*Plant Archives* Volume 20 No. 2, 2020 pp. 9467-9481 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

# TRANSGENIC MANDARIN (*CITRUS RETICULATA* L.) INCLUDING ENDOTOXIN *CRY1AB* GENE FOR RESISTANCE *PHYLLOCNISTIS CITRELLA* STAINTON

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## Abstract

Mandarin, similar to every other, is extremely touched to damage by many insects and pests, which may cause death of the affected plant and reduction in yield. An efficient protocol was successfully achieved for regeneration and genetic transformation using *Agrobacterium*. Mandarin cotyledons (*Citrus reticulata* L.) used as an explant. A binary vector pBI121 containing *Cry1Ab* and kanamycin phospho transferase genes was used in transformation of mandarincotyledons *in vitro*. Cotyledons cultured on MS medium containing 100 mg/l kanamycin recorded 100% mortality thus, it was chosen for the selection of transformed explants. Throughout the selection procedure of transformed explants the minimum inhibitory concentration of the selection marker was used. Suspension culture of *Agrobacterium tumefaciens* strain LBA4404 (OD600=0.4-0.5) was used for explants submersion. The co-cultivation was performed for different hours (0.0, 12, 24, 36 and 48) on LB medium with or without the addition of 100 µM acetosyringone. After the incubation periods, Cotyledon was transferred to MS medium with 2.00 mg/l 2, 4-D, 250 mg/carbenicillin and 100 mg/l kanamycin for detection of transgenic cotyledon. Polymerase chain reaction (PCR) was used for the rapid screening of *Cry 1Ab* gene. Total genomic DNA was isolated from transformants for screening. Using primer specific to *Cry1Ab* gene (forward and reverse), a PCR product with a size of about 1,800bp was amplified when all nucleic acid from the transformants were utilized as templates.

The appearance of the transgene of 1,800bp was confirmed by PCR. Best results for callus induction were observed from cotyledons 100% on MS medium supplemented with 2.00 or 3.00 mg/l 2, 4-D. The highest regeneration efficiency and weight of callus observed by adding with 1mg/l BA plus 1.25mg/l NAA. The shoots of Mandarin (*Citrus reticulata* L.) were then rooted on MS medium containing IBA and NAA at 2.00 mg/l, with high rooting percentage (100%). Regarding the mean number of roots formed/propagule, best results were obtained on full strength MS medium fortified with 2.00 mg/l NAA, which achieved the highest mean value (6.33). Meanwhile, augmenting MS medium with NAA at 2.00 mg/l, which recorded the highest length of roots/ plantlets (6.50 cm). These results signify the successful transfer of *Cry 1Ab* gene into Mandarin plant.

Callus induction (100%) were observed from cotyledons cultured on MS medium supplemented with 2.00 or 3.00 mg/l 2, 4-D. The highest regeneration efficiency and callus weight were observed by adding 1.00mg/l BA and 1.25mg/l NAA. High rooting percentage (100%) of mandarin shoots were scored on MS supplemented with IBA and NAA at 2.00 mg/l,. Our results have provided an insight into the effect of the co-cultivation period on the transformation of mandarin (*Citrus reticulata* L.) using *Agrobacterium tumefaciens*. Acetosyringone was recommended formandar in transformation. Addition of acetosyringone for 48 h has a positive effect on production of transgenic plants for mandarin explants incubated with *Agrobacterium tumefaciens*. After co-cultivation periods, Cotyledons were transferred to MS containing 2.00 mg/l,2, 4-D, 250 mg/carbenicillin and 100 mg/l kanamycin for detection of transgenic cotyledons. Rapid screening of *Cry 1Ab*gene was done using PCR.

***Key words :*** *In vitro*, Mandarin, Rooting and adaptation, Genetic transformation, insect resistance gene, Acetosyringone, *Agrobacterium tumefaciens.*

## Introduction

*Citrus* is one of the most important fruit crops in the world. *Citrus* average cultivated area reached 11.1 million hectares, with a huge production of oranges (75 million tons), followed by clementines, mandarins, tangerines and satsumas (34 million tons), lemons and limes (19 million tons), and grapefruits and pummelos (9 million tons) (FAOSTAT database results 2018).

Due to the great adaptability to different climatic conditions of *Citrus* therefore, they are grown equally successful in tropical, sub-tropical and even in some amenable parts of the temperate regions of the world. The major mandarin producing countries in the world are Indonesia and Turkey (Anonymous, 2012). Unfortunately, several plant pathogens have a negative effect on *Citrus* fruit quality. Moreover, many problems facing *Citrus* species like insect pests, slow growth, long juvenility, insects, pests, diseases, alternate bearing, pre-and post-harvest losses, large number of seeds per fruit, short season of supply and short storage life (Mukhtar *et al.,* 2005). Protection and proper handling of fresh fruit is inadequate, losses during transit and storage can represent in excess of 50% of the harvested crop in developing countries. (Eckert and Ogawa, 1985; Wisniewski and Wilson, 1992).

One of the most important *Citrus* pests worldwide is *Citrus* leaf miner *Phyllocnistiscitrella* Stainton (Lepidoptera: Gracillariidae) (Grafton-Cardwell *et al.,* 2008). Epidermal cells in new leaves is where the larvae feed on, forming serpentine galleries (Achor*et al.,* 1997; Beattie, 2004), where larvee prevents normal leaf development (Garcia Marí *et al.,* 2002), reduces the photosynthetic potential (Schaffer *et al.,* 1997), growth, and productivity of *Citrus* plants (Peña *et al.,* 2000). The presence of *P. citrella* in the leaves ( in addition to the direct damage) increases the incidence and severity of infection by the bacterium *Xanthomonascitri* subsp. *citri* (Hasse) (Xanthomonadales: Xanthomonadaceae), which is the causal agent of *Citrus* canker, one of the main *Citrus* diseases (Jesus Jr. *et al.,* 2006; Hall *et al.,* 2010; Paiva and Yamamoto, 2015).

One of the main aims of *Citrus* breeding programs is the development of novel varieties to improve resistance to various pests and pathogens. *Citrus* conventional breeding strategy has demonstrated numerous limitations which are very common in woody plants (due to their biological characteristics) such as long juvenile period, large size, long generation time, and how the most important horticultural traits are inherited which lack knowledge on it's strategy. (Poles *et al.,* 2020).

A series of genes have been transferred into several major crops which are responsible for important agronomical traits such as insect and viral resistance; stress tolerance and herbicide tolerance (Taylor *et al.,* 1997). Insect resistance genes now can be transferred into plants more quickly and deliberately with the advent of genetic engineering. One example of ways of genetic engineering that may be used to produce insect resistant crops now and in the future is *Bacillus thuringiensis* (Bt) technology. Insect pests can be easily controlled within the orders Lepidoptera, Coleoptera, and Diptera by using Bacillus *thuringiensis* which has been securely utilized throughout the previous 50 years Roh *et al.,* 2007).

Potential effects on non-target organisms, especially predators and parasitoids is one major ecological concern regarding the biosafety of Bt crops on the environment that play an important role in pest control (Martins-Salles *et al.,* 2017). Certain enzymes in the guts of these specific insect species can only digest the Bt endotoxin, hence become toxic to these insects within a few days which prevent the damage done by insects. Thus, due to the lack of specific enzymes in human body, so Bt toxin is not harmful to human (Knowles, 1993).

Enormous, advantages of Bt crops containing Cry proteins, like reduce using synthetic insecticides, improve yield, higher income, lower production costs and compatibility with integrated pest management program. The expression of a toxic protein should be sufficient enough in potentially vulnerable parts and at desired growth stage of the plant for better protection against targeted insect pests, (Naqvi *et al.,* 2017). However, low transformation rate and poor regenerating ability is the big problem of gene transformation in teak. The introduction of useful agricultural genes has been delayed when compared to other species into teak or woody plant species.

The incubation conditions, choice of culture medium, application of growth regulators and their concentrations, and type of explant are the success factors of any *in vitro* regeneration system should depend on (Santiago *et al.,* 2019). A number of complex factors that determine the *in vitro* growth and development of a plant which include the genetic make-up of the plant, nutrients: water, macro-and micro-elements, and sugars, physical growth factors: Light, temperature, pH, $O_2$ and $CO_2$ concentrations and some organic substances: regulators, vitamins, etc. (Pierik, 1987). Many reasons for *Agrobacterium*-mediated transformation to be preferred method for gene transfer like simplicity, cost effective-

ness, little re-arrangement of transgene, ability to transfer relatively long DNA segments (Hamilton *et al.,* 1997), and preferential integration of foreign genes into transcription-ally active regions (Konez *et al.,* 1989; Ingelbrecht *et al.,*1991) thereby ensuring proper expression of transgenes in plants (Hernandez *et al.,* 1999) as compared to other methods.

The aim of this research is to determine the best growth regulators in order to achieve callus induction. Simple quick response to induction of somatic embryogenesis from cotyledon of mature trees in order to obtained healthy and uniform shoots and rooting of regenerated shoots. In this report, we demonstrate a successful *Agrobacterium*-mediated gene transformation of *Cry1Ab* gene into mandarin (*Citrus reticulata* L.) using cotyledon as explant.

## Materials and Methods

This study was carried out during theperiod from 2018 to 2020 in the Laboratory of Gene Transfer and Germplasm Conservation, Department of Plant Biotechnology. Also, in the Tissue Culture Laboratory, Desert Research Center, Cairo, Egypt and the plant material certified immature fruits (90 days after anthesis) were chosen from 10 years old Mandarin (*Citrus reticulata* L.) grafted trees were obtained from the farm at Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt.

**Surface sterilization**

Mandarin fruits were surface-sterilized by soaking in 10% commercial bleach (containing 5 % active chloride) for 20 min, followed by three rinses with sterile distilled water. Fruits were then dipped in 95% ethanol and flamed for a few seconds. After flamed, fruits were cut into two halves for immature seeds collected and juice vesicles were carefully separated for callus induction (Xiao *et al.,* 2004).

***In vitro* seed germination**

Surface sterilization of the given seeds were place dindividually in 350 culture jar containing 40 ml of medium (Murashige and Skoog, 1962) (MS) supplemented with 400 mg/l glutamine, 100 mg/lascorbic acid, 30 g/l sucrose and 7g/l agar. The seeds were maintained at 25 ± 2°C in the dark for three weeks, followed by one week under a 16-h photoperiod 3000 lux. After 5 weeks' cotyledons were excised for callus induction.

**Determination of minimal inhibitory concentration of kanamycin**

The susceptibility trial of kanamycin was carried out to find the inhibitory concentration which arrests cotyledons growth. Sterilized cotyledons were transferred onto plates of MSO (basal MS medium without hormone) containing different concentrations of kanamycin (0.0, 25, 50, 75, 100, 125 and 150 mg/l). Kanamycin was sterilized by filtration through 0.22 um disposable filter and incorporated into pre-cooled (45-50 °C) autoclaved medium. Cotyledons were incubated for 6 months with 5-6 transfers until phenotypic signs of the effect of antibiotic treatment could be detected. The number of survival explants was recorded after eight weeks from cultivation. Three replicates were carried out for each treatment with 25 cotyledons for each replicate. The minimum inhibitory concentration of the selection marker was used throughout the selection procedure of selection transformed explants.

**Bacterial Strain and Plasmid Vector**

*Agrobacterium tumefaciens* strain LBA4404, harbouring a T-DNA, a pBI121 plasmid containing a kanamycin resistance (*npt-II*) gene for bacterial selection and the plasmid including insect resistance (*Cry1Ab*) and *npt-II* genes driven by CaMV and NOS promoters, were kindly provided by Prof. I. Altosaar, University of Ottawa, Canada, under material transfer agreement.

Plasmid construction: A 1.8 kb *cry1Ab* gene from pGEM-4z was subcloned into the BamHI site of pBI121 (Clontech, USA) yielding pBI121-*Cry1Ab*. Gene orientation was verified by double restriction digestion (EcoRI and Hind III). The expression of the *Cry1Ab*

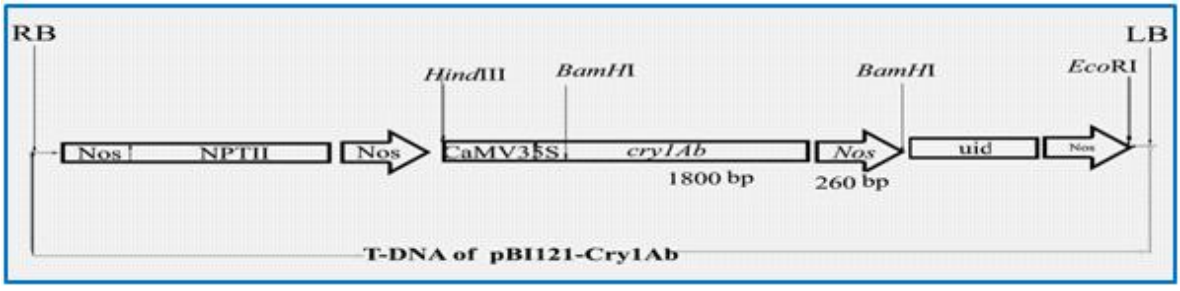


**Fig.1:** The binary Ti based plasmid pBI121 carrying NPT –II as selectable marker for kanamycin resistance and the plasmid including insect resistance (*Cry1Ab*) and *npt-II* genes driven by CaMV and NOS promoters.

gene was under the control of the constitutive CaMV 35S promoter (Fig. 1). The plasmid was transferred into competent cells of *Agrobacterium tumefaciens* (strain LBA 4404) by the freeze-thaw method (An, 1987).

**Growth of *Agrobacterium***

A single colony of LBA4404 was inoculated into 10 ml LB broth, supplemented with 150mg/l kanamycin and the liquid culture was agitated in the dark and grown overnight at 28 °C on a rotary shaker at 200 rpm. Ten percentages of the cultures were added into 100 ml LB broth and cultured under the same conditions. Three milliliters of these liquid cultures were taken for OD reading at 600nm. At time zero, the starting OD600 was 0.006. The OD600 was measured hourly and a growth curve was constructed.

**Pre-culture of cotyledon**

The cotyledons were pre-cultured in fresh MS medium supplemented with 2 mg/l 2,4-D for a week before co-cultivation with *Agrobacterium* pBI121.

**Inoculation and co-cultivation of cotyledons with *Agrobacterium***

A DMSO stock of LBA4404 carrying the pBI121 were streaked onto an LB agar plate with the appropriate antibiotic and incubated at 28°C for 48 hours. A single colony from each plate was inoculated into 3 ml broth and agitated at 200 rpm, 28°C. About 10 %(v/v) of cells from the 48 hours preculture was inoculated into 10 ml fresh LB broth and incubated under the same conditions until the OD600 reached 0.4 to 0.5. Liquid callus induction medium (CIM) was used to dilute the bacteria to $2x10^7$ cells/ml. The cotyledons were co-cultivated with *Agrobacterium tumefaciens* at 25 to 28$^0$C for (12, 24, 36 and 48 hour) along with or without acetosyringone (100 ¼M) (Sigma), with gentle shaking. The cotyledon was then blotted dry on sterile filter paper before plating onto CIM agar. A sterile filter paper disc was placed on the medium, and the cotyledons were spread on the filter paper using a spatulla.

**Elimination of bacteria and selection of transgenic cotyledons**

The co-cultivated cotyledons together with the filter paper were transferred onto the bacterial elimination medium, which was composed of CIM containing 250 mg/l carbenicillin. The cultures were incubated for 7 days in total darkness at 26 ± 1$^0$C. After one week incubation, cotyledons were then transferred to callus induction medium (MS supplemented with 250 mg/l carbenicillin) and incubated for four weeks before being transferred onto selection medium (4.4 g/l MS with 100 mg/l kanamycin plus 250 mg/l carbenicillin). The cotyledons developed to callus after 6 weeks incubation.

**Plant genomic DNA extraction**

Genomic DNA was extracted from cotyledon tissue of transgenic plants and non-transformed control plants of Mandarin (*Citrus reticulata* L.) using the CTAB method references. The cotyledon tissues (2g) were ground in liquid nitrogen. An amount of 12 to 15 ml CTAB extraction buffer (2% (w/v) CTAB, 100 mMTris pH 8.0, 20 mM EDTA pH 8.0, 4% PVP-40, 1.4 M NaCl and 5% (v/v) 2-mercaptoethanol) were added to the ground tissue in 50 ml sterile centrifuge tube. The sample was then incubated at 65°C for 30 to 60 minutes. Ten milliliter of chloroform: isoamyl alcohol 924:1) was added to the grindate and mix gently. The samples were then centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred into fresh tube and 2 or 3 volume of chilled isopropanol was added to precipitate the DNA. The samples were then incubated at -20°C for 30 to 60 minutes. The samples were then spun down at 12,000 rpm for 15 minutes at 4°C. The wash buffer ingredients were added to wash the precipitate and the tube was incubated at room temperature for 60 minutes. The supernatant was discarded and the pellet was dried in the vacuum until all liquid had evaporated. The DNA pellet was then resuspended in 0.5 to 1.0 ml of TE buffer and treated with 2 ¼l of RNase (10mg/ml unit). The sample was then keept at –20°C until needed.

**PCR Analysis**

Confirmation of transformation was conducted by PCR analysis and the amplified products were separated by agarose gel electrophoresis. A total amount of 100 ng of genomic DNA for both putative transgenic plants and untransformed control plants were used as templates. The PCR amplification was carried out using a programmed thermal cycler PTC-200. The primers used for amplification of *CRY* sequence were *CRY* Forward: 5-AGG AAG TTC ATT CAT TTG CAG-3 and *CRY* Reverse: 5-TAA CTT CGG CAG GCA CAAAC-3, respectively. The expected size of the *CRY* sequence is 1.8 kb.

**Effect of different dichlorophenoxy acetic acid (2, 4-D) concentrations in MS medium on callus induction**

Sterilized transformed cotyledons were excised from 5 weeks old *in vitro* raised nucellar seedlings and horizontally divided into two section and cultured in 350 ml culture jars containing 40 ml of MS medium supplemented with 2, 4-D (0.0, 0.5, 1, 2 or 3 mg/l). Explants were incubated in total darkness, in a growth

room 25±2°C for one month for two times. Data was recorded as percentage of callus induction and callus fresh weight (g/explant).

**Impact of MS medium supplemented with different concentrations of NAA on somatic embryos induction from callus derived from cotyledons**

To evaluate the effect of different NAA concentrations on somatic embryos induction, NAA added to the culture medium, as compared with the effect of the standard medium (without NAA addition). NAA was added to the medium at concentrations of 0.0, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/l.

The culture explants were kept under total darkness at 25±2°C. After 8 weeks, the following data were recorded for each studied treatment, *i.e.* somatic embryos formation percentage and the somatic embryos length.

**Influence of 1.00 mg/l BA combination with different concentrations of BA and Kin on shoot proliferation**

Established transgenic embryos were proliferated on MS media containing different concentrations of BA or Kin (0.00[nil], 0.50, 1.00, 1.50 and 2.00 mg/l) individually with NAA at 1 mg/l for proliferation. Explants were cultured in jars and each treatment was represented by 3 replicates, and the following characters were recorded after 30 days of culture:

Mean number of shoots formed/propagule.

Mean shoot length (cm)/propagule.

**Effect of different auxins (IBA and NAA) on root growth characters (root numbers, root length and root formation percentage) *in vitro*.**

Putative transgenic shoots derived from multiplication stage (*ca*. 3-5 cm long) were transferred to half strength MS salts and vitamins containing 1g/l activated charcoal (AC), in addition to 100 mg/l myo-inositol) and 30 g/l sucrose. For rooting, two types of auxins were tested, *viz*., IBA at five concentrations (0.00 [nil], 1.00, 1.50, 2.00 and 2.50 mg/l) and NAA at four concentrations (0.00 [nil], 1.00, 1.50, 2.00 and 2.50 mg/l). The medium was solidified with 2.7g/l phytagel. Cultures were incubated under the same conditions used for shoot proliferation.

The following traits were taken after 14 days, 24 days and 34 days of culture and each treatment consisted of 3 replicates.

Mean number of roots formed/plantlets.

Mean length of roots (cm)/ plantlets.

Percentage of rooted shoots/propagule.

**Acclimatization stage**

Transgenic plantlets were removed from culture vessels (rooted shoots) were washed, thoroughly, with running tap water to discard media residues, and treated with 0.2 % (w/v) Mon cut 25% (a, a, a-trifluoro-3-isopropoxy-o-Toluanilid) solution as a fungicide for 30 sec., then they were transplanted *ex vitro* in plastic pots (8 cm in diameter) containing soil potting mix of peat-moss and sand (v/v). Pots were covered with transparent polyethylene bags and placed in a greenhouse. One week later, the covers were removed, gradually, within one month. The percentage of survived transplants (%) was recorded. After one more month, the acclimatized plantlets were irrigated and fertilized, every week, to be ready for transplanting outside the greenhouse.

**Data collection and statistical analysis**

The data was analyzed as a one-way completely randomized design. This experiment was carried in a completely randomized design (CRD) with three replications. The data were analyzed by one-way analysis of variance, and the mean values were separated using the Fisher's least significant difference test (LSD test at 5%) (Steel and Torrie, 1980). Quantitative data was collected from the results of the Minimum Inhibitory Concentration assay. Data was represented in the form of percentage (%) of cotyledons death cell.

## Results

**Determination of minimal inhibitory concentration of kanamycin**

The results of these minimal inhibitory concentration experiments showed that, after 8 weeks on selection medium, 100 mg/l of kanamycin induced 100% death cell, whereas 75 mg/l of kanamycin induced 0.00 % death cell. Approximately 84.54% of cotyledons survived on 50 mg/l of kanamycin. Meanwhile, 100% cotyledon survived on selection medium containing 0.00 mg/l (without) kanamycin. Based on the MIC results, 100 - 150 mg/l of kanamycin are suitable as the primary selection concentrations before further analyses that was carried out to confirm the integration of foreign genes into the genome using PCR.

Regarding the effect of kanamycin concentrations on mean number of survived cotyledon, table 1 shows that, the highest number of survived cotyledon was recorded with control (30.00), while the lowest number of survival was recorded when using 100 mg/l kanamycin (0.00).

**Transformation, culture selection and explant regeneration of putative transformants**

There were four distinctive co-culture times designed

**Table 1:** Effect of kanamycin concentrations on number of survival of non- transformed cotyledons of mandarin (*Citrus reticulata* L.).

| Kanamycin concentration (mg/l) | Mean number of survived cotyledon | Survival percentage (%) |
|---|---|---|
| Control | 30.00[a] | 100.00[a] |
| 25 | 28.00[b] | 91.30[b] |
| 50 | 23.30[c] | 84.54[c] |
| 75 | 0.00[d] | 0.00[d] |
| 100 | 0.00[d] | 0.00[d] |
| 125 | 0.00[d] | 0.00[d] |
| 150 | 0.00[d] | 0.00[d] |
| L.S.D at 5% | 0.78 | 4.37 |

(12, 24, 36 and 48 hours) to contemplate the impact of co-culture time on cotyledon transformation efficiency (Fig. 2). Fig. 2 demonstrates that, the cotyledon transformation efficiency diminished first and after that increased with the expansion of co-culture time. The transformation efficiency was at a minimum (17.80%) when the cotyledon was co-refined for 12 hour. As the co-culture time went on, the transformation frequency of cotyledon increased step by step and the highest of transformation frequency was 100%, with the co-culture time being 48 hour.

With regard to the principle impact of acetosyringone, the addendum of acetosyringone gave the higher transformation rate (30.00). Transformation trials were intended to improve the successful *Agrobacterium tumefaciens* co-cultivation interval and the topmost addition (acetosyringone) for transformation.

**Determination of the presence of transgenes by PCR technique**

The transgenic nature of the regenerated plants was supported by PCR analysis for DNA obtained from the putative transgenic plants. *Agrobacterium* strain LBA4404 carrying the binary vectors pBI121 was used as a positive control for analysis. Non-transformed control

**Table 2:** Transformation efficiency of Mandarin (*Citrus reticulata* L.) after co-cultivation with *Agobacteriumtumefaciens.*

| Co-cultivation periods (Hours) | Transformation frequency | | | |
|---|---|---|---|---|
| | Number Acetosyringone | | Percentage (%) Acetosyringone | |
| | With | Without | With | Without |
| 12 | 7.67[d] | 5.33[d] | 25.57[d] | 17.80[d] |
| 24 | 20.33[c] | 19.7[c] | 67.77[c] | 65.57[c] |
| 36 | 27.33[b] | 25,0[b] | 91.10[b] | 83.33[b] |
| 48 | 30.00[a] | 28.7[a] | 100.0[a] | 95.53[a] |
| L.S.D at 5% | 1.987 | 1.613 | 6.629 | 5.376 |

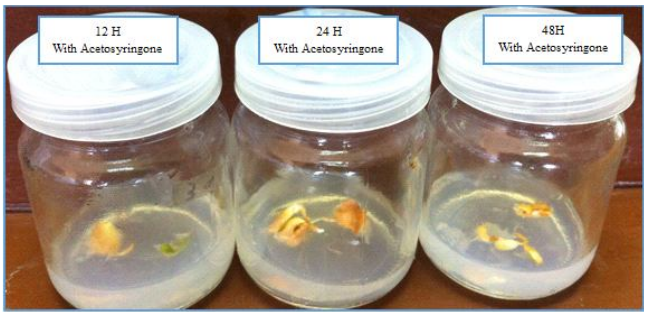


**Fig. 2:** The putative transformed mandarin (*Citrus reticulata* L.) cotyledons after 4 weeksculture on MS selective medium containing 100 mg/l kanamycin along with 250 mg/l carbenicillin.

plants show no amplification of the expected band was observed in (Fig. 3). All genes of interest were introduced into the mandarin genome and the phenotype was studied. Based on the transgenic plants produced, there were no differences in the plant morphology between untransformed and transformed plants so far.

The primer *Cry 1Ab* forward, *Cry 1Ab* reverse and NOS reversed were expected to amplify the *Cry 1Ab* and Nos gene at about 1800bp. This is

because, the primers used were based on

sequences in the *Cry 1Ab* gene and the NOS terminator, thus the DNA fragment did not

contain the NOS region could not be amplified by the PCR.

The expected 1800bp bands were presented in the sample testi n transformed cotyledons. The presence of *Cry 1Ab* in the genomic DNA of putative transformed cotyledons was indicated in (Fig. 3). In contrast, no band was observed for all control samples. Successful gene transfer into mandarin using *Agrobacterium tumefactions* was suggested as the experiments were repeated three times, giving the same result. Therefore, this further confirmed successful transfer of transgenes into the mandarin tissues. DNA of non-transformed explants was used as a negative control and DNA of pBI121 was used as a positive control as shown in (Fig. 3). However, the presence of *Cry 1Ab* in putative transformed plants is no indication of its functionality in its new environment.

**Effect of different dichlorophenoxy acetic acid (2, 4-D) concentrations in MS medium on callus induction**

**Percentage of explants induced callus:**

Data presented in table 3 and Fig. 4 revealed the greatest significant activity of explant induced callus at MS medium supplemented with 2.00

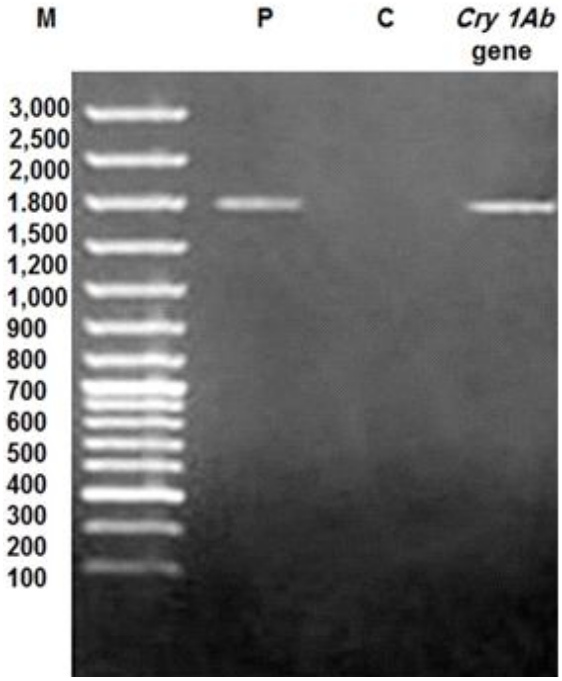


**Fig. 3:** PCR analysis of putative transgenic cotyledon of Mandarin (*Citrus reticulata* L.).
Lane M = 3 kb DNA marker;
P = transforming plasmid (pBI121);
C = nontransformed control; and *Cry1Ab* gene=Putative transformed Mandarin cotyledons.

mg/l 2, 4-D(100%), followed by 3.00 mg/l 2,4 D(86.67%). While the lowest explant induced callus was recorded with MS medium without growth regulator (control) (10%).

**Callus fresh weight/explant(g):**

Concerning the fresh weight data presented in table 3 and Fig. 4 revealed the greatest significant activity of callus fresh weight induction was recorded at the end of 8 weeks on medium containing 1.00 mg/l 2,4 D (4.33 g/culture-compact callus) followed by 2.00 mg/l 2,4-D(3.66 g/culture). On the other side the lowest fresh weight was recorded with MS medium without growth regulator (control) (1.50 g/culture-friable callus).

High callus efficiency was produced at 1.00 mg/l 2, 4-D than other concentrations table 3 and Fig.4, the color of the callus produced was whitish and yellowish white. The lower concentration of 2, 4-D (0.5mg/l) is not sufficient for callus induction (71.67%).

**Impact of MS medium supplemented with different concentrations of NAA on somatic embryos induction from callus derived from cotyledons**

Referring to the specific effect of different concentrations of NAA, on transformed mature embryos number, data showed that, embryos number was

**Table 3:** Effect of MS medium supplemented with different concentrations of 2, 4-D on callus formation from cotyledon of Mandarin (*Citrus reticulata* L.) after 45 days for two times.

| 2, 4-D Concentration (mg/l) | Callus induction percentage (%) | Callus fresh weight |
|---|---|---|
| 0.00 | 10.00$^{d}$ | 1.50$^{d}$ |
| 0.50 | 71.67$^{c}$ | 3.33$^{bc}$ |
| 1.00 | 83.33$^{b}$ | 3.66$^{b}$ |
| 2.00 | 100.00$^{a}$ | 4.33$^{a}$ |
| 3.00 | 86.67$^{b}$ | 3.16$^{c}$ |
| L.S.D. at 0.05% | 6.087 | 0.466 |

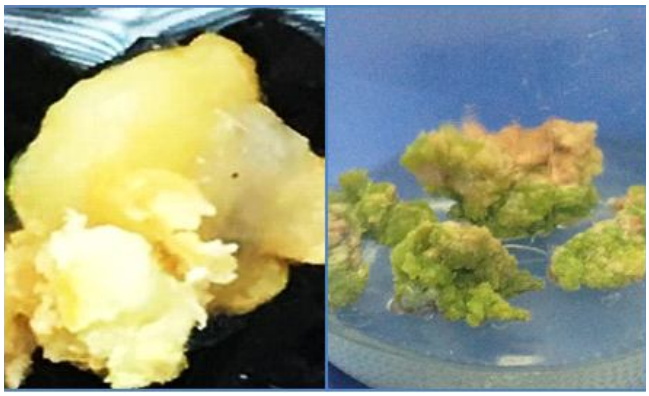

**Fig. 4:** Putatively transgenic calli and the formation of globular shape from Mandarin (*Citrus reticulata* L.) cotyledons on MS medium supplemented with 2 mg/l 2, 4-D.

significantly increased on the medium containing 1.25 mg/l NAA table 4, followed by 1.00 mg/l NAA in a descending order compared with other concentrations and control medium.

In this investigation, high frequency of differentiation was achieved at 1.25 mg/l NAA than different concentrations (Table 4 and Fig. 5).

Higher NAA levels with 1.25 mg/l BA combination showed highest positive effects on embryos number. The number of embryos produced however, was reduced with the increase in the concentration of NAA higher than 1.25 mg/l (Table 4).

Respecting "somatic embryos length", augmenting MS culture medium with NAA at 1.50 or 1.25 mg/l; led to the highest mean value (3.00 and 2.83, respectively without significant difference in between).

**Influence of 1.00 mg/l BA combination with different concentrations of BA and Kin on shoot proliferation**

Data tabulated in table 5 display the effect of MS media supplemented with various levels of both applied growth regulators *viz* BA and Kin on mean number of

**Table 4:** Effect of MS medium supplemented with 1.00 mg/l BA and different concentration of NAA on shoot differentiation from Mandarin (*Citrus reticulata* L.) callus.

| AA Concentration (mg/l) | Shoot differentiation percentage (%) | Shoot length (cm) |
|---|---|---|
| 0.0 | 0.00[e] | 0.00[d] |
| 0.5 | 41.67[d] | 1.60[c] |
| 0.75 | 50.00[c] | 2.00[b] |
| 1.0 | 55.00[b] | 2.30[b] |
| 1.25 | 65.00[a] | 2.83[a] |
| 1.5 | 43.33[d] | 3.00[a] |
| L.S.D. at 0.05% | 4.71 | 0.32 |

**Fig. 5:** Putatively transgenic somatic embryos on 0.50 mg/l NAA in Mandarin (*Citrus reticulata* L.) after three recultures.

shoots formed/propagule and mean shoot length (cm)/ propagule during shoot proliferation.

About the main effect of BA and Kin exerted very highly significant effect ($P$d $\leq$0.05) on the given trait. For instance, augmenting MS medium with BA at 2.00 and 1.50 mg/l, respectively; resulted in the highest mean values as 48.33 and 45, in series.

Regarding mean shoot length (cm)/propagule, fortifying MS medium with Kin at 2.00 mg/l, recorded the highest mean values of the given trait as 6.50, and augmenting MS with BA at 2.00 mg/l, resulted in the lowest mean value (1.16).

**Effect of different auxins (IBA and NAA) on root growth characters (root numbers, root length and root formation percentage) *in vitro***

Results outlined in table 6 and Fig. 7 disclosed the effect of auxin concentration on *in vitro* rooting on mean

**Table 5:** Effect of MS medium supplemented with 1.00 mg/l NAA and combination with Kin and BA at different concentration on shoot proliferation of Mandarin (*Citrus reticulata* L.).

| Cytokinin | Concentration | Shoot number | Shoot length |
|---|---|---|---|
| BA | 0.00 | 20.00[e] | 4.167[c] |
| | 0.50 | 28.67[c] | 3.667[d] |
| | 1.00 | 40.33[b] | 3.000[e] |
| | 1.50 | 45.00[a] | 2.333[f] |
| | 2.00 | 48.33[a] | 1.167[g] |
| Kin | 0.50 | 14.00[f] | 4.500[c] |
| | 1.00 | 20.33[e] | 5.167[b] |
| | 1.50 | 23.67[de] | 5.500[b] |
| | 2.00 | 26.33[cd] | 6.500[a] |
| L.S.D. at 0.05% | | 3.899 | 0.486 |

**Fig. 6:** Proliferation of putatively transgenic somatic embryos of Mandarin (*Citrus reticulata* L.) cultured on MS medium and the best combination 1.00 mg/l NAA plus 2.00 mg/l BA.

number of roots formed/plantlets, mean length of roots (cm)/ plantlets and percentage of rooted shoots/propagule of Mandarin (*Citrus reticulata* L.) during rooting stage.

As for mean number of roots, the main effect of auxin concentration showed that IBA exerted very highly significant effect ($P \leq 0.05$) on the given trait; whereas, augmenting MS medium with 2.00 mg/l, brought about the highest mean numbers of roots/ shoot either 6.33.

Respecting the auxin concentration used with mean length of roots (cm)/ plantlets, the main effect of IBA, showed a very highly significant effect ($P \leq 0.05$) on the given trait, especially when MS medium was augmented with NAA at 2.00 mg/l, which recorded the highest length of roots (cm)/ plantlets as 6.50 compare to the other treatments.

Regarding the auxin concentration used with percentage of rooted shoots/propagule, the main effect

**Table 6:** Effect of MS medium supplementing with IBA and NAA (mg/l) on mean root number, mean length of roots (cm)/plantlets and mean root formation percentage during rooting stage of Mandarin (*Citrus reticulata* L.).

| Auxin | Concentration (mg/l) | Root number | Root length (cm) | Root formation percentage(%) |
|---|---|---|---|---|
| NAA | 0.00 | 1.00$^{f}$ | 2.60$^{f}$ | 25.00$^{f}$ |
| | 0.50 | 2.33$^{e}$ | 4.73$^{c}$ | 68.33$^{d}$ |
| | 1.00 | 3.66$^{cd}$ | 5.63$^{b}$ | 78.33$^{bc}$ |
| | 1.50 | 5.33$^{b}$ | 5.83$^{b}$ | 96.00$^{a}$ |
| | 2.00 | 6.33$^{a}$ | 6.50$^{a}$ | 100.0$^{a}$ |
| IBA | 0.50 | 1.00$^{f}$ | 3.16$^{e}$ | 61.67$^{e}$ |
| | 1.00 | 2.33$^{e}$ | 3.50$^{e}$ | 65.00$^{de}$ |
| | 1.50 | 3.33$^{d}$ | 4.00$^{d}$ | 75.00$^{c}$ |
| | 2.00 | 4.33$^{c}$ | 4.36$^{cd}$ | 81.67$^{b}$ |
| L.S.D. at 0.05% | | 0.806 | 0.480 | 4.374 |

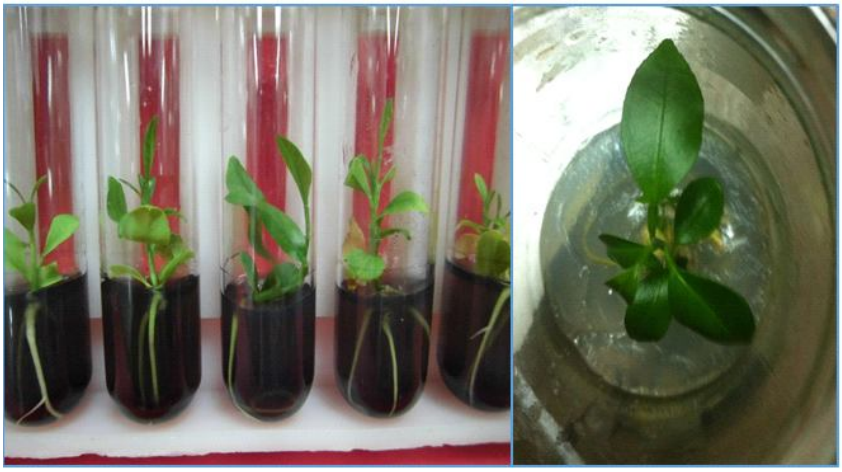

**Fig. 7:** Further growth and root development of transgenic Mandarin (*Citrus reticulata* L.) plantlets on 2.00 mg/l NAA before transferring to soil.

**Fig. 8:** Ex vitro acclimatization of transgenic Mandarin (*Citrus reticulata* L.).

of IBA, showed a very highly significant effect ($P \leq 0.05$) on the given trait, especially when MS medium was augmented with IBA at either 2.00 or 1.50 mg/l, which recorded the highest percentage of rooted shoots/ propagule as either 100% or 96% without significant difference and compare to the other treatments.

## Discussion

One of the main purposes of breeding programs is the development of novel *Citrus* varieties with improved quality and resistance to biotic and abiotic stresses. Thus far, due to the many limitations of typical of tree crops, such as the long juvenility and high heterozygosity, conventional breeding techniques in *Citrus* has been shown to be time consuming and difficult. These problems can be solved by using New Plant Breeding Techniques (NPBTs) which offering new tools that combine site-specific and targeted editing with a reduction in plant breeding time, thus leading to lower production costs (Poles *et al.,* 2020). Genes that confer resistance to selective chemical agents, such as antibiotics or herbicides that are usually co-transformed with a gene of interest are performed in most transformation systems, identification and selection of transgenic shoots. (Orbovicand Grosser 2015; Orbovic *et al.,* 2015); in *Citrus*npt II (neomycin phospho transferase II from *E. coli*), which is commonly used confers resistance to the antibiotic kanamycin, (Peña *et al.,* 2008).

In *Citrus* transformation, it is critical to find suitable selective agents to recover transformed cells, in order to eliminate the high numbers of chimeras and escapes that can be obtained during the process (Gutierrez-E *et al.,* 1997; Moore *et al.,* 1992; Peña *et al.,* 1995a).

Obtaining transgenic lines success greatly depends on the selected procedure adopted. Small numbers of putatively transgenic cells in a large population of non-transformed cells to undergo multiplication is allowed by a slow kill which is preferred over an immediate kill. Hence, to improve transformation recovery an effective selective agent is required. Selection is usually based on antibiotic or herbicide resistance. One of the most widely used selective antibiotics in transformation processes and is most effective when used in concentrations of up to

100 mg/l is Kanamycin. However, at this concentration shoot regeneration may be inhibited. Other antibiotics are not as effective as kanamycin such as geneticin and hygromycin which have also been used, (Costa *et al.,* 2002; Peña *et al.,* 1997). Whether, residual Agrobacterium cells are present or neighboring transformed cells result in the break down or neutralization of the antibiotic the selective antibiotic can be ineffective in these situations.

As a selective agent for transformation Kanamycin was chosen due to higher sensitivity most plants tissue to it (Meijer *et al.,* 1991; Arencibia *et al.,* 1998; Lee *et al.,* 1999; Yara *et al.,* 2001). In the present study, the minimal inhibitory concentration (MIC) was determined for normal, non-transformed cotyledons of Mandarin (*Citrus reticulata* L.). The purpose of conducting these MIC experiment was to determine the minimal level of kanamycin, which could induce cell death in non-transformed cotyledon. MIC is usually the choice of experiment to determine the antibiotic levels which either exert a stringent selection pressure or exert a non-stringent selection pressure for the screening of transformed cotyledon in a population of both transformed and non-transformed cotyledon.

Low yield of kanamycin-resistant cotyledons can be noticed often by transferring cotyledons immediately onto the selection medium after bacterial infection. In the transformation experiments of *Citrus* cotyledons, to allow a slow kill of the untransformed callus, 100 mg/l kanamycin was used in the selection medium.

Kanamycin acts by involving the synthesis of protein. Kanamycin binds the bacterial ribosome through subunit 30g. These results are not true with the mRNA, and finally lead to a misread that causes the incorrect amino acid to be placed into the peptide, thus leading to nonfunctional peptide chains. The success of obtaining transgenic lines greatly depends on the selection procedure adopted. Small numbers of putatively transgenic cells in a large population of non-transformed cells to undergo multiplication is allowed by a slow kill which is preferred overan immediate kill. The transfer of cotyledons immediately onto the selection medium after bacterial infection often resulted in a low yield of kanamycin-resistant cotyledon.

Developing efficient methods for transformation of some plants via *Agrobacterium tumefaciens* was made for many years attempts. It is a soil bacteria which possess a natural ability to infect plants in places of injures which results in arise of cancerous growths (crown gall). This is possible thanks a transfer of fragment of Ti plasmid into plant cells and stable integration with a plant genome. Many factors affect the efficiency of plant transformation that depends on for example: *Agrobacterium* strain, methods and procedures of transformation as well as on plant species, type and age of the explants and regeneration conditions. Increasing the amount of naturally occurring bioactive compounds and the production of biopharmaceuticals is the main goal of plant transformation. Genetic plant transformation using genus *Agrobacterium* is a complex process which requires detailed analysis of incorporated transgene expression and occurs only in the case when the plant cell acquires the ability to regenerate. After applied transformation procedures, the regeneration efficiency observed in medicinal plants are inefficient in many cases (Bandurska *et al.,* 2016).

Different techniques of plant transformation have been created to increase the productivity of change and to accomplish stable expressing of transgenes in plants through the proceed of A. *tumefaciens*-mediated genetic trans-formation in plant, a couple of days of co-culture were required for *Agrobacterium* to contaminate the plant cell; consequently, there was no doubt that the co-culture is an important part for *Agrobacteriumtumefaciens*-mediated transformation and the co-culture time is one of the most imperative variables influencing transformation efficiency in co-culture.

For enabling efficient gene transfer in both monocot and dicots *Agrobacterium*-mediated gene transfer is one such strategy which is well known for that purpose. Because of its versatility, this technique underwent several advancements including development of improved in vitro plant regeneration system, co-cultivation and selection methods, and use of hyper-virulent strains of *Agrobacterium tumefacien s*harbouring super-binary vectors. Using acetosyringone has also been enhanced the efficiency of this method to induce the activity of vir genes, to reduce the *Agrobacterium*-induced necrosis silver nitrate was used while cysteine was used to avoid callus browning during co-cultivation.

Transformation experiments have been carried out using *Agrobacterium*-mediated transformation with numerous hybrids and species of *Citrus*, such as grapefruit, sour orange, sweet orange, trifoliate orange (*Poncirustrifoliata* Raf.), 'Carrizo' citrange, 'Mexican' lime, 'Swingle' citrumelo (*C. paradisi*x *P. trifoliata*), 'Cleopatra' Mandarin, and alemow (*C. macrophylla* Wester) (Dominguez *et al.,* 2000; Ghorbel*et al.,* 2000; Gutierrez-E *et al.,* 1997; Luth & Moore, 1999; Molinari *et al.,* 2004; Moore *et al.,* 1992; Peña *et al.,* 2004, 2007). With the existing protocols transformation of other

economically important *Citrus* cultivars has not yet been successful. Generally, the efficiency transformation protocol of the most *Citrus* cultivars can range from 0 to 45% by using *Agrobacterium.* This is because of a number of limiting factors that can affect the transformation protocol. These include: species or cultivar specificity, type and age of the used explant, *Citrus* cells or tissues competence, strains of *Agrobacterium that were* used and procedure of inoculation, conditions of co-cultivation and pre-culturing, adequate selection conditions and transgenic shoots recovery (Bond and Roose, 1998; Costa *et al.,* 2002; Peña *et al.,* 2007; Yu *et al.,* 2002). Optimizing several factors affecting regeneration, including the quality of the starting plant material, and the composition of the plant growth regulators used during selective regeneration improve the transformation efficiencies. (Wang *et al.,* 2020).

Gene construct that was inserted into the crop, which could be promoter, terminator, structural gene and/or marker gene was detected by PCR.

PCR has confirmed to be a quick and functional and it has to a great extent substituted bioassays in primer grouping of *B. thuringiensis* accumulations. The experience was recurrent three times and a similar outcome was watched, demonstrating that the outside quality was effectively moved into Mandarin (*Citrus reticulata* L.).

A molecular tool broadly used to portray the insecticidal bacterium *Bacillus thuringiensis* is the polymerase chain response (PCR). To enhance. Particular DNA pieces and along these lines to decide the existence or nonattendance of a specific gene this procedure can be utilize. The recognizable proof of *B. thuringiensis* toxin genes by PCR can incompletely anticipate the insecticidal action of a given strain (Porcar and Juarez Perez, 2003).

Several biological and scientific advantages were known for somatic embryogenesis. For instance, plants of commercial importance, it has the potential for its improvement, as well as for the study of the genetic and physiological changes that are related to the fate of a plant cell (Méndez-Hernández *et al.,* 2019).

Uniform in the examples are the external prerequisites for a somatic embryogenesis. It is necessary that the cultures are in the presence of an auxin (particulary suitable is 2, 4-D) at least for a certain period. It is necessary that the cultures are in the presence of an auxin (particulary suitable is 2,4-0) - at least for a certain period (Kohlenbach, 1977).

On this context, somatic embryogenesis induction deals with the primary transformation of vacuolated, parenchymatical cells into densely cytoplasmatic cells with an embryogenic determination. This is, according to all the evidence in literature, only possible in the presence of auxin. Low auxin concentrations (even if they remain present during further culture) allow a more or less direct process of embryo formation. The auxin necessary for the induction of the embryogenic state hinders its defined development as the concentration rises. High auxin concentrations give rise, from the very outset, solely to disorganized embryogenic clumps which only when the auxin has been omitted can develop into organized embryos. This latter process, however, has according to all the foregoing no relation to the induction. It can, therefore, be at least misleading to refer, in connection with the somatic embryogenesis, to a 2,4-D-free medium as inductive and a medium with a 2,4-D content as non-inductive (Matsumoto *et al.,* 1975). It is just as misleading to state that the somatic embryogenesis needs no exogenous auxin (Kartha, 1975).

The results are in conformity with some of the earlier studies on different *Citrus* spp. which showed optimum callus induction response under the influence of in different concentrations 2,4-D. Altaf and Khan, (2009) reported that hormonal combination for good callus induction for seedling leaf of kinnow Mandarin is BA with $GA_3$ (each at 1 mg/l), 2,4-D at 0.5 mg/l and proline at 5 mg/l. Amin and Shekafandeh (2015) found that MS medium supplemented with 0.5mg/l 2,4-D induced maximum embryogenic calli of mexican lime. The best response for primary callus induction of Kinnow Mandarin (90%) was obtained when MS medium was supplemented with 5 mg/l 2, 4-D and 500 mg/l malt extract (Hussain *et al.,* 2016). Mahadi *et al*. (2016) showed that the best a combination of hormones is treatment D2B2 (2 mg/l, 2, 4-D, dan 2 mg/l BAP) producing embryogenic callus of Calamansi (*Citrus microcarpa*).

Amgai *et al.,* (2016) found that shooting from explants was significantly higher (71.72%) on medium level of the BAP (0.5 mg/l) and IAA (0.2 mg/l) using *in-vitro* seedling stem as explant of Mandarin orange. Badr-Elden (2017) showed that Regenerated shoots raised from juice vesicles showed better shoot multiplication and highest length of shoots on MS medium with 2 mg/l BA and 0.4mg/l NAA.

Our results are similar to findings of explants of mature embryos from *Citrus aurantium* var. amara that were cultured on MS medium supplemented with 2 mg/l BAP. Mature embryos could produce multiple shoot- buds (average 2.6 per seedling), hypocotyls of a few seedling

could also from many adventitious buds, Guo Quansheng, (1997). The morphogenetic response related to the epicotyls region and the presence of BAP were genotype-influenced, the presence of BA in the culture medium gave higher number of responsive explants for Citrange. Addition of BAP to the culture medium; provided an increase in the number of shoots per explants for Citrange, Sweet orange and lemon cv. "Volkamer" (Schinor*et al.,* 2006). Recently, (Chamandoosti, 2017) demonstrated that the best result for shoot multiplication an dregeneration was 3.2 and 2.6 shoots per explants with 4.44 µM BA plus 0.053 µM NAA and 4.44 µM BA plus 0.049 µM IBA respectively.

The obtained results in this study are close to those of HuXinXI *et al*., (2007) who indicated that about 97.7% of the adventitious shoots from *Citrus sinensis* was rooted in medium comprise 1/2 MS plus 3% sucrose, 0.7% agar and 2.0 mg/l IBA, pH 5.8. Also, (Kamal *et al.,* 2013) reported that regenerated shoots of *Citrus limon* L. induced roots on MS medium containing 1.0 mg/l IBA. The highest rooting% of 76 and 72 % were scored for MS supplemented with 0.5 or 1 mg $L^{-1}$ NAA, respectively for sweet orange (*Citrus sinensis* L. Osbeck.) (Esmaeilniaand Dehestani, 2015).

Mollika *et al.,* (2020) showed that *in vitro* 80 % rooting of raised shoots in 5.66 days, with highest number of roots (6.16 per shoot) and longest root (3.78cm) of *Citrus indica* when cultured on half strength MS medium supplemented with NAA (1.0 mg/l).

Vigours healthy plantlets of Mandarin (*Citrus reticulata* L.) cultivar were transferred successfully to greenhouse, with "100%" survival percentage.

## Conclusion

An efficient transformation protocol has been established and successfully used to transfer genes of interest (*Cry1Ab* gene) into cotyledons of mandarin (*Citrus reticulata* L.). This protocol does not depend on seedlings, which have genetic variability and the value of which has not been tested when used for transformation. Instead it is based on the regeneration via somatic embryogenesis starting from explants of adult plants which is highly efficient and connected to low percentages of aberrations in some genotypes (Schwenkel and Winkelmann, 1998).

Many factors are very important for the success of transformation procedure, we regard the following: highly embryogenic cell lines, to induce the activity of vir genes acetosyringone should be used, to reduce the *Agrobacterium*-induced necrosis silver nitrate should be used, using cysteine to avoid callus browning, the use of Silwet L-77 as a surfactant during co-culture, kanamycin appropriate selection, and *Agrobacteriumtumefaciens* strain LBA4404. Because somatic embryos develop from few or even single cells within embryogenic callus cultures, we assume a low risk of chimeric regenerants.

Callus induction best results were observed from cotyledons 100% on MS medium supplemented with 2.00 or 3.00 mg/l 2, 4-D. The highest regeneration efficiency and weight of callus observed by adding with 1.00 mg/l BA plus 1.25mg/l NAA. Mandarin shoots (*Citrus reticulata* L.) were then rooted on MS medium containing IBA and NAA at 2.00 mg/l, with high rooting percentage (100%).

The results of this research have provided an insight into the co-cultivation period effect on the transformation of mandarin (*Citrus reticulata* L.) using *Agrobacterium tumefaciens*.

Acetosyringone as a chemical was recommended in most of the transformation of mandarin. Incubation of explants with *Agrobacterium tumefaciens* with the addition of acetosyringone for 48 h was found effective in transgenic plants production. After co-cultivation periods, Cotyledon was transferred to MS medium with 2.00 mg/l 2, 4-D, 250 mg/carbenicillin and 100 mg/l kanamycin for detection of transgenic cotyledon. Polymerase chain reaction (PCR) was used for the rapid screening of *Cry 1Ab* gene. For screening, total genomic DNA was isolated from transformants.

Using primer specific to *Cry1Ab* gene (forward and reverse), a PCR result with a size of about 1,800 bp was magnified when the all nucleic acid from the transformants were utilized as templates. PCR analysis confirmed the appearance of transgene of 1,800 bp in one many plantlets. These results signify the successful introduction of *Cry1Ab* gene into mandarin (*Citrus reticulata* L.) plant.

## "List of Abbreviations"

MS medium: Murashige and Skoog ; MIC: minimal inhibitory concentration ; npt-II gene: kanamycin resistance; BA: Benzyl adenine ; NAA: ² – naphthalene acetic acid ; Kin: Kinetin ; IBA: Indol-3-butyric acid ; 2, 4- D: 2, 4- Dichlorophenoxy acetic acid; AC :activated charcoal ; NPBTs: New Plant Breeding Techniques ; pH : Potential of hydrogen ion ; NaOCl: Sodium hypochlorite ; OD : optical density ; LB : Luria-Bertaini.

**Declarations:**

**Ethics approval and consent to participate**

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are included in this published.

## Competing interests

The authors declare that they have no competing interests.

**Funding**

No fund available.

Authors' contributions

AMBE, MEAEA and AAAN designed and conducted the experiment treatments, AMBE and AAAN carried out statistical analysis, MEAEA and AAAN were responsible for acclimatization stage. EAMA and AMBE wrote the manuscript and reviewed the paper. All the authors have read and approved the final manuscript.

## Acknowledgements

The authors thank Prof. I.Altosaar, University of Ottawa, Canada, for providing Agrobacterium strain.

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