A comparison of callus production from *Moringa oleifera* Lam. leaf, cotyledon and stem explants using 2, 4-Dichlorophenoxyacetic acid and kinetin for media supplementation

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**ABSTRACT**
The study was aimed at investigating the most responsive part of *Moringa oleifera* Lam. that can serve as explant for *in vitro* culture propagation procedure. The phytohormones, 2, 4-Dichlorophenoxyacetic acid (2,4-D) at concentrations of 0.0, 2.5, 5.0, 7.5 and 10.0mg/l and kinetin at concentrations of 0.0, 0.1 and 1.0mg/l, either singly or in combination were used for supplementing Murashige and Skoog’s medium. The test parts of *M. oleifera* plant (leaf, cotyledon and stem) were each used to initiate *in vitro* cultures. After 3 weeks of culture initiation, the effectiveness of the morphogenetic response was determined using an assigned callus intensity scale. By a way of comparison, the results obtained indicated that callus can be generated from all the test plant parts, with the leaf tissue being the most responsive. The optimal amount of phytohormone combination for media supplementation was 5 mg/l 2,4-D and 0.1 mg/l kinetin. The stem explant seemed to be the least responsive for callus production.

**Keywords:** *Moringa oleifera, Callus, 2,4-Dichlorophenoxyacetic acid, Kinetin, Intensity*

**INTRODUCTION**
In recent years, interest has grown in the utilization of what has come to be known as “multipurpose” plants. One of such plants is *Moringa oleifera* Lam (Synonym *Moringa pterygosperma*), the most widely cultivated species of a monogeneric family Moringaceae. *Moringa oleifera* is one of the 14 species of family Moringaceae, native to India, Africa, Arabia, Southeast Asia, South America, and the Pacific and Caribbean Islands (Iqbal *et al.*, 2006). *M. oleifera* is naturalized in many tropics and sub-tropical regions worldwide, and as such, a number of names such as horseradish tree, drumstick tree, ben oil tree, miracle tree and mother’s best friend have been given to the plant. It is called ‘Shagara al Rauwaq’ in Nile valley (Maydell, 1986). The moringa tree was introduced to Africa from India at the turn of the twentieth century where it was used as a health supplement (McBurney *et al.*, 2004).

Every part of moringa tree, from the leaves to the seeds and the bark, down to the roots has beneficial properties that can serve humanity, especially as food and medicine. Elkhalifa *et al.* (2007) studied the nutritive values of the leaves of *M. oleifera* tree and showed that the moisture content was 74.42%, protein 16.7%, fiber 3.5%, ash 8%, and oil 1.7%. In addition, the mineral content was determined and they found that the calcium content was 0.20mg/100g, magnesium 0.13mg/100g, potassium 0.075mg/100g and phosphorus 0.031mg/100g. According to Jed (2005), *M. oleifera* provides 9 times the Iron in Spinach, 14 times the Calcium in milk, 2 times the Vitamin A in carrot, 2 times the protein in yogurt, 4 times the Potassium in bananas and 4 times the fiber
in oats. As a result of these properties, the plant has been used to combat malnutrition especially among infants and breast-feeding women in many developing countries, particularly in India, Pakistan, the Philippines, Hawaii and many parts of Africa (Fahey, 2005).

In order to maintain and sustain cultivation, conventional approaches, like seed planting and cutting have been used for propagation. Nonetheless, these conventional methods of plant propagation and improvement have limited applicability (Yadav et al., 2012). These limitations result in low yield of Moringa due to its low seed germination rate, viability and lack of vegetative propagation methods (Devendra et al., 2012). It has been observed that Moringa seeds should not be kept for more than three months, hence they lose viability as germination rate of seeds was shown to be reduced by 50% after 12 months of storage, with no seeds usually viable after two years (Bosch, 2004). *In vitro* propagation technique may serve as alternative or even complement conventional propagation of *M. oleifera* to make it always available. These limitations have necessitated clonal propagation of *M. oleifera* by *in vitro* technique.

In order to achieve success in the use of *in vitro* culture propagation technique, certain factors which include optimal physiological condition of donor plants, determination of the most productive explants, medium composition and media supplementation as well as the induction conditions must first be investigated (Shittu and Mgbeze, 2012; Can et al., 2008). This study was aimed at comparing callus generation from different parts (leaf, cotyledon, stem petiole and seed) of *M. oleifera* used as explants, employing standard *in vitro* technique and using defined medium, supplemented with various combinations of phytohormones.

**MATERIALS AND METHODS**

**Plant Materials**
Leaf, cotyledon and stem of *Moringa oleifera* Lam. were used as explants for callus induction. These were collected from the premises of Physiology Division, Nigeria Institute for Oil Palm Research (NIFOR), Benin City, Edo State, Nigeria.

**Media preparation and phytohormone supplementation**
Murashige and Skoog (1962) medium was used for callus induction from the various Moringa explants. The stock solution of macro A, micro B, MS vitamins and MS iron were prepared. Each salt was measured and dissolved in distilled water sequentially on a magnetic stirrer. The final solution was made up to 500ml with distilled water. The stock solutions were stored in plastic containers and placed in a refrigerator. The iron solution was wrapped with aluminum foil to prevent degradation.

Murashige and Skoog medium consisting of MS mineral salts, vitamins, 3% sucrose, 0.8% agar supplemented with 2,4-D (0.0, 2.5, 5.0, 7.5 and 10.0mg/l) and kinetin (0.0, 0.1 and 1.0mg/l), singly and in combination. The growth regulators were added to the culture media; pH was adjusted to 5.8±0.02 with 1N NaOH or 1N HCl before autoclaving at 121°C for 20mins. The media were allowed to cool before being used for culture initiation.

**Culture initiation and data collection**
The explants (leaf, cotyledon and stem) were washed thoroughly with distilled water several times and sterilized by placing them in a solution of 10% mercuric hypochlorite and were rinsed three times with sterile water. Scalpels, forceps, Petri dishes, spatulas and inoculation needle were wrapped in an aluminum foil and sterilized at 150°C for 3hours in an oven. During inoculation, the equipment was further sterilized by placing them in ethanol and flamed over a Bunsen burner.
The working surface of the laminar chamber was sterilized by wiping with ethanol and the sterile explants were inoculated into the MS media supplemented with the different concentrations of plant growth regulators. The McCantney bottles containing the media and explants (cultures) were placed in a growth chamber set at 25±2 °C, with 70 % relative humidity for incubation. After 3 weeks of culture initiation, the morphogenetic responses of the various explants were determined using assigned callus intensity on the following scale: -: No callus formation; +: Not profuse; ++: Slightly profuse; +++: Profuse and ++++: Very profuse.

RESULTS AND DISCUSSION

Callogenesis of the explants were observed 3 weeks after culture initiation. The results obtained when the various explants were cultured in MS medium, supplemented with 2,4-D alone (Table 1), kinetin alone (Table 2) and in combination (Table 3) are shown below. Callus was produced when 2,4-D alone was used to supplement MS medium at the various test concentrations, except in the control which had no 2,4-D (Table 1). The stem explant had the least morphogenetic response (+: not profuse). The concentration of 7.5mg/l 2,4-D seemed to be optimum for callus generation from leaf and cotyledon, as both of them gave a profuse (+++) callus intensity. There was a decrease in morphogenetic response of leaf and cotyledon explants at higher concentration (10mg/l) of auxin. Callus was not formed in the in vitro culture without cytokinin as shown in Table 2. Kinetin also induced callus formation in cultures initiated with the various test concentrations and the different types of M. oleifera explants, but at a reduced callus intensity, ranging from not profuse to slightly profuse, when compared with cultures containing 2-4-D. The best response in this category was obtained in the leaf culture when 0.1 mg/l kinetin was used for media supplementation, giving rise to culture of slightly profuse callus intensity.

The efficiency of callus production was much more increased when 2,4-D was combined with kinetin for media supplementation (Table 3). Callus generation was observed in all the test explants, except in the control experiments (cultures without phytohormones). The highest response was observed in the leaf culture when a combination of 5 mg/l 2,4-D and 0.1 mg/l kinetin were used for media supplementation giving rise to a culture that was very profuse (++++). The least responses were observed in the different stem cultures.

Table 1: Effects of 2,4-D in media supplementation on callus production from various Moringa oleifera explants

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>Intensity of callus formed (Assigned)</th>
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<tbody>
<tr>
<td></td>
<td>Leaf</td>
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-: No callus formation; +: Not profuse; ++: Slightly profusued; +++: Profusued; ++++: Very profuse.
Table 2: Effects of kinetin in media supplementation on callus production from various Moringa oleifera explants

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>Intensity of callus formed (Assigned)</th>
<th>Leaf</th>
<th>Cotyledon</th>
<th>Stem</th>
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<tr>
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- : No callus formation  +: Not profuse  ++: Slightly profuse  +++: Profuse  ++++: Very profuse  +++++: Highly profuse  NAA: Naphthalene acetic acid

Table 3: Effects of combinations of 2,4-D and Kinetin on callus production from various M. oleifera explants

<table>
<thead>
<tr>
<th>Concentration of Kinetin (mg/l)</th>
<th>Concentration of 2,4-D (mg/l)</th>
<th>Intensity of callus formed (Assigned)</th>
<th>Leaf</th>
<th>Cotyledon</th>
<th>Stem</th>
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- : No callus formation  ++: Slightly profuse  +++: Very profuse  ++++: Highly profuse  NAA: Naphthalene acetic acid

Callus can be obtained from different plant organs including leaf, seed, stem petiole, pollen, fruit, root and ovary. It is an important material for either directly regenerating plants or vegetative embryogenesis suspension culture. Every differentiated plant tissue is totipotent, but the conditions to dedifferentiate them vary from species to species and even from tissue to tissue within the same plant (Ezhova, 2003). In this study, the best morphogenetic response was obtained from the leaf explant when 2,4-D alone (7.5 mg/l), kinetin alone (0.1 mg/l) or in combination (5.0 mg/l 2,4-D and 0.1 mg/l kinetin) were used to supplement MS medium for callus production in M. oleifera. These gave callus intensities of profuse (+++), slightly profuse (+) and very profuse (++++) respectively. This observation could be due to the fact that the leaf parenchyma cells, especially the mesophylls are easily reprogrammed through dedifferentiation into undifferentiated cells with characteristics similar to meristematic tissues (Twumasi et al., 2009). With the exception of leaf epidermis, leaf cells have thin cell walls which are less lignified (Schadel et al., 2010) and this is likely to dispose them for dedifferentiation and callus formation. Passey et al. (2003) reported that callus induction was dependent on the explants source and types of tissue used. In the present
study, stem explant was the least responsive for callus generation in *M. oleifera* (not profuse in all the concentrations of phytohormone combinations).

Aside from the parts of plants used as explants, also, the types, concentrations and combinations of plant growth regulators are factors affecting callus induction as evident in this study. A combination of both 2,4-D and kinetin gave a better result in callus generation from *M. oleifera*. This observation is in agreement with the finding of Odewale *et al.* (1996) and Shittu *et al.* (2015) that callus induction was promoted on media with high level of auxin without cytokinin in oil palm leaf culture. There have been several reports that 2,4-D induces callus formation in a variety of species (Ma and Xu, 2002; Thao *et al.*, 2003). Callus induction through auxin-cytokinlin combination has been reported for several systems including *Diffenbachia, Chlorophyllum arundinaceum, Dioscoreophyllum cumminsii* and *Brassica napus* (Burbulis *et al*., 2007; Wang *et al*., 2006; Isikhuemen, *et al*., 2016). In this study it was observed that auxin-cytokinlin combinations improved callus formation with respect to explant type, phytohormone combinations and concentrations (Table 3).

**CONCLUSION**
The study showed that callus can be produced from *M. oleifera* Lam leaf, cotyledon and stem explants, when 2,4-D and kinetin are used alone or in combination for supplementing Murashige and Skoog’s medium. The most responsive explant for callus production was the leaf tissue, with optimal phytohormone combination of 5 mg/l 2,4-D and 0.1 mg/l kinetin, while the stem explant is least preferred.

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