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Table of Content: Volume 16 Number 43 25 October, 2017

ARTICLES

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Full Length Research Paper

Impact of trona-aided boiling on the phytochemical constituents and beneficial micronutrients of lima bean (*Phaseolus lunatus* L.)

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The influence of boiling lima bean, using trona solution, on its phytochemical constituents and selected micronutrients was investigated. Lima bean was initially boiled for 3 h using 0.1, 0.2, 0.3, 0.4 and 0.5% trona solution, respectively at ratio 1:5 (bean/trona solution). The boiled lima bean was subsequently dried in an oven at 55°C for 12 h after which it was subjected to various analyses. Maximum reduction of phytochemicals such as trypsin inhibitor activity (74.1%), haemagglutinin (98%), tannin (68.6%), phytic acid (39.6%), and cyanogenic glycoside (73.8%) was obtained at 0.5% boiling with trona-solution. The reduction profile for the B-vitamins includes thiamine (0.58 to 0.13 mg/100 g), riboflavin (0.37 to 0.21 mg/100 g), niacin (0.97 to 0.28 mg/100 g) and pyridoxine (0.41 to 0.11 mg/100 g). Boiling lima bean in trona solution led to significant decrease ($p<0.05$) in the concentration of calcium, magnesium and phosphorus while mineral concentration enhancement was observed with potassium, sodium and iron. Maximum *in vitro* protein and starch digestibilities of 88.6% and 51.9 mg/g, respectively were obtained for 0.5% trona solution boiling of lima bean. Trona usage in the boiling of lima bean therefore served as a beneficial step in the utilization of the legume.

Key words: Lima bean, trona, boiling, phytochemicals, micronutrients.

INTRODUCTION

Leguminous seeds such as soybean, faba bean, pea, mung bean, cowpea, kidney bean, pigeon bean and lima bean constitute an important and inexpensive dietary source for many people particularly in the developing countries (Bello-Perez et al., 2007; Al-Abdalall, 2010). They are regarded as principal sources of macronutrients, micronutrients and phytochemicals containing protein, carbohydrate, vitamins, minerals and polyphenols among others (Zhao et al., 2014). The leguminous seeds are largely utilized as valuable

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ingredients of diverse food products for human consumption as well as for animal feed (Du et al., 2014).

Lima bean (*Phaseolus lunatus*) is regarded as one of the under-utilized legumes which can be grown in many parts of the world including Latin America countries, the United States, Canada, African and Asian countries (Bello-Perez et al., 2007). Lima bean contains a class of chemicals called phytochemicals, the term generally used to describe chemicals from plants that may affect health, but are not essential nutrients (El-Gharras, 2009). Phytochemicals are essentially plant secondary metabolites presents in all plant tissues and their primary role is to protect plants from insects, ultraviolet radiation, and microbial infections and to attract pollinators (Del-Rio et al., 2013). Examples of these phytochemicals are trypsin and chymotrypsin inhibitors, haemagglutinins, cyanogenic glycosides, tannin and phytic acid, which are collectively known as anti-nutrients (Adeniran et al., 2013). These anti-nutritional factors, through their metabolism, interfere with nutrient intake, absorption, utilization and availability in the body systems thereby affecting the health of consumers (Ezeagu and Ibegbu, 2010).

The utilization of lima bean essentially lies in culinary and medicinal uses. These include its uses in soup preparation, making of sandwich filling and salad preparation among others (Sarkar, 2012). Lima bean has been used as an alternative raw material for the production of 'daddawa', a Nigerian condiment (Adeniran et al., 2013). This grain legume has also been recognized to reduce low-density lipoprotein cholesterol thereby leading to the reduction of risk of ischemic heart disease and diabetes (Messina, 2014). Most of the utilization of lima bean fundamentally involves cooking of the grain legume for it to get softened. However, the long cooking duration with concomitant high energy usage is a major drawback and discouragement to lima bean usage. The hard-to-cook characteristic of the grain legume is due to its hard cotyledon which naturally predisposes it to such cooking time elongation (Mamiro et al., 2011).

A lot of attempts have been made by researchers to increase the utilization of lima bean using a wide range of appropriate processing techniques. The elimination methods of the anti-nutritional factors in lima bean had been suggested and these include soaking, autoclaving and toasting (Adeparusi, 2001), boiling (Aremu et al., 2016), heat treatment and fermentation (Adeniran et al., 2013) among others. In dealing with hard-to-cook phenomenon peculiar to lima bean, alkaline cooking with calcium bicarbonate [$Ca(HCO_3)_2$] and a crude rock salt of carbonates (trona), chemically called sodium sesquicarbonate ($Na_2CO_3.NaHCO_3.2H_2O$) are the traditional methods of alleviating this problem in most African households (Mamiro et al., 2011; Olapade and Umeonuorah, 2014). The use of 'trona' for alkaline thermal treatment brings about softness of the bean and reduces the time of boiling which may also have effect on the chemical components of the bean. The process of softening of leguminous seed, during boiling, has been attributed to the disintegration of the cotyledonous tissue in individual cells. According to Belitz et al. (2009), the disintegration of the cotyledonous tissue is usually caused by the conversion of native protopectin to pectin, which quickly depolymerizes on heating. The middle lamella of the cell walls, which consists of pectin and strengthens the tissues, disintegrates in this process.

The objective of this study, therefore, was to investigate the effect of trona-aided boiling on the phytochemical composition and beneficial micronutrients of lima bean (*P. lunatus*).

MATERIALS AND METHODS

Raw lima bean of the red variety was purchased from Odo-Oba market while trona ($Na_2CO_3.NaHCO_3.2H_2O$; sodium sesquicarbonate) was purchased from Sabo market in Ogbomoso, Oyo State, Nigeria.

Cooking of lima bean

Cooking in distilled water

Five hundred grams of lima bean seeds were added to boiling distilled water at ratio 1:5 (bean/distilled water, weight/volume) at 100°C and cooked on a kerosene stove for 3 h. Water was drained off and the seeds were dried in an oven at 55°C for 12 h. The dried seeds were later milled using a hammer mill and sieved to pass through a sieve with a 250 µm mesh size. The sieved flour samples were stored in air tight plastic container for subsequent use.

Cooking in trona solution

Trona was ground into a fine powder and dried at 70°C overnight in an oven. The dried powder was then cooled in a desiccator and kept in a stoppered glass bottle at room temperature (30±2°C). Five hundred grams of lima bean seeds were then boiled in trona solutions at different concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5%, respectively at ratio 1:5 (bean/trona solution; weight/volume) at 100°C for 3 h using a kerosene stove. Thereafter, the boiled seeds were dried, milled and stored as described when boiled in distilled water. The concentrations of trona were so chosen to simulate what is obtainable with traditional processing procedures where very low trona concentration (usually <1%; trona/food material) is commonly used to avoid darkening of cooked product.

Determination of trypsin inhibitor activity

The method of Kakade et al. (1974) was used to determine the trypsin inhibitor activity (TIA) of lima bean flour sample using benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate. A 4.0 g sample was treated with 40 ml of 0.05 M sodium phosphate buffer (pH 7.5) and 40 ml of distilled water. After series of chemical manipulations, the absorbance of end-point solution was read at 410 nm wavelength in a spectrophotometer (UV-160A, Shimadzu, Osaka, Japan). Blank sample was treated similarly through the entire determination. Results were expressed as trypsin inhibitor units (TIU). One TIU was defined as an increase of 0.01 in absorbance units under conditions of assay. Trypsin inhibitory

activity was defined as the number of TIU.

Determination of tannin content

Tannin was determined according to the method of Price and Butler (1977). Sixty milligrams of ground sample was shaken manually for 1 min in 3.0 ml methanol. The mixture was filtered followed by mixing the filtrate with 50 ml distilled water and analyzed within an hour. About 3 ml of 0.1 M $FeCl_3$ in 0.1 M HCl was added to 1 ml filtrate, followed immediately by the addition of 3 ml freshly prepared $K_3Fe(CN)_6$. The absorbance was read on a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan) at 720 nm after 10 min from the addition of 3 ml of 0.1 M $FeCl_3$ and 3 ml of 0.008 M $K_3Fe(CN)_6$. Similar treatments were also carried on the blank. Results were expressed as tannic acid equivalent (mg/100 g sample), calculated from a calibration curve using tannic acid.

Determination of haemagglutinin concentration

Haemagglutinin level of the samples was determined by the method of Arntified et al. (1985). Two grams of each sample was weighed and 50 ml of the solvent of mixture of isobutyl alcohol and trichloroacetic acid was added and allowed to shake on a shaker for 6 h to extract the haemagglutinin. The mixture was then filtered through a double layer filter paper and maintained in a water bath for 2 h at 80°C and the filtrate was allowed to cool. A set of standard solutions of haemagglutinin ranging from 0 to 10 ppm was prepared from the haemagglutinin stock solution. The absorbance of the standard solution as well as that of the filtrate was read at 220 nm on a spectrophotometer (UV-160A, Shimadzu, Osaka, Japan). The result was expressed as haemagglutinin unit (HU)/g sample.

Determination of cyanogenic glycoside content

Determination of cyanogenic glycoside content of the samples was done by the alkaline titration method of the AOAC (1990). Two hundred millilitres of distilled water was added to 1 g of the sample in an 800 cm^3 capacity distillation flask. The flask was fitted for distillation and allowed to stand for 2 h for autolysis to take place. An antifoaming agent (silicon oil) was then added. Steam distillation was carried out and 150 cm^3 of the distillate collected into 250 cm^3 capacity conical flask containing 20 cm^3 of 2.5% sodium hydroxide, then diluted to mark with distilled water. Thereafter, 8 cm^3 of 6 N NH_4OH solution and 2 cm^3 of 5% potassium iodide was added to 100 cm^3 of diluted distillate. This was titrated against 0.02 N silver nitrate ($AgNO_3$) solution using a 10 cm^3 microburette. The end-point was noted as a permanent turbidity against a black background. Titre values were obtained and used to calculate cyanide content (mg HCN/Kg), using the formula:

$$CG = (TV \times 1.08 \times EV / SM \times AL) \times 100 \quad (1)$$

Where, CG is cyanogenic glycoside; TV is titre value (cm^3); EV is extract volume (cm^3); SM is sample mass (g); AL is aliquot (cm^3) used; and 1 cm^3 of 0.02 N $AgNO_3$ = 1.8 mg HCN.

Determination of phytic acid content

The method of Wheeler and Ferrel (1971) was used to determine the phytic acid content of each sample. A 2 g sample was used for the extraction. A standard curve was prepared expressing the results as $Fe(NO_3)_3$ equivalent. Phytate phosphorus was calculated from the standard curve assuming a 4:6 iron to phosphorus molar ratio. The phytic acid content was also calculated by multiplying the amount of phytate phosphorous by the factor 3.55 based on the empirical formula $C_6P_6O_2H_{18}$ and result was expressed as mg/100 g sample.

Mineral content determination

The mineral content was determined according to AOAC (1995). Two grams of lima bean flour was ashed in a muffle furnace at 550°C. The resultant ash was dissolved in 5 ml of $HNO_3/HCl/H_2O$ (1:2:3, v/v/v) followed by heating on a hot plate at the boiling temperature of the solution until brown fumes disappeared. Five millilitres of deionized water was later added to the remaining content in the crucible, and the mixture was heated until a colourless solution was obtained. The colourless solution was then filtered into a 100 ml volumetric flask using a Whatman No. 42 filter paper, and diluted to volume with deionized water. The concentration of the following elements: Ca, Mg and Fe was then determined from the filtered solution using atomic absorption spectrophotometer (Model SP9 Pye Unicam, UK), having initially prepared a standard curve for each element under investigation. The concentration of each element was calculated as mg/100 g of sample.

The analysis of sodium and potassium concentrations of the sample was carried out using flame photometry while the phosphorous content of the filtered solution was determined colorimetrically using vanadate-molybdate reagent according to the method described by Egan et al. (1981). The content of phosphorus in the filtered solution was determined using a standard curve obtained for potassium dihydrogen phosphate (KH_2PO_4) and expressed as mg phosphorus per 100 g of sample.

Thiamine determination

The method of AOAC (1995) was used for the determination. Five grams of lima bean flour was weighed into a 250 ml conical flask and 100 ml 0.1 N H_2SO_4 was slowly added without shaking and left overnight. The mixture was thereafter filtered through Whatman No. 2 filter paper and the first 10 to 15 ml of filtrate was discarded. Ten millilitres of the extract was then pipetted into 100 ml separating funnel. Three millilitres of 15% NaOH was then added into the separating funnel immediately followed by four drops of ferricyanide solution and then shaken gently for exactly 30 s. Fifteen millilitres of isobutanol was rapidly added, followed by vigorous shaken for 60 s to allow the layers to separate and the bottom layer drained off and discarded. One spatulaful of sodium sulphate was directly added into the separating funnel and then swirled gently to clarify the extract. The clear extract was then collected using a Pasteur pipette into a clean dry test tube. The blank sample was similarly prepared by pipetting 10 ml of the extract following the procedures above but with the omission of ferricyanide solution. The thiamine content was estimated using a fluorimeter and results are expressed as mg/100 g sample.

Riboflavin determination

The method of AOAC (1995) was used for the determination. Five grams of lima bean flour was weighed into a 250 ml conical flask and 75 ml 0.1 N H_2SO_4 was added with the flask immersed in boiling water for 30 min and the flask shaken every 5 min. This was allowed to cool at room temperature. Five millilitres of 2.5 M sodium acetate solution was added and allowed to stand for at least 1 h. The solution was then transferred to a 100 ml volumetric flask, made up to the volume with distilled water and filtered through Whatman No. 2 while the first 10 to 15 ml of filtrate was discarded.

The filtrate was then oxidized with potassium permanganate with elimination of its excess with peroxide. The riboflavin content was estimated by interpreting the spectrofluorometry and results were expressed as mg/100 g sample.

Niacin determination

An approximately 0.1 ml of the standard niacin solution was pipetted into two test tubes, respectively. Each of the test tubes was made up to 6 ml with distilled water. Thereafter, 3 ml of cyanogen bromide was added and the content shaken. After 10 min, 1 ml of 4% aniline was added to each tube and the yellow colour developed and after 5 min was read at 420 nm (Spectrophotometer; UV-160A, Shimadzu, Osaka, Japan) against a reagent blank (AOAC, 1995).

Pyridoxine determination

The reversed-phase HPLC method described by Ekinci and Kadakal (2005) was used. The sample treatment consisted of solid phase extraction (SPE) with Sep-Pak C_{18} (500 mg) cartridges that enabled separation of pyridoxine and removed most of the interfering components. Twenty millilitres of distilled water was added to 5 g of the sample. The mixture was homogenized using a homogenizer at 2000 rpm for 1 min. The homogenized sample was centrifuged for 10 min at 14000 × *g* with a centrifuge (model H–2000C, Japan). The stationary phase was prepared by flushing the Sep-Pak C_{18} (500 mg) cartridge with 10 ml methanol and 10 ml acidified water (pH 4.2) to activate the column. The homogenized and centrifuged sample was then loaded on the Sep-PaK C_{18} (500 mg) cartridge at a flow rate of 1 ml min^{-1} using a syringe. The eluent was collected in a bottle and evaporated to dryness in a vacuum evaporator (model Rotavapor R-3000, Bucht, Switzerland). The residue was dissolved in mobile phase (0.1 mol/L KH_2PO_4 (pH 7.0): methanol; 90: 10) and then filtered through 0.45 µm pore size filters. Twenty microlitres of the sample was injected into the HPLC column. The column elute was monitored with a photodiode-array detector at 324 nm for pyridoxine. Identification of compound was achieved by comparing their retention times and UV spectra with those of standards. Five different concentrations of each standard were used to prepare calibration plots for pyridoxine. This was done by plotting concentration (µg/ml) against peak area (mAU). Their correlation coefficients were greater than 0.997. Concentration of pyridoxine was calculated from integrated areas of the sample and the corresponding standards.

Determination of *in vitro* starch digestibility

The enzymatic method of Kumar and Venkataraman (1976) was adopted by using enzymatic glucose oxidase peroxidase kit. Glucose was used as a standard and the degree of hydrolysis was expressed as mg of glucose liberated from the samples after correction for blank values and percent *in vitro* starch digestibility was calculated on the basis of total starch content using the following equation:

$$IVSD = (\text{Glucose released (mg)} \times 0.9 / \text{g of total starch}) \times 100 \quad (2)$$

Determination of *in vitro* protein digestibility

The *in vitro* protein digestibility (IVPD) of lima bean flour samples was measured according to the multienzyme technique (Hsu et al., 1977) and was calculated by using the following equation:

$$y = 210.464 – 18.103x \quad (3)$$

Where, y = the percentage of protein digestibility and x = the pH of the protein suspension after 10 min digestion with a four-enzyme solution.

Statistical analysis

All determinations in this study were done in triplicates. In each case, a mean value and standard deviation were determined. Analysis of variance (ANOVA) was also performed and separation of the mean values was done by Duncan's Multiple Range Test at $p<0.05$ using Statistical Package for Social Scientists (SPSS) software, version 16.0.

RESULTS AND DISCUSSION

Effect of trona-aided boiling on some phytochemicals of lima bean

Table 1 shows the results of the effect of trona-aided boiling on some phytochemicals of lima bean. It was generally observed that boiling (with or without trona) caused reduction in the concentration of phytochemicals in lima bean. However, there were variations in the reduction capacity of different trona concentrations used for boiling. The reduction profile of the phytochemicals from raw lima bean to 0.5% trona-boiling was 29.3 to 7.6 TIU/g (trypsin inhibitor), 61.4 to 1.3 HU/g (haemagglutinin), 22.8 to 7.2 mg/Kg (tannin), 860.4 to 519.8 mg/100 g (phytic acid) and 46.1 to 12.1 mg HCN/kg (cyanogenic glycoside). The reduction in trypsin inhibitor activity can be attributed to the destruction of disulphide bonds which normally guarantee the heat stability of the inhibitor. Therefore, the application of thermal treatment might have destroyed these bonds while boiling in trona solution facilitated further destruction of the bonds and hence the denaturation of the inhibitor (Kalpanadevi and Mohan, 2013). Greater destruction of the disulphide bonds seemed to occur with higher concentration of the trona solution involved in the boiling.

The destruction of haemagglutinin in lima bean was as high as 98% in 0.5% trona-solution boiling. An earlier observation had stated that haemagglutinin could be inactivated at temperature exceeding 50°C (Damang et al., 2017), while it readily dissociates by a change of pH or ionic strength (Belitz et al., 2009). The reduction in tannin concentration by boiling in ordinary distilled water was 30.5%, while that in 0.5% trona-solution boiling was 68.6%. This reduction could be attributed to the solubility property of tannin in water (Ezeocha et al., 2012) while boiling at high temperature could lead to degradation of the phytochemical (Rakic et al., 2007; Udensi et al., 2007). The involvement of trona in the boiling essentially facilitated further destruction of the tannin.

The role of trona boiling on the phytic acid content was relatively low giving a maximum reduction at 39.6 by

Table 1. Effect of trona-aided boiling on selected phytochemicals of lima bean[1].

Type of sample (%)	Trypsin inhibitor activity		Haemagglutinin		Tannin		Phytic acid		Cyanogenic glycoside	
	TIU/g	Reduction capacity (%)[2]	HU/g	Reduction capacity (%)	mg/kg	Reduction capacity (%)	mg/100 g	Reduction capacity (%)	mg HCN/kg	Reduction capacity (%)
Raw	29.3±0.3^{a}	-	61.4±0.2^{a}	-	22.8±0.1^{a}	-	860.4±2.4^{a}	-	46.1±1.2^{a}	-
A (0.0)	23.8±0.2^{b}	18.9	29.3±0.2^{b}	52.3	15.9±0.1^{b}	30.5	801.5±3.8^{b}	6.8	22.2±2.1^{bc}	43.3
B (0.1)	20.5±0.2^{c}	29.9	21.3±0.1^{c}	65.3	14.7±0.2^{c}	35.5	760.4±4.6^{c}	11.6	20.2±1.2^{c}	56.2
C (0.2)	17.6±0.2^{d}	40.1	15.5±0.2^{d}	74.8	12.5±0.1^{d}	45.1	705.3±6.1^{d}	18.0	17.5±1.2^{d}	62.0
D (0.3)	14.7±0.1^{e}	49.9	9.3±1.4^{e}	84.8	10.2±0.2^{e}	55.3	625.2±5.5^{e}	27.3	15.3±0.9^{e}	66.7
E (0.4)	9.9±0.3^{f}	66.2	5.4±0.1^{f}	91.2	8.1±0.1^{f}	64.8	564.2±3.9^{f}	34.4	13.4±0.8^{f}	70.9
F (0.5)	7.6±0.1^{g}	74.1	1.3±0.1^{g}	98.0	7.2±0.1^{g}	68.6	519.8±7.4^{g}	39.6	12.1±1.1^{fg}	73.8

[1]Results are mean values of triplicate determination ± standard deviation. Mean value within the same column having the same letter are not significantly different at $p<0.05$. [2]Reduction capacity (%) was calculated with respect to the initial total value of the respective phytochemical in the raw lima bean. A (0.0%) = Lima bean boiled with distilled water (control); B (0.1%) = Lima bean boiled in 0.1% trona solution; C (0.2%) = Lima bean boiled in 0.2% trona solution; D (0.3%) = Lima bean boiled in 0.3% trona solution; E (0.4%) = Lima bean boiled in 0.45 trona solution; F (0.5%) = Lima bean boiled in 0.5% trona solution.

0.5% trona-solution boiling. The factors that predisposed phytic acid in lima bean to destruction include its leaching tendency into the surrounding solution (Onwuka, 2006) coupled with its heat-labile nature at elevated temperature (Udensi et al., 2007). The reduction in the cyanogenic glycoside concentration in lima bean boiling was also relatively high. Boiling in ordinary distilled water gave 43.3% reduction level while that in 0.5% trona-solution boiling gave about 73.8%. The use of trona solution might have acted the role of complementarity to the heat treatment in the reduction of the cyanogens. Heat treatment had earlier been observed to contribute substantially to the elimination of cyanogenic glycosides in cassava processing for such products as 'akyeke' (Obilie et al., 2004), 'garri' (Agbor-Egbe and Lape-Mbome, 2006) and cassava leaves (Ngudi et al., 2003).

The reduction of phytochemicals as a function of the concentration of trona may be explained as greater impacts (through destruction, dissociation, etc.) being inflicted on the compounds at higher trona concentrations.

Effect of trona-aided boiling on selected vitamin contents of lima bean

Figure 1 shows the selected vitamin contents of lima bean as influenced by trona-aided boiling. The vitamin concentrations were generally decreased with an increase in the trona concentration of the boiling solution. The initial contents of thiamine, riboflavin, niacin and pyridoxine in the raw lima bean were 0.58, 0.37, 0.97 and 0.41 mg/100 g, respectively while at 0.5% trona-boiling, these contents were reduced to 0.13, 0.21, 0.28 and 0.11 mg/100 g, respectively. The reduction in thiamine content may be attributed to its instability in trona solution, which is naturally alkaline. An earlier observation had stated that thiamine stability in alkaline solution is relatively low and is usually influenced by pH, temperature, ionic strength and metallic ions (Gregory, 2008). In the case of riboflavin, its reduction may be attributed to its water solubility property, but relatively stable under normal high temperature food processing conditions (Golbach et al., 2014). The leaching tendency of niacin into the boiling water could be responsible for its reduction level as observed in lima bean processing with trona solution. The involvement of trona in the boiling at high temperature might have contributed substantially to the leaching of niacin. Belitz et al. (2009) had observed that niacin normally occurs in food as nicotinic acid which is quite stable with moderate losses in food processing such as vegetable blanching.

The reduction in the pyridoxine content of food during processing is usually regarded as a complex phenomenon through such factors as elevated temperature and its possible reaction with an amino acid, cysteine (Bui and Small, 2012). The presence of trona during boiling might have contributed to the liberation of the amino acid during the softening process of lima bean seed, thereby facilitating such pyridoxine-cysteine interaction.

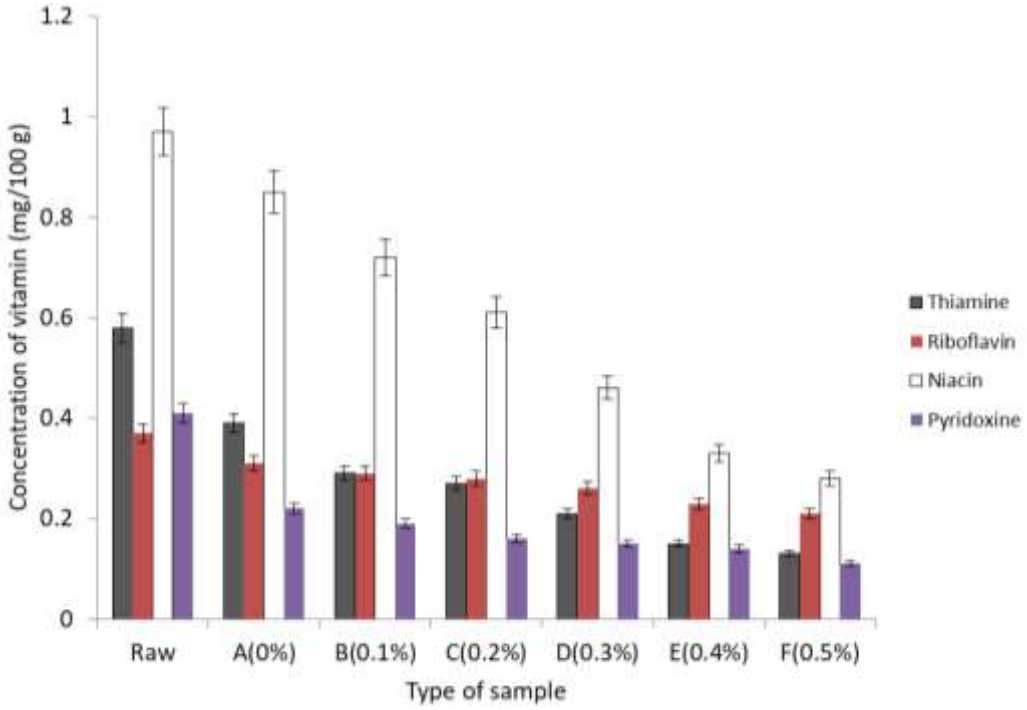

Figure 1. Vitamin concentration in processed lima bean as influenced by trona-aided boiling. A (0.0%) = Lima bean boiled with distilled water (control); B (0.1%) = Lima bean boiled in 0.1% trona solution; C (0.2%) = Lima bean boiled in 0.2% trona solution; D (0.3%) = Lima bean boiled in 0.3% trona solution; E (0.4%) = Lima bean boiled in 0.4% trona solution; F (0.5%) = Lima bean boiled in 0.5% trona solution.

Table 2. Effect of trona-aided boiling on selected mineral contents of lima bean[1].

Type of sample (%)	Mineral (mg/100 g)					
	Calcium	Magnesium	Phosphorus	Potassium	Sodium	Iron
Raw	351.7±4.8^{a}	115.5±2.6^{a}	579.6+3.11^{a}	1629.6±1.1^{c}	9.5±1.1^{e}	6.1±0.8^{f}
A (0.0)	331.7±2.1^{b}	105.1±1.8^{b}	530.1+2.07^{b}	1620.7±2.3^{d}	8.5±0.9^{f}	4.3±0.7^{g}
B (0.1)	326.4±1.8^{c}	104.1±1.3^{b}	525.9+2.01^{c}	1626.8±2.7^{c}	9.6±0.8^{e}	7.2±0.8^{e}
C (0.2)	319.6±1.9^{d}	100.4±1.6^{c}	517.5+0.97^{d}	1628.4±1.7^{c}	10.9±0.6^{d}	9.7±1.1^{d}
D (0.3)	318.7±2.1^{de}	95.4±1.8^{d}	512.7+2.73e	1637.6±1.3^{b}	12.8±1.3^{c}	10.8±0.6^{c}
E (0.4)	315.4±1.2^{e}	93.5±1.1^{de}	505.3+2.09^{f}	1639.6±1.7^{b}	13.8±1.2^{b}	12.1±0.3^{b}
F (0.5)	312.3±1.5^{f}	91.7±1.4^{e}	502.3+0.52^{f}	1642.9±1.9^{a}	15.5±0.9^{a}	13.2±0.8^{a}

[1]Results are mean values of triplicate determination ± standard deviation. Mean value within the same column having the same letter are not significantly different at p<0.05. A (0.0%) = Lima bean boiled with distilled water (control); B (0.1%) = Lima bean boiled in 0.1% trona solution; C (0.2%) = Lima bean boiled in 0.2% trona solution; D (0.3%) = Lima bean boiled in 0.3% trona solution; E (0.4%) = Lima bean boiled in 0.45 trona solution; F (0.5%) = Lima bean boiled in 0.5% trona solution.

Influence of trona-aided boiling on selected mineral contents of lima bean

The effect of trona-aided boiling on selected mineral contents of lima bean is shown in Table 2. The use of ordinary distilled water in the boiling of lima bean generally led to the decrease in the mineral concentration. However, the involvement of trona solution in the boiling caused further decrease in calcium, magnesium and phosphorus while it caused an increase in the concentration of potassium, sodium and iron. The reduction profile of calcium, magnesium and phosphorus was 331.7 to 312.3, 105.1 to 91.7, and 530.1 to 502.3 mg/100 g, respectively while the increment profile of potassium, sodium and iron was 1620.7 to 1642.9, 8.5 to 15.5 and 4.3 to 13.2 mg/100 g, respectively. Minerals

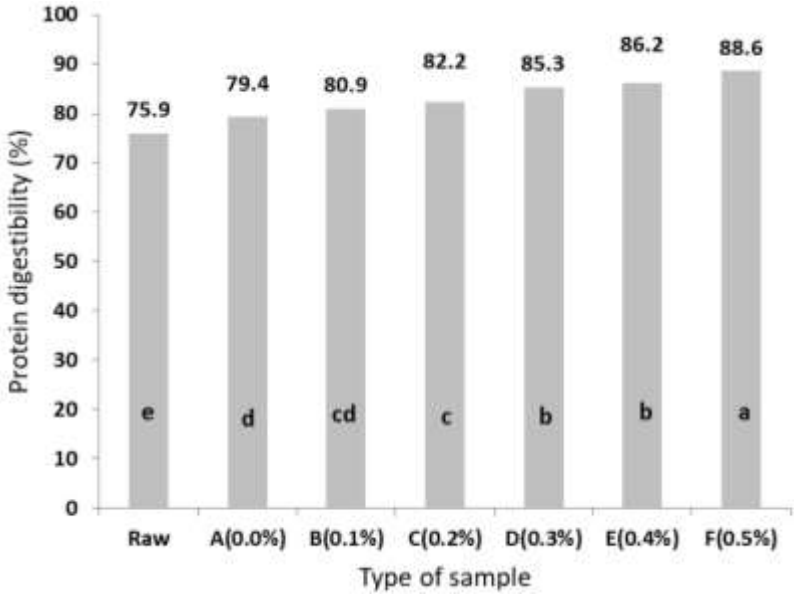

Figure 2. Effect of trona-aided boiling on the *in-vitro* protein digestibility of lima bean. Bars carrying the same letters are not significantly different at $p<0.05$. A (0.0%) = Lima bean boiled with distilled water (control); B (0.1%) = Lima bean boiled in 0.1% trona solution; C (0.2%) = Lima bean boiled in 0.2% trona solution; D (0.3%) = Lima bean boiled in 0.3% trona solution; E (0.4%) = Lima bean boiled in 0.4% trona solution; F (0.5%) = Lima bean boiled in 0.5% trona solution.

generally had been observed to be heat-stable under normal processing conditions (Rickman et al., 2007), but considerable losses could occur through leaching into the cooking water (Mugendi et al., 2010). The increase in the concentration of potassium, sodium and iron could be attributed to their uptake from the trona solution due to possible ionic dissociation at elevated temperature of boiling (Goyal, 2000).

From nutritional standpoint, certain minerals have been recognized to play significant roles in the maintenance of optimal health conditions in humans (Lachance, 1998). Calcium is considered as an essential mineral for human health participating in the biological functions of several tissues such as musculoskeletal, nervous and cardiac systems, bones and teeth, and parathyroid glands (Morgan, 2008; Williams, 2008). Magnesium has been linked to energy metabolism, release of neurotransmitters and endothelial cell functions (Bo and Pisu, 2008) among others. Phosphorus is related to bone and teeth formation and the majority of the metabolic actions in the body including kidney functioning, cell growth and the contraction of the heart muscle (Renkema et al., 2008). Potassium has been implicated for its role in the transmittance of nerve impulses coupled with its relation to heart muscle contraction activity (Lambert et al., 2008). The role of sodium in human physiology is essentially related to the maintenance of the balance of physiological fluids such as in blood pressure, kidney function, nerve and muscle functions (Sobotka et al., 2008). The principal function of iron is connected with the synthesis of haemoglobin and myoglobin coupled with its complementary role of energy production (Huskisson et al., 2007; Shenkin, 2008). Therefore, the loss or gain of some minerals during trona-aided boiling of lima bean might have implications on their beneficial effects when the cooked legume is consumed.

In vitro protein and starch digestibility of lima bean as influenced by trona-aided boiling

The *in vitro* protein digestibility of lima bean boiled in trona solution is shown in Figure 2. Boiling of lima beans in distilled water was observed to increase its digestibility from 75.9 to 79.4% while the involvement with trona caused further increase in digestibility to 88.6% in 0.5% trona-solution boiling. The increase in protein digestibility could generally be attributed to the reduction in inhibitory activity of enzymes and protein-complexing reactions (Embaby, 2010; Pushparaj and Urooj, 2011). The implication of this occurrence is that the reduction in the level of trypsin inhibitor activity (Kalpanadevi and Mohan, 2013) and tannin (Ezeocha et al., 2012) could enhance protein digestibility. Similarly, reduction in the level of phytate, which is capable of forming phytate-protein complex, could also contribute to improved protein

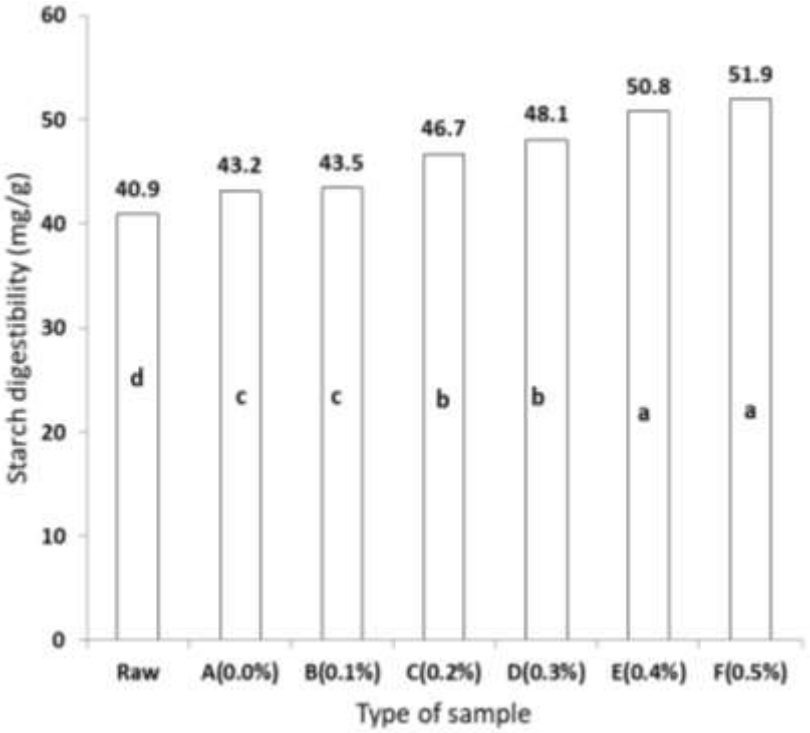

Figure 3. Effect of trona-aided boiling on the *in-vitro* starch digestibility of lima bean. Bars carrying the same letters are not significantly different at $p<0.05$. A (0.0%) = Lima bean boiled with distilled water (control); B (0.1%) = Lima bean boiled in 0.1% trona solution; C (0.2%) = Lima bean boiled in 0.2% trona solution; D (0.3%) = Lima bean boiled in 0.3% trona solution; E (0.4%) = Lima bean boiled in 0.4% trona solution; F (0.5%) = Lima bean boiled in 0.5% trona solution.

digestibility (Selle et al., 2012).

The *in vitro* starch digestibility of lima bean as influenced by trona-aided boiling is as shown in Figure 3. Boiling of lima bean in ordinary distilled water increased starch digestibility from 40.9 to 43.2 mg/g. However, the involvement of trona in the boiling increased starch digestibility to 51.9 mg/g in 0.5% trona-solution boiling. The role of trona in the softening of lima bean had been attributed to the disintegration of the cotyledonous tissue in individual cells. This is caused by the conversion of native protopectin to pectin, which depolymerizes quickly at elevated temperature (Belitz et al., 2009). Therefore, the disintegration of the cotyledonous tissue had, most probably, made starch substrate more accessible to enzymes thereby leading to higher starch digestibility (Ezeogu et al., 2005). The reduction in the phytic acid level could also play a role in enhancing starch digestibility. Singh et al. (2010) had earlier observed that calcium could catalyze amylase activity while phytic acid could form phytic acid-calcium complex. Thus, the reduction in phytic acid level, as a result of trona boiling, will minimize complex formation and hence availability of calcium for effective amylase activity and by extension, an enhanced starch digestibility.

Conclusion

The use of trona in the boiling of hard-to-cook lima bean was highly beneficial. The beneficial effects include the reduction in the phytochemicals traditionally known to interfere in the utilization of protein and minerals, the enhancement of both protein and starch digestibility, and the uptake of certain minerals (potassium, sodium and iron) in the course of boiling. However, marginal losses of certain minerals (calcium, magnesium and phosphorus) were observed while substantial losses of B-vitamins could occur at higher trona concentration of boiling.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Extracellular biogenic synthesis of silver nanoparticles by Actinomycetes from amazonic biome and its antimicrobial efficiency

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The microbial sources used for the production of nanoparticles are important, and one of the reasons for bacterial preference is their ease of manipulation and ecofriendly potential. Three bacteria strains of *Streptomyces* genus were used for the biosynthesis of silver nanoparticles (AgNPs). The aim of the present study was to confirm *Streptomyces* spp. as biological systems in the synthesis and extracellular characterization of silver nanoparticles with antimicrobial activity. The nanoparticles were characterized by ultraviolet–visible spectroscopy (UV-Vis), dynamic light scattering (DLS), RDX, Fourier-transform infrared spectroscopy (FTIR) and transmission electron microscope (TEM). In qualitative antimicrobial test, *Streptomyces parvulus* DPUA 1549 and *Streptomyces owasiensis* DPUA 1748 showed activity against pathogenic microorganisms (bacteria and yeasts) tested. The AgNPs of *S. parvulus* DPUA 1549 showed the most efficient minimum inhibitory concentration (MIC) at low concentrations (1.95 µM) against *Staphylococcus aureus*. They promoted structural damage to the micro-organism cell surface according to AFM images and also reduced the proliferation of NIH-3T3 cells in the cytotoxic assay. Thus, biosynthesis of silver nanoparticles by *Streptomyces* spp. is a potential source to be used in nanomedicine and cosmetology industries.

Key words: *Streptomyces*, tropical region, nanobiotechnology, antimicrobial, cytotoxic test.

INTRODUCTION

Actinomycetes are Gram-positive filamentous bacteria present in marine and terrestrial environment. They have an unparalleled ability to produce diverse active compounds such as antibiotics. Over 500 species of actinomycetes have been described, until now, and among them *Streptomyces* genus provides nearly 80% of

the industrially available antibiotics (Abd-Elnaby et al., 2016; Bhosale et al., 2015).

Nanobiotechnology is an innovative basis in the synthesis of nanomaterials and nanostructures by biological sources. Professional researchers such as microbiologists, biochemists, chemists, physicists and other branches of engineering are contributing new solutions and new sources as micro-organisms through approaches of green biochemistry that provide better efficiency and low cost of processes and materials (Buszewski et al., 2016; Gul et al., 2016; Chemat and Strube, 2015).

In the past years, over 15 billion dollars was invested in nanotechnology and there is a projection of at least 3 trillion Euro until 2020. These investments promote advantages in many industrial processes, biotechnology, information technology, agriculture, food industry and health care (Roco et al., 2011; Schneid, 2014). Reports available have shown the potential of actinomycetes as efficient microbial-mediated candidates for synthesis of extracellular and intracellular silver nanoparticles (AgNPs). The development of novel applications in the pharmaceutical field with metallic nanoparticles makes it an attractive alternative to antimicrobial agents. These AgNPs have antimicrobial action against pathogen Gram-positive and Gram-negative bacteria and multi-resistant strains such as methicillin-resistant *Staphylococcus aureus* (Queiroz et al., 2012; Pantidos and Horsfall, 2014; Durán et al., 2016).

The biosynthesis of nanoparticles by actinomycetes has good stability and polydispersity. They can be genetically manipulated in order to provide better control over the nanoparticles size. In addition, the time required for completion of the reaction using this bacteria ranges between approximately 24 and 120 h (Bhosale et al., 2015). Some species of actinomycetes had shown biological applications of their AgNPs: *Streptomyces djkartnsis* (Biglari et al., 2014), *Streptomyces olivaceus* sp-1392 (Evelyne and Subbiayh, 2014), *Streptomyces coelicolor* (Manikprabhu and Lingappa, 2013), *Streptomyces albogriseolus* (Samundeeswari et al., 2012), *Streoptomyces parvulus* SSNP11 and *Streptomyces albidoflavus* CNP10 (Prakasham et al., 2012) and *Streptomyces rochei* (Selvakumar et al., 2012).

The understanding of the AgNPs biodistribution/accumulation in living systems is important to make them more effective as pharmaceuticals agents. Researches have demonstrated that, at low concentrations, silver is nontoxic to human cells. It also has been reported that Ag^+ ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the cytoplasmic membrane. The interaction of Ag^+ with bacteria is directly related to the size and shape of the nanoparticles (Manivasagan et al., 2013).

In order to contribute to the development of the biogenic nanoparticles applications, the aim of this research was to evaluate the extracellular microbial-mediated synthesis of silver nanoparticles (AgNPs) by *Streptomyces* spp. strains, their characterization, antimicrobial and cytotoxic activities.

MATERIALS AND METHODS

Microorganisms

Three cultures (DPUA 1549, DPUA 1747 and DPUA 1748) of *Streptomyces* genus were selected. They were obtained from DPUA Culture Collection - Federal University of Amazonas, Brazil. All of them had already presented antimicrobial activity by liquid fermentation in previous study (Silva-Vinhote et al., 2011). The actinomycetes were reactivated in ISP2A agar (g/L): (starch, 10; yeast extract, 4; malt extract, 10; dextrose, 4; agar, 20), and adjusted to pH 7.3. The cultures were maintained at 30°C for 21 days and authenticated based on the physiologic, macromorphological and micromorphological characteristics of the group (Lechevalier and Lechevalier, 1989; Waskman and Wodruff, 1941).

Identifications of the micro-organisms

The identification was carried out by polymerase chain reaction (PCR). The DNA strain was excreted using the purification kit of genomic DNA (Promega Corporation, Madison, EUA) and the amplification of 16S ribosomal DNA gene was performed by PCR using universal primers from eubacteria ACT235F (5'-CGCGGCCTATCAGCTTGTTG) and ACT878R (5' - CCGTACTCCCCAGGCGGGG). PCR was conducted in Eppendorf Master Cycle thermocycler. To obtain the final volume of 25 µL, the following were used: 14.2 µL of Milli-Q water, 1.0 µL of denatured cells/DNA, 2.5 µL of DNA polymerase 10× (200 mM Tris-HCl, 500 mM KCl) buffer, 2.5 µL of 25 mM $MgCl_2$, 2.5 µL of 2.5 mM dNTP, 1.0 µL of ACT235F 5' - 3'5 pmol oligonucleotide, 1.0 µL of ACT878R 5' - 3' 5 pmol oligonucleotide and 0.3 µL of 5 U Taq DNA polymerase. The amplification reaction was carried out with the following temperature cycles: (1) 95°C for 5 min, (2) 95°C for 45 s, 72°C for 60 s, 72°C for 60 s (40 times), (3) 72°C for 5 min. The annealing was also made at 68°C. To confirm the amplification, 2.0 µL of the reaction was submitted to electrophoresis in agarose gel with TBE (boric acid-EDTA) and stained with GelRed™. The electrophoresis was developed in 80 V for 10 min and in 100 V for 50 min. The product was sequenced and the resulting sequence was compared with all sequences available in GenBank using BLAST software of the National Center for Biotechnology Information (NCBI).

Synthesis of silver nanoparticles

Submerged fermentation

The actinomycetes were cultivated in Erlenmeyer flasks (125 mL)

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containing 50 mL of MPE broth (g/L): (glucose, 20; soybean flour, 20; NaCl, 5; $CaCO_3$, 2). The process was carried out at 25°C, 200 rpm, for 72 h and the biomass was separated from the extract by vacuum using Whatman nº 1 filter. The biomass was filtrated three times with sterilized water and after the last washing; the liquid solution was recovered for silver nanoparticles biosynthesis process.

Silver nanoparticles biosynthesis

Silver nanoparticles synthesis was made in Erlenmeyer flasks (250 ml) containing 50 mL from the recovered solution. A solution of silver nitrate (1 M) was added until final concentration of 1 mM. The reaction was carried out at 25°C, 200 rpm, for 72 h, in the absence of light. The control sample presented only aqueous solution without the silver ion and maintained at same conditions.

Characterization of silver nanoparticles

UV-Visible spectroscopy analysis

The bio-reduction of silver nitrate and the formation of nanostructures were confirmed by UV–Vis spectra (UV-Vis Cary 50 Probe Agilent) in a range of wavelengths between 350 and 900 nm.

Fourier transform infrared (FTIR) spectroscopy analysis

The AgNPs were also examined for the presence of biomolecules responsible for bioreduction using Fourier transform infrared spectral analysis in a Bomem MB Series-B102 Spectrometer. FTIR analysis reveals the biomolecules responsible for the silver ion reduction and the stabilization of AgNPs in the suspension.

Characterization of AgNPs by XRD

The XRD analysis was obtained by a Shimadzu XRD7000 instrument. The AgNPs diameters were determined using Scherrer equation ($D= 0.9\ \lambda / \beta \cos\theta$), were: D = average of particles diameter; λ = wave-length of electromagnetic radiation; θ = diffraction angle; β (2θ) = width at half height of the diffraction peak.

Particle size (DLS) analysis and potential zeta

Particle size and zeta potential (ζ) measurements were carried out in a Malvern Zetasizer Nano, Model ZS 3600. The hydrodynamic diameter was measured by dynamic light scattering with laser of wavelength 633 nm and a fixed scattering angle of 173°. Particle size was measured considering the particle as spherical like. Each sample was measured three times for two replicates samples at a constant temperature of 25 ± 1°C.

Nanoparticle tracking analysis (NTA)

The intensity analyses of nanoparticles in aqueous suspension were visualized using nano sight-nanoparticle tracking analysis (NTA) version 2.0 Release Version Build 0127.

Characterization of AgNPs by transmission electron microscopy (TEM)

The characterization of silver nanoparticles was done using TEM images to determine the size and shape of nanoparticles by a Zeiss Libra 120 instrument at 120 kv.

Antimicrobial activity

Qualitative assay

The agar diffusion assay was used to screen the AgNPs antimicrobial activity against the following microorganisms test: *Escherichia coli* CBAM 0001, *Staphylococcus aureus* ATCC 25923, *Mycobacterium smegmatis* INCQS 061, *Candida albicans* DPUA 1706, *Candida atlantica* DPUA 1323 and *Candida valderwaltii* DPUA 1327. The bacterial cultures were inoculated on Müller Hinton agar (MHA) and the fungi cultures on Sabouraud agar (SAB) using sterilized cotton swabs (6 mm). In each plate, wells were cut out using a sterilized gel borer and 100 µL of biosynthesized AgNPs were used as a test sample against the clinical isolates. Inoculated plates were incubated at 37°C for 24 h. After incubation, the plates were examined for the presence of an inhibition zone around the wells.

Minimum inhibitory concentration (MIC) determination

The antibacterial activity of AgNPs was tested against three bacterial strains: *S. aureus* ATCC 25923 (Gram-positive), methicillin-resistant *S. aureus* ATCC 43300 (MRSA) (Gram-positive) and *E. coli* CBAM 0001 (Gram-negative). Minimum inhibitory concentration (MIC) was determined according to the method of Quelemes et al. (2013) using 96-well micro dilution plates, where the strains (concentration of 5×10^5 CFU/ml) were exposed to two-fold dilution series of AgNPs solution with concentrations ranging from 0.97 to 500 µM. The same procedure was used to determine the MIC of $AgNO_3$ (control). MIC was defined as the lowest concentration of agent that restricted the bacterial visible growth. All assays were performed in triplicate.

Atomic force microscopy analysis (AFM)

The AgNPs extract with the best MIC results was selected for the atomic force microscopy analysis (AFM) using concentrations ranging from 0.975 to 500 µM. A concentrated inoculum of 1×10^8 CFU/mL was used in each well for better observation by AFM. After incubation for 24 h, 30 µL of the culture media containing MIC-treated or MIC-untreated bacteria were deposited into a clean glass surface and dried in bacteriological incubator at 35°C for 10 min. The samples were gently rinsed twice with 1 mL of deionized water to remove salt crystals and dried again at the same described conditions before AFM analysis. All samples were prepared at the same time, exposed to the same conditions and examined within 8 h of deposition. AFM was carried out with a TT-AFM microscope from AFM Workshop (USA). The analysis of the effect of AgNPs on *S. aureus* cells was carried out in vibrating mode, using NSG10 cantilevers (NT-MDT) with resonant frequency of approximately 280 kHz. Images were analyzed using Gwyddion software 2.33. Multiple areas of each sample were examined and representative images were shown.

Mammalian cell proliferation assay - real-time cell analyzer (RTCA)

To investigate the effects of *Streptomyces* silver nanoparticles on cell proliferation, murine fibroblast cell line NIH-3T3 (ATCC number 1658) was used. Cells were maintained in 25 cm^2 culture flasks (TPP, Switzerland) at 37°C, 5% (v/v) CO_2, in DMEM (Dulbecco's

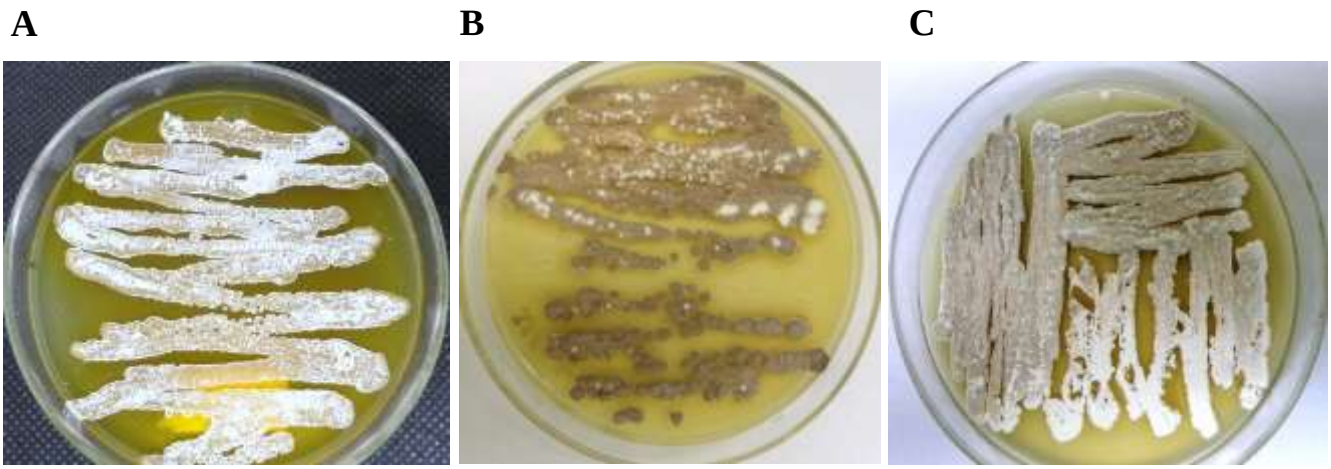

Figure 1. Macroscopic aspects of *Streptomyces parvulus* DPUA 1549 (A), *Streptomyces seoulensis* DPUA 1747 (B), and *Streptomyces owasiensis* DPUA 1748 (C) colonies, respectively.

Modified Eagle's Medium/Sigma-Aldrich) medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (Invitrogen, USA); 100 IU/mL penicillin and 100 µg/mL streptomycin. The proliferation assays were carried out using Real-Time Cell Analyzer (xCELLigence RTCA DP instrument - Roche). Previously in twenty-four hours, the nanoparticle exposure (t = -24 h) in 5000 cells/well were seeded in E-plates. This first procedure aims to allow cell attachment before treatments. At t=0 h, cells (n=3 well per treatment) were exposed to different silver nanoparticle concentrations (control = 0, 1, 10, 50, 100 µM) and the proliferation pattern was followed for 70 h. The primary results were expressed by a cell index that is correlated with the number of cells attached in plate bottom. It is a strong indication of cell proliferation. The statistical analyses were conducted in two ways:

(1) Comparisons of the area under the curve (AUC) of cell index versus time from the different experimental groups. The analyses of variance (ANOVA) were made followed by Dunnett's test ($p < 0.05$) among the experimental groups;
(2) Determination of the $LC_{50\%}$ (lethal concentration for 50% of exposed cells): for that, a nonlinear curve between the cell index and the log of nanoparticle concentration was calculated for different time points. A curve with some of those results was produced to observe the evolution of $LC_{50\%}$ along the exposure time.

RESULTS AND DISCUSSION

Characterization of cultures

The colonies of *Streptomyces* sp. DPUA 1549, *Streptomyces* sp. DPUA 1747 and *Streptomyces* sp. DPUA 1748 were authenticated after reactivation. For *Streptomyces* sp. DPUA 1549, the mycelium colour varied from white to light grey with pigment production; in *Streptomyces* sp. DPUA 1747, there was no observed pigment production; and *Streptomyces* sp. DPUA 1748 showed united colonies without fragmentation, reverse cream, brown mycelium land, without exudate, rough and overlapping edges (Figure 1).

Identification of the microorganisms

The comparison of rDNA sequences is a particularly powerful tool in Streptomycetes family taxonomy (Anderson and Wellington, 2001). Comparison of the 16S rDNA sequences of cultures with the GenBank database showed that these isolates belong to the genus *Streptomyces* with 100% homology. The sequence of *Streptomyces* sp. DPUA 1549, *Streptomyces* sp. DPUA 1747 and *Streptomyces* sp. DPUA 1748 revealed high similarity with *S. parvulus*, *S. seoulensis* and *S. owasiensis*, respectively.

Synthesis and characterization of nanoparticles

The use of UV visible spectra in the AgNPs characterization is made by observation of absorptions from 420 to 445 nm. These references are attributed to plasmonic bands of silver nanoparticles that present spherical shape (Narasimha et al., 2013) (Figure 2). The visible-UV spectrum of DPUA 1549, DPUA 1747 and DPUA 1748 presented values of λ_{max} close to each other. However, a band enlargement of *Streptomyces owasiensis* DPUA 1748 was observed, suggesting a dimension rise of the AgNPs obtained. No color change was observed in the culture filtered without silver nitrate. Transmission electronic microscopy has proportioned a detailed view about size and morphology of AgNPs. TEM images of *Streptomyces* sp. DPUA 1549, DPUA 1747 and DPUA 1748 from aqueous solution are demonstrated in Figure 3A, B and C. The nanoparticles presented spherical shape with larger diameters of DPUA 1748, confirming with data obtained from DLS and UV-vis.

The results obtained in TEM study indicated that the nanoparticles are single spread with average size from 1 to 40 nm. Also, it was observed that the particles involved

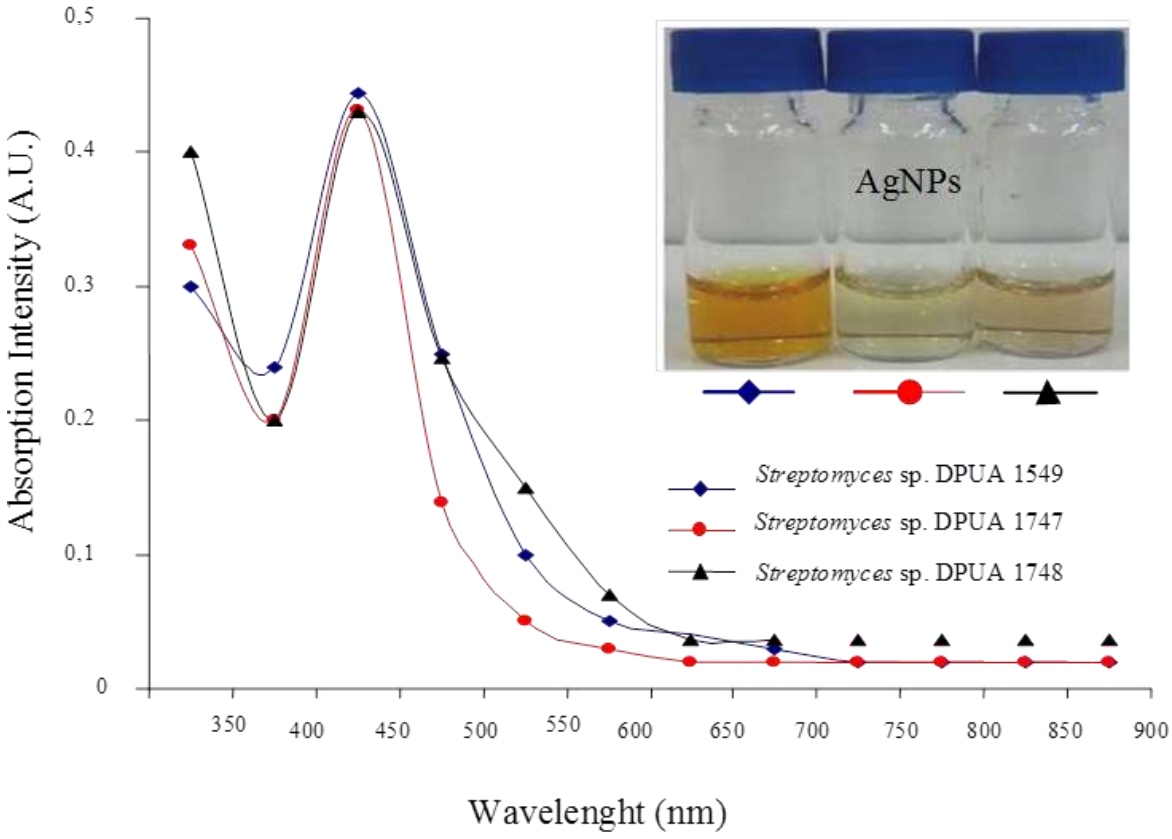

Figure 2. UV-Vis absorption spectra of silver nanoparticles biosynthesized by *Streptomyces* (DPUA 1549, DPUA 1747 and DPUA 1748) with peaks at 444, 431 and 430 nm, respectively.

an organic matrix layer acting as covering agent to the silver nanoparticles (Basavaraja et al., 2008). According to Golinska et al. (2014), nanoparticles stabilization is necessary to avoid aggregation and protect its properties.

Golinska et al. (2014) and Narasimha et al. (2013) reported that metallic nanoparticles biologically synthetized are more stable and disperse better, presenting a secreted coverage by these biological systems. Some studies that used biological systems confirmed the presence of proteins as stabilization agents of metallic nanoparticles (Ingle et al., 2014; Prakasham et al., 2012).

Silver nanoparticles TEM analysis is in agreement with the results presented by Manivasagan et al. (2013), suggesting that the AgNPs were synthetized due to the action of actinomycetes free cells extracts as bio-reducers in this process. Data of DLS demonstrated that the average particles size of *Streptomyces* sp. DPUA 1549, DPUA 1747 and DPUA 1748 were 100, 121 and 160 nm, respectively. An unimodal distribution and low values of polidispersion to the systems was observed (Figure 3D).

Nanoparticle tracking analysis (NTA) was used to confirm the obtained results by DLS (Figure 4A, B and C) and they are in agreement with all samples analyzed. NTA values were 116, 115 and 120 nm to DPUA 1549, DPUA 1747 and DPUA 1748, respectively, confirming the existence of only one population of nanoparticles without significant size variations between them. The value of zeta potential of silver nanoparticles from *Streptomyces* sp. DPUA 1549, DPUA 1747 and DPUA 1748 were -20.3, -6.77 and -7.94 mV, respectively (Table 1).

FTIR spectra of DPUA 1549, DPUA 1747 and DPUA 1748 silver nanoparticles presented bands in 3416, 2940, 1634 and 1054 cm^{-1} that can be attributed to vibrations of symmetric stretching of primary amine, non-symmetric stretching of C-H and stretching due to –C=O and C-O-C groups, respectively. These results also suggest the presence of amino acids and peptides residues which are capable to link to metal, suggesting that biological molecules could play an action in the formation and stabilization of silver nanoparticles in aqueous medium (Basavaraja et al., 2008; Narasimha et al., 2013).

The XRD method was used to determine and confirm the crystalline structure of the synthesized nanoparticles. *S. parvulus* DPUA 1549 showed peaks in 38°, 44°, 66°, 78° and 81° of 2θ which describes the presence of planes (111), (200), (220), (222) and (311) of face-centred cubic silver nanoparticles structure. The presence of silver oxide in 33° and in 55° was described

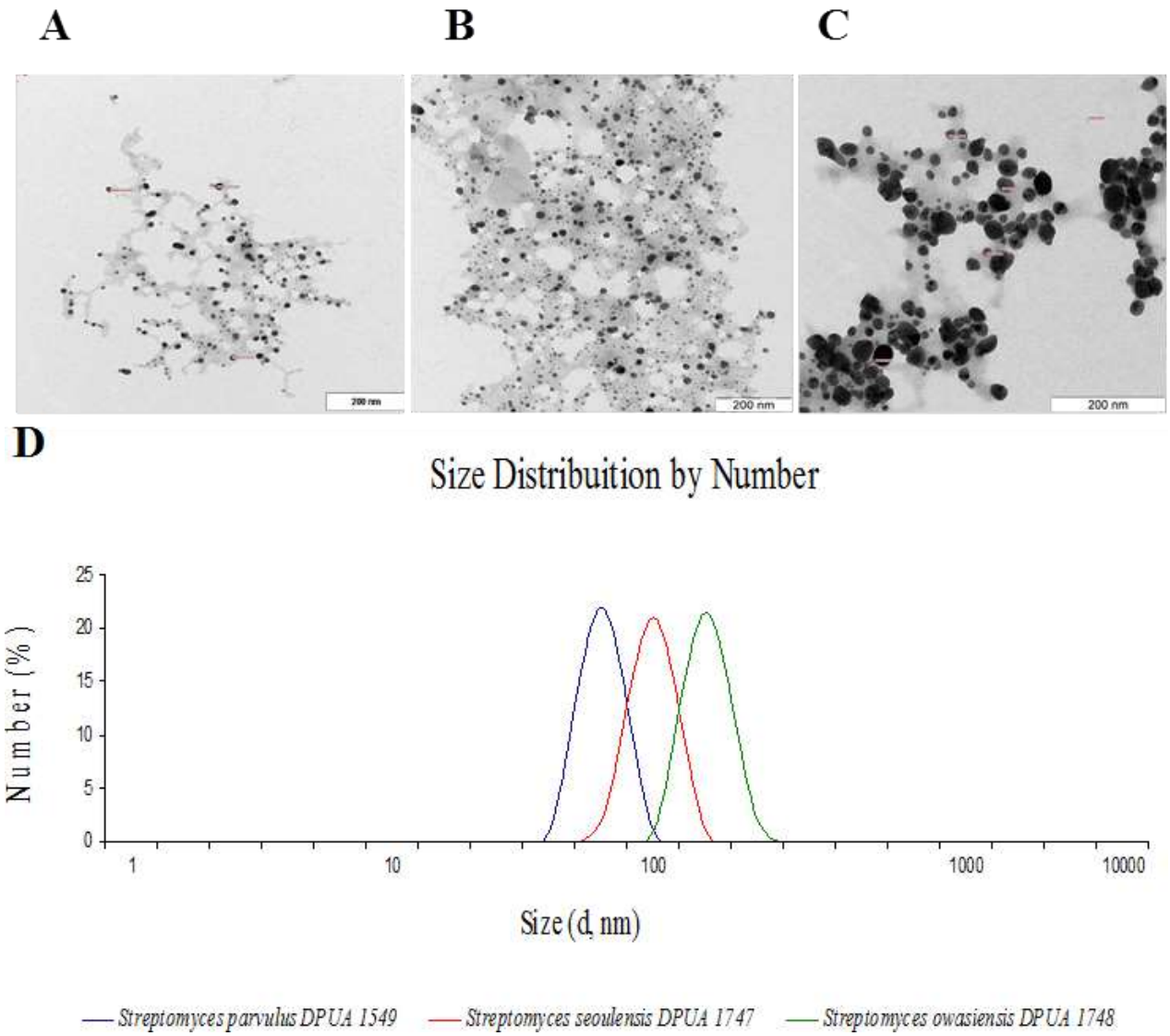

Figure 3. TEM images of AgNPs from *S. parvulus* DPUA 1549 (A), *S. seoulensis* DPUA 1747 (B) and *S. owasiensis* DPUA 1748 (C) (Scale bar=200 nm); (D) Particle size distribution of AgNPs: DPUA 1549 (d, 100.34±0.7 nm), DPUA 1747 (d, 121.20±1.7 nm), DPUA 1748 (d, 160.77±1.65 nm) by DLS.

in the study of Feng et al. (2000). These values are in agreement with those obtained by JCPD (Joint Committee on Powder Diffraction, standard file nº. 04-0783) and a similar XDR pattern with *S. seoulensis* DPUA 1747. In *S. owasiensis* DPUA 1748, a low content of silver nanoparticles and silver oxide mixture indicated a low reactivity of this cultured biocompounds with silver nitrate (Table 3).

Antimicrobial activity

Silver (Ag) is a metallic element presenting antimicrobial properties against several microorganisms as fungi, Gram-positive and Gram-negative bacteria. The antimicrobial activity of AgNPs can be more efficient due to their small size particles, high superficial area and volume ratio (Jung et al., 2008; Tamboli and Lee, 2013). The AgNPs from *S. parvulus* DPUA 1549 and *S. owasiensis* DPUA 1748 presented antagonist action, in a large spectrum, against all pathogenic microorganisms tested (bacteria and yeasts). The inhibition zones varied from 12 to 34 mm (Table 2) with significant values of DPUA 1549 against *S. aureus*. The synergic effect of AgNPs promoted an inhibition raise against the microorganisms test. Manivasagan et al. (2013) and Chauhan et al. (2013) proposed that the antibiotic molecules produced by actinomycetes contain several active groups as hidroxile and amide that can easily react with AgNPs by chelation. It suggests that this reaction

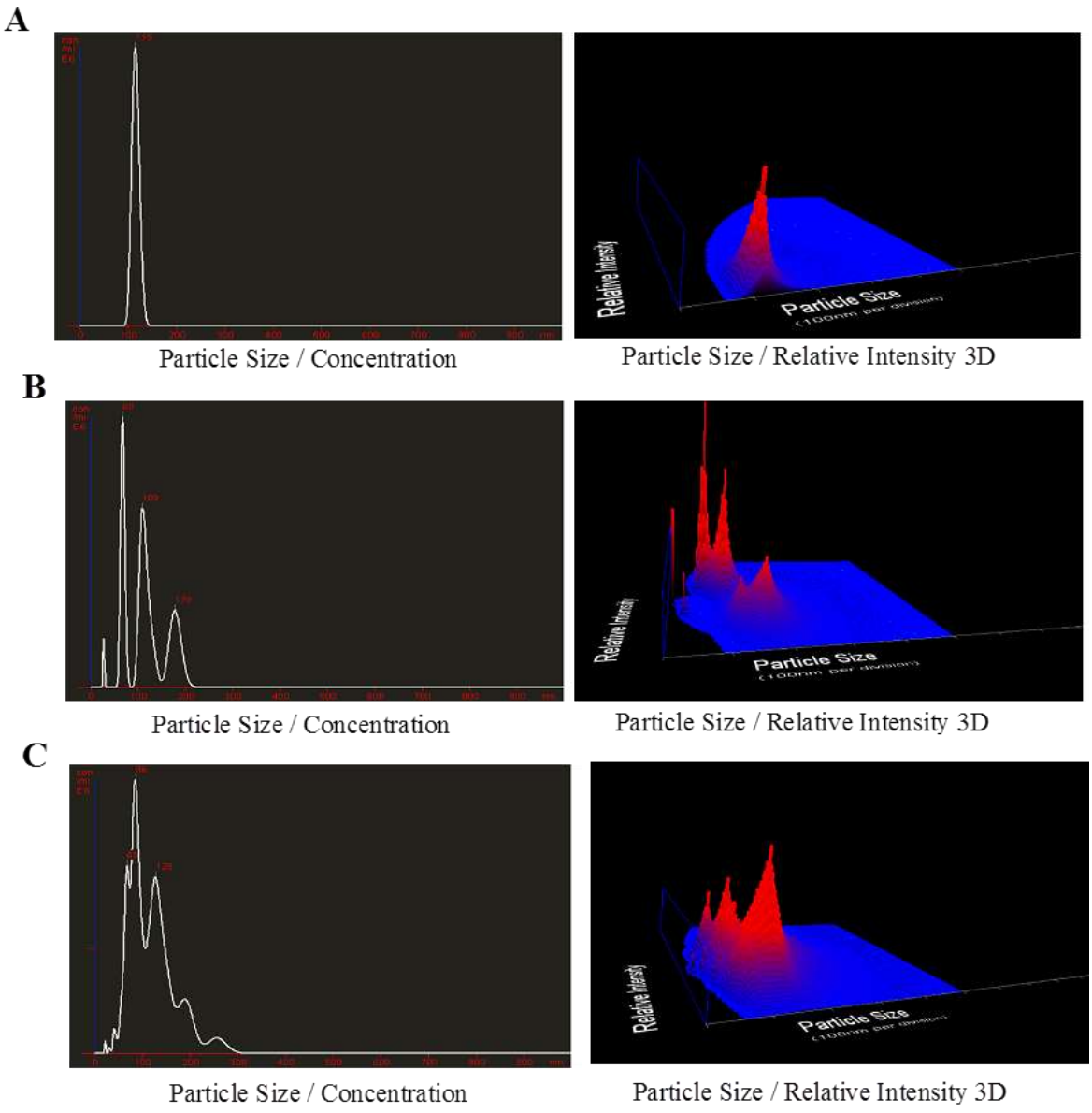

Figure 4. Size distribution graphics by NTA method of AgNPs. *S. parvulus* DPUA 1549 (A); *S. seoulensis* DPUA 1747 (B) and *S. owasiensis* DPUA 1748 (C).

Table 1. Physical chemistry parameters of AgNPs diameter calculated (mean ± standard deviation) by DLS, Zeta potential, and PDI of AgNPs biosynthesized by *Streptomyces* spp.

AgNPs	Diameter (nm)	Zeta potential (mV)	PDI*
Streptomyces parvulus DPUA 1549	100.34±0.7	-20.37	0.180
Streptomyces seoulensis DPUA 1747	121.20± 1.7	-6.77	0.205
Streptomyces owasiensis DPUA 1748	160.77±1.65	-7.94	0.200

*PDI, Polydisperity index.

can raise the growth inhibition of bacterial cells.

MIC assay had the proposal to determine the lowest concentration of AgNPs produced, capable to inhibit microbial growth of pathogens. The AgNPs produced in

Table 2. Qualitative antimicrobial activity assay against micro-organisms tests.

Sample	Micro-organism					
AgNPs	Ec	Sa	Ms	Ca	Cat	Cav
Streptomyces parvulus DPUA 1549	S20	S34	S27	S24	S14	S15
Streptomyces seoulensis DPUA 1747	S13	S23	R	R	S12	S13
Streptomyces owasiensis DPUA 1748	S15	S29	S16	S28	S15	S15

Ec = *Escherichia coli* CBAM 0001; Sa = *Staphylococcus aureus* ATCC 25923; Ms = *Mycobacterium smegmatis* INCQS 061; Ca = *Candida albicans* DPUA 1706; Cat = *Candida atlantica* DPUA 1323; Cav = *Candida valderwaltii* DPUA 1327; R = resistant (there was no development of the inhibition zone); S = sensible (was development of the inhibition zone).

Table 3. Quantitative antimicrobial assays minimum inhibitory concentrations (MIC's) of AgNPs and $AgNO_3$ on *S. aureus* MRSA ATCC 43300, *S. aureus* ATCC 25923 and *E. coli* CBAM 001.

AgNPs and control	*S. aureus* MRSA ATCC 43300 µM	*S. aureus* ATCC 25923 µM	*E. coli* CBAM 001 µM
Streptomyces parvulus DPUA 1549	1.95	1.95	31.25
Streptomyces seoulensis DPUA 1747	250	250	62.5
Streptomyces owasiensis DPUA 1748	125	125	62.5
$AgNO_3$	125	62.5	125

aqueous solution by *S. parvulus* DPUA 1549 showed synergic effect with high efficiency in low values of concentration against the tested Gram-negative bacteria *E. coli* CBAM 001, Gram-positive bacteria *S. aureus* ATCC 25923 and the methicillin-resistant *S. aureus* (MRSA ATCC 43300) (Table 3). Besides, an important result is the most effective action of the AgNPs against these Gram-positive bacteria. Other studies demonstrated an antagonistic action against Gram-negative and Gram-positive pathogens (Kumar et al., 2015; Zonooz and Salouti, 2011). The negative zeta potential (-20.37) and size of nanostructures (100 nm) of *S. parvulus* DPUA 1549 sample was lower when compared to the other tested samples, confirming the results of MIC. According to Kumar et al. (2015) and Kaviya et al. (2011), particles dimensions influence the antimicrobial activity of coloidal AgNPs. Durán et al. (2010) reports that silver nanoparticles obtained in different formulations, sizes and shapes exhibit variable antimicrobial activity.

After previous observation of AgNPs antimicrobial action by MIC, the particles of *S. parvulus* DPUA 1549 were selected to analyze the structural effect against *S. aureus* ATCC 25923 using atomic force microscopy (AFM). The micrographs of *S. aureus* ATCC 25923 incubated without AgNPs showed that the bacteria remained structurally intact (Figure 5A) while the bacteria treated with AgNPs lost the structural integrity of the membrane (Figure 5B). Although the antimicrobial action mechanism of AgNPs is not totally understood, many reports demonstrate that silver interaction and bacterial membrane components caused structural damage to these cells (Jung et al., 2008; Pal et al., 2007).

According to research data, there are some possible action mechanisms to the AgNPs activity, and they include: membrane structural damages; decreasing cell viability due interaction with cofactors and ions; inhibition of DNA replication and, consequently, the protein expression; and the inactivation of some essential enzymes to ATP production (Durán et al., 2010). Li et al. (2011) reported that AgNPs act on membrane permeability. After treatment of bacteria with AgNPs, they seemed to suffer lyses occurring from rupture of wall cell resulting in the release of cellular contents to the external environment.

Some studies report the AgNPs action against *S. aureus*; however, there are no details about all the mechanisms associated to this activity (Jung et al., 2008). For that reason, these particles make it difficult for the microbial ability to enhance defense mechanisms, thus decreasing the resistant strains. Pal et al. (2007) and Shang et al. (2014) affirmed that AgNPs interaction with cell membrane depends on the surface availability. If the nanoparticles are small, the antimicrobial effect can be higher. The AgNPs synthesized by *S. parvulus* DPUA 1549 might be loaded with antibiotic substances during biosynthesis and produce small bionanoparticles that demonstrate a more efficient antibiotic effect.

Cytotoxic effects - mammalian cell proliferation assay

Based on AgNPs antimicrobial activity, cytotoxic activity of the sample was conducted. The mammalian cell proliferation assay after treatment with different

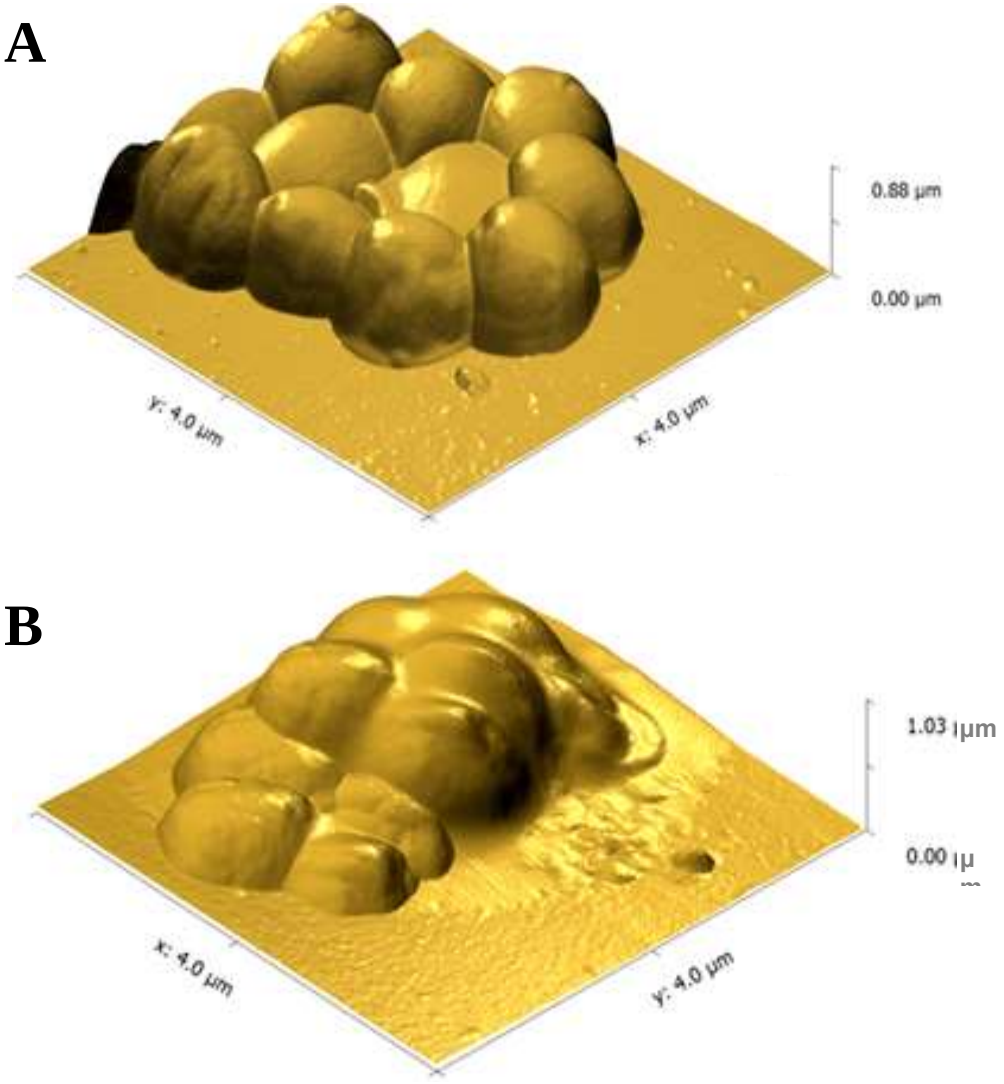

Figure 5. Effect of AgNPs from *S. parvulus* DPUA 1549 against *S. aureus* ATCC 25923 cells. **(A)** Cells control. **(B)** *S. aureus* cells after 24 h of treatment with AgNPs (1.95 µM) at the minimal inhibition concentration (MIC).

concentrations of silver nanoparticles from *S. parvulus* DPUA 1549 showed that all concentrations evaluated impaired NIH-3T3 cell proliferation. In Figure 6A, it is possible to observe a concentration dependent manner of this proliferation impairment however in the area under curve (AUC) analyses (Figure 6B) no significant differences were observed among the nanoparticle treatments. All of them presented significant reduction ($p < 0.05$) compared to the control. In addition, time dependent proliferation impairment was observed. In Figure 6C, the reduction of $LC_{50\%}$ along the time can be observed. The results indicate that the potential cytotoxic activity of this nanomaterial is also time dependent. Silver nanoparticles used as antimicrobial agent are gaining highlight in applications, especially in medical areas (Vivek et al., 2012).

Conclusion

The AgNPs biosynthesis mediated by *Streptomyces* expressed antimicrobial activity against bacteria, especially the Gram-positive ones. The silver nanoparticles from *S. parvulus* DPUA 1549 were the most efficient strain promoting cell superficial damage in tested bacteria; presented low MIC against multiresistant *S. aureus* and cytotoxic action in the reduction of NIH-3T3 cells proliferation. This scientific evidence demonstrates that the AgNPs synthetized by *Streptomyces parvulus* DPUA 1549 are effective and can be considered as a promising antimicrobial agent and a cytotoxic agent. This potential favors the application of these nanoparticles in the elaboration of products to be used in medical and cosmetic industries.

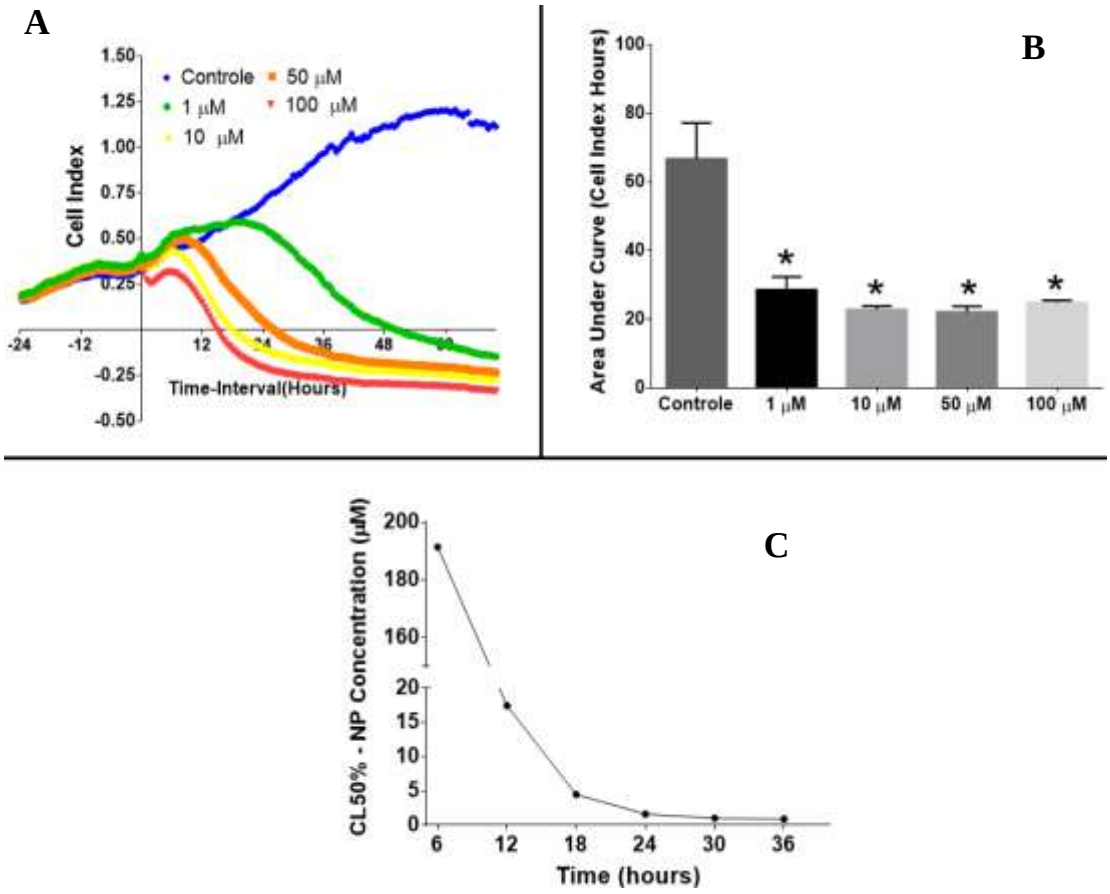

Figure 6. Murine fibroblast (NIH-3T3) proliferation after treatment with different concentrations of silver nanoparticles from *S. parvulus* DPUA 1549. **A,** NIH-3T3 cell proliferation (cell index) after different treatments; area under curve (cell index vs hours) of the different treatments. *Denotes statistically significant difference ($p < 0.05$) compared to control (B). C, Correlation between $LC_{50\,\%}$ and time of exposure.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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In vitro micropropagation of grape vine (*Vitis vinifera* L.) from nodal culture

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The objective of this study was to optimize a protocol for *in vitro* micropropagation of selected grape vine varieties. Preliminary studies were conducted to optimize the duration of sterilization of the explants. For shoot initiation experiment nodes of the three varieties of grape vines were cultured on MS medium (Murashige-Skoog's, 1962) supplemented with five different concentrations of 6-Benzylaminopurine (BAP) and the control. Various experiments were carried out to optimize shoot multiplication using MS medium supplemented with different concentrations of BAP alone or in combination with Indole-3-butyric acid (IBA). To optimize root induction, different concentrations of Indole-3-acetic acid (IAA) were used. Sterilization of explants using 1% of NaOCl for 7 min duration was optimum. Chenin blanc showed high percentage of survival rate (96%) followed by Ugni blanc and Canonannon (88%) at 0.5 mg/L BAP. Among the different concentrations and combination of Plant Growth Regulators (PGRs) used for multiplication, maximum mean number of shoots 7.2, 6.7, and 6.1 was achieved at 1 mg/L BAP combined with 0.1 mg/L IBA for Chenin blanc, Canonnanon and Ugni blanc, respectively. All varieties induced root for all the treatments used including the control but good roots were found on MS medium supplemented with 2 and 4 mg/L of IAA. The plantlets were acclimatized in the glasshouse and survival percentage was 92% for Chenin blanc followed by 78.6 and 73.9% for Ugni blanc and Canonannon, respectively. Thus, the achievements of this study will play a big role in the grape vine culture program.

Key words: *Vitis vinifera* L., acclimatization, explant, cytokinin, auxin.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is perennial woody fruit crop growing in the tropical, subtropical and temperate regions (Anupa et al., 2016). It is one of the most economically important crops in the world with average production of 67.5 million tons each year (Kurmi et al., 2011; Lazo-Javalera et al., 2016). Grapes are used for wine

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production, fresh fruit, dried fruit and juice production. From the total grape production about 71% is used for wine production, 27% as fresh fruit and the remaining 2% as dried fruit (Munir et al., 2015).

Grape has high nutritional value and energy content. It is a rich source of calcium, iron, vitamin C, A, E, amino acid and phosphorous (Jaladet et al., 2009; Yerbolova et al., 2013; Munir et al., 2015). Chemically, grape contains complex compounds which prevent various types of diseases including cancer, heart disease, degenerative nerve disease, Alzheimer disease, retinal disorder, constipation and viral disease (Khan et al., 2015; Munir et al., 2015).

Ethiopia is one of the countries in Africa which have huge potential for the production of grapes due to its favorable climatic condition. Currently in Middle East and Europe, there exist a growing interest for grapes and grape products from Ethiopia (Kassa Melese, 2015). This has a considerable economic significance as a potential source of national revenue. As a result, the government gives high priority to the development and expansion of vineyards to utilize the grape production potential. However, the health and economic significance of grapes might be treated with several types of disease including viral, bacterial and fungal. All these diseases primarily originate from the infected propagating grapevines achieved from the conventional asexual method of propagation. In addition, the conventional method of grapevine propagation (cutting) is time consuming. A planted grapevine needs four to five years to produce propagation materials by cuttings due to its long juvenility period. Grapevine juvenility is one of the principal natural problems hindering grapevine production (Anupa et al., 2016). Hence, the growth of viticulture industry can be maintained through mass production of disease free grapevine materials. *In vitro* propagation methods of grapevine has emerged as a powerful tool for mass propagation and producing disease-free planting material under aseptic condition. Moreover, this allows for large scale production of plantlets with high phytosanitary and genetic quality in limited space, short time and irrespective of the seasons (Melyan et al., 2015).

The propagation of grapes via micropropagation or tissue culture approach has been commercialized around the world. It was applied for selected *Vitis* genotypes using the culture of intact or fragmented shoot apical meristems, axillary-bud microcuttings or through adventitious bud formation (Kurmi et al., 2011; Khan et al., 2015). All types of grapevines do not give similar types of response for specific medium composition. The degree of response is highly dependent on the particular genotype, culture environment, culture medium and hormonal treatment. Hence, it is vital to develop new protocol for rapid multiplication of the available grapes varieties found in Ethiopia. Thus this study aims to develop a protocol for multiple shoot regeneration from node through tissue culture.

MATERIALS AND METHODS

Explant collection and surface sterilization

Three varieties of grape namely Chenin blanc, Ugni blanc and Canonannon, were used in this study. Nodes having 2 to 3 cm length were collected from stock plant grown in greenhouse and thoroughly washed with tap water containing ‘Tween 20’. To identify the optimum sterilization time for sodium hypochlorite (NaOCl) ten nodes of variety Chenin blanc were sterilized for different time duration (5, 7 and 9 min) and cultured on MS medium supplemented with 0.5 mg/L of BAP.

Shoot initiation

Sterilized explants were cultured on MS media supplemented with BAP (0.5, 1, 2, 3 and 4 mg/L) including the control. Then the culture was incubated in a growth room at 27°C for four weeks. Twenty five explants were cultured for each treatment.

Shoot multiplication

The induced plantlets were transferred in to MS medium supplemented with BAP (0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L) and four different concentrations of BAP (0.5, 1, 2 and 4 mg/L) in combination with 0.1 mg/L IBA (Table 6). All the cultures were incubated in a growth room at 27°C for 4 weeks.

Rooting

Shoots having one centimeter and more length were cut 2 mm below their basal node and planted into the medium containing Indole-3-acetic acid (IAA) at different concentrations (0, 0.5, 1.0, 2.0 and 4.0 mg/L). The cultures were incubated in a growth room at 27°C for 4 to 6 weeks.

Acclimatization

Plantlets with well developed roots were transferred into small pots containing sterile soil, compost and sand in the ratio of 2:1:1, respectively and covered by plastic bags for one week in glasshouse.

Experimental design and data analysis

All experiments were replicated and laid out in completely randomized design (CRD). Analysis of variance was conducted using JMP SAS computer software version 8.0. Means were compared using the least squares means procedure. Number of explants survived, number of shoots, nodes, roots per explants, shoots height and root length were recorded in a month interval.

RESULTS AND DISCUSSION

Sterilization of explants

The success of plant tissue culture relies on the sources of the explants and sterilizing periods (Garg et al., 2014; Malyan et al., 2015). The effect of sterilizing duration on the surface sterilization of the explants using 1% sodium

Table 1. Percentage of contaminated and clean cultures sterilized in 1% NaOCl.

Treatment durations (minutes)	No. of explants	No. of explants contaminated	Percentage of contamination	Percentage of clean culture
5	10	8	80	20
7	10	0	0	100
9	10	0	0	100

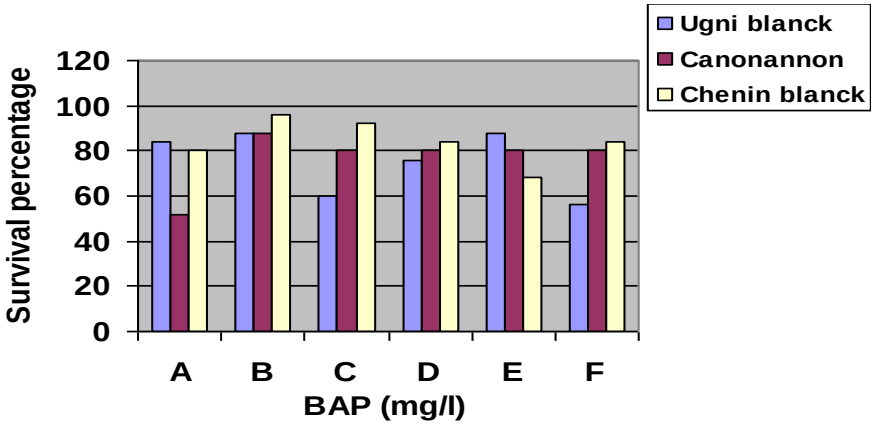

Figure 1. Survival percentage of shoots at different concentrations of BAP. A = control; B = 0.5 mg/L BAP; C = 1.0 mg/L BAP; D = 2.0 mg/L BAP; E = 3.0 mg/L BAP; F = 4.0 mg/L BAP.

Figure 2. Shoot induction on MS medium supplemented with 0.5 mg/L BAP.

hypochlorite is shown in Table 1. The result revealed that, 100% clean cultures were obtained when the explants were treated for 7 and 9 min. However, all explants treated for 9 min died due to prolonged sterilization time.

Effect of different concentration of BAP for shoot initiation and survival rate

Results of the effect of BAP on shoot initiation and survival percentage for the three varieties of grapes (Figures 1 and 2) showed that shoots were induced in all treatments including the control. Khan et al. (2015) also presented similar results in the culture of a grape nodal segment containing an axillary bud with and without plant growth regulator. Even though shoots could be induced on hormone free medium morphologically best shoots were induced in the medium containing 0.5 mg/L BAP. The survival percentage of shoots showed significant variation at different concentration of BAP ($p<0.05$). The maximum percentage of survival was 96% for the variety

Table 2. Effect of BAP on shoot multiplication of the three varieties.

No. of explant	BAP (mg/L)	Mean number of shoots/explant		
		Ugni blanc	Chenin blanc	Canonannon
25	0	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}
25	0.25	2.5 ±1.4^{bc}	2.4 ± 0.8^{b}	2.6 ± 1.4^{b}
25	0.5	5.3 ± 1.9^{a}	5.0 ± 0.9^{a}	5.6 ± 1.7^{a}
25	1.0	2.8 ± 0.1^{bc}	2.9 ± 0.5^{b}	2.8 ± 0.5^{b}
25	2.0	3.4 ± 0.3^{ab}	3.4 ± 0.3^{b}	3.4 ± 0.2^{b}
25	3.0	2.8 ± 0.7^{bc}	3.2 ± 0.3^{b}	3.4 ± 0.4^{b}
25	4.0	2.8 ± 0.6^{bc}	3.3 ± 0.3^{b}	3.0 ± 0.3^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

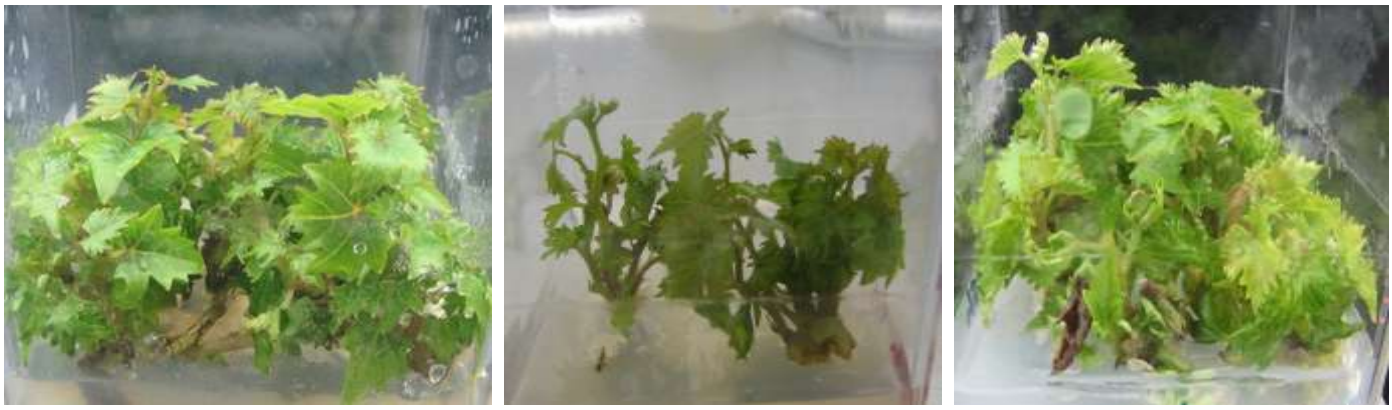

Figure 3. Shoot multiplication of three varieties at 0.5 mg/L BAP.

Chenin blanc and 88% for the variety Ugni blanc and Canonnanon. This study were in contrast with the work of Abido et al. (2013) which evaluated the effects of BAP and Naphtaline Acetic Acid (NAA) and their combinations for shoot multiplication of *V. vinifera* L. cv. Muscat of Alexandria cultured *in vitro*. Their results indicated that the survival rate and the percent of the explants forming growth were not affected significantly by both plant growth regulator and their interaction.

Effect of different concentrations of BAP and in combination with IBA on number of shoot, node and height

Among the seven BAP concentration used for multiplication, 0.5 mg/L of BAP produced significantly maximum mean number of shoots for all the three varieties (Table 2) (Figure 3). On the other hand, Abido et al. (2013) found highest mean number of shoots at 2 mg/L of BAP and the least number was found in the absences of BAP. Canonannon gave maximum mean number of shoots (5.6) followed by Ugnin blanc (5.3) and Chenin blanc (5.0). The best mean height of shoot was attained at 1 mg/L of BAP for Ugni blanc and Chenin blanc moreover Ugni blanc score maximum height on hormone free medium as Canonannon (Table 3). Chenin blanc scored the maximum mean number of node (3.6) at 0.25 mg/L BAP as compared to Ugni blanc and Canonannon (3.1) (Table 4). The results of this study was in agreement with previous studies which confirmed that BAP is the most effective on inducing shoot proliferating among all other cytokinins in *Vitis* (Abido et al., 2013).

Table 3. Effect of BAP on shoot height of the three varieties.

BAP (mg/L)	Mean height of shoots/explant (cm)		
	Ugni blanc	Chenin blanc	Canonannon
0	2.49 ± 0.3^{a}	1.73 ± 0.1^{c}	2.39 ± 0.2^{a}
0.25	2.02 ±0.5^{bc}	2.34 ± 0.4^{ab}	2.02 ± 0.5^{abc}
0.5	1.68 ± 0.2^{c}	1.60 ± 0.1^{c}	1.68 ± 0.2^{bcd}
1.0	2.82 ± 0.3^{a}	2.71 ± 0.2^{a}	2.05 ± 0.2^{ab}
2.0	2.18 ± 0.1^{bc}	2.03 ± 0.3^{bc}	2.08 ± 0.1^{ab}
3.0	1.86 ±0.1^{bc}	1.91 ± 0.1^{bc}	1.47 ± 0.1^{cd}
4.0	1.73 ± 0.1^{c}	1.56 ± 0.1^{c}	1.36 ± 0.0^{d}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 4. Effect of BAP on number of nodes formed for the three varieties.

BAP (mg/L)	Mean number of nodes/explant		
	Ugni blanc	Chenin blanc	Canonannon
0	2.4 ± 0.4^{abc}	1.4 ± 0.1^{c}	2.9 ± 0.9^{a}
0.25	3.1 ± 1.1^{a}	3.6 ± 0.9^{a}	3.1 ± 1.1^{a}
0.5	3.1 ± 0.8^{a}	3.2 ± 0.8^{a}	3.1 ± 0.8^{a}
1.0	2.9 ± 0.3^{ab}	2.8 ± 0.7^{ab}	2.1 ± 0.4^{ab}
2.0	2.0 ± 0.4^{abc}	3.3 ± 0.8^{a}	2.4 ± 0.5^{ab}
3.0	1.2 ± 0.5^{abc}	1.8 ± 0.4^{bc}	1.2 ± 0.4^{b}
4.0	1.6 ± 0.5^{bc}	1.7 ± 0.2^{bc}	1.0 ± 0.3^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 5. Effect of BAP and IBA on shoot multiplication of the three varieties.

BAP (mg/L)	IBA (mg/L)	Mean number of shoots/explant		
		Ugni blanc	Chenin blanc	Canonannon
0.5	0.1	2.2 ± 0.6^{b}	3.4 ± 1.0^{bc}	2.9 ± 0.1^{b}
1.0	0.1	5.2 ± 1.5^{a}	7.2 ± 1.5^{a}	6.7 ± 1.2^{a}
2.0	0.1	6.1 ± 1.6^{a}	4.7 ± 0.7^{b}	4.8 ± 1.3^{ab}
4.0	0.1	1.2 ± 0.3^{b}	2.2 ± 0.5^{c}	3.8 ± 0.9^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Besides development of multi shoots did not occurred in hormone free medium. The presence of BAP, even at relatively low levels (that is, 0.25 mg/L), enhanced shoot multiplication (Tables 2 and 6). As the concentration increases abnormal growth was observed in all varieties. The shoots become short and bunchy; the leaves were thick and fragile. Tehrim et al. (2013) also confirmed that increasing concentration of BAP results a decrease in the shoot length of grape accessions. High cytokinins concentration in the culture media causes production of ethylene that limits the regeneration of shoots and inhibits the elongation of internodes (Khan et al., 2015).

There was significant difference for the response of BAP concentration in number of shoot among the three varieties. The effect of PGRs on shoot number was not significant between the three varieties. Shoot height and number of nodes showed significant variability at various levels of BAP and among the three varieties. The effect of BAP on shoot height and number of node dependent on the type of the variety since the interaction of PGRs (BAP) with varieties was significant. According to this experiment, increased concentration of BAP has a negative effect to lengthen the induced shoots and the number of nodes. Studies indicated that the combination of different growth regulators and their concentration significantly affected shoot length due to their effect on cell division and cell expansion (Aazami, 2010; Khan et al., 2015). In this study, the combination of BAP with IBA showed significant improvement on the length of shoots and number of nodes.

According to the results presented in Table 5, 1.0 mg/L BAP with 0.1 mg/L of IBA gave maximum mean number of shoots (7.2) for the variety Chenin blanc (Figure 4). In this combination, Canonannon had also given 6.7 mean number of shoot which is significantly different from the other combination except 2.0 mg/L BAP with 0.1 mg/L IBA. Kahn et al. (2015) was also reported maximum shoot regeneration frequency (53.33%) at concentrations of BAP and NAA (1.5 and 0.5 mg/L, respectively) and lowest shoot regeneration frequency (6.67%) in control medium (without any growth regulator). Similarly, Abido et al. (2013) achieved the maximum number of proliferated shoots on MS medium containing 3.0 mg/L BAP + 0.2 mg/L NAA.

In this study, the combination having 0.5 and 1.0 mg/L BAP with 0.1 mg/L IBA produced the best height for the variety Chenin blanc, Canonannon and Ugni blanc, respectively (Table 6). Kahn et al. (2015) achieved significantly increased shoot length on growth regulating hormones in the combination of 1.0 mg/L BAP + 0.1 mg/L GA3. The variety Ugni blanc gave more number of nodes as compared to Chenin blanc and Canonannon (Table 7).

Different combination of BAP and IBA showed variability in number of shoot, shoot height and number of

Figure 4. Shoot multiplication of the three varieties on BAP combined with 0.1 mg/L IBA.

Table 6. Effect of BAP and IBA on shoot height of the three varieties.

BAP (mg/L)	IBA (mg/L)	Mean height of shoots/explant (cm)		
		Ugni blanc	Chenin blanc	Canonannon
0.5	0.1	1.8 ± 0.4^{a}	2.2 ± 0.2^{a}	1.9 ± 0.3^{a}
1.0	0.1	2.0 ± 0.2^{a}	1.9 ± 0.2^{a}	1.8 ± 0.1^{ab}
2.0	0.1	1.8 ± 0.1^{a}	1.3 ± 0.4^{b}	1.5 ± 0.1^{bc}
4.0	0.1	1.0 ± 0.2^{b}	0.7 ± 0.2^{c}	1.3 ± 0.1^{c}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Table 7. Effect of BAP and IBA on node formation of the three varieties.

BAP (mg/L)	IBA (mg/L)	Mean number of Nodes/explants		
		Ugni blanc	Chenin blanc	Canonannon
0.5	0.1	2.6 ± 0.5^{a}	3.2 ± 0.8^{a}	2.4 ± 0.6^{b}
1.0	0.1	3.5 ± 0.5^{a}	3.2 ± 0.6^{a}	3.1 ± 0.2^{a}
2.0	0.1	3.0 ± 0.5^{a}	2.0 ± 0.5^{b}	2.3 ± 0.3^{b}
4.0	0.1	1.6 ± 0.6^{b}	0.8 ± 0.1^{c}	2.0 ± 0.2^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

nodes. The ANOVA revealed that there was significant interaction between PGRs and varieties.

Effect of IAA on root induction

The success of tissue culture depends on the rooting ability of the plant. In the micropropagation of grape, auxins like IAA, IBA and NAA was found effective for inducing *in vitro* rooting (Kurmi et al., 2011). The results found in the rooting experiment of this study are presented in Table 8 and Figure 5. The results showed the plantlets cultured on rooting media were induced roots in all media supplemented with IAA including hormone free media. The primary roots were visible after two weeks of culturing on MS medium supplemented by different concentration of IAA. However, more developed and mature roots were obtained after a month.

The maximum mean number of roots was counted for Ugni blanc (4) and Canonannon (5.2) in a medium supplemented with 4.0 mg/L IAA and the number of roots were increased with increasing of IAA concentration. On the other hand, the maximum mean no of root for Chenin blanc (3.7) was obtained in a medium that contains 2.0 mg/L IAA.

Regarding the length of the roots, Chenin blanc and

Table 8. Effect of IAA on root induction of the three varieties.

IAA(mg/L)	Mean no. of roots per explants		
	Ugni blanc	Chenin blanc	Canonannon
0	1.7 ± 0.5^{b}	1.7 ± 0.6^{c}	2.0 ± 0.4^{c}
0.5	2.5 ± 0.2^{ab}	2.4 ± 0.3^{bc}	2.2 ± 0.5^{bc}
1.0	3.4 ± 0.7^{a}	3.3 ± 0.4^{ab}	2.9 ± 0.6^{bc}
2.0	3.8 ± 1.8^{a}	3.7 ± 0.5^{a}	3.6 ± 0.8^{b}
4.0	4.0 ± 0.4^{a}	3.5 ± 0.6^{a}	5.2 ± 1.0^{a}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

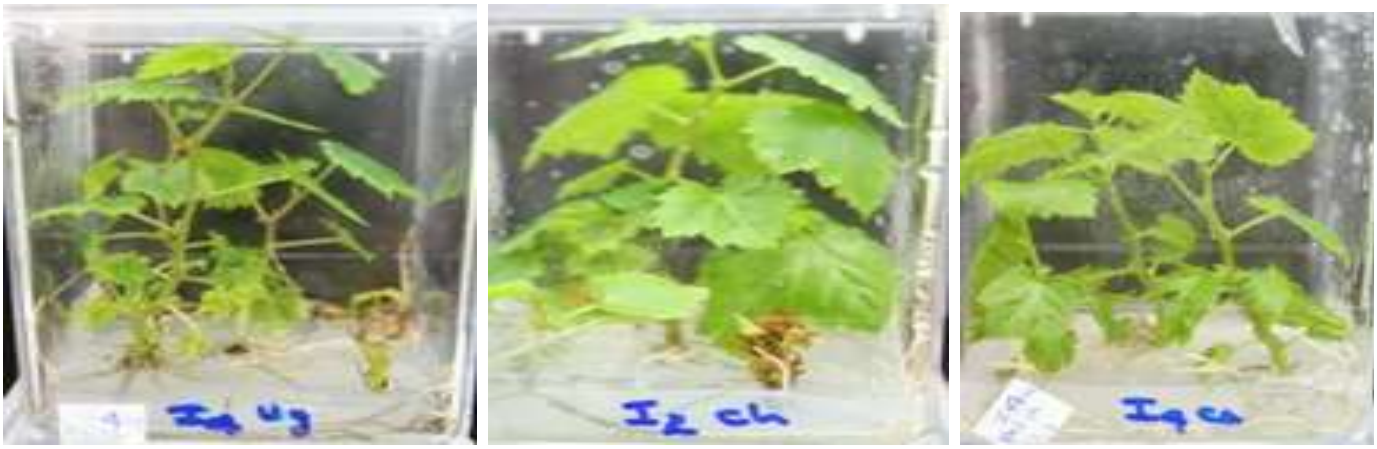

Figure 5. Root induction in MS medium supplemented with different IAA concentrations.

Table 9. Effect of IAA on root length of the three varieties.

IAA(mg/L)	Mean length of roots per explants (cm)		
	Ugni blanc	Chenin blanc	Canonannon
0	4.1 ± 0.8^{a}	4.1 ± 0.5^{c}	5.1 ± 1.5^{a}
0.5	3.8 ± 1.0^{a}	4.8 ± 0.6^{bc}	5.1 ± 4.2^{a}
1.0	5.5 ± 1.7^{a}	7.5 ± 1.3^{ab}	6.8 ± 1.5^{a}
2.0	6.2 ± 2.1^{a}	9.7 ± 0.7^{a}	7.9 ± 4.2^{a}
4.0	5.4 ± 1.3^{a}	6.7 ± 2.8^{bc}	8.6 ± 1.7^{a}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level.

Canonannon induced a maximum mean length of 9.7 cm and 8.6 cm at 2.0 and 4.0 mg/L IAA, respectively. Ugni blanc produced maximum mean length of root (6.2 cm) at 2.0 mg/L IAA. Significant variation in mean number of root among treatments was observed for the varieties Canonannon and Chenin blanc and variety Chenin blanc showed significant variation on the length of roots (Table 9). Shatnawi et al. (2011) found the maximum number of roots per explants in the micropropation of *V. vinifera* L. via meristem culture at 0.6 mg/L of IBA. Number of root induced and length increased with increasing concentration of IAA and the maximum root length was obtained with 0.6 to 0.8 mg/L of NAA. On *in vitro* propagation of grapevine (*V. vinifera* L.) *Muscat of Alexandria* cv, Abido et al. (2013) found the highest rooting percentage, number of roots/shoot and root length (87%, 3.4 and 4.5 cm, respectively) on MS medium supplemented with1.0 mg/L IBA + 0.5 mg/L IAA.

Acclimatization

In this experiment, plantlets about 3 cm tall were transferred into the soil because plantlets less than 3 cm were not survived. Pictures showing the growth condition of grape plantlets in the glasshouse at different time are presented (Figure 6). Moreover those plantlets uncovered with plastic bags started to weaken immediately after being transferred to the soil. This implies the plastic cover protects the plantlets from the external stress for some times until they adapt to the outside environment. After one week, the plastic cover should be removed to avoid fungal development because among the five plantlets remains with the plastic cover all of them were

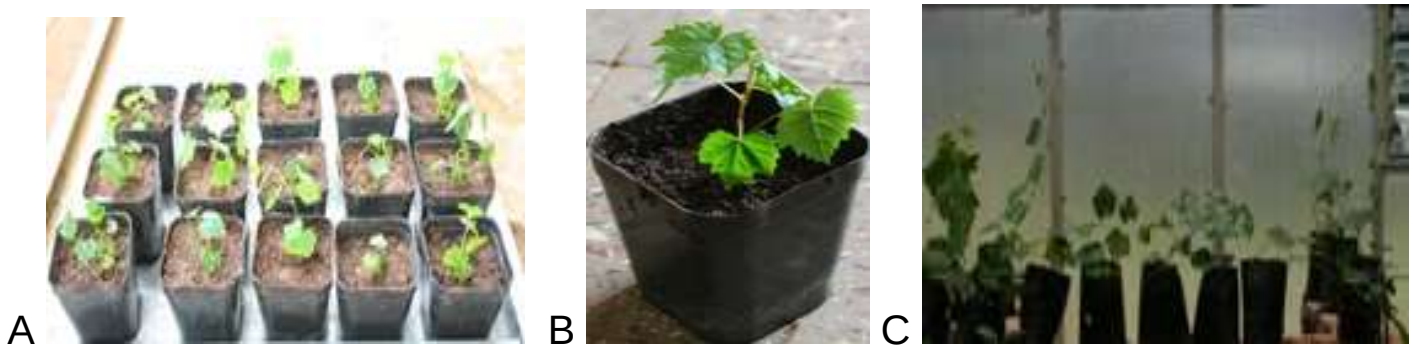

Figure 6. Acclimatization of the *in vitro* plantlets of three varieties of grape vines in the greenhouse under natural sun light condition. (A) Plantlets of grape vine in the glasshouse after one week of removing the plastic bag, (B) Plantlets grown after four weeks of removing the plastic bag and (C) Well - developed plantlets after three month of acclimatization.

contaminated by fungi. Survival rate of the acclimatized plantlets was 78.6, 92.0 and 73.9% for Ugni blanc, Chenin blanc and Canonanoon respectively.

Shatnawi et al. (2011) achieved a survival rate of 95% when the rooted explants were acclimatized *ex vitro* in the study of *in vitro* micropropagation of vitis. Only survival percentage of the variety Chenin blanc were maximum but the rest two varieties have relatively low survival percentages. Laslo et al. (2010) was found also to have 90% survival rate in the *ex vitro* acclimatization of *V. vinifera L*. In addition, Abido et al. (2013) found 80 to 90% survival rate after they kept plantlets in rooting medium for 34 before acclimatization. *In vitro* plantlets of grapevine are very sensitive to *ex vitro* conditions; the success of acclimatization depends on the increases of humidity, reduction of light intensity, and temperature in the first days of acclimatization (Laslo et al., 2010).

Conclusion

The experimental results of this study revealed that seven minutes of sterilization in 1% NaOCl followed by culturing on basal Ms medium supplemented with 0.5 mg/L BAP was found to be effective for sterilization and shoot initiation, respectively. The best multiplication of shoots were obtained at 1.0 mg/L BAP with 0.1 mg/L IBA for the variety Chenin blanc and Canonannon, while 2.0 mg/L BAP with 0.1 mg/L IBA for Ugni blanc. High levels of IAA, 2.0 mg/L for Chenin blanc and 4.0 mg/L for Ugni blanc and Canonannon were found to be effective in enhancing root number and length. In the acclimatization process the survival rate of Chenin blanc was better than the other two varieties. In this *in vitro* micro propagation of grape vine, it is possible to demonstrate culture establishment, shoot proliferation and rooting by using the different concentrations of BAP, IBA and IAA.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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