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Full Length Research Paper

Thermo-chemical sequestration of naphthalene using *Borassus flabellifer* Shell activated carbon: Effect of influencing parameters, isotherm and kinetic study

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The present research describes the removal of naphthalene from aqueous solution using one of the simplest agricultural wastes, *Borassus flabellifer* Shell activated carbon (BFS-AC) by adsorption. Adsorption was chosen due to its cost-effectiveness. The effect of operating parameters such as pH, time, initial concentration of naphthalene, BFS-AC dosage and temperature on the percent removal of naphthalene was determined. The optimum conditions obtained were pH of 7, contact time of 12 h for an optimum concentration of 200 mg/L with the addition of 8 g at a temperature optimum at 40°C attaining the maximum percent removal. The adsorption kinetics supported the Freundlich model with R2 value of 0.995 indicating multilayer adsorption. The chemical kinetic studies followed the pseudo second order mechanism. The characterization of BFS-AC was carried out using scanning electron microscopy (SEM) and X-ray diffraction (XRD) which showed the enhanced porosity and crystallinity of the adsorbent which was mainly due to the surface modification carried out by chemical addition followed by thermal treatment. The initial and final naphthalene concentration after adding the adsorbent was determined by using gas chromatography-mass spectrometry (GC-MS). The characterization thoroughly suggests the efficacy of *B. flabellifer* shell to efficiently sequester naphthalene from aqueous solution.

Key words: Naphthalene, *Borassus flabellifer*, thermo-chemical, activated carbon, adsorption.

INTRODUCTION

Poly-aromatic hydrocarbons (PAHs) are structurally related chemicals consisting of aromatic rings with no substitutions. They are considered to be a very potent class of environmental pollutant causing harmful effects to the environment, which are found out in various environmental conditions (Kafilzadeh, 2015). Global industrialization threatens the globe due to the uncontrolled usage and abuse of chemicals along with the exploitation of natural resources. Rapid industrialization resulted in dumping of large amount of chemical wastes into the environment as well as water resources. Petroleum waste is composed of many

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fractions which includes PAHs as a major constituent (Clinton et al., 2014). The level of PAHs increases due to day to day activities such as mass traffic and variety of industrial processes (de Boer and Wagelmans, 2016). PAHs are the outcomes of various sources such as coal, fossil fuel and peat burning along with mobile sources in the urban regions (Moradi-Rad et al., 2014). PAHs were accumulated in all habitats of environment such as air, water, soil, and food. Many methods were available for the PAHs removal which includes absorption, bio-degradation, ion exchange, chemical precipitation, and high energy irradiation (Balati et al., 2012). The use and management of environment is an important task today (Abdel-Shafy and Aly, 2002). Hence, the contamination of harmful pollutants in environment has become a serious issue, particularly in urban cities where there is more amount of water scarcity. With this aspect, drinking water is said to be contaminated with small amounts of various synthetic compounds which are rich in organic or in-organic contents (Moradi-Rad et al., 2014). Waste waters undergo different stages of treatment which includes primary to tertiary methods depending on availability of source to meet the environmental standards. Anyway, the organic waste such as PAHs are not easily removed with such treatment techniques (Alade et al., 2012). The hydro-phobic nature of PAHs resulted in more amount of toxicity to the environment (Jorfi et al., 2013). The toxic nature, mutagenic and carcinogenic characteristics of PAHs categorized them to be primary pollutants. PAHs are adsorbed easily due to their less solubility tendency in aqueous solutions (Lamichhane et al., 2016). Many compounds were selected as representatives of PAHs, but this study is focused on naphthalene. Naphthalene is present abundantly in waste water, obtained from various industrial activities (Ania et al., 2007). Naphthalene was investigated to be one of the most prominent PAHs in waste water. Naphthalene removal can be carried out using many methods (Aisien et al., 2014) out of which adsorption is found to be a convenient one.

Agricultural materials were found to be more predominant to act as adsorbents due to less cost, large availability and high sorption capacity (Zhu et al., 2016; Pal, 2012). Excess amount of waste materials are generated in the form of agricultural materials such as coconut, corn and palm shell *Borassus flabellifer* (Kumar et al., 2015). *B. flabellifer*, also called Asian palmyra palm, toddy palm, or sugar palm, is native to Indian Subcontinent (Vijayalakshmi and Rajalakshmi, 2010). It is a robust tree, reaching a height of 30 m. The trunk is grey, robust, ringed with leaf scars. The leaves are fan-shaped, 3 m in length. Palm shell is the dried fruit of *B. flabellifer* and it is an agricultural solid material. This is available in plenty from the mills and can be considered as an excellent raw material for the production of activated carbon (Charlesworth et al., 2002; Boving and Zhang, 2004; Ozer and Ozer, 2004; Ayranci, 2005; Tan et al., 2008). Activated carbon has a wide range of applications including the removal of unpleasant odor, taste, color, impurities from domestic and industrial effluents (Kent, 1992; Cooney, 1999; Amarnath and Padmesh, 2009; Yu et al., 2012; Mohan and Singh, 2002; Liao et al., 2011; Momčilović et al., 2011). Activated carbon produced from *B. flabellifer* shell was observed to possess large surface area and pore volume. This feature facilitates its application as an effective adsorbent (Momčilović et al., 2011). Though many activating agents are employed by many researchers for the pretreatment process which involves, carbonization and activation, the present study employs zinc chloride and potassium hydroxide as an activating agent (Jabasingh et al., 2015) followed by thermal pyrolysis.

The precursors include various coal and other biologically derived materials. The activated carbon obtained were subjected to thermal treatment which resulted in the formation of char and other gases (Prabu et al., 2015; Kiruba et al., 2014). This char was able to act as an excellent precursor in the synthesis of activated carbons (Cabal et al., 2009). This study involves the combination of chemical (Gaya et al., 2015) and thermal treatment which was found to be the first in adsorbent preparation. The selection of *B. flabellifer* shell (BFS) mainly depends on the fact that it is much denser when compared with other biological materials (Okoroigwe et al., 2014).

EXPERIMENTAL

Materials

Naphthalene (98% pure), potassium hydroxide (97% pure), zinc chloride (95% purity), and acetone were purchased from Sigma Aldrich, Germany. The stock solution of naphthalene was prepared using acetone and distilled water. The acetone water solution was used to enhance the hydrophobic solubility nature of naphthalene (Felixa et al., 2014). The primary material for activated carbon preparation, *B. flabellifer* shells were procured from Godrej palm plantations, Salem, Tamilnadu, India after palm fruit extraction.

BFS-AC preparation

B. flabellifer shells are collected, washed with distilled water, and dried in oven at 100°C. The dried materials were chopped finely and sieved to mesh size of 2 mm. The resulting materials are subjected to drying at 400°C for 2 h in muffle furnace. After drying, the materials are soaked in 3% KOH solution and 2% $ZnCl_2$ in a ratio of 1:3 for impregnation of the activating agent (Gaya et al., 2015). The chemical activation enhanced the surface area and pore volume to a maximum extent which was confirmed by scanning electron microscopy (SEM) and X-Ray diffraction (XRD) analysis. The impregnation process is followed by thermal treatment at 600°C for 2 h in a muffle furnace. The dried sample is washed repeatedly with de-ionized water until neutrality was attained. The samples are further dried at 200°C for 4 h in a tray dryer to remove the excess moisture content. This treatment was found to be the first one when compared with earlier strategies. This is named as *B. flabellifer* shell activated carbon (BFS-AC) and stored in sealed containers. BFS-AC is used in the present study for the removal of naphthalene from aqueous solution.

Physical and chemical characterization of BFS-AC

Moisture content determination

An empty crucible was weighed and 1 g of BFS-AC was added and the weight of the crucible along with BFS-AC was measured. The BFS-AC loaded crucible was dried in the air oven at 105°C for 24 h and placed in desiccators. The weight of the dried BFS-AC was measured. The moisture content was calculated as follows:

$$\%M = \frac{(W_A - W_C)}{(W_B - W_C)} \times 100 \quad (1)$$

where W_A, W_B and W_C are the empty weight of the container, initial weight of the container with BFS-AC, and final weight of the container with BFS-AC, respectively.

Ash content determination

An empty crucible was weighed and 1 g of BFS-AC was added to it and the weight of empty crucible along with the BFS-AC was measured. The BFS-AC loaded crucible was dried in a muffle furnace for 2 h at 550°C and kept in desiccators for 30 min followed by measuring the weight. The ash content was calculated as follows:

$$\%A = \frac{(W_B - W_C)}{(W_B - W_A)} \times 100 \quad (2)$$

where W_A, W_B and W_C are the empty weight of the container, initial weight of the container with BFS-AC, and final weight of the container with BFS-AC, respectively.

Batch adsorption studies

Stock solution of naphthalene was prepared by dissolving 1 g of naphthalene in 1 L acetone. Diluted concentration of 100, 200, 300 and 400 ppm were prepared from the stock using acetone and distilled water. Batch experiments were conducted in 100 ml conical flasks in a laboratory shaker at 200 rpm in order to determine the effect of pH (2 to 8), temperature (30 to 50°C), time (1, 2, 3, 4, 6, 18 and 20 h), BFS-AC dosage (2 to 8 g/L) and initial concentration of naphthalene (100 to 400 mg/L) on the percentage naphthalene sorption from aqueous solution using BFS-AC. The added adsorbent was separated by centrifuge at 500 rpm. The supernatant was filtered through a 0.45 mm membrane filter and analyzed quantitatively at various time intervals for naphthalene concentration. Supernatant samples after adsorption were subjected to Gas Chromatography–Mass Spectrometry (GC-MS) analysis using purge and trap, GCMATE II GC-MS. GC-MS analysis for detecting naphthalene concentration before and after adsorption was determined by the CMIP5 instrument 210 series equipped with a 100 m Fison DB-5 capillary column operating at a temperature range of 120°C in the splitless mode with an injection volume of 1 µl/min. The adsorption percentage of naphthalene was calculated from the differences between the concentrations of naphthalene in the aqueous solution before and after adsorption process (Jabasingh et al., 2015).

$$\text{Adsorption } \% = \frac{C_0 - C_f}{C_0} \times 100 \quad (3)$$

Adsorption isotherms

The adsorption isotherms were studied in order to show the adsorption behavior and to determine the adsorption capacity. Langmuir and Freundlich adsorption isotherms were fitted to the experimental results for the adsorption of naphthalene. Both isotherms indicate a sharp initial slope indicating the high efficiency of the adsorbent at low concentration and saturation at high concentration (Jabasingh et al., 2010). The linear form of Langmuir isotherm is given by

$$\frac{1}{q_{eL}} = \frac{1}{q_M} + \frac{1}{C_E K_L q_M} \quad (4)$$

And the linear form of Freundlich equation is given by

$$\log q_{eF} = \log K_F + \frac{1}{n} \log C_e \quad (5)$$

where q_{eL}, q_{eF} and q_M are the equilibrium adsorption capacity (mg g^{-1}) according to Langmuir fit, equilibrium adsorption capacity (mg g^{-1}) according to Freundlich fit and the maximum sorption capacity (mg g^{-1}), respectively. K_L (ml mg^{-1}) and K_F (mg g^{-1}) are the Langmuir and Fruendlich constants, respectively. C_e and n are equilibrium naphthalene concentration (mg/L) and sorption intensity, respectively.

Chemical kinetics

Kinetic study was carried out using 50 ml naphthalene solution in the concentration ranging from 100 to 400 mg/L with the addition of 8 g of activated carbon. The pseudo first order and pseudo second order kinetics were determined. A pseudo first order kinetics suggest a constant concentration of any one of the reactants as it is supplied in excess. The sorption kinetic data were treated with pseudo first-order model (Felixa et al., 2014).

$$\log(q_e - q_t) = \log q_e - \left[\frac{k_1}{2.303}\right] t \quad (6)$$

where q_t is the sorption capacity of BFS-AC at any time t and k_1 is the first order rate constant (min^{-1}). The pseudo second order kinetics depend on two reactants and the equation is given as:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left[\frac{1}{q_e}\right] t \quad (7)$$

Where, k_2(g/mg min) is the second order rate constant, determined from the plot of t/q_t versus t. The initial sorption rates were given by

$$h = k_2 q_e^2 \quad (8)$$

Thermodynamic studies

The enthalpy, entropy and Gibbs free energy values were determined using

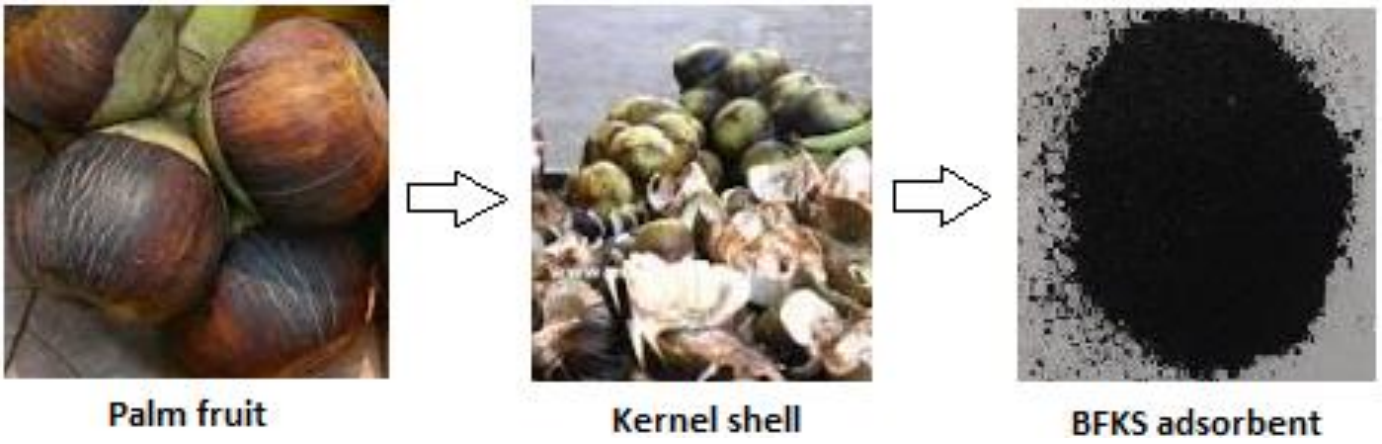

Figure 1. Preparation of BFS-AC.

$$K_D = \frac{q_e}{C_e} \quad (9)$$

$$\log K_D = \frac{-\Delta G}{2.303RT} = \frac{-\Delta H}{2.303RT} + \frac{\Delta S}{2.303R} \quad (10)$$

where K_D is the distribution coefficient in ml mg^{-1}. ΔH, ΔS and ΔG are changes in enthalpy, entropy and Gibbs free energy, respectively (Kiruba et al., 2014).

Analytical methods

The surface morphology of BFS-AC was studied using scanning electron microscope, SEM Supra 55 Carl Zeiss, Germany. The crystallinity of BFS-AC was determined using X-ray diffraction analysis. XRD analysis of the BFS-AC was carried out by the Rikagu laboratory equipment maintained at a voltage of 30 Kv and a current of 20 MA Cu-$K\alpha_2$ radiations. Determination of naphthalene before and after the adsorption process was carried out using GC-MS (CMIP5 instrument 210 series) with a capillary DB-5 column. Naphthalene was quantified using calibration curves by the direct injection of standard mixtures with known concentrations.

RESULTS AND DISCUSSION

Characterization of BFS-AC

Figure 1 shows the activated carbon produced from *B. flabellifer* Shell. The ash content and moisture content of BFS-AC was found to be 10.9 and 3.4%, respectively.

SEM images (Figure 2a to c) show the morphology of the initial shell, shell after its conversion to activated carbon (BFS-AC), BFS-AC after adsorption of napthalene. Large sized pores developed on the shell after the activation process during its conversion to activated carbon. The KOH activation and pyrolysis processes have increased the pore size and pore volume due to the diffusion of KOH and CO_2. This reaction has also resulted in the drastic expansion of the carbon material. The surface of BFS-AC looked like a honeycomb before adsorption process. After the adsorption of naphthalene, the pores were found to expand in their capacity. The fringes disappear after the adsorption process, showing the affinity of naphthalene towards BFS-AC.

Figure 3 gives the crystalline nature and composition of the BFS-AC sample before adsorption of naphthalene. The diffractograms obtained are similar and in better agreement with the XRD pattern of the sample. But, the undesirable peaks in the pattern confirmed the presence some amount of impurity in the sample. XRD indicated the powder pattern of the derived activated carbon under atmospheric conditions. The broad peaks at 2θ, 26.5°, 27.9°, 28.1°, and 28.8° and the peaks at 34.5° and 35.1° confirmed the presence of activated carbon. XRD predicts the particle size of the BFS-AC sample in the range of 2 to 3 mm. GC-MS was used to detect the presence of low molecular weight, naphthalene in aqueous solution before and after the addition of BFS-AC. The reduction in peak confirmed the removal of naphthalene after the addition of adsorbent. Figure 4a and b shows the GC-MS spectra of the naphthalene solution before and after adsorption using BFS-AC.

Effect of various operating parameters

The results of the effect of various operating parameters on naphthalene removal percentage are as shown in Figure 5. The effect of initial naphthalene concentration was determined between 100 and 400 mg/L. The adsorption percentage increases with an increase in the amount of the adsorbent due to the large availability of vacant sites for adsorption. The percentage adsorption was found to decrease beyond 200 mg/L, due to the complete occupation of the vacant sites by naphthalene

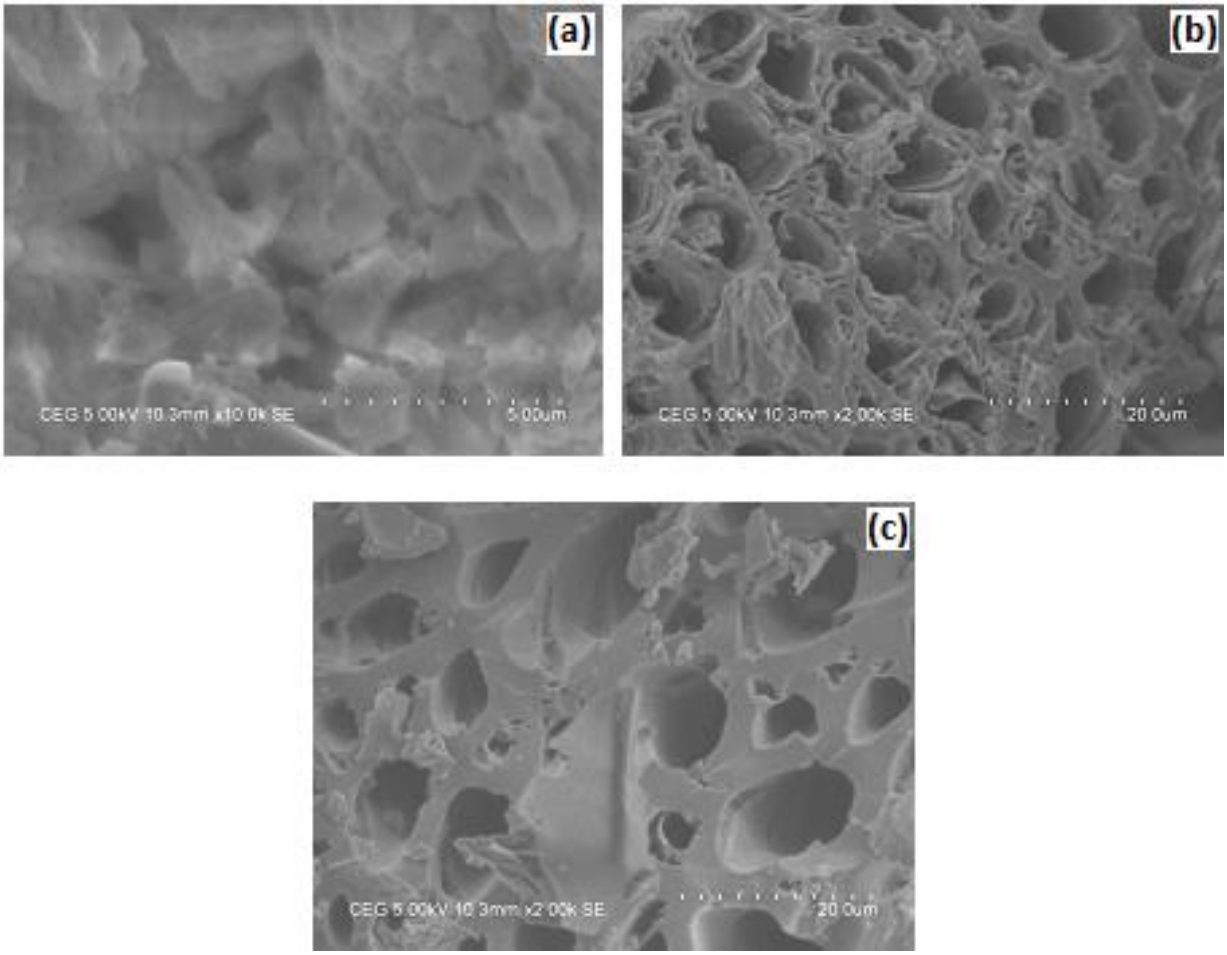

Figure 2. SEM morphology of (a) *Borassus flabellifer* shell, (b) BFS-AC before the adsorption of naphthalene, (C) BFS-AC after adsorption of naphthalene.

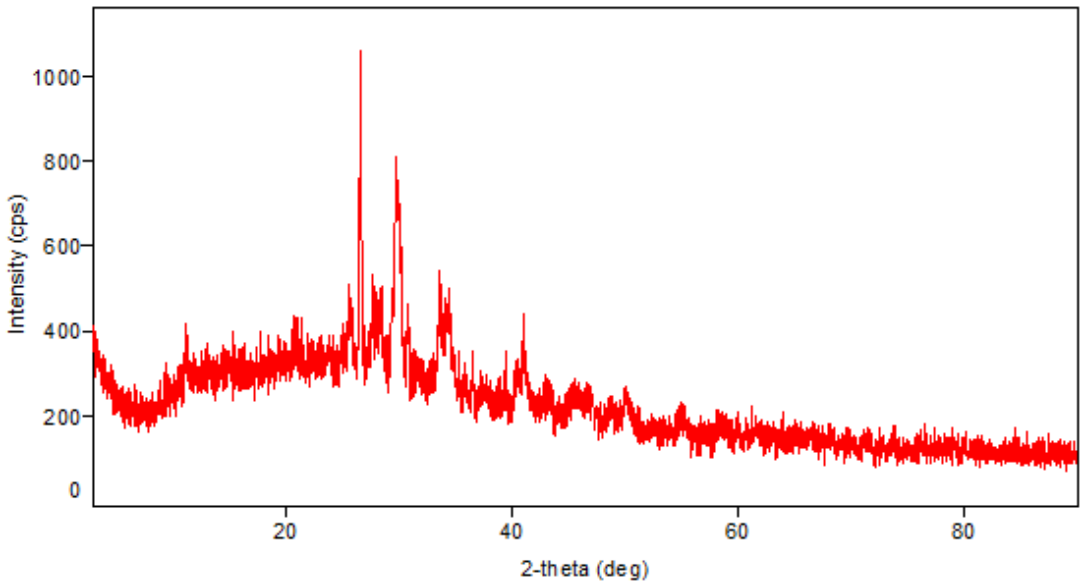

Figure 3. XRD pattern of BFS-AC.

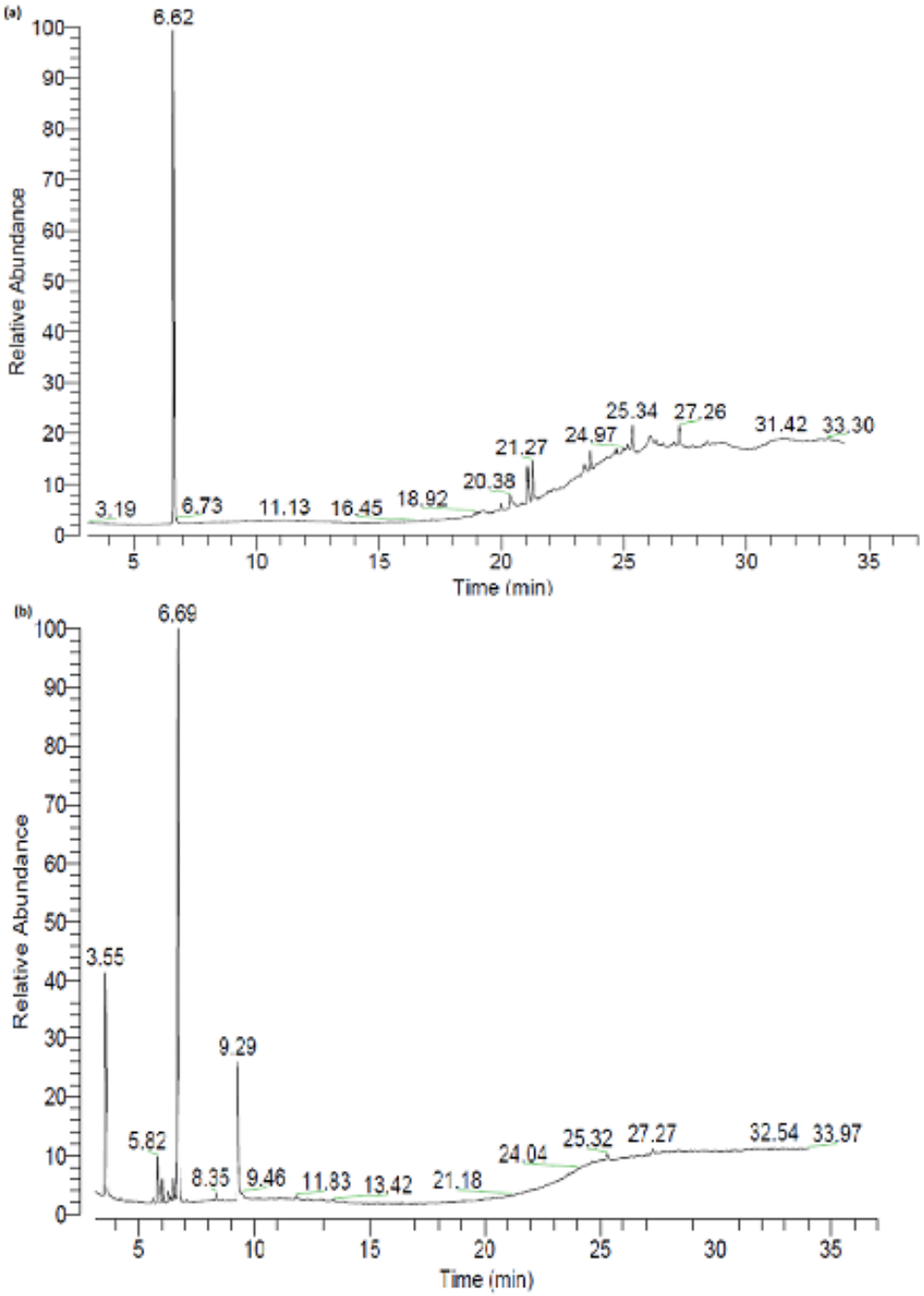

Figure 4. GC-MS spectra of the solution (a) before adding BFS-AC (b) after adsorption using BFS-AC.

molecules. The effect of pH was determined for the solution of concentration 200 mg/L. The solution was tested at various pH levels in the range of 3 to 9. Low pH increased the concentration of H^+ ions and higher pH increased the concentration of OH^- ions. It was clear that increase in pH resulted in more concentration of OH^- ion in naphthalene solution (Momčilović et al., 2011; Jabasingh et al., 2015). More ionized form resulted in less adsorption. The adsorption was maximum at a pH of 7. At this pH, more hydrogen ions were present and this has made the improvement in the activation process by means of triggering the carboxyl groups that are present in the activated carbon. Adsorption was carried out at various temperatures, 30, 35, 40, 45 and 50°C. It was found that as the temperature increases, the adsorption of naphthalene by BFS-AC increased and remained

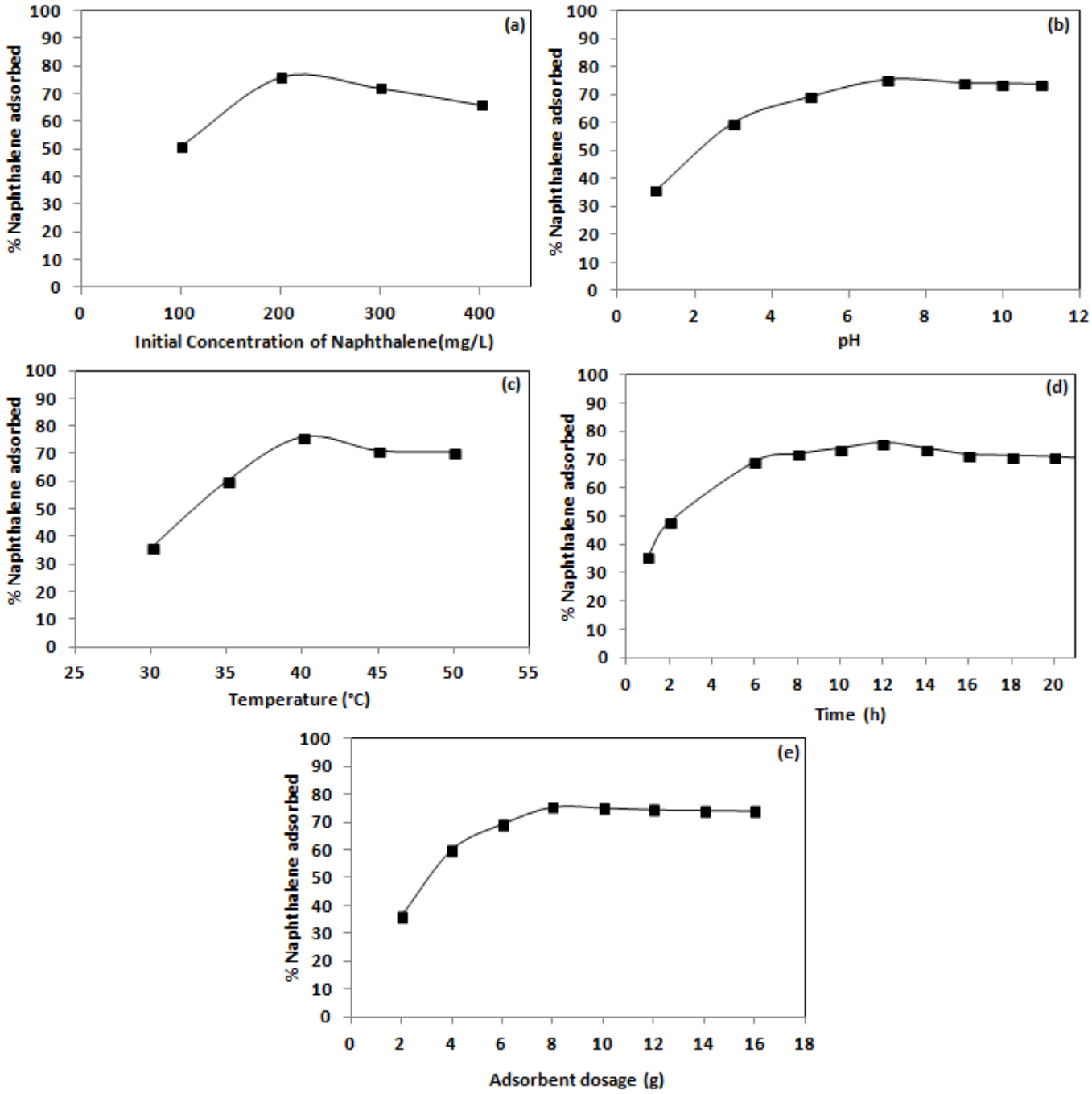

Figure 5. Effect of various operating parameters on naphthalene removal percentage.

constant at 40°C. The samples were subjected to adsorption at different time intervals of 1, 2, 4, 6, 12, 18 and 20 h. The optimum time for naphthalene adsorption by BFS-AC was found to be 12 h with a removal efficiency of 76%. Various dosages of BFS-AC including 2, 4, 6, 8, 10 and 12 g were added to the naphthalene solution of various concentrations. The optimum BFS-AC dosage was found to be 8 g. In order to determine the effect of initial naphthalene concentration on the percentage removal, the naphthalene concentration was varied in the range of 100 to 400 mg/L with a dosage of 8 g adsorbent. At a high naphthalene concentration, the naphthalene molecules present on the surface was high and hence the functional adsorption was dependent on the concentration of initial amount of naphthalene.

Optimum initial concentration of naphthalene that can

Table 1. Adsorption isotherm parameters for naphthalene removal by BFS-AC.

Isotherm model	Parameter	Values	R^2
Langmuir	q_m (mg/g)	0.2256	0.9979
	K_L (L/mg)	16.036	
Freundlich	K_F (L/mg)	0.2455	0.9271
	n (g/L)	4.114	

be effectively adsorbed by BFS-AC was found to be 200 mg/L. Beyond this concentration, the removal percentage was found to decrease.

Adsorption isotherms

The adsorption isotherms were studied in order to show the adsorption behavior and to estimate the adsorption capacity. The fit of Langmuir and Freundlich adsorption isotherms to the experimental results for the adsorption of naphthalene are obtained. Both isotherms indicate a sharp initial slope indicating that the adsorbent operates at high efficiency at low concentration and becomes saturated at high adsorbent concentrations. The Freundlich constant, n, is a measure of both the relative magnitude and diversity of energies associated with the adsorption of naphthalene on palm shells. The adsorption isotherm was used to explain the distribution of solute particles between the liquid and solid phase when an equilibrium state was achieved for the adsorption process. The fitting of the experimental data to the different isotherm equations was an important task in order to find an exact model which is applicable for design purposes. Adsorption isotherms describe the interaction of solutes with the adsorbents and it finds an important application in optimizing the parameters to determine the equilibrium state. Langmuir isotherm describes the monolayer formation and energy term in this equation is expressed as a function of surface coverage. The values of K_L and q_M are listed in Table 1. The popular form of logarithmic equation was related with Freundlich isotherm and this holds good for multilayer formation on the solid surface. The Freundlich constants, K_F and n values are listed in Table 1. The Langmuir and Fruendlich isotherms were validated with their correlation coefficient. The Langmuir isotherm was found to fit the experimental data. Langmuir and Freundlich plots are as shown in Figure 6a and b, respectively.

Adsorption kinetics

The pseudo first order and pseudo second order reaction kinetic constants were evaluated naphthalene adsorption (Table 2). The kinetics of naphthalene adsorption quietly agreed with the pseudo-second order. The controlling step for the pseudo second order was chemisorption mechanism which resulted in the exchange of electrons between the solute particles and solvent (Nasernejad et al., 2005). The results confirmed well to the pseudo second order model equation. If the concentration of one of the reactants remains constant due to its supply in excess, the kinetics could be described as pseudo first order. The second order reaction depends on two reactants (naphthalene and BFS-AC). The pseudo first order and pseudo second order reaction kinetics for naphthalene adsorption onto BFS-AC is as shown in Figure 7a and b.

Adsorption thermodynamics

Enthalpy, entropy and Gibbs free energy values for the naphthalene adsorption onto BFS-AC are shown in Table 3. The thermodynamic parameters (ΔH, ΔS and ΔG) are used to determine the energy changes that occur during the adsorption process. They describe the thermodynamic behavior of naphthalene molecules onto BFS-AC (Figure 8). The feasibility of the adsorption process and its spontaneous nature was confirmed by the negative value of ΔG (Prabu et al., 2015). ΔG increases with the lowering of temperature. Adsorption was favorable at low temperatures.

Chemisorption was found to be predominant in this study. Since ΔH is negative, the reaction was exothermic. The negative value of ΔS indicates a less interaction with the solid-liquid interface and hence a reduction in the adsorption process. This could be improved by providing mild agitation to the adsorption vessel during the adsorption process. Since ΔH^0 are greater than $T\Delta S^0$ for all concentrations of naphthalene at all temperatures, the adsorption process was very much influenced by the enthalpy changes compared to the entropy changes.

Conclusion

The study exploited the efficiency of activated carbon derived from *B. flabellifer* shells for the removal of naphthalene from aqueous solution. Activated carbon obtained from *B. flabellifer* shells was found to be an

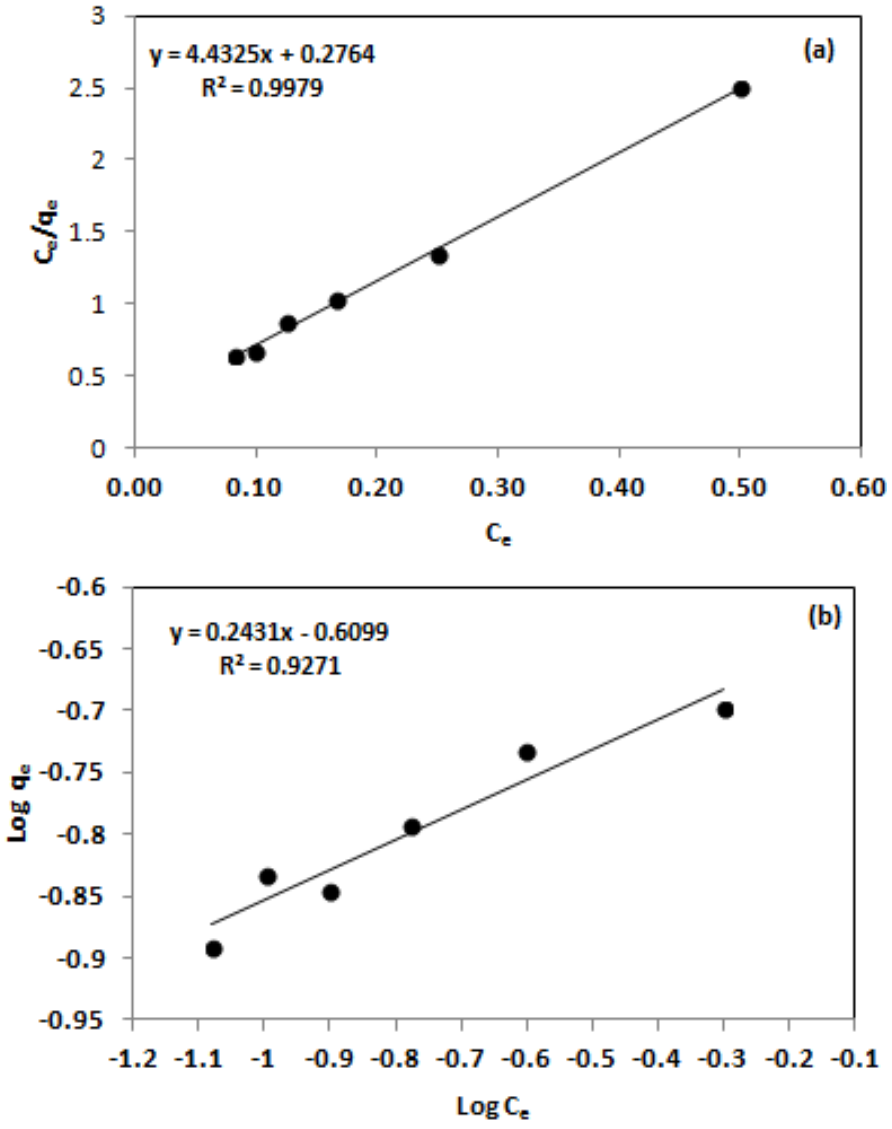

Figure 6. Experimental fit for (a) Langmuir isotherm, (b)Freundlich isotherm.

Table 2. Pseudo first order and pseudo second order adsorption kinetic constants at optimum naphthalene concentration.

Experimental		Pseudo first order kinetics			Pseudo second order kinetics		
C_o (mg/L)	$q_{e(exp)}$ (mg/g)	k_1 (min^{-1})	$q_{e(calc)}$ (mg/g)	R^2	k_2 (g/mg min)	$q_{e(calc)}$ (mg/g)	R^2
200	10.5	0.153	3.855	0.991	0.088	7.299	0.996

excellent adsorbent for naphthalene removal from aqueous solution. The optimum conditions for the removal of naphthalene using *B. flabellifer* shells activated carbon were 8 g BFS-AC at a temperature of 40°C, at an optimum pH 7. This resulted in a maximum efficiency for BFS-AC to remove naphthalene from aqueous solution. Freundlich model provided a good fit to the experimental data with R^2 value of 0.995. The experiments proved a rapid adsorption of naphthalene by BFS-AC. A maximum adsorption capacity was achieved in approximately 12 h. The result of kinetic studies indicates the pseudo second order to be the best fitting kinetic model. The SEM analysis showed that the porosity of BFS-AC and XRD confirmed the crystalline nature of the BFS-AC. GC-MS analysis confirmed the adsorption of naphthalene by the adsorbent.

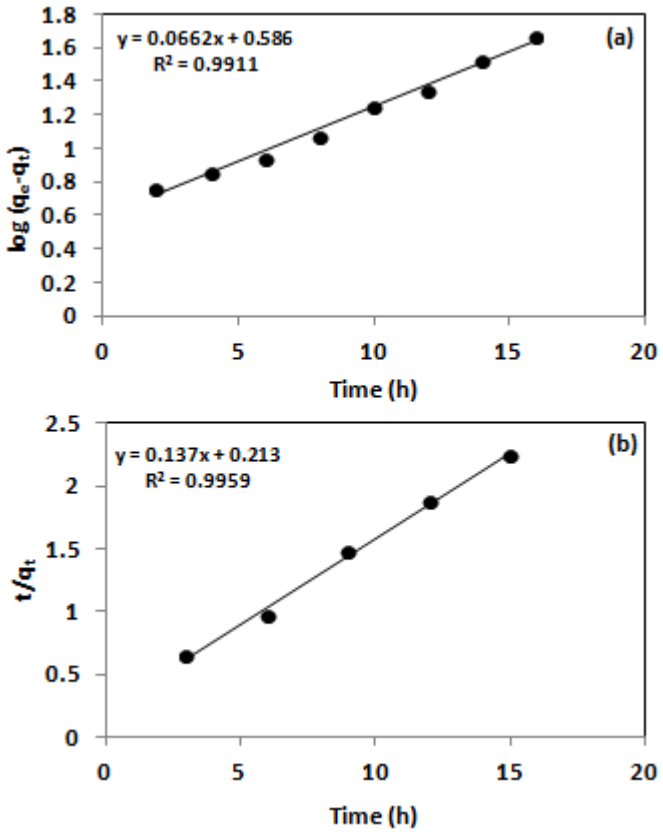

Figure 7. (a) Pseudo first order kinetics; (b) Pseudo second order kinetics.

Table 3. Enthalpy, entropy and Gibbs value for various concentrations of naphthalene

Initial concentration of naphthalene (mg/L)	ΔH^0 (KJ/mol)	ΔS^0 J/mol/K	ΔG^0 (KJ/mol)
100	-608.13	-1164.13	-243.76
200	-544.31	-927.97	-253.86
300	-540.89	-889.15	-277.76

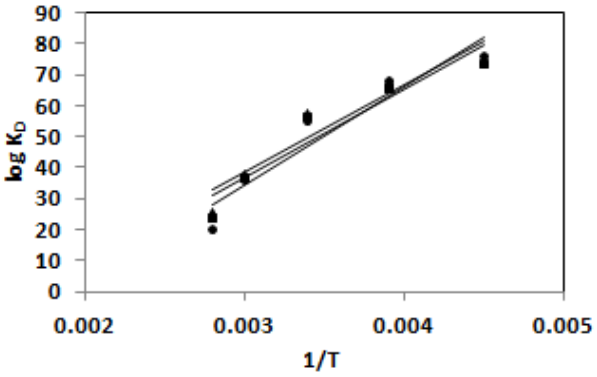

Figure 8. Thermodynamic plot for naphthalene adsorption onto BFS-AC.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of insulin-like growth factor-I gene polymorphism in Egyptian small ruminant breeds

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The genetic improvement of production traits can be developed through marker assisted selection. Insulin-like growth factor I (*IGF-I)* is a member of the somatotrophic axis which has a remarkable variation of its biological effect including protein synthesis and skeletal growth. This study aimed to detect the genetic polymorphism of *IGF-1* in different Egyptian sheep and goat breeds. The amplified fragments at 320-bp were digested with *Hae*III endonuclease and the results show the presence of three different genotypes: CC (15.71%), CG (29.29%) and GG (55.0%). The nucleotide sequence analysis of C and G alleles declared the presence of a single nucleotide polymorphism (C→G) at position 282. The nucleotide sequences of alleles C and G in sheep and goat were submitted to GenBank with the accession number: KX432965, KX432966, KX432967 and KX432968, respectively. In conclusion, a nucleotide substitution (C→G) was detected in *IGF-I* gene in Egyptian sheep and goat breeds resulting in the presence of three different genotypes; CC, CG and GG. The association of *IGF-I* polymorphism with different growth trait parameters were reported at significant levels, so, the genetic and SNP variations in *IGF-I* gene may be a potential molecular marker for growth traits in different Egyptian sheep and goat breeds.

Key words: *IGF-1*, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP), DNA sequencing, sheep, goat.

INTRODUCTION

Molecular genetics techniques are of great interest in the identification of genetic variations in genetic markers which are associated with different production and reproduction traits in farm animals (Jiang et al., 2002; Arora and Bhatia, 2006; Missohou et al., 2006). These genetic variations affect the physiological pathways that consequently lead to quantitative variations in different phenotype characteristics (Schwerine et al., 1995; Lan et al., 2007). In quantitative genetics, there are number of single genes associated with mammary or muscle growth, development and function which were studied as excellent candidates for linkage relationships with quantitative traits of economic importance.

Growth is a process in which the interaction between different neuroendocrine pathways is done and expressed in this phenotype trait. The somatotrophic axis (GH/IGF-I axis) is involved in these pathways and it is considered as the key in postnatal growth and

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metabolism in different mammals including farm animals (Shoshana et al., 2000; Burkhard et al., 2005). One of the most important member of the somatotrophic axis is insulin-like growth factor I (*IGF-I*) which has a remarkable variation of its biological effect like protein synthesis and skeletal growth (Froesch et al., 1985; Baxter, 1985; Clemmons et al., 1987).

In Egypt, there is a shortage in meat production comparing to the nutritional requirements, and there is an increasing gap between dairy products produced domestically and the amount consumed. Production improvements can be achieved by using new genetic technology and linear type appraisal for better selection of heritable traits.There are several indigenous sheep and goat breeds. The common sheep breeds include Barki, Rahmani and Ossimi, while goat breeds include Baladi, Barki and Zaraibi (Galal et al., 2005). The contribution of both species to the total red meat produced in Egypt is about 9.1% (6.4% for sheep and 2.7% for goats). The latest count of slaughtered sheep and goat numbers, represent 41.7% of all slaughtered livestock (27.4% for sheep and 14.3% for goats) (MoA, 2004).

Due to lack of knowledge about the genetic characterization and nucleotide sequence variations of *IGF-1*gene in Egyptian sheep and goat breeds, this study aimed to detect the genetic polymorphism of *IGF-1*/*Hae*III in different small ruminant breeds reared in Egypt and to identify the single nucleotide diversity in different *IGF-1* genotypes.

MATERIALS AND METHODS

Animals and DNA extraction

The blood samples were collected from 140 animals belonging to three sheep breeds; Barki (32 animals), Ossimi (28 animals) and Rahmani (22 animals) in addition to three goat breeds; Baladi (16 animals), Barki (20 animals) and Zaraibi (22 animals). Genomic DNA was extracted from the whole blood according to the method described by Miller et al. (1988) with minor modifications. Briefly, blood samples were mixed with cold 2x· sucrose-triton and centrifuged at 5000 rpm for 15 min at 4°C. The nuclear pellet was suspended in lysis buffer, sodium dodecyl sulfate and proteinase K and incubated overnight in a shaking water bath at 37°C. Nucleic acids were extracted with saturated NaCl solution. The DNA was picked up, washed in 70% ethanol and was dissolved in 1X TE buffer. The DNA concentration was determined, using Nano Drop1000 Thermo Scientific spectrophotometer, and then diluted to the working concentration of 50 ng/µl, which is suitable for polymerase chain reaction.

Polymerase chain reaction (PCR)

The DNA fragment of the studied gene was amplified using polymerase chain reaction technique developed by Mullis et al. (1986). This amplified fragment spans from intron 3 to intron 4 and cover exon 4 of *IGF-1*gene in sheep and goat. A PCR cocktail consists of 1.0 mM primers ((forward primer: 5' - GCT GGG TGT AGC AGT GAA CA -3' and reverse primer: 5' - GTT GCT TCA GCC GCA TAA CT-3'; Zhang et al., 2008), 0.2 mM dNTPs and 1.25 U of Taq polymerase. The cocktail was aliquot into PCR tubes with 100 ng of sheep or goat DNA. The reaction was cycled with the following conditions; initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C, each step for 1 min and the final extension for 5 min at 72°C. The amplification was verified by electrophoresis on 2% agarose gel in 1x TBE buffer using GeneRuler^{TM} 100-bp ladder as a molecular weight marker for confirmation of the length of the PCR products. The gel was stained with ethidium bromide and visualized on UV trans-illuminator.

Restriction fragment length polymorphism (RFLP)

Ten microliter of PCR product were digested with 1 µl of FastDigest *Hae*III restriction enzymes at 37°C for 5 min. The restriction fragments were subjected to electrophoresis in 2 % agarose/ethidium bromide gel (GIBCO, BRL, England) in 1× TBE buffer (0.09 M Tris-boric acid and 0.002 M EDTA). Gels were visualized under UV light and documented in FX Molecular Imager apparatus (BIO-RAD).

Sequence analysis

The PCR products for each genotype of the tested gene were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite and the results of the endouclease restriction were carried out using FastPCR. The nucleotide sequences of the tested gene in Egyptian sheep and goat were submitted to GenBank (NCBI, BankIt).

RESULTS AND DISCUSSION

In quantitative genetics, there are number of single genes associated with mammary or muscle growth, development and function which were studied as excellent candidates for linkage relationships with quantitative traits of economic importance. Among them, a somatotropic axis (SA) contains the most promising candidates (Szewczuk et al., 2012). Insulin growth factor-1 (*IGF-1*) gene is one important gene belonging to the somatotropic axis.

IGF-I has a variety of biological effects which plays an essential role in embryonic and postnatal growth. *IGF-I* concentration is related with fetal size in different species (Baker et al., 1993; Gluckman, 1995; Breier, 1999). *IGF-I* has an important role in growth of fetal organs and skeletal muscles (Lok et al., 1996). *IGF-I* is considered as a key factor in animal growth through its effect on longitudinal bone growth, muscle growth and cartilage growth (Duclos et al., 1999; Zapf and Froesch, 1999; Yakar et al., 2002). *IGF-1* plays an important role in mammalian fertility through the regulation of many hormones which are critical for reproductive system (Miller and Gore, 2001; Velazquez et al., 2008). Due to its important role in growth and reproduction traits, *IGF-1* gene is considered as a candidate marker for these traits and its genetic polymorphism identification is of great interest in Egyptian small ruminant breeds.

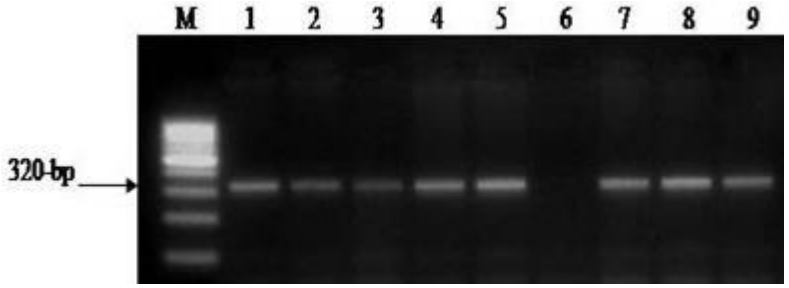

Figure 1. Ethidium bromide-stained gel of PCR products representing amplification of *IGF-1g*ene in Egyptian sheep and goat animals. Lane M, 100-bp ladder marker; lanes 1-5 and 7-9, 320-bp PCR products amplified from sheep and goat DNA; lane 6, negative control.

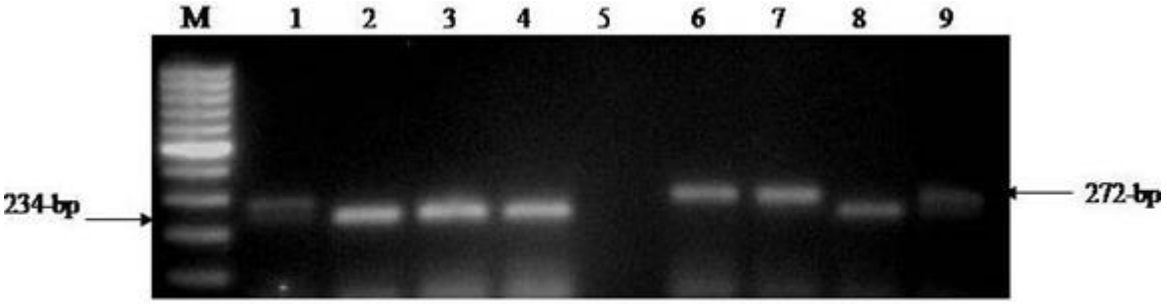

Figure 2. The electrophoretic pattern obtained after digestion of PCR amplified fragment of *IGF-1* gene with *Hae*III restriction enzyme. Lane M, 100-bp ladder marker; lanes 1 and 9: CG heterozygous genotype with four fragments at 272-, 234, 48- and 38 bp; lanes 2-4 and 8: GG homozygous genotype with three fragments at 234, 48 and 38 bp; lanes 6 and 7: CC homozygous genotype with two fragments at 272- and 48-bp; lane 5; negative control * Small sized fragments at 48 and 38 bp are not show in the figure.

The primers used in this study flanked a 320-bp fragment spanning from intron 3 to intron 4 covering exon 4 of *IGF-1*gene in sheep and goat. The amplified fragments obtained from all tested sheep and goat DNA gave the expected fragment at 320-bp (Figure 1).

These PCR amplified fragments (320-bp) were digested with *Hae*III restriction enzyme. Depending on the presence of one or two restriction sites (GG^CC) at positions 48^49 and/or 282^283, the results showed the presence of 3 different genotypes: CC with two digested fragments at 272- and 48-bp, GG with three digested fragments at 234, 48 and 38 bp and CG with four digested fragments at 272, 234, 48 and 38 bp (Figure 2).

The frequencies of GG, CG and CC genotypes were53.125, 37.5 and 9.375% in sheep Barki animals (32 animals), 57.14, 28.57 and 14.29% in sheep Ossimi animals (28 animals) and 59.09, 27.27 and 13.64% in sheep Rahmani animals (22 animals), respectively with the total frequencies of 53.66, 30.49 and 15.85% for GG, CG and CC genotypes, respectively in 82 tested sheep animals for this gene. In tested goat animals, the frequencies of GG, CG and CC genotypes were 56.25, 31.25 and 12.5% for Baladi (16 animals), 55.0, 30.0 and 15.0% for Barki (20 animals) and 59.09, 22.73 and 18.18% for Zaraibi (22 animals), respectively with total frequencies of 56.89, 27.59 and 15.52% for GG, CG and CC genotypes, respectively in 58 tested goat animals for this gene. The total frequencies of G and C alleles in all tested animals (140 animals) were 69.64 and 30.36%, respectively (Table 1).

These three detected genotypes resulted from the presence of two different alleles C and G. The sequence analysis of these two alleles represented a single nucleotide polymorphism (C→G) at position 282 which is responsible for the presence of 2nd restriction site at position 282^283 in the allele G (Figures 3 and 4). The nucleotide sequences of alleles C and G in sheep and goat were submitted to GenBank with the accession nos: KX432965, KX432966, KX432967 and KX432968, respectively.

Several polymorphisms of *IGF-I* gene and their associations with production traits were reported in goats. Zhang et al. (2008) reported the polymorphism in intron 4 of the *IGF-I* gene and its association with growth traits in the Nanjiang Huang goat. The associations of g.5752 G>C and g.1617 G>A mutations with milk yield and body

Table 1. The genotype and allele frequencies of *IGF-1* gene in Egyptian sheep and goat breeds.

Species	Breeds	Number of animals	Genotype frequencies						Allele frequencies	
			GG		CG		CC		G	C
			Number	Frequency (%)	Number	Frequency (%)	Number	Frequency (%)	Frequency (%)	Frequency (%)
Sheep	Barki	32	17	53.125	12	37.5	3	9.375	71.875	28.125
	Ossimi	28	16	57.14	8	28.57	4	14.29	71.43	28.57
	Rahmani	22	13	59.09	6	27.27	3	13.64	72.73	27.27
	Sub-total	82	44	53.66	25	30.49	13	15.85	68.9	31.1
Goat	Baladi	16	9	56.25	5	31.25	2	12.5	71.875	28.125
	Barki	20	11	55.0	6	30.0	3	15.0	70.0	30.0
	Zaraibi	22	13	59.09	5	22.73	4	18.18	70.45	29.55
	Sub-total	58	33	56.89	16	27.59	9	15.52	70.69	29.31
	Total	140	77	55.0	41	29.29	22	15.71	69.64	30.36

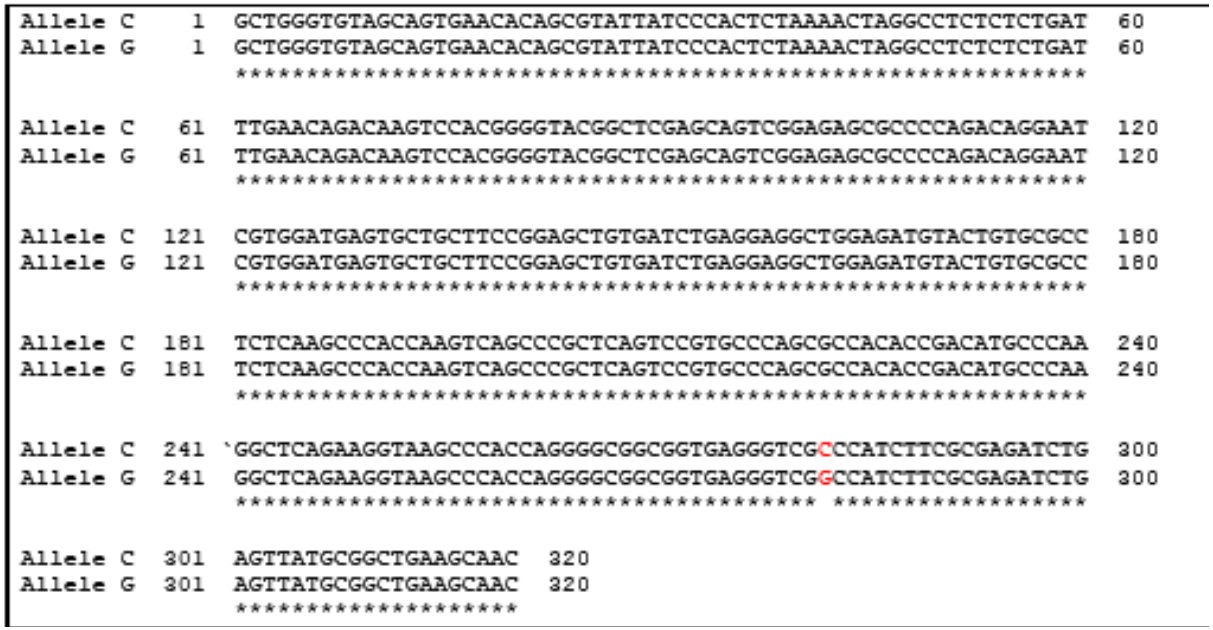

Figure 3. The nucleotide sequence alignment between the two different alleles C and G. Single nucleotide polymorphism (C/G) at position 282 in red.

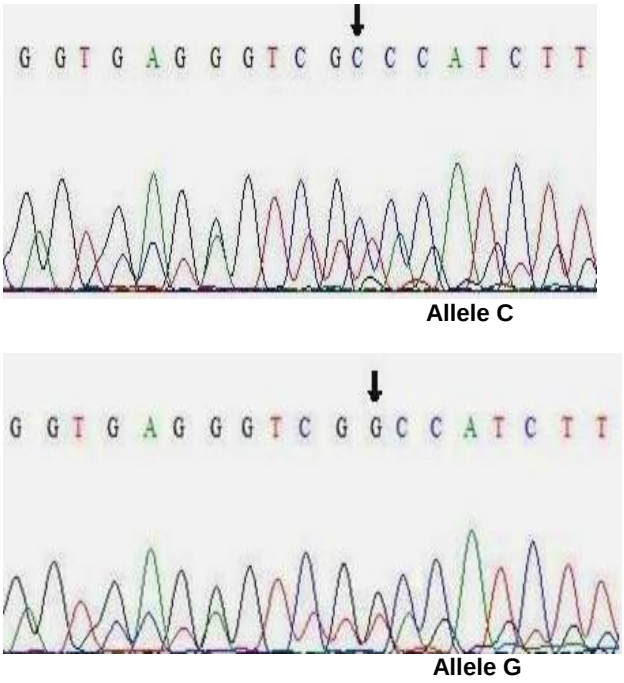

Figure 4. Single nucleotide polymorphism (C→G) at position 282 in alleles C and G.

size in Chinese dairy goats were reported with a significant effect of these variations on the examined productive traits (Deng et al., 2010).

Qiong et al. (2011) evaluated the relation between *IGF-1* variation and cashmere production traits as well as body weight in three Chinese goat breeds. A novel SNP was detected in exon 4 and it is significantly associated with cashmere production traits. The cashmere fineness of BB genotype animals was higher than those of AB and AA genotype individuals.

PCR–SSCP analysis of goat *IGF-I* gene was used to detect the polymorphisms in two Iranian goat breeds (Karimi Kurdistani et al., 2013) which revealed novel G to A transition (g.1617 G >A). Same authors used PCR-RFLP analysis of a part of intron and exon 4 of goat *IGF-I* gene to identify the associations between *IGF-I* /HaeIII polymorphism and growth trait. This polymorphism was significantly associated with different growth parameters which include yearling weight, post-weaning average daily gain and first shearing fleece weight. Animals which possess GG genotype in this site appeared potentially more favorable for these mentioned traits. The frequencies of GG, CG and CC in Iranian goats were 0.61, 0.29 and 0.10% respectively. Our results agree with the findings of this study where, the frequencies of different genotypes of *IGF-1* gene in Egyptian goat breeds were 56.89, 27.59 and 15.52% for GG, CG and CC, respectively. These results also declared the dominance of G allele (70.69%) over C allele (29.31%) in all tested Egyptian goat animals.

The same technique PCR-SSCP in Iranian Makoei sheep breed, Moradian et al. (2013) determined the genetic polymorphism at exon 1 of the *IGF-I* gene and the results reveal the presence of three genotypes; AA (52.0%), AG (42.0%) and GG (6.0%). He et al. (2012) examined the polymorphism of *IGF-1* gene in four Chinese sheep breeds which show the association of different genotypes in ewes with their lams at significant levels.

In conclusion, a nucleotide substitution (C→G) was detected in *IGF-I* gene in Egyptian sheep and goat breeds. Three different genotypes; CC, CG and GG were observed due to the presence of two alleles; C and G. The association of *IGF-I* polymorphism with different growth trait parameters were reported at significant levels. So, the genetic and SNP variations in *IGF-I* gene may be a potential molecular marker for growth traits in different Egyptian sheep and goat breeds and could be used in molecular marker-assisted selection for small ruminant programs.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of organic matter, irrigation and soil mulching on the nutritional status and productivity of okra (*Abelmoschus esculentus* L.) in the semiarid region of Brazil

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Okra (*Abelmoschus esculentus* L) is an important vegetable crop currently cultivated in many parts of the world. The adoption of management strategies to promote the nutritional status and crop productivity in semiarid regions of Brazil is still poorly studied. Thus, this experiment was conducted to evaluate the effect of raising the level of organic matter in the soil, irrigation and soil mulching in the nutritional status and productivity of okra plant in the semiarid region of Brazil. In this study, the experimental design of randomized blocks in a factorial 5×2×2 was used, with four replications. The treatments were five rates of cattle manure, necessary for raising the levels of organic matter in the pits to 1.8, 2.62, 3.44, 4.26 and 5.08%, two water depths (50 and 100%) of crop evapotranspiration, and soil with and without mulch. The elevation of soil organic matter level until 5.08% in conjunction with the implementation of 100% of Evapotranspiration (ETc) water depth and the use of mulch on the soil favored the greatest absorption of nutrients and increased the productivity of okra plant.

Key words: Cattle manure, nutrient absorption, management of water in the soil.

INTRODUCTION

Okra (*Abelmoschus esculentus* L) is a vegetable currently cultivated in many parts of the world (Moyin-

Table 1. Chemical characterization of cattle manure used as a source of organic matter.

N	P	K^+	Ca^{2+}	Mg^{2+}	Na^+	Zn	Cu	Fe	Mn	OM	OC	C/N
g kg^{-1}						mg kg^{-1}				g kg^{-1}		
12.76	2.57	16.79	15.55	4.02	5.59	60.0	22.0	855.0	325	396.0	229.7	18:1

OM = organic matter; OC= organic carbon.

Jesu, 2007). Due to its important role in human nutrition, by providing carbohydrates, proteins, fats, minerals and vitamins, the demand for this culture has increased considerably (Moyin-Jesu, 2007; Phonglosa et al., 2015). Most of the vegetable crops require large amounts of nutrients in a relatively short time, and therefore are considered demanding regarding the nutritional aspect (Coutinho et al., 1993). The nutrient supply is made by means of fertilization, which is often carried out with rates above the recommended, in order to try to avoid nutritional deficiencies (Raij, 1993).

However, prolonged use of mineral fertilizers can increase the acidity of the soil, and reduce the availability of nutrients and organic matter (Agboola, 1974; Aduayi, 1980). The organic matter has buffering action in a wide pH range, due to reactions involving the carboxylic groups of fulvic acids (Mendonça et al., 2006). The elevation of organic matter levels in the soil can promote increased crop yields, especially in sandier soils, by managing to increase the capacity of cation exchange, avoiding major losses by leaching (Santos et al., 2001; Yanfei et al., 2016). In addition, the organic matter is an important way to provide nutrients to plants and may promote greater absorption efficiency, resulting in productivity gain (Sediyama et al., 2009). But the application of large amounts of organic fertilizers can result in high vegetative development, and that may hinder the harvest and cause productivity losses, which explain the importance of studying the optimal rates to be applied (Trani et al., 2008).

For an efficient nutrient absorption by the plants, it is necessary for the soil humidity to be in an adequate amount (Danso et al., 2015). In regions of semiarid climate where low pluviometry is associated with constant irregularity of rainfall, it is necessary to provide water to the culture through the use of irrigation techniques, to increase productivity by optimizing the use of water resources (Barbosa et al., 2015). Another promising alternative to crop management can be the use of mulch on the soil surface, which has been found efficient in reducing hydric loss through evaporation (Teófilo et al., 2012).

However, there are no studies to understand the behavior of okra culture in regions with semiarid climates in Brazil, when adopted this set of techniques. Therefore, this experiment was conducted to evaluate the nutritional status of okra in relation to the elevation of levels of organic matter, irrigation and soil mulching.

MATERIALS AND METHODS

The study was conducted in the field during the period of November 2013 through April 2014, in Paraíba State University UEPB, Campus IV, at the Agroecology sector, in the municipality of Catolé do Rocha (6˚20'38"S, 37˚44'48''W and altitude of 270 m), Paraíba, Brazil. The climate in the region is the BSw'h' type, according to Kõppen classification, characterized as hot semiarid with two distinct seasons, a rainy one with irregular precipitation with an annual average of 800 mm, and another without precipitation. The rainfall (416 mm) in the experiment site was obtained through the meteorological station at UEPB, Campus IV. The rainy season is concentrated between the months of February and April. The average air temperature is 27°C, the soil protected with mulch is around 28ºC and uncovered it is 35ºC. The soil according to Embrapa (2013) was classified as Eutrophic Fluvic Neo-soil. In the first 20 cm of depth it showed 661 and 126 g kg^{-1} of sand, silt and clay, with soil and particle density of: 1.51 and 2.76 g cm^{-3}, respectively, and total porosity 0.45 m^3 m^{-3}. The humidity values at the field capacity, permanent wilting point and available water were 23.52; 7.35 and 16.17%, respectively. As for the chemical characterization, the soil at the same depth presented, according to the methodologies of Embrapa (2011), pH = 7.02; P and K = 53 and 297 mg dm^{-3}; Na^+ = 0.30; Ca^{2+}= 4.63; Mg^{2+}= 2.39; Al = 0.0; H+Al = 0.0 and CTC = 8.08 $cmol_c$ dm^{-3}, respectively; base saturation V = 100% and OM = 1.80%.

The treatments were distributed in randomized blocks in a factorial design 5 × 2 × 2, referring to the following treatments: five rates of cattle manure C/N ratio of 18:1 (Table 1), two water depths (50 and 100%) of crop evapotranspiration (ETc mm day^{-1}) and soil with and without mulching with plant debris of crushed dried parsley (*Ipomoea asarifolia*) in a layer 5 cm thick, with four replications, totaling 80 plots. The plot consisted of three lines 3.2 m long, spaced 1 m, with an area of 6.4 m^2. Each line had nine plants totaling 27 plants per plot.

The pits were opened in the dimensions of 30 cm × 30 cm × 30 cm, with spacing of 1 m between rows and 0.4 m between plants and prepared with soil material from the first 30 cm, along with 16 g pit^{-1} (84 kg ha^{-1} P_2O_5) of simple superphosphate (20% P_2O_5) (Ribeiro et al., 1999) and cattle manure C/N ratio 18:1 (Table 1), in sufficient rates to raise the content of organic matter in the soil 1.80 to 2.62; 3.44; 4.26 and 5.08%. The soil, the fertilizer and the cattle

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Table 2. Values for each rate of organic matter applied and their respective equivalence in the pits.

Rates of organic matter applied (%)	Cattle manure values (g hole^{-1})	Kg ha^{-1} of cattle manure
1.80*	0.00	0.00
2.62	886.00	22.150
3.44	1773.00	44.325
4.26	2659.00	66.475
5.08	3546.00	88.650

*Value existent in the soil.

manure were homogenized and subsequently inserted in the pits. The amount of manure dried in the air (Table 2) with 5% humidity, embedded in each pit was obtained through the expression suggested by Bertino et al. (2015):

$$M = \frac{[(OMR - ROMS) * Vc * Ds * HCM]}{OMCCM}$$

Where M = amount of cattle manure to be applied per pit (g); OMR = organic matter rate to be increased in the soil (g kg^{-1}); Vc = crown volume; Ds = soil density; = ROMS rate of organic matter in soil (g kg^{-1}); OMCCM = organic matter content in cattle manure (g kg^{-1}); HCM = humidity of cattle manure dried in the air.

The sowing was done in the second week of November 2013, with five seeds of okra (*A. esculentus* (L.) Moench) cultivar Santa Cruz 47, per pit. The thinning was performed when the plants had three true leaves in the first week of December, keeping only the strongest plant per pit.

Cover fertilization with nitrogen and potassium was made in function of crop yield and soil analysis at 20, 40 and 60 days after sowing (Ribeiro et al., 1999). The nitrogen and potassium were given in rates of 100 and 75 kg ha^{-1}, respectively, using as source the ammonium sulphate (20% N) and potassium chloride (60% K_2O).

The irrigation of the plants was carried out daily by the method of localized irrigation, adopting the drip system, according to the crop evapotranspiration ETc (mm day^{-1}). The calculation was based on the reference evapotranspiration (Eto, mm day^{-1}), estimated by the class A tank and corrected by the Kc of culture according to the development stage of the plant, obtaining the consumptive use (Cu) considering the wet area percentage (P) = 50%. Thus, for the purposes of calculating the depth of daily net irrigation (DDN = ETc), we used DDN = Cu × P/100 (mm day^{-1}); from this value, we determined the depths applied corresponding to 50 and 100% DDN which were applied daily and the application time used as a way of reducing the water volume (CE_{water} = 0.8 dS m^{-1}), that is, the time was reduced by half of what was offered on the water depth 100% of ETc. The variables assigned in the experiment were: Coefficient of class A tank (Kp) = 0.75; varying crop coefficient according to the culture stage (Kc) = 40 days after sowing was used Kc of 0.68; from 41 to 70 days, 0.79; 71 to 120 days, 1.00, as suggested by Paes et al. (2012). The flow of the drippers (q) = 2.15 L h^{-1} was obtained through field testing with the emitters installed at every 0.2 m on the line, that is, resulting in an area (AS) = 0.2 m^2 per emitter, as suggested by Paes et al. (2012).

In the beginning of flowering, at 65 days after sowing, the third leaf of three central plants was cropped of each plot for determination of N, P, K, Ca, Mg and S of the plant, to assess the nutritional status of the culture (Filgueira, 2007), adopting the methodologies proposed by Malavolta et al. (1997), and at the end of the crop cycle the productivity was evaluated. The results were submitted to variance analysis by the "F" test and polynomial regression, using the statistical software Sisvar 5.0 (Ferreira, 2011).

RESULTS AND DISCUSSION

The interaction between water depths, levels of organic matter and soil mulch is significant, except for the foliar levels of N and S (Table 3). The absorption of nutrients by plants is dependent upon a number of factors, such as the availability of these elements in the soil and appropriate humidity conditions, in function of most being absorbed at a greater proportion by the mass flow, which explains the significant effect of triple interaction, included in the crop yield (Kamaluldeen et al., 2014; Siyal et al., 2016). The exception of the statistical significance of N and S on this interaction may be the result from the spacing adopted in the experiment (1 × 0.4 m), which provided the closure of the entire area at 40 days after sowing, minimizing the effect of this factor. Considering nitrogen responds to the effects of irrigation and soil organic matter, and the accumulated levels of S vary according to the levels of organic matter added to the soil (Table 3).

Increased levels of organic matter stimulate the accumulation of nitrogen in the foliar dry matter of the okra to the highest value of 43.15 g kg^{-1}, the estimated maximum level of 3.80% of the input. The fertilizations with higher levels compromised the absorption of nitrogen (Figure 1A). The increase in leaf nitrogen content to the observed manure level occurs because the organic matter is a nitrogen source, releasing it slowly, avoiding losses of N in the soil by denitrification and leaching, as it happens at a greater proportion when inorganic fertilizers are applied (Barbosa, 2015).

The reduction of the water depth from 100 to 50% ETc resulted in the loss of nitrogen foliar accumulation in the early flowering of the plants from 43.36 to 39.55 g kg^{-1}, resulting in a loss of 8.8% (Figure 1B). This may be the result from decreased contact of nitrate and ammonium ion with the root due to the low humidity (Prado, 2008).

Regardless of the soil with and without cover, higher foliar levels of phosphorus were observed in plants irrigated with the highest water level (100% ETc) (Figure 2). In the treatments with mulch (Figure 2A), the highest P concentrations were 3.79 and 3.44 g kg^{-1} relating to the maximum rates estimated 4.58 and 4.37% in plants with water depths of 100 and 50% of crop evapotranspiration (ETc). It is noticed that the difference in the reduction of

Table 3. Summary of variance analysis related to the variables nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S) and productivity (Prod.) in leaves of okra variety Santa Cruz, when subjected to levels of organic matter in the soil, water depths, with and without soil mulch.

Source of variation	Square mean							Prod.
	DF	N	P	K	Ca	Mg	S	
Block	3	ns	ns	ns	ns	ns	ns	ns
OM	4	*	*	*	**	*	**	**
SM	1	ns	ns	ns	ns	*	ns	**
Depths (D)	1	**	**	*	**	*	ns	**
OM x SM	4	ns	*	**	ns	ns	ns	ns
OM x D	4	ns	ns	ns	**	**	ns	**
SM x D	1	ns	ns	ns	ns	ns	ns	ns
OM x SM x D	4	ns	*	**	*	*	ns	*
Residue	57	-	-	-	-	-	-	-
CV (%)		7.42	7.43	10.07	6.89	7.81	8.52	15.10
				g kg^{-1}				kg ha^{-1}
General average		41.45	3.54	19.40	24.60	6.12	2.05	7736.88

Significant at 5% (*) and 1% (**) of probability by F test; (ns) not significant; DF = degree of freedom; CV% = coefficient of variation; OM= organic matter; SM = soil mulching.

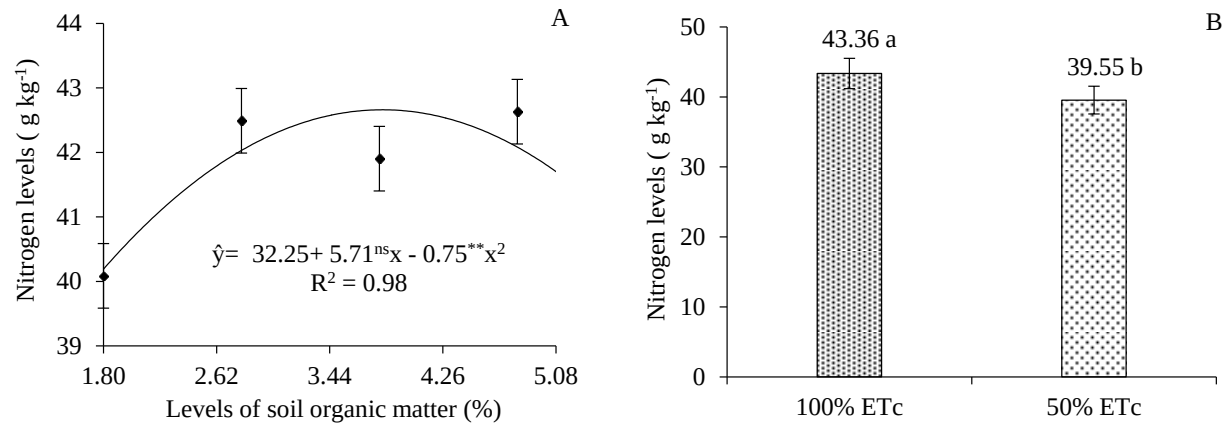

Figure 1. Nitrogen content in okra leaves, depending on the levels of organic matter (A) and water depths (B).

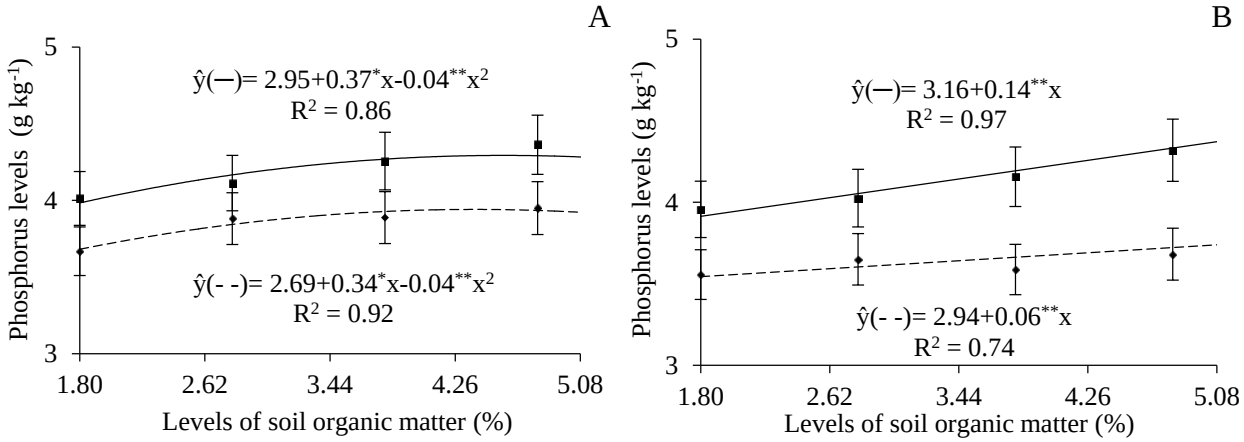

Figure 2. Phosphorus content in okra leaves depending on the levels of organic matter, irrigation with 100% (—) and 50% ETc (---), in a soil with (A) and without mulch (B).

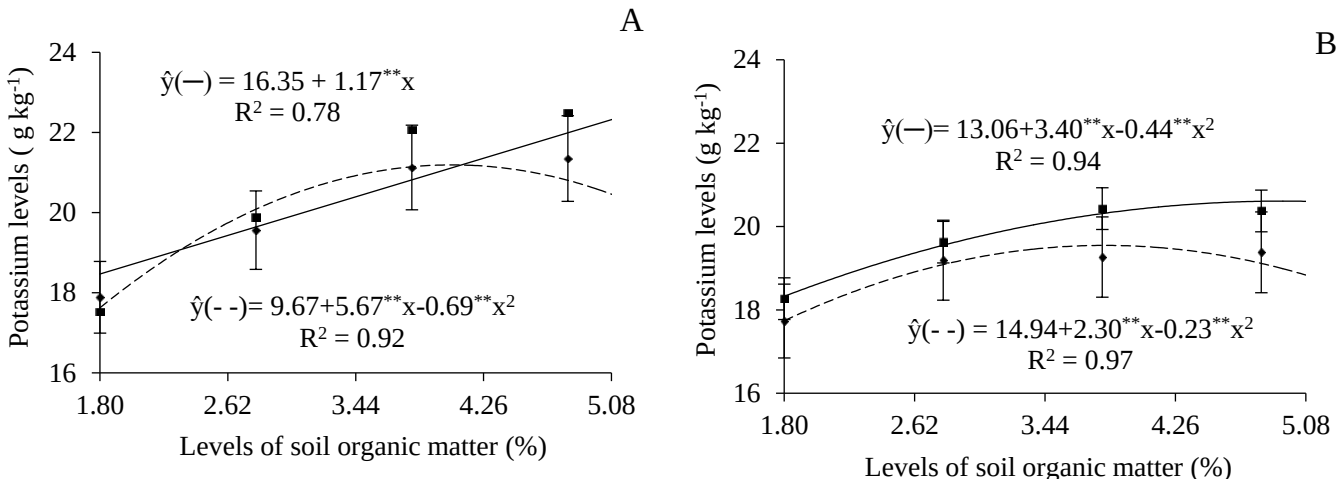

Figure 3. Potassium content in dry matter of okra leaves, depending on the levels of organic matter in the soil, with (A) and without mulch (B), irrigated with 100% ETc (—) and 50% ETc (---).

the water supply from 100 to 50% ETc resulted in a loss of foliar accumulation of P higher than 9%.

In plants from the soil without mulch the rate of organic matter linearly increased phosphorus foliar levels, to the levels of 0.139 and 0.059 g kg^{-1} per unit increase of provided organic matter (Figure 2B). The highest levels were 3.86 and 3.23 g kg^{-1} in the treatments with higher rates of the organic input in plants irrigated with water depth of 100 and 50% of ETc, respectively. As also recorded in plants of the soil with coverage, the reduction of water supply from 100 to 50% ETc resulted in loss of P accumulation of 18.9%.

Cattle manure levels possibly increased the amount of organic phosphorus in the soil, which was then converted to inorganic forms which the plants are able to absorb through the reaction catalysed by enzymes such as phosphatase, produced by soil microorganisms. The metabolic efficiency of microorganisms in the production of this enzyme may improve with increasing soil humidity and temperature, up to a certain limit (Zumsteg et al., 2013; A'Bear et al., 2014), which may have contributed to the increased availability of phosphorus in the soil. This explains the major foliar P in the treatments with 100% of ETc in relation to 50%, and also the small superiority of P value in the treatment that received the highest water depth, without soil mulch.

The results are in accordance with Medeiros et al. (2005), who verified an increase in N and P content in the shoot of rice plants, due to the increase of water content in the soil. In most cases, adequate levels of soil humidity and properly nourished plants express higher photosynthetic efficiency with higher respiration and perspiration rates, and larger energy to overcome the resistance to the penetration of roots into the soil (Hoffmann and Jungk, 1995; Stone, 1985), resulting in an increased nutrient absorption, in general, including the phosphorus that is a not so mobile element in the soil, in which the form of $H_2PO_4^-$ is absorbed on a greater proportion on the diffusion process.

In the plants of the soil with mulch, irrigated with water depth corresponding to 100% of ETc, the potassium content increased linearly in 1.744 g kg^{-1} of K^+ per unit increase of the applied organic input, with the highest value 22.31 g kg^{-1} at the highest rate provided. In the same coverage conditions, the plants treated with irrigation water depth of 50% of ETc had foliar potassium levels elevated to the maximum value of 21.17 g kg^{-1} at the highest estimated rate of 4.1% organic matter applied to the soil (Figure 3A). In plants of the treatments with no mulch, the increase in organic matter stimulated the foliar accumulation of potassium to the values of 20.61 and 19.53 g kg^{-1}, on the maximal rates of 3.82 and 4.88% of soil organic matter, respectively, between the plants irrigated with the water depths of 100 and 50% of crop evapotranspiration (Figure 3B). By relating the major values 21.11 and 23.16 g kg^{-1} (Figure 3A) and 19.53 and 20.61 g kg^{-1} (Figure 3B) between plants irrigated with water depths of 50 and 100% of ETc, it is perceived that the reduction was 8.7 and 5.2% between the plants of the soil with and without mulch.

Comparatively to the phosphorus, the data for potassium indicate similar behavior among plants irrigated with the water depth 50% of ETc, but differentiated between soil with and without mulch. The highest percentage loss of 18.9% of phosphorus foliar content occurred in plants without soil mulching and the greatest potassium loss (8.7%) in the plants on soil protected against losses through evaporation.

The results of 19.53 and 20.61, 21.11 and 23.16 g kg^{-1} are lower than those presented by Cavalcante et al. (2010), at a rate of 15% of cattle manure, when studying rates and sources of organic fertilizers in the

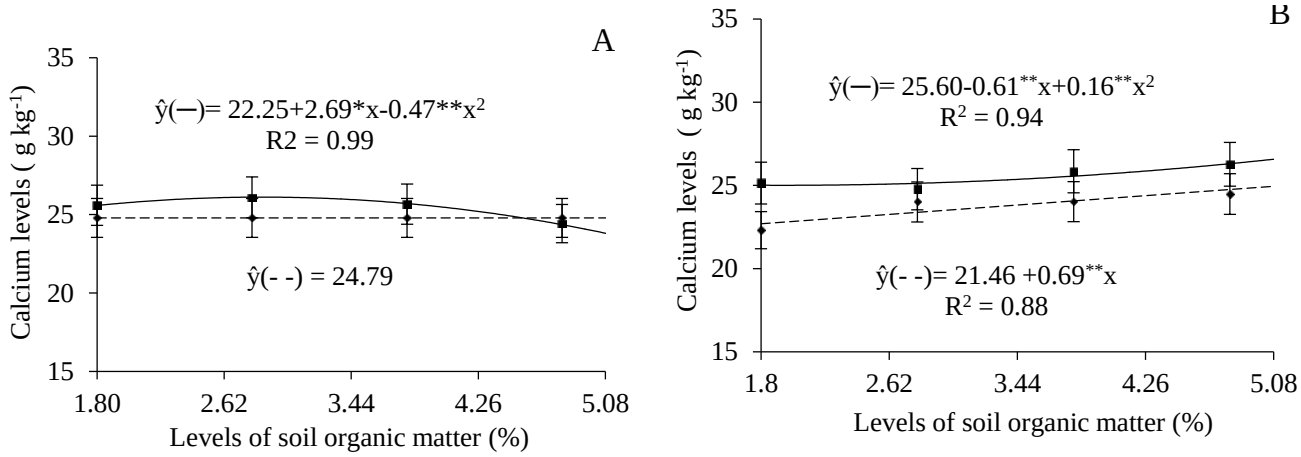

Figure 4. Calcium content in dry matter of okra leaves, depending on the levels of organic matter in the soil, with (A) and without mulch (B), irrigated with 100% ETc (—) and 50% ETc (---).

macronutrient content in okra leaves, possibly due to the cattle manure used in their experiment have higher potassium content (26.2 g kg^{-1}), compared to 16.9 g kg^{-1} K^+ present in the manure used in this experiment (Table 1).

In the soil with mulch and irrigation with water depth of 100% ETc, the levels of organic matter provided increased the foliar contents of Ca^{2+} in the plants to 26.11g kg^{-1} corresponding to the estimated amount of 2.86% of organic fertilizer. However, in plants under irrigation with 50% of ETc, the contents did not fit any mathematical model, so were represented by the average of 24.79 g kg^{-1}. Through the relation of the respective values, we verified that the reduction of water depth from 100 to 50% of ETc caused a 5.1% decline in the foliar accumulation of calcium by the okra plant (Figure 4A).

In the plants on the soil without mulch, the calcium levels were increased linearly with rates of organic matter regardless of irrigated with 50 or 100% of crop evapotranspiration (Figure 4B). The level of 5.8% of organic matter in the soil was responsible for the highest calcium content in the dry matter of okra leaves with averages of 27.38 and 25.43 g kg^{-1}, without mulch on the soil surface, irrigating the plants with 100 and 50% of ETc, respectively. The beneficial effect of organic matter may be related to the improvement of the physical, chemical and biological conditions of the soil, increasing the availability of calcium to the plants, a fact confirmed by Lopes and Guilherme (2007). These results differ from Cavalcante et al. (2010), who observed decrease in calcium content in the dry matter of okra leaves with increase of the cattle manure rate. Comparatively, the plants grown without water stress, regardless of soil cover, had higher levels of N, P, K and Ca^{2+} in the dry matter of okra leaves for the same treatments under water deficit in the soil. The contact ion-root occurs differently for the nutrients, being N, Ca and Mg by mass flow, while P and K through diffusion. Regardless of how the ion-root contact occurs, the elements must be present in the soil solution for the plant absorption to occur (Mauad et al., 2011). In view of this, the reduction of N and Ca^{2+} levels supplied by mass flow was due to the reduction of water content in the soil. On the other hand, for P and K, the decrease in content under increased tension is explained by the fact that the reduction of soil humidity decreases the thickness of the water film, increasing tortuosity, thereby hindering diffusion (Stone, 1985).

In the soil with mulch, foliar contents of Mg^{2+} decreased linearly with the levels of organic matter in plants irrigated with 100% of the ETc. The reduction of 0.285 g kg^{-1} for each unit increase of the organic input, corresponding to the decrease from 6.98 to 6.4 g kg^{-1} and loss of 15.56% between the plants in the soil with 1.80 to 5.08% of organic matter. Probably the greatest soil moisture provided by the mulch plus the water depth of 100% of ETc contributed for the occurrence of Mg^{2+} leaching. In these treatments, in the plants irrigated with water depth of 50% ETc, the Mg^{2+} content increased depending on the organic matter to the greatest value of 5.77 g kg^{-1}, at the maximum rate 3.51% (Figure 5A). These results partly differ from those obtained by Cavalcante et al. (2010) who recorded an increase in Mg^{2+} content in the dry matter of okra leaves with the increment of organic sources in the soil.

In the soil without mulch (Figure 5B), the higher levels of soil organic matter increased the magnesium content in the foliar dry matter of the plants to 6.82 and 6.14 g kg^{-1} corresponding to the estimated rates of 4.32 and 5.02% in plants irrigated with 100 and 50% of ETc. In general, the organic material influences the absorption of Mg^{2+} by the plants in accordance with Malavolta (1997), that organic matter reduces the losses of this secondary macronutrient through leaching by increasing its

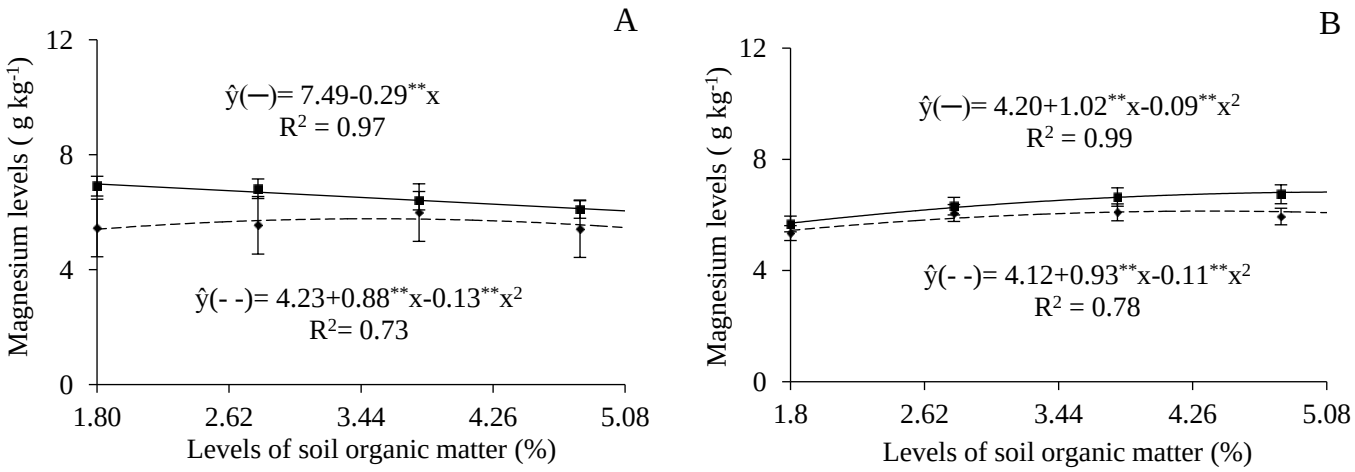

Figure 5. Magnesium contents in the dry matter of okra leaves, depending on the levels of organic matter in soil, with (A) and without mulch (B), irrigated with 100% ETc (—) and 50% ETc (---).

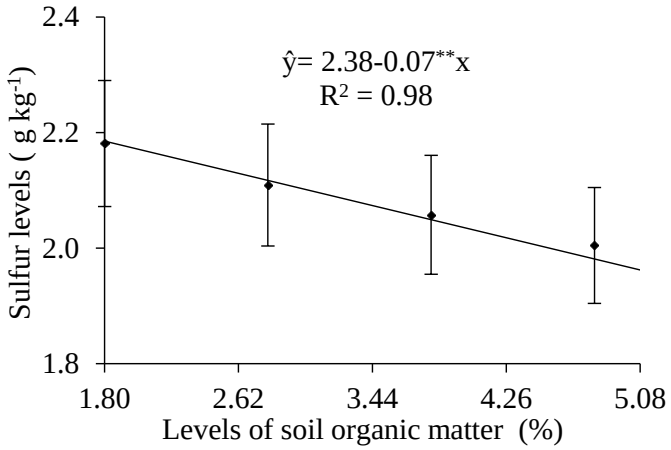

Figure 6. Sulfur content in the dry matter of okra leaves, depending on the levels of organic matter in the soil.

availability to the plants, as concluded also by Souza et al. (2005), with lettuce.

Among the studied nutrients, sulfur was the only one whose foliar levels of okra plant responded only to the levels of organic matter in the soil (Table 3). As shown in Figure 6, the increase in levels of organic matter added to the soil compromised the accumulation of S in the foliar dry matter at the level of 0.068 g kg^{-1} per unit increment of applied organic input. The values decreased from 2.18 to 1.96 g kg^{-1}, causing a reduction of 11.22% between plants treated with 1.80 and 5.08%, respectively. The values oscillated were among the lower limits required by most vegetable and fruit plants (Malavolta et al., 1997). This reduction may be related to the prevalence of the sand fraction in the soil and with the increased organic matter, rich in negative charges and pH dependent, contributed to the repulsion of the sulfate ion, a fact confirmed by Nogueira and Melo (2003), since they found lower sulfur content at 0 to 20 cm of the soil compared to the depth of 0 to 40 cm, after performing liming. The foliar levels of macronutrients in the plants at the beginning of flowering were obtained in the order: N> Ca> K> Mg> P> S, sequence also presented by Cavalcante et al. (2010).

The productivity of okra depending on the levels of organic matter in the soil in the presence and absence of mulch on the soil surface provided productions of (13584.43 and 9292.36 kg ha^{-1}) and (12815.21 and 9159.58 kg ha^{-1}), irrigating plants with 100 and 50% in ETc, respectively, reached the highest level of soil organic matter (Figure 7A and B). The production values obtained in the treatment of plants irrigated with 100% ETc approached the national average, which is 15000.00

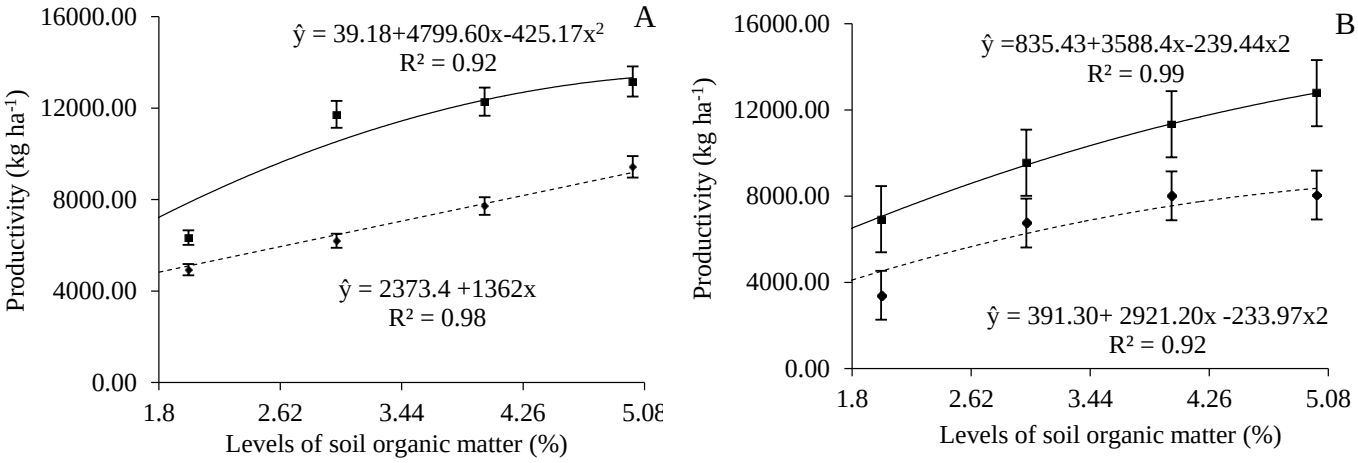

Figure 7. Okra productivity, depending on the levels of organic matter in the soil, with (A) and without mulch (B), irrigated with 100% ETc (—) and 50% ETc (---).

to 20000.00 kg ha^{-1} as reported by Filgueira (2007), and also in agreement with the results of Oliveira et al. (2003), who obtained 16701.00 kg ha^{-1} when assessing the yield of okra, cultivar Santa Cruz, in function of N rates. The increase in the okra productivity in function of organic matter levels in the soil is due to the input be the source of the macronutrients studied (Table 1). Nitrogen is the nutrient that provides greater response towards the productivity of okra (Filgueira, 2007), and foliar content of this element (43.15 g kg^{-1}) on the leaf were above the range considered appropriate (32.6 to 37.1 g kg^{-1}) as reported by Malavolta (1997). We also believe that 100% water depth of ETc has optimized nutrient transport mechanisms, since it is possible to observe higher foliar content of almost all the elements, in a water depth of 100% of ETc, which explains the higher productivity of the crop in this condition, as some studies have found increased the translocation of nutrients to the aerial parts of the plants when soil moisture is above 50% of the water retention capacity (Ruiz, 1986; Costa, 1998).

Conclusion

The results suggest that the management used in this study is an important strategy to promote the nutritional status of the okra plant and increase its productivity. It is recommended to increase the level of organic matter in the soil until 5.08%, the application of a water depth of 100% of ETc and the adoption of mulch to achieve higher productivity. It is important to note that in conditions of low water availability, water depth of 50% of ETc can also be used, in case a previous study was able to verify the economic viability in the region, considering a productivity drop of 31.59%.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Growth promotion mediated by endophytic fungi in cloned seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* hybrids

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Eucalyptus cultivation has expanded considerably in Brazilian systems, leading to the current search for technologies to optimize growing conditions and the production of seedlings in nurseries. Based on the understanding that the development of tree species such as *Eucalyptus* sp. can be influenced by endophytic fungi that act directly as plant growth-promoting species, cloned seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* hybrids grown from minicuttings we stimulated with three species of endophytic fungi and the effects of inoculation on seedling growth was evaluated. Strains of *Trichoderma* sp., *Fusarium* sp. and *Papulaspora* sp. were forced to colonize the root system of the plants, which were continuously maintained under protected cultivation. Inoculation of the symbionts had positive effects on stem length, stem diameter and the fresh and dry biomass of the treated plants. Non-inoculated plants presented a shorter stem length than the plants treated with any of the endophytic species. The cloned seedlings inoculated with *Trichoderma* sp. exhibited the greatest stem measurements at 120 days after transplanting. The seedlings inoculated with *Fusarium* sp. displayed a greater number of leaves than the other seedlings as well as greater amounts of fresh and dry biomass. The authors also conducted quarterly evaluations of the increment in seedling growth promoted by the inoculants, which were more effective in the early stages, up to 60 days after transplanting.

Key words: Inoculants, microorganisms, minicutting, tree species.

INTRODUCTION

Many microorganisms, especially those associated with roots, have historically demonstrated the ability to promote plant growth and productivity (Chang et al., 1986; Kloepper et al., 1988). The numerous effects of

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such plant-microorganism associations include an increased N_2 fixation capacity (Kuss et al., 2007; Lammel et al., 2013; Richardson et al., 2009) and the provision of solubilized nutrients, such as phosphates (Anzuay et al., 2013), where the solubilizing ability of many fungi has been associated with the release of organic acids and a decrease in pH. Gains in plant growth have also been related to facilitated absorption of iron promoted by certain microorganisms (Pii et al., 2015; Sadeghi et al., 2012) that synthesize siderophores (low molecular weight iron-chelating molecules) (Lemanceau et al., 2009; Miethke and Marahiel, 2007). Siderophores act as solubilizing agents of iron from minerals or organic compounds under iron-limited conditions (Rajkumar et al., 2010). The efficiency of siderophores from *Trichoderma asperellum* in donating Fe to plants grown under iron deficiency was demonstrated by De Santiago et al. (2009).

In addition to all of processes described above, some fungi have demonstrated the ability to synthesize phytohormones that are directly associated with plant development, such as auxins and gibberellins. In a study conducted in *Arabidopsis thaliana*, Contreras-Cornejo et al. (2011) found that *Trichoderma virens* produced compounds related to auxin, such as indole-3-acetic acid, indole-3-acetaldehyde and indole-3-ethanol. This phytohormone is known to act in the division and cell elongation process, since it activates enzymes that act on components of the bonds between cell wall cellulose microfibrils, increasing their plasticity (Castro et al., 2001). Consequently, water enters the cells more easily and rapidly increases their size (Taghavi et al., 2009).

There are few studies demonstrating the involvement of endophytes in promoting the growth of perennial plants such as those of the genus *Eucalyptus*. Cultivation of *Eucalyptus* has increased considerably in recent decades, associated with the use of various technologies and information on the edaphoclimatic diversity of cultivation areas (Gonçalves et al., 2014). Large forest stands of *Eucalyptus*, which is wood intended mainly for the civil construction and furniture industry, are distributed across all regions of Brazil, accounting for approximately 70% of the total planted forest area (ABRAF, 2013). Although, vegetative propagation of superior genotypes is employed in intensive clonal Eucalyptus forestry, this type of reproduction causes a number of problems, including variable results for the rooting index of cuttings and diseases acquired during this process (Díaz et al., 2009; Lombard et al., 2010). In this context, all technologies that optimize the growing conditions and production of ministumps directly favor the productive capacity of a nursery. Various agricultural strategies can be employed to improve the production of seedlings, including the use of bio-fertilizers, which are prepared organominerals rich in bacteria (Mehnaz, 2014; Sharma and Chaubey, 2015). Currently, the most widespread bacterial fertilizers are produced based on rhizobacteria (Dias et al., 2012; Melo et al., 2012; Vitorazi Filho et al., 2012). For species of the genus *Eucalyptus*, studies have related the incorporation of rhizobacteria in the substrate with increased rooting of minicuttings, growth after rooting and gains in dry mass (Alfenas et al., 2009; Díaz et al., 2009; Mafia et al., 2009), and these effects are significant when compared with those caused by the treatment of cuttings with indole butyric acid (IBA) (Teixeira et al., 2007). It is also known that there are many environmental and genetically effects such as nursery practice and seed source in tree seedling growth (Dilaver et al., 2015; Yazici et al., 2011; Yazici and Babalik, 2011, 2016).

Since eucalyptus plantations are located in low-fertility soils in Brazil (Gama-Rodrigues et al., 2005), studies that take into account fertilization and nutrient cycling techniques are important for increasing and maintaining forest production. Thus, this study evaluated the effect of inoculation with three endophytic fungi (*Trichoderma* sp., *Fusarium* sp. and *Papulaspora* sp.) on the growth of cloned seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* hybrids grown from minicuttings.

MATERIALS AND METHODS

Acquisition of plant material

The plant material was obtained via the cloning of *E. grandis* x *E. urophylla* hybrids grown from minicuttings. Shoots were collected from a clonal minigarden grown in a sand bed and were approximately one year old. The shoots were maintained in Tri-Mix substrate for the adaptive period of two weeks in a protected environment. To set up the experiment, the seedlings were transferred to 3.5-L pots containing Tri-Mix that had previously been sterilized in an autoclave at 121°C (Figure 1A and D).

Acquisition of fungal isolates

Endophytic isolates were previously obtained from the roots of *Hyptis marrubioides*, a medicinal plant native to the Cerrado (Brazilian savanna) (Vitorino et al., 2012). These isolates were maintained in flasks containing nutrient agar culture medium and stored in the culture collection of the Laboratory of Agricultural Microbiology of the Federal Institute of Goias (IF Goiano), Rio Verde Campus. The isolates were activated in PDA (infusion of 200 g potato, 20 g dextrose, 15 g agar and water up to 1000 mL) (Figure 1C). Mycelial disks of approximately 0.5 cm in diameter were removed from the established colonies and carefully distributed inside the planting holes, where they were placed in contact with the root system of the seedlings at the time of transplantation to the plastic pots (Figure 1D).

Three species of endophytic fungi were tested (*Trichoderma* sp., *Fusarium* sp. and *Papulaspora* sp.), where the control treatment consisted of plants free of microorganism inoculation. The experimental design was completely randomized, and the four treatments (the three fungi and a control) were analyzed in seven replicates. Growth evaluations of stem length (SL), stem diameter (SD), number of leaves (NL), root length (RL), fresh biomass (FB), dry biomass (DB) and leaf area (LA) were performed. The biometric analyses were conducted using a caliper and an analytical balance. To calculate the leaf area, photographs were taken with a digital

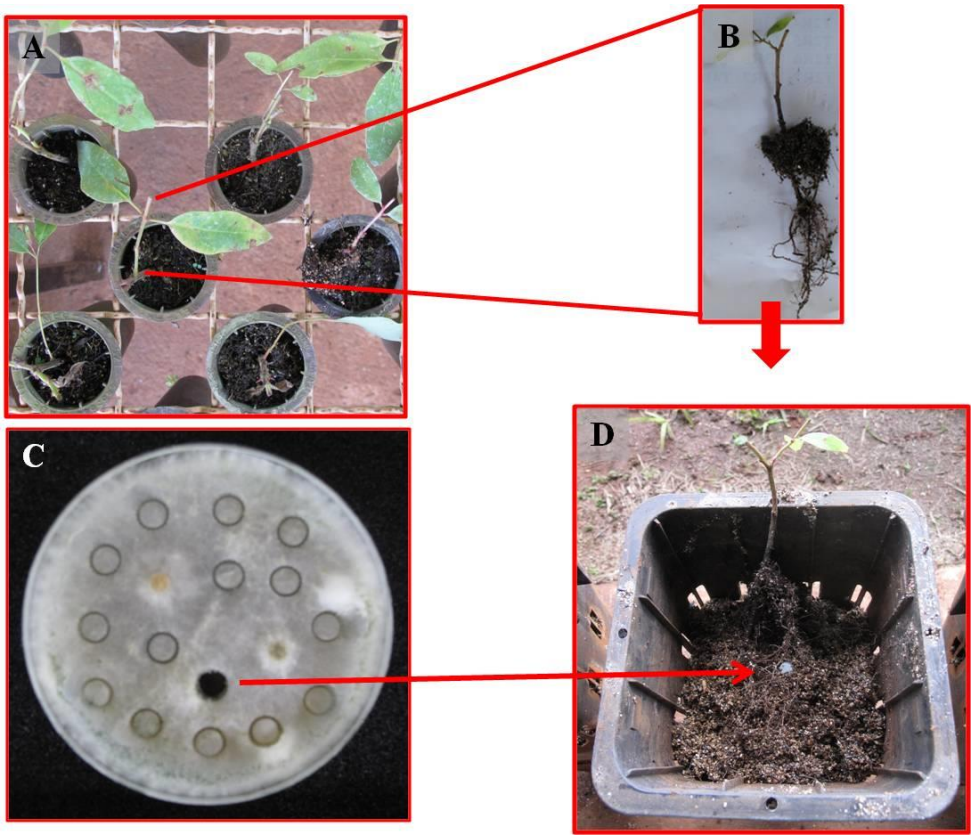

Figure 1. Cloned seedlings of *E. grandis* x *E. urophylla* in the adaptive period (A); seedling to be transplanted (B); Petri dish containing *Fusarium* sp., highlighting the removal of the mycelial discs (C) and mycelial disc inside the planting hole (D).

camera using a reference for size comparison. A white piece of paper with centimeter markings was employed as the reference, upon which the leaves of the plants were placed to take the photographs. The photographs were then analyzed using the software Sigma Scan Pro- V.S. O, Jandel Scientific.

The quarterly values for stem length and stem diameter were used to determine the percentage growth increase of the seedlings via the following equation:

$$x = \frac{100(b-a)}{a}$$

Where, *b* = value of the variable on the assessment day; *a* = value of the variable in the previous assessment.

RL, FB and LA were measured only at 120 days, and mean DB values were obtained after six days of drying in a forced air oven at 65°C, when the samples reached constant weight. The growth data were compared with the values recorded for the seedlings on the day of transplantation. The means for the different treatments were subjected to analysis of variance (ANOVA) using the F-test, and when significant differences were found, the means corresponding to FB, DB and LA were compared using Tukey's test at the 5% probability level, while the means for SL, SD and NL were compared through regression analysis. The data for SL obtained from the inoculation treatments were compared with the control treatment using a *t* test (inoculated plants x non-inoculated plants) for the different evaluation times. Statistical tests were performed with the aid of the R statistical package (R Core Team, 2016).

RESULTS

When evaluating the capacity of the three endophytic fungal isolates to promote the growth of cloned *E. grandis* × *E. urophylla* seedlings, analysis of variance of the means for the biometric variables revealed the significance of the treatments for SL, SD, FB and DB, while no difference was detected between the inoculation treatments and the control for LA. Additionally, differences were observed in relation to the evaluation times (0, 30, 60, 90 and 120 days after transplanting) for SL, SD and NL. The interaction between the different types of fungi used for inoculation and the evaluation times was not significant. This finding indicates independence of these variables, according to the F-test (Table 1).

For SL, the regression analysis was significant for time (R^2 = 95.1) and treatment (R^2 = 30.5). Breakdown of the time variable within the different treatments revealed significance for this growth parameter only at 90 and 120 days after transplanting (p = 0.0003 and p = 0.0000, respectively). For SD, the regression was also significant for time (R^2 = 81.3) and treatment (R^2 = 20.1), where the breakdown was significant at 30, 60 and 90 days (p = 0.0005, 0.0103 and 0.0001, respectively). Regarding NL,

Table 1. Summary of ANOVA results for the biometric measurements obtained from cloned seedlings of *E. grandis* x *E. urophylla* hybrids, grown from minicuttings and inoculated with endophytic microorganisms.

Variable	F			CV (%)
	Treatment	Time	Treatment X time	
Stem length	21.153*	15.559*	1.317	4.38**
Stem diameter	12.768*	10.830*	0.447	4.28
Number of leaves	2.479	11.411*	0.084	7.37
Root length	1.025	-	-	6.65
Fresh biomass	3.986*	-	-	6.61
Dry biomass	4.732*	-	-	6.22
Leaf area	1.828	-	-	7.26

*Significant according to the F-test at 5% probability; **data transformed into $\sqrt{x}$.

the regression analysis was significant for treatment (R^2 = 86.6) and the interaction, where the breakdown showed significance for all time periods evaluated (p = 0.0093, p = 0.0067, p = 0.0072 and p = 0.0006).

The behavior of the different variables as a function of the treatments is shown in Figure 2. A linear effect was observed (a typical demonstration of a temporal increase in SL growth) in all treatments. For SD, linearity was also obtained for the data corresponding to the plants inoculated with *Papulaspora* sp. and *Trichoderma* sp.; however, a quadratic effect on the growth of the control plants as well as those inoculated with *Fusarium* sp. was detected. A quadratic effect was also found for the variable NL in all the treatments as well as the control.

The plants inoculated with the tested endophytic fungi exhibited better stem growth when compared with the control plants (Figure 2A), where the plants inoculated with *Trichoderma* sp. presented the greatest measurements at the end of the evaluation period. The results of the *t* test (Table 2) for SL indicated significant growth of plants inoculated with the endophytes when compared with non-inoculated plants, confirming the benefit of the plant-endophyte association as well as the growth-promoting character of the evaluated species.

The cloned seedlings inoculated with *Fusarium* sp. exhibited more leaves than the other seedlings (Figure 2C) and showed a trend towards high FB and DB values (Table 3).

When evaluating the quarterly increase in the SL of cloned of *E. grandis* x *E. urophylla* seedlings, the highest percentages were obtained 60 days after transplanting (DAT) in association with the endophytes *Fusarium* sp. and *Papulaspora* sp. (Figure 3). Therefore, these fungi (especially *Fusarium* sp.) were effective in stimulating the cloned seedlings in the early stage of their growth. There have been few studies associating the genus *Papulaspora* with growth promotion, and the endophytes of this genus are best known for the biosynthesis of enzymes (Tuppad and Shishupala, 2014) and other metabolites with antimicrobial (Ramos et al., 2010) and/or cytotoxic (Gallo et al., 2014) activity. With respect to SD, the largest increases were also obtained in the early stages, where at 60 DAT, the plants inoculated with the fungus *Trichoderma* sp. exhibited a considerable increase in SD growth; however, in the final assessment at 120 DAT, the plants inoculated with *Papulaspora* sp. exhibited a greater increase in SD development than the plants inoculated with the other fungi (Figure 3).

DISCUSSION

Endophytic microorganisms are currently being evaluated as growth promoters for use in the production of seedlings of various species. It has been suggested that endophytic fungi can act symbiotically with plants, increasing the absorption of nutrients as well as the response to pathogens and stress conditions, which translates into gains in growth (Mandyam and Jumpponen, 2014). In this study, a linear temporal increase was observed for SL in all treatments. In contrast, for the variable NL, the control plants and those subjected to the inoculation treatments exhibited quadratic behavior. The same pattern was detected in the control plants and those inoculated with *Fusarium* sp. with respect to SD. This observation may be explained by a reduction in the values of these variables that occurred at the final evaluation times, possibly related to exhaustion of the nutritional content of the available substrate, or even an absence of symbiotic microflora in the autoclaved substrate, specifically in the case of the control plants for the latter scenario. Symbionts mediate the transfer of nutrients from the soil to plants (Barretti et al., 2008; Behie and Bidochka, 2014), and the presence of endophytic fungi has been linked to mechanisms such as the mineralization of available organic matter (Van Hecke et al., 2005), alteration of the chemical and biological properties of the soil and changes in hydraulic characteristics and aggregate stability (Hosseini et al., 2015). Filamentous fungi, such as those used in this

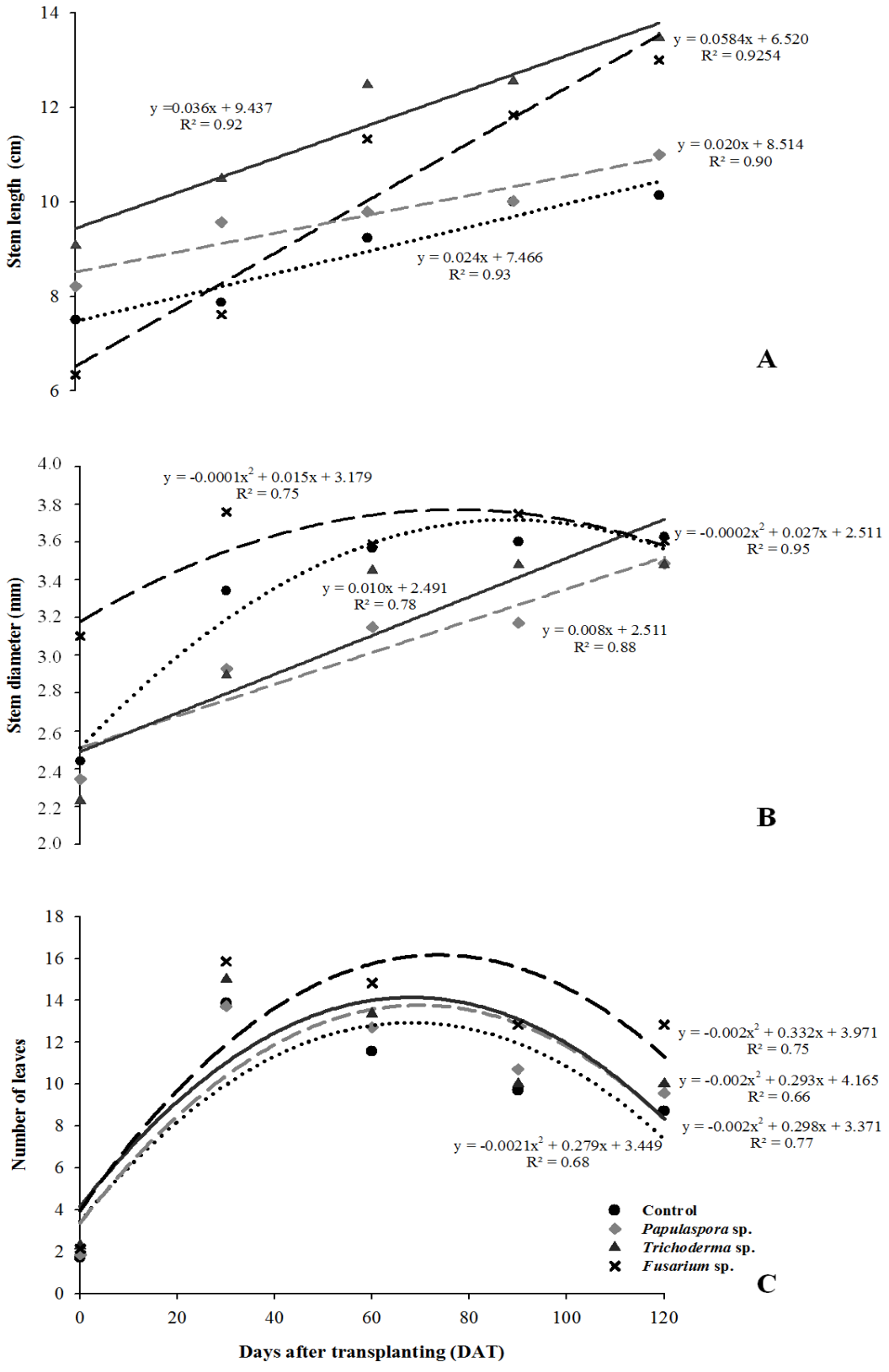

Figure 2. Stem length (A), stem diameter (B) and number of leaves (C) in cloned seedlings of *E. grandis* x *E. urophylla* hybrids grown from minicuttings and inoculated with the endophytes *Papulaspora* sp., *Trichoderma* sp., *Fusarium* sp., assessed at 0, 30, 60, 90 and 120 days after transplanting.

Table 2. Summary of the *t* test results for comparisons of the stem growth of cloned seedlings of *E. grandis* × *E. urophylla* hybrids inoculated with endophytic fungi x control seedlings, evaluated at 0, 30, 60, 90 and 120 days after transplanting (DAT).

DAT	*t*	*p*
0	-0.475	0.645
30	-2.181	0.046
60	-2.315	0.038
90	-1.372	0.198
120	-2.156	0.040

Table 3. Influence of inoculation with endophytic microorganisms on the mean shoot fresh biomass and dry biomass of cloned *E. grandis* x *E. urophylla* hybrid seedlings grown from minicuttings.

Treatment	Fresh biomass (g)	Dry biomass(g)
Control	9.8[b]	5.8[b]*
Papulaspora sp.	8.9[b]	4.8[b]
Trichoderma sp.	10.3[ab]	5.6[b]
Fusarium sp.	17.2[a]	9.5[a]

*Means followed by the same letter in the columns do not differ according to Tukey's test at 5% probability.

work, release enzymes through their hyphae that interact with the organic matter in the substrate (Chigineva et al., 2011) and transform nutrients, such as nitrogen, into forms that are assimilable by plants (Chen et al., 2013). It is possible that endophytic fungi such as mycorrhizae also alter the permeability of roots, facilitating hydraulic conductivity and the absorption of nutrients, which are fundamental processes for the promotion of growth (Ruiz-Lozano and Azcón, 1995).

The cloned seedlings inoculated with *Fusarium* sp. produced more leaves than the control plants or those inoculated with the other fungal species tested. Endophytes of the genus *Fusarium* have attracted the interest of a large number of researchers, who have reported their ability to synthesize bioactive secondary metabolites, including exopolysaccharides (Mahapatra and Banerjee, 2012), naphthoquinones (Kornsakulkarn et al., 2011) and cytotoxic alkaloids (Musavi et al., 2015; Venugopalan and Srivastava, 2015). When the potential of this symbiont as a growth promoter was evaluated, its action was found to result in the highest FB and DB values in the cloned *E. grandis* x *E. urophylla* seedlings (Table 3). In *Annona squamosa*, a *Fusarium* sp. strain endophytic to *Annona* spp. was also described as a growth promoter due to increase in the DB of the shoots of seedlings cultivated in a greenhouse (de Oliveira Silva et al., 2006). Similar results were observed in seedlings of *Passiflora edulis* f. *flavicarpa* Deg, in which this fungus stood out among other endophytes based on promoting increases in shoot FB (Luz et al., 2006). According to Magalhães et al. (2003), the dry matter production rate is strongly affected by leaf area, which is influenced by factors such as environment and management. The amount of biomass produced by the plant can therefore be defined by a simple physiological relationship, based on the amount of radiation intercepted and the efficiency of its conversion into dry matter (Charles-Edwards, 1982). Therefore, the increase in the shoot dry biomass of cloned *E. grandis* x *E. urophylla* seedlings may indicate an improvement in the quality of seedlings, which would allow a reduction in the time spent maintaining them in nurseries.

Endophytic fungi have only recently been recognized for their importance in improving the overall fitness of host plants. Despite the prevalence and diversity of plant-endophytic fungus associations, studies have sought to document the impact of using these fungi on plants of agronomic or medical interest; however, such research is still in its incipient stages when compared with the number of studies on growth promotion conducted with bacteria, especially rhizobacteria, such as *Rhizobium*, *Pseudomonas*, *Bacillus*, *Azotobacter* and *Azospirillum* (Ahemad and Kibret, 2014; Bashan et al., 2014; Dutta and Khurana, 2015; Egamberdieva and Lugtenberg, 2014; Sharma et al., 2015). Studies using endophytic fungi as growth promoters have thus far been restricted to investigation of their antagonist activity against pathogens (Aktar et al., 2014; Parmar et al., 2015a) and have been much more restrictive than studies conducted with mycorrhizae and non-root rhizosphere fungi. However, new endophytic fungi are frequently isolated and identified as possible inoculants, such as *Penicillium funiculosum*, *Sordariomycetes* sp. and *Fusarium* spp., which were shown to stimulate growth in *Glycine max* L. (Khan et al., 2011), *Oryza sativa* L. (Li et al., 2012) and *Hordeum vulgare* (Maciá-Vicente et al., 2009), respectively.

Fungal strains of the genus *Fusarium* can be found in symbiotic associations with the internal tissues of most plants (Demers et al., 2015; Singh et al., 2015). However, these strains can also act as pathogenic fungi (Sobowale et al., 2005), triggering *Fusarium* wilt, or fusariosis, which is characterized by xylem hypertrophy (Pinto et al., 2010) and wilting followed by death of the affected plants (Costa et al., 2010). The high pathogenicity of *Fusarium* strains in some plants of agronomic interest (Gásperi et al., 2003; Nascimento et al., 2014), together with the great economic losses triggered by fungi of this type in stored fruits (Dantas et al., 2003) or grains (Barros et al., 2005; Ramos et al., 2014), has limited the number of studies attempting to use *Fusarium* strains as species that promote plant growth. Based on the absence of symptoms caused by infestation with pathogenic *Fusarium* strains as well as the growth response observed in the plants evaluated in this study, it can be concluded that the tested *Fusarium* strain is, in accordance with strict criteria, a plant growth-promoting

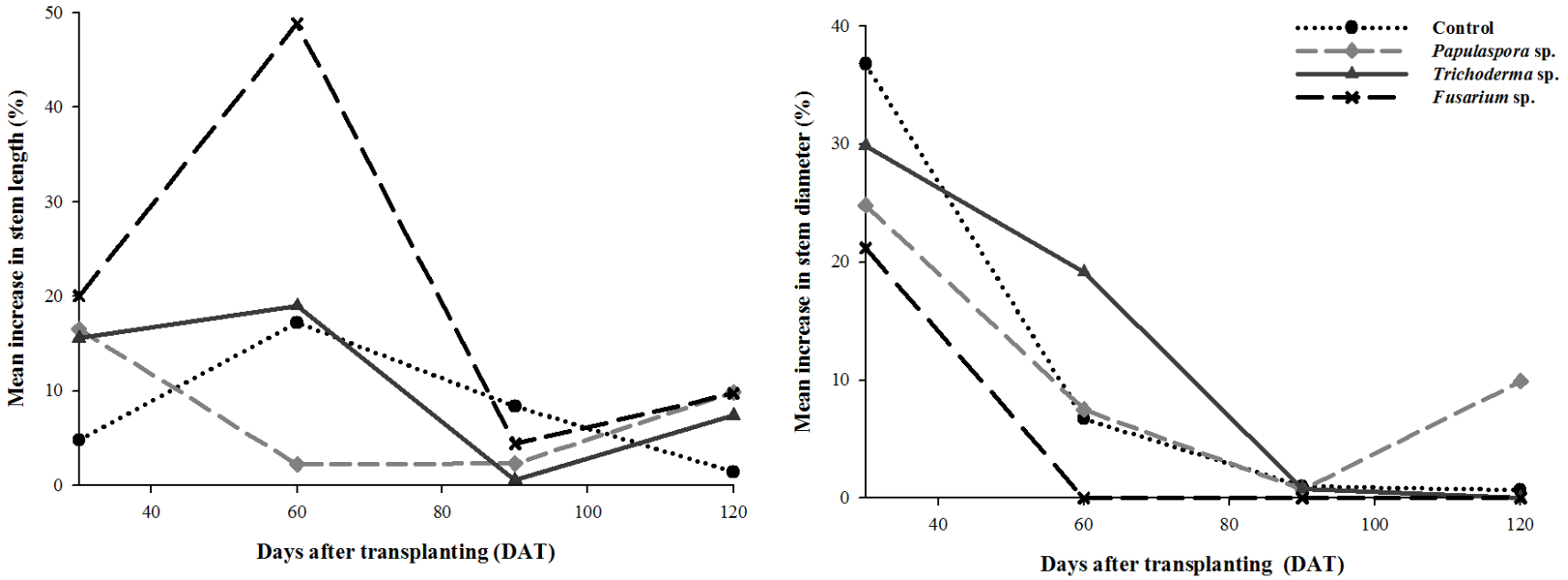

Figure 3. Quarterly increase in the stem length and stem diameter of cloned seedlings of *E. grandis* x *E. urophylla* hybrids grown from minicuttings inoculated with the endophytes *Papulaspora* sp., *Trichoderma* sp. and *Fusarium* sp.

endophytic strain. Many strains of the genus *Fusarium* and other endophytic fungi can produce phytohormones (Nassar et al., 2005) that directly influence plant development. It is well established that some *Fusarium* strains are capable of producing the plant hormone gibberellin (GA) (Troncoso et al., 2010; Tudzynski, 2005). Working with strains of *Fusarium proliferatum* that are symbiotic with orchids, Tsavkelova et al. (2008) identified an isolate that is a significant producer of GAs, and strains symbiotic with *Physalis alkekengi* have been shown to synthesize a wide range of GAs, including GA_3 (Rim et al., 2005). GAs are ubiquitous substances that elicit several important metabolic functions during plant growth (Bomke et al., 2008), which could potentially represent the means by which *Fusarium* sp. stimulated the cloned *E. grandis* x *E. urophylla* seedlings, promoting their growth.

The plants inoculated with *Trichoderma* sp. exhibited the greatest mean SL at the end of the evaluation period. In *E. urograndis*, Paz et al. (2012) tested the EUCB 10 strain of the endophytic bacterium *Bacillus* sp., which was also shown to be efficient in promoting height gains in the evaluated plants, in addition to increasing the dry mass of the roots and shoots. With respect to the increases in stem length and diameter, the seedlings subjected to the isolate tested in the present study responded positively only in the early stages of development (60 DAT). Many studies have associated *Trichoderma* sp. with growth promotion, though this effect is almost always related to the ability of these fungi to act as an antagonist of plant pathogens (Druzhinima et al., 2011; Kumar et al., 2015; Parmar et al., 2015b), which was not the case in the present study. However, strains of *Trichoderma* used in biocontrol may stabilize in the rhizosphere, subsequently stimulating plant growth and eliciting plant defense reactions against pathogens (Harman et al., 2011). *Trichoderma* spp. have been shown to induce growth and control *Sclerotinia sclerotiorum* in seedlings of the bean plant cv. Carioca grown in nurseries (Aguiar et al., 2012). However, in seedlings of *Eucalyptus benthamii*, it was indicated that this fungus shows antagonistic potential for the biological control of

Botrytis cinerea (Sbravatti Júnior et al., 2013). Research involving species of this genus as well as other fungi that are considered to be potential plant growth promoters is still in the exploratory stages, and studies associating endophytic microorganisms with *Eucalyptus* species have been conducted with the sole intention of isolation and characterization (Kaewkla and Franco, 2011; Taylor et al., 2009). Therefore, a greater number of studies will be required to improve the understanding and application of these microorganisms in agriculture on a large scale as well as in other areas that rely on biotechnological advances.

Conclusions

From the study, it can be concluded that: 1. plants inoculated with endophytic fungi exhibited better stem growth than that of control plants; 2. cloned *E. grandis* x *E. urophylla* seedlings inoculated with *Fusarium* sp. presented a greater number of leaves as well as higher FB and DB values when compared with the other plants; 3. endophytic fungi were effective in stimulating the growth of cloned *E. grandis* x *E. urophylla* seedlings in the early development stage (60 DAT).

Conflict of interests

The authors have not declared any conflict of interests.

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Short Communication

Primary study on the components and main physico-chemical as well as biological properties of the oil of *Alpinia galanga* (L.) Wild in Phu Tho-Vietnam

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The oil of *Alpinia galanga* (L.) Willd in Phu Tho was obtained by steam distillation and dried with Na_2SO_4. By Gas chromatography-mass spectrometry (GC-MS) method, 29 components in the oil were predicted by comparing their retention times and molecular weights with the standards'. In particular, there were 10 hydrocarbons such as monoterpenes: 29.15%, sesquiterpenes: 21.06%, and 19 oxygenated components like aldehydes (7.29%), alcohols (32.43%), ketones (1.09%), and esters (7.57%). Physico-chemical properties, antioxidant activities as well as antimicrobial activities of the oils were also analyzed. The density (at 20°C), acid index and ester index of the oil were 0.812 g/ml; 0.653 mg KOH/g and 0.728 mg KOH/g, respectively. The antioxidant activity was determined by using 1,1-diphenyl-2-picrylhydrazol (DPPH) radical percentage inhibition and it was 47.15±0.34%. Antimicrobial activity against *Salmonella typhi, Bacillus cereus, Staphylococcus aureus* and *Escherichia coli* of the oil was identified by agar diffusion method.

Key words: Oil of *Alpinia galangal* (L.) Willd in Phu Tho-Vietnam, components, physico-chemical, biological activity.

INTRODUCTION

The *Alpinia galanga* (L.) Willd is planted in mountainous areas of the Doan Hung, Ha Hoa, Lam Thao, Phu Ninh district of the Phu Tho province. *A. galanga* (L.) Willd shows effects in medical field. It has been used to weld and increase digestion, reduce swelling pain and fever. In particular, in South East Asia, *A.galanga* (L.) Willd is used to treat skin diseases, dyspepsia, some symptoms of digestive tract, flu, malaria, rheumatoid arthritis and some other kinds of infections. *A. galanga* (L.) Willd is also used to produce medicines to treat stomach cancer and throat cancer (Moi et al., 2002).

The components of different varieties of this plant have

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shown variability (Scheffer et al., 1981). The components and their bioactivities of some *A. galanga* (L.) Willd have been reported (Loi et al., 2015). However, the components as well as their main physico-chemical and biological properties of *A. galanga* (L.) Willd in PhuTho, Vietnam have not been evaluated yet. Therefore, the aim of this study is to primarily analyze the components, physico-chemical indexes and antibacterial activity of the oil of *A. galanga* (L.) Willd in Phu Tho.

MATERIALS AND METHODS

The *A. galanga* (L.) Willd, identified by Assoc. Prof. Dr. Tran Huy Thai, Institute of Ecology and Biological Resources, was harvested from Phu Tho province in 2015. The essential oil was obtained by steam distillation after drying with Na_2SO_4. The sample was stored in the Department of Biotechnology and Food Processing, Hanoi University of Industry, Vietnam. The sample has been stored at the Department of Biotechnology and Food, Hanoi University of Industry.

The tested bacterial strains (*Staphylococcus aureus*, *Escherichia coli, Salmonella typhi* and *Bacillus cereus*) were obtained from School of Biotechnology and Food Technology, Hanoi University of Science and Technology. All chemicals and media were purchased from Sigma.

Oil extraction

The oil was extracted by using rhizome of the plant with water in oil distillation equipment Clevenger (Germany) in the ratio of 1:4 (w/v) respectively for 180 min.

Gas chromatography mass spectrometry (GC-MS)

The sample and standards were run parallelly in the *GC-MS* experiment. Gas chromatography (GC) analysis was performed by using Agilent Technologies HP 6890 Plus Gas chromatograph system equipped with Flame Ionization Dectector (FID) and fitted with HP-5MS columns (30 m × 0.25 mm, film thickness 0.25 µm). The temperature was programmed as follows: The column temperature was programmed from 80 to 150°C in 23.3 min at a rate of 3°C/min and then from 150 to 220°C in 8.75 min at a rate of 8°C/min. The used injector temperature was 230°C. The *MS* conditions were as follows: Ionization voltage was 70 eV, transfer temperature was 250°C, the carrier gas was helium used at a flow rate of 0.5 ml/min, and the split ratio of the injector was 1:5 (Loi et al., 2015; Charles et al., 1992; Thang and Loi, 2016; Chau et al., 2014). The *MS* fragmentation patterns were compared with known patterns of other oils and with those in the literature by using Wiley (Wiley 9thVersion), NIST 08Libraries (on ChemStation HP). The percentage of each component was calculated by the percentage of its peak area.

Determination of physico-chemical properties of oil

The density, angle of rotation, refraction index, acid index and ester index of the oil were determined by using ISO 8444: 2010, ISO 8446: 2010, ISO 8445: 2010, ISO 8450: 2010 and ISO 8451: 2010, respectively (Anthology of the National Standards for essential oil - testing methods, 2010).

Determination of antioxidant activity using free radical scavenging activity

The free radical scavenging activity of the oil was measured by using 1,1-diphenyl-2-picrylhydrazol (DPPH) *(*Molyneux, 2004; Matook and Fumio, 2006; Shyu and Hwang, 2002*)*. A 0.5 mM solution of DPPH in methanol and 0.005 M acetate buffer (pH 5.5) were prepared. An aliquot of 0.1 ml of the sample solution was added to the tube containing 2 ml of acetate buffer, 1.9 ml of methanol and 1 ml of DPPH solution. In the blank tube, DPPH was removed; in the control tube, 1 ml of DPPH was added to the tube containing 2 ml acetate buffer and 2 ml methanol. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in the dark. The decrease in absorbance at 517 nm was measured after 30 min until the reaction reached plateau. Vitamin C with the concentration of 0.5 mM was used as a positive control and its free radical scavenging activity was performed in parallel in the same experiment. These experiments were run in duplicate.

The inhibitory percentage of DPPH was calculated as follows:

$$\text{Scavenging effect (\%)} = [(A_o - (A - A_b)) / A_o] \times 100\%.$$

Wherein A_o is the value of absorbance of the control at the wavelength of 517 nm; A is the value of absorbance of the sample at the wavelength of 517 nm; and A_b is the value of absorbance of the blank at the wavelength of 517 nm.

Determination of antibacterial activity using agar diffusion method

Antibacterial activity was roughly determined by agar diffusion method. 50 µl of the oil was put into wells on the plates containing tested bacterial strains. The activity was roughly estimated by the diameter of the antibacterial round (mm), which was calculated by the formula D- d (mm), wherein D was the diameter of the antibacterial round (mm) and d was the hole diameter (mm) (Perez et al., 1990).

RESULTS AND DISCUSSION

The components of the oil

GC-MS of the sample was performed in order to roughly determine the components of the oil. Based on the retention times and molecular weights of the sample and the standards (the GC profile was not shown here), 29 components and their percentages in the oil were evaluated and shown in the Table 1. The table showed that 29 components were predicted in the oil of *A. galanga* (L.) Willd in Phu Tho-Vietnam. Ten out of them were hydrocarbons (such as monoterpenes: 29.15% and sesquiterpenes: 21.06%) and the rest were oxygenated ones (like aldehydes: 7.29%, alcohols: 32.43%, ketones: 1.09% and esters: 7.57%). The results provided additional evidence to show varied percentages of the components of the oils of *A. galanga* (L.) Willd. Notably, the amounts of aldehydes and alcohols in the oil were higher than those of the oil in Malaysia (De Pooter et al., 1985). Probably, the differences were due to the geographical conditions such as the soil factors, weather,

Table 1. The components of the oil of *Alpinia galanga*(L.) Willd in Phu Tho-Viet Nam.

S/N	Components	Retention time (min)	Proportion (%)
Monoterpenes			**29.15**
1	α-Pinene	3.456	2.14
2	Camphene	4.177	5.98
3	β-Pinene	4.643	1.93
4	α-Terpinene	4.868	6.72
5	Limonene	8.054	8.32
6	p-Cymene	8.370	2.17
7	Terpinolene	14.368	1.89
Sesquiterpenes			**21.06**
8	β-cubebene	12.114	2.21
9	α-Humulene	15.658	5.62
10	Valencene	15.714	3.67
11	α-Farnesene	15.857	3.89
12	δ-Cadinene	16.183	5.67
Aldehydes			**7.29**
13	Octanal	6.015	3.18
14	Nonanal	13.718	1.15
15	Citronellal	14.104	1.89
16	Neral	14.816	1.07
Alcohols			**32.43**
17	α-Farnesol	5.335	3.16
18	β-Farnesol	7.116	5.59
19	Citronellol	7.532	2.17
20	Geranyllinelol	7.601	6.37
21	Borneol	7.843	7.09
22	Ascaridol	8.875	1.97
23	Terpinen-4-ol	9.925	3.04
24	α-Terpineol	13.043	1.92
25	Nerolidol	14.906	1.12
Ketones			**1.09**
26	α-Thujone	15.443	1.09
Esters			**7.57**
27	Linalyl acetate	6.856	4.12
28	Neryl acetate	13.945	1.67
29	Genaryl acetate	15.515	1.78
	Total		**98.59**

% was calculated by the percentage of chromatographic peak area.

climate, growing conditions and harvesting time (Charles et al., 1992).

The physical-chemical indexes of the oil of *A.galanga* (L.) Willd in Phu Tho-Vietnam

The density, angle of rotation, refraction index, acid index and ester index of the oil were presented in Table 2.

These results were consistent with those of the oils of *A. galanga* (L.) Willd from Malaysia (De Pooter et al., 1985). In particular, the oil had a density (0.821), which was smaller than 0.9 and refractive index (1.415), which was smaller than 1.47. However, no significant differences were observed in these values. The value of the angle rotation of the sample showed that the oil was

Table 2. Physico-chemical indexes of the oil of *A. galangal* (L.) Willd in Phu Tho-Viet Nam

S/N	Physical-chemical indexes	Result
1	Density at 20°C	0.812
2	Anglerotationα^{20}_{D}	84° 35’
3	Refractive indexn^{20}_{D}	1.458
4	Acid index (mg KOH/g)	0.653
5	Ester index (mg KOH/g)	0.728

Table 3. The diameters of antibacterial activity rounds of the oil *of A. galangal* (L.) Willd in Phu Tho-Viet Nam

S/N	Tested microorganisms	Diameter of antibacterial round (mm)
1	*Salmonella typhi*	29.17
2	*Bacillus cereus*	31.42
3	*Staphylococcus aureus*	26.25
4	*Escherichia coli*	27.12

capable of being dissolved in both polar organic and nonpolar organic solvents. The acid index of the sample showed that the oil could be less of an oxidation. This result was coincident with the percentage of the components of the oil. In particular, the total oxygenated components determined in this research were less than 50% (Table 1).

The biological activities of the oil of *A. galanga* (L.) Willd in Phu Tho-Vietnam

The free radical scavenging activity DPPH of the oil

The DPPH free radical scavenging activity of the oil of *A. galanga* (L.) Willd was 47.15±0.34% and this value was a bit higher than that of 0.5 mM vitamin C (39.65±0.42%). These activities of the oils of the leaves of *Liquidambar formosana* Hance in Bac Giang and *Citrus sinensis* peel were found to be 41.13 ± 0.22% and 45.32 ± 0,18%, respectively (Loi et al., 2015; Matook and Fumio, 2006). Therefore, we could say that The DPPH free radical scavenging activity of the oil of *A. galanga* (L.) Willd is higher than that of the leaves of *L. formosana* Hance in Bac Giang and *C. sinensis* peel.

Antibacterial activity of the oil of Alpinia galangal (L.) *Willd in Phu Tho*-Vietnam

In order to estimate the antibacterial potentials of the oil of *A. galanga* (L.) Willd in Phu Tho-Vietnam, agar diffusion method was used in this experiment. Tested microorganisms used in this experiment were *S. aureus, E. coli, S. typhi* and *B. cereus.* The diameters of antibacterial activity rounds of the oil against these bacteria were shown in Table 3. The results showed that the oil of *A. galangal* (L.) Willd in Phu Tho–Vietnam possessed antibacterial activity against all of the four microorganisms tested. Among them, the antibacterial activity against *B. cereus* was the highest one. The activity of the oil of *A. galanga* (L.) Willd in this research is similar to that of the oils of the leaves of *L. formosana* Hance in Bac Giang as these oils were found to possess antibacterial acitivities against all of the four tested microorganisms (Loi et al., 2015).

Conflict of Interests

The authors have not declared any conflict of interests.

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