Websites:
http://www.sciencepub.net
http://www.sciencepub.net/rural

Emails:
editor@sciencepub.net
sciencepub@gmail.com

World Rural Observations

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Development of micro-propagation protocol for Apple Balady cultivar using leaf and barcoding using rbcL gene

Sayed, A. A. Elsayh[1]; Rasha, N. Arafa[2] and Shafik, D. Ibrahim[3]

[1]Agricultural Research Centre, Horticulture Institute, Department Breeding, Agricultural Research Center, Egypt
[2]The Central Laboratory of Date Palm Researches and Development, Agricultural Research Center, Giza, Egypt
[3]Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, Giza, Egypt
Email: rashanagyarafa@gmail.com

Abstract: The aims of this work are to develop a tissue culture and speedy micro-propagation technical system, as well as an effective shoots regeneration process using apple Balady cultivar leaf explants. *In vitro* leaves would be used as explants for the induction of adventitious shoots, and the impact of various kinds and concentricity of cytokinins as well as carbon sources on shoots regeneration were investigated. *In vitro* leaves were being used as explants for adventitious shoots induction and the impacts of various cytokinins quantity and type, as well as various carbon sources, on shoots regeneration, were investigated. MS medium that contained BA at 2.0 + NAA at 5.0 (mg/l) proved beneficial for inducing callus from leaf explants. Addition 3.0 mg/l BA + 1.0 mg/l Kin + 0.5 mg/l NAA on MS medium shown to become the preferable differentiation and proliferation medium for apple shoots. MS basal medium mixed with 2.0 mg/l TDZ + 0.1 mg/l NAA was confirmed to really be the most effective shoots regeneration medium, yielding 20.32 shoots per jar. *In vitro* shoots proliferation was most successful when sorbitol at a concentration of 30 g/l was included in the culture medium. When shoots were grown with a mix of 2.0 mg/l GA_3 plus 3.0 mg/l BA, the greatest growth and elongation results were obtained. ½ MS supplemented with 1.5 mg/l IBA was shown to be the best appropriate option rooting medium, with a rooting rate of 87.6%. The objective of this research is to use ribulose-1,5-bisphosphate carboxylase/oxygenase vast portion to do DNA barcoding on *Malus domestica* (rbcL). In compare to a database of the same species, the similarity level for rbcL was maximum (99.44%).

[Sayed, A. A. Elsayh, Rasha, N. Arafa and Shafik, D. Ibrahim. **Development of micro-propagation protocol for Apple Balady cultivar using leaf and barcoding using rbcL gene**. *World Rural Observ* 2021;13(4):1-17]. ISSN: 1944-6543 (Print); ISSN: 1944-6551 (Online). http://www.sciencepub.net/rural 1. doi:10.7537/marswro130421.01.

Keywords: *Malus domestica*, leaf explants, medium composition, callus induction, differentiation, multiplication, carbon sources, rooting and DNA extraction.

1. Introduction

In temperate zones, apple is one of the most extensively cultivated and commercially significant fruit crops **(Kovalchuk *et al.*, 2009)**. Malus is a member of the rose family (Rosaceae). The apples were the third most significant fruit crop in the planet, with 64.3 million trees planted each year **(FAO, 2009)**. Malus species are economically important because they are a rich source of antioxidants, cancer-fighting chemicals, and fibers in low-fat, low-calorie produce, all of which are beneficial to human health **(Volk *et al.*, 2015)**.

The asexual propagation of apple rootstocks is prevalent (sucker, hardwood cutting, semi-hardwood cutting and budding). These techniques are inefficient and time-consuming and they are often connected with issues such as stock plant limitations and extended better product quality **(George, 1993)**. The traditional propagation techniques stifle productivity and increase the cost of the final product. Tissue culture may be able to solve this problem since continual subcultures *in vitro* have been shown to result in increased rooting potential **(Hammatt and Grant, 1993)**.

When compared to traditional clonal propagation techniques, the *in vitro* propagation approach has a high potential for multiplying plant material for breeding projects in an extremely short amount of time with less effort and at cheaper prices **(Bommineni *et al.*, 2001)**. The rapid multiplication of rootstocks with desired features may offer a quick and reliable strategy when it comes to development of an extremely large number of unified plantlets in a stumpy period year round. Various phases of micro-propagation, include the production of *in vitro* cultures, the regeneration of shoots, the micro-shoots rooting and acclimation, have particular medium composition needs **(Dobránszki *et al.*, 2010)**. Many publications have described *in vitro* propagation of apple rootstocks as an effective and alternate approach

for commercial multiplication of plants and fruits **(Dalal *et al.*, 2006; Ciccotti *et al.*, 2008; Silva *et al.*, 2019)**.

Direct and indirect processes for the formation of adventitious apical meristem exist. The development of a meristem happened without the proliferation of undifferentiated calli tissue via the direct approach. However, callus or suspension culture may produce meristems in an indirect way **(Gahan and George, 2008)**. A rapid rise in the number of new shoots that lengthen enough to transition to the rooting stage is required for the proliferation stage to be effective **(Dobranszki and Silva, 2010)**. MS basal salt medium **(Murashige and Skoog, 1962)** that is the greatest often utilized basic growth medium for tissue culture Apple growth. **(Boudabous *et al.*, 2010; Geng *et al.*, 2015)**.

The apple shoot multiplication is based on a medium that contains cytokinins as the primary plant growth regulator, as well as auxin and gibberellin at lesser quantities. 6-Benzylaminopurine (BA) was employed as a cytokinin source in most investigations on apple shoot multiplication **(Sharma *et al.*, 2000; Dobránszki and Silva 2010)**. Apple callus has been used to induce adventitious shoots development and plant regeneration **(Caboni *et al.*, 2000)**. The adventitious buds were regenerated directly from inter-nodal segments of *in vitro* produced shoots, according to another research **(Belaizi *et al.*, 1991)**.

In laboratory circumstances, the shoots development and output are influenced by a variety of variables, one of these factors is the kind and concentricity of externally provided carbon sources in the basal media **(De-Neto and Otoni, 2003)**. The carbohydrates regulate growth and morphogenesis via their nutritional value as well as their various osmotic potentials, which influence cell division and formation rates **(Sotiropoulos *et al.*, 2006)**. Carbon sources also play a role in the synthesis of various chemicals as well as the regulation of many cell development processes **(Karami *et al.*, 2006)**. As a result, carbohydrates are critical for shoots growth *in vitro*, a high-energy process **(Jain and Babbar, 2003)**.

The many woody species are hard to root by cuttings, hence the production of adventitious roots is a critical stage in micro-propagation **(Tereso *et al.*, 2008)**. Furthermore, essential properties such as the roots count and the roots lengths for every shoot are required for effective adaptation of plants **(Sharma *et al.*, 2007)**. *In vitro* interventions have a genotype-dependent results. During establishment, proliferation and rooting *in vitro* on a given medium, various apple genotypes behave differently **(George and Debergh, 2008)**. DNA barcoding is a technology for identifying species that amplifies and sequences DNA in a specified location to produce a worldwide database of living creatures **(Hebert *et al.*, 2003)**. Many sites have been widely employed as DNA barcodes in plants across the earth, including rbcL (ribulose bisphosphate carboxylase), ITS2 (internal transcribed spacer 2), psbA-trnH (intergenic spacer area), and matK (MaturaseK). The Plant Working Group of the CBOL **(Group *et al.*, 2009)**. Because this coding facility is available universality and simplicity in amplifying and analysing, rbcL genes have been frequently employed for phylogenetic study within the angiosperm family and subclass, and even within the diverse groupings of seed plants **(Newmaster *et al.*, 2006)**.

The main objective of this research paper seems to found the best medium composition for callus formation, proliferation and regeneration in apple cultivar Balady, as well as to promote axillary shoot proliferation. In the regeneration and elongation phases, varied doses of plant growth regulators such as cytokinins BA, Kin and TDZ were combined with auxin NAA. We also looked at the impact of various carbon sources on the multiplication stage. The effectiveness of varying auxin concentrations on roots development and elongation was investigated. *Malus domestica* DNA barcoding with the important constituents of ribulose-1, 5-bisphosphate carboxylase/oxygenase (rbcL).

2. Material and Methods

Throughout 2019 and 2021, this research has been carried out at the Fruit and Ornamental Breeding Department, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt.

2.1- Sterilization and explants material

The leaves of an apple genotype Balady (*Malus domestica*) tree that was approximately 15 years old were taken as explants for callus initiation, regeneration, multiplication and roots in this research. They came from Nag Hamady city, Qena, Egypt.

The leaves were washed under flowing water from the faucet, then immersed for 15 minutes in antioxidant solutions (150 mg citric acid and 100 mg ascorbic acid), followed by a one-minute immersion in 70% ethanol, thereafter dipped in sodium hypochlorite treatment (20 % v/v Clorox solution) with two drops of tween 20 throughout 15 minutes. The leaves then were properly cleaned multiple times utilizing sterile water. The leaves' edges (10-12 mm long) were removed, and the remaining of the leaf was sliced horizontally to the midrib into two pieces. The leaves were then divided into little pieces (0.3 cm) and cultivated on the surface of MS medium treated with various plant growth regulator combinations.

Fig (1): The leaf explants of apple Balady cultivar

2.2- Establishment medium

MS basal medium supplemented with MS vitamins, 0.1 mg/l myo-inositol, 0.5 mg/l pyridoxine-HCl, 0.5 mg/l nicotinic acid, 2.0 mg/l glycin, 0.1 mg/l thiamine-HCL, 30 g/l sucrose, and 7.0 g/l agar are often used to culture leaves pieces. Before putting the agar, modify the pH of the basal medium to 5.7 0.1 via placing KOH or HCl solutions. The media were distributed at 40 ml each jar into small culture jars (150 ml) and capped with polypropylene closures. After that, the medium was autoclaved for 20 minutes at 1.5 kg/cm^2 at 121°C.

2.3- Initiation stage

The prior phase's leaf segments were employed as initial explants for callus formation. The impact of two distinct cytokinin sources, BA and kinetin (Kin), in varied doses (0.0, 0.5, 1.0, 1.5, and 2.0 mg/l) and in conjunction with 0.5 mg/l naphthalene acetic acid (NAA), was studied at this experiment. Free medium of the plant growth regulators are used as control treatment. For 16 hours each day, the cultures were grown in a growth chamber fitted at a temperature of 26 ±1 degrees Celsius with a light levels of 2000 lux supplied by cool white fluorescent lamps. For two subcultures, the explants were re-cultured on the same fresh medium every month. At the conclusion of the experiment, the data were calculated.

2.4- Differentiation stage

For shoots proliferation, the callus was transferred to MS basal medium supplemented with two kinds of cytokinins BA at concentrations level higher than tested in the previous stage (0.0, 1.0, 2.0, 3.0 and 4.0 mg/l) with Kin at 1.0 mg/l and NAA at 0.5 mg/l. For three subcultures, the explants were re-cultured within the same medium each month. The shoots number per callus piece and regeneration percentage (%) was recorded after three months from callus transferring to differentiation medium. For 16 hours each day, the cultures were incubated in a growth conditions at 25 ±1 °C with a light intensity of 2000 lux.

2.5- Shoot multiplication

2.5-1 The impact of combing various amounts of a sub stance

The effects of Thidiazuron (TDZ) and NAA on apple shoots proliferation. Following shoots proliferation, the shoots 0.8–1.0 cm long produced from callus were grown vertically in MS medium containing varied amounts of TDZ with NAA. Shoots were regeneration in MS basal medium contained 30 g/l sucrose, 7 g/l agar and 0.0, 0.5, 1.0, and 2.0 mg/l of TDZ combining with 0.1 mg/l NAA. For this experiment, basal media (medium without any growth regulators) was employed as a control.

2.5. 2- The effect of different sources of carbon on multiplication of apple shoots.

The micro-shoots were transplanted to MS medium with varied concentrations of sucrose, sorbitol, mannitol and glucose at (0.0, 5.0, 10.0, 20.0, 30.0 and 40.0 g/l) to assess the effect of different carbon sources on shoots regeneration. Before autoclaving at 121°C for 15 minutes, the pH of the media was corrected to 5.8. The shoots were multiplied and re-cultured each month on the same medium for two subcultures after four weeks and put in a chamber with a 16-hour photoperiod of 2000 lux light intensity and an 8-hour dark period at 25 ±2. Three explants per culture jar and five replicates for each treatment were used. The shoots number (shoot/explant), shoots length (cm) and leaves number (leaf/shoots) were determined after culturing for three months.

2.6- Shoot elongation

The effect of combinations between GA_3 and BA on shoots regeneration of apple.

After shoots multiplication, 4.0 cm long shoots were chosen for the elongation stage and grew in MS basal medium supplemented with Gibberellic acid (GA_3) at (0.0, 0.5, 1.0 and 2.0 mg/l) and BA at (0.0, 1.0, 2.0 and 3.0 mg/l) in varying combinations. The shoots placed into the chamber with 16 hours photoperiod 2000 lux light intensity and 8 hours dark at 25 ± 2º. Three explants per culture jar and five replicates for each treatment were used. The shoots number (shoot/explant), shoots length (cm) and leaves number (leaf/shoots) were determined after 6 weeks of culturing.

2.7- Root formation

The types and concentrations of plant growth regulators have an influence on roots growth and development. In this experiment, the result of auxin varieties and concentrations on roots growth and development was studied. The multiplicate shoots were then cultured for two weeks on PGRs free MS medium before transferring to root induction and development medium to prevent the carryover effect of shoot multiplication hormones. Approximately 3 to 5 cm of well-regenerated shoots were cultivated on ½ strength MS medium, that included 30 g/l sucrose, 6 g/l agar, and NAA or Indole butyric acid (IBA) at (0.5, 1.0, and 1.5 mg/l) individual. As a control treatment, ½ strength MS medium without hormone was employed. The plantlets were kept in a growth chamber at 25 ±2°C with a 16-hour photosynthetic rate supplied using a white light source, and the shoots were re-cultured on a same medium every month for two subcultures. The data were recorded after 2 months in culture as follows, rooting percentage, number of roots and root length. In all experiments, there were six treatments, each treatment contained five replicates and each jar contains 6 explants.

2.8- Acclimatization

The tissue culture plantlets are transplanted and acclimatized in the last phases of the process. The plantlets that formed roots with three or four leaves were surface-disinfested for 15 seconds with a fungicide liquid (Rizolex-T 50% 2.0 g/l) and thereafter transferred into plastic pots contained sterile soil. Peat moss and vermiculite were used as the potting media in a 3:1 (v/v) ratio. To help achieve a high degree of moisture content, the pots contains plantlets were wrapped film. Water was first delivered in sufficient quantities. The intensity of the sunlight was half that of the *in vitro* interval. The film had been removed midway after ten days, thus the intensity was adjusted to regular illumination settings. The water necessary for growing plants was sprayed behind the film throughout this time period until the plantlets' significant new leaves completely grown. After three weeks, the film was removed, and the plantlets were required to maintain for two months under the greenhouse environments (25 °C day, 20 °C night, 16/8 day/night photoperiod and 75% moisture content) with regular irrigation.

2.9- DNA barcoding

DNA extraction and purification

DNeasy Plant Kit is being used to extract total DNA from *Malus domestica* leaf pieces (QIAGEN, Germany). The quantity and purity of the isolated DNA were determined using NanoDrop.

Gene sequencing and PCR

The PCR reaction was performed in accordance with the instructions **Ibrahim *et al.* (2016)** in a 50 response container comprising 25 of Master Mix (sigma), 2 of each primer (10 pc mol) (*In vitro* gen), USA, 3 of template DNA (10 ng), and 18 of dH_2O. The primer pairs rbcL-F (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3'), rbcL-R (5'-TCG CAT GTA CCT GCA GTA GC-3') were applied for PCR expansion and sequencing of the rbcL gene from chloroplast DNA (cpDNA). In order to do the PCR, using a Perkin-Elmer/GeneAmp PCR System 9700 (PE Applied Biosystems, USA) with a 40-cycle protocol following a 5-minute denaturation cycle at 94^{o}C. Each cycle includes a 30 second denaturation stage at 94 ^{o}C, a 30 second annealing step at 50^{o}C, and a 30 second elongation step at 72 degrees Celsius. During the last cycle, the primer expansion section was prolonged to 7 minutes at 72 ^{o}C. Electrophoresis of the amplification products on a 1.5% agarose gel containing ethidium bromide (0.5 ug ml/l) in 1X TBE buffer at 95 volts resolved the amplification results. The size of PCR products may be determined using this method, a 100 bp DNA ladder (Promega, USA) was employed as a molecular size reference. A UV transilluminator was used to see the gel pictures, and a Gel Documentation System was used to photograph them (BIO-RAD 2000, USA).

A QIAquick PCR Purification Kit was used to purify PCR products (Qiagen, USA). The dideoxynucleotide chain termination technique was used to sequence the PCR product using a DNA sequencer (ABI 3730XL, Applied Biosystems) (Microgen, Korea) and a BigDye Terminator version 3.1 Cycle Sequencing RR-100 Kit (Applied Biosystems, USA) to adhere to the directions of the manufacturer.

Species classification

The important Local Alignment Tool (BLAST) offered on the National Centre of Biotechnology Information (NCBI) website was used to assign DNA barcoding to *Malus domestica*. All of the sequences were display to GenBank in the United States. The nucleotide sequences of **"OK349169"** were assigned accession numbers by GenBank. All or any created sequences were subjected to BLAST searches employing online databases (DDBJ/EMBL/GenBank) and examined using the BLASTN 2.8.1 software (http://www.ncbi.nlm.nih.gov/BLAST) and aligned with Align Sequences Nucleotide BLAST. Only the best maximum percent identity comprised one species and scored >99 percent was regarded successful for species identification.

Top of Form

Bottom of Form **(De Groot *et al*., 2011)**. Phylogenetic analysis was conducted using MAFFTv6.864, http://www.genome.jp/tools-bin/mafft, then the phylogenetic trees were generated.

2.10- Analytical statistics

According to, this research seemed to be constructed as a randomized full block design **(Gomez and Gomez, 1984)**. Using the MSTAT computer program, the acquired data were statistically evaluated **(MSTAT Development Team, 1989)**. Duncan's Multiple Range Analysis was used to evaluate the averages of several treatments to see whether there were any changes **(Duncan, 1955)**.

3. Results and Discussions

3.1- Initiation stage

3.1-1 Influence of different cytokinin types and concentrations in combination with NAA on callus initiation of leaves explants of apple.

It was observed that the explants initiated in MS medium at different hormonal concentrations showed different responses of callus formation from leaf explants **Table (1 and 2)**. The observations for callus formation began three weeks after culture. Growth of the callus increased significantly with the incubation period 8 weeks. Degree of callus induction was comparatively low in medium supplemented with different concentrations of kin with NAA. Explants initiated on the control culture which were devoid of hormone did not produce any callus.

The ratio of leaves number that initiated callus after 60 days of inoculation compared to the starting leaves number was used to measure the callus induction rate (%). All two PGR combinations tested induced callus formation compared to the control treatment. As shown in **Fig. (2)**, MS contained 2.0 mg/l BA + 0.5 mg/l NAA formed compact light green calli with the greatest callus induction rate (80.4%) and callus fresh weight 0.87 gm. As demonstrated in **Fig. (3)**, adding Kin at 2.0 mg/l + NAA at 0.5 mg/l on MS medium yielded calli with a 55.8% induction rate and a callus fresh weight of 0.48 gm.

The treatment consisting of MS basal medium accompanied with kin at 0.5 mg/l + NAA at 0.5 mg/l had the lowest calli formation rate of 27.1% with callus fresh weight 0.29 gm, and also the treatment consisting of MS basal medium supplied with kin at 0.5 mg/l + NAA at 0.5 mg/l seemed to have the least callus fresh weight 0.26 gm. The variations in formation rates were statically worthy, indicating that MS + 2.0 mg/l BA + 0.5 mg/l NAA is the ideal culture medium for inducing callus in apple leaves.

Table 1: Impact of various combinations of BA and NAA on the callus formation from leaves of apple after two subcultures.

Treatments (mg/l)	% of explant induced callus	Callus F.W.
0.0 BA + 0.0 NAA	0.0 e	0.0 e
0.5 BA + 0.5 NAA	27.1 cd	0.29 cd
1.0 BA + 0.5 NAA	35.0 c	0.38 bc
1.5 BA + 0.5 NAA	63.2 b	0.58 b
2.0 BA + 0.5 NAA	80.4 a	0.87 a

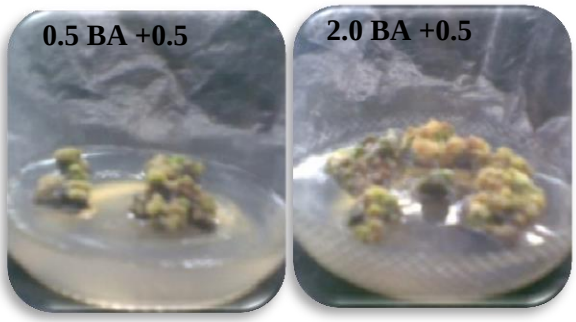

Fig. (2): From leaf explants, the apple Balady cultivar had the maximum callus initiation rate with BA at 2.0 mg/l + NAA at 0.5 mg/l and the minimum callus formation rate with BA at 0.5 mg/l + NAA at 0.5 mg/l.

Table 2: Influence of various combinations of kin and NAA on the callus established from leaves of apple after two subcultures.

Treatments (mg/l)	% of explant induced callus	Callus f.w.
0.0 kin + 0.0 NAA	0.0 d	0.0 d
0.5 kin + 0.5 NAA	22.0 bc	0.26 c
1.0 kin + 0.5 NAA	26.0 bc	0.29 c
1.5 kin + 0.5 NAA	38.7 b	0.35 b
2.0 kin + 0.5 NAA	55.8 a	0.48 a

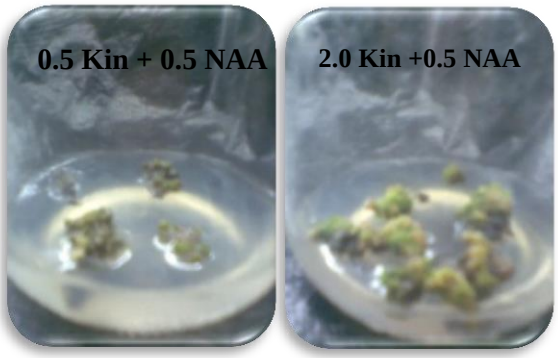

Fig (3): Influence of various Kin doses in combined with 0.5 mg/l NAA on calli formation in leaf explants.

These findings confirmed with **Compton and Gray's (1993)** who observation that adding IAA to the medium with BA enhanced callus development. Leaf segments seem to be the most often utilized explants in *Malus* spp. to promote adventitious shoots. The adventitious shoots are often generated either directly without formed calli **(Pawlicki and Welander, 2015)** or indirectly via calli formation **(Dufour, 1990)**. BA was just necessary for the early growth and development of apple shoot tip explants, which often resulted in proliferating cultures **(Jabbarzadeh and Khosh-Khui, 2005)**. For callus formation, NAA and 2,4-D are routinely used in varying combinations with BA **(Maheshwari and Kumar, 2006)**. The mix of BA with NAA produced organogenic callus in *Momordica dioica* **(Devendra *et al.*, 2009)**. Callus induction was enhanced by the addition of NAA 0.5-5.0 and BA 0.5-5.0 mg/l. The results of the first subculture showed that both BA and NAA were necessary for callus induction from all explant types as callus regeneration, within the absence of NAA in medium turned brown/black and showed no more growth **(Neelam *et al.*, 2011)**. The several explants, including that of the leaf, meristems and shoot, have been shown to be beneficial in apple micro-propagation in previous researches **(Zhang *et al.*, 2014; Wang *et al.*, 2016)**. For callus initiation, the cleansed leaves were sliced into 5x5 mm parts and maintained in culture jars that contain 50 ml of MS medium supplied with BA at (0.50-1.25), NAA at (0.40-1.25), IBA at (0.0-0.5) (mg/l), and Kinetin at (1.0-2.0 mg/l) in mixtures **(Kumar *et al.*, 2016)**.

3.2- Differentiation stage

3.2- 1 Influence of different concentrations of BA with added kin and NAA on shoots proliferation from callus of apple after three subcultures.

The MS media, several combinations of BA, kin and NAA that were shown to proliferation the healthy adventitious shoots from callus. In **Table (3)** indicate the proliferation results after three sub - cultures. There were considerable variations in the treatments. The MS medium that included 3.0 mg/l BA + 1.0 mg/l kin + 0.5 mg/l NAA had the maximum number of shoots on callus (12.7 shoots/callus) and also the best proliferation percentage (91.6%), whereas the treatments of MS medium containing 1.0 and 2.0 mg/l BA plus 1.0 mg/l kin and 0.5 mg/l NAA only responded 5.3 shoots/callus (41.5 %) and 7.6 shoots/callus (68.2%), as shown in **Fig. (4)**.

The proliferation number of shoots on the callus won't increase with the control treatment, remaining at 2.4 shoots/callus with a proliferation percentage of 12.4%. The number of induced shoots developing on the callus was decreased to 9.3 shoots/callus, when the BA concentration was raised above 3.0 mg/l, with a proliferation proportion of 80.3%.

In conclusion, MS basal medium with 3.0 mg/l BA, 1.0 mg/l kin and 0.5 mg/l NAA has been the ideal medium for proliferation shoots from calli.

Table 3: Impact of the various combinations among BA, Kin and NAA on differentiation and proliferation of apple callus after three subcultures.

Treatments (mg/l)	Number of shoots/callus	Proliferation %
0.0 BA+1.0 Kin+0.5 NAA	2.4 f	12.4 e
1.0 BA+1.0 Kin+0.5 NAA	5.3 de	41.5 d
2.0 BA+1.0 Kin+0.5 NAA	7.6 c	68.2 c
3.0 BA+1.0 Kin+0.5 NAA	12.7 a	91.6 a
4.0 BA+1.0 Kin+0.5 NAA	9.3 b	80.3 b

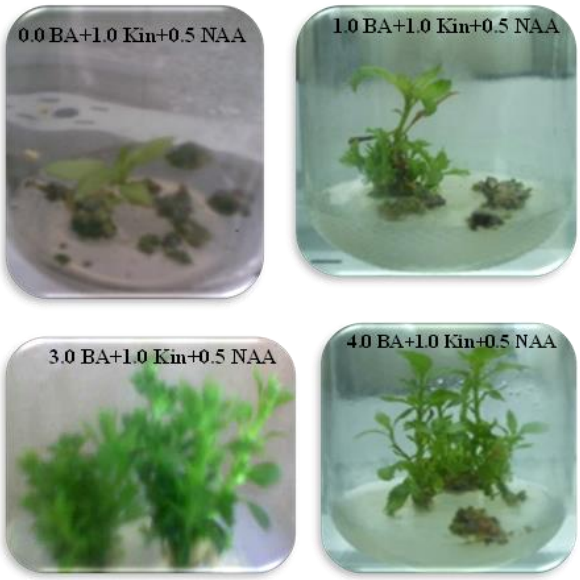

Fig (4): The growth vigor compare of the apple shoots multiplication on various combinations of BA, Kin and NAA in MS medium.

These results were in agreement with **Welander and Maheswaran (1992)** who reported that, utilizing MS basal media containing 10 µM BAP, 1 µM NAA and 3% sucrose, the maximum regeneration occurred. The numerous variables impact regeneration in

renewal studies utilizing apple leaf tissues as explants, includes the presence of PGRs, the duration of dark culture, and the nutritional value of the media **(Wei *et al.*, 2009; Magyar-Ta'bori *et al.*, 2010; Mitic *et al.*, 2012)**. The cytokinins include N6-substituted adenins that stimulate division of cells in plants and may also act a contribution in cells differentiation **(Magyar-Ta'bori *et al.*, 2010)**. In MS media with particular doses of the cytokinin BA and the auxin NAA, the majority of apple types regenerate quickly **(Wei *et al.*, 2009)**. Because there was no endogenous auxin, cytokinin and auxin must be equiponderant during shoots regeneration from leaf explants, which did not display regeneration or callus production when treated with cytokinin alone. Somatic embryogenesis, which generally needs both hormones, is used to regenerate shoots from leaves **(Shukla *et al.*, 2014)**.

Kumar et al. (2016) who reported that for the stimulation of shoots from leaf-derived callus, 45 day-old calli being transported to MS nutrient medium that contain BA at (0.75-3.00 mg/l), NAA at (0.01-0.03 mg/l), and Kin at (1.0-3.00 mg/l) in various mixture and doses. The effects of BA at (2.0 mg/l) and NAA at (0.02 mg/l) augmented MS media on callus were investigated. The findings revealed that when callus was cultivated horizontally linked with the medium, the highest rate of shoots induction regeneration (85.90 %) and hence the highest number of shoots (8.0) were achieved.

When BA was used at a lower concentration of 0.5 mg/l, the proliferation of *in vitro* shoots was poor. Proliferation was significantly improved when BA was used at the highest concentration of 1.0 mg/l. The optimal medium for regenerating shoots was 3.0 mg BA, 0.3 mg IBA and 30 g sucrose/l, with a regeneration rate of 71.6% **(Qing-Rong *et al.*, 2019)**. The apple cultivar 'Red Chief,' a prominent apple cultivar, was revealed to have highly excellent *in vitro* shoot regeneration. The frequency of shoot proliferation ranged from 26.47 - 96.35% from leaf explants cultured in a medium containing combinations of BA and NAA with significant differences between the treatments. The preferable treatment was BA at 5.0 mg/l and NAA at 0.2 mg/l, which produced the highest multiplication average as well as 5.23 shoots per explant **(Kumar *et al.*, 2021)**.

3.3- Shoot multiplication

In the regeneration stage, the most often employed cytokinins are (TDZ and BA), however their efficacy is dependent on genotype and other parameters. Other cytokinins, such as zeatin and kinetin, were shown to be less active in general. A well-chosen cytokinin pre-treatment also may raise the organogenic capacity of the explants. The cytokinins used throughout the pretreatments may have an influence on leaves structure, which then in turn affects the ability of the explants to regenerate **(Magyar-Tábori *et al.*, 2010)**.

3.3- 1 Effect of TDZ in combination with NAA on apple shoots multiplication after 2 subcultures.

The outcomes of the numbers of shoots each jar as well as the height of shoots were both extremely relevant, are shown in **Table (4)**, number of shoots per jar of all treatments was significantly higher under TDZ in combination with NAA than the control treatment. MS basal medium contained of 2.0 mg/l TDZ in conjunction with 0.1 mg/l NAA was proven to be the best medium for shoot multiplication, which produced an average shoots number 20.32 shoot/jar with shoot length 6.76 cm and the leaves number was 60.2 leaf/shoots and followed by 13.5 shoot/jar with shoot length 4.32 cm and the leaves number 51.4 leaf/shoot were recorded at medium supplemented with 1.0 mg/l TDZ in combination with 0.1 mg/l NAA. However, the number of shoots per jar was significantly reduced to 9.26 shoots/jar with 3.8 cm length and 25.3 leaf/shoot when the TDZ concentration was reduced to 0.5 mg/l, as shown in **Fig. (5).**

Table 4: Impact of the various levels of TDZ with NAA on regeneration of apple shoots after two subcultures.

Treatments (mg/l)	Shoots number	Shoots length (cm)	Leaves number
0.0 TDZ + 0.0 NAA	3.33 d	1.50 d	8.76 d
0.5 TDZ + 0.1 NAA	9.26 bc	3.80 c	25.3 c
1.0 TDZ + 0.1 NAA	13.50 b	4.32 b	51.4 ab
2.0 TDZ + 0.1 NAA	20.32 a	6.76 a	60.2 a

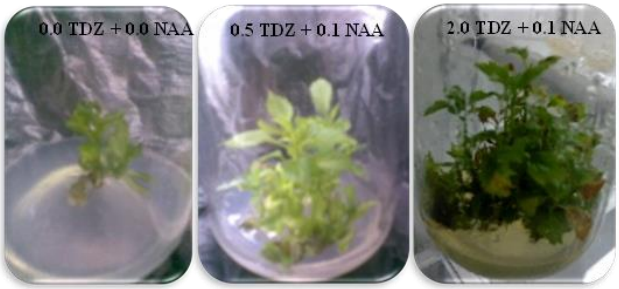

Fig (5): Shoots multiplication of apple Balady cultivar on MS medium contained various combinations of TDZ and NAA, the best results were obtained with TDZ at 2.0 mg/l with 0.1 mg/l NAA.

These results were in approval with **Matand and Prakash (2007)** who demonstrated that a brief period of culturing in medium containing TDZ was the most successful in enhancing shoots development in geranium and peanut. This suggests that TDZ may have a residual impact in explants after a small period of usage, and that continuous utilization may not even be necessary to promote morphogenesis. When TDZ was treated at 5.0 µM, the vast majority of the remaining cultivars reacted most well **(Li *et al.*, 2014)**. The most often employed cytokinins in apple regeneration are BA and TDZ, which have been compared in various investigations. TDZ was shown to be more practicable than BA for shoots multiplication in various studies. The several cultivars, including 'Royal Gala' and 'Dayton,' reacted similarly to TDZ concentrations ranging from 5 to 20 µM **(Magyar-Ta'bori *et al.*, 2010)**. MS nutrient medium with TDZ at 5.0 mg/l and IBA at 0.3 mg/l was optimal for regeneration and continued the shoots multiplication in 'Golden Delicious,' with a regeneration frequency of 95% **(Mitic *et al.*, 2012)**.

Modgil and Pathania (2018) suggested that, the quantity and type of cytokinins and auxins, as well as the period of explant exposure to plant growth regulators, impacted the frequency of regeneration. On light-incubated regeneration media, the shoots were regenerated by callus or directly from leaves. However, with explants cultured under light conditions, the total count of regenerated explants and the shoots number were the largest. The optimal shoots regeneration (34%) was achieved with BA treatments of 3.0 mg/l BA with 1.0 mg/l NAA, whereas, the TDZ treatment (0.6 mg/l TDZ with 0.5 mg/l NAA) provided the maximum regeneration ratio (44%) as well as yielded more shoots with the longest lengths. The probability of regeneration was raised by utilizing a two-stage approach in which leaves were cultivated on a regeneration medium for 10 days and then moved to a MS medium at 0.6 mg/l TDZ with 0.5 mg/l NAA. In this research, 49%t of shoots were started directly with a maximum of 6 shoots per regenerated explant in a much shorter time frame, but MS medium contained BA, had no effect on shoots induction. In eight Galician traditional apple varieties, **Lizárraga *et al.* (2017)** found that TDZ was more successful in multiplying shoots than BA, although the ideal dose varied on the cultivar.

TDZ at 2.0 mg/l combined with IBA at 0.2 mg/l led to a guaranteed regeneration rate 100% for apple adventitious shoots. Raised TDZ doses, on the other hand, impeded shoot regrowth **(Jin *et al.*, 2014)**. For regeneration from leaf explants, TDZ is more effective than BA, as **Li *et al.* (2014)** proven in apple. TDZ-induced adventitious buds protocols have been devised for a variety of *Malus* species and genotypes **(Podwyszyńska *et al.*, 2017).**

3.3-2 The influence of various sources of carbon on regeneration of apple shoots after two subcultures.

For shoots regeneration, MS nutrient medium contained 3.0 mg/l BA + 0.5 mg/l NAA, four diverse kinds of carbon sources were utilized at five different doses. The effects of specific sugars on shoots regeneration from apple leaf explants were evaluated using a basal medium supplemented with sucrose, sorbitol, mannitol and glucose at various doses (5 to 40 g/l). After 8 weeks of inoculation, the data was collected. Sorbitol outperformed all other carbon sources at varying amounts, followed by sucrose, glucose and mannitol.

In a medium containing 3.0 mg/l BA and 0.5 mg/l NAA with 30 g/l sorbitol, the greatest shoots number was reported (10.33 shoots/jar), with the maximum shoot length (4.6 cm) and the leaves numbering 40 leaf/shoots. The second best carbon source was found to be 30 g/l sucrose which resulted in shoots number 8.0 shoot/jar with length 3.5 cm and leaves number was 25 leaf/shoots. The increased concentration of sucrose and sorbitol over 30 g/l reduced the properties of shoots to shoots number 4.5 and 2.76 shoots/jar with lengths 1.18 and 2.7 cm and the leaves number were 16 and 10 leaf/shoots, respectively.

In low concentrations of carbon sources were achieved the lowest results as at 5.0 g/l sucrose which recorded shoots number 1.5 shoots/jar with length 0.91 cm and the leaves number were 3.0 leaf/shoots, the treatment of sorbitol at 5.0 g/l resulted in low response as shoots number 1.8 shoots/jar with length 1.20 cm and leaves number 4.0 leaf/shoots, as shown in **Fig. (6)**.

The number and length of shoots obtained in the mannitol carbon source were very low with one concentration at 10.0 g/l, but none of the other concentrations induced any shoots. At 30 g/l glucose, the least number of shoots (3.6 shoots/jar) and shoot length (1.4 cm) is obtained, and the number of leaves dropped to 8.0 leaf/shoots. However, the concentration of glucose at 5 g/l gave the worst results as shoots number 1.2 shoots/jar with length 0.75 cm and leaves number 3.0 leaf/shoots.

From the above results, it is clear that, the frequency of shoots regeneration as measured by the number of shoots, shoot length and number of leaves in all carbon sources, while rise profusely at 30 g/l.

Table (5): Impact of various sources of carbon on multiplication of shoots in apple after two subcultures.

Carbon source (g/l)	Number of shoots	Shoots length (cm)	Number of leaves
Sucrose			
0	0.00 p	0.00 o	0.00 l
5	1.50 jk	0.91 mn	3.00 k
10	1.77 i	1.04 m	4.26 i
20	2.80 e	1.66 e	8.00 e
30	8.00 b	3.50 b	25.00 b
40	4.50 c	1.18 kl	16.00 c
Sorbitol			
5	1.80 i	1.20 k	4.00 ij
10	2.60 ef	1.40 h	6.00 g
20	3.50 d	1.56 g	12.00 cd
30	10.33 a	4.60 a	40.00 a
40	2.76 e	2.70 c	10.00 d
Mannitol			
5	0.00 p	0.00 o	0.00 l
10	1.00 o	0.70 n	3.00 k
20	0.00 p	0.00 o	0.00 l
30	0.00 p	0.00 o	0.00 l
40	0.00 p	0.00 o	0.00 l
Glucose			
5	1.20 n	0.75 n	3.00 k
10	1.30 lm	1.20 k	5.00 h
20	2.20 h	1.30 ij	7.00 ef
30	3.60 d	1.40 h	8.00 e
40	2.46 g	2.19 d	5.00 h

This was not the case at concentrations of 40 g/l, where regeneration frequency, the number of shoots and shoot length were all reduced. These results are consistent with those which reported that *in vitro* plants, carbohydrates must be continuously supplied since photosynthetic activity is inhibited owing to low light intensity, high humidity and restricted gaseous exchanges **(Kozai, 1991)**. Efficient shoot regeneration is a prerequisite before producing any transgenic plant for improved qualities. Many variables influence the successful development and multiplication of shoots *in vitro*. The concentration and kind of exogenous carbohydrate source supplied in the MS medium is one of these parameters **(Lipavska and Konradova, 2004)**. It is found that sucrose had a maximum positive effect. Plant cells depend on carbon sources for energy and osmotic regulation. Due to insufficient levels of carbon dioxide *in vitro*, an effective carbon source is needed to promote efficient shoot regeneration **(Faria *et al.*, 2004)**. Additional carbon sources, including as sorbitol and mannitol, have been reported to play an important role in *Zea mays in vitro* growth **(Gauchan, 2012).**

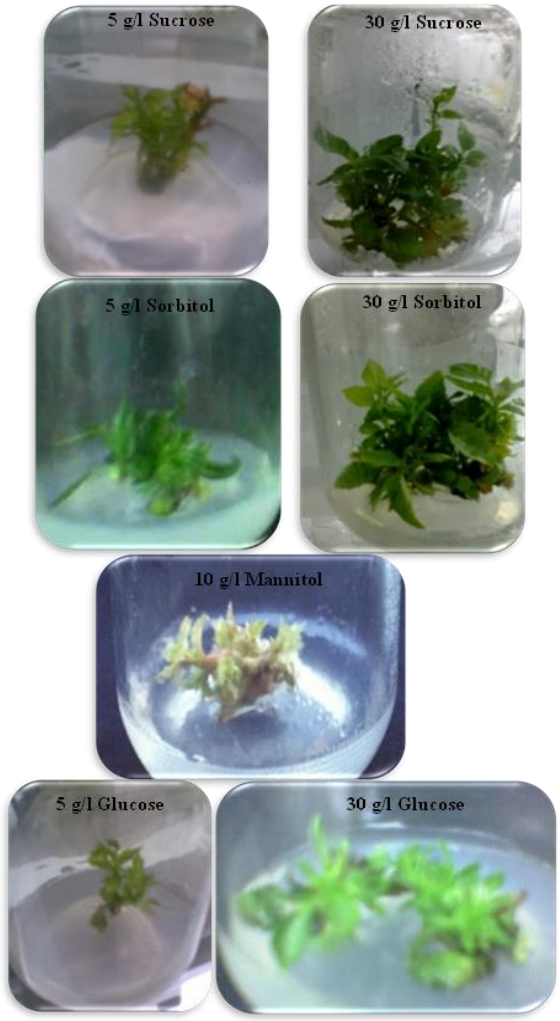

Fig (6): Effect of different carbon source and concentrations on the number and length of shoots in the apple Balady cultivar.

The degradation of sucrose results in the formation of glucose and fructose, which are very important in breaking dormancy. Glucose has given the least effect on the regeneration of shoots, it is due to being metabolized at a low level but they are important in osmotic regulation. The positive effect of glucose was also reported in *Prunus mume* **(Harada and Murai, 1996)**. This might be related to limited uptake and low activity of the enzyme in question.

Four diverse carbon sources were employed in the current study (Sucrose, Sorbitol, Mannitol and Glucose). In our research, the sorbitol outperformed sucrose, mannitol and glucose in terms of shoots regeneration. Young leaves in a medium supported by MS macro-elements contained 22.0 µM BAP and 0.1 µM NAA coupled with sorbitol, at concentrations of 165.0 µM or 220.0 µM, had the

optimum regeneration rate and the most shoots per leaf explant, according to **Pawlicki and Welander (2015)**. Sorbitol found to outperform sucrose, glucose, fructose or a combination of those sugars.

The shoots regeneration rates were the highest in the N6 medium paired with BA on multiple treatments combining of basal media, LS and N6 media, and varied kinds and concentrations of cytokinins, BA and TDZ. The optimum plant hormone and carbon source combination treatment for shoots regeneration was 5.0 mg/l BA and 0.1 mg/l NAA with 40 g/l sorbitol. The sorbitol doses of 40 g/l and 60 g/l were also shown to be the ideal for shoots regeneration. With 40 g/l sorbitol, the maximum regeneration (81.8 %) was obtained. On the rooting medium, which consisted of ¼ MS medium with 0.2 mg/l indole-3-butyric acid (IBA), the regenerated shoots lengthened and rooted. The plantlets had been acclimatized, and the regenerated plants had normal phenotypes as well **(Lee *et al.*, 2019)**. The composition of basal medium influenced *in vitro* shoots growth behavior and the cytokinin and carbon source had a synergic effect on shoots regeneration. Both cytokinins and carbon source influenced shoots regeneration. From leaf explants, when BA was used, the sucrose was more effective than the sorbitol, especially when BA was at a higher concentration of 3 mg/l or 4 mg/l, shoot regeneration rate was significantly higher on the sucrose than that on the sorbitol (**Qing-Rong *et al.*, 2019)**.

3.4- Shoots elongation

When shoots were excised from multiple shoot cultures, the influence of diversified media with plant growth regulators combinations on shoots multiplication and elongation were shown in **Table (6)**. The multiplication rate is calculated as the numeral of shoots, shoots length and number of leaves after three months of culture. The number of shoots was 6.76 shoot/jar with an average of shoot length 2.63 cm and a number of leaves of 20 leaf/shoots were recorded with MS basal medium contained 0.5 mg/l GA_3 and 1.0 mg/l BA. While increasing the concentrations of GA_3 to 1.0 mg/l and combining it with BA at 2.0 mg/l yielded significantly higher values, the number of shoots was 10.33 shoots/jar, shoots length 5.36 cm and number of leaves 35.0 leaf/shoots, as shown in **Fig. (7)**.

The greatest number of shoots (15.66 shoots/explant) with 7.82 cm long shoots, as well as the maximum number of leaves (54.0 leaf/shoot) were produced on MS medium contained 2.0 mg/l GA_3 and 3.0 mg/l BA, as shown in **Fig. (7)**. the control treatment had no effect on shoot multiplication or elongation.

Table 6: Effect of the different combinations between GA_3 and BA on elongation and multiplication of apple shoots after a 6-week cultural.

Treatments (mg/l)	Number of Shoots	Shoot length	Number of Leaves
0.0 GA_3 + 0.0 BA	4.00 c	1.50 d	10.00 cd
0.5 GA_3 + 1.0 BA	6.76 b	2.63 c	20.00 c
1.0 GA_3 + 2.0 BA	10.33 b	5.36 b	35.00 b
2.0 GA_3 + 3.0 BA	15.66 a	7.87 a	53.00 a

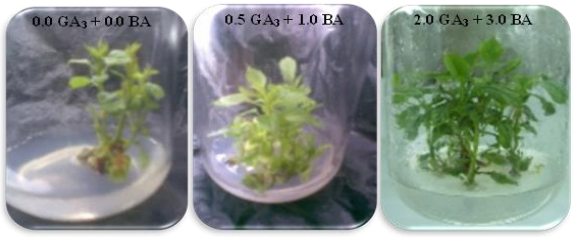

Fig (7): Influence of growth regulators on the regeneration rate and elongation of shoots produced from leaf explants on different concentrations of GA_3 with BA. The best results were obtained using GA_3 at 2.0 mg/l + BA at 3.0 mg/l.

These results are in agreement with **Dastjerd *et al.* (2013)** who revealed that only when the medium included a mixture of BA and GA_3 was it feasible to induce and lengthen M26 rootstock shoots. **Noormohammadi *et al.* (2013)** noted that for optimization of the elongation shoot and proliferation medium, the 2-isopentyl adenine (2-ip) and BA are the cytokinins that are most often utilized, moreover, their efficacy is genotype-dependent as well as dependent on other variables. For the proliferation of M26 and MM106 apple rootstocks, phyto-hormones (2.0 mg/l BA and 2.0 mg/l 2ip with 0.1 mg/l IBA) were created. In general, the MS medium containing BA (2.0 mg/l) generated the greatest shoots development and elongation for both rootstocks. **Dobránszki and Teixeira da Silva (2010)** demonstrated the bud elongation is effectively achieved by adding GA_3 in the medium, but the required concentration depends on the genotype. **Jing *et al.* (2015)** the role of GA_3 for shoots development has been noted.

The micro-shoots obtained from callus were cultivated on MS medium containing GA_3 at (0.03-0.04), BA at (1.0-3.0), NAA at (0.01-0.04) and Kin (1.0-3.0) (mg/l) in various combinations and doses for multiplication and elongation **(Kumar *et al.*, 2016)**. MS contained 3.0 mg/l BA, 0.3 mg/l IBA, and 30.0 g/l sucrose was shown to be the optimal shoots regeneration medium, with a 71.6% regeneration rate **(Qing-Rong *et al.*, 2019)**. Previous research has

shown that twelve varied combinations of BA and NAA with various concentrations resulted in the best medium for M9T337 shoots regeneration. The findings showed that MS nutrient medium augmented with 2.0 mg/l BA + 0.1 mg/l NAA + 0.3 mg/l GA_3 achieved the simplest multiplication range (3.93%). The amount of plant hormones may be a significant factor in determining explant organogenic ability **(Shi *et al.*, 2021)**. The medium contained 0.8 mg/l BA, 0.5 mg/l GA_3 and 0.1 mg/l IBA, the shoots regenerated were micro-propagated by axillary branching **(Kumar *et al.*, 2021)**.

3.5- Rooting

Several healthy shoots were isolated and placed to MS media containing various auxins, as shown in **Tables (7 and 8)**. Compared to the control treatment, NAA or IBA indicated a significant favorable effect mostly on roots growth of apple.

The proportion of roots, the count of roots in each shoot and the length of roots were significantly varied between treatments. The highest roots percentage, number of roots and length were recorded at 1.5 mg/l IBA rather than any concentration of NAA. After 6 weeks of *in vitro* cultivation, roots were seen on regenerated shoots. Adding 1.5 mg/l IBA increased the proportion of tissue culture shoots that rooted substantially, providing the greatest rooting (87.6%), as shown in **Table (7)**. The roots reached a maximum number of 6.4 root/shoot in this concentration, with the longest root length of 4.53 cm, as shown in **Fig. (8)**. Hardened and field-grown plants have the highest survival percentage when their roots are longer.

The proportion of roots was lowered to 44.3% due to a low concentration of IBA. The least length of 1.8 cm was accomplished with the fewest number of roots (2.6 root/shoot). When the IBA concentration was increased to 1.0 mg/l, the root properties improved, resulting in a 52.0% root percentage, 3.1 root/shoot with a length of 2.67 cm. The MS basal medium contained NAA at 0.5 mg/l, rooting from shoots occurred with a frequency 25%, yielding 1.46 roots per shoot with a length of 0.36 cm. When NAA concentration rises to 1.0 mg/l, however, it increased to 30.2 %, resulting in 1.83 root/shoots with a length of 1.17 cm.

The best results with the NAA concentrations were 1.5 mg/l NAA which produced a rooting percentage of 36.7%, the number of roots 2.57 root/shoot and root length 1.36 cm, as shown in **Table (8)**. Apple micro-shoot cultured on control medium (media without any supplement of growth hormone) produce callus-like structure around the base of the shoot and couldn't produce root on hormone-free media. IBA (1.5 mg/l) was determined to be the most suited auxin for roots induction of the two auxins tested (87.6%).

Table 7: The effect of varying IBA concentrations on apple shoots rooting after two months of culture.

IBA (mg/l)	Rooting %	Number of Roots	Root length (cm)
0.0	0.0 d	0.0 e	0.0 c
0.5	44.4 bc	2.6 cd	1.83 b
1.0	52.0 b	3.1 b	2.67 b
1.5	87.6 a	6.4 a	4.35 a

Table 8: The effect of varying NAA doses on apple shoots rooting after two months of culture.

NAA (mg/l)	Rooting %	Number of Roots	Root length (cm)
0.0	0.0 d	0.0 d	0.0 d
0.5	25.0 c	1.46 bc	0.36 c
1.0	30.2 ab	1.83 b	1.17 ab
1.5	36.7 a	2.57 a	1.36 a

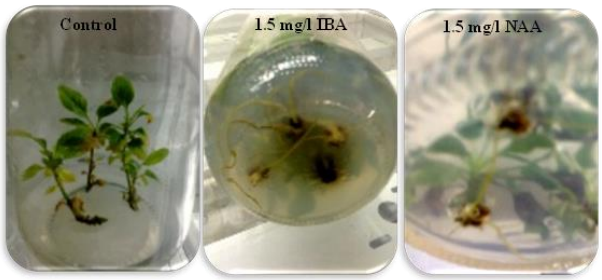

Fig. (8): The impact of varying amounts of IBA or NAA in 12 MS media on apple rooting.

The findings similar to these were published with **Savitha *et al.* (2010)**, who discovered that rooting occurred at all doses but at varying rooting percentages. At 2.0 mg/l IBA and 1.5 mg/l NAA, the most roots were formed. The shoots become necrotic, lose leaves and die gradually after subjected to large concentrations over 3.0 mg/l IBA/NAA. At dosages less than 1.0 mg/l, NAA and IBA produced a small roots number. **Amiri and Elahinia (2011)** it was discovered that IBA was required for M9T337 rooting, and that the optimal IBA concentration was the key to effective rooted.

After direct regeneration, rooting and acclimatization of the shoots was still not satisfactory. Sub-culturing of buds on hormone-free medium did not increase rooting potential, but the shoots regenerated on media with BA increased rooting ability of young shoots and had the largest rooting rate (up to 76%). The amount of cytokinin for regeneration

medium and the various culture media used affected the rooting proportion of shoots. After acclimatization, all rooted shoots survived **(Magyar-Tábori and DobránszkiI-Hudák, 2011)**. When cultivated on ½ MS medium containing two auxins (IBA and NAA; 2-8 mg/l), well-developed shoots rooted. The medium supplemented with IBA, had better rooting than NAA-supplemented medium. For the induction of roots in legumes, IBA is thought to be the most potent growth regulator **(Ozean *et al.*, 1992)**. The rooting was stimulated with used IBA which observed in *Clitoria ternatea* **(Barik *et al.*, 2007)**, *Cotinus coggygria* **(Metivier *et al.*, 2007)** and *Aegle marmelos* **(Nayak *et al.*, 2007)**. IBA dosing in during roots formation, had the maximum results for roots induction. The impact of IBA on roots production has been described for various plants, including *Hemidesmus indicus*, which is similar to our findings **(Sreekumar *et al.*, 2000)** and *Cunila galiodes* **(Fracaro and Echeverrigary, 2001).**

Modgil *et al.* (2010) compared to NAA, IBA achieved a higher rooting rate (81-82%) than using NAA (71%). On half strength medium contained 3.0 mg/l of IBA, the healthy thick roots were produced with a high frequency of 66.7%, and their length raised to 4.1 cm in 30 days **(Boudabous *et al.*, 2010)**. The micro-shoot rooting consists of a brief roots formation step (maximum 1 week) generally followed by a prolonged roots extension stage (many weeks). In the roots induction phase, IBA was usually employed, whereas NAA was mostly used and then an auxin-free medium was used in the roots step **(Dobránszki and Teixeira da Silva, 2010)**. IBA is the greatest widely used auxin for rooting in various genotypes of apple, having rooting percentages ranging from 18 to 100% depending on the dose used and genotype **(Jaime *et al.*, 2019)**.

IBA has been successfully used in several plant species that are traditionally difficult to rooting to encourage the roots of cuttings. Both greenwood and hardwood cuttings were successfully encouraged to produce adventitious roots when treated with IBA **(Yang *et al.*, 2019)**. Used ½ MS supplemented with 0.3 mg/l IBA, achieved the greatest roots number per adventitious shoot, 13.00 root, resulting in a rooting proportion of 100%. The IBA concentration of 0.6 mg/l resulted in the greatest rooting number of 11.33 per adventitious shoot **(Shi *et al.*, 2021)**. The rooting rate was improved by IBA than by NAA. But rooting rate didn't show a significant difference when the shoots were cultured on ½ MS with 0.3 mg/l NAA or 0.5 mg/l NAA and ¼ MS with any auxin or any concentration. Root number was higher when the shoots were cultured on ½ MS with IBA than that on NAA, The optimal rooting medium was ½ MS contained 0.3 mg/l IBA and 20 g/l sucrose, and the highest rooting rate was 69.8% **(Qing-Rong *et al.*, 2019)**. Approximately 85% of micro-shoots were established in auxin-free medium after a 10-day initial liquid culture with 3.0 mg/l IBA was used to establish the roots. Rooted plantlets were effectively acclimated to their new environment **(Kumar *et al.*, 2021)**.

In opposition to this discovery, **Mehta *et al.* (2014)** found the rooting of MM106 and B9 rootstocks at 98 and 92%, respectively, in PGR-free media. IBA was ineffective in inducing roots in 'Jarka' and 'Mivibe' (8% and 1% rooting of *in vitro* shoots, respectively); whereas, 1.0 mg/l NAA and 1.0 mg/l IAA effectively caused rooted (44 and 22%, respectively) **(Paprštein and Sedlák, 2015)**.

3.6- Acclimatization

Apple plantlet acclimatization process carried out under plastic house with some modification of humidity by using locally available materials. In this study, at the stage of hardening, some plantlets were shown wilting in the first week of transferring and the result shows some leaves were dried up. This is due to open water loss from their leaves or the roots' and root-stem connections' poor hydraulic conductivity **Kumar and Rao (2012)**. However, after 20 days of hardening original leaves were grown from each apple shoot. Progressively the plantlets started developing and the number of leaves increased as the plant height increased. After 8 weeks plantlet's survival percentage data was taken. The high survival percentage (76.3%) was recorded, as shown in **Fig (9)**.

Fig. (9): Successful acclimatization of apple Balady cultivar plantlets.

After 3 weeks, the plantlets that formed roots were put inside pots contained a sterilized combination of sand and soil at ratio (3:1) as well as kept in the growing chamber until two weeks before being relocated to shade and finally to field settings, according to a prior research. The survival percentage of plantlets in the field was 80 ±5% **(Kumar *et al.*,**

2016). Plants including well roots were first transplanted to plastic pots that containing peat and sand (2/3:1/3), and then the plants that acclimatized were transplanted to the soil under climatic conditions. 60% of the plants that were transplanted to the field survived **(Boudabous *et al.*, 2010)**. The *in vitro*-rooted shoots were effectively toughened (80–100%) on a variety of substrates, including peat, coco-peat, soil: sand (3:1 v/v), a mix of vermiculite and soil, or coco-peat: peat. (1:1:1 v/v/v) perlite: vermiculite **(Jaime *et al.*, 2019)**. It was then necessary to move the rooted plantlets into the laboratory greenhouse for acclimation and growth for 3 days, after which they were transferred into plastic pots with saucers filled with sterile water and sterilized culture substrate and placed in an environment with 30–60 percent relative humidity **(Shi *et al.*, 2021).**

3.7- DNA barcoding of *Malus domestica*

The purpose of this work was to look into the possibility of employing rbcL DNA barcodes to identify *Malus domestica* plants. A short genetic sequence from a typical portion of the genome is adequate for plant species identification, suggesting that DNA barcoding might be a valid method of certifying plant identity. The outputs of BLAST matching and tree analysis of *Malus domestica* are represented in **Table (9)** and **Fig. (10)** for the plant species that have the best percentages of similarity. The newly generated sequence of the cpDNA marker; rbcL was used as a barcode.

A fan shape may be seen in the phylogenetic trees that include the plant species with the greatest percentage of similarity (see **Fig. 10**), with closely related species clustered together and more distantly related species scattered throughout the tree.

The universality of DNA barcode markers is restricted due to geographical and morphological variations and plant species evolution **(Roy *et al.*, 2010)**. The current study's findings demonstrate that *Malus domestica* may be successfully identified at the species level, and it is advised that other markers, such as nuclear gene markers, be investigated in order to more properly identify the plant as a whole.

Table (9): *rbcL* DNA barcode of related plant species with similarity percentage of more than 98.3%, downloaded from GenBank database

Plant species	Accession no.	E-value	Query coverage (%)	Similarity (%)
Malus domestica	MK434916	0.0	100	100.00
Malus domestica	KM360872	0.0	100	100.00
Malus domestica	OU745006	0.0	100	100.00
Malus domestica	OU745006	0.0	100	100.00
Malus domestica	OU744968	0.0	100	100.00
Malus domestica	LT996898	0.0	98	100.00
Malus domestica	MH536583	0.0	100	97.23
Malus domestica	OU744552	0.0	40	92.03
Malus domestica	OU744959	0.0	68	92.23
Malus domestica	OU696683	0.0	68	92.23

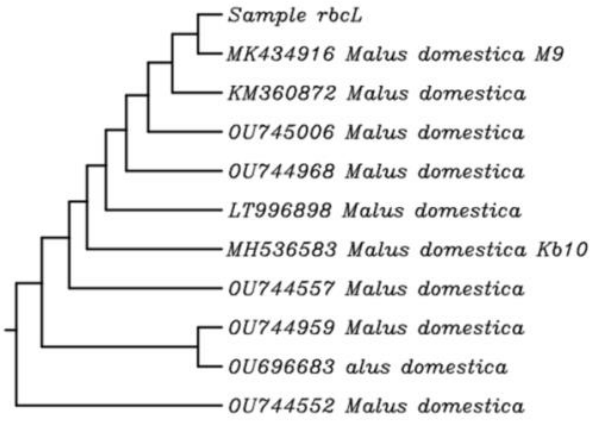

Fig. (10): Phylogenetic tree of *Malus domestica* using the cpDNA marker; rpcl, showing names of plant species and accession numbers.

4. References

Amiri, E. M. and Elahinia, A. (2011). Optimization of medium composition for apple rootstocks. Afr. J. Biotech., 10: 3594–3601.

Barik, D. P.; Naik, S. K.; Mudgal, A. and Chand, P.K. (2007). Rapid plant regeneration through *in vitro* axillary shoot proliferation of butterfly pea (*Clitoria ternatea* L.) a twinning legume. *In Vitro* Cellular and Developmental Biology, 43: 144–148.

Belaizi, M; Paul, H.; Sangwan, R. S. and Sangwan-Norreel, B. S. (1991). Direct organogenesis from internodal segments of *in vitro* grown shoots of apple cv. Golden delicious. Plant Cell Reports, 9: 471-474.

Bommineni, V.; Mathews, H.; Samuel, S.; Kramer, M. and Wagner, D. (2001). A new method for rapid *in vitro* propagation of apple and pear. HortScience, 36: 1102–1106.

Boudabous, M.; Mars, M.; Marzougui, N. and Ferchichi, A. (2010). Micro-propagation of apple (*Malus domestica* L. cultivar Douce de Djerba) through *in vitro* culture of axillary buds. Acta Botanic Gallica, 157 (3): 513-524.

Caboni, E.; Lauri, P. and D'Angeli, S. (2000). *In vitro* plant regeneration from callus of shoot apices in apple shoot culture. Plant Cell Reports, 19: 755–760.

Ciccotti, A. M.; Bisognin, C.; Battocletti, I.; Salvadori, A.; Herdemerteus, M. and Jarausch, W. (2008). Micro-propagation of apple proliferation resistant apomictic *Malus sieboldii* genotypes. Agron. Res., 6: 445- 458.

Compton, M. E. and Gray, D. J. (1993). Shoot Organogenesis and Plant Regeneration from Cotyledons of Diploid, Triploid, and Tetraploid Watermelon. Journal of the American Society for Horticultural Science, 118 (1): 151–157.

Dalal, M. A.; Das, B.; Sharma, A. K.; Mir, A. A. and Sounduri, A. S; (2006). *In vitro* cloning of apple (*Malus domestica* Borkh) employing forced shoot tip cultures of M9 rootstock. Indian J. Biotechnol., 5: 543-553.

Dastjerd, Z. H.; Jabbarzadeh, Z. and Marandi, R. J. (2013). Interaction effects of chitosan, benzyladenine and gibberellic acid on *in vitro* proliferation of M26 apple rootstock. Hortic. Env. Biotechnol., 54: 538– 547.

De-Nato, V. B. P. and Otoni, W. C. (2003). Carbon sources and their osmotic potential in plant tissue culture: does it matter? Scient. Hort., 97: 193-202.

Devendra, N. K.; Subhash, B. and Seetharam, Y. N. (2009). Callus growth and plant regeneration in *momordica dioica* (roxb.). willd cucurbitaceae. Am. Eurasian. J. sustain agric., 3: 743- 748.

Dobránszki, J. and Silva, J. A. (2010). Micro-propagation of apple— a review. Biotechnol. Adv., 28: 462–488.

Dufour, M. (1990). Improving yield of adventitious shoots in apple. Acta Hortic., 280: 51–60.

Duncan, D. B. (1955). Multiple range and multiple F test. Journal of Biometrics, 11: 1-42.

Fao (2009) http://faostat.fao.org/site/567/Desktopdefault.aspx ?Pag eID=567.

Faria, R. T.; Rodrigues, F. N.; Oliveira, L. V. R. and Muller, C. (2004). *In vitro Dendrobium nobile* plant growth and rooting in different sucrose concentrations. Horticultura Brasileira, 22: 780-783.

Fracaro, F. and Echeverrigaray, S. (2001). Micro-propagation of *Cunila galioides*, a popular medicinal plant of south brazil. Plant Cell, Tissue and Organ Culture, 64: 1–4.

Gahan, P. B. and George, E. F. (2008). Adventitious regeneration. In: George EF, Hall MA, De Klerk GJ (eds) Plant propagation by tissue culture, 3rd edn. Springer, Dordrecht, pp 355–401.

Gauchan, D. P. (2012). Effect of different sugars on shoot regeneration of maize *Zea mays* (L.). Kathmandu University. Journal of Science, Engineering and Technology, 8: 119-124.

Geng, F.; Moran, R.; Day, M.; Halteman, W. and Zhang, D. (2015). *In vitro* shoot proliferation of apple rootstocks 'B.9', 'G.30' and 'G.41' grown under red and blue light. HortScience, 50: 430–433.

George, E. F. (1993). Plant Propagation by Tissue Culture; Part 1: The Technology. 2nd edition. Great Britain: Exegetics Ltd. 574p.

George, E. F. and Debergh, P. C. (2008). Micro-propagation: Uses and Methods. In: George EF, Hall MA, De Klerk GJ, editors. Plant Propagation by Tissue Culture. 3rd Edition. Springer, Dordrecht, Netherlands. pp. 29-64.

Gomez, K. A. and Gomez, A. A. (1984). Statistical Procedures for Agricultural Research. John Wiley and Sons, New York, USA, pp: 680.

Groot, G. A.; During, H. J.; Maas, J. W. and Schneider H. (2011). Use of rbcL and trnL-F as a two-locus DNA barcode for identification of NW-European ferns: an ecological perspective. PLoS One 6: e16371.

Group, CBOL Plant Working (2009). A DNA barcode for land plants. Proc. Natl. Acad. Sci., USA 106:12794- 12797.

Hammatt N. and Grant, N. J. (1993). Apparent rejuvenation of mature wild cherry (*Prunus avium* L.) during micro-propagation. J. Plant Physiol., 141: 341-346.

Harada, H. and Murai, Y. (1996). Micro-propagation of *Prunus mume* koehne. Plant cell, Tissue and organ culture, 40 (2): 159-167.

Hebert, P. D.; Cywinska, A.; Ball, S. L. and Dewaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270 (1512): 313- 321.

Ibrahim, S. D.; Adawy, S. S.; Atia, M. A. M.; Alsamman, A. M. and Mokhtar, M. M. (2016). Genetic diversity, variety identification and gene detection in some Egyptian grape varieties by SSR and SCoT markers. Plant Omics., 9 (5): 311-318.

Jabbarzadeh, Z. and Khosh-Khui, M. (2005). Factors affecting tissue culture of Damask rose (*Rosa damascena* Mill.). Scientia. Hort., 105: 475-482.

Jaime, A.; Silva, T. D.; Gulyás, A.; Magyar-Tábori, K.; Wang, M. R.; Wang, Q. C. and Dobránszki, J. (2019). *In vitro* tissue culture of apple and other *Malus* species: recent advances and applications. Planta, 1-32.

Jain, N. and Babbar, S. B. (2003). Effect of carbon source on the shoot proliferation potential of epicotyle explant of *Syzygium cuminii*. Biologia Plantarum, 47 (1): 133-136.

Jin, W.; Wang, Y. and Wang, H. (2014). Adventitious shoot regeneration from leaves of apple rootstock 'Pingyitiancha' (*Malus hupehensis* var. pinyiensis) and genetic fidelity of regenerated plantlets using SSR markers. Can. J. Plant Sci., 94: 1345-1354.

Karami, O.; Deljou, A.; Esna-Ashavi, M. and Ostad-Ahmadi, P. (2006). Effect of sucrose concentration on somatic embryogenesis in Carnation (*Dianthus carophyllus* L.). Scient. Hort., 110: 340-344.

Kovalchuk, I.; Lyudvikova. Y.; Volgina, M. and Reed, B. M. (2009). Medium, container and genotype all influence *in vitro* cold storage of apple germplasm. Plant Cell Tissue Organ Culture, 96: 127–136.

Kozai, T. (1991). Micro-propagation under photoautotrophic conditions. – In: Debergh PC and Zimmerman RH, (eds) Micro-propagation Technology and Application. Dordrecht: Kluwer Academic Publishers, pp. 447-469.

Kumar, A.; Singh, Y.; Shalini, V. and Modgil, M. (2021). Optimization of *in vitro* adventitious shoot regeneration from leaf explants of apple cultivar 'Red Chief'. Research Journal of Biotechnology.

Kumar, K. and Rao, I. U. (2012). Morpho physiologicals problems in acclimatization of micro-propagated plants in *ex vitro* conditions- A Reviews. Journal of Ornamental and Horticultural Plants, 2 (4): 271-283.

Kumar, R. S.; Joshi, C. and Nailwal, T. K. (2016). Callus Induction and Plant Regeneration from Leaf Explants of Apple (*Pyrus malus* L.) cv. Golden Delicious. International Journal of Current Microbiology and Applied Sciences, 5 (2): 502-510.

Lee, Y. K.; Kwon, Y. W. and Hyung, N. (2019). Optimal Medium Compositions for Plant Regeneration via Adventitious Shoot Formation Using 'Fuji' Apple Leaf Explants. Journal of Plant Biotechnology, 46: 310–317.

Li, B. Q.; Feng, C. H.; Hu, L. Y.; Wang, M. R.; Chen, L. and Wang, Q. C. (2014). Shoot regeneration and cryopreservation of shoot tips of apple (*Malus*) by encapsulation–dehydration. *In Vitro* Cell Dev. Biol. Plant, 50: 357–368.

Lipavska, H. and Konradova, H (2004). Somatic embryogenesis in conifers: the role of carbohydrate metabolism. *In vitro* cellular & Developmental Biology, 40: 23.

Lizárraga, A.; Fraga, M.; Ascasíbar, J. and González, M. L. (2017). *In vitro* propagation and recovery of eight apple and two pear cultivars held in a germplasm bank. Am. J. Plant Sci., 8: 2238–2254.

Magyar-Tábori, K. and DobránszkiI-Hudák, J. (2011). Effect of cytokinin content of the regeneration media on *in vitro* rooting ability of adventitious apple shoots. Scientia Horticulturae, 129 (4): 910-913.

Magyar-Tábori, K.; Dobránszki, J.; Bulley, S. M.; Silva, J. A. and Hudák, I. (2010). The role of cytokinins in shoot organogenesis in apple. Plant Cell, Tissue and Organ Culture, 101: 251–267.

Maheshwari, P. and Kumar, A. (2006). Organogenesis, shoot regeneration and flowering response of *Vernonia cinerea* to different auxin/cytokinin combinations. *In Vitro* Cellular & Developmental Biology, 42: 589–595.

Matand, K. and Prakash, C. S. (2007). Evaluation of peanut genotypes for *in vitro* plant regeneration using Thidiazuron. J. of Biotech., 130: 202-207.

Mehta, M.; Ram, R. and Bhattacharya, A. (2014). A simple and cost effective liquid culture system for the micro-propagation of two commercially important apple rootstocks. Indian J. Exp. Biol., 52: 748–754.

Metivier, P. S. R.; Yeung, E. C.; Patel, K. R. and Thorpe, T. A. (2007). *In vitro* rooting of micro-shoots of *Cotinus coggygria* Mill, a woody ornamental plant. *In Vitro* Cellular & Developmental Biology, 43: 119–123.

Mitic, N.; Stanisic, M.; Milojevic, J.; Tubic, L.; Cosic, T.; Nikolic, R. and Ninkovic, S. (2012). Optimization of *in vitro* regeneration from leaf

explants of apple cultivars Golden Delicious and Melrose. HortScience, 47: 1117-1122.

Modgil, M. and Pathania, S. (2018). Plant regeneration from leaf explants in apple rootstock MM111: Effect of cytokinins and auxins. Indian Journal of Biotechnology, 17 (2): 370-378.

Modgil, M.; Gupta, R. and Thakur, M. (2010). *In vitro* rooting and hardening in apple rootstock EMLA111—influence of some factors. Acta Hortic., 865: 339–344.

Modgil, M.; Sharma D. R. and Bhardwaj S. V. (2012). Micro-propagtion of apple cv. Tydeman's Early Worcester. Scientia Horticulturae, 81: 179–188.

MSTAT Development Team (1989). MSTAT user's guide: a microcomputer program for the design management and analysis of agronomic research experiments. Michigan State University, East Lansing, USA.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., 15: 473–497.

Nayak, P.; Behera, P. R. and Manikkannan, T. (2007). High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *In Vitro* Cellular & Developmental Biology, 43: 231–236.

Neelam, S.; Mukesh, K. M. and Vidya, P. (2011). Effect of plant growth regulators, explants type and efficient plantlet regeneration protocol through callus induction in *Naringi crenulata* (Roxb.) Nicolson and its biochemical investigation, African journal of biotechnology, 10 (77): 17769-17777.

Newmaster, S. G.; Fazekas, A. J. and Ragupathy, S. (2006). DNA barcoding in land plants: evaluation of rbcL in a multigene tiered approach Canadian Journal of Botany, 84: 335–341.

Noormohammadi, Z.; Farahani, F. and Safarzadeh, **M. (2013).** Study of Morphological Traits Changes in Different Media Culture of Two Apple Rootstocks (M26 and MM106). Malaysian Applied Biology, 42 (1): 25–33.

Ozean, S.; Burghehi, M.; Firek, S. and Draper, J. (1992). High frequency adventitious shoot regeneration from Immature colyledons of Pea (*Pisum sativum* L.). Plant Cell Reports, 11: 44–47.

Paprštein, F. and Sedlák, J. (2015). Micro-propagation of Czech apple cultivars. Acta Hortic., 1083: 267–271.

Pawlicki, N. and Welander, M. (2015). Adventitious shoot regeneration from leaf segments of *in vitro* cultured shoots of the apple rootstock Jork 9. Journal of Horticultural Science, 69 (4): 687-696.

Podwyszyńska, M.; Sowik. I.; Machlańska, A.; Kruczyńska, D. and Dyki, B. (2017). *In vitro* tetraploid induction of *Malus×domestica* Borkh. using leaf or shoot explants. Sci. Hortic., 226: 379–388.

Qing-Rong, S.; Qiu-Zhu, G.; HaiBo, W.; Lin-Guang, L. ; Ji-Han, T. and Hong-Yan, S. (2019). Tissue culture and induction of adventitious shoot regeneration from leaf explants of cold-hardy dwarfing apple rootstock 'BP-176'. Journal of Fruit Science, 36 (6) 812-818.

Roy, S.; Tyagi, A.; Shukla, V.; Kumar, A.; Singh, U. M. and Chaudhart, L. B. (2010). Universal plant DNA barcode loci may not work in complex groups: A case study with Indian berberis species. Plos One, 5 (10): e 13674.

Savitha, R.; Shasthree, T.; Sudhakar, S. and Mallaiah, B. (2010). High frequency of plantlet regeneration and multiple shoot induction from leaf and stem explant of *Citrullus colocynthis* (L.) Schrad, an endangered medicinal cucurbit. Int. J. Pharma boil, Sci, 6 (2): 1-8.

Sharma, M.; Modgil, M. and Sharma, D. (2000). Successful propagation *in vitro* of apple rootstock MM106 and influence of phloroglucinol. Indian J. Exp. Biol., 38: 1236–1240.

Sharma, T.; Modgil, M. and Thakur, M. (2007). Factors affecting induction and development of *in vitro* rooting in apple rootstocks. Indian J. Exp. Bio., 45: 824-829.

Shi, J.; Dong, Z.; Song, C.; Xie, B.; Zheng, X.; Song, S.; Jiao, J.; Wang, M. and Bai, T. (2021). Establishment of an efficient micro-propagation system in enhancing rooting efficiency via stem cuttings of apple rootstock M9T337. Horticultural Science (Prague), 48 (2): 63–72.

Shukla, P. S.; Das, A. K.; Jha, B. and Agarwal, P. K. (2014). High-frequency *in vitro* shoot regeneration in *Cucumis sativus* by inhibition of endogenous auxin. Vitro Cell Dev. Biol. Plant, 50: 729–37.

Silva, J. T.; Gulyás, A.; Magyar-Tábori, K.; Wang, M. R.; Wang, Q. C. and Dobránszki, J. (2019). *In vitro* tissue culture of apple and other Malus species: recent advances and applications. Planta, 249: 975–1006.

Sotiropoulos, T. E.; Molassiotis, A. N.; Mouhtaridou, G. I.; Papadakis, I.; Dimassi, K. N.; Therios, I. N. and Diamantidis, G. (2006). Sucrose and sorbitol effects on shoot growth and proliferation *in vitro*, nutritional status and peroxidase and catalaze isoenzymes of M 9 and MM106 Apple (*Malus domestica* Borkh.) rootstocks. Europ. J. Hort. Sci., 71 (3): 114-119.

Sreekumar, S.; Seeni, S. and Pushpangadan, P. (2000). Micro-propagation of *Hemidesmus indicus*

for cultivation and production of 2- hydroxy 4-methyl benzaldehyde. Plant Cell, Tissue and Organ Culture, 62: 211–218.

Tereso, S.; Miguel, C. M.; Mascarenhas, M.; Roque, A.; Trindade, H. and Maroco, J. (2008). Improved *in vitro* rooting of *Prunus dulcis* Mill. cultivars. Biol. Plantarum, 52: 437-444.

Volk, G. M.; Chao, C. T.; Norelli, J.; Brown, S. K.; Fazio, G.; Peace, C.; McFerson, J.; Zhong, G. Y. and Bretting, P. (2015). The vulnerability of US apple (Malus) genetic resources. Genet. Resour. Crop Evol., 62: 765–794.

Wang, M. R.; Li, B. Q.; Feng, C. H. and Wang Q. C. (2016). Culture of shoot tips from adventitious shoots can eradicate apple stem pitting virus but fails in apple grooving virus. Plant Cell Tissue and Organ Culture, 125: 283–291.

Wei, G.; Liang, M.; Li, D.; Sun, H. and Dai, H. (2009). Adventitious bud regeneration from leaves *in vitro* of *Malus hupehensis* Rehd and M7. Acta Qingdao Agric. Univ., 26: 103 108.

Welander, M. and Maheswaran, G. (1992). Shoot regeneration from leaf explants of dwarfing apple rootstocks. J. Plant Physiol., 140: 223–228.

Yang, H.; Klopotek Y.; Hajirezaei, M. R.; Zerche, S.; Franken, P. and Druege, U. (2019). Role of auxin homeostasis and response in nitrogen limitation and dark stimulation of adventitious root formation in petunia cuttings. Annals of Botany, 124: 1053–1066.

Zhang, X.; Qin, Y.; Liang, D.; Zou, Y. and Ma, F. (2014). Enhancement of *in vitro* shoot regeneration from leaf explants of apple rootstock G.41. *In Vitro* Cellular & Developmental Biology Plant, 50: 263–270.

11/2/2021