Micropropagation in *Moringa oleifera* Lam for high-throughput multiplication

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**Abstract**

*Moringa oleifera* is the most widely cultivated species of the Moringaceae family. Attributed to several medicinal uses and a high nutritional value. An efficient *in vitro* clonal propagation methodology for *Moringa oleifera* (PKM1 Variety) was developed using nodal explants of young seedlings grown ex vitro. Nodes were cultured in Murashige and Skoog (MS) medium with different combinations of plant growth regulators. Multiple shoots were successfully achieved by culturing nodal explants in a medium containing different concentrations of 6-Benzylaminopurine (BAP) and Kinetin (Kin) in combination with naphthalene acetic acid (NAA). BAP at 2mgL\(^{-1}\) was considered optimal for generating a maximum an average of 6.03±0.21 auxillary shoots per explants after 60 days of culture inoculation. A high rate of multiplication has been established by routine subculture on a similar shoot induction medium. Maximum numbers of individual roots were established on a medium containing indole-3-butyric acid (IBA) at 1.5mgL\(^{-1}\). Primary hardening was done in pots containing the potting mixture and transferred plantlets were covered with clear polythene bags. Seventy per-cent of the rooted plants survived and secondary hardening was carried out after 15 days in a shaded greenhouse.

**Keywords**

*Moringa oleifera*, Micropropagation, Benzylaminopurine, Naphthalene acetic acid, Kinetin

**INTRODUCTION**

*Moringa oleifera* Lam. is the most commonly grown species of the genus moringa, and it belongs to the family Moringaceae (Sabale *et al*., 2008). Native to India distributed to tropical and sub-tropical regions of the world (Balaguru *et al*., 2020). It has application in food and pharmaceutical industries. It is also used for purification of water, bioremediation and in agriculture management (Gupta *et al*., 2018). This tree is considered to be a peculiar plant species as it has vast nutrient and metabolite profile, which is rarely found in other plants (Abdull Razis *et al*., 2014).

Moringa is considered as a most nutrient-dense yet discovered where fruits, leaves, flowers, and immature capsules of this tree are highly nutritious and used in many countries in the world (Anwar and Bhanger, 2003). Every part of the moringa tree is edible and it is promising food source when other foods are generally scarce and it has the maximum leaves at the end of the dry season (Flugie, 1999).

*Moringa oleifera* is multi-season and one of the most helpful trees. Along these lines, it can be portrayed as a ‘miracle tree’ (Ashfag *et al*., 2012). Moringa is likewise proposed as a viable supplement of dietary minerals. The pods and leaves are abundant in minerals such as Ca, Mg, K, P, Zn, Na, Cu, and Fe (Aslam *et al*., 2005). Moring leaves have higher concentrations
of vitamin A and C, potassium, calcium and iron (Balaguru et al., 2020). Ayurvedic conventional medication says that *Moringa oleifera* can forestall 300 illnesses and its leaves have been abused both for preventive and remedial (Ganguly, 2013). Moringa has been seen as a good source of polyphenols and antioxidants (Mishra et al., 2011). Additionally, *Moringa oleifera* is likewise utilized for biogas production, domestic cleaning agents, food for animals, gum, fertilizer, manufacture of perfume, and hair care items. Moringa seed oil (yield 30-40% by weight), sweet non-sticking, non-drying oil as it stands to rancidity (Tsaknis et al., 1999).

*Moringa oleifera* has become very important, so it contains 18 of the 20 amino acids needed by the human body and is one of the plant species with a 30-40% higher seed oil content (Oriabi, 2016). The plant has found wide uses in industries because of its nutritional and medicinal value (Shank et al., 2013). In order to reach commercial demand, this plant has to be preserved and multiplied. Conventional plant propagation methods, such as seed planting and stem cutting, have limited applicability (Yadav et al., 2012). However, increasing demands for uniform and quality seeds, lead to looking for viable and affordable alternative planting materials.

The technique of plant tissue culture was extensively used for *Moringa oleifera* using a direct micropropagation pathway (Marfori, 2010; Saini et al., 2012). Also, a few reports have described the use of indirect micropropagation techniques (Devendra et al., 2012; Shittu et al., 2017). The micropropagation of *Moringa oleifera* was successfully established by Ravi et al. (2019) through nodal culture and 17.6 shoots were produced in MS medium fortified with 2.5 µM BAP and significant reduction in leaf fall (20.6%) was observed by adding 2.5 µM AgNO₃ as an anti-ethylene agent. The highest shoot multiplication by nodal segments obtained from upper parts of ex vitro grown plants was reported by Salem (2015). Abbas (2014) studied the development of multiple shoots through the nodal segment obtained from seedlings grown in vitro. To this end, employing plant tissue culture for the supply of large numbers of high-quality planting materials in a medium containing different combinations of growth regulators is proposed in this study.

**MATERIALS AND METHODS**

The current research work was performed at the Plant Tissue Culture Laboratory, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore during November month of the academic year 2019-20.

The MS (Murashige and Skoog medium) was sterilized at 121°C temperature (15 psi pressure) for 20 minutes in a vertical type autoclave. Different concentrations of growth regulators were added after filter sterilization. Medium was dispensed into pre-sterilized test tubes and stored at 25±2°C until inoculation. The control used for this experiment was basal MS medium.

Morphologically uniform seeds of *Moringa oleifera* (PKM1 variety), were collected from Horticultural College and Research Institute, Periyakulam. To establish a nodal culture of *moringa* plant, seeds were germinated in plastic pots filled with clay soil in greenhouse condition. Nodal segments obtained from these three week old soil growing seedlings were surface disinfected with two drops of Tween 20 and 1% Bavistin for five minutes. Surface sterilization for the nodes was carried out inside the laminar air flow using 0.1% mercuric chloride for 1 min. and 6% sodium hypochlorite for 3 min. Finally, the explants were thoroughly rinsed thrice with sterile water.

Individual nodal segment of an approximate 2 cm length was inoculated aseptically on sterilized MS (Murashige and Skoog, 1962) nutrient medium containing 30 gL⁻¹ of sucrose, 8gL⁻¹ of agar, for shoot initiation. The medium pH was set at 5.8 before autoclaving. The effect of different concentrations of 6-Benzylaminopurine (BAP, 0, 0.5, 1.0, 1.5 and 2) alone or combination of naphthalene acetic acid (NAA) at 0.2 mg and Kinetin (KIN, 0, 0.5, 1.0, 1.5 and 2) alone or in combination of naphthalene acetic acid (NAA) at 0.2 mg was studied on multiple shoot induction. All growth regulators used in this study were obtained from sigma chemicals. Nodal cultures were incubated at 25±2°C and 16-h photoperiod. The number of shoots formed per explant and shoot length was recorded.

For root induction, micro shoots induced were sub cultured to a root inducing medium (RIM-MS + indole-3-butyric acid (IBA)) at 0, 0.5, 1.0, 1.5and 2.0 mgL⁻¹ of naphthalene acetic acid (NAA) at 0.2 mg. Number of roots/shoot and the roots length were recorded 30 days after transfer. Well rooted plantlets were weaned for hardening in potting mixture.

Well rooted plantlets were hardened in plastic bags containing sterilized mixture of soil and coco peat (1:1). Plants were covered with punch held polythene bags to improve humidity, watered, and left in 26°C under partial sunlight. The polythene bags covered were removed after 15 days and the established plants were transferred to the greenhouse for secondary hardening.

The data recorded were subjected to statistical analysis by using Completely Randomized Design and the treatments were replicated thrice. Values were represented as Means±Standard Deviation (SD). The data were analysed with MS Excel spread sheet and WASP software.

**RESULT AND DISCUSSION**

The number of multiple shoots per nodal explant stimulated by BAP, Kinetin, and NAA at different concentrations after 60 days of inoculation was recorded. The application of 2mgL⁻¹ BAP resulted in the maximum number of axillary shoots (6.03±0.21) per nodal explants with maximum shoot formation.
length (Fig. 1cd). MS basal medium was less effective in inducing axillary shoots from explant (Table 1). The shoot multiplication of moringa in the culture medium was strongly under the influence of concentration of nutrients in the medium, where the rate of shoot multiplication varied with the different concentrations of the growth regulators in the MS medium. The better response was observed in the full strength MS medium as it included all the essential elements needed for the dedifferentiation of cells leading to the better induction of adventitious shoots (Hassanein et al., 2019).

Similarly, in kinetin containing medium (1.5 mgL⁻¹), the maximum number of axillary shoots per explant recorded was 3.93±0.06 (Table 2). The axillary shoots were sub cultured to the root induction medium with IBA and NAA. Application of 1.5mgL⁻¹IBA produced the maximum number of roots (5.70±0.36) per shoot after 30 days and medium without plant growth regulators recorded the lowest number of roots (2.00±0.1) per shoot (Fig. 2).

Table 1. Effect of BAP and NAA on shoot proliferation from nodal explant

| Sl. No | BAP (mg) | NAA (mg) | Number of shoots* | Shoot length (cm)* |
|--------|----------|----------|-------------------|--------------------|
| T₁     | 0.00     | 0.00     | 1.67±0.12         | 2.18±0.04          |
| T₂     | 0.50     | 0.00     | 2.37±0.06         | 2.91±0.10          |
| T₃     | 1.00     | 0.00     | 4.43±0.21         | 3.46±0.04          |
| T₄     | 1.50     | 0.00     | 4.03±0.06         | 3.13±0.10          |
| T₅     | 2.00     | 0.00     | 6.03±0.21         | 5.17±0.09          |
| T₆     | 0.50     | 0.20     | 2.03±0.21         | 2.21±0.04          |
| T₇     | 1.00     | 0.20     | 3.73±0.32         | 3.01±0.08          |
| T₈     | 1.50     | 0.20     | 2.90±0.10         | 3.73±0.15          |
| T₉     | 2.00     | 0.20     | 2.50±0.10         | 3.01±0.15          |

Experiments were conducted with three replicates with 5 explants in each replication. *Values represents the means ± Standard Deviation (SD)of three replicates. In a column, different letters indicate statistically significant differences between the mean (p<0.05).

Table 2. Effect of Kinetin and NAA on shoot proliferation from nodal explant

| Sl. No | Kinetin (mg) | NAA (mg) | Number of shoots* | Shoot length (cm)* |
|--------|--------------|----------|-------------------|--------------------|
| T₁     | 0.00         | 0.00     | 1.67±0.12         | 2.22±0.01          |
| T₂     | 0.50         | 0.00     | 1.30±0.10         | 2.00±0.10          |
| T₃     | 1.00         | 0.00     | 3.93±0.06         | 2.63±0.15          |
| T₄     | 1.50         | 0.00     | 3.00±0.10         | 4.04±0.06          |
| T₅     | 2.00         | 0.00     | 3.50±0.20         | 3.73±0.15          |
| T₆     | 0.50         | 0.20     | 2.53±0.47         | 2.40±0.20          |
| T₇     | 1.00         | 0.20     | 3.10±0.10         | 3.27±0.06          |
| T₈     | 1.50         | 0.20     | 2.73±0.21         | 3.10±0.10          |
| T₉     | 2.00         | 0.20     | 2.93±0.06         | 3.00±0.20          |

Experiments were conducted with three replicates with 5 explants in each replication. *Values represents the means ± Standard Deviation (SD)of three replicates. In a column, different letters indicate statistically significant differences between the mean (p<0.05).

Previous studies showed that multiple shoot induction and rooting in Moringa oleifera has been documented with different growth regulators. Riyathong et al. (2010) observed 10.8 shoots per cultured stem in medium containing 2 mgL⁻¹BAP, similarly rooting was obtained in MS containing 1 mgL⁻¹NAA. Islam et al. (2005) showed that BA at 4.44 to 6.66µM was optimal for shoot formation. Saini et al. (2012) obtained 9.01±1.0 shoot per cultured node in a medium containing 4.44µM BA and 100% rooting was obtained in MS medium supplemented with indole-3-acetic acid (IAA) at 2.85µM and indole-3-butyric acid (IBA) at 4.92µM. Marfori (2010) reported that 2.4µM BAP to be the best for producing an average of 4.6 axillary shoots per explant. At mild ventilation conditions, Salem (2016) cultured the nodal section of Moringa oleifera on the Murashige and Skoog medium with 6-Benzylaminopurine (2.5µM) as the strongest multiple shoot formations and adventitious root formation was obtained when shoot cuttings were cultured on medium supplemented with 4.92µM IBA. Hassanein et al. (2019) used nodal culture for in vitro micropropagation of moringa and tested different concentrations of BAP and kinetin for shoot proliferation. The result showed that BAP was superior in shoot multiplication compared with kinetin.
Fig. 1. *In vitro* regeneration of *Moringa oleifera*. a Plants grown in greenhouse. b Initiation of multiple shoots in MS medium fortified with 2 mg L\(^{-1}\) BAP. c and d multiple shoots obtained from nodal explants on MS medium fortified with 2 mg L\(^{-1}\) BAP. e Rooted plant obtained on MS medium fortified with 1.5 mg L\(^{-1}\) IBA. f Primary hardening of rooted plant. g Hardened plant in greenhouse.

Fig. 2. Effect of different concentrations of IBA and NAA on the *in vitro* rooting.
Stephenson and Fahey (2004) found that 4.44 μM BAP supplementation in MS medium as the best combination for shoot induction and 2.68 μM NAA for root induction. Gupta et al. (2020) obtained 18 shoots per cultured node in a medium containing 3 mgL⁻¹ BA, similarly maximum number of roots (14.1±2.9) was obtained in MS containing 0.2 mgL⁻¹ IBA. Chand et al. (2019) examined the effect of 6-benzyladenine (BA) and naphthalene acetic acid (NAA) in inducing multiple shoots from nodal explants and reported that woody plant medium (WPM) fortified with BA at 4.44 μM as optimal (3.22±0.17 shoots per explant) in inducing multiple shoots. The ability to produce multiple shoots from different parts of Moringa oleifera was studied by Förster (2013) and basal parts of in vitro plant showed that the highest number of shoots in the medium fortified with 0.5 mgL⁻¹ BAP. Ridzuan et al. (2020) found that the MS medium fortified with 0.5 mgL⁻¹ and 1 mgL⁻¹ BAP separately as the optimum for shoot multiplication (3.1±0.16 and 3.5±0.51 shoots respectively) and 1 mgL⁻¹ IBA for root induction. Stem explants (Riyathong et al., 2010) and node explants (Salem 2016) were used from in vitro seedlings and field grown plants, respectively, to obtain in vitro organogenesis in moringa. Our results of shoot induction were found similar to Riyathong et al. (2010).

These variable responses may be attributed to differences in the plant source and the type of explant used to establish tissue cultures. Therefore, according to our results, the nodal segment of moringa, variety PKM1 cultured on MS medium containing 2 mgL⁻¹ BAP can be defined as suitable for M.oleifera micropropagation compared to medium fortified with different concentrations of kinetin and good root formation was observed when shoots were sub cultured on MS medium with 1.5 mgL⁻¹ IBA(fig 1e).

Micropropagation in moringa variety, PKM1 was attempted and demonstrated the effect of various combinations of plant growth regulators. The multiple shoot induction, BAP alone without any other hormonal combination is preferable. Among the different combinations of BAP, kinetin and NAA tested for shoot proliferation, BAP was better than kinetin in shoot multiplication. BAP is the most important cytokine in for shoot multiplication of Moringa oleifera. The MS medium fortified with 2mgL⁻¹ BAP resulted in the more number of shoots per explant (6.03±0.21) with maximum shoot length. The highest average numbers of roots per shoot (5.70±0.36) were from the MS medium supplemented with 1.5 mgL⁻¹ IBA.

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