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Improving micropropagation of *Moringa oleifera*: The use of semi-solid medium for rooting and sucrose-free liquid medium combined with temporary ventilation for hardening

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ABSTRACT

Moringa oleifera is a multi-purpose tree that is now becoming popular as an economic crop due to increasing discoveries of its nutritional and medicinal properties. The present investigation is aimed at improving the micropropagation of moringa, particularly the acclimatization of plantlets, which is still problematic due to the high mortality observed when the micropropagated plantlets are transferred in the field. Rooting of plantlets was done using a semi-solid medium solidified with 4 g/L agar, which was found to support growth and minimize root injury when the plantlets were taken out of the medium. The rooted plantlets were then subjected to *in vitro* hardening using a sucrose-free liquid medium combined with temporary ventilation through the uncapping of the culture tubes for 20 min in three consecutive days. This has effectively solved the rapid wilting observed when tissue culture-derived moringa plantlets were transferred into the soil. The improved protocol described in the present paper resulted in a very high survival rate (>98%) and is expected to facilitate micropropagation of *M. oleifera* on a commercial scale.

1. INTRODUCTION

Moringa oleifera Lam. is a multi-purpose tree belonging to the family *Moringaceae* [1]. Its immature pods, flowers, and leaves are consumed as vegetables and medicine [2]. The popularity of *M. oleifera* in recent years was due to increased knowledge and discoveries of its nutritional and medicinal properties [3]. The leaves are the most nutritious part of the plant, containing 9 times more protein than yoghurt, 10 times more vitamin A than carrots, 7 times more vitamin C than oranges, 25 times more iron than spinach, 17 times more calcium than milk, and 15 times more potassium than bananas [4].

M. oleifera is steadily gaining popularity as an economic crop, with market demand for moringa products increasing annually. In 2021, the world market for moringa products was estimated at USD 7.79 billion. With an annual growth rate of 9.63%, it is expected to grow to USD 14.80 billion in 2028 [5]. To attain moringa industrialization, commercial farms are needed to be established [3,6]. Consequently, this will require a large number of planting materials for its rapid mass propagation.

M. oleifera is conventionally propagated using seeds [7] or stem cuttings [8]. Plants obtained from seeds vary in genotype and phenotype, which leads to variations in yield quality and quantity [9]. They also have a relatively long juvenile phase [10]. In addition, the viability of seeds tends to decline rapidly [11]. The use of stem cuttings, on the other hand, is not practical because of the limited number of moringa trees currently available for use as mother plants. Moreover, obtaining stem cuttings from the mother plant often reduces growth and yield [12]. An alternative approach to meet the large requirement for planting materials is through tissue culture. There are a number of published reports on the plant tissue culture of *M. oleifera* for its mass propagation [8,9,12-20], but their application on a commercial scale has not been realized because the acclimatization of plantlets transferred *ex vitro* is still problematic due to the high mortality rate of more than 50% contributed by root injury and rapid wilting.

Tissue culture-derived moringa plantlets have a very weak root system. During *ex vitro* transfer, their roots are easily injured when taken out of the agar-solidified medium, resulting in poor water and nutrient absorption. They also wilt rapidly just minutes after uprooting from the culture medium, primarily due to their thin leaves with less cuticular waxes and opened stomates, resulting in poor regulation of water loss. Hence, moringa plantlets transferred *ex vitro* are initially hardened by maintaining them in a condition with high relative humidity, either in a mist or humidity chamber [17,20] or by covering individual plantlets with a polythene bag [13-15,18-19]. Both of these hardening techniques are labor-intensive and time-consuming, requiring at least 3 months to complete.

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The root injury incurred in the micropropagation of moringa can be solved by using a softer, semi-solid medium. The rapid wilting, on the other hand, can be solved by *in vitro* hardening, wherein the rooted plantlets are transferred into a liquid medium without sucrose to induce photoautotrophic growth. Removal of sucrose is also to reduce the chance of microbial contamination when the plantlets are gradually exposed to the ambient environment for ventilation. This study was aimed to improve the acclimatization of tissue culture-derived *M. oleifera* plantlets by using a semi-solid medium for rooting, then transferring the rooted plantlet to a sucrose-free liquid medium combined with temporary ventilation for hardening prior to transfer to soil.

2. MATERIALS AND METHODS

2.1. Culture, Medium, and Growth Condition

Except when otherwise stated, all culture media used were Murashige and Skoog's (MS) basal medium, [21] supplemented with 30 g/L sucrose and 0.1 mg/L naphthaleneacetic acid. The solid medium was supplemented with 8 g/L of agar. The pH of the medium was adjusted to 5.8, and 10 mL of the culture medium was dispensed into culture tubes (15 cm height × 2.5 cm diameter), covered with plastic caps, and then autoclaved at 121°C for 20 min. All cultures were kept in a growth room and maintained under 16-h/day light conditions at an intensity of 50 μmol/m^2/s from cool white fluorescent lamps at 25 ± 2°C.

2.2. Establishment and Maintenance of *M. oleifera* Tissue Cultures

Establishment and maintenance of moringa tissue cultures were performed as described previously [13]. Unopened mature moringa pods were collected, washed with liquid soap, and rinsed in running tap water. Inside a clean bench, the pods were surface-sterilized by wiping with 95% ethanol and flaming for 10 s. The pods were opened, and then the seeds were taken aseptically and planted on a solid medium until germination. From the germinated seedlings, nodal explants were taken and planted in a fresh, solid medium. Repeated subculture of nodal explants on a fresh solid medium after every 2 weeks was done for continuous production of plantlets.

2.3. Optimization of Agar Concentration in the Solid Medium to Avoid Root Injury

Nodal explants from tissue culture-derived plantlets were planted on a medium solidified with varying amounts of agar, as follows: 2, 4, 6, and 8 g/L. Two weeks after transfer, the following growth responses were taken: (1) shoot length, (2) root length, and (3) number of roots. The shoot and root lengths were measured with a ruler, and the number of roots was counted manually. The number of plantlets with and without injured roots was also counted, and the percentage of plantlets with injured roots was computed using the following formula: Percentage of plantlets with injury (%) = [A/B] × 100, where A is the number of plantlets with root injury and B is the total number of plantlets.

2.4. *In vitro* Hardening of Rooted Plantlets to Avoid Rapid Wilting

Two-week-old rooted plantlets from semi-solid medium were transferred into capped culture tubes with 10 mL of sucrose-free liquid MS basal medium. The following day, they were temporarily ventilated by the uncapping of the culture tubes at varying durations of 10, 20, 30, 40, 50, and 60 min, which was done daily for three consecutive days during the daytime. On the 4th day, they were permanently uncapped, then maintained in the culture room for 1 week. They were individually transferred in a plastic bag with soil and maintained for another 2 weeks in the greenhouse prior to transfer in the field. The percentage of survival was taken 1 month after transfer in the field and computed using the following formula: Percentage of survival (%) = [A/B] × 100, where A is the number of plantlets that survived and B is the total number of plantlets.

2.5. Experimental Design and Data Analysis

All experiments were laid out in a completely randomized design. For the rooting experiment, there were four treatments replicated 3 times, with each treatment consisting of 10 plantlets. For the hardening experiment, there were seven treatments replicated 3 times, with each treatment consisting of 20 plantlets. Data were analyzed by one-way analysis of variance followed by an LSD test at 5% level of significance using the STAR 2.0 statistical software (IRRI, Philippines).

3. RESULTS

3.1. Optimization of Agar Concentration in the Solid Medium to Avoid Root Injury

The solid medium for *in vitro* rooting was prepared with varying gel strengths using varying amounts of agar. A soft semi-solid medium was obtained when the medium was solidified with 2 g/L agar; a semi-solid medium was obtained when the medium was solidified with 4 g/L agar; a soft solid medium was obtained when the medium was solidified with 6 g/L agar; and a hard solid medium was obtained when the medium was solidified with 8 g/L agar. Figure 1 shows the moringa plantlets grown on these solid media. As shown in Figure 1, the differences in all growth responses, *i.e.,* shoot length, root length, and root number, of the plantlets as influenced by different treatments were very visible 2 weeks after transfer. Table 1 shows the data on these growth responses as influenced by varying agar concentrations. The highest shoot length was observed in plantlets grown on the semi-solid medium. A lower shoot length was observed in plantlets grown on the soft solid medium or on the hard solid medium. The lowest shoot length was observed in plantlets grown on the soft, semi-solid medium. Similarly, the highest root length was observed in plantlets grown on the semi-solid medium. Root length was lower when the plantlets were cultured on the soft semi-solid medium or when they were cultured on the soft solid medium. The lowest root length was observed in plantlets cultured on the hard, solid medium. A similar trend was observed for the number of roots, wherein plantlets grown on the semi-solid medium had the highest number of roots. Plantlets grown on the soft semi-solid medium had a lower number of roots as compared with plantlets grown on the semi-solid medium. Plantlets grown on the soft solid medium and on the hard solid medium had the lowest number of roots.

The percentage of plantlets with root injuries is also shown in [Table 1]. It was found to be proportional to the amount of agar used to solidify the medium, wherein an increasing percentage of plantlets with injured roots was observed with an increasing amount of agar. The soft, semi-solid medium resulted in all plantlets without injured roots. No significant difference was observed in the percentage of plantlets with root injury when agar was increased to 4 g/L. Further increasing the amount of agar to 6 and 8 g/L resulted in increasing the gel strength of the culture medium and consequently increasing the percentage of plantlets with injured roots.

Table 1: Influence of varying amounts of agar on some growth parameters of moringa plantlets and on percentage of plantlets with root injury.

Amount of agar (g/L)	Medium description based on gel strength	Growth parameter			% of plantlets with root injury
		Shoot length (cm)	Root length (cm)	Root number	
2	Soft semi-solid	7.03±0.21[d]	3.60±0.20[b]	7.70±0.61[b]	0.0±0[c]
4	Semi-solid	15.40±0.36[a]	4.03±0.15[a]	15.33±1.15[a]	6.7±5.8[c]
6	Soft solid	11.17±0.23[b]	3.43±0.06[b]	5.07±0.15[c]	66.7±5.8[b]
8	Hard solid	8.47±1.21[c]	0.63±0.06[c]	4.00±0.10[c]	100.0±0[a]

*Within a column, treatment means followed by a common letter are not significantly different at 5% by LSD.

Figure 1: Tissue culture-derived *Moringa oleifera* plantlets 2 weeks after transfer in a solid medium with varying amounts of agar 2, 4, 6 and 8 g/L.

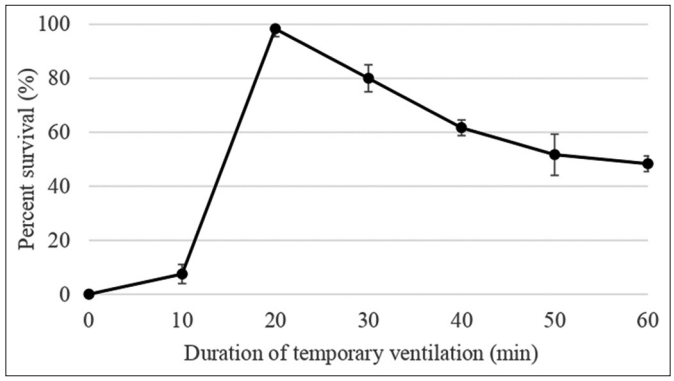

Figure 2: Influence of varying durations of temporary ventilation on survival of *Moringa oleifera* plantlets.

3.2. *In vitro* Hardening of Rooted Plantlets to Avoid Rapid Wilting

For the hardening experiment, the rooted plantlets were transferred to a sucrose-free liquid medium. They were initially covered with plastic caps for 1 day to condition them in the liquid medium. The following day, they were temporarily ventilated by uncapping the culture tubes at varying durations from 10 to 60 min for three consecutive days. Figure 2 shows the influence of varying durations of temporary ventilation on the survival of *M. oleifera* plantlets 1 month after transfer in the field. Without temporary ventilation (0 min), all the plantlets died; thus, the survival rate was 0%. Temporary ventilation for 10 min yielded a survival rate of only 7.5%. Increasing the duration of temporary ventilation to 20 min resulted in the highest survival rate of 98.3%. Further increasing the temporary ventilation beyond 20 min resulted in a decreasing percentage of survival of the plantlets, with 30, 40, 50, and 60 min of temporary ventilation resulting in 80.0, 61.7, 51.7, and 48.3% survival, respectively.

4. DISCUSSION

The weak root system of tissue culture-derived plantlets is one of the major problems in the micropropagation of moringa [20]. Their roots are very brittle and easily break when the plantlet is taken out of the agar-solidified medium. This causes root injury, leading to poor water and nutrient absorption, and the injuries created are very susceptible to infection by soil pathogens, contributing to the high percentage of mortality after soil transfer. One strategy to minimize root injury is to use a softer medium for rooting the plantlet, as has been done in the micropropagation of *Centella asiatica* [22], *Corema album* [23], *Capparis spinosa* [24], and various orchid species [25]. Results of the rooting experiment showed that the root injury observed in plantlets grown on the semi-solid medium solidified with 4 g/L agar was lower than that observed in plantlets grown on the harder solid medium solidified with 6 and 8 g/L agar. The use of the soft semi-solid medium solidified with 2 g/L agar yielded the lowest root injury, but the growth response in this medium was also the lowest likely due to the negative effect of hyperhydricity, as has been observed in the micropropagation of banana [26], *Malus* spp. and *Arabidopsis thaliana* [27], and *Salvia santolinifolia* [28], in which hyperhydric shoots formed were very small in size, showing distorted growth and a glassy, curled, wrinkled, waterlogged and translucent appearance. Similarly, all observed growth parameters, *i.e.,* shoot length, root length, and root number, of moringa plantlets were lower in the solid medium than the semi-solid medium. Harder medium causes the slow uptake of nutrients, leading to lower nutrient availability for the plants and hence a reduction in growth rate [29]. Therefore, the semi-solid medium solidified with 4 g/L agar was found to be the best medium for *in vitro* rooting of *M. oleifera* based on the ability to support growth and the minimal root injury upon uprooting of the plantlet.

Another major problem in the acclimatization of *M. oleifera* is the rapid wilting of the *in vitro*-rooted plantlets transferred into the soil. This is a phenomenon commonly observed in tissue culture-derived plantlets since their leaves have non-functional stomates and poorly developed cuticles [30-32]. In the present protocol, a modification was introduced to maintain the plantlets in an environment with high relative humidity. Instead of maintaining the rooted plantlets in a mist chamber after transplantation or covering the individual plantlet with a polythene bag, the rooted plantlets from the semi-solid medium [Figure 3a] were transferred individually in a sucrose-free liquid medium in a culture tube [Figure 3b] covered with a plastic cap to retain high humidity. This is the 1st time that *in vitro* hardening was done in *M. oleifera* using a liquid medium. *In vitro* hardening is

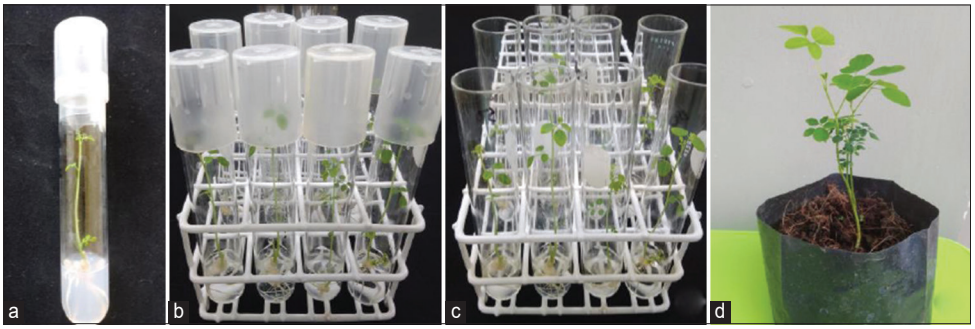

Figure 3: Improved acclimatization of micropropagated *Moringa oleifera*. (a) Rooted plantlet in semi-solid medium ready for hardening. (b) Plantlets transferred in sucrose-free liquid medium in capped culture tubes. (c) Plantlets subjected to temporary ventilation by uncapping of the culture tubes. (d) Hardened plantlet transferred to soil in a plastic bag ready for field transplantation.

more cost-efficient than the common practice of using an expensive mist chamber [33,34]. It is also less tedious than the practice of individually covering the plantlets with a perforated polythene bag since it reduces the labor, time, and space required to transfer the plantlets individually into the soil in plastic pots [35]. In *in vitro* hardening, as used in the present protocol, sucrose was removed from the liquid medium to prevent microbial contamination. The removal of sucrose also deprived the plantlets of their carbon source. Thus, for their survival, they need to switch from a condition dependent on an exogenous supply of carbon as a source of energy (heterotrophy) to a condition capable of producing its own carbohydrate in the presence of light (photoautotrophy). Photoautotrophy requires functional stomates to allow the entry of carbon dioxide, which becomes available to the plantlets by ventilation through the uncapping of the culture tubes [Figure 3c], thereby exposing the plantlets to the ambient environment. Since the leaves of tissue culture-derived plantlets were initially devoid of cuticles and had non-functional stomates, they are not yet capable of regulating water loss. Therefore, their exposure to the ambient environment must be done gradually until their leaves develop cuticles and their stomates acquire the ability to close. In a previous study, moringa plantlets were cultured in loosely- and tightly-sealed containers [9]. Under tightly sealed containers with a low ventilation rate, *in vitro*-cultured plantlets exhibited retarded shoot growth. In contrast, the plantlets in loosely sealed containers with a high ventilation rate exhibited stimulated shoot growth. In *Gynerium sagitatum*, successful acclimatization was also achieved through ventilation done by the periodic removal of culture covers every 2 h for 5 days [36]. Similar to this, ventilation in the present study was done by temporary removal of the plastic cover of the culture tube, but the duration was shortened to 10–60 min since the initial experiment using 2 h of cap removal resulted in the rapid wilting of the plantlet. Based on the results of the hardening experiment in the present study, temporary ventilation of 20 min was optimal for the survival of moringa plantlets. Temporary ventilation lower than 20 min resulted in a low survival rate, likely due to their inability to sequester enough carbon dioxide in the air as a source of energy. On the other hand, increasing the temporary ventilation beyond 20 min also resulted in a decreasing percentage of plantlets survival due to acclimatization shock. In a span of <1 month, the hardened plantlets were successfully transferred individually into the soil in a plastic bag [Figure 3d] and finally into the field under full sunlight.

5. CONCLUSION

The use of a semi-solid medium for rooting and a sucrose-free liquid medium combined with temporary ventilation for the hardening of plantlets improved the micropropagation of *M. oleifera*. Compared with the previous protocols, the acclimatization stage of the present protocol requires a shorter time, *i.e.,* from more than 3 months in the previous protocols to less than a month in the present protocol. The latter is also less labor-intensive than the former, as the rooted plantlets are transferred into liquid medium in culture tubes rather than soil in plastic bags individually covered with polythene bags to retain high humidity. Furthermore, the present protocol does not require special facilities such as a mist or humidity chamber because the rooted plantlets are hardened *in vitro* in culture tubes, so more plantlets can be hardened even in a smaller space. This improvement in the acclimatization of moringa plantlets resulted in a very high survival rate and is therefore expected to facilitate micropropagation of *M. oleifera* on a commercial scale.

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7. AUTHOR'S CONTRIBUTION

The author has solely made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, including drafting and revising the article, agreeing to submit it to the current journal, giving final approval of the version to be published, and agreeing to be accountable for all aspects of the work. The author is eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements and guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author on request.

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