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OPTIMIZATION OF THE MICRO-CLONING SYSTEM OF THREATENED *Moringa oleifera* LAM.

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For the efficient mass propagation of *Moringa oleifera*, an *in vitro* regeneration protocol was optimized through direct and indirect regeneration. The callus was initiated within four days on MS medium supplemented with 2.0 mg/L of 2,4-D, from cotyledonray explants. IAA and NAA took more days (18.17±1.17 and 17.80±0.90, respectively) to initiate callus as compared to 2,4-D and TDZ (7.98±0.41 and 9.15±0.55, respectively). 2,4-D and Kinetin in-combination also initiated callus earlier (9.75±0.52 days). In direct regeneration, the maximum shoot length (7.0±0.58 and 6.27±0.41cm) and number of leaves (12.0±1.15 and 16.0±0.50) were recorded in hypocotyl explants when cultured on MS medium supplemented with 0.1 and 0.5 mg/L of Kinetin, respectively. On average nodes produced more number of shoots (1.65±0.21) as compared to hypocotyl explants. The number of roots (3.83±0.40 and 3.67± 0.21) and the root length (7.15±0.62 and 9.33±1.14) by node and hypocotyls was good on MS and the half MS media as compared to the other auxins used in this study. While the hypocotyl explants gained maximum root length (9.33±1.14) and the number of roots were higher (3.83±0.40) than node explants. The 85% plantlets were acclimatized successfully for the field conditions.
Keywords: *Moringa*, *in vitro*, regeneration, callus, micropropagation

INTRODUCTION

The role of trees for supplying the various human and animal nutritional requirements is as old as mankind itself. Moreover, trees are commonly used for pharmaceutical properties. In modern era, despite tremendous advancement in synthetic chemistry, several human health problems are dominantly solved by herbal drugs and possess no or rare side effects. With increase in world population, the use of trees for nutrition, anthapogenic activities, there is rapid demolishing of natural ecosystems leading to precious and useful medicinal plants as dwindling and endangering (Kamboj, 2000). Presently, there are no well-organized practices for growing of nutritional tree farming and natural habitats are disappearing particularly for *Moringa*. The *Moringa oleifera* is native to sub-Himalayan tracts of India and Pakistan (Shahzad *et al*., 2013). All of its parts are highly nutritious not only for the human beings but also for the animals to increase their milk production. But it is considered as an ignorant plant tree even at its place of origin due to under utilization and consequently this germplasm is depleting from this area.

*Moringa*ceae is an old world, a perennial soft wood tree family, distributed in tropical regions. It is an indigenous to western and sub-Himalayan tracts including India, Pakistan, Asia Minor, Africa and Arabia (Somali *et al.,* 1984). Thirteen tropical and subtropical species of this genus are known of which many are in danger of extinction. Genetic conservation status of *Moringa arborea*, *Moringa borziana*, *Moringa longituba*, *Moringa rivae*, *Moringa ruspoliana*, and *Moringa stenopetala* is endangered (Stephenson and Fahey, 2004). There is a great concern in the conservation of the *Moringa* species from biodiversity, ethno-botanical, dietary and pharmacological perspectives. Only *Moringa oleifera* L. is cultivated and its cultural practices have been developed (Sanchez *et al.,* 2006). *Moringa oleifera* is a valuable food that has drawn attention as the 'natural nutrition of the tropics. The fruit, leaves, flowers and immature pods of this tree are highly nutritious and are used as vegetable in many parts of the world, especially in under developed countries like Africa, India, Pakistan, the Philippines and Hawaii (Anwar and Bhanger, 2003). Propagation is usually done by growing cuttings of 3-4 feet shoot or through seed (Nouman *et al*., 2012). Sexual propagation of some of these species is tedious and not even be possible without having enough individual plants for cross-pollination. Thus seed germination is low while conventional propagation system in Pakistan is through cuttings of the mother plant limbs which ultimately cause the death of the mother plant.

Tissue culture techniques are based on the totipotency concept of cells and play important role in plant micropropagation, and conservation of plants materials (Jaskani *et al*. 2008). In addition to these, tissue culture techniques are important tools in the fundamental and applied research. These are helpful in the conservation of genetic resources, understanding of gene structure, function in molecular biology and plant improvement through transgenic technology.

Since flowering of a number of the large tree species of *Moringa* does not even commence until a critical size is attained, highly unlikely to occur with multiple trees in a greenhouse, tissue culture may be the only practical way to cultivate these trees out-side the tropics. Amplification of these rare individuals by tissue culture propagation would make them more widely available and less likely to become lost to cultivation. Thus developing tissue culture methods for this genus is urgently required (Stephenson and Fahey, 2004). The aim of the current study was to establish direct and in direct *in vitro* regeneration protocol of *Moringa oleifera* which is mostly present in the southern Punjab (Pakistan).

MATERIALS AND METHODS

***Plant material and sterilization*:** Healthy uniform seeds of *Moringa* were obtained from different selected districts of Punjab province (Pakistan). Seeds were surface sterilized inside the laminar flow hood by immersion in 0.1% mercuric chloride (w/v) for 2 min and 20% sodium hypochlorite (v/v) for 5 min, followed by rinsing three times in sterile distilled water. Seed coats were removed aseptically and seeds were again surface sterilized by immersion in 20% sodium hypochlorite (v/v) for 2 min, followed by rinsing three times in sterile distilled water (Savita *et al*., 2010). De-coated seeds were planted aseptically in MS basal medium (Murashige and Skoog, 1962). Seed cultures were maintained in the dark at 26±2°C for 15 days. Upon germination, seedlings were transferred under continuous light at 2,500 lux intensity produced from cool white fluorescent tubes.

***Culture media*:** MS (Murashige and Skoog, 1962) organic and inorganic salts and vitamins were used as a basal medium. Growth regulators were made with different workable concentrations by dissolving them in 1N NaOH and made up the required volume with the distilled water and stored at 4°C refrigerator for future use.

Indirect regeneration:

***Explants*:** The explants were taken from the *in vitro* grown plantlets through seeds. Different explants including leaves, epicotyl, hypocotyl and cotyledons were used for the callus induction.

***Callus induction*:** The explants for callusing were cultured on MS media enriched with different levels (0, 0.5, 1.0, 1.5 and 2.0 mg/L) of 2,4-D, TDZ (Thidiazuron), IAA (Indole acetic acid) and NAA (Naphthalene acetic acid). MS media alone or supplemented with different growth regulators was used in combinations 2.0+0.5 and 2.5+1.0 mg/L, respectively, in each combination of IAA+BAP, 2,4-D+Kinetin and NAA+Kinetin; and also 0.1 g/liter myoinositol was added in each media. For further proliferation the callusing explants were sub-cultured on fresh media after 3-4 weeks with the same composition. The observations were made for callogenesis after 1 week interval. The cultures were incubated for 4 weeks under cool white fluorescent lights with a 16 h photoperiod at temperature 26±2°C. Callus cultures were sub-cultured two times on the same fresh media at 3-4 weeks interval (Saini *et al.,* 2012). All the *in vitro* experiments were conducted with 5 replicates per treatment and the data for days to initiate callus was recorded.

***Somatic embryogenesis and shoot regeneration*:** Different growth regulators (individually and in-combination) were used for the somatic embryogenesis and regeneration. MS media supplemented individually with BA, Kinetin and NAA at different levels (0.5, 1.5, 2.0 and 2.5 mg/L) and also in combination MS + BAP (3.0 mg/L) + NAA (1.0 mg/L) and MS + Kinetin (1.0 mg/L) + NAA (3.0 mg/L).

Direct regeneration:

***Shoot induction*:** Germinated seedlings consisting of 3–4 nodes (3–4 weeks after inoculation) were used in the experiment. Nodes and hypocotyl explants were prepared and transferred to a multiple shoot induction medium, consisting of MS salts and benzyl adenine purine (BAP), Kinetin and benzyl adenine (BA) with different concentrations (0, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/L) to determine their effect on multiple axillary shoot formation. Data were recorded regarding, shoot length, number of leaves and number of shoots per explants.

***Root induction*:** Micro shoots obtained were repeatedly subcultured in MS basal medium supplemented with root inducing hormones. Axillary shoots induced from nodal and hypocotyl explants were transferred to a root induction medium, consisting of MS salts and indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) with different concentrations (MS 0, half MS, 0.05, 1.0, 1.5, and 2.0 mg/L of each growth regulator). Data regarding number of roots per shoot and root length were recorded after 7 days of transfer to root inducing media. Rooted plantlets were transferred to the pots for hardening.

***Hardening of the rooted plantlets*:** The rooted plantlets were transferred in plastic bags containing sterilized sand, watered, covered with transparent polythene bags, and kept under partial light inside a growth room at ambient temperature (26±2°C). Gradually holes were made in polythene bags to reduce humidity and after 15 days, the polythene bags were removed and the survived plants were maintained inside the greenhouse for another 15 days. These hardened plantlets were then transferred into the soil.

***Data analysis*:** Experiment was replicated three times with 25 tubes per treatment per replication. The experimental design was Completely Randomized Design (CRD) with three factors. The data were analyzed for variance and significant treatment means were compared using Duncan Multiple Range (DMR) test.

RESULTS

Indirect regeneration:

***Days to initiate callus*:** The interaction of growth regulators at different levels with different explant types showed significant association for induction of callus within a given time frame. Different explants (hypocotyls, epicotyls, cotyledon and leaf) varied significantly in their potential to initiate and produce calli on media containing different growth regulators.

When 2,4-D was used in 2.0 mg/L concentration it proved the most efficient growth regulator for the callus initiation which started callus just in 4.20±0.20 days in the cotyledon explants (Table 1). Overall mean of the callus initiation showed that good callus initiation was observed in the cotyledons with 2,4-D (6.10±0.26 days) whereas the hypocotyls also showed good response of the callus initiation (7.45±0.48 days) but the delay in callus initiation was examined in the leaf explants (10.20±0.33 days). The TDS also performed good for the quick callus initiation; on average all the explants on TDZ media took 9.15±0.55 days to initiate callus while epicotyl explant at the higher level of 2.0 mg/L initiated callus in 4.40±0.24 days. Contrary to the 2,4-D and the TDZ, the IAA and the NAA for all the explants, took much time for the callus initiation (18.17±1.17 and 17.80±0.90 days, respectively). It was also observed, with increasing the concentration of all the growth regulators there was reduction in the time to initiate callus (Table 1).

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05); Small letters represent comparison among interaction means and capital letters are used for overall mean; Values are mean±SE.

All the explants were cultured on media containing different combinations of growth regulators and a strong association was observed between auxins and cytokinins. 2,4-D and Kinetin used collectively gave efficient callus initiation in 9.75±0.52 days as compared to the other two combinations of the growth regulators, while hypocotyl and epicotyl explants initiated callus in less number of days (7.60±0.69 and 9.30±0.47 days, respectively) (Table 2). 2,4-D and

Table 1. Interactive effect of explants, growth regulators and their levels on days to initiate callus in *Moringa*

Explant x Level (mg/L)	Growth regulators			
	2,4-D	**TDZ**	**IAA**	**NAA**
Epicotyl x 0.5	9.60±0.51c	11.40±0.51a	11.20±1.69j	16.20±0.37e
Epicotyl x 1.0	8.00±0.45d	8.20±0.37e	14.40±2.38h	15.60±0.40f
Epicotyl x 1.5	6.80±0.58e	5.60±0.24g	18.80±0.58e	14.20±0.58g
Epicotyl x 2.0	8.20±0.66d	4.40±0.24h	21.60±0.60c	11.00±0.55i
Mean Epicotyl	**8.15±0.55C**	**7.40±0.34D**	**16.50±1.31A**	**14.25±0.48B**
Hypocotyl x 0.5	10.20±0.58b	10.40±2.69c	21.20±0.73c	20.00±0.45c
Hypocotyl x 1.0	6.00±0.45f	11.20±0.80a	19.60±0.51d	19.80±0.49c
Hypocotyl x 1.5	8.20±0.37d	9.60±0.24d	25.40±0.68b	17.00±0.45d
Hypocotyl x 2.0	5.40±0.51g	8.60±0.40e	27.00±0.32a	15.80±0.58f
Mean Hypocotyl	**7.45±0.48D**	**9.95±1.03C**	**23.3±0.56A**	**18.15±0.49B**
Cotyledon x 0.5	8.20±0.37d	11.00±0.84b	13.20±1.77i	20.20±0.73c
Cotyledon x 1.0	6.60±0.24e	9.80±0.37d	16.40±1.44f	20.20±0.86c
Cotyledon x 1.5	5.40±0.24g	8.80±0.20e	18.80±2.56e	12.80±2.11h
Cotyledon x 2.0	4.20±0.20h	7.40±0.24f	19.00±2.77d	10.20±0.58j
Mean Cotyledon	**6.10±0.26D**	**9.25±0.41C**	**16.85±2.14A**	**15.85±1.07B**
Leaf x 0.5	13.20±0.37a	10.80±0.37c	15.40±1.21g	27.60±0.75a
Leaf x 1.0	10.20±0.37b	10.40±0.51c	18.40±0.51e	22.00±4.28b
Leaf x 1.5	9.40±0.24c	9.00±0.32d	19.20±0.50d	22.40±0.60b
Leaf x 2.0	8.00±0.32d	9.60±0.40d	19.80±0.37d	19.80±0.66c
Mean Leaves	**10.20±0.33C**	**9.95±0.40D**	**18.20±0.67B**	**22.95±1.57A**
Overall Mean	**7.98±0.41D**	**9.15±0.55C**	**18.71±1.17 A**	**17.80±0.90B**

Table 2. Effect of growth regulators on explants regarding days to initiate callus in *Moringa*

Explant	Growth regulator		
	2, 4-D+Kin	**IAA+BAP**	**NAA+Kin**
Leaf	10.60 ± 1.61b	10.50 ± 0.27d	9.60 ± 0.48b
Epicotyl	9.30 ± 0.47c	15.70 ± 0.68a	18.00 ± 0.58a
Hypocotyl	7.60 ± 0.69d	15.10 ± 0.46b	9.30 ± 0.47c
Cotyledon	11.50 ± 0.62a	13.40 ± 0.69c	18.20 ± 0.55a
Mean	9.75 ± 0.52B	13.68 ± 0.42A	13.78 ± 0.74A

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean. Values are mean ±SE.

Kinetin at 2.0+1.0 mg/L initiated callus in less time period (8.3±0.71 days) as compared to the other higher concentrations (Table 3, Fig. 1).

Table 3. Effect of growth regulators and their levels on days to initiate callus in *Moringa*

Level	Growth regulator		
(mg/L)	2, 4-D+Kin	IAA+BAP	NAA+Kin
2.0+1.0	8.30±0.71b	14.35±0.60a	13.50±1.05b
2.5+1.0	11.20±0.61a	13.00±0.55b	14.05±1.06a
Mean	9.75±0.66B	13.68±0.58A	13.78±1.58A

Auxins and cytokinins were used individually and in combination with different concentrations for the regeneration from the callus. Most of the callus produced was of loose structure and did not respond further for regeneration. So the direct regeneration was done using the hypocotyls and the nodes.

Direct regeneration:

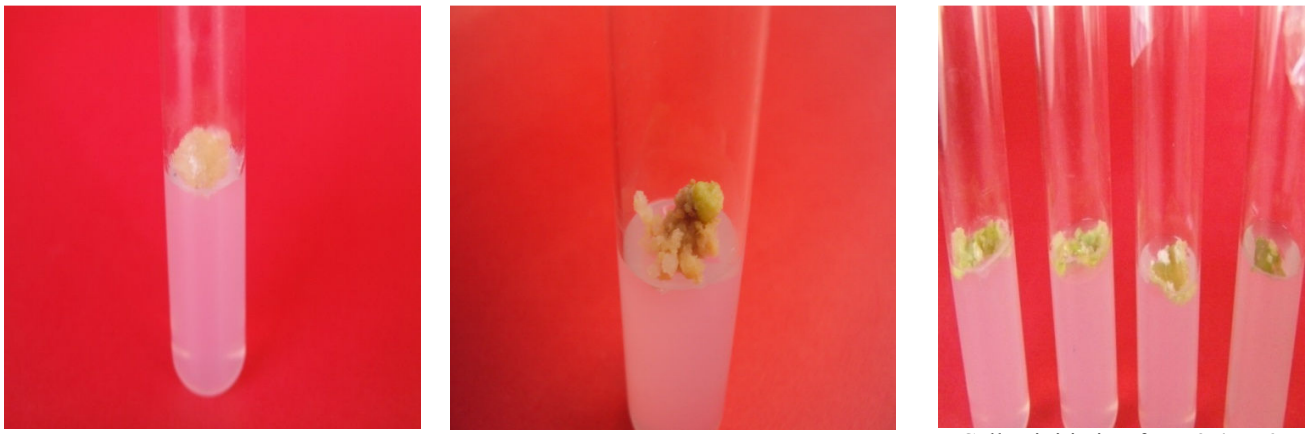
***Shoot length (cm)*:** The direct regeneration of the *Moringa* was accomplished by direct culturing of the *in vitro* grown seedling explants, the hypocotyls and the nodes. The interaction among the explants, growth regulator and the levels also had the significant effect on the shoot length of the *Moringa*.

The shoot length was affected by the interaction among the growth regulators and its all levels as well as the explants (Table 4). The maximum shoot length (4.31±0.49 cm) was attained when the hypocotyl explants were cultured on the MS medium supplemented with Kinetin. The least shoot length (2.18±0.31cm) was attained when these hypocotyls were cultured on the MS medium supplemented with BAP while BA gave the medium shoot height (2.55± 1.51cm).

Similar results were observed when the node explants were cultured on the same media and the Kinetin gave the best results with maximum shoot length as compared to the BAP (2.39±0.39 cm) and BA (2.44±0.27 cm), respectively. The Kinetin at lower concentration of 0.1 mg/L and 0.5 mg/L attained the maximum shoot height of 7.0±0.58 and 6.27±0.41 cm, respectively (Fig. 2). The nodes attained the less height (5.00±0.29 cm) as compared to the hypocotyls at the concentration of 0.5 mg/L of the Kinetin. The increase in the concentrations reduced the shoot length in both the nodes and the hypocotyl explants. Same trend of the shoot length was recorded both in the BAP and BA but the shoot length was almost half as compared to the Kinetin (Table 4).

***Number of leaves*:** The different growth regulators and their levels individually and also in combination had a highly significant effect on the number of the leaves. While the interaction between the explants, growth regulator and the levels also had a highly significant effect on the number of the leaves.

The interaction among the growth regulators and their levels with the explants (Table 4) revealed that the hypocotyls produced the maximum number of leaves (16.0±0.5) when cultured on Kinetin at 0.5 mg/L followed by 0.1 mg/L which produced 12.0±1.15 leaves while on the highest level of Kinetin (2.0 mg/L) the number of leaves were decreased 3.00±0.58. With the increase in the concentration of the Kinetin there was reduction in the number of leaves. The hypocotyls produced the maximum number of leaves (17.67±1.45) at the concentration of 1.0 mg/L of BAP. Kinetin and BAP produced statistically similar number of leaves on all the levels of the growth regulators (9.67±0.63 and 10.0±1.04, respectively) while BA produced the least number of leaves (6.53±0.77) on all the levels of BA used. The nodes produced the maximum number of leaves (19.33±0.67) when cultured on 1.0 mg/L of Kinetin while at



Callus initiated after 4.20 days of culture at 2.0 mg/L of 2,4-D from cotyledons

Callus initiation after 5.4 days from hypocotyls at 2.0 mg/L of 2,4-D

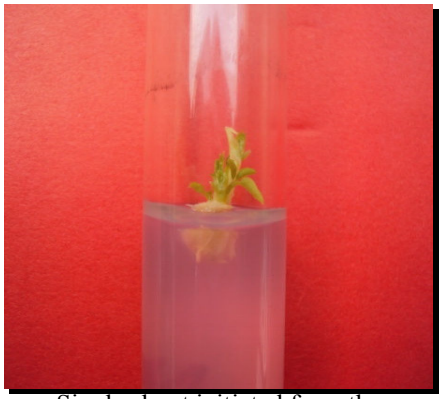
Callus initiation from 2,4-D & Kinetin from hypocotyl explant

Figure 1. Callus initiation in different explants of *Moringa* in media having different growth regulators.

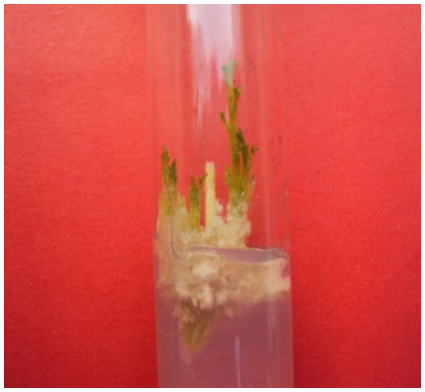
Table 4. Effect of growth regulators and their levels on explants regarding shoot length (cm) and number of leaves in *Moringa*

Growth regulator x Level (mg/L)	Shoot length (cm)		Number of leaves	
	Hypocotyl	Node	Hypocotyl	Node
Kinetin x 0.1	7.00±0.58a	3.30±0.47b	12.00±1.15c	11.67±0.88c
Kinetin x 0.5	6.27±0.41b	5.00±0.29a	16.00±0.50b	17.33±1.20b
Kinetin x 1.0	3.40±0.67d	4.23±0.43a	10.00±0.58d	19.33±0.88a
Kinetin x 1.5	3.37±0.59d	3.23±0.72b	7.33±0.33f	9.33±0.67d
Kinetin x 2.0	1.50±0.23f	1.80±0.15d	3.00±0.58h	2.33±0.33f
Mean (Kinetin)	**4.31±0.49A**	**3.51±0.41A**	**9.67±0.63B**	**11.99± 0.79 A**
BAP x 0.1	0.83±0.17g	1.13±0.19d	4.00±0.58h	3.00±0.58f
BAP x 0.5	2.93±0.07e	3.30±0.36b	10.00±1.73d	5.67±0.88e
BAP x 1.0	2.60±0.46e	2.40±0.56c	17.67±1.45a	9.33±0.88d
BAP x 1.5	2.40±0.45e	2.90±0.46c	9.33±0.88e	6.67±0.88e
BAP x 2.0	2.13±0.38e	2.20±0.36c	9.00±0.58e	6.67±1.20e
Mean (BAP)	**2.18±0.31C**	**2.39±0.39C**	**10.0±1.04A**	**6.27±0.88C**
BA x 0.1	3.13±0.23d	3.20±0.26b	7.00±0.58g	7.33±0.88e
BA x 0.5	5.47±0.32c	4.60±0.49a	8.33±0.88f	10.00±0.58d
BA x 1.0	3.13±0.52d	2.87±0.20c	8.33±0.88f	6.33±1.45e
BA x 1.5	0.87±0.41g	1.17±0.18d	5.67±1.20g	5.00±1.15e
BA x 2.0	0.17±0.03g	0.37±0.3	3.33±0.33h	3.00±0.58f
Mean (BA)	**2.55±1.51B**	**2.44±0.27 B**	**6.53±0.77C**	**6.33±0.93B**
Overall means	**3.01±0.77 A**	**2.78±0.36 B**	**8.73±0.81A**	**8.19±0.87B**

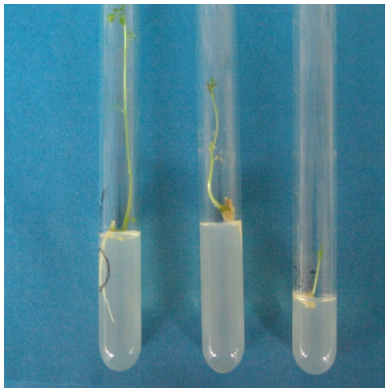
Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05); Small letters represent comparison among interaction means and capital letters are used for overall mean; Values are mean ±SE.



Single shoot initiated from the hypocotyl



Multiple shooting from node at 2.0 mg/L of Kinetin



shoot length at BAP, BA and Kinetin from right to left at 0.5 mg/L

Figure 2. Shoot length initiated at different levels of growth regulators in *Moringa*

low level of Kinetin + 0.1 mg/L or at 0.5 mg/L, produced the good number of leaves (11.67±0.88 and 17.33±1.20, respectively).

On average all the growth regulators in combination with different levels, the node explants produced the maximum number of leaves (11.99±0.79). The BAP and BA with all the levels of the growth regulators produced statistically similar number of leaves (6.27±0.88 and 6.33±0.98, respectively). BAP produced the maximum leaves (9.33±0.88) at 1.0 mg/L concentration while BA produced maximum leaves (10.00±0.58) at 0.5 mg/L concentration. With the increase in the concentration (beyond 1.0 mg/L) there was reduction in the number of the leaves (Table 4).

***Number of shoots per explant*:** The interaction of means of the growth regulators and the explants (Table 5) revealed that the node explants produced more number of shoots as compared to the hypocotyls. The hypocotyls cultured on MS medium supplemented with Kinetin, produced the lowest number of shoots (0.99±0.10) followed by BAP (1.67±0.16) while maximum number of shoots (2.07±0.25) were produced with hypocotyls explants when cultured on MS medium supplemented with BA. The node explants

produced the least number of shoots (1.61±0.27) when cultured on MS medium supplemented with Kinetin while BAP and BA produced the same number of shoots (1.67±0.16 and 1.67±0.19, respectively).

Table 5. Effect of growth regulators on explants for number of shoots in *Moringa*

Growth regulators	Explants	
	Hypocotyl	Nodes
Kinetin	0.99 ± 0.10c	1.61 ± 0.27b
BAP	1.67 ± 0.16b	1.67 ± 0.16a
BA	2.07 ± 0.25a	1.67 ± 0.19a
Mean	1.57 ± 0.12B	1.65 ± 0.12A

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05); Small letters represent comparison among interaction means and capital letters are used for overall mean; Values are mean ±SE.

***Number of regenerated roots*:** The growth regulators and the levels individually had the significant effect on the number of the roots. While the interaction of the growth regulators (NAA, IAA and IBA) and their four levels (0.05, 1.0, 1.5 and 2.0 mg/L) had the highly significant effect on the number of the roots produced while media without the growth regulators, MS alone and the half strength MS were also used for the root regeneration as a control.

The means of the interaction between the levels of the growth regulators and the explants revealed that both the growth regulators (hypocotyls and nodes) produced more number of roots at 1.0 mg/L (3.22±0.43 and 3.56±0.38, respectively). In both the explants the increase in the level of growth regulators negatively impacted the number of the roots produced. The maximum roots (3.56±0.38) were produced in the nodes at the 1.0 mg/L of growth regulators. On average the nodes showed more response (2.50±0.26) as compared to the hypocotyls (2.25±0.28) for the number of the roots (Table 6A).

Table 6A. Effect of growth regulators and their levels on explants for number of roots in *Moringa*

Level (mg/L)	Explants	
	Hypocotyl	Node
0.05	2.11±0.20	2.78±0.22
1.0	3.22±0.43	3.56±0.38
1.5	2.00±0.24	2.11±0.26
2.0	1.67±0.24	1.56±0.18
Mean	2.25±0.28B	2.50±0.26A

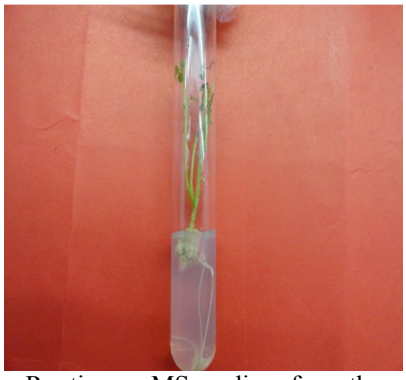
Table 6B. Media without the growth regulators and explants for number of roots in *Moringa*

Level (mg/L)	Explants	
	Hypocotyl	Node
MS	3.67±0.33a	3.00±0.00b
Half MS	3.67±0.33a	4.67±0.33a
Mean	3.67±0.21B	3.83±0.40A

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05); Small letters represent comparison among interaction means and capital letters are used for overall mean; Values are mean ±SE.

The mean number of the roots produced on the MS and the half MS media (without growth regulators) was more as compared to the number of roots produced on the media with the root inducing growth regulators. The hypocotyls produced the same number of roots on both the MS and the half MS media (3.67±0.33). The node explants produced more roots on half MS media (4.67±0.33) as compared to the MS media (3.0±0.0) (Table 6B).

***Root length (cm)*:** The means of the interaction between the growth regulators and the explants of root length were analyzed statistically and their means were compared by the Dumcan's Multiple Range test. The hypocotyl explants had the more root length (3.29±0.42 cm) when cultured on MS medium supplemented with IAA (Fig. 3) followed by those from IBA (2.30±0.32cm) and least root length was recorded with NAA (1.95±0.32 cm). The node explants produced



Rooting on MS medium from the hypocotyls explant

Rooing on IAA medium from hypocotyls

Root length from node explants on MS media

Figure 3. Root initiation from hypocotyl and node explants on different growth regulators

statistically similar root length when cultured on MS medium supplemented with IAA and IBA (3.02±0.34 and 3.01±0.39 cm, respectively) while nodes produced the least root length (2.41±0.34) when cultured on MS medium supplemented with NAA (Table 7A, Fig. 3).

The interaction between the explants and the media without the addition of any growth regulators revealed that the root length was significantly more as compared to the media with the growth regulators (Table 7B). The hypocotyl explants showed more root length when cultured on MS media (11.83±0.44 cm) (Fig.3) and half MS media (6.83±0.17cm).

The node explants also showed the similar pattern that root length was more on the MS media (8.50±0.29 cm) as compared to the half MS media (5.80±0.15cm). On average the hypocotyls explants showed the more root length (9.33±1.14 cm) as compared to nodes (7.15±0.62 cm) cultured on both MS and half MS media (Table 7B).

Table 7A. Effect of growth regulators on explants for root length (cm) in *Moringa*

Growth regulator	Explants	
	Hypocotyl	Node
NAA	1.95±0.32c	2.41±0.34b
IAA	3.29±0.42a	3.02±0.34a
IBA	2.30±0.32b	3.01±0.39a
Mean	2.51±0.35B	2.81±0.36A

Table 7B. Interaction of growth regulator and explants without growth regulators for root length (cm) in *Moringa*

Growth regulator	Explants	
	Hypocotyl	Node
MS	11.83±0.44a	8.50±0.29a
Half MS	6.83±0.17b	5.80±0.15b
Mean	9.33±1.14A	7.15±0.62B

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05); Small letters represent comparison among interaction means and capital letters are used for overall mean; Values are mean ±SE.

***Acclimatization*:** The plantlets after attaining the roots were shifted to the pots having sterilized sand cups. The plantlets were removed from the test tubes and washed with the autoclaved distilled water to remove the agar from the roots. Then the plantlets were shifted to the pots and irrigated with the Hoagland solution (Fig. 4). These pots were then covered with the polythene to maintain the humidity and were placed in the growth room at 26±2°C. After three weeks some holes were made in the polythene to reduce the humidity and then polythene was completely removed. These plantlets were shifted to the greenhouse with 85% transplanting success.

DISCUSSION

Moring, a genus with charismatic properties for nutrition and medicinal application and some species are in danger of extinction, specially *M. hildebrandtii* is now extinct in the wild (Olson and Razafimandimbison, 2000; Steinitz *et al.,* 2009). *Moringa*, the group of plant unexplored to unravel its potential in human food, ethnobotanical and pharmaceutical use can be improved, conserved through tissue culture techniques. A mass production is also need of the time for the fodder purpose which increases the milk production in cattle (Sanches *et al*., 2006), and this milk can be available to the people of underdeveloped countries to combat their nutritional requirements.

It is very important to develop a rational combination among growth regulator, level of growth regulators and explants types to initiate calli in minimum possible time which may be healthy, proliferative and more likely possess high embryogenic potential. Callus induction is desirable when it is initiated in less time period. Different growth regulators effected differently for callogenesis with respect to explants. When the cotyledons and hypocotyls were cultured on the MS medium supplemented with 2,4-D, initiated callus within one week. The leaf explants took more than 10 days to initiate callus. The epicotyls initiated callus within a week when cultured on TDZ media while hypocotyls, cotyledons and leaf explants took about ten days for callus initiation.

Whole plantlet shifted to pots having sand

Plantlet covered with polythene bag to maintain humidity

Growth of acclimatized plantlet with more number of leaves

Figure 4. Acclimatization of *Moringa* plantlets in pots

Abdellatef and Khalafalla (2010) also used 2,4-D for callus induction using leaves and hypocotyl explants and evaluated that 2,4-D had a significant effect on callus induction. They also found that low concentration of 2,4-D (0.1 mg/L) proved to be good for callus induction from leave explants contrary to our results which revealed that high level of 2,4-D (2.0 mg/L) initiated callus in less number of days. Many other scientist evaluated that 2,4-D gave best callusing response in the plants (Evans *et al.,* 1984). Khalafallah *et al.* (2011) again used 2,4-D for callus induction in *Moringa* leaves and found the same results as previous that low concentration proved to be more efficient in callus induction of *Moringa*.

Among all the auxins the 2,4-D response towards callus is more good. Nieves and Aspuria (2011) used the increased concentrations of the 2,4-D (1.0, 2.5 and 5.0 mg/L) for callus induction in cotyledon explants of the *Moringa* and found that callus was initiated in 14 to 28 days after culture.

The cotyledonry explants initiated callus with four days using 2.0 mg/ L concentration of 2,4-D. Nieves and Aspuria (2011) used cotyledon explants for the callus initiation on the MS medium supplemented with different concentrations of the 2,4-D for *Moringa*. Contrary to the present study they found no variation in the different levels of the 2,4-D while they combined 2,4-D with BA and then further enriched with TDZ which gave the profuse callus. The hypocotyls initiated callus within five days using 2.0mg/L concentration of 2,4-D. The epicotyls also initiated callus within four days when cultured on 2.0 mg/L of the TDZ media. The other concentrations took an average of nine days for callus initiation. IAA and NAA took more than two weeks for callus initiation in almost all the concentrations. All of these calli which were produced were non-embrygenic and this was tried many times with different growth regulators (used alone and in combinations) but no shoot regeneration was achieved.

In case of micro-cloning, regeneration of shoots from *Moringa* explants were found highly dependent on level and type of growth regulators. All growth regulators showed shoot regeneration and varied in their potential and efficiency by inducing shoot production. The hypocotyls and nodes both regenerated shoots but the hypocotyls explants regenerated shoots more quickly as compared to the nodes. Islam *et al.* (2005) used BAP for *in vitro* shoot regeneration of the *Moringa oleifera* and found that BAP at 1.0 and 1.5 mg/L concentration produced good regeneration response. While Abdellatef and Khalafalla (2010) used node explants and found that BA proved more effective as compared to Kinetin for shoot induction. Hypocotyl were more responsive to callusing in less time period while in other study nodes produced good shoots in *Moringa* (Islam *et al.,* 2005)

The induction of the multiple shooting is also a desirable characteristic in micropropagation. The nodes produced more number of shoots (1.65) as compared to the hypocotyls (1.57) on average of the all the three cytokinins. Hypocotyls gave the maximum shoots (2.07) when cultured on the BA and the kinetin initiated the least number of shoots (0.99) while nodes produced good number of shoots at all the growth regulators. Low concentration of the BA and Kinetin also produced more number of shoots in the other studies conducted on *Moringa* by Riyathong (2010) and Abdellatef and Khalafallah (2010). The higher concentration of the cytokinin increases the callus initiation at the cut ends of the shoots which hinders the development of the shoots. The node explants had the axillary shoots which induced more multiple shoots as compared to the hypocotyl. Induction of multiple shoots is more successful in nodes as compared to other explants. Most of the shoots were observed with the callus development at the base and this phenomenon was most prominent when the concentration of the growth regulator was increased. Islam *et al*. (2005) also used the node explants for the regeneration of *Moringa* and got the same findings.

In tissue culture system, formation of roots is very controversial for successful establishment of micro-cloning system. In present study, root formation correlated with explants type, growth regulator type and level as well as combination of growth regulators.

The roots produced on the MS and the half MS media (without growth regulators) was more as compared to the number of roots produced on the media with the auxins. The hypocotyls produced same number on roots on both the MS and the half MS media (3.67) .These number of roots are almost equal to the number of roots obtained by Islam *et al*., (2005) who observed 4 roots on MS media alone. The node explants produced more roots on half MS media (4.67) as compared to the MS media (3.0). Islam *et al.* (2005) also concluded that the MS and the half MS media gave the more number of roots while Stephenson and Fahey (2004) evaluated that good rooting response was present in the half MS media.

The root length was significantly more on the media without growth regulators as compared to the media with the growth regulators. The hypocotyl explants showed more root length when cultured on MS media (11.83 cm) and half MS media (6.83 cm). The node explants also showed the same pattern that root length was more on the MS media (8.50 cm) as compared to the half MS media (5.80 cm). On average the hypocotyls explants showed the more root length (9.33 cm) as compared to nodes (7.15 cm) cultured on both MS and half MS media. Siani *et al.* (2012) revealed the observations when established *in vitro* regeneration system for *Moringa* oleifera and evaluated that maximum rooting was recorded when auxins were used in combination (IAA and IBA at 2.85 and 4.92 μM respectively). After the MS and the half MS media good root regeneration was recorded on media

containing NAA which was similar to the findings of the Riyathong (2010).

***Conclusion*:** The indirect and direct regeneration of the *Moringa* was made by the establishment of the regeneration protocol *in vitro*. The indirect regeneration (callogenesis) was achieved by using the 2,4-D, TDZ, NAA and IBA and the 2,4-D effect towards callogenesis was good as compared to the other two growth regulators. Hypocotyl and cotyledons gave the early response of callusing with 2,4-D at the higher concentration while NAA and IAA induced callus after long time as compared to the 2,4-D and TDZ. Higher concentration of 2,4-D gave more quick result towards callus as compared to the low concentration. Three combinations of growth regulators (2,4-D+Kinetin), (IAA+BAP) and (NAA+Kinetin) at two different levels (2.0 and 1.0 mg/L) and it was evaluated that the lower concentration (2.0 and 1.0 mg/L respectively) respond more quickly in short time period to callus induction as compared to the higher concentration. The direct regeneration involved the regeneration through the hypocotyl and the nodes. The hypocotyl regenerated earlier as compared to the nodes. The higher concentration of the growth regulators (2.0 mg/ L) started shooting in less number of days. Kinetin at 0.1 and 0.5 mg/L gave more shoot length both in the epicotyl and node explants. Kinetin and BAP produced more number of leaves and the shoot length at the lower concentration of the growth regulators while at higher concentrations there was gradual reduction in the number of leaves. The rooting of the regenerated shoots was the best in the MS and half MS media without the addition of the growth regulators. The rooted plantlets were acclimatized successfully.

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