.

INTRODUCTION

Flaxseed is the seed produced by flax (*Linum usitatissimum* L.) belonging to the Flax family, probably originated in Asia (Vieira et al., 2012). It is a herbaceous variable height of between 40 cm and 1 m erect stems, branches to the top of the plant. Varieties for food consumption are brown and golden (Lima, 2007; Morris, 2007).

Flaxseed was introduced in Brazil early XVII century in the state of Santa Catarina and later spread to the states of Rio Grande do Sul, Paraná and São Paulo, these regions which present climate necessary for flowering flax (0 to 2°C) and has a 150-day cycle (Marques, 2008).

Flaxseed presents in its composition omega 3, omega 6 and omega 9, as well as other basic nutritional components and antioxidant compounds. The seeds are between 39 and 45% oil. (Oliveira et al., 2012).

In South America, the largest producer is Argentina, with about 80 tons per year, while Brazil produces only 21 tons. According to the IBGE (2010), the planted area was 16.584 ha and the average yield per hectare was 974 kg, but the production can reach up to 1.5 tons (Oliveira et al., 2012).

The determination of the amount of water lost by evapotranspiration is essential to establish the water

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balance in the region. Thus, one can determine if this region is suitable for the cultivation of certain plant species and quantity irrigation, using efficiently water and energy (Kobiyama and Vestena, 2006).

The potential evapotranspiration (ETp), which is needed rain, is the water loss is the process into the atmosphere through a natural grassy surface, standard, without water restriction to meet the needs of soil evaporation and transpiration. The real evapotranspiration (ETr) is the water loss from a natural surface, in any condition humidity and vegetation cover and the reference evapotranspiration is a parameter that relates the ETp with a reference culture the reference. Rain and ETp are meteorological elements of opposite directions, expressed in millimeters rainfall per day (Thornthwaite, 1944; Ponce, 1989).

In irrigation management driven through evapotranspiration estimation, the most commonly used devices are the Class A tank and automatic meteorological station. The Class A tank is further used to estimate ETp when the irrigation management relies on measurement of water evaporation, where the amount of evaporation is converted into reference evapotranspiration by specific coefficients (Doorenbos and Pruitt, 1977; Allen et al., 1998).

The equation to estimate the potential evapotranspiration is presented in Equation 1, using the analytical method and based on results the estimated ETp for more than a hundred locations (Camargo, 1971).

$$ETp = Qo. T. K. D \quad (1)$$

ETp = potential evapotranspiration in $mm.dia^{-1}$; Qo = extraterrestrial solar radiation above the atmosphere, on the 15th of Each month, in $mm.dia^{-1}$, equivalent evaporation (get it in special tables); T = period of the average daily temperature, in °C; K = adjustment factor: 0.01 for Ta (temperature annual average) to 23.5°C; 0.0105 for Ta 23.6 24.5°C; 0.011 for Ta 24.6 to 25.5°C; 0.0115 for Ta 25.6 to 26.5°C; 0.012 for Ta 26.6 to 27.5; 0.013 Ta for more than 27.5°C, and D = number of days in the period.

There are other methods for determination of evapotranspiration, among them we can mention the Thornthwaite, Jensen and Haise, Blaney-Criddle, Ivanov, Penmann-Montheith and for each evaporímeter model, different parameters apply to determine the evapotranspiration (Souza et al., 2009).

Although the Penman (1948) is based on principles right physical, it is not operationally perfect, but it can be used as a standard model to estimate the ETP. The success of this method is due to the strong influence of solar radiation in the process of evapotranspiration. The limitation of its use is the difficulty of the necessary meteorological data. This has led some researchers to suggest changes aiming to improve and simplify the Penman method (Tucci and Beltrame, 2000; Vanzela et al., 2007).

Fernandes et al. (2004), showed a correlation 0.76 and concordance of 0.94, using a mini tank to determine the evapotranspiration in melon culture compared to that obtained with an automatic weather station, through the Penman-Monteith-FAO equation, showing to be feasible use of the methodology by small producers.

The use of alternative evaporimeters is considered feasible in small irrigation systems, and the variation between the Class A tank and the evaporimeter alternative is acceptable, being approximately 18%. The over estimation provided by mini-tank was 6.6%. The lower surface of the tank, leading to a higher evaporation is compensated due to the lower thermal energy storage to be transferred to the body of water in the plastic walls (Cruz and Barreto, 2002; Lopes Filho, 2000).

The fertilization with urea and ammonium sulfate provided a significant increase in phenotypic characteristics of flaxseed. Fluid replacement resulted in a significant increase in height, fresh and dry weight of sunflower plants. (Werner et al., 2012; Bassegio et al., 2012).

Assuming the report, the goal to achieve the current study was to evaluate the characteristics of golden flax cultivation, after flowering and producing, with nitrogen application influence and different amounts of irrigation.

MATERIALS AND METHODS

The work was conducted in the experimental field of the State University of Western Paraná, in an area of 2.5 and 2.5 m, located in Cascavel, Paraná, Brazil, located at latitude 24º53'47 "S and longitude 53º32'09 "W, altitude of 781 m, with the average annual rainfall of 1640 mm and average temperature of 19°C. The climate is temperate mesothermal and super humid (IAPAR, 2011).

Golden flax seeds were planted on 04/22/2013, with and without the influence of nitrogen on the lines and applying different amounts of treatment daily irrigation, according to evapotranspiration. The soil of the region is classified as Typical Red Hapludox (EMBRAPA, 2006).

The germination of the crop came four days after planting, then immediately was made a thinning, leaving a plant spacing of about 2 cm. The spacing between the planting rows was 40 cm and the size of 1.25 m treatment line, totaling for each irrigation hose an area of 1 m^2. The flow in each hose was 75 $l.h^{-1}$ thus representing the time required in each irrigation line treatment.

The treatments were separated into 0 (T1), 0.5 (T2), 1.0 (T3), 1.5 (T4), 2.0 (T5) and 2.5 (T6), the multiplication factor amount evaporated daily in culture, using the value of treatment T0 as witness measured daily in a mini-tank evaporimeter (EVM) installed on the site of cultivation, which has been inserted a graduated ruler and with a diameter and height of 20 cm × 20 cm, converted in proportion to the area under cultivation.

After flowering, six plants were taken for each treatment and evaluated the following characteristics: plant height, number of siliques, fresh and dry weight of silica, fresh and dry weight of the branches and the number of branches. Analyses were made on 08/20/2013.

On 10/14/13 cultivars were susceptible to harvest. Six plants from each treatment were taken and analyzed the following data: number of siliques, seed number per plant, total weight of seeds, weight of 100 seeds, after drying in an oven at 60°C for 48 h.

Until the beginning of drip irrigation, irrigation was done manually,

of 15 min per day in the whole area (2.5 x 2.5 m). Drip irrigation started from May 1.

To analyze the flow of water, measurements were made in each dripper, containing in each irrigation line 25 drippers. The flow rate was nearly constant in each dripper, with a value of 2.083×10^{-6} m^3 s^{-1}. Only in rainfall days were not performed irrigations.

The application of nitrogen (N) in the form of urea $(NH_2)_2CO$ in the growing area have been made in the proportion of 50 kilograms per hectare. Two applications were made during the experiment, the 1st on 06/05/2013 and the 2nd on 20/06/2013.

The experimental arrangement was completely randomized design, using five treatments and the witness, and removing the six most representative samples. The results were submitted to analysis of variance (ANOVA) and means compared by Tukey test, adopting the level 1-5% significance, with degree of freedom of the residue (GLR = 30) and using the statistical package version Assistat® 7.7 beta (Silva, 2014).

The equation that best characterized the production was polynomial regression 2nd degree, removing 1 m in treatments and adding to the 6 samples that had previously been taken for analysis, estimating through this, output per hectare.

RESULTS AND DISCUSSION

During the development of the golden flaxseed culture data were observed daily evaporation, the amount of rainfall and irrigation performed in different treatments in different months is represented in Table 1. The increase in rainfall results in a lower evapotranspiration and irrigation needs. Kobiyama and Vestena (2006) report that the potential evapotranspiration (ETP) annual average in Cascavel - PR was 1271 mm, with lower rates in the months of May, June and July, which differs from the results obtained due to the reduced number rains.

Abumanssur (2006) using data obtained by IAPAR in 25 meteorological stations located in the state of Parana for more than 17 years determined an average evapotranspiration in the region of 87 mm.mês, with 0.05 significance level, representing 57.85% below that obtained during the experiment.

After flowering, 6 samples of golden flaxseed were collected, treatments with (DCN) and without application of nitrogen (DSN) for analysis and are shown in Tables 2 and 3.

The difference of plant height (AP) between T1 and T5 treatments, where there was the highest rate, no nitrogen was 16.67%, which corroborates the results obtained by Bassegio et al. (2012), which found a water replacement value of 1.81 Evm and plant height 78 cm, this value increased to 6.37% obtained in the experiment.

Carvalho et al. (2006) reported increases in plant height with the increase in water availability. Santos et al. (2010) obtained maximum values for cultivation of *Jatropha curcas*, with replacement of 2.9 times. Taiz and Zager (2006) point out that fluid restriction can affect the metabolic processes of growth.

Werner et al. (2012) had greater plant height, in his experiments with linseed and different amounts of urea application of the rate of 50 $kg.ha^{-1}$, an amount used in the experiment. It was found that urea application resulted in an increase in height of only 2.92% of the plant, however, is combined with 1.5 Evm results in an increase of 36.95%.

The number of branches (NR) increased 23.04% with fluid replacement without nitrogen, between T1 and T3-T4. When added to fertilization, increased 215.21% in T3, which matches Biscaro et al. (2012), who found 1.5 ET in the cultivation of castor and Werner et al. (2012), which were obtained by culturing 50% flax increased by applying the urea 50 kg ha^{-1}.

The number of siliques (NS) is directly influenced by irrigation, an increase of 225.42% between T1 and T5, but if added to application of nitrogen, this percentage reaches 488.58% in T4, which is confirmed by Bassegio et al. (2012) and Werner et al. (2012).

Fresh mass of siliques (MFS) in the treatments without nitrogen increased from 1.38 to 3.92 g in T1 at T5, and applying nitrogen increased from 3.94 to 8.45g in T1 at T4, which is similar to the trend obtained on the siliques dry mass (MSS), consistent to Werner et al. (2012) and Bassegio et al. (2012), however, larger when treatments are combined.

An increase of 488.77% occurred between T4 and T1 with nitrogen and without nitrogen, to fresh mass of the plant (MFP). The same tendency occurred for the dry mass of the plant (MSP), which went from 1.2 g to 6.05 g, corroborating Bassegio et al. (2012) that by applying a polynomial model, achieved the highest response in 1.8 Evm to golden flaxseed.

The results show that the fluid resuscitation and fertilization improves plant response in its development. But with excess water is decreased due to the reduction in gas exchange between the plant and the environment, which reduces the supply of oxygen to the root system and limits breathing and nutrient absorption (Armstrong et al., 1994; Pardos, 2004).

To evaluate production were collected 6 plants per treatment and analyzed: number of siliques (NS), seeds (NSEM) and mass (M) and the weight of 100 seeds (M100).

These data for the different treatments applied on Golden flaxseed (DCN) and without nitrogen (DSN) are shown in Tables 4 and 5.

The number of siliques (NS) collected in production was on average for all treatments without application of nitrogen (DSN), 19.41% lower than in the samples taken after flowering, due to losses by climatic phenomena and animals that may fed. With application of nitrogen (DCN), the lost NS was even greater, with 30.66%.

Santos et al., (2013) studied the golden flaxseed, found between 40 and 80 siliques the harvest period, more responsive applying nitrogen between 200 and 300 kg ha^{-1}, while Viera et al. (2012) obtained value 53.17 most siliques applying 40 kg ha^{-1} of potassium (K).

The number of seeds (NSEM) of the treatments did not differ statistically analyzed. The T5 treatment without application of nitrogen showed an increased number of

Table 1. Evapotranspiration – ET, Precipitation - P and irrigation in treatments (mm.month^{-1}).

Date	ET	T1	T2	T3	T4	T5	T6	P
05/13	109.43	0	54.21	108.43	162.64	216.86	271.07	330.00
06/13	75.41	0	38.71	77.42	116.13	154.84	193.55	365.50
07/13	216.84	0	109.98	219.96	329.94	439.92	549.90	26.00
08/13	220.38	0	111.43	222.86	334.29	445.72	557.16	55.50
09/13	129.90	0	63.94	127.89	191.83	255.78	319.72	200.00

Table 2. Golden flaxseed after flowing without application of nitrogen (DSN).

Treatment	AP	NS	MFS	MSS	NR	MFP	MSP
T1	62.67^{b}	19^{b}	1.38^{c}	0.29^{b}	2.17^{a}	3.74^{c}	1.20^{b}
T2	63.33^{b}	22.67^{ab}	1.97^{bc}	0.54^{ab}	2.17^{a}	4.62^{bc}	1.4^{b}
T3	69.00^{ab}	36.50^{ab}	3.32^{ab}	0.84^{ab}	2.67^{a}	8.70^{a}	2.66^{a}
T4	69.17^{ab}	39.33^{ab}	2.66^{abc}	0.68^{ab}	2.67^{a}	8.01^{ab}	1.89^{ab}
T5	73.17^{a}	42.83^{a}	3.92^{a}	0.95^{a}	2.33^{a}	9.33^{a}	2.84^{a}
T6	70.33^{ab}	22.50^{ab}	1.92^{bc}	0.49^{ab}	2.33^{a}	6.12^{abc}	1.86^{ab}
F	3.28*	4.54**	5.39**	3.35*	0.62^{ns}	6.41**	6.60**
dms	9.77	20.65	1.78	0.568	1.24	3.88	1.10
CV%	8.19	38.61	40.03	51.24	29.60	32.69	31.81
GA	67.94	30.47	2.53	0.632	2.39	6.75	1.97

[a]Means followed by the same letter within each analyzed parameter (column), do not differ by Tukey test at 5% error probability. (**) = Significant at 1% probability, (*) = significant at the 5% probability, (NS) = not significant. CV% = coefficient of variation. dms = least significant difference. GA = general average. AP = plant height (cm), NS = number of siliques, MFS = fresh mass siliques (g), MSS = dry mass siliques (g) NR = number of branches, MFP = fresh mass of plants (g) MSP = dry mass of the plant (g), T1 (0%), T2 (50%), T3 (100%), T4 (150%), T5 (200%) and T6 (250%).

Table 3. Golden flaxseed after flowing with application of nitrogen (DCN).

Treatment	AP	NS	MFS	MSS	NR	MFP	MSP
T1	64.5^{c}	44.83^{a}	3.94^{a}	0.98^{a}	3.00^{ab}	8.50^{b}	3.02^{a}
T2	74.17^{b}	61.17^{a}	4.76^{a}	1.13^{a}	3.67^{ab}	10.94^{ab}	3.84^{a}
T3	78.17^{ab}	90.83^{a}	7.98^{a}	2.09^{a}	4.67^{a}	16.99^{ab}	5.78^{a}
T4	85.83^{a}	92.87^{a}	8.45^{a}	2.20^{ab}	3.67^{ab}	18.28^{a}	6.05^{a}
T5	82.67^{ab}	66.00^{a}	5.73^{a}	1.50^{a}	2.5^{b}	13.31^{ab}	4.37^{a}
T6	80.67^{ab}	54.67^{a}	4.85^{a}	1.18^{a}	2.83^{b}	12.02^{ab}	3.82^{a}
F	12.85**	2.55*	2.54*	1.72^{ns}	3.60*	3.23*	2.36^{ns}
dms	9.08	52.49	4.99	1.70	1.76	8.85	3.35
CV%	6.67	43.73	47.72	63.82	29.67	37.82	42.54
GA	77.67	68.39	5.95	1.51	3.39	13.34	4.48

[a]Means followed by the same letter within each analyzed parameter (column), do not differ by Tukey test at 5% error probability. (**) = Significant at 1% probability, (*) = significant at the 5% probability, (NS) = not significant. CV% = coefficient of variation. dms = least significant difference. GA = general average. AP = plant height (cm), NS = number of siliques, MFS = fresh mass siliques (g), MSS = dry mass siliques (g) NR = number of branches, MFP = fresh mass of plants (g) MSP = dry mass of the plant (g), T1 (0%), T2 (50%), T3 (100%), T4 (150%), T5 (200%) and T6 (250%)

seeds, while with application took place in T4. The average number of seeds, with and without nitrogen, was 6.03 and 7.01, respectively, and excels in 24.80% less than relates Morris (2007) maximum value.

The seed mass (M) increased 245.10 and 322.53% respectively between T1 and T5 treatments without nitrogen (DSN) and with nitrogen (DCN), which encourages an improvement 449.02% between planting

Table 4. Golden flaxseed production without application of nitrogen (DSN).

Treatment	NS	NSEM	M	M100	M1L
T1	16.33[b]	111.33[a]	0.51[a]	0.46[a]	19.49
T2	21.33[ab]	114.67[a]	0.61[bc]	0.53[a]	21.12
T3	34.5[ab]	192.67[a]	1.07[ab]	0.56[a]	25.66
T4	36.00[a]	191.00[a]	1.15[a]	0.55[a]	26.59
T5	37.67[a]	212.17[a]	1.25[a]	0.60[a]	27.47
T6	16.5[b]	123.50[a]	0.78[abc]	0.53[a]	25.61
F	5.09**	3.07*	6.32**	1.52[ns]	-
dms	19.19	112.25	0.52	0.16	-
CV%	40.40	40.59	33.10	16.69	-
GA	27.05	157.55	0.90	0.54	24.32

[a]Means followed by the same letter within each analyzed parameter (column), do not differ by Tukey test at 5% error probability. (**) = Significant at 1% probability (*) = significant at the 5% probability (NS) = not significant. CV% = coefficient of variation. dms = least significant difference. GA = general average. NS = number of siliques, NSEM = seed number, M = the mass of seeds (g) M100 = mass of 100 seeds (g) m1L= mass of 1 linear meter of planting (g). T1 (0%), T2 (50%), T3 (100%), T4 (150%), T5 (200%) and T6 (250%)

Table 5. Golden flaxseed production with nitrogen application (DCN).

Treatment	NS	NSEM	M	M100	M1L
T1	22.83[a]	146.67[a]	0.71[b]	0.49[a]	34.80
T2	39.67[a]	287.67[a]	1.49[ab]	0.54[a]	41.12
T3	44.67[a]	314.33[a]	1.67[ab]	0.55[a]	44.47
T4	61.50[a]	433.17[a]	2.22[ab]	0.35[a]	50.46
T5	61.33[a]	415.50[a]	2.29[a]	0.55[a]	48.87
T6	54.5[a]	409.83[a]	2.14[ab]	0.53[a]	47.13
F	2.16[ns]	2.55*	2.81*	0.52[ns]	-
dms	43.62	294.35	1.55	0.12	-
CV%	52.41	50.12	50.33	13.25	-
GA	47.42	334.53	1.76	0.53	44.47

[a]Means followed by the same letter within each analyzed parameter (column), do not differ by Tukey test at 5% error probability. (**) = Significant at 1% probability (*) = significant at the 5% probability (NS) = not significant. CV% = coefficient of variation. dms = least significant difference. GA = general average. NS = number of siliques, NSEM = seed number, M = the mass of seeds (g) M100 = mass of 100 seeds (g) m 1 L= mass of 1 linear meter of planting (g). T1 (0%), T2 (50%), T3 (100%), T4 (150%), T5 (200%) and T6 (250%).

without fluid replacement and application of urea (worst result) and adding the best result (T5) obtained.

Merging the results with those found by De Rossi et al. (2012) on population density in the same cultivar, which had the highest rates of fresh and dry mass of siliques with 100 plants per m^2, it leads up to a point great production.

Mass measurements of 100 seeds (M100) did not differ significantly between treatments, which are backed by Ambrosano (2012), which had the same answer. The same author states that the mass 100 flax seeds varies between 0.63 and 0.67 g, which differs from what was found, ranging between 0.35 and 0.63 g.

The mass data per hectare (Mha), with and without nitrogen, for the different treatments are shown in Figure 1, stipulated by the removal of 1 m and dried in an oven at 60°C for 48 h, using a 2nd degree polynomial regression that best relates the behavior of production.

Note that, an increase in productivity between T1 and T5 treatments at 40.94%, but in T6, there was a decrease due to water excess. The combined fertilization can raise productivity by 258.91%, between treatments with smaller and higher performance and reduces the water requirement because the summit took place in T4.

The average yield obtained flaxseed is 974 kg ha^{-1}, but can reach 1.5 tons ha^{-1}, confirming the results obtained, taking maximum values of up to 2 h^{-1} ton by optimization of resources used in cultivation. (Grant et al., 1999; IBGE, 2010; Oliveira et al, 2012.).

Santos et al. (2013) obtained from 600 to 1000 kg ha^{-1} for Golden flaxseed applying different amounts of nitrogen, but decreased slightly by applying excessive

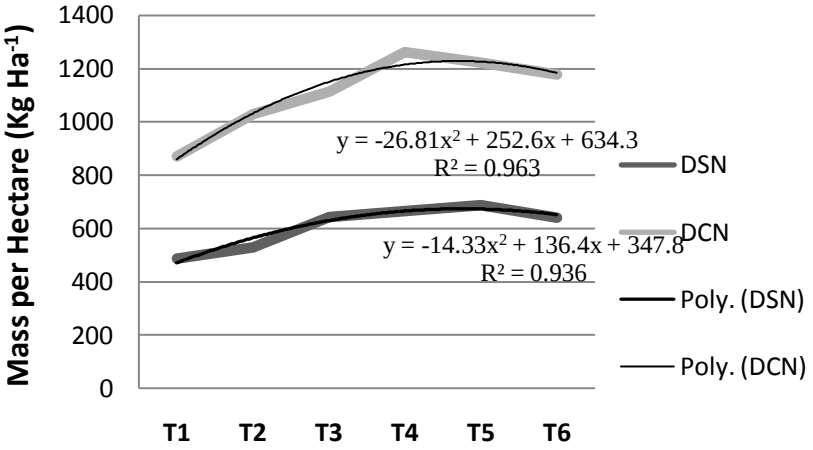

Figure 1. Mass per hectare in treatments T1 to T6, with (DCN) and without application of nitrogen (DSN), 0 (T1), 50 (T2), 100 (T3), 150 (T4), 200 (T5) and 250% (T6) of fluid replacement

amounts to 300 kg ha^{-1}, similar to the values obtained, but lower, due to the addition of irrigation.

Conclusions

By measuring the daily evapotranspiration, determined the amount of fluid replacement required for the culture of golden flax, which represented an increase of the phenotypic characteristics. However, the excess water leads to productivity losses. Allied irrigation, the application of urea $(NH_2)_2CO$ also resulted in an increase in the phenotypic characteristics.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Nutritional valorization of ginger lily forage (*Hedychium gardnerianum*, Sheppard ex Ker-Gawl) for animal feeding: Treatment with urea

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Fiber availability is not always ensured in all year long grazing systems. In this context, low quality fiber feeds may be of relevance for bovine feeding. We propose, thus, to study the effect of adding 5% of urea (on a dry matter basis) on the chemical composition and nutritional value of *Hedychium gardnerianum*, Sheppard ex Ker-Gawl (ginger lily or Kahili ginger), a traditional fiber source used by Azorean farmers. Treatments were: green ginger lily as a control, addition of 5% urea on a DM basis to green ginger lily, with treatments lasting 0, 5, 10, 15 and 30 days and addition of 5% urea on a DM basis to previously dried ginger lily, at day 0. The treatment afforded a significant (P<0.05) increase in crude protein, while maintaining the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents, and a variation in the acid detergent lignin (ADL) content. No significant improvement in *in vitro* dry matter digestibility. The treatment with urea of the green forage does not have the same effect as in other low quality fiber feeds, namely straw, since ginger lily has much higher a Crude Protein value than those other fibrous feeds.

Key words: *Hedychium gardnerianum, in vitro* digestibility, urea treatment, roughage.

INTRODUCTION

Livestock production under grazing regimes is confronted often with periods of fiber shortages, which are motivated by normal production curves of pasture or climate change that have significant influences in grass production. Current production concepts, using a minimum of concentrates while maximizing available food and low digestibility of fibrous feedstuffs gained some importance in the diet of ruminants.

However, and due to the low feeding value of these foods together with low voluntary intakes combined to low digestibility, they fail to meet the maintenance needs of ruminants. In order to improve the nutritional value of low-quality fibrous food, various treatments have been proposed: physical, biological and chemical (Jarrige, 1987).

The presence of ruminants in the Azores archipelago

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dates back to European settlement (with cattle, sheep and goats). However, it was for the purpose of the known cultural and economic cycle called the "industrial crops" in the late nineteenth century and especially in the second half of the twentieth century, that cattle farming, particularly dairy, has had a major expansion, making it the dominant economic activity in the archipelago.

For centuries, semi-natural grasslands (implanted after cutting the primeval forests and with a mixture of native and exotic plants) were the basis of the Azorean animal husbandry. Installed in acidic soils, these were composed essentially of grasses, including *Holcus mollis* L. and *Anthoxanthum odoratum* L. (Davies, 1962).

In a traditional grazing system in which milk production accompanied the grass production cycle, good management of pastures represented a gain for farm's profitability. In general, these pastures are located in mid/high altitudes, being therefore subject to the action of winds, high rainfall and low temperatures, which favors a main grass production period in spring with two clear periods of scarcity in both summer (particularly August and September) and winter (November through February). On the islands with the lowest average altitudes, and/or in the lowlands, there is a single famine period of forages – summer - which is nevertheless quite long (Borba, 2007). In periods of fodder shortage alternatives are common in some of the islands. These include mainly *Pittosporum undulatum* Vent. (Incense), *Hedychium gardnerianum* Sheppard ex Ker-Gawl. (ginger lily), *Morellia faya* Aiton (firetree) and *Ilex perado* Aiton ssp. *azorica* (Loes.) Tutin (Holly) (Borba, 2007). In general, the cows were fed during the winter with poor food, which does not allow them to cope with calves growth and restore the necessary weight to prepare for the next lactation, resulting in low milk yields which are then limited to just a few months.

H. gardnerianum Sheppard ex Ker-Gawler is a rhizomatous perennial herb of the Zingiberaceae family, it is known as the ginger lile. It has a stalk which can extend up to 2 m long, with oblong leaves reaching 30 cm and several yellow-orange flowers in a spike of 20 to 30 cm in length. It is an aggressive invasive weed capable of spreading rapidly and dominating large areas in the Azores (Portugal). Moreover, *H. gardnerianum* out-competes many native plants and has become a significant threat to the survival of many of them (Sjögren, 1984).

A fiber deficiency has demanded for Azorean farmers to import raw materials that could however be produced within the region. Traditional fiber sources used by Azorean farmers gain thus a new importance which requires urgent studies of its feasibility both in production and nutritious value. Ginger lily, an unconventional forage of traditional use in the Azores has a low nutritional value. Among the available treatments to increase the nutritional value, urea treatment was selected due to its cheap price and availability. Furthermore, it attacks the cell wall and provides nitrogen enrichment, making it available to use in the protein synthesis by the rumen microbiota. The urea treatment was carried out with the aim of improving the nutritional value of the ginger lily, like for other roughages.

MATERIALS AND METHODS

Forage collection and preparation

The current study was conducted in the Animal Nutrition Lab, Department of Agricultural Sciences, University of the Azores, located in Angra do Heroísmo, Terceira, Azores, Portugal. The whole-plant (leaves and pseudo stems) was manually harvested at the beginning of the flowering stage (April, 2014) about 15 cm above the soil, in Mata das Veredas (295 m altitude), Terra Chã, Municipality of Angra do Heroísmo. The forage was chopped using a laboratory type chopper at length of 2 to 3 cm.

Experimental design

The authors studied the effect of treatment time and form on the nutritional value of ginger lily. For this purpose, the following treatments were performed (in triplicate):

(C) Ginger lily control,
(U) Ginger lily treated with 5% urea (DM basis).

The ginger lily treatment duration times were 0 (U0), 5 (U5), 10 (U10) and 15 (U-15) days.

The treatment was performed by spraying the samples with a urea solution. For each treatment, 3 kg sample were placed in a plastic box with lid. All treatment was made in green ginger lily. After treatment, the samples were dried in a forced air oven at 65°C for 72 h.

Chemical analysis

Dried samples were then ground through 1 mm screen using a Retsch mill (GmbH, 5657 HAAN, Germany). Ground samples were analyzed for dry matter (DM, method 930.15), crude protein (CP, method 954.01) and total ash method (942.05) according to the standard methods of AOAC (1995). Briefly, the dry matter content of forage was determined by placing samples in a forced air oven at 105°C for 24 h. Total ash was evaluated by igniting samples in a muffle furnace at 600°C for 12 h. Crude protein was determined by standard micro-Kjeldahl method using digestion equipment (Kjeldatherm System KT 40, Gerhart Laboratory Instruments, Bonn, Germany) and an automated Kjeltec 2300 Auto-analyzer apparatus for distillation and titration (Foss Electric, Copenhagen, Denmark). Where, neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to Goering and Van Soest (1970). Both NDF and ADF were expressed without residual ash. *In vitro* digestibility was determined by (Tilley and Terry, 1963) method and modified by Alexander and McGowan (1966).

Statistical treatment

ANOVA was performed, followed by the Scheffe multiple comparison test whenever significant ($p<0.05$) were detected.

RESULTS AND DISCUSSION

The *H. gardnerianum* is a poor forage with low digestibility *in vivo* and low dry matter intake, measured in sheep (Borba, 1991). The tests carried out by the authors are used to select a method that allows a nutritional valorization of this fodder, which is a major source of fiber in cattle's feed in some Azorean islands. From the methods used, the authors chose the treatment with urea, together with the chemical treatment carried out, allows an enrichment of forage's nitrogen levels.

Much has been speculated about the mode of action of ammonia on the straw. It is accepted that reticulum-rumen's bacteria attack the free cellulose by cellulases, but are not able to break the connection lignin-cellulose. Thus, the ammonia will have on the cell walls an action which results in the rupture of the xylan chains and a physical action whose effect is an increase in the capacity of absorption of water. Since the cell wall carbohydrates and lignin account for over 70% of the organic matter of straw, those combined effects lead to increased solubility of the organic matter in the reticulum-rumen, therefore, its availability for microbial fermentation, increasing the nutritional value (Borba, 2006).

The ammonification of low quality forages results in an increased intake (20 to 40%) due to the decrease in rumen retention time and increased rate of passage (Balch and Champling, 1965; Thornton and Minson, 1973; Ogi et al., 1979). The increasing digestibility of dry matter, organic matter and cellulose are generally small or even zero in medium quality forage. The increase in digestibility of organic matter resulting from the ammonification, due to the increased digestibility of ADF and NDF fraction by breaking the links between hemicellulose and lignin (Ogi et al., 1979). According Fadel et al. (2004), the rice straw ammonification translates into an increase of digestibility of dry matter of 55.16 to 62.12%, which may be due to increased hydration rate of the treated straw and / or the effect on flexibility, solubility and fragility of treated straw.

The authors' treatments with urea used a concentration of 5% of the dry matter. Ideal urea application rates are arguable. Among the concerns urea utilization raises are its toxicity (Brandini, 1996) and its influence on rumen motility (Goularte et al., 2010). It is widely accepted that the optimal application rates are between 4 and 6 kg urea per 100 kg DM. The most used application rate is 5 kg (Schiere and Ibrahim, 1989). However, Quashie (2014) states that for the treatment of rice straws, the optimum concentration of urea is 6.5% DM, in a treatment of 21 days at a humidity of 40%. Carvalho et al. (2006) concluded that when treating the sugarcane with increased levels of urea, the minimum level of PB that provided a rumen function was 7%, obtained with the addition of 2.62% urea. But also they found that levels of 5.0 and 7.5% urea (9.91 and 12,985 respectively) have contributed to the growth of microbial population and thus to a better feed efficiency.

After data analysis, concerning dry matter values (Figure 1), there is a statistically significant decrease ($P<0.05$) of dry matter content with the treatment: urea 5 days of treatment (U5) to urea 10 days of treatment (U10) and 15 days of treatment (U15) as compared to the control (C). Similar results were found by other authors, including Kohdaparast et al. (2011), Wanapat et al. (2013) and Hassan et al. (2011). For the zero hour treatment level (U0) there were no significant differences ($P>0.05$) in the dry matter content, which also is pointed out by different authors (Oluokun, 2005; Aregawi et al., 2013) and even Akraim et al. (2013) reported an increase in dry matter of barley straw treated with urea. t refer to the appearance of molds to a greater or lesser extent in prolonged treatments.

The crude protein shows an increase with the addition of urea (Figure 2), and it was found that this treatment is greatest in U0, similar results were found by Moselhy et al. (2015). The explanation for this in our opinion is due to the fact of not having been a ureolysis so intense, since there was no time treatment, the sample was dried over urea immediately after addition. Long treatments with urea originate proteolysis, which leads to a loss of nitrogen in ammoniacal form by evaporation.

The increase in the content of crude protein in samples treated with urea is mentioned by several authors being one of the advantages of this method of chemical treatments in relation to others, such as treatment with sodium hydroxide (Sirohi and Rai, 1999; Oluokun, 2005; Kohdaparast et al., 2011; Akraim et al., 2013; Aregawi et al., 2013).

Treatments with other fibrous forages including straw were carried out in a sealed environment to prevent volatilization of the ammonia. In our case, the boxes where the treatments were performed were not airtight, making gas exchange with the exterior possible and ammonia volatilization may thus have occurred.

Regarding fiber in particular NDF and ADF values (Figure 3 and 4), in general, there was not a noticeable action of urea, Oluokun (2005) also did not found changes on fiber while Kohdaparast et al. (2011) disclose variations of NDF from 79.2 to 78.8% and of ADF from 58.8 to 60.6%, in canola straw treated with 4% urea. Other authors have reported significant decreases in the two fractions is the case of Hassan et al. (2011), Aregawi et al. (2013) and Akraim et al. (2013).

With regard to the ADL values (Figure 5), they revealed a significant increase ($P<0.05$) in U0 and U5 treatments compared to control. Aregawi et al. (2013), report a non-significant decrease in ADL and Reis et al. (2001) reported significant decreases ($P<0.05$) ADL, in hay treated with urea in a 5.4% DM.

Figures 6 and 7 refer to cellulose and hemicellulose respectively, the authors can see a trend of decreased cellulose, relative to the control, although this is not

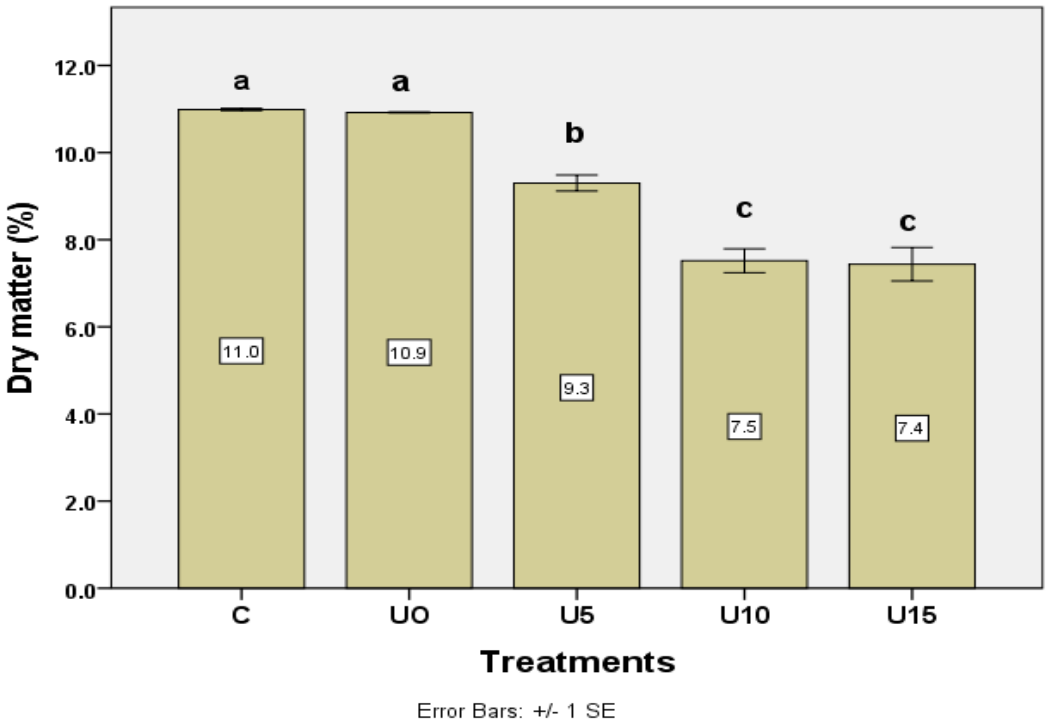

Figure 1. Effect of treatment on the average dry matter content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

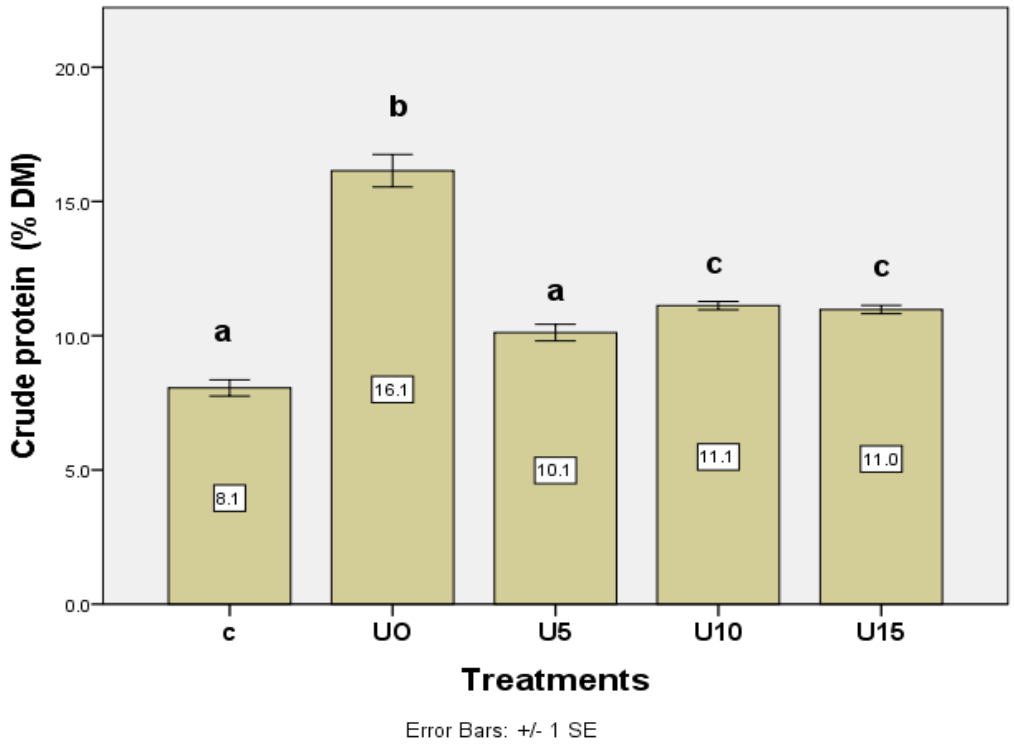

Figure 2. Effect of treatment on the average Crude Protein content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

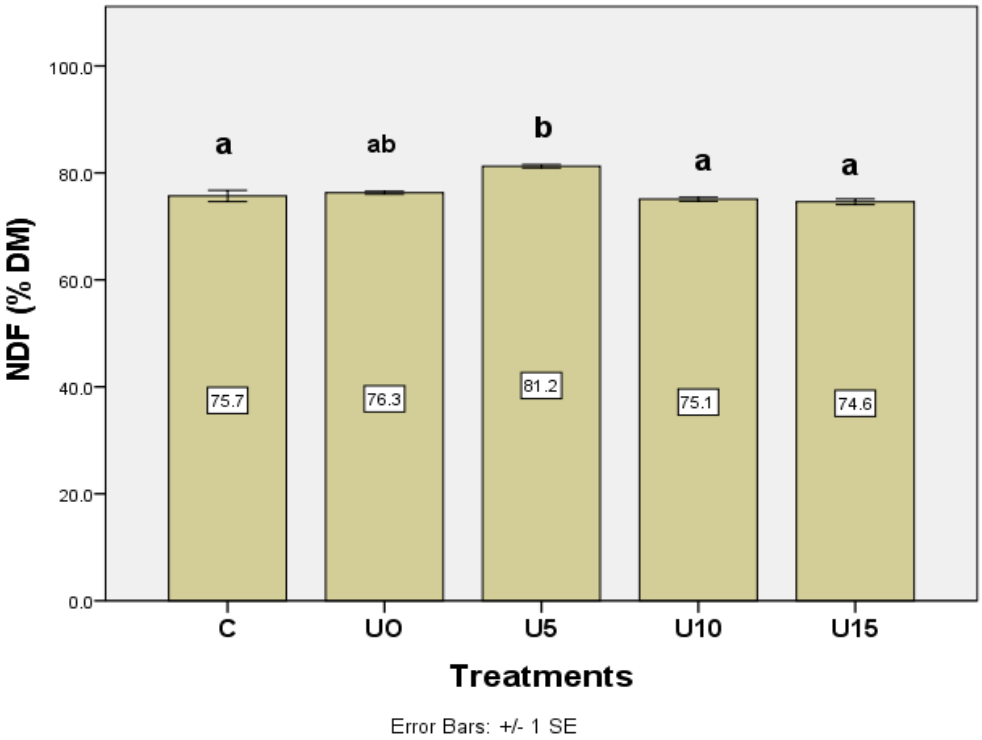

Figure 3. Effect of treatment on the average NDF content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

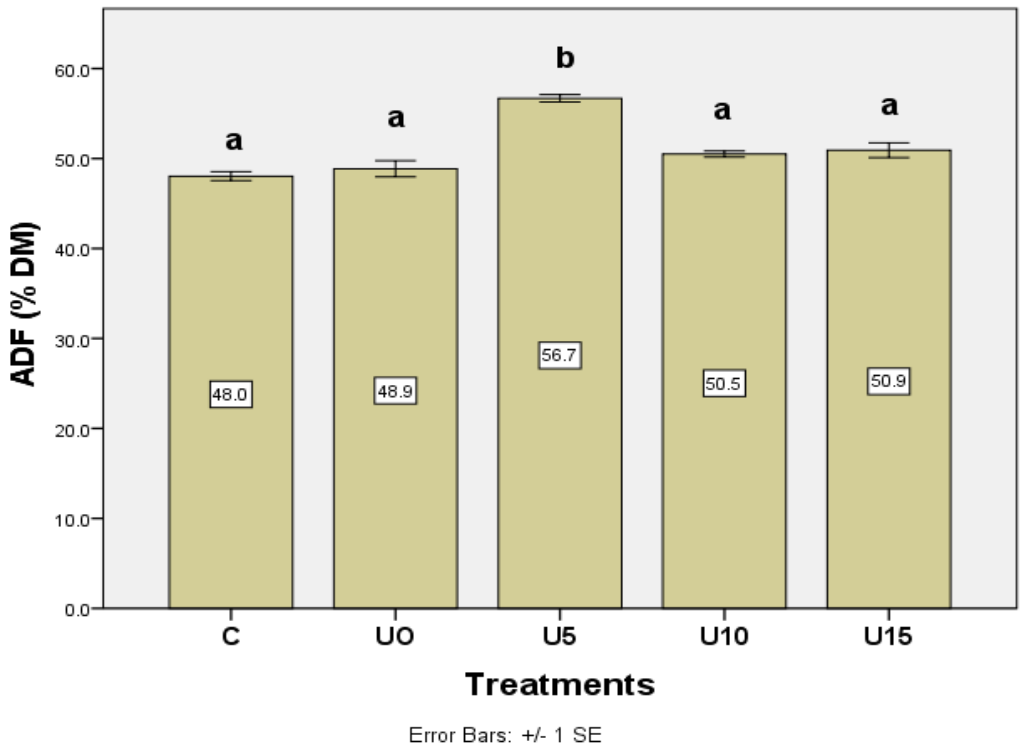

Figure 4. Effect of treatment on the average ADF content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

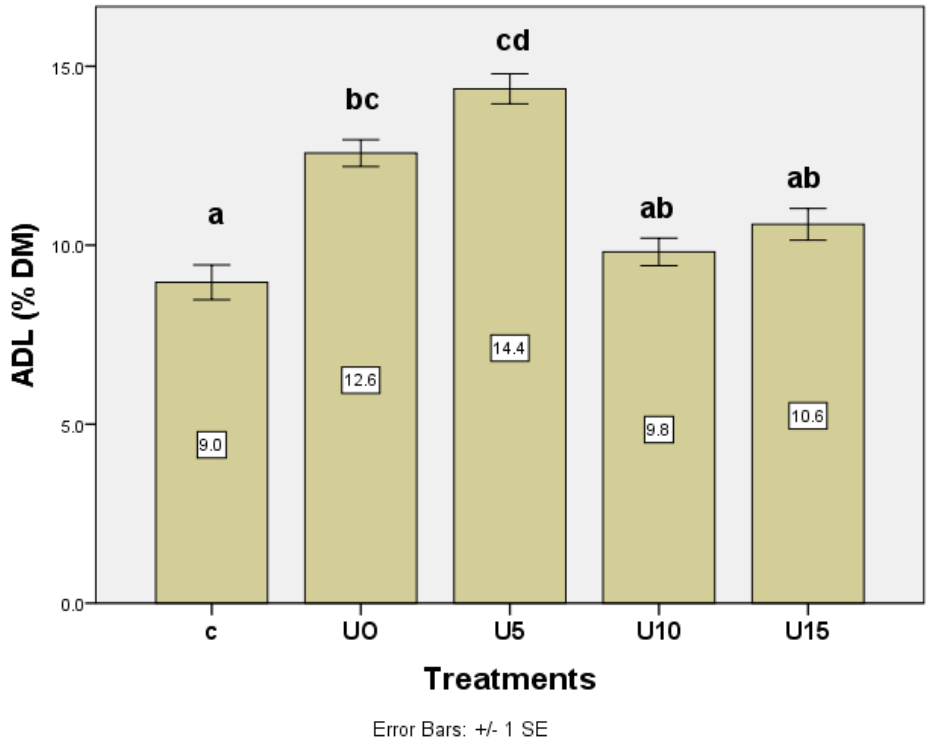

Figure 5. Effect of treatment on the average ADL content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

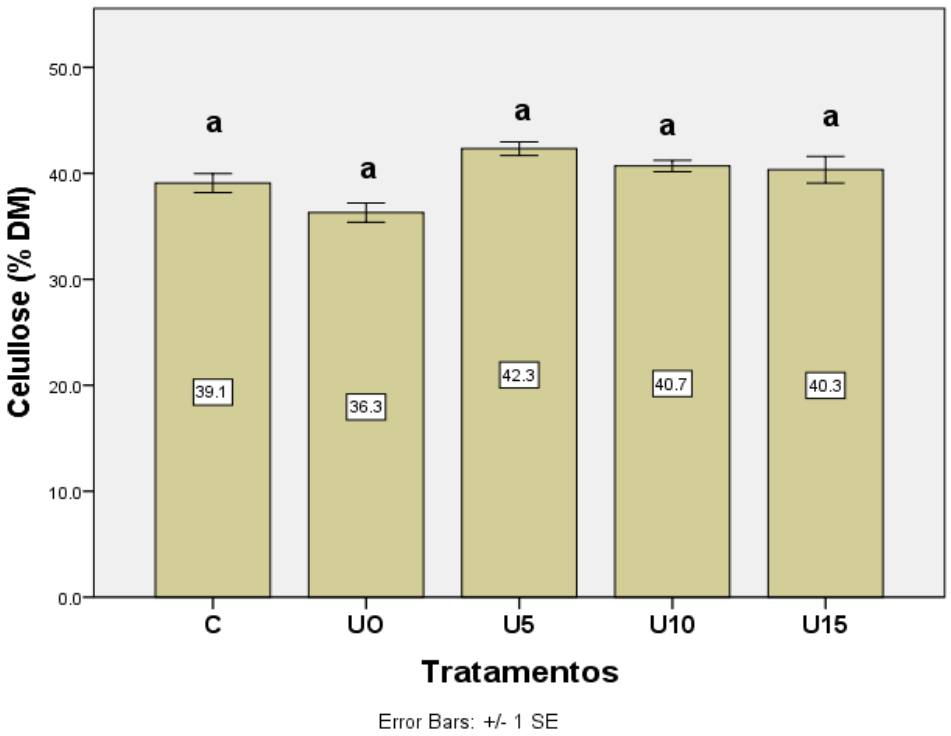

Figure 6. Effect of treatment on the average cellulose content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

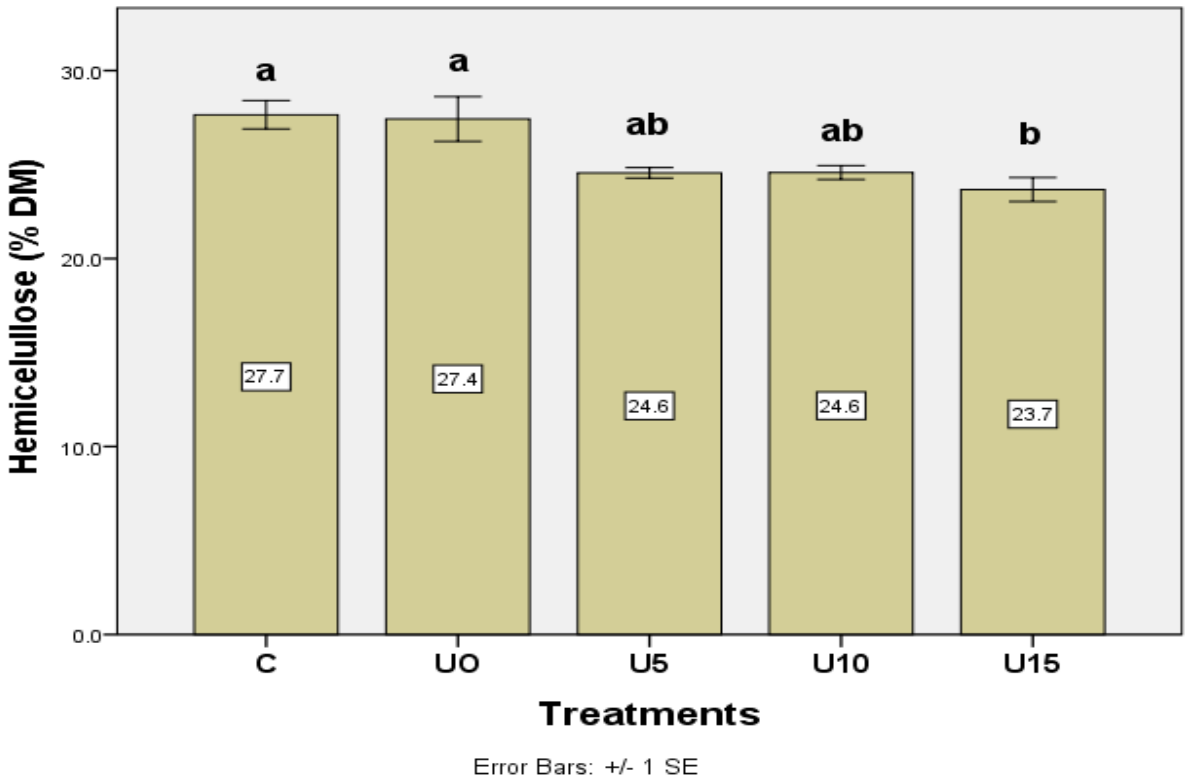

Figure 7. Effect of treatment on the average Hemicellulose content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

statistically significant. The same tendency is observed for hemicellulose, most relevant in the sample treatment U15, the one that is statistically (P<0.05) different from control. Aregawi et al. (2013) who observed a significant (P<0.01) decrease in the hemicellulose and cellulose on their treatments of sesame straw. Reis et al. (2001) reported significant decreases (P<0.05) from the hemicellulose but not observe any effect of the treatment on the content of the hay cellulose and lignin.

In Figure 8, the Ash values of U10 and U15 treatments showed a statistically different percentage (P<0.05) increase in the treatment with urea when compared to U5 treatment. These results are in agreement with report by some authors such as Oluokun (2005), Hassan et al. (2011) and Akraim et al. (2013). However, Kohdaparast et al. (2011) observed maintaining the Ash values of canola straw treated with 4% urea.

As regards to digestibility of dry matter (Figure 9), a significant difference (P<0.05) between the U0 and U5 treatments may be explained by the content of crude protein, as indicated by Jarrige (1987), which argues the complement of fibrous foods with nitrogen increases the nitrogen constituents degraded in the reticulum-rumen, which provides a nitrogen source for microbial population, thus increasing their activity. This trend is also observed for the digestibility of organic matter (Figure 10) in a more relevant way.

Treatment with ammonia will result in additional enrichment in non-protein nitrogen of importance for the reticulum-rumen microbiota in feeds with low nitrogen content such as straw. Treatment with ammonia almost doubles the nitrogen content in straw. However, other authors mention that the effects of urea treatment are more visible when the forage has crude protein content below 7% (Lazzarini et al., 2009). Below this level, the microbial activity in the rumen is severely limited. For this reason, the treatment with urea results in straws in a significant increase in digestibility. Wanapat et al. (2013), observed a significant increase (P<0.01) in Dry Matter digestibility when treating rice straw (2.7% CP on a DM basis) with 3% urea on a DM basis. Hassan et al. (2011) describe a significant increase in DM digestibility (P<0.05) in wheat straw fermented with 4% urea and 4% molasses, with an initial CP value of 2.90 and a final value of 15.18. Sirohi and Rai (1999) observed synergy between urea and lime (powder) in the treatment of wheat straw, which efficiently increased the content of CP, the *in vitro* and *in sacco* digestibility of DM and OM, in an optimal treatment concentration of 4% urea and 4% lime. Aregawi et al. (2013) indicate an increase from 34.8 to 43.5% when treating sesame straw with 4% urea.

Urea treatment is regarded in general as an effective treatment method for low quality fiber foods such as straw and thus upgraded their nutritional value. As

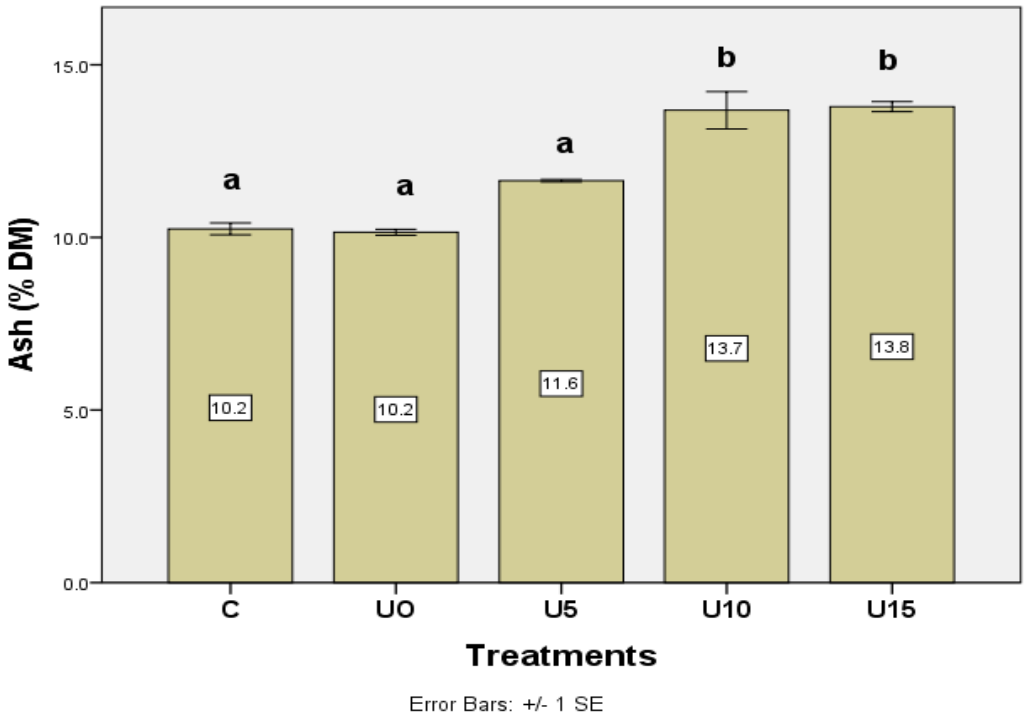

Figure 8. Effect of treatment on the average Ash content of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

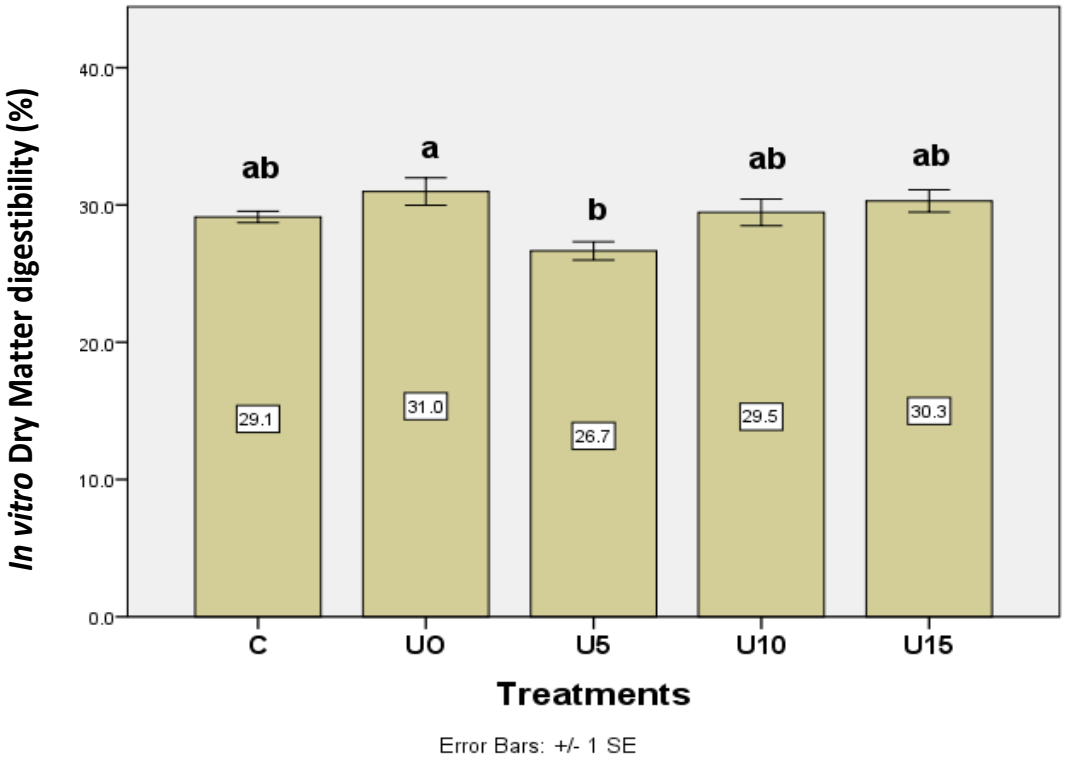

Figure 9. Effect of treatment on the average *in vitro* digestibility of dry matter of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

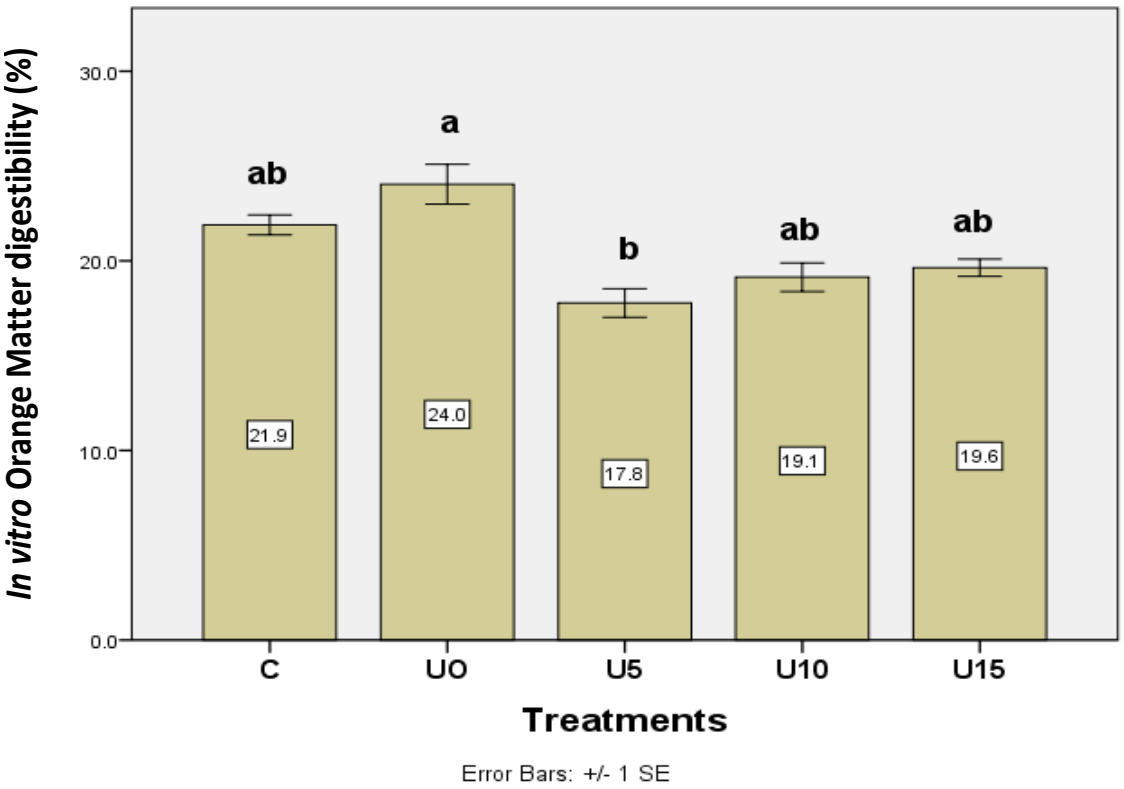

Figure 10. Effect of treatment on the average *in vitro* digestibility of organic matter of *Hedychium gardnerianum* forage. Averages that display the same index are not significantly (P<0.05) different. Error bars represent the standard error.

mentioned in Jabbar et al. (2009), different variants have been used in this treatment with the aim of simplifying their usage by farmers. Polyorach and Wanapat (2014) concluded that this is a simple, affordable method for the nutritional valorization of straw in an assay that involved treatment with urea and calcium hydroxide.

In a comparative study on the effect of various treatments (anhydrous ammonia, urea, sodium hydroxide and calcium hydroxide) on the nutritional value of roughage, Pires et al. (2010) concluded that sodium hydroxide and calcium hydroxide show a higher efficiency on reducing the content in cell wall materials and increasing the digestibility of the treated roughage than anhydrous ammonia and urea.

The crude protein content of ginger lily is above 7% level required for the normal functioning of the rumen microbiota, as reported by Lazzarini et al. (2009). In our understanding, this accounts for the low effect of urea addition on its nutritional value.

In the present assay, urea treatment was carried out on green forage with high water content, a CP content of 8.05% DM in non-hermetic plastic containers according with the methodology of Quashie (2014). Treatments with sodium hydroxide shall also be performed in the future to assess whether treating with this alkali would afford a greater efficiency in the degradation of *H. gardnerianum* cell wall.

Conclusions

H. gardnerianum is poor and low nutritional level forage. To allow for its use as a fiber feed for ruminants, it is important to find a simple method to improve its nutritional value. When used in green forage, urea treatment does not have the same effect as when it is applied to low-quality fiber feeds, such as straw, since ginger lily has a much higher Crude Protein value than straw. This is possibly the main conclusion that can be drawn from this present work.

New treatments should be envisaged using dried ginger lily, not only by applying urea, but also sodium hydroxide, which should have a more pronounced effect on the degradation of the cell wall of this forage, thus improving its nutritional value.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Heat treatment to overcome seeds dormancy of *Panicum maximum* cultivars (Poaceae)

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The guinea grass *Panicum maximum*, an African grass, is one of the main forage grasses in tropical America. Its propagation is mainly carried out by seeds, but seed dormancy hampers good pasture establishment. The aim of this study was to evaluate the use of heat treatment to overcome seed dormancy of *P. maximum* cultivars Milênio, Tanzânia and Mombaça. Seeds of each cultivar have been subjected to heat treatments at temperatures of 50, 60 and 70°C with exposure time for 5, 10 and 15 h. Afterwards, they were placed in germination test. The data were subjected to analysis of variance and, when significant, polynomial regression was performed, with up to 5% of probability. The variables analyzed were germination percentage, germination speed index (GSI) and average germination time (AGT). *P. maximum* seeds of different cultivars showed distinct responses to heat treatment. The cultivar Tanzânia responds positively to different combinations of temperature and periods of seeds exposure, and the treatment at 70°C for about 8 h is recommended to overcome dormancy with better germination performance. For cultivar Milênio, it is recommended the exposure of seeds at 70°C for 15 h. Cultivar Mombaça is negatively influenced by the heat treatment, so this treatment is not recommended.

Key words: African grasses, germination, pasture, temperature.

INTRODUCTION

The guinea grass (*Panicum maximum* Jacq.), originating in Africa, is one of the main forage grasses in tropical America, with great importance in pasture establishment. It presents good range of adaptation to tropical and subtropical conditions, high forage production of good quality, good resistance to grazing, being well accepted by animals. Its propagation is mainly carried out by seeds (Dias and Alves, 2008). The use of quality seeds with high seed germination is essential to a good pasture establishment, with high technological level (Chiodini and Cruz-Silva, 2013). However, *P. maximum*, and most forage species have low seed germination, which may be

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related to the presence of seed dormancy (Martins and Silva, 2003).

Dormant seeds are those which, although viable, do not germinate in a specific period of time under any combination of normal physical environmental factors (temperature, light/dark, etc.) which, on the other hand, is favorable to its germination, that is, after the seed becomes non-dormant (Baskin and Baskin, 2004). This mechanism is one of the main strategies of plant species to increase their survival rates and seedling establishment, because it allows germination to occur only when it is more likely that the conditions for seedling establishment are appropriate (Finch-Savage and Leubner-Metzger, 2006). However, dormancy affects emergence speed of plants in the field, resulting in an irregular stand, which slows pasture establishment and favors the appearance of invasive plants (Martins and Silva, 2003; Almeida and Silva, 2004; Lacerda et al., 2010).

Dormancy expression in tropical grasses is associated with physiological causes (present in freshly harvested seeds and suppressed during storage) or physical causes (related to restrictions imposed by the seed coat to oxygen and water uptake by seeds) (Whiteman and Mendra, 1982). However, the current state of knowledge is unsafe to indicate procedure settings that are capable of preventing the expression of dormancy on pasture establishment. Several studies have shown the efficiency of the chemical treatment use, with immersion of seeds in sulfuric acid for different time periods, in order to overcome seed dormancy of forage grasses species such as *P. maximum* (Smith, 1979; Martins and Silva, 1998), *Brachiaria brizantha* (Garcia and Cicero, 1992; Montório et al., 1997; Lago and Martins, 1998; Munhoz et al., 2009), and *B. dyctioneura* (Almeida and Silva, 2004). However, this method presents technical and operational risks to the environment (Almeida and Silva, 2004; Martins and Silva, 2006) and it is of difficult adaptation to seeds which, because of structural features, are easily damaged by the process (Martins and Silva, 1998). Thus, considering risk reduction, heat treatment to overcome dormancy has been presented as an alternative.

Heat treatment can be applied via moist heat (hot water or steam) or dry heat. This last one has a lower heat capacity and heat exchange than the wet one, requiring therefore longer exposure time (Lazarotto et al., 2013). However, further studies are needed to better define the binomial temperature/time for each species for the use of the dry heat as a method to overcome seed dormancy (Alencar et al., 2009). This study evaluated the use of heat treatment to overcome seeds dormancy of *P. maximum* cultivars Tanzania, Mombaça and Milênio, preserving their germination and vigor.

MATERIALS AND METHODS

This study was carried out at Embrapa Beef Cattle, Campo Grande(MS), Brazil. Three *P. maximum* cultivar seeds have been used (Tanzânia, Mombaça and Milênio), just after harvest. After harvesting and processing, the seeds of each cultivar were placed in beakers and subjected to heat treatments at temperatures of 50, 60 and 70°C, with time exposure for 5, 10 and 15 h in a kiln with forced air circulation. The zero time, without application of heat, was considered as control. For each treatment combination (temperature and exposure time) 1.0 g of seed of each cultivar was used. Just after the heat treatment, the seeds were put to germinate.

A completely randomized design was used. For each treatment (combination of temperature and time exposure), four repetitions of 100 seeds were used, put in plastic boxes (gerbox) with germitest paper, previously moistened with distilled water (2.5 times the weight of germitest paper). The seeds were then incubated in germination chamber Biochemical Oxygen Demand (BOD) with photoperiod of 8 hours and alternated temperatures of 15/35°C during 28 days, according to the Seed Analysis Rules (Brazil, 2009). Evaluations of germinated seeds were carried out daily. The variables evaluated were:

Germination (%): Considering as germinated seeds the ones which presented at least 2.0 mm of seminal root (Juntila, 1976).

Average germination time – AGT (days): Estimated by the formula of Krzyzanowski et al. (1999): $AGT = (\Sigma n_i t_i)/ \Sigma ni$, where: n = number of germinated seeds per day and t_i = incubation time (days).

Germination speed index – GSI: determined according to the formula of Maguire (1962), GSI = Σ(n/t), where: n= number of germinated seeds in the computed first, second, …, and last count; t= number of days from sowing to first, second, …, and last count.

At the final count tests, the seeds remaining in the substrate and not germinated were tightened with a pincer. The ones with the seed coat softened, which broke with the pressure were considered dead.

The data (germination percentage, germination speed index and average germination time) were subjected to analysis of variance and, when significant, polynomial regression was performed (Pimentel Gomes, 2009), with up to 5% of probability. Germination percentage data were transformed into arcsine $\sqrt{x/100}$ (Sokal and Rohlf, 1995).

RESULTS

Heat treatment influenced the seed germination of the three *P. maximum* cultivars evaluated. For cultivar Tanzânia, all evaluated temperatures showed quadratic adjustments between the exposure times to treatment and the variables analyzed. Except to 70°C/15 h treatment, all other treatments enhanced germination of this cultivar as it provided an increase in the percentage of germination, AGT reduction and increased GSI compared to control (Figure 1). The higher germination percentage (54.4%) was obtained in the treatment at 70°C/7.56 h, representing an increase of almost nine times the germination obtained in the control (6.25%). Treatments with temperatures 50 and 60°C showed similar germination maximum point (49.6%), and, thus, lower percentage than in treatment at 70°C (Figure 1A). However, these two treatments (50 and 60°C) showed better results for vigor than 70°C treatment, once they had lower AGTs (Figure 1B) and higher GSIs (Figure 1C).

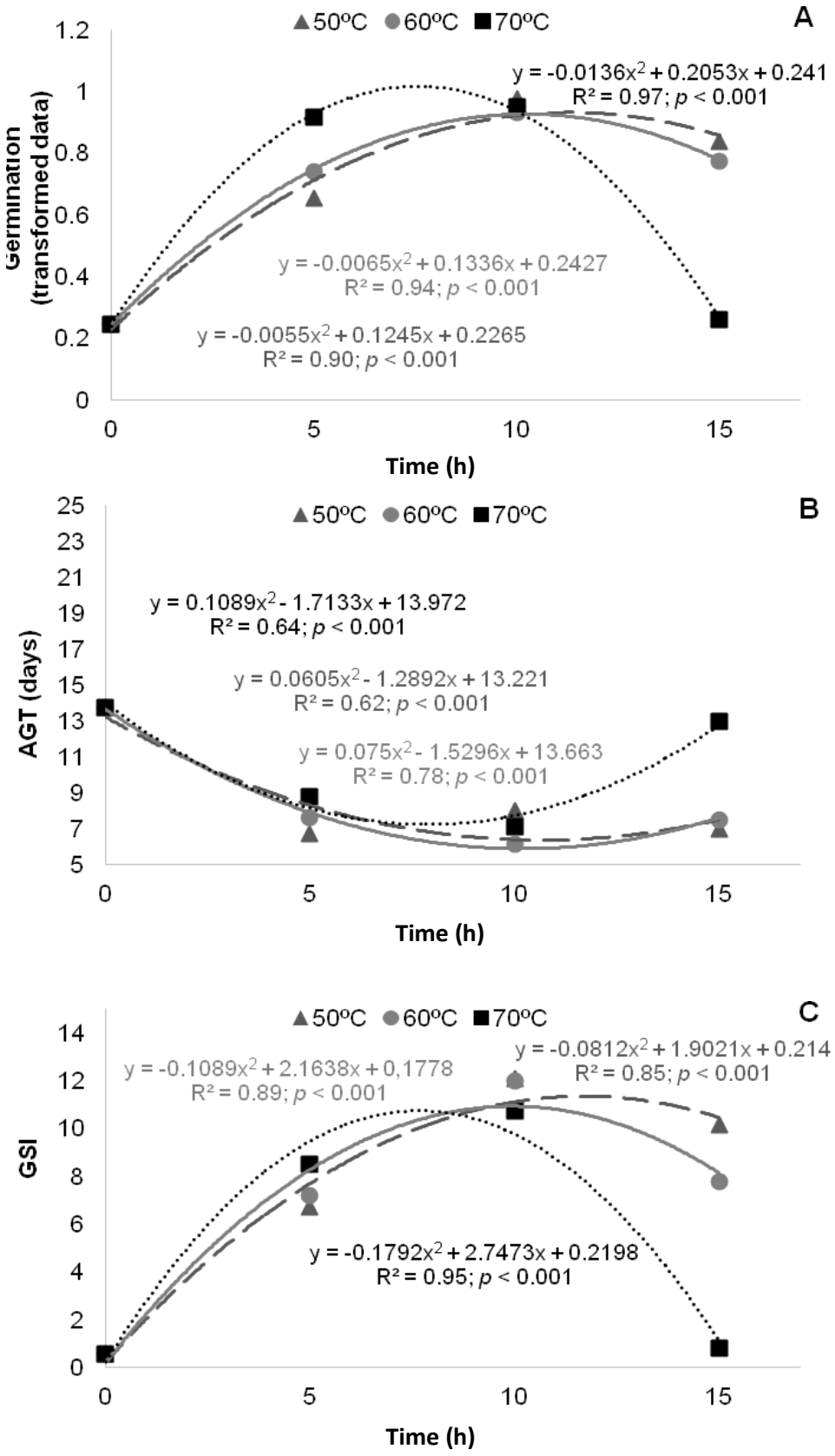

Figure 1. Germination (A), Average germination time (B) and Germination speed index (C) of *P. maximum* seeds cultivar. Tanzânia treated with temperatures (50, 60 and 70°C), by different exposure times (0, 5, 10 and 15 h).

For Milênio cultivar, only the treatment at 70°C resulted in response of all variables (Figure 2). At this temperature, a linear increase of germination percentage was obtained with increasing treatment time, and the exposure of the seeds for 15 h at this temperature resulted in an increase of about 3.2 times in germination (Figure 2A). Furthermore, treatment at 70°C/15 h resulted also in maximal reduction AGT (Figure 2B) and increased GSI (Figure 2C), indicating that this treatment positively influenced the seed vigor. Although the treatment at 50°C has shown a tendency to reduce AGT with increasing exposure time of seeds (Figure 2B), the low regression adjustment found ($R^2 = 0.30$), combined with the lack of response of this temperature for the other variables suggest the ineffectiveness of this treatment to overcome seed dormancy. Treatment at 60°C was not effective in overcoming seed dormancy of Milênio cultivar either (Figure 2).

The Mombaça cultivar, in general, was negatively affected by the heat treatment for overcoming seeds dormancy (Figure 3), resulting in reduced germination (to 50 and 60°C) (Figure 3A), increase in AGT (Figure 3B) and reduction of GSI for all temperatures evaluated (Figure 3C). Despite the fact that treatment at 70°C has resulted in a linear increase of germination in relation to the exposure time (Figure 3A), the detrimental effect in terms of seed vigor (increase of AGT and reduced GSI) suggests that it should not be used.

DISCUSSION

The study of alternatives to overcome fodder seed dormancy can contribute to the development of methods capable of industrial application, enabling their commercialization with dormancy partial or totally eliminated (Martins and Silva, 2003). The most indicated method would be the one which requires less time and economic investment, coupled with its easy handling in laboratory routines. Since dormancy overcoming treatments should simulate the environmental conditions in which the seeds are subjected in their natural habitat, tropical species tend to respond well to methods which use heat exposure (Garcia and Baseggio, 1999). Specifically in the case of grasses such as *P*. *maximum*, the seeds after releasing the parent plant are located predominantly on the soil surface, being exposed to soil action and fire (natural or artificial). Thus, these species have been selected as expression of survival and adaptation mechanisms at high temperatures (Martins and Silva, 1998).

In one of the few studies reported in the literature about the application of heat treatment in *P. maximum* seeds, Martins and Silva (1998) submitted seeds of the species to temperatures of 40, 55, 70 and 85°C for 5, 10 and 15 h. The results showed that exposure of the seeds to 40°C for 5 and 15 h were more favorable treatments because they resulted in overcoming seed dormancy and caused less damage during storage. Higher temperatures, despite being able to reduce the percentage of dormant seeds had latent deleterious effects on the storage.

The decrease in *P. maximum* seed dormancy rate during storage is a characteristic variable with the lot, harvest season and the genotype (Smith, 1979; Harty et al., 1983; Condé and Garcia, 1985). If this variation among genotypes of the species is expected in the natural seeds dormancy overcoming, it may be possible that it can be also found when using artificial treatments to overcome it. The study of Martins and Silva (1998) makes no reference to which cultivar was used and the present study found significant variation in the response to heat treatment between different cultivars of *P. maximum*. Cultivars Tanzania and Milênio had better germination response (percentage and speed) to 70°C temperature, although there are differences in exposure time of seeds suitable for each cultivar (Figures 1 and 3). On the other hand, Mombaça cultivar responded negatively to the tested temperatures and, in this case, it is possible that the use of temperatures below the tested in the present study may represent a strategy for overcoming dormancy of this cultivar, as found by Martins and Silva (1998).

There is little information available in the literature about the mechanisms that regulate the physiological response of seeds when exposed to high temperature. One possibility is related to the synthesis of proteins. Di Nola et al. (1990) found that exposure of *Echinochloa crus-galli* seeds for up to two hours at 46°C after soaking for 96 h at 36°C stimulated seed germination, affecting the composition of soluble proteins linked to cell membranes during the transition from the dormant stage to non-dormant. Furthermore, Martins et al. (1998) found that the glumes of *P. maximum* seeds submitted to thermal treatments showed shrinkage of their cells (stress resulting from the application of heat), which could interfere with seed permeability to gases and water. Thus, treatments that promote physical disintegration of the pericarp, eliminating their vulnerability, could be agents of overcoming seed dormancy.

In addition to dormancy, the incidence of pathogens in fodder species can negatively influence the establishment of pastures. This second factor promotes the reduction of productivity and quality, as well as a significant impediment to exports due to phytosanitary barriers (Marchi et al., 2008; Mallmann et al., 2013). High incidence of pathogen levels can reduce the viability of seeds or even promote their death even before germination (Mallmann et al., 2013). Some studies have shown that heat treatments can be an alternative for the control of pathogens (Muniz, 2001; Oliveira et al., 2011; Gama et al., 2014). However, this treatment is a measure that causes physiological and biochemical changes in the seeds at different intensities and may compromise their performance (Coutinho et al., 2007). Thus, the initial application of heat to the seeds for the purpose of determination of the temperature and heat

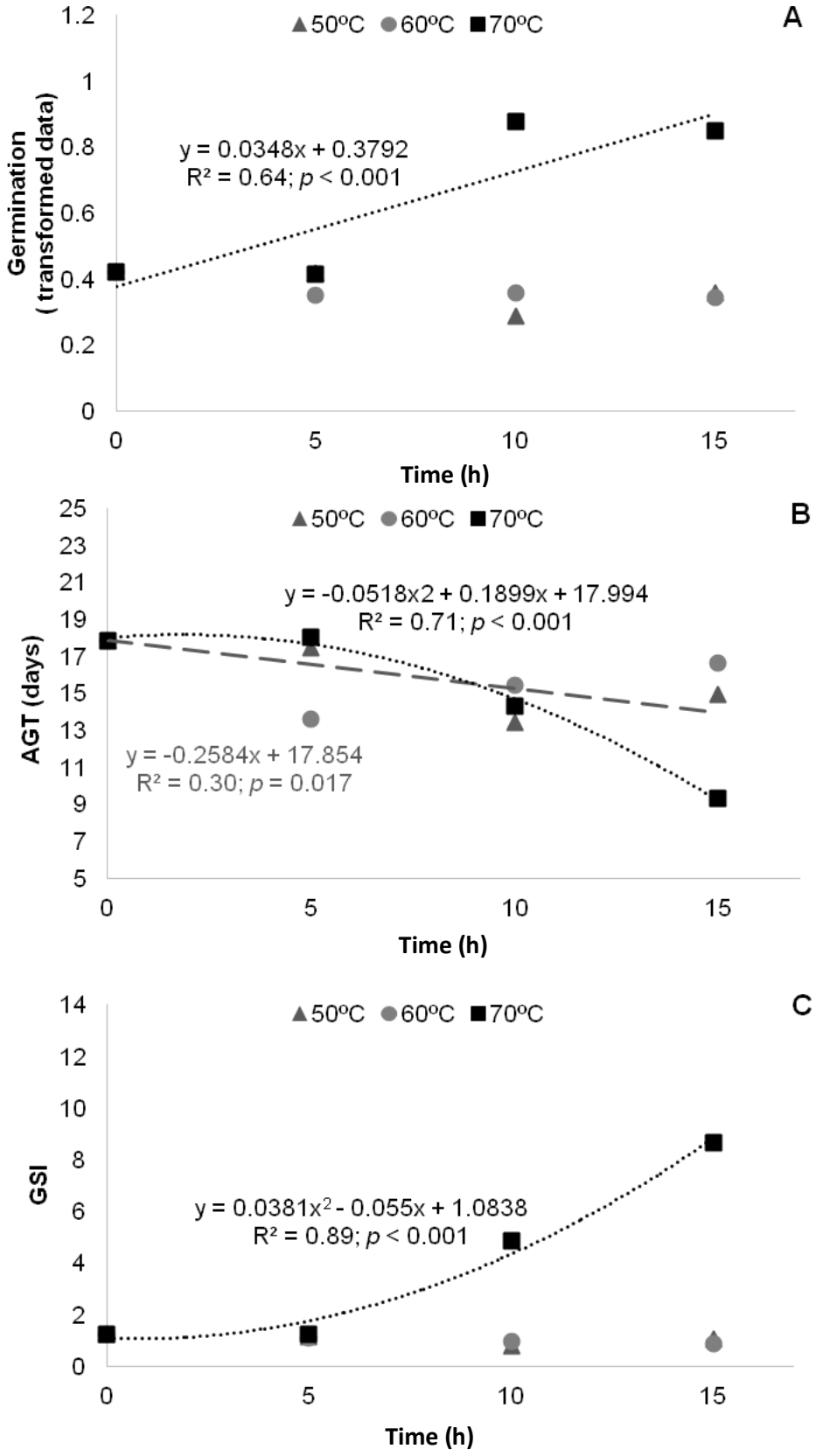

Figure 2. Germination (A), Average germination time (B) and Germination speed index (C) of *P. maximum* seeds cultivar. Milênio treated with temperatures (50, 60 and 70°C), by different exposure times (0, 5, 10 and 15 h).

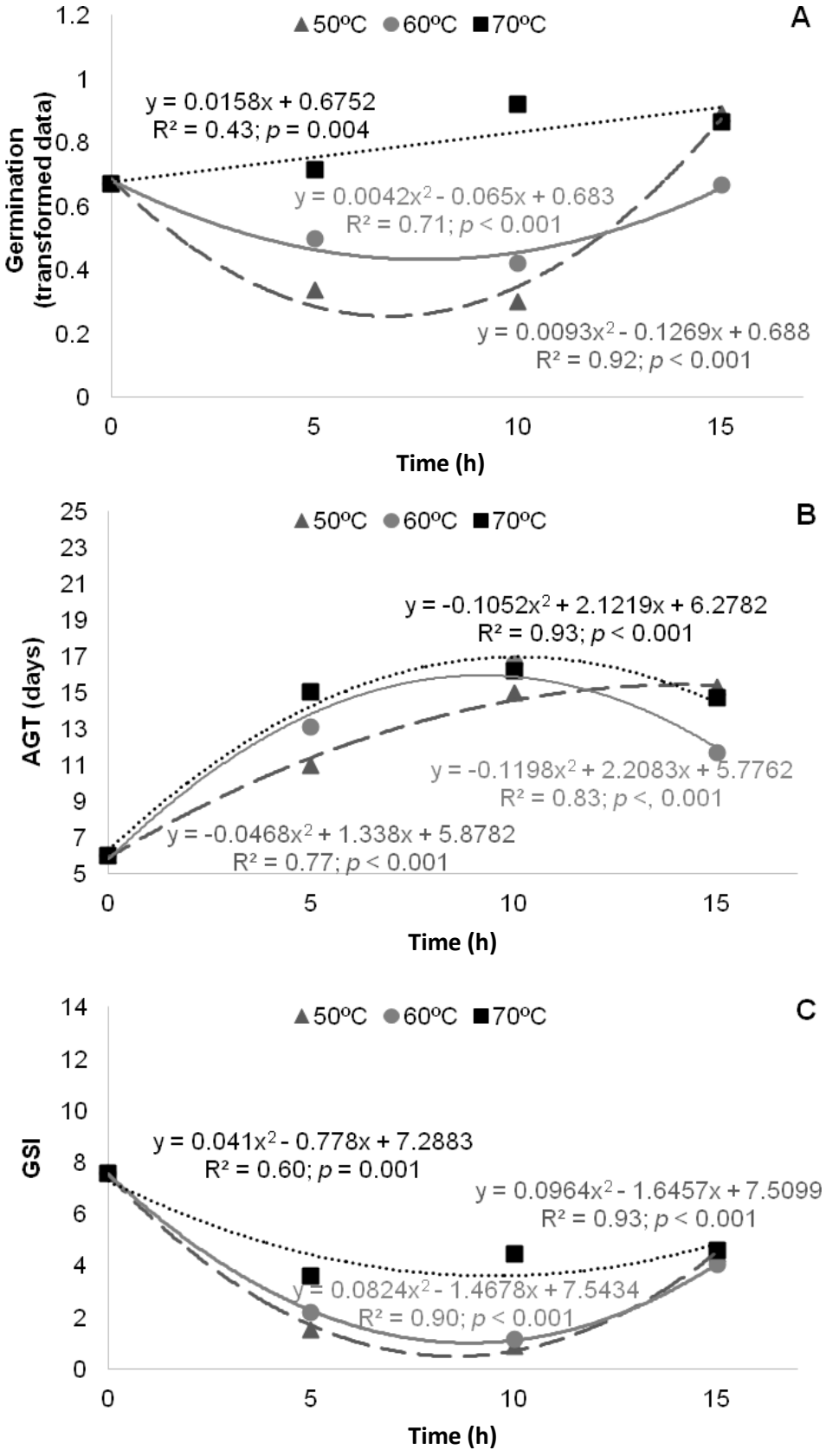

Figure 3. Germination (A), Average germination time (B) and Germination speed index (C) of *P. maximum* seeds cultivar Mombaça treated with temperatures (50, 60 and 70°C), by different exposure times (0, 5, 10 and 15 h).

application time in different species and varieties which do not reduce germination may represent a starting point for the phytosanitary control.

Conclusion

P. maximum seeds of different cultivars have distinct responses to heat treatment. The cultivar Tanzânia responds positively to different combinations of temperature and period of seeds of exposure, and the treatment at 70°C for about eight hours is recommended to overcome dormancy with better germination performance. For cultivar Milênio, it is recommended the exposure of seeds to 70°C for 15 h. Cultivar Mombaça is negatively influenced by the heat treatment, so this treatment is not recommended for this cultivar. Thus, treatments to overcome dormancy of seeds should also take into consideration the specificity of different cultivars of a particular species.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Procedure for collecting milk sample and the number of milkings in relation to chemical composition and somatic cells of the fresh milk

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This study was carried out to determine which system of milkings and sampling methodology presenting chemical composition results safer and lower variation of the results. In Properties I and II, the fresh milk samples were collected in 40 ml flasks containing bronopol in order to compare the chemical composition of the milk, as adopting two sample collection methods, that is two and three milkings. In order to compare the chemical composition of milk and SCC, between two sampling methodologies, in two daily milkings (Property I) and three daily milkings (Property II), fresh milk samples were collected randomly totaling 300 samples on the property I and 312 samples on the property II. The milk chemical components were determined through analytical principle that is based on differential absorption of infrared waves by components of the milk. By analyzing the treatments, experimental design was used entirely randomized and averages were compared by Tukey test using the statistical program SISVAR (statistical analysis system). When evaluating the data obtained through variation coefficient (VC), the occurrence of experimental was observed, since most results showed low variation. However the distinct procedures for sampling of the fresh milk may present varied levels of the chemical composition and SCC, therefore overestimating or underestimating the values of the milk composition, when samples are collected at different times of the day and different number of milkings.

Key words: Cows of high production, isolates samples, period, pool, sampling methodology.

INTRODUCTION

The samples of the fresh milk collected for analyses of the chemical composition and somatic cell count (SCC) may suffer various kinds of changes before arriving at laboratory, since the collection method, time, storage

temperature, transportation and the milk homogenization for complete dissolution of the preservative may cause component changes.

The most common factors for variation of the milk components are: the animal individuality, breed, feed, lactation stage, age, environmental temperature, season, physiological factors (pregnancy, estrous cycle) and pathological ones (mastitis), persistency of lactation, cow size, cow mammary quarters, milking portion and interval between milkings (Weiss et al., 2002; Rangel et al., 2009). Although different factors may affect the composition of the milk, it is very important to repair the errors and to avoid any adulteration that may occur at the time of collection. The detection of the critical points that affect the quality and production of the milk is very important in controlling the herd.

In order to maintain the legitimacy of the samples in obtainment of the milk, they should be standardized, since the influence from milking type (mechanical or manual) and the method used to obtain the sample can alter the outcome of the chemical composition of milk (Reis et al., 2007). Taking into account some criteria such as the interval between milkings and the method for collecting milk samples in order to perform laboratorial analyses, it is possible to observe the occurrence of variation in composition of the milk (Cabral et al., 2013). According to Friggens et al. (2001), the milk component presenting more variability is fat, whereas protein and lactose do not differ according to criteria under evaluation. Therefore, only a single sample of milk is sufficient to obtain reliable results, when those variables are under evaluation.

When evaluating the quality of the milk, as taking into account some criteria such as fractioned collection of the milk sample in automatic milking systems, a high interference degree is observed in some milk components (Nielsen et al., 2005). However, the management practices adopted in rural properties and the milking type should be taken into account.

It is possible to assert the occurrence of variation in some components of the milk, according to the method used in obtaining the milk sample, the milking type adopted in the property, whether manual or mechanical, and the interval of eight or twelve hours between milkings.

In this context, this study was carried out to determine if two distinct procedures in collecting milk samples, such as pool of sample and independent samples, interfere with the chemical composition and somatic cell count of the fresh milk. If different number of milkings occasion variation in the chemical composition of the milk, when samples are collected at different times of the day, and which sampling method provides security for laboratorial analyses.

MATERIALS AND METHODS

The research was conducted in two Dairy Properties located in Rio Verde municipality on Southwestern Goiás, according to ethical standards and approved by the Ethics and Biosafety Committee of the, Federal Institute of Education, Science and Technology of Goiano – Rio Verde, Campus – Go. The region has tropical climate, 740 m altitude, and average annual pluviometric index of 769 mm, with two distinct periods: rainy from October to April and dry from May to September. The research was conducted during the crop cycle period from May to October 2012.

The region has slightly sloping relief and the soil is red latosol, which favors the planting of annual crops and the livestock.

Rural properties

In rural Property I, the herd consisted of 90 crossbred cows and the milkings were accomplished in two periods of the day at 5:30 and 16:30 h. The cows were divided in three lots and maintaining the criterion of production, as being the most productive (>20 L) medium production (15 to 20) and less productive (<15 L). In afternoon milking, during the experimental period, the lots of cows entered the contrary, that is, the less productive (<15 L) the average production (15 to 20 L) and the most productive (>25 L) in order to increase the interval between milkings of the highly productive cows.

The collection procedure followed the norms of good milking practices: teats were washed with water and the first three milk jets were discarded in a black bottom mug to verify the presence of lumps, then, with the aid of an applicator, teats were immersed in pre-milking solution based on sodium hypochlorite and waiting 25 s to obtain total production efficiency. After cleaning, teats were dried with paper towels for the coupling of teatcups. At the end of milking, post-milking solution was used, whose base was 0.25% glycerin iodine.

In rural Property II, the herd consisted of 180 crossbred cows and three daily milkings were accomplished. The first milking began at 4:00 h in the morning, the second at midday and the third at 18:00 h. The cows were divided in lots and milked according to production. The highly productive cows (>20 L) corresponded to first lot, whereas the medium-production cows (15 to 20 L) corresponded to second lot and the lowest productive cows (<15 L) corresponded to third lot. The property had the fish scale-type automatic milking machine, which worked in loop and had a milking line with twelve teatcup sets with individual collectors to measure daily milk production and sampling collections.

In preparing the cows for daily milking, the traditional method prevailed. So, the black-bottom bucket test was performed, by using the first three milk jets, pre-dipping with towels imbibed in bactericidal disinfectant solution based on 30% Di(aminopropyl)

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laurylamine solution. One tip of the towel was used for each teat. For drying the teat, the towel reverse corners were used. After sanitary procedure, the set of teatcups were coupled, and the milking was continuously performed.

After the milking process, the set of teatcups were automatically removed and the estimated milk production (in kg) was displayed on individual digital reader of the milking machine. Then, the post-dipping with 0.25% iodine was accomplished.

Feeding the cows

The experimental period occurred during crop cycle, as taking into account the dry season of six months. During this time interval, the cows were kept under feedlot system in both rural properties. On rural Property I, the cows were sheltered in collective feedlots under shady environment, whereas on Property II the animals were housed in free-stall.

In Property I, during the experimental period, the cows were given corn silage and cottonseed-based concentrate, soybean bran, corn germen, urea, vitamin and mineral premix and water ad libitum. In Property II, the diet consisted of corn silage and concentrate with corn, soybean meal, vitamin and mineral premix, urea and water ad libitum.

Collecting the samples

In Properties I and II, the samples of the fresh milk were collected in 40 ml flasks containing the conservative bronopol. In Property I, the milking was performed twice. Some months earlier, it was observed the variation in volume of the milk produced daily to obey the following proportion: 2/3 on morning and 1/3 on afternoon. So, the same proportions were adopted in pool sampling. In the first milking, the milk samples reached 2/3 of the flask and 1/3 in the second milking that represented the pool sample. The independent samples, corresponding to 100% milk of the flask, were collected together with the pool in each milking of the day (morning and afternoon). From each milked cow, three milk samples were obtained (one pool sample, one sample on morning and one sample on afternoon).

In Property II, a partial sample of the fresh milk was obtained in each milking (three daily milkings) that represented a pool, as following the proportion: 1/3 milk on morning, 1/3 milk on afternoon and 1/3 milk on evening added in the same 40 ml flask. Together with pool, an independent sample was collected in each milking, as totalizing 100% milk in the flask. For each milked cow, a total of four milk samples were obtained (a pool sample, a morning sample, an afternoon sample and an evening sample).

Electronic analysis of the milk chemical composition

The contents of fat, protein, lactose and nonfat dry extract (NDE) were determined, by adopting the analytical principle which is based on differential absorption of the infrared waves by milk components, as using the equipment Milkoscan 4000 (Foss Electric A/S. Hillerod, Denmark). The results were expressed as percentages (%).

Somatic cell count

The analysis of the somatic cells (SC), from which the analytical principle is based on flow cytometry was performed through the equipment Fossomatic 5000 Basic (Foss Electric A/S, Hillerod, Denmark). The results were expressed in SC/ml.

Statistical analysis

With a view to comparing the chemical composition of the milk, between the sampling methodologies, in two daily milkings (Property I) and three daily milkings (Property II), the milk samples were collected from 50 cows in Property I. Cows were randomly allocated to three treatments with two replicates, as totaling 300 samples. In Property II, the milk samples were collected from 26 cows randomly allotted to four treatments with three replicates, as totaling 312 samples.

In analyzing the treatments, the entirely randomized experimental design was used and the averages were compared by Tukey test at ($P<0.05$) significance level, as using the statistical program SISVAR (Statistical Analysis System) (Ferreira, 2003).

In order to verify the relationship between milk chemical composition and milk production and SCC, the simple correlation among variables was performed through ASSISTAT program, according to Silva and Azevedo (2006), by applying the t-Test at ($P<0.05$) and ($P<0.01$) significance levels.

RESULTS AND DISCUSSION

The average results of the chemical composition and somatic cell count (SCC) of the milk samples collected under different sampling and number of daily milkings are presented in Tables 1 and 2. According to the obtained data and the evaluation of the variation coefficient (VC), the experimental accuracy was confirmed, since 50% from the total results showed low variation, as demonstrating the variables that showed greater instability in Property I were the production of milk, fat and somatic cell count (SCC) with VC corresponding to 28.45, 23.76 and 22.89% respectively (Table 1) whereas, in Property II, the variables with greater variation were fat and SCC with VC corresponding to 31.84% and 24.40% respectively (Table 2), whereas the ideal VC to animals is lower than 20%.

During the experimental period, the milk production (milk kg/milking) of the highly productive cows, within the range of two and three milkings, showed average results with ($P<0.05$) difference (Tables 1 and 2).

In Property I, the daily milk production representing the average pool showed the highest value, as differing ($P<0.05$) from morning and afternoon milkings that did not differ from each other at ($P<0.05$) significant level. In the morning, the milk volume was numerically higher than afternoon milking. This difference occurred because the pool average results from the daily average production of milk (Reis et al., 2007) (Table 1).

In Property II, the average values of the milk production differed ($P<0.05$) (Table 2). The average of the pool samples presented the highest value, since it represents

Table 1. Average values of the chemical composition and SCC in samples of the fresh milk obtained in two daily milkings, in high production dairy systems.

Variable	Property I			VC (%)
	Two milkings			
	Pool	Morning	Afternoon	
Milk production (L)	28.62[a]	14.35[b]	14.27[b]	28.45
Fat (%)	3.43[ab]	3.38[b]	3.68[a]	23.76
Protein (%)	3.15[a]	3.16[a]	3.16[a]	10.02
Lactose (%)	4.63[a]	4.64[a]	4.63[a]	5.14
NDE (%)	8.77[a]	8.80[a]	8.80[a]	5.00
SCC (x1000 SC/ml)	462[a]	467[a]	499[a]	22.89

Lowercase letters in the line differ significantly at 5% probability. NDE: Nonfat dry extract. SCC: Somatic cells count. VC: Variation coefficient.

Table 2. Average values of the chemical composition and SCC in samples of the fresh milk obtained in three daily milkings, in high production dairy systems.

Variable	Property II				VC (%)
	Three milkings				
	Pool	Morning	Afternoon	Evening	
Milk production (L)	22.57[a]	9.55[b]	7.33[c]	5.69[d]	2.13
Fat (%)	3.79[bc]	3.54[c]	4.08[ab]	4.48[a]	31.84
Protein (%)	3.45[a]	3.48[a]	3.50[a]	3.49[a]	10.31
Lactose (%)	4.68[a]	4.63[a]	4.65[a]	4.62[a]	5.34
NDE (%)	9.08[a]	9.04[a]	9.10[a]	9.06[a]	4.81
SCC (x1000 SC/ml)	241[a]	241[a]	332[a]	318[a]	24.40

Lowercase letters in the line differ significantly at 5% probability. NDE: Nonfat dry extract. SCC: Somatic cells count. VC: Variation coefficient.

the daily milk production. In the sampling on morning, afternoon and evening, a gradual reduction occurred in production of the milk, which is probably due to interval between milkings. This occurrence was expected due to irregular schedules, that is, 8 h interval between first and second milking, 6 h between second and third milkings and 10 h between third to first milking of the subsequent day.

The averages of the variable fat, under different sampling methods and analyzed in two milkings, differed ($P<0.05$) (Table 1). The fat content increased gradationally, as milkings were performed. The lowest fat average was observed on morning period, as compared with afternoon period. The fat average, obtained by sample pool method, differed from fat contents obtained in the other periods over which the samples were collected by using the independent sample method, respectively, since the pool represents the general average of the values in both periods (Table 1).

In Property II, the average results for fat content of the milk samples differed from each other ($P<0.05$). The fat content increased on increasing order of the samples, according to accomplishment of the milkings from morning to night. In the morning period, the lowest average was observed. This one differed from samples obtained in other collection periods, besides presenting a result equal to the average of pool. In the afternoon period, the fat contents were equal to those obtained in the night period and the pool. In the night period, the fat content was equal to that obtained in the afternoon period and differentiated from other samples.

Probably, this effect occurs because the greater volume of the milk produced by the animal during the morning period and, consequently, the chemical components will be lower. Therefore, corroborating with the results found by Nielsen et al. (2005) who showed the milk component which has a greater variation range, when applied to the milking interval to be the fat. This

result can be explained by the well-known dilution effect because with an increased volume of the milk, a reduction of the chemical components occurs (Mollenhorst et al., 2011).

Mendes et al. (2010) associated this effect with nutrition of the cows, that is, when a larger amount of the ration in relation to grass is supplied, an increase occurs in proportion of the propionic acid in relation to both acetic and butyric acids, therefore causing a reduction in fat by dilution. Different results were observed by Lammers et al. (1996). When relating the nutrition with fat contents, those authors affirmed the reduction of the fiber content in the diet to decrease the fat content in milk.

Contradicting the results obtained by the above mentioned author, Kargar et al. (2012) used different fat sources in the diet of Holstein cows and they observed the production and fat of the milk to be not affected by supplementation with high ration level in relation to forage levels, since the authors used a ration: grass proportion of 66:34.

The comparison between milk production and fat demonstrates that the greater is the production of the lactating cows, the lower is the percent fat. So, the lowest average of milk production occurred at night and, consequently, the highest percentage of fat (4.48%). The same result was observed by Friggens et al. (2001), who reported that the larger is the volume of milk the lower is the fat content.

With the results obtained in this study, it is possible to infer that in the collection of milk samples, as aiming at fat analysis, the sampling should be done by the method of pool. In this method, the collection of the milk samples is performed at ratio 2/3 and 1/3 of milk when in two milkings, or at ratio 1/3 of milk in each milking when performing three milkings.

Reis et al. (2007) evaluated the procedures for collecting milk. They reported that lower fat levels may be due to greater volume of milk accumulated inside the udder on the morning, as causing the dilution of fat. For validation of the results, however, the authors concluded that would be correct to accomplish a pool of the milk sample because the fat amount found (3.81%), which showed median value, when compared with the content observed in the morning period (3.44%) and in afternoon period (4.49%). However, it should be taken into account that the milk samples obtained by those researchers correspond to first jets of milk, which denotes the components were less concentrated.

According to Friggens et al. (2001), it is impossible to collect independent milk samples and provide daily estimates of the percent milk fat in conventional milking systems.

Wall and McFadden (2008) stated that milk samples used in studies to investigate the influence from milking interval and herd management on milk composition and SCC should undergo standardization in order to make possible the collection of accurate data.

The average results of protein, lactose, NDE and SCC showed no difference ($P>0.05$) (Table 1 and Table 2). Therefore, it is possible to infer that only one milk sample obtained at any milking period is sufficient for reliable results issued for these variables.

Concerning to SCC, Brasil et al. (2012) found that, in mechanical and manual systems, there was a difference in SCC levels. The mechanical milking provided considerable increase in the SC number (545.000 SC/ml), whereas SCC obtained in the manual milking was much lower (253.000 SC/ml).

When evaluating the milk composition in two and three milkings, Österman and Bertilsson (2003) observed that differences in percent fat increased, whereas the milk protein content has a tendency to be more similar at the beginning and at the end of lactation. The results from analyses of the milk samples should be real and reliable because, as dairies pay for quality, the reliability of the results is fundamentally important to avoid frauds and errors in payments to producers and the demerit of the milk quality.

Figure 1 shows the averages of the production in the Properties respective. When comparing the trajectory of the milk production in Properties I and II, between the collection methods, it was observed the production index to be similar. This represents slight regression in liters, since it is observed that, when the practice of milking is constant, with less than eight-hour interval between milkings, the dairy capacity of the cows decreases.

Figure 1 shows that the production of milk, when milking was performed two times a day with 12 h interval, the average production showed a slight and not statistically significant reduction. However, when milking was performed three times a day at irregular intervals of eight, six and ten hours respectively, an abrupt drop in milk production occurred. This behavior is probably due to higher interval between the last milking of the day and the first one of the next day.

Results similar to those found in the present study were observed by Ouweltjes (1998), who demonstrated the relationship between milk production and the interval between milkings of dairy cows to occasion a greater milk production on the morning than on afternoon.

When comparing a milking system similar to the one adopted in the present study, that is two and three daily milkings, Wall and McFadden (2008) observed the lactation curve of the cows milked three times a day or more to maintain a prolonged lactation peak, which probably maintains the sanity of the mammary glands.

Different results were observed by Marnet and Komara (2008), when evaluating the amount of milking performed

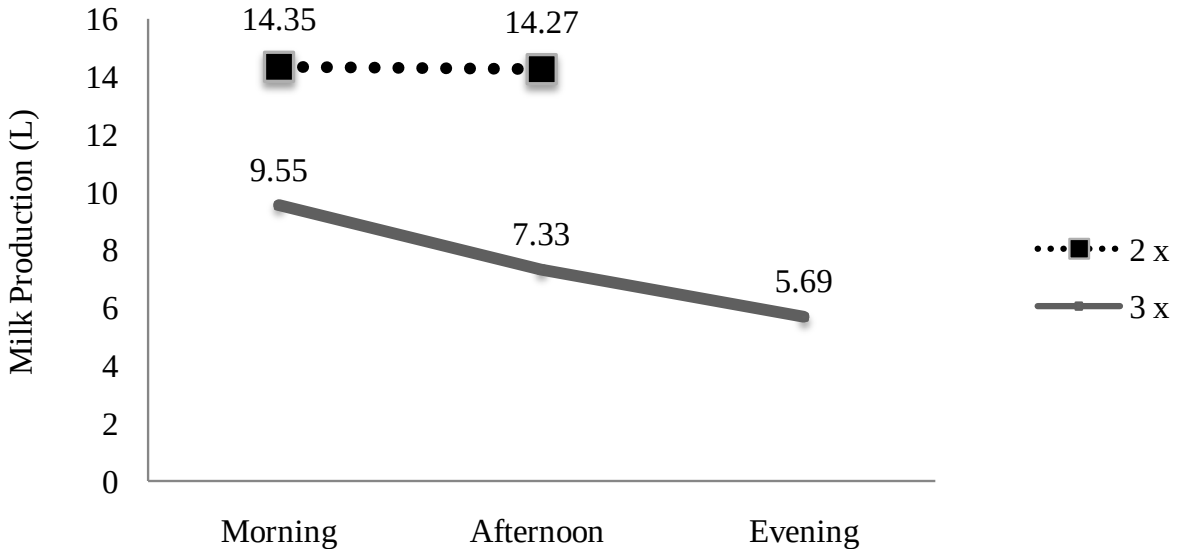

Figure 1. Average results of the milk production in Dairy Farms with two and three milkings.

Table 3. Simple correlation among milk production and SCC with chemical composition of the milk samples obtained in two and three milkings.

Correlation	Milk production		SCC	
	2 milkings	3 milkings	2 milkings	3 milkings
Fat	-0.2811**	-0.2412**	0.0642^{ns}	0.2061**
Protein	-0.3132**	-0.0061^{ns}	0.2535**	0.3102**
Lactose	-0.0903^{ns}	0.0124^{ns}	-0.4202**	-0.4374**
NDE	-0.2941**	-0.0158^{ns}	-0.0446^{ns}	-0.0570^{ns}
SCC	-0.0676^{ns}	-0.0922^{ns}	-	-

** Significant at 1% probability level (P<0.01). ns = non-significant (P>0.05).

daily and the profitability. They observed the system of milking only once a day in order to save time and flexibility to be not the best for cows, since only one daily milking causes greater impact upon physiology of the udders and udder health, therefore limiting the use of this system. Table 3 shows the results from simple correlation among variables of the chemical composition in dairy systems with two and three milkings.

In dairy farms adopting two and three daily milkings, the fat content was negatively correlated with the volume of milk production, which is related to an increase in milk volume and a decrease in milk fat concentration, as presenting highly significant values at (P<0.01) probability level.

The results of simple correlation in the range between two and three milkings with twelve hours apart (Property I) and 8, 6 and 12 h (Property II), respectively, corroborate with Nielsen et al. (2005) who found that fat content varies when milk is milked at intervals of six and twelve hours, since higher results were observed at six hours interval, as compared to the result observed in the milking interval of twelve hours.

When the protein values are compared between two daily milkings, the correlation with milk production was negative at 1% significant probability level; when samples were obtained at three milkings, the correlation between production and protein was negative, however not significantly different at (P<0.05). According to Dürr (2004), the changes in the milk protein content are less significant than the changes in fat, although the fat and protein influence the milk production; however, the protein content has lower variation in the results than fat.

The results from simple correlation of fat and protein, observed in two daily milkings were negative at (P<0.01) probability level. Similar results were reported by Friggens et al. (2001), who also found negative correlation of the variables fat and protein with milk production. According to Santos et al. (2001), the increase of the milk production in relation to percent fat and decreased percent protein is related to the use of supplementary fats in the diet, which is due to substitution of the rumen-available carbohydrates by lipids, as causing toxic effects to microorganisms of the rumen, which causes the reduction in microbial growth and consequently affecting the transport of amino acids to mammary glands. Thus, the concentration of the milk protein may decrease because the deficiency of one or more amino acids.

The fat content of the milk decreases with increasing volume of the milk obtained in the milking procedure, regardless of the number of milkings accomplished per day. Concerning to protein content and NDE, however, the correlation was significant only in dairy system with two daily milkings.

The lactose content was not significantly different between dairy systems of two and three milkings in relation to milk production. Therefore, it should infer that milk production varies as a function of the variable lactose. These results differ from those reported by Meyer et al. (2006), who affirmed the levels of lactose to be highly correlated to milk production.

The study carried out by Silva et al. (2000) on mastitic milk and non-mastitic milk revealed a decrease of the lactose contents in the mastitic milk. However, such a relation was already expected because the infection leads to destruction of the secretory tissue. So, there is a reduction in the synthesis of the mammary glands, which leads to reduction of the lactose contents and consequently the decrease in milk production.

According to Santos and Fonseca (2007), milk that has mastitis presents several alterations mainly in the physical and chemical composition. Mendes et al. (2010) reported that the components undergoing more changes are proteins, that is, the casein decreases and the serum proteins increase, whereas 10% reduction occurs in fat and lactose.

According to Eifert et al. (2006), the lactose is the primary and most important osmotic component of the milk, since it is associated with secretion of water and the volume of the milk produced. It is a component depending on glucose in its synthesis and, when it occurs at lower proportion in milk, may suggest insufficiency of glucose in the animal, as a resulting tendency to lower milk production.

For NDE in two-milkings dairy systems, the correlation is negative and the results were significant at (P<0.01) in three-milkings, the correlation presented no significant differences at (P<0.05).

The correlation of SCC with milk production was not different at (P<0.05) level in two and three daily milkings. Numerically, SCC of the cows in lactation had a gradual increase as milkings were accomplished, and SCC levels increased with decreased milk production.

This performance of SCC increase was different from that found by Mollenhorst et al. (2011), who observed that the parameters under study, such as milk production, calving order and interval between milkings, showed low relationship, since a very low SCC variation was observed when the intervals between milkings were lower than six hours.

The results found by Takahashi et al. (2012) did not differ from those found in the present study. No effects of the production upon variation of SCC occurred. However, when SCC was compared as function of the seasons, the effect was with changes mainly during the summer. The author relates this effect with the thermal stress that affects the animals, as making them more susceptible to infections in the mammary glands. However, the author also states that, in numerous herd, the animal SCC affects just slightly the count of the tank as compared to a small herd, which can probably be related to technological level of the Property (Zanela et al., 2006).

According to Coldebella et al. (2004), the SC increase occasion absolute losses in milk production, however it is independent from production level of the animal, where those losses occur from the count of 17.000 SC/ml. When comparing the reduction of the milk production with SCC/ml, the authors observed that, in the first lactation, there was a reduction of 0.30 kg milk per 100,000 SC/ml and 0.61 kg per 200.000 SC/ml. In the second lactation, the cows with SCC of 200.000 cells/ml had a reduction of 0.63 kg in milk production at 50th day postpartum, 0.92 kg at day 150th and 1.77 kg at day 250th postpartum. The cows at 3rd lactation or higher, with this same SCC showed a reduction of 0.60 kg, 1.09 kg and 1.85 kg on days 50, 150 and 250 postpartum, respectively.

Heuven et al. (1988) evaluated the inheritance of SCC and its genetic relationship with milk production and order of births. They found the inheritance of the somatic cell count to be low in early lactation and the variation of SCC is likely to alter from the second delivery, since it is correlated with milk production.

When observing the correlation between SCC and fat, it is observed that no differences (P<0.05).occurred in dairy systems adopting two milkings. In the systems adopting three milkings, however, the relationship between SCC and fat was highly (P<0.01) as showing a positive correlation. This suggests that, with the increased SC contents, an increase in the fat levels occurs.

The SCC results, in relation to protein, were highly significant (P<0.01) and presented positive correlation in two and three milkings. Results differing from the present study were reported by Bueno et al. (2005), who observed a reduction in protein level of the refrigerated raw milk, when SC levels increased. Lacerda et al. (2010) obtained similar results to the present study, since the relationship between protein and SCC were correspondingly higher between properties with SCC increased.

The values of the lactose in milk correlated negatively with SCC, since they were highly (P<0.01) in two and three milkings. Those values also proved the increase in somatic cells to cause reduction in the synthesis of lactose. Results similar to the present study were found by Bueno et al. (2005), who observed a reduction in lactose content as SCC increased. The same was observed by Auldist (1995), when verifying the reduction in concentration of lactose to be related with increased SCC.

The reduction of the lactose contents would result from either lower synthesis of this milk component in infected mammary glands and the loss of lactose from gland to bloodstream, due to increased permeability of the membrane separating the milk from blood, as leading to its excretion in urine (Pereira, 2000). Lacerda et al. (2010) reported different results for relationship between lactose and SCC, when presenting results of increase in lactose content.

The relationship between SCC and NDE showed no significant difference (P>0.05), as demonstrating that SCC probably does not interfere in NDE contents. Lacerda et al. (2010) obtained different results for relationship between SCC and NDE, since they stated that the average NDE increased when SCC increased during summer and winter.

The sanity of the lactating cows may cause alteration in chemical composition of the milk. However, Brasil et al. (2012) reported the incidence of mastitis in the herd to result in increased SCC, which is one of the main parameters used for evaluation of the milk quality, since it is related to either decreased concentrations of the milk components and changes in sensory characteristics of the dairy products.

The results of this study, conducted in Properties I and II, showed values of the centesimal composition and SCC/ml according to the parameters required by legislation (IN/62 2001) (Brazil, 2011). Because being according to legislation, the milk produced in both rural properties under different milking and management systems, as one property being high-tech and the other one low-tech but presenting result according to legislation, the quality of the product commercialized in the region is adequate to consumption.

Conclusion

The distinct procedures for sampling of the fresh milk may present varied levels of the chemical composition and SCC, therefore overestimating or underestimating the values of the milk composition, when samples are collected at different times of the day and different number of milkings.

So, the pool sample collection would be the procedure indicated, since the result for composition presents a daily relative average, therefore a homogenous and legitimate result. The sampling for issuing reports concerning to milk quality should be judiciously performed in order to representatively express the average values of the milk chemical constituents.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of sweetpotato accessions for end-user preferred traits improvement

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This study assessed the genetic diversity and differentiation in sweetpotato accessions in Ghana to guide selection for genetic improvement on beta-carotene, dry matter and sugar contents to promote increased utilization. One hundred and fifteen sweetpotato accessions from four different sources, which were the International Potato Centre (CIP) collection, local collection from farmers' field, local improved varieties, and local and exotic collections from the National Agricultural Research Programmes were studied using 40 agro-morphological and physico-chemical traits, and 25 SSR markers. Variability was obtained for 13 agro-morphological traits and all the physico-chemical traits. Significant genetic diversity indicates existence of a high degree of agro-morphological and physicochemical variation. Within Group variation (97%) accounted for most of the diversity indicating a broad genetic base. The divergence indicates that breeders can form different populations with significant levels of genetic variation to exploit heterosis and improvement of populations. A strong negative relationship was found for sugar content and dry matter content and indicates a possible development of non-sweet high dry matter sweetpotato varieties. However, developing non-sweet, high dry matter and high beta-carotene sweetpotato varieties could be challenging due to the strong negative association between dry matter content and beta-carotene content, and the positive association existing between beta-carotene and sugar content. This study has in addition confirmed the breeding potential of sweetpotato accessions in Ghana and the probability of providing useful genetic variation for the development of farmer preferred cultivars.

Key words: Analysis of Molecular Variance (AMOVA), diversity, end-user, simple sequence repeats (SSR) markers, Sweetpotato, traits.

INTRODUCTION

Sweetpotato is a major staple crop in developing countries all over the world because of its diverse uses. These include use in many food and industrial products such as starch, sweeteners, noodles, citric acid, soft

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drinks, desserts, flour, industrial alcohol, ethanol fuel and livestock feed. Despite its importance, the level of utilization in Ghana is very low and it is not well integrated into Ghanaian diets (Adu-Kwarteng et al., 2002). This is because consumers in Ghana prefer sweetpotato with dry mealy flesh, non-sweet, and high nutritive value (Sam and Dapaah, 2009; Baafi et al., 2015), but locally available varieties are sweet that limits consumption as a staple food (Missah and Kissiedu, 1994). In addition, the recently introduced orange-flesh genotypes, identified as a cheapER source of Vitamin A, are low in dry matter content. These factors have led to the low adoption of the 13 varieties released to date. There is, therefore, the need to incorporate non-sweetness, high dry matter, and/or high beta-carotene contents into the existing genetic background of high yielding and early maturing cultivars which are resistant to biotic and abiotic stresses.

A prerequisite for genetic improvement of sweetpotato is knowledge of the extent of genetic variation present in the germplasm. Information on genetic diversity guides selection of divergent parents to broaden genetic base of a breeding population and produce progenies with heterosis (Manosh et al., 2008). Identification of populations with high frequencies of favourable alleles for desirable traits is an important step in the development of improved varieties (Gasura et al., 2008). Understanding the genetic diversity is also critical to find new alleles for desirable traits (Warburton et al., 2002). Since the amount of genetic diversity within populations determines the extent of response in traditional breeding through selection, genetically diverse breeding populations are needed (Bos et al., 2000). Morphological characterization has been used extensively in diversity studies for various crop plants including sweetpotato (Bos et al., 2000; Kaplan, 2001; K'opondo, 2011). Agro-morphological and physicochemical traits are important diagnostic features for distinguishing among sweetpotato accessions. The use of these traits as genetic markers can speed up selection in sweetpotato improvement. SSR markers have been used to study genetic diversity in sweetpotato (Buteler et al., 1999; Diaz and Gruneberg, 2008; Tumwegamire et al., 2011; Somé et al., 2014). SSR markers are multi-allelic, highly polymorphic, highly reproducible, co-dominant and provide rich genetic information with good genome coverage (Kawuki et al., 2009; Sree et al., 2010). The SSR markers are affordable and amenable to most breeding procedures and applicable in public breeding programmes that may not be able to afford expensive diversity assessment techniques (Turyagyenda et al., 2012). Application of both phenotypic and genetic markers is important in obtaining full knowledge of genetic diversity in sweetpotato germplasm.

The objective of this work was to characterize sweetpotato germplasm in Ghana using phenotypic and SSR markers with focus on enhancing end-user characteristics of sweetpotato for increased utilization in Ghana.

MATERIALS AND METHODS

Agro-morphological and physico-chemical characterization

Germplasm collection and evaluation

Germplasm was collected from the major sweetpotato growing areas in Ghana in 2010. These were the Northern, Upper East, Upper West, Volta, Eastern, Central and the Brong Ahafo Regions. Collections from the CSIR-Crops Research Institute, Kumasi and the CSIR-Plant Genetic Resource Institute, Bunsu, were also included. In addition, accessions were collected from the Crop Science Department, University of Ghana and the International Potato Centre (CIP) gene bank in Accra and Kumasi. Thus, a total of 115 sweetpotato accessions (Table 1) were collected. These represent four groups, which were local accessions (32), local improved varieties (13), exotic and local accessions in National Agricultural Research Systems (NARS) or programmes (43), and exotic accessions from CIP, Kumasi germplasm (27). Evaluation of the sweetpotato germplasm was carried out under rain-fed conditions using Randomised Complete Block Design (RCBD) in three replications at CSIR-Crops Research Institute research fields at Fumesua (forest ecozone) in 2011, after carrying out planting material multiplication in 2010. Planting distance was 1 m between ridges and 0.3 m within row of ridge length 3.6 m.

Data collection

Data collection was done based on the sweetpotato descriptor for field phenotyping (CIP/AVRDC/IBPGR, 1991) as well as storage root quality traits as shown in Table 2. Harvesting was done at three and half months after planting. At harvest, data were taken on storage root yield and its components and a random sample of storage roots (one small, one medium and one large) were taken for physico-chemical analysis. Storage roots considered for the yield data were those over 0.3 m in diameter and without cracks, insect damage or rotten parts (Ekanayake et al., 1990). With the exception of the dry matter content, all the storage root quality traits were determined using the near-infrared reflectance spectroscopy (NIRS) which uses the work flow of the Quality and Nutrition Laboratory of CIP Lima, Peru. Fifty grams fresh sample was used. It was freeze-dried for 72 h using a freeze dryer. Dry matter content was determined after freeze drying as ratio of dry weight to fresh weight of sample expressed as a percentage. These were determined at CIP Laboratories in Kumasi, Ghana and Lima, Peru.

Data analysis

Data were subjected to Principal Component Analysis (PCA) and Cluster Analysis using Genstat version 9.2.0.152 (Genstat, 2007). The PCA was done based on the correlation matrix. Data for beta-carotene, dry matter and total sugar contents were subjected to an Analysis of Variance (ANOVA) using Genstat version 9.2.0.152 (Genstat, 2007). Based on the mean performance of these traits, the top 10 and the bottom 10 accessions were selected to construct a dendrogram and a GGE Biplot using the most important traits for PC1 and PC2. The dendrogram was constructed based on the hierarchical, single link method using Euclidean test. The biplot was constructed to depict the phenotypic relationships among the accessions, their correlation with the traits significant for PC1 and PC2, as well as the association among the traits. The biplot was

Table 1. List of the 115 accessions collected and their source.

Local accessions	Local improved accessions	NARS accessions		CIP accessions
CRIWAC 01-10	SANTOMPONA*	TAG 03-019*	B-REGARD*	CIP 442903
CRIWAC 02-10	FARAA*	NS 001*	FIASO RED*	CIP 442291*
CRIWAC 03-10	TEKSANTOM	OK 03-015	TAG 03-030*	CIP 440069
CRIWAC 04-10	OGYEFO*	DOS 03-021	GWERI	CIP 440390*
CRIWAC 05-10*	OKUMKOM*	CARROT C	BD 96-029*	CIP 442462*
CRIWAC 06-10*	OTOO*	HUMBERCHERO*	FREMA*	CIP 442776
CRIWAC 07-10*	HISTARCH*	B/FASO 002*	DOS O3-006*	CIP 440062*
CRIWAC 08-10*	SAUTI*	FA 10-026*	NS 003	CIP 442589*
CRIWAC 09-10	APOMUDEN*	RESISTO*	AAT 03-004	CIP 442145
CRIWAC 10-10*	LIGRI*	NASPOT 1*	OK 03-021	CIP 442147*
CRIWAC 11-10*	BOHYE*	AAT 03-017	BOT 03-030*	CIP 440095*
CRIWAC 12-10*	PATRON*	OK 03-014	OK 03-017	CIP 441771
CRIWAC 13-10*	DADANUIE*	JONATHAN*	KAYIA WHITE	CIP 442901*
CRIWAC 14-10		H-ASIATOR*	UKEREWE*	CIP 443016*
CRIWAC 15-10*		TANZANIA	OK 03-018	CIP 440071*
CRIWAC 16-10		NINGSHU 1*		CIP 442896*
CRIWAC 17-10*		BOT 03-021		CIP 442162*
CRIWAC 18-10		KEMB 37		CIP 442775
CRIWAC 19-10*		BOT 03-028*		CIP 443027*
CRIWAC 20-10		BOT 03-020*		CIP 443129*
CRIWAC 21-10		J-ORANGE*		CIP 442264*
CRIWAC 22-10		BOT 03-027*		CIP 442654
CRIWAC 23-10*		ADA 001		CIP 443035*
CRIWAC 24-10*		DOS O3-017*		CIP 442913*
CRIWAC 25-10*		NAV 001		CIP 442237*
CRIWAC 26-10		AAT 03-025*		CIP 443019
CRIWAC 27-10*		B/FASO 001*		CIP 442850*
CRIWAC 28-10*		ZAMBEZI*		
CRIWAC 29-10*				
CRIWAC 30-10				
CRIWAC 31-10*				
CRIWAC 32-10*				

*List of the 76 sweetpotato accessions used for the molecular characterization.

constructed using GGE Biplot software (Yan and Kang, 2003).

Molecular characterization using SSR markers

Genetic material

A total of 76 sweetpotato accessions were used for the study (Table 1). These represent four groups, which were collections from International Potato Centre (CIP) gene bank in Ghana (19), local collection from farmers' field (19), local improved varieties (12), and local and exotic collections sourced from the National Agricultural Research Systems (NARS) or Programmes (26). These were planted at the CSIR-Crops Research Institute research field at Fumesua which is in the forest ecozone.

DNA extraction

This was done at the Molecular Laboratory of the CSIR-Crops Research Institute, Fumesua using the method of Egnin et al. (1998), in 2012. Two hundred milligram of young tender leaf tissue was weighed into 2 ml Eppendorf tube and was ground to powder after freeze drying with liquid nitrogen. Eight hundred microliter (800 µl) of buffer A [1M Tris HCl (pH 8) = 50 mM, 5 M NaCl = 300 mM, 0.5M EDTA (pH 8) = 20 mM, PVP = 20%, Sodium Metabisulphate = 1 g/100 ml, 20% Sercosine = 1.5] was added and incubated at 90°C for 10 min, and vortexed every 5 min. The suspension was cooled at room temperature for 2 min after which 400 µl of 5 M potassium acetate was added and then gently mixed by inversion 5 to 6 times. The suspension was then incubated on ice for 30 min with continuous shaking, followed by centrifuging at 13,000 rpm for 10 min. The upper phase was transferred to a new Eppendorf tube. One volume of cold isopropanol and $1/10^{th}$ of 3 M sodium acetate was added and mixed 10X by inverting the tube. This was followed by incubation at -20°C for 1 h, and centrifuging at 13,000 rpm for 10 min. The supernatant was poured off, the pellets were washed with 800 µl, 80% ethanol, and centrifuged at 14,000 rpm for 5 min. The alcohol was then discarded and the pellets were dried. Five

Table 2. List of agro-morphological descriptors and root quality traits.

Foliage descriptors	Agronomic descriptors and storage root morphology	Storage root quality descriptors
Vine inter-node length	Storage root shape (1 - 9)	Dry matter
Vine inter-node	Variability of storage root shape (3 - 7)	Fructose
Diameter	Storage root surface defects (0 - 8)	Glucose
	Storage root cortex thickness (1 - 9)	Sucrose
Vine colour (1 - 9)	Storage root skin colour (1- 9)	Maltose
Vine tip pubescence (0 - 7)	Storage root flesh colour (1 - 9)	Total sugars
Mature leaf size	Storage root formation (1 - 7)	Beta-carotene
Petiole length	Storage root stalk (0 - 9)	Starch
Petiole pigmentation (1 - 9)	Number of storage roots/plant	Protein
Vine weight	Number of storage root (marketable)	Calcium
General outline of leaf (1 - 7)	Number of storage root (unmarketable)	Magnesium
	Weight of storage root	Iron
	Weight of storage root (Marketable)	Zinc
	Weight of storage root (Unmarketable)	
	Variability of storage root size	
	Harvest index	
	Latex production in storage roots (3 - 7)	
	Oxidation in storage roots (3 - 7)	

Values in parenthesis indicate scale of measurement

hundred microliter (500 µl) of 1X TE buffer was used to dissolve the pellets, followed by the addition of 4 µl RNase A, and incubation at 37°C for 30 min. This was followed by addition of 250 µl of 7.5 M ammonium acetate. The suspension was incubated on ice for 3 min, and centrifuged at 13,000 rpm for five minutes, and then transferred into a new 1.5 ml tube. Seven hundred microliters (700 µl) of isopropanol was added, mixed by inversion (ice inversion), and centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the pellets were washed with 1 ml 80% ethanol by centrifuging at 14,000 rpm for five minutes. Again the supernatant was discarded, followed by drying of the pellets at room temperature. The DNA pellets were then dissolved in 200 µl 1X TE buffer, and its quality was checked on 0.8% agarose gel.

Genotyping with simple sequence repeats (SSR) markers

The genotyping was carried out at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India in 2012. A 3 ng sample of total genomic DNA from each of the samples was used for the polymerase chain reactions (PCRs). Twenty-five pairs of SSR markers confirmed for sweetpotato DNA amplification (Buteler et al., 1999; Diaz and Gruneberg, 2008; Tumwegamire et al., 2011) were used (Table 3). A final volume of the reaction mixture of 10 µL, which contains 25 mM MgCl2, 10x buffer, 10 mM deoxyribonucleotide triphosphate (dNTPS), 1 µM M13 FORWARD 700/800, 1 µM forward primer, 1 µM reverse primer, 5 U µL–1 *Taq* polymerase, 3 ng µL–1 DNA, and a double distilled water were used for the PCR. The amplification conditions were set up at 94°C for four minutes and denaturation at 94°C for one minute; annealing at between 56.0 to 62.0°C (depending on the annealing temperature of the primer); and polymerization at 72°C for one minute. Step 2 annealing was 56.0 to 62.0°C (depending on the annealing temperature of the primer) and was repeated 30 times, and a final extension at 72°C for 7 min. Amplification products were analyzed and read on a computer automated Licor (4300) DNA Analyzer (Licor Biosciences, Lincoln, NE) for 25 pairs of SSR primers.

Simple sequence repeats data scoring and analysis

Accessions amplified were noted and used to estimate percent accessions amplified. The number of alleles for each marker was noted and recorded. Markers that showed variation in at least 25% of the accessions were noted and their alleles were recorded as unique alleles. Percent unique alleles were computed as the ratio of number of unique alleles to the total number of alleles. Genotypes were scored for the presence (1) or absence (0) of each fragment. NTSYSpc software version 2.1 (Rohlf, 1993, 2002) was used to run the binary data. Jacard's coefficients (Jaccard, 1908) were used to construct a similarity matrices from the binary data by using SIMQUAL algorithm. This was followed by construction of a dendrogram using the unweighted paired group method average (UPGMA) applying the SHUAN algorithm. Principal Coordinate Analysis (PCoA) was performed from Jacard's coefficients using Genstat (Genstat, 2007). The polymorphic information content (PIC) was determined based on the approach and method of Weir (1996) as presented below:

$$PIC = 1 - \Sigma P_i^2$$

Where, Pi is the frequency of the ith allele.

Analysis of Molecular Variance (AMOVA) was also performed using Arlequin 3.1 version computer software (Excoffier et al., 2005), to quantify the genetic variation and relationship existing between and among the sweetpotato and the four population groups studied.

Table 3. List and description of the 25 SSR markers used to characterize the sweetpotato accessions.

Marker	Repeat	Primer F	Primer R	Size	Temperature (°C)	Reference
Ib3/24	Not determined	TTTGGCATGGGCCTGTATT	GTTCTTCTGCACTGCCTGATTC	-	56	Tseng et al. (2002)
Ib-316	(CT)3C(CT)8	CAAACGCACAACGCTGTC	CGCGTCCCGCTTATTTAAC	150	58	Buteler et al. (1999)
Ib-242	(CT)3CA(CT)11	GCGGAACGGACGAGAAAA	ATGGCAGAGTGAAAATGGAACA	135	58	Buteler et al. (1999)
Ib-297	(CT)13	GCAATTTCACACACAAACACG	CCCTTCTTCCACCACTTTCA	134	58	Buteler et al. (1999)
IBCIP-1	(ACC)7	CCCACCCTTCATTCCATTACT	GAACAACAACAAAAGGTAGAGCAG	140-153	63	Yañez (2002)
IBCIP-2	(ACC)2+6	GTAACCTGTCAGCCATCTGT	CCTAGTGGGTATTTGCAGAG	268-290		Yañez (2002)
IbC12	(TTC)6	TCTGAGCTTCTCAAACATGAAA	TGAGAATTCCTGGCAACCAT	94-108	55	Solis et al. (2009)
IbS01	(AGA)10	TCCTCCACCAGCTCTGATTC	CCATTGCAGAGCCATACTTG	210-228	56	Benavides et al. (2005)
IbR03	(GCG)5	GTAGAGTTGAAGAGCGAGCA	CCATAGACCCATTGATGAAG	245-263	56	Benavides et al. (2005)
IbS07	(TGTC)7	GCTTGCTTGTGGTTCGAT	CAAGTGAAGTGATGGCGTTT	177-194	55	Benavides et al. (2005)
IbS10	(CT)12	CTACGATCTCTCGGTGACG	CAGCTTCTCCACTCCCTAC	253-298	60	Benavides et al. (2005)
IbS11	(TTC)10	CCCTGCGAAATCGAAATCT	GGACTTCCTCTGCCTTGTTG	217-242	60	Benavides et al. (2005)
IbS17	(GGA)4	CAGAAGAGTACGTTGCTCAG	GCACAGTTCTCCATCCTT	158-198	58	Benavides et al. (2005)
IbS18	(TAGC)4	CTGAACCCGACAGCACAAG	GGGAAGTGACCGGACAAGA	232-242	58	Benavides et al. (2005)
IbR12	(CAG)5A	GATCGAGGAGAAGCTCCACA	GCCGGCAAATTAAGTCCATC	331-393	60	Benavides et al. (2005)
IbR13	(TTC)6	GTACCGAGCCAGACAGGATG	CCTTTGGGATTGGAACACAC	205-258	60	Benavides et al. (2005)
IbR14	(CCT)6	CCTATGGCAATTCGGTCACT	GGAACATTGCCTACACTCTG	216 -222	58	Benavides et al. (2005)
IbR16	(GATA)4	GACTTCCTTGGTGTAGTTGC	AGGGTTAAGCGGGAGACT	196-215	60	Benavides et al. (2005)
IbR19	(CAG)5b	GGCTAGTGGAGAAGGTCAA	AGAAGTAGAACTCCGTCACC	192-213	60	Benavides et al. (2005)
IbR21	(GAC)5	GACAGTCTCCTTCTCCCATA	CTGAAGCTCGTCGTCAAC	169-186	58	Benavides et al. (2005)
IbR20	(GGC)5	CTTCACTCTGCTCGCCATTA	GTACTTGGACGGGAGGATGA	194-212	48	Benavides et al. (2005)
J175	(AATC)4	ATCTATGAAATCCATCACTCTCG	ACTCAATTGTAAGCCAACCCTC	-	58	Solis et al. (2009)
J10A	(AAG)6	TCAACCACTTTCATTCACTCC	GTAATTCCACCTTGCGAAGC	-	58	Solis et al. (2010)
J67	(GAA)5	CACCCATTTGATCATCTCAACC	GGCTCTGAGCTTCCATTGTTAG	-	58	Solis et al. (2011)
J116A	(CCT)6	TCTTTTGCATCAAAGAAATCCA	CCTCAGCTTCTGGGAAACAG	-	58	Solis et al. (2012)

RESULTS

Phenotypic variation

The first six Principal Components (PCs) with Eigen values greater than 1.0 jointly explained 54.86% of the total variation in the accessions based on the 40 agro-morphological and physicochemical traits studied (Table 4). The traits of importance for the first component involved root traits of commercial interest. Beta-carotene, dry matter and total sugar contents were of importance for PC2.

The mean performance of the top 10 and the bottom 10 selected accessions for beta-carotene, dry matter and sugar contents are presented in Table 5. Significant differences were observed between the accessions for the traits. The range of values obtained for beta-carotene content was 6.83 - 33.67 (mg/100 g) DW. For dry matter content the range was 27 - 50%, and for sugar content the range was 9.83 - 30.34%. Ogyefo and Apomuden had the lowest and highest values for beta-carotene content. Apomuden had the lowest dry matter content whilest FA-10-026 had the highest dry matter content. CRIWAC 19-10 and CIP 442850 gave the lowest and highest sugar

Table 4. Principal component analysis of the agro-morphological and physico-chemical traits.

Trait	PC1	PC2	PC3	PC4	PC5	PC6
Root weight	-0.371	-0.091	-0.133	0.005	-0.028	-0.033
Marketable root wgt.	-0.362	-0.082	-0.139	0.021	-0.003	-0.008
Unmarketable yield.	-0.370	-0.094	-0.128	-0.002	-0.038	-0.043
β-carotene	0.168	-0.310	-0.128	-0.050	-0.030	0.024
Calcium	0.035	-0.300	0.205	0.155	-0.143	-0.063
Dry matter	0.168	-0.310	-0.128	-0.050	-0.030	0.024
Iron	0.172	-0.035	-0.416	0.063	-0.126	-0.099
Fructose	0.041	-0.259	-0.056	-0.257	0.343	0.173
Glucose	0.023	-0.316	0.020	-0.212	0.307	0.143
Maltose	-0.046	-0.284	0.310	0.119	-0.197	0.009
Magnesium	0.086	-0.308	-0.140	0.043	-0.049	-0.086
Rt. Oxidation	-0.064	0.020	-0.111	0.069	-0.032	0.414
Protein	0.114	0.071	-0.401	0.107	-0.178	-0.125
Starch	-0.141	0.144	0.361	0.201	-0.133	-0.018
Sucrose	0.018	-0.235	0.145	0.191	-0.336	-0.143
Total sugar	0.029	-0.404	0.133	0.030	-0.012	-0.025
Zinc	0.157	-0.021	-0.364	0.147	-0.249	-0.140
Outline of leaf	0.087	0.017	0.091	0.106	-0.052	0.090
Harvest index	-0.245	0.016	-0.012	-0.323	-0.165	-0.165
Latex in roots	-0.001	0.037	-0.015	-0.005	-0.153	0.128
Mature leaf size	-0.165	-0.031	-0.010	0.301	0.125	0.037
Storage root no.	-0.371	-0.091	-0.133	0.005	-0.028	-0.033
Marketable roots no.	-0.325	-0.043	-0.097	-0.087	-0.089	-0.26
Unmarketable rt. no.	-0.144	-0.101	-0.087	0.164	0.102	0.094
Petiole length	-0.119	0.079	-0.085	0.184	0.226	-0.156
Petiole pigmentation	-0.021	0.115	-0.001	-0.185	-0.153	0.260
Cortex thickness	-0.007	0.122	-0.051	0.104	-0.006	0.014
Flesh colour	0.118	0.124	0.061	0.023	0.040	-0.335
Root formation	0.070	0.014	-0.022	-0.025	-0.150	0.039
Root shape	-0.038	0.034	0.062	0.045	0.056	-0.310
Root skin colour	-0.011	-0.013	-0.114	0.008	-0.239	0.346
Root stalk	0.113	0.075	-0.029	0.129	0.146	0.144
Root surface defects	-0.056	-0.066	-0.131	-0.020	0.113	-0.083
Root shape	0.057	0.070	0.011	0.038	0.047	-0.148
Root size variability	-0.079	0.038	0.053	0.116	-0.201	0.168
Vine colour	-0.032	0.164	-0.009	-0.116	-0.261	0.254
Inter-node diameter	-0.105	-0.012	-0.051	-0.116	0.194	0.015
Inter-node length	-0.037	-0.067	-0.075	0.200	0.011	0.185
Vine tip pubescence	0.013	-0.017	-0.026	0.300	0.129	0.096
Vine weight	0.023	0.001	-0.039	0.441	0.156	0.137
Latent roots (Eigen vectors)	6.304	4.501	3.688	2.817	2.419	2.215
Variability (%)	15.76	11.25	9.22	7.04	6.05	5.54
Cumulative (%)	15.76	27.01	36.23	43.27	49.32	54.86

*Values in bold indicate the most relevant characters (>0.3) that contributed most to the variation of the particular component.

contents, respectively.

The dendrogram separated the selected accessions with a Euclidean similarity distance ranging from 1.00 to 0.93 (Figure 1). At 1.00 level of similarity, all the accessions were distinct from each other except BOT 03-030 and CIP 442896. Conversely, at about 0.93 levels of significance, two clusters were identified with all the accessions being similar except for CRIWAC 12-10. Five

Table 5. Performance of the top 10 and bottom 10 accessions selected based on beta-carotene, dry matter and sugar contents for construction of dendrogram and GGE biplot.

Accession	Total sugars (%)	Accession	Beta-Carotene (mg/100 g) DW	Dry matter (%)
Top 10 accessions			**Top 10 accessions**	**Bottom 10 accessions**
CIP 442850	30.34	APOMUDEN	33.67	27
APOMUDEN	28.97	RESISTO	27.53	38
B/FASO 002	24.04	B-REGARD	24.31	32
CIP 440062	23.30	CRIWAC 03-10	23.32	32
B-REGARD	22.90	CIP 442850	20.21	27
CRIWAC 12-10	22.84	CIP 443035	19.75	36
B/FASO 001	22.69	CRIWAC 05-10	19.00	39
TAG 03-030	21.92	BOT 03-028	17.83	38
CIP 440071	21.84	ZAMBEZI	17.58	40
UKEREWE	21.10	BOT 03-020	17.35	39
Bottom 10 accessions			**Bottom 10 accessions**	**Top 10 accessions**
CRIWAC 25-10	12.54	FA 10-026	16.75	50
CRIWAC 30-10	12.45	HISTARCH	9.85	45
DOS O3-006	12.35	CIP 442264	7.74	45
AAT 03-025	12.26	ABAIDOO 01	7.00	44
CRIWAC 11-10	12.26	BD 96-029	12.97	43
CIP 440095	12.06	OGYEFO	6.83	42
OGYEFO	11.67	FARAA	12.27	42
CIP 442264	11.06	CRIWAC 31-10	9.74	41
HISTARCH	10.43	CIP 442896	11.27	40
CRIWAC 19-10	9.83	BOT 03-030	17.35	39
SED (P<0.05)	2.62	SED (P<0.05)	1.52	3.00

main clusters A, B, C, D, and E at 94.5% (0.945) level of significance were identified. The first four clusters contained 1 to 5 accessions per cluster while the fifth cluster (E) had 26 accessions.

The distribution of PC1 and PC2 among the correlated traits, the selected accessions as well as between the selected accessions and the correlated traits are shown in Figure 2. Three groups were observed for the correlated traits. Beta-carotene, fructose, total sugars, calcium (Ca), and magnesium (Mg) were grouped together in Quadrant 1. Storage root yield traits were grouped in Quadrant 2, while only dry matter was found in Quadrant 3. Four groups were detected for the accessions. Beauregard and Apomuden were the most distantly related accessions in Quadrant 1, whilest CIP 440032 and CIP 442264 were the most distantly related accessions in Quadrant 2. The most distantly related accessions in the third and fourth quadrants were Histarch and Ogyefo, and CIP 442850 and TAG 03-030, respectively.

Genotypic variation

Out of the 25 SSR markers used to assess the genetic diversity of the sweetpotato accessions, only 20 produced amplifications. The five markers that did not produce amplification were IbS01, IbS07, IbS10, IbCIP2 and IbR20. A total of 87 polymorphic alleles were observed across the accessions and loci. These ranged from two to six with mean of 4.25. Markers IbS18 and IbR21 recorded the lowest number of alleles while Ib3/24, Ib316, Ib-297, IbC12, IbS11, J10A and J116A recorded the highest number of alleles (Table 6). Out of the 87 alleles revealed by the 20 SSR markers across accessions and loci, 40 (45.98%) were unique alleles and the average number of unique alleles was two. IBCIP-1, IbC12 and J67 produced no unique alleles while Ib3/24 recorded the highest number (5) of unique alleles followed by Ib-297 and J10A with 4 unique alleles. However, Ib3/24 obtained the highest percent polymorphism (83.33%), followed by IbR14 (75.00%). The range and the average percent polymorphism were 0 to 83.33 and 45.50%, respectively. The PIC values were high and ranged between 0.62 for J67 and 0.96 for IbR16 and IbR19, with a mean of 0.84. The highest amplification was recorded by IbR14 (90.91%) followed by IbR316 and J67 with value of 77.92%. IbR16 recorded the lowest amplification. Base range for the markers was highest and lowest for IbR03 (262-277) and J175 (133-147).

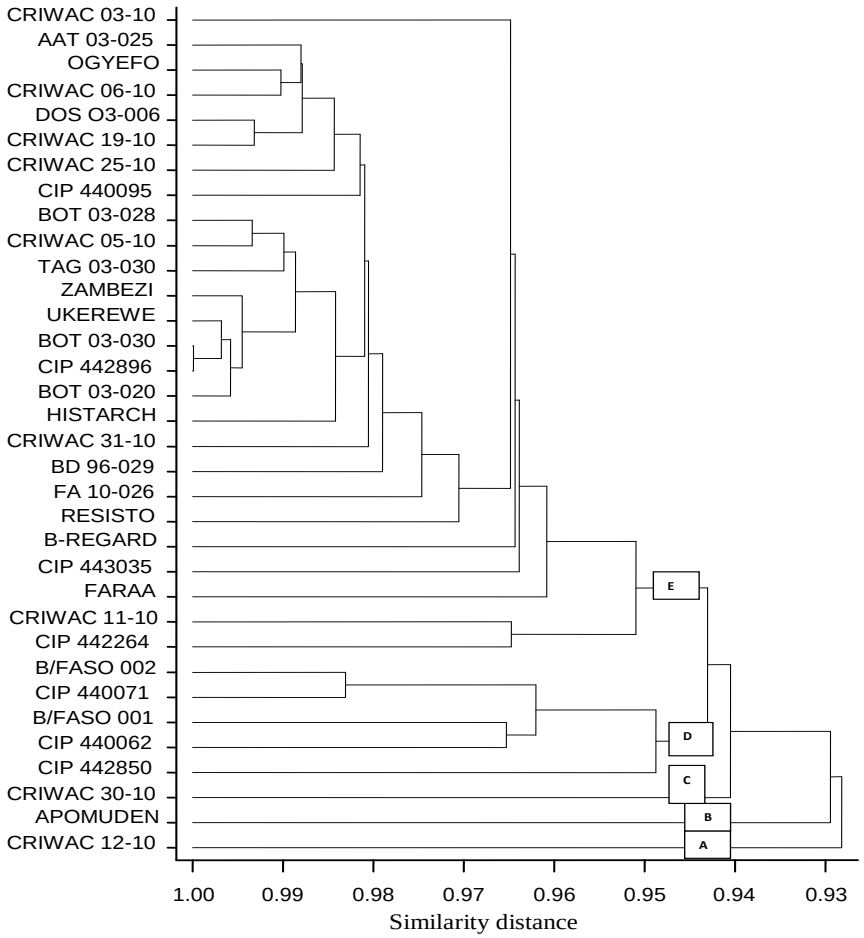

Figure 1. Dendrogram constructed based on the selected accessions and traits important for PC1 and PC2.

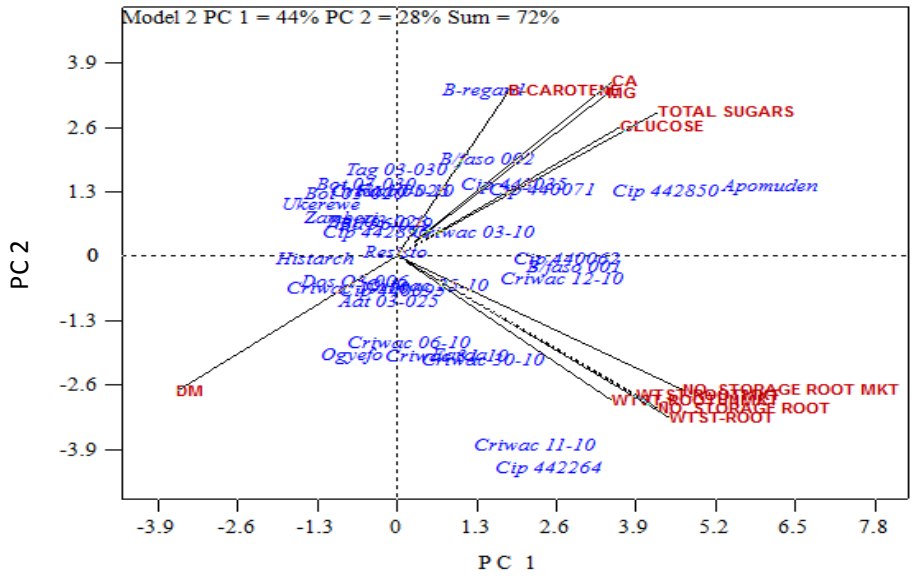

Figure 2. Biplot showing relationship between correlated traits and selected accessions.

Table 6. Polymorphism and base range of the 20 SSR markers.

Marker	Accessions Amplified	Accessions Amplified (%)	No. of Alleles	Loci across Accessions	No. of unique Alleles	Percent Polymorphism	PIC	Base Range
Ib3/24	47	61.04	6	1 - 2	5	83.33	0.87	136 - 150
Ib-316	60	77.92	6	1 - 4	2	33.33	0.66	152 - 168
Ib-242	39	50.65	4	1 - 4	2	50.00	0.90	135 - 155
Ib-297	40	51.95	6	1 - 4	4	66.67	0.86	151 - 183
IBCIP-1	38	49.35	4	1 - 4	0	0.00	0.89	154 - 166
IbC12	54	70.13	6	2 - 6	0	0.00	0.72	108 - 123
IbR03	43	55.84	4	1 - 4	1	25.00	0.86	262 - 277
IbS11	47	61.04	6	1 - 6	2	33.33	0.88	241 - 256
IbS17	51	66.23	5	1 - 3	3	60.00	0.84	181 - 202
IbS18	40	51.95	2	1 - 2	1	50.00	0.87	249 - 253
IbR12	57	74.03	4	1 - 3	2	50.00	0.73	336 - 357
IbR13	32	41.56	4	1 - 4	2	50.00	0.91	222 - 231
IbR14	70	90.91	4	1 - 2	3	75.00	0.75	179 - 188
IbR16	30	38.96	3	1 - 3	2	66.67	0.96	220 - 230
IbR19	31	40.26	3	1 - 3	1	33.33	0.96	212 - 223
IbR21	42	54.55	2	1 - 2	1	50.00	0.84	182 - 203
J175	46	59.74	3	1 - 3	2	66.67	0.93	133 - 147
J10A	38	49.35	6	1 - 4	4	66.67	0.91	192 - 220
J67	60	77.92	3	1 - 3	0	0.00	0.62	191 - 212
J116A	50	64.94	6	1 - 5	3	50.00	0.84	206 - 229
Mean	45.75	59.42	4.35	1.1 - 3.4	2	45.50	0.84	192.1 - 208.7

IbS11 recorded the highest number of loci (1- 6) across accessions followed by IbC12 (2 - 6). The lowest number of loci (1-2) across accessions was produced by Ib3-24, IbS18, IbR14 and IBR21.

Principal coordinate analysis (PCoA), which was determined from the similarity coefficients is graphically presented in Figure 3 (showing diversity in sweetpotato accessions), and Figure 4 (showing diversity in the group structure of the sweetpotato accessions). The two axes explained 45.21% of the total similarity (54.79% of total variation) with the first axis (PCoA1) accounting for 28.08% and the second (PCoA2) accounting for 17.13%. The 76 sweetpotato accessions investigated by PCoA did not form clear groups according to the group structure both within and between.

The dendrogram constructed separated the 76 sweetpotato accessions into major clusters at different similarity levels ranging from 0.00 to 1.00 (Figure 5). At slightly greater than 0.00 similarity level, two major clusters were observed. CIP 6 (CIP 442462) constitutes the first cluster while the second cluster consisted of the other 75 accessions. At 0.25 similarity level, seven major clusters were observed while 17 were found at 0.50 similarity level. The markers fully discriminated the 76 sweetpotato accessions by the 1.00 level of similarity except for two improved cultivars LOCIMP2 (Santompona) and LOCIMP10 (Otoo). The primers, however, did not fully discriminate the accessions into the different group structures.

Significant differences were observed between the sweetpotato accession within the groups ($P<0.01$) as well as between the groups ($P<0.05$)

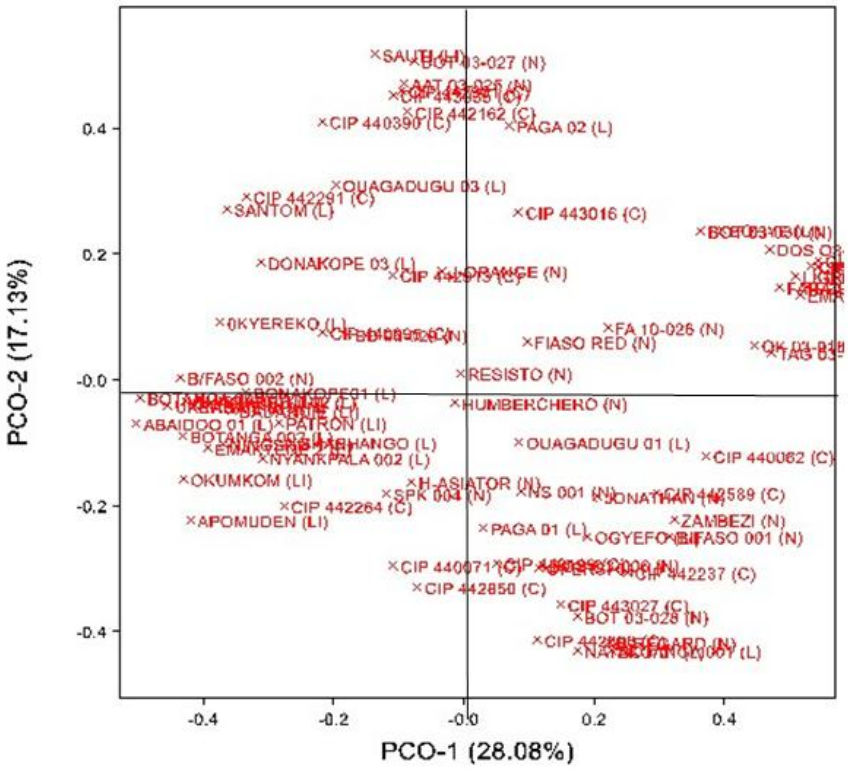

Figure 3. Principal coordinates analysis from similarity coefficients showing diversity in the 76 sweetpotato accessions.

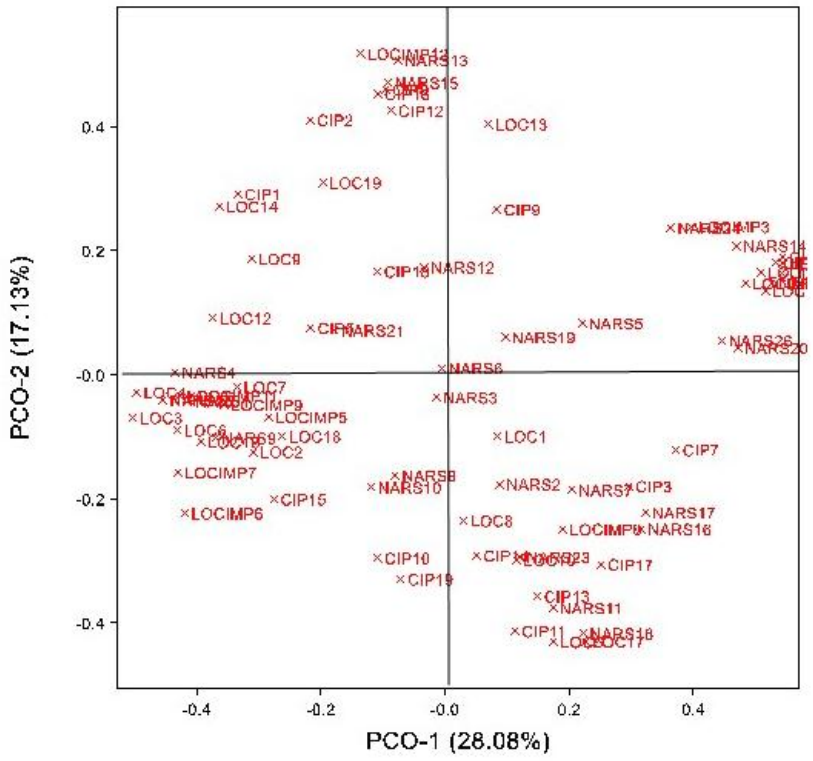

Figure 4. Principal coordinates analysis from similarity coefficients of 76 sweetpotato accessions showing the diversity in the group structure.

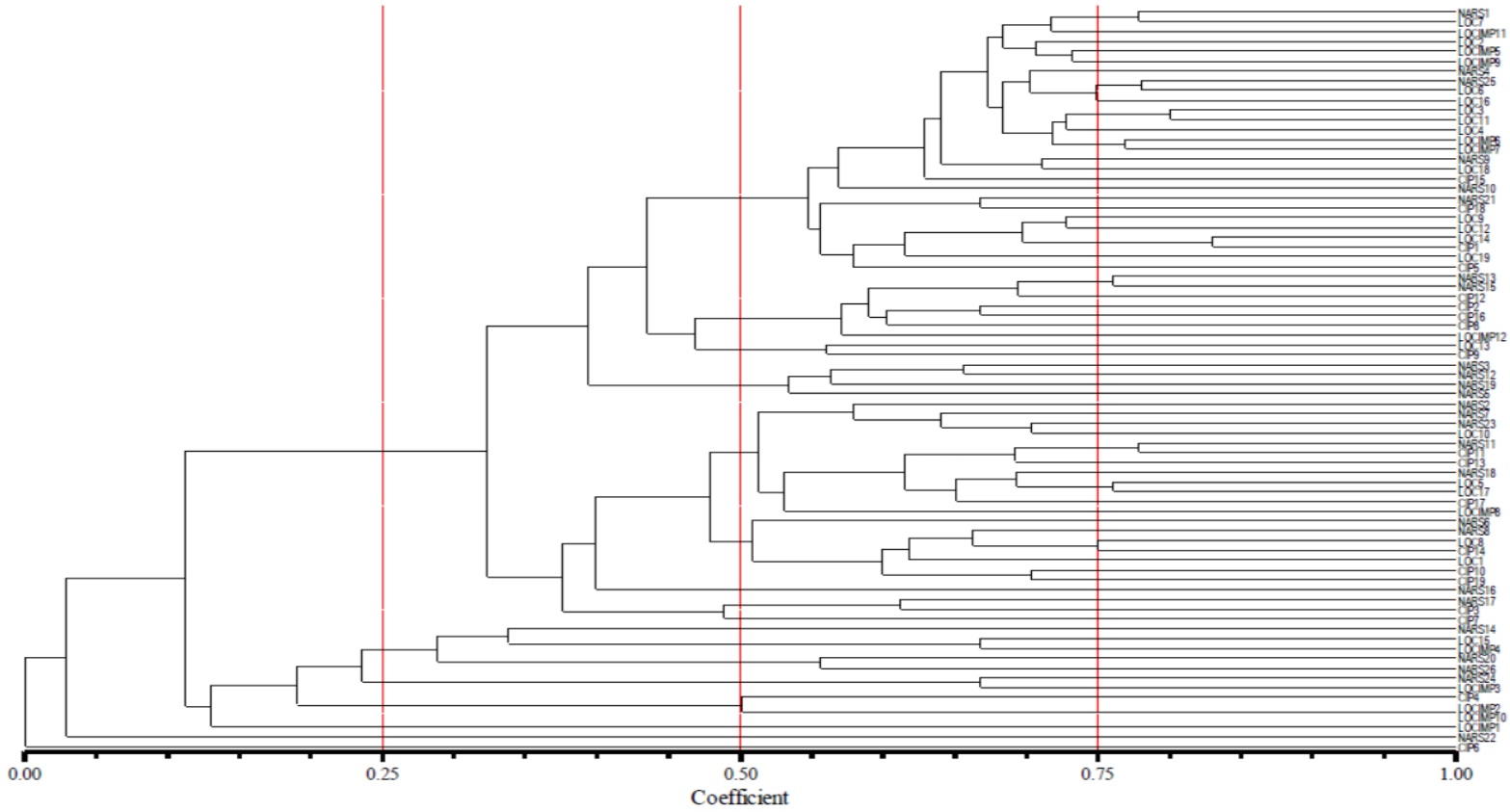

Figure 5. Dendrogram showing genetic relationships among 76 sweetpotato accessions.

as shown in Table 7. The differences observed within the groups however accounted for a greater percentage (97.12%) of variation observed than that found between the groups (2.88%).

DISCUSSION

Variability was observed in all the physico-chemical traits and 20 out of the 27 agro-morphological traits. This indicates a high degree of agro-morphological and physicochemical polymorphism among the accessions. Diversity in flesh colour (beta-carotene content) of sweetpotato cultivars has been reported (Warammboi et al., 2011). Sugar content in sweetpotato is also reported to be cultivar-dependent (Ravindran et al., 1995; Aina et al., 2009), and showed high levels of polymorphism with SSR markers. This confirms the discriminatory capacity of the SSR markers on sweetpotato (Gichuru et al., 2006; Tumwegamire et al., 2011). High level of polymorphism was observed in this study with an allele range of two to six alleles per SSR marker and this is in agreement with Yada et al. (2010). Buteler et al. (1999) obtained high polymorphism with an allele range of 3 to 10. Somé et al. (2014), also reported 1 to 8 alleles. A range of 2 to 11 alleles was reported by Tumwegamire et al. (2011). A lower level of polymorphism, ranging between one and

Table 7. Analysis of molecular variance (AMOVA) for the 76 sweetpotato accession.

Source of variation	Df	Sum of squares	Variance components	Percentage of Variation
Among groups	3	55.894	0.35619*	2.88
Within groups	72	865.198	12.01664**	97.12
Total	75	921.092	12.37284	-

*Significant at 0.05 **Significant at 0.01.

four alleles per SSR locus has also been reported (Hwang et al., 2002). Differences observed may be attributed to the use of different SSR primers, sweetpotato genotypes and annealing temperatures. Varying number of SSR primers used in diversity studies may also account for the differences in observations.

Hwang et al. (2002) attributed high level of polymorphism to large genome size and heterozygosity of sweetpotato. It should also be noted that genetic diversity due to heterozygosity in sweetpotato is driven by both the mating system (outcrossing in combination with self-incompatibility) and the high ploidy level of the crop (autohexaploid) (Tumwegamire et al., 2011). The AMOVA and ANOVA results also indicated significant differences within and between the different sweetpotato groups studied. These results demonstrate significant genetic diversity and indicates that meaningful selection and improvement of these traits is possible (Mohammed et al., 2012; Nwangburuka and Denton, 2012). Furthermore, these demonstrate the existence of diversity at the individual genotype level that can be exploited to obtain trait combinations in specific varieties. In addition, the divergences indicate that it is possible to select contrasting parents from these accessions for improvement of beta-carotene, sugar and dry matter contents in sweetpotato. These results agree with results of other researchers (Zhang et al., 2000; 2001; Gichuki et al., 2003; Gichuru et al., 2006; Abdelhameed et al., 2007; Grüneberg et al., 2009; Tumwegamire et al., 2011).

PIC is a measure of the discriminatory capacity of a marker (Jia et al., 2009). According to Heng-Sheng et al. (2012), a PIC value greater than 0.5 is high, and any marker with such value may be effective in genetic diversity study. In this study, the PIC value for all the markers that showed amplification were greater than 0.5. This implies that the values which ranged from 0.62 to 0.96 with mean of 0.84 were very high indicating a high discriminating power of the SSR markers used. These values are greater than range and mean of 0 to 0.88, and 0.72 reported by Somé et al. (2014). Based on the number of unique alleles and the PIC values, all the SSR markers that showed amplification were very effective in discriminating among the sweetpotato accessions. In spite of this, the markers did not discriminate between cultivars LOCIMP2 (Santompona) and LOCIMP10 (Otoo) at 1.00 level of similarity even though these cultivars are agro-morphologically distinct. It is probable that no were repeats found that could differentiate the two cultivars and therefore, more SSR markers need to be used in the future to have a full diversity study.

Genetic relationships between traits may result from pleiotropic gene effects, linkage of two genes, linkage disequilibrium and epistatic effects of different genes or environmental influences (Falconer and Mackay, 1996). The strong negative relationship found for sugar content and dry matter content as depicted in the GGE biplot indicates that it is possible to develop non-sweet high dry matter sweetpotato varieties. A similar observation was made by Gruneberg et al. (2009), who also reported that development of non-sweet sweetpotato varieties should not be too difficult. However, developing non-sweet, high dry matter and high beta-carotene sweetpotato varieties could be challenging due to the strong negative association between dry matter content and beta-carotene content, and the positive association existing between beta-carotene and the sugar content. Breeding for such cultivars may require many cycles of selection and hybridization to break genetic linkages associated with the traits. However, beta-carotene seems to be controlled by a limited number of genes and should be easy to manipulate.

Conclusion

This study provides estimate on the level of genetic variation among sweetpotato accessions in Ghana. Significant genetic diversity was found between the accessions for dry matter, beta-carotene and sugar content. This information can be used in sweetpotato germplasm management and improvement in Ghana. The study also affirmed the discriminatory capacity of the SSR markers, and the agro-morphological and physico-chemical markers for sweetpotato characterization especially for breeding programmes with limited resources. Sufficient useful genetic variation is present in the accessions studied which may be exploited to provide for substantial amount of improvement through selection of superior genotypes. The strong negative association between dry matter and sugar content indicates that it is feasible to develop non-sweet high dry matter sweet potato cultivars which are the preferred sweetpotato varieties in Ghana. However, developing non-sweet, high dry matter and high beta-carotene sweetpotato varieties

may require many cycles of selection due to the strong negative association between dry matter content and beta-carotene content.

Conflict of Interests

The authors declare they have no conflict of interests.

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