# DEVELOPMENT OF *in vitro* REGENERATION AND *Agrobacterium* MEDIATED TRANSFORMATION SYSTEMS FOR *Moringa oleifera* PLANT

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*Moringa oleifera* Lam. commonly known as the drumstick or ben oil tree is a widely cultivated species of monogeneric family Moringaceae. It is a fast-growing tropical perennial soft-wooded tree with a long history of traditional medicine and culinary uses adding that it is very nutritious and has a variety of potential uses (Verma *et al*., 1976; Fahey, 2005). Much of the plant is edible by humans or by farm animals. The leaves are rich in protein, vitamin A, vitamin B, vitamin C and minerals (Jules and Paull, 2008). The tree is also a rich source of antioxidants. The seeds contain 30 to 40% oil which is highly valued in the cosmetic industry for its unique property beside having exceptional anti ageing and anti cancer properties due to the antioxidants and the nutrients present in it that help to curb the activity of the free radicals on the skin. It is widely cultivated in India, the Philippines, Sudan, South Africa, tropical Asia, Latin America, the Caribbean, and in the Pacific islands (Verdcourt, 1985; Palada, 1996). Other species of genus Moringa are*: M. stenopetala* is an important crop in Kenya and Ethiopia (Verdcourt, 1985). Similarly, *M. peregrine* was known to the ancient Egyptians who utilized its seed oil. Moringa trees are usually propagated by seeds or cuttings. However, not enough seeds or cuttings are available for use as planting materials as moringa still has not been cultivated in commercial quantity in Egypt. Around 500,000 ha of land will be needed to produce moringa oil at a commercial scale (Marfori, 2010). At a planting distance of 1 m x 1 m, 5 billion seedlings will be needed as planting materials for 500,000 ha. Very limited information is available on genetic variability and availability of superior genotypes for commercial cultivation. The traditional way to improve the desired traits is by breeding techniques, but these techniques have limitations as they depend on sexual compatibility, and takes considerable time (10-15 years) to release a new variety. Alternatively, genetic manipulation tools, such as, genetic transformation methods are representing valuable tool for the functional study of genes as well as the production of genetically improved plants by introducing genes that responsible for increasing oil content and genes that regulate the production of endogenous plant antioxidants that leads to obtain plants with enhanced contents of agronomical, nutritional and

pharmaceutical traits. There are extensive reports that demonstrate successful attempts for the modification of plant oil composition *via* metabolic engineering as well (Abbadi *et al*., 2004; Hoffmann *et al*., 2008; Lopez *et al*., 2009; Cheng *et al*., 2010; Petrie *et al*., 2011; Lopez *et al*., 2012; Haslam *et al*., 2013; Lopez *et al*., 2014). Building on all previous considerations adding the tremendous potential opportunities with *M. oleifera* for sustainable agriculture and the development of cash crops in semiarid regions as Egypt, the plant was selected for this regeneration and transformation work. The aim of the present study was to optimize the regeneration and mass *in vitro* propagation of Moringa plants as well as to establish a reliable genetic transformation protocol as a preliminary request for further genetic improvements of moringa plant *via* genetic engineering approaches.

## MATERIALS AND METHODS

### *Plant material and explant preparation*

Healthy uniformed seeds of *M. oleifera* were obtained from Faculty of Agricultural Science, Menofiya, Egypt. Seeds were surface sterilized inside the laminar flow hood with 75% ethanol for one minute followed by immersion in 30% sodium hypochlorite (v/v) for 10 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 5 min, followed by rinsing three times in sterile distilled water. Seeds were planted aseptically in MS basal medium (Murashige and Skoog, 1962) containing 30 g/l sucrose and solidified with 8 g/l agar (Himedia). The pH was adjusted to 5.8, after which the medium was dispensed at 40 ml each in culture bottles and sterilized by autoclaving at 121°C for 20 min. Seed cultures were maintained in the dark at 27 ± 1°C for 15 days. Upon germination, seedlings were transferred under continuous light at 2,000-Lux intensity produced from cool white fluorescent tubes. Young, aseptically grown shoots (6 weeks old) were cut into nodal segment explants each bearing one or two axillary buds were inoculated on shoot induction media supplemented with different concentrations of growth regulators.

### *Bacterial strain and transformation vector*

*Agrobacterium tumefaciens* strain EHA105 was used for plant transformation. The *Agrobacterium* cells were directly transformed with the *Agrobacterium* binary vector pBI121 that harbors both the *uidA* gene, coding for *β*-glucuronidase (GUS) (Jefferson *et al.*, 1987) as a reporter gene under CaMV 35S promoter and neomycin phosphotransferase II (*NPTII*), kanamycin resistant gene (Bevan *et al.*, 1983) as a selectable marker gene under NOS promoter in order to select for the transformants onto selective regeneration medium. Both *Agrobacterium* strain and plant transformation vector obtained from Clontech Laboratories.

***Induction of multiple shoots***

Nodal segment explants were prepared and inoculated onto multiple shoot induction media (SIM) consisting of MS salts and different concentrations of plant growth regulators of benzylaminopurine (BAP) and zeatin at (1.0, 1.5 and 2.0 mg/l) and their combinations with naphthaleneacetic acid (NAA) at (0.0, 0.3 and 0.6 mg/l) to determine their effect on multiple axillary shoot formation. All growth regulators used in the study were obtained from Sigma Chemicals Co., St. Louis, MO, USA. Percentage of response and the number of shoots per explants was recorded throughout three weeks after inoculation onto shoot induction (SIM) media. All cultures of regeneration treatments were carried out on MS salts supplemented with sucrose (3%), agar (8 g/l) and the pH was adjusted to 5.8 before autoclaving (121°C, 20 min). *In vitro* cultured plant materials were incubated in a controlled growth chamber at 25±2°C and 8/16 hr (dark/light) photoperiod.

***Shoot elongation***

Young developed shoots were transferred to shoot elongation media consisting of the same concentrations and combinations of SIM media supplemented with 0.5 mg/l gibberellic acid ($GA_3$) for encouraging shoot elongation. Shoot clusters were maintained on this media two weeks until young well developed shoots reached around 2 cm long.

***Rooting of shoots***

Regenerated shoots were excised and then transferred to a root induction medium (RIM) consisting of MS salts, indole-3-acetic acid (IAA) at concentrations of 0.5 and 1.0 mg/l or indol-3-butyric acid (IBA) or different combinations of them as well as a free hormone media as a control. Percentages of response were recorded 7 days after transfer to RIM. Rooted plantlets were transferred to the soil for hardening.

***Hardening of the regenerated plantlets***

Successfully rooted plantlets were hardened in pots containing autoclaved mixture of soil, sand and petmos (3:1:1 v/v). Plants were watered, then covered with transparent plastic bags, and kept under partial sunlight inside a greenhouse at ambient temperature (26-28°C). After 15 days, the plastic bags were removed and the survived plants were maintained inside the greenhouse.

***Transformation procedure and conditions***

***Pre-culture step***

Nodal segments explants were prepared as described in the regeneration experiment, then they were precultured on MS medium supplemented with 39.28 mg/l (3,5,Dimethyl-4-hydroxy acetophenone) acetosyringone (Aldrich) and 1.0 mM galacturonic acid then incu-

bated over night at 25°C and 16/8 photoperiod.

***Inoculation***

The inoculium of *Agrobacterium tumefaciens* EHA105 cells harboring pBIN121 was prepared from a freshly streaked LB plate culture containing kanamycin and streptomycin 50 mg/l each. A loop from the plate culture was inoculated into 5 ml LB liquid medium containing the same antibiotics as LB plate with the same concentrations and grown for 16 h at 28°C with vigorous shaking at 250 rpm. An aliquot of 100 µl from the culture was used to inoculate 10 ml of a LB broth that contained the same mentioned antibiotics as LB plate with the same concentrations in addition to 39.28 µg/ml acetosyringone then incubated in a shaker incubator at 28°C at 250 rpm for 3-4 hours. Optical density (OD) was measured by spectrophotometer at 660 ηm and the bacterial concentration was adjusted to approximately $5\times10^8$ cfu/ml by MS basal liquid medium prior to use. Nodal explants were immersed in the diluted bacterial solution for 5-10 minutes under sterilized conditions then the explants were blotted dry on sterile filter paper, then explants were kept for 2 days co-cultivation onto the same preculture medium in the incubator at 25°C and cool white light. Light intensity was 45-50 $m^{-2}s^{-1}$ under continuous light.

***Selection and regeneration***

After cocultivation, the nodal segment explants were subsequently transferred to the selective shoot induction plates containing suitable regeneration media supplemented with 50 mg/l kanamycin, to select for transformed shoots in addition to 500 mg/l carbenicillin and 250 mg/l cefotaxim to prevent *Agrobacterium* growth (all antibiotics were added as filter sterilized solutions after autoclaving) then cultures were incubated under the previous optimized conditions. After additional two weeks of incubation, the number of developed shoots was recorded. Will developed shoots were excised individually from the explants and subcultured on suitable rooting media that containing 50 mg/l kanamycin. The experiment had three replicates. Finally, Successful rooted plantlets were hardened in pots containing sterilized mixture of soil and sand v/v and were acclimatized in the greenhouse. The established putatively transgenic Moringa plants were designated as $T_0$-generation plants.

***Confirmation of successful transformation***

***Polymerase chain reaction (PCR) analysis***

Putative transgenic Moringa $T_0$ plantlets were verified for the presence of the transgenes by PCR analysis using *GUS* and kanamycin (*Km*). Gene specific primers. Total genomic DNA was isolated from leaves of randomly selected putative transgenic plants as well as the untransformed control plant using modified CTAB protocol (Puchooa, 2004). The *GUS* specific primer sequences; sense (*GUS*-F): 5'-CATGTCGCGCAAACTGTAAC-3' and

anti-sense (*GUS*-R): 5'-AATCGCCTGTAA GTGCGCTTG-3') while the kanamycin specific primer sequences; sens (*Km*-F):5'-GCATACGCTTGATCCGGCTAC-3'and anti-sense (*Km*-R): 5'-TGATATTCG GCAAGCAGGCAT-3'), were used in the PCR analysis. PCR was performed in a total volume of 25 µl and the reaction mixture consisted of 10x PCR reaction buffer, 50 ng template DNA, 0.2 mmol/l dNTPs, 1.5 mM $MgCl_2$, 0.2 mM of each primer and 1 unit of *Taq* DNA polymerase. The PCR reaction was started by an initial denaturing step at 94°C for 2.5 min, followed by 35 cycles of the following profile: denaturing at 94°C for 30 sec, annealing at 56°C for 1 min, synthesis at 72°C for 1 min followed by an extension at 72°C for 10 min. The amplification mixture was analyzed by electrophoresis in 1% agarose ethidium bromide gels.

### *Histochemical GUS assay*

Histochemical analysis of GUS activity in putative transgenic Moringa plantlets was carried out using 5-bromo-4-chloro-3-indolyl-b-d-glucuronide (X-Glu) as a substrate as described by Jefferson *et al*. (1987) using different parts of the plant. Following overnight tissue-staining with X-Glu at 37°C, chlorophyll was removed from plant parts by soaking in a mixture of 70 % ethanol and 10% commercial bleach Clorox for 4-6 hrs. The ethanol bleach mixture was replaced three to four times then washed with double distilled water.

## RESULTS AND DISCUSSION

### *Influence of growth regulators on shoot regeneration and root induction*

The percentages of responded explants as well as the mean number of axillary shoots per explant induced by BAP and Zea at different concentrations and their different 18 combinations with NAA after 3 weeks were represented in Table (1). The SIM4 medium resulted in the highest number of responded explants as the application of 1.0 mg/l BAP gives rise of shoots on 95.2% of the inoculated explants with an average of 6.6 axillary shoots per explant with direct shoot development without any formation of callus structures (Fig. 1). SIM5 medium that contained a combination of BAP and NAA at 1.0 and 0.3 mg/l, respectively, enhanced axillary shoot proliferation at 93% of inoculated nodal explants with an average of 5.3 shoots per explants. The same concentrations of zea in SIM13 and SIM14 media was much less effective than BAP in respect of the responded explants which was only 57.3% and 52% with an average of 3.1 and 2.9 axillary shoots per explant, respectively with more tendency for callus formation than shoot proliferation. It was noticed that almost all media that were including both Zea or NAA or their combinations had developed more or less callus structures (data not shown). Higher concentration of BAP was inhibitory, inducing 2.7 axillary shoots per explants when used alone at 1.5 mg/l or in combination with 0.3 and 0.6 mg/l NAA inducing 1.4 and 0.7 axillary shoots per

explants, respectively. Regenerated shoots were subcultured on the same shoot induction media supplemented with gibberellic acid ($GA_3$) at 0.5 mg/l to enable shoot elongation. Well developed shoots were transferred to root induction medium supplemented with IAA or IBA or their combination as well as free hormone medium, Figure 1D. Induction of roots was recorded as a percentage after two weeks from inoculation and data were represented in Table (2). Application of 1.0 mg/l IBA along with 0.5 mg/l IAA resulted in the highest percent of responded shoots (100%) with direct root developments and no callus formation (Fig. 1 E1 & E2), while there were 95% responds among shoots when media containing IBA alone. However, application of IAA alone resulted in root induction (75%) with more tendencies for callus formation at the base of the young shoots (Fig. 1 E3). The *in vitro* regenerated plantlets were successfully hardened and established on the soil with normal growth pattern (Fig. 1 F).

*In vitro* regeneration and multiplication of *M. oleifera* has been previously investigated using different growth regulators. Our results were in consensus with Stephenson and Fahey (2004) as they obtained 4.7 shoots per cultured seed in medium containing 1 mg/l BA with 1 mg/l $GA_3$, while rooting was obtained in MS media containing 0.5 mg/l NAA. At similar result of ours Saini *et al*. (2012) reported that 1 mg/l BA was found to be optimal in producing maximum number of shoots per explants while efficient in vitro rooting of individual shoot culture was obtained in 0.5 mg/l IAA plus 1mg/l IBA treatment. Islam *et al*. (2005), demonstrated that using BAP at concentrations 1.0-1.5 mg/l was found to be best for shooting response, whereas rooting was efficient on MS basal medium. The efficiency of BA and NAA for organogenesis was further supported by similar reports. Riyathong *et al*. (2009) found that shoot multiplication were successfully carried out in the presence of BA 2.0 mg/l giving average number of 10.8 shoots per explant. In the same time they found that regeneration of *in vitro* explants was initiated by using the medium containing 0.5 mg/l NAA that produced the shoots and roots from callus culture. Marfori (2010) reported that of the three cytokinins tested, namely BAP, kinetin (Kin) and thidiazuron (TDZ), BAP at 0.5 mg/l was found to be optimal in inducing bud break, producing an average of 4.6 axillary shoots per explant after two weeks, whereas optimal rooting of individual shoot culture was obtained with application of NAA at 0.05 mg/l.

### *Statistical analysis for the efficiency of shoot regeneration*

To compare the efficiency of the different treatments on explant responds and shoot regeneration statistical analysis has been conducted on the obtained results to evaluate the degree of significance between the different combinations of BAP, Zea and NAA. The data obtained were subjected to statistical analysis of variance as described by Steel and Torrie (1980).

The treatment means were compared using LSD test at 0.05 level of significant.

The results of this analysis are summarized in Table (1). The results proved the previous conclusion that the SIM4 and SIM5 medium were the most efficient treatments in respect to the percentages of responded explants while in terms of the highest number of regenerated shoots, SIM4 media was the best. It is also clear from the table that there were no significant differences at P= 0.05 level for the other treatments.

***Agrobacterium-mediated transformation***

Nodal segment explants of young moringa seedlings were prepared as previously described and then were infected with *A. tumeficiense* strain EHA105 cells harboring pBI121. Then explants were maintained for two days cocultivation period with *Agrobacterium* then they were transferred to selective regeneration medium that contained SIM4 medium supplemented with 50 mg/l Kanamycin 500 mg/l carbincillin, and 75 mg/l cefotaxim and incubated until the putative transformed shoots were developed. It was found to be important that the explants must undergo in several subculture steps onto a fresh selection medium at least once after 10 days and in each time both abnormal structures and dead tissues must trimmed out to promote faster shoot formation, and to prevent *Agrobacterium* regrowth. Results showed that the surviving explants on kanamycin SIM4 medium ranged among the replica from 19 to 46 explants with a total number of 235, representing 39.6% transformation efficiency (Table 3). To the best of our knowledge and after extensive searches conducted *via* published article databases, we could not find articles on the transformation of *M. oleifera*. On the other hand there are many successful attempts to produce different transgenic oilseed crops that are successfully engineered to accumulate high levels of different fatty acids as well as to elevate the oil contents of its seeds (Abbadi *et al*., 2004; Lopez *et al*., 2009; Lopez *et al*., 2012; Lopez *et al*., 2014; Haslam *et al*., 2013).

***Analysis of the putative transgenic moringa plantlets***

***a. PCR analysis***

The explants that were able to survive on the selective media supplemented with Kanamycin and were able to develop the putative transgenic shoots were examined to verify the presence of both *GUS* and *Km* gene *via* PCR analysis. Plant genomic DNA was isolated from five samples of randomly selected *Km*-resistant shoots that were transformed with pBI121 *via Agrobacterium*-mediated transformation as well as the untransformed plantlet controls that were grown on SIM4 medium without antibiotics as negative control while using pBI121 DNA as a positive control, then subjected to PCR analysis using the specific primers of both *GUS* and *Km* genes. Results showed that out of

the selected five samples of *km*-resistant plantlets, four samples showed the expected amplified fragment sizes of 875 bp and 1822 bp corresponding to both *Km* and *GUS* genes, respectively, while they were absent in untransformed plant control (Fig. 2). Further PCR analysis could be conducted on large scale to evaluate the transgenic lines obtained.

***b. Histochemical GUS assay***

The *uid*A gene or *gus* gene is one of the most widely used reporter genes in plant transformation due to its accurate fluorimetric assays and precise histochemical localization of GUS in transgenic tissues. The explants that were able to grow on the selective media supplemented with Km were examined histochemically for *GUS* activity. Out of the total number of 93 *Km*-resistant shoots that were survived on selective shoot induction medium, only 20 young shoots were randomly selected and subjected to a histochmical *GUS* assay and visually compared with non-transformed plant materials. A percentage of 80 % of the tested plant materials developed blue color that indicating *GUS* expression in various parts of Moringa plantlets whereas no blue color was observed untransformed plants (Fig. 3).

## SUMMARY

The present investigations were aimed to develop a high efficiency of *in vitro* regeneration and genetic transformation systems of *Moringa oleifera* Lam from nodal segments of young aseptically grown seedlings using *Agrobacterium*-mediated transformation approach. Frequency of responded explants and number of shoots per explant were recorded during the course of the regeneration experiment. Regeneration capacity of nodal segments was evaluated on Murashige and Skoog (MS) media supplemented with 18 different combinations of plant growth regulators of benzylaminopurine (BAP), Zeatine (Zea) and naphthaleneacetic acid (NAA). Application of 1.0 mg/l BAP individually was found to be superior in terms of highest number of responded explants (95.7%) as well as the highest average (6.6) of axillary shoot developments per explant with direct emerging of adventitious shoots escaping callus formations. Well developed shoots subjected to rooting media supplemented with IAA or IBA or their different combinations. The most successful rooting events (100%) for regenerated shoots were obtained on rooting media containing ½ MS salts and supplemented with 1.0 mg/l IBA along with 0.5 mg/l IAA within three weeks maximum. The plant transformation vector pBIN121 harbors both the *uidA* (*GUS*) and *NPTII* (kanamycin resistant) genes were used to establish the *Agrobacterium*-mediated transformation experiment. Number of Putative transformed young shoots that developed onto Kanamycin selective regeneration medium were recorded representing 39.6% transformation efficiency. PCR analysis was carried out to verify successful transformation and gene integration for both *GUS* and *NPTll* genes in randomly selected

young shoots while Histochemical *GUS* assay confirmed the successful expression of *GUS* gene in different parts of the putative transgenic plantlets.

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Table (1): Statistical analysis for the efficiency of different concentrations and combinations of growth regulators on shoot regeneration and number of shoots per nodal segment explants**.**

| Media | BAP (mg/l) | Zea (mg/l) | NAA (mg/l) | Mean of Responds% | Mean of Shoots/explants |
|---|---|---|---|---|---|
| SIM1 | 0.5 | 0.0 | 0.6 | 80.2b | 4.3c |
| SIM2 | 1.0 | 0.0 | 0.0 | 46.0fg | 3.2d |
| SIM3 | 1.0 | 0.0 | 0.3 | 30.0 j | 0.8k |
| SIM4 | 1.0 | 0.0 | 0.6 | 95.2a | 6.6a |
| SIM5 | 1.5 | 0.0 | 0.0 | 93.0a | 5.3b |
| SIM6 | 1.5 | 0.0 | 0.3 | 81.6b | 2.1efg |
| SIM7 | 1.5 | 0.0 | 0.6 | 78.1b | 2.7de |
| SIM8 | 0.0 | 0.5 | 0.0 | 72.6c | 1.4hi |
| SIM9 | 0.0 | 0.5 | 0.3 | 70.3c | 0.7jk |
| SIM10 | 0.0 | 0.5 | 0.6 | 55.8de | 2.3ef |
| SIM11 | 0.0 | 1.0 | 0.0 | 43.0fgh | 2.1efg |
| SIM12 | 0.0 | 1.0 | 0.3 | 31.2 j | 0.7jk |
| SIM13 | 0.0 | 1.0 | 0.6 | 57.3d | 3.1d |
| SIM14 | 0.0 | 1.5 | 0.0 | 52.0e | 2.9d |
| SIM15 | 0.0 | 1.5 | 0.3 | 37.4i | 1.6ghi |
| SIM16 | 0.0 | 1.5 | 0.6 | 47.0f | 2.0fgh |
| SIM17 | 0.0 | 0.0 | 0.0 | 42.0ghi | 1.7fghi |
| SIM18 | 0.5 | 0.0 | 0.6 | 40.0hi | 1.1ij |
| SIM19 | 1.0 | 0.0 | 0.0 | 36.0i | 0.4k |

Values within a column followed by the same letter(s) are not significantly different at the P=0.05 level according to the least significant difference test as described by Steel and Torrie (1980).

Table (2): Influence of IBA and IAA and their combinations on root induction.

| IBA (mg/l) | IAA (mg/l) | Rooting% |
|---|---|---|
| 0.5 | 0.0 | 70.0 |
| 1.0 | 0.0 | 95.0 |
| 0.0 | 0.5 | 68.0 |
| 0.0 | 1.0 | 75.0 |
| 0.5 | 0.5 | 90.0 |
| 1.0 | 0.5 | 100.0 |
| 0.5 | 1.0 | 62.0 |

Table (3): *Agrobacterium*-mediated transformation frequency of the moringa nodal segment explants obtained on selective regeneration medium.

| Replicates | Number of explants | Surviving explants | |
|---|---|---|---|
| | | No. | % |
| R1 | 54 | 19 | 35.2 |
| R2 | 106 | 46 | 43.4 |
| R3 | 75 | 28 | 37.3 |
| Total | 235 | 93 | 39.6 |

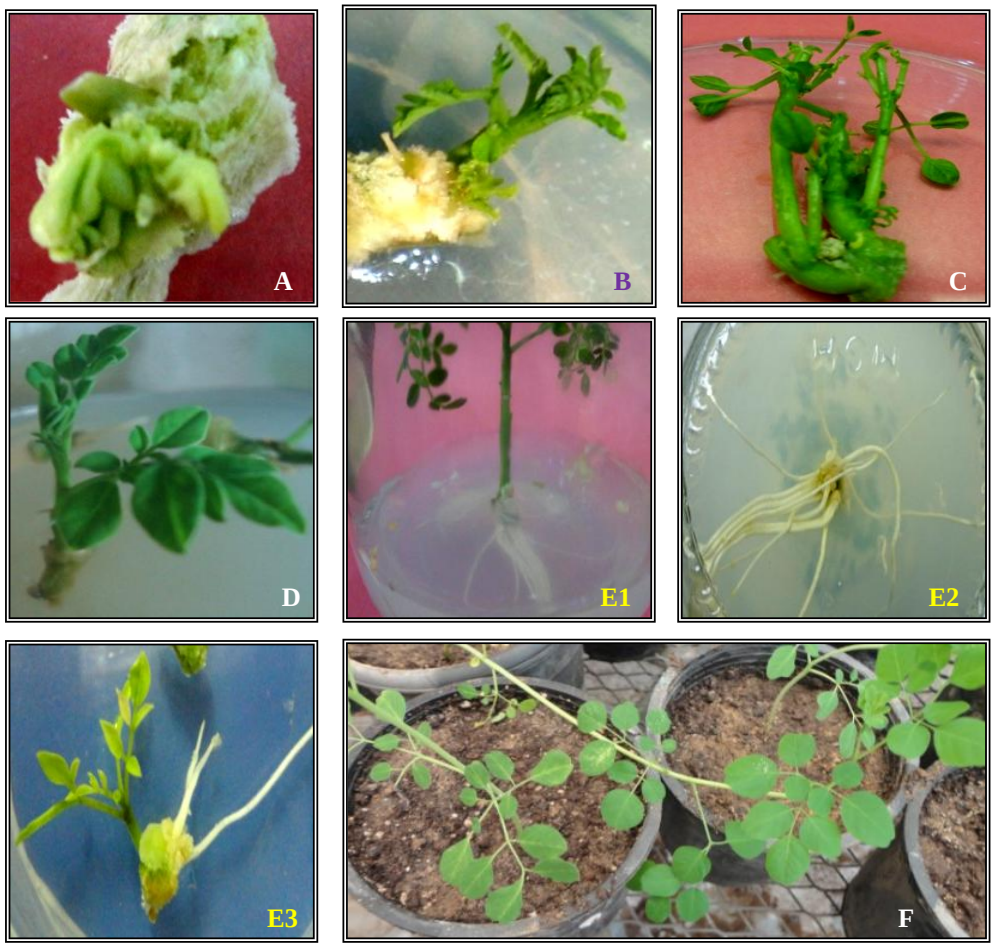


Fig. (1): *In vitro* regeneration stages of *M. oleifera*.(A) nodal segment explants with multiple axillary shoots developed two weeks after inoculation on SIM4 (B) well developed young shoots after three weeks (C) multiple shoots elongation (D) subculture on the same shoot induction medium supplemented with 0.5 mg/l $GA_3$ (E1&E2) well developed roots on ½ MS medium containing 1.0 mg/l IBA along with 0.5 mg/l IAA showing no callus structures (E3) root developments on IAA alone with high tendency for callus structure formations (F) *in vitro* regenerated Moringa plantlets acclimatization under controlled greenhouse conditions.

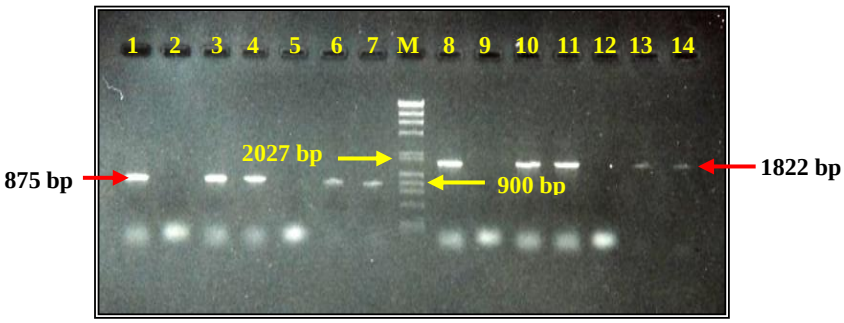


Fig. (2): 1% agarose gel electrophoresis showing the results of PCR analysis for DNA extracted from putative transformed moringa plants with the plasmid pBI121. Lanes 3-7 represent the amplified PCR fragments obtained using *Km* specific primers, while lanes 10-14 for the *GUS* specific primers. Lanes 1&8 represent +ve control PCR products produced from pBI121. Lanes 2&9 represent –ve control of untransformed plants (M= λ DNA marker digested with *Hin*d III & $\phi$ digested with *Hae*III).

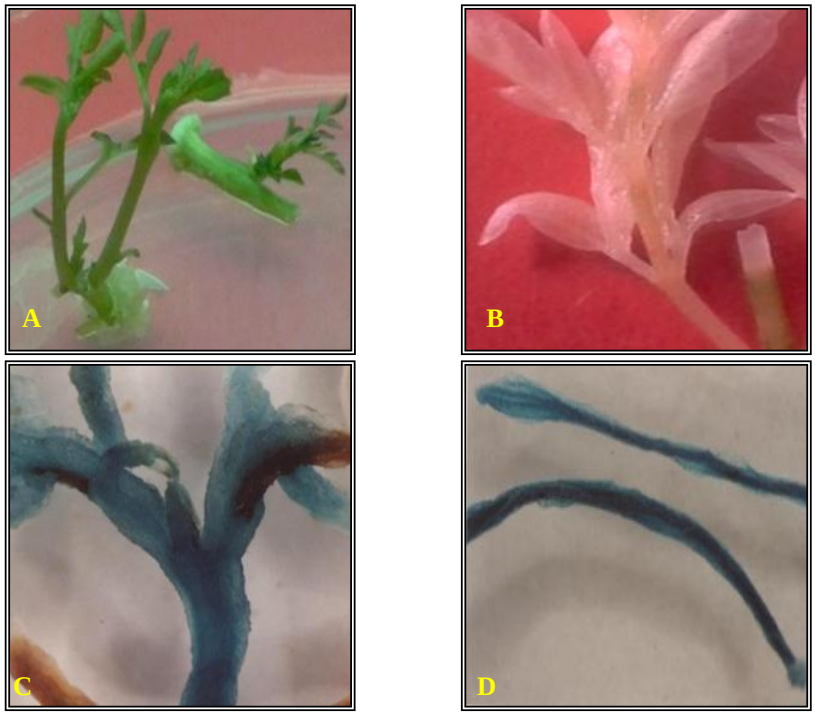


Fig. (3): Kanamycin resistant young shoots developed on selective shoot induction plates containing SIM4 medium supplemented with 50 mg/l kanamycin (A) Histochemical *GUS* assay showing blue color indicating *GUS* expression in different transgenic Moringa parts, Moringa stem and young leaves (B) Moringa roots (C) while blue colour was absent in untransformed control plants (D).