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In vitro callogenesis of *Moringa oleifera* Lam. using seed explant

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ABSTRACT: The use of plant growth regulators in tissue culture media is critical for callogenesis. The type and concentration of auxin and cytokinin used have profound effect on callus induction. In this study, three types of auxin and one cytokinin were investigated for media supplementation. The auxins were naphthalene acetic acid (NAA), indole acetic acid (IAA) and 2,4-dichlorophenoxyl acetic acid (2,4-D), each at concentrations of 0, 2.5, 5.0, 7.5 and 10 mg/l. The cytokinin used was benzyl amino purine (BAP) at concentrations of 0, 0.1, 1.0 and 10.0mg/l. The possible combination of each auxin and BAP were observed for the best growth regulator when used singly or in combination to supplement Murashige and Skoog (MS) medium for callogenesis of *Moringa oleifera* seed culture. Callus was observed in medium fortified with all three auxins when used singly and in combination. The most effective auxin was 2,4-D. The rate of callogenesis increased when the auxins were in combination with BAP at (0-1.0 mg/l). There was no formation of callus in the medium supplemented with BAP at 10 mg/l when used singly or in combination with the various auxins. The maximum callogenesis observed was in medium fortified with 2,4-D at 2.5 mg/l and BAP at 1.0mg/l.

Key words: Moringa, Callogenesis, *In vitro*, Auxin, Cytokinin

Introduction

Moringa oleifera Lam. belongs to the flowering plant family, Moringaceae, order Brassicales and has its origin from Arabia and India. Common names include moringa, malunggay, benoil tree, West Indian ben, drumstick tree and horseradish tree (Nieves and Aspuria, 2011; Morton, 1991). The tree is now found in many semiarid, tropical and sub-tropical regions of the world. *Moringa* is a small, fast growing deciduous tree or shrub that reaches 12 m in height and 0.3 m in diameter. It has a wide, open, typically umbrella-shaped crown and usually a single stem. Its wood is soft and its bark is light. It tends to be deeply rooted (Olson and Carlquist, 2001; Olson, 2001).

M. oleifera can be taken as an example of a so called "multipurpose tree" and is usually referred to as "a miracle plant", because every part of the tree is said to have beneficial properties. It has been used for various purposes which include human food, animal fodder, natural medicines (Fahey, 2010; Peixoto *et al*., 2011), stimulant of milk production in lactating mothers (Stephenson and Fahey, 2004), water purification (Madsen *et al*., 1987; Mangale *et al*., 2012), fertilizer, living fence, alley cropping, natural pesticide, malnutrition relief, wood fuel and biodiesel (Rashid *et al*., 2008). Also, it has been found to be the most nutrient rich plant ever discovered. *Moringa* can be cultivated by direct seeding, transplanting and the use of hard stem cuttings, but there are several limitations associated with these propagation methods. Large area of farm land is required for its cultivation, because it thrives well at a planting distance of 1m x 1m. Using transplanting propagation method, very large number of planting materials is required. For example, 5 billion seedlings are required as planting materials for 500,000 ha of land. The conventional method of cultivating the plant using seeds is often associated with much genetic variability. More so, when stem cuttings are used for its propagation, it often requires hard labour, can lead to reduction in growth and yield, and at times death of mother plant can result (Islam *et al*., 2005). These limitations have necessitated clonal propagation of *M. oleifera* by *in vitro* technique.

The term "*in vitro*" culture is used broadly to refer to the culture of living materials such as seeds, embryos, organs, tissues, cells and protoplasts on nutrient media under sterile conditions (Pierik, 1984). Plant tissue culture is another term often used to refer to this procedure (Street, 1979). Basically, there are four types of *in vitro* cultures which include: callus, cell suspension, protoplast and organ cultures. Callus refers to rapidly proliferating undifferentiated mass of cells arising from an isolated differentiated tissue cultured on a nutrient medium containing specific growth hormones such as auxins and cytokinins (Skoog and Miller, 1957). Callus is produced on explants *in vitro* as a response to wounding and growth substances, either within the tissue or supplied in the medium. Auxin at a moderate to high concentration is the primary growth substance used to produce callus (Odewale, *et al*., 1996). The most common cell culture medium used in plant tissue culture is Murashige and Skoog (1962).

In vitro technique is rapidly becoming a commercial method for propagating new cultivars, rare species and difficult-to-propagate plant (Preece and Read, 1993). There have been few reports on the *in vitro* cultures of *M. oleifera*. Saini *et al*. (2012) developed a rapid *in vitro* micropropagation of *M. oleifera* (Variety- PKM-1). Callus has also been induced from the cotyledons (Nieves and Aspuria, 2011) and nodal explants taken from young seedlings or mature plants (Stephenson and Fahey, 2004).

The objective of this study was to induce callus from *M. oleifera* seed explants, employing standard *in vitro* techniques and using defined medium, supplemented with various combinations of phytohormones.

Materials and Methods

Plant materials

Matured pods containing viable seeds of *M. oleifera* were obtained from a home garden at Ikpoba Hill, Benin city, Edo State.

Establishment of *in vitro* culture of *Moringa oleifera*

This study was carried out using the facilities of Tissue Culture Laboratory of the Nigerian Institute for Oil Palm Research (NIFOR), Benin City. Full strength Murashige and Skoog (1962) medium, supplemented with 3% sucrose, phytohormones (as indicated in the next section) and 0.8% agar were prepared and the pH was adjusted to 5.7. An aliquot of about 10 ml was dispensed into 80 screw-cap McCartney bottles, autoclaved and allowed to cool at ambient temperature (28^{o}C). *M. oleifera* seeds were dehull from the pods and seed coats were later removed to expose the seeds. The seeds were surface sterilized using 10% hypochlorite solution (containing a drop of Tween-20) for 10 min. Traces of the sodium hypochlorite were removed by rinsing with sterile distilled water several times to get rid of the sterilizing solution which is toxic to the seeds at prolonged exposure. Each seed was cut into four parts and each part was aseptically transferred into the screw-cap McCartney bottles containing the culture medium using sterilized forceps (i.e. inoculation) and covering them up immediately. They were then incubated in the dark at 27 ^{0}C in the growth room for 4 weeks.

Phytohormone combination and supplementation

Three types of auxin and one cytokinin were used. The auxins were naphthalene acetic acid (NAA), indole acetic acid (IAA) and 2,4-dichlorophenoxyl acetic acid (2,4-D), each at concentrations of 0, 2.5, 5.0, 7.5 and 10 mg/l. The cytokinin used was Benzyl amino purine (BAP) at concentrations of 0, 0.1, 1.0 and 10.0mg/l.

(a) Testing the effects of 2,4-D and BAP

The auxin, 2,4-D at concentrations of 2.5, 5.0, 7.5 and 10.0 mg/l and BAP at concentrations of 0.1, 1.0 and 10.0 mg/l were combined. Control experiment without 2,4-D and BAP was also included. The possible combinations for the various concentrations of the two plant growth regulators (2,4-D and BAP) led to an experiment with 20 treatments, with each treatment having 3 replicates.

(b)Testing the effects of NAA and BAP

NAA at concentrations of 2.5, 5.0, 7.5 and 10.0 mg/l, and BAP at concentrations of 0.1, 1.0 and 10.0 mg/l were added. Control experiment without NAA and BAP was also included. The possible combinations for the various concentrations of the two plant growth regulators (NAA and BAP) led to an experiment with 20 treatments, with each treatment having 3 replicates.

(c) Testing the effects of IAA and BAP

IAA at concentrations of 2.5, 5.0, 7.5 and 10.0 mg/l, and BAP at concentrations of 0.1, 1.0 and 10.0 mg/l were combined. Control experiment without IAA and BAP was also included. The possible combinations for the

various concentrations of the two plant growth regulators (IAA and BAP) led to an experiment with 20 treatments, with each treatment having 3 replicates.

Results

In this study, callogenesis of the seeds of *M. oleifera* was detected two weeks after culture initiation. The results obtained on the effect of different concentrations of BAP and 2,4-D when used to supplement MS medium are shown in Table 1. The cytokinin, BAP was effective alone in the initiation of callus. Similarly, callus was also initiated with 2,4-D alone at the various concentrations (0-10 mg/l), but optimal concentration for callus initiation was obtained at 2,4-D concentration of 7.5 mg/l. Callus was induced with 2,4-D in combination with BAP at the various levels of concentration. The addition of BAP (0-0.1 mg/l) to the culture medium containing different 2,4-D levels greatly stimulated further callus proliferation beyond the levels obtained for 2,4-D alone. Fresh weight of callus (assigned) or callus intensity was highest at 2.5 mg/l 2,4-D concentration in combination with 1.0 mg/l of BAP. However, there was no callus formation when the concentration of BAP was increased to 10 mg/l at all 2,4-D concentration.

Table 1: Effects of different concentrations of BAP and 2,4-D media supplementation on callogenesis of *Moringa oleifera* seed explant two weeks after culture initiation

Concentration of phytohormones (mg/l)		Weight of callus generated (assigned)	Intensity of callus formed
BAP	**2,4 D**		
0.0	0.0	0.15	+
	2.5	0.21	+ +
	5.0	0.23	+ +
	7.5	0.23	+ +
	10.0	0.12	+
0.1	0.0	0.21	+ +
	2.5	0.33	+ + +
	5.0	0.31	+ + +
	7.5	0.22	+ +
	10.0	0.21	+ +
1.0	0.0	0.33	+ + +
	2.5	0.55	+ + + + +
	5.0	0.42	+ + + +
	7.5	0.33	+ + +
	10.0	0.25	+ +
10.0	0.0	0.0	-
	2.5	0.0	-
	5.0	0.0	-
	7.5	0.0	-
	10.0	0.0	-

-: No callus formation; +: Not profuse; ++: Slightly profuse; +++: Profuse callus; ++++: Very profuse; +++++: Highly profuse; BAP: benzyl amino purine; 2,4-D: 2,4-dichlorophenoxyl acetic acid

Callus initiation was effective when either BAP or NAA was used alone or in combination at the various range of concentrations (0-10 mg/l) for media supplementation (Table 2), but the optimal concentration of NAA for callus initiation was obtained at concentration of 10 mg/l. Callus was induced with NAA in combination with BAP at the various concentrations. The combination of BAP (0-0.1 mg/l) and NAA in the culture media greatly stimulated further callus proliferation more than that obtained for NAA alone. Mean fresh weight of callus or callus intensity was highest at NAA concentration of 2.5 mg/l in combination with 1.0 mg/l of BAP. Callus was not formed at any BAP concentration beyond 1.0 mg/l.

Table 2: Effects of different concentrations of BAP and NAA media supplementation on callogenesis of *Moringa oleifera* seed explant two weeks after culture initiation

Concentration of phytohormones (mg/l)		Weight of callus generated (assigned)	Intensity of callus formed
BAP	**NAA**		
0.0	0.0	0.12	+
	2.5	0.13	+
	5.0	0.11	+
	7.5	0.11	+
	10.0	0.20	++
0.1	0.0	0.20	+ +
	2.5	0.21	+ +
	5.0	0.21	+ +
	7.5	0.21	+ +
	10.0	0.20	+ +
1.0	0.0	0.22	+ +
	2.5	0.40	+ + + +
	5.0	0.30	+ + +
	7.5	0.21	+ +
	10.0	0.20	+ +
10.0	0.0	0.10	+
	2.5	0.00	-
	5.0	0.00	-
	7.5	0.00	-
	10.0	0.00	-

-: No callus formation; +: Not profuse; ++: Slightly profuse; +++: Profuse callus; ++++: Very profuse; +++++: Highly profuse; BAP: benzyl amino purine; NAA: naphthalene acetic acid

Table 3 shows the results obtained on the effect of different concentrations of BAP and IAA. Callus was also initiated with IAA alone at the various ranges of concentration (0-10 mg/l), but optimal concentration of IAA for callus initiation was obtained at concentration of 10 mg/l. When the hormones were combined, **c**allus was induced with BAP at the various concentrations. The addition of BAP (0-1.0 mg/l) to the culture medium greatly stimulated further callus proliferation beyond the levels obtained for IAA alone. Fresh weight of callus (assigned) or callus intensity was highest at IAA concentration of 5.0 mg/l in combination with 1.0 mg/l of BAP. Callus was not formed at all BAP concentration beyond 1.0 mg/l.

Table 3: Effects of different concentrations of BAP and IAA media supplementation on callogenesis of *Moringa oleifera* seed explant two weeks after culture initiation

Concentration of phytohormones (mg/l)		Weight of callus generated (assigned)	Intensity of callus formed
BAP	**IAA**		
0.0	0.0	0.10	+
	2.5	0.20	+ +
	5.0	0.20	++
	7.5	0.21	+ +
	10.0	0.23	++
0.1	0.0	0.20	+ +
	2.5	0.21	+ +
	5.0	0.30	+ ++
	7.5	0.31	+ ++
	10.0	0.21	+ +
1.0	0.0	0.10	+
	2.5	0.30	+ + +
	5.0	0.40	+ + ++
	7.5	0.20	+ +
	10.0	0.10	+
10.0	0.0	0.00	-
	2.5	0.00	-
	5.0	0.00	-
	7.5	0.00	-
	10.0	0.00	-

-: No callus formation; +: Not profuse; ++: Slightly profuse; +++: Profuse callus; ++++: Very profuse; +++++: Highly profuse; BAP: benzyl amino purine; IAA: indole acetic acid

The effects of the various auxins in combination with 1 mg/l of BAP in the callogenesis of *M. oleifera* seed explants are shown in Table 4. From the results, 2.4-D was the most effective auxin on callogenesis. IAA was less effective than NAA, whereas 2,4-D and NAA had their optimal callus formation at 2.5mg/l, optimal callogenesis of IAA was at 5.0mg/l.

Table 4: Callus obtained from *Moringa oleifera* seed explants with either 2,4-D, NAA or IAA in combination with 1mg/l BAP used for media supplementation

Auxins (2.5 mg/l)	Intensity of callus formed
2,4-D	+++++
NAA	++++
IAA	+++

-: No callus formation; +: Not profuse; ++: Slightly profuse; +++: Profuse callus; ++++: Very profuse; +++++: Highly profuse; 2,4-D: 2,4-dichlorophenoxyl acetic acid; NAA: Naphthalene acetic acid; IAA: Indole acetic acid; BAP: Benzyl amino purine.

Discussion

The growth regulators (auxin and cytokinin) used in this study for callus induction were effective when used alone and also in combination. Callus generation increased in the medium supplemented with each of the auxins when BAP (0 -1.0 mg/l) was added. It can be deduced from the work of Eufrocinio (2010) that BAP is very effective in *in vitro* multiplication of *Moringa oleifera* from nodal sections of young, aseptically-grown seedlings as it was the best for inducing axillary shoots. The use of auxins in the supplementation of MS medium for callogenesis of *Moringa oleifera* is effective but the addition of BAP at 0-1.0 mg/l to the auxins used in this experiment was critical for profuse callus formation. This finding is evident from Table 1-3 presented. It is also important to note that all concentrations of BAP beyond 1.0 mg/l did not induce callus. High amount of cytokinin in culture medium has been implicated for shoot induction. For callus generation, a balanced amount of auxin and cytokinin is required. In this study, BAP concentrations beyond 1.0 mg/l inhibited callus generation from *M. oleifera* seed explant. There was no callus proliferation in the medium supplemented with 10 mg/l BAP when used alone or in combination with 2,4-D, NAA or IAA. From the results it can be inferred that the levels of BAP concentration can either be a booster or a retardant when combined with auxins in the callogenesis of *M. oleifera* seeds. Low concentrations of BAP between 0-1.0 mg/l increased the effectiveness of the auxin present in the medium to induce callogenesis, while high concentration of 10 mg/l BAP did not support tissue dedifferentiation of explants. A similar observation has been reported on *M. oleifera* cotyledon (Nieves and Aspuria, 2011).

The most effective concentration of BAP for callogenesis of moringa seed is 1.0 mg/l, as the highest fresh weight and callus intensity were observed at this concentration, when combined with any of the three auxins used in this experiment. According to Islam *et al.,* (2005), BAP at 1.0mg/l is the most effective for callus and shoot initiation of *Moringa oleifera* nodes. Their results showed that BAP at 1.5mg/l and 1mg/l aided callus and shoot proliferation but more shoots were observed in the medium supplemented with 1mg/l BAP. The auxins used in this study were all effective for callus initiation and proliferation at the various levels (0-10 mg/l) but 2,4-D was the most effective. This finding is in line with the study of Lalida *et al*. (2013) in their experiment to induce callus from the leaves, stem and root of sterile *M. oleifera* plantlets where 2,4-D was selected and used to supplement the medium. The auxin, 2,4-D was selected because of its well known ability to induce callus.

The results obtained from this study suggested that callus can be induced from seeds of *Moringa oleifera* using standard MS medium supplemented with 2,4-D, NAA and IAA individually, and in combination with BAP at 0-1.0 mg/l. For optimal callus proliferation 2.5 mg/l 2,4-D and 1 mg/l BAP should be used to supplement MS basal medium.

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