African Journal of Biotechnology

Volume 16 Number 9, 1 March 2017

ISSN 1684-5315

Academic Journals

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African Journal of Biotechnology

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academicJournals

Vol. 16(9), pp. 380-387, 1 March, 2017
DOI: 10.5897/AJB2016.15643
Article Number: CA73EA662992
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Comparative study of native microorganisms isolated from watermelon (*Citrullus lanatus*) waste and commercial microorganism (*Clostridium thermocellum*) used for bioethanol production

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Received 2 September, 2016; Accepted 16 January, 2017

This study was to examine the efficiency of native microorganisms, in relation to the commercial bacterium *Clostridium thermocellum* ATCC 27405 for bioethanol production from waste watermelon on a pilot scale. The raw material was subjected to a grinding process for the later stage of hydrolysis under different temperatures using *Aspergillus fumigatus* and *Leuconostoc dextranicum* native microorganisms. This was followed by fermentation using control strains of *Zymomonas mobilis* (native microorganism) and *C. thermocellum* ATCC 27405. After hydrolysis, the fermentation product was evaluated by physico chemical analysis, pH determination and reducing sugars concentration using refractometry. Finally, the product obtained was subjected to vacuum distillation to increase the grade of purity. It was there after analyzed using pycnometry and gas chromatography (GC). GC results revealed that the yield of ethanol from hydrolysis and fermentation by native strains was improved by 27.62%, compared to yields of ethanol obtained from *Z. mobilis* (11.62%) and *C. thermocellum* (3.10%). It was therefore concluded that the native strains were more efficient in hydrolysing and fermenting watermelon compared to the commercial *C. thermocellum* and *Z. mobilis*.

Key words: *Aspergillus niger*, *Clostridium thermocellum* ATCC 27405, *Zymomonas mobilis*, hydrolysis, fermentation processes.

INTRODUCTION

Global warming and environmental pollution daily increases with the use of fossil fuels; to mitigate this problem, it is necessary to investigate new alternative energy sources, especially those derived from plant biomass that do not affect ecosystems and plants production for agricultural use. Currently, there is ongoing search for new and better ecological strategies to produce bioethanol, using agricultural waste with high

fermentable sugars content, such as watermelon waste fruits. Global warming is a phenomenon of significant increase in average temperature caused by the use of fossil fuels, oil industrialization, burning forests, industry and carbon monoxide fumes caused by the automotive park. These activities have caused an imbalance between land, ocean and atmosphere, which has changed global air circulation and water patterns. In recent years, there has been a gradual increase in temperature and it is estimated between 0.19 and 0.38°C (Melendo, 2014). According to databases, 1998 was the warmest year. As a result of global warming, snow-covered areas have been considerably reduced (Melendo, 2014).

Dependence of world society on fossil resources is high, reaching 80% of primary energy consumed in 2004 and 2006 in Europe and Spain; consumption was 79 and 83%, respectively (Eritja, 2015). The use of fossil fuels and global energy engine is cost effective and causes depletion; its effects on global warming and urban pollution attributed to combustion have forced research on the use of alternative energy sources, especially those derived from plant biomass. In this context, bioethanol obtained from sugars, starches or cellulosic material is an alternative to be used as a supplement or substitute for gasoline, engine power, thus reducing energy dependence in addition to the social benefits derived from their production (IRENA, 2015). The main sources of bioethanol production worldwide are: sugar cane, beets, wheat, corn, barley, cassava, sweet sorghum, etc. Also, agricultural residues such as olive residues, rice bran, fruit peel, etc. are included. Among the most commonly used for ethanol production energy crops, sugarcane is the raw material most commonly used in tropical countries such as Brazil and India. In North America and Europe, ethanol fuel is derived from corn starch and cereals (Colina, 2000).

Fermentation musts' sugar production is faster and adequate when further bacterial and fungal microorganisms are used, which by their rapid consumption of glucose help better develop the fermentation processes of plant materials (Mariscal, 2011). Ecuador began exporting oil from the Amazon rainforest since 1972, the date from which has been extracted more than 4 billion oil barrels. As a result of the activities of oil exploration, continued deforestation which affects ecosystems, causing loss of water in the Ecuadorian Sierra and the rest of the country is produced; so that Amazonian clouds are becoming less compact and this leads to a reduction in rain water flow (Baquero and Mieles, 2015). In this regard, the Department of Life Sciences and Agriculture at the Universidad de las Fuerzas Armadas (ESPE) seeks new and better ecological strategies to produce ethanol by hydrolysis and fermentation processes, using agricultural waste with high fermentable sugars content; this is the case of waste watermelon, Glory Jumbo variety. Watermelons can be grown in open fields and in greenhouses, so it is easy to dispose of waste fruits during the whole year. For this reason, fuel development from waste watermelon would not only have positive environmental effects, but would give an additional economic output to watermelon farmer (Salazar, 2010).

In Ecuador, ethanol can be produced on a large scale since there exist approximately 1513 ha. To obtain ethanol, watermelon cultivation in coast region which serve as raw materials were used, and this can provide individual, cheap and renewable energy source. Watermelon is grouped into the Cucurbitaceae botanical family, and comprises about 850 species of herbaceous plants that produce large fruits and protected by a hard crust. Watermelon plant has large and weighty fruits, reaching up to 30 cm in diameter and weighing between 15 to 20 kg. For domestic trade, watermelons usually weigh between 3 to 8 kg (Canales, 2003). *Citrullus lanatus* species is highly fermentative as per kg of fruit, 50% ethanol is obtained. The replacement of gasoline by ethanol is currently an economic problem rather than technical, to reverse this, it is necessary to find more efficient and better economic biotechnological alternatives (Alban et al., 2003).

Watermelon valued for its nutritional qualities, is very important in Ecuador, South America. However, about 20% of the harvest of this highly perishable fruit is lost due to post harvest decay. Decayed watermelons may be an important source of industrially-produced bioethanol. Industrial bioethanol production requires the use of efficient bacteria for fermentation. Therefore, the aim of this study was to produce bioethanol as clean energy alternative using bacteria, fungi, and native fruit with the purpose of fermenting watermelons waste as an energy material. The native microorganisms (bacteria and fungi) obtained from watermelon waste have equal or better efficiency in producing bioethanol compared with commercial microorganisms.

MATERIALS AND METHODS

Sample collection

Watermelon fruit waste was collected from the Membrillo locality; Manabi province, Tosagua Canton, Ecuador. Tosagua canton located northwest of Manabi province. Fruit that has no external damage was selected. Fruits collected in the field were used for the preparation of plant samples in laboratory. Experiments related to preparation of aqueous extracts, isolation of native microorganisms,

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biochemical tests, enzymatic hydrolysis, fermentation, distillation and quantification of bioethanol by gas chromatography were conducted under controlled conditions.

Sample preparation

Plant material was sterilized thoroughly with sterile water followed by removal of excess moisture. The sterilized material was immediately used. To obtain the microorganisms, triturated 100 g pulp and rinds was applied and this material was placed in Erlenmeyer flasks by adding 50 ml of distilled water and allowed to stir at 80 rpm and 50°C for bacterial and 25°C for fungal development through 8 days to obtain high levels of biomass with beneficial microorganisms (fungi and bacteria). Upon completion of the required time, serial dilutions were made to 1×10^{-4} and the last dilution was depth seeded in Potato dextrose agar (PDA) (Camacho et al., 2009).

Bacteria isolation and identification

For *Leuconostoc dextranicum* isolation, the Mayeux culture medium was used, containing (g): peptone 10, yeast extract 5, sucrose 100, sodium citrate 1, glucose 5, gelatin 2.5, agar 15 per liter of distilled water. Subsequently, the bacterial medium was autoclaved at 120°C during 20 min, dispensed and gelled in petri dishes at room temperature. For bacterial identification, Blast program was used (Bailón et al., 2003). *Zymomonas mobilis* isolation was performed in WL differential agar specific culture medium for 4 days and incubated at 25°C temperature. Bacteria obtained were massively multiplied in fermentation medium (g/L): glucose-100; yeast extract-10; KH_2PO_4- 2; (NH_4) $2SO_4$- 1; $MgSO_4.7H_2O$ - 0.5. *C. thermocellum* strain was purchased commercially (*C. thermocellum* ATCC 27405).

Fungi isolation and identification

Fungal colonies with different macroscopic characteristics were isolated, and then they were seeded in Petri dishes with PDA and antibiotic to inhibit bacterial growth. All fungal cultures were incubated at 25°C for 7 days. Once fungal colonies were fully developed, monosporic cultures were made in PDA to obtain axenic cultures. To identify fungal morphology, keys techniques were used. For the isolation of *Aspergillus niger* and *Aspergillus fumigatus*, the following culture media were used: agar Czapek, potato dextrose agar (PDA), agar broth nitrates and Bacto Peptone. To this, 30 mg of terramicina and cycloheximide antibiotics were individually added. 3,5-Dintro salicylic acid (DNS), sodium and potassium tartrate, with sodium hydroxide for the quantification of reducing sugars reagents were used. For DNS reagent, preparation of glucose stock was carried out, followed by washing of material with distilled water. To evaluate the effectiveness of *A. niger* relative to the native microorganisms (bacteria and fungi), the hydrolysis step was subjected to incubation for 8 days at temperatures (25, 30 and 35°C). Subsequently, *C. thermocellum* ATCC 27405 effectiveness and *Z. mobilis* with the native microorganism (bacteria) in the fermentation phase for 8 days at a temperature of (25, 30 and 35°C) were compared. Finally to obtain and determine ethanol concentration, distillation equipment and pump was used. Bioethanol production was quantified bygas chromatography.

Experimental design

Completely randomized design (CRD) experimental design was used with 2 × 3 + 1 factorial arrangement to compare hydrolysis ability of each treatment. Where M has two factors: microorganism type and C temperature, with three levels each. M_1 (bacterium) - C_1=25; M_2 (fungus) - C_2=30; C_3=35 [Data were analyzed using the INFOSTAT package (INFOSTAT, 2000) version].

Table 1. Morphological characteristics of *Aspergillus* sp.

Aspergillus sp.	Conidiophore		Phialides (diameter in mm)
	Height (mm)	Width (mm)	
Aspergillus fumigatus	17.5	22.5	7.5
	17.5	20	7.5
	20	22.5	7.5
	12.5	20	7.5
	20	25	7.5
Average	17.5	22	7.5
Aspergillus niger	20	25.2	8.75
	21.2	27.5	8.75
	22.5	22.7	8.75
	23.5	26.7	8.75
	18.2	23.8	8.75
Average	21.1	25.2	8.75

Statistical analysis

Statistical analysis was determined by DMS Test 5% for microorganisms; Duncan test 5% overall treatment and temperature levels, regression and correlation of the temperature levels with each variable under study for each microorganism in the hydrolysis step. Statistical analysis for the fermentation phase was determined using tests by comparing average Duncan 5% for each treatment, normality and homoscedasticity test were used to verify the assumptions of ANOVA. For analysis of variance the quantity which must ferment in each experimental unit was used; that is regression and correlation between temperatures with each of the variables under study. To compare the ability of synthesis, fermentation and distillation amount of each treatment design was completely randomized. Where one factor is C levels and comprised: temperature and the microorganism (bacteria) which was more efficient than the control over a total of four treatments.

RESULTS AND DISCUSSION

After watermelon waste treatment, a high diversity of native beneficial microorganisms was obtained.

Fungi identification

Table 1 shows *A. niger* and *A. fumigatus* fungi macroscopic and microscopic identification. *A. niger* macroscopic characteristics were black or dark brown colonies, the reverse is colorless to yellow, dense colonies, flocosa granular, compact and white mycelium. Biseriate microscopically and radial conidial heads, stipes thick-walled, smooth, hyaline, yellowish or pale brown observed, almost spherical vesicles, brown globose

Table 2. Biochemical tests of *Leuconostoc dextranicum* and *Zymomonas mobilis*.

Zymomonas mobilis	*Leuconostoc dextranicum*
Gram-	Gram+
Catalase+	Fructose+
Oxidase-	Galactose+
Gelatinase-	Lactose+
Urease+	Maltose+
Tween 80-	Starch-
D-Glucose +	Sucrose+
Lactose+	Trehalose+
Citrate-	Dextran+
Mobility-	Arabinose-

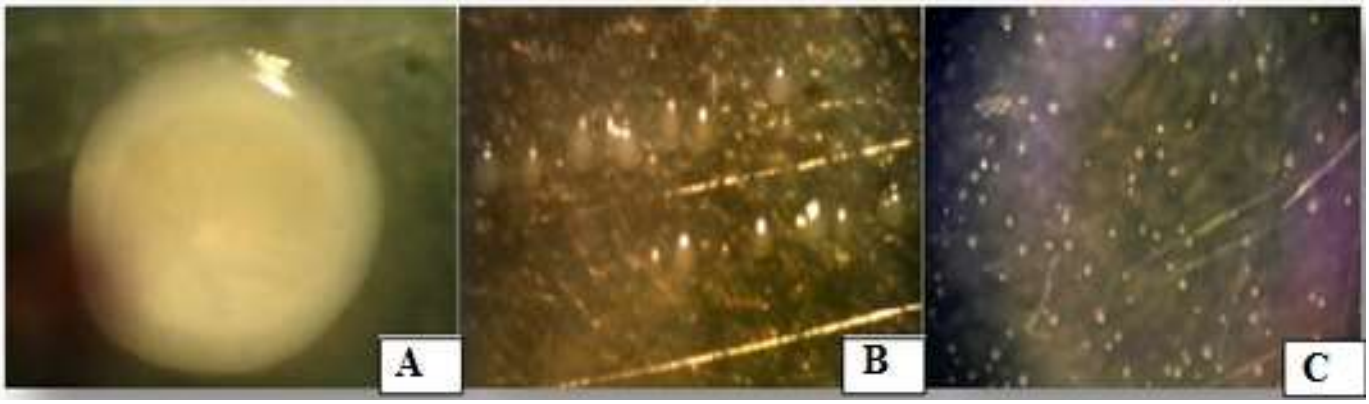

Figure 1. A. *Leuconostoc dextranicum*. **B.** *Zymomonas mobilis.* **C.** *Clostridium thermocellum* (ATCC 27405).

conidia, irregular roughness with ridges and bumps. *A. fumigates* macroscopic characteristics were blue-green to gray-greencolonies, whitemiocelio, colorless back, yellowish, reddish brown or green, velvety flocosa, flat or radial grooves texture. Uniseriate microscopic and predominantly columnar conidial heads, hyaline and smooth stipes, vesicular pear-shaped or spoon-shaped is observed, phialides occupying half or two thirds of the gallbladder, globose to oval, smooth or slightly rough conidia. These results are similar to those obtained by Abarca (2000), which corroborates the views expressed by Loustau (1964) and Tangarife (2011).

Bacteria identification

The two different bacterial isolates such as *Leuconostoc dextranicum* and *Z. mobilis* were characterized by their morphological and biochemical properties as shown in Table 2, where the results are consistent with studies reported by Matiz (2002), with *Zymomonas* sp. gender classification, however, *L. dextranicum* microscopic identification Gram positive forming short chains was observed unlike described by García (2007), who reported that *L. dextranicum* is a gram-positive coccobacillus and form short chains. In catalase and oxidase tests, positive results was obtained unlike the results obtained by API test identification where the results of oxidase and catalase tests were negative, with positive anaerobiosis and positive citrate; these characteristics are consistent with the reports described by Gonzáles (2013). The morphological difference microscopically observed in *L. dextranicum* is because growth ions are present in the culture medium; according to Fernandez (2010) cells grown on glucose or on solid media can have an elongation at bar, morphology formed, which occurred in the present study since the medium of solid and liquid culture had a source of sugar dextrose. *C. thermocellum* ATCC 27405 is a commercial strain classified as Biosafety classification on U.S. Public Health Service Guidelines; it is a gram positive bacilli with spore forming, which is consistent with the results reported by Demain et al. (2005). Cervantes (2007) states that the positive bacterial growth is due to the addition of carbohydrate, proteins, amino acids, vitamins and essential cofactors. Figure 1 shows the three types of bacterial colonies obtained.

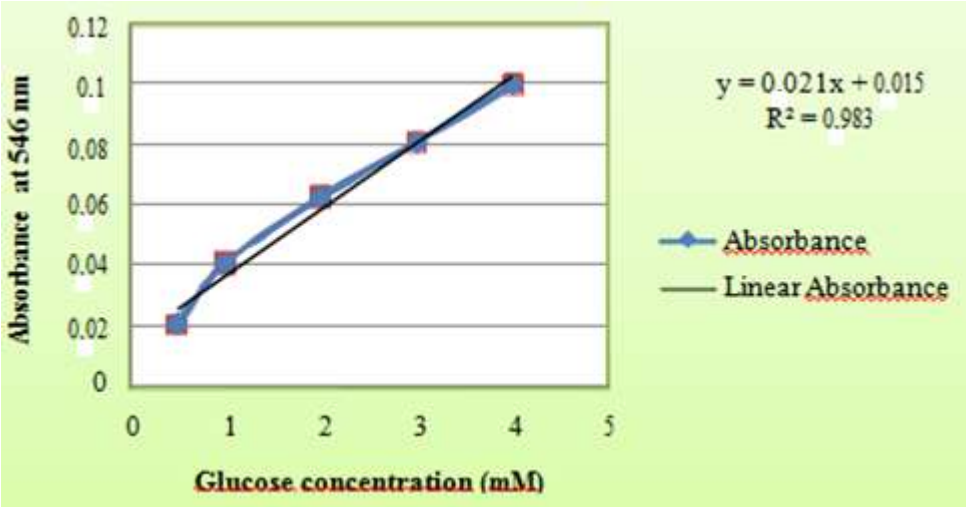

Figure 2. Reducing sugars concentration in waste watermelon fermentation - hydrolysis.

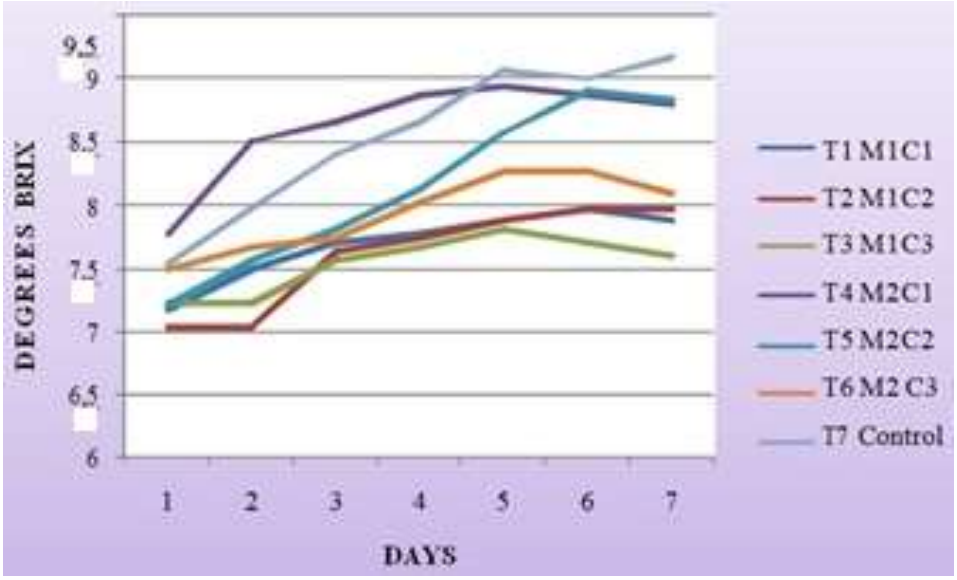

Figure 3. Microorganisms and temperature activity on watermelon degrees brix in the hydrolysis step.

Bacterial fermentation - hydrolysis

Figure 2 shows the amount of reducing sugars obtained with 3,5-dinitrosalicylic acid (DNS) (glucose) technique content in watermelon fermentation - hydrolysis by comparing these results with those obtained by Matiz et al. (2002), a 10 to 20% total sugars increase in relation to 3.878 g/L of initial amount was obtained. This demonstrates that the substrate used is nutritionally enriched and promotes native microbial populations growth. Figure 3 shows reducing sugars (Brix) production in enzymatic hydrolysis step using three native microorganisms: *L. dextranicum*, *A. fumigatus* and *A. niger* (control) at different temperatures (25, 30 and 35°C).

Compared to acid hydrolysis, *A. fumigatus* produced the greatest amount of reducing sugars as these fungi have ability to produce endoglucanase enzymes, which remove beta-1,4-glucosidic links chain cellulose (Landazábal, 2004; Cortínez, 2010). The enzyme activity was measured using refractometer. According to the results presented in Figure 3, in the hydrolysis process there were significant differences so that none of its values is discarded, however the sugars percentage was

found worthy (reducing sugars 8.38) and were higher than those obtained in the untreated raw material; these sugars were obtained using 3,5-dinitrosalicylic acid (DNS) technique, where the initial amount of reducing sugars is 3.88 g/L, demonstrating increased unfolded sugars and justifying the process carried out with any of the microorganisms *L. dextranicum* and *A. fumigatus*. Control was more functional because it presents greater degrees of brix number in relation to other treatments.

Fermentation

Hydrolysis yields enabled microorganisms used in fermentation step to convert the reducing sugar(s) to ethanol. According to Cervantes (2007), three types of bacteria occur in the must fermentation: acidic, alcoholic and viscous. The interaction of the three types determines an increase in microbial population counts, consumption of sugars, proteins and formation of ethanol gave a viscous appearance. *Z. mobilis* followed the metabolic pathway of Entner-Doudoroff pathway from which ethanol was obtained; *L. dextranicum* used heterolactate route for obtaining carbon and energy. Pyruvate as an end product of a biochemical pathway was converted to ethanol by the action of pyruvate decarboxylase and alcohol dehydrogenase; *C. thermocellum* ATCC 27405 uses acetone butanol route fermentation to complete energy metabolism. On the other hand, Cervantes (2007) reported that lactic acid bacteria such as *L. dextranicum* are responsible for viscose and alcoholic fermentation of fermented beverages. An interesting finding was the increase in gram-positive bacillus *L. dextranicum* being native bacteria that is more efficient at room temperature 25°C; which could correlate with increased viscosity, physical change in the mature leaven. Time and fermentation, pH 4.5, were within 8 days for all treatments since a decrease was observed in the bubbles produced during fermentation by CO_2 evolution.

Distillation

Distillation semi-micro equipment was used. By setting the variance analysis for the distillation volume obtained no significant differences was found. Never the less with T4 treatment (Control$_1$ *C. thermocellum* ATCC 27405), highest volume distillation was obtained (Table 3).

Ethanol quantification by pycnometry

When comparing between T_1 and T_2 treatments (with *A. niger* hydrolyzed and fermented with *C. thermocellum* ATCC 27405 and *Z. mobilis*) it was determined that these treatments generated an average of 11.78 ± 0.059% lower alcohol which was produced by *A. fumigatus* (11.87 ± 0.059%) with T_1 and T_2 treatments (hydrolyzed with *A. fumigatus* and *C. thermocellum* fermented with ATCC 27405 and *Z. mobilis*). These values demonstrate that the activity of synergism between these microorganisms is developed to complete the procedure. These results agree with those obtained by Escudero (2015). Although in treatments not high percentage of alcohol is obtained, it is confirmed that the improvement in controlling process variables increases the efficiency in obtaining alcohol (Table 4). Ethanol percentages shown in Table 4 out-performed those obtained by Alban and Freire (2009), which means that the conditions for initial treatment and time directly influenced process quality. In fermentation process, using *L. dextranicum* native strain at 30°C, the highest percentage of 15.18 ± 0.059 was obtained and at 35°C, 13.26 ± 0.059 was achieved. With *Z. mobilis* ATCC 27405 and *C. thermocellum* control strains at 35°C, percentages of 13.09 ± 0.059% and 13.21 ± 0.059 were obtained, respectively.

Table 3. Distilled volume in watermelon fermentation.

Treatments	Distilled volume (mL)
T1 BC_1	15.25
T2 BC_2	15.50
T3 BC_3	14.75
T4 Control$_1$ *C. thermocellum*	16.25
T5 Control$_2$ *Z. mobilis*	15.00

Table 4. Ethanol percent determination by pycnometry method. Watermelon fermentation after 8 days of incubation under anaerobic conditions with controlled temperatures.

Treatments	% of ethanol by pycnometry		
	25°C	30°C	35°C
Ld+Ld	11.38	15.18	13.26
Ld+T_1	11.384	11.36	13.09
Ld+T_2	11.36	11.94	13.21
Af+Ld	11.98	11.95	12.48
Af+T_1	11.65	11.37	12.47
Af+T_2	11.87	11.372	12.53
An+Ld	11.38	12.48	12.51
An+T_1	11.37	11.64	11.94
An+T_2	11.35	12.47	11.92

Ld = *Leuconostoc dextranicum*; Af = *Aspergillus fumigatus*; An = *Aspergillus niger.*

Ethanol quantification by gas chromatography

When gas chromatography was performed, it was

Table 5. Ethanol percentage determination by Gas Chromatography. Watermelon fermentation after 8 days, under anaerobic conditions (25; 30; 35°C) with native and control strains.

Sample	Parameter	Method	Units	Results
Extract 1 NB+NBT° 25°C	Ethanol	Gas chromatography	g/100 ml	0.12
Extract NB+NBT° 30°C	Ethanol	Gas chromatography	g/100 ml	27.62
Extract 1 Af+NBT°25°C	Ethanol	Gas chromatography	g/100 ml	1.58
Extract An+LdT° 30°C	Ethanol	Gas chromatography	g/100 ml	0.42
Extract 1 NB+NBT° 25°C	Ethanol	Gas chromatography	g/100 ml	11.00
Extract Ld+LdT° 35°C	Ethanol	Gas chromatography	g/100 ml	7.89
Extract Af+NBT° 35°C	Ethanol	Gas chromatography	g/100 ml	3.14
Extract An+T2 T° 35°C	Ethanol	Gas chromatography	g/100 ml	3.10

NB = Native bacteria; Ld = *Leuconostoc dextranicum*; Af = *Aspergillus fumigatus*; An = *Aspergillus niger.*

determined that values decreased progressively, which coincides with Alban and Freire (2009) who reported that, synergism occurs when microorganisms occupy the same substrate. As reported by Bustamante et al. (2000), when the pH decreases, the *A. niger* growth increases, preventing other microorganisms growth, so the fermentative capacity of *Z. mobilis* also decreased. In the same way, increased pH caused decreased fermentation efficiency. So, optimum pH is best suitable for production of bioethanol by *Z. mobilis* (Khoja et al., 2015). Finally when comparing ethanol amount obtained by gas chromatography and pycnometry, different values were obtained (Table 5), however, the advantages of use of pycnometer is highlighted. When performing gas chromatography of all treatments after 8 days fermentation and at temperatures (25, 30, 35°C); highest concentration [27.62% (v/v)] was achieved with treatment NB+NB (*L. dextranicum*) at 30°C.

Conclusions

*L. dextranicum*n, *A. fumigatus* and *A. niger*, were identified as *C. lanatus* native microorganisms and have hydrolytic and fermentative capacity. Enzymatic hydrolysis with native microorganisms was the best method for obtaining ethanol at a temperature of 25°C, with this temperature a complete conversation of reducing sugars of 9 g/L was obtained, which are transformed into alcohol in the fermentation step. In fermentation stage at different temperatures (25, 30 and 35°C), *L. dextranicum* was more efficient than *C. thermocellum* ATCC 27405 commercial strain and *Z. mobilis.* With *L. dextranicum,* 27.62% was achieved as ethanol maximum concentration. Watermelon wastes are suitable for production of clean bioethanol, abundant, inexpensive and help to reduce the negative effect in production of biofuels from sugarcane monocultures and corn.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge the Department of Life Sciences, Universidad de lasFuerzas Armadas-ESPE for providing all the necessary facilities throughout this research work. We would also like to acknowledge the contributions of colleagues in our respective institutes for helpful comments on the manuscript.

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Vol. 16(9), pp. 388-399, 1 March, 2017
DOI: 10.5897/AJB2017.15881
Article Number: 22E331862997
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of the essential oils of *Thymus vulgaris*, *Cinnamomum zeylanicum* and *Mentha piperita* on fungal growth and morphology

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Received 9 January, 2017; Accepted 27 January, 2017

This study aimed to evaluate the antifungal activity of essential oils of *Thymus vulgaris* L., *Cinnamomum zeylanicum* B. and *Mentha piperita* L. on some saprophytic fungi. Essential oils were extracted by hydro-distillation, and chemical composition was analysed by gas chromatography coupled with mass spectrometry (GC-MS). *T. vulgaris* had as major components, Thymol (35.12%), *p*-cymene (25.36%) and γ–terpinene (12.48%). E-B-Caryophyllane (21.82%), E-Cinnamaldehyde (13.03%) and eugenol (12.15%) were primary in *C. zeylanicum*. Menthol (33.59%), menthone (18.47%) and α-pinene (8.21%) were primary in *M. piperita*. Applying the micro-atmospheric method, essential oils were tested against *Rhizopus oryzae* Went & Prins, *Rhizopus stolonifer* Ehrenb, *Aspergillus tamarii* Taka, *Aspergillus parasiticus* Speare, *Aspergillus flavus* Link and *Talaromyces purpureogenus* purpureogenum. The minimum inhibitory concentrations were 3 to 8, 5 to 16 and 13 to 23 µL/75mL air space for *T. vulgaris, C. zeylanicum* and *M. piperita*, respectively. Means of percentage inhibition were compared through one-way ANOVA by the Tukey test. Scanning electron microscopy revealed fungal cell wall deformation after exposure to essential oil vapour. These essential oils can be exploited as alternatives to synthetic food preservatives.

Key words: Essential oil, *Aspergillus*, *Rhizopus*, *Talaromyces,* fungal morphology, food preservation.

INTRODUCTION

Food spoilage initiated by fungi infestation dates back to the time when primitive man began to cultivate crops and store food (Pitt and Hocking, 2009). Research into fungal food spoilage and its prevention, therefore, had clearly

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been that of urgent necessity: lacking in spectacular appeal, it is, however, often neglected. Synthetic fungicidal powders often used include Mancozeb, Thirame, methyl Thiophanate, etc. However, the use of these chemicals as food preservatives poses several problems particularly, environmental pollution, toxicity to living organisms, persistence as residues in food products, resistance of pathogenic agents (Etter et al., 2003).

It is well established that certain plants and their metabolites possess antimicrobial properties (Nguefack et al., 2004). These properties are significantly due to their volatile fraction, that is, essential oils (Hulin et al., 1998) and the presence of other bioactive substances such as phenols. Essential oils have a very broad spectrum of action, since they inhibit the growth of microorganisms (Ambindei et al., 2014) as well as insects (Tatsadjieu et al. 2007).

Thymus, Mentha and Cinnamum species are essential oil producing plants, and are being exploited for different properties. In native medicine, flowering parts and leaves of *Thymus* species have been extensively used as herbal tea, tonic, carminative, antitussive and antiseptic, as well as for treating colds (Maksimovic et al., 2008; Rota et al., 2008). Several studies have revealed the anti- oxidant, viral, inflammatory and microbial potential of the essential oil of *Thymus vulgaris* (Nickavar et al., 2005; Sessou et al., 2012).

Essential oils of *Mentha* species are generally used to flavor liquors, breads, salads, soups and cheese, as well as in cosmetics (Yadegarinia et al., 2006). Herbalists consider peppermint (*Mentha piperita*) as an astringent, antipruritic, antispasmodic, antiemetic, carminative, analgesic, antimicrobial and a stimulant (Hoffman, 1996; Jiofack et al., 2010).

Cinnamon can serve as a blood and digestive tonic, as a natural food preservative, and also exhibits antibacterial as well as antifungal properties (Kalemba and Kunicka, 2003; Jazet et al., 2007).

The main objective of this study was to evaluate the antifungal activity of the essential oils of *T. vulgaris, Cinnamomum zeylanicum* and *M. piperita* against some food spoilage fungi. This is based on the hypothesis that essential oils of these plants can inhibit fungal growth and development. Specifically, the chemical composition of the essential oils, the percentage inhibition, the nature of inhibition, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and effect of essential oils on fungal morphology were evaluated.

MATERIALS AND METHODS

Whole mature plant of *T. vulgaris* L., was harvested in Dschang (geographical coordinates: 5°27' North and 10°04' East); leaves of *C. zeylanicum* B. and whole plant of *M.a piperita* L. were harvested in Mbouda (5°38' North and 10°15' East) in the West Region of Cameroon. These plants were harvested in September 2013 and were identified in the National Herbarium in Yaounde - Cameroon where voucher specimens were kept.

Fungal species

Fungal species were isolated from contaminated stored grains from Ngaoundere-Cameroon, their DNA were extracted, amplified and sequenced. The corresponding DNA sequence was blasted in the NCBI gene bank (Ambindei et al., 2016). The six fungal strains used in this study were a strain each of *Rhizopus oryzae, Aspergillus tamarii, Aspergillus parasiticus, Rhizopus stolonifer, Aspergillus flavus* and *Talaromyces purpureogenus*.

Extraction and chemical composition analyses of essential oils

Essential oils from the different plants were extracted by hydrodistillation with the help of a Clevenger apparatus. Essential oils obtained were analysed by gas chromatography and gas chromatography coupled with mass spectrometry (GC/MS) (Jazet et al., 2010).

Gas chromatography was done in a Varian CP-3380 GC with flame ionization detector (FID) fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25 µm). The operating conditions were: Injection temperature: 200°C; detection temperature: 200°C; temperature program 50 – 200°C at 5°C/min; carrier gas nitrogen, with a flow rate of 1 mL/min.

GC-MS analyses were performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 m) and interfaced with a quadrupole detector (GC quadrupole MS system, model 5970). Column temperature was programmed from 70 – 200°C at 10°C/min; injector temperature was 200°C. Helium was used as carrier gas at a flow rate of 0.6 mL/min. The mass spectrometer was operated at 70 eV. The operating conditions were: temperature programming: 70 to 200°C at 10°C/min; Injection temperature: 200°C; Flow rate of vector gas (helium): 0.6 mL/min, injection volume: 0.1 µL of essential oil solution diluted at 10% in hexane.

Compounds were identified by comparing the calculated retention indices and the mass spectrum with those given in literature (Adams, 2007).

Antifungal activity

The micro-atmosphere method described by Sulaiman (2013) was used at varying amount of essential oils. The inhibiting action of the essential oil vapour is highlighted. The inoculum is placed at the center of a solidified medium in a Petri dish. A given quantity of essential oil is placed on the lid of the petri dish and the dish incubated in reverse position. The solution to be tested evaporates and the volatile phase carries on an inhibiting activity on the germ tested. The essential oil has no direct contact with the inoculum, only the essential oil vapour.

Preparation of culture medium

A potato dextrose agar w/chloramphenicol HIMEDIA brand culture medium was used. According to the manufacturer's instructions, 39.05 g of medium was suspended in 1000 mL distilled water. The suspension was heated to boil so as to enable complete dissolution of medium. It was then sterilised by autoclaving at 121°C at a pressure of 1 bar for 15 min.

Inoculation and application of essential oil

20 mL of culture medium was poured in a Petri dish of diameter 90 mm and height 10 mm. The resultant air space volume in the Petri dish was calculated to be 75 ml. After solidification, a 6 mm well

was created at the centre of the medium and a mycelial disc from a two-day old pure culture of the respective fungus was deposited into the well. Pure essential oil at varying volumes of 5, 10, 15, 20 and 25 µL were placed on the lid of the Petri dish with the help of a micropipette, giving corresponding concentrations of 5, 10, 15, 20 and 25 µL/75 ml air space. Placed in a reverse position, the Petri dishes were sealed with parafilm so as to prevent cross contamination. Each concentration was repeated thrice. A negative control was carried out with no essential oil. No reference volatile antifungal substance was available, hence the absence of a positive control. The Petri plates were then incubated at 28±2°C. The incubation duration was dependent on the growth rate of the specific fungus. Mycelial growth diameter (in mm) was recorded after every 24 h until the control plates were completely covered.

Expression of results

Percentage inhibition

The percentage inhibition of fungal growth was calculated as compared to the control without essential oil by the formula:

$$\text{Inhibition } (\%) = \frac{D_c - D_t}{D_c} \times 100$$

Where D_c (mm) = diameter of fungal growth in the control dish; D_t (mm) = diameter of fungal growth in the test dish

Nature of inhibition

The fungistatic or fungicidal nature of the active essential oil was determined by transferring the inoculated discs from the Petri dishes which had 100% inhibition to a new plate with fresh PDA/chloramphenicol agar medium without essential oil. The absence of growth signifies death of inoculum hence the essential oil was fungicidal; the presence of growth signifies that essential oil was fungistatic.

For each fungal strain, a 3 x 6 factorial design (three essential oils, six concentrations) was used for data analyses. Means of percentage inhibition were compared by the Tukey test through one-way ANOVA using OriginPro 8.0 software. Graphs were plotted using Microsoft Excel 2016.

Effect of essential oil on fungal morphology

The effect of essential oil vapour on fungal morphology was studied by observation of exposed and non-exposed fungi species with a scanning electron microscope (Hafedh et al., 2010). At the end of incubation, fungal mycelia bearing distinct features were harvested with the help of sterile forceps and air-dried. This was to eliminate water from fungal hyphae and to preserve surface structure and prevent collapse of the cells when exposed to the SEM's high vacuum. Before viewing, dried samples were mounted in a JEOL JSC 1200 model coater and coated with a thin layer of gold to prevent the build-up of an electrical charge on the surface and to give a better image. The SEM used was a JEOL scanning electron microscope, JSM 5600 LV model.

RESULTS AND DISCUSSION

Extraction yield of essential oils

M. piperita had the highest extraction yield (4.20%), followed by *T. vulgaris* (2.93%) and *C. zeylanicum* (1.44%). These differences in yield might be due to the different metabolic rates and specific intrinsic properties of the plants. *M. piperita* and *T. vulgaris* from Pančevo, Serbia, (Soković et al., 2007) had yields of 3.2 and 3%, respectively. *C. zeylanicum* from Cocotomey-Atlantique, Southern Benin had a yield of 1.1% from dried leaves (Yehouenou et al., 2012). Differences among same plants might be as a result of differences in harvest time, the agro-ecological zones, postharvest treatments and processing conditions.

Chemical composition of essential oils

The compounds and their respective percentages present in the different essential oils as analyzed by GC/MS are shown in Table 1. All three essential oils were each composed of more than thirty compounds, most of which were monoterpenes, especially oxygenated monoterpenes.

T. vulgaris had 46.91% of oxygenated monoterpenes with major ones being thymol (35.12%), linalool (4.72%), camphor (2.38%) and carvacrol (2.01%), against 45.00% monoterpene hydrocarbons. Sesquiterpenes hydrocarbon made up 6.68% while oxygenated sesquiterpenes were only 1.25%. As for monoterpene hydrocarbons, the major constituents were *p*-cymene (25.36%), γ–terpinene (12.48%), myrcene (1.38%) and α-terpinene (1.09%). The main sesquiterpene was E-B-caryophyllane (4.72%), with the others below 1%.

The essential oil of the leaves of *C. zeylanicum* had 35 constituents. The dominant groups of the compounds were oxygenated monoterpenes (50.96%) and sesquiterpene hydrocarbons (27.91%). Monoterpene hydrocarbons constituted 17.74% while oxygenated sesquiterpene were only 2.18%. For individual constituents, E-cinnamaldehyde, eugenol and 2-hexyl-(Z)- cinnamalehyde were the dominant oxygenated monoterpenes with 13.03, 12.15 and 10.66%, respectively. Other oxygenated monoterpenes were eugenyl acetate (4.3%), E-cinnamic acid (3.26%) and linalool (2.8%). The major sesquiterpene hydrocarbons were E-B-caryophyllane (21.82%) and β-cubebene (4.22%). As for monoterpene hydrocarbons, the majority were α–pinene (6.31%), camphene (2.76%), limonene (2.5%) and β–pinene (2.24%). There were only two constituents of oxygenated sesquiterpenes: caryophellene oxide (1.94%) and guaiol (0.23%).

With respect to *M. piperita* essential oil, of the 34 identified compounds, oxygenated monoterpenes constituted 68.46%, monoterpene hydrocarbons constituted 26.30% while sesquiterpene hydrocarbons had 2.47%. Unlike *T. vulgaris* and *C. zeylanicum,* there was no oxygenated sesquiterpene. The major oxygenated monoterpenes were menthol (33.59%), menthone (18.47%), piperitone (4.66%), menthyl acetate (3.73%) and isoborneol (3.5%). Limonene (13.36%), α- pinene

Table 1. Chemical composition of essential oils of the studied plants.

S/N	RI	Compounds (in order of elution)	Relative percentage		
			T. vulgaris	*C. zeylanicum*	*M. piperita*
		Non-terpenes	**0.14**	**1.06**	**0.18**
1	-	Heptanal*	0.08	0.15	-
2	989	3 – Octan-2-one	0.06	-	0.18
3	956	Benzaldehyde	-	1.06	-
		MTH	**45.00**	**17.74**	**26.30**
4	922	α – Thujene	0.94	0.17	0.23
5	930	α – Pinene	0.89	6.31	8.21
6	945	Camphene	0.99	2.76	0.32
7	971	Sabinene	0.52	-	-
8	974	β – Pinene	0.19	2.24	0.69
9	985	Myrcene	1.38	0.42	1.24
10	1002	α – Phellandrene	0.11	1.25	-
11	1008	δ – 3 – Carene	-	0.16	0.90
12	1014	α-Terpinene	1.09	-	0.21
13	1021	*p* – Cymene	25.36	1.75	0.28
14	1025	Limonene	0.46	2.50	13.36
15	1031	(Z)-β-ocimene	-	-	0.09
16	1056	γ – Terpinene	12.48	-	0.34
17	1063	p – menthe-3,8-diene	0.57	-	-
18	1085	Terpinolene	-	0.19	0.44
		MTO	**46.91**	**50.96**	**68.458**
19	1028	1,8-Cineole	0.12	0.56	1.28
20	1095	Linalool	4.72	2.80	0.40
21	1122	(Z)-Epoxy-ocimene	-	-	0.44
22	1137	E-pinocarveol	-	-	0.22
23	1143	Camphor	2.38	-	0.23
24	1151	Nerol oxide	-	0.50	-
25	1154	Menthone	-	-	18.47
26	1159	Z-Isocitral	-	0.80	-
27	1163	Isoborneol	-	-	3.50
28	1169	Borneol	1.14	0.95	-
29	1174	Terpinen-4-ol	0.97	0.23	-
30	1176	Menthol	-	-	33.59
31	1177	Isomenthol	-	-	0.60
32	1187	α – Terpineol	-	0.49	0.55
33	1195	Myrtenol	-	0.19	-
34	1226	Cis-Carveol	-	0.31	-
35	1249	Geraniol	-	0.28	-
36	1253	Piperitone	-	0.29	4.66
37	1270	E - Cinnamaldehyde	-	13.03	-
38	1287	Safrole	-	0.21	-
39	1291	Menthyl acetate	-	-	3.73
40	1293	Thymol	35.12	-	-
41	1294	Carvacrol ethyl, ether	0.30	-	-
42	1299	Carvacrol	2.01	-	-
43	1355	Eugenol	0.16	12.15	-
44	1446	E-Isoeugenol	-	-	0.80
45	1456	E-Cinnamic acid	-	3.26	-

Table 1. Contd.

46	1525	Eugenyl Acetate	-	4.30	-
47	1765	2-hexyl-(Z)- Cinnamalehyde	-	10.66	-
		STH	**6.68**	**27.91**	**2.44**
48	1346	α-Cubebene	-	-	0.21
49	1371	α – Copaene	-	0.93	-
50	1378	β – Cubebene	-	4.22	-
51	1385	β – Bourbonene	0.21	-	-
52	1408	Z-Caryophyllene	-	-	0.30
53	1420	β-Cedrene	-	-	0.31
54	1423	E-B-Caryophyllane	4.72	21.82	-
55	1464	(E)- 9-epi-Caryophyllene	-	-	0.76
56	1477	D – Germacrene	0.21	-	0.24
57	1483	α – Murolene	0.78	-	-
58	1498	α – selinene	-	0.95	0.26
59	1515	γ – Cadinene	0.27	-	-
60	1524	δ – Cadinene	0.49	-	0.35
		STO	**1.25**	**2.18**	**0.00**
61	1587	Caryophellene oxide	0.98	1.94	-
62	1613	Guaiol	-	0.23	-
63	1621	Iso caryophyllene	0.03	-	-
64	1763	14-oxy-α-Muurolene	0.23	-	-

* Identified tentatively; - = absent; MTH = monoterpenes hydrocarbons; MTO = oxygenated monoterpenes; STH = sesquiterpenes hydrocarbons; STO = Oxygenated sesquiterpenes.

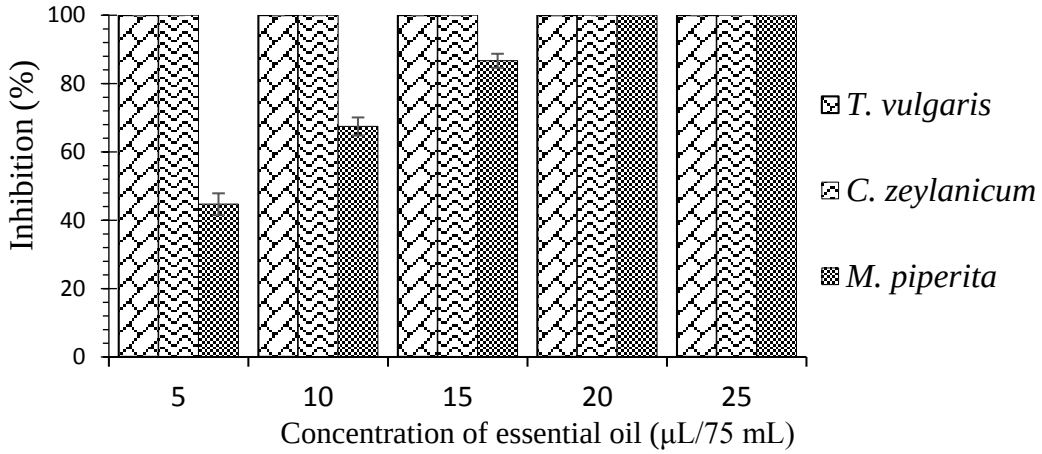

Figure 1. Percentage inhibition of essential oils on *R. oryzae* after 5 incubation days.

and myrcene (1.24%) were the major monoterpene hydrocarbons. All identified sesquiterpenes were below 1%.

In vitro inhibitory effect of essential oil vapour on fungal growth

Not all essential oil concentrations showed total inhibition; growth rate was different based on the essential oil type and the concentration. Incubation duration for *R. oryzae* lasted for 5 days as the control petri dish was completely covered. The percentage inhibition of mycelial growth of *R. oryzae* by different concentrations of essential oil vapour is as shown on Figure 1. Inhibition increased with increase in essential oil concentration. After five incubation days, all the essential oils showed total

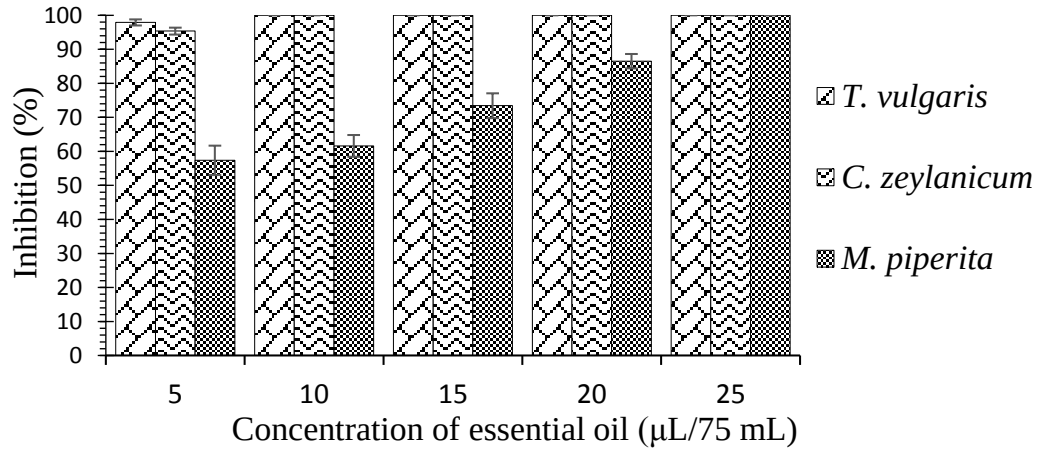

Figure 2. Percentage inhibition of essential oils on *A. tamarii* after 9 incubation days.

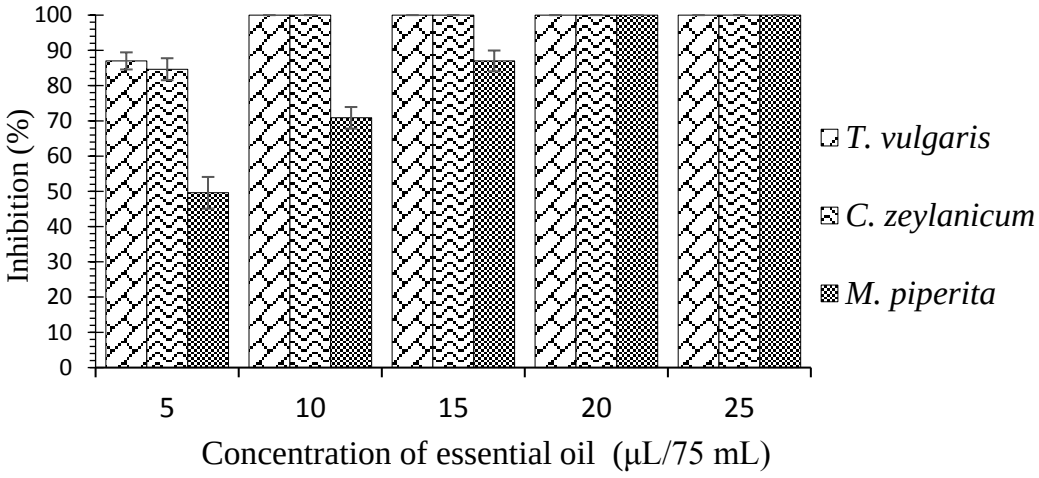

Figure 3. Percentage inhibition of essential oils on *A. parasiticus* after 8 incubation days.

inhibition at 5 µL for *T. vulgaris* and *C. zeylanicum*, and at 20 µL for *M. piperita*. According to the post-hoc Tukey test, there was no significant statistical difference (p>0.05) per concentration of *T. vulgaris* and *C. zeylanicum,* whereas *M. piperita* showed significant statistical difference (p<0.05) in inhibition rate at volumes up to 15 µL. Specifically, the percentage inhibition of *M. piperita* varied from 44.71 ± 3.21 (for 5 µL) to 100% (20 µL).

As illustrated in Figure 2, the higher the amount of essential oil, the greater the percentage inhibition, with 100% inhibition being attained with 10 µL essential oil of *T. vulgaris* and *C. zeylanicum*, and 25 µL for *M. piperita*. For *M. piperita*, there was a positive correlation between percentage inhibition and amount of essential oil. After nine incubation days, the Petri plates with 5 µL essential oil had the least percentage inhibition (57.38±4.32% for *M. piperita*). Differences in percentage inhibitions of *T. vulgaris* and *C. zeylanicum* were not significant statistically (p>0.05) at all amount of essential oils within the incubation period.

Figure 3 is an illustration of the inhibition of growth of *A. parasiticus* by different volumes of essential oils after eight days of incubation. As in the other cases, the least percentage inhibition was obtained at 5 µL with values of 49.61±4.50, 84.65±3.12 and 87.01±2.42% for *M. piperita, C. zeylanicum* and *T. vulgaris*, respectively. Differences in percentage inhibitions at all amounts of essential oils were not significant statistically for *T. vulgaris* and *C. zeylanicum*, meanwhile *M. piperita* showed statistically different (p<0.05) results per amount of essential oil up to 15 µL. The result of the percentage inhibition of essential oils on *R. stolonifer* after five incubation days is as illustrated in Figure 4.

T. vulgaris and *C. zeylanicum* exhibited total inhibition at 10 µL, while *M. piperita* showed total inhibition at 15

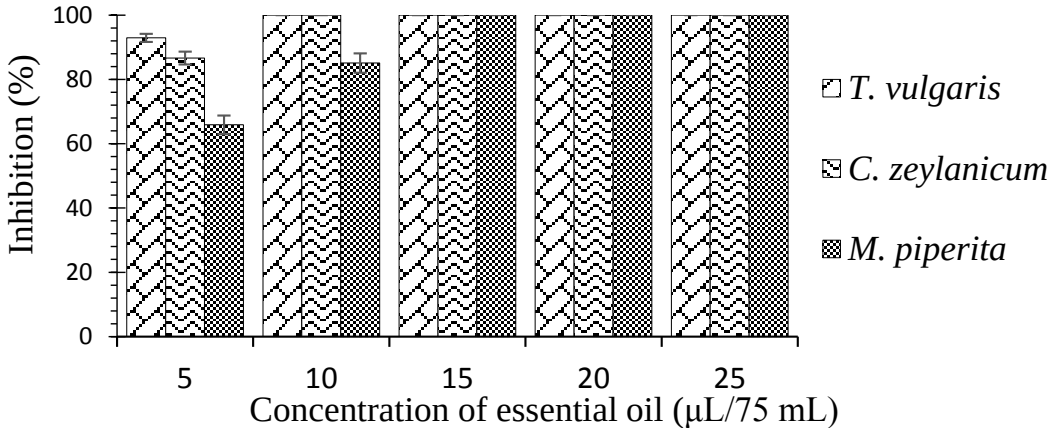

Figure 4. Percentage inhibition of essential oils on *R. stolonifer* after 5 incubation days.

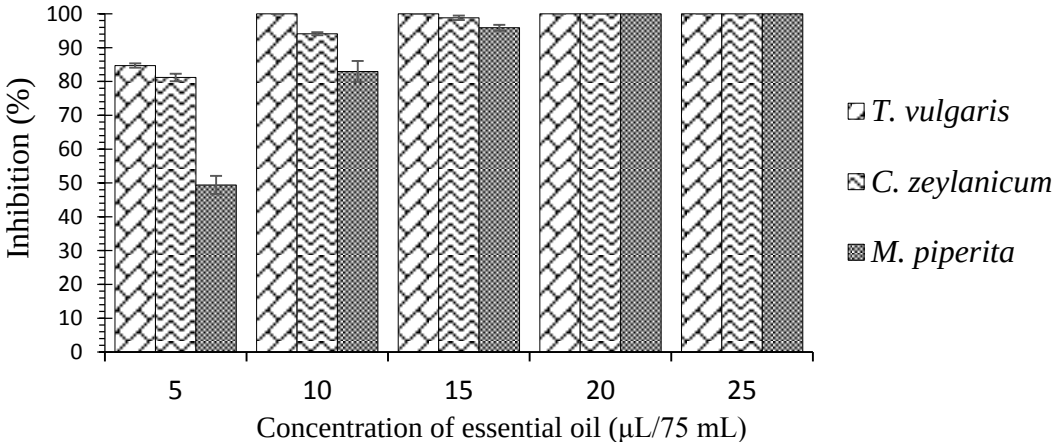

Figure 5. Percentage inhibition of essential oils on *A. flavus* after 8 incubation days.

µL. The minimum percentage inhibitions were 65.88±2.88, 86.67±2.02 and 92.94±1.24% for *M. piperita*, *C. zeylanicum* and *T. vulgaris*, respectively, values significantly different from each other (p<0.05). As in the other cases, especially as demonstrated by *M. piperita*, an increase in the amount of essential oil increased the inhibition rate. As illustrated on Figure 5, percentage inhibition of *A. flavus* increased with increasing amount of essential oils. Incubation lasted for eight days. Total inhibition was exhibited at 10 µL for *T. vulgaris* and 20 µL for *C. zeylanicum* and *M. piperita*. In contrast to the other fungal species, at volumes of 5 and 10 µL, all essential oils showed significantly different percentage inhibition from each other (p<0.05). The least percentage inhibition per essential oil was 49.41±2.66, 81.18±1.12 and 84.71±0.64 for *M. piperita, C. zeylanicum* and *T. vulgaris,* respectively. Incubation of *T. purpureogenus* lasted for nine days, and the percentage inhibition is as reported in Figure 6.

Essential oils of *T. vulgaris* and *C. zeylanicum* exhibited total inhibition at 5 µL, while *M. piperita* exhibited total inhibition at 20 µL. A minimum inhibition of 60.71±2.34% was recorded by *M. piperita* with a volume of 5 µL. There was no significant difference (p>0.05) in the activity of *T. vulgaris* and *C. zeylanicum* as both showed total inhibition at all volumes applied. However, at amounts of 15 µL and lesser, *M. piperita* showed statistically significant (p<0.05) results from the other essential oils, and at higher volumes, all three essential oils had similar outcomes. After noting the volume of essential oil for which total inhibition was exhibited from the preliminary tests, the minimum amount of each essential oil required for total inhibition was determined and is shown in Table 2.

Except for *A. tamarii* and *A. flavus,* there was no significant statistical difference between the MIC values for *T. vulgaris* and *C. zeylanicum.* Meanwhile, MIC values for *M. piperita* were statistically different from the other essential oils for all fungal species except for *A. flavus* were results with *C. zeylanicum* were statistically the

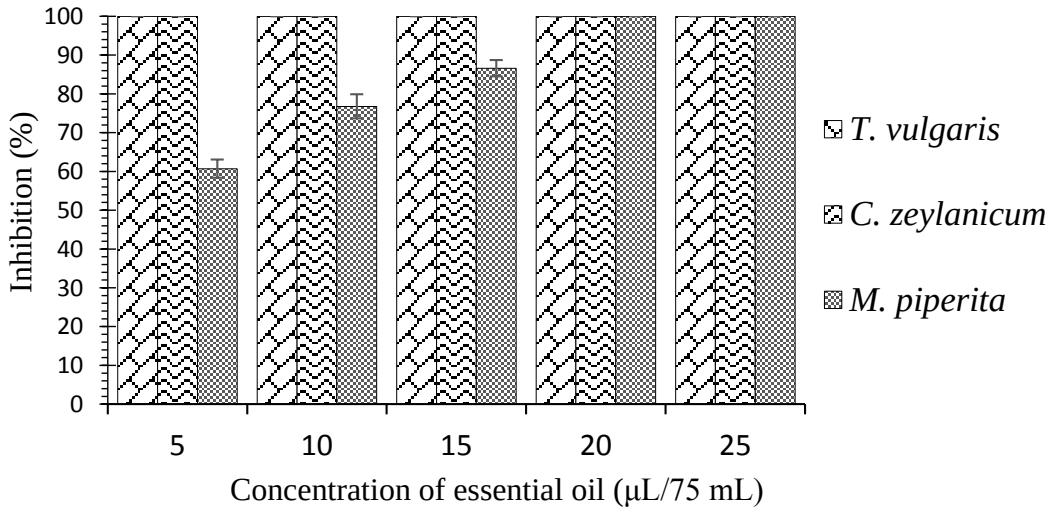

Figure 6. Percentage inhibition of essential oils on *T. purpureogenus* after 9 days.

Table 2. Minimum inhibitory concentration **(**MIC**)** of essential oils.

	MIC per respective fungus (µL/75 mL air space)					
	R. oryzae	***A. tamarii***	***A. parasiticus***	***R. stolonifer***	***A. flavus***	***T. purpureogenus***
T. vulgaris	3.7±0.6[a]	6.0±0[a]	9.0±1[a]	7.3±0.6[a]	8.3±0.6[a]	4.3±0.6[a]
C. zeylanicum	4.7±0.6[a]	9.0±1[b]	8.7±0.6[a]	8.0±0[a]	16.3±0.6[b]	5.3±0.6[a]
M. piperita	18.0±0[b]	23.0±1[c]	18.0±1[b]	12.7±0.6[b]	17.3±0.6[b]	17.7±0.6[b]

Values bearing same letter on the same column are not statistically different (p<0.05) according to the Tukey test.

same (p>0.05). On a general note, *T. vulgaris* was the most active, followed by *C. zeylanicum* then *M. piperita*.

The antifungal activity of these essential oils is a function of their individual chemical compositions. Essential oil components either exhibit synergism, additive, antagonistic or can portray individual properties (Burt, 2004). The presence of phenolic compounds in the different essential oils renders them good antifungal agents. Thymol, linalool, carvacrol and eugenol are indications of an outstanding antifungal potential (Hyldgaard et al., 2012) as in the case of *T. vulgaris* essential oil. The relative high percentage of cinnamaldehyde and its derivatives in *C. zeylanicum* essential could be responsible for its antifungal activity (Carmo et al., 2008). In addition, the presence of other components such as caryophyllane (21.82%), α-pinene (6.31%), eugenol acetate (4.3%) could also confer antifungal activity.

The carvacrol precursor *p*-cymene, on its own, is not an excellent antifungal agent (Aligiannis et al., 2001; Bagamboula et al., 2004), but will boost the activities of components with functional side groups (Ultee et al., 2000; Rattanachaikunsopon and Phumkhachorn, 2010). The presence of *p*-cymene therefore in the essential oils, especially in *T. vulgaris* and *C. zeylanicum* is a strong indication of increased antifungal activity. Menthol and menthone components of essential oils are very good antifungal compounds (Sulaiman, 2013). These two components, in addition to α-pinene, limonene may be responsible for the antifungal activity of *M. piperita.* The presence of phenolic compounds like carvacrol, thymol, eugenol and menthol increase the antimicrobial activity of essential oils. This is attributed to the presence of an aromatic nucleus and a phenolic -OH group known to be reactive and to form hydrogen bonds with active sites of target enzymes (Velluti et al., 2003).

Nature of inhibition

Upon re-inoculation of mycelial discs of Petri dishes that showed total inhibition in freshly prepared culture medium, the three essential oils showed different nature of inhibitions, depending on their concentration. Even at the maximum tested concentration of 25 µl/75 ml air space, the action of *M. piperita* essential oil was fungistatic on all tested fungi. *C. zeylanicum* was fungicidal at 25 µl/75 ml air space to *R. oryzae* and *T. purpureogenus*, and fungistatic to the other tested fungi species. *T. vulgaris* was fungicidal to *T. purpureogenus* at

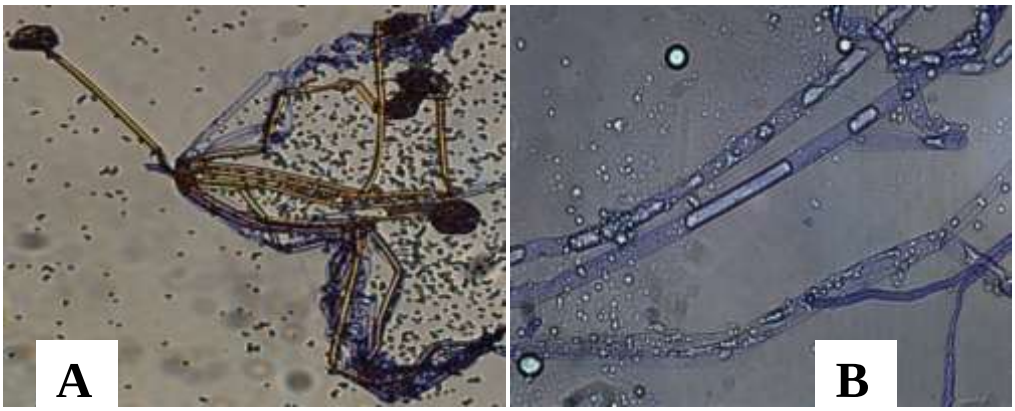

Figure 7. Unexposed (A) and exposed (B) *R. oryzae* to *C. zeylanicum* essential oil vapour as viewed with an optical microscope at 20 and 40x magnification, respectively after staining with lactophenol cotton blue.

20 µl/75 mL air space, and at a concentration of 25 µl/75 mL air space, was fungicidal to *R. oryzae, A. tamari* and *A. flavus.* Its action on *R. stolonifer* and *A. parasiticus* was only fungistatic.

Morphological change of fungi exposed to essential oil vapour

Morphological change due to exposure to essential oil vapour was studied in order to determine the site of action. Because of their relatively rigid vegetative and distinct features (Sporangiophores (specialised aerial hyphae), sporangia and spores), *R. oryzae* and *R. stolonifer* were used to elaborate the change in fungal morphology after exposure to essential oil vapour. Figure 7 shows images of *R. oryzae* unexposed and exposed to *C. zeylanicum* vapour as taken by an optical microscope.

R. oryzae not exposed to essential oil vapour displayed characteristic rigid hyphae bearing sporangia and spores. This rigid vegetative body was evident by the lactophenol cotton blue that stains the chitin portion of the fungal cell wall, giving it a good contrast. Upon exposure to essential oil vapour, the vegetative bodies of the fungi lost their rigidity, leading to poor or no development of sporangia. Since these spore forming bodies were absent, it justified the complete absence of fungal spores. At the level of the hyphae, treated samples showed poor colouration when stained with lactophenol cotton blue dye, because a higher microscopic magnification (40×) was used. The major component of the fungal cell wall, chitin, was therefore not well developed.

The 3-dimensional views of *R. oryzae* and *R. stolonifer* unexposed and exposed to *C. zeylanicum* essential oil vapour as viewed with a scanning electron microscope (SEM) are as shown in Figures 8 to 11. In the absence of essential oil (Figures 8 and 10), *R. oryzae* and *R. stolonifer* showed distinct fungal structures: rigid hyphae, bearing sporangia with spores. The presence of essential oil vapour led to abnormal fungal growth. As a result, hyphae were not well developed, lost fungal rigidity, had no sporangium formation and hence, complete absence of spores. The lost in rigidity implies poorly developed cell wall, leading to a change in morphology. These findings are complementary with that of other researchers who put to evidence the poor development of *A. niger* by *C. zeylanicum* essential oil (Carmo et al., 2008). This disruption of the cell wall will definitely lead to leakage of cytoplasmic content of cell. These modifications in the cytological structure may be related to the interference of the essential oil with enzymes responsible for cell wall synthesis (Shukla et al., 2000).

The antifungal property of essential oils could involve inhibition of extracellular enzymes synthesis and the disruption of the cell wall structure resulting in lack of cytoplasm, damage of integrity and ultimately mycelial death. Cytoplasm granulation, cytoplasm membrane rupturing, cytoplasm hyperacidity and break down of the electron transport chain are some structural and metabolic events possibly related to the antifungal property of essential oils (Lopez-Diaz et al., 2002; Hyldgaard et al., 2012). It is also reported that essential oils are able to interfere with the mitochondrial membrane system by a membrane-disruptive activity closely associated with the enzymatic reactions, such as respiratory electron transport, protein transport and coupled phosphorylation (Atanda et al., 2006; Rasooli et al., 2006).

Conclusion

The antifungal activity of the vapour phase of the essential oils of *T. vulgaris*, *C. zeylanicum* and *M. piperita* could be attributed to major components present in the respective essential oils. The high concentration of

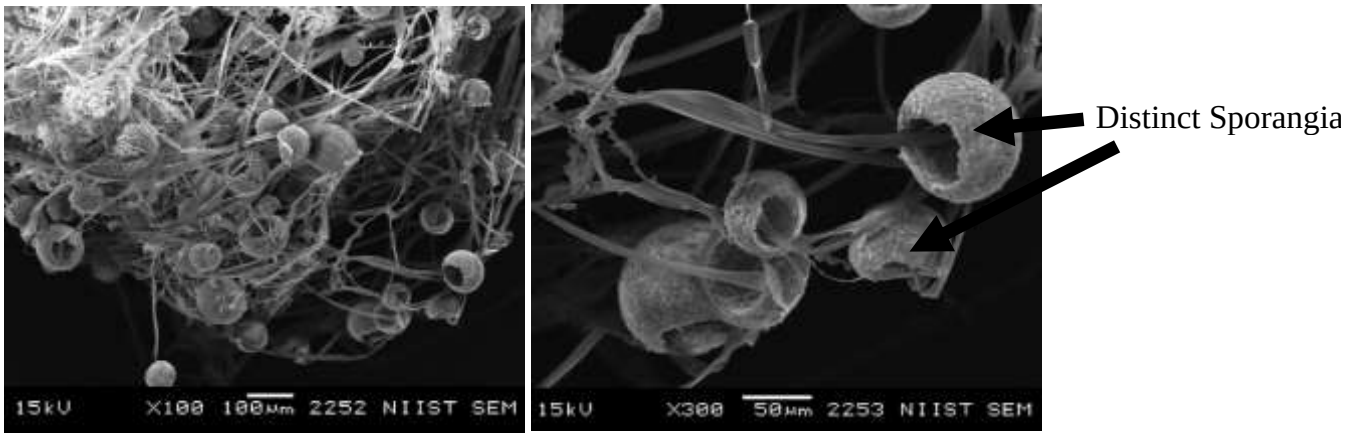

Figure 8. *R. oryzae* unexposed to essential oil vapour as viewed with a SEM.

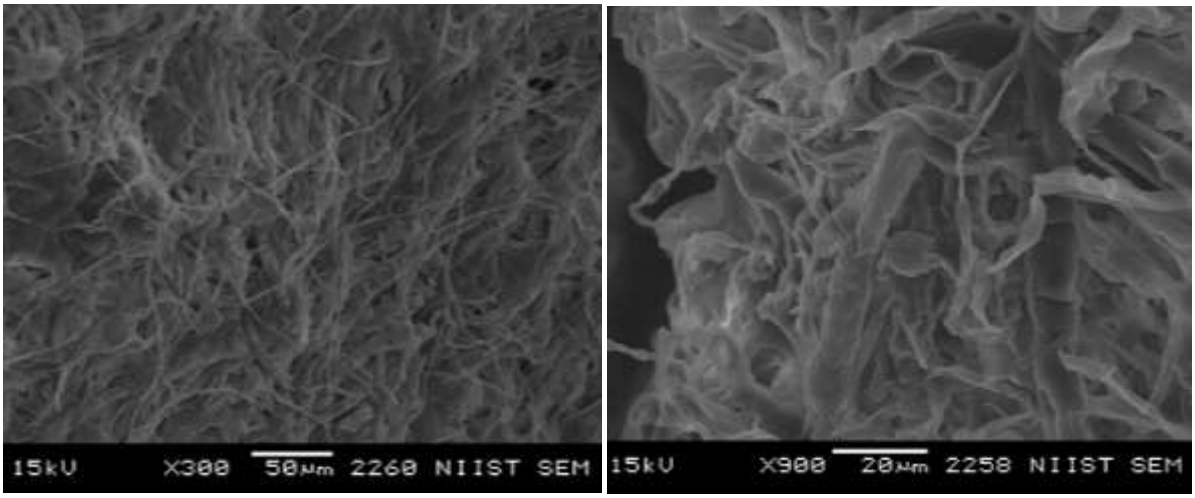

Figure 9. *R. oryzae* exposed to *C. zeylanicum* essential oil vapour as viewed with a SEM.

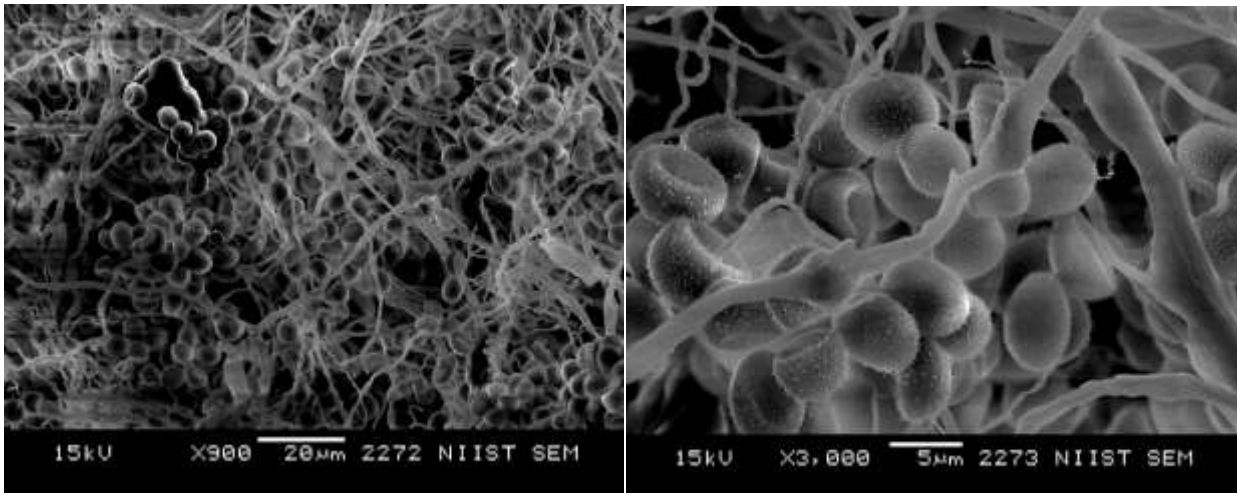

Figure 10. *R. stolonifer* non-exposed to essential oil vapour as viewed with a SEM.

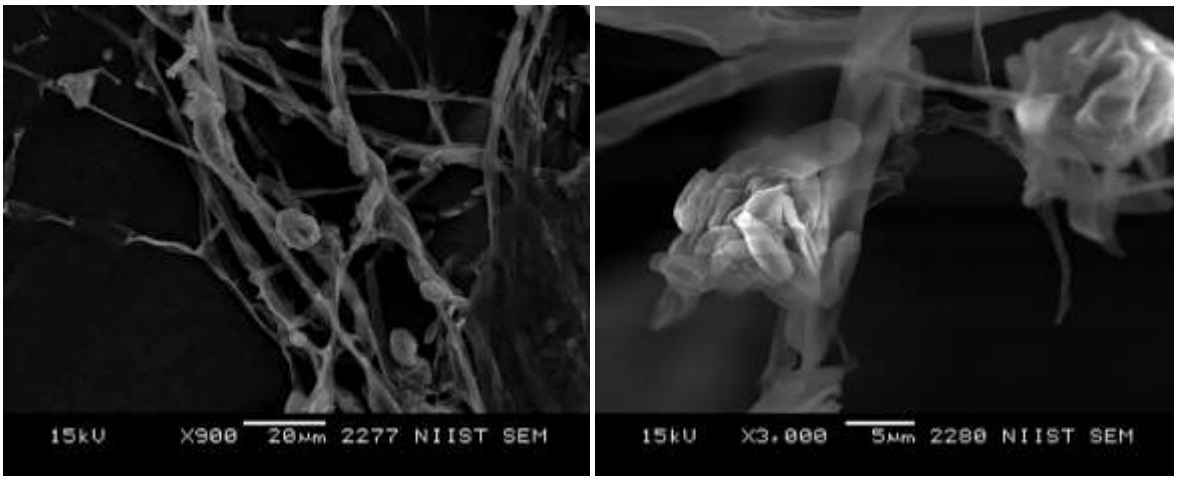

Figure 11. *R. stolonifer* exposed to *C. zeylanicum* essential oil vapour as viewed with a SEM.

oxygenated monoterpene contributed to the increased antifungal activity of the essential oil against tested fungi. Improper development of fungi in the presence of essential oils portrays the fungicidal potential of these essential oils. The fact that essential oils of *T. vulgaris*, *C. zeylanicum* and *M. piperita* are generally recognised as safe gives more virtue for these natural products to be applied conveniently, especially in the vapour phase, as a food preservative instead of synthetic fungicides with multiple site effects.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors sincerely acknowledge The World Academy of Science (TWAS) and the Council of Scientific and Industrial Research (CSIR) India for financial support; the National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), particularly, the Division of Process Engineering and Environmental Technology (PEET) for enabling them carry out this work in their laboratory; and ENSAI of the University of Ngaoundere for laboratory analysis and supervision.

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Vol. 16(9), pp. 400-407, 1 March, 2017
DOI: 10.5897/AJB2016.15840
Article Number: E0E064C63001
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Silver nanoparticles biosynthesized by secondary metabolites from *Moringa oleifera* stem and their antimicrobial properties

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Received 16 December, 2016; Accepted 10 February, 2017

Biological extracts have recently shown a great potential for rapid biosynthesis of silver nanoparticles (AgNPs) with antibacterial activity. AgNPs were synthetized by reduction effect of secondary metabolites produced by the *Nigrospora* sp. fungus, from *Moringa oleifera* stem as a reducing agent and silver nitrate ($AgNO_3$) (1 mM) as salt precursor. The synthesis of nanoparticles (NPs) was monitored through analysis of the UV-Vis spectroscopy absorption in the 436 to 440 nm range indicating the presence of AgNPs in the colloidal aqueous solutions. Fourier transform infrared spectroscopy (FTIR) spectra were performed to identify the compounds responsible for the bio reduction of the Ag^+. The morphology and sizes of AgNPs were characterized by scanning electron microscopy (SEM), energy dispersive of X-ray spectroscopy (EDS), dynamic light scattering (DLS), and colloid stability by zeta potential measurements. The NPs obtained were spherical in shape with size in the 3 to 70 nm range. Antibacterial activity was confirmed by evaluation of their effect against *Escherichia coli, Klebsiella cloacae* and *Staphylococcus epidermidis*. The proposed green synthesis of AgNPs from secondary metabolites produced by the *Nigrospora* sp. fungus from *M. oleifera* stem can be strongly recommended as a potential method for biomedical application.

Key words: Silver nanoparticles, secondary metabolites, green chemistry, antibacterial activity.

INTRODUCTION

One of the most important areas of nanotechnology is biomedicine, where metallic nanoparticles have been used for different applications such as controlled release of drugs, synthesis of new medicines, tumor cells detection, among others (Ghazwani, 2015). AgNPs are gaining particular attention due to their desirable properties like optical, magnetic, electronic, biological, catalytic and antibacterial activity. Chemical and physical

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methods have been used to synthetize silver nanoparticles but most of these techniques are not environmentally friendly (Suarez-Cerda et al., 2015). Biological methods currently employ microorganisms, plants and their extracts (Kuppusamy et al., 2014). Many studies have proven that the plant extracts act as potential precursors for the synthesis of nanomaterials in a non-hazardous way. Plant extracts contain metabolites which act as reducing and stabilizing agents for bioreduction of metallic NPs (Anjum and Abbasi, 2016; Kuppusamy et al., 2014). Recent reports reveal green synthesis of silver nanoparticles using primary metabolites of the plants extracts as reducing agents, for example leaf extract of *Moringa* (Prasad and Elumalai, 2011), extracts of *Prosopis glandulosa* (Abdelmoteleb et al., 2016), and of *Vitex negundo* plants. Antimicrobial activity against human pathogens has also been found suitable for the biosynthesis of AgNPs (Kathireswari et al., 2014). However, most of these techniques do not use secondary metabolites as reducing agents. Silver nanoparticles can acts as effective and alternative bactericide agents for combating bacterial drug resistance problems (Ahmed et al., 2015). The surface area of the NPs ensemble becomes larger as the particle size decreases, which in turn increases the total surface available for contact with bacteria, increasing the antibacterial efficiency of AgNPs (Ghazwani, 2015).

The aim of this paper was to synthesize AgNPs by the reducing effect of secondary metabolites produced by the activity of fungi and bacteria, particularly the *Nigrospora* sp. fungus from *M. oleifera* stem, in an aqueous solution of silver nitrate. The AgNPs formation was evaluated by UV-Vis, fourier transformed infrared (FTIR) spectroscopy, dynamic light scattering (DLS), scanning electron microscopy (SEM) and electron dispersive X-ray (EDS). The synthetized AgNPs exhibit antibacterial properties against different pathogenic microorganisms.

MATERIALS AND METHODS

Stem and reagents/reactants

The chemicals used in this study were from Sigma (Bangalore, India) and Merck (Mumbai, India) with specifications of the American Chemical Society (ACS). *M. oleifera* stem samples were harvested from trees cultivated in Mexicali, Baja California, Mexico. The stem samples were previously washed with 1% v/v hypochlorite aqueous solution and distilled water to remove the dust. The sample was macerated in a mortar and dried at room temperature.

Preparation of the reductant precursors

Plant extract was prepared according to the methods of Prasad and Elumalai (2011) with some modifications. Ten grams of fresh-dried macerated *M. oleifera* stem in 1 L of water were heated for 20 min at 60°C to extract the active ingredients into the aqueous phase. After that, the solution was kept in incubation to permit the growing of microorganisms contained in the plant; endophytic bacteria and fungus. The main developed microorganism in the culture was the *Nigrospora* sp. fungus, which has been reported to produce bioactive secondary metabolites from *M. oleifera* stem extracts, mainly griseofulvin, dechlorogriseofulvin, 8-dihydroamulosin and mullein (Zhao et al., 2012). The culture was grown during 10 days and then the supernatant with metabolites was employed for the synthesis.

Biosynthesis of AgNPs

Working solutions with different v/v ratios of supernatant from *M. oleifera* stem ferments were prepared using 1 mM silver nitrate. The synthesis of AgNPs was conducted at room temperature with ratios of 1:1 (A), 1:5 (B) and 1:10 (C) between the silver nitrate and the supernatant. The reaction was carried out during 10 min. The bioreduction of Ag^+ ions was observed by color change from faint yellow to yellow-brown, indicating the formation of AgNPs (Bello et al., 2015; Netala et al., 2016). The reaction mixtures were poured into a test tube and the separation was carried out using a centrifuge (10000 rpm/10 min). The supernatant was extracted with a micropipette and the pellet was suspended in 10 mL acetone and then in distilled water. This centrifugation and resuspension processes were repeated 3 to 4 times. The resulting AgNPs were air dried to evaporate excessive liquid and these were used for further characterization.

Characterization of AgNPs

The progress of reaction was followed spectrophotometrically using a Perkin-Elmer UV-Vis Lambda 25 spectrophotometer. UV-visible spectroscopy is an important technique to establish the formation and stability of NPs in solution (Dhand et al., 2016). The scan was recorded from 300 to 600 nm, showing a characteristic peak appearing within the range of 440 to 450 nm. FTIR was carried out to identify the molecules corresponding to the metabolites involved in reduction, capping and stabilization of the synthetized AgNPs (Haghighi Pak et al., 2016). AgNPs were recorded using a Perkin-Elmer Spectrum One in ATR mode in the 4000 to 500 cm^{-1} range. The presence of AgNPs was confirmed by EDS, and the surface characterization was performed by SEM analysis. This was done using a JEOL JSM-6010L, with an accelerating voltage of 10 kV and a STEM support. Zeta potential and hydrodynamic sizes of the synthesized NPs were determined by introducing 3 mL of sample in the Nanotrac Wave instrument.

Determination of antimicrobial activity by well-diffusion method

The antimicrobial activities of the NPs were confirmed by well diffusion method against pathogenic microorganisms (Kim et al., 2007). Pure cultures of *Escherichia coli* (ATCC-25922), *Klebsiella cloacae* (ATCC-23355) and *Staphylococcus epidermidis* (ATCC-12228), Manassas, VA, USA were used for antibacterial analysis. Approximately, 80 mL of trypticase soy were dispersed on sterilized Petri dishes. One hundred micro liter (about 100 CFU/mL) of each bacterium was spread uniformly onto the individual plates using sterile cotton swabs. The dried AgNPs were dissolved in distilled water and used immediately. 30 µL of different ratios of AgNPs, 1:10 (A), 1:5 (B) and 1:1 (C) were loaded to each well through a micropipette (positive control), 30 µL of $AgNO_3$ solution and a blank was prepared without AgNPs; all sets were incubated at 37°C for 24 h. Afterwards the zone of inhibition (ZOI) was measured (Haghighi Pak et al., 2016; Lokina et al., 2014). The antimicrobial activity was realized for triplicate for each microorganism.

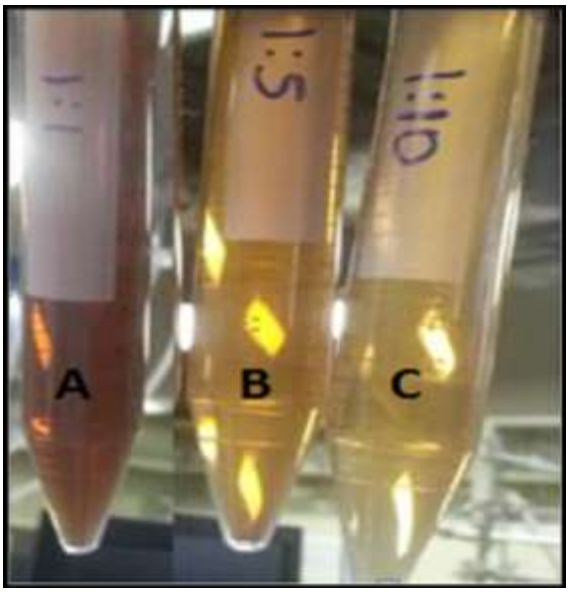

Figure 1. Color variation of AgNPs reaction with different ratios: supernatant/$AgNO_3$ (a) 1:1, (b) 1:5, (c) 1:10.

RESULTS AND DISCUSSION

The collaborative action of biomolecules in the supernatant was visualized through change of the color of the solutions. The supernatant presented the usual faint yellow color of media before reacting with $AgNO_3$. The color tones changed, turning from yellow to brown, indicating that NPs with different sizes were formed (Figure 1). This result indicates that the secondary metabolites obtained from the metabolic activity of *Nigrospora* sp. fungus in *M. oleifera* stem aqueous extracts were able to reduce the silver nitrate precursor to AgNPs.

Characterization by UV-Visible and FTIR spectroscopy

AgNPs have free electrons, which give an increment of the surface plasmon resonance (SPR) absorption band due to the interaction of electrons of metal nanoparticles with the light wave (Elumalai et al., 2015; Ghaedi et al., 2015; Gurunathan et al., 2009). A shift of the band maximum from 440 to 436 nm is observed in the UV-Vis spectra depending on the supernatant/$AgNO_3$ ratio (Figure 2). The shift corresponds to change of color, which is due to reduction of Ag^+ and formation of AgNPs. The results show that the ratio of supernatant plays an important role in the control of nanoparticle formation. The narrow peak was observed when supernatant was mixed with $AgNO_3$ solution in a 1:10 ratio, indicating yield of nanoparticles. As the supernatant/$AgNO_3$ ratio decreases, the absorption signal increases because more Ag^+ are available for interaction with biomolecules from the supernatant, thereby a more intense color results. Also, increasing number of silver nanoparticles were formed as a result of reduction of Ag^+. We can affirm that bioactive compounds in the ferment in the *Nigrospora* sp. fungus were responsible for the accelerated reduction and stabilization of Ag-NPs.

Figure 3 shows the FTIR spectrum of colloid suspension with AgNPs synthetized using *M. oleifera* stem ferment. The broad band at 3593 cm^{-1} may be related to OH^- groups present in biomolecules. The band observed at 2917 to 2843 cm^{-1} was assigned to the aliphatic C–H group. The peak around 1624 cm^{-1} corresponds to C=O stretching vibration, while the peaks observed at 1541 and 1341 cm^{-1} correspond to secondary amine group and to symmetric bending of CH_3, respectively. The peak at 1274 cm^{-1} is due to SO_3^- stretching vibration and the one at 1077 cm^{-1} corresponds to C=O bonds of ether, ester or phenol. Comparison with standard library reveals the presence of characteristic peaks of β-carotenes, flavonoids and tannins, which are actively involved with enzymes in the reduction and stabilization of the AgNPs.

Zeta potential

Zeta potential measurement is an important analysis because it complements the other characterizations and gives information for the solubility, physical stability, colloidal dispersity, and velocity dissolution of the NPs in the suspensions. The measured Zeta potential was -200 mV, indicating physical stability of nanoparticles synthetized.

Analysis of AgNPs size distribution

Samples A and B present size distributions with maximums of ~15 and 6.5%, respectively, centered at approximately 35 nm (Figure 4). The distribution maximum of sample C is ~38% at ~3 nm. In addition, the nanoparticles in sample C exhibit better uniformity compared to samples A and B seen as much narrow distribution. The average size of the AgNPs in samples A, B and C was 70, 25 and 3 nm, respectively. The obtained results demonstrate that the supernatant/$AgNO_3$ ratio has an important role in controlling the growth and size of AgNPs during the green synthesis.

SEM and EDS analysis of AgNPs

SEM results on Figure 4 show that the nanoparticles were spherical in shape. Sample A contains particles with larger sizes due to the increment of silver nitrate. This

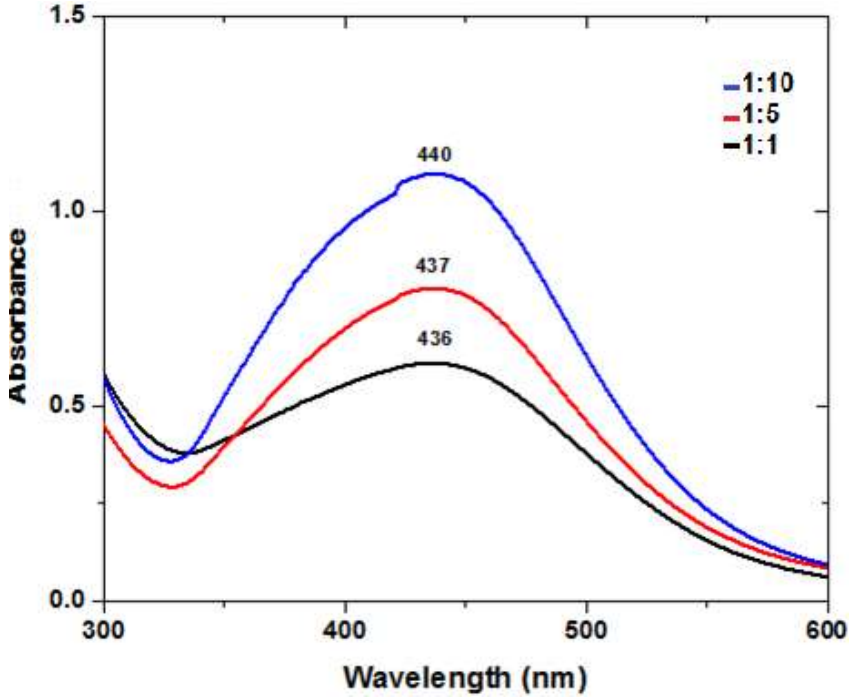

Figure 2. UV-Vis absorption spectra of synthesized AgNPs biosynthesized.

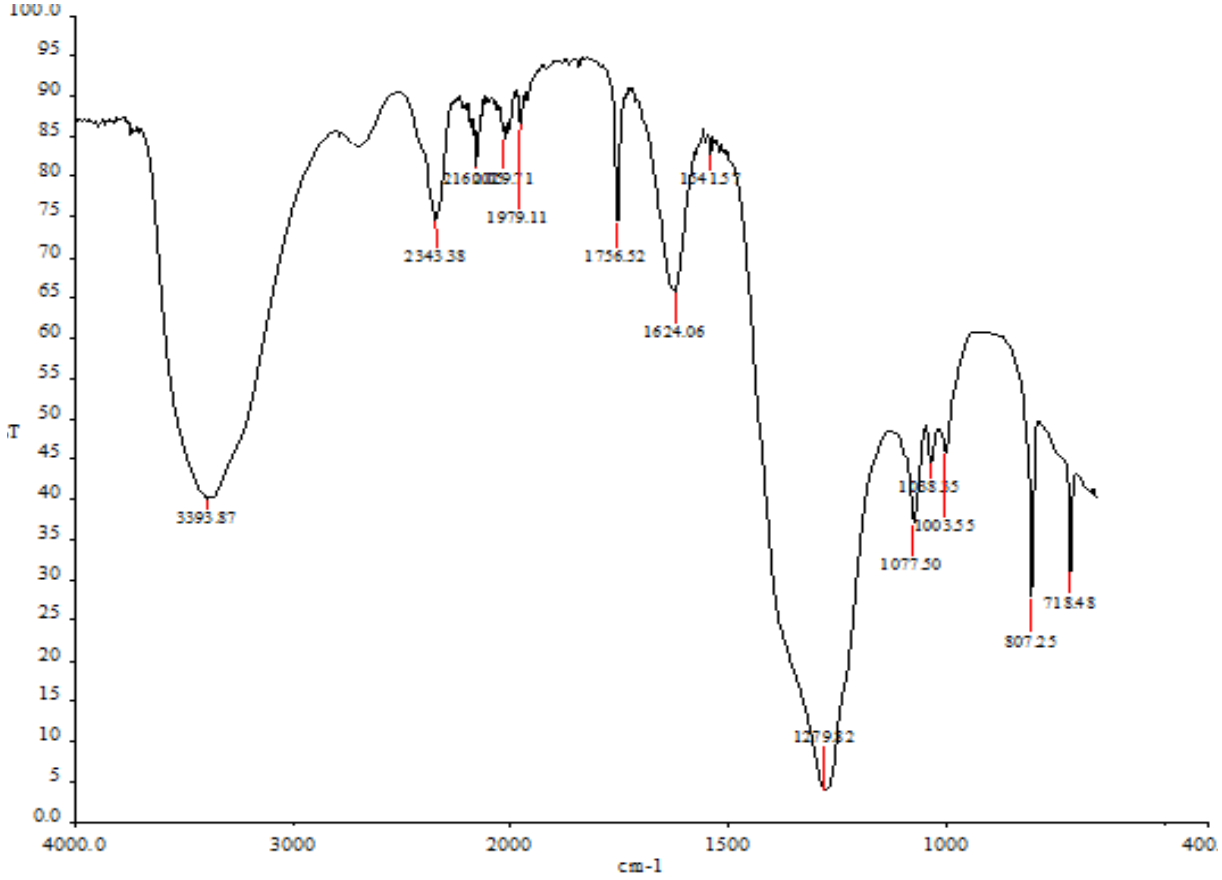

Figure 3. FTIR spectrum *Moringa Oleifera´s* stem of synthetized AgNPs.

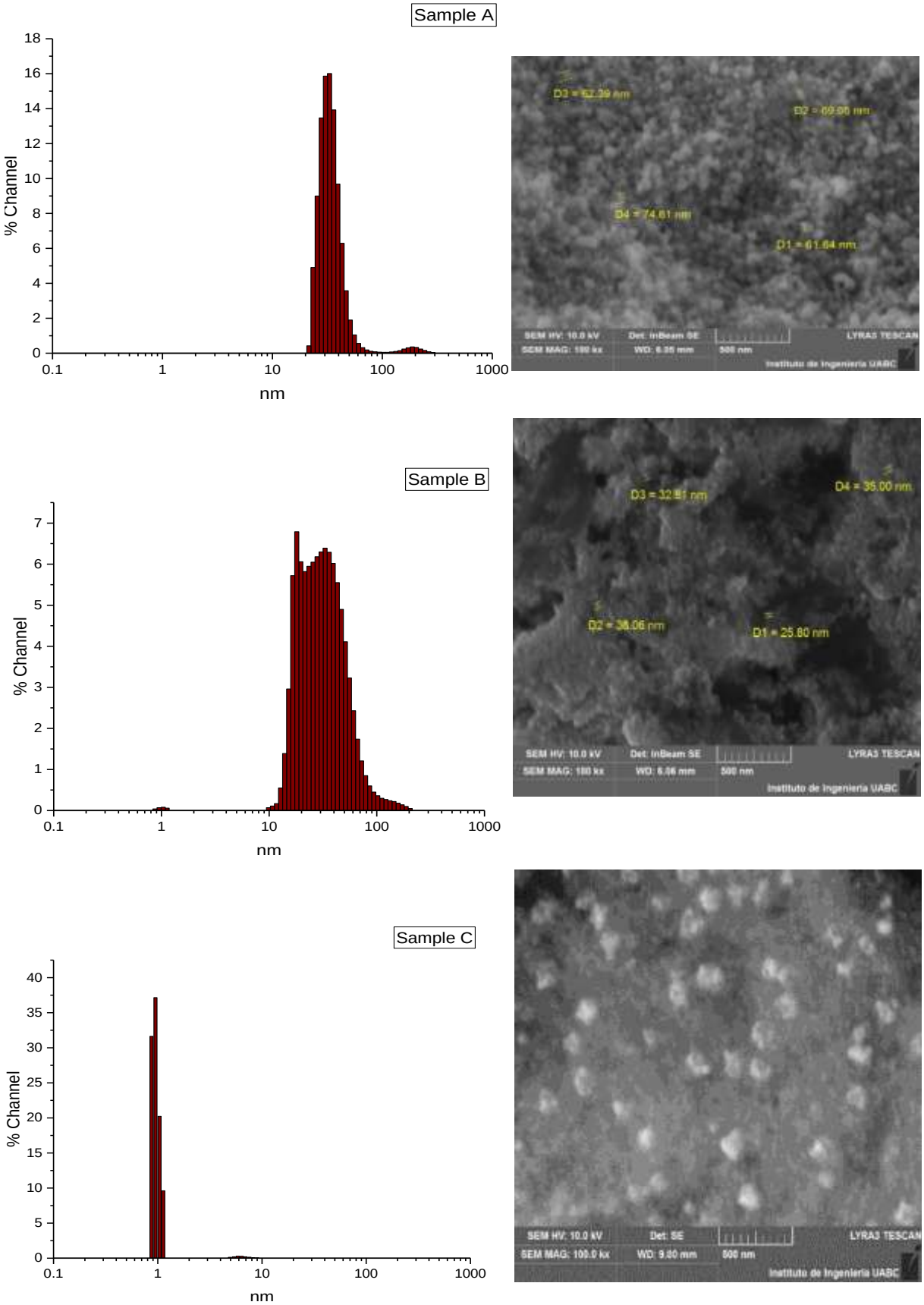

Figure 4. DLS analysis samples A, B and C, respectively, and SEM images of synthesized AgNPs.

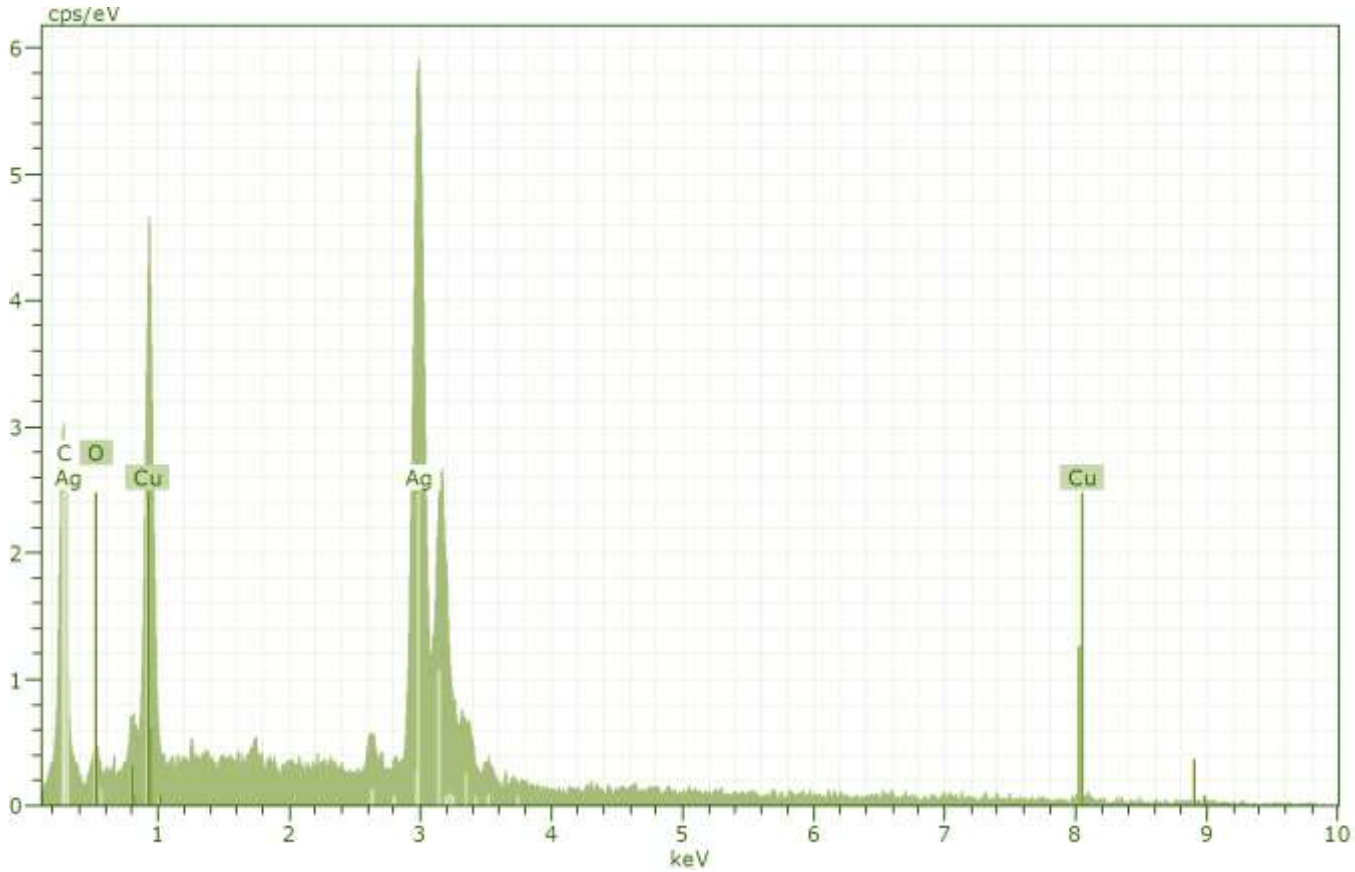

Figure 5. EDS spectrum of synthetized AgNPs.

favors the silver ions reduction, which leads to nanoclusters of AgNPs and formation of larger agglomerates in this solution. The EDS results for the three types of colloidal solutions were very similar. In Figure 5 the EDS spectrum of sample A is presented. The main peak is due to silver; the peaks of copper are result of samples preparation on a copper grid. The analysis of SEM and DLS are congruent.

Antimicrobial activity of AgNPs against pathogenic microorganism

Zone of inhibition found for *E. coli* (*E. coli*), *S. epidermis* (*S. epidermidis*) and *K. cloacae* (*K. cloacae*) were 19.5, 19 and 18 mm respectively (Figure 6). Results in Table 1 shows minimal level bacterial inhibition of $AgNO_3$ solutions and blank (ferments alone) in comparison to obtained silver nanoparticles in this study. Results confirm that biosynthesized AgNPs presents antibacterial properties. The antibacterial activity is similar to the reference antibiotic reported (Prasad and Elumalai, 2011). The mechanism bactericide of AgNPs is not fully explored. Maybe antibacterial activity is due to the AgNPs bind of the cell membrane through covalent and ionic bonding, causing changes in its permeability which affects their respiratory functions. They penetrate bacteria, damaging structures containing sulfur and phosphorus based functional groups such as the DNA chains; it also contributes to the bactericidal effect of the Ag^+ ions emerging from the surface of the AgNPs (Abdelmoteleb et al., 2016).

Conclusion

This study demonstrated the synthesis of AgNPs by the reducing effect of secondary metabolites produced by the activity of *Nigrospora* sp. fungus, based on a simple, safe and green method. AgNPs synthesis was achieved in a short time after mixing the supernatant with the silver nitrate solution. The NPs obtained were characterized by UV-Vis, DLS and SEM-EDS spectroscopy. The characteristic signal in the colloidal suspensions is proven to be sensitive to the detection of AgNPs because they showed a strong absorption peak because the silver nanoparticles exhibit excitation of SPR. UV-Visible analysis showed that the absorption peaks depends on the ratios employed due to the absorbance peaks that

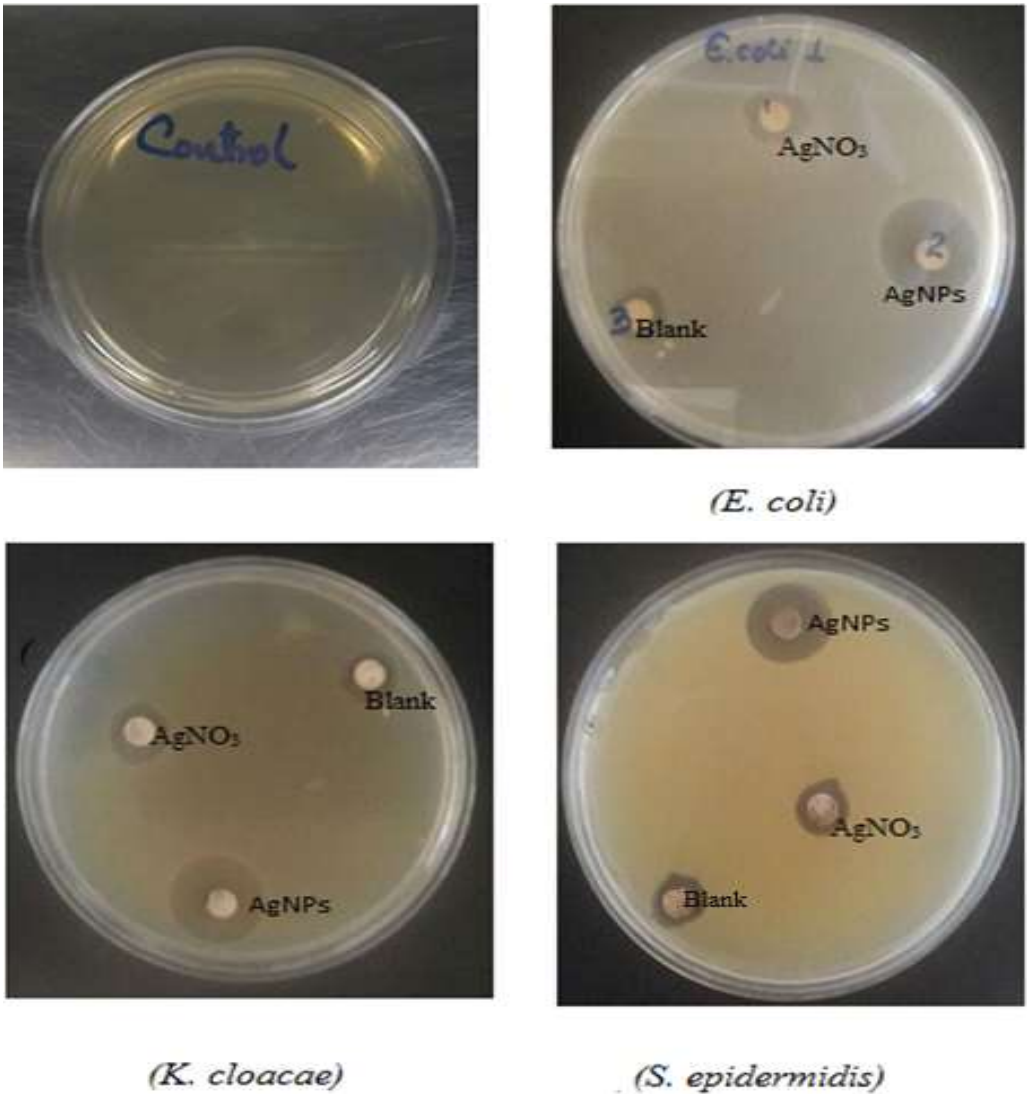

Figure 6. Antibacterial activity of synthetized AgNPs.

Table 1. Inhibition performance of synthetized AgNPs against pathogenic microorganisms.

Organism	Zone of inhibition (mm) (30 µL)		
	Blank (supernatant alone) (mean±SD)	1 mM $AgNO_3$ (mean±SD)	AgNPs (1:1 ratio) (mean±SD)
E. coli	1.2±0.01	2.3±0.01	19.5±0.09
K. cloacae	1.1±0.04	2.9±0.06	18±0.12
S. epidermidis	2.3±0.02	3.4±0.02	19±0.10

Values, mean + SD indicates the replicates of three tests. AgNPs, silver nanoparticles; SD, standard deviation.

decreased as the size of the nanoparticles increased. SEM images shows that sizes of particles observed were well-defined in the 3 to 70 nm range with spherical shapes. EDS analysis of AgNPs confirmed the presence of elemental silver. AgNPs obtained showed antibacterial activity; inhibiting the growth of *E. coli*, *K. cloacae* and *S. epidermidis.* Results contribute to the development of new routes of synthesis utilizing secondary metabolites. Bioactive compounds by *Nigrospora* sp., fungus from *M.* oleifera stem stands as potential candidates for biosynthesis and stabilizer of AgNPs in biomedical applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

E. C. Arrieta gratefully acknowledges the financial support of CONACYT-Mexico, for Master´s Fellowship (No. 599211) and Universidad Autónoma de Baja California for the use of their facilities.

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Vol. 16(9), pp. 408-417, 1 March, 2017
DOI: 10.5897/AJB2015.14885
Article Number: 43907DC63005
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Biodegradation of monocrotophos by bacteria isolated from soil

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Received 24 July, 2015; Accepted 13 October, 2016

Bacteria were isolated by enrichment culture technique from groundnut (*Arachis hypogaea* L.) soils and tested for their ability to degrade monocrotophos in mineral salts medium under aerobic conditions in the laboratory. Based on some of the morphological and 16S rRNA gene sequence analysis, the isolates were identified as *Rhodococcus phenolicus* strain MCP1 and *Rhodococcus ruber* strain MCP-2. The initial (0-day) recovery of monocrotophos in the culture medium was 94%; and by the end of 4th day, about 21% of added monocrotophos was lost from the uninoculated medium. By the end of 1st, 2nd, 3rd and 4th day 13, 20, 24, 30% and 18, 33, 37, 45% of monocrotophos was degraded, by *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, when the mineral salts medium was supplemented with monocrotophos as a C source. Simultaneously 12, 22, 26, 30% and 18, 26, 37, 40% of N-methylacetoacetamide a metabolite of monocrotophos was recovered in the media inoculated with the *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, during the same period. Decrease in the amount of monocrotophos with a concomitant increase in the level of N-methylacetoacetamide clearly indicates the degradation of parent compound.

Key words: Monocrotophos; N-methylacetoacetamide, biodegradation; isolation, identification.

INTRODUCTION

Monocrotophos ((3-hydroxy-N-methyl-cis-crotonamide) dimethyl phosphate) is an organophosphorus (OP) insecticide widely used to control aphids, leaf hoppers, mites and other foliage pests on crops, such as cotton,

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sugarcane, peanuts (groundnut) and tobacco because of its low cost and effectiveness. It was first produced by Ciba AG and Shell Development Co. in 1965 and has been classified as extremely hazardous, with an LD_{50} value of 20 mg kg^{-1} for mammals. Monocrotophos has contact, systemic and residual activities; the prolonged use of this compound has recently found its way into the water ways in the proximity of its application (Palmer et al., 2007). OP pesticides are widely used in India for protection of agricultural yields. However, these pesticides pose serious threats to organisms, including humans, and hamper soil microbial activity; thus, they are a cause for concern. Monocrotophos is a systemic pesticide and its action is mainly on organs such as skin, eyes and central nervous system. The half-life of monocrotophos in soil was reported to be 40 to 60 days (Sha, 1999; Tomlin, 2000; Bhadbhade et al., 2002a). Continuous and excessive use of OP compounds has led to the contamination of several ecosystems in different parts of the world (Cisar and Snyder, 2000; Tse et al., 2004). For example, surveys revealed that 100% of sampled catchments in Scotland and 75% of sampled aquatic sites in Wales were contaminated with OP compounds used in sheep dips (Boucard et al., 2004). OP compounds are highly toxic to mammals and are toxic to other non-target animals, and the toxic effects of OP compounds on invertebrates, vertebrates and wildlife are well documented (Galloway and Handy, 2003). The pesticides in aquatic environment are potentially toxic and are difficult to degrade by conventional treatment processes (Colin et al., 2004; Abdullah and Ling, 2010; Avasarala et al., 2011). Growing public concern on the contamination of drinking water supplies and the aquatic environment with organic pollutants has stimulated research activity for their treatment.

Monocrotophos insecticide during sexual development causes the feminization/demasculinization of the reproductive traits. Reproductive toxicity caused by organophosphates (monocrotophos) at cellular and molecular level in the ovaries of rat. The reproductive toxicity of monocrotophos has also been observed in bobwhite quail (Tian et al., 2012; Vijay et al., 2014). Organophosphorus pesticides have been extensively used in the area of agriculture to manage insect or pests of a number of economically important crops. Organophosphate pesticides are well known as the inhibitor of acetylcholinesterase activity, not only in insects, it can also affect central nervous system of other organisms (Vijay et al., 2013). Hence, the degradation of monocrotophos is imperative.

Metabolic reactions, such as N-demethylation, O-demethylation, hydroxylation of N-methyl groups and cleavage of the phosphate-crotanamide linkage, occur during the metabolism of monocrotophos by microbial cultures and in soils (Guth, 1994; Bhadbhade et al., 2002a) with the formation of O-desmethylmonocrotophos monomethyl phosphate, dimethyl phosphate, N-methylacetoacetamide and N-methylbutyramide. The use of pesticide-degrading microbial systems for the removal of pollutants from contaminated systems requires an understanding of the ecological requirements of degrading organisms. Sunlight significantly influences the degradation of monocrotophos in soil but not in water, indicating the involvement of components of soil in photolytic degradation (Dureja, 1989; Lee et al., 1990). The rate of hydrolysis of monocrotophos in soil and aqueous environments is pH dependent, and the half-lives of monocrotophos are 131 days at pH 3, 26 days at pH 9 and 30 days at neutral pH at 25°C in the dark (Lee et al., 1990). Monocrotophos is weakly sorbed by soil particles because of its hydrophilic nature. Leaching of monocrotophos may pollute the groundwater, ultimately resulting in adverse effects on biological systems (Singh and Singh, 2003; Bhalerao and Puranik, 2009).

Together with its high mammalian toxicity, these characteristics make monocrotophos an ideal compound for decontamination and detoxification. Rangaswamy and Venkateswarlu (1992) isolated a monocrotophos degrading *Bacillus* sp. from previously treated soil. Several microorganisms have been isolated which are able to utilize pesticides as a source of energy. There are some reports of fungi including *Trametes hirsutus*, *Phanerochaete chrysosporium*, *Phanerochaete sordia* and *Cyathus bulleri* that are able to degrade lindane and other pesticides (Singh and Kuhad, 2000, 1999). However, most evidence suggests that soil bacteria are the principal agents responsible for enhanced biodegradation (Walker and Roberts, 1993). On numerous occasions, mixed bacterial cultures with pesticide degradation ability were isolated but their individual components are unable to utilize the chemical as an energy source when purified (Roberts et al., 1993); an example is the OP nematicide fenamiphos (Singh et al., 2003).

Bacteria capable of degrading several pesticides have been isolated from soil. *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* sp. insidiosum SBL 11 were found capable of utilizing monocrotophos as a source of phosphorus but not as a carbon source (Singh and Singh, 2003). *Rhodococcus phenolicus* strain $G2P^{T}$ utilize phenol, chlorobenzene and chlorobenzoic acid as source of carbon (Marc and James, 2005). As a measure of bioremediation, soil fungi capable of degrading monocrotophos were isolated from various geographical and ecological sites (Bhalerao and Puranik, 2009).

Although few microorganisms capable of degrading monocrotophos have been isolated, most of studies concerning on the degradation of monocrotophos with bacterial cultures focusing on a parent compound without prominence on its degradation products. In the present study we report the accumulation of N-methylacetoacetamide in significant proportions as degradation product in the mineral salts media inoculated with *Rhodococcus* sp. under aerobic conditions. Besides

there are no reports available on the degradation of monocrotophos by a novel bacterial strains, *Rhodococcus phenolicus* strain MCP1 and *Rhodococcus ruber* strain MCP-2.

MATERIALS AND METHODS

Chemicals

Monocrotophos with 99% purity was purchased from Sigma-Aldrich Company, Bangalore, India. All other chemicals and reagents used in this study were analytical grade.

Isolation and identification of bacteria

To isolate soil bacteria capable of degrading selected insecticide, monocrotophos enrichment culture technique was opted (Jayamadhuri and Rangaswamy, 2009). Commercial formulation of monocrotophos was added separately to 50 g portions of the soil samples to provide a final concentration of 50 µg g^{-1} soil. The soil samples were maintained at 60% water holding capacity (WHC) and incubated at room temperature (28 ± 4°C) under aerobic conditions. After five such additions with monocrotophos at 10-day intervals, triplicate soil samples were withdrawn for the isolation of soil bacteria by serial dilution agar plate method (Vijay et al., 2015).

Initially bacteria in the enrichment culture showing ability to degrade monocrotophos were grown on mineral salts media. After about 24 to 48 h of incubation at 37°C, several well-separated, individual colonies of different morphological types appeared and were further streaked onto fresh mineral salts media plates for further purification. Morphological features were studied using light microscope.

Degradation of monocrotophos residues by selected bacterial strains

The ability of the selected bacterial strains, *R. ruber* strain MCP-2 and *R. phenolicus* strain MCP1 to degrade the organophosphate insecticide, monocrotophos was tested under aerobic conditions in the laboratory. Aliquots from stock solutions, prepared in acetone, of the technical grade monocrotophos were added to 250 ml sterilized Erlenmeyer flasks to provide a final concentration of 50 µg ml^{-1} keeping in view of their toxic levels to the bacteria. The carrier solvent was completely evaporated to dryness and 50 ml portions of steam sterilized mineral salts medium (NH_4NO_3 1.5 g, $K_2HPO_4$1.5 g, KH_2PO_4 0.5 g, Mg SO_4 0.2 g, NaCl, 0.5 g, distilled water 1000 ml, pH 7.0) (Kai et al., 2006) was added into each flask under aseptic conditions.

The residues were then equilibrated for a day to obtain aqueous solutions of the monocrotophos and inoculated with an inoculum density of 1.0 OD cells measured at 600 nm using a Spectronic-20D spectrophotometer (Milton Roy) and incubated under continuous shake culture condition (150 rev min^{-1}) in an orbital shaking incubator at 37°C. Triplicate test samples were withdrawn from Erlenmeyer flaks after 1st, 2nd, 3rd and 4th day of incubation for solvent extraction and estimation of parent compound and metabolite by TLC and HPLC analysis.

Extraction of monocrotophos residues

The residues of monocrotophos from the triplicate samples of mineral salts media were extracted with equal volume of ethyl acetate (Bhadbhade et al., 2002b). At the desired intervals (1st, 2nd, 3rd and 4th day) the culture filtrate supernatant (CFS) was extracted with equal volume of ethyl acetate for the residues of monocrotophos**.** The solvent was evaporated and the residue obtained was redissolved in approximately 10 ml of methanol for the High performance liquid chromatography (HPLC) analysis. Organophosphate insecticide, monocrotophos was detected at 214 nm.

Quantification of monocrotophos residues by thin layer chromatopgraphy (TLC) and high performance liquid chromatography (HPLC)

For identification of degradation products of monocrotophos, the methanolic fraction was spotted along with authentic compounds N-methylacetoacetamide and monocrotophos on 300 µm thick silicagel-G plates. The plates were developed for a distance of 15 cm with chloroform-methanol-diethyl ether (10:1:1) and air-dried. The authentic compound and metaboloites were located by spraying the plates with ferric chloride reagent (27.0 g $FeCl_3.6H_2O$ in 1000 ml 96% ethanol). The silica gel of the samples alongside to the authentic compound N-methylacetoacetamide were scrapped off and carefully transferred to centrifuge tubes and 5 ml ethanol was added. After vigorous agitation for 2 min on a vortex mixture, contents were centrifuged at 4000 rpm for 15 min and the supernatant was collected for the estimation of N-methylacetoacetamide by a Spectrophotometer (Gundi and Reddy, 2006). Suitable aliquots of supernatant were treated with 0.5 ml ferric chloride reagent (as described above) and the volume was made up to 5 ml with distilled water, and the absorbance was measured at 510 nm in a Spectronic-20D spectrophotometer (Milton Roy). The quantity of metabolite, N-methylacetoacetamide was calculated by comparing the absorbance values with a standard curve prepared with authentic N-methylacetoacetamide.

The residues of the parent insecticide, monocrotophos have been determined by a slightly modified method of Rajendra et al. (2013) by HPLC analysis. The residues of the moncocrotophos redissolved in methanol was analysed in Agilent High Performance Liquid Chromatography (HPLC) system (1100 series), Ascentis® Express C18 HPLC Column (250 × 4.6 mm), Variable wavelength detector. The mobile phase was methanol: water (70:30, v/v), and the flow rate was 1 ml min^{-1}. A 20 µl of sample was injected into the column and the monocrotophos residues were monitored at 214 nm. Under these conditions the retention time of the parent insecticide, monocrotophos was 159 s.

Statistical analysis

All data are averages of three replicates. The data were analyzed for significant differences ($P \leq 0.05$) between inoculated and uninoculated pesticide samples in mineral salts media using Duncan's multiple range (DMR) test (Megharaj et al., 1993; Srinivasulu et al., 2012).

RESULTS AND DISCUSSION

Isolation and identification of monocrotophos degrading bacterial strains

Bacterial cultures were isolated by enrichment culture technique, after treating the soil samples five times with an insecticide, monocrotophos at 50 µg g^{-1} level. Primarily the bacteria designed as MCP1 and MCP2 and were tested for their ability to degrade the

(a)

TGGGCGAAGCCTTTTCCAGCGACGCCGCGTGAGGGATGACCGCCTTCGGGTTGTAAACCTCTTTCAG
CAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC
GTCGTCTGTGAAAACCCGCAGCTCAACTGCGGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCA
GGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAG
GCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAG
CTAACGCATTTAAGCGTCCCCGCCTGGGGAGTTACGGCCGCAAGGCTAAAAACTCGAAAGAGTTGAC
GGGGGGCCCCCCGCAAAACCGCGGGAGCATGTGGATTAATTCCNANGCCNAGNCGAAAGAACCTTA

(b)

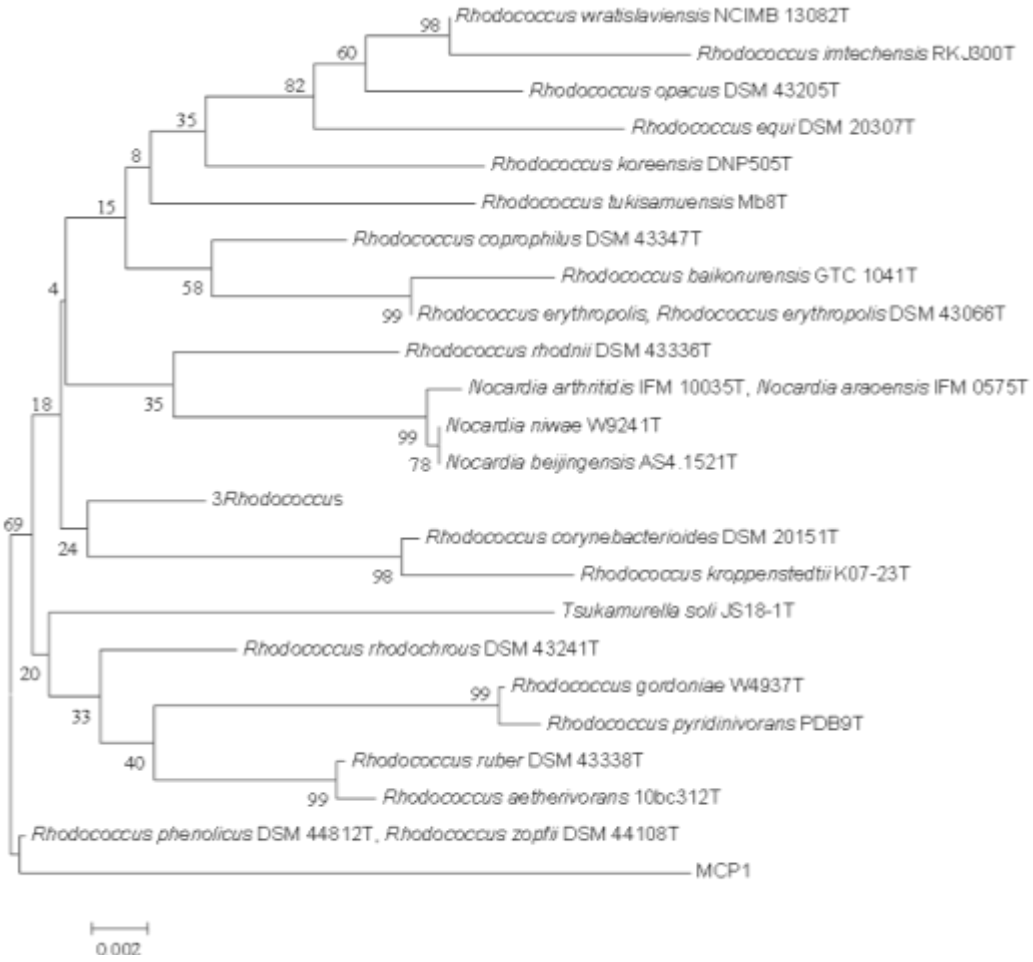

Figure 1. (a) 16S rRNA gene sequence; (b) Phylogram of *Rhodococcus phenolicus* strain MCP1.

monocrotophos in mineral salts medium. Both the bacterial strains, MCP-2 and MCP1 on mineral salts agar media after 24 hours showed opaque and round morphology, whereas MCP-2 colour has changed to salmon pink if incubation period was extended to 48 to 72 h. The bacterial strains grew well in mineral salts media supplemented with monocrotophos as carbon source at 37°C. The morphological features under light microscope were all Gram positive short rod shaped. Based on the partial 16S rRNA gene sequence, MCP1 belonged to *Rhodococcus phenolicus* sp. while MCP-2 belonged to *Rhodococcus ruber* sp The 16S rRNA gene sequences of the bacteria are represented in Figures 1a and 2a. Figures 1b and 2b illustrates the phylogenetic relationship between MCP-2, MCP 1 and their close relatives.

Degradation of monocrotophos by bacterial strains

After the incubation at desired intervals (1st, 2nd, 3rd and

(a)

```
TGGGCGAAGCCTTTTCCAGCGACGCCGCGTGAGGGATGACCGCCTTCGGGTTGTAAACCTCTTTCAG
CAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC
GTCGTCTGTGAAAACCCGCAGCTCAACTGCGGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCA
GGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAG
GCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAG
CTAACGCATTTAAGCGTCCCCGCCTGGGGAGTTACGGCCGCAAGGCTAAAAACTCGAAAGAGTTGAC
GGGGGGCCCCCCGCAAAACCGCGGGAGCATGTGGATTAATTCCNANGCCNAGNCGAAAGAACCTTA
```

(b)

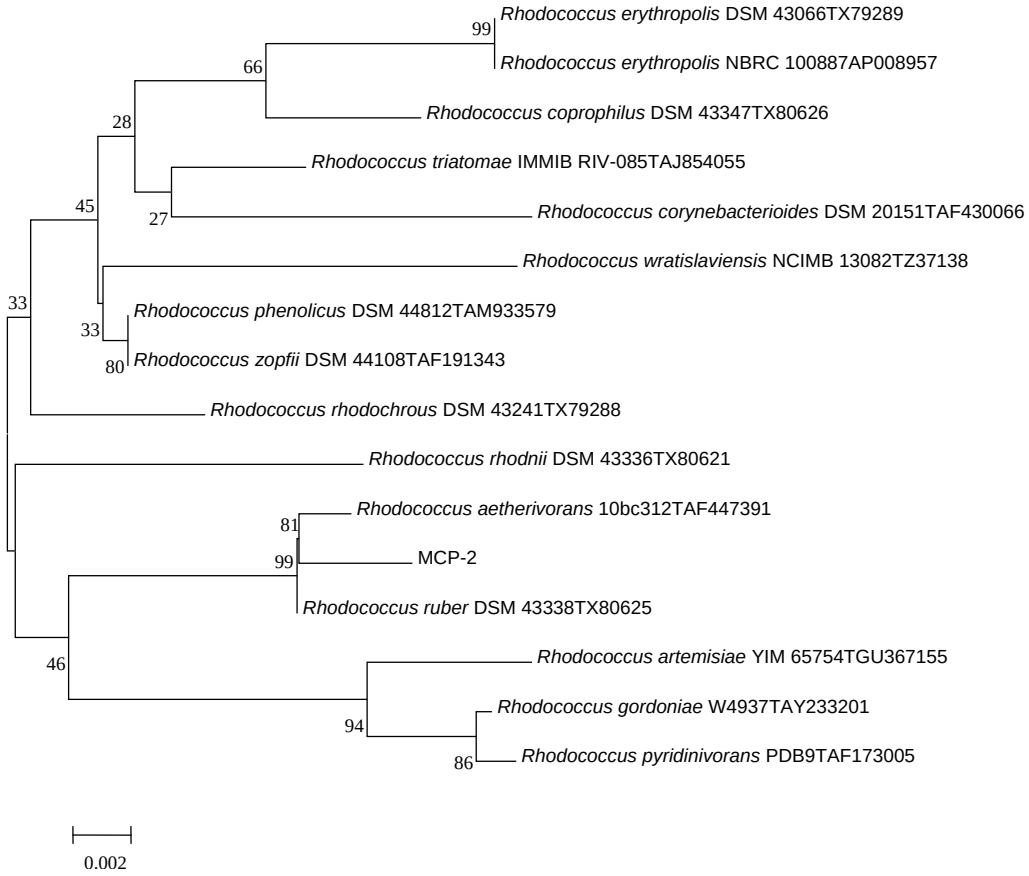

Figure 2. (a) 16S rRNA gene sequence; **(b)** Phylogram of *Rhodococcus ruber strain* MCP-2.

4th day), triplicates of the bacterial inaculated and uninoculated samples of mineral salts media were withdrawn from Erlenmayer flasks for the estimation of monocrotophos residues by TLC and HPLC analysis. The initial (0-day) recovery, with complex extraction and analytical procedures employed, of monocrotophos immediately after its application to the mineral salts medium was 94%. There was an appreciable decrease in the levels of monocrotophos during the incubation period even in uninoculated control. Thus, by the end of 4th day, about 21% of added monocrotophos was lost from the uninoculated medium and this observation reveals that abiotic degradation of monocrotophos also occurred (Figure 3). Conversely, Gundi and Reddy (2006) noticed significant decrease in the monocrotophos in sterile soils.

By the end of 1st, 2nd, 3rd and 4th day 13, 20, 24, 30% and 18, 33, 37, 45% of monocrotophos was degraded, by *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, when the mineral salts medium was supplemented with monocrotophos in comparison to uninoculated controls (Figure 3). Simultaneously, 12, 22, 26, 30% and 18, 26, 37, 40% of N-methylacetoacetamide was recovered from the media inoculated with the *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, at the same incubation period (Figure 4). Simultaneous decrease in the amount of monocrotophos

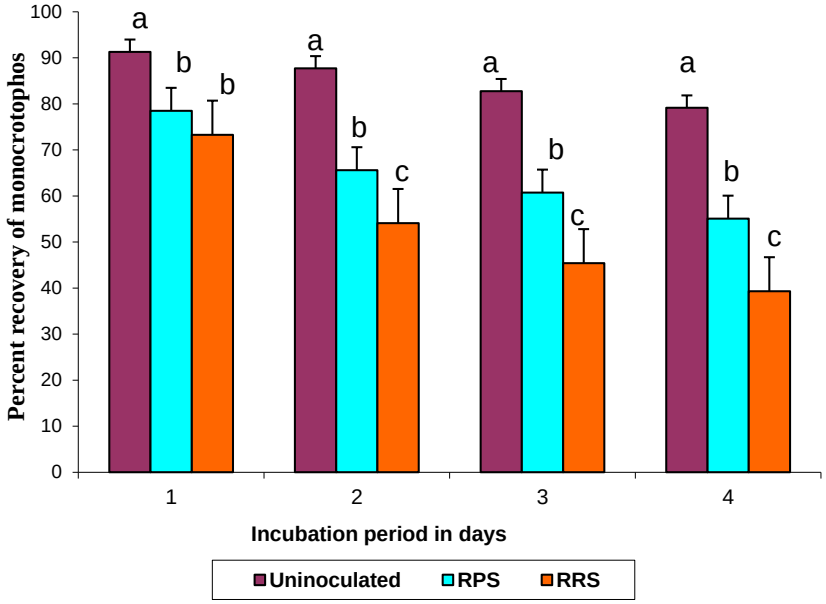

Figure 3. Degradation of monocrotophos by bacteria isolated from soil. Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test**.** Values represented in figure are means of three replicates. RPS = *R. phenolicus* strain MCP1; RRS = *R. ruber* strain MCP-2.

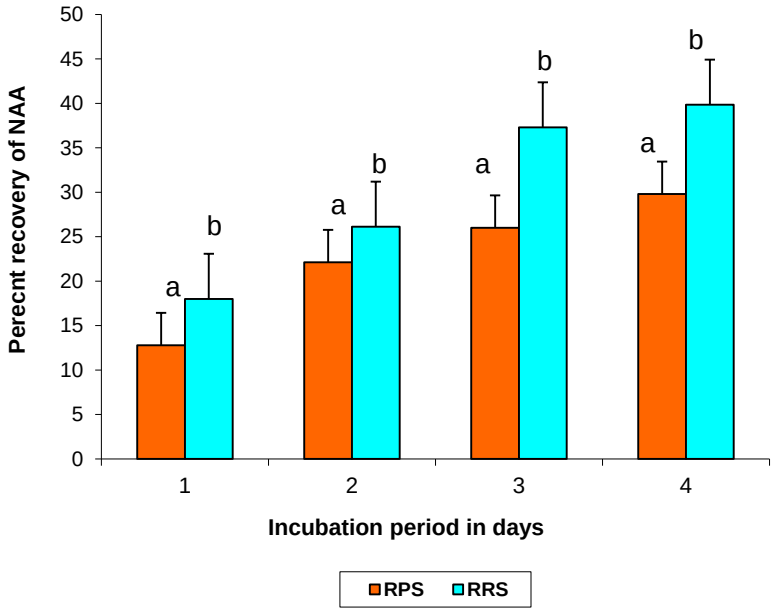

Figure 4. Percent recovery of N-methylacetoacetamide released from monocrotophos by solvent extraction from the culture media after incubation period. Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test**.** Values represented in figure are means of three replicates. RPS = *R. phenolicus* strain MCP1; RRS = *R. ruber* strain MCP-2.

with a concomitant increase in the level of its metabolite, N-methylacetoacetamide clearly indicates the degradation of parent compound. There was a significant decrease in the amount of monocrotophos in the media

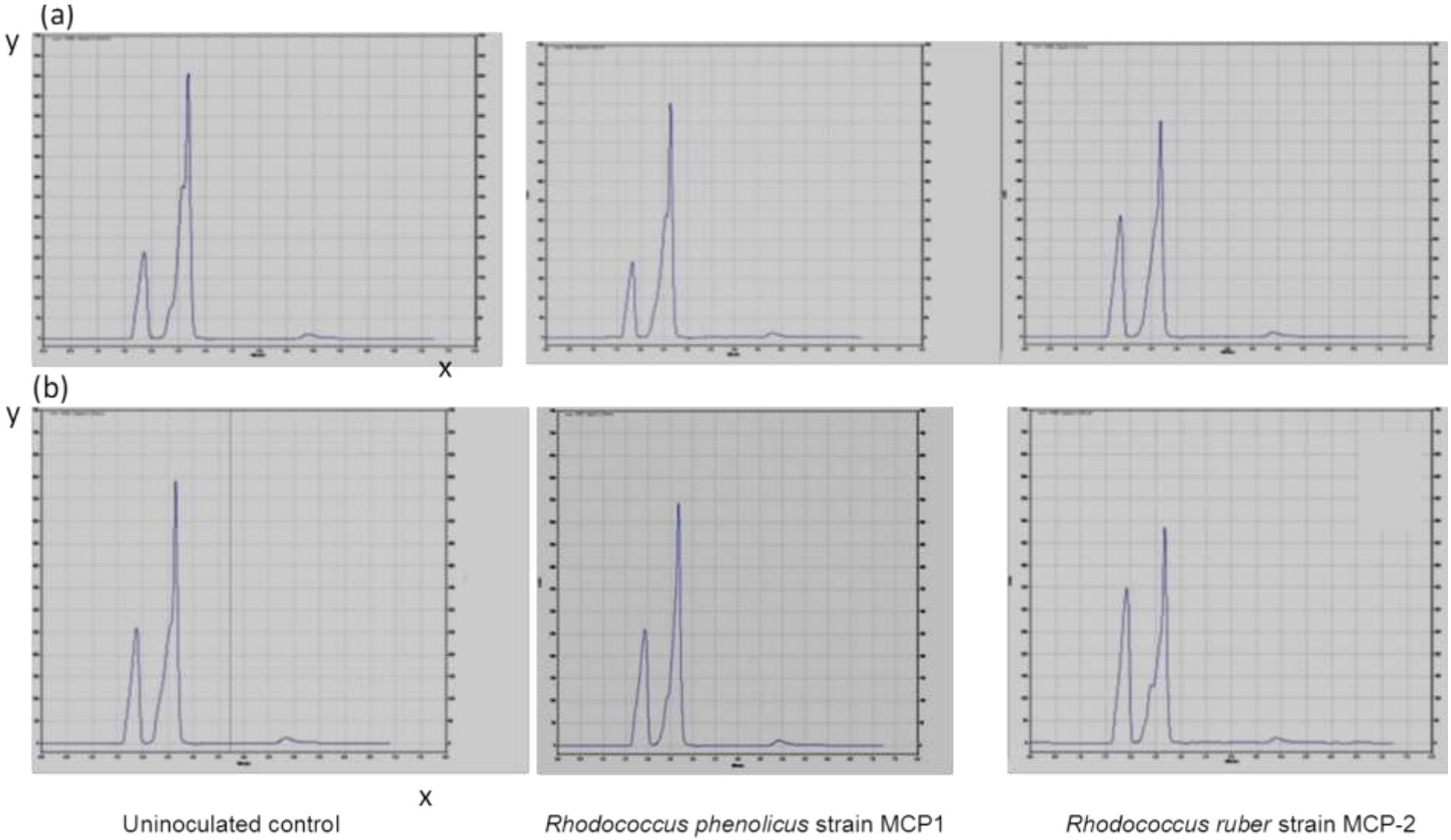

Figure 5. HPLC chromatograms of monocrotophos residues, obtained by solvent extraction from the culture media after (a) First day; (b) Second day of incubation. x = Retention time (RT), 2.5 - 2.7 min; y = Milli absorbance units (mAU).

inoculated with bacteria in comparison with uninoculated control as revealed by the HPLC chromatograms (Figures 5 and 6). Quantitative analysis of the residues of monocrotophos in organic solvent extraction of the medium by thin-layer chromatography showed the presence of

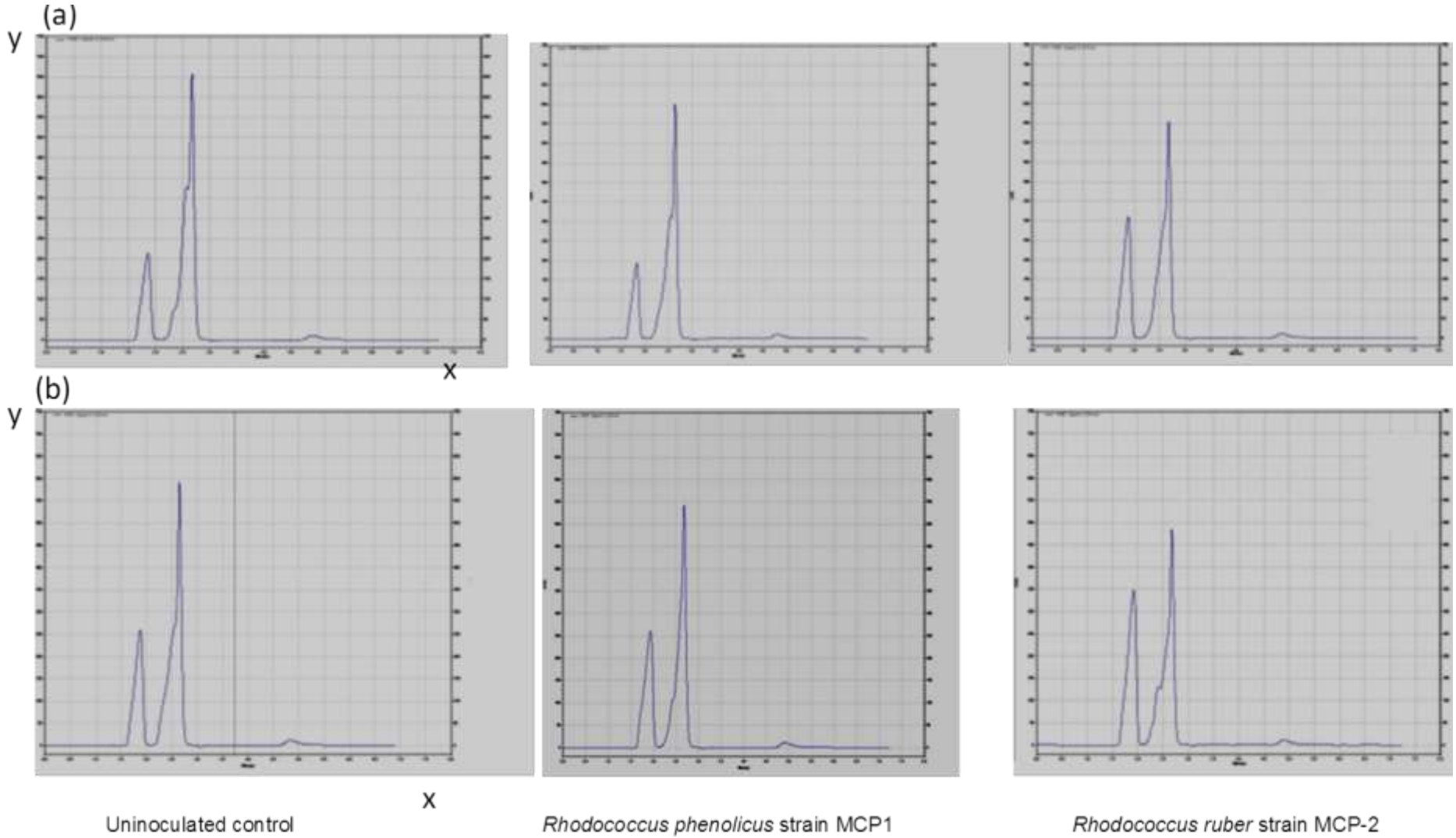

Figure 6. HPLC chromatograms of monocrotophos residues, obtained by solvent extraction from bacterial culture media after (a) third day; (b) Fourth day of incubation. x = Retention time (RT), 2.5 - 2.7 min; y = Milli absorbance units (mAU).

N-methylacetoacetamide in samples inoculated with the *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2. Formation of N-methylacetoacetamide by the hydrolysis of monocrotophos is the major route of degradation. Comparatively *R. ruber* strain MCP-2 showed

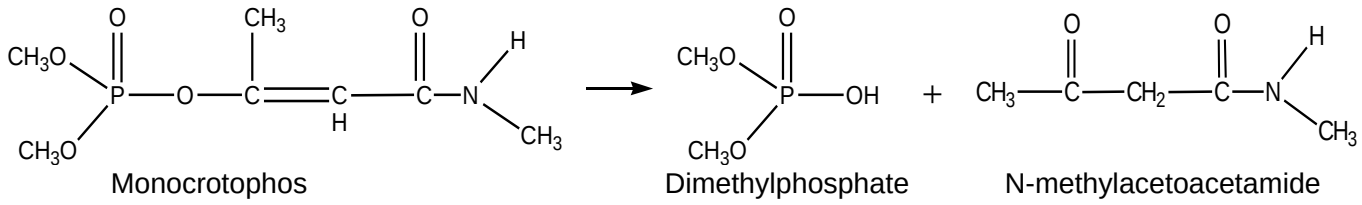

Figure 7. Hydrolysis of monocrotophos in mineral salts media.

highest degradation rate of monocrotophos than *R. phenolicus* strain MCP1 (Figure 3).

Metabolic reactions such as N-demethylation, O-demethylation, hydroxylation of N-methyl groups and cleavage of phosphate-crotanamide linkage occurred in the metabolism of monocrotophos by plants (Skripsky and Loosli, 1994), animals and in microbial cultures (Bhadbhade et al., 2002a, b) with formation of O-decimethyl monocrotophos monomethyl phosphate, dimethyl phosphate, N-methylacetoacetamide and N-methlybutyramide. In the current study, recovery of N-methylacetoacetamide in significant percentages (30-40%) from both the bacterial strains inoculated mineral salts media clearly indicates hydrolytic cleavage of bond between phosphate and crotonamide as shown in Figure 7. This metabolite was not toxic to animals in comparison with monocrotophos and O-desmethylmonocrotophos (Guth, 1994). However, the fate of N-methylacetoacetamide is exactly not known. This metabolite appears to be mineralized to CO_2 and NH_3 through the formation of methylamine and acetoacetate in the environment (Gundi and Reddy, 2006).

Studies on insecticide biodegradation revealed useful information for development of efficient technologies by manipulating suitable microbial strains for environmental development. Soil fungi capable of degrading monocrotophos were isolated from various geographical and environmental sites (Bhalerao and Puranik, 2009). *Rhodococcus* sp. are the potential degraders of persistent environmental pollutants/pesticides. Biotechnological application of this group of bacteria is based on the peculiaraties of their metabolism. Single strain can degrade multiple organic pollutants, for example, *Rhodococcus* strain DEE5151 utilize a broad range of alkyl ethers including diethyl ether, di-*n*-propyl ether, di-*n*-butyl ether, phenetole as sole carbon/energy sources (Kim and Engesser, 2004; Solyanikova and Golovleva, 2011). Annamaria et al. (2010) studied the degradation of carcinogenic/toxic compounds, RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine), by *Rhodococcus* sp. strain DN22.

Conclusions

The results of the present study indicate that the hydrolysis appeared to be a major pathway of degradation of monocrotophos with formation of N-methylacetoacetamide as degradation product. *R. ruber* strain MCP-2 showed degradation of 15% higher rate of monocrotophos than *R. phenolicus* strain MCP1. At the end of 4th day incubation around 30 to 45% of monocrotophos was mineralized by the isolates in comparison to uninoculated control. These data showed the efficiency of monocrotophos degradation by a bacterial isolates over a short time period. The results indicate the promise of the bacterial isolates *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 in the bioremediation of organophosphorus pesticide-contaminated soils.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The work was supported by the University Grants Commission (UGC), New Delhi, India; and in part the Secretaria Nacional de Educación Superior Ciencia y Tecnología (SENESCYT) Ecuador.

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Vol. 16(9), pp. 418-428, 1 March, 2017
DOI: 10.5897/AJB2016.15831
Article Number: EC56A6863009
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

An improved protocol for *in vitro* propagation of the medicinal plant *Mimosa pudica* L.

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Received 14 December, 2016; Accepted 10 February, 2017

This work aimed to develop a protocol for the *in vitro* establishment, multiplication, rooting and *ex vitro* acclimatization of *Mimosa pudica* L., a species used in folk medicine and with pharmacological activity. Aseptic cultures were established from seeds inoculated in MS medium, without growth regulators, followed by an *in vitro* stabilization phase in culture medium supplemented with 2.22 µM BAP. The cultures were transferred to MS medium supplemented with different cytokinins, combined or not with an auxin, aiming its large-scale propagation. The culture medium supplemented with 5 µM BAP plus 0.5 µM NAA provided the highest multiplication rate and top quality plantlets. The combination of 0.6 µM TDZ plus 0.05 µM NAA resulted in higher multiplication rates than in response to combination of BAP plus NAA, although the subsequent maintenance of the cultures in a medium without growth regulators has resulted in low regenerative response. *In vitro* rooting of micro-cuttings was high even in the absence of auxins. Over 90% of plantlets transferred to the greenhouse survived after the acclimatization phase. Acclimatized plants presented normal vegetative and reproductive development. The procedures established in the present study allow a massive production of *M. pudica* plants for further pharmacological studies.

Key words: Biodiversity conservation, *ex vitro* acclimatization, *in vitro* rooting, micropropagation, *Mimosa pudica.*

INTRODUCTION

Mimosa pudica L., popularly known in Brazil as *dormideira*, *sensitiva*, *mimosa*, and *maria-fecha-a-porta*, is a perennial and native species of Tropical America, which also can be found in Tanzania, South Asia, South-East Asia and many Pacific Islands having near-frequent rainfall (Patra et al., 2016). Mimosa plants present

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herbaceous habit, with the presence of thorns throughout the stem. The growth is annual and, despite presenting erect stem when young, they become creeper plants at the adult phase. The leaf sensitiveness to mechanical, electrical or thermal stimuli is a feature of this species, evidenced by the fast closure of leaflets when disturbed (Jensen et al., 2011). *M. pudica* is used in folk medicine, mainly in Asian countries, whereas its extracts are attributed diuretic, astringent and antispasmodic activities. Its roots and leaves are used in hemorrhoids and fistula treatments and against convulsions (Hassan et al., 2010). *In vitro* and *in vivo* analysis of flavonoids and phenolic compounds of leaves, stems, and seeds of the *M. pudica* have found high antioxidant, antimicrobial, anticancer and antidiabetic activities, justifying its use in different diseases treatments (Doss et al., 2011; Kaur et al., 2011; Zhang et al., 2011; Joseph et al., 2013; Gunawardhana et al., 2015; Tunna et al., 2015; Jose et al., 2016; Muhammad et al., 2016; Patro et al., 2016).

Mimosa is the main genus of legumes associated to β-rhizobia, bacteria related to soil fertility increase, due to its N_2 fixing action (Elliott et al., 2007; Reis Jr. et al., 2010). Being a nutrient cycle promoter, their species are used in recovery of natural and degraded ecosystems (Camargo-Ricalde et al., 2004). On the other hand, *M. pudica* is described as a highly invasive pantropical weed, having a negative impact on natural and agricultural ecological systems (Klonowska et al., 2017). Although presenting fast and easy propagation in nature, the seeds of *M. pudica* are subject to biotic and abiotic agents, which affect the germination success (Jensen et al., 2011).

Considering its pharmacological and medicinal properties and its ecological relevance, there are, relatively, few studies involving large-scale micropropagation of *M. pudica* (Ramakrishna et al., 2009; Hassan et al., 2010; Ramesh et al., 2013). Micropropagation allows the production of genetically uniform plants in reduced space and in controlled conditions of temperature, photoperiod and luminosity (Parveen and Shahzad, 2011; Varshney and Anis, 2012). Studies related to *in vitro* cultivation of the *M. pudica* are very important, especially because it is a non-domesticated species and an environment facilitator, allowing the comprehension of specific biochemical and physiological mechanisms and the use of the characters that make it a medicinal species, as well as for its control as a weed.

The present study aimed to establish an efficient protocol for large-scale propagation of aseptic cultures of *M. pudica*, assessing the effects of different plant growth regulators on *in vitro* multiplication and rooting phases, as well as on acclimatization to *ex vitro* conditions. Plants produced through tissue culture can be used in pharmacological and ecophysiological researches, avoiding the need to collect specimens in a natural environment, in which genetic variability plays a fundamental role.

MATERIALS AND METHODS

Plant material and establishment of *in vitro* micropropagation procedure

In vitro establishment of aseptic cultures of *M. pudica* was carried out by seeds harvested from dry and mature fruits, from plants developed in the natural environment. After collection, the seeds were subjected to surface asepsis by rinsing in tap water for 60 min. Then, the seeds were immersed in ethanol 70% (v/v) for 30 s and, later, in a bleach solution with 2% of active chlorine, diluted at 30% (v/v) for 15 min. Finally, the seeds were washed in distilled and autoclaved water. After asepsis, the seeds were inoculated in MS basal medium (Murashige and Skoog, 1962), without growth regulators, in a laminar flow hood. The success of *in vitro* establishment was estimated 30 days after inoculation, considering the percentages of seeds contamination and total germination. In order to obtain stabilized cultures, 60 days after *in vitro* establishment, nodal segments (2-3 cm) from seedlings were transferred to MS culture medium supplemented with α-naphthalene acetic acid (NAA: 0.107 µM) or 6-benzylaminopurine (BAP: 2.22 µM), besides the control group (without growth regulators). After 60 days, the *in vitro* cultures were evaluated regarding the number of shoots per explants, height of shoots, and percentage of rooted plantlets.

In vitro multiplication

Aiming to eliminate the residual effects of previous culture media, after *in vitro* stabilization phase, the explants were transferred and kept for 30 days on MS medium without growth regulators. Then, nodal segments (2-3 cm) were obtained from plantlets maintained in this condition and transferred to MS culture medium supplemented with BAP, kinetin (KIN), adenine sulphate (AS), diphenylurea (DFU) or thidiazuron (TDZ), at 0, 2.5, 5 or 7.5 µM, totaling 16 treatments. The cultures were kept in these culture media for 30 days and evaluated regarding the number of shoots and roots per explant, height of shoots, and callus development. Root quality was also evaluated with scores, ranging from 1 to 5 given by three independent evaluators, with 5 corresponding to the root system that presents the best development, and 1 for explants that did not present root development.

After we found that BAP was the cytokinin that provided the highest *in vitro* multiplication rates, new assays were carried out aiming to further increase culture proliferation. Nodal segments (2-3 cm) obtained from explants aseptically established in MS medium without growth regulators, were inoculated in MS medium supplemented with BAP (0, 5 or 7.5 µM) and NAA (0, 0.05, 0.25 or 0.5 µM), in all possible combinations, totaling 12 treatments. The cultures were evaluated after 45 days regarding percentage of explants presenting three or more shoots, number and height of shoots.

Aiming to obtain elongated shoots, nodal segments (2-3 cm) from the best treatment in the previous assay (5 µM BAP plus 0.5 µM NAA) were inoculated in MS medium supplemented with gibberellic acid (GA_3: 0, 0.28, 1.44, 2.89 or 4.53 µM), in presence or absence of 5 µM BAP, totaling 10 treatments. After 45 days, the cultures were evaluated regarding number and height of shoots and the number of roots per explant.

Considering the results found in the assay with the different cytokinins, another set of explants, previously kept in MS medium without growth regulators, was transferred to MS medium supplemented with TDZ (0.6, 0.9 or 1.2 µM) plus NAA (0, 0.05, 0.25

or 0.5 µM), in all possible combinations, totaling 12 treatments. After 45 days, the cultures were evaluated considering the number of shoots higher than 0.5 cm. As the new shoots, in this assay, did not show suitable elongation, the explant clusters (rosettes) produced (0.5 ± 0.1 cm) were transferred from MS culture media with 0.6 µM TDZ, singly or combined with NAA, to MS culture media without growth regulators or to MS culture media supplemented with 0.25 µM NAA. After 45 days, the cultures were evaluated considering the percentage of regenerated shoots and height of shoots.

In vitro rooting

Aiming to stimulate the rooting of micro-cuttings, nodal segments from plantlets (2-3 cm, excluded the apical part) were transferred to MS culture media supplemented with NAA, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0.1, 0.2, 0.3 or 0.4 µM, besides the control). After 40 and 60 days of inoculation, the cultures were evaluated regarding the number of roots and the length of the largest root.

In vitro culture conditions

At all stages of *in vitro* culture, the plantlets were kept in 2.5 × 15 cm test tubes. The MS culture media was supplemented with MS vitamins, sucrose (30 g L^{-1}), and agar (7 g L^{-1}). The culture media pH was adjusted to 5.7 ± 0.1 before autoclaving, carried out for 20 min at 120°C and 1 atm of pressure. The test tubes were capped with autoclaving polyethylene closures, and sealed with PVC film (Vitaspenser, Goodyear, 15 µm). The cultures were kept in a growth room under controlled conditions of temperature (26 ± 1°C), photoperiod (16 h) and luminosity (40 µmols m^{-2} s^{-1}).

Ex vitro acclimatization

After 60 days of cultivation in rooting medium, the plantlets were removed from test tubes and their roots were washed in running tap water to remove culture media debris. Later, the plantlets were transplanted to polystyrene trays with 128 cells, filled with commercial substrate Plantmax Hortaliças HT®. The trays were covered and wrapped with transparent plastic, remaining for 20 days in shadowed environment. After this period, the trays were transferred to a greenhouse covered with transparent plastic and Sombrite® 70%, and maintained under programmed micro sprinkler system undertaken for 5 min twice a day. After 40 days, the plants were transferred to pots with a mixture of soil/washed sand/cattle manure at the proportion of 3:2:1 (v/v/v). The efficiency of acclimatization procedures was evaluated taking in account the plantlets final survival percentage.

Statistical analysis

All experiments were conducted in a completely randomized design, with five replicates, except for cases that percentages were compared, in which ten repetitions per treatment were used. Linear or polynomial regression in accordance with residual requirements in assays related to multiplications and rooting phases was employed. In some of the experiments, counting data were normalized by the equation $\sqrt{x+0.5}$ and the results submitted to analysis of variance (ANOVA). The obtained means were compared through Scott-Knott test at 5% probability, using SAEG software (version 9.1).

RESULTS

In vitro establishment and stabilization

In this work, the *in vitro* disinfection procedures of *M. pudica* seeds were very effective, with less than 2% of microbial contamination and high germination rates (87%). Figure 1a presents features of *M. pudica* plantlets, 15 days after *in vitro* germination in MS medium without growth regulators.

In stabilization phase of *M. pudica* cultures, the multiplication rate was 4.5 shoots/explant in response to 2.22 µM BAP; the best result among the three treatments used. Micro-cuttings rooting in this condition was around 40% less than that found in plantlets kept in culture medium with 0.107 µM NAA. In contrast, the supplementation of NAA to the culture medium did not significantly affect the number of shoots, rooting percentage or shoots height, with results similar to the control, without BAP. Although BAP has stimulated shoot multiplication, a negative correlation was observed between elongation and culture propagation rates (Figure 2a).

In vitro multiplication

At the *in vitro* multiplication phase, the supplementation of 5 µM BAP to MS medium resulted in the production of approximately 5 shoots/explant (Figure 2b), the highest multiplication rate among all cytokinins used. Nevertheless, at 7.5 µM BAP, an inhibition was observed in shoots production. *M. pudica* plantlets kept *in vitro* in the presence of cytokinins are shown in Figure 1b. The increase in BAP concentration resulted in inhibition of shoots elongation, with plantlets reaching, in average, 2.5 cm height in culture media supplemented with 5 µM BAP (Figure 2c). In this condition, plantlets showed poor root quality (Figure 1b 4-6 and Figure 2d).

In contrast to what was observed in relation to BAP, the supplementation of KIN did not significantly stimulate *M. pudica* shoots proliferation (Figure 1b: 7-9). The development of more than one shoot/explant was observed only in response to KIN higher than 5 µM. However, there were no differences compared to the control. In relation to TDZ, the concentrations evaluated initially (2.5, 5 and 7.5 µM) did not promote significantly shoot proliferation, besides having promoted an excessive callus proliferation (Figure 1b 10-12).

In this study, the best shoot multiplication was found when 5 µM BAP was associated to 0.5 µM NAA, with 90% of explants presenting three or more shoots (Table 1). Nevertheless, in the higher BAP concentration, the number of shoots reduced. Additionally, NAA exclusion of culture media resulted in impaired morphogenetic response of the *M. pudica* cultures. As it does not affect significantly the number of shoots (Table 2), the

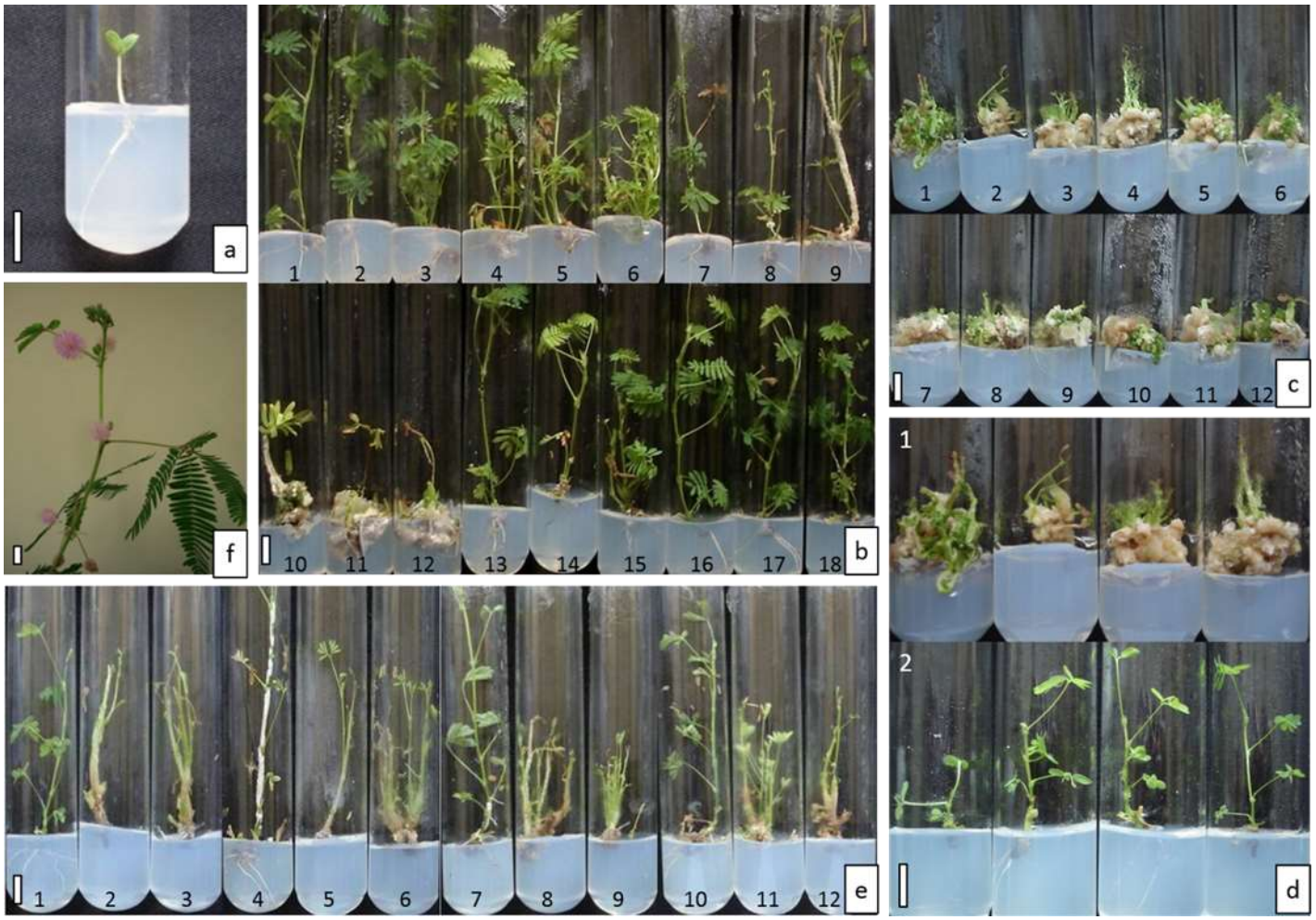

Figure 1. Different stages of micropropagation and acclimatization of *M. pudica* plants: **(a)** *In vitro* stabilization stage. **(b)** *In vitro* multiplication stage in response to different cytokinins: 1, 2, and 3 - control; 4 - BAP 2.5 µM; 5 - BAP 5 µM; 6 - BAP 7.5 µM; 7 - KIN 2.5 µM; 8 - KIN 5 µM; 9 - KIN 7.5 µM; 10 - TDZ 2.5 µM; 11 - TDZ 5 µM; 12 - TDZ 7.5 µM; 13 - DFU 2.5 µM; 14 - DFU 5 µM; 15 - DFU 7.5 µM; 16 - SA 2.5 µM; 17 - SA 5 µM; 18 - SA 7.5 µM. **(c)** *In vitro* multiplication stage in response to TDZ and to NAA: 1 - TDZ 0.6 µM; 2 - TDZ 0.6 µM + NAA 0.05 µM; 3 - TDZ 0.6 µM + NAA 0.25 µM; 4 - TDZ 0.6 µM + NAA 0.5 µM; 5 - TDZ 0.9 µM; 6 - TDZ 0.9 µM + NAA 0.05 µM; 7 - TDZ 0.9 µM + NAA 0.25 µM; 8 - TDZ 0.9 µM + NAA 0.5 µM; 9 - TDZ 1.2 µM; 10 - TDZ 1.2 µM + NAA 0.05 µM; 11 - TDZ 1.2 µM + NAA 0.25 µM; 12 - TDZ 1.2 µM + NAA 0.5 µM. **(d)** *M. pudica* plantlets in presence of TDZ 0.6 µM (1) and 45 days after transfer to MS medium (2). **(e)** *In vitro* multiplication stage in response to BAP and NAA: 1 - control; 2 - BAP 5 µM; 3 - BAP 7.5 µM; 4 - NAA 0.05 µM; 5 - NAA 0.05 µM + BAP 5 µM; 6 - NAA 0.05 µM + BAP 7.5 µM; 7 - NAA 0.25 µM; 8 - NAA 0.25 µM + BAP 5 µM; 9 - NAA 0.25 µM + BAP 7.5 µM; 10 - NAA 0.5 µM; 11 - NAA 0.5 µM + BAP 5 µM; 12 - NAA 0.5 µM + BAP 7.5 µM. **(f)** Details of reproductive development in acclimatized plants of *M. pudica.* Scale-bar: 1 cm.

supplementation of NAA to the culture medium increased the number of plantlets responding to treatments (Table 1). The combination of 5 µM BAP with 0.5 µM NAA resulted in production of more vigorous explants, without callus development or hyperhydricity symptoms (Figure 1e).

When shoots height was analyzed (Table 3), negative effects of BAP supplementation were observed, regardless of its concentration. Positive effects of NAA supplementation were only observed in media without BAP. The joint supplementation of NAA and BAP did not influence significantly shoots elongation, showing negative correlation between the multiplication rate and shoots length.

The use of GA_3 was ineffective to stimulate *M. pudica* plantlets elongation and multiplication. Although GA_3 supplementation has not promoted significant increases in adventitious roots proliferation when compared to control, in the concentration of 0.28 µM roots formation was observed in some plantlets even in presence of BAP (data not shown).

Due to the hyperhydricity and callus development at the micro-cuttings basis in response to TDZ doses used in the previous essay (2.5, 5 and 7.5 µM), a second investigation was undertaken with lower concentrations (0.6, 0.9, and 1.2 µM), in combination with NAA (0, 0.05,

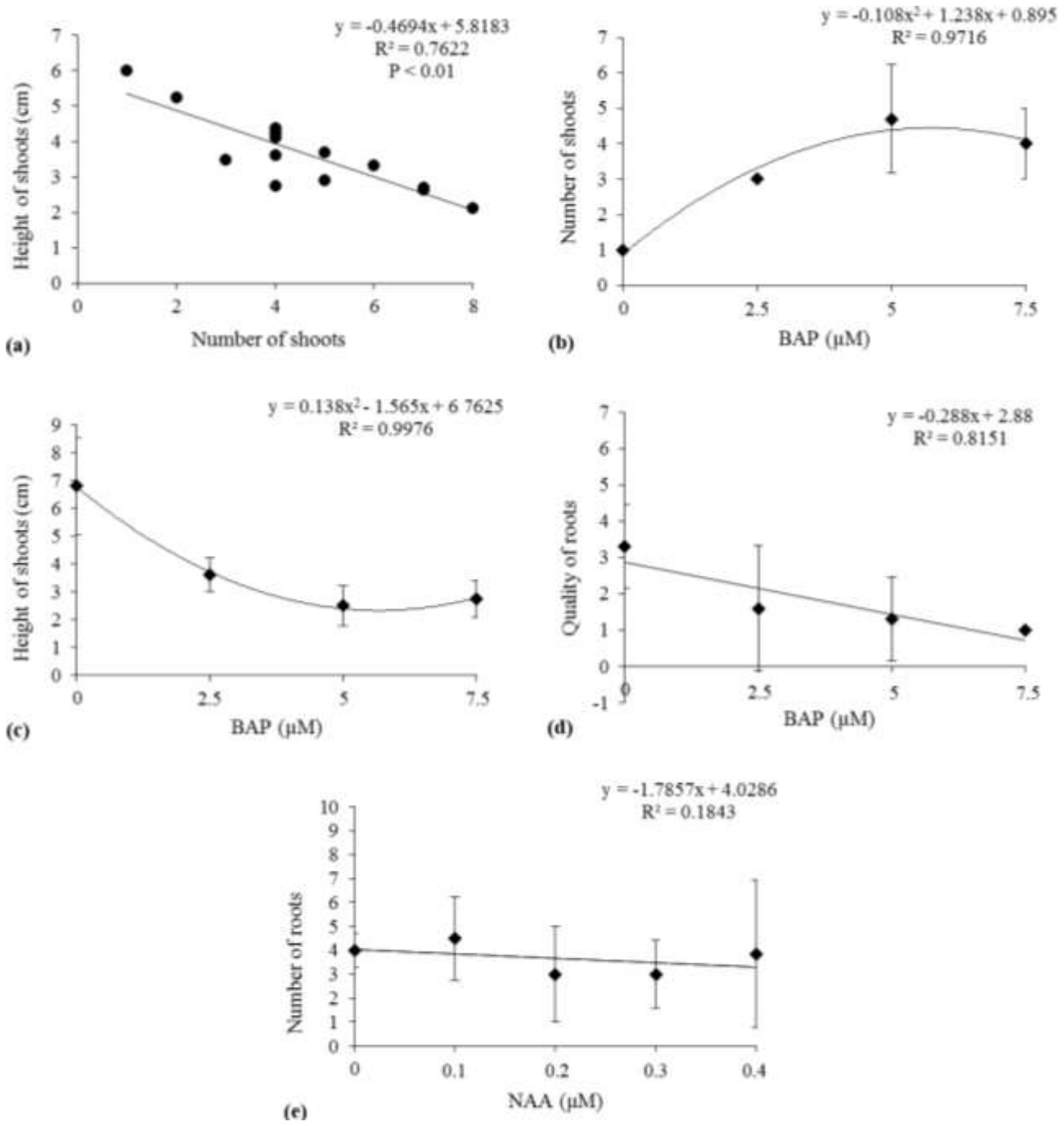

Figure 2. Relation between height and number of shoots in *M. pudica* explants cultivated for 60 days in media with 2.22 µM BAP, in the stabilization phase **(a)**, relation between BAP concentration and the number of shoots **(b),** height of shoots **(c)**, and roots quality **(d)** in the multiplication phase, 30 days after *in vitro* cultivation, and relationbetween increasing NAA concentration and number of roots in the rooting phase, after 60 days of *in vitro* cultivation **(e)**. Bars = mean standard error. n = 5.

0.25, and 0.5 µM). Although callus development and hyperhydricity had been observed in response to 0.6 µM TDZ, approximately ten shoots higher than 0.5 cm were obtained in culture media without NAA. The shoots obtained in response to 0.6 µM TDZ were qualitatively better than shoots produced in response to higher concentrations, due to less severe symptoms of hyperhydricity (Table 4 and Figure 1c).

In our study, the supply of NAA to the culture medium resulted in significant reduction in the number of shoots

Table 1. Percentage of *M. pudica* explants that presented three or more shoots in each treatment 45 days after beginning of experiments.

Treatment	% of explants with three or more shoots
MS without growth regulator	NR
BAP 5 µM	45
BAP 7.5 µM	50
NAA 0.05 µM	NR
BAP 5 µM + NAA 0.05 µM	50
BAP 7.5 µM + NAA 0.05 µM	40
NAA 0.25 µM	NR
BAP 5 µM + NAA 0.25 µM	70
BAP 7.5 µM + NAA 0.25 µM	60
NAA 0.5 µM	NR
BAP 5 µM + NAA 0.5 µM	90
BAP 7.5 µM + NAA 0.5 µM	40

NR = No response to treatment.

Table 2. Number of shoots in *M. pudica* explants cultivated *in vitro* for 45 days in response to different BAP and NAA concentrations.

BAP (µM)	NAA (µM)			
	0	0.05	0.25	0.5
0	1^{Ab}	1^{Ab}	1^{Ab}	1^{Ab}
5.0	3.6^{Aa}	3^{Aa}	4.3^{Aa}	5.3^{Aa}
7.5	4^{Aa}	4.6^{Aa}	5^{Aa}	2^{Aa}

Upper and lower case letters denote significant differences in each line and each column respectively, attested by the Scott-Knott test ($P < 0.05$).

Table 3. Height of shoots (cm) in *M. pudica* explants cultivated *in vitro* for 45 days in response to different BAP and NAA concentrations.

BAP (µM)	NAA (µM)			
	0	0.05	0.25	0.5
0	3.83^{Ba}	6.83^{Aa}	8^{Aa}	8.2^{Aa}
5.0	2.8^{Aa}	3.83^{Ab}	2.3^{Ab}	2.5^{Ab}
7.5	2.75^{Aa}	2.25^{Ab}	2^{Ab}	1.75^{Bb}

Upper and lower case letters denote significant differences in each line and each column respectively, attested by the Scott-Knott test ($P < 0.05$).

Table 4. Number of shoots higher than 0.5 cm in *M. pudica* explants cultivated *in vitro* for 45 days in response to different TDZ and NAA combinations.

TDZ (µM)	NAA (µM)			
	0	0.05	0.25	0.5
0.6	10^{Aa}	7^{Ba}	6^{Ba}	4^{Ba}
0.9	3.67^{Ab}	2.3^{Ab}	1.67^{Ab}	1.3^{Ab}
1.2	$0.67^{A}c$	$0^{A}c$	0.3^{Ab}	1^{Ab}

Upper and lower case letters denote significant differences in each line and each column respectively, attested by the Scott-Knott test ($P < 0.05$).

Table 5. Regeneration rate of *M. pudica* feasible explants after 45 days of *in vitro* cultivation. Arrows represent transfer to a new culture medium.

Treatment	Regeneration rate (%)
TDZ 0.6 µM → MS medium	20
TDZ 0.6 µM → NAA 0.25 µM	0
TDZ 0.6 µM + NAA 0.05 µM → MS medium	40
TDZ 0.6 µM + NAA 0.05 µM → NAA 0.25 µM	0
TDZ 0.6 µM + NAA 0.25 µM → MS medium	0
TDZ 0.6 µM + NAA 0.25 µM → NAA 0.25 µM	10
TDZ 0.6 µM + NAA 0.5 µM → MS medium	30
TDZ 0.6 µM + NAA 0.5 µM → NAA 0.25 µM	0

higher than 0.5 cm only in treatments containing 0.6 µM TDZ (Table 4 and Figure 1c). In spite of promoting shoots proliferation, TDZ does not stimulate elongation, resulting in shoot in the rosette feature (Figure 1c). The subdivided rosettes transferred to MS culture media without growth regulators presented better regeneration than those transferred to culture media having 0.25 µM NAA (Table 5 and Figure 1d). In this study, the best regeneration of cultures derivate from rosettes explants was observed in

treatments that culture medium was supplemented with 0.6 µM TDZ combined with NAA at 0.05 or 0.5 µM. The plantlets from rosettes explants regenerated in MS medium showed normal morphology (Figure 1d).

In vitro rooting

The analysis related to number of roots did not evidence significant differences between control and treatments in which NAA was added, showing that regardless of concentration, this growth regulator did not increase shoots rooting (Figure 2e). Also, differences were not found when the largest root length was evaluated, although the treatment with 0.4 µM NAA resulted in more vigorous and longer roots in the end of the first 40 days of *in vitro* cultivation. However, these differences vanished after 60 days. No significant difference was also observed in response to IBA between control and treatments regarding the number and elongation of adventitious roots in micro-cuttings of *M. pudica* (data not shown).

Despite higher IAA concentrations (0.4 µM) in the first days of cultivation promoted the production of branched and elongated roots, no significant difference was found between this treatment and the control after 60 days of *in vitro* cultivation. Additionally, IAA concentration between 0.1 and 0.3 µM inhibited adventitious roots development (data not shown).

Ex vitro acclimatization

Plantlets from *in vitro* cultivation developed very well in *ex vitro* conditions, with average survival of over 90%. 20 days after the beginning of the acclimatization phase, plants were already in conditions to be transferred to the greenhouse, presenting suitable vegetative development (around 15 cm height). After 40 days at the greenhouse, period in which all plantlets survived, they were transferred to pots under natural conditions. Acclimatized plants of *M. pudica* presented normal vegetative and reproductive development (Figure 1f). After 60 days of acclimatization, they presented blossoming, showing that the reproductive development was not affected by procedures of the *in vitro* cultivation.

DISCUSSION

An efficient establishment of *M. pudica in vitro* cultures

Although the *Mimosa* species produced large amounts of seeds, they are generally preyed and lose their viability when exposed to extreme conditions (Camargo-Ricalde et al., 2004). The *in vitro* establishment of *M. pudica* cultures from seeds was very successful, with only 2% microbial contamination and 87% germination. For *Carya illinoinensis* (Wangenh.) K. Koch, similar disinfection procedure showed contamination level of 5% after one week of establishment, reaching 18 to 20% after three weeks (Renukdas et al., 2010). According to George and Sherrington (1984), 10% is the acceptable contamination threshold in the *in vitro* establishment phase, which shows the efficiency of disinfection procedures used in this work.

The multiplication rate achieved in stabilization phase in response to BAP (4.5 shoots/explant) was similar to the results observed for *Uraria picta* (Jacq.) Desv. ex DC. (Anand et al., 1998) and for *Albizia falcataria* (L.) Fosberg (Widiyanto et al., 2008), two other Fabaceae. The rooting in this condition was approximately 40% less than in culture medium supplemented with 0.107 µM NAA, which can be attributed to the inhibitory effects of BAP on the rhizogenesis. Besides, a negative correlation was observed between elongation and cultures propagation, similar to that found for *in vitro* cultures of *Salvadora persica* L. (Phulwaria et al., 2011) and *Albizia falcataria* (L.) Fosberg (Widiyanto et al., 2008).

The use of BAP and NAA combination, essential for *M. pudica* shoots multiplication

In the multiplication phase, the inhibitory effect observed in the shoot propagation at 7.5 µM BAP shows that for the *M. pudica* cultures, high BAP concentration may be harmful to shoot proliferation. In studies related to *in vitro* cultivation, BAP is one of the most important cytokinins in shoot induction (Janarthanam and Seshadri, 2008). Positive effects of BAP supplementation at 5 µM were also reported in the multiplication phase for *Artemisia vulgaris* L. (Sujatha and Kumari, 2007) and *Acacia mangium* (Shahinozzaman et al., 2012) cultured *in vitro*.

While cytokinins are widely used in the morphogenesis and in *in vitro* proliferation phase, other substances have been employed in some researches. Ramakrishna et al. (2009) reported promising results for *in vitro* proliferation of *M. pudica* in response to the addition of melatonin and serotonin to the culture media, producing 15 and 22 shoots/explants, respectively. Although that analysis had been carried out after two months, twice the evaluated period in our work, those results suggest an alternative for *in vitro* shoots induction, since those substances may be used alone or combined with cytokinins and/or auxins, aiming to increase the effectiveness of the micropropagation process.

The increase in BAP concentration inhibited the shoots elongation and produced plants with reduced root quality, which, however, is not a limiting factor at this *in vitro* cultivation phase. The results found in this study related to BAP effects on *M. pudica* culture proliferation are typical of the effects of cytokinins in *in vitro* morpho-

genesis control. As predicted by the classic morphogenesis model suggested by Skoog and Miller (1957), beyond a certain concentration, the cytokinins promote an unbalancing in the hormonal metabolism. The results from this study show that *M. pudica* explants are adversely affected by excessive concentrations of BAP that, however, differs from the findings for *Justicia gendarussa* Burm. f. (Thomas and Yoichiro, 2010) and *Acacia tortilis* subsp. *raddiana* (Savi) Brenan (Nandwani, 1995), species that showed high multiplication rates in response to BAP concentrations that caused inhibition in *M. pudica* shoot proliferation. Banu et al. (2014) obtained a high proliferation rate (about 19 shoots/seed) after *in vitro* cultivation of *M. pudica* in media with approximately 8.9 µM (2 mg L^{-1}). They used synthetic seeds produced both with shoot tips and nodes, demonstrating that this technique can be effectively used to propagate this species. However, the production of synthetic seed can raise the cost of procedures and the time required for propagation.

The results obtained with the supplementation of KIN, wherein no differences were found among the control and other treatments, suggest that it may be required higher levels of this cytokinin to stimulate shoots proliferation. According to Shiva et al. (1994), cytokinins relative effectiveness in shoots induction follows the BAP > KIN > zeatin > AS order. Studying plant of *Psoralea corylifolia* L., a leguminous used in Indian and Chinese medicine, Baskaran and Jayabalan (2008) also verified low effectiveness of the KIN in promoting *in vitro* proliferation. This same result was found by Banu et al. (2014), after germination of synthetic seeds of *M. pudica*.

Several studies used TDZ as cytokinin to stimulate *in vitro* cultures proliferation (Rolli et al., 2012). In our assessment, however, TDZ did not stimulate *M. pudica* shoots proliferation. These results contradict, in certain extent, the expectations related to the beneficial effects of the phenylurea derivatives in promoting the organogenesis and shoots proliferation, compared to cytokinins derived from adenine, such as BAP and KIN (Khurana-Kaul et al., 2010). TDZ, in addition to acting as synthetic cytokinin, also promotes the overexpression of natural cytokinins, and therefore is successfully used in shoots proliferation in concentrations below 1 µM (Varshney and Anis, 2012). TDZ effects seem to be related to its influence in the synthesis of *IPT* genes, stimulating the natural production of zeatin, in addition to promote upstream regulation of the genes related to specific responses to cytokinins, resulting in cell division increase and, often in the stimulus to callus development (Taiz and Zeiger, 2010), an unwanted morphogenetic response observed in this work.

The use of NAA in association with BAP is widely reported in the literature. In this study, the shoots multiplication increased when these growth regulators were combined. Differences were observed for the *Acacia mangium* Willd for which the supplementation of 0.5 µM NAA to the culture medium for multiple shoots induction (4 µM BAP) was harmful, leading to the reduction of shoots proliferation (Shahinozzaman et al., 2012). For *M. pudica* cultures, Hassan et al. (2010) found high multiplication rates when 6.6 µM BAP and 2.6 µM NAA were combined, although the response percentages have been smaller. Also, studying *M. pudica* micropropagation, Ramesh et al. (2013) achieved an increase in number of shoots/explant using simultaneously IAA and NAA in culture media supplemented with BAP. The isolated supply of BAP in culture media promoted a less intense proliferative response, showing that these growth regulator effects are related to the balance between auxins and cytokinins, which is crucial to break the apical dominance and for the synthesis and activation of cyclin-dependent kinases (CDKs), enzymes related to the cell division (Taiz and Zeiger, 2010).

Despite the use of gibberellins be reported for the promotion of *in vitro* shoot elongation (Chen et al., 2008) and low doses of GA_3 associated to BAP affect positively the *in vitro* rooting formation and elongation (Magyar-Tábori et al., 2010), for *M. pudica* the GA_3 was ineffective in this process. Nevertheless, root formation was observed in culture media supplemented with 0.28 µM GA_3 even in the presence of BAP. This result was corroborated by Žiauka and Kuusienė (2010), who observed complete inhibition in root formation when *Populus tremula* L. nodal segments were treated with high doses of paclobutrazol, an antagonist molecule of gibberellin synthesis, indicating that GAs, in low concentrations, may become beneficial to root development. Apparently, reduced doses of GA_3, when associated with BAP, increase the effectiveness of *in vitro* propagation processes, particularly in explants elongation and root formation phases (Pati et al., 2006; Magyar-Tábori et al., 2010). However, in this study, the beneficial effects of gibberellins were not observed for *M. pudica*.

Although TDZ stimulates hyperhydricity more intensively than BAP and KIN (Kadota and Niimi, 2003), in the present study, 0.6 µM TDZ stimulated the proliferation of greater number of elongated shoots than in presence of other cytokinins. Low concentrations of TDZ were effective in inducing multiple *in vitro* shoots in peanuts cultures (Joshi et al., 2008). In *Lens culinaris* Medik., 0.5 µM TDZ also stimulate *in vitro* shoots formation (Chhabra et al., 2008). However, in response to 2.5 µM TDZ, they noticed complete inhibition in the shoots proliferation.

There was a decrease in the number of shoots higher than 0.5 cm in response to addition of NAA to the culture medium supplemented with 0.6 µM TDZ. According to Yucesan et al. (2007), high TDZ concentrations might reduce the shoots proliferation, regardless of the presence of auxins. According to Murthy et al. (1996), the ineffectiveness of auxins in stimulating shoots

proliferation may be attributed to TDZ effects on the modulation of its endogenous concentrations, suggesting that TDZ stimulates the *de novo* synthesis of natural auxins, dismissing its inclusion in the culture medium.

According to Ahmad et al. (2006), the transference of shoots cluster to a culture media without cytokinins reverse the rosette feature and stimulates the shoots elongation. A successful regeneration of the subdivided rosettes from TDZ was observed when they were transferred to MS culture media without growth regulators, showing that subcultivation in these conditions is crucial for later development of these explants (Khalafalla and Hattori, 1999; Joshi et al., 2008). Although Prathanturarug et al. (2005) has suggest that a second treatment with TDZ may increase culture multiplication, the results from our work do not corroborate with this possibility, since explants regeneration in culture media supplemented with high doses of TDZ was very low. The plantlets regenerated in MS medium presented normal morphology and similarity of plants not subjected to TDZ treatment, a result also found by Siddique and Anis (2007) in *Ocimum basilicum* L. cultures. Although the cultivation in culture media supplemented with 0.6 µM TDZ produced ten shoots higher than 0.5 cm, a number twice as high as that obtained with the best treatment using 5 µM BAP plus 0.5 µM NAA, the percentage of explants regeneration in subcultures was very low (20%). In fact, any treatment with TDZ, supplemented or not with NAA, did not reach 50% of regeneration. These results confirm the use of BAP as the best cytokinins among the tested for multiplication of *M. pudica*.

Auxin type and concentration effect on *in vitro* rooting process in *M. pudica*

The induction, initiation, and expression are the major phases of adventitious rhizogenesis (Kose et al., 2011). The auxins are important in all stages, presenting a crucial role in stimulating the pericycle cells determination (Hartmann et al., 1990). The majority of studies employing auxins are species dependent (Gürel and Wren, 1995; Parveen and Shahzad. 2011). Nevertheless, in our study, the supplementation with NAA did not increase shoots rooting. The same response was observed for IBA, with no significant differences between control and other treatments. Nevertheless, studying *Acacia tortilis* subsp. *raddiana* (Savi) Brenan micropropagation, Nandwani (1995) found the best rooting responses in medium supplemented with 14.7 µM IBA, suggesting that, for woody species, it may be necessary the addition of auxins at high concentrations. Despite that IBA has been successfully used in studies related to promotion of *in vitro* rooting (Shahinozzaman et al., 2012), it is more effective when employed to stimulate new rooting formation in cuttings (Hartmann et al., 1990; Ahmad et al., 2006).

In contrast to what was observed in relation to the NAA and IBA, some effects on rooting micro-cutting of *M. pudica* were observed in response to the IAA. In the first days of cultivation, the higher IAA concentration (0.4 µM) promoted the formation of branched and elongated roots. However, no significant differences between this dose and the control were found after 60 days of *in vitro* cultivation. IAA concentration ranging from 0.1 and 0.3 µM inhibited adventitious roots formation. The IAA also was the most efficient auxin for rhyzogenesis in *Cichorium intybus* L., although in a concentration tenfold lower than used in this study (Yucesan et al., 2007). It is known that in excessive concentrations, the IAA may affect the metabolism of endogenous auxin, promoting the activation of the *GH3* genes, encoding proteins involved in auxin homeostasis, making them inactive (Ludwig-Müller, 2011). The IAA endogenous level, in response to IAA doses up to 0.3 µM, was probably changed due to inactivation of this molecule by conjugation, which results in lower free contents of auxins in the bioactive form and, consequently, reducing adventitious roots formation. Nevertheless, at concentrations of 0.4 µM, despite possible activation of the *GH3* genes has occurred, IAA content was probably not fully conjugated, resulting in levels of auxins close to those found in the control.

The results from this study showed that, for *M. pudica in vitro* cultures, both type and concentration of auxins added to culture medium did not interfere significantly on *in vitro* rooting. The requirement of adventitious rooting formation in micropropagation protocols is estimated by success of the acclimatization stage, since some species are able to establish in *ex vitro* conditions even presenting poor *in vitro* rooting (Meiners et al., 2007; Parveen et al., 2010).

The procedures of acclimatization

The results found in this study show that *M. pudica* is a species of easy acclimatization, with survival of over 90% and with excellent vegetative development after transference to the greenhouse. The results found in our study show that the adventitious rooting developed at *in vitro* phase under stimulus of auxins was irrelevant, since both rooted and non-rooted micro-cuttings of *M. pudica* presented high survival rate to acclimatization. Moreover, the success in the acclimatization phase found in the present study was better than those found in other studies with *M. pudica* (Hassan et al., 2010; Ramesh et al., 2013). The plantlets mortality observed during the *in vitro* rooting stage was higher than in acclimatization phase. This result suggests that cultures of *M. pudica* exhibit high sensitivity to the ethylene generated *in vitro*, due to auxins added in the culture medium.

After 40 days in greenhouse conditions, period in which

all plantlets survived, they were transferred to pots under field conditions. *M. pudica* acclimatized plants presented normal vegetative and reproductive development (Figure 1f). After 60 days of acclimatization, the plants presented blossoming, showing that the reproductive development had not been affected by procedures of the *in vitro* cultivation.

Conclusion

The culture medium supplemented with 5 µM BAP plus 0.5 µM NAA is recommended for high multiplication rates and for providing top quality plantlets. *In vitro* rooting of micro-cuttings is high for *M. pudica*, even in the absence of auxins. Over 90% of plantlets transferred to the greenhouse can survive after the acclimatization phase. The procedures established in the present study allow a massive production of *M. pudica* plants for further pharmacological and ecophysiological studies on a species which has ecological, ornamental and medicinal importance.

ACKNOWLEDGEMENTS

We thank the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Programas de Apoio à Publicação/Pró-Reitorias de Pesquisa (PROPESQ) e Pós-gradução (PROPG)/Universidade Federal de Juiz de Fora (UFJF) for financial support for the development and publication of this work.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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academicJournals

Vol. 16(9), pp. 429-433, 1 March, 2017
DOI: 10.5897/AJB2017.15895
Article Number: 5691F1563013
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Quality characteristics of dried persimmon (*Diospyros kaki* Thunb) of different fruit sizes

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Received 16 January, 2017; Accepted 10 February, 2017

Fruits of persimmon are considered as one of the health promoting foods which has been established as one of the major fruits in Korea. Persimmon is one of the fruits processed mainly by drying. Quality characteristics of dried persimmon prepared with different fruit sizes of cultivar Sanggandungsi were investigated. Smaller sized fruits showed significantly high weight loss as compared to bigger ones due to drying. On the other hand, bigger sized fruits possessed significantly high value for hardness after 35 days of drying. Size of fruit was not found to be vital for soluble solid contents of dried fruits. One of the important bioactive molecules of persimmon fruits (tannin content) is significantly high in bigger sized fruits as compared to smaller ones. Significant differences in various parameters among different sized fruits indicate that, physicochemical characteristics of dried persimmon fruit could be affected by the fruit size.

Key words: Drying, fruit size variation, physicochemical properties.

INTRODUCTION

Persimmon (*Diospyros kaki* Thunb.) fruit is rich in health promoting substances. It contains health promoting bioactive compounds like, ascorbic acid, condensed tannins, and carotenoids, which have antioxidant properties (Rao and Rao, 2007; Shahkoomahally et al., 2015; Fu et al., 2016). Consumption of persimmon fruit could be useful for the prevention of atherosclerosis (Zhang et al., 2016) because of its plasma lipid-lowering ability and antioxidant properties.

In China, it is widely consumed against health problems such as coughs, hypertension, dyspnoea, paralysis, burns, and bleeding because the fruit is rich in nutrients such as vitamin C, vitamin A, calcium, iron, and phenolic compounds (Nicoleti et al., 2007). It also shows inhibitory effect on human lymphoid leukemia cells, mutagenicity of C-nitro and C-nitroso compounds (Achiwa et al., 1997) as

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well as the incidence of stroke and the extension of the lifespan of hypertensive rats (Uchida et al., 1995; Xie et al., 2015). Reports suggest that consumption of the persimmon fruits is good for human health (Lia et al., 2013; Butt et al., 2015). However, regular availability of fruits is difficult since persimmon is grown only in a particular season in specific climatic regions. Moreover, some consumers are reluctant to buy fresh fruits because of the possibility of having its poor quality when consumed fresh (Byrne, 2002). Such circumstances favor producing high quality dried fruits.

Drying is one of the oldest methods of food preservation (Dehydration, 1998). Fruits can be dried to preserve and allow them to be available during the period, when fresh fruits could not be harvested. The dry fruits may retain the characteristics of natural products, offer reduced transportation cost due to reduced mass, and resist growth of mold and other microorganisms which cause deterioration in fresh fruits (Marques et al., 2006). Drying of persimmon fruits also reduces the astringency that comes from the phenolic compounds of some varieties. Astringency and color have been reported as important quality characteristics of dried persimmon (Akyildiz et al., 2004).

Persimmon, one of the best health promoting fruits, is processed mainly by drying. Few reports have been published describing the effect of drying on some fruits. However, there was no published reference report describing the quality characteristics of dried persimmon of different fruit sizes. The objective of this study was to investigate the quality characteristics of dried persimmon of different fruit sizes. The present study may provide information about selection of proper sized persimmon fruits, for drying.

MATERIALS AND METHODS

Preparation of dried persimmon

Persimmon fruits were dried following the method of Kim et al. (2014). The fruits were washed with tap water and peeled off manually using a peeler. The pedicels of peeled fruits were tied to string and hanged for natural drying. Before peeling and subsequent drying, the fruits were divided into 4 categories based on their mass as: S-1 (200±5 g), S-2 (240±5 g), S-3 (280±5 g), and S-4 (320±5 g).

Seven fruits were tied on 1 m long strings and 2 to 3 strings were tied together while hanging for drying. The strings of persimmons were hung under the eaves of a roof, where they got plenty of sun and breeze but were protected from rain. The strings of persimmons fruits kept for drying were hung more than 3 m above the ground. In the present experiment, fruits were dried for 35 days. Average temperature and relative humidity during the fruit drying period were 7.2°C and 78.79%, respectively.

Fruit weight and soluble solid

Weight of raw and dried persimmon fruits (with pedicel) was measured using a digital balance (GT 480, Ohaus, Florham Park, NJ, USA). Soluble solid content was determined using a refractometer (RX-5000α, Atago, Tokyo, Japan) and expressed as °Brix. All measurements were made in triplicate and average values were reported.

Determination of moisture and hardness

Moisture content determined was measured following standard method (AOAC, 1995 method 950.46). The weight loss was calculated by subtracting the weight of the fresh peeled fruits and the weight of the dried peeled fruits, dividing the difference by the weight of the fresh peeled fruits, and then multiplying the quotient by 100. Hardness was measured using rheometer (Compac-100, Sun Scientific Co. LTD, Japan) under the following operational conditions: test type, mastication; adaptor type, circle; adaptor area, 0.20 cm^2; and table speed, 60 mm/min.

Color measurement

L*(lightness), a*(redness, + or greenness, -), and b*(yellowness, + or blueness,-) values of dried persimmon were measured using a chroma meter (CR-300; Minolta Corp., Osaka, Japan). A Minolta calibration plate (YCIE=94.5, XCIE=0.3160, YCIE=0.330) and a Hunter Lab standard plate (L*=97.51, a*=-0.18, b*=+1.67) were used to standardize the instrument using a D65 illuminant. Color values were measured directly on 3 zones, of each dried persimmon and average values were reported.

Tannin content

Tannin content of dried persimmon was analyzed according to the method described by modified Prussian Blue assay (Graham, 1992; Price et al., 1988). One hundred milliliters of sample extract was added to 3 mL of distilled water, centrifuged at 10000× *g* for 15 min, and supernatant was collected. Absorbance was measured at 700 nm using a spectrophotometer (Hewlett- Packard, model 8452A, Rockvile, USA). The total phenolic contents were calculated from the calibration curve prepared from the absorbance of gallic acid standard solutions (Park, 1999). After which, phenolic content was expressed as tannin content.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and differences between means at $P<0.05$ were analyzed using the Tukey test. The statistics package version 4.0 (Analytical Software,Tucson, AZ, USA) was used for statistical analysis. Average values of triplicate measurements were considered for statistical analysis unless otherwise mentioned.

RESULTS AND DISCUSSION

Weight loss

Four sorted persimmon fruit samples for each group with average weights of 200, 240, 280, and 320 g were peeled and dried at natural condition for 35 days. Weight loss of peeled persimmon at the end of drying was significantly ($P \leq 0.05$) high for fruits of lower weights, S-1 (55.5%) and S-2 (55.7%) as compared to that of S-3 (52.0%) and

Table 1. Weight loss of dried persimmon after 35 days of drying.

Sample[1)]	Weight (g) of peeled and unpeeled fruits (in parentheses) at different drying period (days)		Weight loss of peeled fruit at day 35 (%)
	0	35	
S-1	160.3±2.3[2)] (200.2±5.6)[3)d]	71.3±1.9[d]	55.5±0.6[a]
S-2	201.4±3.1 (242.4±3.6)[c]	89.3±2.3[c]	55.7±0.5[a]
S-3	240.2±2.1 (280.5±3.2)[b]	115.2±1.8[b]	52.0±0.3[b]
S-4	281.1±3.3 (320.4±4)[a]	141.2±3[a]	49.8±0.5[c]

[1)]Sanggamdungsi persimmon fruits of different weights. S-1, (200±5 g); S-2, (240±5 g); S-3, (280±5 g); S-4, (320±5 g).[2)]Each value represents mean±SD of triplicate measurements and value with different superscripts within a column indicate significant differences (P ≤ 0.05).

Table 2. Moisture content, hardness, and soluble solid content of dried persimmon after 35 days of drying.

Sample[1)]	Moisture content (%)	Hardness (Kg/5 mm)	Soluble solid (°Bx)
S-1	49.0±0.1[b]	0.5±0.001[d]	32.3±0.2[b]
S-2	48.0±0.3[d]	0.93±0.002[c]	31.1±0.1[c]
S-3	48.5±0.2[c]	1.25±0.03[b]	34.1±0.1[a]
S-4	50.1±0.2[a]	1.56±0.01[a]	31.1±0.2[c]

[1)]Sanggamdungsi persimmon fruits of different weights. S-1, (200±5 g); S-2, (240±5 g); S-3, (280±5 g); S-4, (320±5 g). [2)]Each value represents mean±SD of triplicate measurements and value with different superscripts within a column indicate significant differences (P ≤ 0.05).

S-4 (49.8%) (Table 1). Lower wet loss, in the present study, in the bigger fruits might be due to higher dry matter contents in bigger sized fruits. Results of Tittonell et al. (2001) suggest that, bigger sized leaves contain more dry matters as compared to that in smaller ones. Moreover, the weight loss increased with a decreasing sample size of apple fruit slices (Van Nieuwenhuijzen et al., 2001).

Hardness and moisture and soluble solid contents

Hardness of the small sized fruits was significantly low as compared to bigger fruits (Table 2). Hardness of S-4 (1.56 Kg/5 mm) was almost three times higher than that of S-1 (0.5 Kg/5 mm). The reason behind higher hardness value in bigger sized fruits might be due to, higher dry matter content in them. The results are in agreement with those of Tittonell et al. (2001) which suggest that, bigger sized leaves contain more dry matters as compared to that in smaller ones. Moisture contents of S-4 (50.1%) were significantly higher than that of other samples (Table 2). Higher moisture content in the bigger size fruits might be explained by the fact that, a smaller sample has a larger surface area/volume ratio as compared to the larger samples (Van Nieuwenhuijzen et al., 2001).

Fruit size could directly affect drying characteristics such as drying rate and moisture diffusivity in blueberry (Shi et al., 2008). MacGregor (2005) found that under similar drying conditions, larger fruits of blueberry took longer to reach the final moisture content than smaller ones, but the larger berries had a higher mass-losing rate than the smaller ones. Sample S-3 (34.1 °Bx) contained significantly high soluble solid contents as compared to other samples (Table 2). Blažková et al. (2002) also found positive correlations between the weight (size) of fruits and soluble solid contents.

Hunter color value and tannin content

Color value of the dried persimmon is known to play an important role in the consumer's acceptability. Hunter's color value of the dried persimmon of different size is shown in Table 3. S-4 possessed the highest (36.5) value for lightness and yellowness (26.2) while S-1 scored the highest value for redness (16.0). Fruit color changes to yellow or red according to kind and amount of carotenoid as persimmon matures and dried persimmon surface possesses more redness than unripe fruit (Kim et al., 1986). The highest tannin content was found in S-3 (0.1%) followed by S-4 (0.09%) (Table 3).

Tannins are one of the important bioactive molecules present in persimmon (Ahn et al., 2002; Vázquez-Gutiérrez et al., 2013). In the present study, bigger fruits were found to contain higher amount of tannins as compared to smaller ones. Results of the present study

Table 3. Hunter color value and tannin content of dried persimmon after 35 days of drying.

Sample[1)]	Color value			Tannin content (%)
	L*	a*	b*	
S-1	36.4±0.03[b]	16.0±0.02[a]	25.9±0.01[b]	0.04±0.01[d]
S-2	34.6±0.08[d]	13.9±0.03[d]	23.9±0.02[c]	0.06±0.01[c]
S-3	35.4±0.04[c]	14.1±0.01[c]	23.3±0.03[d]	0.1±0.01[a]
S-4	36.5±0.03[a]	14.9±0.02[b]	26.2±0.02[a]	0.09±0.01[b]

[1)]Sanggamdungsi persimmon fruits of different weights. S-1, (200±5 g); S-2, (240±5 g); S-3, (280±5 g); S-4, (320±5 g). [2)]Each value represents mean±SD of triplicate measurements and value with different superscripts within a column indicate significant differences (P ≤ 0.05). [3)]L*, lightness (100, white; 0, black); a*, redness (-, green; +, red); b*, yellowness (-. blue; +, yellow).

also agree with those of Roby et al. (2004) who also found higher tannin content in bigger sized wine grapes.

Conclusion

Different size of persimmon fruits varied in their physicochemical characteristics. Weight loss due to drying was high in smaller size fruits. Hardness value was high for bigger size fruits. However, soluble solid content was not found to be affected by fruit size.

Tannins, which are one of the vital bioactive molecules in persimmon, were higher in bigger fruits than in smaller ones. Results of the present study provide information about selection of proper sized fruits for drying. Overall results of the present study indicate that, physicochemical characteristics of dried persimmon fruit are affected by the fruit size.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work was financially supported by the Rural Development Administration, Republic of Korea (PJ010239022016).

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