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RESEARCH ARTICLE

OPTIMIZATION OF TISSUE CULTURE PROTOCOL FOR REGENERATION AND DIRECT MULTIPLICATION OF *MORINGA OLEIFERA* BY SEED GERMINATION

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Abstract

Moringa oleifera (G.H. No.: 95436, Dr. Muneeba Khan, Centre for Plant Conservation, University of Karachi) known as the miracle tree, is a plant of great importance. The plant is well known for its diverse medicinal and nutritional properties. The objective of the research is focused on tissue culture of *Moringa oleifera* through callus induction, direct multiplication as well as from seed culture. The study is also focused on optimization of sterilization protocol and media for the tissue culture processes. As expected, *Moringa oleifera* gave promising results using MS Media supplemented with different concentrations of Plant Growth Regulators such as IAA, NAA, BAP, Kinetin and 2,4-D. Seed initiation gave optimum results on MS Basal media. Direct multiplication of the plant was observed with optimum results on MS media supplemented with 0.1 mgL⁻¹ BAP. For callus induction, effects of Plant Growth Regulator and photoperiod were observed. In Dark conditions, maximum growth was seen on MS media supplemented with 2, 4-D (2 mgL⁻¹), and that in light conditions, MS media with 2,4-D (2 mgL⁻¹ and 3 mgL⁻¹) gave almost similar results. Initially, seed coats were removed inside the Laminar Flow Hood but this was affecting the sterilization negatively. Seed sterilization was then modified by removing seed coats and sterilizing them outside the Laminar Flow Hood. The seeds were then transferred to the LFH for further sterilization, and then cultured on the growth media. For the sterilization of the explant for direct multiplication and callus induction, firstly Ethanol was used which caused bleaching effect. Later, Ethanol was removed from the protocol, reducing the bleaching effect to zero, and sterilization was maximized.

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Introduction:-

Since the beginning of time, we have relied on other sources for food and life supporting material. Plants have been a major provision for human nutrition and medications throughout history. Medicinal plants are still important as they were in the previous eras when modern medicine was not as popular as today's time (Al Khateeb et al., 2013).

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Around the world, especially countries that have an old cultural background, such as Pakistan, India, and China (Stephenson and Fahey, 2004), a significant percentage of population depend on the plants for the cure for certain type of ailments, ranging from slight allergic reaction of the skin to the serious conditions such as cancer (Förster et al., 2013). Through advance testing techniques, modern medicine has validated those plants, which have been previously known to have certain medicinal effects (Kingham, 2001; Mathur et al., 2014). Main active ingredients have also been identified by biochemical testing, and those compounds have been used to make medicines used for diagnosis and treatment in today's time (Wagner and Bladt, 1996; Balunas and Kinghorn, 2005).

Moringaceae family has total of 13 species, which grow in tropical and sub-tropical climates, and range in size from small herbs to gigantic trees. The trees of this family grow very quickly, and are drought resistant so they can withstand harsh environments. Native to the Sub-Himalayan tracks of Pakistan and India, and widely cultivated in the Philippines, South Africa, Sudan and Latin America (Verdcourt, 1985; Palada, 1996), *Moringa oleifera* (G.H. No.: 95436, Dr. Muneeba Khan, Centre for Plant Conservation, University of Karachi) is a fast-growing perennial tree, with a history of traditional medicine and culinary uses. Almost all parts of Moringa tree are edible and useful to humans (Fuglie, 1999; Anwar et al., 2007; Ferreira et al., 2008; Banerji et al., 2009). Seeds can be eaten raw green, powdered or roasted or steeped for use in curries or tea (Gassenschmidt et al., 1995). Seeds of *Moringa oleifera* have also been used as a natural flocculants to clarify water for drinking purposes (Bichi, 2013). The impressive range of medicinal uses and high nutrition makes it a plant of interest for the international pharmaceutical and agricultural community. It is highly nutritious for humans and animals, especially in life stock to increase their milk production (Shahzad et al., 2013). It is a potential food source, especially the leaves which are rich in nutrients, vitamins and as well as minerals (Fuglie, 1999). Previously, reported to have contained certain metabolites, having hypotensive activity (Faizi et al., 1995), anti-tumor activity (Bennett et al., 2003; Murakami et al., 1989), hepatoprotective activity (Pari and Kumar, 2002), *Moringa oleifera* has tremendous opportunities for sustainable agriculture and development as a cash crop. Thus, preservation of this plant is of great importance for dietary and pharmacological perspectives.

Tissue culture is the growing of living tissue in an artificial medium, away or apart from the source of isolated tissue (Ahloowalia et al., 2004). Micropropagation can be defined as the type of tissue culture that uses plant material such as leaves or other meristematic tissues as explant to grow the plant under aseptic environment (Debergh and Read, 1991). Micropropagation can either be direct or indirect. Direct multiplication initiates rooting and shooting in the explant, whereas in the indirect micropropagation callus formation is initiated followed by morphogenesis (Kadhimi et al., 2014). All this transformation of the plant is controlled by the manipulation of concentration and combination of different plant growth regulators.

Based on the concept of totipotency, tissue culture plays a major role in plant micropropagation and conservation (Jaskani et al., 2008). Techniques of tissue culture are important in applied and fundamental research, proving helpful in understanding gene structure, genetic resource conservation, functions in molecular biology and plant improvement through transgenic technology. Moreover, tissue culture has previously been used for mass propagation of elite and economically important plants (Preece and Read, 1993), also for the endangered species on the verge of extinction (Islam et al., 2005). Previously, *Moringa oleifera* has been cultured using nodal explants from non-aseptic sources from either mature plants or young seedlings (Stephenson and Fahey, 2004; Islam et al., 2005; Marfori, 2010).

Moringa is traditionally propagated by stem cutting. This method adversely affects the mother plant's growth, yield and even causes death (Islam et al., 2005). Tissue culture is an alternate method for its regeneration and multiplication. The objective of the research is focused on tissue culture of *Moringa oleifera* through callus induction, direct multiplication as well as from seed culture. The study is also focused on optimization of sterilization protocol and media for the tissue culture processes.

Materials and Methods: -

Plant material was collected from the Department of Pharmacy, University of Karachi and identified by taxonomist, Dr. Muneeba Khan at the Centre for Plant Conservation, University of Karachi and a voucher specimen number (G.H. No.: 95436) was deposited in the Herbarium of the University of Karachi. Seeds were harvested from Bahawalpur region of Punjab province, Pakistan, and stored at normal room temperature in a glass jar until further use. The leaves used for the callus induction purpose were further collected from the plants grown in the green house.

Seed Initiation:

The seed initiation of *Moringa oleifera* was done as per described below:

Media Preparation:

The basal media used for seed initiation was the Murashige & Skoog Medium (Murashige and Skoog, 1962) was prepared using the stock solutions of MS Macro, MS Micro, MS Vitamins and Fe-EDTA as per the recipe. Two types of media were prepared: one with the plant growth regulators (PGRs) and the other without PGR i.e. Basal media. The PGRs used were: Indole 3-Acetic Acid ($1-3 \text{ mgL}^{-1}$), α -Naphthalene Acetic Acid ($1-3 \text{ mgL}^{-1}$) and 2, 4-Dichlorophenoxyacetic acid ($1-3 \text{ mgL}^{-1}$). The media was autoclaved at 121°C at 20 psi for 15 min. The sterilized media was stored in the tissue culture lab at $25\pm 2^\circ\text{C}$ until further use.

Sterilization

In Protocol 1, surface sterilization of seeds was done in Laminar Flow Hood with 0.1% Mercuric Chloride solution for 2 min. Then the seeds were transferred into 20% Sodium Hypochlorite solution and kept for 5 min. Then the seeds were thrice rinsed with sterilized distilled water. The seed coats were removed, and again surface sterilized by immersion in 20% Sodium Hypochlorite Solution for 2 min. The seeds were then rinsed again thrice with sterilized distilled water (Shahzad et al., 2014). In Protocol 2, changes were made in the second step in which the timing of sterilization with 20% Sodium Hypochlorite was increased to 10 min, and in fourth stage where after the removal of the seed coats surface sterilization by immersion in 20% Sodium Hypochlorite Solution was increased to 5 min (Saini et al., 2012). In Protocol 3, the outer hard coats of the *Moringa oleifera* seeds were manually removed under normal bench top environment. The seeds were then transferred into the Laminar Flow Hood. Inner seeds (white in color) were then first washed in sterilized distilled water. The remaining sterilization was done as per the steps of protocol 2 with the omission of the second use of sodium hypochlorite for seed coats' surface sterilization.

Initiation:

All the seeds after sterilization were transferred to the MS Basal media and MS media with different PGRs aseptically. The cultured seeds were kept in dark at $25^\circ\text{C} \pm 2$ for 15 days. On germination, the seeds were shifted to light zone of 2000 lux intensity with photoperiod of 16/8 (light/dark) hours.

Direct Multiplication and Callus Induction:

The direct multiplication and callus induction of *Moringa oleifera* was done as per described below:

Media Preparation:

For direct multiplication and callus induction, MS Basal media was used as a control, and MS Media supplemented with plant growth regulators was used for experimental purpose. The plant growth regulators used were: Indole 3-Acetic Acid ($1-3 \text{ mgL}^{-1}$), α -Naphthalene Acetic Acid ($1-3 \text{ mgL}^{-1}$), 2, 4-Dichlorophenoxyacetic Acid ($1-3 \text{ mgL}^{-1}$), Kinetin ($1-3 \text{ mgL}^{-1}$) and 6-Benzylaminopurine-BAP ($0.1-1 \text{ mgL}^{-1}$). The media was autoclaved at 121°C at 20 psi for 15 min. The sterilized media was stored in the tissue culture lab at $25\pm 2^\circ\text{C}$.

Sterilization:

In Protocol A, the explants (stalks with leaves) were washed with tap water, and then transferred to sterilizing solution, which consisted of 20% sodium hypochlorite. It was kept on shaker for 15 minutes. After 15 minutes, the jar was taken into Laminar Flow Hood. The explants were transferred to sterilized distilled water and washed for 2 - 3 minutes. Again, they were transferred to another bottle of sterilized distilled water and washed for 2 - 3 minutes. The explants were then transferred to 70% ethanol for 1 min and then again in sterilized distilled water. UV was turned on for 10 minutes. After this, they were washed again for the last time with sterilized distilled water for 2 to 3 minutes. In Protocol B, the timing of sterilization with ethanol was increased to 5 min. In protocol C, no ethanol was used for sterilization of the explants.

Direct Multiplication:

For Direct Multiplication, the leaves were carefully removed from the stalks. The stalks were then cut at both ends diagonally at the meristematic regions. They were then planted in the media supplemented with different

concentrations of Plant Growth Regulators (IAA, NAA, 2, 4-D, Kinetin and BAP) and placed at an angle of 60° in the light /dark photoperiod (16/8hrs) and light zone of 2000 lux intensity.

Callus Induction:

For Callus Induction, the leaves were carefully cut from all four sides and then cultured in the media of different concentrations of IAA, NAA and 2, 4-D. The cultured explants were kept in dark and light zones separately with light zone of 2000 lux intensity and photoperiod of 16/8 (light/dark) hours.

Statistical analysis:

Ten replicates were produced for all the tests using different media. The results were observed after one week's interval. For statistical analysis, the standard deviation was set as ± 1 and error bars were used for the analysis.

Results:-

Optimization of Sterilization Protocol:

For the sterilization of seeds and explants, three different protocols were used for each of them namely as Protocol 1, 2 and 3 for seed and protocol A, B and C for the explants. Ten bottles were cultured with each protocol separately and rate of contamination was checked for each method carried out. Protocol 3, in case of seed sterilization, gave the best result as it gave less contamination rate as compared to the other two protocols used. The removal of seed on bench top prior to transfer of seed in the hood proved to be beneficial and gave promising results. Similarly, for direct multiplication and callus induction, the explants were sterilized using different protocols. Protocol A gave high rate of contamination every time when used. Protocol B although helped to reduce the rate of contamination to some extent but it also produced bleaching effect in leaves and stalks which were to be used for callus induction, turning them from green to yellow and eventually colorless leading to complete death of the explant. Protocol C helped in sterilization and reducing the bleaching effect. Less bottles were contaminated and the bleaching effect was totally reduced and the explant grew into desired part i.e. callus, and produced roots and shoots in case of direct multiplication.

Optimization of Media for Seed Initiation:

Several types of media were used for the seed initiation such as MS Basal Media, MS Media with Plant Growth Regulators (IAA, NAA and 2, 4-D). MS Basal media showed promising results of seed germination as the seed produced huge number of roots and shoots. Almost, similar results were observed when seed was germinated on MS Media with IAA (1 mgL^{-1}). Hence, MS Basal Media was considered best for seed initiation as it produced better results than the MS Media with IAA.

Optimization of Media and Photoperiod for Callus Induction:

For callus induction, different concentrations of plant growth regulators were used initially. The Plant Growth Regulators used in MS Media were IAA, NAA, and 2, 4-D, all with concentrations from 1 mgL^{-1} to 3 mgL^{-1} . The best results for callus induction were seen on 2, 4-D with 2 mgL^{-1} and 3 mgL^{-1} . After identifying the optimum concentration of PGR, the effect of light on callus induction was checked. It was seen that in dark conditions, the MS Media with 2, 4-D (2 mgL^{-1}) gave enhanced growth of callus from leaves as that seen in callus grown on 2, 4-D (3 mgL^{-1}). Same results for 2, 4-D (2 mgL^{-1} and 3 mgL^{-1}) were observed in light conditions. But, dark conditions gave enhanced callus growth.

Optimization of Media for Direct Multiplication:

The media used for direct multiplication was MS Media having Plant Growth Regulators namely IAA, NAA, 2, 4-D, BAP and Kinetin with concentrations 0.5 mgL^{-1} to 3 mgL^{-1} . It was seen that results of direct multiplication were obtained on MS media having BAP 0.1 mgL^{-1} . The media with the least concentration of BAP gave the best results (Riyathong et al., 2010).

Discussion:-

Optimization of Sterilization Protocol:

For the sterilization of seed, different protocols were used. Each protocol was giving different contamination rate. The protocol 1 gave a contamination rate of 100%. Total ten seeds were cultured by using protocol 1 and none of the seed survived. High bacterial contamination was seen. After these results, the protocol 1 was modified by increasing the time for first washing with sodium hypochlorite solution from 5 minutes to 10 minutes, and for the second wash from 2 minutes to 5 minutes. This was protocol 2. Same contamination rate was also observed by this protocol i.e. 100%. All the three seeds sterilized with protocol 2 showed high rate of contamination. After all these results, protocol 3 was designed in which the seed coats were removed on the bench top before transferring the seeds into the laminar flow hood. Another change made was that the washing of seeds with sodium hypochlorite solution was done once after the washing with mercuric chloride solution. Initially, three seeds were sterilized using this protocol and no contamination was observed. Later on, all the seeds were sterilized using the protocol 3. Contamination rate observed in protocol 3 was 20%. This might be possible due to the removal of source of contamination from internal soft seed coat. Thus, protocol 3 proved to be optimum for sterilization of the seeds.

The sterilization of explants for callus induction and direct multiplication was done by performing three different protocols namely: Protocol A, B and C. Different rate of contamination and bleaching effect was observed in all the protocols. For all the three protocols, ten bottles of each concentration for every PGR were cultured. Protocol A gave a contamination rate of 100% and bleaching effect of 80%. After these results, the protocol B was used in which the time for ethanol was increased to 5 min to avoid any further contamination. The results obtained from this protocol were that the contamination rate decreased by 75% but bleaching effect increased by 20%. It was proposed that the bleaching effect was due to the use of ethanol for extended time, which led the explants towards chlorosis. To avoid contamination and bleaching effect, Protocol C was used. In this case, no ethanol was used. The contamination rate reduced to 5%. No bleaching effect was seen in this case and the explants remained green after sterilization. The results thus supported the Protocol C as the optimum sterilization method for explant without any bleaching effect.

Optimization of Media for Seed Initiation:

For seed initiation, MS Basal media and MS media supplemented with IAA, NAA and 2, 4-D (1 mgL^{-1} and 3 mgL^{-1}) was used. The number of roots and shoots from seeds on MS Basal media were 19 and 6, respectively. For IAA (1 mgL^{-1}), the roots were 18 and shoots were 8. Whereas, for the rest of the media used, the number of roots and shoots were 1. Thus, it showed that the suitable media for seed initiation are MS Basal media and MS Media with IAA (1 mgL^{-1}). As both the media gave the same results evidently, so it can be proposed that MS Basal media should be used for this purpose as addition of any PGR did not produce a significant increase in number of roots and shoot observed after germination.

Optimization of Media and Photoperiod for Callus Induction:

For the callus induction, the explant was cultured on different media. First of all, the optimum media was checked. The MS Media supplemented with 2, 4-D (2 mgL^{-1} and 3 mgL^{-1}) were found to give the best results of callus. Rest of the MS Media containing IAA and NAA (1 mgL^{-1} to 3 mgL^{-1}) did not show any callus growth. Later on, the factor of photoperiod was investigated. The explants cultured in the optimum media (2, 4-D 2 mgL^{-1} and 3 mgL^{-1}) were kept in both the dark and light conditions. Similar rate of callus growth was seen when the explant was cultured on 2, 4-D (2 mgL^{-1} and 3 mgL^{-1}) when kept in light conditions. Whereas, in dark conditions, MS Media supplemented with 2, 4-D (2 mgL^{-1} and 3 mgL^{-1}) gave better results than light conditions. MS Basal Media was used as control, which gave no callus production. Callus induction was done as it has many beneficial uses i.e. it can be used for biotransformation, secondary metabolites production and analysis, and genetic engineering.

Optimization of Media for Direct Multiplication:

The direct multiplication was done by culturing the explant on media having IAA, NAA, 2, 4-D (1 mgL^{-1} to 3 mgL^{-1}) and, BAP (0.1 to 3 mgL^{-1}) and Kinetin (0.5 mgL^{-1} to 3 mgL^{-1}). Results of direct multiplication were observed on the MS media supplemented with BAP 0.1 mgL^{-1} . Surprisingly, it was observed that the lowest concentration of PGR such as BAP was suitable for the direct multiplication of *Moringa oleifera*. Whereas, the higher concentrations of BAP

inhibited the process of direct multiplication. Similar inhibition of direct multiplication was seen when the explant was grown in the MS media supplemented with other PGRs such as IAA, NAA, 2, 4-D and Kinetin. Direct multiplication was done as it is an important part of plant tissue culture and can further be used for acclimatization.

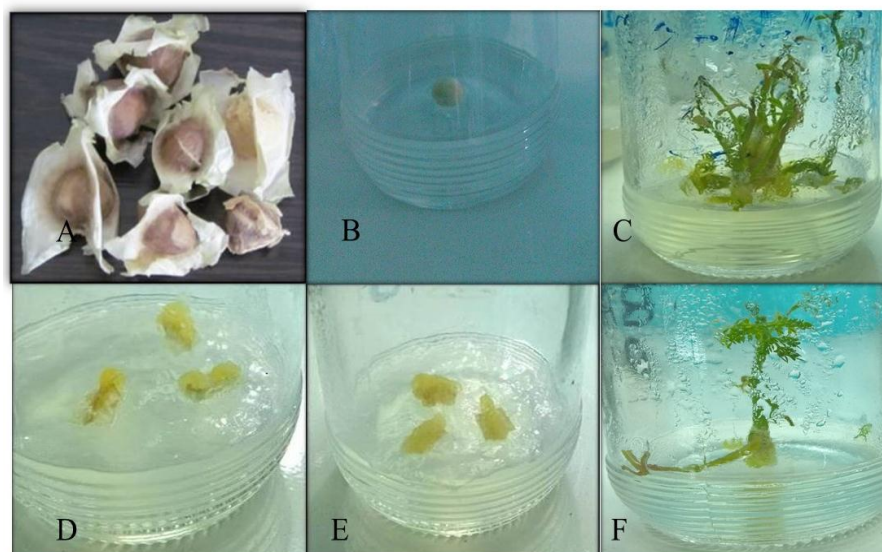


Fig 1:-A. Seeds of *Moringa oleifera* **B.** Seed Sterilization by Protocol 3 **C.** Seed Initiation after 4 weeks **D.** Callus Induction on 2, 4-D (2 mgL^{-1}) in Light Conditions **E.** Callus Induction on 2, 4-D (2 mgL^{-1}) in Dark Conditions **F.** Explant for Direct Multiplication on MS Media with BAP 0.1 mgL^{-1}

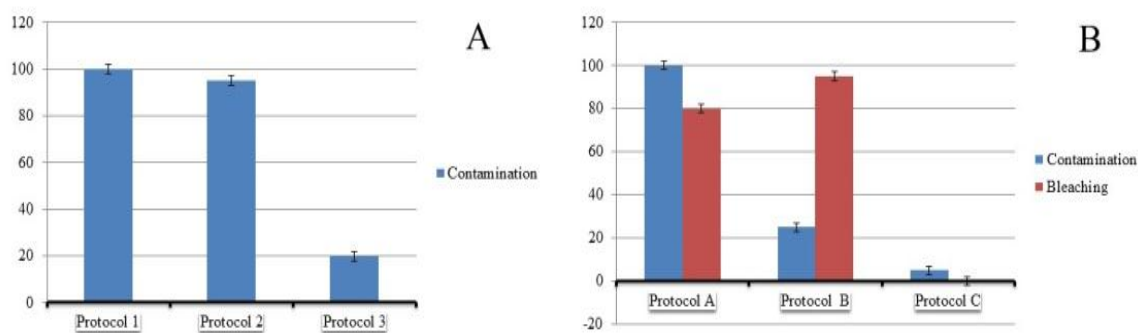


Fig 2:-A. Contamination Rate in Seed Sterilization **B.** Contamination Rate in Explant Sterilization and Bleaching Effect Produced

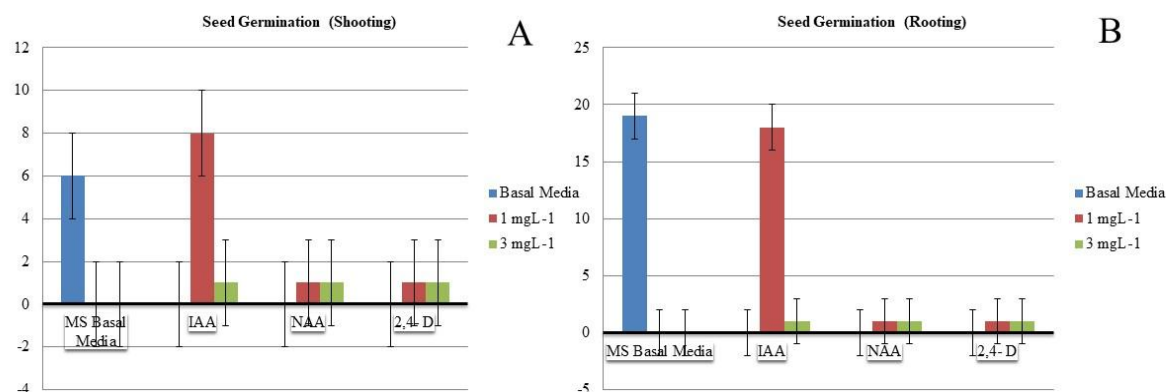


Fig 3:-A. Shooting from seed in MS Media supplemented with Different PGRs **B.** Rooting from seed in MS Media supplemented with Different PGRs

Table 1A	
PGRs IN MEDIA	No. of Shoots
MS Basal	0
BAP (0.1 mg L ⁻¹)	15
BAP (0.5 mg L ⁻¹)	5
BAP (1 mg L ⁻¹)	3

Table 1A		
PGRs IN MEDIA	LIGHT	DARK
MS Basal	--	--
2, 4-D (2mg L ⁻¹)	+	+
2, 4-D (3mg L ⁻¹)	+	+

Table 1:-A. Direct Multiplication in MS Media supplemented with Different concentrations of BAP
B. Callus Induction on 2, 4-D (2 mgL⁻¹ and 3 mgL⁻¹) in Light and Dark Condition

Conclusion: -

The research revolves around the different techniques of tissue culture applied to grow *Moringa oleifera* and optimizing the sterilization protocol for the seeds and explants. The seeds were germinated on various media and optimum results were obtained on MS Basal media. The Callus induction was highly observed when kept in dark conditions, cultured on 2, 4-D (2 mgL⁻¹) whereas the direct multiplication of the explant was seen better on MS media supplemented with 0.1 mgL⁻¹ BAP.

Author contributions:

This work was carried out in collaboration between all authors. AC, KA and NF designed the experiments and did the result analysis. AC and NF carried out the lab experiments. NF and NM contributed to writing the paper.

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Conflict of interest:

The authors declare that they have no conflict of interest.

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