Physiological potential and fungi control of *Moringa oleifera* Lamark seeds treated with essential oils

Potencial fisiológico e controle de fungos de sementes de *Moringa oleifera* Lamark tratadas com óleos essenciais

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*Moringa oleifera* Lam. is a fast-growing, drought-resistant, rustic plant with high nutritive value. The aim of the present study was to determine the effectiveness of essential oils from citronella grass (*Cymbopogon winterianus*), lemongrass (*Cymbopogon citratus*) and thyme (*Thymus vulgaris*) on the reduction of the incidence of fungi associated with *Moringa* seeds as well as the influence of these oils on the physiological quality of the seeds. Two experiments were conducted, both with an entirely randomized design. The seeds were treated with the citronella, lemongrass and thyme essential oils at concentrations of 250 and 500 µL (100 seeds per treatment). The fungicide Captain was used as the control treatment. The incidence of fungi on the treated seeds was evaluated in the first experiment. The germination percentage, shoot length, primary root length, shoot dry mass and root dry mass of the seedlings were evaluated in the second experiment. The most incident fungus was also isolated for the transmission test. The following fungi were identified on the *Moringa* seeds: *Fusarium* spp., *Nigrospora* sp., *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Phomopsis* sp., *Curvularia* sp., *Colletotrichum* sp., *Pestalotipsis* sp., *Lasiodiplodia* sp. and *Chaetomium* sp. The essential oils significantly reduced the incidence of the fungi. However, changes in the physiological quality of the seeds were associated with the use of the oils. No transmission of *Fusarium* spp. from the seeds to the seedlings occurred, but this fungus caused the rotting of seeds in the germination phase.

Keywords: *moringa*, vigor, incidência de fungos.

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*Moringa oleifera* Lam. é uma planta rústica de crescimento rápido, resistente à seca e com alto valor nutritivo. O objetivo do presente estudo foi determinar a eficiência de óleos essenciais de capim-citronela (*Cymbopogon winterianus*), capim-limão (*Cymbopogon citratus*) e tomilho (*Thymus vulgaris*) na redução da incidência de fungos associados às sementes de *Moringa*, bem como a influência desses óleos na qualidade fisiológica das sementes. Foram realizados dois experimentos, ambos com delineamento inteiramente casualizado. As sementes foram tratadas com os óleos essenciais de citronela, capim-limão e tomilho nas concentrações de 250 e 500 µL (100 sementes por tratamento). O fungicida Captain foi utilizado como tratamento controle. A incidência de fungos nas sementes tratadas foi avaliada no primeiro experimento. A porcentagem de germinação, comprimento da parte aérea, comprimento da raiz primária, massa seca da parte aérea e massa seca da raiz das plântulas foi avaliada no segundo experimento. O fungo mais incidente também foi isolado para o teste de transmissão. Os seguintes fungos foram identificados nas sementes de *Moringa*: *Fusarium* spp., *Nigrospora* sp., *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Phomopsis* sp., *Curvularia* sp., *Colletotrichum* sp., *Pestalotipsis* sp., *Lasiodiplodia* sp. e *Chaetomium* sp. Os óleos essenciais reduziram significativamente a incidência dos fungos. Entretanto, alterações na qualidade fisiológica das sementes foram associadas ao uso dos óleos. Nenhuma transmissão de *Fusarium* spp. das sementes às plântulas foi constatada, mas esse fungo causou o apodrecimento das sementes na fase de germinação.

Palavras-chave: *moringa*, vigor, incidência de fungos.

1. INTRODUCTION

*Moringa oleifera* Lamark. belongs to the family *Moringaceae*, is native to India and grown in tropical regions throughout the world [1]. The average height of the plant is about 5 m; however, under favourable condition it reaches up to 10 m [2]. *Moringa oleifera* trees are characterized by its divided leaves, thin crown, whitish flowers, fruit capsule and winged type with a dehiscent aspect of a pod having markings for measuring seed up to 35 cm [3]. This fast-growing rustic
plant have low water requirements, which is a very relevant feature for a region with sparse rains events, in which plants are under severe environmental stresses. Thus, the cultivation of moringa has been suggested as a viable alternative for the semiarid region of northeastern Brazil.

In addition to its use as an ornamental tree, the specie is known for its great versatility [4]. The leaves are supplied to animals as forage while the seeds are used for medicinal and industrial purposes. The wood is used to produce paper and textile fibers. The roots are considered abortive [5] and have been widely used by the pharmaceutical industry to combat in deficiency of vitamins A and C, in rheumatism and gout treatments, such as wound healing. In addition, the plant is known to have several pharmacological benefits for human consumption including growth promotion, antimicrobial, therapeutic and antioxidant effects [6].

The interest in the production of forest seedlings has increased in recent years due to the use of these plants for recovering degraded areas and the re-composition of the landscape [7]. The establishment of moringa can be done through direct sowing, planting with cuttings and transplanting seedlings produced in nurseries. Studies carried out with this species have shown that sowing in the final field is advantageous, since the moringa seedlings are fragile and losses are registered during transplantation [8]. Thus, this species can be cultivated by planting seeds that germinate easily. The plant produces many seeds and this production starts after six months to one year after planting [9].

For the successful production of seedlings and plant establishment, it is essential to use high quality seeds, expressed by genetic, physical and physiological attributes that are responsible for originating plants with high vigour [10]. One of the factors that can affect the quality of the seedlings is the difficulty of obtaining pathogen-free seeds, once those are one of the main sources of the dissemination and transmission of several pathogens associated with diseases that directly impact plant establishment [11, 12].

Seed-associated pathogens can cause different forms of harm, such as the loss of germinative power and a reduction in the vigour of the seedlings, including the possibility of pre-emergence and post-emergence death, root rot and infection of the sprout [11]. Studies carried out with *Moringa oleifera* found several fungal species to be seed transmitted, such as *Aspergillus niger*, *Aspergillus flavus*, *Alternaria sp*, *Fusarium sp*, *Penicillium sp* and *Phomopsis sp*. [13, 14].

The treatment of seeds is generally performed with synthetic fungicides. Despite the importance of these products, their continual and inadequate use causes harm to the environment as well as both human and animal health and the high cost of these substances leads to a considerable increase in production costs [11, 15]. The use of alternative methods from natural sources, such as essential oils, constitutes a sustainable replacement for chemical products in the management of pathogens on seeds [16].

Studies have demonstrated the effectiveness of different essential oils for the control of seed-associated pathogens, such as the oil from the andiroba (*Carapa guianensis* Aubl) and diesel tree (*Copaifera langsdorffii* Desf.) on cowpeas (*Vigna unguiculata* L. Walp) [14], clove oil (*Syzygium aromaticum* (L.) Merr. & L. M. Perry) and diesel tree oil (*Copaifera langsdorffii* Desf) on “fava d’anta” seeds (*Dimorphandra gardneriana* Tuslane) [11], and clove oil (*Syzygium aromaticum* L.) and diesel tree oil (*Copaifera langsdorffii*) on the “Sodom apple” tree seeds (*Calotropis procera* Ait. R. Br.) [17].

Despite the effectiveness in controlling seed-associated pathogens, these essential oils can also exert a negative impact on germinative power and plant vigour [14]. However, oils can also preserve the physiological quality of the treated seeds [11]. Therefore, the aim of the present study was to determine the effectiveness of different concentrations of the essential oils from citronella grass (*Cymbopogon winterianus* Jowitt ex Bor), lemongrass (*Cymbopogon citratus* (DC.) Stapf) and thyme (*Thymus vulgaris* L.) regarding the reduction in the incidence of fungi associated with *Moringa oleifera* Lam. seeds as well as the effect of these oils on physiological quality of the seeds and seedlings.
2. MATERIAL AND METHODS

2.1 Experiment location

The experiment was conducted at the Phytopathology Laboratory of the Center for Agrarian Sciences of the Universidade Federal da Paraíba (UFPB), Campus II, Areia, PB, Brazil.

2.2 Collection and sorting of moringa seeds

Moringa seeds (*Moringa oleifera* Lam.) were collected from a matrix located in *Parque dos Coqueiros* in the city of Natal, RN, Brazil. The seeds were sorted manually. Those with malformations or attacked by pests were discarded and the rest were stored in a cold chamber at $15 \pm 2^\circ C$ for 30 days.

2.3 Control of fungi associated with moringa seeds treated with essential oils

The seeds (100 per treatment) were treated with three essential oils obtained commercially by immersion in the different solutions for five minutes. The treatments were composed as follows: T1 – Negative control (seeds disinfected with 1% sodium hypochlorite for three minutes); T2 – citronella oil at 250 µL; T3 – citronella oil at 500 µL; T4 – lemongrass oil at 250 µL; T5 – lemongrass oil at 500 µL; T6 – thyme oil at 250 µL; T7 – thyme oil at 500 µL; T8 – Captan fungicide (240 g/100 kg of seeds).

In the detection of microorganisms, the paper substrate incubation method ("blotter test") was used [18], which consists of fungal growth on seeds submitted to a humid environment. For the method, Petri dishes (Ø 15 cm) previously disinfected with a double layer filter paper that was previously sterilized and moistened with 10 mL of sterile distilled water (SDW) were used. The seeds were divided into 10 replications of 10 units, which were individually distributed on Petri dishes, following aseptic conditions. The dishes were incubated at $25 \pm 2^\circ C$ for seven days. After this period, quantification and identification of fungi were performed, in gender level, based on its morphological characteristics visualized with the aid of stereoscopic, optical microscopes and specialized literature [19], the seeds being observed individually. The results were expressed as percentage of the incidence of fungi per seed.

2.4 Physiological quality of moringa seeds treated with essential oils

The following tests were performed to evaluate the influence of the citronella, lemongrass and thyme oils on the physiological quality of the moringa seeds:

Germination test: 100 seeds were distributed into four repetitions of 25 seeds each and placed in “germitest” paper rolls moistened to 2.5 times the dry weight of the paper. The rolls were placed in transparent plastic bags to avoid the loss of water and then placed into biochemical oxygen demand (BOD) chamber set at 25°C with an eight-hour photoperiod. Counts of normal seedlings were performed on the 4th and 10th day after sowing [18].

First germination count: conducted concomitantly to the germination test; germinated seeds were counted on the 4th day after sowing [18].

Germination rate index (GRI): The number of germinated seeds was recorded daily from the 4th to 10th day. The GRI was determined based on the equation proposed by Maguire [20].

Length and dry mass of seedlings: The length of 100 seedlings into four repetitions of 25 seedlings was assessed in both tests individually. With a digital caliper (0.001 mm accuracy), the length of the shoot and root of the seedlings was gauged, and the results were delivered in centimeters. Subsequently, shoots and roots were separately packed in Kraft paper bags, and then taken to a forced air convection oven set at 65 °C for 48 h. After that time, having the samples reached a constant weight, the dry mass of each seedling part was weighed in an analytical balance (0.001 g accuracy) and the results were expressed in grams [21].
2.5 Transmission of *Fusarium* spp. from moringa seeds to seedlings

After the identification of the fungi, the species having the highest incidence on the moringa seeds (*Fusarium* spp.) was isolated from infected seeds for the subsequent transmission test, following the methodology of Sousa et al. (2008) [22]. To isolate *Fusarium* spp., fragments of the mycelia growing on the seeds were transferred to a potato-dextrose-agar (PDA) medium and incubated in Petri dishes at 25 ± 2°C for seven days in BOD. After confirmation of *Fusarium* sp., the fungus was multiplied in another medium containing PDA at 25 ± 2°C for the transmission test.

For the transmission test, 100 seeds were disinfected with a 1% solution of sodium hypochlorite for three minutes [10], followed by three successive rinses with sterilized distilled water (ADE) and placed in plastic trays within sterilized filter paper to remove the excess moisture.

Inoculation with *Fusarium* spp. was performed by direct contact of the seeds with the fungal colony on Petri dishes with exposure for 24 hours [22]. The seeds were then sown at a depth of 2 cm into plastic trays containing a commercial substrate that had previously been autoclaved twice (1 atm, 120°C for 60 min, with a 24-hour interval between autoclaving sessions). The trays were kept in a greenhouse for 20 days with the temperature ranging from 28 to 30°C during the day and 18 to 20°C at night. Irrigation was performed manually once a day. During the period of emergence and at the final evaluation, the seedlings with pre-emergence and post-emergence symptoms of infection were counted, removed and placed in a humidity chamber for the isolation of the pathogen and confirmation of the causal agent.

2.6. Statistical Analysis

The treatments were arranged in a completely randomized design with four replications. The normality of sample means was determined by the Shapiro–Wilk test. The fungal infection data were transformed into √y+0.5. The infection and physiological quality data were compared using Tukey’s test with a 5% probability level. The Sisvar® software was used for the statistical analysis [23].

3. RESULTS

Table 1 shows the results of analyses of variance for first germination count (FGC), germination (G), germination velocity index (GRI), shoot length (SL), root length (RL), shoot dry matter (SDM), root dry matter (RDM) and for the fungal species *Fusarium* spp. (FUS), *Nigrospora* sp. (NIG), *Aspergillus* sp. (ASP), *Penicillium* sp. (PEN), *Alternaria* sp. (ALT), *Phomopsis* sp. (PHO), *Curvularia* sp. (CUR), *Colletotrichum* sp. (COL), *Pestalotipsis* sp. (PES), *Lasiodiplodia* sp. (LAS) and *Chaetomium* sp. (CHA). The analyses of variance showed statistically significant effects of treatments for all traits evaluated, except for the fungal species *Curvularia* sp., *Colletotrichum* sp., *Pestalotipsis* sp., *Lasiodiplodia* sp. and *Chaetomium* sp.

| Source of Variation | DL | FGC Mean Squares | G Mean Squares | GRI Mean Squares | SL Mean Squares | RL Mean Squares | SDM Mean Squares |
|---------------------|----|------------------|----------------|------------------|----------------|----------------|-----------------|
| Treatment Error     | 7  | 55.13**          | 997.71**       | 135.76**         | 2.77**         | 6.71**         | 1E-03**         |
|                     | 24 | 1.29             | 46.00          | 6.48             | 0.72           | 2.04           | 1E-04           |
| Treatment Error     | 7  | 2E-04**          | 1.34**         | 0.59**           | 0.28**         | 0.13**         | 0.05*           |
|                     | 24 | 6E-05            | 0.09           | 0.03             | 0.20           | 0.02           | 0.02            |
| Treatment Error     | 7  | 0.04**           | 0.04 NS        | 0.01 NS          | 0.01 NS        | 0.01 NS        | 0.01 NS         |
|                     | 24 | 0.01             | 0.03           | 0.01             | 0.01           | 0.01           | 0.01            |

**, *, NS - Significant at 0.01, 0.05 probability levels and not significant by the F test, respectively.
The respective fungi and incidence rates on the moringa seeds (Negative control) were *Fusarium* spp. (31.0%), *Nigrospora* sp. (17.0%), *Aspergillus* sp. (10.0%), *Penicillium* sp. (7.0%), *Alternaria* sp. (4.0%), *Phomopsis* sp. (4.0%), *Curvularia* sp. (2.0%), *Colletotrichum* sp. (2.0%), *Pestalotipsis* sp. (2.0%), *Lasiodiplodia* sp. (1.0%) and *Chaetomium* sp. (1.0%) (Figure 1).

Among the fungi, *Fusarium* spp., *Nigrospora* sp., *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp. and *Phomopsis* sp. had the statistically relevant incidences to enable comparisons between treatments (Figure 2).
All treatments led to a significant reduction in *Fusarium* spp. in comparison to the negative control (Figure 2A). A 96.9% reduction in the incidence of this fungus was found when the thyme oil was used, independently of the concentration. These results did not differ significantly from those achieved with the commercial fungicide, lemongrass oil at 250 and 500 µL and citronella oil at 500 µL, which led to reductions of 87.0%, 74.1%, 83.8% and 83.8%, respectively. The citronella oil at 250 µL led to a 61.29% reduction in the incidence of *Fusarium* spp. compared to the negative control (Figure 2A). The inhibitory effect demonstrates the fungitoxic potential of the essential oils, suggesting that these oils are potential substitutes for commercial fungicides in the control of these pathogens.

A 100% reduction in the incidence of *Nigrospora* sp., *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp. and *Phomopsis* sp. was found with the essential oils (Figures 2B, 2C, 2D, 2E, 2F). The only exception was the citronella oil at a concentration of 250 µL.

Despite the effectiveness in inhibiting or reducing the growth of the pathogens identified on the moringa seeds, the citronella, lemongrass and thyme essential oils had a phytotoxic effect on the first germination count and GRI, with statistically significant differences in comparison to the negative control. The citronella oil at 250 µL, lemongrass oil at 250 µL and thyme oil at 500 µL had the greatest effect on the seeds in terms of the first germination count and GRI. The effect of these oils did not differ significantly from the commercial fungicide with the exception of the germination using thyme oil at 500 µL (Table 2).
Table 2. First germination count (FGC), germination (G), and germination rate index (GRI) of moringa (Moringa oleifera Lam.) seeds treated with essential oils of citronella, lemongrass and thyme in different concentrations.

| Treatments            | FGC (%) | G (%)  | GRI   |
|-----------------------|---------|--------|-------|
| Negative control      | 11.0 a  | 55.0a  | 19.5a |
| Citronella (250 µL)   | 0.5c    | 22.0c  | 1.7c  |
| Citronella (500 µL)   | 3.7b    | 50.0ab | 8.1b  |
| Lemongrass (250 µL)   | 0.7c    | 42.0abc| 6.9bc |
| Lemongrass (500 µL)   | 1.2bc   | 31.0abc| 3.9b  |
| Thyme (250 µL)        | 1.7bc   | 35.0abc| 4.6bc |
| Thyme (500 µL)        | 0.5c    | 6.0d   | 1.1c  |
| Fungicide             | 1.5bc   | 27.0abc| 5.3bc |
| CV (%)                | 24.67   | 14.52  | 21.18 |

Means with same letter in column do not differ significantly from each other at 5% probability level (Tukey’s test).

In the germination test (Table 2), only 55% of the untreated seeds germinated, not achieving the minimum germination rate required for the commercial production of basic seeds, which is 65% [24]. The citronella oil at 500 µL, lemongrass oil at 250 and 500 µL and thyme oil at 250 µL had germination rates similar to the negative control, with no statistically significant differences, suggesting that the adjustment of the concentrations of these oils could generate more satisfactory results in the treatment of the seeds.

Regarding the shoot and root length of the seedlings, no significant differences were found between the negative control and the oils, however there was a difference between the oils and the concentrations tested. The lowest values for these variables were found for the lemongrass oil at 500 µL and thyme oil at 500 µL, respectively, with significant differences only from citronella oil at 500 µL (Table 3).

Table 3. Shoot length (SL), root length (RL), shoot dry matter (SDM) and root dry matter (RDM) of moringa (Moringa oleifera Lam.) seedlings treated with essential oils of citronella, lemongrass and thyme in different concentrations.

| Treatments   | SL (cm) | RL (cm) | SDM (g) | RDM (g) |
|--------------|---------|---------|---------|---------|
| Negative control | 3.0ab   | 1.75ab  | 0.040ab | 0.020ab |
| Citronella (250 µL) | 1.3ab   | 2.25ab  | 0.013c  | 0.017ab |
| Citronella (500 µL) | 3.2a    | 5.4a    | 0.053a  | 0.032a  |
| Lemongrass (250 µL) | 1.9ab   | 2.5ab   | 0.026bc | 0.024ab |
| Lemongrass (500 µL) | 1.0b    | 1.5b    | 0.013c  | 0.021ab |
| Thyme (250 µL)    | 1.6ab   | 2.2ab   | 0.014c  | 0.020ab |
| Thyme (500 µL)    | 1.1b    | 1.4b    | 0.008c  | 0.007b  |
| Fungicide         | 1.9ab   | 1.9ab   | 0.018c  | 0.021ab |
| CV (%)            | 21.11   | 24.68   | 20.80   | 20.51   |

Means with same letter in column do not differ significantly from each other at 5% probability level (Tukey’s test).

The citronella oil at 500 µL showed a higher accumulation of dry matter in the shoot and root, with a significant difference between all treatments, with the exception of the negative control in the shoot dry matter and only between the thyme oil at 500 µL in the root dry matter (Table 3).

No transmission of Fusarium spp. from the seeds to the seedlings occurred. However, this fungus caused the rotting of seeds (57%) in the germination phase (Figure 3). These results confirmed the results of the germination test, demonstrating that the lot of seeds had low germinative power.
4. DISCUSSION

Fusarium spp. was the fungus with the greatest incidence on the moringa seeds. This genus is responsible for the reduction of the germinative capacity of seeds, root rot and consequent death of infected seedlings [25]. Among the fungi identified on the seeds, Nigrospora sp. [26], Aspergillus sp. and Penicillium sp.) [27] are frequently found on seeds, causing deterioration and reducing the germinative potential and vigor of seedlings.

Several species of the genera Alternaria [28], Colletotrichum [29], Curvularia Silva et al. [30] and Pestalotiopsis [31] are reported causing leaf disease on different crops, including forest species. Phomopsis sp. is responsible for causing rot in seeds and infecting seedlings, significantly compromising the initial phase of the development and establishment of plants [32].

Fusarium spp. and Alternaria spp. are “field” fungi and Aspergillus spp. and Penicillium spp. are “storage” fungi that produce mycotoxins that cause harm to plants, animals and humans [27].

Chaetomium sp. occurred with a low frequency on the moringa seeds. Several studies report the considerable potential of this genus for use in biological control, since it is efficient at colonizing soil and substrates, playing a role in the control of various pathogens [33].

According to Costa et al. (2011) [34], the antifungal activity of essential oils is related to their hydrophobicity, which enables the interaction between the oil and lipids of the cell wall, membrane and mitochondria of the pathogen, leading to a change in permeability and causing damage to these structures. The thyme oil contains the phenols thymol and carvacrol [35], whereas the major component of the citronella and lemongrass oils is citral [36], all of which have effective fungicidal activity.

On the other hand, the results of the germination test, first germination index and GRI reveal a phytotoxic effect of the essential oils on the moringa seeds (Table 2). This effect has previously been observed on seeds of Vigna unguiculata treated with citronella oil Xavier et al. (2012) [37]. Analyzing the effect of essential oils on the control of pathogens and the germination of corn seeds, Jardinetti et al. (2011) [38] found that the thyme oil reduced the GRI when compared to the negative control. According to Reigosa et al. (1999) [39], the allelochemical effects on different physiological processes of a plant are dependent on the concentration. Thus, the fact that the treatments affected this index may be related to the high concentration of the oil and further studies are needed to test the effects of lower concentrations.

Studying the effects of essential oils from cinnamon (Cinnamomum zeylanicum Blume syn. C. verum J. Presl), pepper-rosmarin (Lippia sidoides Cham.), citronella (Cymbopogon citratus), clove basil (Ocimum gratissimum L.) and jaborandi (Pilocarpus microphyllus Stapf ex Wardleworth) on lettuce leaves, Alves et al. (2004) [40] found a concentration-dependent allelopathic effect (reducing or inhibiting plantlet growth) for the majority of the oils studied, with the exception of the jaborandi oil, which stimulated plant growth.
The results of the transmission test underscore the evidence that the considerable incidence of pathogens may have compromised the physiological quality of the seeds, which would explain the low germinative power. Several studies have reported that, besides problems in the field, the genus *Fusarium* is also responsible for the rotting of seeds [41]. Silva et al. (2017) [42] also found no transmission of *Fusarium* spp. from the seeds to the seedlings of *Pinus taeda*, but this microorganism caused the rotting of seeds in the germination phase.

In this study, no lesions were found on the seedlings in the post-emergence period. However, several studies have reported the transmission of *Fusarium* spp. from seeds to seedlings. Using the transmission test, Walker et al. (2016) [25] found that *Fusarium acuminatum* and *Fusarium verticillioides* were pathogenic to *Cordia americana* L. Gottshling & J.E.Mill. and Maciel et al. (2012) [43] found that *Fusarium* sp. was pathogenic to *Parapiptadenia rigida* Bentham. Brenan. moreover, Lazarotto et al. (2012) [44] found that *Fusarium* spp. isolated from *Cedrela fissilis* Vell. Meliaceae. seeds were pathogenic to the seedlings, causing root damage and subsequent lack of vigor of the seedlings.

5. CONCLUSION

All treatments achieved control of up to 100% of the pathogens found on the moringa seeds, except the genus *Fusarium* spp. However, a 96.9% reduction in the incidence of this microorganism was achieved with the thyme oil at concentrations of 250 and 500 μL.

The essential oils at the concentrations tested exerted a negative influence on the physiological quality of the moringa seeds.

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