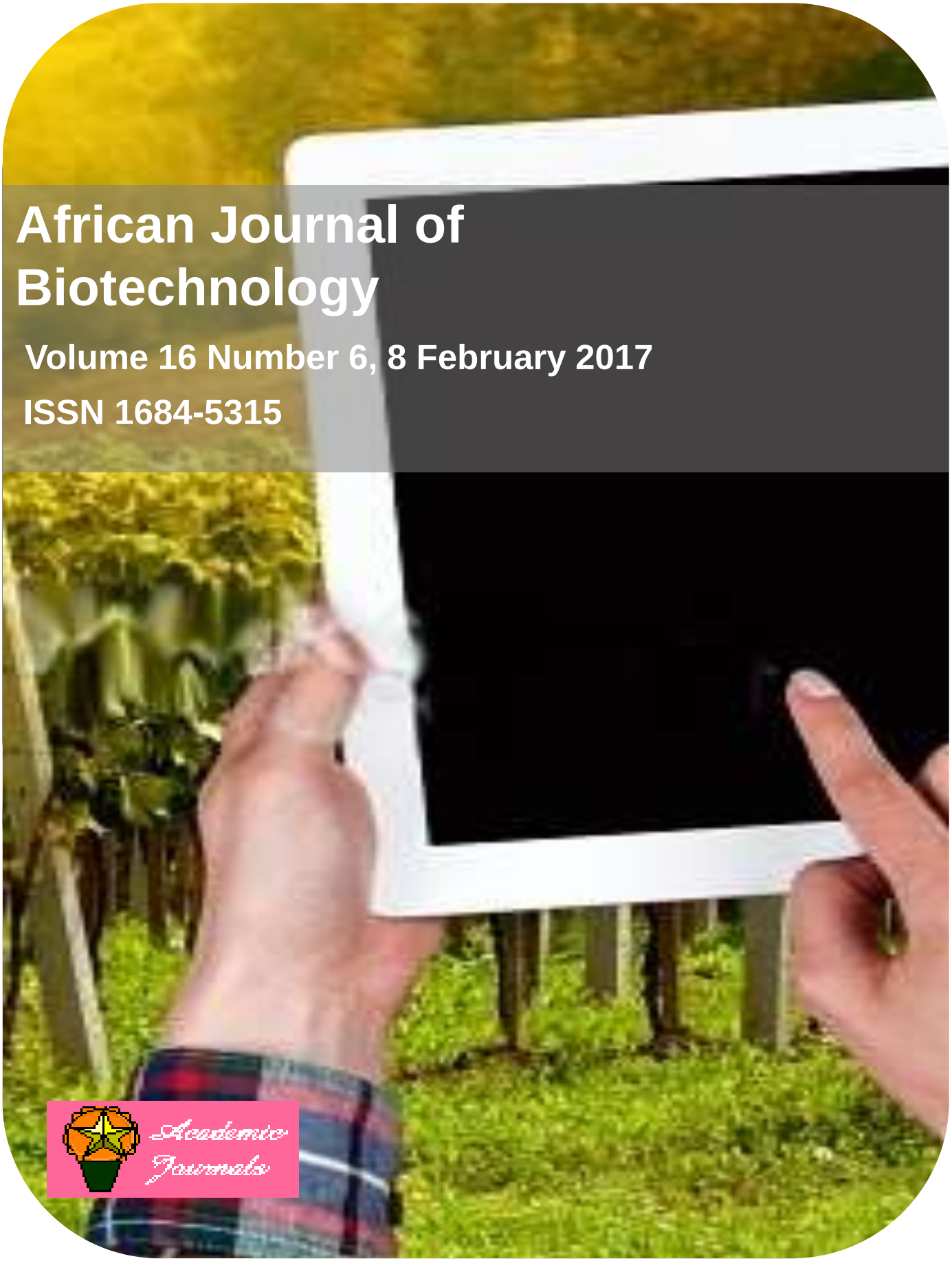

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Review

The potential of *Aspergillus* species in transformation of agricultural products for sustainable production of textile and leather industries in Tanzania

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Manufacturing industries contribute about 8% of the gross domestic products (GDP) whereas leather and textile industries supply 18% employments of manufacturing industries in Tanzania. Manufacturing industries merely depend on agriculture for raw materials and other inputs. However, processing of leather and textiles requires a lot of inputs, many of them supplied from agricultural produce and some are imported from developed nations. In vast developing countries, including Tanzania, manufacturing industries are constrained by limited production technology and the allied costs than raw materials. The conventional processing of leather and textiles requires immense technological investment that is associated with high production cost. In Tanzania, inputs such as soaking, bating and tanning agents for tannery industries are expensive and sometimes not readily available due to importation costs. On the other hand, management of waste effluents from leather and textile processing is the major impediment for development of these industries. However, natural processing of covering materials by using fungal biotechnology is of great concern in Tanzania to avert the prevailing constrains. The application of fungal based biotechnology would reduce production cost and health consequences resulting from chemicals, particularly, chromium. The effects of toxic chemicals from leather and textile industries would be mitigated by employing non-viable *Aspergillus* biomass in the industrial processes. This would minimize production of the harmful wastes from the industries. As a result, leather and textile production would be achieved at low cost, without hazardous waste production, resulting to safer products for both human and the environment. This review evaluates current production of leather and textile industries and highlights the potentials of microbial biotechnology.

Key words: Biotechnology, *Aspergillus*, leather and textile, industrial effluents, agriculture.

INTRODUCTION

Agriculture remains the major supplier of food and income, to majority of rural communities though its production is less than demands (Korotayev and Zinkina, 2015; Shombe, 2008). In order to sustain life of both rural and urban inhabitants, agricultural produce must be processed and the value added will obtain new goods or increase the shelf life of existing ones (Shombe, 2008). Industrialization is the key for transforming agricultural

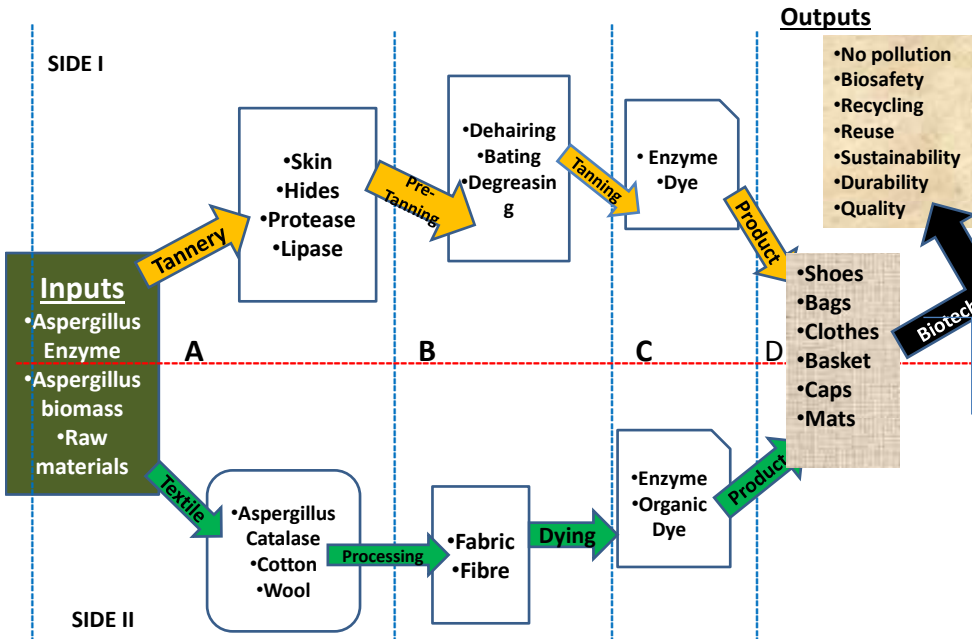

Figure 1. *Aspergillus* enzymic processing of textile and leather products.

produce by processing them into worthy products (Mwaigomole, 2014). Textile and leather are important ancient industries in Tanzania that contributed to, youth employment and exportation of goods in 1980's (Mwaigomole, 2014). Tanzania is the major producer of skin and hides which supply the industry with raw material and could export the excess.

However, the quality of hides and skin produced is squat due to poor handling and processing technologies (China and Ndaro, 2015). On the other hand, pest damage to agricultural produce weakens the quality of produce prior to processing (Kanui et al., 2016; Sertse and Wossene, (2007)), who reported the economic impact of ticks in tannery industries, in Ethiopia. Moreover, transformation of hides and skin into leather requires chemicals or enzymes as depicted in Figure 1, but chemicals are the current option in Tanzania. The cost of chemicals to the environment led to closure of some industries and is the main hindrance to production and exportation of goodsin Tanzania (ITC, 2016; China and Ndaro, 2015). Shutting down of these industries causes losses to owners and denies the national revenue. Other constrains challenging leather and textile industries are management of waste, that are claimed to be toxic to the environment. However, microbial industrial processing of skin hides and fibre into products is reported to be effective and safe.

Microbes are free living organisms found almost everywhere in the environment (Finlay, 2002). Their abundance ranges from marine, terrestrial, to fossils (Foissner, 2006). This is because they are well adapted to various environmental conditions (Finlay, 2002). Microbes are the ancient organisms that colonized the land and claimed to be the origin of life on earth (Falkowski et al., 2008; Mileikowsky et al., 2000). Nevertheless, microbes are mostly condemned for their negative impacts to human and other life forms, particularly disease-causing pathogens (Klich, 2007; Blumenthal, 2004).

On the other hand, whatever causes effect to human should be exploited on its potentials for industrial application (Zafar et al., 2007). For instance, the mycotoxins produced by *Aspergillus* spp. have economic importance in biotechnology industries (Cotty and Jaime-Garcia, 2007) despite the negative impacts caused to humans. It is clearly known that microbes play a greater role in sustaining lives of other creatures on earth (Schuster et al., 2002). They serve as sources of medicine for various infectious diseases, industrial processing enzymes, formation and balancing of nutrient composition in soil (Amaike and Keller, 2011).

Fungi play role in varied range of economic and development sectors including agriculture, textile and leathery industry (Cleveland et al., 2003). For instance,

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Aspergillus flavus is abundantly available in soil, agricultural produce and processed food (Perrone et al., 2007). Despite its availability in wide range of environment, it is complained by its ability to produce toxic metabolites such as Afflation that may lead to liver cancer (Klich, 2007). In Tanzania for instance, *A. flavus* has not been utilized for biotechnological industries rather complained and avoided for health concerns (Klich, 2007). However, the good side of *A. flavus* is its industrial records in other developed nations (Cardamone, 2002). *Aspergillus* species is essential for synthesis of proteolytic enzyme required by various industries (Chellapandi, 2010).

It is in this vein that *A. flavus* locally available in Tanzania could be used for industrial processing of skin, hides, wool and cotton for production of good leathery and cotton clothes. Moreover, effluent emitted by tanning and dying agents could safely be treated with *Aspergillus* biomass. Thus this article reviewed the potential of *Aspergillus* species with the main focus on *A .flavus* as an available option for safer leather and textile products for both human and the environment in Tanzania.

ASPERGILLUS IN INDUSTRIAL APPLICATION

The genus *Aspergillus* comprises of many economic important species with industrial applications (Oyeleke et al., 2010; Chellapandi, 2010). *Aspergillus* species are sources of vast industrial enzymes with varied application. Aspegillus species was reported to produce extracellular enzyme that is said to be effective in biocontrol of pest in cotton fields (Liu et al., 2001). *Aspergillus* species have been documented to produce many industrial benefits including tannery and textile processing products (Batra and Saxena, 2005). For the purpose of this review, only textile and leather industry is focused on. This is because tannery and textile processing are closelyrelated and they both produce covering materials.

Since the dressing materials need to be safe and comfortable to users, production of such products from natural processing should be safe. Conversely, textile and leather industries use toxic chemicals for tanning or dying raw fiber which cause environmental and health problems (Costa and Klein, 2006). However, most of *Aspergillus* species have multiples applications in these industries thus its application would avert such problem (Bayramoglu et al., 2006). For instance *A. flavus, Aspergillus niger* and *Aspergillus fumigutus* were reported to produce enzymes essential in tannery industry (Barthomeuf et al., 1994). Thanikaivelan et al. (2004) reported the potential of *Aspergillus tamarii* in tannery biotechnology whereas *Aspergillus terreus* was reported to produce tannery enzyme for processing leather (Tang et al., 2004).

More interestingly, *A. flavus* was revealed to produce proteolytic enzyme in processing of wool for textiles (Cardamone, 2002). On the other hand, Deepa et al. (2007) demonstrated the sorption role of *A. flavus* in textile effluents. Additionally, Sivakumar et al. (2014) reported the activity of *Aspergillus* in remediation of textile wastes for safe environment. Similarly, the most toxic Azo dyes from textile wastes were acted upon by *A. flavus* biomass (Akar et al., 2009; Ali et al., 2009; Singh and Singh, 2010). Various applications of *A. flavus* have been reported including pretreatment of industrial wastes before they are released to the environment (Esmaeili and Kalantari, 2012). Hence harnessing of local *Aspergillus*, species would decrease industrial production cost particularly in leather and textiles in Tanzania.

CULTIVATION, OPTIMIZATION AND ADAPTATION TO ENVIRONMENT

Aspergillus species are almost found in every environment on earth (Srividya et al., 2009; Ramirez-Camejo et al., 2012). They can easily adapt to the environment and simply be cultured to optimize them for commercial uses in industries (Guinea et al., 2006). Laxman et al. (2005), revealed how fungi populations could be optimized to increase production of enzymes that are essential for industrial manufacturing of leathery products. Many *Aspergillus* species have been optimized by using local materials for production of industrial enzymes (Devi et al., 2008).

There are several ways of developing and multiplying fungal colonies (Gervais and Polin, 2003). However, selection of culturing method usually depends on the desired products. The solid state fermentation has been pointed to be the best method of increasing production of alkaline enzymes from *Aspergillus* (Ellaiah et al., 2002; Muthulakshmi et al., 2010). This technique has many advantages over submerged method as it produces enough alkaline protease (Kumar and Takagi, 1999).

In addition to that, *Aspergillus* produces enzymes at low cost in which locally available materials from food remains can be used to produce, high amount of enzyme with reduced environmental pollution. This is because local materials like rice and wheat bran produce little waste on environment (Paranthaman et al., 2009). Another study done on *Aspergillus foetidus* revealed the ability of cheaply cultured fungus, in improving tannery processing (Purohit et al., 2006). Another study revealed that, *A. terreus* can be fermented on cheaply available materials and optimized to produce desired quality (Gao et al., 2008). Hence, use of locally available material such as wheat, rice and other cereals by cheap technology in optimization of *Aspergillus* would enhance activity at low costs.

APPLICATION IN LEATHER INDUSTRY

Leathery industry requires many inputs and involves a

series of pre-tanning processes (Suresh et al., 2001). Prior to tanning, animal skins and hides are exposed to high enzymic processes or chemical treatments that are quite expensive (Thanikaivelan et al., 2005). A series of pre tanning processes such as dehairing and bating of raw skin and hides can be performed by using microbial enzymes to reduce costs (Macedo et al., 2005). Use of organic and microbial inputs, tanneries is not practiced in large scales though it is essential for biotechnological development.

However, use of chemicals such as chromium as tanning agents have a lot of negative implications and costly to the environment (Fahim et al., 2006). This chemical accumulates in the environment and may cause carcinogenic effect (Dayan and Paine, 2001) and has currently been implicated as one of the challenges in running leathery industry in developing and developed world (Fahim et al., 2006).On the other hand, application of enzyme in tannery processing is cheaper and has less or no toxic wastes (Aravindhan et al., 2007). Thus an option to use natural tanning agents and microbial enzyme for pre-tanning, offers more sustainable production of leather materials (Batra and Saxena, 2005). Various species of *Aspergillus* have been reported to produce enzymes for chemical reactions in industrial processing of raw skin and hides into leather (Thanikaivelan et al., 2004). *A. niger* was potentiated as good source of lipase for digestion of animal protein in leathering process (Paranthaman et al., 2009).

Additionally, it was reported that lipase from *A. niger* had recognizable action in digestion of animal fats from skin (Houde et al., 2004; Hasan et al., 2006). Production of protease by *A. flavus* has demonstrated the role of fungi in manufacturing industries (Kamini et al., 1999). Another research done by Malathi and Chakraborty (1991), reported the new alkaline protease produced by *A. flavus* useful in tannery industry.Kim (2007), revealed the ability of *A. flavus* in production of keratinase that is used in enzymic catalysis of keratin from feather. Moreover, Chellapandi (2010), revealed the production of tannery protease by *A. flavus* and *A. terreus*. Though many studies have been conducted on *Aspergillus* species, none has been documented as a potential of fungal biotechnology in textile and leather industries in Tanzania.

ASPERGILLUS SPECIES FOR TEXTILE PROCESSING

Textile industry is among the oldest industries that requires multiple inputs with processes (Chandra and Kumar, 2000). Wool and cotton undergoes various processes to obtain fabric for making clothes (Sousa et al., 2007). Microbial lacasses play a role in processing raw materials into fabric (Couto and Toca-Herrera, 2006) from *Aspergillus* species. However, this industry is constrained by management of dye wastes (Sawhney et al., 2008). Management of dye waste particularly the Azo dye is the main challenge (Mu et al., 2009). However, Araujo et al. (2009), revealed the ability of *A. niger* proteases in textile processing. More studies done on *Aspergillus* species revealed production of industrial catalytic enzymes for textile processing (Bhat, 2000; Parvinzadeh et al., 2008). Various classes of catalytic enzymes have been studied from *Aspergillus* so far (Kirk et al., 2002; Sukumaran et al., 2007). Catalytic enzyme such as amylase produced by Aspergilllus member was documented for its application in textiles (Alva et al., 2007).

On the other hand, enzymatic treatments by *A. flavus* have been evaluated and validated in textile wastes (Sawada et al., 2007). McMullan et al. (2001) reported the biodegradation activity of *Aspergillus* on textile dyes. Even the dead biomasses of *Aspergillus* species are useful in the elimination of accumulated industrial waste of heavy metals. *A. flavus* is also known as good biosorption of heavy metals that are harmful to human such as lead and copper (Akar and Tunali, 2006). The dead biomass of *A. niger* has high ability to absorb dye wastes from textiles (Khalaf, 2008). Other studies have also revealed its biosorption of complicated dye waste from textile industries especially the azo dye (Ranjusha et al., 2010). Thus, harnessing fungal products for industrial processing of textiles in developing countries including Tanzania would improve and promote sustainable production.

INDUSTRIAL WASTE MANAGEMENT

Leather processing uses chromium sulphate and other chemicals for tanning skin or hides (Srivastava et al., 2007). This chemical generates huge amount of wastes that are very toxic (Gotvajn et al., 2009) and are directed into public sewage system and finally, accumulate in soil and environment leading to health problems. Costa and Klein (2006) reported that chromium is one of very toxic chemicals that can cause carcinogenic problem to human. Removal of chromium from tannery waste by conventional methods is tedious, expensive and causes much hazardous impact to environment (Dayan and Paine, 2001).

However, *Aspergillus* species have good record in the management of industrial wastes and effluents (Joshi et al., 2011). *Aspergillus* species are capable of removing chromium from leathery wastes (Srivastava and Thakur, 2006). Sandana et al. (2006) revealed that *A. niger* absorbed chromium sulphate from tannery wastes. *A. niger* has been reported to absorb heavy metals such as copper and zinc from industry wastes (Prasenjit and Sumathi, 2005; Price et al., 2001). On the other hand, Srivastava et al. (2007) reported the role of *Aspergillus* in absorbing chromium sulphate from tannery effluent. Another study by Nasseri et al. (2002) revealed the

potential of *Aspergillus oryzae* in absorbing chromium IV from tannery wastes. Furthermore, other studies revealed the ability of *Aspergillus candidus* in bioremediation of colored and toxic waste from tanneries (Murugan and Al-Sohaibani, 2010). Additionally, Prigione et al. (2009) reported the ability of *A. flavus* in the absorption of hazardous tannery waste.This finding revealed the potential of *A. flavus* in treatment of waste from industrial effluents.

POSSIBLE IMPLICATION IN LEATHERY INDUSTRY IN TANZANIA

Leather and textile are ancient industries in Tanzania and contribute to the national earnings. However, their production is currently decreasing due to a number of factors. One of the limiting factors is scarce and it limited inputs technologies including the use of microbial biotechnology that is cheaply available. *Aspergillus* species are the good source of industrial catalysts for processing of raw skin and hides. On the other hand, some *Aspergillus* members can produce tannery enzymes as well as absorbing tannery effluents, before they are released to soil and agricultural fields. Since vast majority of *Aspergillus* have demonstrated many industrial applications in both leather processing and removing of tannery wastes, optimization through mass culturing by using locally available resources would eliminate its scarcity. Most of *Aspergillus* species are naturally found in the environments and can also be cultured in large scale biotechnological laboratory to optimize the supply for industrial production. For instance, large amount of biomass of *Aspergillus* species was reported to absorb and eliminate large amount of chromium compound from tannery effluents (Sharma and Goyal, 2010).

Aspergillus is very abundant in Tanzania but not utilized for industrial production. The potential advantages of using native *Aspergillus* species, is low in production cost, technology and adaptability of products to local environments as postulated in Figure 1. Hence, screening and commercialization of *A. flavus* enzymes is of economic importance and would improve biotechnology development in Tanzania and across Africa continent.

Figure 1 represents the enzymatic processing of leather and textile products by using *A.flavus* products. The diagram consists of four main processing stages; A to D in both Side I and II, represent tannery and textile processing of raw materials, respectively. In Side I, skin and hides are the inputs (A) for processing leather which undergoes pre-tanning processes (B) by using alkaline proteases and lipase to obtain raw leather. Finishing is done by enzymic tanning and dying by *A. flavus* (C). Thereafter, desired product and other out puts are reached and viable and dead. *A. flavus* biomass absorbs wastes and release safe effluent to the soil in (D). On the other side II, processing of cotton and wool to get textile material is done in similar way using *A. flavus* but different raw materials. Cotton, wool and catalase (A) are the input for processing fibre and fabric (B).These materials are dyed under enzyme mild conditions (C), toxic waste is absorbed by *Aspergillus* dead mass, while products are produced in safety way (D).

CONCLUSION

Textile and leather industries remain the major producer of wear material in Tanzania. Hence, sustainable production requires efficient supply of quality skin, hides and fiber from well agricultural practices. Though, enzyme application processing of these industries is still underutilized in Tanzania.

Harnessing of *A. flavus* would improve industrial production and enhance safety of the products. This study evokes the attitude of harnessing natural and locally available resources such as *A.flavus* in development of biotechnology industries in Africa, to achieve desired products at low costs using locally available resources and technology.

Conflict of Interests

The authors have not declared any conflict of interests.

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Expression stability of reference genes in the skeletal muscles of beef cattle

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The objectives of this study were to evaluate the relative expression stability of five candidate reference genes in the *semimembranosus* (SM) and *longissimus thoracis* (LT) of beef steers, heifers and young bulls. The mRNA levels of Beta-Actin, eukaryotic initiation factor-2B Subunit 2, glyceraldehyde-3-phosphate dehydrogenase, peptidylprolyl isomerase-A and succinate dehydrogenase complex-subunit A were quantified using real-time polymerase chain reaction (qPCR). The combined analysis using the geNorm algorithm revealed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and eukaryotic initiation factor-2B subunit 2 (EIF2B2) were the most stable gene pairs. However, individual experimental conditions showed that succinate dehydrogenase complex-subunit A was most stably expressed in bulls and heifers SM, and in bulls and steers LT. Glyceraldehyde-3-phosphate dehydrogenase was most stably expressed in bull SM, steer SM, bull LT and heifer LT. The expression stability ranking order differed between experimental conditions, but all genes had low expression variability. Therefore, using the two most stable reference genes, namely GAPDH and EIF2B2, would result in more accurate normalizations for quantitative real-time PCR studies in the SM and LT muscles of beef cattle. The need for prior evaluation of candidate reference genes in different muscles and sex groups of beef cattle is thus emphasized by the present results.

Key words: Bovine, gene expression, *longissimus thoracis,* normalization, reference genes, *semimembranosus*, geNorm.

INTRODUCTION

Gene expressions of proteolytic enzymes are routinely analyzed to provide insight into the factors that influence the quality of meat. Variations in the mRNA levels of a specific gene are best studied using quantitative real-time

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PCR (qPCR) because the method has proven to be more accurate, reliable and the enhanced sensitivity means that even extremely low levels can be quantified (Bustin et al., 2005). Detected variations are usually due to biological changes within the cell, as well as the compounding experimental errors arising from the qPCR process (Bustin, 2010). These technical variations can be controlled through normalization of qPCR data using a set of housekeeping and reference genes. This is a highly recommended approach because it accounts for differences in RNA quantity and handling and as well as downstream errors that arise during cDNA synthesis (Bustin et al., 2005; Stürzenbaum and Kille, 2001). It is expected that these reference genes are stably expressed under various experimental treatments and physiological conditions (Huggett et al., 2005). This assumption is generally violated due to the fact that the various housekeeping genes used as reference controls can be regulated because of their other functions besides basal cellular metabolism (Thellin et al., 1999).

Indeed, tissue specific differences in expression stability of reference genes have been reported in bovine *longissimus* muscle (Pérez et al., 2008), liver (Lisowski et al., 2008) as well as in visceral fat tissues, subcutaneous fat and mammary glands (Saremi et al., 2012) and many other experimental conditions (Vorachek et al., 2013). This indicates that there is no consensus regarding the expression stability of a given reference gene. Moreover, information regarding the stability of reference genes in various bovine skeletal muscles and from beef cattle of different sex-age groups is currently limited. Considering that muscle type and sex of the animal will bring about differential expression levels, it is necessary to determine the most stable gene and the number of genes that would be required for accurate normalization in each condition. Thus a systematic analysis of reference genes remains the most suitable strategy towards a robust normalization of qPCR data (Vandesompele et al., 2002). This study therefore aimed to evaluate the expression stability of selected reference genes in the *semimembranosus* and *longissimus thoracis* of beef steers, heifers and young bulls.

MATERIALS AND METHODS

Study animals and sample collection

This study used 18 Hereford-cross cattle that consisted of 6 young bulls, 6 steers and 6 heifers. The steers were young bulls which were surgically castrated when they were 2 months old. All animals were weaned at 10 months of age and then allowed to feed on high quality grass and grass silage *ad libitum.* The animal were slaughtered in a licensed commercial abattoir in West Yorkshire, UK, in accordance with the European Community Directive, 86-609-EC. The young bulls, steers and heifers were marketed when they attained 550 kg live weight at the average age of 547, 764, and 889 d, respectively. Meat samples were excised from the *semimembranosus* (SM) and the *longissimus thoracis* (LT) of each animal and then submerged in individual tubes containing RNA*later*™ reagent to stabilize the RNA. The tubes containing the samples were incubated at 4°C for 24 h and then preserved at -20°C until required for downstream processes that were carried out within one month.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of RNA*later*™ preserved samples using the RNeasy fibrous tissue extraction kit (Qiagen, Germany) according to the manufacturer's protocol. The eluted RNA was thereafter DNAse-treated using the SIGMA AMP-D1 kit (Sigma-Aldrich, St. Louis, USA) to eliminate any carryover genomic DNA. The NanoDrop ND-1000 spectrophotometer was used to determine the concentration and quality of the eluted RNA where 1 unit at 260 nm is equivalent to 40 ng μl^{-1} of RNA and the 260/280 nm ratio provided an estimate of the quality. The integrity was assessed by performing electrophoresis on a 2% agarose gel and by exposure to UV light using the SYNGENE system (SynGene Ltd., Cambridge, UK). The components of the Verso cDNA synthesis kit (Abgene Ltd., Epsom, UK), which consisted of 1× cDNA buffer, Verso reverse transcriptase, oligo-dT primers and dNTPs, were prepared as a mastermix and then aliquot into thin-walled PCR tubes. 500 ng of total RNA was added to each PCR tube and topped with RNase-free water to make up a 20 µl reaction. Each PCR run also had a tube for the no-enzyme control and a non-template control. All tubes were then placed in an Eppendorf Mastercycler Gradient 5331 thermal cycler (Eppendorf AG, Hamburg, Germany) programmed at 42°C for 30 min and 95°C for 2 min for the reverse transcription step and enzyme deactivation step, respectively. Several replicates were prepared for each sample in order to prepare sufficient cDNA for all selected genes. The cDNA was then stored at -20°C until required for downstream processes.

Quantitative real-time PCR

The primers for and Beta-Actin (*ACTB)*, Eukaryotic initiation factor-2B subunit 2 (*EIF2B2),* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)*, peptidylprolyl isomerase-A (*PPIA)* and succinate dehydrogenase complex-subunit A (*SDHA)*, were obtained from a Bovine HKG gene set designed, optimized and supplied commercially by PrimerDesign Ltd (Southhampton, UK). The genes were selected for evaluation as they have distinct functions and their details are outlined in Table 1. An aliquot was taken from each cDNA tube, pooled and then serially diluted for a 5-point standard curve. Triplicate reactions were run for the standard curve analysis and duplicates for all other samples in a 25 µl amplification reaction mixture containing 5 µl of cDNA, 12.5 µl ABsoluteTM Blue QPCR SYBR® Green master mix (ABgene Ltd., Epsom, UK), 300 nM (final concentration) of gene specific primers and PCR-grade water. For the negative control, 5 µl of PCR-grade water was used instead of the cDNA.

qPCR reactions were performed in the Mastercycler® ep realpex thermal cycler (Eppendorf AG, Hamburg, Germany), using the following conditions: 1 cycle of enzyme activation for 15 min at 95°C, 40 cycles of 15 s denaturation at 95°C, 30 s annealing at 60°C and 30 s elongation at 72°C as well as a melting curve analysis step. The resultant PCR products were subjected to electrophoresis on a 2% agarose gel with 1 × TBE buffer. The amplifications were confirmed specific by the presence of a single band of expected size as visualized by exposure to UV light with the SYNGENE bio-imaging system (SynGene Ltd., Cambridge, UK).

Data analysis using geNorm

The qPCR data in the form of Ct values were exported to Microsoft

Table 1. GeneBank accession numbers, functions and amplification information of the 5 bovine reference genes.

Gene symbol	Accession Number	Full gene name	Function	Amplicon length (bp)	Slope	Efficiency (%)
ACTB	[GenBank:NM_ 173979]	Beta-Actin	A ubiquitous cytoskeletal protein involved in cellular structure, integrity and motility	130	-3.532	92
EIF2B2	[GenBank:NM_ 001015593]	Eukaryotic initiation factor-2B, Subunit 2	Key regulator of translation that catalyses the exchange of GTP during protein synthesis	111	-3.310	100
GAPDH	[GenBank:NM_ 001034034]	Glyceraldehyde-3-phosphate dehydrogenase	Involved in carbohydrate metabolism as an oxidoreductase during glycolysis and the reverse reaction gluconeogenesis.	136	-3.496	93
PPIA	[GenBank:NM_ 178320]	Peptidylprolyl isomerase A (Cyclophylin A)	Involved in cell cycle, isomerisation of peptide bonds and enhances protein folding	81	-3.386	97
SDHA	[GenBank:NM_ 174178]	Succinate dehydrogenase complex, subunit A	Involved in the mitochondrial electron transport chain during the conversion of succinate to fumarate in the Tricarboxylic acid cycle	126	-3.427	96

Table 2. Mean Ct values and standard deviations of the candidate reference genes tested.

Gene Symbol	Bulls SM		Bulls LT		Heifer SM		Heifer LT		Steers SM		Steers LT	
	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
ACTB	21.12	0.23	21.53	0.29	21.22	0.61	21.02	0.26	20.98	0.51	21.13	0.30
EIF2B2	23.99	0.43	24.31	0.73	23.89	0.27	23.96	0.18	24.03	0.26	24.24	0.32
GAPD	15.30	0.26	15.04	0.24	15.11	0.27	15.21	0.22	15.10	0.25	15.20	0.47
PPIA	22.77	0.41	23.06	0.42	22.17	0.42	22.24	0.35	22.66	0.38	22.82	0.16
SDHA	19.43	0.19	20.31	0.22	20.13	0.25	20.36	0.23	19.76	0.13	20.05	0.19

Excel spreadsheet for subsequent processing and analysis. Amplification efficiency (*E*) of each reference gene was derived from the slope of the standard curve using the formula, $E = (10^{(-1/slope)} -1)*100$. The Ct values were then transformed into relative quantification values (Q) following the delta-Ct formula, $Q = E^{(\Delta Ct)}$, where *E* is the efficiency of a specific gene and ΔCt is the difference between the lowest intra-gene Ct value and the mean Ct of each technical replicate sample (min Ct - sample Ct). These Q-values were then used to obtain the gene expression stability measure (M) by calculating pair-wise variation with the geNorm applet. The gene with the lowest M-value is the most stably expressed gene in a given environmental or experimental condition. Furthermore, the geNorm applet calculated the V-score, which indicates the benefit of including a 3rd, 4th and 5th gene in the calculation of a normalization factor (NF). The authors (Vandesompele et al., 2002) stated that a V-score less than 0.15 indicate that using additional reference gene to calculate the normalization factor would not result in significant increase in accuracy. The analysis was carried out to rank all the 5 HKGs under six different conditions resulting from 3 groups of animals (bulls, steers and heifers) and 2 muscles (LT and SM).

RESULTS

RNA quality and PCR performance

The RNA extract was of acceptable quality as indicated by the high (> 1.9) A260/A280 absorbance ratios. A single band of expected size was observed on the agarose gel indicating a lack of primer dimer formations and the specificity of the primers. Moreover, the efficiencies of amplification (Table 1) derived from the 5-point standard curve of each gene were greater than 90 % with correlation coefficients (R^2) above 0.99. The mean Ct values and standard deviations of each candidate gene tested are as shown in Table 2. Ct values in range of 15 To 24.31 were observed with *EIF2B2* being the least expressed while GAPDH appears to be highly transcribed. The most variation was associated with *EIF2B2* expression in Bull LT (24.31 ± 0.73) and the least

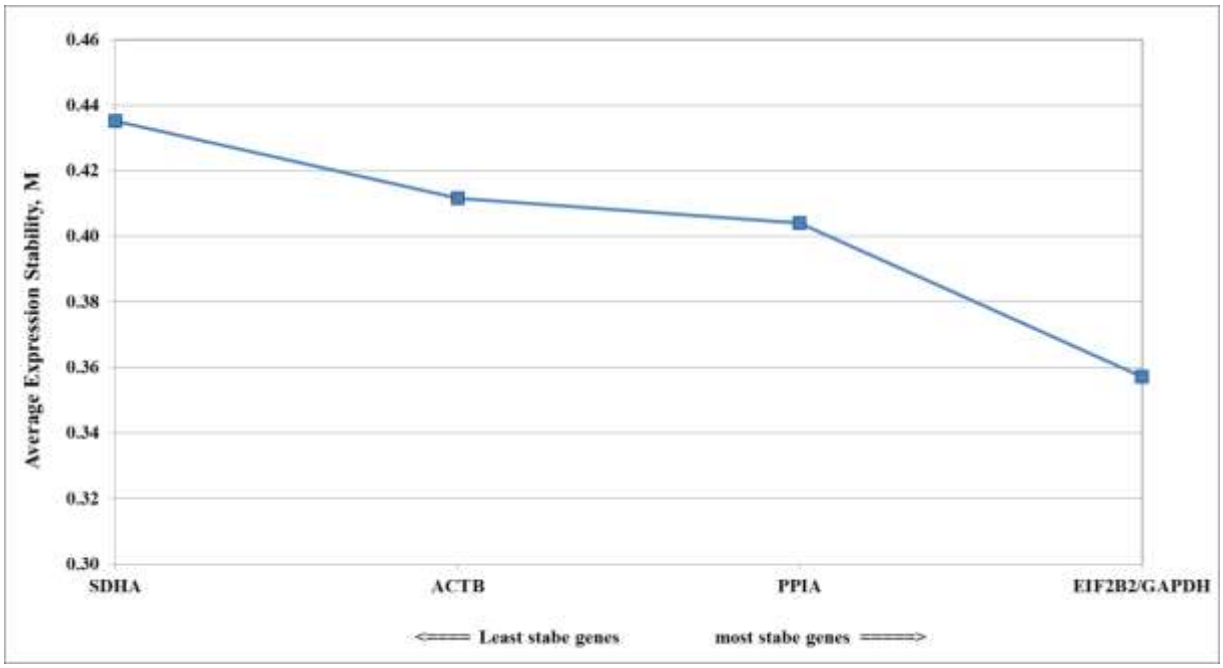

Figure 1. A combined expression stability analysis of reference genes in bovine skeletal muscles according to geNorm. Higher M-values indicate lower expression stability.

Table 3. Expression stability ranking of reference genes in the longissimus and semimembranosus muscles of beef cattle.

parameter	*Semimembranosus*		*Longissimus thoracis*	
	Ranking	**Stability value (M)**	**Ranking**	**Stability value (M)**
Bulls	*GAPDH*/*SDHA*	0.1327	*GAPDH*/*SDHA*	0.1212
	ACTB	0.1669	*ACTB*	0.2624
	PPIA	0.2183	*PPIA*	0.2938
	EIF2B2	0.2610	*EIF2B2*	0.4065
Heifers	*EIF2B2*/*SDHA*	0.1951	*ACTB*/*GAPDH*	0.0941
	PPIA	0.2847	*SDHA*	0.1342
	GAPDH	0.3124	*PPIA*	0.1757
	ACTB	0.3786	*EIF2B2*	0.1985
Steers	*EIF2B2*/*GAPDH*	0.1649	*PPIA*/*SDHA*	0.2062
	PPIA	0.1775	*EIF2B2*	0.2491
	ACTB	0.2203	*ACTB*	0.3011
	SDHA	0.2609	*GAPDH*	0.3748

variation was associated with SDHA expression in steer SM (19.76 ± 0.13).

Variations in gene expression stability

The overall gene expression stability rankings of the 5 reference genes are illustrated in Figure 1. This shows that *GAPDH* and *EIF2B2* were the most stable gene pair. However, the M-values of the remaining genes were below the 1.5 mark suggested by Vandesompele et al. (2002), an indication that in the present study *PPIA*, *ACTB* and *SDHA*, were also stably expressed, and in that order. On the other hand, the analysis of individual conditions revealed a different pattern of expression stability (Table 3). The expression stability of the reference genes were generally reversed between the SM and LT muscles of steers and heifers. In the SM of

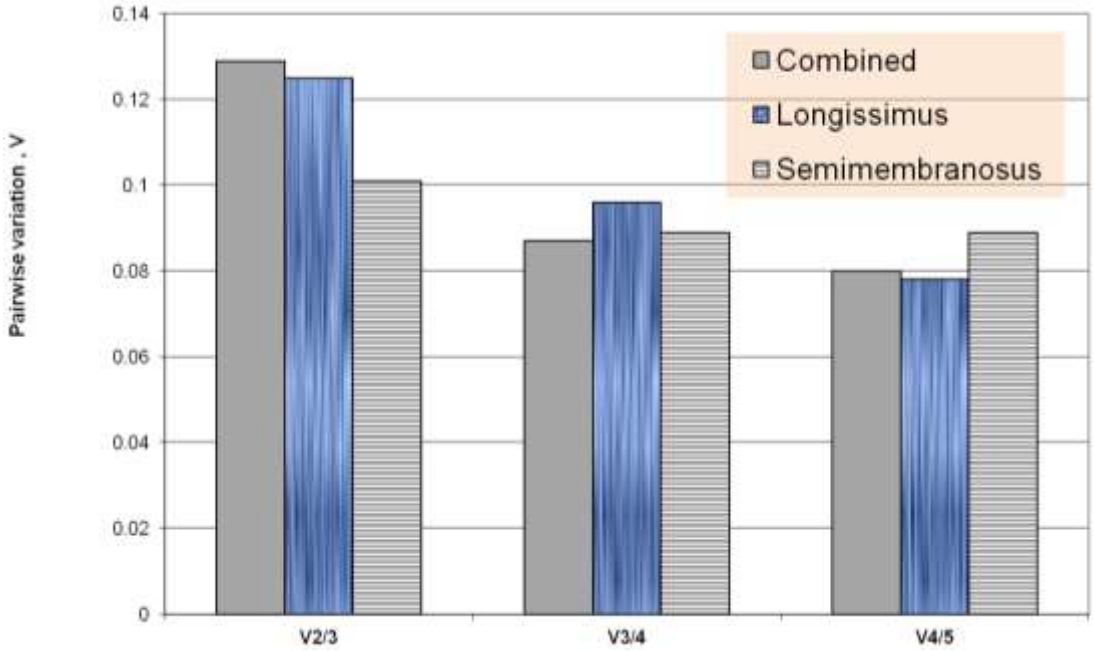

Figure 2. Evaluation of the optimum number of reference genes for accurate normalization determined by geNorm software. The Vn/Vn+1 (V) ratio indicates the benefits in the accuracy of normalization for each additional gene (n) included in the calculation of the normalization factor.

steers, *GAPDH* was the most stable gene together with *EIF2B2* but it was more regulated in the LT. In contrast, the *GAPDH* was more regulated in the SM of heifers as compared to the LT. *EIF2B2* also had the highest M-values in the SM and LT of bulls, and the LT of heifers, but it was found to be stably expressed as shown by the lowest M-values in the SM of steers and heifers. In contrast, *SDHA* was ranked highest in 4 experimental conditions except in the steer SM where it appeared to be variably expressed (Table 3). *ACTB* and *PPIA* displayed intermediate stability under most conditions but were both found to be stably expressed in the LT of heifers and steers, respectively. Therefore, the order of expression stability differed between the 2 muscles and 3 sex groups (Table 3).

Optimal number of reference genes for normalization

The optimum number of reference genes required for accurate normalization of gene expression data for SM and LT muscles in beef cattle were illustrated in Figure 2. In this study, all experimental conditions revealed a $V_{2/3}$ less than 0.15, indicating that the addition of more than 2 HKGs in the calculation of a normalization factor would not offer significant benefits (Figure 2). These results indicated that the geometric average of at least 2 most stable genes would produce an accurate normalization factor and reliable results for the analysis of gene expression in the LT and the SM muscle. The inclusion of a third reference gene reduced the pairwise variation further in the combined analysis and in the LT analysis but not in the SM (Figure 2). Thus the results indicate that the inclusion of a 4th relatively less stable gene would have no significant beneficial effect on the normalization factors as compared to using 3.

DISCUSSION

The present study was conducted to validate the expression stability of a set of HKGs in the skeletal muscles of cattle from a commercial beef herd that comprised of different gender groups, specifically, young bulls, heifers and steers. The *longissimus thoracis* and the *semimembranosus* were chosen as economically important muscles in the beef industry with relatively distinct meat quality characteristics. The analysis revealed that the expression stability of the 5 reference genes investigated here varied across all experimental conditions. This variability may be attributed to differences in gene regulation and metabolic activities in the two muscles in interaction with gender. Although the expression of many reference genes in bovine have been evaluated in various tissues and under different experimental treatments, the information on their expression stability in skeletal muscle is limited (Bahar et al., 2007; Bonnet et al., 2013; Pérez et al., 2008).

According to Goossens et al. (2005), as well as De Ketelaere et al. (2006), *SDHA* was found to be one of the

most stable reference genes in bovine embryos and in polymorphonuclear leukocyte cells of lactating cows, respectively. In contrast, SDHA was ranked highest in term of expression stability in Bovine neutrophils. However, the higher variability in the overall analysis in the present study is probably due to the variable expression levels of *SDHA* in various muscle fibre types, a reflection of differential demand for the action of the encoded *SDHA* enzyme in the metabolism of glucose derivatives in various samples.

GAPDH and *ACTB* are generally thought of as the most stable genes and are frequently used as reference genes for normalization in more than 90% of the studies (Suzuki et al., 2000). However, other studies have shown that these too can be affected by various experimental conditions and requires prior validation (Bionaz and Loor, 2007; Bustin, 2000; Chapman and Waldenström, 2015; Glare et al., 2002; Stamova et al., 2009). The present study found *GAPDH* to be a suitable endogenous in the SM and LT of young bulls, SM of steers and the LT of heifers. This is in agreement with Bahar et al. (2007) who found *GAPDH* as the most stable gene in the *Longissimus* muscle of Limousine cross heifers. Moreover, *GAPDH*, and *ACTB* were found to be stably expressed in the livers of cows subjected to different physiological and dietary treatments (Janovick-Guretzky et al., 2007).

In contrast, the expression stability of *GAPDH* in steer LT of the present study was ranked lowest. Similar results were demonstrated by analysis of the *Longissimus* muscles of pigs (Erkens et al., 2006; Nygard et al., 2007) as well as in cattle (Pérez et al., 2008), where *GAPDH* was found to be highly regulated. Indeed, a review by Chapman and Waldenström, (2015), showed that there are only a few studies that found *GAPDH* and ACTB to be optimal for normalization of gene expression data. *GAPDH* expression is regulated by a variety of factors including calcium (Chao, et al., 1990) and insulin levels (Nasrin et al., 1990). DeGraff et al. (2010) further demonstrated that androgen treatment regulates the expression of insulin-like growth factor binding protein (IGFBP-2) and *GAPDH*. These findings suggest a possible sex induced regulation of *GAPDH* for the steer muscles in the present study.

With regards to *ACTB*, the gene encodes an important component that is required for muscle contractions and shape. The SM, being a leg muscle undergoes relatively more frequent contractions than the LT. The present analysis indicated a stably expressed *ACTB* in the LT but a regulated gene in the SM. In the same view, *ACTB* was ranked fourth most stable gene in bovine *Longissimus* muscle (Pérez et al., 2008), but highly variable in bovine embryos (Goossens et al., 2005). Therefore, *ACTB* may not be a suitable reference gene in active muscles and tissues were it appears to be more regulated. *EIF2B2* was also found to be stably expressed in the skeletal muscles of steers but slightly regulated in muscles of young bulls. Differences in protein synthesis efficiencies between bulls, steers and heifers as well as between different muscles have been documented. In general, bulls have an advantage over steers and heifers as they gain much faster and produce leaner carcasses (Seideman et al., 1982; Venkata et al., 2015), suggesting enhanced protein synthesis in growing bulls. The *EIF2B2* protein may, depending on the circumstances, slow or enhance protein synthesis (Abbott and Proud, 2004) and this could be associated with the differences in the stability of *EIF2B2* expression observed in the present study.

Conclusions

Taking into consideration the performance of all the 5 reference genes studied in the skeletal muscles of beef cattle, more accurate normalization factors can be calculated using the geometric means of *GAPDH*, *PPIA* and *EIF2B2*. The present study also suggests that *ACTB* is not a suitable reference gene especially in more active muscles such as the SM. Thus the expression stability was influenced by muscle type and the sex of the animal. These results contribute to a list of HKGs that may be useful in gene expression studies in *Semimembranosus* and *Longissimus* of beef cattle. Further studies are therefore warranted in other economically important muscles of livestock.

Conflict of Interests

The authors have not declared any conflict of interests.

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Influence of *Suillus luteus* on *Fusarium* damping-off in pine seedlings

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The role of the ectomycorrhizal (Myc) fungus *Suillus luteus* as a biological control agent against damping off caused by *Fusarium verticillioides* (Fo) and *Fusarium oxysporum* (Fm) on Scots (*Pinus sylvestris* L.) and Stone pine (*Pinus pinea* L.) was studied in a greenhouse experiment. The vegetative mycelium of *S. luteus* in a vermiculite/peat carrier was added to potting substrate before inoculation with Fo or Fm spores (macro and microconidia). Also, seedlings were inoculated only with the Myc, Fo, Fm or water for treatment comparisons. The seedling disease index (SDI) of seedlings varied significantly among pine, *Fusarium* and Myc treatments. Scots pine seedlings inoculated with *F. verticillioides* and *F. oxysporum* had a reduced SDI when co-cultured with *S. luteus*. Damage in Stone pine seedlings inoculated with *F. oxysporum* was significantly reduced in the presence of Myc fungus, but no reduction of disease symptoms was observed when inoculated with *F. verticillioides*. Mycorrhizal formation in co-cultures with *F. verticillioides* was low and absent in co-cultures with *F. oxysporum*, although *S. luteus* inoculation resulted in a greater antagonism against this latter pathogen. The protective effect of *S. luteus* against damping-off by *Fusarium* species was not related to the percent of mycorrhizal apexes in the roots of Stone and Scots pine seedlings.

Key words: Forest nurseries, ectomycorrhiza, *Suillus luteus,* damping off, *Fusarium oxysporum, Fusarium verticillioides,* biological control, *Pinus* species.

INTRODUCTION

Fusarium oxysporum Schlecht. Emend. Snyd. & Hans. and *Fusarium verticillioides* (Sacc) Niremberg (*F. moniliforme* Sheldon) are important soil borne pathogens with worldwide distribution and large host range. In forest nurseries, both species are involved in damping off disease, occurring in germinating seeds and first-year seedlings of most conifers and broadleaves around the world (Dick and Dobbie, 2002). In Spain, *F. oxysporum* and *F. verticillioides* are the main causal agents of damping off in forest nurseries (Martín-Pinto et al., 2006a,b), responsible for considerable losses, particularly in conifer species.

Several fungicides are used to control this disease, though many of them are not effective and do not protect

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the seedlings in the nurseries (Machón et al*.,* 2006). Furthermore, reducing residual toxicity from chemicals in the soil is also demanded for an environmentally acceptable nursery management (Hwang et al*.,* 1995) and therefore biological approaches to disease control are sought.

Ectomycorrhizal fungi (Myc) have a beneficial relationship with plants by improving nutrient uptake and plant growth (Smith and Read, 1997), thus enhancing the establishment of forest seedlings (Guerin-Laguette et al., 2004). Several studies have documented the protective role of ectomycorrhizae against fungal pathogens (Aleksandrowicz-Trzcinska, 2008; Manka, 2010; Zhang et al., 2011; Diez and Alves-Santos, 2012), nematodes (Diedhiou et al*.,* 2003), and insects (Halldorsson et al*.,* 2000).

Suillus luteus (L. Ex Fr.) Gray is a common agariocoid fungus in Europe (Lamaison and Polese, 2004) where it regularly forms ectomycorrhizal relationships with many pine species. In Spain, it is frequently associated with Scots pine (*Pinus sylvestris* L.) and Stone pine (*Pinus pinea* L.), two widespread tree species with quite different habitats.

Thus, the goal of this work was to study the protective effect of *S. luteus* against damping-off damage caused by *F. oxysporum* and *F. verticillioides* to Scots and Stone pine seedlings under greenhouse conditions.

MATERIALS AND METHODS

Organisms

The plant material used in the assays consisted of Scots pine ("Montaña Soriano-Burgalesa" provenance, ES.08) and Stone pine ("Meseta Castellana" provenance, ES.01) seeds provided by Fuenteamarga forest nursery (Valladolid, Spain). The damping off pathogens *F. verticillioides* (Fm-6P) and *F. oxysporum* (Fo-4P) were freshly isolated from diseased seedlings growing in commercial nurseries located at the Provinces of León (Imave nursery) and Soria (Indesfor nursery), respectively. The Myc fungi *S. luteus* (Sl-1 strain) was isolated from a fruiting body collected in a *P. sylvestris* stand located at Palencia Province. The cultures of *Fusarium* species and *S. luteus* were maintained during two months on Komada (K) medium (Komada, 1975) and modified Melin Norkrans (MMN) medium (Marx, 1969), respectively.

Greenhouse experiments

Inoculum of *Fusarium* spp. was produced by culturing the fungus in liquid Potato Dextrose Agar (PDA) medium for 7 days in the dark. Spores (macro- and microconidia) were obtained by filtration and re-suspension at a concentration of 10^6 spores/ml. Inoculum of *S. luteus* was prepared by culturing the fungus in a mixture of 1000 ml vermiculite and 100 ml peat, twice sterilised at 120°C for 60 min, and moistened with 500 ml of MMN liquid medium (pH adjusted to 5.0) in 2 L flasks. Once the culture medium was added, the flasks were autoclaved for 20 min at 121°C, and after cooling, the flasks were inoculated with the ectomycorrhizal fungus by adding 20 agar plugs (5 mm in diameter) from solid cultures on MMN medium. All the flasks were maintained at 25°C in the dark for two months. Non-inoculated flasks were also prepared for control treatments.

Twelve treatments were compared, 6 treatments for each pine species: (1) control (non-inoculated), (2) *S. luteus* (Myc), (3) *F. verticillioides* (Fm), (4) *F. oxysporum* (Fo), (5) Fm+Myc, and (6) Fo+Myc. In early February 2004, pine seeds were surface sterilized by dipping in 30% H_2O_2 for 30 min, then washed 10 times with sterile distilled water to eliminate disinfectant before sowing in multipot trays (250 cm^3 per pot) containing a mixture of 215 cm^3 of peat most and vermiculite (1:1), previously sterilized twice at 121°C for 90 min. In the treatments with Myc, 50 ml of *S. luteus* inoculum was transferred to the pots prior to seedling and laid on the surface of the sterilized peat moss (Pindstrup Mosebrug S.A.E., Burgos) mixture (165 cm^3). Immediately after sowing, a 5-ml spore suspension (10^6 spores ml^{-1}) of *F. verticillioides* or *F. oxysporum* was pipetted to each pot on the Fm and Fm+Myc, or on the Fo and Fo+Myc treatments, respectively. Control seedlings were inoculated with 5-ml of distilled water. Finally, the seeds of all the treatments were covered with 15 cm^3 of sterile peat most and irrigated daily with 20 ml of sterile distilled water during two weeks. After that, watering and other procedures were routine greenhouse practice, except that no fertilization or fungicides were applied.

Every treatment consisted of 3 replicates (trays) of 48 seeds each (one seed per pot) resulting in a total of 864 seeds (=6 treatments × 3 replicates × 48 seed per replicate) assayed for each pine species. The trays were randomly arranged on the bench and maintained in a greenhouse environment without light or temperature regulation until early July (18 weeks). Temperature during the experiment was: February (Average minimum temperature, Tmin = 1.1°C; Average maximum temperature, Tmax = 10.7°C; Average temperature, T = 5.4°C); March (Tmin = 2.5°C; Tmax = 16.1°C; T = 9.8°C); April (Tmin = 5.3°C; Tmax = 17.7°C; T = 11.5°C); May (Tmin = 8.3°C; Tmax = 22.7°C; T = 15.5°C); June (Tmin = 12.2°C; Tmax = 27.7°C; T = 20.5°C), and July (Tmin = 14.1°C; Tmax = 31.7°C; T = 23.4°C). Care was taken to minimize contamination between trays during watering and tray maintenance on the greenhouse bench.

Damping off was analysed by recording the damage to the seedlings at the end of the experiment. Three damage classes were established: (1) none to light damage (less than 10% shoot affected); (2) moderate to severe damage (over 10% of shoot affected); (3) dead seedling. Within each replication (tray), seedling damage was obtained as the mean value of the 48 seedlings and a seedling disease index (SDI) was calculated for each treatment as the mean value of seedling damage in its three replications.

Fifteen randomly selected seedlings for each treatment (dead seedlings were not included) were then taken to the laboratory and shoot height, shoot dry weight, root collar diameter, root length, and root dry weight were measured. Excised, soil-washed roots were examined for ectomycorrhizal short roots and placed in vials containing a FAA preserving solution (5 ml of formaldehyde, 5 ml of acetic acid and 90 ml of ethyl alcohol). Intensity of root colonization was expressed as the percentage of mycorrhized apexes within 250 observed plant apexes.

Statistical analysis

Untransformed data were subjected to analysis of variance (ANOVA) procedures ($p<0.05$) using Statistica 7.0 (StatSoft Inc., 1984-2005, Tulsa, Ok) software. Least significant difference (LSD) Fisher test ($p<0.05$) was applied to compare mean values when significant differences were found.

RESULTS AND DISCUSSION

The SDI of seedlings varied significantly among pine,

Table 1. ANOVA table for pine seedling disease index (SDI).

Source	d.f.	MS	F-Value	p-value
Pine	1	3.48	163.48	0.000*
Myc	1	0.34	16.20	0.000*
Fusarium	2	1.30	61.02	0.000*
Pine × Myc	1	0.08	3.93	0.058
Pine × *Fusarium*	2	0.60	28.39	0.000*
Myc × *Fusarium*	2	0.06	3.14	0.061
Pine × Myc × *Fusarium*	2	0.02	0.98	0.389

d.f.: Degrees of freedom; MS: means squares; Myc: ectomycorrhizal fungus *Suillus luteus*.

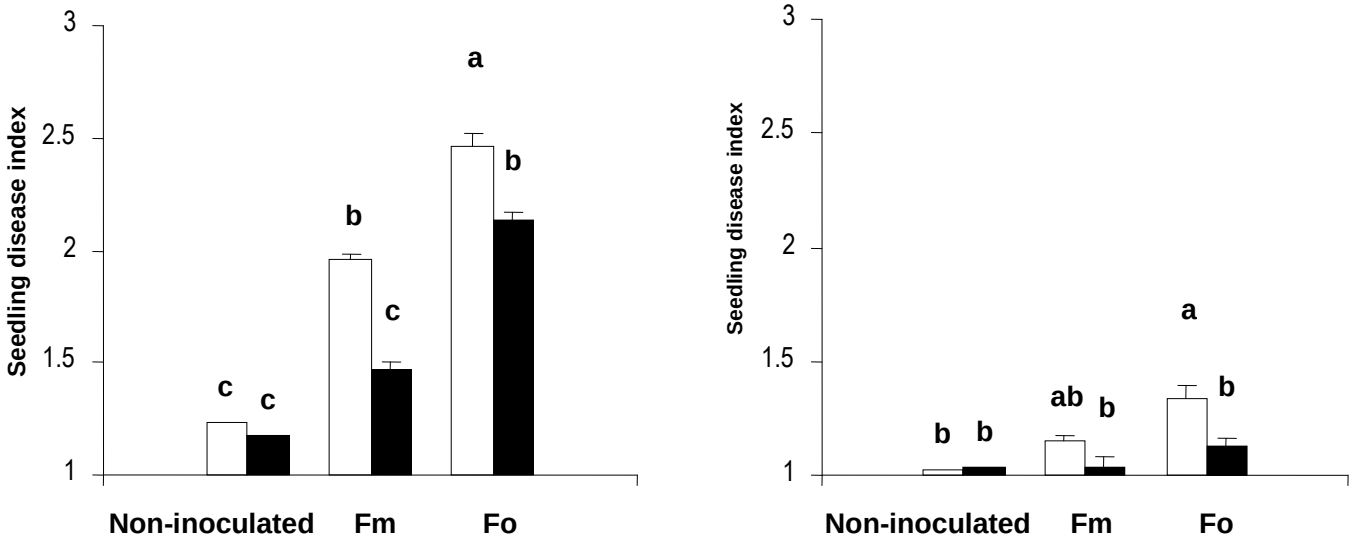

Figure 1. Effect of *Suillus luteus* inoculation (dark bars), on seedling damage Index (SDI) of Scots (left) and Stone (right) pine seedlings inoculated with *Fusarium oxysporum* (Fo) and/or *F. verticillioides* (Fm) (white bars). Vertical bars followed by different letters are significantly different (LSD Fisher test, P=0.05).

Fusarium and Myc treatments (Table 1). Moreover, SDI differences between pine species were related to the *Fusarium* isolates (Pine × *Fusarium* interaction: p<0.01). The LSD Fisher test showed *F. oxysporum* to produce a significantly higher SDI than *F. verticillioides* (Figure 1). Stone pine seedlings were less affected by *Fusarium* than those of Scots pine.

Inoculation of pine seedlings with the ectomycorrhizal fungus *S. luteus* reduced damage by *Fusarium* damping off (Figure 1). Thus, Scots pine seedlings inoculated with *F. verticillioides* and *F. oxysporum* had a reduced SDI when co-cultured with *S. luteus*. Damage in Stone pine seedlings inoculated with *F. oxysporum* was significantly reduced in the presence of Myc fungus, but no reduction of disease symptoms was observed on those inoculated with *F. verticillioides*. A similar protective effect was also obtained by other Myc fungi on Douglas-fir, Jack pine (Chakravarty and Hwang, 1991; Hwang et al., 1995), and Scots pine (Machón et al., 2006, 2009). This is the first time that *Fusarium* antagonism has been demonstrated with *S. luteus.* However, not all the Myc tested against damping-off pathogens showed a protective effect. Thus, Hwang et al. (1995) working with a close species, *Suillus tomentosus*, failed to demonstrate a positive outcome (clearly showed with the other Myc tested, *Paxillus involutus*) on Jack pine (*Pinus banksiana* Lamb.), despite the significant level of mycorrhization obtained in the plants treated with *F. verticillioides* (10%). It seems that the damping off antagonisms by Myc may be related to the mycorrhizal species selected.

Root colonization was 3.68% in the Myc treated Stone pine plants (Table 2). Mycorrhizal formation was significantly reduced when seedlings were co-inoculated with *F. verticillioides* (1.25%, p=0.049), and no mycorrhizal apexes at all were found in co-inoculations with *F. oxysporum*. Root colonization was absent in the Stone pine treatments without the Myc inoculum. Colonization in Scots pine roots was 9.34% in the Myc

Table 2. Number of mycorrhizal short roots (%) on Stone pine and Scots pine seedlings.

Treatment	Scots pine	Stone pine
Not inoculated	16.24[a]	0[b]
Fm	0[b]	0[b]
Fo	0[b]	0[b]
Myc	9.34[a,b]	3.68[a]
Fm+Myc	15.24[a]	1.25[b]
Fo+Myc	0[b]	0[b]

Fm: *Fusarium verticillioides;* Fo: *F. oxysporum;* Myc: ectomycorrhizal fungus *Suillus luteus*. Means followed by different letters within each column are significantly different. LSD Fisher test, P=0.05, n=15.

treatment, and it was apparently increased (p=0.324) when the pots were co-inoculated with *F. verticillioides* (15.24%), but no mycorrhizal apexes were found in the seedlings co-inoculated with *F. oxysporum*. As expected, no mycorrhizal formations were found in seedlings from the Fm and Fo treatments. Nevertheless, Scots pine non-inoculated treatment showed a significant number of mycorrhizal short roots, similar to those of the Myc treated seedlings (16.24%, p=0.258), despite the precaution measures used to avoid contamination. This might be one of the reasons why there were no differences between non-inoculated and Myc treated seedlings neither in their SDI nor in the plant growth variables. However, in Stone pine, where some mycorrhization was achieved, there were no differences compared to controls either.

The interaction of Myc fungi with root pathogens of pines is still not well understood. Duchesne (2000) hypothesized that root protection by the Myc may be the result of three effects: a protective barrier caused by the presence of a fungal mantle around the roots, the production of antimicrobial substances either by the mycosymbiont or by the host plant, and the competition for nutrients in the rhizosphere. Morin et al*.* (1999) found a negative correlation between the percentage of infected plants by *Cylindrocladium floridanum* and the intensity of mycorrhizae formation in the roots of black spruce seedlings. In our study, the antagonism caused by *S. luteus* was not related to the percentage of mycorrhizal apexes formed in the host roots. Thus, in Stone pine seedlings this value was only 1.25% in the Fm+Myc treatment and no mycorrhizal apexes were found in the Fo+Myc treatment, although *S. luteus* was only effective against *F. oxysporum* (Figure 1). In Scots pine seedlings, mycorrhization appeared only in the Fm+Myc (15.24%), but not in the Fo+Myc treatment, however, *S. luteus* antagonism was significant for both pathogens. It seems then that the protection exerted by *S. luteus* against Scots and Stone pine damping-off was not associated with a protective barrier by the Myc fungal mantle around the roots, as hypothesized by Duchesne (2000). Similar results were obtained by Diedhiou et al. (2003) on *Meloidogyne incognita*, where a clear relationship between mycorrhyzation and nematode control could not be established.

Several authors have reported disease suppression by Myc fungi associated to fungal-produced antimicrobial substances (Chakravarty and Hwang, 1991; Zhang et al., 2011; Diez and Alves-Santos, 2012). Toxic effects of mycorrhizal fungi have been described not only against plant pathogens, but also against insects (Halldorsson et al., 2000) and nematodes (Diedhiou et al., 2003). Antagonism of *Boletus edulis*, *Rhizopogon roseolus*, *Laccaria laccata* and *Lactarius deliciosus* against spore germination of *F. oxysporum* and *F. verticillioides* by toxic-like compounds released to the culture media has been previously confirmed in our lab (Martín-Pinto et al*.,* 2006a), besides *S. luteus* (Olaizola et al*.,* 2003). Therefore, suppression of *Fusarium* spp. damping off by *S. luteus* could be due in part to the production and release to the peat moss of antifungal compounds by this Myc.

One mycorrhizal morphotype was identified on the roots of Scots and Stone pine seedlings showing dichotomously branched mycorrhizae. This was similar to one of the three morphotypes obtained by Kieliszewska-Rokicka et al. (1998) after inoculation of Scots pine with *S. luteus in vitro*. In our study, the number of mycorrhizal short roots formed in the root of Scots (9.34%) and Stone pine (3.68%) was lower than those obtained by these authors (that reached the 90%) or by Hwang et al. (1995) with Jack pine and *S. tomentosus* (51.5%). Similar low values of mycorrhization for *S. luteus* (2.87% for Stone pine and 2.46% for Scots pine) were reported in post-emergence damping off assays using two-month old seedlings (Mateos et al., 2004).

The number of mycorrhizal apexes was higher in Scots than in Stone pine seedlings, similarly to that obtained with *Laccaria laccata* in a similar assay (Machón et al., 2004, 2009). Mycorrhization ability might be related to the host, as shown in another ectomycorrhizal fungus (Parladé et al., 2004).

Contrary to other reports (Smith and Read, 1997; Kieliszewska-Rokicka et al., 1998; Guerin-Laguette et al., 2004), a positive effect on plant growth (shoot height, shoot dry weight, root collar diameter, root length, root dry weight) in mycorrhized seedlings was not clearly shown in our study (Tables 3 and 4). This fact may be related to the poorly colonized root systems in the plants inoculated with the Myc fungi. However, Hwang et al*.* (1995) and Diedhiou et al*.* (2003) working with heavily mycorrhized seedlings also failed to demonstrate this positive effect on plant growth.

Hwang et al. (1995) observed a decrease to one-fifth of the number of mycorrhizal short roots formed in Jack pine seedlings mycorrhized with *S. luteus* after *F. verticillioides* inoculation. In our work, the presence of *F. verticillioides* lowered to one-third the percentage of

Table 3. Effect of *Suillus luteus* (Myc), *Fusarium oxysporum* (Fo) and/or *F. verticillioides* (Fm) on shoot height, shoot dry weight, root collar diameter, root length and root dry weight of Scots pine seedlings 18 weeks after sowing.

Treatment	Shoot Height (cm)	Shoot dry weight (g)	Diameter of root collar (mm)	Root length (cm)	Root dry weight (g)
Not inoculated	6.01^{a}	0.05^{a,b}	0.54^{b}	14.46^{a}	0.03^{a}
Fm	5.53^{a,b}	0.06^{a,b}	0.74^{a}	14.16^{a}	0.03^{a}
Fo	5.65^{a,b}	0.04^{b}	0.60^{b}	13.63^{a,b}	0.03^{a}
Myc	5.89^{a,b}	0.06^{a}	0.65^{a,b}	13.83^{a}	0.03^{a}
Fm+Myc	5.63^{a,b}	0.05^{a,b}	0.57^{b}	13.76^{a}	0.03^{a}
Fo+Myc	5.41^{b}	0.04^{b}	0.57^{b}	12.57^{b}	0.02^{a}

Fm: *Fusarium verticillioides;* Fo: *F. oxysporum;* Myc: ectomycorrhizal fungus *Suillus luteus*. Means followed by different letters within each column are significantly different. LSD Fisher test, P=0.05, n=15.

Table 4. Effect of *Suillus luteus* (Myc), *Fusarium oxysporum* (Fo) and/or *F. verticillioides* (Fm) on shoot height, shoot dry weight, root collar diameter, root length and root dry weight of Stone pine seedlings 18 weeks after sowing.

Treatment	Shoot Height (cm)	Shoot dry weight (g)	Diameter of root collar (mm)	Root length (cm)	Root dry weight (g)
Not Inoculated	12.39^{a,b}	0.40^{a,b}	1.48^{a}	13.46^{a,b}	0.12^{a,b}
Fm	11.38^{b,c}	0.33^{b}	1.35^{a}	13.42^{a,b}	0.09^{c}
Fo	11.61^{a,b,c}	0.34^{b}	1.49^{a}	12.67^{b}	0.09^{c}
Myc	12.97^{a}	0.45^{a}	1.52^{a}	14.27^{a}	0.13^{a}
Fm+Myc	10.47^{c}	0.32^{b}	1.44^{a}	13.40^{a,b}	0.09^{c}
Fo+Myc	12.11^{a,b}	0.37^{b}	1.34^{a}	14.43^{a}	0.10^{b,c}

Fm: *Fusarium verticillioides;* Fo: *F. oxysporum;* Myc: ectomycorrhizal fungus *Suillus luteus*. Means followed by different letters within each column are significantly different.

mycorrhizal short roots in the Myc treated stone pine plants, whereas in the seedlings inoculated with *F. oxysporum*, no mycorrhizae formation was observed, so it appears that the presence of this pathogen inhibited the mycorrhizal process. However, this effect may possibly be only linked to the pathogenic isolates of *Fusarium*, since other studies (García-Romera et al*.,* 1998; Diedhiou et al*.,* 2003) described a stimulation of mycorrhization in the presence of saprophytic *F. oxysporum* strains.

Results indicate a positive role of *S. luteus* for biological control of damping off in forest nurseries. However, the protective effect provided by *S. luteus* against damping-off by *Fusarium* spp. was not related to the percent of mycorrhizal apexes in the roots of Stone and Scots pine seedlings. More work has to be done to explore the active mechanism of disease suppression by this ectomycorrhizal fungus and also for considering its practical application in commercial nurseries.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

The ameliorative role of cranberry extract and bone marrow cells against chlorambucil cytotoxicity in rat fertility

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The objective of the current study was to evaluate and compare the effectiveness of cranberry extracts and bone marrow cells against chlorambucil (CHB) effect on rats' fertility. Forty adult male albino rats were divided randomly into eight equal groups as the following; normal control, rats injected orally with 0.2 mg/kg of CHB for 14 days, rats injected orally with 100 mg/kg of cranberry extract (CB) for ten days, rats intravenously injected with bone marrow cells (BMC) through tail vain, rats protected with both CB and BMC, rats treated with CHB+CB, rats treated with CHB+BMC and rats treated with CHB+BMC+CB. Genotoxicity were evaluated by counting and comparing the value of sperm abnormalities and normal sperm count. Results show that rats injected with CHB had remarkable increase in sperm head abnormalities as without hook, banana shape and hummer shape. Admission of cranberry extract and bone marrow cells after chemotherapy improved the frequency of the sperm abnormalities.

Key words: Chlorambucil, cranberry, bone marrow, sperm.

INTRODUCTION

Chlorambucil (CHB) is a potent chemotherapy bifunctional alkylation agent which is commonly known as Leukeran and known chemically by IUPAC system as 4-[p-[bis (2chloroethyl) amino] phenyl] butyric acid (Evert et al., 1953). CHB is used as treatment for chronic lymphatic leukemia and other clinical applications such as Hodking's and non-Hodking's lymphoma (Wohrer et al., 2005). Although it has a high therapeutic activity, CHB is a potential human carcinogen with increasing risk towards the development of secondary cancer in patients (Neugut and Ulpric, 2008). The cytotoxicity of CHB is caused by its ability to form a strong covalent bond with proteins, RNA and DNA double strand which leads to structural and functional DNA damage that is considered one of the leading causes of mutagenic and carcinogenic effects (Tew, 2008). CHB also has damaging effects on gonads by causing temporary azospermia and damage on the Leydig cells of male testis. Hence, it causes a malfunction in the reproductive system as well as permanent azospermia when the drug has been taken for long periods or in high doses (De Vita et al., 2001). Bone marrow originated from hematopoietic stem cell and

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mesenchymal stem cells which give the bone marrow its importance and function; where it has the replacement ability for various types of damaged cells (Teitelbaum, 2000). Bone marrow transplantation is considered as an effective method in the treatment of different diseases like leukemia (Gratwohl et al., 2006), accelerate wound healing in diabetic rats (Mcfarlin et al., 2006), and it is used after a high dose of radiation or chemotherapy to help the body recover from the damaging effects of chemotherapy.

Cranberry (*Vaccinium macrocarpon*) is a small, dark red fruit that is known as a very rich source of phytochemicals (Cunningham et al., 2003). It contains many active components such as flavonols, sugar, organic acid and flavonoids as anthocyanins and proanthocyanidis (Ariga, 2004). Nowadays, using cranberry has been expanded from using it as a source of healthy food into medical one. Cranberry components exhibit various health benefits including prevention of adhesion of microbes in the urinary tract (Reid et al., 1992) and cholesterol reduction (Reed, 2001). Moreover, it has a very strong antioxidant activity (Yan et al., 2002) and anticancer effects because its activity prevents tumor cells from proliferating (Sun et al., 2002). The mammalian sperm is a terminally differentiated cell which appears deceptively simple (Ramalho-Santos et al., 2002). It has a limited function in the vast majority of creatures which is delivering the haploid genome to the oocyte during fertilization and this function is associated with many physiological, cytological and molecular biology changes that is able to alter animal production, infertility and toxicology. Sperm morphology is used to study the medical and chemical effects on an experimental animal body before it can be implemented into human body (Hu and Yan, 2002). Hence, numerous drug discoveries are tested in rats before approval for human clinical trials (Alias et al., 2011). The purpose of the present study was to evaluate the protective effect of the cranberry extract and bone marrow cells on sperm abnormalities induced by CHB on Swiss albino rats (*Rattus norvigicus*).

MATERIALS AND METHODS

Chemicals

CHB (leukeran) was purchased from (Excella pharma GmbH, Germany) as 2 mg tablet which is dissolved in distilled water and given to animals through gastric tubes. Each animal received (0.2 mg/kg/day) for body weight for fourteen days daily according to Olyinka et al. (2014). Cranberry was purchased from EMA pharma found in capsule form that contains 270 mg of cranberry extraction. Each rat was given cranberry through gastric tube as dose (100 mg/kg/day) for body for ten days daily according to Elberry et al. (2010).

Bone marrow preparation

The bone marrow was taken from young male Swiss albino rats, approximately six weeks old. Recipients and donors were chosen from the same inherited strain (brother to brother) which were sacrificed. Both femurs ends were cut off with sharp bone cutter and then by applying air pressure gently the bone marrow was forced out into small sterile test tube. It was possible to obtain 80:100 mg bone marrow (wet weight) from both femurs, then the weighted bone marrow was suspended in appropriate volume of cold M/15 phosphate buffer (pH=7.2). Rats were injected with 1 ml of bone marrow suspension via tail vein by using a 26 gauge needle (Zowail et al., 2012).

Experimental design

Forty male Swiss albino rats (*R. norvigicus*) (180 to 200 g) were used in this study. Animals were obtained from the National Research Center in Dokki, Cairo, Egypt. The experimental study was performed as international followed by ethical standards according to the guide for the care and use of laboratory animals of the National Institutes of Health. Animals had free access to water and complete food supplied *ad libitum* during the whole experimental period and kept under suitable conditions.

The experimental animals were categorized in 8 equal groups, 5 animals each. The animal received the various treatments for different periods as shown in Table 1.

Sperm head morphology assay

The sperm suspension was obtained from rats by excised both caudal epidedymis then they were minced together in isotonic solution and filtered to exclude the large tissue fragments. The sperm suspension was stained with 5% Eosin Y stained (aqueous), then spread on clean glass slides (Mukherjee et al., 1988). One thousand were examined directly under light microscope (100X) to detect the morphological abnormalities in sperm according to the criteria of Wyrobek and Bruce (1975). Any overlay or contact sperms or heads without tails were ignored.

Statistical analysis

Statistical analysis was performed on SPSS software (version18) using one-way analysis of variance (ANOVA). Significance was considered when P values are less than 0.05. Mean standard error (SE) was expressed to all values where 5 animals were evaluated in each group.

RESULTS

Various types of sperm head abnormalities appeared in Swiss albino rat injected with CHB. These were identified and quantified relatively to control group. The sperm head abnormalities represented as without hook (Figure 1b), banana shape (Figure 1c), and hummer sperm (Figure 1d).

The results illustrated in Table 2 and Figures 2 and 3 show the sperm head abnormalities and the number of normal sperm per five thousand in five rats. The result revealed that CHB when given at the dose of 0.2 mg/kg body weight daily for 14 days induced a high frequency of sperm head abnormalities when compared with control group. Without hook, sperm head shape was the most frequent abnormalities followed by banana and hummer shape. There is a significant increase in the total sperm

Table 1. Experimental group.

Group	Treatments
G1	Negative control, receiving 0.9% NaCl (0.5 ml intraperitoneal injection (i.p) twice/week for 14 days)
G2	Chlorambucil (0.2 mg/kg b.w./ml) (1 ml oral intubation daily for 14 days)
G3	Cranberry (100 mg/kg b.w./ml) (1 ml oral intubation daily for 10 days)
G4	Bone marrow single dose (1 ml of bone marrow suspension injected intravenously via tail vein)
G5	Bone marrow cells as single (1 ml) dose after cranberry (100 mg/kg b.w./ml) for 10 days
G6	Chlorambucil (0.2 mg/kg b.w./ml) for 14 days before cranberry (100 mg/kg b.w./ml) for 10 days
G7	Chlorambucil (0.2 mg/kg b.w./ml) for 14 days then bone marrow (1 ml) single dose
G8	Chlorambucil (0.2 mg/kg b.w./ml) for 14 days then cranberry (100 mg/kg b.w./ml) for 10 days after that bone marrow single dose

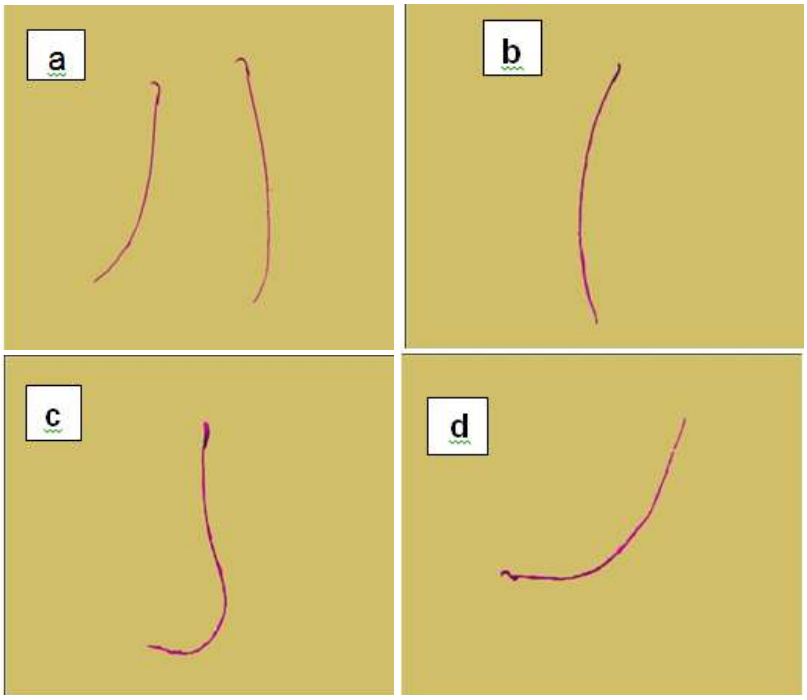

Figure 1. (a) Normal sperm. **(b)** Without hook. **(c)** Banana shape. **(d)** Hummer sperm.

Table 2. Average of sperm head abnormalities of male rat treated with chlorambucil, cranberry and bone marrow.

Group	Normal	Without hook	Banana	Hummer	TSA
Control[a]	935±8.22[b]	35.4±7.79[b]	15±4.64[b]	9.4±2.06	59.8±14.49[b]
CHB[b]	834.4±16.49[a,c,d,e,f,g,h]	108.8±16.03[a,c,d,e,g,h]	44.6±4.41[a,c,d,e,f,g,h]	9±1.45[d]	162.4±21.89[a,c,d,e,f,g,h]
CB[c]	936±4.56[b]	30.4±2.56[b,g]	17.2±4.14[b]	8±0.93	55.6±7.63[b]
BM[d]	947±3.68[b,g]	24.6±1.36[b,g]	16.2±0.97[b]	4.6±1.21[b]	45.4±3.54[b,f]
CB+BM[e]	964±3.99[b]	28.6±3.58[b]	13.6±0.98[b,f]	9±2.91	51.2±8.77[b,d,h]
CHB+CB[f]	926.8±8.78[b]	40±5.33[h]	20±2.32[b,e]	10.6±2.91	70.6±10.53[b,d]
CHB+BM[g]	924.4±4.88[b,d]	42.24±2.9[b,c,d]	15.4±1.44[b]	7.8±1.56	65.6±5.9[b]
CHB+CB+BM[h]	926.8±4.87[b]	24.4±0.81[b,f]	19±1.76[b]	5.6±1.33	49±3.9[b,f]

TSA, Total sperm abnormalities; CHB, chlorambucil; CB, cranberry; BM, bone marrow. Average was expressed as mean ± standard error. The significant were indicated as follow: [a]Significant with control; [B]significant with CHB; [c]Significant with C.B; [D]significant with (BM); [E]significant with (CB+BM); [F]significant with (CHB+CB); [G]significant with (CHB+BM); [h]significant with CHB+CB+BM; significant means $p<0.05$.

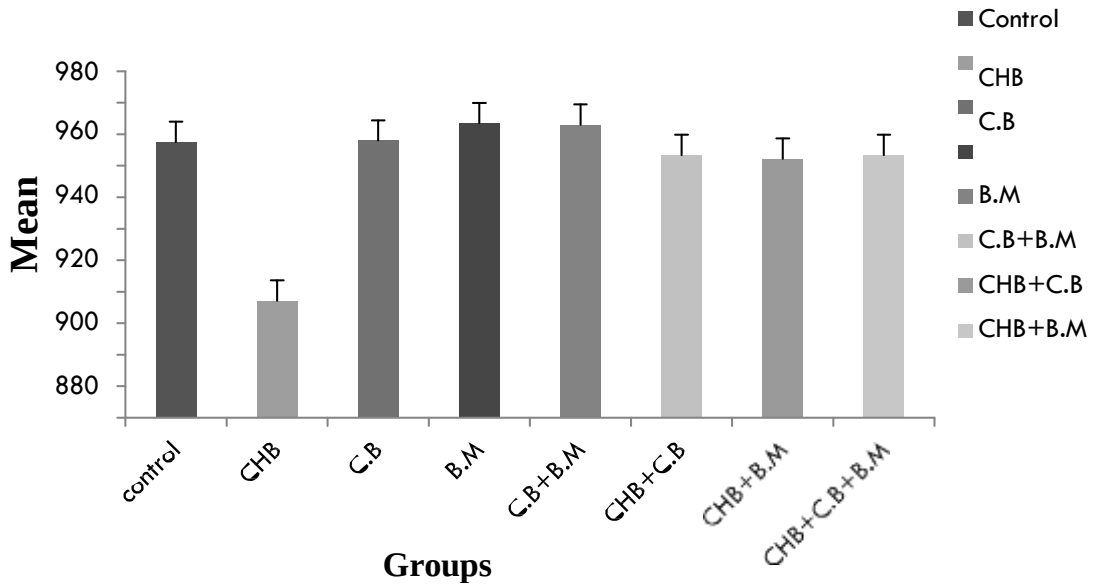

Figure 2. The mean value of normal sperm heads of rats treated with CHB, CB and BM.

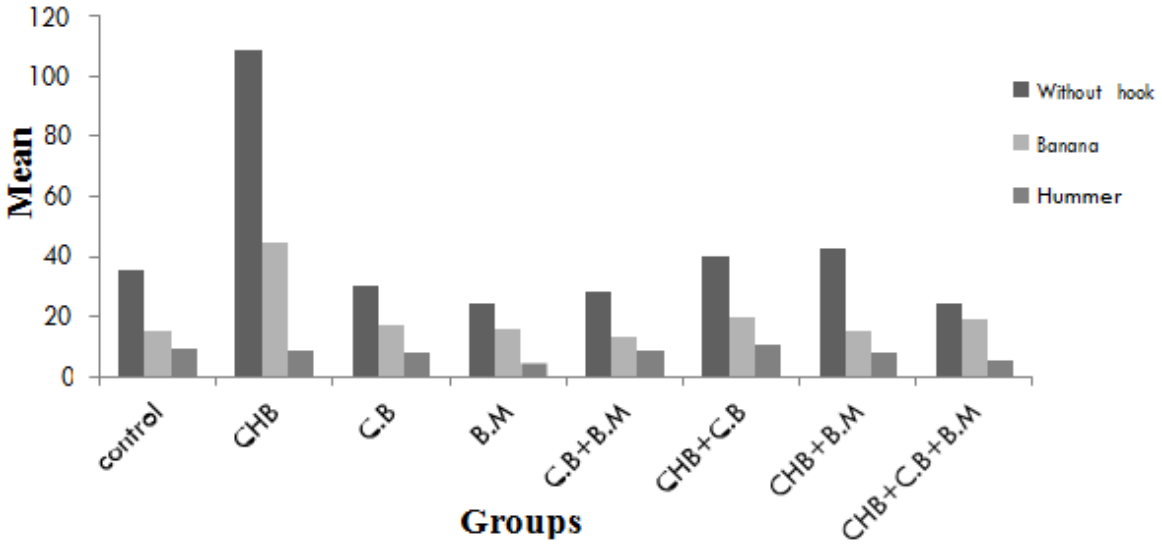

Figure 3. Average of total sperm head abnormalities observed in male rat treated with CHB, CB and BM. CHB, Chlorambucil; CB, cranberry; BM, bone marrow.

abnormalities in CHB treated group at P<0.05 over the control group where the mean value of total head abnormalities of CHB compared to the control were (162.4±21.89 vs. 59.8±14.49).

The result in Table 2 shows that when cranberry extract (CB) is injected at given dose (100 mg/kg body weight) daily for ten days and bone marrow (BM) as single dose injection was given separately or together did not induce significant difference of sperm abnormalities or normal sperm count compared to control group. Interestingly, the result indicates that when cranberry and bone marrow were administrated separately with CHB showing significant decrease in the rates of rats sperm head abnormalities changes lower than those treated with CHB only. There is a remarkable significant decrease in the mean value of sperm head abnormalities that appeared in the group treated with CB and BM after CHB compared with CHB group, where the mean value of CHB+CB+BM was 49±3.9 which is lower than CHB group mean value (162.4±21.89).

DISCUSSION

There are ongoing trials to reduce and eliminate the harmful side effects of chemotherapy on the fertility of patients. Nowadays, the objective of most research is to find out the most effective natural products and strategies to use it for treatment of cancer.

The present study aimed to investigate the protection action of cranberry extract and bone marrow transplantation as the way to reduce the side effects of CHB on sperm abnormalities and total normal sperm count.

Cranberry is considered one of the useful natural products that is used as antibacterial and anticancer drug (Yan et al., 2002) as it has antipoliferative activity to protect body cells from damage (Sun et al., 2002). Cranberry extract has a great ability to inhibit more than 50% of prostate cancer, where polyphenols fraction is the most effective cranberry component against prostate tumor (Seeram et al., 2004). Bone marrow transplantation is considered a useful method that helps in body treatment from malignance tumor as they contain both mesenchymal stem cells that is able to differentiate various types of body cells like hepatic cells (Dai et al., 2009).

The present study results indicate that CHB has a damaging effect on the normal sperm by decreasing its count and increasing the presence of rats sperm head abnormalities as those without hooks, banana shape and hummer head. These results agree with those reported by Viviani et al. (1985) that the combination between alkylating agents as CHB and procarbazine for treating solid Hodgkin's lymphoma showed an excellent high survival rate but the majority of male patients later developed permanent azospermia (Viviani et al., 1985). Wallace et al. (2005) mentioned that cytotoxic chemotherapy such as CHB and melphalan have the ability to cause gonad injury and illustrated that damage nature and extend depends on the dose of the drug given and the period of administration and this injury may be developed to oligospermia or worse condition as azospermia and it indicates that this effect is a result for Leydig cell dysfunction especially after the increasing accumulative dose of gonad toxicity chemotherapy (Wallace et al., 2005). Testes germinal epithelium cells is characterized by rapid divining rate so they are the most sensitive organ to cytotoxic chemotherapeutic agents which lead to permanent damage and disorder in spermatogenesis process (Howell et al., 1999). Haskell study has indicated that chemotherapy targets rapidly dividing cells which causes germinal cell aplasia that quilts the seminiferous tubules and reduces sperm production which may cause oligospermia or azospermia, thereby, affecting the patient's fertility (Haskell, 2001). The current study result shows a decrease in sperm head abnormalities and an increase in normal sperm count in the group which is protected by cranberry and/or bone marrow separately or in the animals group that received CB and/or BMC after CHB administration. This result agrees with another study was done by Mohammadi who indicated that phytochemical medicines have a potent positive impact on human sperm parameters such as motility, count and viability. Increase in Leydig cell count and seminiferous tubule diameters, improve histopathological recovery of testis, able to decrease abnormal sperm count, enhance sperm motility and increase concentration in ejaculation volume (Mohammadi et al., 2013). Moreover, the study done by Dobrzynska (2013) indicated that resveratol as natural polyphenol (found in cranberry) is considered a promising hope to treat male infertility as it enhances sperm motility and count in rodents. Resveratol was able to inhibit the toxic effect of other agents. Moreover, it has the ability to modulate cell behavior in response to the damaging effects that are induced by radiation. Therefore, using cranberry might be very useful in cancer therapy (Dobrzynska, 2013).

Conclusion

The current study indicates that using cranberry extraction and bone marrow transplantation after CHB administration reduces sperm head abnormality rate and increases the count of normal perm.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Tradescantia pallida as a biomonitoring tool to assess the influence of vehicle exhaustion and benzene derivatives

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Air pollutants affect their quality, making, inappropriate, harmful and damaging to health in general. The air quality can be measured by the use of higher plants as bioindicators in environmental biomonitoring. In this study, we evaluated the increased frequency of micronuclei (MN) in *Tradescantia pallida* exposed to potentially toxic environments. The vegetables were implanted in five points (4 test and 1 control) for 15, 30 and 60 days. After this period of exposure, the young inflorescences were analyzed and the observation frequency of MN using the technique of *Tradescantia-micronucleus* (TRAD-MCN). The results showed an increased frequency of MN in pollen grains of *T. pallida* in environment with severe vehicular exhaust and manipulation of benzene derivatives, indicating that the compounds present in the atmosphere of these environments have genotoxic potential.

Key words: Atmospheric pollutants, bioindicators; genotoxicity, pollen grains, *Tradescantia pallida*.

INTRODUCTION

According to Conama Resolution (n° 3) 28/06/1990, smog refers to the presence of compounds or substances that promote interference in the air quality. The air quality standards, primary and secondary, suffer direct

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interference from vehicular exhaust, considered the main cause of air pollution. Volatile organic compounds, particulate materials, nitrogen oxides, carbon and sulfur, and benzene derivatives, such as polycyclic aromatic hydrocarbons (PAHs) cause damage to the environment, affecting the flora, fauna, and people, for making improper air, inconvenient, harmful and detrimental to health in general (Pereira et al., 2013; Inomata et al., 2015).

The chemical modification air quality standard can be measured through the use of bioindicators, plant or animal beings, characterized by the use in biomonitoring of air quality (Billet et al., 2015). As a result of, the plants are inexpensive, easy to use and cultivation, have shown as the most effective means of an assessment and response quality of the environment, presenting practical use mainly related to air pollution control (Rodríguez et al., 2015).

The variation in biological monitoring techniques is sufficient to encompass the use of various species, believed to be active biological monitors (Güez et al., 2012; Ávila et al., 2013). The use of superior plants such as angiosperms, in biomonitoring studies has grown sharply (Santos, 2015). The *Tradescantia pallida*, is popularly known as tetrapoeraba-roxa. It is an ornamental plant widely distributed in Brazil, as it has a great adaptability to climate variations. It is a small angiosperm in the Commelinaceae family for presenting genetic characteristics favorable to studies, such as large chromosomes, it is often used as a test system in biomonitoring (Carvalho, 2005; Crispim et al., 2012).

One of the parameters for biomonitoring is the comparative analysis of the structural pattern of leaves with anatomical features arising from the exposure to a particular agent. These features can be macro or microscopic, as the decrease in leaf size and stomata, organelles responsible for gas exchange (Mott et al., 2014).

Other trials from *Tradescantia* are cytogenetic tests, related to the analysis of the cellular genome. Two of these tests are widely accepted on the observation of somatic mutations or changes in the structure of chromosomes: The test with analysis of mutations in stem hairs (Trad-SHM) and bioassay of MN in pollen grains of stem cells (Trad-MCN) (Carvalho, 2005; Savóia et al., 2009; Andrade-Vieira et al., 2011).

Thus, this study aimed to evaluate the toxic effects by increasing frequency of micronuclei (MN) in *T. pallida* exposed to few zones in the city of Teresina, Piauí, Brazil.

MATERIALS AND METHODS

T. pallida was identified by the Herbarium Graziela Barroso - TPB (Federal University of Piauí) with the voucher number 28339. Then 50 seedlings of *T. pallida* were cultivated in small pots with fertile soil and regular irrigation. We distributed the plants in 4 points in the city of Teresina - Piauí, during the period of August to October in 2012. It is because, August to October is the dry season with more dust, temperature, and vehicle exhausts in this state of Brazil. The municipality is located on the right bank of the Parnaíba River and occupies a total area of 1,809 km^2, with geographical coordinates: 05º05'12" south latitude and 42º48'42" west longitude (Branco, 2003).

The areas selected for the exhibition were: a petrol filling station (5°04'12.54"S, 42°81'26.22"W), an auto body repair of vehicles (5°11'21.47"S, 42°79'13.78"W), responsible for handling solvents and coating materials, to be submitted to exhaust environments of automotive vehicles and handling benzene derivatives; a via of access, avenue Frei Serafim (5°08'37.82"S, 42°79'75.33"W), and a vehicle parking in an institution of higher education (5°06'89.23"S, 42°74'70.64"W); by having heavy automobile traffic, thus being subjected to oxidation fuel and large amounts of particulate materials (Figure 1). In addition to the 4 test points, 1 was also exposed to an area with low frequency of urban pollution, operating as a negative control (NC) (5°11'73.89"S, 42°70'98.56"W).

After the cultivation, 10 seedlings were sent for display in each of their previously cited environments, and they were weekly watered with deionized water. Plant structures used in laboratory analysis were young inflorescences (Figure 2), where pollen grains appear more abundantly on tetrad stage. The inflorescences withdrawals were carried in aqueous solution (for maintenance adequate humidity and integrity) to the laboratory and analyzed in the range of periods: 15, 30 and 60 days after implantation. This procedure related to frequency of MN found in pollen grains of stem cells with the time of exposure to clastogenic effects of pollutants being studied.

Once young inflorescences were removed, they were fixed in methanol: acetic acid (3:1) for 24 h and stored in ethanol (70%). The flower bud clumps were dissected out and macerated in glass slide for staining acetic carmine (2%) and further heating to about 60°C for impregnating the dye. In an optical microscope at magnification of 400 X, 3000 tetrads were viewed for each exposure period. The occurrence frequency of MN was obtained from the ratio between the number of MN in 300 tetrads for each exposure place at all points, including the NC, in each time period described above, and follows the method described by Andrade-Vieira et al. (2011).

Statistical analysis was performed using GraphPad Prism (version: 5.0). values are mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Bonferroni and Dunnett's test, considering $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

The results allowed the increase in the frequencies of MN in *T. pallida*, from exposure to gaseous pollutants originating from the vehicle combustion and benzene derivatives action in periods of 15, 30 and 60 days of exposure as shown in Figures 3 to 5.

Figure 6 shows all points with the NC, total period of 60 days, allowing an analytical correlation between the genotoxic influence of air pollutants, absorption time and accumulation of these air pollutants by plants and environments more significance for induction of MN.

The median frequency of MN in *T. pallida* obtained by tetrads counts 3000 per point in each period demonstrated great variance as compared to the NC as shown in Table 1. Exposure times were compared to determine the presence or absence of relevant statistics on the test points as shown in Table 2.

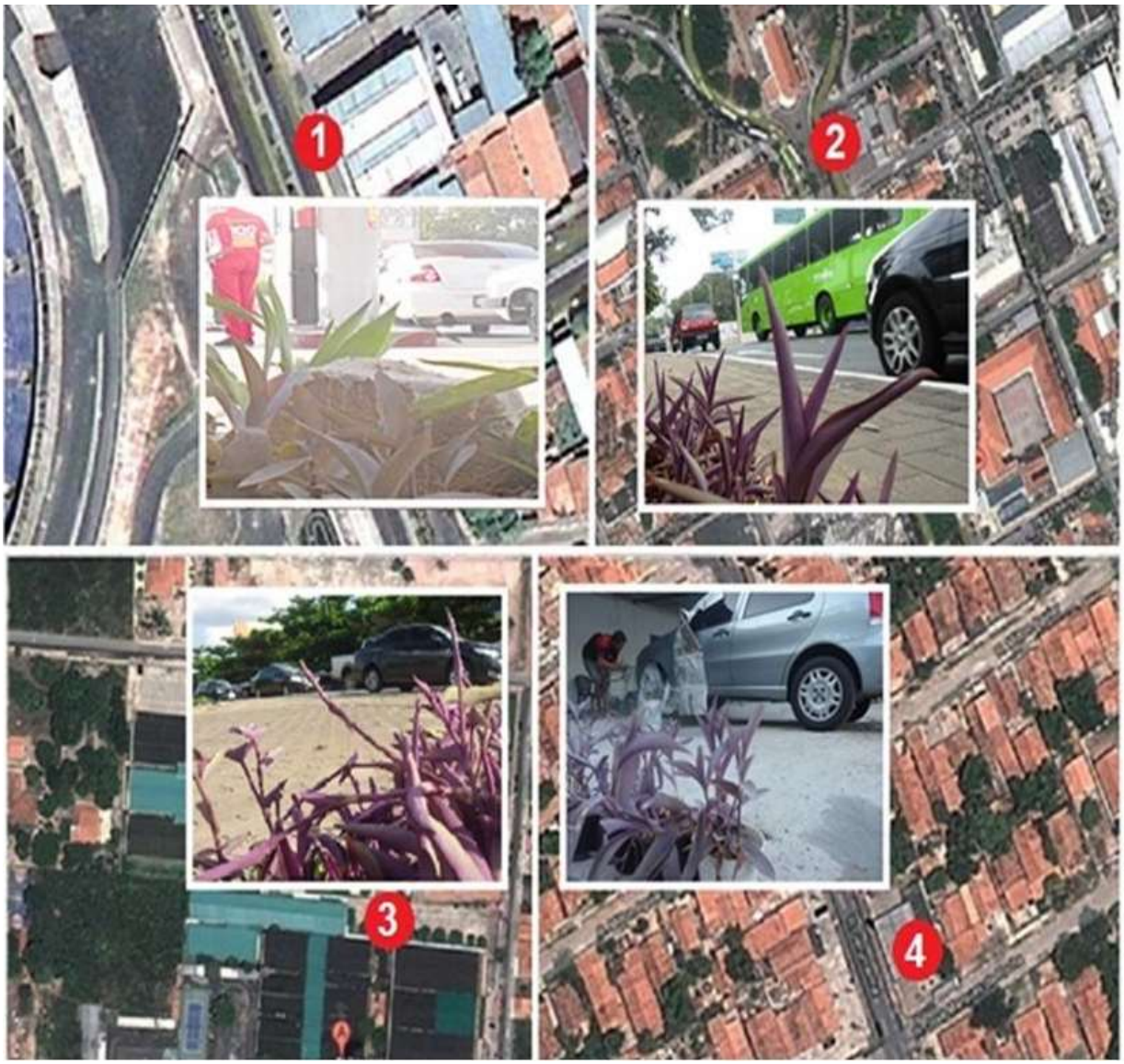

Figure 1. *Tradescantia pallida* exposure places in the city of Teresina: fuel refueling point (point 1), access way, avenue Frei Serafim (point 2), vehicle parking lot of a Higher Education Institution (point 3), and workshop manipulation of coating materials and solvents (point 4).

DISCUSSION

The plants are more sensitive to pollution than animals and man. Thus, they favor subsidies and an easier study of the effects of pollutants in the environment (Klumpp et al., 2006; Maioli et al., 2008). Chemical and physical agents exert influence vastly on the rate of change of biochemical activities in plants, thus increasing the frequency of changes in DNA molecules has been widely studied (Carvalho, 2005).

Frequency of MN showed elevated in all points when compared to the NC group. According to Figure 1, although the increase in the percentage of this type of chromosomal damage was not significant in points 1 and 3 within 15 days after implantation of *T. pallida*, points 2 and 4 had high statistical significance.

Low MN rates found in sections 1 and 3, respectively petrol station and parking, are justified by the reduced exposure to vehicle exhaust, the main cause of air pollution. Since the high frequency of MN in points 2 and 4, respectively an avenue of the great movement of vehicles and a garage, it is justified by the greater presence of toxic compounds due to the intense vehicular exhaust and direct manipulation of benzene derivatives in these environments.

The amount of carrier flow and handling content of compounds derived from benzene led to a wide variability in the results between the test points. The tests, as well

Figure 2. Young inflorescence of *Tradescantia pallida* (white arrow).

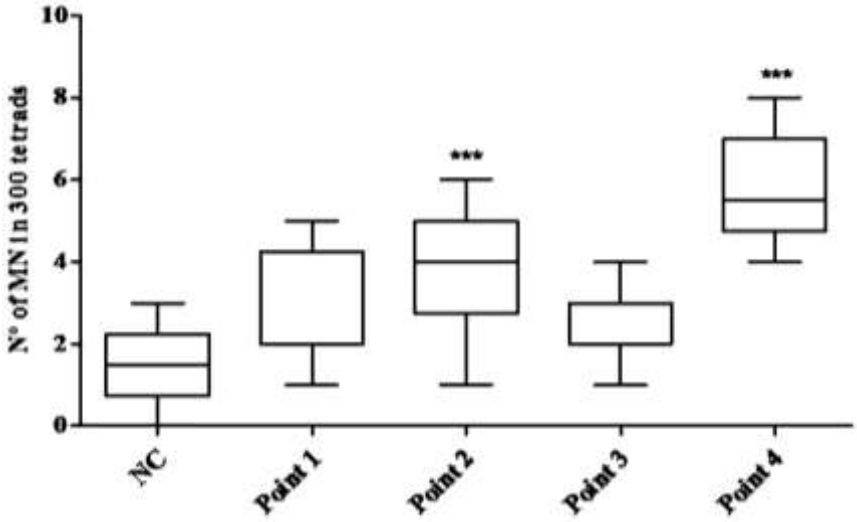

Figure 3. Frequency the micronuclei (MN) in (mean ± SD) of young *T. pallida* inflorescences after 15 days of seedlings exposure to local testing and control test. ANOVA followed by Dunnett's multiple comparison test; frequency of MN at significance of p<0.001*** compared to the NC.

literature showed a direct and proportional relation between the significant number of chromosomal damage observed in *T. pallida* and elevation in the presence of these toxic gases, according to Figures 2 to 4 (Gábelová et al., 2004).

It was evident that local with exceeding the flow of vehicles has higher clastogenic effects and aneugenic indices correlated to Table 1. The longer period of exposure to polluting compounds induced an increase in the percentage of genetic damage of *T. pallida* as is

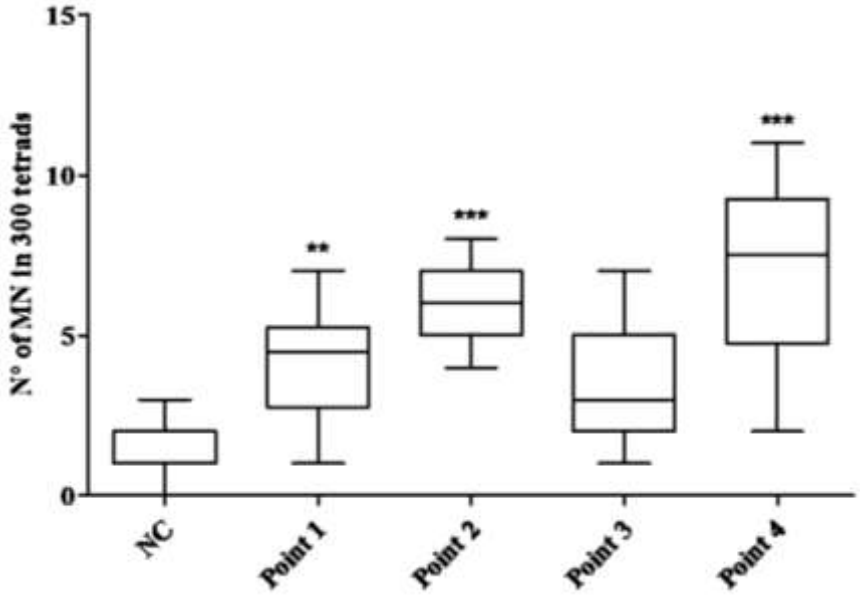

Figure 4. Frequency the (MN) in 300 tetrads (mean ± SD) of young *T. pallida* inflorescences after 30 days of seedlings exposure to local testing and control test. ANOVA followed by Dunnett's multiple comparison test; frequency of MN at significance of p<0.001** and p<0.001*** compared to the NC.

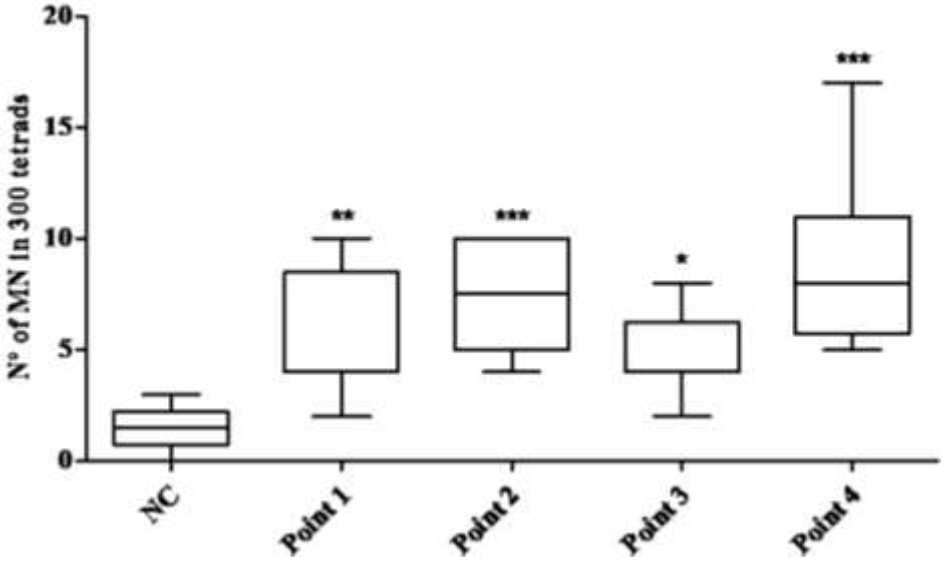

Figure 5. Frequency the (MN) in 300 tetrads (mean ± SD) of young *T. pallida* inflorescences after 60 days of seedlings exposure to local testing and control test. ANOVA followed by Dunnett's multiple comparison test; frequency of MN at significance of p<0.01** and p<0.001*** compared to the NC.

shown in Table 2. In the 15 days analysis only point 4 was significant in relation to the other, as in the analysis of 30 days was observed that point 2 became significant to the point 4. In consonance, the analysis to 60 days of exposition shown that all the points has become significant according to the increasing on MN.

Points 2 and 4 showed high relevance, the fact can be proven by the index and type of pollutants in these places is more intense. What determines the influence of the amount and the constant movement of vehicles in point 2

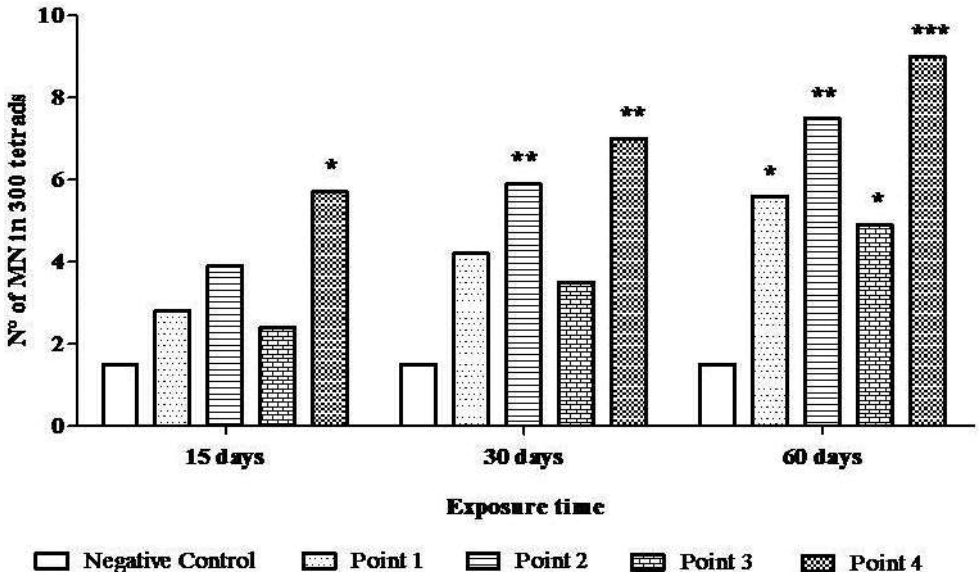

Figure 6. Frequency of MN in 300 tetrads of young (mean ± SD).*T. pallida* inflorescences in all periods of exposure, 15, 30 and 60 days. Two-way ANOVA followed by Bonferroni *post-hoc* test; comparison between the exposure days and points at p<0.05*, p<0.01** and p<0.001*** compared to the NC.

Table 1. Micronuclei (MN) formation in tetrads of young *T. pallida* inflorescences in all periods of exposure, 15, 30 and 60 days.

Point	15 days	30 days	60 days
	MN (3000 tetrads)	MN (3000 tetrads)	MN (3000 tetrads)
NC	1.54 ± 1.08 (0.0 - 3.0)	1.54 ± 1.08 (0.0 - 3.0)	1.54 ± 1.08 (0.0 - 3.0)
Point 1	2.8 ± 1.39 (1.0 - 5.0)	4.2 ± 0.81** (1.0 - 7.0)	5.6 ± 2.79** (2.0 - 10.0)
Point 2	3.9 ± 1.52 *** (1.0 - 6.0)	5.9 ± 1.19 *** (4.0 - 8.0)	7.5 ± 2.27 *** (4.0 - 10.0)
Point 3	2.4 ± 0.84 (1.0 - 4.0)	3.5 ± 1.78 (1.0 - 7.0)	4.9 ± 1.79 * (2.0 - 8.0)
Point 4	5.7 ± 1.33 *** (4.0 - 8.0)	7.0 ± 2.78 *** (2.0 - 11.0)	9.0 ± 3.9 *** (5.0 - 17.0)

Directed using ANOVA and Dunnett's multiple comparison test. Mean ± standard deviation (SD) with a significance of 15-days p<0.001*** in points 2 and 4; for 30 days of p<0.01** in point 1 and p<0.001*** in points 2 and 4; and 60-days p<0.05* in point 3, p<0.01** in point 1 and p<0.001*** in points 2 and 4 in the test group, compared to the NC.

is the constant manipulation of benzene derivatives at point 4, that influence the expressively increase in MN frequency.

The frequency of MN found at points with higher vehicular traffic is according to records of Carreras et al. (2006) and Klumpp et al. (2006) who observed a direct relationship between mutagenic index and expressively increase of population concentration in urban areas. Metropolitan areas tend to present more atmospheres contaminated by pollutants than areas with less flow of vehicles and people, leading to a higher incidence of genetic damage in plants used as bio-indicators.

The data cited by Carreras et al. (2006) from a study with the same focus, using 3 test points in the city of Cordoba, Argentina, showed values between 2.4 ± 2.08 in distant area from the center and 4.2 ± 2.6 in region central city, revealing lower rates in inducing genetic damage in *T. pallida* compared with our study. The results showed mean values of 5.6 ± 0.7 and 7.1 ± 1.0, equating to the average percentage of this study, since the highest percentage found in sections that had only the pollutant factor of vehicle exhaust it was 7.5 ± 2.27, as shown in Table 1.

In a study, Costa and Droste (2012) suggested that rainfalls may decrease the genotoxic impacts in *T. pallida*. For this reason, we targeted dry season in this study to avoid the rain effects on this plant. Our study confirms the genotoxic effects in *T. pallida* at the points exposed in the city of Teresina.

It is noteworthy that during the analysis of *T. pallida* other structural changes in the cells of pollen grains were identified. These findings understood increased formation

Table 2. Influence of exposure time to compare the percentage of micronuclei (MN) in *T. pallida* between test groups, assuming the significant value of each.

Days	Point 1	Point 2	Point 3	Point 4
15	NS	NS	NS	p<0.05 *
30	NS	p<0.01 **	NS	p<0.01 **
60	p<0.05 *	p<0.01 **	p<0.05 *	p<0.001 ***

Performed using two-way ANOVA and Bonferroni posttests. Comparison between days of exposure and exposure points, with significant results at p<0.05*, p<0.01** and <0.001***, compared to the negative control. NS, Non-significant.

of non-viable pollen (characterized by the absence or reduction in the cytoplasm and a weak staining), which involves the interference of the fertility of the plant organism. According to Costa and Droste (2012), reduced pollen viability and increased in the frequency of MN, it is resulting from the influence of the vehicle pollution and extent of drought, factors that correlate to the points tested in research and high temperature in the city, Teresina.

In urban areas, air pollution is demonstrated by the photochemical smog resulting from the interaction between nitrogen oxides, hydrocarbons such as benzene and sunlight to form oxidation products, which can cause harm to humans and plants. As cars emit carbon monoxide, sulfur dioxide, aromatic compounds and hydrocarbons, contribute directly to the formation of photochemical smog and other pollution (Proncove, 2006; Semace, 2011).

The growing demand for new technologies makes the benzene is one of the industrial substances produced in higher volume. It comes mainly in production and oil refining, one of the constituents of gasoline. The exhaust of automotive vehicles accounts for about 5% of total hydrocarbons emitted into the environment. It is understood that the primary means of benzene poisoning in the workplace, either by direct contact with the substance or its derivatives (Abiquim, 1999; Costa and Costa, 2002). Thus, the high frequency of MN in *T. pallida* is related in point 4 with the studies of Alves et al. (2011), which found a positive association between high frequency of MN in pollen mother cells *T. pallida* exposed at different times of the large amount of polycyclic aromatic hydrocarbons - HPA's. Among the molecules considered mutagenic and / or carcinogenic, the HPA's have a prominent place in environmental and biological studies (Conpet, 2006; Jung et al., 2009; Brito et al., 2013; Silva et al., 2015).

When exposed to environmental pollutants, *T. pallida* suffers in short, physiological and structural changes resulting from the genotoxic potential. Thus, many injuries can be caused by these compounds, serving as an excellent indication, for example, its clastogenic effects, chromosomal breakage, and aneugenic, partial or total loss of chromosomes, detected by exposing the plant to the environment (Ma and Grant, 1982; Savóia, 2008; Sisenando, 2011; Crispim et al., 2014).

According to Thewes et al. (2011), chromosomal variations found in this experiment are explained by environmental changes that affect the errors in the process of formation and development of cellular structures of these vegetables (Rodríguez, 2015)

Identification of the potential harm in the genome of *T. pallida*, through exposure of polluted air *in situ* may be an important indicator for genotoxic events in mammals, since these eukartotic cells are continuously entering into different divisional phases and constantly mutate (Carvalho, 2005).

Conclusion

Air pollution is an emerging problem in society; it's leading to major changes in ecosystems thus a significant number of diseases in humans, animals and plants in general. The *T. pallida* is an important form to detect air pollution. TRAD-MCN bioassay is a relatively simple technique, easy reproducibility and high efficiency in the evaluation of chromosomal damage arising from the interference of the pollutants. The results showed the genotoxic potential of the four tested environments when compared to the negative control. Thus, increased frequency of micronuclei was correlated with the local exposure time and, as the largest potential to cause chromosomal aberrations occurred in most vehicle flow and manipulation of benzene derivatives environments. These data, although relevant, are early. Necessitating the need for investigations in different seasons and chemical analyzes of atmospheric air of the places to be studied.

Conflicts of Interests

The authors have not declared any conflict of interests.

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