Optimization of Growth Regulators on In vitro Propagation of Moringa stenopetala from Shoot Explants

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Abstract

Background: *Moringa stenopetala* is belongs to flowering family Moringaceae and genus Moringa. It is often referred to as the East African Moringa tree because it is native only to southern Ethiopia and northern Kenya. The expansion of its cultivation and utilization throughout the world especially in Africa is becoming important. For such expansion, the existing propagation method is limiting, so it needs good propagation system to supply enough planting material with uniform genotype. Therefore, the main objective of this study was to optimize an in vitro shoot multiplication protocol for M. stenopetala by using shoot tip as explants.

Results: Shoots were sterilized and cultured on Muraghige and Skoog (MS) medium for in vitro shoot initiation. For multiple shoot induction, the explants were cultured on MS medium supplemented with different concentrations of kinetin (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) along with Indole-3- butyric acid (IBA) or α - naphthalene acetic acid (NAA) (0.01, 0.1, 0.5mg/l) and maintained at 25 ± 2°C for four weeks. Rooting was achieved by culturing well developed shoots in half strength MS medium containing IBA (0.1, 0.5, 1.0, 1.5, 2.0 mg/l), NAA (0.1, 0.5, 1.0, 1.5, 2.0 mg/l) and 0.5 mg/l IBA in combination with NAA (0.1, 0.5, 1.0, 1.5, 2.0 mg/l). Statistical analysis revealed that there was significant difference among all treatments applied in both shoot multiplication and rooting experiments. Maximum number of shoots per explant (3.43±1.41) and 7.97±4.18 leaves per explant were obtained on MS medium containing 0.5 mg/l kinetin in combination with 0.01mg/l NAA. The highest mean number of roots per shoot (1.63±1.03) and mean root length (0.87±1.22 cm) were obtained on MS medium containing 1.0 mg/l NAA and 0.1 mg/l IBA alone respectively. After acclimatization, 76% plants survived in greenhouse.

Conclusions: In general, using NAA along with kinetin for shoot multiplication was better than kinetin along with IBA and application of NAA alone at concentration of 1.0 mg/l and 1.0 mg/l NAA along with 0.5 mg/l IBA were more effective for root induction.

1. Background

Moringa stenopetala belongs to family *Moringaceae* that is represented by a single genus *Moringa*. The genus *Moringa* is represented by 14 species to which *M. stenopetala* belongs. The genus *Moringa* originated from sub-Himalayan tracts of Northern India, distributed worldwide in the tropics and sub-tropics [25, 27]. *Moringa stenopetala* is often referred to as the East African Moringa tree because it is native only to southern Ethiopia and northern Kenya [21]. Though it grows in many other parts of the tropics, it is not as widely known as its close relative, *Moringa oleifera* but often considered generally more desirable than *M. oleifera.* [7, 21] stated that the taxonomic position of the family is not clear. It has some features similar to those of *Brassicaceae* and *Capparidaceae* but the seed structure does not agree with either of the above families. Pollen studies have not provided any other suggestions and recent molecular studies have pointed to relationship with the *Carricaceae.* This indicates that the taxonomic position of the family is not yet settled and is open for further studies. Its seed physiology is also yet studied in the tropics in general and Ethiopia in particular [8].

The habitat where the genus occur in Ethiopia includes: rocky areas along rivers, dry scrub land, Acacia-Commiphora woodland, water courses with some evergreens, open Acacia-Commiphora bush land on gray alluvial soil and in cultivation around village. It is cultivated in terr aced fields, gardens and small towns [7, 11]. It is dominantly found in well-drained soils of southern Ethiopia at an altitudinal range of about 1100–1600 m.a.s.l. with annual rainfall ranging from 500–1400 mm and annual temperature ranging from 24–30 °C [34]. Due to its water storage capacity in the bottle-shaped stem, it is drought tolerant and remains green when the other forage is scarce during the dry season [1] and due to this character, it is called 'camel crop'. Besides, one of the nicknames of the tree is "never dying" due to its incredible ability to survive in harsh climate and drought condition [29]. Cold temperatures are limiting factor for the cultivation of the species in Ethiopia because it does not tolerate frost or freeze may cause it to die back to ground level, where new sprouts may be produced. The species does not have any specific soil requirements, except it does not grow on waterlogged or swampy soils. The soil pH ranges from acidic to alkaline but mostly exhibit neutral reaction [26].

Moringa stenopetala has the following features: it is fast growing evergreen perennial flowering plant or deciduous soft tree, well adapted to semi-arid areas with annual rainfall as little as 500 mm and poor soil [30]. It is more drought tolerant - but less frost-resistant than *M. oleifera* [23]. Even though, *Moringa stenopetala* is more resistant to insect pests than other species in its family. It is affected by a caterpillar of Noorda trimaculalis [6].

M. stenopetala is propagated both by direct sowing of the seeds without pretreatment and vegetatively using branch cuttings [14]. The optimum temperature for the germination of *M. stenopetala* seeds was reported to be about 25 °C [5]. Optimum light for germination of all *Moringa* species is half shade. When sown in the hotter weather of mid-April, germination percentages for *M. stenopetala* and *M. oleifera* were only 54 and 40 percent, compared to 92 and 94 percent in half shade. Seeds should be planted about 2 cm deep in soil that is moist but not too wet [22]. The best suited season for sowing the seeds is March to August under Southern Ethiopian conditions. The time of sowing must be strictly adhered to because the flowering phase should not concede with rainy seasons, which results in heavy flower shedding [18].

M. stenopetala is one of the world's most nutritious crops: All parts of the tree except the wood are edible. The leaf of *Moringa* is very popular vegetable in southern Nation Nationalities and Peoples Regional State of Ethiopia and valued for its special flavor [30]. The leaf is rich in carbohydrate, proteins, minerals and essential amino acids. It has more betacarotene than carrots, more protein than peas, more calcium than milk, more potassium than bananas, and more iron than spinach [1].

Many parts of the *M. stenopetala* plant have been used in medicinal preparations traditionally against different disease. The seed can be used for various purposes such as for food, medication, water purification and oil extraction [4]. Besides, its vital value for human and livestock nutrition, this plant is largely uninvestigated [3, 19]. Due to this, *M. stenopetala* is not known in most part of the world other than its area of cultivation (Southern Ethiopia and Northern Kenya). Currently, it attracted the attention of scientists across the globe for health management due to its nutritional and medicinal properties as well

as easy of propagation and ability to thrive under harsh environments. It has the potential to end malnutrition, starvation, as well as prevent and treat many diseases. It has got a nick name as ‘truly a miracle plant’, ‘ mothers’ best friend’ and a ‘God gift’ for the nourishing and healing of man. Thus, it is emerging as a future crop considering its wider adaptability and tolerance to many abiotic stresses. Accordingly, it became essential to take stock of knowledge and develop a road map to harness its potential for the benefit of farmers as well as consumers [29]. Therefore, expansion of cultivation and utilization of this tree throughout the world especially in Africa is important. However, for such expansion, it needs good propagation system to supply adequate planting materials of superior genotypes.

Moringa species are associated with many production constraints, such as a relatively long period of fruit bearing, non-availability of planting materials, lack of alternative method of propagation, requirement for long rainy period in regions where water is scarce and vulnerability to pests and diseases [11, 35]. [6] reported that the pest destroys the leaves of the entire plant population of a village within a week. This pest could be a drawback for in situ conservation of the plant. Moreover, flowering does not commence until a critical tree size is attained, and after some years the tree branches stop producing fruits/seed. Therefore, vegetative propagation like cutting is a necessity to obtain uniformity in yield and quality. However, it is less successful due to its slow regeneration and also requires large size cuttings (1-1.5 m long) as well as trees grown from cuttings are known to have much shorter roots or a poor root system [16]. As a result, *in vitro* propagation methods are the best alternative for propagation of this plant with uniform genotypes within a relatively short period of time. Thus, this study was intended to optimize *in vitro* propagation protocol for *Moringa stenopetala* so that to evaluate the combined effect of NAA and kinetin and IBA and kinetin on shoot multiplication. As well, to evaluate the effect of NAA and IBA on rooting of multiplied shoots.

2. Results

2.1. Shoot Multiplication

The effect of NAA along with kinetin on shoot multiplication after four weeks of culture was highly significant ($P \leq 0.05$). The results of the present study showed that using NAA in combination with kinetin for shoot multiplication was better than kinetin in combination with IBA.

In all treatments, there were differences in the rate of shoot multiplication. The mean number of shoots per explant range from 1.00 ± 0.48 to 3.43 ± 1.41 (Table 1; Table 2; Fig. 1). Among all the treatments, 0.5 mg/l kinetin in combination with 0.01 mg/l NAA resulted in the highest number of shoots per explant (3.43 ± 1.41) (Table 2). The MS medium supplemented with 0.5 mg/l kinetin combined with 0.1 mg/l NAA, 0.5 mg/l kinetin combined with 0.1 mg/IBA and 0.5 mg/l kinetin combined with 0.5 mg/l NAA gave the second (2.47 ± 1.36) and the third (2.30 ± 1.90), (2.33 ± 1.85) maximum mean shoot number respectively. Shoot explants cultured on MS medium supplemented with 2.5 mg/l kinetin in combination with 0.5 mg/l NAA and 2.5 mg/l kinetin in combination with 0.5 mg/l IBA produced the lowest mean number of shoots per explant, 1.00 ± 0.48 and 1.00 ± 0.54 respectively. Shoot length was significantly

higher on medium without any plant growth regulator (control) as compared to media containing IBA and Kinetin and NAA and kinetin but it produced the lowest mean number of shoots per explant (1.2 ± 1.00). The maximum shoot length (1.10 ± 0.97 cm) and number of leaves (9.33 ± 10.56) were recorded on MS medium supplemented with 0.5 mg/l Kinetin combined with 0.01 mg/l IBA and 1.0 mg/l kinetin combined with 0.01 mg/l NAA respectively. With increasing the concentrations of IBA and NAA, the number of shoots per explant decreased.

Table 1
The effect of different combination of kinetin and IBA on shoot multiplication

KN(mg/l)	IBA(mg/l)	Shoot no. per explant	leaf no. per explant	Shoot length (cm)
0.0	0.0	1.20 ± 1.00^{bc}	2.33 ± 1.15^{def}	2.67 ± 1.13^{a}
0.5	0.01	2.27 ± 1.62^{a}	6.60 ± 8.30^{bcd}	1.10 ± 0.97^{b}
0.5	0.1	2.30 ± 1.90^{a}	7.73 ± 7.57^{a}	1.08 ± 1.07^{b}
0.5	0.5	2.03 ± 1.40^{bc}	5.77 ± 6.15^{bcde}	0.61 ± 0.31^{bc}
1.0	0.01	1.83 ± 1.34^{bc}	5.83 ± 7.01^{bcde}	1.01 ± 0.72^{bc}
1.0	0.1	2.20 ± 1.21^{a}	6.77 ± 4.83^{bc}	0.90 ± 0.55^{de}
1.0	0.5	2.20 ± 1.73^{a}	7.40 ± 7.14^{a}	0.93 ± 0.98^{bc}
1.5	0.01	2.03 ± 1.50^{bc}	7.47 ± 6.50^{a}	0.99 ± 0.84^{bc}
1.5	0.1	$1.23 \pm .94^{bc}$	3.83 ± 4.31^{bcdef}	0.82 ± 0.67^{bc}
1.5	0.5	$1.50 \pm .94^{bc}$	2.15 ± 1.45^{def}	0.89 ± 0.88^{bc}
2.0	0.01	2.20 ± 3.39^{a}	2.50 ± 2.08^{cdef}	0.66 ± 0.36^{bc}
2.0	0.1	1.23 ± 1.14^{bc}	2.77 ± 3.90^{cdef}	0.60 ± 0.43^{bc}
2.0	0.5	1.67 ± 1.30^{bc}	3.40 ± 3.77^{bcdef}	0.71 ± 0.40^{bc}
2.5	0.01	$1.13 \pm .94^{bc}$	2.57 ± 2.27^{cdef}	0.53 ± 0.40^{bc}
2.5	0.1	$1.00 \pm .64^{bc}$	1.53 ± 1.78^{ef}	0.57 ± 0.46^{bc}
2.5	0.5	$1.00 \pm .54^{c}$	1.00 ± 1.15^{f}	0.44 ± 0.42^{c}
Over all means		1.70 ± 1.58^{B}	4.49 ± 5.55^{A}	0.79 ± 0.70^{C}

Table 2
Effect of different combination of kinetin and NAA on shoot multiplication

KN(mg/l)	NAA (mg/l)	Shoot no. per explant	Leaf no. per explant	Shoot length (cm) (no. ±SD)
0	0	1.20 ± 1.00^{efg}	2.33 ± 1.15^{f}	2.67 ± 1.14^{a}
0.5	0.01	3.43 ± 1.41^{a}	7.97 ± 4.18^{bc}	0.68 ± 0.26^{b}
0.5	0.1	2.47 ± 1.36^{bc}	5.33 ± 3.65^{bcde}	0.68 ± 0.24^{b}
0.5	0.5	2.33 ± 1.85^{cd}	7.20 ± 7.44^{bcd}	0.50 ± 0.28^{bc}
1.0	0.01	2.20 ± 1.81^{cde}	9.33 ± 10.56^{a}	0.59 ± 0.39^{bc}
1.0	0.1	$1.20 \pm .96^{efg}$	3.10 ± 3.28^{df}	0.36 ± 0.22^{c}
1.0	0.5	1.23 ± 1.04^{efg}	3.67 ± 5.06^{cde}	0.57 ± 0.40^{bc}
1.5	0.01	1.57 ± 1.28^{cdefg}	4.23 ± 6.63^{cde}	0.46 ± 0.28^{bc}
1.5	0.1	1.97 ± 1.50^{cdef}	5.10 ± 6.58^{bcde}	0.47 ± 0.20^{bc}
1.5	0.5	1.43 ± 1.31^{defg}	2.23 ± 3.10^{f}	0.48 ± 0.40^{bc}
2.0	0.01	$1.23 \pm .73^{efg}$	1.90 ± 1.03^{f}	0.50 ± 0.21^{bc}
2.0	0.1	1.10 ± 0.96^{fg}	2.40 ± 4.42^{f}	0.47 ± 0.28^{bc}
2.0	0.5	1.10 ± 0.183^{fg}	2.57 ± 2.08^{f}	0.53 ± 0.22^{bc}
2.5	0.01	1.00 ± 0.31^{fg}	1.63 ± 1.03^{f}	0.55 ± 0.28^{bc}
2.5	0.1	1.00 ± 0.38^{fg}	$1.53 \pm .94^{f}$	0.47 ± 0.27^{bc}
2.5	0.5	1.00 ± 0.48^{fg}	1.23 ± 1.19^{c}	0.36 ± 0.29^{c}
Overall means		1.57 ± 1.36^{B}	3.96 ± 5.40^{A}	0.51 ± 0.30^{C}

Numbers within the same column with different letter(s) are significantly different from each other according to Tukey's multiple range test at $P \leq 0.05$. The upper-case letters indicate the overall means. The values represent mean ± standard deviation.

Numbers within the same column with different letter(s) are significantly different from each other according to Tukey's multiple range test at $P \leq 0.05$. The upper case letters indicate the overall means. The values represent mean ± standard deviation.

2.2. Rooting

Application of NAA alone exhibited the maximum mean root number per shoot as compared to IBA alone and IBA in combination with NAA. The highest mean root number per shoot (1.63 ± 1.03) and mean root length (0.87 ± 1.22 cm) were obtained on medium containing 1.0 mg/l NAA and 0.1 mg/l IBA, respectively (Table 3). The second (1.50 ± 0.38) and third (1.23) highest mean root number were obtained on medium containing 1.0 mg/l IBA alone and 0.5 mg/l IBA and NAA with 0.67 ± 0.28 cm and 0.52 ± 0.51 cm mean root length respectively. The lowest mean number of roots per explant was produced on growth regulator free medium (0.26 ± 0.57), 2.0 mg/l NAA (0.27 ± 0.45), and 2.0 mg/l NAA along with 0.5 mg/l IBA (0.27 ± 0.58).

Table 3
Effect of NAA and IBA on rooting of micro shoots

NAA (mg/l)	IBA (mg/l)	Root no. per explant	Root length (cm)
0	0	0.26 ± 0.57^{bc}	0.12 ± 0.21^{bc}
0	0.1	1.03 ± 1.47^{c}	0.87 ± 1.22^{a}
0	0.5	1.23 ± 0.70^{b}	0.43 ± 0.43^{b}
0	1.0	1.50 ± 0.38^{a}	0.67 ± 0.28^{c}
0	1.5	0.13 ± 0.35^{bc}	0.15 ± 0.48^{bc}
0	2.0	0.07 ± 0.25^{bc}	0.05 ± 0.20^{bc}
0.1	0	1.07 ± 1.86^{bcd}	0.34 ± 0.55^{cd}
0.5	0	1.23 ± 1.19^{bc}	0.52 ± 0.51^{bc}
1.0	0	1.63 ± 1.03^{a}	0.84 ± 0.54^{a}
1.5	0	0.67 ± 0.84^{cd}	0.23 ± 0.28^{cd}
2.0	0	0.27 ± 0.45^{d}	0.14 ± 0.24^{d}
0.1	0.5	0.50 ± 0.97^{bc}	$0.13 \pm 0 .21^{c}$
0.5	0.5	0.67 ± 1.16^{bc}	0.24 ± 0.37^{bc}
1.0	0.5	1.20 ± 1.16^{a}	0.43 ± 0.40^{a}
1.5	0.5	1.03 ± 1.13^{a}	0.41 ± 0.46^{a}
2.0	0.5	0.27 ± 0.58^{f}	$0.09 \pm 0 .19^{c}$

Numbers within the same column with different letter(s) are significantly different from each other according to Tukey's test at $P \leq 0.05$. The values represent mean ± standard deviation.

2.3. Acclimatization

Among 50 plantlets acclimatized in the green house, 38 (76%) survived.

3. Discussion

3.1. Effect of kinetin, IBA and NAA on shoot multiplication

In vitro propagation of *M. oleifera* was investigated well, but for M. stenopetala there are few available reports. Therefore, in the present study efforts have been made to optimize *in vitro* propagation protocol for *M. stenopetala*. Surface sterilization of seeds with 10% NaOCl solution for 25 minutes was effective to reduce microbial contaminants. In this study, there was 100% initiation of shoot after four weeks of culture of the shoot tip in shoot initiation media supplemented with BAP alone. This is similar to the finding of [15] who got 100% initiation of shoots from stem segment explants taken from 10-day old seedlings of *M. oleifera*.

In this study the effect of cytokinin in combination with auxin were compared. Application of 0.5 mg/l kinetin along with 0.01 mg/l NAA resulted in the maximum mean numbers of shoots (3.43 ± 1.41) with 7.97 ± 4.18 mean number of leaf and 0.67 ± 0.26 cm means shoot length per explant. In addition, there was continuous decrease in the number of shoots when the concentration of NAA increased from 0.01 to 0.1 mg/l combined with kinetin. Moreover, a high cytokinin to auxin ratio favours shoot formation.

Induction of multiple shoots in *Moringa concanensis* has been previously characterized with different growth regulators. [9] on nodal explants of *Moringa concanensis* obtained 11.00 ± 1.15 mean shoot number with 5.00 ± 1.95 cm mean length on MS medium supplemented with 0.1 mg/l kinetin along with 0.05 mg/l NAA. The finding of these authors is contrasting to the present one. In the present study, the maximum mean number of shoots per explant (3.43 ± 1.41) with mean shoot length of 0.68 ± 0.26 cm was recorded at the concentration of 0.5 mg/l kinetin along with 0.01 mg/l NAA. Even though, the result reported by this author is greater than the present study, the trend was similar. In both studies relatively at low concentration of auxin (NAA) and high concentration of cytokinin (kin) best result was obtained. The difference is due to the genotype difference, source of explants, and difference in concentration of cytokinin (kinetin) and auxin (NAA). In addition, the performance of kinetin in shoot length is further supported by sister family (*Brassicaceae)* of *Moringa*. The study in *Matthiola incana (Brassicaceae*) showed that MS medium supplemented with 2.0 mg/l kinetin without NAA resulted in the best shoot length (1.20 cm) [17]. In the present study the medium containing 2.0 mg/l kinetin along with 0.01 NAA produced 1.23 ± 0.73 mean number of shoot with 0.50 ± 0.21 cm means shoot length. Even if the concentration of kinetin is the same in both studies, the length of shoot reported by these authors is greater than the present study. This is may be due to the genotype difference of the explants, source of explants and the authors used kinetin alone.

Shoot explants cultured on MS medium supplemented with 0.5 mg/l kinetin in combination with 0.1 mg/l IBA exhibited both the maximum number of mean shoot number (2.30 ± 1. 90) and mean number of leaves (7.73 ± 7.57) per explant. The result from combined effect of IBA and kinetin was found to vary with the concentration of IBA and kinetins in all three parameters of shoot growth (means number of shoot, number of leaf and shoot length). When the concentration of kinetin was greater than that of IBA, relatively better result was recorded. This is due to the effect of cytokinin as it promotes the axilliary branching or axilliary bud proliferation [32]. Although both auxin and cytokinin are usually required for growth or morphogenesis, auxin inhibits cytokinin accumulation [12] while cytokinin can inhibit at least some of the action of auxin. There was continuous decrease in number of shoots when concentration of IBA increased from 0.01 to 0.5 mg/l combined with kinetin.

The finding of [2] on *Moringa peregrina* (Forsk) showed that MS medium supplemented with 1.0 mg/l kin resulted in 2.6 mean numbers of shoots per explant. Higher levels of cytokinin (2.0 mg/l kinetin) significantly reduced the number of leaves per micro-shoot. Micro-shoots grown on growth regulators free MS medium and that supplemented with kinetin were similar in length. The finding of this author agrees with the present study except the result obtained in hormone free medium. In the present study, the MS medium supplemented with 0.5 mg/l kin along with 0.1 mg/l IBA resulted in both maximum number of shoot (2.30 ± 1.90) and mean number of leaves (7.73 ± 7.57). In addition, when the concentration of kin raised from 0.5 mg/l to 2.5 mg/l along with increasing IBA concentration from 0.01 mg/l to 0.5 mg/l lower number of shoot per explant (1.0 ± 0.54) with 0.44 ± 0.42 cm shoot length were obtained. The explants on MS medium without growth regulators (as a control) were elongated compared to MS medium supplemented with growth regulator hormones. This finding is in agreement with the finding of [13], who got simultaneous increase number of nodes from 3.61 to 4.64 when the concentration of kinetin increased from 0.5 to 2.0 mg/l in *Matthiola incana (Brassicaceae)*. In conclusion, type and concentration of growth regulators and species (genotype) are the most important factors in *in vitro* shoot multiplication.

In general, the combined effect of kinetin and NAA exhibited more effect as compared to the combined effect of IBA and kinetin.

3.2. The effect of NAA and IBA on rooting

The analysis of variance revealed that root number and root length varied significantly with half strength MS medium supplemented with NAA, IBA and the combination of both. Application of NAA alone exhibited the maximum mean root number per shoot as compared to IBA alone and IBA in combination with NAA. The highest number of mean roots per shoot (1.63 ± 1.03) and mean root length (0.87 ± 1.22 cm) were obtained at 1.0 mg/l NAA and 0.1 mg/l IBA respectively.

Furthermore, increasing NAA from 0.0 mg/l to 1.0 mg/l by keeping IBA concentration to 0.0 mg/l showed a significant increase in the mean number of roots per shoot from 0.26 ± 0.57 to 1.63 ± 1.03 and mean root length from 0.12 ± 0.21 to 0.84 ± 0. 54 cm. However, further increase in the concentration of NAA from 1.0 to 2.0 mg/l showed a reduction in the mean root number per shoot and mean root length from

1.63 ± 1.03 to 0.27 ± 0.45 and 0.84 ± 0.54 to 0.14 ± 0.24 cm respectively. The same trend was observed in both treatments (the effect of different concentration of IBA alone and IBA in combination with NAA). Increasing IBA from 0.0 mg/l to 1.0 mg/l increased both the number of roots per shoot from 0.26 ± 0.57 to 1.50 ± 0.38 and mean root length from 0.12 ± 0.21 to 0.67 ± 0.28 cm. Further increase in the concentration of IBA from 1.0 mg/l to 2.0 mg/l, mean root number were reduced from1.50 ± 0.38 to 0.07 ± 0.25 and mean root length from 0.67 ± 0.28 to 0.05 ± 0.20 cm, respectively. This result revealed that when the concentration of both growth regulators (NAA and IBA) was greater than 2.0 mg/l, mean root numbers and mean root length were decreased. In agreement with the finding, [10] reported the decreasing of root number when the concentration of IBA is greater than 2.0 mg/l. They stated the inhibitory effect of high concentration of auxin on root formation of plants as a cause for such decreasing. However, the concentration varies with genotype. Moreover, the optimum concentration is between1.0 and 2.0 mg/l as the present result indicated.

The combined effect of NAA and IBA on root induction was intermediate effect of the separate effect of each of them. Increasing NAA from 0.1 mg/l to 1.0 mg/l by keeping IBA concentration to 0.5 mg/l showed a significant increase in the mean number of roots per shoot from 0.50 ± 0.97 to 1.20 ± 1.16 and mean length of root from 0.13 ± 0.21 cm to 0.43 ± 0.40 cm. Further increase in the concentration of NAA above 1.0 mg/l by keeping IBA concentration to 0.5 mg/l reduced mean root number from 1.20 ± 1.16 to 0.27 ± 0.58.

In this combination, the maximum result was obtained at 1.0 mg/l NAA in combination with

0.5 mg/l IBA, it resulted in 1.20 ± 1.16 mean number of roots per shoot and 0.43 ± 0.40 cm mean root length. The minimum result was observed at 2.0 mg/l NAA in combination with 0.5 mg/l IBA, which resulted in 0.27 ± 0.58 mean numbers of roots per shoot and 0.09 ± 0.19 cm mean root length.

[31] obtained 4.7 roots per explant in ½ strength MS medium supplemented with 0.5 mg/l NAA in *M. oleifera*. In contrast, [15] found that hormone-free medium was the best rooting medium. In the present study, less mean root numbers (1.23 ± 1.19) were obtained in this concentration (0.5 mg/l) and growth regulator free medium (0.26 ± 0.57) as compared to the result reported by these authors. But the highest mean number of root and mean root length per explant obtained in the present study was 1.63 ± 1.03 cm and 0.87 ± 1.22 cm in MS medium containing 1.0 mg/l NAA and 0.1 mg/l IBA alone respectively. [15] Stated that medium supplemented with 1.0 and 2.0 mg/l NAA was not inducing rooting at all. In addition, these authors also stated that the medium supplemented with 0.05 mg/l, 1.0 mg/l and 2.0 mg/l IBA produced 0.0, 2.0 and 2.8 mean numbers of roots per explant respectively. In the present study, the first highest mean root number were produced at concentration of 1.0 mg/l NAA and the second highest mean number of root per explant (1.50 ± 0.38) and the least mean number of root (0.07 ± 0.25) were obtained in these concentrations (1.0 mg/l and 2.0 mg/l IBA) respectively. When the concentrations of IBA increased from 0.05 mg/l to 2.0 mg/l, mean number of root per explant also increased. The trend reported by these authors agrees with the present study particularly in the effect of IBA rather than NAA. The result of the present study indicated that when the concentration of IBA increased from 0.1 mg/l to 1.0 mg/l, mean

number of roots per explant continuously increased but further increasing of IBA concentration beyond 1.0 mg/l led to decreasing mean number of roots per explant. [15] found the highest number of roots per explant at 2.0 mg/l IBA which showed the least mean number of root in the present study.

In agreement with the finding of [2] on *Moringa peregrina (*Forsk) who got the maximum number of roots (44.0) per explant in MS medium supplemented with 1.0 mg/l IBA and MS medium containing 0.5 mg/l IBA produced longer roots than control or medium supplemented with different levels of NAA. Here, medium supplemented with1.0 mg/l IBA alone showed highest rate of root induction (1.50 ± 0.38) with longer roots at 0.1 mg/l IBA (0.87 ± 1.22) compared to control medium. As the present data shows that using lower levels of auxin (either NAA or IBA) is significantly better than higher levels for root induction and elongation. [33] also reported the inhibition of root elongation by higher concentration of growth regulators and stated ethylene deposition as the reason. Auxins of all types stimulate plant cell to produce ethylene, especially when high number of synthetic auxins are used. Ethylene retard root elongation. According to this author, the other reason for reduced response of root number and root length at higher concentration of auxin may be poor vascular connection of the root with the stem because of the interventions of callus.

In an experiment for *in vitro* rooting of *M. oleifera*, when micro-shoots were cultured on a medium containing different level of IAA and IBA, medium supplemented with 0.5 mg/l IAA along with IBA at 1.0 mg/l resulted in the highest number of induced roots [28]. These variable responses could be due to different factors including genetic differences, differences in the explant source, the concentration difference of growth regulators and the type and/or age of explants used to establish the cultures [20].

3.3. Acclimatization

Among 50 plantlets acclimatized in the green house, 38 (76%) survived. Which is similar to the result of [20, 28] on *Moringa oleifera*. [20] transferred plantlets of *Moringa oleifera* to small plastic pots containing fumigate soil and by covering potted plantlets with clear polythene bags and kept in a shaded greenhouse for 2–4 weeks before exposure to ambient conditions. The author got 80% of survived plants. [28] also obtained the same result as [10], provided that the potted plantlets were covered with clear polythene bags and kept in a shaded greenhouse for 15 days before exposure to ambient conditions.

4. Conclusions

Results of this study indicate that large- scale propagation of *M. stenopetala* by tissue culture methods is feasible and several plantlets can be regenerated from a single shoot tip explant. The analysis of variance revealed that the effect of NAA along with kinetin on shoot multiplication after four weeks of culture was highly significant ($P \leq 0.05$). Results indicate that using NAA along with kinetin for shoot multiplication was better than kin along with IBA. Therefore, 0.5 mg/l kinentin combined with 0.01 mg/l NAA was found to be optimal in producing maximum number of shoots per explant (3.43 ± 1.41). Application of NAA alone at concentration of 1.0 mg/l (1.63 ± 1.03) and 1.0 mg/l IBA (1.20 ± 1.16) were

more effective for root induction. This implies that this protocol enables mass propagation of *M. stenopetala* from shoot tip explant.

According to the result obtained in this study, MS medium supplemented with 0.5 mg/l kinetin along with 0.01 mg/l NAA for shoot multiplication and ½ MS medium supplemented with 1.0 mg/l NAA alone and 1.0 mg/l NAA combined with 0.5 mg/l IBA for rooting is recommended for in vitro propagation of *M. stenopetala* from shoot tip explant. Therefore, further optimization of this protocol may be required for mass propagation of this plant. Further optimization of this protocol may also require low cost mass propagation of this plant by reducing the media components used in this protocol or substituting with cheaper components and methods. It is also recommended to practice other techniques like culturing of explants in liquid medium reduces the cost of agar for mass propagation of *M. stenopetala.*

5. Methods

5.1. Source of explant and surface disinfection

Matured seeds of *M. stenopetala* were collected from Merab Abaya, Arba Minch area, in the Southern Nation Nationalities and peoples Regional State of Ethiopia during November and December of 2015. We got permission to collect the sample from local authorities and oral consent form local societies, after we did discussion about the benefit the research. The collected specimens are identified and confirmed by botanist before we did further experimental analysis. Fruits were cut open and the seeds were separated from pods and washed with local detergent (omo) for 10 min and rinsed in running tap water for five minutes then thoroughly washed and rinsed again in sterilized distilled water for about 24 hours to speed up seed germination.

Seeds were surface sterilized with 10% NaOCl solution for 25 min followed by five washings with sterile distilled water and sown in culture jars containing 50 ml plant growth regulators-free MS [24] medium under aseptic conditions. The MS medium was enriched with 30 g/l sucrose (w/v), 8 g/l agar (w/v), pH was adjusted to 5.8 and autoclaved at 121ºC with a pressure of 105 KPa for 15 min. The cultures were maintained in growth room under light intensity of 16-hour photoperiod provided by cool-white fluorescent lamps at a temperature of 25 ± 2 ºC.

5.2. Preparation of Stock Solution and Culture Media

5.2.1. MS Stock Solution Preparation

The stock solution of macronutrients, micronutrients and vitamins were prepared separately by weighing the recommended amount of powder by dissolving in distilled water and stored them in refrigerator at a temperature of + 4 °C until used. The prepared stock solutions were stored at + 4 °C for a maximum of one month.

5.2.2. Growth regulators stock solution preparation

In this study, different plant growth regulators namely cytokinins (kinetin) and two auxins (IBA, NAA) were used with different concentrations and combinations. Each of these growth regulator stock solutions was prepared by weighing and dissolving the powder in distilled water at a concentration of 1.0 mg/ml. To begin the dissolving process 3–4 drops of 1M NaOH or 1M HCl were added based on the requirement of the growth regulators (NaOH for auxin, HCl for cytokinin). Then, the volume was adjusted by adding distilled water. Finally, growth regulators stock solutions were stored in a refrigerator at a temperature of + 4 °C until used.

5.2.3. Culture Media Preparation

Culture media were prepared by mixing the proper amount of MS stock solutions (50 ml/L macro, 5 ml/L micro, 5 ml/L iron EDTA and 5 ml/L vitamin) then 30 g/l sucrose was added to the solution as energy and carbon source. After mixing all the components and adjusting the volume, growth regulators were added as required and pH was adjusted to 5.8 using 1N NaOH or 1N HCl. Finally, 8.0 g/l agar was added. After melting the agar by using hot plate, 50 ml medium was dispensed into each Magenta GA-7 culture vessel. The medium was sterilized by autoclaving at a temperature of 121 °C at a pressure of 105 KPa for 15 min. Finally, the medium was allowed to cool in the laminar air flow cabinet.

The media compositions that were used for different experiments were as follows:

Media for shoot Multiplication

- MS + IBA and Kinetin + 30 g/L sucrose + 8 g/L agar
- MS + Kinetin + NAA + 30 g/L sucrose + 8 g/L agar

Media for Rooting

- 1/2 MS + IBA + 30 g/L sucrose + 4 g/L agar
- 1/2MS + NAA + 30 g/L sucrose + 4 g/L agar
- 1/2MS + IBA + NAA + 30 g/L sucrose + 4 g/L agar

5.3. Culture Conditions

All types of cultures were kept in a growth room at a temperature of 25 ± 2 °C and under light intensity of 16-hour photoperiod provided by cool-white fluorescent lamps.

5.4. Shoot initiation

Shoot tips obtained from *in vitro* germinated seedlings were used for culture initiation. Shoot tips from *in vitro* germinated seedlings were excised and cultured on MS medium supplemented with BAP (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) alone and in combination with NAA (0.1, 0.5 and 1.0 mg/l). The medium was supplemented with 30 g/l sucrose (w/v) and the pH was adjusted to 5.8 before addition of 8 g/l agar (w/v). Six shoots per Magenta culture vessel was considered as a unit of replication and there were five

replications for each treatment. The cultures were maintained in culture room under light intensity of 16-hour photoperiod provided by cool-white fluorescent lamps at a temperature of 25 ± 2 ºC.

5.5. Shoot Multiplication

The *in vitro* initiated shoots were cultured on MS medium supplemented with different concentrations of kinetin (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) in combination with IBA (0.0, 0.01, 0.1, 0.5) or kinetin (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) in combination with NAA (0.01, 0.1, 0.5 mg/l) to determine their effect on multiple axillary shoot formation. Growth regulators free MS basal medium was used as a control. Six shoots per Magenta culture vessel was considered as a unit of replication and there were five replications for each treatment. The cultures were maintained at 25 °C ± 2 °C with 16 h photoperiod and subcultured every four weeks. Shoot length, number of leaves and number of shoots per explant were recorded after four weeks.

5.6. Rooting

Micro shoots obtained from shoot multiplication medium were transferred to rooting medium. The rooting medium was half strength MS medium containing different concentrations of NAA and IBA. Six shoots were cultured in each culture vessel and a total of five replications were designed for each treatment. The cultures were maintained in growth room under the same condition as of shoot multiplication. Number of roots per shoot and root length were recorded after 4 weeks.

5.7. Acclimatization

Plantlets with well-developed shoots and roots were taken out of the culture jars and the roots were washed thoroughly with running tap water and transferred into pots containing autoclaved (sterilized) sand, red soil and compost at a ratio of 2:1:1 respectively. The plantlets were covered with transparent plastic bag to maintain moisture for two weeks and placed them in the shade region of tissue culture room prior to their transfer to the greenhouse condition and watered within two days interval (morning and evening).The plastic cover was gradually removed after one month and the plantlets were successfully established in greenhouse.

5.8. Data Analyses

After four weeks of transferring explants into multiplication media, number of shoots per explant, mean shoot length and number of leaves were recorded. After four weeks of transferring well developed shoots into rooting media, number of roots per shoot, and mean root length were recorded. All data were subjected to analysis of variance (ANOVA) to quantify the differences between applied treatments. Treatment means were separated using the least significance differences (LSD) at probability level of $p \leq 0.05$. ANOVA table was constructed using SPSS computer software of version 20 and Tukey's multiple range test was used.

Abbreviations

2, 4-D

2, 4-Dichlorophenoxyacetic acid
2-ip
Isopentenyl-adenine
BAP
6- Benzylamino purine
IAA
Indole acetic acid
IBA
Indole-3- butyric acid
Kin
Kinetin
MS
Murashige and Skoog
NAA
α -naphthalene acetic acid
PGR
Plant growth regulator
TDZ
Thidiazuron
CRD
Complete Random Design
ANOVA
Analysis of Variance
SPSS
Statistical Package for Social Science
LSD
Least Significance Difference

Declarations

I declare that this thesis is my original work and has not been submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. All sources of materials used for the thesis have been duly acknowledged.

Ethical Approval

Not applicable

Consent for publication

Not applicable

Competing interest

All authors declared that they have no competing interests to their work.

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Author contributions

A Y carried out this study by preparing proposal, conducting laboratory activities to collect all necessary data, finalized it through statistical analysis and prepared manuscript. Upon this process my respected and humble advisor, T F, contributed his beneficial guidance, constructive comments and experiences throughout my study and full write up of the thesis including manuscript preparation. Moreover, F S also contributed his professional guidance in commenting and editing the manuscript of this thesis work. All authors have read and approved the manuscript.

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Availability of Materials

Materials related to this paper are shared to the journal editorial board.

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Figures

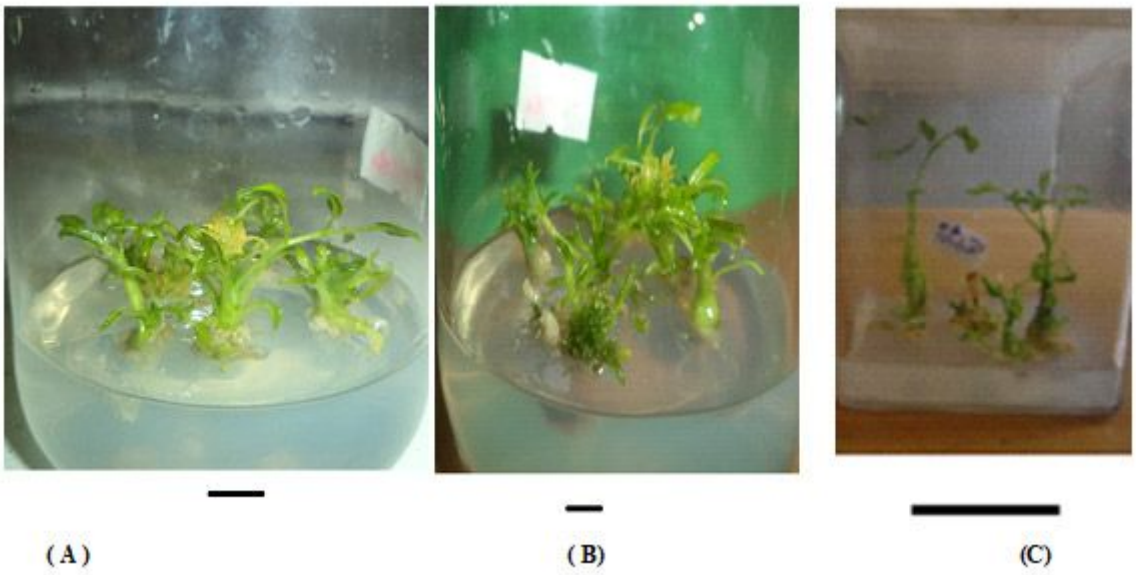

Figure 1

The effect of different growth regulators on shoot multiplication after four weeks of culture; A) 0.5 mg/l kinetin + 0.01 mg/l NAA, B) 0.5 mg/l Kinetin + 0.1 mg/l IBA, C) PGR free. Bar =2cm

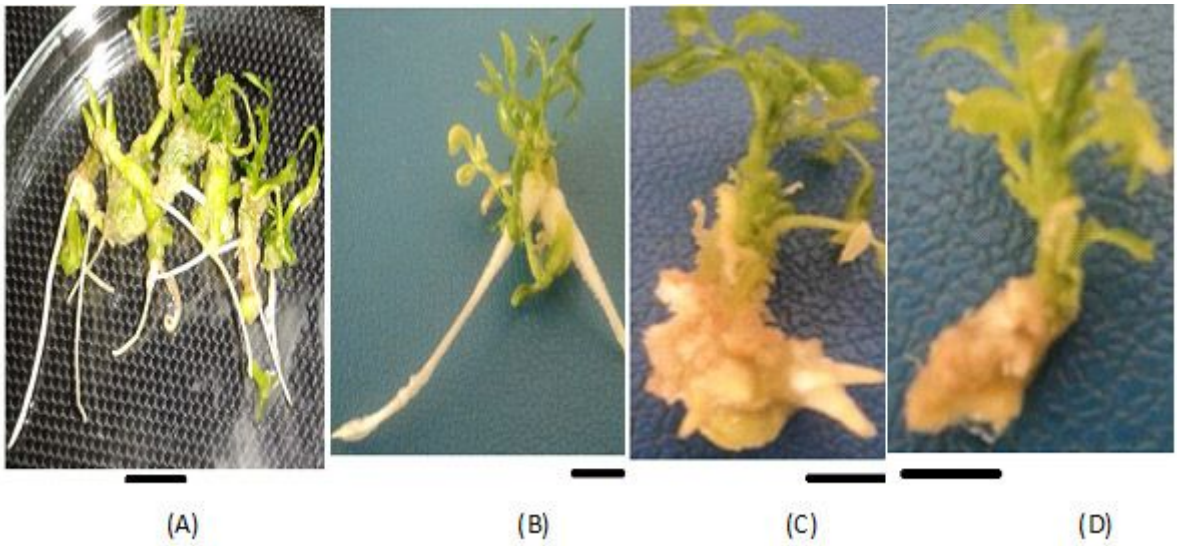

Figure 2

The effect of NAA and IBA on rooting of micro shoots after four weeks of culture. A) 1.0 mg/l NAA, B) 1.0 mg/l IBA, C) 1.0 mg/l NAA in combination with 0.5 mg/l IBA, D) PGR free medium. Bar = 2 cm

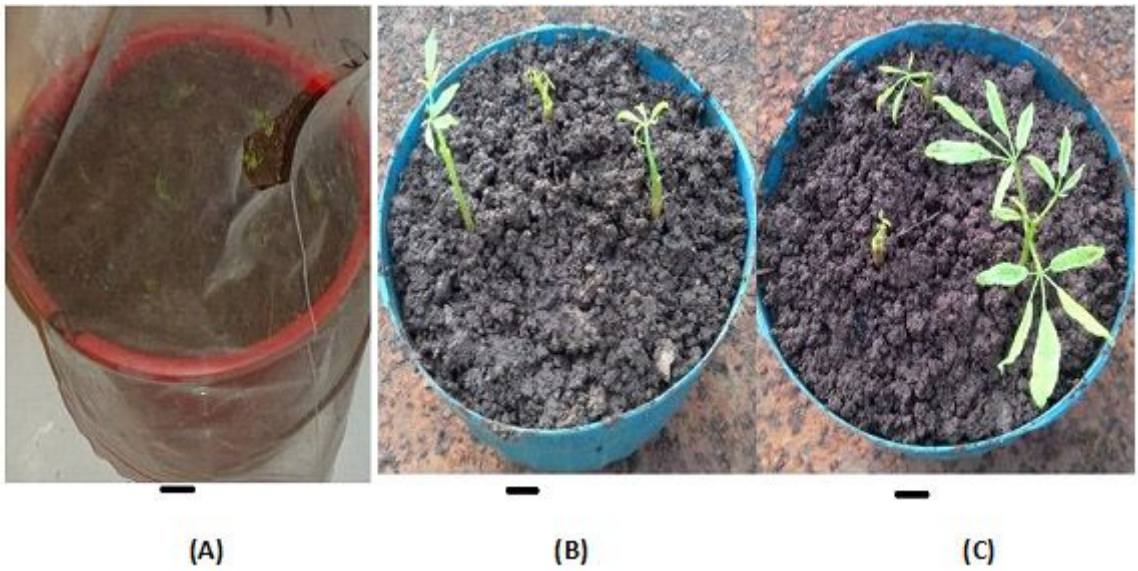

Figure 3

Acclimatization; A) During transplanting, B) After three weeks, D) After four weeks. Bar = 2 cm