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CLONAL MICROPROPAGATION OF THREATENED *MORINGA CONCANENSIS* NIMMO (MORINGACEAE): SUSTAINABLE ALTERNATIVE

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ABSTRACT: Moringa is one of the medicinally important genera and *Moringa concanensis* Nimmo is a threatened species of it. Clonal micropropagation has been used to produce true to type multiple copies and help to conserve a number of plants. A comprehensive micro-propagation protocol has been developed in the present study. It was found that Murashige and Skoog (1962) (MS) basal elements enriched with 0.05 mg/L of 1-naphthalene acetic acid (NAA) in combination with 0.3 mg/L of 6-benzyl amino purine (BAP) induced 93.3% shooting and maximum (5.5 ± 1.63) number of micro-shoots. Shoot elongation and multiplication were recorded to enhance by making use of 1.0 mg/L of Gibberellic acid (GA_3). Maximum rooting (83.3%) was observed on half-strength MS basal media supplemented with 0.5 mg/L of Indole 3-Butyric Acid (IBA) in combination with 0.5 mg/L NAA. Even though the success of this protocol is varied according to genotype, it still has high applicability for the conservation of germplasm of plant.

INTRODUCTION: The use of plants as medicine is an age-old practice in India. The Vedas and Upanishads have descriptions of plants that are used for the treatment of various ailments of men, animals, and plants. Details about the plants, its parts, and the method of its use for treating a variety of diseases are described in literature like Charak Samhita. Increasing demand and the establishment of manufacturing units of herbal medicines resulted in an indiscriminate collection from wild habitat in the absence of cultivation resulted in depletion of the stock.

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Also, due to a lack of knowledge about the medicinal properties of many plants and their subsequent neglect, a large number of plants are at the risk of getting threatened and later on extinction. Moringa is one of such plants having medicinal properties that are being used on a large scale. The genus Moringa is native to parts of Africa and Asia [1], and at present, its 13 species are widely distributed in the World. In India species of Moringa can be found in every eco-climatic zone from desert to rainforests. *Moringa concanensis* is one of the species having its uses recorded in traditional as well as Unani literature. Vernacular names of *M. concanensis* are Kattumurungai or Peyimurungai, Murinna, Kokan Moringa, Kadu sevga, Nugge, Muva, Jangli or Dungrau Saragvo and Kadvo Saragvo in Gujarat [2]. All parts of the plant are used for treating rheumatism, venomous bites, painful swellings and gastrointestinal motility disorders.

It is also used as circulatory stimulant [3]. Its roots are utilized to relieve spinal cord pain [3]. Dry leaves and dried seeds are used for ophthalmic preparations and treatment of venereal infection, goiter, glycosuria, diabetes, jaundice, some skin tumors, sore throat and lipid disorder [3, 4]. Its flowers are known to cure thyroid while, powder of seeds is used internally for treating intestinal worms. The stem bark is used to relieve bloating and the gum is used for the treatment of headache and dental problems [4]. It is also used as an abortifacient. Fatty oil extracted from the seed kernels can be utilized in the formulation of the topical sunscreen lotion for protection against Ultraviolet (UV) radiation from sun rays [4].

Like other medicinal plants due to its overexploitation, neglect and changes in the soil topography, *M. concanensis*, once distributed in tropical dry forest from southeastern Pakistan to the southern tip of India is now restricted to a few patches of India and Pakistan. It is a wild plant in nature. Earlier, *Moringa concanensis* Nimmo was reported to grow in Gujarat. Due to indiscriminate cutting of forests, clearing of pastures and other common land resources, it has been reported by many scientists to have become rare and endangered plants [4, 5]. *Moringa concanensis* is reported to be threatened by prolonged drought and poor soil conditions. *Moringa concanensis* Nimmo is communicated as a vulnerable species of Gujarat as it has disappeared completely from wild [6].

Scientists have developed methods of vegetative propagation for conservation, propagation, and protection of genetic uniformity in *M. concanensis,* using stem cuttings of a mature tree. The use of plant tissue culture methods is well documented for the conservation of threatened and rare plants. The technique can be used for clonal propagation and also for retrieving somaclonal variants [7]. The present paper is an attempt to formulate a reproducible protocol for micro-propagation of threatened medicinal plants, *M. concanensis.*

MATERIALS AND METHODS:

Collection of Plant Material and Source of Explant: Sources of explants were collected from trees of *M. concanensis* located at the primary government school campus in Vyra and farmhouse in Surat, Gujarat. Dried seeds were harvested from collected matured and dried drumstick pods. Each plant sample was authenticated by Dr. M. N. Reddy, Professor Dr. M. N. Reddy, Department of Bioscience, VNSGU, Surat and plant authentication numbers for samples 1 and 2 are BVBRC1401 and BVBRC1402 respectively.

Explant Preparation and Culture Condition: Harvested matured and dried seeds were washed thoroughly in running tap water, seed coat was removed aseptically, and seeds were washed using liquid detergent Teepol (Himedia, India) added with one drop of Tween 20 (Himedia, India) for 15-20 min, followed by rinsing thrice with sterile distilled water. Later on, these seeds were treated with 10 ppm solution of fungicide-Carbendazim (Sigma Aldrich, USA) for 15 min followed by rinsing thrice with sterile distilled water. Subsequently, seeds were treated with a 10% solution of NaOCl (Merck, Germany) for 10 minutes, followed by rinsing twice with sterile distilled water. Finally, seeds were surface sterilized with 0.1% (w/v) mercuric chloride solution for 5 min and rinsed three times with sterile distilled water.

All the chemicals and plant growth regulators used here as a component of media were procured from Himedia (India) except Indole 3-Butyric Acid (IBA) purchased from Sigma Aldrich (USA). Finally, surface-sterilized seeds were inoculated on Murashige and Skoog (MS) basal medium [8] containing 3% sucrose and 0.75% agar (w/v). The pH of the media was adjusted to 5.7-5.8 using 0.1 N NaOH and HCl. The medium was autoclaved at 121 °C and at 1.0546 Kg/cm^2 for 15 min. One seed per culture tube was inoculated and culture tubes were incubated at 24 ± 1 °C temperature in a growth chamber under cool-white light (about 50 $\mu mol/m^2/s$) at 16/8 h photoperiods. Parts of *in-vitro* grown seedlings were used after it reaches a height of 8 cm.

Shoot Regeneration, Multiplication and Elongation: Stem nodes, internodes, hypocotyls and epicotyls segments of *in-vitro* grown seedling were used as a source of explants. For shoot multiplication and elongation, various parts of *in-vitro* grown seedlings were transferred to MS medium supplemented with different growth hormones in combination. MS media enriched with

different concentration of BAP (0, 0.3, 0.5, 1.0) mg/L in combination with (0, 0.05, 0.5, 1.0) 1-Naphthalene Acetic Acid (NAA) mg/L was used for multiple shoots generation. After the multiplication of shoots from elite and quiescent explants, shoots were further subcultured on MS medium supplemented with 0.3 mg/L BAP along with (0, 0.5, 1.0) mg/L of Gibberellic acid (GA_3) for elongation of shoots. Subculturing was done at an interval of 4-5 weeks.

Data on % shoot formation; the number of shoots per explants; the height of micro shoots per explant and time taken for shoot initiation were recorded, analyzed, and presented in **Tables 1** and **2**.

Root Induction: The multiple adventitious shoots were separated and cultured on MS basal and half-strength MS media supplemented with different concentrations (0, 0.5, 1.0, 1.5, and 2.0) mg/L of NAA along with 0.5 mg/L of IBA for aiding in optimum rooting response. Data were recorded after 2 weeks, and a number of roots per shoots, % root formation, and length of roots per shoot observed and presented in **Table 3**. Rooted plantlets were transferred to the pots for hardening.

Acclimatization: Well rooted plantlets were carefully taken out and rinsed thoroughly with sterile distilled water to remove any remaining medium. Subsequently, plantlets were transferred in plastic bags filled with autoclaved planting/potting mixture: soil: vermicompost: perlite + vermiculite: sand (2:1:1:1).

Plants were irrigated with half-strength MS medium and covered with perforated polythene bags to reduce humidity loss. These plantlets were kept in a growth chamber for 15 days. After 15 days, polythene bags were removed and survived plants were maintained in a greenhouse for more 15 days. Hardened plantlets were then transferred to the field.

Data Analysis: Experiments were set in a complete randomized design (CRD), and each treatment consisted of 10 replicates and was repeated thrice. Statistical analysis was done using SPSS 22.0 software. Duncan's multiple range test was used to detect a significant difference and variance among mean [9]. A P-value <0.05 was considered significant.

RESULTS AND DISCUSSION:

***In-vitro* Seed Germination:** Lack of awareness and urbanization affected the habitat of *Moringa concanensis. M. concanensis* Nimmo has been reported as threatened species of Moringa genus, thus attempts should be made to rescue germplasm of the plant. Vegetative propagation approach like clonal micropropagation can be used to rescue threatened species of Moringa. The procedure of clonal micropropagation provides elite disease-free plantlets and yields multiple copies of a plant in less space and inlimited duration. Seeds were collected from wild habitat, so there are high chances of seeds is getting infested with microbes. *In-vitro* process of seed germination eliminates chances of contamination and provides elite seedling that will serve as a source of explants in the present study. For raising healthy and elite seedling, seeds were treated with different surface sterilizers.

In present study seeds only treated with NaOCl led to failure in seed germination due to contamination present. The existence of seed-borne bacteria had been reported by Steinitz *et al.* (2009) [10] and also noted that NaOCl alone was not found enough to eradicate microbial contamination in *Moringa oleifera*. Steinitz *et al.,* (2009) reported that microbial contamination at the shoot base interfered with rooting [10]. Due to microbial infestation seeds were either unable to germinate, died or led to the growth of infected seedling. To get completely rid of microbial infection, seeds had been treated with 10 ppm solution of fungicide carbendazim for 15 min in the present study. Treatment of seeds with higher concentration of fungicide for more than 15 min causes damage to the seeds. Use of 0.1% mercury chloride for 5 min lead to 90% seed germination rate during the present study. Similar results were recorded by Jun-jie *et al.,* (2017) in the case of *M. oleifera* [9]. The use of mercury chloride in higher concentrations found phytotoxic to seed growth.

The high success rate of seed germination can be achieved by treating seeds with a series of disinfectants. In the case of *Moringa oleifera* 84% seed germination was observed by making use of a variety of disinfectants [11]. The viability of seed is a potent factor in the germination of seed. In Moringa species, 80% of seed remains viable up to

one year [11]. In the present study, germination began 7 days after inoculation on MS media **Fig. 1A**. Different parts of 28 days old, healthy, and elite seedlings were used for clonal-propagation. MS basal medium without growth hormones was used for seed germination in the present study. Similar results were reported by Avila-Treviño *et al.,* (2017) [12].

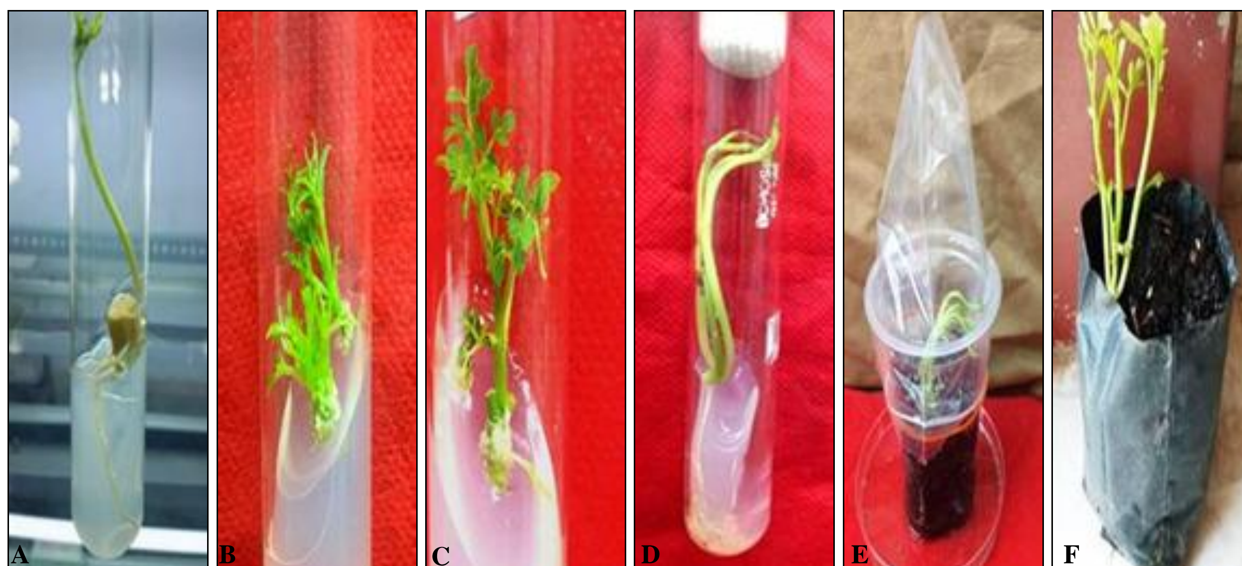

FIG. 1: CLONAL MICRO-PROPAGATION OF *MORINGA CONCANENSIS* AND ACCLIMATIZATION OF REGENERATED PLANTLETS: (A) 24 days old seedling; (B) Multiple shoots induced from *in-vitro* grown seedlings on ms media containing 0.3mg/L BAP and 0.05 mg/L of NAA After 28 days; (C) Elongated shoots observed on MS medium enriched with 0.3 mg/L BAP and 1.0 mg/L GA_3; (D) Rooting of regenerated micro shoots on MS medium supplemented with 0.5 mg/L of IBA and NAA; (E) *In vitro* regenerated elite and healthy plantlet shifted for acclimatization in plastic glass filled with autoclaved potting mixture and covered with polythene bag; (F) Acclimatized plant.

Shoot Regeneration, Multiplication and Elongation: Cytokinin is responsible for cell division, multiple shoot induction, and elongation. During the present study, multiple shoots developed within a fortnight from various parts of seedling like hypocotyls and shoot nodes, sub-cultured on shoot induction medium. Shoot regeneration efficiency was greatly improved when BAP used in combination with NAA in the case of *in-vitro* propagation of *M. concanensis* **Table 1**.

It can be seen from **Table 1** that 0.3 mg/L of BAP in combination with 0.05 mg/L of NAA was found best as 93.3% shooting was observed with 5.5 ± 1.63 number of shoots and with shoot height 5.40 ± 1.53 cm using nodal and hypocotyls regions of *in-vitro* grown seedling in 28 days of inoculation as seen in **Fig. 1B**. Fatima *et al*., (2016) reported 0.1 mg/L BAP and 0.5 mg/L NAA found best for the proliferation of shoots using *M. concanesis* [3].

TABLE 1: EFFECT OF DIFFERENT CONCENTRATION OF BAP AND NAA ON SHOOT REGENERATION AND MULTIPLICATION

S. no.	BAP(mg/L)	NAA (mg/L)	% Shoot formation	Number of shoots/explant	Shoot length (cm)
1	0.0	0.05	20 ± 0.41d	0.2 ± 0.41d	0.21 ± 0.45g
2	0.3	0.05	93.3 ± 0.25a	5.5 ± 1.63a	5.40 ± 1.53a
3	0.5	0.05	73.3 ± 0.45ab	2.5 ± 1.26c	2.45 ± 1.13bc
4	1.0	0.05	56.7 ± 0.50bc	1.4 ± 1.3c	1.30 ± 1.17ef
5	0.0	0.5	40.0 ± 0.49cd	0.8 ± 0.96cd	0.79 ± 0.92f
6	0.3	0.5	73.3 ± 0.45ab	2.3 ± 1.4b	1.60 ± 0.9e
7	0.5	0.5	63.3 ± 0.49bc	0.93 ± 0.86c	0.86 ± 0.72f
8	1.0	0.5	60.0 ± 0.49bc	0.83 ± 0.79cd	0.91 ± 0.78f
9	0.0	1.00	56.7 ± 0.50bc	1.0 ± 0.99c	1.06 ± 0.95ef
10	0.3	1.00	70.0 ± 0.47ab	2.83 ± 1.76b	2.89 ± 1.78b
11	0.5	1.00	63.3 ± 0.49bc	2.27 ± 1.68b	1.92 ± 1.38cd
12	1.0	1.00	56.7 ± 0.50bc	1.00 ± 0.98c	1.19 ± 0.99ef

Each value in Table 1 represents the mean ± SD of three replicates, each with 10 explants. Means followed by the different letters in the same column are significantly different from each other at P≤0.05 level, according to Duncan's multiple range test.

Earlier studies on *Moringa oleifera* achieved optimum shooting by using 1.0 mg/L of BAP [12]. Avila-Treviño *et al*., (2017) used 1 mg/L of BAP, resulted in the production of the highest sprout number but the shoots were very thin and vitrified, which was not suitable for multiplication [12].

In the present study, it was noted that the height of the plants' main shoot and offshoots decreased with an increased BAP concentration. In contrast to the present study Gayathri *et al*., (2015) reported that BAP at 2 mg/L produced 80% of shoots and 6 numbers of axillary shoots [13]. An increase in the BAP level in MS media can lead to a decrease in induction of shoots. Head wilt was very often found in all plant parts in BAP free medium. The high concentration of BAP causes a decrease in the number of shoot formation per explants.

During the regeneration process, a small amount of callus was first formed along the cut surface in about 1 week and then numerous shoot bud primordia appeared in another two weeks. For further multiple shoot elongation combination of gibberellic acid and cytokinin was used in the present study.

TABLE 2: EFFECT OF DIFFERENT CONCENTRATION OF GA_3 AND COMBINATION OF BAP ON SHOOT INDUCTION AND ELONGATION

S. no.	MS+BAP (mg/L)	GA_3 (mg/L)	% shoot formation	Number of shoot/explants	Shoot length/explants (cm)
1	0.3	0.0	33.3 ± 0.48^c	0.33 ± 0.48^c	0.24 ± 0.36^c
2	0.3	0.5	73.3 ± 0.45^b	2.0 ± 1.29^b	2.34 ± 0.38^b
3	0.3	1.0	96.6 ± 0.18^a	7.57 ± 1.13^a	6.82 ± 0.60^a

Each value in the table 2 represents the mean ± SD of three replicates, each with 10 explants. Means followed by the different letters in the same column are significantly different from each other at P≤0.05 level, according to Duncan's multiple range test.

Multiple micro-shoot containing explants when transferred on MS media with 0.3 mg/L of BAP along with 1.0 mg/L of Gibbrelic acid-(GA_3) found best. Around 96% shooting was observed with 6.82 ± 0.60 cm of shoot height as seen in **Fig. 1C** and **Table 2**. Gibberellic acid was found responsible for cell elongation and cell division [14]. Well-developed micro shoots were eventually transferred onto the root induction medium.

Root Induction: Micro shoots were separated and transferred carefully to root induction media. In present study highest rooting (83.3%) was observed on half-strength MS media supplemented with 0.5 mg/L of IBA in combination with NAA resulted in 6.5 ± 0.50 roots per shoot and root length was about 5.6 ± 0.50 cm as seen in **Fig. 1D** and **Table 3**. In the case of *M. concanensis,* full-strength MS basal media was also found excellent with a combination of growth hormones IBA and NAA, but a maximum number of rooting was achieved on half-strength MS media **Table 3**. The similarity was observed in the results of Marfori (2010) reported half-strength MS media with NAA found optimum for vigorous root induction [15]. Other researchers stated that maximum rooting can be archived by incorporating IBA or NAA alone or in combination with the half-strength MS media [14, 16].

Fatima *et al*., also observed maximum rooting on growth hormone free half-strength MS media in the case of *M. concanesis*, but roots found slender and short [3]. In *M. olieifera* 100 % *in-vitro* rooting on growth hormone-free MS basal media was observed 12 weeks later [14].

TABLE 3: EFFECT OF NAA, IBA AND STRENGTH OF MS MEDIA ON ROOT INDUCTION

S. no.	MS	NAA (mg/L)	IBA (mg/L)	% Roots formation	Number of roots/ shoots	Root length per shoots (cm)
1	Full	0.0	0.5	60 ± 4.54^c	1.80 ± 0.40^d	3.20 ± 0.41^d
2	Full	0.5	0.5	80 ± 3.71^a	5.00 ± 0.45^a	5.33 ± 0.48^a
3	Full	1.0	0.5	63.3 ± 4.79^b	3.20 ± 0.41^b	4.10 ± 0.40^b
4	Full	1.5	0.5	52.3 ± 4.3^d	2.06 ± 0.37^c	3.33 ± 0.48^c
5	Full	2.0	0.5	46.3 ± 4.90^e	1.40 ± 0.49^e	2.23 ± 0.43^e
6	Half	0.0	0.5	53.3 ± 4.79^d	2.26 ± 0.45^c	2.23 ± 0.48^e
7	Half	0.5	0.5	83.3 ± 4.79^a	6.5 ± 0.50^a	5.6 ± 0.50^a
8	Half	1.0	0.5	69.7 ± 4.13^b	3.57 ± 0.50^b	4.30 ± 0.47^b
9	Half	1.5	0.5	57.33 ± 4.49^c	2.26 ± 0.52^c	3.23 ± 0.43^c
10	Half	2.0	0.5	50.0 ± 5.25^e	0.93 ± 0.36^d	2.63 ± 0.49^d

Each value in the table-3 represents the mean ±SD of three replicates, each with 10 explants. Means followed by the different letter in the same column are significantly different from each other at P<0.05 level, according to Duncan's multiple range test.

In the present study, slender and weak roots were developed on growth hormone-free medium. So, during the transfer of plantlets, root damage was observed. The length of the root was increased by using IBA, while the number of root formation declined and callus differentiation, increased by using high concentrations of NAA. The high concentration of IBA is harmful for multiple root induction from shoots. Well rooted plantlets had been preceded for acclimatization. Plantlets produced under the present protocol looked healthy.

Acclimatization: The healthy plantlets after successful rooting were shifted to a plastic glass filled with an autoclaved media. The *in-vitro* grown plantlets were removed from test tubes and washed with sterile distilled water to remove traces of agar from plantlets. Those plantlets were then switched to a plastic glass filled with an autoclaved mixture of potting mix material; soil: vermicompost: perlit+ vermiculite: sand (2:1:1:1:1) and irrigated with half-strength MS medium. The plastic glass was then covered with polythene to maintain the humidity and was placed in a growth chamber at 25 ± 2 °C [14] as seen in **Fig. 1E**. Plastic glasses were transferred to the greenhouse after a period of three weeks. These *in-vitro* regenerated plantlets were subsequently transferred with an 80% transplanting rate into pots containing soil **Fig. 1F**. Abbas (2014) and Steinitz *et al*., (2009) reported 85% and 95% success rate by transplanting survived plantlets to the soil after 4-5 weeks [10, 14].

CONCLUSION: *Moringa concanensis* Nimmo is one of the potent medicinal plants proclaimed as threatened species of genus Moringa. The present study aims to establish a sustainable, efficient and highly reproducible clonal propagation protocol for conservation and multiplication of this plant. The purpose behind selecting *in-vitro* grown seedling, as a source of explants, was to eliminate the chances of contamination. Surface sterilization and disinfection method optimized during the study found highly effective. Moreover juvenile and quiescent explants propagated on MS media with BAP, and NAA developed into 5.5 ± 1.6 number of shoots, and shoot height was 5.40 ± 1.53 cm. Maximum shoots (96.6%) were synthesized on MS+BAP and GA_3 medium. We were able to achieve long and maximum rooting on half-strength MS medium supplemented with growth hormones NAA and IBA. Healthy and uniform plantlets produced during this protocol. Further, by using the molecular marker technique, researchers can check the genetic fidelity of plant tissue culture raised plantlets. This research paper can be used in genetic transformation studies and can assist in the conservation of multipurpose plants.

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