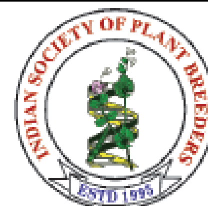
Received: 24 Nov 2020 | Revised: 19 Dec 2020 | Accepted: 20 Dec 2020

Electronic Journal of Plant Breeding



Research Article

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 subtropical regions of the world (Balaguru *et al*., 2020). Ithas 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 apeculiar 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 fount 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 micro-propagation 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 $AgNO_3$ 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 concentrationsof6-Benzylaminopurine (BAP, 0, 0.5, 1.0, 1.5 and 2) alone or combination of naphthalene acetic acid (NAA) at 0.2mg and Kinetin (KIN, 0, 0.5, 1.0, 1.5 and 2) alone or in combination of naphthalene acetic acid (NAA) at 0.2mg 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}andnaphthalene acetic acid (NAA) at 0.2mg. 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 holed 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 Mean±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

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^{-1}IBAproduced 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)*
T_1	0.00	0.00	1.67±0.12^{g}	2.18±0.04^{f}
T_2	0.50	0.00	2.37±0.06^{e}	2.91±0.10^{e}
T_3	1.00	0.00	4.43±0.21^{b}	3.46±0.04^{c}
T_4	1.50	0.00	4.03±0.06^{c}	3.13±0.10^{d}
T_5	2.00	0.00	6.03±0.21^{a}	5.17±0.09^{a}
T_6	0.50	0.20	2.03±0.21^{f}	2.21±0.04^{f}
T_7	1.00	0.20	2.37±0.32^{e}	3.01±0.08^{de}
T_8	1.50	0.20	2.90±0.10^{d}	3.73±0.15^{b}
T_9	2.00	0.20	2.50±0.10^{e}	3.01±0.15^{de}

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)*
T_1	0.00	0.00	1.67±0.12^{f}	2.22±0.01^{fg}
T_2	0.50	0.00	1.30±0.10^{g}	2.00±0.10^{g}
T_3	1.00	0.00	3.93±0.06^{a}	2.63±0.15^{e}
T_4	1.50	0.00	3.00±0.10^{cd}	4.04±0.06^{a}
T_5	2.00	0.00	3.50±0.20^{b}	3.73±0.15^{b}
T_6	0.50	0.20	2.53±0.47^{e}	2.40±0.20^{f}
T_7	1.00	0.20	3.10±0.10^{c}	3.27±0.06^{c}
T_8	1.50	0.20	2.73±0.21^{de}	3.10±0.10^{cd}
T_9	2.00	0.20	2.93±0.06^{cd}	3.00±0.20^{d}

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^{-1}BAP, similarly rooting was obtained in MS containing 1 mgL^{-1} NAA.Islam *et al*. (2005) shown 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.

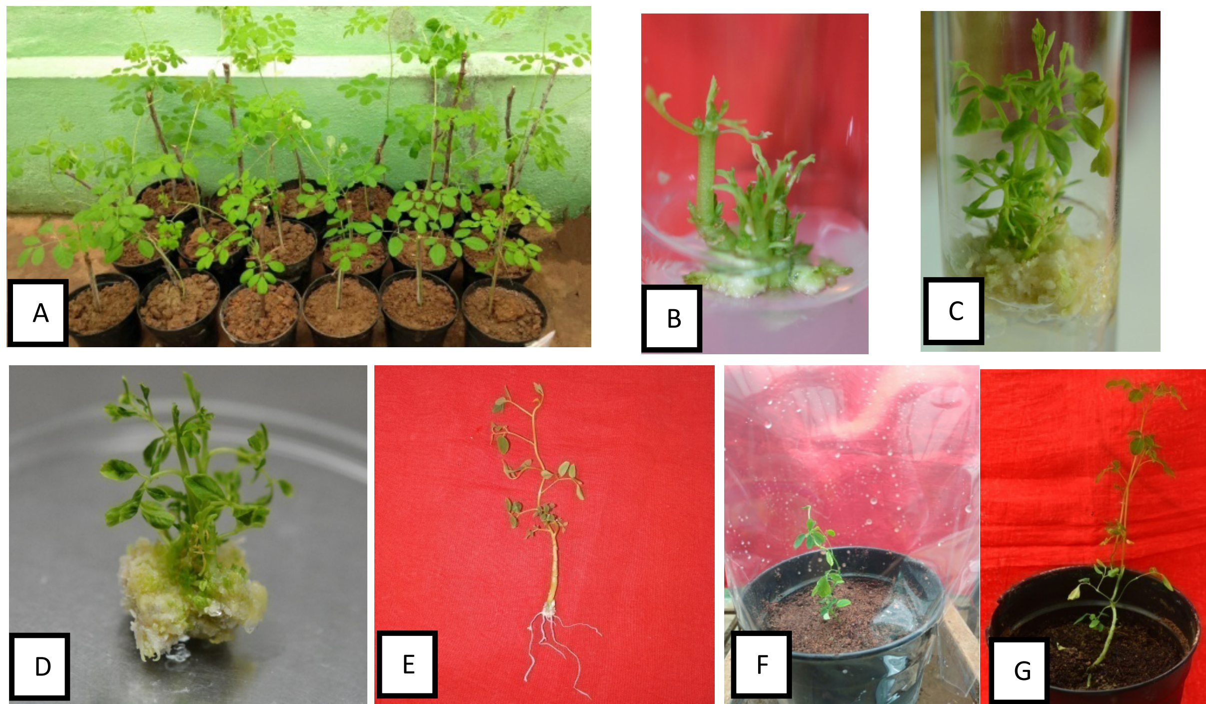
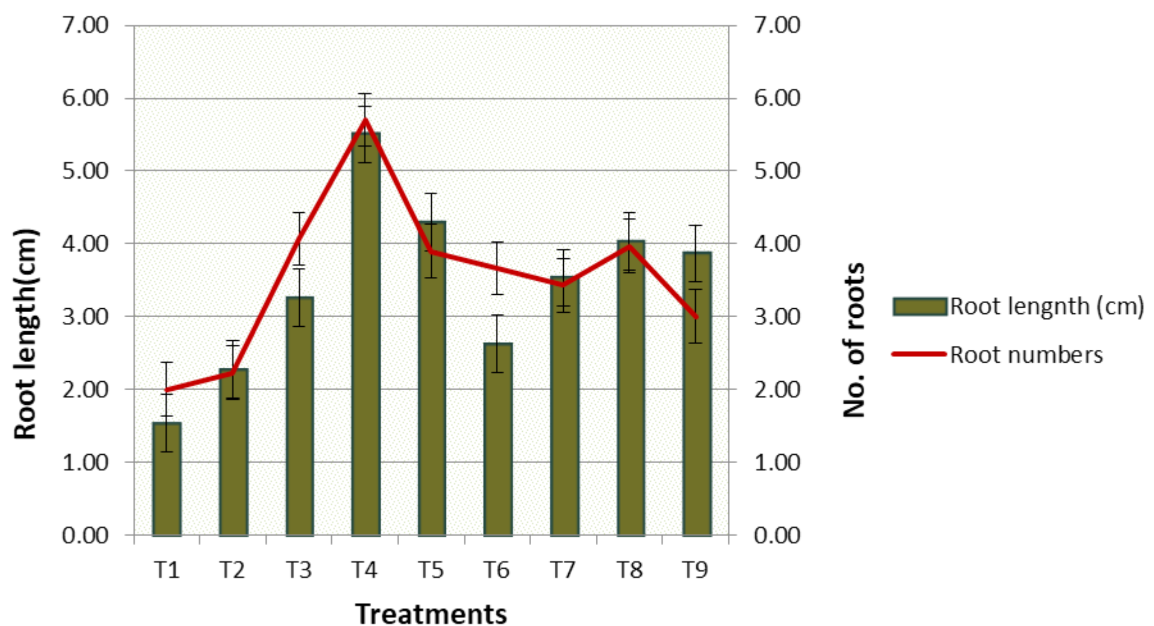


Fig. 1*In vitro* regeneration of *Moringa oleifera*.a Plants grown in green house. b Initiation of multiple shoots in MS medium fortified with 2mgL^{-1} BAP.c and d multiple shoots obtained from nodal explants on MS medium fortified with 2 mgL^{-1} BAP.eRooted plant obtained on MS medium fortified with 1.5 mgL^{-1} IBA.f Primary hardening of rooted plant.g Hardened plant in greenhouse.



T1 - MS basal
T2 - MS+0.5 mgL^{-1} IBA
T3 - MS+1.5 mgL^{-1} IBA
T4 - MS+2 mgL^{-1}IBA
T5 - MS+2 mgL^{-1}IBA
T6 - MS+0.5 mgL^{-1}IBA+0.2 mgL^{-1} NAA
T7 - MS+1 mgL^{-1}IBA+0.2 mgL^{-1} NAA
T8 - MS+1.5 mgL^{-1}IBA+0.2 mgL^{-1} NAA
T9 - MS+2 mgL^{-1}IBA+0.2 mgL^{-1} NAA

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 with0.5 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.

ACKNOWLEDGMENTS

J.D. Harshitha acknowledge Department of Biotechnology, Government of India, for providing fellowship for pursuing M.Sc.(Biotechnology) Program.All the authors are thank Department of Plant Biotechnology, Centre for Plant Molecular Biology & Biotechnology, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, for providing Laboratory facilities.

REFERENCES

Abbas, D. 2014. Development of *in vitro* regeneration and Agrobacterium mediated transformation systems for *Moringa oleifera* plant. *Egyptian Journal of Genetics and Cytology*, **43**(1): 99-111. [Cross Ref]

AbdullRazis, A. F. Ibrahim, M. D. and Kntayya, S. B. 2014. Health benefits of *Moringa oleifera*. *Asian pac J cancer prev*, **15**(20): 8571-8576. [Cross Ref]

Anwar, F. and Bhanger, M. I. 2003. Analytical characterization of *Moringaoleifera* seed oil grown in temperate regions of Pakistan. *J. Agric. Food. Chem*, **51**(22): 6558-6563. [Cross Ref]

Ashfaq, M. Basra, S. M. andAshfaq, U. 2012. Moringa: a miracle plant for agro-forestry. *J. Agric. Soc. Sci*, **8**: 115-122.

Aslam, M. Anwar, F.Nadeem, R. Rashid, U.Kazi, T. G.andNadeem, M. 2005. Mineral composition of *Moringaoleifera* leaves and pods from different regions of Punjab, Pakistan. *Asian J. Plant Sci*, **4**(4): 417-421. [Cross Ref]

Balaguru, P.Sathiyamurthy, V. A.Janavi, G. J. and Santha, S. 2020. Variability in perennial moringa (*Moringaoleifera* Lam.) genotypes for quantitative and qualitative traits. *Electron. J. Plant Breed*, **11**(02): 515-520. [Cross Ref]

Chand, S. Pandey, A. and Verma, O. 2019. *In vitro* regeneration of *Moringaoleifera* Lam.: A medicinal tree of family Moringaceae. *Indian J Genet Plant Breed.*,**79**(3): 606-613. [Cross Ref]

Devendra, B. N.Talluri, P. and Srinivas, N. 2012. Callus induction and somatic embryogenesis of *Moringaoleifera*Laman anti-radiation plant. *Journal of Agricultural Technology*, **8**(6): 1953-1963.

Förster, N.Mewis, I. and Ulrichs, C. 2013. *Moringaoleifera*—establishment and multiplication of different ecotypes *in vitro*. *GesundePflanz.*, **65**(1): 21-31. [Cross Ref]

Fuglie, L. J. 1999. The miracle tree: *Moringaoleifera*, natural nutrition for the tropics.

Ganguly, S. 2013. Indian ayurvedic and traditional medicinal implications of indigenously available plants, herbs and fruits: A Review. *Int. J. Res. Ayurveda Pharm*, **4**(4). [Cross Ref]

Gupta, S. Jain, R. Kachhwaha, S. and Kothari, S.L. 2018. Nutritional and medicinal applications of *Moringa oleifera* Lam.—Review of current status and future possibilities. *J.hermed*, **11**: 1-11. [Cross Ref]

Gupta, S. Kachhwaha, S. Kothari, S. L. and Jain, R. 2020. Synergistic effect of cytokinins and auxins enables mass clonal multiplication of drumstick tree (*Moringaoleifera* Lam.): a wonder. *In Vitro Cellular & Developmental Biology-Plant*, 1-12. [Cross Ref]

Hassanein, A. Salem, J.Faheed, F. and EL-Nagish, A. 2019. Some important aspects in *Moringa oleifera*Lam. micropropagation. *Acta Agric.Slov.*, **113**(1). [Cross Ref]

Islam, S.Jahan, M. A. A. and Khatun, R. 2005. *In vitro* regeneration and multiplication of year-round fruit bearing *Moringa oleifera* L.*J Biol Sci*, **5**: 145-148. [Cross Ref]

Marfori, E. C. 2010. Clonal micropropagation of *Moringaoleifera*L.*Philipp Agric Sci*, **93**: 454-457.

Mishra, G. Singh, P. Verma, R. Kumar, S. Srivastav, S.Jha, K. K. andKhosa, R. L. 2011. Traditional uses, phytochemistry and pharmacological properties of *Moringaoleifera* plant: An overview. *Der Pharmacia Lettre*, **3**(2): 141-164.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, **15**(3): 473-497. [Cross Ref]

Oriabi, A. G. 2016. *Moringaoleifera in vitro* culture and its application as anti-diabetic in alloxan induced diabetic albino mice. *Int. J. Curr. Microbiol. App. Sci*, **5**(2): 43-49. [Cross Ref]

Ravi, R. D.Siril, E. A. and Nair, B. R. 2019. The effect of silver nitrate on micropropagation of *Moringaoleifera* Lam. an important vegetable crop of tropics with substantial nutritional value. *PhysiolMolBiol Plants*, **25**(5): 1311-1322. [Cross Ref]

Ridzuan, N. I. Abdullah, N.Vun, Y. L. and Supramaniam, C. V. 2020. Micropropagation and defence enzymes assessment of *Moringaoleifera* L. plantlets using nodal segments as explant. *S. Afr. J. Bot.*, ***129***: 56-61. [Cross Ref]

Riyathong, T.Dheeranupattana, S. Palee, J. and Shank, L. 2010. Shoot multiplication and plant regeneration from *in vitro* cultures of drumstick tree (*Moringa oleifera* Lam.). In *The International Symposium on Biocontrol and Biotechnology* (pp. 154-159).

Sabale, V. Patel, V.Paranjape, A. Arya, C.Sakarka R. S. and Sabale, P. 2008. *Moringaoleifera* (Drumstick): an overview. *PharmacognRev*, **2**(4): 7.

Saini, R.K. Shetty, N.P.Giridhar, P. and Ravishankar, G.A. 2012. Rapid *in vitro* regeneration method for *Moringaoleifera* and performance evaluation of field grown nutritionally enriched tissue cultured plants. *3 Biotech*, **2**(3): pp.187-192. [Cross Ref]

Salem, J. M. 2016. *In vitro* propagation of *Moringaoleifera* L. under salinity and ventilation conditions. *Genet. Plant physiol*, **6**(1-2): 54-64.

Shank, L. P.Riyathong, T. Lee, V. S. and Dheeranupattana, S. 2013. Peroxidase activity in native and callus culture of *Moringaoleifera* Lam. *J Med Biol Eng*, **2**(3). [Cross Ref]

Shittu, O. H.Akaluzia, H. C. and Chibuogwu, M. O. 2017. A comparison of callus production from *Moringaoleifera* Lam. leaf, cotyledon and stem explants using 2, 4-Dichlorophenoxyacetic acid and kinetin for media supplementation. *SAU Sci-Tech. J*, **2**(1): 1-6.

Stephenson, K. K. and Fahey, J. W. 2004. Development of tissue culture methods for the rescue and propagation of endangered Moringa spp. germplasm. *Econ. bot*, **58**(1): S116-S124. [Cross Ref]

Tsaknis, J. Lalas, S. Gergis, V.Dourtoglou, V. and Spiliotis, V. 1999. Characterization of *Moringaoleifera* variety Mbololo seed oil of Kenya. *J. Agric. Food chem*, **47**(11): 4495-4499. [Cross Ref]

Yadav, K. Singh, N. and Verma, S. 2012. Plant tissue culture: a biotechnological tool for solving the problem of propagation of multipurpose endangered medicinal plants in India. *Journal of Agricultural Technology*, **8**(1): 305-318.