Development of *in vitro* clonal propagation protocols for *Moringa oleifera*

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Abstract

Conventional propagation of *Moringa oleifera* involves the use of seeds and cuttings. However, being a crop of high economic value and demand, it is required to apply fast and efficient biotechnological methods to propagate this crop and improve traits of interest in order to ensure its availability in the market. As such, *in vitro* clonal propagation protocols for *Moringa oleifera* were developed with an objective of determining early graft compatibility between *M. oleifera* and *M. stenopetala.* Leaves obtained from greenhouse grown *Moringa oleifera* and *Moringa stenopetala* plants were used for callus induction and fusion experiments. Leaves from both species were cultured on Murashige and Skoog (MS) medium supplemented with six different growth regulators [(0.2 mg $^{-l}$ α-naphthalene acetic acid (NAA)), (0.2 mg $^{-l}$ Dichlorophenoxyacetic acid (2,4D)), (0.02 mg $^{-l}$ Thidiazuron (TDZ)), (0.2 mg $^{-l}$ NAA + 0.02 mg $^{-l}$ TDZ), (0.2 mg $^{-l}$ 2,4D + 0.02 mg $^{-l}$ TDZ) and (0.2 mg $^{-l}$ NAA + 0.2 mg $^{-l}$ 2,4D + 0.02 mg $^{-l}$ TDZ)]. Two pieces of callus were excised and co-cultured on MS medium supplemented with 0.02 mg $^{-l}$ TDZ in combination with 0.2 NAA mg $^{-1}$. After a month of culturing, co- cultures were prepared for viewing under a light microscope to determine compatibility by the presence of a necrotic line, union line and phenolic compounds. Combinations of 0.02 mg $^{-l}$ TDZ and 0.2 mg $^{-l}$ NAA were effective supplements for callus induction. *Moringa oleifera* and *Moringa stenopetala* co-cultures had high phenolic deposits at the graft interface. The high accumulation of phenolic compounds at graft interfaces is highly associated with incompatibility since these compounds prevent auxin transport. Consequently, the species were regarded as incompatible.

Keywords: growth regulators, callus, induction, compatibility

INTRODUCTION

Moringa oleifera is a crop of the tropics and sub-tropics native to the Sub- Himalayan tracts of India, Bangladesh and Afghanistan (Förster et al., 2013). It belongs to the family Moringaceae which consists of 12 other species (Pandey et al., 2012). As the most popular and most cultivated crop amongst its close relatives, *M. oleifera* has spread to various tropical destinations in the African continent (Förster et al., 2013).

Historically, the leaves, seeds, barks and roots of *M. oleifera* have been consumed as food or utilised as traditional medicine (Jaja-Chimedza et al., 2017). Ever since its discovery, *M. oleifera* has received enormous scientific attention due to its significant nutritious and medicinal value (Kayalto et al., 2013). The presence of secondary metabolites in rich amounts, forms the bases for this plant's powerful healing potential (Förster et al., 2013). According to Bennett et al. (2003) all plant parts of *M. oleifera* contain glucossinates which have a reputable value in detoxifying the human body against carcinogens. Moreover, scientific studies prove that *M. oleifera* has anti-inflammatory (Jaja-Chimedza et al., 2017), antioxidant (Ademiluyi et al., 2018), and anti-microbial activities (Karim and Azlan, 2012).

For a crop displaying so much potential, it is imperative to find ways of accelerating its production and maintain it throughout the year in order to satisfy its demand. In addition, the threats of climate change accentuate the need to apply different technologies such as clonal propagation and breeding to develop plants that will withstand the harsh and long dry periods currently tormenting crop production. Micropropagation presents an effective system to produce crops throughout the year since growing conditions are controlled hence production is independent of seasons.

Studies show that rapid and mass propagation of *Moringa oleifera* through tissue culture is feasible (Ibrahim and Ameen, 2017; Jun-jie et al., 2017; Li et al., 2016) but so far, no information has been reported on compatibility within the moringa genus. Grafting *M. oleifera* with a more drought tolerant specie such as *Moringa stenopetala* is crucial and will help counteract the effects of climate change. Grafting is a traditional agricultural practice for crop improvement commonly applied by commercial fruit tree growers to improve fruit quality, improve disease resistance and hardiness amongst other reasons (He et al., 2018). However, this technique has an inevitable drawback known as graft failure which is more prominent in distantly related species (Gainza et al., 2015). This is a limiting factor in orchard development and the breeding of rootstocks mainly because it takes long periods to be fully expressed, sometimes taking longer than five years (Assunção et al., 2016; Mahunu et al., 2013). Hence, the recognition of graft incompatibility at an early stage is necessary.

The cause of graft failure is complex and varies from being triggered by physiological changes, a biochemical change and/or anatomical defects of the vascular systems (Mahunu et al., 2013). According to He et al. (2018) auxins play a significant role in regulating developmental growth and metabolic reactions hence could be one of the mechanisms involved in the physiological effects of grafting.

Although graft incompatibility is regarded as complex, early detection methods have been developed and include *in vitro* techniques such as callus fusion and micro grafting, histological studies and phenolic examination (Errea et al., 2001). Mng'omba et al. (2008) detected graft compatibility of *Uapaca kirkiana* provenances and species using callus fusion techniques where compatibility was indicated by the presence of a union line, phenolic deposit at the graft interface and necrotic layers in a short period. In a different approach Zarrouk et al. (2010) evaluated peroxidase activities at the graft interfaces of Prunus species providing early diagnosis of graft incompatibility which was determined by the display of high peroxidase activity in incompatible grafts.

This study was thus conducted with the aim of establishing an efficient protocol for callus induction and results were further used for detecting early graft incompatibility between *Moringa oleifera* and *Moringa stenopetala* using a callus fusion technique.

MATERIALS AND METHODS

Plant material

The experiment was conducted in a tissue culture laboratory situated in the Hatfield Experimental farm of the University of Pretoria. Species and varieties selected for this study were; *Moringa oleifera* seeds (PKM-1) sourced from India and *Moringa stenopetala* seeds sourced from Hawaii. Seeds of these species were germinated in a greenhouse and leaf explants for callus induction were derived from these pot plants.

Surface decontamination of leaf explants, culture media and growth conditions

Leaves sourced from green house plants were sterilised following a series of washing starting with a quick wash in a 50% ethanol solution for 30 seconds, followed by another wash with 10% detergent solution for 5 minutes and lastly rinsing with water for 10 minutes 3 times. Half strength MS medium (Murashige and Skoog, 1962) was used for all experiments. MS medium was autoclaved at 121°C for 20 min and all cultures were kept under a daily

illumination of 16 hours using cool -white fluorescent tubes which provided a photosynthetically active radiation of 60 µmol m^{-2} s^{-1}. Temperature was maintained at 25± 2°C.

Callus induction

Leaves were cut into sections and cultured abaxially on 10ml MS medium dispensed in test tubes. Concentrations and combinations of the growth regulators used were 0.2 mg $^{-l}$ α-naphthalene acetic acid (NAA), 0.2 mg $^{-l}$ Dichlorophenoxyacetic acid (2,4D), 0.02 mg $^{-l}$ Thidiazuron (TDZ), 0.2 mg $^{-l}$ NAA + 0.02 mg $^{-l}$ TDZ, 0.2 mg $^{-l}$ 2,4D + 0.02 mg $^{-l}$ TDZ and 0.2 mg $^{-l}$ NAA + 0.2 mg $^{-l}$ 2,4D + 0.02 mg $^{-l}$ TDZ . The experimental layout was a completely randomised design. Each treatment consisted of 10 explants and replicated 4 times. Data collected included number of contaminated cultures and callus fresh mass.

Callus fusion

Two pieces of callus (2mm) each from both species were excised and placed side by side in a thermal polyethylene ring half inserted on MS medium. The medium was supplemented with 0.02 mg $^{-l}$ TDZ and 0.2 NAA mg $^{-l}$. Co-cultures were arranged in a completely randomised design with each treatment replicated 10 times. Cultures were incubated in a growth chamber set at 25± 2°C for a month.

Callus co-culture fixation and staining

Co-cultures were removed from MS medium and placed in clean polyethylene tubes. The tubes were filled with Formalin acetic acid and left for 7 days. Samples were dehydrated in series of ethanol (30%, 50%, 70%, 100% and 100%) each lasting for a week. This was followed by dehydration in a series of graded xylene following the same procedure. Specimens were then filled with paraffin wax and placed in an oven at 40 ^{o}C for 3 days after which the wax was discarded. This process was repeated four times and immediately after the fourth trial, specimens were embedded in paraffin wax and dissected using a thermolyne sabron equipment and a Stereo star zoom autocut 2040 model respectively. Cutting was done at a right angle to the callus union and union cuts were prepared on microscope slides. Slides were stained by dipping into safranin for 2 hours, dehydrated in a series of graded ethanol (30%, 60%, 100% and 100%) and dipped into fast green for 2 minutes followed by dewaxing in a series of graded xylene (30%, 60%, 100% and 100%). Specimens were then viewed under a light microscope connected to a digital camera and microphotographs of callus union interfaces were taken.

Statistical analysis

Data on culture contamination and callus fresh mass were subjected to analysis of variance using SAS statistical software and significant differences among treatments means were determined using Duncan's test at 5% level ($p \leq 0.05$). Visual scoring was done for the micro-graft union line where 1 represented visibly high, 2 = faint, 3 = absent), for necrotic layer; 1 represented visible, 2 = faint, 3 = absent) and for phenolic; 1 represented high, 2 = medium, 3 = low, 4 = absent). The scores were converted into percentages and then subjected to correspondence analysis using R studio data analysis software. A perceptual map was drawn to show distribution and association of different callus co-cultures with respect to the presence of necrotic layer, phenol deposit and the union line.

RESULTS AND DISCUSSIONS

Effect of surface decontamination

Contamination is one of the limiting factors in generating *in vitro* plantlets. Hence, it is very crucial to optimise sterilising procedures in order to avoid losses. In this study, there

were no significant differences (P ≤ 0.05) between species and culture media with regards to contamination. Contamination was low and the protocol presented contamination percentages varying from 5% to 45% respectively (Figure 1). This result shows that *M. oleifera* leaf explants can be sterilised effectively using this protocol. However, other authors have reported a contamination percentage as low as 2% when using other protocols.

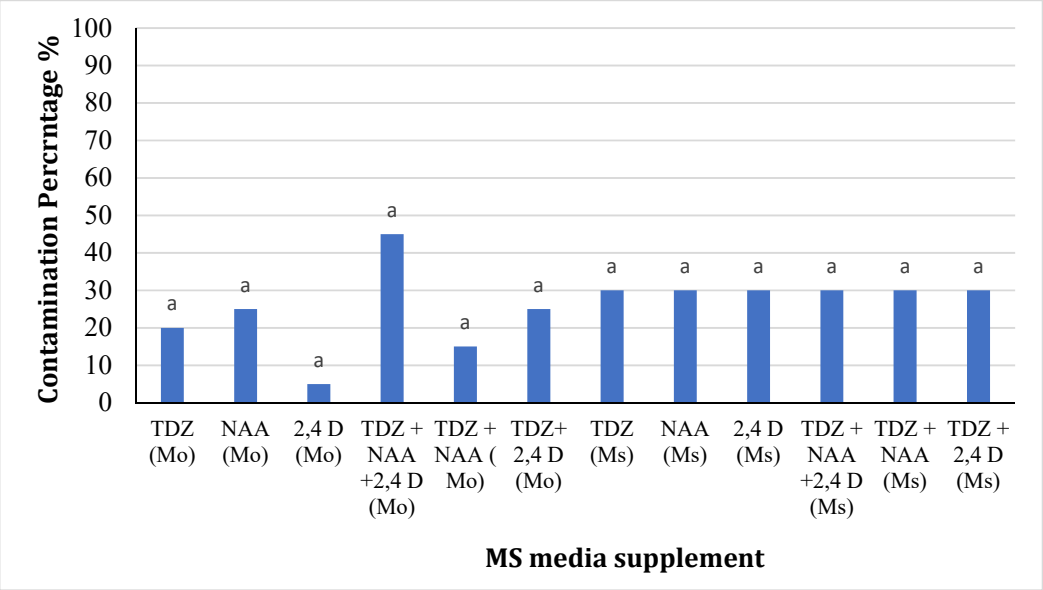

Figure 1: Contamination percentage of *Moringa oleifera* (Mo) and *Moringa stenopetala* (Ms) callus cultures. TDZ, NAA and 2,4D represent Thidiazuron, α-naphthalene acetic acid and Dichlorophenoxyacetic acid, respectively. Bars with the same letter are not significantly different according to Duncan's multiple range test at 5% level.

Effect of plant growth regulators on callus induction for *M. oleifera* and *M. stenopetala* leaf explants

The addition of growth regulators in a culture medium is a technique commonly applied to optimise the regeneration of plant tissues (Li et al., 2016). Different growth regulators were used to induce callus from leaf explants. The results showed significant differences (P ≤ 0.05) among species and the different treatments of growth regulators used. The use of TDZ in combination with either NAA or 2,4-D led to vigorous callus formation in both *Moringa species* with *M. stenopetala* (Ms) leaf explants inducing the highest callus fresh mass (1.99g) (Figure 2). Callus induction was observed in all *Moringa oleifera* (Mo) treatments however, biomass accumulation was significantly low compared to *M. stenopetala*. The significant difference in callus formation between the two species could be attributed to the fact that the amount of endogenous plant growth regulators found in a plant together with the exogenous application of growth regulators have an equally important effect to the response of tissues in a culture medium. Huang et al. (2012) reported that endogenous and exogenous growth regulators are both key factors in triggering cell differentiation *in vitro.* Similarly, Huang et al. (2012) found that rice callus with high levels of endogenous growth regulators have a high regeneration ability. *M. oleifera* probably has less endogenous growth regulators compared to *M. stenopetala.* This suggest that a higher concentration of growth regulators will have to be applied in *M. oleifera* culture media inorder to achieve a result like that of *M. stenopetala.*

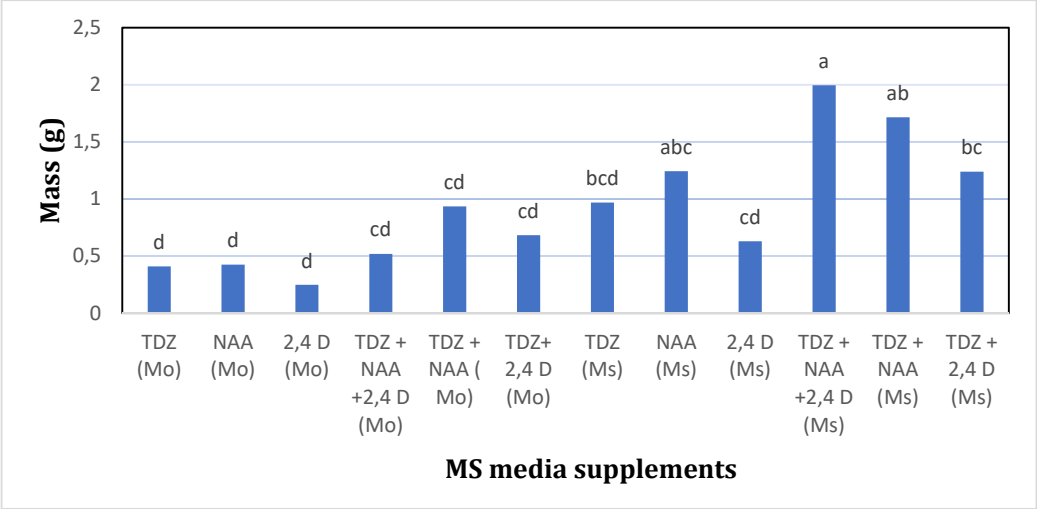

Figure 2. Fresh mass (g) of *Moringa oleifera* (Mo) and *Moringa stenopetala* (Ms) callus cultures. TDZ, NAA and 2,4D represent Thidiazuron, α-naphthalene acetic acid and Dichlorophenoxyacetic acid, respectively. Bars with the same letter are not significantly different according to Duncan's multiple range test at 5% level.

Callus union interfaces of *M. oleifera* and *M. stenopetala* homografts.

According to Musacchi et al. (2000) phenolic compounds are involved in the chemical defence strategy of plants. However, they also could inhibit regeneration, development and differentiation of cells, thus conferring their production very crucial in the grafting of plants. In the present study, the presence of phenolic compounds and a visible union line at the contact surfaces of two pieces of callus fused together were detectors of compatibility and incompatibility. Distribution and association of the different callus combinations with respect to the presence of phenol deposit, union line and cell necrosis is shown in Figure 3. *Moringa oleifera* homografts and *Moringa stenopetala* homografts are close together in the top and bottom right quadrants indicating a strong association and compatibility.

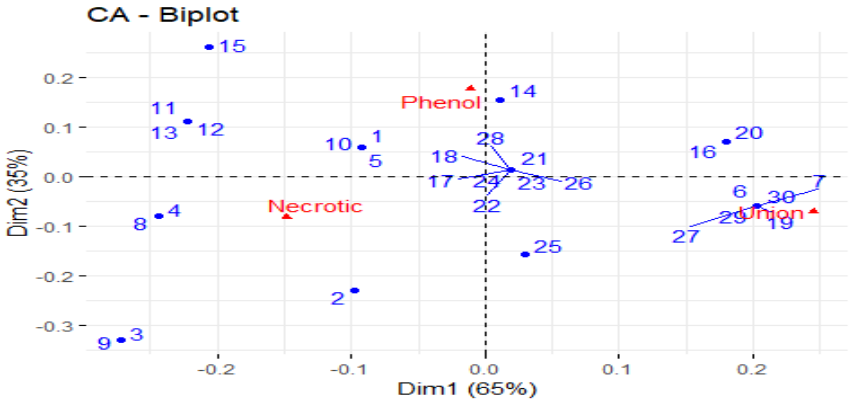

Figure 3. Distribution and association of *M. oleifera* homografts (11-20), *M. stenopetala* homografts (21-30) and *M. oleifera + M. stenopetala* grafts (1- 10) with respect to union line, necrosis and phenolic deposits from correspondace analysis.

Graft interfaces of *M. oleifera* homografts (Figure 4) show a faint union line aligned diagonally from the bottom left corner to the top right corner implying an outright compatibility. Similarly, *M. stenopetala* homograft also shows a complete cell union with a

visible union line aligned vertically in the middle. For that reason, both homografts were considered compatible when grafted within the same species.

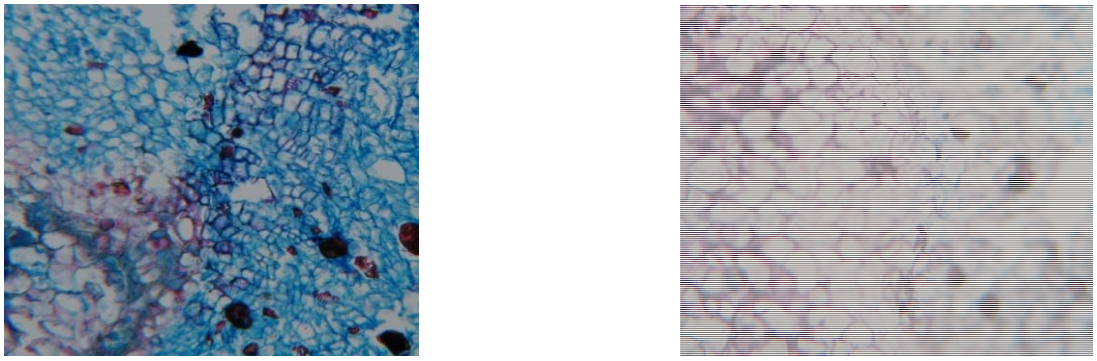

Figure 4. Callus union interfaces for *Moringa oleifera* (left)and *Moringa stenopetala* (Right)

Callus union interfaces of *M. oleifera* and *M. stenopetala* heterografts

Co- cultures of *M. oleifera* and *M. stenopetala* were highly saturated with phenolic compounds indicated by the browning of callus in Figure 5. As a result, callus cells at the graft interface were supressed and could not grow any further to unify. When removed from the rings*, M. oleifera* and *M. stenopetala* heterografts could easily separate (Figure 5) suggesting that they were not firmly attached at the contact surfaces. The browning of callus at the graft interfaces was also evident in the separated grafts.

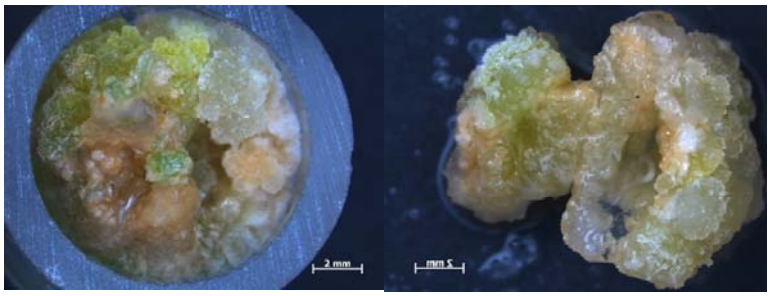

Figure 5. *M. oleifera* and *M. stenopetala* co-cultures in polyethylene rings (left) and after they were removed from rings (right)

Figure 6 shows a visible seperation at the union running diagonally from the bottom left corner with necrotic layers on either ends, highly accumulated with phenolics. Consequently, *Moringa oleifera and Moringa stenopetala* graft partners are spread in the top and bottom left quadrants (Figure 3) signaling high association with incompatibility. According to Gainza et al. (2015) it is common for heterografts to be incompatible and this compatibility is mainly caused by biochemical and functonal alterations at the graft interface. As already highlighted, phenolics implicate a lot of biochemical pathways responsible for graft union formation. Auxin translocation towards the union site is one of the important processes hindered by a pronounced accumulation of polyphenols at the graft interface (Errea et al., 2001). According to Koepke and Dhingra (2013) the lack of auxins at the graft interface renders graft union formation impossible as these hormones are key factors in triggering differentiation of vascular tissues, aswell as lignification. The influence of phenolic compounds towards graft union formation is thus indisputable. However, the quality and quantity of phenols at the graft surface is also important to consider as phenols may be present in both compatible and incompatible combinations (Mng'omba et al., 2008). According to Gainza et al. (2015) perfoming a metabolomic analysis to study the nature and

quantity of phenolic compounds at the graft interface is an accurate approach. Moreover, the production of secondary metabolites e.g. phenols by plants varies with seasons hence it is very imperative to investigate graft compatibility at different seasons before declaring the species incompatible.

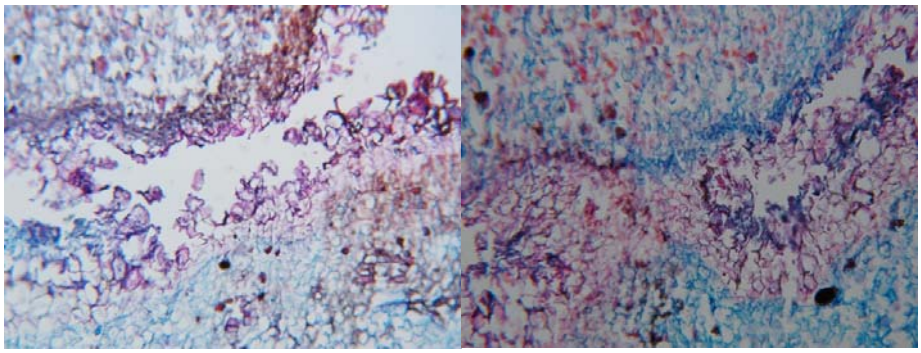

Figure 6: Graft interfaces of *M. oleifera* and *M. stenopetala* heterografts

CONCLUSIONS

In the present study, *in vitro* propagation of moringa through callus cultures was investigated using different growth regulators. Lower concentrations of TDZ (0.02 mg $^{-l}$) had a synergistic effect towards callus induction when combined with either NAA or 2,4D hence the addition of TDZ is recommended in order to achieve optimal proliferation of Moringa callus cultures. Graft compatibilty was consequently assesed and the presence of a union line and the presence of phenolic compounds at the graft interface were the main indicators. *M. oleifera* and *M. stenopetala* homografts were compatible despite the presence of phenolic compounds in some of the grafts. Callus fusion between *M. oleifera* and *M. stenopetala* was unsuccesful and this was caused by the excess accumulation of phenolic compounds at the graft interfaces which suppressed growth of callus and prevented auxin transport. Further investigation on the nature and quantity of phenols causing disfunctions at the graft interface are required to provide a solid credence to this phenomenon.

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