DEVELOPING A STANDARDIZED *IN VITRO* STERILIZATION METHOD FOR FIELD-GROWN *MORINGA OLEIFERA* EXPLANTS

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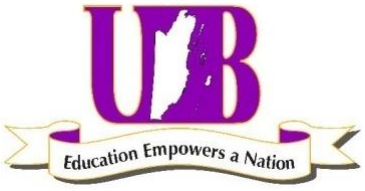

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Abstract

Moringa oleifera or the "miracle tree" is a tropical tree whose properties are of great value to humans and which has sparked great interest among the business and scientific communities for its economic potential. Micropropagation is one way to exploit the properties of this valuable tree and satisfy the demand for its planting material. The present investigation aims at devising a standardized *in vitro* sterilization method for *Moringa* explants obtained from field-grown trees and investigates the effect of donor plant age of in regards to contamination levels. Contamination in this paper refers to fungi and bacteria, primarily those that are visible to the naked eye. Explants were obtained from nodal sections of juvenile and mature field-grown/naturally-grown *Moringa* plants. Four (4%) Sodium hypochlorite (NaClO) and 70% ethanol (C_2H_6O) were used as the primary surface sterilizing agents and the duration of exposure was varied. Aseptic transfer of explants in Murashige and Skoog (MS) medium was done under the laminar flow chamber using standard tissue culture techniques. Sixty two (62%) percent of juvenile explants exposed *only* to NaClO experienced high levels of contamination. On the other hand, 65% of the juvenile explants exposed to NaClO and C_2H_6O experienced *very* low levels of contamination. Of the mature explants exposed to NaClO and the C_2H_6O, 55% had high contamination levels while the remaining 45% experienced low contamination. Exposure of explants to 4% NaClO along with dipping in 70% C_2H_6O proved to be more effective at reducing contamination. Juvenile explants respond better to this sterilization method than mature explants. The results of the present study will be very useful in the micropropagation of *M. oleifera* particularly since the sterilization phase is crucial in establishing disease-free cultures.

Acknowledgement

This project would not have been successful without the help and support of a number of people. Dr. Dion Daniels, through his course of Plant Tissue Culture, taught me all the necessary material on the subject including basic laboratory techniques used in micropropagation. He also supplied plant material and gave suggestions based on his experience in micropropagation. Several unnamed people, especially family and friends, who gave me their support and encouragement throughout this project. The University of Belize, Central Campus, for granting me the opportunity to work on this project; and finally, I would like to acknowledge the Moringa trees from which the samples were taken and for their wonderful properties that give hope to many people.

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Introduction

Moringa oleifera (hereafter referred to simply as 'Moringa") is the best known and most widely cultivated tree species in the family Moringaceae. It is native to the sub-Himalayan parts of Northern India, Pakistan, Bangladesh and Afghanistan, but has gradually proliferated and today can be found in tropical and sub-tropical regions all over the world. The distinctive features of the Moringa include the fact that it is a highly resilient tree that grows fast and is easy to propagate, as well as it is drought resistant and has very low requirements in terms of nutrients, water and management. For this reason, in many parts of the world such as India, Moringa is often grown in plantations to satisfy the demands for its planting material. In Belize, Moringa began gaining popularity until recently and is now one of the most demanded trees for its planting material, used mainly for its pharmaceutical properties. Micropropagation, through the use of plant tissue culture techniques, is one way to satisfy the demands for Moringa and exploit the many properties of this valuable tree.

Moringa is considered to be one of the most useful trees in the world, including by the World Health Organization (WHO). It is a highly impressive plant since almost every part of the tree is edible and has powerful medicinal properties. Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act on cardiac and circulatory stimulants, possess antitumor antipyretic, antiepileptic, anti-inflammatory (Kumar et al., 2009), antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepato-protective, antibacterial and antifungal activities and are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in south Asia (Anwar et al., 2007; Paliwal et al., 2011). It is generally known in the developing world as

a vegetable, a medicinal plant and a source of vegetable oil (Bennett et al., 2003; Paliwal et al., 2011)

In Belize, as is in other countries, extensive research on plant tissue culture is still being conducted to improve the technology and make good use of available equipment and resources without affecting the quality of plants that are being micropropagated. Quality is one aspect in plant tissue culture that is of utmost importance because it is not only about the amount of plants produced but about the superiority and value that comes along with it. Among the various research topics that are currently available on plant tissue culture, the main focus of this study is on *one* of the most important objectives of micropropagation: to develop standardized sterilization protocols in order to produce disease-free plants. The main objective of this study is to develop a standardized sterilization method for field-grown *Moringa oleifera* explants, while at the same time investigate if there is an effect of donor plant age on contamination levels.

Literature Review

Explant contamination depends on several plant and environmental related factors such as species, age, explant source and prevailing weather condition (Singh et al., 2011). Research shows that despite the best timing and selection efforts it is almost impossible to eliminate contamination from *in vitro* grown plants. In fact, according to (Leifert et al., 1990) losses due to contamination *in vitro* average 3 and 15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories, the majority of which is caused by fungal, yeast and bacterial contaminants. If this is the case for *in vitro* grown plants, field-grown plants present an even greater challenge and, hence the reason for this study involving field-grown *Moringa oleifera* plants as a source of explants. Reducing contamination at the whole plant level as described by Matthews and Duncan (1993) is a laborious and drawn-out process. For this reason, the main objective in this study is to devise a simple and effective sterilization method to decontaminate explants of *M. oleifera* and to investigate the effect of source and age of donor plants on contamination levels.

Through the course of time and extensive studies, disinfection protocols have been developed for several plant species. An important "rule" per say, is the selection of pathogen-free donor plants that are of optimum quality. However, many times these disinfection protocols are designed to include and favor plants that come from a germplasm bank which guarantee the plant's genetic and phytosanitary quality. Field-grown or naturally grown plants are not preferred sources of explants but they are still considered a source and used quite often when aseptically-grown plants are not available. According to Chern et al., (1993), different explant sources have different growth potential due to differences in age, endogenous metabolic status and differential genome. The younger the tissues the better is the response *in vitro*. Explants

derived from mature plants have been reported to be highly recalcitrant *in vitro*. For *in vitro* culture establishment, it is of prime importance to find safe sterilization agents that can remove the fungus and bacteria from the explant tissue (Ahmad et al., 2003). Surface sterilization is perhaps the most important sterilization treatment prior to culture initiation. Since *in vitro* conditions provide bacteria and fungi with an optimal growth environment, unsuccessful sterilization hinders the progress of tissue culture studies. Surface-sterilization processes aim to eliminate all microorganisms that can easily grow under *in vitro* conditions; on the other hand, it should guarantee the explant's viability and regeneration capacity, which are known to be affected by the concentration, application period (Allan, 1991) and temperature of disinfectant. Since direct contact of explant with disinfectant during the sterilization process may have a severe effect on regeneration capacity of the tissue (Yildiz, 2002), using aseptic tissues as source of explant is highly recommended (Yildiz et al., 1997).

Several sterilizing methods and laboratory practices exist to reduce or eliminate contamination and to ensure the establishment of disease-free cultures. In this study, sodium hypochlorite (NaClO) was used along with ethanol (C_2H_6O) as the main surface sterilizing agents. Aseptic laboratory techniques were also used to ensure minimal contamination as possible. The most widely-used surface sterilizing agent however, is sodium hypochlorite (NaClO), which when dissolved in water forms two substances: hypochlorous acid (HOCl) and the less active hypochlorite ion (OCl^-). These two substances play a key role in oxidation and disinfection. The advantages of using sodium hypochlorite as a disinfecting agent include the fact that it is quite inexpensive and can easily be stored and transported. Several studies have been done on the effectiveness of sodium hypochlorite as a decontaminant. Colgecen et al (2009) found out that almost all explants exposed to low concentrations of sodium hypochlorite became

contaminated, whereas nearly all explants exposed to high concentrations of sodium hypochlorite lost their viability. Similar results were reported by Moutia & Doukin (1999) that high concentration of sodium hypochlorite proved to be toxic resulting in 100% necrosis and death of explants. Possible reasons for these results may be due to the species of plant under study and the source and age of explants which have been found to be factors related to explant contamination. Other research shows that when ethanol is used apart from sodium hypochlorite in the sterilizing procedure, contamination levels decrease significantly than when sodium hypochlorite is used alone. Ndakidemi et al (2013) in their studies found out that the best sterilization is achieved when explants are immersed in sodium hypochlorite followed by dipping in ethanol. They also found out that all explants in the control group were contaminated after five days of inoculation. Apparently, this is an obvious result for explants collected from naturally grown plants. In fact, Rout et al. (2000), Odutayo et al. (2007), and Webster et al. (2003), found out that the use of field grown plants as direct sources of explants for the production of "clean" *in vitro* plantlets, presents a major challenge in *in vitro* cultures.

Uses and Benefits of M. oleifera

M. oleifera is a valuable tree for its many benefits and has an impressive range of medicinal uses. It has direct impact on health, nutrition, agriculture, water, sanitation, biodiversity, and the environment. Moringa is considered the "miracle tree" in some parts of the world because all its parts can be used, from the flowers to the roots. A wide variety of nutritional and medicinal virtues have been attributed to its roots, bark, leaves, flowers, fruits, and seeds (Ramachandran et al., 1980; Anwar et al., 2007; Kumar et al., 2010). Leaves are eaten as vegetables, and pressed or dried to serve in traditional pharmacology to treat many ailments or to be used as condiment. Flowers produce nectar and have anti-inflammation properties.

Moringa seeds are rich in proteins and oil, and are used for beauty care as well as for water purification. The wood provides a blue dye and is used for live fences. Medicinal qualities offer to treat diabetes, to enrich anemic blood, to staunch a skin infection, to be an antibiotic, to heal gastric ulcers, and to care eyes. In Africa, 25g of Moringa powder is administered to pregnant women daily to improve prenatal nutrition (Diatta, 2001). In Northern Nigeria, the fresh leaves are used as a vegetable, roots for medicinal purposes and branches for demarcation of property boundaries and fencing.

In many countries, the seeds of the Moringa tree are used to purify water. In the Sudan, dry *Moringa oleifera* seeds are used in place of alum by rural women to treat highly turbid Nile water (Jahn, 1986). Studies have revealed that this process of water purification not only removes solid contaminants, but also greatly reduces amounts of harmful bacteria (Trees for Life International, 2011). Madsen et al. (1987) carried out coagulation and bacterial reduction studies on turbid Nile water in the Sudan using *Moringa oleifera* seeds and observed turbidity reduction of 80-99.5% paralleled by a bacterial reduction of 1-4 log units (90-99.9%) within the first one to two hours of treatment, the bacteria being concentrated in the coagulated sediment. The University of Leicester (UK) and the UK's Overseas Development Administration experimented with Moringa seeds for treating water on a commercial scale in Malawi, Africa. Folkard et al. (1990) found that Moringa powder treated water just as well as imported commercial chemicals such as alum for far less cost. A study at the University of Newcastle-upon-Tyne (UK) found that Moringa seeds not only purify water but also soften it. The softening property of *Moringa oleifera* was also accidentally discovered in a study done by Sani (1990) and is the only one documented to date. It was observed that in addition to turbidity reduction of 92-99%, the hardness was also reduced to between 60-70% after coagulation and two hours settling.

Moringa trees have been used to combat malnutrition, especially among infants and nursing mothers (Fahey, 2005). In Africa and India Moringa is used in feeding programs to fight malnutrition. The immature green pods (drumsticks) are prepared similarly to green beans, while the seeds are removed from the mature pods and cooked like peas or roasted nuts. The leaves are cooked and used like spinach, and they are also dried and powdered for use as a condiment. *Moringa oleifera* is a promising food source especially it's leaves which are rich in nutrients and minerals and the tree has maximum leaves at the end of the dry season when other foods are typically scarce (Fuglie 1999). International organizations and institutions are exploring the best ways on how to use Moringa as a nutritional supplement and in food fortification. Three non-governmental organizations that have promoted Moringa are Trees for life, Church World Service, and Educational Concerns for Hunger Organization.

Phytochemistry

Phytochemicals are chemical compounds produced by plants. Phytochemical analyses have shown that the Moringa leaves are particularly rich in potassium, calcium, phosphorus, iron, vitamin A and D, essential amino acids, as well as known antioxidants such as β-carotene, vitamin C, and flavonoids (Bennett et al., 2003; Aslam et al., 2005; Manguro and Lemmen, 2007; Amaglo et al., 2010; Gowrishankar et al., 2010). Moreover, Fahey (2005) mentions that this plant family is rich in compounds containing the simple sugar, rhamnose, and rich in a fairly unique group of compounds called glucosinolates and isothiocyanates which have been reported to have hypotensive, anticancer, and antibacterial activity. Studies by Eilert et al. (1981) identified the presence of an active antimicrobial agent in Moringa oleifera seeds. The active agent isolated was found to be 4α L-rhamnosyloxy-benzyl isothiocyanate.

Environmental Preferences and Tolerances

The Moringa tree grows mainly in semi-arid tropical and sub-tropical areas. Optimum temperature for growth is between 25-35^{o}C (77-95^{o}F), however it will tolerate 48^{o}C (118^{o}F) in the shade. Moringa also requires well distributed rainfall of 1,000-2,000 millimeters (40-80 inches), high solar radiation and well drained soils (Radovich, 2011). Altitudes below 600 meters (1,970 feet) are optimal, but it has been known to grow at altitudes as high as 1,200 meters (3,940 feet) in the tropics (Trees for Life International, 2011).

Moringa is relatively tolerant of drought and poor soils, and responds well to irrigation and fertilization (Radovich, 2011). Moringa tolerates a wide range of soil tpes and pH (4.5-9), but prefers well drained soils in the neutral pH range (Palada and Chang, 2003). It can grow well in heavy (clay) soils provided that they do not become saturated for prolonged periods of time. Light (sandy) soils are preferred for rooting branch cuttings directly in the ground (Radovich, 2011).

Growth and Development

Moringa oleifera can grow at a remarkable rate when young, with 3-4 meters of growth in the first years provided that conditions are favorable. In cultivation, trees raised from seeds start flowering after 2 years of growth while trees grown from large cuttings begin to produce fruit 6-12 months after planting (Bosch, 2004). Mature trees eventually reach a height of 6-15 meters when growing in good conditions. However, plants growing under hostile conditions grow much slower and can have a stunted appearance, only reaching 3 meters in height.

Moringa species is deciduous during the dry season and has an enlarged underground rootstock. It is these two characters that make *M. oleifera* drought tolerant. In the Northern hemisphere, *M. oleifera* loses its leaves from December to January, though during droughts it

may also lose its leaves at other times of the year. New growth usually begins to appear in the months of February to March and flowering often precedes or coincides with the appearance of the new leaves (Bosch, 2004).

The flowering seasons typically continues through March while its fruit ripen from April to June. The bisexual flowers of *M. oleifera* are highly cross-pollinated and pollination is mainly facilitated by animals such as bees and birds. *M. oleifera* does not seem to require any specific pollinators as it readily produces viable seed in all parts of the world where it has been introduced.

A study done by Muluvi et al. (2004) confirms that *M. oleifera* has a mixed mating system and is capable of reproducing from a single individual. A single tree can produce 300-400 fruit per year within 3 years of planting while a mature tree can produce up to 1000 fruit per year. As each fruit contains approximately 20 seeds, a mature tree can therefore produce about 20,000 seeds per year.

Commercial Production

The current volume of Moringa sold internationally is not sufficient to qualify it as a commodity on the global market, and hence the trade statistics for Moringa products are only available in an aggregated form (SFA, 2015). In different parts of the world, such as India, Moringa is an industry producing tons of plant material annually. This is influenced by the demand for natural products mainly driven by consumers in developed and emerging economies. Southern India appears to be the most developed region worldwide in terms of commercial Moringa production, although there are also some functioning farming systems in Nigeria and Northern Nigeria (Saint Sauveur, 2001).

According to Radovich (2011) commercial production of Moringa pods in India is about 1.2 million metric tons produced annually on 38,000ha. The Tamil Nadu University in India has done extensive research on commercial Moringa varieties and developed two new strains ('PKM-1' and 'PKM-2') as high yielding varieties with regards to vegetable pod production. These selected lines are early flowering varieties which can produce market ready pods within six months (Radovich, n.d.). The demand for Moringa products, such as Moringa leaf powder and Moringa oil has been growing worldwide. Over the past few years, a large variety of Moringa products have spread into many markets and are now available on most health food websites and in many health stores globally.

Moringa oleifera and Plant Tissue Culture

The few reports on the tissue culture of malunggay (Stephenson and Fahey 2005; Islam et al. 2005; Xiang et al. 2007) described clonal propagation through the use of nodal explants taken from non-aseptic sources – either from young seedlings or mature plants.

Materials & Methods

This study was conducted during the period of March 12 to April 30, 2015 in the micropropagation lab at the University of Belize, Central Campus. The explants for this study were excised from juvenile (4 months old) and mature (4 years old) field-grown Moringa trees. These explants were exposed to two sterilization treatments. Treatment 1 consists of exposing explants to a solution of sodium hypochlorite (4%) with a drop of liquid soap for 20 minutes and then rinsing with sterile distilled water three times before inoculation. Treatment 2 consists of Treatment 1 followed by dipping in ethanol (70%) for 10 minutes and then rinsing with sterile distilled water three times before inoculation. Success was based on whether contamination was avoided. Explants were considered unviable if the live tissue was no longer green or were contaminated.

The first batch consisted of 34 juvenile explants. In order to establish aseptic cultures, these 34 explants were exposed to Treatment 1. To enhance the sterilization procedure the beaker containing the explants was shaken; all sterilization procedures were done under the laminar flow chamber. Prior to inoculation, the pH of the MS (Murashige and Skoog) medium was adjusted to 5.7 using and/or sodium hydroxide and hydrochloric acid and then sterilized in the autoclave at $121^{o}C$; $1.2Kg/cm^2$ for a period of 15 minutes. The explants were then inoculated in sterilized MS nutrient media containing 100% of each MS salt, 0.5mg/L thiamine, 3mg/L 6-BAP (Benzylaminopurine), 1mg/L NAA (Naphthaleneacetic acid), 50mg/L myoinositol, 15g/L sucrose and 7g/L agar. Inoculation of explants in MS medium was done under the laminar flow hood using sterile tools such as forceps and cutting blades. The explants were observed after 10 days of inoculation and contamination was recorded.

The second batch of explants consisted of 20 juvenile explants and 20 mature explants. These 40 explants were exposed to Treatment 2 at a later date. The surface sterilization procedure took place once again under the laminar flow chamber. As done for the previous batch, the pH of the MS (Murashige and Skoog) medium was adjusted to 5.7 using and/or sodium hydroxide and hydrochloric acid and then sterilized in the autoclave at $121^{o}C$; $1.2Kg/cm^2$ for a period of 15 minutes before inoculation. Explants were then inoculated in sterilized MS nutrient media containing 100% of each MS salts, 1mg/L thiamine, 12mg/L 6-BAP, 4mg/L NAA, 100mg/L myoinositol, 30g/L sucrose and 8g/L agar. Inoculation of explants in MS medium was done under the laminar flow chamber using sterile tools. The explants were observed after 10 days of inoculation and contamination was recorded.

The percentage of explants contaminated was obtained and categorized as either low contamination (C= $\leq$43.96mm) or high contamination (C= >43.96mm) based on the circumference that was contaminated around the explant. The data was analyzed by using the Chi-Square (x^2) tests with contingency tables.

Results

The first data comparison is between juvenile explants exposed to two different sterilization treatments (Table 1).

Table 1 Contamination of juvenile explants based on treatment method

	Juvenile Explants	
Contamination Levels	Treatment 1	Treatment 2
Low Contamination	13 or 38.2%	13 or 65%
High Contamination	21 or 61.8%	7 or 35%
Total Explants	34 or 100%	20 or 100%

Treatment 1 consists of exposing explants to a solution of sodium hypochlorite (4%) with a drop of liquid soap for 20 minutes and then rinsing with sterile distilled water 3 times before inoculation. Treatment 2 consists of Treatment 1 followed by dipping in ethanol (70%) for 10 minutes and then rinsing with sterile distilled water 3 times before inoculation (Table 2). Low contamination was considered as microbial growth with a circumference of 43.96mm or less. Any contamination above that level was considered as high contamination (Figure 1).

Table 2 Contamination of *M. oleifera* explants as affected by two surface sterilizing treatments

Disinfection Treatment		Low contamination (≤ 43.96mm in circumference)		High Contamination (>43.96mm in circumference)		Percentage (%) of viable plants
		Juvenile Tissue	Mature Tissue	Juvenile Tissue	Mature Tissue	
Treatment 1	Sodium hypochlorite (4%) and a drop of liquid soap for 30 minutes followed by rinsing with sterile with distilled water	13	0	21	0	94%
Treatment 2	Treatment 1 + dipping in ethanol (70%) for 10 minutes followed by rinsing with sterile distilled water	13	9	7	11	15%

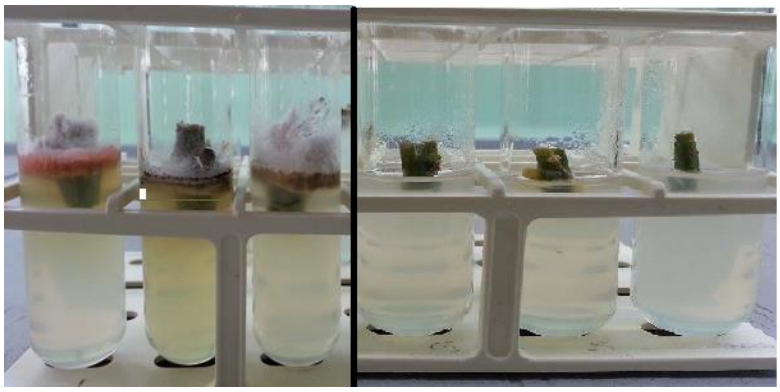

Figure 1 Classification of low contamination and high contamination of explants after 10 days of inoculation

The first batch of juvenile explants consisted of 34 explants exposed to Treatment 1 only. The Chi-Square tests showed that 38% of these juvenile explants experienced low contamination levels; while the remaining 62% experienced high contamination levels (Figure 2).

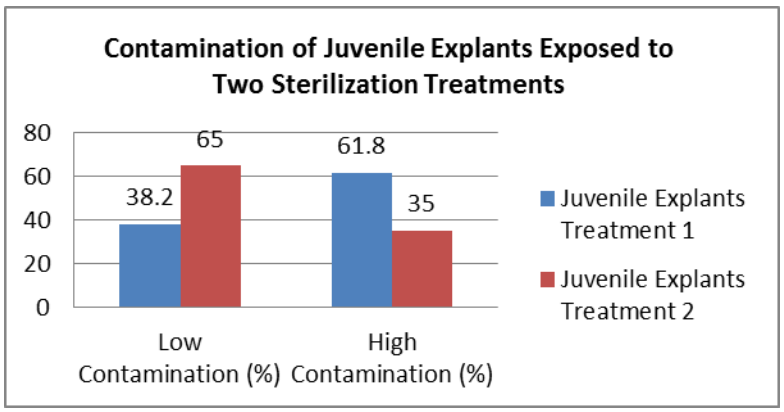

Figure 2 Effect of sterilization treatments on juvenile explants

The second batch of juvenile explants consisted of 20 explants exposed to Treatment 2. The Chi-Square tests show that 65% of these explants experienced low contamination levels and 35% were highly contaminated (Figure 2).

Based on the results from the Chi-square test for independence, the Pearson Chi-square statistic of X^2=3.613, and p=0.057, there is statistical evidence to support the null hypothesis that there is no relationship between treatment methods of juvenile explants and levels of contamination. Therefore, contamination levels and treatment methods are independent. Overall, there was 100% contamination of juvenile explants exposed to both treatment methods.

Based on the percentages (Figure 2), it can be seen that contamination decreased significantly when the juvenile explants were treated with Treatment 2. It was noted, however, that the majority of juvenile and mature explants exposed to Treatment 2 lost a significant amount of their live surface tissue due to longer exposure to sodium hypochlorite (4%) followed by dipping in ethanol (70%). The live tissue was exhibited by the green plant tissue. Juvenile explants exposed to treatment 1 experienced high contamination levels (61.8%) but surface tissue was not lost as compared with those explants exposed to Treatment 2.

The second data comparison is between juvenile and mature explants exposed to Treatment 2 only (Table 3 and Figure 3). Based on the Chi-Square contingency table, juvenile tissue experienced 65% low contamination and 35% high contamination. On the other hand, mature tissue was found to have 45% low contamination and 55% high contamination.

Table 3 Juvenile and mature tissues exposed to Treatment 2

	Treatment 2	
Contamination Levels	**Juvenile Tissue**	**Mature Tissue**
Low Contamination	13 or 65%	9 or 45%
High Contamination	7 or 35%	11 or 55%
Total Explants	20 or 100%	20 or 100%

Based on the Pearson Chi-Square statistics, X^2=1.616, and p=0.204, there is statistical evidence to support the null hypothesis that donor plant age (juvenile and mature) and contamination levels are independent.

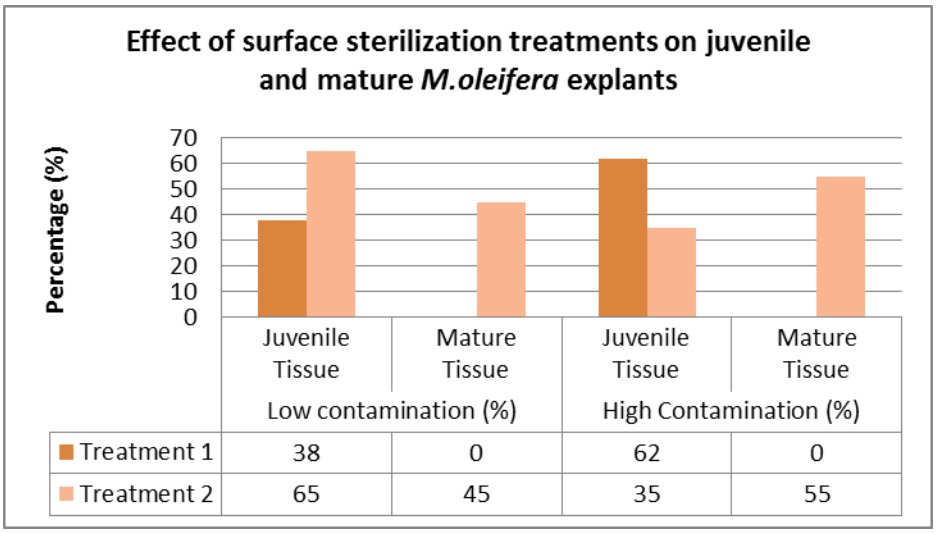

Figure 3 Effect of sterilization treatments on juvenile and mature explants of *M. oleifera*

Discussion

Based on the results, it can be seen that contamination was not avoided by using either of the two sterilization treatments; 100% contamination of explants was obtained in both treatment methods. It is very difficult to determine exactly how contamination might have gotten introduced into the micropropagation system. Fortunately, because all non-organic materials used, such as tools, vessels and rinse water were completely sterilized using an autoclave, they can be ruled out as probable sources of contamination. Contamination however, is more difficult to remove from organic material because there is a risk of killing the cells or causing irreversible chemical changes in hormones from the treatment. Thus, the prevention of contamination requires that *enough* growth inhibiting agents be applied to kill the contaminants without killing the explant. In other words, explants should be surface sterilized only by treatment with disinfectant solutions at suitable concentrations for a specific period of time.

Keeping in mind that the explants were obtained from field-grown Moringa trees, it is no doubt that these plants would present a major challenge when it comes to contamination. Contamination and the unviability of explants could be as a result of the presence of endogenous microbes. It has been reported that endogenous or endophytic fungi become pathogenic to the host plants when the plants are stressed, for example, when the cell walls are weakened or under other unfavorable *in vitro* conditions (Darworth & Callan, 1996). The endogenous or endophytic microbes are often hard to decontaminate. According to Cassells (1991), culture asepsis is important in all plant culture protocols. However, many trees in the tropics live and survive in association with endogenous or cryptic microbes (fungi) in the wild (Darworth & Callan, 1996).

This could be the case for *Moringa oleifera* but more research needs to be done to prove this hypothesis.

Based on the results of this study, the effect of the age of the donor plant on contamination levels is not significant. Other factors such as explant source, explant size, explant age and species can also influence contamination levels *in vitro*. Also, although the use of a two-step (two-source) sterilization procedure has proven beneficial with certain species, perhaps it is not suitable for Moringa species. Or, the concentration and exposure times at which they were used was not adequate for this species.

Conclusion

The overall objective of this project was to develop a standardized *in vitro* sterilization method for field-grown *Moringa oleifera* explants. The results of this study indicate that field-grown Moringa explants, both juvenile and mature, have to be exposed to various *other* sterilization treatments until a proper one can be found that eliminates pathogens yet preserves the viability of the explants. Field-grown Moringa trees are not the best explant source so in order to establish culture *in vitro* different sterilization treatments have to be employed at different concentrations and different exposure times. This study provides insight in the use of sodium hypochlorite (4%) and ethanol (70%) as the main sterilizing agents for *M. oleifera*. It is evident that these two sterilizing agents, at the concentrations and exposure times used, are not suitable for the sterilization of *Moringa oleifera* field-grown plants. Oyebanji (2009) suggested that a balance between concentration and time must be determined empirically for each type of explant because of phytotoxicity.

Developing a standardized sterilization method for micropropagation of field-grown Moringa is not an easy task, but it is definitely not impossible. The results of this study provide baseline data for further research on the topic especially since sterilization protocols for the micropropagation of Moringa have not yet been established. As a matter of fact, one of the major challenges in this study was the lack of detailed reports on previous studies. Now that this study has come to an end, the next step towards finding the appropriate sterilization treatment for field-grown *M. oleifera* explants would be to test other treatment methods and/or vary the concentrations and exposure times of those sterilizing agents used in this study.

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