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EVALUATING VIABLE CELL INDICATORS FOR FILAMENTOUS CYANOBACTERIA AND THEIR APPLICATION

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ABSTRACT

Filamentous cyanobacteria, such as *Anabaena* 7120 have great industrial potential due to their capability to be genetically engineered to produce next-generation biofuels while using minimal nutrients. One challenge of working with these microbes is that classical methods of quantifying cell viability are not effective due to their filamentous morphology. Therefore, fluorescent assays were evaluated to determine if they could be used as a reliable indicator of cell viability. Initially, a dual-stain assay using SYTO® 9 and SYTOX® Blue was investigated. Fluorescence from SYTO® 9 was accurately correlated with viable cells; however, SYTOX® Blue did not work as a non-viable cell indicator due to non-specific binding in both viable and non-viable cells. Autofluorescence from light harvesting pigments was also evaluated as a viable cell indicator, but unfortunately these pigments resulted in several emission peaks that couldn't be captured by a single emission filter. Moreover, certain light harvesting pigments continued to fluoresce after the cell became non-viable. SYTO® 9 was then compared to absorbance and chlorophyll content to quantify viable *Anabaena* 7120 in a chemical inhibition testing protocol. This protocol requires a low initial biomass concentration to prevent binding of the chemicals to cell biomass, and at low cell densities SYTO® 9 was superior to absorbance and chlorophyll content in quantifying viability. It was also determined that SYTO® 9 allows for the evaluation of different cultivation media on the growth of cyanobacteria in photobioreactors. SYTO® 9 is a reliable, accurate indicator of viability of filamentous cyanobacteria and can be used in a high-throughput manner via a microplate reader.

Keywords: Biofuel, Chemical inhibition test, Filamentous cyanobacteria, Photobioreactor, SYTO® 9

INTRODUCTION

Developing processes to produce renewable fuels and chemicals remains important, as fossil fuel reserves are finite and the adverse effects of fossil fuel generated greenhouse gases are well documented **(Chen *et al*., 2011; Von Blottnitz and Curran, 2007)**. Using filamentous cyanobacteria for this purpose is an attractive option as they are capable of producing biofuels and chemicals from CO_2 and solar energy **(Machado and Atsumi, 2012)**. For example, filamentous cyanobacteria have already been engineered to produce next-generation biofuels such as limonene **(Halfmann *et al*., 2014a)**, farnesene **(Halfmann *et al*., 2014b)**, and linalool **(Gu *et al*., 2012)**. Many strains of filamentous cyanobacteria are also diazotrophic, using specialized cells called heterocysts to fix atmospheric nitrogen. These attributes have led to cyanobacteria emerging as a promising platform organism for production of fuels and chemicals **(Schoepp *et al*., 2014)**.

Classical methods of monitoring cell viability, such as viable cell counts and flow cytometry, will not work for filamentous cyanobacteria due to their filamentous morphology **(Johnson *et al*., 2015)**. **Sarchizian and Ardelean (2012)** do report using the direct viable count method with epi-fluorescence microscopy on filamentous cyanobacteria isolated from a mesothermal spring for quantification of viable cells. However, many strains of filamentous cyanobacteria tend to aggregate in liquid media making accurate direct counts via microscopy quite difficult. Cell viability information is critical for research purposes (e.g., screening mutants for increased tolerance to biofuels they are engineered to produce), as well as to monitor cyanobacteria performance in photobioreactor (PBR) systems **(Jin *et al*., 2014)**.

One potential option for quantifying viability of filamentous cyanobacteria is the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Life Technologies™, Carlsbad, CA, USA), which has been successfully used to quantify viability of several types of bacteria **(Magajna and Schraft, 2015; Zeidán-Chuliá *et al*., 2015)**, including cyanobacteria **(Zhu and Xu, 2013)**. Previously, we determined that this kit does not allow accurate viability estimates of filamentous cyanobacteria, since the non-viable cell indicator propidium iodide (PI) also crossed the intact membranes of viable filamentous cyanobacteria, thereby leading to erroneous results **(Johnson *et al*., 2015)**. Thus, alternative methods to quantify viability in filamentous cyanobacteria need to be developed.

SYTOX® Blue is one of several SYTOX® Dead Cell Stains that should not be able to cross intact cell membranes. This fluorochrome has been successfully used to quantify non-viable cells in bioaggregates **(Chen *et al*., 2007)** and in aerobic granules **(Adav *et al*., 2007a)**. In this study, SYTOX® Blue was tested as a method to quantify non-viable filamentous cyanobacteria. To attempt to quantify viability in filamentous cyanobacteria, autofluorescence from the light harvesting pigments phycobiliproteins (PBS) and chlorophyll α were also tested in this study. Fluorescence from the viable cell indicator SYTO® 9 was compared to absorbance and chlorophyll α content to determine which is a superior method for monitoring viability in conditions even when biomass content is minimal. Finally, SYTO® 9 was evaluated for monitoring the effect of different cultivation media on cyanobacterial growth in 40 L PBRs.

MATERIALS AND METHODS

Microbial strains, maintenance, and culture conditions

The filamentous, diazotrophic cyanobacterial strain *Anabaena* sp. PCC 7120, a model species for cyanobacteria **(Bryant, 2006; Rippka *et al*., 1979)**, was obtained from the Pasteur Culture Collection (Paris, France). In our previous study, 3 strains of filamentous cyanobacteria were investigated and all had similar results regarding interactions with viable cell indicators **(Johnson *et al.*, 2015)**. Therefore, in this study we only investigated *Anabaena* 7120, but are confident the results described herein would be similar for most diazotrophic strains of filamentous cyanobacteria. A putative spontaneous mutant of *Anabaena* 7120, referred to hereafter as A7120.(0.32t).farn, was isolated during prior directed evolution trials in which *Anabaena* 7120 was acclimated to 0.32 g/L farnesene **(Johnson *et al*., 2016a)**. For long term storage, strains were frozen at -80°C in 5% v/v methanol. For short term maintenance, the cyanobacteria were grown on BG11 agar (1.5% agar) **(Allen and Stanier, 1968)** at pH 7.1 and

incubated at room temperature under constant illumination of 24 µE m^{-2} s^{-1}, and then stored at room temperature. Light intensities were measured with a Heavy Duty Light Meter with PC Interface (Extech Intsruments, Waltham MA, USA).
In the experiments described below, cyanobacterial cultures were grown either in 27 ml screw capped test tubes, 250 ml Erlenmeyer flasks, or in 40 L PBRs. The 27 ml test tubes had an open top cap with PTFE/silicone septa to allow inoculation and sampling via syringe and needles, and yet prevent the loss of volatile chemicals that were being tested for cell toxicity. Tubes were filled with ~27 ml BG11 with 20 mM HEPES buffer and 0.5 g/L $NaHCO_3$ for a carbon source. The tubes were incubated at room temperature under constant illumination of approximately 24 µE m^{-2} s^{-1} using fluorescent lights while rotating at 8 rpm in a Thermo Fisher Scientific™ Labquake™ Tube Rotator (Thermo Fisher Scientific™, Waltham, MA, USA). The 250 ml Erlenmeyer flask trials contained 100 ml of BG11 broth at pH 7.1 supplemented with 20 mM HEPES buffer. Flasks were stoppered with a foam stopper and the opening was covered with aluminum foil. The flasks were incubated in a Lab-Line® Incubator-Shaker (Lab-Line® Instruments, Melrose Park, IL, USA) at 30°C and 100 rpm under constant illumination of 19 µE m^{-2} s^{-1} using fluorescent lights.
PBR trials were conducted in 40 L transparent fiberglass flat bottom tanks (Solar Components Corp., Manchester, NH, USA) that were sparged from the bottom with a mixture of 95-5% air-CO_2 at a rate of 0.25 L/L/min. The culture medium consisted of 30 L of cultivation medium and was inoculated with 1.5 L (5%) of an *Anabaena* 7120 culture that had been grown to mid-log phase. The reactors were incubated at room temperature (20-22°C) under constant illumination of approximately 40 µE m^{-2} s^{-1} using fluorescent lights until ~2 days after stationary phase was reached.

Statistical analyses

To conduct *post hoc* power analysis and to ensure the sample size of each treatment was large enough to achieve statistical power of ≥ 0.95, the G*Power statistical power analysis program was utilized **(Faul *et al.*, 2007)**. To determine if significant differences existed among the treatments (different cultivation media) on filamentous cyanobacterial growth, a one-way ANOVA was performed using the R statistical software program **(R Core Team, 2013).** If it was determined by the ANOVA that significant differences did occur, then the Tukey's test was performed to determine among which treatments the statistical differences occurred.

Evaluating SYTOX® Blue and SYTO® 9 as a dual-stained assay to quantify viability

SYTOX® Blue and SYTO® 9 were purchased from Thermo Scientific (Waltham, MA, USA). In a previous study by our research group, it was determined that SYTO® 9 stains viable filamentous cyanobacteria cells as expected **(Johnson *et al.*, 2015)**. To determine if SYTOX® Blue fluoresces non-viable cyanobacterial cells at the expected excitation/emission wavelengths, 1 ml of a mid-log phase culture of *Anabaena* 7120 was chemically killed by exposing it to 10 ml of 70% isopropanol in a 15 ml conical test tube that was incubated at room temperature for 1 h with manual mixing every 15 min. The cells were then recovered by centrifuging at 10,000 rpm for 10 min at room temperature. The cell pellet was then washed in 1 ml BG11 and centrifuged at 10,000 rpm for 1 min at room temperature. Next, the washing step was repeated two additional times and the final cell pellet was resuspended in 1 ml BG11. SYTOX® Blue was added to the cell suspension at a final concentration of 5 µM. A 100 µl aliquot of the culture was then transferred to a microscope slide for examination by a Cytation™ 3 Cell Imaging Multi-Mode reader (BioTek® Instruments, Inc., Winooski, VT, USA). Cyanobacterial cells were observed for fluorescence in the blue spectra by the reader at the maxima excitation/emission for SYTOX® Blue when the dye is bound to DNA (444/480 nm).
To evaluate a dual-stain assay using SYTO® 9 (for viable cells) and SYTOX® Blue (for non-viable cells), *Anabaena* 7120 was grown to mid-log phase in a 250 ml Erlenmeyer flask under the conditions described above. The following protocol was adapted from **Johnson *et al.*, (2015).** Ten ml of the culture was transferred to a 15 ml conical and processed as shown in Figure 1 to obtain solutions containing equal numbers of viable and non-viable cells. The solutions were mixed in 2 ml ratios of 0:100, 10:90, 50:50, 90:10, and 100:0. In this study, SYTOX® Blue was used as a replacement for PI as a non-viable cell indicator. In this assay, per the manufacturer's protocol **(Molecular Probes, 2004)**, mid-log cyanobacterial culture is assumed to contain 100% viable cells. Obviously this isn't entirely accurate, but is the basis of the assay that has been used successfully in previous studies **(Adav *et al.*, 2007a; Chen *et al.*, 2007)**. One hundred µl of viable: non-viable cell solutions were pipetted into wells of a 96-well plate in triplicate. SYTO® 9 and SYTOX® Blue were added to each well at a concentration of 5 µM for each stain. The plates were then wrapped in aluminum foil and incubated in the dark at room temperature for 15 min. After incubation, fluorescence was measured by a Synergy 2 Multi-Mode Microplate Reader (BioTek®, Winooski, VT, USA). To measure green fluorescence from SYTO® 9 stained cells, a fluorescence filter with excitation wavelength 485 ± 20 nm; emission wavelength 528 ± 20 nm was used. To measure blue fluorescence from SYTOX® Blue stained cells, a fluorescence filter with excitation wavelength 440 ± 30 nm; emission wavelength 485 ± 10 nm was used. For each trial, an empty well was used as a negative control to ensure the microplate reader was working properly.

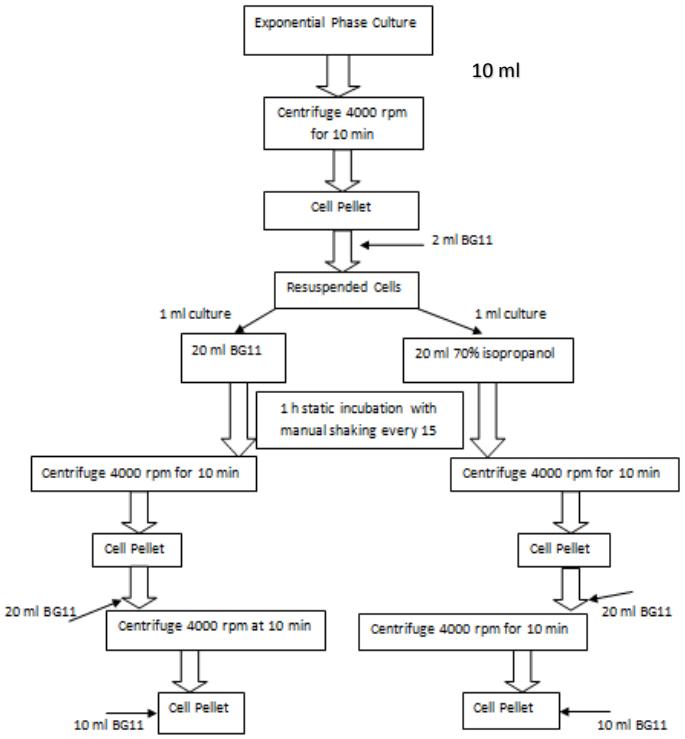


Figure 1 Process used to prepare viable and non-viable cells for use in developing calibration curve

Figure adapted from **Johnson *et al.*, (2015)**. The resulting samples were mixed in ratios of 0:100, 10:90, 50:50, 90:10, and 100:0. (Isopropyl alcohol used to make cells non-viable).
Linear regression was performed to plot the ratio of green and blue fluorescence *vs.* cell viability percentage. The data was analyzed by dividing the green fluorescence (F) emitted (em) by the blue fluorescence emitted, ($Ratio_{G/B}$ = $F_{cell,em1}/F_{cell,em2}$). The ratio was plotted as $Ratio_{G/B}$ versus cell viability percentage. Linear regression analysis was performed to calculate and assess the correlation coefficient.
To further investigate SYTOX® Blue as a non-viable cell indicator, fluorescence data was analyzed from the microplate reader. Each well contained 100 µl of either *Anabaena* 7120 or BG11, as well as both SYTO® 9 and SYTOX® Blue, or one stain individually at a final concentration of 5 µM. An empty well was used as a control to ensure the microplate reader was working properly. To measure fluorescence from the stains, the filters that were described above were used.

Comparison of SYTO® 9 and autofluorescence as viable cell indicators

To compare SYTO® 9 and autofluorescence as viability assays in filamentous cyanobacteria, a 40 L PBR containing 30 L BG11 was inoculated as described above. The inoculum had been grown in a 4 L Erlenmeyer flask containing 2 L BG11 in a Lab-Line® Incubator-Shaker under the conditions described above. After inoculation, the PBR was incubated under the conditions described above, and samples were taken daily for absorbance (OD_{700}) and viability until stationary phase was reached. The samples were taken from a homogenous suspension in the PBR caused by air sparging from the bottom of the reactor. For absorbance, 1 ml samples were measured using a Thermo Fisher Scientific™ Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA).
To measure viability via SYTO® 9, 100 µl samples were transferred to a 96-well plate and SYTO® 9 was added to each well at a concentration of 5 µM. Fluorescence was then measured by the microplate reader using the excitation/emission wavelength spectra described above. For viability measurements via autofluorescence, 100 µl samples were transferred to a 96-well plate. There are various wavelengths used in the literature, for example, various emission spectra have been used for chlorophyll α determination, including 681 nm, **(Guzmán *et al.*, 2015)** 672 nm, **(Lozano *et al.*, 2013)** and 660 nm **(Ogawa and Sonoike, 2015)**. Also, PBS have fluorescent peaks at 635, 645, 654, 659, and

673 nm **(Gryliuk *et al*., 2014)**. Thus, several excitation/emission filters were tested on the microplate reader to determine which yielded the greatest fluorescence intensity of a mid-log phase culture of *Anabaena* 7120. The filters tested were (excitation/emission): 520 ± 20 / 620 ± 40; 485 ± 20 / 620 ± 40; 540 ± 25 / 620 ± 40 (all units = nm).

Assessing cell viability in a chemical inhibition test

To investigate if fluorescence from SYTO® 9 allows for the screening of putative filamentous cyanobacterial mutants that potentially have developed an increased tolerance to toxic chemicals, SYTO® 9 stain was evaluated for use in measuring the viability of filamentous cyanobacteria that were exposed to farnesene in chemical inhibition tests. Farnesene is volatile **(Asai *et al*., 2016; Ruther and Hilker, 1998)**, thus screening and acclimation trials needed to be conducted in sealed test tubes to maintain a constant titer of the chemical. As no atmospheric CO_2 was able to enter the test tube, 0.5 g/L $NaHCO_3$ was added to the BG11 medium after autoclaving to serve as the carbon source **(Hailing-Sørensen *et al*., 1996; Johnson *et al*., 2016c; Mayer *et al*., 2000)**.
Twenty-seven ml test tubes containing ~27 ml of $NaHCO_3$-supplemented BG11, along with 0.032 g/L farnesene, were inoculated with 270 µl (1%) of either a mid-log phase *Anabaena* 7120 culture or a putative spontaneous mutant A7120(0.32t).farn. Cultures were incubated under conditions described above. Daily samples were taken via a needle and syringe in order to maintain constant titers of farnesene. For viability determinations, 100 µl samples were transferred to a microplate. SYTO® 9 was added to each well at a concentration of 5 µM, and fluorescence was determined as described above. Absorbance and chlorophyll α were measured by diluting 100 µl samples with 900 µl BG11 in a cuvette. Absorbance was measured at an optical density (OD) of 700 nm. Chlorophyll α was determined using the equation: Chl α content (µg/ml) = 14.96 (OD_{678} – OD_{750}) – 0.616 (OD_{720} – OD_{750}), and the protocol described by **Guoce *et al.* (2011)**.

Assessing cell viability in photobioreactors

To investigate if fluorescence from SYTO® 9 allows for the comparison of different treatments on the viability of filamentous cyanobacteria in PBRs, *Anabaena* 7120 was cultivated in various growth media. The cultivation media evaluated were as follows: BG11 (standard BG11 includes sodium nitrate), BG11 supplemented with urea rather than sodium nitrate ($BG11_U$), BG11 without a nitrogen source ($BG11_0$), and tap water. The cultivation media containing nitrogen sources (BG11 and $BG11_U$) contained 0.248 g/L nitrogen. The PBRs described above were inoculated with 1.5 L (5%) of a mid-log phase culture of *Anabaena* 7120. The PBRs were incubated in the conditions previously above described. For daily viability determinations, 100 µl samples were transferred to a 96-well microplate. SYTO® 9 was then added to each well at a concentration of 5 µM, and fluorescence was determined as described above.

RESULTS AND DISCUSSION

Evaluating SYTOX® Blue and SYTO® 9 as a dual-stain assay for quantifying viability

SYTOX® Blue is a nucleic acid binding fluorochrome commonly used to stain non-viable bacterial cells, as it should not be able to penetrate an intact cell membrane **(Adav and Lee, 2008; Krause *et al*., 2007; Truernit and Haseloff, 2008)**. SYTO® 9 is a fluorescent dye capable of penetrating most cellular membranes, causing all cells containing nucleic acids to fluoresce green **(Lee and Rhee, 2001; Shi *et al*., 2007)**. SYTOX® Blue was used as an alternative to the non-viable cell indicator PI. PI was previously shown by **Johnson *et al*., (2015)** to not function properly as a non-viable cell indicator in filamentous cyanobacteria because it also stained viable cells. We postulated that this was due to intercellular channels that allow nutrient passage between cells in the filaments **(Mullineaux *et al*., 2008)**.
Previously, we had confirmed that SYTO® 9 caused viable *Anabaena* 7120 cells to fluoresce in the green spectra **(Johnson *et al*., 2015)**. Using the Cytation™ 3 microplate reader, we confirmed that SYTOX® Blue caused non-viable *Anabaena* 7120 cells to fluoresce in the blue spectra (data not shown). These observations were consistent with other studies and information from the stain's manufacturer **(Adav *et al*., 2007b; Chen *et al*., 2007; Filoche *et al*., 2007; Sato *et al*., 2004; Zhu and Xu, 2013)**.
To determine if a dual-stain assay consisting of SYTO® 9 and SYTOX® Blue is capable of accurately quantifying the viability of filamentous cyanobacteria, calibration curves were generated to correlate the ratio of green (SYTO® 9, viable cells) to blue (SYTOX® Blue, non-viable cells) fluorescence against known mixtures of viable and non-viable cells. Figure 2 shows the plots and regression lines for 3 trials. All trials had high R^2 values (0.96-0.98). Trials 1 (m = 0.0129) and 3 (m = 0.0101) had similar slopes, compared to Trial 2 (m = 0.006). Trials 1 (b = 0.7186) and 2 (b = 0.8457) had virtually identical y-intercepts compared to Trial 3 (b = 1.6693). The variability in the results between the 3 trials indicated that there was a problem with the dual-stain assay.

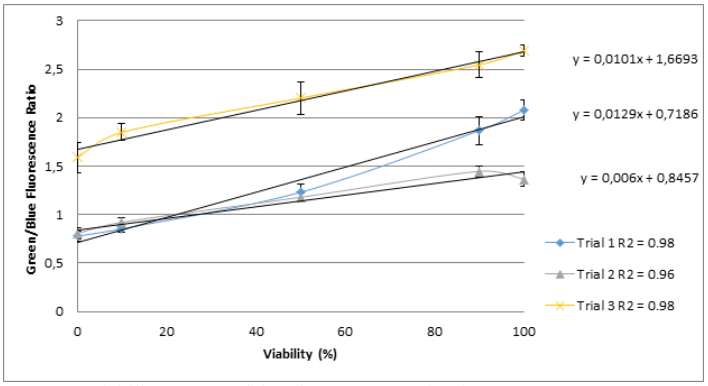


Figure 2 Viability *vs.* green/blue fluorescence ratios for *Anabaena* sp. PCC 7120

In each trial, triplicate SYTO® 9 green and SYTOX® blue fluorescence measurements were taken for each mixture of viable and non-viable cells. This data was used to calculate the green/blue fluorescence ratio. Error bars represent the standard deviation. The black lines represent the least-squares fit.

To investigate why the correlation equations were not identical for the 3 trials shown in Figure 2, we assessed the underlying data. Figure 3 shows the separate plots for green (SYTO® 9) and blue (SYTOX® Blue) fluorescence *vs.* % viability from Trial 1 (Trial 2 and Trial 3 had virtually identical results to Trial 1), as well as the calculated ratio. Fluorescence from the SYTO® 9 stain provided the expected positive slope of green fluorescence intensity over percent viability. However, fluorescence from the SYTOX® Blue stain resulted in a relatively flat slope instead of the anticipated strong negative slope. The flat slope indicates that SYTOX® Blue was staining both non-viable and viable cells, which is similar to what occurred with PI in filamentous cyanobacteria **(Johnson *et al*., 2015)**. Thus, it appears that the SYTO® 9 and SYTOX® Blue dual-stain assay is not precise in this application. Other researchers have noted the occurrence of misinterpretations in live/dead staining results for various reasons **(Bridier *et al*., 2015; Johnson and Criss, 2013; Lu *et al*., 2014; Stiefel *et al*., 2015)**.

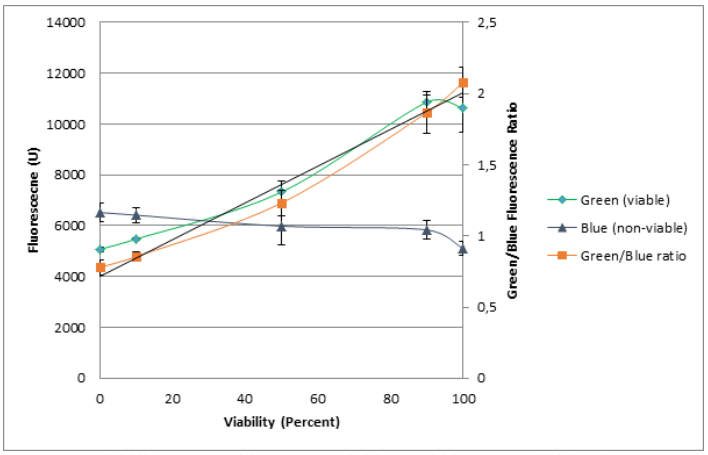


Figure 3 Viability assessed by green (viable) and blue (non-viable) fluorescence for *Anabaena* sp. PCC 7120

Each data point represents the average of triplicate readings, and error bars represent the standard deviation. This data was used to calculate the green/blue fluorescence ratio, which is also shown, along with the least-squares fit line (black line).
To ensure that the BG11 medium and the BG11 medium with fluorochromes added was not the cause of inaccuracy in the dual-stain assay, raw data from the microplate reader was also analyzed for samples containing the medium only, as well as the medium with 100% viable cells (Table 1). For green fluorescence, the average fluorescence intensity readings (arbitrary unit: U) were 433 for the empty well, which was considered background fluorescence. For BG11 + SYTO® 9 the average intensity was 1,835 U, which is about 4x higher than the empty well and represents the baseline green fluorescence of the medium and the SYTO® 9 dye. BG11 + SYTOX® Blue average intensity was 325 U, which suggested that the SYTOX® Blue dye did not provide any green fluorescence, since the intensity was approximately the same as the background fluorescence reading. BG11 + SYTO® 9 + SYTOX® Blue resulted in a fluorescence reading of 1,767 U, which was approximately the same as observed when just SYTO® 9 was added. The data from the controls described above is what would be expected.

Table 1 Microplate readings of Anabaena sp. PCC 7120 evaluating fluorescence intensity [arbitrary units (U)] of SYTO® 9 and SYTOX Blue Averages and standard deviations are representative of a triplicate.

	Empty Well	BG11 + SYTO 9	BG11 + SYTOX Blue	BG11 + 2 dyes	*Anabaena* 7120 + SYTO 9	*Anabaena* 7120 + SYTOX Blue	*Anabaena* 7120 + 2 dyes
SYTO® 9 (Ex 485 ± 10 Em 528 ± 20 nm)	433.00 ± 9.84	1835.00 ± 84.18	325.33 ± 9.71	1767.00 ± 105.51	26585.33 ± 1373.56	303.00 ± 6.92	28218.67 ± 2752.60
SYTOX (Ex 440 ± 30 Em 485 ± 10 nm)	2745.67 ± 8.62	2095.00 ± 21.07	1830.33 ± 44.16	4160.00 ± 898.86	4609.00 ± 354.23	3051.33 ± 109.74	6111.00 ± 544.47

Viable *Anabaena* 7120 cells in BG11 + SYTO® 9 yielded an intensity of 26,585 U, and this high green fluorescence reading strongly suggested that SYTO® 9 accurately measured viable cells. *Anabaena* 7120 in BG11 + SYTOX® Blue resulted in a reading of only 303 U, which was approximately the same as the background fluorescence reading. Thus, the SYTOX® Blue dye did not provide any green fluorescence. *Anabaena* 7120 in BG11+ SYTO® 9 + SYTOX® Blue yielded a reading of 28,218 U, and this green fluorescence intensity was approximately the same as observed when only SYTO® 9 was added. The above data yielded from the treatments with viable cells was expected. Therefore, we concluded that SYTO® 9 and the resulting green fluorescence correlate accurately with cell viability, and SYTOX® Blue does not interfere with the green fluorescence.

For blue fluorescence, the average fluorescence intensity for the empty well was 2,745 U, which was background fluorescence. BG11 + SYTO® 9 yielded an intensity of 2,095 U, which suggested that the SYTO® 9 dye did not provide any blue fluorescence, since the intensity was ~25% lower than the background fluorescence reading. The data also showed that the background fluorescence intensity decreased when the culture medium was added to the well. BG11 + SYTOX® Blue resulted in an intensity of 1,830 U, which was ~33% less than the background fluorescence reading, but almost identical to the BG11 + SYTO® 9 reading. Therefore, we concluded that this SYTOX® Blue dye by itself did not add any additional blue fluorescence, which was expected as the stain is supposed to only yield blue fluorescence when bound to DNA or RNA **(Estevez *et al.*, 2011)**.

As BG11 + SYTO® 9 and BG11 + SYTOX® Blue yielded virtually identical blue fluorescence, it can be concluded that neither stain contributes to blue fluorescence, and that BG11 medium causes a decrease in background fluorescence compared to the empty well. BG11 + SYTO® 9 + SYTOX® Blue fluorescence intensity was 4,160 U, and this intensity was higher than what was observed due to the background or presence of either SYTO® 9 or SYTOX® Blue alone. The data from this part of the experiment is logical as the summation of blue fluorescence from BG11 + SYTO® 9 and BG11 + SYTOX® Blue (3,925 U) was virtually identical to fluorescence from BG11+ SYTO® 9 + SYTOX® Blue (4,160 U).

Viable *Anabaena* 7120 in BG11 + SYTO® 9 yielded a fluorescence of 4,609 U, which was approximately double of what was observed in the same test without viable cells present. The green fluorescence from viable cells in BG11 + SYTO® 9 was 26,585 U. The high intensity of this fluorescence, coupled with the close proximity of green and blue on the light scale, was the reason for this increase in background fluorescence. *Anabaena* 7120 in BG11 + SYTOX® Blue intensity was 3,051 U, which was 1,200 U higher than in the same test without viable cells present. Similar results were also observed with PI in our previous study, and was confirmed via microscopy to be caused by PI binding to nucleic acids in viable cells **(Johnson *et al.*, 2015)**. Since PI and SYTOX® Blue have identical modes of action for staining non-viable cells, this study strongly suggests that SYTOX® Blue was staining viable cells, otherwise the fluorescence intensity wouldn't have increased.

Anabaena 7120 in BG11+ SYTO® 9 + SYTOX® Blue resulted in a fluorescence intensity of 6,111 U, which was ~50% higher than in the same test without viable cells present. Again, this suggests SYTOX® Blue was staining viable cells, however the presence of SYTO® 9 may have also contributed to this increase as it did in the identical treatment without cells. The above data from the treatments with viable cells is not what would be expected. Thus, it can be concluded that SYTOX® Blue also stains viable filamentous cyanobacterial cells, which is a similar result to what we observed with PI (another non-viable cell indicator) in filamentous cyanobacteria **(Johnson *et al.*, 2015)**.

Both PI and SYTOX® Blue have now been shown to be ineffective as non-viable cell indicators with filamentous cyanobacteria. Thus, it can be assumed that none of the other SYTOX® dead cell stains will work for this purpose as they are all supposed to be impermeant to viable cell membranes. This conclusion concurs with a study by **Sato *et al.* (2004)** in which strong correlations between expected and measured values in mixtures of live and dead *Anabaena* 7120 cells could not be made when SYTOX® Green was the non-viable cell indicator.

While, the SYTOX® Dead Cell Stains have been showed to work with certain species of cyanobacteria **(Sato *et al.*, 2004; Zhu and Xu, 2013)**, none were heterocyst-forming strains of filamentous cyanobacteria. **Tashyreva *et al.* (2013)** report using a SYTOX® dead cell stain to stain *Phormidium* populations. However, this stain was not used in conjunction with SYTO® 9, thus the results cannot be compared. Using SYTO® 9 and SYTOX® Blue as a dual-stain viability assay does not work as expected with filamentous cyanobacteria, however this experiment showed evidence that fluorescence from SYTO® 9 appeared to be an accurate viable cell indicator of filamentous cyanobacteria.

Comparison of SYTO® 9, absorbance, and autofluorescence as viable cell indicators

Cyanobacteria are known to autofluoresce in the red spectrum **(Caiola *et al.*, 1996; Dagnino *et al.*, 2006)** when excited in the green spectrum due to chlorophyll α **(Aiken, 1981; Lichtenthaler *et al.*, 1986)** and PBS **(Baier *et al.*, 2004)**. We hypothesized that autofluorescence from these light harvesting pigments could be used to assess cell viability, with the additional benefit that no external stains would be needed. The complicating factor of this approach is that photosynthetic pigments can have multiple emission wavelengths. For example, various emission spectra have been used for chlorophyll α determination, including 681 nm, **(Guzmán *et al.*, 2015)** 672 nm, **(Lozano *et al.*, 2013)** and 660 nm **(Ogawa and Sonoike, 2015)**. Also, PBS have fluorescent peaks at 635, 645, 654, 659, and 673 nm **(Gryliuk *et al.*, 2014)**. For this study, filters with several wavelengths were tested on a mid-log culture of cyanobacteria and it was determined than an excitation wavelength of 540 ± 25 nm and an emission wavelength of 620 ± 40 nm yielded the highest fluorescence values.

Figure 4 shows that SYTO® 9 is a better indicator of viability of filamentous cyanobacteria than autofluorescence from light harvesting pigments. While there are a couple data points in the SYTO® 9 plot that cause the plot to not have the standard smooth sigmoidal shape expected with microbial growth curves, this plot has a similar shape to another plot in the literature, where SYTO® 9 was used to monitor cell viability of a microorganism **(Yagüe *et al.*, 2010)**. Both plots have data points that alter the expected smooth shape of the plot, yet still allow for adequate interpretation of the growth dynamic that is occurring.

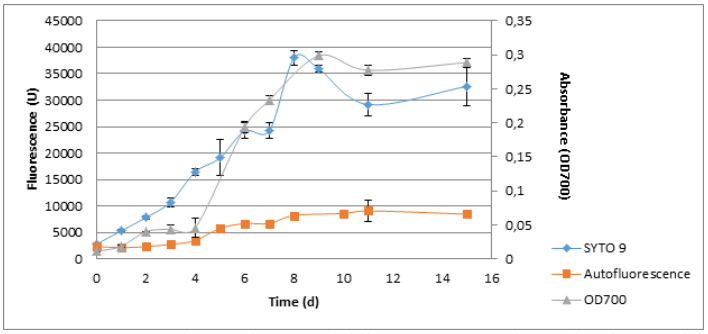


Figure 4 Comparison of two viability assays against absorbance for quantification of *Anabaena* sp. PCC 7120 in a 40 L PBR. The data represents the mean (n = 3). Error bars represent the standard deviation.

A possible reason for the weak autofluorescence in Figure 4 was that the emission peaks from each of the pigments is different, thus the assay loses sensitivity. Another reason could be that there are no differences in fluorescence from the pigments in viable *vs.* non-viable cells. Regardless of the reason, autofluorescence from light harvesting pigments is not a reliable indicator of viability. This concurs with a study by **Sato et al. (2004)**, which concluded that the intensity of fluorescence from the light harvesting pigments was not related to the relative percentages of viable *vs.* non-viable cells. The fact that the plot generated from fluorescence from SYTO® 9 is similar to the plot generated by absorbance readings provides further evidence that SYTO® 9 can be used as a reliable indicator of viability in filamentous cyanobacteria.

Assessing cell viability in a chemical inhibition test

High cell densities can affect the bioavailability and toxicity of chemicals that are being assessed for cell toxicity. This is caused by the adsorption of the chemical onto the living or dead cell biomass. The result can be a reduction in the effective dosage of the chemical, leading to an over-estimation of the tolerance of the

organism to the chemical. Thus, it is desirable to use minimal biomass levels in these tests so that the dosage and toxic effect of chemicals can be assumed to be constant **(Nyholm and Peterson, 1997; Peterson and Nyholm, 1993)**. If the chemicals being tested are volatile, then it is also important that the testing be performed in sealed vessels with minimal headspace, which may limit the volume of sample that can be withdrawn. Larger sample volumes would affect the headspace and potentially alter the results of the chemical inhibition test. Unfortunately, many cell quantification methods are not very accurate at low cell densities and with low sample volumes, and this may be more problematic if one is trying to assess viable cell numbers, rather than total cell biomass.

To determine which viability assay was the most accurate and reproducible under low cell density conditions, we evaluated absorbance, chlorophyll α, and fluorescence from SYTO® 9 in chemical inhibition tests involving farnesene. Farnesene is a long-chain hydrocarbon that filamentous cyanobacteria are capable of being genetically engineered to produce **(Halfmann et al., 2014b)**. The antimicrobial property of farnesene, generally as a component of plant oil, is well established **(Agnihotri *et al*., 2011; Aligiannis *et al*., 2004; Caccioni *et al*., 1998; Gudžić *et al*., 2002; Lopes-Lutz *et al*., 2008)**. Farnesene has many applications including biofuels, lubricants, cosmetics, and fragrances **(Buijs *et al*., 2013; Halfmann *et al*., 2014b)**, thus would be a valuable product of an industrial strain of filamentous cyanobacteria. We hypothesized that the fluorescence assay would provide necessary sensitivity compared to absorbance and chlorophyll α when biomass content and sample volumes are low.

Figure 5 shows the comparison of 3 cell quantification methods [2 for quantifying viable cells (SYTO® 9 fluorescence and chlorophyll content) and 1 for quantifying total cells (absorbance)] for screening cyanobacteria in BG11 medium containing 0.5 g/L $NaHCO_3$ as a carbon source and 0.032 g/L of farnesene. For each method, growth of a wildtype *Anabaena* 7120 strain was compared to a putative mutant *Anabaena* 7120 strain previously acclimated to tolerate 0.32 g/L farnesene (A7120.(0.32t).farn). A lower titer of farnesene (0.032 g/L) was used compared to what the strain was able to tolerate (0.32 g/L), as this allowed for monitoring growth rather than just survivability. These trials used a low density starting cell inoculum, and were conducted in sealed test tubes where sample volume was limited to 100 µl daily samples. The test tubes contained 27 ml culture, thus the small daily samples had minimal impact on the headspace of the vessel and the concentration of biomass in the test tube.

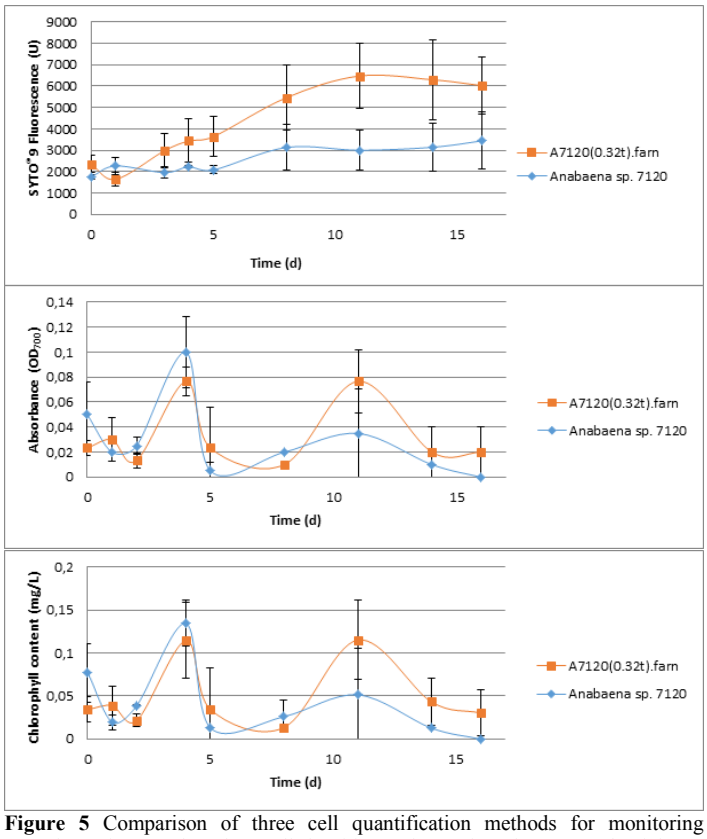


Figure 5 Comparison of three cell quantification methods for monitoring cyanobacteria growth at low cell densities and sample volumes. Each trial was completed in triplicate. Error bars represent the standard deviation.

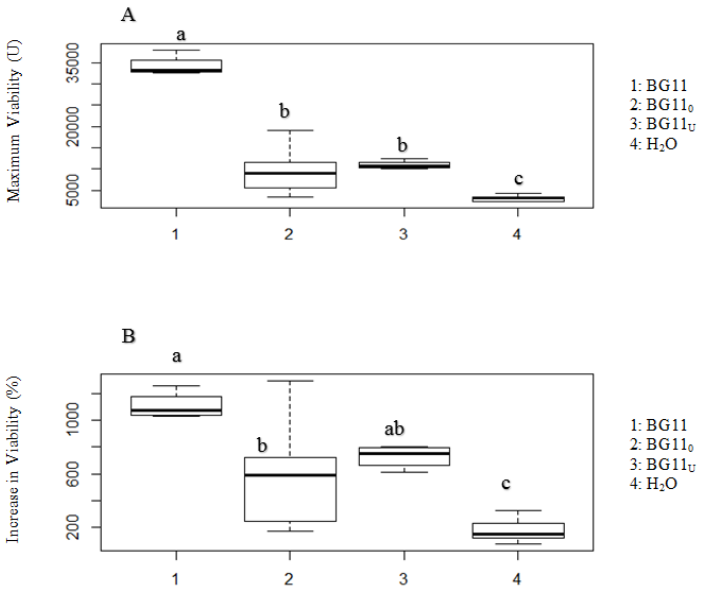
All samples for all assays were taken from the same tube at the same time in these trials. As only 100 µl samples were taken, the cultures had to be diluted with BG11 in order to have enough volume to accurately use the spectrophotometer. Fluorescence from SYTO® 9 was the only method that provided typical growth curves under these conditions of low cell density and small sample size. Even though the error bars are large in the SYTO® 9 plot, the assay was still able to distinguish a difference between the acclimated strain and the wildtype. As the daily sample size was limited, it was necessary to dilute the sample to obtain sufficient volumes for measurements in the spectrophotometer, which likely diluted the biomass (measured as absorbance) and chlorophyll α levels to or below the sensitivity level of the spectrophotometer. Presumably, this is at least partially responsible for the high degree of variability shown in the absorbance and chlorophyll α plots. The large error bars in Fig. 5 probably occurred due to the small biomass levels. Any amount of random or experimental error with values that low would yield large error bars. Further evidence that low biomass content caused the large error bars can be observed by comparing Fig. 5 to Fig. 4, which had greater biomass content. The error bars are much smaller in Fig. 4 even though the method for measuring viability was identical.

As mentioned earlier, high cell densities will yield inaccurate results in chemical inhibition tests, thus the biomass concentration must be kept to a minimum. The conclusion reached in this part of the study concurs with a study by **Mayer *et al.* (1997)**, that states that absorbance is considered to have borderline sensitivity and precision at the low biomass levels required for toxicity tests. All 3 growth parameter measurements were taken from the same culture at the same time, thus fluorescence from SYTO® 9 is a superior method when cell density and/or sample size are limited.

Assessing cell viability in photobioreactors

It was previously shown in this study (Fig. 4) that absorbance (total cell biomass) and the fluorescence assay (viable cell biomass) are capable of quantifying cyanobacterial growth when biomass and sample volumes are not limited. We postulated the fluorescence assay would be a preferred alternative to absorbance, because the former only measures viable cells. There is also the potential to use smaller sample volumes and detect smaller differences in cell numbers. Therefore, we evaluated the use of the fluorescence assay to monitor viable cyanobacterial cells in 40 L PBR trials that compared various cultivation media.

Figure 6 shows the effect of different cultivation media on the growth of *Anabaena* 7120 as measured by fluorescence from SYTO® 9. The definitions for the 3 growth parameters measured from the fluorescence assay are as follows: maximum viability is the maximum fluorescence value during a trial, percent increase in viability is the final fluorescence divided by the initial fluorescence, and viability rate of change is the maximum viability divided by the incubation time. The 3 parameters measured allowed for a more robust analysis of the dynamics of filamentous cyanobacterial growth. In all cases, BG11 (contains sodium nitrate) resulted in the most growth. Tap water resulted in the least growth, and BG11 supplemented with urea ($BG11_U$) and BG11 with no combined nitrogen source ($BG11_0$) were statistically similar. Replacing sodium nitrate with urea resulted in reduced growth compared to BG11 in 2 of the 3 parameters measured. Cultivating *Anabaena* 7120 in BG11 supplemented with urea rather than sodium nitrate also led to decreased growth in studies by **Wang and Liu, (2003) and Johnson *et al*., 2016b.** The results of this experiment allowed us to conclude that SYTO® 9 was capable of detecting differences in cyanobacterial growth due to differences in growth media. Thus, this assay is an attractive option for industrial microbiologists interested in evaluating large-scale processes involving filamentous cyanobacteria.



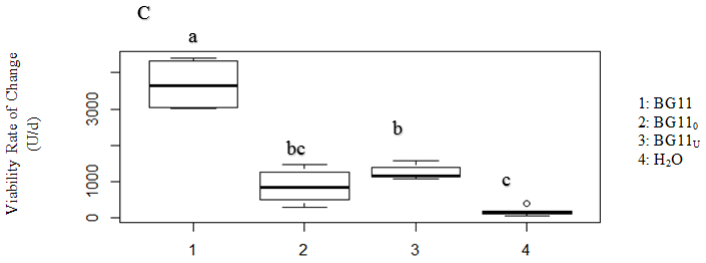


Figure 6 Comparison of different cultivation media on 3 growth parameters of *Anabaena* sp. PCC 7120 using fluorescence from SYTO® 9 as a viable cell indicator. Each trial contained ≤ 4 replicates. A: maximum viability during a trial; B: percent increase in viability; C: viability rate of change. Different lower case letters indicate a statistical difference occurred among the treatments as determined by a Tukey's test. BG11: standard cyanobacterial growth medium containing sodium nitrate; $BG11_0$: BG11 with no combined nitrogen source; $BG11_U$: BG11 supplemented with urea rather than sodium nitrate; H_2O: tap water.

CONCLUSIONS

The study described herein showed that SYTO® 9 is a reliable and accurate indicator of filamentous cyanobacteria viability. SYTOX® Blue did not work as a non-viable cell indicator in filamentous cyanobacteria and led to erroneous results similar to those we previously reported for the non-viable cell indicator PI **(Johnson *et al*., 2015)**. Thus, it can be assumed that the other SYTOX® Dead Cell Stains will also not work as non-viable cell indicators in filamentous cyanobacteria because they have the same mode of action. Potentially, this could be caused by the intercellular modes of transporting nutrients, allowing PI and SYTOX® Blue to penetrate viable cell membranes. Determining the cause of non-specific binding of non-viable cell indicators in filamentous cyanobacteria is a future direction of this research. Microscopic evidence of non-specific binding of PI in filamentous cyanobacteria can be observed in a study by **Johnson *et al.* (2015)**. Autofluorescence from light harvesting pigments was also not a practical method to monitor viability, presumably due to the intensity of fluorescence from the pigments not relating to the relative percentages of viable *vs.* non-viable cyanobacterial cells.

Fluorescence from SYTO® 9 is preferred for monitoring viability of filamentous cyanobacteria under conditions of low biomass concentrations compared to absorbance and chlorophyll α. A sealed system with low biomass content is needed for various applications, such as screening mutants for increased tolerance to next-generation biofuels. Small sample volumes should also be taken in these tests so that the headspace of the system does not affect the chemical titer in solution. With small sample volumes and the low biomass content, it is not possible to obtain accurate absorbance and chlorophyll α content results. However, it is possible to use SYTO® 9 as a viability indicator under these conditions.

In 40 L PBR trials where sample size was not limited, fluorescence from SYTO® 9 was capable of detecting differences in cyanobacterial growth caused by differences in growth media. We believe this method is preferred compared to absorbance due to the fluorescence assay's capability of measuring only viable cells, whereas absorbance measures total cells and cell debris. The results of this study and our prior work **(Johnson *et al*. 2015)** show that SYTO® 9 is a reliable, accurate indicator of viable cells in filamentous cyanobacteria. While this study only used *Anabaena* 7120, it is a model species for filamentous cyanobacteria **(Bryant, 2006; Rippka *et al*., 1979)**. Also, previous work by our research group investigating a fluorescence assay used several filamentous cyanobacterial strains, including *Anabaena* 7120, and they all produced identical results with respect to the fluorescence assay **(Johnson *et al*., 2015)**. Thus, we are confident the results of this study are applicable to other strains of filamentous cyanobacteria. Measuring fluorescence from SYTO® 9 in a microplate reader allows for high-throughput data collection, which further adds to its potential. Applications of the SYTO® 9 described in this study include monitoring cultures in PBR systems and screening mutants for increased tolerance to next-generation biofuels.

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ENHANCED PRODUCTION OF SINGLE CELL PROTEIN FROM *M. capsulatus* (BATH) GROWING IN MIXED CULTURE

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ABSTRACT

The growing global demand for nutritional protein means that a sustainable source such as Single Cell Protein from *Methylococcus capsulatus* (Bath) can become a potential replacement for fishmeal and other animal feeds. Improving biomass concentrations using statistical optimization during synergistic fermentations with a mixed consortium of the three heterotrophic bacteria *Alcaligenes acidovorans, Aneurinibacillus danicus,* and *Brevibacillus* sp. can increase the feasibility of the industrial process. The medium components Mg^{2+}, Ca^{2+}, Fe^{3+}, Cu^{2+}, PO_4^{2-}, NO_3^-, MoO_4^{2-}, trace metals, and process temperature were screened using a two-level Plackett-Burman Design in shake flasks which resulted in Cu^{2+} being the only significant factor. The optimum level of $CuSO_4.5H_2O$ was found to be 40 µM using One Factor Response Surface Methodology, which was three times higher than the typical values of Cu^{2+} used previously. These combined strategies led to a 265% increase in biomass, with final cell concentration of 10.3 g/L, up from 2.8 g/L in fed batch fermentations over 48 hours. The heterotrophic bacteria did not grow on NMS or methane but increased biomass concentration when added to *M. capsulatus* (Bath) cultures.

Keywords: *Methylococcus capsulatus* (Bath), Heterotrophic bacteria, Single Cell Protein, Plackett-Burman, Response Surface Methodology

INTRODUCTION

Single Cell Protein (SCP) from *M. capsulatus* (Bath), which is cell biomass containing 70% protein, has been produced as a protein supplement for various animal feed applications. There is renewed interest in the industrial production of SCP since global food and protein demands are projected to rise significantly by 2050 in line with population growth **(Alexandratos & Bruinsma, 2012; United Nations 2012)**. Its application as a solution to the impending world protein shortage depends on the development of the industrial bioprocess, which includes reactor design, efficient utilization of substrates, and medium development.

The fermentation of *M. capsulatus* (Bath) is conducted either as a monoculture or in a mixed culture with heterotrophic bacteria, and involves the use of Nitrogen Mineral Salts (NMS) medium and the input of air, natural gas, and carbon dioxide (**Eccleston & Kelly, 1972; Stanley & Dalton 1982)**. A previous study **(Harwood & Pirt, 1972)** attempted to increase biomass concentrations from *M. capsulatus* (Bath) in a monoculture in bench scale experiments by investigating the effects of several ions, trace metals, and process parameters on biomass concentration. However, maximum cell concentration never increased beyond 0.4 g/L. Values for maximum cell concentration from other studies **(Carlsen, Joergensen, & Degn, 1991; Furigo & Jorgensen, 1993; Larsen & Dalton, 1986; Leak & Dalton, 1986; Zhivotchenko, Nikonova, & Jorgensen, 1995)** never exceeded a maximum of 6 g/L under various conditions. There has never been any attempt to optimize the medium for *M. capsulatus* (Bath) fermentations where heterotrophic bacteria are included.

The use of a mixed bacterial culture was found to be critical in the industrial production process used by Norferm Danmark A/S where three heterotrophic bacteria – DB 3 (*Alcaligenes acidovorans),* DB 4 (*Aneurinibacillus danicus*), and DB 5 (*Brevibacillus* sp.) – would "constantly invade" *M. capsulatus* cultures **(Bothe *et al.*, 2002)**. This study identified the heterotrophic strains, quantified the growth of the bacteria on substrates found in supernatant fractions, and determined that all strains were non-toxic. DB 3 and DB 4 exhibited high growth on acetate, DB 5 was best at reducing free amino acids, and all strains reduced total organic carbon. However, there was no indication from this study that these heterotrophs acted on the oxidation products of hydrocarbons other than ethane. Previous studies have indicated that the heterotrophic bacteria act in synergy with an obligate methylotroph like *M. capsulatus* (Bath) by scavenging the oxidized by-products of natural gas, as well as the metabolic by-products of the obligate methylotroph, thereby bringing a measure of stability to the overall process while maximizing growth rates and yield coefficients **(Harrison, 1978; Linton & Buckee, 1977)**. Thus it is important that the media for this process is optimized as a mixed culture since the presence of the heterotrophic bacteria is crucial for its industrial application.

In order to increase the volumetric productivity of SCP during continuous fermentations, there is a requirement for achieving high cell concentrations during the batch and fed-batch stages. The first optimization step must therefore involve improving the composition of the growth medium and optimizing other process parameters for achieving maximum cell concentrations in the shortest possible time. The simplest strategy for medium development is the 'one at a time' method where one component is varied while the others are kept constant. In this way, the individual effects of each component on the medium are determined, but it can be time-consuming due to the large number of experiments involved **(Kennedy & Krouse, 1999)**. A better strategy for optimization is the Response Surface Methodology (RSM) where a functional relationship between the medium components and the response of interest can be determined **(Khuri & Mukhopadhyay, 2010)**. It involves the use of a design matrix for screening along with an optimization technique in which a mathematical model is used to determine the optimized medium composition **(Kennedy & Krouse, 1999)**.

Typically, a first-degree design (full or partial factorial) is used to screen and determine the significance of the factors. The most widely used screening method is the Plackett-Burman Design (PBD) which is a 2-level factorial design where the factor levels are set to a high and low value. In PBD, the number of experimental runs n is equal to $k + 1$, which is the same as the number of factors in the model, and the design only applies when n is a multiple of 4. This design does not account for interaction effects but if the levels are chosen correctly, it is a useful screening tool for determining the most significant factors, thereby reducing the number of factors to be optimized. The first-degree design is then followed by a second-degree design (single or multi-factor) which determines the optimum settings of each factor that result in either the maximum or minimum response over a certain range of interest. The most popular second-order design is Central Composite Design (CCD) which involves a 2^k factorial design with

factors set at five levels and the addition of an axial portion consisting of 2*k* axial points chosen based on the distance of the axial parameter (α) from the design center. In the case where the screening process results in only one factor being significant, a One Factor Response Surface Methodology (OFRSM) can be used to determine the optimal level of the variable.

OFRSM has been used to model methylene blue degradation **(Attarchi, Montazer, & Toliyat, 2013)**, dynamic viscosity of a micro-emulsion **(Jeirani *et al.*, 2013)**, and to optimize biosurfactant production **(Chen *et al.*, 2012)**. These combined RSM techniques have been used for a wide range of applications including enhancing biomass from fungi **(Zhang *et al.*, 2014)**, microalgae **(Zarate-Chaves *et al.*, 2013)**, and yeast **(Li *et al.*, 2009)**. It has also been used to enhance ethanol production **(Manwar, Mahadik, & Paradkar, 2013; Walia *et al.*, 2014)**, and other high value products **(Kosmider *et al.*, 2012; Mokhtari-Hosseini *et al.*, 2009; Yatsyshyn, Fedorovych, & Sibirny, 2010)**.

The objective of this study was to increase biomass concentration from *M. capsulatus* (Bath) in a mixed culture with three heterotrophic bacteria using statistical experimental design. This approach has not been previously used to optimize media for production of biomass by this microbe, either in a mono- or mixed culture. PBD was used as a screening tool for the medium components, followed by a second-degree RSM design chosen based on the resultant number of significant factors. Since only one factor was found to be significant, OFRSM was used to determine the optimal level. The predicted response determined from the OFRSM was then validated in shake flasks and fed-batch fermentations over 48 hours.

MATERIALS AND METHODS

Growth of *M. capsulatus* (Bath)

The methylotrophic microorganism used in this study was *Methylococcus capsulatus* (Bath) NCIMB 11132 (NCIMB Ltd. Culture Collection, Aberdeen, Scotland) obtained as freeze-dried vials. The cells were resuscitated using NCIMB Nitrogen Mineral Salts (NMS) Medium 131 (Salts per L: $MgSO_4.7H_2O$ 1.0 g, $CaCl_2.2H_2O$ 0.26 g, KNO_3 1.0 g, $C_{10}H_{12}FeN_2NaO_8$ 0.0038 g, $Na_2MoO_4.2H_2O$ 0.00026 g, $Na_2HPO_4.2H_2O$ 0.356 g, KH_2PO_4 0.026 g). Trace metals solution was added at final concentrations: $CuSO_4.5H_2O$ 0.8 µM, $FeSO_4.7H_2O$ 1.8 µM, $ZnSO_4.7H_2O$ 1.4 µM, H_3BO_3 0.24 µM, $CoCl_2.6H_2O$ 0.21 µM, EDTA disodium salt 0.67 µM, $MnCl_2.4H_2O$ 0.1 µM, and $NiCl_2.6H_2O$ 0.04 µM. All chemicals were of ACS Analytical grade sourced from SIGMA ALDRICH CO (St. Louis, MO), MALLINCKRODT BAKER (New Jersey, USA), and FISHER SCIENTIFIC (New Jersey, USA). Cells were stored in 15% glycerol at -80^0 C and were also maintained on agar plates made from NMS medium with 0.02% v/v methanol added after sterilization. Cells were spread onto the plates aseptically using a vial from glycerol stock, after which the plates were placed in an airtight gas jar and left in an incubator set at 45^{0}C, under an atmosphere of CH_4: Air: CO_2 of 9:9:2. Plates were then stored at 4^{0}C until ready for use.

To prepare seed cultures for experiments, colonies from agar plates were inoculated into 500 mL baffled shake flasks containing 100 mL aliquots of NMS medium and 20 µL methanol. Each flask was sealed with a two-holed rubber stopper fitted with stainless steel tubing connected to silicone tubing with a sterile filter (Sartorius Midisart 2000) attached at one end to allow sparging with 45% CH_4 and 4.5 % CO_2. Both pieces of tubing were clamped and the shake flasks were placed into a digital incubated shaker (ThermoScientific MaxQ Mini SHKE 4450 Digital) at 45^{0}C and 180 rpm for 48 hours. Cells were centrifuged at 5000 g for 10 minutes, after which the cell pellet was re-suspended in NMS medium and used in further experiments. OD at 540 nm was measured using a ThermoScientific Genesys 6 UV/Visible Spectrophotometer with deionized water as the blank. Dry cell weight was found by vacuum filtering a known volume of cell culture through a Merck Millipore cellulose acetate 0.45 µM filter and dried to constant weight.

Growth of heterotrophic bacteria

For shake flask investigations, the three heterotrophic bacteria - NCIMB 13287 *Alcaligenes acidovorans*, NCIMB 13288 *Aneurinibacillus danicus*, and NCIMB 13289 *Brevibacillus* sp. were added to the culture. *A. danicus* and *Brevibacillus* sp. were cultured in 3 g/L Yeast extract broth (Lab M) and *A. acidovorans* was cultured in 3 g/L Nutrient broth (Lab M). 50 mL of each respective media was placed in a 250 mL shake flask which was fitted with a foam stopper and sterilized at 121^{0}C for 15 minutes. Once cooled, the flasks were inoculated with the respective organisms and placed in the shaker at 45^{0}C, 180 rpm, for 24 hours. The cells were centrifuged, re-suspended in NMS before combining with *M. capsulatus* (Bath) for fermentation. For investigation of heterotrophic growth on NMS, each strain was added to 50 mL shake flasks containing 10 mL NMS to a start OD of 0.1. Flasks were sparged with air, methane, and carbon dioxide in the ratio described previously and incubated on the shaker and OD 540 nm was checked after 24 hours.

Plackett-Burman Design

Generation of the design matrix and statistical analysis for PBD was done using Minitab 16 (MINITAB Inc.). Media recipes used in previous literature (Table 1) were used to select the high (+) and low (-) levels for each variable. Nine (9) variables (Table 2) were selected as the most relevant to screen using a 12-run PBD (Table 3) for increasing biomass production in the fermentation broth. In the NCIMB medium, $CuSO_4.5H_2O$ is only present in a small amount in the trace metal solution. However, it was included here as a major factor since it is included in other fermentation media for growth of *M. capsulatus* (Bath) in varying concentrations. The concentration and composition of the trace metals solution was kept constant but the volume added was modified. In the PBD model, all interactions were ignored and it was assumed that a first order regression model was applicable.

Each run in the design matrix was conducted in 250 mL shake flasks with 50 mL of medium corresponding to the specific run in the design matrix and inoculated with 1 mL of seed culture. Flasks were sparged and placed in an incubated shaker set at the appropriate temperature (either 37 ^{0}C or 45 ^{0}C) with a shaking speed of 180 rpm. After 24 hours, the OD_{540} was measured. All experiments were carried out at least in triplicate and the results were the average of the replicate experiments with standard error ≤ 5%. The confidence level was set at 95% and the significant factors were selected based on the *p*-value falling below 0.05. The Effect (E) of each factor was determined from the regression analysis. The effects of the non-significant variables were used to set the new concentrations of these variables for validation experiments in shake flasks and fed-bath fermentations.

One Factor Response Surface Methodology

Based on the outcome of the PBD, OFRSM was used to determine the optimum concentration of the significant factor using Design Expert 8.0 (STAT-EASE Inc.). The factor was examined at five concentration levels in an eight run design generated by the statistical software to determine the optimum concentration. Shake flasks were prepared as previously described for PBD with the appropriate concentrations of the medium components.

Validation experiments

All experiments with the optimized NMS medium were performed in shake flasks in triplicate as previously described for 24 hours. The optimized medium was also tested via fed-batch fermentations in a 5L Sartorius B. Braun Biostat B-plus fermenter (SARTORIUS STEDIM). Start volume was 2L of the optimized NMS medium, with a ten-fold concentrated feed stream of the media at flow-rate 4 m L/min connected to low flow peristaltic pumps (Watson Marlow 101U/R) for fed-batch operation. pH was controlled automatically using 1M solutions of NaOH and H_2SO_4 and temperature was automatically controlled at 43^{0}C in the double jacketed vessel. Agitation rates were selected based on achieving a mass transfer coefficient (kLa) value of 0.05 s^{-1} for 0.5 vvm air in NMS medium (500 rpm), and 0.12 s^{-1} (800 rpm) for higher cell densities (3-4 g/L). kLa was determined using the Hydrogen Peroxide-Catalase Method (**Hickman, 1988; Cooke *et al.*, 1991**). Air flow rate was set at 0.5 vvm, CH_4 at 0.1 vvm, and CO_2 at 0.0012 vvm. *M. capsulatus* (Bath) cells and the heterotrophic bacteria were inoculated into the fermenter in the ratio 75:25 respectively, with *A. acidovaorans* comprising 10% of the mixture, and 7.5% each of *Brevibacillus* sp. and *A. danicus*. All fermentations were stopped after 48 hours with final ODs recorded and compared to ODs at 48 hours for fed-batch fermentations using the un-optimized media. Results were converted to cell concentration (g/L) using a linear relation for OD versus dry cell weight (DCW) determined using cell suspensions of *M. capsulatus* (Bath):

$$OD = 2.6184\, DCW + 2.0139 \quad (1)$$

Table 1 Media compositions for Nitrogen Mineral Salts medium from previous literature

Reference	(Foster & Davis, 1966)	(Whittenbury, Phillips, & Wilkinson 1977)	(Harwood & Pirt 1972)	(Dalton & Whittenbury, 1976)	(Carlsen, Joergensen , & Degn, 1991)	(Larsen & Dalton 1986)		(Furigo & Jorgensen, 1993)	(Zhivotchenko, Nikonova, & Jorgensen, 1995)
Methane %	50	30-50	50	20	2.4 g/L	Excess		40	66
Methanol v/v	-	0.01-0.1 (w/v)	-	-	-	-		-	-
Salt Concentration (g/L) unless otherwise stated							**Concentration mM**		
KNO_3	-	1.0	-	1.0	1.0	-	**NH_4^+**	25	20
$NaNO_3$	2.0	-	-	-	-	-	**SO_4^{2-}**	12.56	-
NH_4Cl	-	0.5	0.15	-	-	-	**PO_4^{3-}**	7.81	-
NH_4NO_3	-	-	-	-	-	1.88	**Cl^-**	1.91	-
$MgSO_4.7H_2O$	0.2	1.0	0.075	1.0	0.25	0.38	**Mg^{2+}**	1.27	0.34
$CaCl_2.2H_2O$	0.015	0.26	0.001	0.200	0.05	0.146	**K^+**	1.86	-
[a]FeNaEDDHA	-	0.004	-	-	-	-	**Ca^{2+}**	0.94	0.59
[b]NaFeEDTA	-	-	-	0.005	-	-	**Na^+**	0.002	-
$FeSO_4.7H_2O$	0.001	-	-	-	0.014	0.075	**Fe^{3+}**	0.018	0.031
$Na_2MoO_4.2H_2O$	-	0.00026	-	0.0002	0.001	0.0000012	**Cu^{2+}**	0.0085	0.00037-0.128
MoO_3	0.00001	-	-	-	-	-	**Ni^{2+}**	0.00005	-
$CuSO_4.5H_2O$	0.000005	0.00075	0.0003	-	0.0025	0.0035	**Mn^{2+}**	0.00013	0.027
NaH_2PO_4	0.09	-	-	-	-	-	**Co^{2+}**	0.00053	0.00071
$Na_2HPO_4.12H_2O$	0.21	0.1	1.2	0.33	-	0.356	**Zn^{2+}**	0.0017	0.004
KH_2PO_4	-		0.7	0.26	0.13	0.26	**B^{3+}**	0.00026	0.08
Citric Acid	-	-	0.2	-	-	-	**Mo^{7+}**	0.002	-
Ferric Citrate	-	-	0.002	-	-	-	**Mo^{6+}**	-	0.00083
HCl	-	-	0.003 ml	-	-	-	**NaOH**	-	22.5-23.5
KCl	0.04	-	-	-	-	-	**KH_2PO_4**	-	2.64
							NaH_2PO_4	-	3.28

[a] NaFeEDTA = Ethylenediaminetetraacetic acid ferric sodium salt $C_{10}H_{12}N_2NaFeO_8$
[b] FeNa-EDDHA = "Sequestrene Iron complex" Sodium Ferric ethylenediamine BIS-(2-hydroxyphenylacetate) $C_{18}H_{16}O_6N_2NaFe$

Table 2 List of variables and their respective coded names for PBD screening

Variable	Variable code	Levels (g/L)		
		Low (-)	Center point (0)	High (+)
KH_2PO_4	A	0.1	0.45	0.8
KNO_3	B	1	1.5	2
$MgSO_4.7H_2O$	C	0.5	1	1.5
$CaCl_2.2H_2O$	D	0.1	0.3	0.5
$C_{10}H_{12}FeN_2NaO_8$	E	0.005	0.05	0.1
$CuSO_4.5H_2O$	F	0.003	0.011	0.018
$Na_2MoO_4.2H_2O$	G	0	0.0005	0.05
Trace Metals[a]	H	0	1	2
Temperature[b]	I	37	41	45

[a]Trace metals concentrations in mL/L
[b]Temperature units = ^{0}Celcius

Table 3 Variables for PBD (in coded levels) with corresponding OD_{540} response values for biomass concentration

Run #	Coded variables and levels											Response (OD_{540})
	A	B	C	D	E	F	G	H	I	*J	*K	
1	+	-	+	-	-	-	+	+	+	-	+	0.519
2	+	+	-	+	-	-	-	+	+	+	-	0.320
3	-	+	+	-	+	-	-	-	+	+	+	0.159
4	+	-	+	+	-	+	-	-	-	+	+	0
5	+	+	-	+	+	-	+	-	-	-	+	0.431
6	+	+	+	-	+	+	-	+	-	-	-	0
7	-	+	+	+	-	+	+	-	+	-	-	0.078
8	-	-	+	+	+	-	+	+	-	+	-	0.607
9	-	-	-	+	+	+	-	+	+	-	+	0.120
10	+	-	-	-	+	+	+	-	+	+	-	0.055
11	-	+	-	-	-	+	+	+	-	+	+	0
12	-	-	-	-	-	-	-	-	-	-	-	0.355

* J and K are dummy variables

RESULTS AND DISCUSSION

Screening of the factors by PBD

Nine variables were screened according to the PBD experimental runs shown in Table 3 along with the corresponding OD response values for each of the runs. The results show that runs 1, 5, and 8 gave the maximum yields while some runs showed no growth, or the combination of salts caused precipitation (represented as '0'). These results were then subjected to regression analysis (Table 4) where the effects of each factor are shown. Most effects were slightly positive, meaning that a higher level would produce a more favorable response while for $CuSO_4.5H_2O$, KNO_3, and temperature, the effects were negative indicating that a value lower than the maximum level would be more favorable.

Table 4 Regression analysis for results of PBD screening

Term	Effect	Coefficient	Standard error	t	P
Constant		0.2203	0.02398	9.19	0.012
KH_2PO_4	0.0010	0.0005	0.02398	0.02	0.985
KNO_3	-0.1113	-0.0557	0.02398	-2.32	0.146
$MgSO_4.7H_2O$	0.0137	0.0068	0.02398	0.28	0.802
$CaCl_2.2H_2O$	0.0780	0.0390	0.02398	1.63	0.245
$C_{10}H_{12}FeN_2NaO_8$	0.0167	0.0083	0.02398	0.35	0.761
$CuSO_4.5H_2O$	-0.3563	-0.1782	0.02398	-7.43	**0.018**
$Na_2MoO_4.2H_2O$	0.1227	0.0613	0.02398	2.56	0.125
Trace Metals	0.0813	0.0407	0.02398	1.70	0.232
Temperature	-0.0237	-0.0118	0.02398	-0.49	0.671

S = 0.0830763 PRESS = 0.49692 R-Sq = 97.34% R-Sq (pred) = 4.11 % R-Sq (adj) = 85.35%

The results show that only $CuSO_4.5H_2O$ was significant with a *p*-value less than 0.05, which is confirmed in the half normal effects plot (Fig. 1) so the null hypothesis was rejected. $CuSO_4.5H_2O$ also had the largest coefficient and *t*-value (with effect in the negative direction). Even though the other eight variables were insignificant to the model with *p*-values above 0.05, the overall model is significant with a *p*-value of 0.012 and the R-Sq value showed that 97.34% of the total variation was explained by the model.

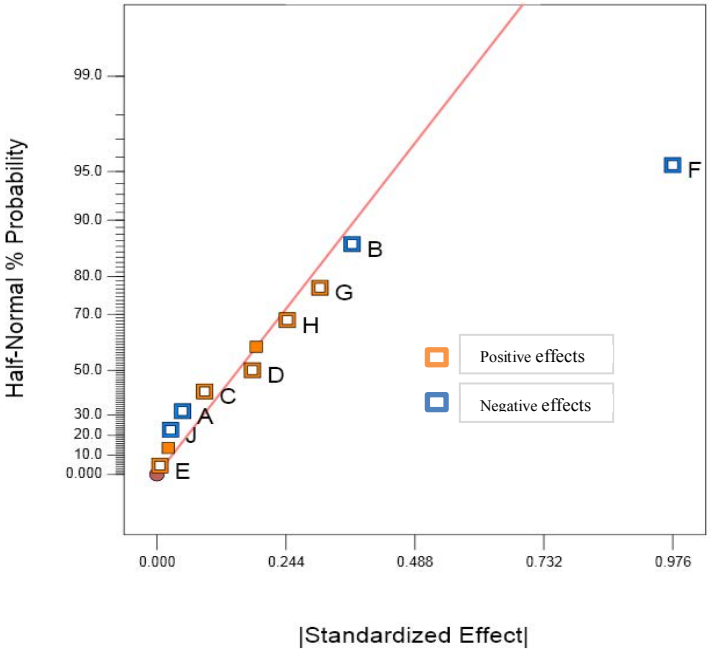

Figure 1 Half Normal plot of standardized effects showing significance of $CuSO_4.5H_2O$ (Variable F)

Optimization with One Factor Response Surface Methodology

Since only one variable was significant, the CCD could not be performed, hence OFRSM was used to determine optimal $CuSO_4.5H_2O$ concentration. The OFRSM run design consisted of eight runs at five concentration levels of $CuSO_4.5H_2O$ in the range 0.003 – 0.018 g/L, with two replicates at the low, center, and high concentrations (Table 5). The concentration values for the other factors were set based on the direction and size of the effect values and were kept constant throughout the OFRSM optimization trials. The average ODs obtained from the OFRSM are also shown in Table 5. The highest ODs were observed around the midpoint value of the concentration range. As seen in the response surface map (Fig. 2) showing the distribution of the data between the limits of the confidence intervals (blue dotted lines). The prediction statistics generated from the software estimated that an OD of 0.804 would result when the concentration of $CuSO_4.5H_2O$ is set at 0.0107 g/L. The confidence interval (CI) showed that 95% of the probable response values were contained within the interval 0.422 to 1.19.

Table 5 OFRSM design for $CuSO_4.5H_2O$ at five concentration levels with OD_{540} responses

Run	Concentration, (g/L)	Average OD_{540}
1	0.003	0.188
2	0.003	0.563
3	0.00675	0.106
4	0.0105	0.886
5	0.0105	0.717
6	0.0142	0.197
7	0.018	0.045
8	0.018	0.109

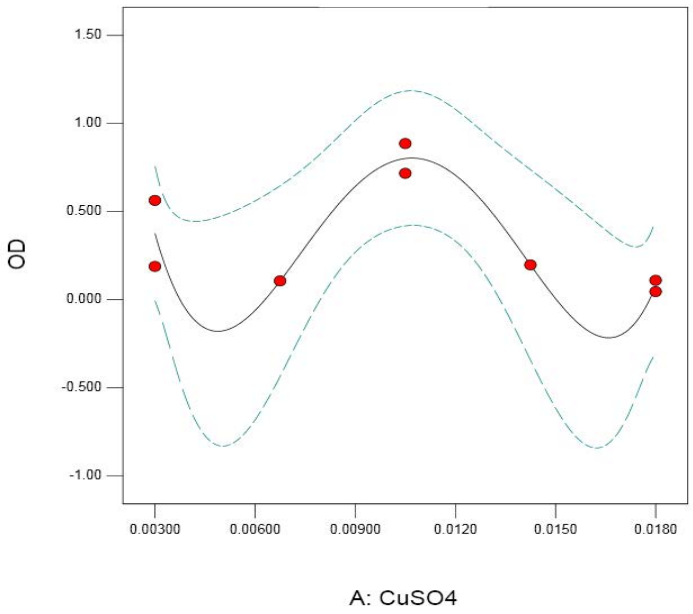

Figure 2 Response surface for $CuSO_4.5H_2O$ showing distribution of OD_{540} at the five design levels
Legend: - - - - - - - - - Confidence intervals ——————Surface response plot

Confirmation experiments: Fed-batch fermentations

The shake flask experiments were performed using concentration of 0.01 g/L $CuSO_4.5H_2O$ (40 µM) at a temperature of 43 ^{0}C.This resulted in an average OD of 0.935 ± 0.040 with a standard deviation (SD) of 0.057. This result was higher than the predicted value from OFRSM but was still within the range of both the confidence and prediction intervals, showing that the model was acceptable for forecasting the result with a certain degree of accuracy. The fed-batch fermentations over 48 hours showed an average OD of 29.0 or average cell concentration of 10.30 g/L (SD 0.24). With the un-optimized NMS medium, the average OD and cell concentration were 9.4 and 2.80 g/L (SD 0.18) respectively for the same time interval. This represented a 265% increase in cell concentration based on this new concentration level for $CuSO_4.5H_2O$.
In the fed-batch fermentations, the feed stream was typically started between OD 4 -7 when it was estimated that nutrients had become limited. In the optimized OPT run shown in Figure 3, the feed stream is started approximately 14 hours earlier than the UNOPT run, indicating a shortened lag phase due to the extra copper. The slope of the OPT run is also steeper, indicating a faster growth rate than for the UNOPT run. Another validation experiment in the fermenter was performed using the new concentration values of the other medium components with the initial un-optimized Cu^{2+} concentration value (0.00256 g/L). The resulting cell concentration was 5.60 g/L (SD 0.70), a 100% increase from the initial value of 2.80 g/L. This means that the overall increase in cell concentration was also affected by changes in values of the other factors. However, the medium composition was still limiting since the increase in copper allowed growth to further increase to 10.30 g/L for the same number of fermentation hours.

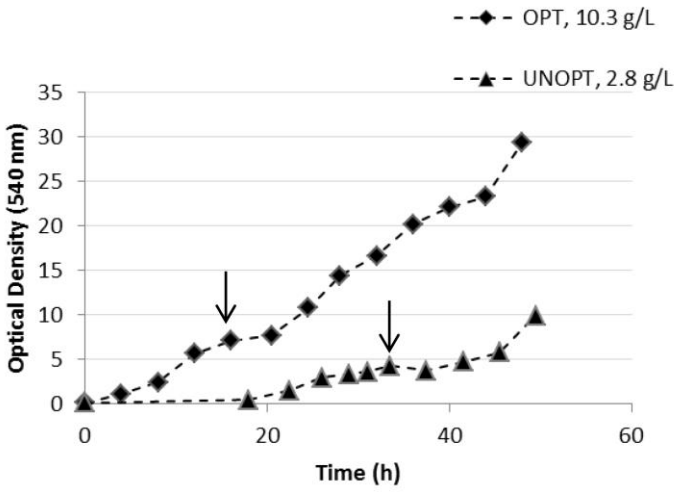

Figure 3 OD over 48 hours for fed-batch mixed culture
Legend: Before (UNOPT) and after (OPT) optimization. Arrows indicate where feed-streams were started.

Heterotrophic bacteria

All three bacteria demonstrated no growth on the NMS medium with CH_4 as carbon source but showed excellent growth in shake flasks in complex media when OD 540 nm was measured after 24 hours (*A. acidovorans*, 3.145; *A. danicus*, 1.823; *Brevibacillus* sp., 1.531). If the heterotrophs were CH_4 dependent, their growth would likely outpace that of *M. capsulatus* (Bath) since growth rates for the heterotrophs on acetate ranged from 0.45 h^{-1} - 0.6 h^{-1} **(Stanley & Dalton, 1982)** while the maximum growth rate recorded for *M. capsulatus* (Bath) in a monoculture was 0.37 h^{-1} **(Joergensen & Degn, 1987)**. Therefore the requirement for extra Cu^{2+} in the medium is not a requirement for heterotrophic growth but rather, it is necessary for optimal growth of *M. capsulatus* (Bath). When the three heterotrophs were combined with *M. capsulatus* (Bath), the OD increased from 0.349 ± 0.52 SD before addition to 0.807 ± 0.032 SD under the same conditions. The contribution of the heterotrophic cells to the overall OD in shake flasks was not determined.

It is well known from previous studies that the amount of copper in the medium influences the expression of either the soluble (sMMO) or particulate (pMMO) forms of Methane monooxygenase **(Prior & Dalton, 1985; Semrau *et al.*, 1995)**, with the pMMO being activated at Cu^{2+} concentrations greater than 1.5 µM (**Zhivotchenko *et al.* ,1995; Stanley *et al.*, 1983**. In the un-optimized medium used in this study, the Cu^{2+} concentration was 11 µM which indicates that the pMMO was already activated. However, the significant increase in growth when copper was increased to 40 µM in the optimized medium suggests that even though the pMMO was active, the Cu^{2+} concentration was still limiting. Zhivotchenko *et al*. **(1995)** indicated that 6 µM Cu^{2+} is the optimum level required for every 1 g/L biomass. Using this correlation for 10.30 g/L biomass concentration resulting from this study, the Cu^{2+} requirement would be approximately 62 µM (0.015 g/L). This concentration was investigated in the range of the OFRSM experiments in shake flasks but was not found to be optimal. This suggests that the relationship between biomass concentration and copper requirement is not a simple linear relation as the previous study suggests. From the data generated in this study, and assuming that 6 µM Cu^{2+} is required for 1 g/L biomass; the Cu^{2+} requirement can be modeled according to:

$$[Cu^{2+}] = e^{\left(\frac{[X]+6.72}{4.70}\right)} \qquad (2)$$

The heterotrophic bacteria used for the mixed culture fermentations used previously by Norferm Danmark A/S was not planned but was found to be a necessary addition to the process. Their contribution to the cell population during batch culture was estimated by microscopic analysis to be approximately 5% of the cell population at an approximate cell concentration of 1 g/L. At Norferm Danmark A/S, the final heterotroph population, which was likely determined after extended continuous production runs, ranged between 13 % - 19.8 % of the total cell population, depending on the composition of natural gas used **(Bothe *et al.*, 2002)**. The activity of the heterotrophic bacteria as a function of dissolved oxygen concentration may help elucidate the function of these bacteria during different phases of the fermentation Although the heterotrophs can function as natural gas oxidation product scavengers, their main purpose seems to be scavenging of dead cells since oxygen becomes limiting at higher cell concentrations resulting in less oxidation by-products of natural gas but increasing numbers of cells in death phase. The mixed culture growth is a complex process since the heterotrophs do not grow on NMS. Hence, modeling would have to include monitoring of production and consumption rates of all metabolic products during fermentation, natural gas oxidation products, and monitoring of cell population and viability, both as a consortium and individually.

Surprisingly, despite its crucial role in cell energetics, phosphate concentration was not a significant factor. Although calcium was also insignificant, the effect value dictated that a higher value was preferred so the concentration was increased to the mid-range value. Ca^{2+} is known to have an important role in the active sites of Methanol Dehydrogenase (MDH) and the MMO, allowing the prosthetic group pyrrolo-quinoline quinone (PQQ) to maintain the correct configuration for its role in substrate oxidation. The concentration of MoO_4^{2-} was doubled for similar reasons and this is understandable since molybdenum is an important element for biological processes. Overall, the statistical optimization allowed the system to be examined as a whole, and combined with the other changes to the media, this method worked well for detecting the optimum operating levels.

The impact of other fermentation parameters on cell density needs to be further investigated since the biomass concentration also depends on the availability of dissolved oxygen and methane. At low cell densities, these substrates are present in excess but as cell density increases, oxygen and methane become limiting. However, it is highly possible that the cultures in this study were grown under oxygen limitation since similar methanotrophic organisms have exhibited substrate inhibition as dissolved oxygen increased **(Wendlandt, Jechorek, & Bruhl, 1993)**. The optimal agitation and gas supply rates need therefore to be investigated, and the point at which mass transfer becomes limiting needs to be determined for the overall optimization of this bioprocess.

CONCLUSION

The medium components for biomass production from a mixed culture of *M. capsulatus* (Bath) and three heterotrophic bacteria were screened by PBD and optimized with OFRSM. The use of these two experimental design techniques allowed for investigation of multiple process parameters in a condensed format. By using these tools, the optimum level of Cu2+ was found, but its effect at various concentrations could also be observed on the surface response map. The increase in growth rates and the almost tripled cell concentrations observed using the optimized medium shows the important role of copper in the operation of the methane oxidation enzyme systems. The complex nature of the heterotrophic synergy needs further investigation to fully understand their specific role in *M. capsulatus* (Bath) fermentations.

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EVALUATION OF THE IMMUNOGENICITY OF EACH OF L-AMINO OXIDASE- AND L-ASCORBIC ACID-INACTIVATED HEPATITIS A VIRUS IN MICE AS POTENTIAL VACCINE CANDIDATES

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ABSTRACT

Hepatitis A virus (HAV) is one of the most common causes of acute viral hepatitis worldwide. Formaldehyde is the currently used inactivating agent in HAV vaccine processing despite of its adverse effects. The current study aimed to evaluate both L-amino acid oxidase (LAO) and L-ascorbic acid (LAA) as alternative inactivants for HAV and the immunogenicity of inactivated HAV in mice. Vero cell line was used for cultivation of HAV. The cytotoxicity of LAO and LAA on Vero cells was evaluated using 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide (MTT) assay. The immunogenicity of each LAO- and LAA-inactivated HAV was examined in parallel with reference HAV vaccine in mice. Humoral (total IgG) and cellular immune responses (IFN-γ and IL-5) were evaluated in mice sera using ELISA. Both LAO and LAA could efficiently inactivate HAV within 30 and 36 hrs post treatment, respectively, at concentrations of 0.4 µgm/ml of LAO and 1.5 mg/ml of LAA. Inactivated vaccines were immunogenic to mice on both the humoral and cellular levels. LAO prepared vaccines showed a more promising immune reactivity than LAA prepared ones and alum-adsorbed vaccines were more immunogenic than non-adjuvanted ones. In conclusion, data recorded suggest that both LAO and LAA can be used as inactivating agents for HAV grown in cell culture. LAA- and LAO-inactivated HAV can be potential vaccines as they provide effective humoral and cellular immune responses comparable to that of the reference vaccine. The stability of test vaccines is recommended to be traced at different thermal conditions, in addition to different stabilizers and different pharmaceutical formulations must be tested trying to produce a lyophilized formula for long-term stability.

Keywords: Hepatitis A, vaccine, virus inactivation, L-amino oxidase, L-ascorbic acid, alum

INTRODUCTION

Hepatitis A virus (HAV) is the most common cause of infectious viral hepatitis which is acquired via the faecal-oral route. Hepatitis A is a common infection worldwide that is associated with unsafe drinking water, inadequate sanitation and poor personal hygiene **(Wu and Guo, 2013)**. It is estimated that approximately 1.5 million clinical cases of HAV infection occur worldwide per annum. In addition, HAV infection is very common in underdeveloped countries such as Africa, parts of South America, the Middle East and India. In high endemic areas, hepatitis A occurs early in childhood; however, the development in sanitary conditions has resulted in a shift of the age groups affected by hepatitis A with increasing incidence in older age groups **(Sartori *et al*., 2012)**. In Egypt, a study that has been performed in 2008 revealed that the frequency of HAV infection in children from low social class was high (81 %), while the prevalence rate was low (27.3 %) in those of higher classes **(Franco *et al*., 2012)**. The disease is self-limited however HAV infection may result in acute liver failure and death, while risk increases with age and the presence of chronic liver disease **(Wu and Guo, 2013)**. Prevention of HAV infection could be achieved by avoidance of exposure to contaminated food and water, proper disposal of excreta as well as administration of vaccine to those risk groups, such as health care workers **(Zuckerman *et al*., 2009)**. Indeed, universal immunization would successfully control hepatitis A, although high costs and limited availability of vaccines preclude such a recommendation **(Tahaei *et al*., 2012)**.

Vaccines against viral infections consist mostly of live attenuated or inactivated viruses **(Stauffer *et al*., 2006)**. In 1992, two HAV formalin-inactivated vaccines, namely Havrix (GSK) and VAQTA (Merck), were available in United States and some other developed countries. These vaccines were developed by growing the virus in human diploid cell lines and then inactivated by treatment with formaldehyde **(Karayiannis *et al*., 2004)**. Other formalin-inactivated HAV vaccines include Avaxim (Sanofi Pasteur) and Epaxal (Crucell Switzerland) **(Glück *et al*., 1992; Vidor *et al*., 1996)**. These vaccines are administered intramuscularly as a two-dose regimen, given at zero and six to 12 months. They generally well tolerated, with occasional reports of mild local reactions or, more rarely, fever and malaise **(Karayiannis *et al*., 2004; Tahaei *et al*., 2012)**. Immunity induced by these vaccines is achieved in approximately 100 % of immunocompetent patients one month after receiving the recommended two doses **(Fiore *et al*., 2006)**.

Several inactivating agents have been described to successfully inactivate viruses for vaccine purposes. Still, formaldehyde is the most widely used inactivating agent in vaccine industry for decades **(Madhusudana *et al*. 2004)**. However, formaldehyde inactivation efficacy varies between vaccines concerning formalin concentration, time of inactivation and temperature. Generally, the higher the formalin concentration and temperature the faster is the inactivation, although this may adversely affect the antigenicity owing to thermal degradation and destruction of important epitopes **(Sanders *et al*., 2015)**. As the integrity of the immunological epitopes of inactivated vaccines is virus-inactivant related. Therefore, it is a matter of interest to evaluate cheap and easily available alternative chemicals for fast and efficient inactivation of viruses without affecting its antigenicity **(Madhusudana *et al*., 2004)**. Some studies have revealed that L-ascorbic acid (LAA) or vitamin C can be used as an inactivating agent for both DNA and RNA viruses while retained good antigenicity **(White *et al*., 1986**; **Rawal *et al*., 1995; Madhusudana *et al*., 2004; Abd El-Razek *et al*., 2011)**. In addition, several studies reported that L-amino acid oxidas (LAO) present in animal secretary fluids, scorpion and snake venoms have strong antimicrobial activities against various pathogenic bacteria and viruses **(Meenakshisundaram *et al*., 2009; Alyan *et al*., 2014; Kasai *et al*., 2015)**. **Izidoro (2014)** reported the possible inhibition of HIV-1 replication by LAO isolated from *Trimeresurus stejnegeri* venom. Based on the available information on virus inactivation efficacy of both LAO and LAA, the objective

of our study was to evaluate them as alternative inactivants to HAV and related immunogenic efficacy of both LAO- and LAA-inactivated HAV in mice.

MATERIAL AND METHODS

Hepatitis A virus strain and cell line

HAV strain HM175 was kindly supplied from water pollution department, National Research Centre, Cairo, Egypt. African green monkey kidney cell line [Vero cells, clone CCL-81] was kindly provided by Research and Development Sector, the Egyptian Holding Company for Biological Product and Vaccines (VACSERA, Giza, Egypt).

Maintenance of Vero cell line

The 199 Eagle [E-199] medium (GIBCO, USA) supplemented with 200 mM L-glutamine, 10 % foetal calf serum (FCS) (Sigma Aldrich, USA), 100 IU/ml of penicillin and 100 mg/ml of streptomycin (Invitrogen, USA) was used to maintain Vero cell line in tissue culture flasks (TPP, Switzerland) according to **Doyle and Bryan (1998) and Mather and Roberts (1998)**, where the growth medium of the mother bottle was decanted. It was incubated at 37°C in 5 % CO_2 incubator (Jouan, France) until monolayer was developed. The monolayer was washed gently using sterile phosphate buffered saline [PBS] of pH 7.2. PBS was decanted and the cell monolayer was washed with 10 ml pre-warmed 0.25 % (W/V) trypsin-EDTA solution [Invetrogen, USA] and left in contact with cells for 15 - 30 seconds. Trypsin was decanted, the TC flasks were incubated with the trace trypsin at 37°C until the cells detach from the surface. Cells were dispensed in number of flasks to maintain cell count of ~ 2×10^5/ml. TC flasks were kept till monolayers were developed. The actual cell number in the suspension was calculated by counting the cells using the haemocytometer and trypan blue dye (Sigma Aldrich, USA) exclusion method.

Virus seed stock preparation.

Maintained Vero cells were inoculated with the HAV. The growth medium was carefully decanted and HAV as 0.1 MOI (multiplicity of infection) was inoculated onto Vero cells. HAV infected flasks were shaken for 15 minutes intervals to assure well virus distribution. Maintenance medium (100 ml) was then added to each infected flask. Infected flasks were incubated at 37°C and examined microscopically every day till the development of cytopathic effect (CPE). Flasks developed CPE were subjected to freezing and thawing three times to extract both cell free and cell associated virus from cells **(EL-Karamany, 1987)**.

HAV seed stock infectivity titration

HAV harvest was titrated on Vero cells, where HAV seed was 10-fold serially diluted using sterile E-199 medium. Dilutions of HAV were dispensed to Vero cells pre-cultured 96-well plates as 0.1 ml/well. Plates were incubated at 37°C for 7 days with daily microscopic examination using inverted microscope (Hund, Germany). Infectivity titre was determined according to **Reed and Muench (1938)** equation:

PD Index = (A-50 %) / (A-B) × Log dilution (10)

Where A is the percentage of CPE at dilution immediately above 50 % and B is the percentage of CPE at dilution immediately below 50 %. After that index was applied to the dilution that produced the percentage of cytopathic effects immediately above 50 %.

Chemical inactivants

L-amino oxidase (LAO) and L-ascorbic acid (LAA), used for HAV inactivation in this study, were purchased from Sigma Aldrich, USA. They were prepared at a concentration of 1 mg/ml and processed for evaluation of their safe concentrations. LAA solution contained $CuSO_4$ at a final concentration of 5 µg/ml.

MTT assay

Cytotoxic effects of both LAO and LAA was determined on Vero cells using 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide (MTT), where Vero cells were dispensed in 96 well plates. Plates were incubated till confluency. Test inactivants were 2-fold serially diluted and 24 hrs post incubation at 37°C, dead cells were washed out using PBS. Remaining viable cells were stained with MTT stain as 50 µl (5 mg/ml)/*well*. Plates were incubated at 37°C for 3-4 hrs. Developed crystals were dissolved using 0.4 % acidified iso-propanol or Dimethyl sulphoxid (DMSO). Developed colour was read at 570 nm wave length using Biotek- ELx-800 ELISA microtiter plate reader. The viable cell number was calculated according the equation: Viable % = OD Test × 100 / OD of cont. The safe concentration of test inactivant was determined.

Determination of inactivation kinetics of HAV

Inactivation kinetic relative to time post treatment with LAO and LAA was determined according to **Madhusudana (2004)** and **El-Karamany (1987)** where 1 ml of chemically treated virus was collected at time interval of one hour. Virus samples were 10-fold serially diluted 10^1 - 10^8 in 199-E medium. Prepared dilutions were dispensed onto 24 hrs pre-cultured Vero cells in 96 well plates (TPP-Swiss). Infected plates were kept at 37°C in 5 % CO_2 (Jouan–France) with daily microscopic observation using inverted microscope for detection of the CPE. The 50 % end point induced CPE was determined according to **Reed and Muench (1938)**.

Acute toxicity of test inactivants

Acute toxicity of each test inactivants was performed according to **Abd El-Razek *et al*. (2011)**. Intraperitoneal acute toxicity was studied in Swiss Webster male mice. The animals had free access to feed and drinking water. Mice were allocated into groups (10/cage). Test chemicals safe concentrations were administered intraperitonealy. General symptoms of toxicity and mortality were observed for 24 hrs, after which the animals were left for further 7 days for delayed toxicity.

Aluminum phosphate (Alum) adjuvant

The solutions of each of 0.63 M $AlCl_3.6H_2O$ and 0.3 M $Na_3PO_4.12H_2O$ were prepared in 40 ml normal saline. Prepared solutions were 0.2 µm filtered. Contents were stirred continuously during the procedure at 40 to 60 rpm. After wards, 0.3 M $Na_3PO_4.12H_2O$ solution was added to the mixing bottle, and then 300 ml normal saline was added. The antigen was also added followed by addition of 0.63 M $AlCl_3.6H_2O$ solution to the mixing bottle. The pH was maintained between 6.5 – 6.8. The final volume was adjusted with sterile normal saline and the suspension was mixed for 2 hrs at 37°C **(Gupta, 1998; Lindblad, 2004)**.

Mice immunization

Six to eight weeks old Swiss Webster male mice, housed at the animal facility of VACSERA, Giza, Egypt, were used in this study according to the recommendations of Animal Care and Use Committee (ACUC). Alum was used as adjuvant at concentration of 0.35 mg/ml to enhance the immune response to the injected inactivated virus. In this study, five groups of mice, 10 each, were immunized subcutaneously. Four groups were immunised individually with LAO- and LAA-inactivated HAV, alum adsorbed and none-adsorbed, while the fifth group was immunised with the currently marketed HAV vaccine Havrix. Havrix is an alum-adsorbed commercial vaccine (Glaxo SmithKline) containing 1440 ELISA units [EU] of formalin-inactivated HAV as a positive control. Immunized mice were bled through retro orbital plexus. Immune sera prepared from blood samples collected from each group at a two-week interval post-immunization. Antibody level [IgG] and cytokines [IFN-γ and IL-5] produced post-immunization were monitored.

Detection of HAV-specific antibodies in mice sera

Antibodies against HAV vaccine were detected in post-immunization sera samples using enzyme-linked immunosorbent assay (ELISA) according to **Abd El-Razek et al. (2011)**, where sera samples were diluted as 1/100 in dilution buffer (PBS + 1 % BSA) and added to HAV antigen pre-coated 96-well maxisorb ELISA plates. Sera samples were serially diluted and plates were incubated for an hour at 37°C. Plates were washed three times with 300 µl of 1× wash buffer (PBS + 0.05 % Tween 20) using automated ELISA plate washer for better washing performance. Then, 100 µl of anti-mouse IgG-HRP conjugate were added to all wells leaving one empty for the substrate blank. Plates were mixed gently for 5-10 seconds and then incubated for 60 minutes at 37ºC. Plates were washed as previous. Tri Methyl Benzdeine (TMB) substrate buffer (Sigma Aldrich, USA) was added as 100 µl/well and plates were kept in dark for 20 minutes at room temp. The reaction was stopped using 100 µl of 2N H_2SO_4. Optical density of developed colour was measured at 450 nm using BioTek-XL-800 –USA) ELISA reader within 60 min.

Cytokines determination

The amounts of IFN-γ and IL-5 in mice immune sera were assayed using specific sandwich ELISA kit for each cytokine (eBiosciences, USA) according to the manufacturers' instructions.

Statistical analysis

Results are expressed as mean values for three three independent experiments. Comparison between the different groups was made using unpaired student *t*-

tests to assess significance using GraphPad Prism 5 software. Differences at *P* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Treatment with LAA or LAO could successfuly inactivate HAV

Testing the cytotoxic effects of test inactivants LAO and LAA showed that the safe concentrations were 0.4 µg/ml and 1.5 mg/ml, respectively. These concentrations were used to inactivate HAV, which showed no toxicity to mice as all inoculated tmice were alive throughout the designed 7-day period of test. Data recorded revealed that HAV was completely inactivated within 30 hrs and 36 hrs post-treatment with LAO and LAA, respectively, and no residual infective virus was detected (Figure 1).

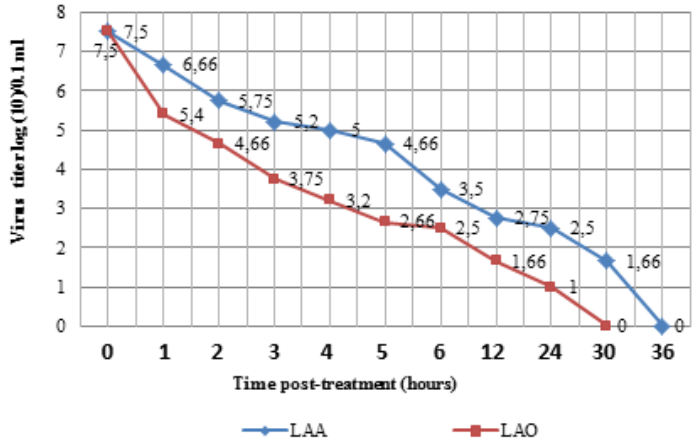

Figure 1 Evaluation of inactivation kinetics of HAV post-treatment with each LAO and LAA relative to time using cell culture assay.

Each of LAA- and LAO-inactivated HAV induced humoral and cellular immune responses in mice

Regarding the humoral immune response, total HAV-specific IgG response was monitored. Data recorded revealed that HAV-specific IgG antibodies were detectable in the mice immune sera as early as two weeks and the peak was detected at the 6th week and 8th week post-immunization with test LAO-inactivated vaccines either alum adjuvanted or non-adjuvanted and LAA-inactivated vaccines either alum adjuvanted or non-adjuvanted, respectively. There was a significant difference ($P < 0.05$) in the level of the antibody production, which was detected 14 weeks post-immunisation with LAO prepared vaccine than those detected post-immunization with LAA prepared vaccines. Alum adjuvanted vaccines showed a significant elevated antibody level ($P < 0.05$) than non-adjuvanted ones throughout the immunization course. Based on the antigen content, the purchased reference vaccine showed the highest level of antibody response (Figures 2, 3).

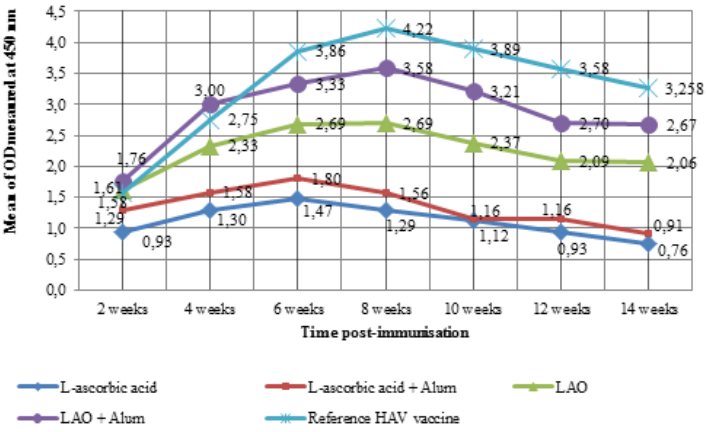

Figure 2 IgG immune response of mice immunised with alum adjuvanted and non-adjuvanted LAA- and LAO-inactivated HAV vaccines, relative to time post-immunisation.

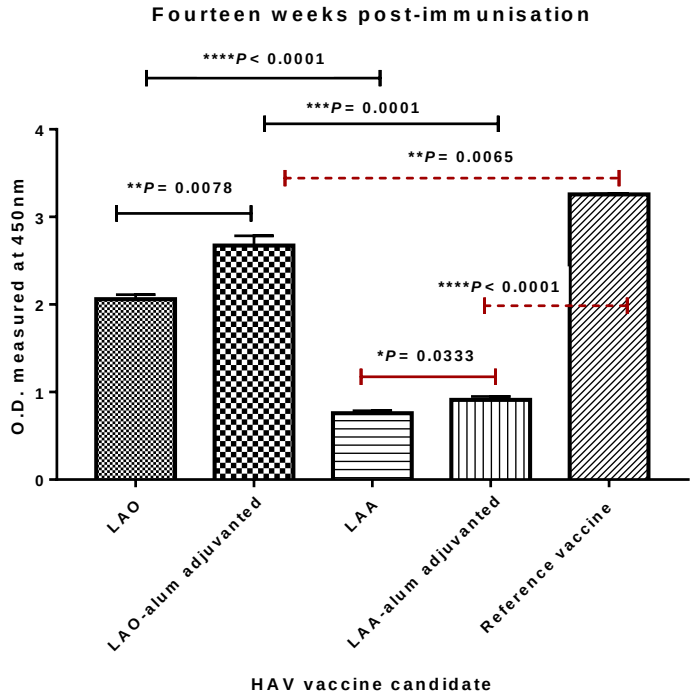

Figure 3 IgG immune response in mice 14 weeks post-immunization with HAV vaccines. Data presented as mean ± SE of three replicates.

Concerning the cellular immune response, IFN-γ and IL-5 cytokines were estimated in immunised mice sera. The production of these cytokines could be detected as early as 3 days post-immunisation with HAV-inactivated vaccines. The level of IFN-γ was significantly higher ($P < 0.05$) in case of both alum adjuvanted LAA- and LAO-inactivated HAV vaccines than non-adjuvanted ones until 28 days pos-immunisation. The alum adjuvanted LAO- and LAA-inactivated vaccines showed high levels than that produced by purchased positive control vaccine ($P < 0.05$) at 14 days and 21 days post-immunisation. At 28 days post-immunisation, there was no significant difference ($P < 0.05$) between the level produced by LAO-adjuvanted vaccine and that of the purchased vaccine ($P > 0.05$), while that of LAA-adjuvanted vaccine was slightly higher at $P < 0.05$ indicated by one asterisk. LAO-inactivated vaccines produced significantly higher levels of IFN-γ ($P < 0.05$) than that produced by LAA-inactivated ones at 14, 21 and 28 days post-immunisation denoted by four asterisks, except at 28 days, there was no significant difference ($P < 0.05$) between adjuvanted ones (Figures 4, 6).

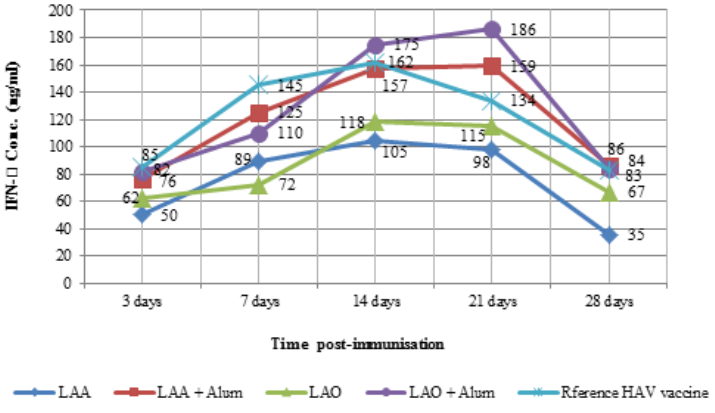

Figure 4 Estimation of IFN-γ concentration in mice sera post-immunisation with LAO- and LAA-inactivated HAV vaccines using specific sandwich ELISA.

IL-5 level was significantly higher ($P < 0.05$) in both LAO- and LAA-inactivated vaccines adjuvanted with alum than those non-adjuvanted ones at 14, 21 and 28 days post-immunisation. LAO-inactivated vaccines produced significantly higher levels of IL-5 ($P < 0.05$) than that produced by LAA-inactivated ones. The IL-5 level produced by purchased vaccine was significantly higher ($P < 0.05$) than that produced by test vaccines at 14, 21 and 28 days post-immunization except LAO-adjuvanted one which was significantly higher ($P < 0.05$) than control vaccine at 21 days (Figures 5, 6).

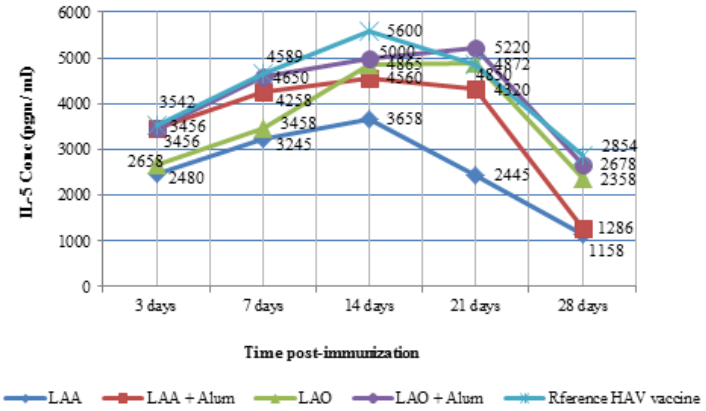

Figure 5 Estimation of IL-5 concentration in mice sera post-immunization with LAO- and LAA-inactivated HAV vaccines using specific sandwich ELISA.

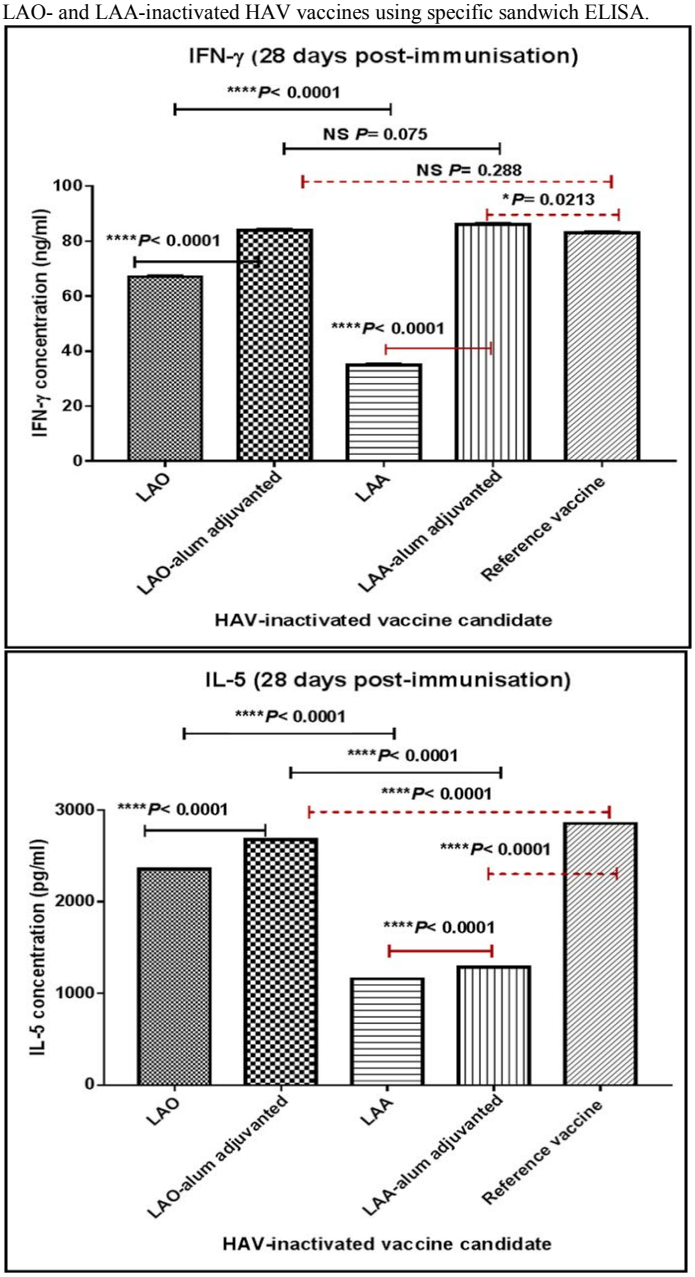

Figure 6 IFN-γ and IL-5 concentrations in mice sera at 28 days post-immunization with LAO- and LAA-inactivated vaccines.

In the past two decades, the HAV cases have declined in several parts of the world due to partly the developed vaccines **(Kanyenda *et al*., 2015)**. The commercially available HAV vaccines contain formalin-inactivated HAV, grown on human cell lines and purified, that has been adsorbed with alum to enhance its immunogenicity. While they are considered efficacious, often multiple boosters are required to confer protection against HAV. These vaccines are also very expensive to produce, available in limited quantities, and must be kept cold until they are administered by injection **(Mitchell and Galun, 2003)**. Still in developing countries, such as Egypt, HAV vaccination may be considered on the basis of epidemiological and cost-effectiveness concerns. Thus, the available HAV vaccines are not yet commonly used **(Franco *et al*., 2012)**. Viral inactivation is an important procedure in vaccine development against viral infections **(Stauffer *et al*., 2007)**. However, the inactivating agent may have an effect on the viral epitopes pattern to which the antigenicity and/or immunogenicity of vaccine is related **(Blackburn and Besselaar, 1991)**. Thus, the inactivation procedures may drastically impair induction of neutralizing IgG antibodies responses for most viruses **(Bachman *et al*., 1994)**. Formaldehyde is the inactivating agent used for HAV vaccines **(Chowdhury *et al*., 2015)**. However, it was observed that for formalin inactivation concentration, pH, temperature and medium composition are extremely critical factors. Formalin was found to require at least 24 – 96 hrs at 4°C –37°C for inactivation. In addition, the higher concentrations of formaldehyde and temperature used to speed up the inactivation may harmfully affect the immunogenicity of inactivated virus **(Chowdhury *et al*., 2015; Sanders *et al*., 2015)**. Seriously, an incomplete formaldehyde inactivation procedure may be fatal for public health. For example, incomplete inactivation of the Venezuelan equine encephalitis vaccines, prepared by formalin inactivation, was the cause of the outbreak of the disease during the 1969 – 1972 pandemic in Central America **(Brown, 1992)**. Furthermore, formaldehyde which is an alkylating agent inactivates viruses via chemical reaction with viral capsid proteins and nucleic acid **(Budowsky *et al*., 1991)**, and it has been classified by International Agency for Research in Cancer (IARC) under group 2A. However, there is no epidemiological data referring to the carcinogenic risk of the alkylating agents on humans **(Chowdhury *et al*., 2015)**. Owing to previous data, there is a need for available, cheap and efficient alternatives for fast inactivation of HAV without affecting its immunogenicity allowing HAV vaccines to be less expensive to be manufactured and more amenable to mass vaccination programs. Thus, the main objective of the present study was to evaluate the efficiency of LAO and LAA as inactivants of HAV and examine the immunogenicity of each LAA- and LAO-inactivated HAV compared with the currently available formaldehyde prepared vaccine.

In the present study, both test inactivants could successfully inactivate HAV using safe concentrations (0.4 µgm/ml of LAO and 1.5 mg/ml of LAA), within 30 hours and 36 hours post-treatment, respectively. LAO has previously showed remarkable virucidal activities to viruses such as varicella-zoster virus and herpes simplex virus type 2 **(Zedan *et al*., 2003; Alyan *et al*., 2014)**. In addition, data recorded of HAV inactivation by LAO in this study is consistent with results of **Aly Mohamed** and his colleagues (unpublished data), despite the use of another virus model namely Rift Valley fever virus that was completely inactivated with LAO within 6 hrs, and the immune potential of this vaccine was better than that of the known β-propiolactone (BPL)-inactivated vaccine. Recently, snake venom LAO has been recognized as a multifunctional protein with promising biomedical applications because of its antimicrobial, anti-HIV, anticoagulant, and inducing of platelet aggregation. LAO enzyme is acting specifically on L-amino acids and generally on hydrophobic amino acids **(Du *et al*., 2002)**. The inactivation of HAV by LAO is possibly due to the oxidizing stress of LAO attributed to the ability of the enzyme to localize H_2O_2 to the target cells through channels in its structure that would direct the H_2O_2 product to the exterior surface of the protein, near the glycan moiety. Thus, the glycan moiety is thought to be involved with LAO-target cell interaction. Accordingly, the virucidal activity of LAO was the base on which it was used as virucidal agent to prepare an improved HAV vaccine **(Shebl *et al*., 2012)**.

Regarding the cytotoxicity of LAO, our findings were found to be in agreement with **Shebl *et al*. (2012)** study which revealed that LAO showed significant cytotoxicty to Vero normal cells at concentrations higher than 0.4 µgm/ml. The LAO cytotoxicity may be attributed to the released H_2O_2 enhancing the oxidative stress on cell nucleus causing cell death and progressive apoptosis. Concerning the use of LAA as an inactivant for HAV, our findings are supported by **Madhusudana *et al*. (2004)** that ascorbic acid can be used as an inactivating agent for viruses grown in cell line although the authors used it for rabies virus. In addition, earlier experiments used LAA as an inactivating agent for both RNA and DNA viruses, confirmed its efficiency as inactivating agent for number of viruses including vaccinia virus **(Turner *et al*., 1964)** HIV virus **(Rawal *et al*., 1995)** and *in vitro* infectivity of herpes viruses and paramyxoviruses **(White *et al*., 1986)**. **Abd El-Razek *et al*. (2011)** study revealed also the complete effective inactivation of Rift Valley fever virus using LAA for vaccine development. The mechanism whereby ascorbic acid inactivates viruses is not fully understood. Although, it was partly explained by the presence of oxygen which is essential and ascorbic acid undergoing auto-oxidation results in the formation of OH groups that could bring about the inactivation of the cell free viruses **(Murata *et al*., 1986)**.

The mice immunisation experiments performed in this study revealed that both inavtivated HAV vaccines induced a good antibody (humoral) immune response as indicated by the results of the HAV-specific IgG ELISA. Regarding the immunogenicity of LAO-inactivated vaccine, the results revealed that HAV-specific antibodies were detected 14 days post-immunization and this was in agreement with **Keeffe *et al*. (1989)**, despite their trial was conducted on the

clinical level. Whilst, the immune response peak was on the 8th week post-immunization with LAO or LAO alum adjuvanted vaccine, similar to the antibody response produced by HAV reference vaccine, and on the 6th week post-immunization for LAA or LAA alum adjuvanted vaccine. Subsequently, the level of antibody production starts to decrease. Natural infection with Hepatitis A virus leads to life long detectable antibody in most individuals, whereas vaccine induced antibody levels wane over time. In healthy adults, vaccine induced anti-HAV has been observed to decrease rapidly from one month after the booster vaccination until six month later, followed by a rather constant decrease over the subsequent two years, approximately 14 % per year **(Van Damme *et al*., 1994)**. Thus, in most countries, booster-vaccination policy is guided by manufacturers' recommendations, national authorities, or both. Data showed that after a full primary vaccination course, protective antibody amounts persist beyond 10 years in healthy individuals, and underlying immune memory provides protection far beyond the duration of anti-HAV antibodies **(Van Damme *et al*., 2003)**.

One of the major cellular effectors is the CD4+ helper T cells that elaborate cytokines which enhance both antibody and cellular immune responses. For investigating the cellular immune response in immunised mice and the predominant phenotype, cell-mediated (Th1) or humoral (Th2) immune responses elicited by immunization with LAO- or LAA-inactivated HAV, the level of two cytokines IFN-γ (Th1 cytokine) and IL-5 (Th2 cytokine) were estimated in the post-immunisation sera. The cytokine-specific sandwich ELISA results revealed that the mice immune response generated with both LAA- and LAO-inactivated HAV immunisation was a mixed Th1/Th2 immune response profile similar to that produced by HAV reference vaccine. However, the quantitative measurement of the level of each cytokine indicated that the concentrations of Th1 cytokine IFN-γ (cellular immunity) was higher than that of Th2 cytokine IL-5 (humoral immunity). These results indicated that the extent of up-regulation of IFN-γ production was the highest when compared to that of IL-5, confirming a significant Th1 immune response. Accordingly, there was distinct humoral and cellular immune responses post-immunisation with both LAA- and LAO-inactivated HAV. Consistent with our results, **Schmidtke *et al*. (2005)** study results which revealed that both distinct B and T cell responses were determined within 14 days after immunisation with formalin-inactivated vaccine (Havrix) in humans. However, the cellular immune response in the current study might be the predominant one which is fitting for vaccination against viral infections by HAV. That is because the cellular immune response is very vital for combating viral infections **(Lappin and Campbell, 2000)**. In addition, clinical and experimental evidence proved the hypothesis that HAV hepatocellular damage and the efficient elimination of virus-infected hepatocytes are mediated by virus-specific, proliferating T lymphocytes derived interferon **(Schmidtke *et al*., 2005)**. The data recorded in this study concerning type of immune responses was also in agreement with **Cederna *et al*. (1999)**, despite their trial was on the clinical level, which revealed that HAV antibody and proliferative effective T cell response were elicited by a formalin-inactivated HAV vaccine in the immunized subjects. However, antibodies produced against HAV are present over an extended period in the human sera, indicating the likely importance in maintaining providing a long-term immunity **(Wang *et al*., 1996)**. These results indicated the high and good immunogenicity of each of LAA- and LAO-inactivated HAV in Swiss Webster mouse strain. Although, there was no significant difference in the immunogenic potential between the LAO- and LAA-inactivated HAV; both showed equivalent antigenic potency as measured by indirect ELISA.

The concentrations of Th1 cytokine IFN-γ in alum adsorbed LAA- and LAO-inactivated HAV was higher in those non-adsorbed ones. This finding is consistent with the practically established concept that alum adjuvant is administered with antigens in experimental murine and rabbit immunization studies to enhance immunity **(Gupta, 1998; Lindblad, 2004)**. Indeed, all HAV commercially available formalin-inactivated vaccines are adsorbed onto alum as an adjuvant **(Karayiannis *et al*., 2004; Tahaei *et al*., 2012)**. Previous published studies concerned the cellular immune response to vaccine prepared using LAO as a natural product is very rare as the majority of inactivating agents are chemicals in nature not natural derivatives. However, the use of LAA as an inactivating agent showed similar immune response, and particularly the cellular immune response pattern as in case of inactivation of rabies virus reported by **Madhusudana *et al*. (2004)** and **Abd El-Razek *et al*. (2011)**. Thus, it was hardly to compare HAV vaccine potentials to other prepared vaccines using another inactivating agent but using the same technique except in case of preparing Rift Valley fever virus vaccine inactivated with both BPL and LAO despite the previous reporting of LAO as a virucidal agent to Rift Valley fever Virus, Vesicular Stomatitis Virus (VSV), Herpes and Adeno viruses **(Abd El-Razek *et al*., 2011; Alyan *et al*., 2016)**. Accordingly, data recorded regarding the immune response is in agreement with one of the authors of this study, **Aly Mohamed** and his colleagues (unpublished data), that LAO-inactivated Rift Valley fever vaccine was high immunogenic than the BPL-inactivated one. The use of alum-adjuvanted vaccines has showed a better immune response than non-adjuvanted ones.

CONCLUSION

It can be reported that both LAA and LAO are promising and equally efficient for inactivation of HAV. Each of LAA- and LAO-inactivated HAV appears to provide potentially effective anti-HAV vaccines owing to the finding that they induced both good humoral and cellular immune responses. The immune potentials of both LAA- and LAO-inactivated vaccines are almost equally to that of formalin-inactivated HAV vaccines currently available in the Egyptian market. There was no distinguished difference in the values of cellular immune response parameter of interest. Both HAV-inactivated vaccines were biologically of near bio-reactivity to immune system. Alum as adjuvant is useful to enhance the immune potentials of inactivated HAV on the experimental level. However, the present study is limited by showing the immune response only for days and/or weeks, thus long-term tracing of immune response on both the cellular and humoral levels is warranted. The HAV-inactivated vaccines in the current study were not challenged in experimental animals as human is the only reservoir host of HAV and the lack of animal model that mimics the human infection. An extensive investigation of the HAV vaccine-induced immune response should include the analysis of circulating HAV-specific T lymphocytes. As storage, handling and the heat stability of vaccines are consequently matters of great concern. Thus, studying the stability either real time or accelerated one and tracing the accumulative effect of residual LAO is recommended on the biochemical pathological level. Both LAO and LAA should be further compared with other inactivating agents along with testing different inactivation agents and/or vaccine adjuvants to maximize the immune response and formulate the best suitable method for HAV vaccine preparation.

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INDUCTION OF CALLUS FROM LEAF AND STEM TISSUES OBTAINED FROM *CAPSICUM ANNUUM* EXPLANT GROWN ON WASTE ENGINE OIL-POLLUTED SOILS

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ABSTRACT

The present study investigated callusing frequency of explants from leaf and stem tissues obtained from *Capsicum annuum* explant exposed to waste engine oil contamination. The explants obtained from 21-31 days old seedlings were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of BAP (0, 1, 2, 3, 4, 5 and 6 mg/l) and NAA, IAA, 2, 4-D and IBA (0, 5, 10, 15 and 20 mg/l). Results showed that efficient callusing in leaves and stems explants (21 days old) was achieved after explants were cultured on MS semi-solid media containing 1-5 mg/l BAP and 0.5-1.5 mg/l IBA for 4 weeks; while callus formation was hindered in older tissues. An effective protocol for callogenesis of 21 day-old explants (stems) of *C. annuum* obtained from oil-polluted soil in a semi-solid state MS medium using BAP and IBA was thus developed. The study also documented the ability of IBA to induce callus.

Keywords: Callus, Callogenesis, morphogenesis, explant, tissue culture, oil-polluted soil

INTRODUCTION

In order to survive poor economic conditions, there is usually the option of diversification of income by poor locals. One of such is the proliferation of alternative employment like artisans including auto mechanics. In many poverty-impoverished countries, particularly in the third world, there seem to have been an increase in the siting of mechanic workshops as well workshops of other artisans which use and dispose of engine oils and other waste petroleum products (**Ikhajiagbe, 2010; Ikhajiagbe and Anoliefo, 2012a)**. These workshops range from repairers of automobiles, industrial machines and engines, as well as generating sets. These mechanics replace engine oil from motor vehicle and generator engines, and other machines. The engine oil wastes are in most cases disposed inappropriately into open vacant plots and fields as well as drainages. This action directly or indirectly leads to soil pollution with oil and its constituents; thus leading to phytotoxicity. The adverse impact oil and water-soluble factions of oil have on plant growth and development, as well as oversell crop yield has been reported (**Vwioko and Fashemi, 2005; Ikhajiagbe, 2010**).
The impact of oil on plants starts from germination. **Ikhajiagbe *et al.* (2014)** reported effects of oil on germination parameters of *Vigna unguiculata.* **Anoliefo and Vwioko (1995)** also reported reduced germination rate of *Capsicum frutescens* and *Zea mays* due to oil pollution. The inhibitory effects of oil on seed germination may be due to a number of reasons including physical limitations and biological impairment on the seeds (**Amakiri and Onofeghara, 1984**). When this occurs, crop sustainability can no longer be guaranteed, particularly during the time when the clamour for food security seems loudest. The idea of possible regeneration and proliferation of seedlings to guarantee the sustenance of species and species lines is thus imperative. This informs the basis of the study.
Recent works by **Ikhajiagbe (2010)** and **Ikhajiagbe and Anoliefo (2012a, b)** showed that soil polluted by waste engine oil resulted in chlorosis and the death of the crop plants sown within five (5) months of pollution. This finding was for current oil-in-soil concentrations of above 5% w/w. This begs the question whether it was possible for any crop plant that was sown in oil-polluted soil within 5 months of pollution to be regenerated from explants of such plants. If this were achieved, then it would be possible to save the oil-threatened plants. In the present study, pepper (*Capsicum annuum L.*) has been selected. *Capsicum annuum L.* plays a vital part in many cuisines. Apart from their use as spices, pepper contains capsaicin which has use in many pharmaceutical preparations. This plant species is usually propagated by seed; however the use of tissue culture has become an important tool in clonal propagation and breeding programs.
Although there are not yet documented evidences that oil pollution currently threatens the *in vitro* regeneration of peppers, the possibility is not far-fetched, especially in a world that now revolves round the petroleum sector. The need to address this possible threat is not the only rationale for the study; there is also the need to satisfy the ever-increasing demand for the crops. This therefore calls for more dependable breeding methods for mass proliferation of the crop in the face of the present challenges.
Most plants in oil-contaminated soils accumulate heavy metals and toxic poly aromatic hydrocarbons leading to chlorosis and eventually, necrosis (**Ikhajiagbe, 2010**). When this happens, harvesting explants for the purpose of regeneration or other in vitro studies can become very challenging. The present study intends to find out at what time in the pepper's developmental phase where successful explants exposed to waste engine oil polluted soil can be regenerated using tissue culture techniques. The objective is to induce callus from leaf and stem tissues obtained from *C. annuum* explant grown on polluted soils, employing standard *In vitro* techniques and using defined medium, supplemented with various combinations of plant growth hormones and to determine the growth rate of callus formation.

MATERIAL AND METHODS

This study was carried out in the Tissue Culture Laboratory, Physiology and Tissue Culture Division, Nigerian Institute for Oil Palm Research (NIFOR), located at 1735 land area, 29km off the City centre, off Benin - Akure Road, Benin, Edo State, Nigeria.

Collection and preparation of materials for the experiment

Top soil (0 – 10 cm) was collected from a cleared field, beside Tissue Culture Laboratory (NIFOR) and sun-dried to constant weight. Thereafter, 12.5kg of the sun-dried soil was collected using shovel and hand trowel, weighed (using measuring scale) and poured into ten (10) perforated nursery bags with three replicates labeled as control. Waste engine oil obtained as pooled from an automechanic workshop was added to the soil; this was thoroughly mixed to obtain a constant concentration of 5%w/w WEO-in-soil. The bags of soil were left for one month to attenuate, after which physiochemical parameters of polluted and control soils were determined (Table 1). Thereafter, planting of

viable pepper seeds (which were pre-soaked in water for ten hours) were carried out in the morning (7am) and left to grow for a month.

Collection of Explants

Stem and leaf explants were randomly collected at three different ages; 21, 28 and 31 days old. These three (3) age groups were preferred because previous reports by **Ikhajiagbe (2010)** revealed that plants sown in 2.5 – 10.0% w/w oil-polluted soil began to show signs of physiological stress within 2 weeks (or 14 days) of exposure to polluted soils. These explants were cultured *In vitro*.

Development of MS protocol for explants

The callusing efficiencies of *Capsicum annuum* leaf and stem explants were examined by using Murashige and Skoog (MS) basal medium (**Murashige and Skoog, 1962**) with different concentrations of plant growth regulators (PGRs). However, modified MS protocols were developed for the purpose of the study for explant regeneration. In developing the MS protocols, stock solutions were first prepared using the facilities of Tissue Culture Laboratory of the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Nigeria. All the apparatus used were thoroughly washed, rinsed twice and dried using the oven at 65^0C for 1 hour. After preparation, the solutions were stored in a refrigerator at 60^0C.

Stock Solutions

MS Macro x10 (500ml): Two hundred (200) ml of distilled water was poured in a 400ml Pyrex bottle and placed on a magnetic stirrer with the stirring bar and allowed to stir for about 5mins. A measured 9.5g of potassium nitrate (KNO_3), 8.25g of ammonium nitrate ($NH_4 NO_3$), and 0.85g of potassium dihydrogen orthophosphate ($KH_2 PO_4$), 2.2g of calcium chloride dehydrate ($Cl.2H_2O$) and 1.85g of Magnesium sulphate ($MgSO_4.7H_2O$) were weighed and poured into the bottle. After pouring the first compound, it was allowed to stir for 5 - 7mins before the other compounds were added. The solution was poured into a 500ml measuring cylinder and rinsed with distilled water. It was then made to 500ml and stored for use in a 1-litre flask.

MS Micro A x10 (500ml): A measured 250ml of distilled water was poured into a 400ml Pyrex bottle, which was placed on a magnetic stirrer (with the stirring bar) and allowed to stir. Thereafter, 1.11g of Maganous sulphate ($MnSO_4.4H_2O$), 0.315g of Boric acid (H_3BO_3), 0.43g of zinc sulphate ($ZnSO_4.7H_2O$) and 0.415g of potassium iodide were weighed and added into the stirring bottle. After 10mins, it was turned into a 500ml measuring cylinder and then made up to the mark. The solution was stored for use.

MS Micro B (500ml): Distilled water (300ml) was measured into a 400ml Pyrex bottle which was placed on a magnetic stirrer (with the stirring rod). Measured qualities (0.125g) of sodium molybdate ($Na_2MOD_4.2H_2O$), 0.0125g of cupric acid ($CuSO_4.5H_2O$) and 0.0125g of cobalt chloride ($Cl_2.6H_2O$) were poured into the bottle. After 10mins, it was turned into a measuring cylinder and then made up to the 500 ml mark.

MS Iron x100 (500ml): Fifty (50) ml of distilled water was poured into a conical flask (containing stirring bar) and placed on a hot plate. Measured 1.865g of ethylene diamine tetraacetic acid (EDTA) was weighed into a conical flask and allowed to heat at 260^0C for 10mins. Thereafter, 1.39g of ferrous sulphate ($FeSO_4.7H_2O$) was weighed into a beaker containing 30ml of water which was placed on a stirrer machine and allowed to dissolve. This was poured into the EDTA solution and allowed to heat for 10mins, and thereafter made up to the 500ml mark.

MS Vitamins x100 (500ml): 200ml of distilled water was poured into a 400ml Pyrex bottle which was placed on a magnetic stirrer and allowed to stir. Then 0.25g of thiamine-HCL, 0.125g of pyridoxine-HCL, 0.25g of Nicotinic acid, 0.1g of Glycine acid and 0.125g of calcium parthotenate were weighed and poured into the Pyrex bottle. After 10mins the entire solution mixture was turned into a measuring cylinder and made up to the 500ml mark.

Establishment of MS protocol (1000ml) for 21 and 28 day-old explants

Macro x10 ---------- 100ml
Micro (A and B) x100 ---------- 10ml
Iron x100 ---------- 10ml
Vit. x100 ---------- 10ml
Inositol ---------- 0.1g
Ascorbic acid ---------- 0.28g
Sucrose ---------- 30g

The media were divided into 28 bottles of 36ml each

MS Medium for pepper (28days old) callogenesis

Full strength MS medium supplemented with 30g sucrose, macro x10, micro x10 A and B, iron x100,vitamins x100, plant growth hormones (PGH). 36ml of the solution was dispensed into 28 bottles, each containing 0.35g of agar pH of 5.8. An aliquot of 12ml was dispensed into 84 labeled (control and polluted) screw-cap McCartney bottles, three replicates of each concentrations of PGH, autoclaved and allowed to cool at ambient temperature (28^0C). The explants (stems and leaves) were removed from the plants, rinsed with tap water; surface sterilized using 5% hypochlorite solution (containing a drop of $Tween_{20}$) for 5mins. Sterile water was used to remove traces of sodium hypochlorite solution five times to get rid of the sterilizing solution which is toxic to the stems and leaves on delayed exposure. The explants were cut into thin sections (using sterilized forceps and surgical blades on sterilized Petri dishes) and transferred aseptically into the test tubes containing the culture medium using sterilized forceps (i.e. inoculation) and covering them up immediately with aluminium foil passed over flame. They were then incubated in the dark growth room at 27^0C for 6 weeks.

MS Medium for pepper (21days old) callogenesis

The medium was in semi-solid state. The amount of agar used was reduced to 0.28g for 36ml of the solution. The explants (stems) were removed from the plants, rinsed with tap water; surface sterilized using 0.4% mercuric chloride ($HgCl_2$) solution containing a drop of $Tween_{20}$ for 5mins. Traces of the $HgCl_2$ were removed by rinsing with sterile distilled water five times to get rid of the sterilizing solution which is toxic to the stems and leaves on prolonged exposure. The explants were cut into thin sections (using sterilized forceps and surgical blades on sterilized Petri dishes) and transferred aseptically into the test tubes containing the culture medium using sterilized forceps (i.e. inoculation) and covering them up immediately with aluminium foil passed over flame. They were then incubated in the dark growth room at 27^0C for 8weeks.

Establishment of MS protocol (1350ml) for 31day-old explants

Macro x10 ---------- 135ml
Micro (A and B) x100 ---------- 13.5ml
Iron x100 ---------- 13.5ml
Vit. x100 ---------- 13.5ml
$NaH_2PO_4.2H_20$ ---------- 0.229g
Inositol ---------- 0.135g
(ASP, ARG, GLU) ---------- 0.135g
Ascorbic acid ---------- 0.338g
Sucrose ---------- 40.5g

Then, the media were divided into 45 bottles of 30ml each

MS Medium for pepper (31 days old) callogenesis

Standard MS medium is supplemented with 40.5g sucrose, macro x10, micro x10 A and B, iron x100,vitamins x100, plant growth hormones (PGH). Measured quantity (30ml) of the solution was dispensed into 45 bottles, each containing 0.28g of agar pH of 5.8. An aliquot of 10ml was dispensed into 135 labeled (control and polluted) test tubes (50ml), three replicates of each concentrations of PGH, autoclaved and allowed to cool at ambient temperature (28^0C). The explants (stems and leaves) were removed from the plants, rinsed with tap water; surface sterilized using 5% hypochlorite solution (containing a drop of $Tween_{20}$) for 5mins. Sterile water was used to remove traces of sodium hypochlorite solution five times to get rid of the sterilizing solution which is toxic to the stems and leaves on delayed exposure. The explants were cut into thin sections (using sterilized forceps and surgical blades on sterilized Petri dishes) and transferred aseptically into the test tubes containing the culture medium using sterilized forceps (i.e. inoculation) and covering them up immediately with aluminium foil passed over flame. They were then incubated in the dark growth room at 27^0C for 8weeks. The media were prepared in the main laboratory and the initiation was carried out in the Laminar flow cabinet.

Plant growth hormones (PGH) modification and supplementation for 31days old pepper

Three types of auxins and one cytokinin were used. The auxins used were Indole acetic acid (IAA), Naphthalene acetic acid (NAA) and 2,4 dichlorophenoxyl acetic acid (2,4-D), each at concentrations of 0, 1.5, 3, 4.5 and 6 mg/L. The cytokinin used was Benzyl amino purine (BAP) at concentrations of 0, 0.1 and 1 mg/L (Table 2).

Testing the effects of IAA and BAP

IAA at concentrations of 1.5, 3, 4.5 and 6 mg/L as well as BAP at concentrations of 0.1 and 1 mg/L was added. Control experiment without IAA and BAP was

also included. The possible combinations for various concentrations of the two PGH (IAA and BAP) led to an experiment with 15 treatments.

Testing the effects of NAA and BAP

NAA at concentrations of 1.5, 3, 4.5 and 6 mg/L as well as 0.1 and 1 mg/L concentrations of BAP were added. Control experiment without NAA and BAP was also included. The possible combinations for various concentrations of the two PGH (NAA and BAP) led to an experiment of 15 treatments.

Testing the effects of 2, 4-D and BAP

2, 4-D at concentrations of 1.5, 3, 4.5 and 6 mg/L and BAP at concentrations of 0.1 and 1 mg/L were added. Control experiment without 2, 4-D and BAP was also included. The possible combinations for various concentrations of the two PGH (2, 4-D and BAP) led to an experiment with 15 treatments.

Plant growth hormones (PGH) modification and supplementation for 28 and 21days old pepper

One type of auxin and one cytokinin were used. The auxin used was Indole butyric acid (IBA) at concentrations of 0, 0.5, 1 and 1.5 ppm. The cytokinin used was Benzyl amino purine (BAP) at concentrations of 0, 1, 2, 3, 4, 5 and 6 ppm (Table 3).

Testing the effects of IBA and BAP

IBA at concentrations of 0.18, 0.36 and 0.54 mg/L as well as 0.36, 0.72, 1.08, 1.44, 1.8 AND 2.16 MG/L concentrations of BAP were added. Control experiment without IBA and BAP was also included. The possible combinations for various concentrations of the two PGH (IBA and BAP) led to an experiment of 28 treatments.

Determination of source of contamination in inoculum

In other to ascertain contamination rate for explants obtained from control and polluted soils, swaps from the contaminated media were taken and cultured on PDA and nutrient agar media, using the spread plate method (Cheesebrough, 2001).

Experimental Design

The experimental design chosen was the completely randomized design (CRD). As a result, treatments were randomized over the media. Each treatment consisted of 3 replicates. The treatments were the various levels of Indole acetic acid (IAA), Naphthalene acetic acid (NAA), 2,4 dichlorophenoxyl acetic acid (2,4-D), Indole butyric acid (IBA) and Benzyl amino purine (BAP) in the media. In order to avoid bias and misidentification, treatment bottles were properly labeled according to a given treatment name and replicate number. Results were presented as mean ± standard error.

RESULTS

The aim of this experiment was to study the growth of callus formation from pepper (*Capsicum annuum* L.) leaf and stem explants, obtained from polluted soils (see Table 1), employing standard *in vitro* techniques and using defined medium, supplemented with various combinations of cytokinin and auxins. Results showed that naphthalene acetic acid (NAA) and Benzyl amino purine (BAP) supplemented on solid media resulted in the callogenesis of the control 31 day-old stem explants. Callus initiation was obtained at the concentrations of 1.5 and 4.5 (mg/l) after 8weeks. This treatment showed a low percentage of callogenesis. Callogenesis did not occur in the Indole acetic acid (IAA) and Benzyl amino purine (BAP)-amended media, as well as in the 2, 4 dichlorophenoxyl acetic acid (2,4-D) and Benzyl amino purine (BAP) media. However, 33.3% callogenesis was obtained in the control explants in the 0.1 mgl^{-1} BAP/1.5 mgl^{-1} IAA combination (Table 4). No callus was obtained in the polluted treatments. Similarly, 66.7% callogenesis was obtained in 1.0 mgl^{-1} BAP/4.5 mgl^{-1} NAA combination. Appropriate combinations of 0.1 mgl^{-1} BAP/1.5 mgl^{-1} IAA as well as 1.0 mgl^{-1} BAP/4.5 mgl^{-1} IAA showed 33.3% and 66.7% callogenesis respectively in control explants (Table 5). No callus was obtained in the polluted experiments. Similar results were obtained with the BAP/2, 4 - D combinations (Table 6). The addition of IBA (0 and 1.0 mg/l) to the culture medium containing different BAP levels greatly stimulated further callus proliferation beyond the levels obtained for IAA and 2, 4-D (see Table 7). However, there was no callus formation when the concentration of IBA was increased to 1.5mg/l at any given BAP concentration.

Callus induction with IBA/BAP-supplemented semi-solid medium at various levels of concentration of both the control and polluted explants source (21days old) after 2weeks of initiation was reported (Table 8). The age of the explants and medium state greatly stimulated further callus proliferation beyond the levels obtained for other treatments. Fresh weight of callus was obtained at increased concentrations (3, 4, 5 and 6) of BAP in polluted plant source, and control source (1, 2, 4 and 5). Calli formations were effective when the plant's (pepper) age was 21days old, medium state (which was in semi-solid state) and the type of sterilant ($HgCl_2$) used. Callus was induced with IBA in combination with the cytokinin, BAP at various levels of concentration. The use of semi-solid medium containing different levels of concentrations stimulated further callus proliferation beyond the levels obtained from other treatment.

Results also showed that the contamination rate for leaf and stem explants (31, 28 and 21days) obtained from control and polluted soils (Table 9). The rate shows seven days interval after inoculation of the explants. For the 31day-old leaf explants; the contaminated media after 8 weeks indicates fungal infection. This observation is similar to the 31day-old stem explants. The contaminated media for both 28 day-old leaf and stem explants indicates bacterial infection after 8 weeks. Results for the 21 day-old leaf and stem explants indicate bacterial and fungal infections respectively. Young plant tissues possess higher capability for response to *in vitro* culture, compared to older plant materials. This may explain the favourable responses recorded for the young stem explants. The standard medium for callogenesis of *Capsicum annuum* has been presented to be the semi-solid state MS medium that is enhanced with BAP 1, 2, 3, 4 and 5 and IBA 0.5 and 1.5, 0 and 1, 0, 0.5, 0.5 and 1.5 mg L^{-1}. Survival rate of the established calli ranged from 60 – 80%, and were kept in a dark growth room. The study confirms attainment of maximum callogenesis as long as proper concentrations of plant growth hormones were applied.

Table 1 Physicochemical properties of soil at one month after pollution, prior to sowing of pepper

Parameters	Unpolluted soil	Oil-polluted soil
pH	5.59	5.47
Electric conductivity (µs/cm)	289	339
Total organic carbon (%)	0.59	2.32
Total nitrogen (%)	0.15	0.29
Na `(meq/100 g soil)	11.10	11.10
K (meq/100 g soil)	1.16	1.16
Ca (meq/100 g soil)	22.30	22.30
Mg (meq/100 g soil)	15.40	15.40
Fe (mg/kg)	720.34	997.64
Mn (mg/kg)	13.64	11.70
Zn (mg/kg)	10.36	21.80
Cu (mg/kg)	<0.01	2.60
Cr (mg/kg)	<0.01	1.60
Cd (mg/kg)	<0.01	0.12
Pb (mg/kg)	<0.01	1.26
Ni (mg/kg)	<0.01	0.70
V (mg/kg)	<0.01	0.64
Total hydrocarbon content (mg/kg)	100.78	1268.00

Table 2 A schematic arrangements of treatments used for explants obtained at 31 days. pH - 5.8, Agar - 0.28g

	0ppm	5ppm	10ppm	15ppm	20ppm
NAA_{100ppm}/BAP_{100ppm}					
0ppm	0ml / 0ml	1.5ml / 0ml	3ml / 0ml	4.5ml / 0ml	6ml / 0ml
0.1ppm	0ml / 0.03ml	1.5ml/ 0.03ml	3ml/ 0.03ml	4.5ml/0.03ml	6ml/0.03ml
1ppm	0ml/0.3ml	1.5ml/0.3ml	3ml/0.3ml	4.5ml/0.3ml	6ml/0.3ml
IAA_{100ppm}/ BAP_{100ppm}					
0ppm	0ml / 0ml	1.5ml / 0ml	3ml / 0ml	4.5ml / 0ml	6ml / 0ml
0.1ppm	0ml / 0.03ml	1.5ml/ 0.03ml	3ml/ 0.03ml	4.5ml/0.03ml	6ml/0.03ml
1ppm	0ml/0.3ml	1.5ml/0.3ml	3ml/0.3ml	4.5ml/0.3ml	6ml/0.3ml
$2,4\text{-}D_{100ppm}$/BAP_{100ppm}					
0ppm	0ml / 0ml	1.5ml / 0ml	3ml / 0ml	4.5ml / 0ml	6ml / 0ml
0.1ppm	0ml / 0.03ml	1.5ml/ 0.03ml	3ml/ 0.03ml	4.5ml/0.03ml	6ml/0.03ml
1ppm	0ml/0.3ml	1.5ml/0.3ml	3ml/0.3ml	4.5ml/0.3ml	6ml/0.3ml

Table 3 A schematic arrangements of treatments applied to explants obtained from 21 and 28 days old plants. pH - 5.8; Agar - 0.35g (for 28day-old explants) and 0.28g (for 21day-old explants).

IBA_{100ppm}/ BAP_{100ppm}	0ppm	0.5ppm	1ppm	1.5ppm
0ppm	0ml/0ml	0.18ml/0ml	0.36ml/0ml	0.54ml/0ml
1ppm	0ml/0.36ml	0.18ml/0.36ml	0.36ml/0.36ml	0.54ml/0.36ml
2ppm	0ml/0.72ml	0.18ml/0.72ml	0.36ml/0.72ml	0.54ml/0.72ml
3ppm	0ml/1.08ml	0.18ml/1.08ml	0.36ml/1.08ml	0.54ml/1.08ml
4ppm	0ml/1.44ml	0.18ml/1.44ml	0.36ml/1.44ml	0.54ml/1.44ml
5ppm	0ml/1.8ml	0.18ml/1.8ml	0.36ml/1.8ml	0.54ml/1.8ml
6ppm	0ml/2.16ml	0.18ml/2.16ml	0.36ml/2.16ml	0.54ml/2.16ml

Table 4 Effects of BAP and NAA in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	NAA mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0	100±0	0.429±0.012 (B)	0.424±0.0.098(C)
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
0.1	0	0	0	-	
	1.5	33.3±0.0	0	0.350±0.0.035(LB)	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
1.0	0	0	0	-	-
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	66.7±0.0	0	0.082±0.016 (DB)	
	6.0	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 5 Combination of different concentrations of BAP and IAA in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	IAA mg	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0	100±0	0.510±0.035 (B)	0.452±0.083(C)
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
0.1	0	0	0	-	
	1.5	33.3±0	0	0.054±0.009 (B)	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
1.0	0	0	0	-	-
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	66.7±0	0	0.499±0.031 (DB)	
	6.0	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 6 Effects of different concentrations of BAP and 2, 4-D in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	2,4-D mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0	100±0.0	0.481±0.078 (B)	0.462±0.0.046 (C)
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
0.1	0	0	0	-	
	1.5	33.3±0.0	0	0.114±0.027 (B)	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
1.0	0	0	0	-	-
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	63.2±1.8	0	0.231±0.019 (C)	
	6.0	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 7 Shows the effects of appropriate combinations of BAP and IBA in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	IBA mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0.0	100±0	0.444±0.102 (B)	0.520±0.114 (C)
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
1.0	0	0	0	-	
	0.5	0	0	-	-
	1.0	66.7±0.0	0	0.343±0.083 (C)	-
	1.5	0	0	-	-
2.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
3.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
4.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 8 Effects of different concentrations of Benzyl amino purine (BAP) and Indole butyric acid (IBA) in a semi-solid state, callogenesis (%) and weight (colour) of callus of *C. annuum L.*

BAP mgL^{-1}	IBA mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0.0	100±0.0	0.544±0.102 (B)	0.613±0.11 4 (C)
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
1.0	0	0	0	-	
	0.5	30.9±1.3	0	0.213±0.092 (B)	-
	1.0	33.3±0.0	62.9±4.2	0.280±0.045 (C)	0.333±0.10 0 (B)
	1.5	0	0	-	-
2.0	0	0	100±0.0	-	0.563±0.13 2 (C)
	0.5	33.3±0.0	0	0.034±0.008 (B)	-
	1.0	97.7±2.1	0	0.251±0.074 (B)	-
	1.5	33.3±0.0	0	0.232±0.062 (B)	-
3.0	0	0	66.7±0.0	-	0.182±0.07 5 (LB)
	0.5	0	0	-	-
	1.0	0	33.3±0.0	-	0.222±0.07 3 (B)
	1.5	0	0	-	-
4.0	0	0	0	-	-

	0.5	33.3±0.0	0	0.138±0.088 (C)	-
	1.0	33.3±0.0	0	0.031±0.011 (B)	-
	1.5	0	0	-	-
5.0	0	0	0	-	-
	0.5	33.3±0.0	0	0.227±0.052 (B)	-
	1.0	31.7±1.9	0	0.125±0.037 (DB)	-
	1.5	33.3±0.0	66.7±0.0	0.097±0.024 (C)	0.572±0.102 (C)
6.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

DISCUSSION

The most significant characteristic in plant tissue culture is the explant; and until the appropriate choice of explant effective regeneration may not be achieved. **Bhojwani and Dantu (2013)** reported that the most commonly used explant for most micropropagation work is either apical bud or nodal segment. In the present study, regeneration potential of leaf and stem explants was investigated. Auxins like Indoleacetic acid (IAA) and napthaleneacetic acid (NAA) generally stimulate cell expansion, particularly cell elongation; thus promoting adventitious root development. The capacity for NAA to initiate rapid tissue response even in very small amounts has been reported (**Mineo, 1990)**. This auxin is relatively stable in plant tissues, and their hormonal influences last for extensive periods.

The functionality of most phytohormones, whether synthetic or natural, is not to the extent of acting in isolation within the plant body; the hormones rather function in relation to each other (**Mineo, 1990**). This may account for improved weights of calluses, as reported in the study, than when the auxins were used in isolation. **Sharma and Shahzad (2008)** also earlier reported no callus formation or seed germination in MS medium alone, during the induced regeneration from cotyledonary node explant of *Abelmoschus moschatus.* The capacity for NAA and BAP-supplemented MS medium to initiate callogenesis was reported. Although the popularity of use of both indolebutyric acid and naphthaleneacetic acid for propagation of plants from stem and leaf cuttings have been well documented (**Hopkins & Hüner, 2009; Taiz & Zeiger, 2010)**; the supplementation of the culture media with the hormones did not initiate callogenesis in polluted explants, except in the unpolluted. The presentation of differential callus colour as reported in the study is most likely due to differences in type of explant used, the solid state of the medium, as well as possible presence of contamination of explant or medium. **Jin et al. (2014)** reported significant changes in callus colour with increase in age of *Pogostemon cablin* callus, from white to green. Initial contamination of explant is a significant factor that determines the success of callus formation. Such contamination may be of biological origin, for example microbial contamination, or chemical. In the study, plants from which explants were obtained were originally exposed to waste oil pollution, and as such the possibility for contamination from phyto-accumulated oil constituents like heavy metals and polyaromatics suffice as like possible factors for poor callus initiation. This was generally reported in the study. However, in the BAP/IBA-supplement semi-solid medium, significant callogenesis was reported. Generally, the use of semi-solid medium containing different levels of concentrations stimulated further callus proliferation beyond the levels obtained from other treatment. Hormone stability is ostensibly more significant than the total concentration of any single hormone. It is also an important factor in considering the overall effects of hormones on plant growth and morphological changes. This therefore means that the hormone variances in the experimental media states (solid or semi-solid state) ought to generate rather dissimilar effects on the growth and development of excised explants. This may be responsible for the differences in percentage callogenesis and callus weight gain in the BAP/IBA-supplemented media in solid and semi-solid states (**Mineo, 1990**). **Aghaei et al. (2013)** earlier reported significant callus induction of stem explants in *Pistacia atlantica* subsp. Kurdica inoculated in IBA-supplemented medium. Similar reported were provided by **Jin et al. (2014)** in the *in vitro* propagation of *Pogostemon cablin*, and **Caraballo et al. (2010)** *in Agave fourcroydes*. **Agrawal and Chandra (1983); Phillips and Hustenberger (1985); Agrawal *et al.* (1989); Harini and Sita (1993); Christopher and Rajam (1994, 1996); Hyde and Phillips (1996); Hassan *et al.* (1999)** reported that at elevated concentrations of BAP and low levels of auxins, *Capsicum annuum* could be regenerated from explants including hypocotyls and cotyledons. The study also reported significant callogenesis of the 21 day-old stem explants. Contamination rate for this treatment also gave a low result. The effectiveness of use of IBA in culture media has been further buttressed in this study. According to **Sharma and Shahzad (2008)**, however, the inclusion of either (0.05 mg/L) IBA or (0.05 mg/L) NAA in BA-containing media did not improve the regeneration efficiency of *Abelmoschus moschatus.* In many other species similar combinations were advantageous for shoot multiplication as reported in *Macuna pruriens* (**Bretagne et al., 1994**). The magnitude of broad differentiation in tissue culture is subject to the hormonal balance of the support medium as well as the physiological state of the tissue, which usually swings in the direction of the young tissues than older ones **(Mineo, 1990).** Explants from numerous intact plant parts can be used to form callus. However, the most successful explants are often young tissues of one or a few cell types. More so, given the fact that some nutrients like nitrogen, phosphorus and potassium can readily relocate from older to younger leaf, leaving the possibility for deficiency symptoms in older leaves. Young plant tissues possess higher capability for response to *in vitro* culture, compared to older plant materials. This may explain the favourable responses recorded for the young stem explants in this study.

Results of **Debauza and Pena (2001); Peddabonia *et al.* (2006)** on the organogenesis of *C. annuum* confirmed the claim in this study that the standard medium for callogenesis of *Capsicum annuum* is a semi-said state MS medium that is enhanced with BAP and IBA. **Hassan *et al.* (1999); Dabauza and Peña (2001)** however reported MS medium supplemented with silver nitrate to be suitable as an elongation medium, whereas NAA- and IBA – supplemented MS media enhances rooting in sequential experiments. Survival rate of the established calli ranged from 60 – 80%.

Table 9 Contamination rate for explants obtained from control and polluted soils

No. of days after initiation	Leaf explants		Stem explants		Contamination source			
					Leaf explant		Stem explant	
	Control	Polluted	Control	Polluted	Control	Polluted	Control	Polluted
31 days-old explants								
1 – 7	-	+	-	-	-	B,F	-	-
8 – 14	-	++	-	++	-	B,F	-	B,F
15-21	+	+	-	+	B,F	B,F	-	B,F
22-28	+	++	-	+	B,F	B,F	-	B,F
29-35	+	++	++	+++	B,F	F	B,F	F
36-42	+	+++	++	+++	F	F	B,F	F
43-49	++	++++	+++	+++++	F	F	F	F
50-56	+++	++++	++++	+++++	F	F	F	F
28 days-old explants								
1 – 7	-	-	-	++	-	-	-	F
8 – 14	-	++	-	+++	-	B,F	-	F
15-21	-	+	+	++	-	B,F	B,F	F
22-28	-	+	++	+	-	B,F	B,F	F
29-35	-	+	+	++	-	B,F	B,F	B,F
36-42	+	+++	++	++	+	B	B,F	B
43-49	++	+++	++	+++	++	B	B	B
50-56	+++	+++++	++++	+++++	+++	B	B	B
21days-old explants								
1 – 7	-	-	-	-	-	-	-	-
8 – 14	-	++	-	+	-	B	-	+
15-21	+	++	-	+++	+	B	B	B,F

Legend: - No contamination; + not profuse; ++ slightly profuse; +++ profuse; ++++ very profuse; +++++ highly profuse. F majorly fungal infection, B majorly bacterial infection.

CONCLUSION

In this study, an effective procedure for callogenesis of *Capsicum annuum* explants obtained from pepper plants sown in oil-polluted soil had been developed, using BAP - and IBA – supplemented MS media in a semi-solid state. This protocol could be very useful for callus production which could be used in large scale production of this cultivar of pepper from polluted soils over a short period of time; and offers a potential method for the genetic enhancement of the crop. Similarly, the ability of IBA to induce callus was documented. However, a lot of difficulties were observed during the induction of callus, over all the experiments, therefore a further investigation is needed to overcome these problems.

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IN VITRO EFFECTS OF THE *CHLAMYDOMONAS REINHARDTII* EXTRACT ON BOVINE SPERMATOZOA

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ABSTRACT

In vitro storage and processing of animal semen is represents a risk factor to spermatozoa vitality, potentially leading to reduced fertility. A variety of substances isolated from natural sources may exhibit protective or antioxidant properties on the spermatozoon, thus extending the lifespan of stored ejaculates. *Chlamydomonas* has been shown to possess antimicrobial, anti-inflammatory and antioxidant properties, turning the extract into a potential candidate for preserving liquid animal semen during in vitro storage. This study compared the ability of different concentrations of the *Chlamydomonas reinhardtii* extract on the motility, viability and superoxide production of bovine spermatozoa during different time periods (0, 2, 6, 12 and 24h) of *in vitro* culture. Spermatozoa motility was assessed using the SpermVision™ CASA (Computer aided sperm analysis) system. Cell viability was examined using the metabolic activity MTT assay and the nitroblue-tetrazolium (NBT) test was applied to quantify the intracellular superoxide formation. The CASA analysis revealed that the *Chlamydomonas* extract supplementation was able to prevent a rapid decline of spermatozoa motility, especially in the case of concentrations ranging between 1 and 5 µg/mL ($P<0.001$ with respect to Times 6h, 12h and 24h). At the same time, concentrations ranging between 5 and 10 µg/mL of the extract led to a significant preservation of the cell viability throughout short-term ($P<0.05$ in case of Time 6h) as well as long-term periods of the experiment ($P<0.01$ with respect to Time 12h, and $P<0.001$ in case of Time 24h). 5 and 10 µg/mL of the extract exhibited antioxidant characteristics, translated into a significant reduction of the intracellular superoxide production, particularly notable at Times 12h ($P<0.01$ with respect to10 µg/mL and $P<0.05$ in case of 5 µg/mL) and 24h ($P<0.01$). The results indicate that the *Chlamydomonas* extract is capable of delaying the damage inflicted to the spermatozoon by the *in vitro* environment.

Keywords: Chlamydomonas, spermatozoa, motility, viability, superoxide production

INTRODUCTION

Over the last decade, diverse studies have reported about the beneficial effects of oral supplementation of energetic or antioxidant substances on semen quality and male fertility in animals and humans (**Donnely *et al.*, 1999**; **Agarwal and Sekhon, 2010**). Nevertheless, knowledge concerned with the *in vitro* effects of stimulating or protective molecules on the spermatozoon is still substantially limited or controversial. Meanwhile, in vitro data are crucial for further progress in practical andrology, as it has been systematically shown that diverse biologically active compounds may protect the spermatozoon against the loss of motility or viability. Subsequently, this information may be viable for spermatozoa handling protocols in medical and veterinary laboratories for long-term semen preservation (cryoconservation) or artificial insemination.

The *in vitro* environment represents a hazard to the sperm survival, as it provides suitable conditions for ROS (reactive oxygen species) overproduction and a subsequent structural and/or functional damage to the cell (**Saleh and Agarwal, 2002**). Administration of synthetic supplements to cell cultures is an effective way to prevent structural or functional alterations to spermatozoa. However, the safety of synthetic additives has been questioned leading to the renaissance of naturally occurring substances with numerous beneficial properties. The chemical diversity, structural complexity, availability or lack of substantial toxic effects of natural products converts them into ideal candidates for new therapeutic strategies (**Alarcón de la Rastra, 2008**).

Algae, such as *Chlamydomonas*, *Chlorella* or *Gelidiella* are a rich source of novel biologically active metabolites with various application in pharmaceutical industries. These are a pool of antioxidants such as carotenoids, astaxanthin, phenol and flavonoid derivatives. Many research studies suggest that the biological composition of microalgae including proteins, carbohydrates, minerals and bioactive compounds has a potential medical value (**Fuentes *et al.*, 2000; Kightlinger *et al.*, 2014**). Correspondingly to the present urge to discover novel and effective biologically active agents, algal derived compounds have a broad range of antibiotic, antiviral, antioxidant, antifouling, anti-inflammatory, cytotoxic and antimitotic activities which could be explored further (**Salvador, 2007**). Bioactive compounds such as polyphenols, catechin, flavonols, glycosides, and phlorotannins discovered from methanol extract of red, green and brown algae have been reported to have a uniqueness in their molecular skeleton and structures contributing to the strong antioxidant activity (**Khoddami *et al.*, 2013**).

Based on this body of evidence, this *in vitro* study was aimed to assess the efficacy of the *Chlamydomonas reinhardtii* extract on bovine spermatozoa motility, viability and superoxide radical formation during a 24 hour *in vitro* cultivation, in order to provide information on its behavior in the male reproductive cell, as well as to define optimal concentrations of this extract for further experiments in veterinary andrology.

MATERIAL AND METHODS

Chlamydomonas reinhardtii was grown aseptically on an agar medium (1/2 Murashige- Skoog, 10 % sucrose, 0.6 % plant agar, pH 5.2) in 225 mL plastic boxes and growth chamber under a 16/8 day/night period at 22°C (**Michalko and Matušíková, 2012**). After collection and drying, the algal tissues were crushed, weighed and soaked in ethanol p.a. (96 %, Sigma-Aldrich, St. Louis, USA) during two weeks at room temperature in the dark. Exposure to sunlight was avoided to prevent the degradation of active components. The ethanolic algal extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove ethanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vaccum pump KNF N838.1.2KT.45.18, KNF, Germany). Crude extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA)

to equal 100.4 µg/mL as a stock solution (**Michalko and Matušíková, 2012**; **Tvrdá *et al.*, 2015**).

Bovine semen samples were obtained from 10 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic) on a regular collection schedule using an artificial vagina. The ejaculates had to accomplish the basic criteria given for the corresponding breed. After collecting the samples were stored in the laboratory at room temperature (22–25°C). Each sample was diluted in physiological saline solution (PS; sodium chloride 0.9 % w/v; Bieffe Medital, Italy) containing various concentrations of the *Chlamydomonas* extact (A – 50; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 µg/mL) using a dilution ratio of 1:40. The samples were cultured at room temperature (22-25°C). We compared the control (Ctrl) group (medium without *Chlamydomonas* supplementation, containing 0.5% DMSO) with the experimental groups.

Spermatozoa motility (percentage of spermatozoa with a motility >5 µm/s; %; MOT) was examined with the help of the Computer-aided sperm analysis (CASA) system using the SpermVision^{TM} program (Minitube, Tiefenbach, Germany) and Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed into the Makler Counting Chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. At least 1000 cells were evaluated in each sample (**Massanyi *et al.*, 200**8).

Viability of the cells exposed to *Chlamydomonas in vitro* was evaluated by the metabolic activity (MTT) assay. This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan can then be measured spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, Thermo Fisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed to *Chlamydomonas*). Results from the analysis were collected during five repeated experiments at each concentration (**Knazicka et al., 2012**).

The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of superoxide radical. This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium chloride (2,2′-bis(4-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy- 4,4′-diphenylene)ditetrazolium chloride; Sigma-Aldrich,) and superoxide radical. Formazan can be measured spectrophotometrically at a measuring wavelength of 620 nm against 570 nm as reference by a microplate ELISA reader (Multiskan FC). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to *Chlamydomonas*). Results from the analysis were collected during five repeated experiments at each concentration (**Tvrdá et al., 2013**).

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. One-way ANOVA with Dunnett's post test was used for statistical evaluations. The level of significance was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

RESULTS AND DISCUSSION

Over the past years, algae have emerged exhibiting a complex biological activity. Due to their broad range of effects, particularly with respect to antibacterial, anti-inflamatory protection and antioxidant mechanisms, *Chlamydomonas*, *Chlorella* or *Gelidiella* have attracted a widespread scientific and consumer interest (**Fuentes *et al.*, 2000**; **Annamalai and Nallamuth, 2014**; **Kightlinger *et al.*, 2014**). Different studies have reported that *Chlamydomonas* extracts are well absorbed and rapidly metabolized, while being well tolerated and no distinct toxicity was reported (**Annamalai and Nallamuth, 2014**; **Kightlinger *et al.*, 2014**; **Sobhani *et al.*, 2015**).

The CASA assessment showed a continuous decrease of spermatozoa motility in all groups over the course of a 24h *in vitro* culture (Table 1). The initial (Time 0h) MOT was higher in the experimental groups B-D (0.5-5 µg/mL *Chlamydomonas* extract) when compared to the control group (0 µg/mL *Chlamydomonas* extract), although without any statistical significance (P>0.05). A statistically significant motion-promoting effect of the *Chlamydomonas* extract became visible after 2h, specifically in the group C (P<0.05). Moreover, 50 µg/mL *Chlamydomonas* extract (group A) caused a non-significant decrease of the spermatozoa motility (P>0.05). After 6h, the decline of spermatozoa MOT became significant in the group A (P<0.001) in comparison with the control, while we observed a significantly higher spermatozoa motion in the experimental groups C and D (P<0.001). Examination at 12h of *in vitro* culture showed that the spermatozoa motility was significantly increased in groups C and D (P<0.001) when compared to the control. At the same time, a significantly decreased motion was detected in the group A (P<0.001) in comparison to the control. At the end of the experiment (24h), the motility observed in the experimental groups supplemented with 0.1-10 µg/mL *Chlamydomonas* extract (experimental groups B-F) was significantly higher in comparison with the control (P<0.05 in case of group B; P<0.001 with respect to groups C-E; P<0.01 in relation to the group F). Meanwhile, MOT was significantly decreased in the group A (P<0.001), supplemented with the highest concentration of the *Chlamydomonas* extract (50 µg/mL) after a comparison with the Ctrl group (Table 1).

Table 1 Spermatozoa motility (%) in the absence (Ctrl) or presence (A-F) of the *Chlamydomonas reinhardtii* extract during different time periods (Mean±SEM; n=10)

	Ctrl	A	B	C	D	E	F
0h	90.11±2.17	83.02±1.19	90.90±1.17	90.20±1.44	91.06±1.29	88.25±1.98	89.53±2.15
2h	82.48±3.15	75.40±1.46	86.64±1.58	91.91±1.79*	87.23±1.41	84.15±1.60	83.01±1.13
6h	62.61±1.14	38.65±3.73***	68.01±1.32	83.92±1.52***	80.55±1.66***	66.52±1.98	64.46±2.12
12h	53.08±3.04	21.88±2.07***	55.80±2.37	73.92±2.11***	71.88±2.22***	56.73±3.14	54.57±1.54
24h	41.15±1.81	10.45±1.67***	49.82±2.61*	66.99±3.24***	65.34±2.23***	57.12±2.12***	52.03±2.27**

*** (P<0.001); ** (P<0.01); * (P<0.05). Ctrl – 0; A – 50; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 µg/mL *Chlamydomonas reinhardtii* extract

According to the MTT assay, instant *Chlamydomonas* supplementation (Time 0h and 2h) had no significant effects on the sperm cell viability in any of the experimental groups (P>0.05; Figure 1).

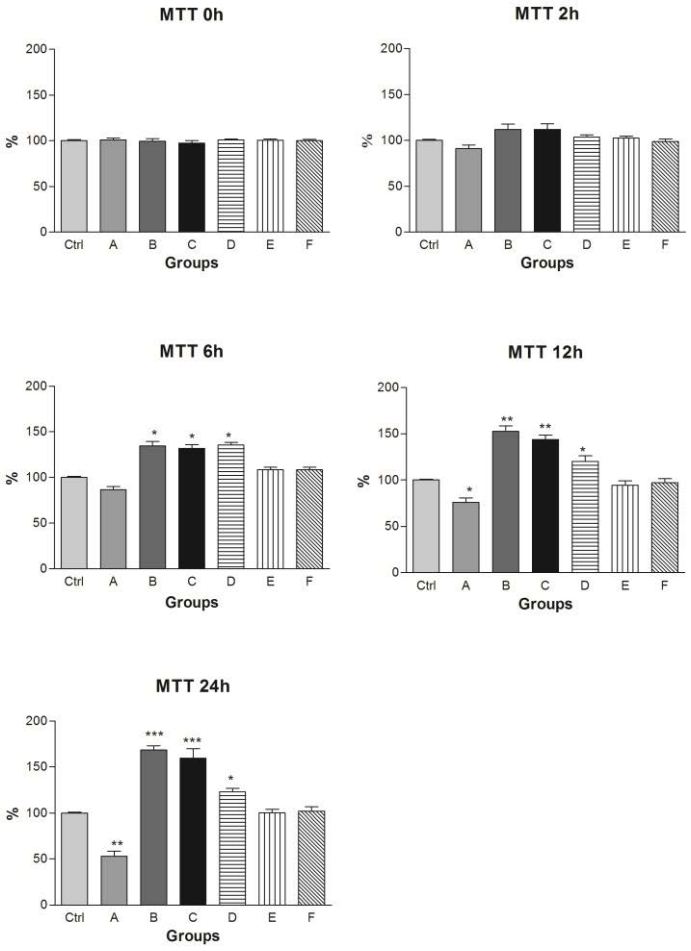

Figure 1 The effect of various concentrations of the *Chlamydomonas reinhardtii* extract on the viability of bovine spermatozoa (n=10) at 0h, 2h, 6h, 12h and 24h. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolize 100%. The data were obtained from five independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05. Ctrl – 0; A – 50; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 µg/mL *Chlamydomonas reinhardtii* extract.

At 6h it was revealed that 1-10 µg/mL extract (groups B-D) had a viability promoting effect on the bovine spermatozoon, alongside with statistically significant results (P<0.05) when compared to the control group (Figure 1). These stimulating effects remained visible and statistically relevant throughout time periods of 12h (P<0.01 in relation to groups B and C; P<0.05 with respect to group D), as well as 24h (P<0.05 in case of group D; P<0.001 with respect to groups B and C). Similar to the CASA analysis, the MTT test revealed an inhibition of the cell viability followed by the administration of 50 µg/mL *Chlamydomonas* extract (group A), particularly during long-term timeframes of the in vitro culture (P<0.05 with respect to Time 12h; P<0.01 with regards to Time 24h).

It has been previously stated that *Chlamydomonas* contains a variety of flavonoids, such as isoflavones, flavanones, flavonols and dihydrochalcon (**Khoddami *et al.*, 2013**), all of which have been extensively studied for their potential roles on spermatogenesis or in vitro sperm survival. Improved spermatozoa motility and mitochondrial activity after flavonoid administration was recorded in different studies on fresh as well as frozen goat, mouse and human semen (**Purdy *et al.*, 2004**; **Mazzi *et al.*, 2011**; **Tung *et al.*, 2014**). Furthermore, **Ibrahim *et al.* (2014)** have shown that flavonoids isolated from diverse natural sources possess a protective effect against DNA damage in murine sperm. At the same time, a sulfono glycolipid (S-ACT-1) isolated from *Gelidiella acerosa* has shown to possess a potent human sperm motility stimulating activity *in vitro* with the potential to be developed into a sperm stimulant (**Premakumara *et al.*, 2001**). The analysis of sperm parameters in the study of **Sobhani *et al.* (2015)** demonstrated that the general and advanced motility of frozen-thawn human spermatozoa significantly increased following incubation with the extract of the *Sargassum* brown algae. On the other hand, twelve seaweeds were screened for *in vitro* spermicidal activity in the report by **Prakash *et al.* (2014)**. Among these twelve seaweeds, *Halimeda gracilis* showed 100% inhibition of human spermatozoa at 10 µg/ml in 20 s. Furthermore, dose- and time-dependent spermicidal assay revealed that the sperm was completely immobilised for 20 s. Plasma membrane of sperm was damaged due to the exposure of *H. gracilis* extract. MTT assay with *H. gracilis* extract showed 88.5% of cytotoxic incidence.

Although the *Chlamydomonas* extract had no instant effects on the oxidative balance within the *in vitro* spermatozoa culture (P>0.05; Time 0h and 2h; Figure 2), experiments following a 6h cultivation revealed that the administration of 5 and 10 µg/mL extract led to a significant decline of the superoxide formation in comparison to the control (P<0.05). *Chlamydomonas* extract concentrations ranging from 1 to 10 µg/mL (groups B-D) exhibited a long-term and statistically significant antioxidant protection of spermatozoa and a subsequent prevention of the escalating intracellular superoxide production, considered to be the first step in the generation of oxidative stress (P<0.01 in case of 10 µg/mL, and P<0.05 with respect to 5 µg/mL at Time 12h; P<0.01 in terms of 5-10 µg/mL, P<0.05 with respect to 1 µg/mL at Time 24h). On the other hand, high (group A) concentrations of *Chlamydomonas* exhibited pro-oxidant properties reflected in a significant superoxide overgeneration, staring at Time 12 (P<0.05) and deepening the detrimental effects in a time-dependent manner (P<0.01 with respect to Time 24h; Figure 2). Numerous studies have emphasized on the fact that algae possess significant antioxidant activities (**Annamalai and Nallamuth, 2014**; **Kightlinger *et al.*, 2014**). The antioxidant ability could be attributed to the exceptionally high content of phenolic compounds, particularly flavonoids with potent ROS-scavenging activities (**Anandakumar *et al.*, 2009**). Thus, *Chlamydomonas* extracts could be a promising natural source of antioxidants, possibly used in nutritional or pharmaceutical industry for the prevention of ROS-mediated diseases. Our NBT results complement the report by **Sobhani *et al.* (2015)** showing that following in vitro administration of 250 or 500 pg/ml of *Sargassum* extract the level of ROS notably declined in frozen-thawn human semen. Phenolic compounds have been repeatedly shown to have beneficial effects of the oxidative balance in male reproductive tissues and cells.

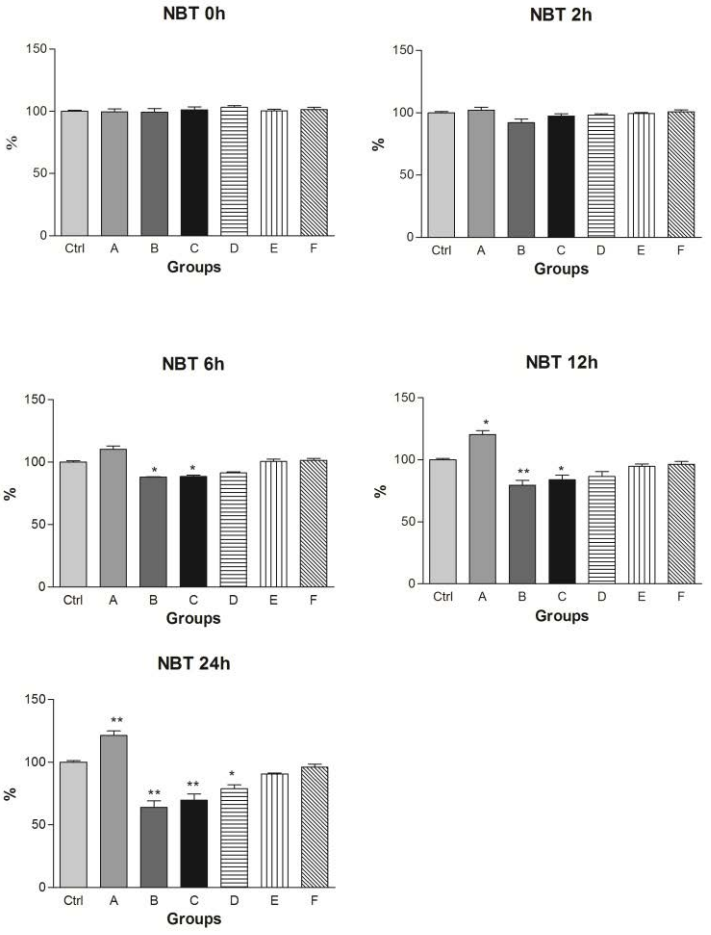

Figure 2 The effect of various concentrations of the *Chlamydomonas reinhardtii* extract on the spermatozoa (n=10) superoxide production at 0h, 2h, 6h 12h and 24h. Each bar represents the mean (±SEM) optical density as the percentage of controls, which symbolize 100 %. The data were obtained from five independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05. Ctrl – 0; A – 50; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 µg/mL *Chlamydomonas extract*.

As shown by **Ateşşahin *et al.* (2010)** biologically active compounds frequently found in *Chlamydomonas* were able to significantly decrease lipid peroxidation, restore glutathione synthesis and catalase activity, associated with normal spermatogenesis and sperm viability. In a different study (**Çeribaş *et al.*, 2012**),

polyphenol administration led to significantly increased total antioxidant capacity, superoxide dismutase levels, as well as sperm percentage, viability, motility, accompanied by a decrease of malondialdehyde in rats, hence suggesting that flavonoids could be effective in enhancing healthy semen parameters.

CONCLUSION

Our results, although preliminary, support the evidence for the dose-dependent *in vitro* biological activity and scavenger potential of the *Chlamydomonas reinhardtii* extract against oxidative stress induced in bovine spermatozoa. The development of new culture media offering a better protection to spermatozoa from the oxidative damage and improve their energy requirements is absolutely necessary. *Chlamydomonas* extracts, in small amounts, could be used as a ROS scavenging and a metabolic promoting supplement, especially in routine andrology techniques such as in vitro fertilization, artificial insemination or spermatozoa cryopreservation. These results obviously cannot foresee a definitive *in vivo* outcome, since a direct impact of *Chlamydomonas* extract supplementation on male subfertility needs to be explored further. To translate our findings into routine practice, studies on the toxicity, pharmacokinetics and bioavailability of *Chlamydomonas* extracts in male reproduction are critical.

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MICROSATELLITE DNA MARKER FOR MOLECULAR CHARACTERIZATION OF AFRICAN MAIZE (*Zea mays* L.) LANDRACES

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ABSTRACT

Maize (*Zea mays* L) originated from Mexico but has been introduced and domesticated in various parts of the world. Maize is an important cereal crop having subsistent, commercial and industrial uses. The aim of this study was to characterize genetic diversity in some African maize landraces using microsatellite DNA markers. Maize landraces were collected from some parts of Edo State, Nigeria, while others were sourced from IITA (International Institute of Tropical Agriculture), Ibadan, Nigeria and Premier Seed Company, Nigeria. Fourteen populations of 15 plants each were characterized, the application of 7 microsatellite markers sufficiently provided information on genetic diversity of all 14 populations investigated. The study revealed a total number of 21 alleles across all loci, with a mean number of 3 alleles per locus. Polymorphic information content (PIC) ranged from 0.26 (umc1161) to 0.71 (umc1196), with a mean of 0.52, indicating that markers used were polymorphic. The dendrogram displayed two main clusters; cluster 1 had most populations from Nigeria grouped together with populations from Malawi and Togo, while populations from Guinea, Chad, Tzm-ese, Tzm-enee and Tzm-1340 from Kano, Nigeria grouped together in cluster 2. Population Tzm-1413 from Somalia in East Africa was a complete outlier, as revealed by the dendrogram. Results suggest that the populations studied were not greatly diverse but can be used to establish a field trial, where germplasm will be scored based on adaptation, tolerance and resistance to biotic and abiotic factors, which will furthermore validate the genetic variability revealed by the microsatellite markers.

Keywords: Maize, landraces, SSR, microsatellite, characterization

INTRODUCTION

Maize (*Zea mays* L.) is a cereal crop of the Poaceae family widely cultivated in most parts of the world due to its adaptability and productivity **(Gerpacio & Pingali, 2007)**. Maize introduction from the centre of origin in Mexico into different growing conditions in the tropical, sub-tropical, and temperate regions has led to the advent of hundreds of diverse landraces **(Dubreuil *et al*., 2006)**.
Maize is the most important cereal crop in Sub-Saharan Africa (SSA) and an important staple food for more than 1.2 billion people in SSA and Latin America **(IITA, 2015)**. Landraces are heterogeneous populations which are genetically diverse and are typically selected by farmers for their adaptability potentials **(Prasanna & Sharma, 2005)**. They are important genotypes for crop breeding owing to their ability to adapt to specific environmental conditions and the large source of genetic variability that they provide **(Paterniani *et al*., 2000)**. Maize landraces are valuable sources of genetic variability and have been intensively used in breeding programs **(Udry & Duarte, 2000)**.
Research developing new biotechnological techniques has provided increased support to assess genetic diversity at both phenotypic and the genotypic levels **(Sajib *et al*., 2012)**. The advancement in the use of molecular markers has proved to be valuable for genetic diversity analysis at DNA level in plant species **(Melchinger & Gumber, 1998)**. Unlike morphological markers, molecular markers allow great number of lines to be characterized; hence, increasing the efficiency of maize breeding programs **(Choukan *et al*., 2006)**.
Microsatellites are di-, tri- or tetra nucleotide tandem repeats in DNA sequences. These are also known as SSRs (simple sequence repeats), STRs (short tandem repeats), STMs (sequence tagged microsatellites) and VNTRs (variable number of tandem repeats). SSRs are co-dominant; highly polymorphic and specific **(Jones *et al.*, 1997)**; little DNA is required; very repeatable; so cheap and easy to run; need a small amount of medium quality DNA; the analysis can be semi-automated and performed without the need of radioactivity **(Guilford *et al.*, 1997)**, and are highly transferable between populations **(Gupta *et al*., 1999)**. They have been used in mapping the genome of corn **(Taramino & Tingey, 1996)**, genetic fingerprinting **(Senior *et al*., 1998)**, and to characterize landraces **(Aci *et al.*, 2013)**.
Characterization of genetically heterogeneous populations using molecular markers has before now been very expensive and time consuming **(Prasanna, 2012)**. A method for microsatellite analysis of pools of individuals from a population has proven to be cost effective than genotyping multiple individuals per population, and much more accurate than genotyping only one individual per population **(Dubreuil *et al*., 2006)**. **Liu *et al.* (2005)** studied sampling method with SSR markers and showed that bulk DNA from 15 individuals could help to assess genetic diversity of maize accessions.
Studies using bulking method and SSR markers are more efficient ways to study open pollinated varieties and populations of maize, and they have allowed the elucidation of the origin of European landraces **(Rebourg *et al.*, 2001, 2003)**, genetic diversity of Southwest China landraces **(Yao *et al*., 2007)**, relationship between Latin American landraces **(Warburton *et al.*, 2008)**, population genetic and diversity analysis of Indian landraces **(Wasala & Prasanna, 2012)**, genetic characterization of Ghanaian landraces **(Oppong *et al.*, 2014)**. Moreover, research work on measuring genetic diversity and characterizations of maize landraces of African countries are few, hence this study.
The objective of this study was to characterize genetic diversity in some African maize landraces using microsatellite DNA markers.

MATERIALS AND METHODS

Plant materials

Landraces were collected from parts of Edo State (Egor, Ehor, Ekpoma, Uromi, Sobe and Okpella), and others were sourced from IITA (International Institute of Tropical Agriculture), Ibadan, Nigeria. An OP (open-pollinated) variety (Suwan-1SR) and a hybrid variety (Oba 98) from Premier Seed Company, Zaria, Nigeria were included as controls (Table 1).

Table 1 Accessions of maize landraces used in this present study

Population	Collection Country	State/Province	Remarks
Tzm-Esu	Nigeria	Edo	White grain
Tzm-Ese	Nigeria	Edo	White grain
Tzm-Ecew	Nigeria	Edo	white/ yellow grain
Tzm-Ecene	Nigeria	Edo	White grain
Tzm-Enow	Nigeria	Edo	White small grain
Tzm-Enee	Nigeria	Edo	Yellow grain
Tzm-307	Chad	Mayo, Kebbi	White small grain
Tzm-1340	Nigeria	Kano	Reddish-black grain
Tzm-1097	Malawi		White grain
Tzm-1413	Somalia	Lower Shebelle	White/yellow/purple grain
Tzm-1276	Togo		White grain
Tzm-1545	Guinea	Baguiherd	Yellow grain

Two hundred and ten individuals were used for this study; these samples were bulked subset from 14 main populations (Table 1). Fifteen individuals were randomly selected per population for bulking. These samples were planted in a 1kg plastic bag filled with sieved topsoil in the screen house of the Department of Soil Science, Faculty of Agriculture, University of Benin, Edo State, Nigeria for young leaves to emerge for DNA extraction.

DNA extraction

Fresh young leaves 200mg (0.2g) were harvested from 15 individuals per population. Bulk samples were prepared by adding the same amount of leaf materials excised from fifteen samples in a population to form a composite sample **(Wasala & Prasanna, 2012)**. DNA was extracted from the bulked fresh leaf samples using Dellaporta method with little modification **(Dellaporta *et al*., 1983)**. DNA quality was checked by DNA quantification using a Nanodrop Spectrophotometer (Thermo scientific, ND 1000 Spectrophotometer).

Microsatellite analysis

SSR primer pairs provided by the International Institute of Tropical Agriculture were screened with the maize DNA sample using TD (touch down) SSR-1 protocol in a Thermocycler. PCR (Polymerase chain reaction) cocktail mix was prepared in 10 µl tube containing reaction mixtures, 1 µl of 10X buffer, 0.4µl of 50Mm $MgCl_2$, 0.8 µl of 2.5ml dNTPs, 0.8 µl of DMSO, 0.5 µl of forward primer, 0.5µl of reverse primer, 0.1 µl of Taq, 2.9µl of ddH_2O, 3.0 µl of diluted DNA. The amplification conditions with the touchdown thermal cycling protocol were 95°C for 1 min, 30 cycles at 94°C for 1 min, 67°C annealing temperature decreasing 0.4°C per cycle for 2 min, 72°C for 2 min, and a terminal extension step at 72°C for 1 hour.
PCR products were electrophoresed, separated on 1% Agarose gel and visualized after staining with Ethidium Bromide using Uvitech gel documentation system (ENDURO^{TM} GDS, Labnet International, Inc.).

Data analysis

Each individual band was considered as a single loci/allele. Allele/loci (bands) were scored as present (1) or absent (0). Genetic analysis was conducted based on the scoring. Average number of alleles (n_a), number of effective alleles (n_e), expected heterozygosity (H_e), Shannon's index (I), the dendrogram was constructed by the Unweighted pair-group method with arithmetic averages (UPGMA) showing distance based inter relationship among samples using Nei's distance matrix (**Nei, 1972**). All parameters were computed and analyzed using Population Genetic Analysis Software (POPGENE Version 1.31) (**Yeh *et al*., 1999**). Polymorphic information content was calculated as:

$$PIC = 1 - \sum_{i=1}^{n} Pi^2$$

Where P = allele frequency; n = number of alleles of the jth marker and i = the ith allele of the jth marker.

RESULTS AND DISCUSSION

Seven SSRs used for this study are distributed on 5 maize chromosomes (Table 2). A total of 21 alleles were observed across all SSR loci used for the study, the number of alleles per locus varied from 2 (umc1161) to 4 (umc1196), with a mean of 3 alleles per SSR locus (Table 2). This was lower than the results reported by **Aci *et al*. (2013)** who observed a total of 87 alleles and a mean of 5.8 alleles across 18 loci on 15 Algerian accessions; **Oppong *et al*. (2014)** who detected a total of 145 alleles and a mean of 7.3 SSR alleles per locus (across 20 loci) in a study of over 500 Ghanaian maize landraces (bulked DNA), The differences in the number of alleles recorded in this study compared to other works could be due to the size of the sample studied and probably, the fewer number of loci analyzed.

Table 2 Summary statistic of the markers used in the study

Marker	Bin	N_a	N_e	H_e	I	PIC
Phi96100	2.01	3	1.98	0.52	0.85	0.49
Phi093	4.08	3	3	0.71	1.10	0.67
Phi087	5.06	3	2.52	0.63	1.01	0.43
Umc1161	8.06	2	1.35	0.27	0.43	0.26
Phi059	10.02	3	2.97	0.69	1.09	0.67
Phi084	10.04	3	1.78	0.46	0.76	0.44
Umc1196	10.07	4	3.43	0.74	1.31	0.71
Mean		3	2.43	0.57	0.94	0.52

N_a = Observed number of alleles; N_e = Effective number of alleles; H_e = Expected heterozygosity
I = Shannon's Information index; PIC = Polymorphic information content

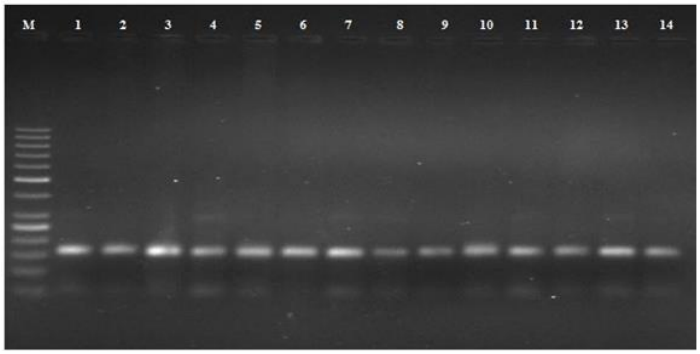

Figure 1 SSR bands for Phi084 viewed under UV light. M – 50bp DNA ladder, 1 – Tzm-esu, 2 – Tzm-ese, 3 – Tzm-ecew, 4 – Tzm-ecene, 5 – Tzm-enow, 6 – Tzm-307, 8 – Tzm-1340, 9 – Tzm-1097, 10 – Tzm-1413, 11 – Tzm-1276, 12 – Tzm-1545, 13 – Swam-1SR, 14 – Oba98

Number of effective alleles (N_e) is a function of the proportion of polymorphic loci, the number of alleles per locus, and the evenness of the allelic frequencies, thus it is a measure of the genetic information in a population or species **(Wasala & Prasanna, 2012)**. Number of effective alleles (N_e) for this study ranged from 1.35 (umc1161) to 3.43 (umc1196), with a mean of 2.43 across the loci analyzed (Table 2).The mean effective number of alleles across genotypes analyzed was lower than that recorded by **Wasala & Prasanna (2012)** and **Yao *et al*. (2007)**, of 3.85 and 3.90 respectively on 42 SSR loci each, which may be due to the number of SSR loci used as 7 SSR loci were deployed for this study. The Shannon–Weaver/wiener index is a measure of heterogeneity that include evenness and richness of species **(Hollenbeck and Ripple, 2007)**. A mean value of 0.94 for Shannon information index was detected in this study (Table 2); this was higher than the mean of 0.52 reported by **Salami *et al*. (2016)**.
H_e averaged 0.57 and ranged from 0.27 for umc1161 to 0.74 for umc1196 (Table 2). Heterozygosity mean value of 0.69 reported by **Yao *et al*. (2007)** on 42 SSR loci was higher than the mean of 0.57 recorded in this present study. **Aci *et al*. (2013)** reported a mean of 0.57; this is consistent with the heterozygosity mean obtained in this study. The genetic diversity mean of 0.46 reported by **Salami *et al*. (2016)** across 3 loci on 185 Benin landraces was lower than that observed in this study. All the SSR loci were able to detect genetic diversity which is defined as the probability that 2 randomly chosen alleles from the population are different among the maize genotypes **(Liu & Muse, 2005)**.
PIC values of each marker varied for all SSR loci, from 0.26 to 0.71, with a mean of 0.52 (Table 2). The highest mean value of 0.71 was obtained from umc1196, and the lowest value was detected in umc1161 (0.26). PIC of the SSRs was relatively high with a mean of 0.52. Similar PIC mean have been recorded by other researchers in genetic diversity and characterization studies of *Zea mays* L. **Banisetti *et al*. (2012)** reported an average PIC value of 0.49 on 22 maize genotypes, this is comparable to PIC average detected in this study. PIC average of this study indicate that markers were polymorphic and informative.
The trend across all loci studied showed marker umc1196 having the highest value across all loci for number of alleles, number of effective alleles, Shannon's information index, PIC, and heterozygosity, while umc1161 had the lowest values. **Oppong *et al.* (2014)** recorded umc1196 also having higher values than umc1161, this is an indication that although both markers are polymorphic, Umc1196 is more polymorphic compared to Umc1161.
Dendrogram displayed that the genotypes from Edo state, Nigeria fell into the same cluster (cluster 1). Genotypes from the Central part of Edo state, Nigeria (Tzm-ecew and Tzm-ecene) were more related than other genotypes in the cluster as expected. Tzm-1276 from Togo, and Suwan-1SR an open pollinated variety from Nigeria were also grouped in cluster 1, Tzm-1097 from Malawi grouped within the sub clusters of cluster 1 and displayed genetic similarity with populations in cluster 1 and 2 (Figure 2). Tzm-ese from the Southern part of Edo state and Tzm-enee from the Northern part of the state grouped together in cluster 2 displaying no genetic difference, this could be because most farmers tend to introduce their superior local genotypes to their peers during visit or farmers association meetings. Tzm-307 from Chad and Oba 98 a hybrid variety also

displayed no genetic difference as they were genetically similar based on the dendrogram. Nevertheless, it is imperative to state that 7 SSR loci might be insufficient in distinguishing the populations sufficiently.

Tzm-1413 from Somalia was a complete outlier. The outlier (Tzm-1413) as revealed by the UPGMA-based dendrogram (Figure 2) being from East Africa, a different agro-ecological zone could be the reason while they were more distantly related to all other genotypes, which were basically from West Africa.

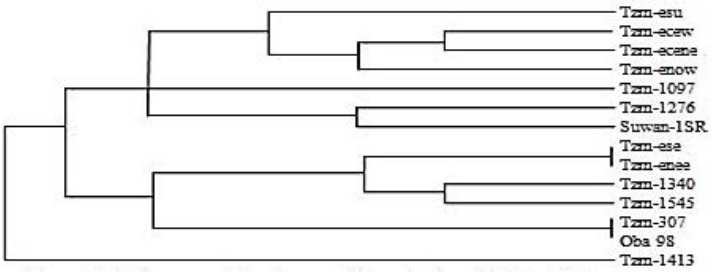

Figure 2 Dendrogram of 14 maize population based on data from SSR markers

CONCLUSION

This research has shown that the populations studied were not greatly diverse, though some of the landraces analyzed were from distinct areas where they have acclimatized; Maize remains a universal crop that possess similar traits but respond to domestication mostly based on ecology.

The result of this study will be useful to guide an oriented breeding program aimed at the improvement of populations studied. This result can thus be used to establish a field trial, where germplasm will be scored based on adaptation, tolerance and resistance to biotic and abiotic factors, which will furthermore validate the genetic divergence and similarity revealed by the microsatellite markers. Phenotypic characterization/morphological marker analysis is recommended to demonstrate the phenotypic variation (genotype + environment) of the maize germplasm studied. Genetic characterization of more numbers of maize landraces from divergent zones using high-throughput marker platforms such as SNP platforms is also recommended for a better precision.

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INFLUENCE OF GENTAMICIN ON THE SPECIFIC CELL CULTURE (BHK-21) *IN VITRO*

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ABSTRACT

Gentamicin (GENT) is an aminoglycoside antibiotic commonly used against Gram-negative bacterial infections. GENT is probably the most commonly used antibiotic of all aminoglycosides. The aim of our study was to evaluate the *in vitro* toxicity of different concentrations of GENT on selected mammalian cell culture (BHK-21 – baby hamster kidney cells). After application of various concentrations of GENT, we controlled the condition of cells in the wells microscopically (magnification x 400). Based on the structure of cells, we evaluated the presence of vital, subvital and dead cells. Cell medium was used for biochemical analyses (Calcium - Ca, Magnesium – Mg, total proteins - TP, Sodium - Na, Potassium - K and Chloride – Cl). Viability of the cells exposed to selected antibiotic *in vitro* was evaluated using the metabolic activity (MTT) assay. BHK-21 cells were able to survive at a concentration 187.5; 500; 1500; 4500 µg/mL. We found statistically significant decrease ($P<0.001$) of vital cells in comparison with control in all concentrations of GENT higher than 500 µg/mL. We also found significant increase in the number of subvital and death cells compared to control group in all concentrations of GENT higher than 500 µg/mL. Biochemical parameters observed in the medium were significantly affected in all concentrations of GENT. Content of Na^+ and Cl^- was the most importantly affected in all observed groups against control group ($P<0.001$). A statistically significant decrease of Ca ($P<0.01$) was detected (control vs 937.5 µg/mL resp. 7500 µg/mL of GENT). The mitochondrial activity of the BHK21 cells was significantly ($P<0.001$) decreased after the administration of all concentrations of GENT when compared to the Control. In conclusion, the exposure of Baby Hamster Kidney fibroblasts (BHK-21) to gentamicin at our concentrations resulted in severe cell damage. Acquired knowledge is possible to apply in toxicity evaluation of pharmacological effective substances in vitro.

Keywords: Gentamicin, BHK21, cell morphology, biochemistry, mitochondrial activity

INTRODUCTION

Fifty years of experience with aminoglycoside antibiotics has confirmed their usefulness in many infections with gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp., *Klebsiella* spp., *Serratia* spp., *Morganella* spp., and *Pseudomonas* spp. as well as *Staphylococcus aureus* and some streptococci (**Vakulenko and Mobashery, 2003**). The increased knowledge about molecular structure, pharmacology and pharmacokinetics has resulted in reduced risks for severe toxic damage in kidneys (nephrotoxicity) (**Mingeot-Leclercq and Tulkens, 1999; Khan *et al.*, 2009; Com *et al.*, 2012**) and in the ear (ototoxicity) (**Wersäll, 1995; Garetz and Schacht, 1996; Forge and Schacht, 2000; Selimoglu, 2007; O´neil, 2008**). Despite their toxicity, aminoglycosides play an increasingly important role in the management of serious infections. Their toxicity has led to comparatively restrained usage, but they remained effective against many pathogens. Aminoglycosides are valuable drugs for symptomatic treatment of gram-negative sepsis, for the management of serious infections caused by *Pseudomonas aeruginosa*, and as agents in the treatment of endocarditis (**Turnidge, 2003**).

Gentamicin is an aminoglycoside antibiotic commonly used against Gram-negative bacterial infections (**Priuska and Schacht, 1995; Rao *et al.*, 2006**). Gentamicin (GENT) is probably the most commonly used antibiotic of all aminoglycosides (**Balakumar *et al.* 2010**). GENT is administered for meningitis, pneumonia, Pseudomonas infections, septicemia, E. coli infections, Staphylococcus infections, listeria, tularemia, brucellosis, endocarditis, respiratory tract infections, urinary tract infections, bone infections, cystic fibrosis, diverticulitis, neutropenia, and sepsis and necrotizing entercolitis in newborns, for peritonitis, topical treatment for burns and skin infections, opthamalic drops for eye infections, intratympanic injection for Meniere's disease (**Xie *et al.*, 2011**). Numerous studies documented cytotoxicity of GENT (**Cuppage *et al.*, 1977; Spiegel *et al.*, 1990; Crann *et al.*, 1992; Dehne *et al.*, 2002; Chung *et al.*, 2006**). The aim of our study was to evaluate the *in vitro* toxicity of different concentrations of GENT on selected mammalian cell culture (BHK-21 – baby hamster kidney cells).

MATERIAL AND METHODS

Cell culture

In our experiment we used BHK-21 (Baby Hamster Kidney fibroblasts) cell line stored at the Department of Bio Preparations, Institute for State Control of Veterinary Bio preparations and Medicines in Nitra. Cells were revived according to relevant protocols. Cells were transferred into the sterile Roux flasks (DMEM/F12 supplemented with 20% FCS, non-essential amino acids, glutamine, LIF, fibroblast growth factor-2, beta-mercaptoethanol and antibiotics for FE cells) following revival and cultivated at the 37°C. After 24 hours, the monoculture assessed and cell density was determined. Cell suspension was prepared by dilution of the cells using FBS enriched culture medium. Prepared suspensions were transferred into 48 well plates at 500 µl per well. After further incubation in FBS enriched culture media, the cells were assessed microscopically. When a single-layer was coherent, the medium was discarded and freshly prepared antibiotics were layered on cells (**Fülöpová *et al.*, 2012; Tvrdá *et al.*, 2016**).

Antibiotic

For testing of BHK-21 cells we chose gentamicin-GENT (Intervet, MSD Animal Health, South Africa). Concentrations, used in our experiment, were obtained on the basis of knowledge of the minimum inhibitory concentrations of gentamicin effect on bacteria and LD50 for laboratory animals. These concentrations are non-toxic for eukaryotic cells therefore we raised them 1000-times. Consequently, they were modified to concentration, which is toxic for all cells (LD100). These concentrations were used as zero dilution, titration continued with a decimal dilution. Selected concentrations used in our experiment are displayed in Table 1 (**Fülöpová *et al.*, 2012; Tvrdá *et al.*, 2016**).

Table 1 Concentrations of gentamicin used for the BHK21 cell line experiments

	Cytomorphology	Biochemistry	Viability Test
Cell culture	**Concentrations (µg/mL) of gentamicin (GENT)**		
BHK-21	0; 187.5; 500; 1500; 4500; 7500	0; 937.5; 1875; 3750; 7500	0; 1500; 4500; 6500

Cell morphology

After application of various concentrations of GENT, we controlled the condition of cells in the wells microscopically (magnification x 400). Based on the structure of cells, we evaluated the presence of vital, subvital and dead cells (**Fülöpová *et al.*, 2012**).

Biochemical test

After 24 hours exposure of selected cells to GENT, cultivating medium was drained out by pipette and frozen in micro tubes to -20 °C. Frozen medium was used for biochemical analyses for the purpose of determination of possible antibiotic effect on cell metabolism. Quantification of Calcium (Ca), Magnesium (Mg) and total proteins (TP) was performed using photometry. Analyses were realized in the biochemical and hematological laboratory at the Department of Animal Physiology of SUA using commercial sets DiaSys (Diagnostic Systems GmbH, Germany) on semi-automatic analyzer Rx Monza (Randox Laboratories Ltd., United Kingdom). Quantification of Sodium (Na), Potassium (K) and Chloride (Cl) was performed by the automatic analyzer EasyLyte (Medica, Bedford, USA) (**Kováčik *et al.*, 2012**).

Cell viability (MTT)

Viability of the cells exposed to selected antibiotic *in vitro* was evaluated using the metabolic activity (MTT) assay (**Tvrdá *et al.*, 2015**). This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The resulting formazan can be measured spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, Thermo Fisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed to the antibiotic) (**Tvrdá *et al.*, 2016**). Results from the analysis were collected during three repeated experiments at each concentration.

Statistical methods

The significance of differences between the control and experimental groups was evaluated by one-way analysis of variance (ANOVA), with the Scheffe's test. The level of significance for the comparative as well as correlation analysis was set at ***(P<0.001); **(P<0.01); *(P<0.05).

RESULTS AND DISCUSSION

Morphology and survival of the BHK-21 cell line were affected by the concentration higher than 500 µg/mL of GENT. Number of subvital and death cells were directly proportional to elevation of the gentamicin content in the culture medium. We recorded a lethal dose for all cells in the medium with the highest content of GENT (7500 µg/mL). Analysis of morphological changes of BHK-21 cells is shown in Figure 1.

Figure 1 Changes of BHK-21 cell morphology after exposure to gentamicin. Concentrations of gentamicin: A) 0 µg/mL (control); B) 1500 µg/mL; C) 4500 µg/mL (magnification x 400)

BHK-21 cells were able to survive at a concentration 187.5; 500; 1500; 4500 µg/mL. We found statistically significant decrease (P<0.001) of vital cells in comparison with control in all concentrations of GENT higher than 500 µg/mL. We also found significant increase in the number of subvital and death cells compared to control group in all concentrations of GENT higher than 500 µg/mL (Figure 2).

Biochemical parameters observed in the medium were significantly affected in all concentrations of GENT. Content of Na^+ and Cl^- was the most importantly affected in all observed groups against control group (P<0.001). A statistically significant decrease of Ca (P<0.01) was detected (control vs 937.5 µg/mL resp. 7500 µg/mL of GENT) (Figure 3).

The mitochondrial activity of the BHK21 cells was significantly (P<0.001) decreased after the administration of all concentrations of GENT when compared to the Control (Figure 4).

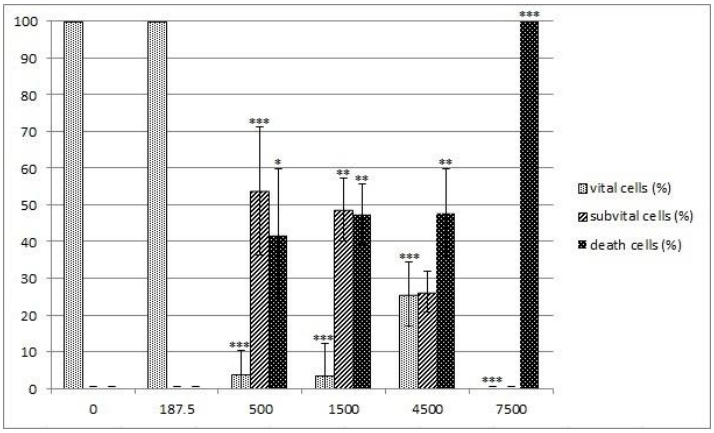

Figure 2 Values (%) of BHK-21 cell morphological changes after GENT application (GENT concentrations: 187.5; 500; 1500; 4500; 7500 µg/mL) against control (GENT concentration: 0 µg/mL)
***(P<0.001); **(P<0.01); *(P<0.05).

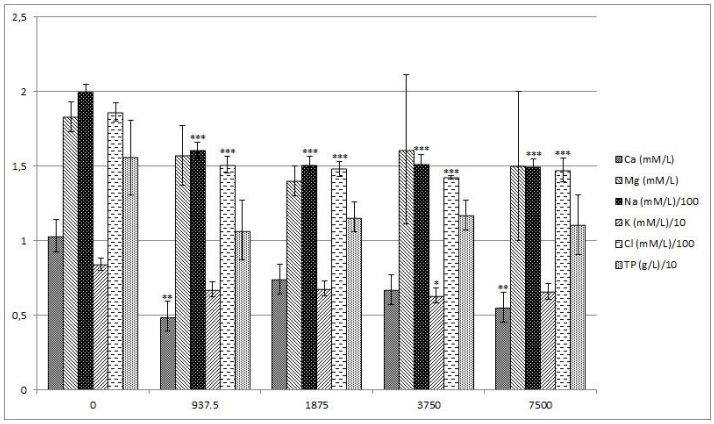

Figure 3 Biochemistry parameters levels in medium after GENT application (GENT concentrations: 937.5; 1875; 3750; 7500 µg/mL) against control (GENT concentration: 0 µg/mL)
***(P<0.001); **(P<0.01); *(P<0.05).

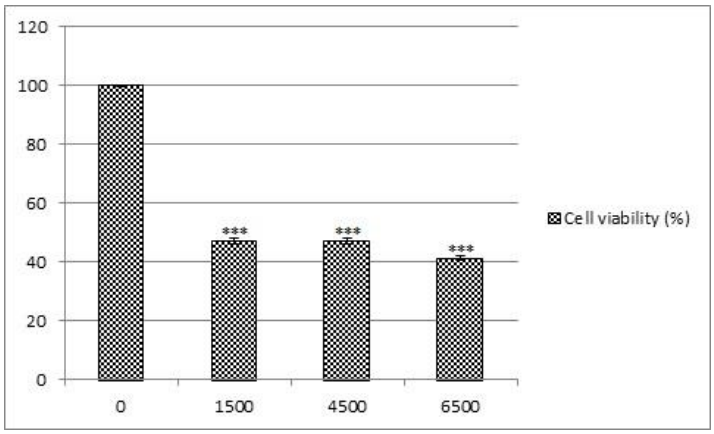

Figure 4 Effect of gentamicin on the viability of BHK-21 cells (MTT test) (GENT concentrations: 1500; 4500; 6500 µg/mL) against control (GENT concentration: 0 µg/mL)
***(P<0.001); **(P<0.01); *(P<0.05).

Aminoglycoside antibiotics are substances with relatively narrow spectrum of activity. Antibacterial activity of aminoglycoside antibiotics depends on their effective concentration in extracellular space. Nephrotoxicity induced by aminoglycosides manifests clinically as renal failure (**Mingeot-Leclercq and Tulkens, 1999**). GENT has been tested as a typical model for the study of nephrotoxicity (**Cuppage *et al.*, 1977; Mondorf *et al.*, 1978**). There are a few data in the literature about the effect of the gentamicin and other aminoglycosides on the cell lines metabolic activity (**Ford *et al.*, 1994; Yagi *et al.*, 1999; El Mouedden *et al.*, 2000; Duewelhenke *et al.*, 2007**). We demonstrated that GENT in high concentrations may be cytotoxic for Baby Hamster Kidney cells (BHK-21). The MTT assay provided information about the overall metabolic activity (**Berridge *et al.*, 2004**).
Yu *et al.* (2014) tested GENT on vestibular hair cells (VHCs II) and their findings indicated that increasing of Ca^{2+} could antagonize gentamicin blocking effect; also gentamicin may block the dependent K^{+} channels by impairing calcium influx. The effect of GENT to organisms and cell lines have been claimed – some studies have reported negative significant effects (**Isefuku *et al.*, 2003**), whereas other studies have not (**Duewelhenke *et al.*, 2007**).
In previous studies (**Fülöpová *et al.*, 2012; Kováčik *et al.*, 2012; Tvrdá *et al.*, 2016**), the effect of macrolide antibiotics (tilmicosin, tylosin and spiramycin) was tested on the specific mammalian cell lines (BHK 21, FE, VERO) *in vitro*. Effects of these antibiotics have a similar tendency for all measured parameters as GENT, but at lower concentrations (150 µg/mL; 500 µg/mL).
El Mouedden *et al.* (2000) tested exposure of GENT to three cell types (Embryonic Rat Fibroblasts, MDCK and LLC-PK_1 cells) and confirmed intrinsic capability of inducing apoptosis in cells after systematic administration. The murine C2C12 cells cultured with different concentrations of gentamicin (12.5 - 800 µg/ml) for 48 days showed negative changes in cell viability and alkaline phosphatase activity, although the cell number showed no significant changes (**Ince *et al.*, 2006**).

CONCLUSION

Aminoglycoside antibiotics were discovered in the middle of the bygone century. Their antimicrobial activity found wide use in humane and veterinary medicine. Their use was markedly limited after determination of toxicity on vestibular and glomerular apparatus.
In conclusion, the exposure of Baby Hamster Kidney fibroblasts (BHK-21) to gentamicin at our concentrations resulted in severe cell damage. The cytotoxicity of antimicrobial agents evaluated in mammalian cell cultures enables us to provide better understanding to their specific *in vitro* and *in vivo* properties. These results raise questions as to the feasibility of using gentamicin. Acquired knowledge is possible to apply in toxicity evaluation of pharmacologically effective substances *in vitro*. In this regard we must be aware that any biologically active substance, antibiotics, toxicants, heavy metals, natural extracts behave differently in *in vivo* experiments in comparison to *in vitro* conditions.

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ROLE OF NATURAL SUBSTANCES AND VITAMIN SUPPLEMENTATION IN TINNITUS PREVENTION AND TREATMENT

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ABSTRACT

The aim of this review is to refer a possibility of using natural substances for treating or reducing the symptoms of tinnitus. Tinnitus is a sensation of sound without an external source. It often manifests as a ringing in the ears, but it may also sound like a buzzing, hissing, whistling or even roaring in the head. Tinnitus is a symptom of an underlying condition. It can be linked to hearing loss, stress, ear damage, blood pressure, tumours and atherosclerosis. Exposure to loud noise is one major cause of tinnitus, since it wears down the delicate hair cells in inner ear that translate sounds into nerve impulses. Potential therapy of tinnitus is a pharmacological treatments. Fortunately, there are many effective natural alternatives to drugs that can bring considerable relief and help cope. The potential form of treatment is vitamins and natural flavonoids therapy. Low levels of melatonin and vitamin B12 in body have a significant correlation with the development of tinnitus. It was reported that melatonin is useful in the treatment of tinnitus, even in cases associated with sleep disturbance. There are relationship between vitamin B12 deficiency and dysfunction of the auditory pathway. Antioxidants are another substances which have a promising effect in the treatments of tinnitus. The constituents of *G. biloba* are potent scavengers of free radicals and has been prescribed their positive effect on treat of central nervous system disorders and cognitive deficits. Positive antioxidant effects have vitamin C, hesparidin and diosmin also.

Keywords: Tinnitus, vitamins, antioxidants, bioflavonoids, natural substances

INTRODUCTION

Tinnitus

Tinnitus is defined as the perception of sound in the absence of external auditory stimulation **(Hoekstra, 2013)**. Although the experience of short bursts of noise is almost universal, tinnitus is typically defined as noise that lasts at least 5 minutes **(Davis, 1995)**.This is the most common statement among researchers in audiology and related fields, stemming from basic neurosciences **(Kaltenbach, 2011)** to applied psychophysiology **(Kropp *et al.*, 2012)**, audiology **(Caffier *et al.*, 2006)**, and behavioural psychology **(Westin *et al.*, 2008)**. Among severe sufferers, tinnitus causes disability associated with concentration deficits, insomnia, hypersensitivity to sounds, anxiety and depression. Often a combination of several complaints leads to a diminished quality of life **(Erlandsson and Hallberg, 2000; Bauch *et al.*, 2003)**. It poses a significant clinical problem for millions of people and is proportionally problematic in countries where epidemiological data have been reported **(Henry *et al.*, 2005; Erlandsson and Dauman, 2013).**

The overall prevalence of tinnitus in adult populations ranges from 7 % to 19 %. The prevalence of tinnitus increases with age and seems to attain a plateau or even decrease at around 60–80 years **(Henry *et al.*, 2005)**. Within the group of treatment-seeking patients, the male-female ratio is 2:1. In up to 5% of the adult population, tinnitus interferes negatively with the ability to lead a normal daily life, and in 2%, it has a severe effect on daily life **(Nondahl *et al.*, 2002)**. The most common additional complaints are sleep problems, depression and anxiety **(Zoger *et al.*, 2006)**. Patients report limitations in activity and restrictions to participation in work and employment, and in social and civic life **(Tyler and Baker, 1983)**. The distress can become so intense as to drive patients to suicide **(Pridmore *et al.*, 2012)**.

Hearing loss is presumably the most important risk factor for tinnitus, but the association is complex. Tinnitus is reported in individuals with apparently normal hearing, and only some hearing-impaired persons report tinnitus **(Aarhus *et al.*, 2015, Tyler, 2006)**.

For those whose tinnitus has significant clinical impact, a number of therapeutic approaches have been described and employed, from cognitive-behavioral therapies and sound enrichment, to drug approaches. Some studies have shown favorable results, while others did not result in benefits **(Baguley *et al.*, 2013)**.

Treatments proffered for tinnitus can be grouped into four main classes: pharmacological, acoustic–physical, psychological, and some combination of elements from at least two of these three. Pharmacological and physical treatments principally aim to affect the tinnitus itself, ideally to eliminate it or reduce its prominence to the point that it is no longer troublesome **(Noble, 2008)**.Various substances have been used and tested as drug treatments. Among them, antioxidants have appeared promising **(Polanski *et al.*, 2016)**. Oxidative stress is a consequence of the inefficient utilization of molecular oxygen (O_2) by cells **(Reiter *et al.*, 2004)**. ROS including the superoxide anion radical (O_2•−) and hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), and singlet oxygen (IO_2) are generated as by-products of cellular respiration and other metabolic processes. They damage cellular macromolecules including DNA, proteins, and lipids **(Kozina *et al.*, 2007)**. Additionally, however, there are also highly devastating agents which are nitrogen based e.g. nitric oxide (NO•) and especially the peroxynitrite anion (ONOO−) **(Tengattini *et al.*, 2008)**. Oxidative stress is thought to play an important role in atherosclerotic vascular disease. Thus, dietary antioxidants such as ascorbate (vitamin C) can protect against the development and progression of atherosclerosis in experimental models. Numerous observational studies have shown an inverse association between antioxidant intake of body status and the risk of cardiovascular diseases **(Cares *et al.*, 2000)**. Antioxidant vitamins may reduce risks of cardiovascular disease has been the subject of considerable research attention in recent years. Basic research studies have provided evidence of possible mechanisms for an effect of antioxidants on atherosclerosis, and several observational epidemiologic studies have suggested that risk of coronary heart disease (CHD) may be 20 – 40 % lower among those with high dietary intake or serum levels of antioxidant

vitamins. CHD remains the leading cause of death in the United States, as well as most developed countries, accounting for approximately one of every four deaths. For this reason, even the modest reductions in CHD risk suggested by studies to date, if real, could yield substantial public health benefits. Due to the changing environment including a lot of noise pollution is very important **(Ayepola *et al.*, 2014)**. Hearing protection is very important as the hearing right after eyesight is one of the most important senses. Numerous neurological, vascular and other somatic disorders have been linked to the development of the tinnitus. Therefore no single treatment will be effective for treating all tinnitus patients **(Loockwood *et al.*, 2002)**. It was reported that the several natural substances have a potential benefit effect on the cause of this disorders.

Effects of selected natural substances at the cell level

Melatonin

Melatonin is an evolutionally phylogenic old molecule, which can be traced back to the ancient photosynthetic prokaryotes. It is a tryptophan derivative that was first isolated from bovine pineal glands **(Lerner *et al.*, 1958)**. Melatonin was later found to be also present or synthesized in extrapineal tissues such as retina, Harderian gland, gastrointestinal tract, testes and lymphocytes **(Reiter *et al.*, 2013)**. Melatonin is a functionally diverse molecule **(Reiter *et al.*, 2010)**, its originally described mission was the regulation of circadian and circannual cycles **(Marczynski *et al.*, 1964; Reiter, 1991, 1993; Zhang and Zhang, 2014)**. This molecule, acting through the melatonin receptor, seems to affect sleep, mood, sexual maturation and reproduction, immune function, aging and the antioxidative defense system. Clinical research has explored several influences that melatonin could exert on a wide range of disorders, symptoms and pathologies **(Altun *et al.*, 2007, Lanfumey *et al.*, 2013)**. A clinical study conducted on healthy volunteers revealed that low plasma melatonin concentrations may significantly correlate with the development of subjective idiopathic tinnitus **(Lasisi *et al.*, 2012).** Melatonin is thought to produce therapeutic effects through different mechanisms such as antioxidant and free radical scavenger activities. Furthermore, melatonin appears to interfere with the peripheral and central autonomic systems, with a subsequent decrease in tone of the adrenergic system and increase in cholinergic activity **(Simko *et al.*, 2010)**. Melatonin exerts advantageous vascular changes that improve labyrinth perfusion, thus protecting the inner ear from hypoxia. Melatonin can reduce muscular tone, and it may relieve tensor tympani muscle spasms, thus improving symptoms. In addition to relieving tinnitus, melatonin improves sleep quality **(Hurtuk et at., 2011; Miroddi *et al.*, 2015; Pinpdda *et al.*, 2010).**

Melatonin, dubbed the hormone of darkness, is known to regulate a wide variety of physiological processes in mammals. **Dubocovich and Markowska (2005)** describes well-defined functional responses mediated through activation of high-affinity MT_1 and MT_2 protein coupled receptors viewed as potential targets for drug discovery. MT_1 melatonin receptors modulate neuronal firing, arterial vasoconstriction, cell proliferation in cancer cells, and reproductive and metabolic functions. Activation of MT_2 melatonin receptors phase shift circadian rhythms of neuronal firing in the suprachiasmatic nucleus (SCN), inhibit dopamine release in retina, induce vasodilation and inhibition of leukocyte rolling in arterial beds, and enhance immune responses. The melatonin-mediated responses elicited by activation of MT_1 and MT_2 native melatonin receptors are dependent on circadian time, duration and mode of exposure to endogenous or exogenous melatonin, and functional receptor sensitivity. Together, these studies underscore the importance of carefully linking each melatonin receptor type to specific functional responses in target tissues to facilitate the design and development of novel therapeutic agent **(Dubocovich and Markowska, 2005)**.

In humans, expression of the MT1-receptor subtype in the SCN was first shown by Weaver and Reppert 1996. Transcripts for the MT2-receptor were not detected in humans but in mice (**Dubocovich *et al.*, 1998)**. The role of melatonin in the SCN has been described in several rodent studies and may be also valid for humans. The general opinion is that melatonin is an endogenous synchronizer **(Cajochen *et al.*, 2003)**. It provides the SCN with the information about the night and timed secretion of melatonin adjusts to the light/dark cycle. In rats it is able to stabilize circadian rhythms, reinforce them and maintain their mutual phase relationship. Furthermore, melatonin entrains free running activities in rodents **(Korf *et al.*, 2003)**. The mechanisms behind these effects are an inhibiting of neuronal firing, which might be important for defining SCN sensitivity to entraining stimuli. In humans this might contribute to the regulation of sleep. **Ekmekcioglu *et al.* (2003)** identified MT1 and MT2 receptors in human coronary arteries from pathological samples and also from healthy controls. Furthermore, **Ekmekcioglu *et al.* (2001a)** presented preliminary evidence for a circadian variation of the MT1-receptor in coronary arteries. The role of melatonin in human coronary arteries needs to be evaluated. **Ekmekcioglu *et al.* (2003)** and **Ekmekcioglu *et al.* (2001b)** showed that both types of MT-receptors are present in the human aorta. **Monroe and Watts, (1998)** assume that melatonin has vasodilatory effects, since studies in aortic rings from rat and rabbits showed that melatonin induces vasodilatation.

Melatonin, the main hormone produced by the pineal gland, displays a circadian rhythm peaking at night **(Arendt, 1995)**. Pinealocytes uses tryptophan as substrate for melatonin synthesis, and melatonin levels change as a function of tryptophan availability **(Yaga *et al.*, 1993)**. Pyridoxine is converted to its active coenzyme form, pyridoxal phosphate (PLP). More than 60 PLP-dependent enzymes are known, including enzymes that participate in decarboxylation reactions such as the decarboxylation of DOPA to dopamine and 5-hydroxytryptophan to serotonin **(Abou-Saif and Lipman, 2001, Salzmann *et al.*, 2000)**. The activity of pyridoxine as a coenzyme in the tryptophan metabolism was described in the kinurenine and methoxyindole pathways. Pyridoxine acts as a coenzyme of 5-hydroxytryptophan decarboxylase. The enzyme carboxylates 5-hydroxytryptophan to serotonin, the immediate precursor of melatonin. The effect of pyridoxine on aromatic amino acid decarboxylase activity supports a regulatory role of pyridoxine on the synthesis of neurotransmitters **(Dolina *et al.*,1993, Geng *et al.*, 1995)**. Melatonin was shown to increase brain pyridoxal phosphokinase activity, inhibition of glutaminergic neurotransmission, resulting in inhibitory effects on central nervous system activity **(Acuna-Castroviejo *et al.*, 1986 Luboshitzky *et al.*, 2002)**.

In study **Lasisi *et al.* (2012)**, the main finding is that low plasma melatonin and vitamin B12 have significant correlation with the development of tinnitus among the elderly. Melatonin is a neurohormone produced centrally by the pineal gland; it regulates the sleep-wake cycle by inducing sleepiness and reducing body temperature through its effects on the circadian clock **(Megwalu *et al.*, 2006; Saunders, 2007)**. Several researchers have reported that melatonin is useful in the treatment of tinnitus, even in cases associated with sleep disturbance **(Megwalu *et al.*, 2006; Simko and Paulis, 2007)**.

***Ginkgo biloba* L.**

Ginkgo biloba L., also popularly known as living fossil, possesses a variety of biological and pharmacological activities **(Singh *et al.*, 2008)**. The 2 main pharmacologically active groups of compounds present in the *Ginkgo* leaf extract are the flavonoids and the terpenoids **(Smith and Luo, 2004)**. Flavonoids, also called phenylbenzopyrones or phenylchromones, are a group of low molecular weight substances that are widely spread in the plant kingdom. Flavonoids present in the *Ginkgo* leaf extract are flavones, flavonols, tannins, biflavones (amentoflavone, bilobetol, 5-methoxybilobetol, ginkgetin, isoginkgetin and sciadopitysin), and associated glycosides of quercitin and kaempferol attached to 3-rhamnosides, 3-rutinosides, or p-coumaric esters **(McKenna *et al.*, 2000)**. These compounds are known to act mainly as antioxidants/free radical scavengers, enzyme inhibitors, and cation chelators **(DeFeudis and Drieu, 2000)**. Two types of terpenoids are present in *Ginkgo* as lactones (nonsaponifiable lipids present as cyclic esters): ginkgolides and the bilobalide **(Mahadevan and Park, 2008; Smith and Luo, 2004)**.

The extracts of the leaves of *Ginkgo biloba* have been found to possess cardioprotective, antiasthmatic, antidiabetic, hepatoprotective and potent CNS activities **(Liebgott *et al.*, 2000; Naik and Panda, 2007)**.

The constituents of *G. biloba* are potent scavengers of free radicals **(Naik *et al.*, 2006; Pietri *et al.*, 1997)**. By scavenging free radicals and ROS, *G. biloba* inhibits lipid peroxidation and augments levels of endogenous antioxidants. Literature reports extensive work on the cardioprotective activity of *Ginkgo biloba* extracts (EGb). Most studies have shown EGb to improve the recovery of post ischemic cardiac function (coronary flow, aortic flow, LVdP and its first derivative) in the ischemic reperfused myocardium **(Bao *et al.*, 2008; Clostre, 2001)**.

It has been demonstrated that EGb protects the heart by its antioxidant properties and its ability to adjust fibrinolytic activity **(Panda and Naik, 2014)** In study **Haramaki *et al.* (1994)**. EGb diminished the decrease of myocardial ascorbate content after 40 minutes of ischemia and 20 minutes of reperfusion and also suppressed the increase of dehydroascorbat.

Clinically, it has been prescribed to treat CNS disorders such as Alzheimer's disease and cognitive deficits. It exerts allergy and changes in bleeding time. While its mutagenicity or carcinogenic activity has not been reported, its components, quercetin, kaempferol and rutin have been shown to be genotoxic. There are no standards or guidelines regulating the constituent components of *Ginkgo biloba* leave extract nor are exposure limits imposed **(Chan *et al.*, 2007)**.

The standardized *Ginkgo biloba* extract (EGb 761) is recommended for the treatment of geriatric memory disorders including vascular and neurodegenerative dementia. Its use is steadily increasing around the world **(Alber Kader *et al.*, 2007)**. Clinical efficacy in cognitive decline and dementia has been confirmed by a series of randomized, double-blind, placebo-controlled clinical trials **(Beck *et al.*, 2016; Gauthier and Schlaefke, 2014; Janssen *et al.*, 2010; Weinmann *et al.*, 2010; Tan *et al.*, 2015)**. Improved microcirculation, enhanced neuroplasticity and support of mitochondrial energy production have been discussed as underlying mechanisms of action **(Spieß *et al.*, 2014)**. However, these suggested modes of action are mainly based on animal and in-vitro-data and have not been verified in human **(Beck *et al.*, 2016)**.

Hesperidin

Fortunately, organisms are endowed with a series of agents that can either directly detoxify radicals or their associated reactants (free radical scavengers) or

they metabolize them to innocuous molecules (antioxidative enzymes) **(Kozina *et al.*, 2007; Tengattini *et al.*, 2008)**.

Hesperidin is a naturally occurring flavonoide that exists in citrus and other plants and can be isolated in large amounts from the peels of *Citrus aurantium* (bitter orange), *Citrus sinensis* (sweet orange), and *Citrus unshiu* (satsuma mandarin) **(Crozier *et al.*, 2009)**. Hesperidin is reported to exert a wide range of pharmacological effects such as antioxidant, anti-inflammatory, anti hypercholesterolemic and anticarcinogenic properties **(Chen *et al.*, 2010)**. It has also been demonstrated that hesperidin can protect neurons against various types of insults associated with many neurodegenerative diseases **(Cho, 2006)**. In study **Tamilselvam *et al.* (2013)** investigated the neuroprotective effect of hesperidin on rotenone-induced cellular model for Parkinson disease by analysing its effect on rotenonemediated oxidative stress generation, mitochondrial dysfunction and apoptosis in human neuroblastoma SK-N-SH cells. Their data suggests that hesperidin exerts neuroprotective effect against rotenone due to its antioxidant effect, maintenance of mitochondrial function, and antiapoptotic properties in a neuroblastoma cell line.

Phytochemicals, particularly antioxidants from natural sources such as fruits, vegetables and herbs have gained popularity due to their protective properties against several chronic diseases such as cancer and cardiovascular diseases **(Temple, 2000)**. Among the natural compounds extracted from plants, polyphenols have received much attention due to their powerful antioxidant,antimicrobial and antiviral activities as well as their capacity to inhibit the proliferation of cancer cells, protect neuron against oxidative stress, stimulate vasodilation, reduce vascularization and improve insulin secretion **(Del Rio *et al.*, 2010)**. Polyphenols are adiverse class of chemical compounds that share the ability to act as chain breaking antioxidants, which are proposed to protect against the damage caused by free radicals to DNA cell membrane and cell components **(Dziri *et al.*, 2012)**. Moreover, they exhibit antibacterial, antiinflammatory, antiallergenic, antiarthrogenic and antithrombotic effects **(Ajila *et al.*, 2010)**. Recent research on the nutritional aspects have shown that polyphenols are able to modulate nutrient availability through the inhibition of digestive enzymes involved in lipid and starch break down, which could lead to beneficial effects on calorie intake, obesity, and bloodglucose **(McDougall *et al.*, 2009, Nagella *et al.*, 2014)**.

Hesperidin exerts protective action in cardiac tissue by its antihypertensive and antioxidant properties **(Wilmsen *et al.*, 2005)**. Some reports evidenced that hesperidin targets peroxisome proliferator-activated receptor-gamma (PPAR-γ) to exert biological actions **(Salam *et al.*, 2008)**. PPAR-γ being a member of the ligand-dependent nuclear receptor category regulates glucose, lipid and energy homeostasis **(Hihi *et al.*, 2002; VandenHeuvel, 1999)**. In addition, PPAR-γ regulates cellular proliferation and differentiation inducing apoptosis in a wide spectrum of human tumor cell lines **(Ondrey, 2009; VandenHeuvel, 1999)**. Flavonoids like hesperidin are reported to possess satisfactory capability to neutralize free radicals. This antioxidant property may be related to their pharmacological actions and they may be used as protective agents in a number of cardiac diseases **(Agrawal *et al.*, 2014)**.

Diosmin

The second natural bioflavonoids is diosmin (3′,5,7-trihydroxy-4′-methoxyflavone) which is is the aglycone of the flavonoid glycoside diosmin (3′,5,7-trihydroxy-4′-methoxyflavone-7-ramnoglucoside). Diosmin is hydrolyzed by enzymes of intestinal microflora before absorption of its aglycone diosmetin. Diosmin is abundant in the pericarp of various citrus **(Campanero *et al.*, 2010; Del Bano *et al.*, 2004; Nogata *et al.*, 2006)** and is considered a vascular-protecting agent in the treatment of hemorrhoids, lymphedema, varicose veins and different types of cancer **(Camarda *et al.*, 2007; Cesarone *et al.*, 2006; Le Marchand *et al.*, 2000)**. As a flavonoid, it also possesses a multitude of biological activities including anti-inflammatory and antioxidant properties **(Jean and Bodinier, 1994; Guillot *et al.*, 1998)**. However, its anti-inflammatory and protective mechanisms on PC12 cells, a model of phenotypic neuronal cells, have not been studied to date **(Milano *et al.*, 2014)**.

Diosmin is a natural flavone glycoside which can be obtained by dehydrogenation of the corresponding flavanone glycoside, hesperidin that is abundant in the pericarp of various citrus fruits **(Campanero *et al.*, 2010)**. Diosmin treatment of streptozotocin-nicotinamide induced diabetic rats, ameliorated oxidative stress in plasma and tissues as evidenced by improved glycemic and antioxidant status along with decreased lipid peroxidation **(Srinivasan and Pari, 2012)**. Experimental evidence showed the potential of rutin, a flavonol to delay glomerulosclerosis of diabetic nephropathy (DN) due to its ability to inhibit cell hypertrophy and the accumulation of ECM mediated by TGF- β1/Smads and ROS signals in mesangial cells cultured by high glucose **(Tang *et al.*, 2011)**.

***Ruscus aculeatus* L.**

Ruscus aculeatus L. (butcher's broom), belonging to the family of Liliaceae, appears in a great number of dietary supplement patents **(Engl, 2006; Rizza *et al.*, 2011)** present in literature, referring *R. aculeatus* rhizomes extract as an active ingredient to enhance microcirculation. Indeed, *R. aculeatus* preparations are widely distributed in Europe, and have been hardly used for more than 40 years to treat chronic venous insufficiency and vasculitis **(Bouskela *et al.*, 1994; Capra, 1972; Huang *et al.*, 2008)**. Therefore, oral supplementation with *R. aculeatus* extracts may prevent time-consuming, painful, and expensive complications of varicose veins and other venous insufficiency, representing an alternative to traditional treatments which require a high degree of patient compliance to be effective **(MacKay, 2001)**. While anthocyanins are the main compounds of *R. aculeatus* skin berries **(Longo and Vasapollo, 2005)**, steroidal saponins represent the main class of chemical compounds isolated from rhizomes and roots of *R. aculeatus* and are considered to be the active compounds of *R. aculeatus* commercial products **(de Combarieu *et al.*, 2002; Mimaki *et al.*, 1998a)**. *R. aculeatus* saponins are characterized by spirostanol or furostanol aglycones, bearing a sugar chain at C-1 or at C-3 **(de Combarieu *et al.*, 2002; Kite *et al.*, 2007; Mimaki *et al.*, 1999)**. In particular, a mixture of two spirostane aglycons, neoruscogenin and its (25*R*)-Δ25,27 dihydro derivate ruscogenin, is considered the active ingredient of some *R. aculeatus* commercial drugs **(de Combarieu *et al.*, 2002)**.

L- ascorbic acid - Vitamin C

Vitamin C or ascorbic acid is a water-soluble vitamin, critical for collagen and L-carnitine biosynthesis, for the conversion of dopamine to norepinephrine; it also improves iron absorption. Under physiological conditions,this vitamin also acts as a potent antioxidant **(Li and Schellhorn, 2007)**. Papaverine hydrochloride is a synthetic alkaloid that exerts a tissue protective effect correlated to antioxidants, because this substance promotes non-specific smooth muscle relaxation,leading to vasodilation **(Mathis *et al.*, 1997)**. Antioxidants act synergistically with other agents or in isolation, functioning in different ways, protecting cell membranes and also eliminating oxygen free radicals **(Polanski *et al.*, 2015; Seidman, 2000)**.

The beneficial effects of vitamin C supplementation in humans are controversial. A study reported that vitamin C may improve glycemic control, lowering both fasting blood glucose and glycated haemoglobin (HbA1c) **(Eriksson and Kohvakk, 1995)**. Chronic oral administration of vitamin C to patients with type 2 diabetes causes a decline in plasma free radicals that is associated with improved whole body glucose disposal **(Mullan et a., 2002; Paolisso *et al.*, 1995)** and improved endothelial function **(Regensteiner *et al.*, 2003)**. Recently, another study reported a reduction in the malondialdehyde (MDA) level, a major product of oxidative damage in both fasting and postprandial states of type 2 diabetic patients after vitamin C (1000 mg day-1) supplementation for 6 weeks although no effect was observed on lipid profiles **(Mazloom *et al.*, 2011)**. Some studies have indicated that the intra-arterial infusion of vitamin C restores endothelium-dependent vasodilation in patients with type 1 or type 2 diabetes **(Timimi *et al.*, 1998; Ting *et al.*, 1996)** suggesting that hyperglycemia-induced oxidative stress mediates endothelial dysfunction in diabetic patients **(Ayepola *et al.*, 2014)**.

B Vitamins

Except the typical antioxidants for the proper functioning of the nervous and vascular system in the body are also important B-group vitamins. Maintaining the proper functioning of the nervous system is very important because it affects the function of other systems. The nervous system, is responsible for sensing the internal and external environmental stimulus and as well as coordinating muscles and organs activities. Thiamine (Vitamin B1) is a coenzyme in the pentose phosphate pathway, which is a necessary step in the synthesis of fatty acids, steroids, nucleic acids and the aromatic amino acid precursors to a range of neurotransmitters and other bioactive compounds essential for brain function **(Kerns *et al.*, 2015)**. Thiamine playsa neuromodulatory role in the acetylcholine neurotransmitter system, distinct from its actions as a cofactor during metabolic processes **(Hirsch and Parrott, 2012)** and contributes to the structure and function of cellular membranes, including neurons and neuroglia **(Ba, 2008)**.

The two flavoprotein coenzymes derived from riboflavin, FMN and FAD are crucial rate limiting factors in most cellular enzymatic processes. As an example, they are crucial for the synthesis, conversion and recycling of niacin, folate and vitamin B6, and for the synthesis of all hemo proteins, including hemeglobin, nitric oxide synthases, P450 enzymes, and proteins involved in electron transfer and oxygen transport and storage **(Rivlin, 2007)**. The flavoproteins are also co-factors in the metabolism of essential fatty acids in brain lipids **(Sinigaglia-Coimbra, 2011)** the absorption and utilisation of iron **(Mushtaq, 2011)** and the regulation of thyroid hormones **(Rivlin, 2007)**. Dysregulation of any of these processes by riboflavin deficiency would be associated with its own broad negative consequences for brain function. Riboflavin derivatives also have direct antioxidant properties and increase endogenous antioxidant status as essential cofactors in the glutathione redox cycle **(Ashoori and Saedisomeolia, 2014)**.

Vitamin B6 sufficiency is required for optimal health. This is due to the participation in many different biochemical reactions. Vitamin B6 and its derivatives are needed, especially for coenzyme functions in main metabolic pathways in the human body. For that reason, it is clear that a vitamin B6 deficiency, even in mild forms, has effects on the human metabolism. Several

diseases and impairments of health are connected to the wide variety of B6 functions in suboptimal status. This can also be worsened through ageing **(Spinneker *et al.*, 2007)**.Vitamin B6 has an important role in the process of melatonin biosynthesis. Journal of Gergion Med News reported the study on the 30 laboratory white rats which were divided into two groups - experimental and control groups. The animals in the first group were treated with vitamin B6 injection. Every other day at 22 00, melatonin concentration was defined by means of ELISA. The experiment has lasted for two months. At the end of the experiment, the plasma level of melatonin increased by 35,95% in the first group of animals in comparison with the second control group. It is found that, B6 vitamin injections strengthens melatonin biosynthesis; consequently strengthening of melatonin biosynthesis influences positive therapeutic effects. One of the reasons for pathological processes, developed in organism on the background of B6 vitamin deficiency, is reduction of endogen melatonin production **(No authors listed, 2007)**.

Vitamin B12 is a largest known biomolecule and the only nutrient with a stable carbon-metal bond. One molecule of cobalt lies at the centre of each B12 molecule. Isolated B12 is a crystalline compound with a bright red colour, due to the presence of cobalt. Vitamin B12 works with folic acid in many body processes including synthesis of DNA, red blood cells and the insulation sheath (myelin sheath) that surrounds nerve cells and facilitates the conduction of signals in the nervous system. Severe depletion manifests as pernicious anaemia which was invariably fatal until the discovery of B12 in liver. But long before anaemia sets in, other conditions may manifest, most often neurological problems (numbness, pins and needles sensations a burning feeling in the feet, sharing muscle fatigue, sleep disorders, memory loss, irrational anger, impaired mental function and Alzheimers or psychological conditions (dementia, depression, psychosis and obsessive-compulsive behaviour) **(Fallon, 1987; Singh and Sachan, 2011)**. There are many reasons for reviewing the neurology of vitamin-B12 and folic-acid deficiencies together, including the intimate relation between the metabolism of these two vitamins, their morphologically indistinguishable megaloblastic anaemias, and their overlapping neuropsychiatric syndromes and neuropathology, including their related inborn errors of metabolism. Folates and vitamin B12 have fundamental roles in CNS function at all ages, especially the methionine-synthase mediated conversion of homocysteine to methionine, which is essential for nucleotide synthesis and genomic and non-genomic methylation. Folic acid and vitamin B12 may have roles in the prevention of disorders of CNS development, mood disorders, and dementias, including Alzheimer's disease and vascular dementia in elderly people. Vitamin-B12 and folic-acid deficiency and related inborn errors of metabolism may result in similar megaloblastic anaemias and overlapping neuropsychiatric complications. In the early stages there is often dissociation between the neuropsychiatric and haematological manifestations, as occurs in other general metabolic disorders that affect the CNS. The occurrence of CNS complications is influenced by the duration as well as the severity of either deficiency, by predisposing genetic factors, including polymorphisms of folate or vitamin-B12 dependent enzymes, and by any associated metabolic disorders. The administration of folic acid in the presence of vitamin-B12 deficiency may be harmful to the nervous system, after brief temporary improvement, and ultimately harmful to the blood, after more striking but suboptimal temporary improvement. In the CNS, as in the blood, failure or blocking of the supply of methyl folate will result in impaired purine, thymidine, nucleotide, and DNA synthesis, as well as disruption of DNA transcription, methylation, gene expression, and other epigenetic mechanisms affecting tissue growth, differentiation, and repair. There is now substantial interest in the role of folic acid, vitamin B12, and related pathways in nervous-system function and disease at all ages and the potential use of the vitamins, especially folic acid, in the prophylaxis of disorders of CNS development, mood, and cognitive decline, including some dementias **(Reynolds, 2006)**.

Concerning vitamin B12, finding **Lasisi *et al.* (2012)** is supported by the report of **Shemesh *et al.* (1996)**. They reported that the incidence of vitamin B12 deficiency is significantly higher among patients with tinnitus and noise-induced hearing loss (47 %) compared with those with noise induced hearing loss alone and normal subjects who exhibited vitamin B12 deficiency in 27 % and 19 %, respectively. In addition they reported some improvement in tinnitus and associated complaints in 12 patients following vitamin B12 replacement therapy. These suggest a relationship between vitamin B12 deficiency and dysfunction of the auditory pathway; hence authors recommended that routine vitamin B12 serum levels be determined when evaluating patients for chronic tinnitus.

The presence of tinnitus as the only features in these subjects with low plasma vitamin B12 suggest that perhaps tinnitus may be one of the early features of the various neurological abnormalities associated with B12 deficiencies **(Lasisi *et al.*, 2012)**.

The B vitamins folate, vitamin B_6 (pyridoxine), and vitamin B_{12} (cobalamin) are important regulators of homocysteine metabolism in the body, and randomized controlled trials have demonstrated that supplementation with folate (natural dietary folate or the synthetic folic acid) alone or in combination with vitamins B_6 and B_{12} significantly reduces blood homocysteine concentrations **(Bønaa *et al.*, 2006, Lonn *et al.*, 2006)**. Although increased intakes of these B vitamins could plausibly reduce the risk of stroke, findings from observational studies on folate **(Van Guelpen *et al.*, 2005)**, vitamin B_6 **(He *et al.*, 2004)**, and vitamin B_{12} **(Virtanen *et al.*, 2005)** in relation to stroke risk have been inconsistent. Likewise, randomized clinical trials examining the effects of supplemental folic acid and other B vitamins on stroke incidence among individuals with preexisting cardiovascular or renal disease have produced conflicting results **(Bazzano *et al.*, 2006, Wang *et al.*, 2007, Larsson *et al.*, 2008)**.

Globally 24 million people have some form of dementia, with 4.6 million new cases diagnosed each year. It is estimated that the number of people affected will double every 20 years and reach 81 million by 2040. Pharmacotherapy of Alzheimer disease and other dementias can provide only modest cognitive or disease-modifying benefits. However, even modest benefits may have significant effects on quality of life, caregiver burden, and societal economic costs. Increased homocysteine levels in conjunction with low levels of folate, vitamin B6, and vitamin B12, which interact to control homocysteine, have been reported to correlate with decreased performance on cognitive tests. For these reasons, B vitamin supplementation has been proposed to prevent or reverse cognitive decline. Several studies examined whether supplementation with pyridoxine hydrochloride (hereinafter “vitamin B6”), cyanocobalamin or hydroxycobalamin (hereinafter “vitamin B12”), and folic acid can prevent, decrease the progression rate of, or reverse the neurologic changes associated with age-related neurodegenerative retinal blood flow via the diacylglycerol-protein kinase C pathway **(Agarwal, 2011)**.

CONCLUSION

Due to multifactorial mechanisms behind formation of tinnitus it is difficult to determine the most appropriate treatment. Pharmacological treatments is one of several potential method of therapy. This review described positive effects of natural substances on various types of underlying condition that cause tinnitus or can alleviating symptoms. It was determined that low plasma melatonin and vitamin B12 have significant correlation with the development of tinnitus among the elderly. Melatonin exerts advantageous vascular changes that improve labyrinth perfusion, thus protecting the inner ear from hypoxia. Melatonin can reduce muscular tone, and it may relieve tensor tympani muscle spasms, thus improving symptoms. In addition to relieving tinnitus, melatonin improves sleep quality. The large group of substances with potential positive effect on tinnitus or for alleviating the symptoms are substances with antioxidant action. Hesperidin exerts protective action in cardiac tissue by its antihypertensive and antioxidant properties. The compounds of *Ginkgo Biloba* L. are known to act mainly as antioxidants/free radical scavengers, enzyme inhibitors, and cation chelators. Diosmin as a flavonoid, also possesses a multitude of biological activities including anti-inflammatory and antioxidant properties. In the pharmacologic therapy is important not only directly effect of some substances but also their synergistic action. Synergistic effect can bring different results. Therefore is necessary evaluating their effect with the combinations with another substances.

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