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Optimization of High Frequency Somatic Embryogenesis and Plant Regeneration Method in *Moringa oleifera* Lamk

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ABSTRACT

Moringa oleifera Lam. belongs to the Moringaceae family which grows in a dry tropical area. Besides being a great food, it has horticulture, industrial and pharmaceutical potential. A protocol was designed for this esteemed plant for the callus formation and plant regeneration via somatic embryogenesis. Murashige and Skoog's (MS) medium and B5 medium fortified with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) alone or in combination with 0.1-0.2 mg/l 6-Benzylaminopurine (BAP) was used. The synthetic auxin, 2, 4-D at 2 mg/l and a cytokinin BAP 0.1 mg/l were used to induce callus from shoot apical meristem explant. This media preparation was found to be best combination for the induction of somatic embryo. About 44% of the thriving embryos were germinated on B5 medium containing 0.5 mg/l BAP and subsequently regenerated on B5 medium containing 1 mg/l BAP. The B5 medium supplemented with hormones was found to be better for germination and regeneration of somatic embryos as compared to the MS medium. The presented tissue culture protocol will be helpful for the improvement of genetic resources via approaches like somaclonal variations, wild hybridization and genetic engineering in *Moringa* species.

Key words: *Moringa oliefera*, somatic embryogenesis, regeneration, tissue-culture protocol

INTRODUCTION

Moringa oleifera Lam. belongs to Moringaceae family, distributed in India, Middle East and in tropical belt. It is an important food commodity in the tropical areas, and commonly known as drumstick tree (Fig. 1). It is a versatile tree having nutritional, pharmaceutical, industrial and socio-economic properties. Except wood all parts of the tree like fresh and dried leaves, immature pods, flowers, fruits, etc. are edible and exceptionally alimental (Hamza and Azmach, 2017). The immature green pods, also called "drumsticks" are exceptionally nutritious and widely used as vegetable. The leaves of Moringa are highly nutritive, being a rich source of antioxidants, vitamin A, B, C, D, E, K, iron, zinc, magnesium, manganese and potassium, beta-carotene and protein (Meghwal *et al.*, 2022).

As Moringa is an enriching plant with distinct nutritive properties, it is especially recommended for the nursing mothers and infants for the constant supply of nutrients in the malnourished areas. Non-governmental organizations like Church World Service (CWS) and Educational Concerns for Hunger Organizations (ECHO) have been considering Moringa as "natural nutrition for the tropics" (Singh *et al.*, 2018). Moringa leaves consist of bioactive components including phytochemicals and glucosinolates, etc. which have several health advantages thereafter used in nutraceuticals at large scale (Kashyap *et al.*, 2022).

It is known to provide energy drinks, food supplement for fortification, cosmetics (cosmetics and shampoos), and oil which has potential as biofuel. Moringa seeds can produce considerable amount of oil which could be used for cooking or as biofuel or lubricating agents

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Fig. 1. Green *Moringa oleifera* plant.

in manufacturing. After oil extraction, the remaining pressed cake has potential to be used as fertilizer. Parts of the Moringa tree are useful in different industries like dried leaves are useful for animal feed to increase milk production, leaf extract in foliar spray to improve plant growth, seeds in the purification of water, bark in tanning industry, and wood in paper and textile industries (Mulugeta and Fekadu, 2014). All parts of this magic tree have been considered for the different purposes. It possesses excellent medical properties like anti-tumor, anti-inflammatory, antipyretic, antiulcer, antispasmodic, antiepileptic, diuretic, antidiabetic, antihypertensive, cell reinforcement, and in cholesterol reduction. It has been used traditionally to cure vivid health issues like cardiac problems. It is also been used in the wide range of treatment due to its antifungal properties (Prajapati *et al.*, 2022).

M. oleifera is a well-known food of tropics due to its availability in all seasons when the supply of other food source becomes limited (Kushwaha *et al.*, 2015). Annual production of Moringa in India is 2.2 to 2.4 million tonnes which makes it largest producer in the world (Sekhar *et al.*, 2017). However, the production of this crop has remained low to fulfil the higher market demands. Moringa plant can be propagated by stem cuttings or through seed. The viability of Moringa seeds tends to decline rapidly. Moreover, very less number of seedlings is obtained through seeds because of the poor germination capacity. There are remarkable opportunities with *M. oleifera* for development as cash crop in semi-arid areas and sustainable agriculture (Fotouo *et al.*, 2015; Pokhrel *et al.*, 2016, Thapa *et al.*, 2019). Therefore, a rapid and uniform multiplication of this plant is required and this could be achieved with the help of tissue culture as it can be utilized in the successful propagation of other important crop trees as well like mango, apple, date palm, coconut etc.

Tissue culture is not a new technique to the plant breeder as it has been routinely used for the many crop species (Tazeb, 2017). Micro-propagation is a well-known alternative being widely used in the laboratory for the purpose of multiplication of selected plant genotypes of horticulture and medicinal trees (Chandana *et al.*, 2018). Therefore, it is advocated that tissue culture is one of the most suitable and practical ways to cultivate Moringa tree outside the tropic area. Only limited studies are available which advocate tissue culture of *M. oleifera* from immature embryo, seedling and mature tree derived explants (Shittu *et al.*, 2016; Muslihatin *et al.*, 2017; Boopathi *et al.*, 2021). Coetser *et al.* (2022) reported the somatic embryogenesis in *M. pterygosperma* using zygotic embryos and callus as explants. Commercial propagation of Moringa by tissue culture has not been achieved yet due to limited success in the protocols. Therefore, developing a suitable tissue culture method for Moringa genus is an urgent need (Okafor, 2020; Boopathi *et al.*, 2021). The present study describes rapid, easy and efficient protocol for regeneration of *M. oleifera* by using *in vitro* raised somatic embryos.

MATERIALS AND METHODS

M. oleifera (var. PKM-1) growing in a field nearby Pune, MS, India, was selected as a source of explants and plant identification was confirmed by using flora and by referring to experts from botanical garden. A sterile blade was used to excise shoot apical meristem from mother plant (from 4-5 trees) (Caggiano *et al.*, 2021). The meristem is then sterilized as per Oo *et al.* (2018) with little modifications. Meristem surface sterilization was performed by 70% alcohol for 1 min, followed by 0.5%

mercuric chloride for 15 min. These explants were then rinsed properly with sterile distilled water.

The surface sterilized explants of size 0.5 to 1 cm were placed on autoclaved Murashige and Skoog's (MS) basal medium and Gamborgs B5 medium having different concentrations of 2,4-D and BAP (Table 1) to induce callus. The MS media was supplemented with 3% (w/v) sucrose, whereas all B5 media was supplemented with 2% (w/v) sucrose and 0.7% agar was used as gelling agent in both the medias. Culture conditions were maintained similar to Jeyaram *et al.* (2022) with little modifications. The cultures were maintained at 25±2°C temperature, 60% relative humidity, 2000 lux light intensity and a photoperiod of 16 h light and 08 h dark. For each treatment 50 explants were used with three replicates.

Calluses thus produced were sub-cultured 5-6 times at interval of seven days on fresh MS and B5 medium separately with same hormones combinations. After six weeks callus producing somatic embryos were transferred for germination on basal media (MS or B5) containing BAP (0, 0.1, 0.5 and 1.0 mg/l) for one week. Three replicates each of 20 somatic embryos were used for germination of somatic embryo for each treatment. The frequency of embryo germination was recorded after one week of culture period. Since the number of responding somatic embryos was more in B5 medium, this medium was used for further experiment. After two weeks, germinated embryos were transferred for regeneration on B5 medium fortified with different concentrations of BAP (0, 0.5, 1 and 2 mg/l). To induce roots the regenerated healthy shoots were inoculated in ½ B5 medium containing 0.5 mg/l IBA.

For histological studies, somatic embryos were fixed in formalin, acetic acid and alcohol. Subsequently, the fixed tissues were dehydrated with serial rinsing with tertiary butyl alcohol. The dehydrated samples were embedded with paraffin wax (MP 58°C). About 15 µm serial sections were cut and then the micro-sections were stained with hematoxylin and mounted on DPX. Studies were conducted as per Asthana *et al.* (2017) with some modifications.

Mean values of the treatment were compared by Least Significance Difference (LSD) by using Duncan's Multiple Range test (DMRT). The statistical analysis was performed with MSTAT-C (version 1.42) software package.

RESULTS AND DISCUSSION

In the preliminary studies, several hormones

Table 1. Effect of different concentrations and combinations of growth regulators on callus induction and somatic embryo formation from shoot apical meristem of *M. oleifera*

Growth regulators (mg/l)		Callus induction (%)*		No. of somatic embryos/calli**	
2-4 D	BAP	B5 medium	MS medium	B5	MS
0	0	00±00^{a}	00±00^{a}	00±00	00±00
0.5	0	00±00^{a}	05±0.4^{a}	00±00	00±00
1	0	11±0.9^{b}	13±0.3^{b}	00±00	00±00
1.5	0	14±0.9^{b}	24±1.7^{bc}	00±00	00±00
2	0	23±0.9^{c}	47±1.3^{e}	00±00	00±00
2.5	0	22±2.0^{c}	53±2.1^{f}	00±00	00±00
3	0	23±0.6^{c}	34±2.4^{d}	00±00	00±00
0.5	0.1	00±00^{a}	05±0.2^{a}	00±00	00±00
1	0.1	11±0.9^{b}	15±1.0^{bc}	02±0.3	03±0.6
1.5	0.1	32±1.1^{d}	23±0.2^{c}	02±0.3	05±0.2
2	0.1	24±0.5^{c}	67±1.8^{fg}	04±0.5	08.3±1.0
2.5	0.1	22±0.3^{c}	25±1.2^{c}	03±0.2	10±0.3
3	0.1	16±1.5^{bc}	15±0.5^{bc}	00±00	00±00
0.5	0.2	00±00^{a}	00±00^{a}	00±00	00±00
1	0.2	06±1.2^{a}	09±0.9^{b}	00±00	00±00
1.5	0.2	23±0.4^{c}	23±1.2^{c}	00±00	00±00
2	0.2	29±0.5^{d}	39±1.1^{de}	02±0.2	04±0.4
2.5	0.2	12±0.5^{b}	15±1.7^{bc}	02±0.1	03±0.2
3	0.2	06±0.3^{a}	09±0.8^{b}	00±00	00±00

Same superscript is not significantly different at P=0.05 level. DMRT. *recorded after three weeks and **recorded after six weeks.

(NAA, Picloram, 2, 4-D) were used to induce callus formation, however, only 2, 4-D was found to be effective. Out of different combinations tried, 2, 4-D in combination with BAP, was statistically significant in terms of induction of callus and somatic embryo formation in both basal medias viz., MS and B5 (Table 1). The media without BAP also produced callus (highest percentage was 53+2.1) but the somatic embryo formation was not observed. Callus formation was initiated after first subculture (7 d) and was allowed to grow for six weeks on same hormone combination with frequent sub-culturing on fresh media (Fig. 2A). The callus formed was compact and green in colour. In case of 2, 4-D supplementation, it was observed that less than 0.5 mg/l and more than 2.5 mg/l concentration had inhibitory effects on the callus formation. High callus and somatic embryos were obtained with 2, 4-D at 2 mg/l and 2.5 mg/l in presence of BAP (0.1 mg/l) on both the basal media. Somatic embryos were observed after six weeks of culture. The frequency of callus formation was highest (i.e. 67%) in MS medium containing 2.0 mg/l 2,4-D and 0.1 mg/l BAP as compared to other media composition, however, the number of somatic embryo/calli was more (i.e. 10 embryo/calli) in MS medium containing 2.5 mg/l 2,4-D and 0.1 mg/l BAP (Table 1). Somatic embryos differentiated from all sides of callus (Fig. 2B). Fig. 2C represents the individual cotyledonary shaped embryo. Histological sections of embryo confirmed mature cotyledonary shaped embryo with the vascular connection and shoot poles (Fig. 3). Regular sub-culturing of callus at 7-day interval also influenced the embryo formation as compared to sub-culturing at 15-day interval.

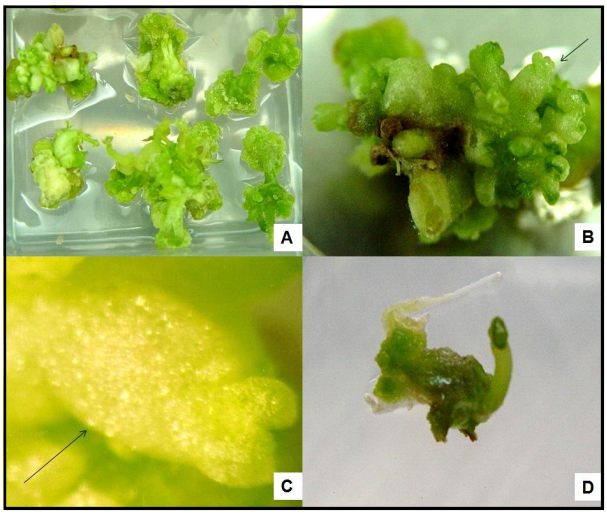

Fig. 2. Somatic embryogenesis; A. callus formation on MS+2 mg/l 2,4-D + 0.1 mg/l BAP; B. somatic embryos after six weeks of culture; C. individual somatic embryo and D. germinating somatic embryo on B5+0.5 mg/l BAP.

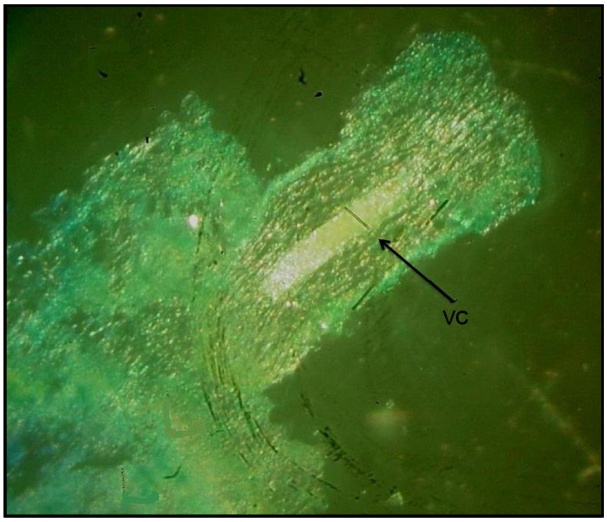

Fig. 3. Histological section of somatic embryo (VC– Vascular connection).

In view of somatic embryogenesis, the use of 2, 4-D alone or in combination has become a routine method (Yang *et al.*, 2021). Out of different combinations tried, the combination of 2, 4-D with BAP showed better results in callus induction and somatic embryos (Table 1). Although auxin has important role in pattern formation in embryo, still a specific concentration of auxin is required for the proper development of embryos and any inadequate or higher concentration of hormones may inhibit proper development. Studies reported that concentration of 2, 4-D was important in determining proper callus growth. Inadequate amount of 2, 4-D could have inhibitory effect on callus growth (Damayanti *et al.* 2020; Karami *et al.* 2023). In our study, we found that 2, 4-D at 2 mg/l with BAP induced high frequency of callus formation. The explants cultured only on BAP containing media showed embryogenesis, whereas no embryogenesis was observed from explants cultured devoid of BAP, suggesting that BAP plays important role in somatic embryogenesis of drumstick (Table 1). Mayerni *et al.* (2020) observed shoot induction in all the plants when 1.0 and 1.5 mg/l BAP concentration was supplied. Similar results were obtained in our

study as indicated in Table 2, where highest shoot elongation was observed at 1.0 mg/l BAP i.e. 97±4.1. In many studies, somatic embryogenesis was induced from callus of different explants, namely, zygotic embryo, leaf explant, meristimatic shoot, hypocotyle (Custódio *et al.* 2022; Ferreira *et al.* 2022). Experimental data presented by Gantait *et al.* (2018) and Venkatachalam *et al.* (2015) advocated that basal medium, MS organic salts, complemented with sucrose and growth regulators (2, 4-D, NAA and 2-iP), was best formulation for plant regeneration in palms through somatic embryogenesis. Zang *et al.* (2016) reported that MS basal media was best for the callus formation and subsequent shoot growth among several other basal media. In present investigation as well, MS media was observed with higher suitability in view of callus induction as compared to the B5 medium. However, among two tested basal medium, B5 medium was found to be more efficient for embryo germination as compared to the MS medium. Shoot organogenesis was stimulated by the organic nitrogen compounds when the concentration of inorganic N-source (NH_4NO_3) got reduced to 1/20 of that in standard MS medium. Adugna *et al.* (2020) achieved *in vitro* propagation of *Moringa* species to sustain biodiversity by axillary shoot growth from single node shoot segments and the regeneration was accomplished on basal MS medium by taking cotyledonary node of decapitated seedlings. Avila-Treviño *et al.* (2017) published report on the *in vitro* propagation of *M. oleifera* by using bud and apex as explants. Findings observed multiple budding of *M. oleifera* in the absence of growth regulators in MS medium. The regeneration of stem and leaf explants was performed in MS media with 1/mg/l BAP and 0.2 mg/l of AIA.

Table 2. Effect of different concentrations of BAP containing B5 medium on shoot elongation

BAP mg/l	Per cent shoot elongated
0	50±1.0
0.5	83±3.1
1	97±4.1
2	79±1.4

These embryos were subsequently germinated with root and shoot pole (Fig. 2D). The mature somatic embryos germintated with high frequency (44%) in B5 medium supplemed with 0.5 mg/l BAP (Fig. 2), suggesting the positive role of B5 basal medium on germination of embryos. Frequency of somatic embryo germination on B5 + 0.5 mg/l BAP was 44% in comparison to MS+0.5 mg/l BAP which was only 12%. Thus, all further experiment was conducted with B5 as basal medium for shoot elongation and rooting experiment.

High frequency (44%) of somatic embryo germination was successfully achieved in the experiment. Boopathi *et al.* (2021) suggested that tissue culture protocol developed for clonal multiplication of *Moringa* species could be employed for germplasm maintenance of endangered *Moringa* species. The protocol developed herein can also be exploited for maintenance of other endangered species of *Moringa.*

Two weeks after embryo germination shoots with at least 0.5-2 cm long were transferred for shoot elongation in B5 medium containing 0, 0.5, 1 and 2 mg/l BAP. The percentage of shoot elongation was 100% in 1 mg/l BAP, followed by 0.5 mg/l BAP. Shoots with at least 5 cm were transferred to rooting media containing ½ B5 + 0.5 mg/l IBA produced high frequency of rooting. *In vitro* roots were morphologically normal with frequency of 83+4%.

CONCLUSION

In conclusion, the study revealed that successful regeneration of *M. oleifera* plantlets could be obtained via somatic embryogenesis using shoot apical meristem. Effect of different basal media (B5 or MS) was found to influence callus induction, somatic embryo formation and their subsequent germination. The protocol mentioned herein can be used for successful rapid and uniform multiplication of *M. oleifera* to fulfil the tremendous requirement of *M. oleifera*. The successful regeneration protocol *via* somatic embryogenesis can also be exploited in multiplication of elite or genetically engineered *M. oleifera* plants.

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