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Isolation of Heterotrophic and Hydrocarbon-Utilizing Fungi from Selected Mechanic Workshops in Port Harcourt

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Spent engine oil wastes in soils are currently considered one of the most serious environmental problems. This type of pollution decreases or fully destroys soil fertility, changes the elemental composition of soil. After their introduction into soil, hydrocarbons affect soil microorganisms directly or indirectly. In this study the population of heterotrophic and hydrocarbon utilizing fungi was investigated in soils from different mechanic workshop in Port Harcourt, Rivers State. Soil physicochemical parameters such as pH, temperature, nitrate, phosphorous, potassium, total hydrocarbon content and heavy metals like Pb and Cd were also determined. Standard procedures were followed in the mycological and physicochemical parameters determination. In the soil samples, counts of the total heterotrophic fungi ranged from 0.87±3.62 to 6.9±3.37 $\times 10^4$ cfu/g soil while counts of the hydrocarbon utilizing fungi ranged from 0.85 ±1.91 to 2.75±1.26 $\times 10^3$ cfu/g soil. The control soil sample recorded more total heterotrophic fungal counts with significant difference while the soil from the mechanic workshops recorded more hydrocarbon utilizing fungal counts than the control soil sample and was significantly different. Eight fungal genera were isolated and they include *Mucor*, *Aspergillus*, *Penicillium*, *Blastomyces*, *Scedosporium*, *Microsporium*, *Candida* and *Scopulariopsis*. Fungal genera such as *Microsporium*, *Candida* and *Scopulariopsis* were not isolated from soils from the mechanic workshops but only isolated from the control soil sample. The

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pH values ranged between 5.81 to 7.91, temperature ranged from 27.7 to 30 oC, nitrate value ranged from 0.04 to 0.21 mg/kg, PO4 ranged from 1.10 to 3.42 mg/kg, total hydrocarbon content (THC) value ranged from 0 to 170.01 mg/kg, potassium (K) value ranged from 5.063 to 17.013 mg/kg. The heavy metals analyzed were Pb (Lead) and Cd (Cadmium). The Pb ranged from 0.10 to 5.12463 mg/kg, cadmium ranged from 0.13 to 1.65072 mg/kg. The soil samples from mechanic workshops were contaminated with hydrocarbons, and the fungal isolates were primarily hydrocarbon utilizers that may be exploited for contaminated soil bioremediation.

Keywords: Bioremediation; fungi; hydrocarbon; mechanic workshops; spent engine oil; soil.

1. INTRODUCTION

Motor mechanics usually discard used engine oil into gutters, water drains and soil, this is a common practice in Nigeria [1]. Spent engine oil is described as used lubricating oils recovered after servicing and afterwards draining from vehicle and generator engines. Spent oils have a higher percentage of aromatic and aliphatic hydrocarbons, nitrogen and sulphur compounds, and metals (Mg, Ca, Zn, Pb) than fresh oils since these metals are introduced into the oil due to engine wear and tear [2]. Spent engine oil has a significant negative impact on soil and soil microorganisms. Due to inadequate aeration, immobilization of soil nutrients, and decrease of soil pH, it provides unfavorable conditions for life in the soil [3]. It has been demonstrated that soil polluted with hydrocarbons undergoes significant changes in characteristics, affecting the physical, chemical, and microbiological aspects of the soil [1]. Some of these heavy metals are required micronutrients for plants at low concentrations, but at large concentrations, they can induce metabolic problems and growth suppression. The soil environment is the most dynamic location of interactions in nature, and it is also where numerous biochemical events involved in the breakdown of organic matter and the sustenance of plants, especially agricultural crops, take place [4]. One of the causes of environmental degradation is soil pollution from petroleum and its byproducts [5,6].

Microorganisms and their activities have frequently been recommended in tests for the impacts of a specific chemical component in soil, as well as in studies of soil pollution [5,6]. Soil microbial flora, such as the fungal community, often known as "mycoflora of the soil," may be affected by spent oil pollution. This could lower the number of microorganisms in polluted soil to levels comparable to non-polluted soil. Organic matter, including hydrocarbons, is broken down by soil microbes, while inorganic components are converted from one form to another [6]. The soil is home to a variety of fungus species, which can cause spoiling when there is a lot of water activity. However, several members of the genera are known to be useful in man's daily existence. Oil pollution's impact on the soil ecosystem is mostly governed by the soil's biotic and abiotic elements. The persistence of oil pollutants in the environment is determined by these biotic and abiotic features of soil, albeit this can be influenced to some extent by the quality and mixing of the hydrocarbon. Exposure to crude and refined oils has been shown to have measurable effects on ecologically important microbial communities [7]. Once crude oil is discharged into the soil, it quickly sinks, with the volatile fraction escaping and the less volatile fraction being degraded by microbes [6]. This is due to a complicated hydrocarbon mixture that includes paraffins, olefins, kerosene, and octane [8]. The amount and quality of oil spilt, as well as past exposure of native soil microbes to oil, influence the sensitivity of soil microflora to petroleum hydrocarbons. Fungi, for example, are incredibly varied microorganisms that can adapt to survive in hostile conditions. Microbes have adapted their degradative enzyme system to break down a wide range of complex compounds [9]. The ability of these microorganisms to form endospore and vegetative cells, which can withstand harsh and unfavorable environments, is crucial to their survival in a changing environment. The study areas are known are in the city of Port Harcourt with lots of automobile activities resulting to the generation of wastes that are indiscriminately poured on the bare soil. This study was therefore carried out to isolate and identify fungal isolates in soil samples from mechanic workshops.

2. MATERIALS AND METHODS

2.1 Study Site

The study site was five different mechanic workshops in Obio/Akpor Local Government Area of Rivers State. The control site was

collected from an uncontaminated soil at Rukpokwu. The locations and their coordinates are as follows;

Mechanic village point 1 (MVP1): N 4^{o} 48'16, E 6^{o}59'06
Mechanic village point 2 (MVP2): N 4^{o} 48'16, E 6^{o}59'12
Mechanic village point 3 (MVP3): N 4^{o} 48'18, E 6^{o}59'14
Woji Mechanic workshop (WMW): N 4^{o} 48'16, E 6^{o}59'12
Chinda Mechanic workshop (CMW): N 4^{o} 50'20, E 6^{o}56'45
Uncontaminated soil (control): N 4^{o} 54'30, E 7^{o}0'17

2.2 Sample Collection

Soil samples (100 g each) were collected from give different soils sites (designated (MVP1, MVP2, MVP3, WMW, CMW, control). The soil samples were collected at 0-15cm depth from different point to form a composite using an auger borer and put into sterilized polythene bags labelled appropriately and stored in an ice box to avoid contaminations, then taken immediately to the department of laboratory for analysis 0-15 cm depth.

2.3 Media Preparation

Normal Saline: This was prepared by dissolving 8.5 g of NaCl (Sodium chloride crystals) that was weighed with the help of a weighing scale into 1000 ml of distilled water into a beaker we inserted an air-tight stopper into the mouth of the volumetric flask and shake it; then autoclaved at 121°C at 12PST pressure for 15 mins and cooled 2 room temperature and then dispensed into test tube for serial dilution of the soil samples. It was used as diluent to resuscitate stressed micro-organisms for isolation.

Serial Dilution: Serial dilution was done by dispensing 9 ml of sterilized normal saline using a sterile pipette into sterile test tubes. 1 g of the soil samples was weighed using a weigh balance and placed into the test tube. This produces 10ml of the dilute solution, this dilute solution has 1ml of extract/10 ml, producing a 10-foud serial dilution (i.e the amount of stock in each ml of the diluted solution was 0.1 ml) this procedure was repeated for all six soil samples.

2.4 Enumeration and Isolation of Total Heterotrophic Fungi

Aliquot (0.1ml) of 10^{-2} and 10^{-3} dilutions were transferred on prepared Sabouraud Dextrose agar (SDA) plates which have been fortified with tetracycline antibiotics for the inhibition of bacterial growth. The plates were later spread evenly using sterile bent glass rod. Inoculation was done in duplicates and after inoculation, plates were incubated at room temperature (22-25℃) for 4 days. Enumeration of fungal counts was carried out after incubation, while distinct fungal colonies were morphologically characterized and sub-cultured on fresh SDA plates for further identification.

2.5 Identification of Fungal Isolates

The fungal isolates were identified using cultural and morphological features such as colony growth pattern, conidial morphology, and pigmentation. The technique of Sarah et al. [10] was also adopted for the identification of the isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the aerial mycelia from the representative fungi cultures was removed and placed in a drop of lactophenol. The mycelium was well spread on the slide with the needle. A cover slip was gently placed with little pressure to eliminate air bubbles. The slide was then mounted and viewed under the light microscope with ×10 and ×40 objective lenses. The morphological characteristics and appearance of the fungal organisms seen were identified in accordance with Sarah et al. [10].

2.6 Mineral Salt Agar

The mineral salt agar was prepared by adding 0.5 g of Dipotassium Phosphate and 0.3 g of Sodium Chloride and 0.02 of iron (II) Sulphate hexahydrate and 0.3 g of Zinc Chloride and 0.3 g of magnesium sulfate heptahydrate and 0.3 g of Sodium nitrate and 0.2 g of manganese (II) sulfate in 1000 ml of distilled water 15.0 g of agar – agar was added to the mineral salt medium and thoroughly shaken. The mixture was sterilized in an autoclave at 121°C for 15 minutes and allowed to cool 0.1 ml of tetracycline (antibiotics) were added to suppress bacterial growth, shaken and then poured into sterile petri

dishes and allowed to solidify, and dried in hot air oven before inoculation.

2.7 Isolation of Hydrocarbon Utilizing Fungi

The vapour phase transfer method as described by Aleruchi and Obire [7] was used after serial dilution, an aliquot (0.1 ml) was transferred aseptically from dilutions 10^{-1} into mineral salt agar and filter paper dipped in crude oil was used to cover the top and 10^{-2} and 10^{-4}. The inoculums were spread using a sterile bent glass rod. The inoculated plates were incubated plates were incubated at 30°C for 5 to 7 days after which the plates were observed for growth. The colonies which developed were counted and record taken. Pure cultures of fungi were obtained by subculturing discrete colonies onto freshly prepared sabouraud dextrose agar plates and incubated at 30°C for 5 to 7 days.

2.8 Determination of Physiochemical Parameters and Heavy Metals

pH: To calibrate the pH meter at 7.0, two grams (2 g) of phosphate power seeded in 20ml of deionized water was used as a buffer; the reference electrode was lowered into the liquid sample. The mean of two readings from the samples was calculated. After rinsing with distilled water, the electrode was cleaned with blotting paper.

Temperature: The temperature of the samples was determined using mercury-in-glass thermometer at the collection by dipping the thermometer into the container which holds the soil samples.

Phosphate (APHA 4500PC): A quarter (25 g) of the sample was decanted into a 250 ml conical flask and the volume was reconstituted to the final volume. 0.5 g potassium persulphate and 2ml of 2M tetraozosulphate (VI) acid were added to five millilitres of the mixture in a 250 ml conical flask. The mixture was heated until it reached the digest condition. One drop of phenolphithalein indicator was added, and the solution was neutralized with 1.0 N sodium hydroxide base. Five millilitres (5 ml) of standard solution was added and brought to the final concentration before homogenization and spectrometry at a wavelength of 650nm (spectronic 20, Genesys, Thermos, USA). The relationship was used to calculate the phosphate concentration.

Nitrate (APHA 4500-E): About 1 g of the sample soil was transferred into a vial. About 0.5 ml of the 2.5% brucine reagent was added to the sample. About 2.0 ml of concentrated sulphuric acid was added and mixed for 30 seconds. The mixture was allowed to stand for 45 minutes, mixed again and 2.0 ml of distilled water was added again and mixing continued for about 30 seconds. The vial was allowed to stand in cold water for 15minutes. The absorbance was measured at 610 nm using 10mm cell in an ultra-violet spectrometer (SPECTRUMLAB 25A).

Total Hydrocarbon Content (THC): The total hydrocarbon content (THC) in the soil samples were determined using the standard method recommended by ASTM D 9071B – 7. In addition, the residual total hydrocarbon content in the soil were quantified by using the standard method of the American Petroleum Institute.

2.9 Methods for the Heavy Metal Analysis

Heavy metal analysis was conducted using Agilent FS240AA Atomic Absorption Spectrophometer according to the method of APHA [11].

Working Principle: Atomic absorption spectrometer's working principle was based on the sample being aspirated into the flame and atomized when the AAS's light beam is directed through the flame into the monochromator, and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Since metals have their own characteristic absorption wavelength, a source lamp composed of that element is used, making the method relatively free from spectral or radiational interferences. The amount of energy of the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample.

2.10 Sample Digestion

1. 5 g of the dried sample was weigh and transferred in to a digestion flask and 20ml of the acid mixture (650 ml conc HNO_3; 80 ml perchloric acid; 20 ml conc H_2SO_4) was added
2. The mixture was heated in a digesting flask until a clear digest was obtained.
3. It was diluted with distilled water to the 50 ml mark.

2.10.1 Sample digestion for water sample

Procedure: The sample is thoroughly mixed by shaking, and 50 ml of it is transferred into a glass beaker of 250 ml volume, to which 5 ml of conc. nitric acid is added and heated to boil for 30 mins, properly filtered.

The sample is aspirated into the oxidising air-acetylene flame. When the aqueous sample is aspirated, the sensitivity for 1% absorption is observed.

Table 1. Standard flame emission conditions for Pb, Cd

Metals	Wavelength nm	Slit (nm)	Flame
Lead	217.3	0.2	Air-acetylene
Cadmium	228.8	0.2	Air-acetylene

2.11 Stock Standard

Solution: 1000 mg/l, of stock metal solution was dissolved in a minimum volume of (1+1) HNO_3. Dilute to 1 liter with 1% (v/v) HCL, appropriate dilution were carried out to produce 2, 4 and 6ppm working solution

2.12 Statistical Analysis

One way ANOVA was used for the analysis while Tukey Pairwise Comparisons at 95% Confidence interval was used in separating the means. Analysis was done using Minitab 19.

3. RESULTS

The total heterotrophic fungal (THF) counts and the hydrocarbon utilizing fungal (HUF) counts obtained in this study are presented in Table 2. The highest total fungal count of 6.9±3.37 cfu/g were obtained from the control soil sample. While the least value of 0.87±3.62 cfu/g was observed in MVP1 soil sample. There was significant difference between the total fungal counts from the soil samples from mechanic workshops and the control soil. The hydrocarbon utilizing fungi recorded a least value of 0.85±1.91 cfu/g in the control sample while the highest value of 2.75±1.26 cfu/g was observed in CMW soil sample.

Table 3 showed the total number of fungal species that were isolated and their percentage distributions. The following fungal isolates were obtained; *Mucor* sp, *Aspergillus* sp, *Penicillium* sp, *Blastomyces* sp, *Scedosporium* sp, *Microsporium* sp, *Candida* sp and *Scopulariopsis* sp. For the percentage distribution, Mucor sp recorded highest distribution of 30.6% from MVP2, while the control soil recorded 0% distribution. *Aspergillus* sp recorded the same distribution of 20% from MVP1, MVP2, WMW and CMW. *Penicillium* sp was not recorded in control soil. The highest distribution of 24.1% was recorded from CMW soil sample. *Blastomyces* sp recorded highest distribution of 31.6% from MVP2 while the control soil recorded 0%. *Scedosporium* sp was not observed in MVP3 and the control soil. *Scedosporium* sp recorded 35.0% distribution from CMW soil sample. *Microsporium*, Candida and *Scopulariopsis* sp were not isolated from any mechanic workshop soil samples, but were obtained from the control soil.

The physiochemical parameters from the soil samples are presented in Table 4. The highest pH value of 7.91 was recorded in the control sample, while the least value of 5.81 was recorded in sample MVP3. The temperature was highest in sample control (30^oC) while the least value of 27.7^oC was recorded in WMW. The highest Nitrate value of 0.21 mg/kg was observed in CMW. The least nitrate value of 0.04 mg/kg was recorded in WMW. PO_4 recorded highest value of 3.42 mg/kg in the control and

Table 2. Mean total heterotrophic fungal counts (CFU/G) and hydrocarbon utilizing fungal counts of various soil samples

Sample	THF ($\times10^4$)	HUF ($\times10^3$)
Uncontaminated soil (Control)	6.9 ± 3.37^{c}	0.85 ± 1.91^{b}
CMW	1.69 ± 7.06^{a}	2.75 ± 1.26^{a}
MVP1	0.87 ± 3.62^{ab}	1.75 ± 1.26^{a}
MVP2	1.21 ± 6.03^{ab}	2.25 ± 5.00^{a}
MVP3	1.04 ± 2.83^{ab}	2.50 ± 1.29^{a}
WMW	1.60 ± 6.05^{b}	1.00 ± 8.16^{a}

** Means with the same superscript across the column are not significantly different at P>0.05*

Table 3. Fungal Isolates and their Percentage (%) distribution in the soil samples

Isolate	MVP1 (%)	MVP2 (%)	MVP3 (%)	WWM (%)	CMW (%)	Control (%)
Mucor sp	13.9	30.6	13.9	25.0	16.7	0
Aspergillus sp	20.0	20.0	10.0	20.0	20.0	10.0
Penicillium sp	13.8	31.0	13.8	17.2	24.1	0
Blastomyces sp	15.8	31.6	5.3	21.1	26.3	0
Scedosporium sp	15.0	25.0	0	25.0	35.0	0
Microsporium sp	0	0	0	0	0	100
Candida sp	0	0	0	0	0	100
Scopulariopsis sp	0	0	0	0	0	100

Table 4. Physiochemical parameters of soil samples

Parameter	MVP1	MVP2	MVP3	WMW	CMW	Control
pH	5.90	5.82	5.81	7.01	6.53.	7.91
Temperature (0°C)	29.0	29.2	27.8	27.7	28.8	30.6
Nitrate (mg/kg)	0.08	0.06	0.10	0.04	0.21	0.10
PO_4 (mg/kg)	1.10	1.21	1.71	1.53	2.14	3.42
THC (mg/kg)	140.07	170.01	112.04	120.13	53.32	0
K (mg/kg)	17.013	14.017	15.121	9.252	5.063.	6.012
Pb (mg/kg)	4.10213	3.21676	4.00341	2.14613	5.12463	0.10
Cd (mg/kg)	1.13604	0.97452	1.02134	0.42873	1.65072	0.13

least value of 1.10 mg/kg in the MVP1 soil sample. The THC value of 170.01 mg/kg recorded in MVP2 was the highest amount recorded in all the samples. The control sample recorded no value of THC. K recorded the highest value of 17.013 mg/kg in MVP1 soil sample while the least value of 5.063 mg/kg was recorded in sample CMW. The heavy metals analyzed were Pb (lead) and Cd (Cadmium). The highest value of Pb was recorded in soil sample CMW (5.12463 mg/kg) while the least value of 0.10 mg/kg was recorded in the control. Cadmium recorded highest value of 1.65072 mg/kg in CMW sample while the least of 0.13 mg/kg was recorded in the control soil.

4. DISCUSSION

The total heterotrophic fungal count was higher in the control soil sample than the soil samples obtained from the various mechanic workshops and was significantly different. The presence of heterotrophic fungi signifies healthy soil. Microbes are microorganisms, like bacteria, fungi and protozoa, and are essential components of healthy soil. Fungi decompose dead plants and animals, manure, and pesticides; protect water quality; increase the ability of soil to retain water; and make critical nutrients bioavailable to living plants and animals [12]. The hydrocarbon utilizing fungi recorded higher counts in the mechanic workshops than the control soil sample. the low heterotrophic fungal counts in the mechanic workshops could be as a result of the effect of pollution on the soil samples in the mechanic workshops. Pollution of the soil samples in the mechanic workshops was due to the indiscriminate release of various wastes such as paints, hydraulic fluids, lubricants, solvents, oil spills, carbide and batteries. This has been an ongoing practice in most mechanic workshops in Nigeria. Some organisms are killed or controlled by toxic components of crude oil while other oil degrading heterotrophic organisms are increased in number [13]. The was in agreement with the finding in this study. The high population of hydrocarbon utilizing fungi in the mechanic workshops could also be attributed to the adaptation of the hydrocarbon utilizing fungi to the amount of hydrocarbon in the environment. The fungal isolates from the mechanic workshops have adopted to the nature of the soil that has been polluted with oil from the activities going on there. Some of the fungal isolates from the mechanic workshops have earlier been reported as hydrocarbon utilizers by April et al., [14] Obire et al., [15] and George – Okafor et al., [16]. Indigenous fungi become highly adapted to survival in hydrocarbon-contaminated terrestrial environments through selective enrichment and genetic modifications that enable them to catabolize xenobiotic chemicals [17,18]. The pH of the various soil samples was slightly acidic to alkaline. Fungal isolates are known to thrive in

diverse environmental conditions and the form of asexual reproduction which they undergo is one of the major attributes for their inhabitations of different environment. Although, they thrive better in acidic environments but growth in other environmental pH have been reported. According to Smith and Read [19], soil fungi can grow in a wide range of soil pH but their population is more under acidic conditions because of severe competition with bacteria at neutral pH. The varied temperature of the soil could be attributed to the amount of heat exchange as well as other factors like shade which limits the soil from receiving direct radiant energy from the sun. According to Elias et al. [20] the temperature of the soil is determined by heat flow in the soil and heat exchanges between the soil and the atmosphere while Onwuka and Mang [21] opined that Solar radiation is the primary source of soil temperature. The temperature of soils is a very important parameters as it controls many activities in the soil including nutrient availability and enzymatic activities of the microorganisms and other biotic life forms existing in the soil. Furthermore, most soil microorganisms require temperatures ranging from 10°C to 35.6°C to function well [21]. The soil temperature in the current study is within the required temperature for microbial activities and plant growth. The highest Nitrate value was observed in CMW. The least nitrate value was recorded in WMW. PO_4 recorded highest value in the control and least value in the MVP1 soil sample. Higher available phosphorus in unpolluted soils than polluted soils could be attributed to higher acidity of polluted soils which causes the fixation of available phosphorus [22]. However, the content of available phosphorus in the study sites was below the critical level (10 -17 mg/kg). K recorded the highest value of 17.013 mg/kg in MVP1 soil sample while the least value of 5.063 mg/kg was recorded in sample CMW. The highest total hydrocarbon content value recorded in MVP2 as compared to all the other mechanic soil samples could be as a result of the high level of activities going on in this particular location resulting in the more discharge of engine oil in the environment. The control sample recorded no value of THC, probable because no form of activity occurred in that area that could result to the discharge of petroleum product that would have resulted to contamination. Higher values of total hydrocarbon content recorded in mechanic soils could be attributed to the possession of non-volatile and poly-cyclic hydrocarbons which alters the chemical properties of soils and also due to the existence of anaerobic condition which resulted into insufficient oxygen supply and hence anaerobic decomposition ensued resulting in organic materials (methane and carbon dioxide, the former – a hydrocarbon) being produced and hence the increase in the THC. Ayotamuno et al., [23] made a similar observation. However, the values of THC did exceed the critical level of 6.1 – 7.3 mg/kg [24]. Higher values of heavy metals in mechanic soils could be attributed to the fact that crude oil contains Pb and Cd [25], and these were possibly added to the soil during spillage. But the values of these heavy metals did not exceed their critical levels for crop production [26]. *Scedosporium* sp had the highest percentage distribution generally and was observed in chinda mechanic workshop (CMW). The growth of these fungi could be because of its ability to adapt to the pollutants.

5. CONCLUSION

In conclusion, fungi isolated from mechanic workshop were characterized. The total fungal counts observed in the soils from mechanic workshops were significantly lower than the control. In contrast, hydrocarbon-utilizing fungi were higher in the mechanic workshops than the control. The Fungal isolated from the mechanic workshops were mainly hydrocarbon utilizers and could be used for bioremediation of contaminated soil. *Scedosporium* sp can also be further studied since they grew well in Pd and chromium contaminated soil.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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