Article

# *In vitro* propagation of apple tree from mature zygotic embryos

Juan Pablo Cabral-Miramontes[1]
Jorge Armando Chávez-Simental[2§]
Cecilia Pulido-Díaz[2]
Manuel González-Portillo[3]
José Rodolfo Goche-Télles[4]
Víctor Manuel Barragán-Hernández[2]

[1]Institutional Master's Degree in Agricultural and Forestry Sciences-Juarez University of the State of Durango. Durango, México. (juan.cabralmiramontes@gmail.com). [2]Institute of Forestry and Wood Industry-Juarez University of the State of Durango. Durango, México. (ceccy_ndd@hotmail.com; vmbh70@yahoo.com.mx). [3]Faculty of Chemical Sciences-Juarez University of the State of Durango. Durango, México. (mgportillo2000@yahoo.com). [4]Faculty of Forest Sciences-Juarez University of the State of Durango. Durango, México. (jgoche@ujed.mx).

[§]Corresponding author: jorge.chavez@ujed.mx.

## Abstract

The municipality of Nuevo Ideal is one of the most important regions for apple production in the state of Durango, Mexico. There are scattered *Malus domestica* trees from extinct orchards that were part of a production system and that today are abandoned without agronomic management, but that have shown an efficient adaptability to the conditions and continue to produce good quality fruit. The objective of this study was to develop an *in vitro* propagation protocol of *M. domestica* using seeds of feral trees from this region. Germination and production of adventitious shoots were evaluated using Murashige & Skoog (MS) medium and woody plant medium (WPM) supplemented with phytohormones 6-Benzylaminopurine (BAP) and indole butyric acid (IBA) at different doses. For rooting, indole acetic acid (IAA), naphthaleneacetic acid (NAA) and kinetin (KIN) were also used in combination with the previous ones. Germination and shoot formation obtained better results with the concentration of 0.5 mg $L^{-1}$ of BAP in MS medium at 60 days. In the leaf development, the treatment with 1.5 mg $L^{-1}$ of BAP in MS medium stood out, with 21.07 leaves on average. The plants of all the treatments had roots, however, the best development was shown by the treatment with 1.5 mg $L^{-1}$ of NAA and 0.15 mg $L^{-1}$ of BAP in WPM medium. Through the protocol generated in this research, it is possible to massively propagate the *M. domestica* species for purposes of germplasm conservation and subsequent exploitation of the crop.

**Keywords:** culture of plant tissues, micropropagation of fruit trees, vitroplants.

Reception date: March 2022
Acceptance date: May 2022

## Introduction

In the state of Durango there are 48 486 ha destined for perennial crops, of which 13.33% are used to produce apples, which places this perennial crop in the third place of importance after green alfalfa and walnut. In 2019, the apple harvested area obtained a total production of 21 020 t of different cultivars, such as: Delicous, Starking, Doble red, Top red, Winter banana and Winter pearmain, concentrated mainly in the municipalities of Canatlán, Nuevo Ideal and Santiago Papasquiaro (FAO, 2014; SIAP, 2019).

The micropropagation of perennial and temporary crops has contributed to the development of agronomic production in recent decades. Thanks to this technique, clones of outstanding varieties can be generated in large quantities and with additional benefits (Hoyos *et al*., 2008). The *in vitro* organogenesis technique is a process during which organs, buds, shoots and roots are produced from plant tissues grown in an artificial medium of defined chemical composition and incubated under controlled environmental conditions (Zhao *et al*., 2008; Levitus *et al*., 2010).

According to Dobránszki and Teixeira Da Silva (2010), cited by Teixeira da Silva *et al.* (2019), the *in vitro* propagation of apple trees, as in other species, includes four stages; the first stage consists of the *in vitro* establishment of cultures from living tissue of donor plants. At this stage, it is important to consider the phytosanitary conditions of the donor plant, since it may contain agents that cause contamination, coupled with the fact that the selection of the plant tissue to be used must have an optimal physiological state due to the capacity of totipotency and differentiation of tissues. Stage two refers to the regeneration or multiplication of adventitious shoots. In woody species such as the genus *Malus*, tissue oxidation is common, exhibiting blackening of cells, tissue and plant organs.

When cuts are made in the explant, oxidative stress is produced caused by the metabolism of oxygen that produces free radicals known as reactive oxygen species (ROS) and these in turn can be generated in other cellular organelles, such as peroxisomes and lysosomes, as a result of the cuts made in the explants that cause oxidation in the area of the cut (Azofeifa, 2009). This stage is where a large number of disease-free clones are generated in a short period of time, therefore, overcoming this *in vitro* stage is of vital importance (Pancaningtyas, 2015).

Stage three consists of the rooting of the shoots, which determines the success and survival of the plants due to the absorption of nutrients and the stability it provides to the specimens, and finally stage four, which consists of acclimatizing the plants generated *in vitro* and their establishment in an open environment ready to be planted in the field, a procedure that consists of subjecting the plant from an aseptic environment to simulation of an endemic environment (Tandon *et al*., 2021).

The establishment of mass multiplication protocols for different species of fruit trees through embryogenesis of mature zygotes from seed is an alternative where it is common to use growth regulators (auxins/cytokinins) to promote vegetative development (Dobránszki and Teixeira Da Silva, 2010). However, *in vitro* techniques are expensive as they require skilled labor, as well as specific equipment and the high cost of plant material, so the effectiveness of any *in vitro* propagation protocol should be optimized and improved whenever possible (Teixeira *et al*., 2019).

In the municipality of Nuevo Ideal, Durango, there are specimens of this species considered as 'feral' because they come from plant material selected from orchards that were productive for several years and are now abandoned. The trees that still exist in a very dispersed way show an adaptation to the conditions where they are located without receiving any type of agronomic management such as fertilization, irrigation, pruning, among others, and they still have an acceptable production of apple of good quality that sometimes even exceeds the quality of the orchards currently in production.

Therefore, in order to preserve the genetic quality of this species, the present study was developed with the aim of generating an efficient protocol as a strategy for the conservation and *in vitro* propagation of *Malus domestica* using seeds from these trees from the region of Nuevo Ideal, Durango, as initial material.

## Materials and methods

This work was conducted in the Laboratory of Genetics and Plant Production of the Institute of Silviculture and Wood Industry of the Juárez University of the State of Durango.

### Plant material

The apple tree is commonly propagated by means of techniques such as rooting of cuttings, grafts, among others; however, it was decided to implement the plant tissue culture technique due to the remoteness of the donor trees and the difficulty of following up on any of the techniques mentioned. With this technique, different sources of plant material can be used, such as buds, explants, among others, however, in this research, it was decided to use of mature zygotic embryos from the seed.

The obtaining of plant material was made from the selection of apple (*Malus domestica*) fruits with desired phenotypic characteristics, visibly healthy and without pests, collected from feral trees from the municipality of Nuevo Ideal, Durango, during the harvest time of 2015. The seeds were extracted in the laboratory and kept in refrigeration at a temperature of 4 °C in hermetically sealed sterile bottles. For its establishment in culture medium, the seed was previously disinfected with 70% ethanol, 20% sodium hypochlorite and sterile distilled water. The experiment was developed in two phases: a) germination and production of shoots; and b) rooting.

### Germination and production of shoots

A cut was made on the longitudinal part of the testa of the seed, to immediately remove the tegmen and leave the embryo and cotyledons exposed in direct contact with the culture medium. Fourteen treatments composed of 25 repetitions each were proposed to evaluate germination and the production of adventitious shoots, for which Murashige and Skoog (1962) (MS) medium and woody plant medium (WPM) (Mc Cown and Lloyd, 1981) were used, complemented with growth regulators such as 6-Benzylaminopurine (BAP) as cytokinin and indole butyric acid (IBA) as auxin at different doses. Two control treatments were considered, one of each culture medium without the addition of growth regulators (T1 and T8) (Table 1). Twenty-five grams of sucrose per liter and 5.5 g $L^{-1}$ of gelling agent (agar) were added to the culture medium, adjusting the pH to 5.7 before being sterilized in autoclave for 20 min at 115 °C and 1.5 kg $cm^{-2}$.

**Table 1. Treatments evaluated in the *in vitro* germination and production of shoots of *Malus domestica*.**

| Treatment | Culture medium | BAP (mg $L^{-1}$) | WAS (mg $L^{-1}$) |
|---|---|---|---|
| T1 | MS | - | - |
| T2 | MS | 0.5 | - |
| T3 | MS | 1.5 | - |
| T4 | MS | - | 0.5 |
| T5 | MS | - | 1.5 |
| T6 | MS | 0.5 | 0.5 |
| T7 | MS | 1.5 | 1.5 |
| T8 | WPM | - | - |
| T9 | WPM | 0.5 | - |
| T10 | WPM | 1.5 | - |
| T11 | WPM | - | 0.5 |
| T12 | WPM | - | 1.5 |
| T13 | WPM | 0.5 | 0.5 |
| T14 | WPM | 1.5 | 1.5 |

The establishment was performed under aseptic conditions using a laminar flow chamber and sterile instruments. The embryos were placed in glass bottles with 25 ml of culture medium corresponding to the treatments. Subsequently, they were transferred to the culture chamber in dark conditions during the first seven days, starting on day eight, they underwent a photoperiod of 16 h light and eight h of darkness at a constant temperature of 25 ±2 ºC. The variables evaluated at this stage were: percentage of germination, number of shoots, seedling height (mm), leaf development (number of leaves) and stem diameter (mm) by morphological analysis applying the technique of image analysis described by González *et al*. (2005).

## Rooting

At 60 days, the shoots produced in the previous stage were brought to the rooting phase. They were established in culture medium MS and WPM reduced to 50% of their nutrients as described by Guadie (2011), added with different doses of growth regulators based on auxins and cytokinins, such as: naphthaleneacetic acid (NAA), indole acetic acid (IAA) and indole butyric acid (IBA), with or without combination with cytokinins: 6-benzylaminopurine (BAP) and kinetin (KIN). Two control treatments were considered, one of each culture medium without the addition of growth regulators (T1 and T8) (Table 2). The variables evaluated were: rooting percentage and root length (mm) by morphological analysis 30 days after the start of this phase.

**Table 2. Treatments evaluated for *in vitro* rooting of *Malus domestica.***

| Treatment | Culture medium | NAA (mg $L^{-1}$) | BAP (mg $L^{-1}$) | IAA (mg $L^{-1}$) | IBA (mg $L^{-1}$) | KIN (mg $L^{-1}$) |
|---|---|---|---|---|---|---|
| T1 | MS | - | - | - | - | - |
| T2 | MS | 0.5 | 0.05 | - | - | - |
| T3 | MS | 1.5 | 0.15 | - | - | - |
| T4 | MS | - | - | - | 0.5 | 0.05 |
| T5 | MS | - | - | - | 1.5 | 0.15 |
| T6 | MS | - | - | 0.5 | - | 0.05 |
| T7 | MS | - | 0.15 | 1.5 | - | - |
| T8 | WPM | - | - | - | - | - |
| T9 | WPM | 0.5 | 0.05 | - | - | - |
| T10 | WPM | 1.5 | 0.15 | - | - | - |
| T11 | WPM | - | - | - | 0.5 | 0.05 |
| T12 | WPM | - | - | - | 1.5 | 0.15 |
| T13 | WPM | - | - | 0.5 | - | 0.05 |
| T14 | WPM | - | 0.15 | 1.5 | - | - |

### Statistical analysis

A completely randomized experimental design with 14 treatments and 25 replicates each was used, the results were evaluated using the Kruskall-Wallis statistical analysis. The data obtained were subjected to the analysis of variance with the Wilcoxon test, with the statistical package R version 3.2.2.

## Results and discussion

### Germination and formation of shoots

The average germination obtained in the treatments was 32.3% 60 days after the *in vitro* establishment of the seed. Nevertheless, the highest percentage of germinated seeds (56%) was obtained with the treatment composed of MS culture medium supplemented with 0.5 mg $L^{-1}$ of BAP (T2), which is consistent with the research developed by Rustaei *et al*. (2009), who worked with *Malus domestica*. Because wild donor plants were used in this research, which lacked rigorous health conditions, the disinfection protocol was not as effective in some treatments because there was an average of 31.7% contamination by unidentified fungi and bacteria. This coincides with what is established by Da Câmara *et al*. (1991); Keresa *et al*. (2012), who argue that the establishment of plant material depends largely on the phytosanitary care of seed donor plants (Figure 1).

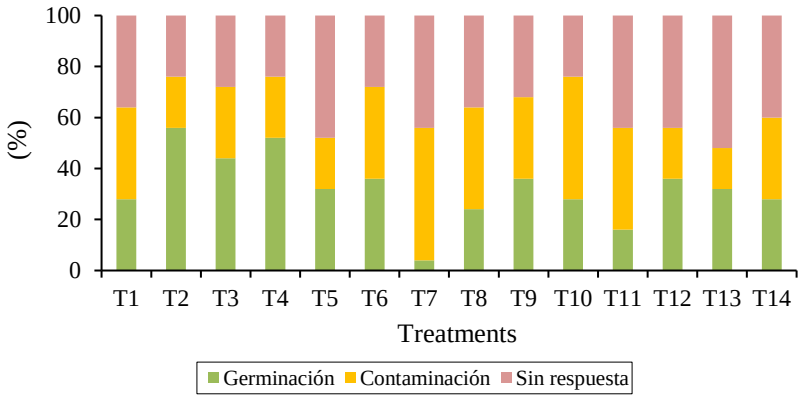


**Figure 1. Percentage of germination, contamination and unresponsive seed of *M. domestica* 60 days after the establishment in culture medium.**

The situation observed in this experiment coincides with what was argued by Soni *et al.* (2011), who point out that it is less likely to find contaminated explants collected in summer, while explants collected in spring, autumn and winter show a higher percentage of contamination, which ranges from 12 to 54%. Therefore, it is important to implement preventive disinfection treatments of the donor plant, because despite the abundant existence of asepsis protocols, there are microorganisms of difficult control that can get to lodge in the internal parts of the fruit and vascular bundles due to the presence of endogenous contaminants acquired by the plant in its natural environment, which explains the incidence of contaminants in this experiment.

The results indicated good shoot induction; however, variation in their number according to the type and dose of the growth regulator applied was detected. The dose of 1.5 mg $L^{-1}$ of BAP (T3 and T10) had the highest number of shoots per explant (36 shoots each, respectively) (Figure 2 y 3) regardless of the culture medium in which it was established.

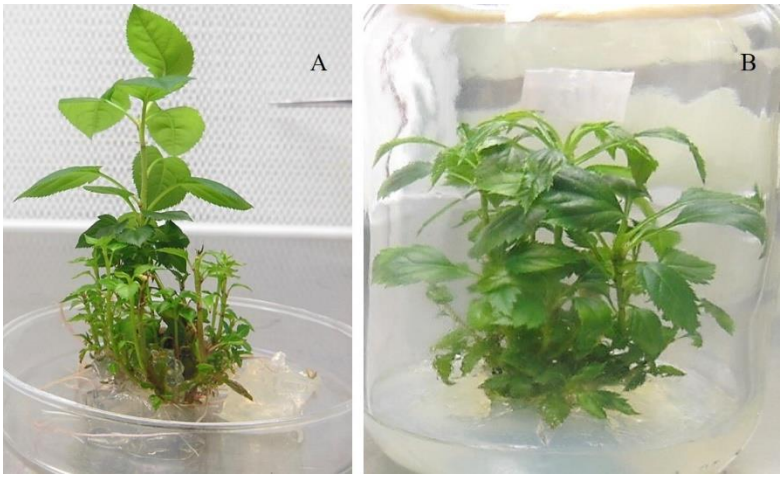


**Figure 2. *In vitro* production of adventitious shoots of *M. domestica* in two culture media 60 days after establishment: A) T3 (MS with 1.5 mg $L^{-1}$ of BAP); B) T10 (WPM with 1.5 mg $L^{-1}$ of BAP).**

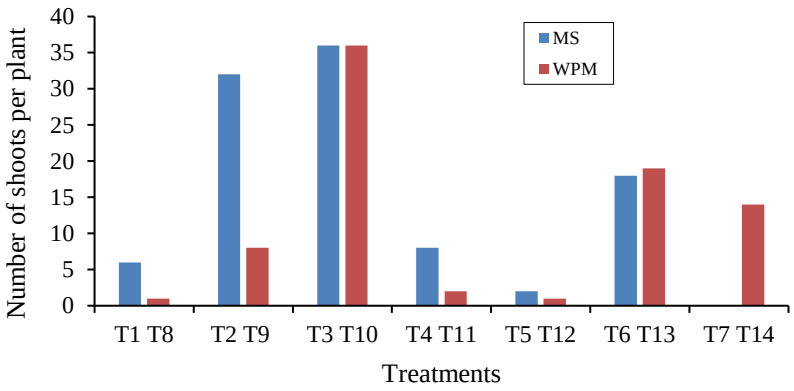


**Figure 3. Number of shoots per plant of *M. domestica* produced *in vitro* in two culture media and different doses of growth regulators.**

Although the response was the same in both media with the indicated dose, it was observed that the culture medium significantly influenced vegetative development, since, in the variables of plant height, stem diameter and number of leaves, the MS culture medium exhibited the best results (Figures 4, 5 and 6). Some authors have proposed different doses of growth regulators in the culture media evaluated here (Vilchez *et al*., 2014; Montes-Salazar *et al*., 2016), where it is argued that the WPM culture medium is used for woody species (Mc Cown and Lloyd, 1981). However, the MS culture medium is considered a broad-spectrum basal medium that can be used in different types of plants with favorable results. Unlike other studies on *in vitro* propagation in species, in this work, the WPM culture medium was surpassed by the MS culture medium in most of the variables evaluated.

Hoyos *et al.* (2008) reported that this hormone stimulates the development of lateral shoots, cell multiplication in apical meristems and leaf expansion. Likewise, they argue that, since it does not have roots, the explant does not have the ability to synthesize BAP, therefore, its addition in the multiplication stage is fundamental.

For their part, Dalal *et al*. (2006) argue similar results in the multiplication of apple rootstocks (M9), where they observed a notable increase in the formation of multiple shoots by axillary branching in MS medium supplemented with BAP 2.22 µM, IBA 0.49 µM and Kn of 2.32 µM, reaching a 4:1 multiplication rate. This behavior is due to the presence of cytokinin BAP, which has a direct effect on the generation of shoots.

Likewise, Murashige (1974); Chaturvedi *et al*. (2004), emphasize that the BAP hormone establishes a particular superiority in the induction of axillary shoots in the case of woody species. For their part, Kepenek and Karoğlu (2011) increased the multiplication rate in the M9 rootstock and the varieties 'Starking Delicious' and 'Amasya' when they applied growth retardants (paclobutrazol and daminozide), but these atrophied causing a null effect on root development.

## Plant height

The results of the analysis of variance indicate that the values of plant height and number of leaves obtained at 60 days showed no significant differences ($p \geq 0.05$) between the culture media MS and WPM. The only variable that showed significance was stem diameter (Table 3).

**Table 3. Analysis of variance with the Wilcoxon test in height, stem diameter and number of leaves of *M. domestica*.**

| Variable | MS | | WPM | | *P*-value |
|---|---|---|---|---|---|
| | Median | Rank | Median | Rank | |
| Height of seedlings (mm) | 16.954 | 107.785 | 16.436 | 103.214 | 0.563 |
| Stem diameter (mm) | 1.771 | 116.066 | 1.097 | 94.933 | 0.0076* |
| No. of leaves | 8.561 | 105.342 | 9.009 | 105.657 | 0.967 |

Although the analysis did not show significant differences between culture media, it was observed that plants of different sizes were produced, with the plants with the MS medium showing greater height, with T3 (1.5 mg $L^{-1}$ of BAP), which obtained an average of 27.59 mm, and T4 (0.5 mg $L^{-1}$ of IBA) with 27.36 mm (Figure 4) standing out.

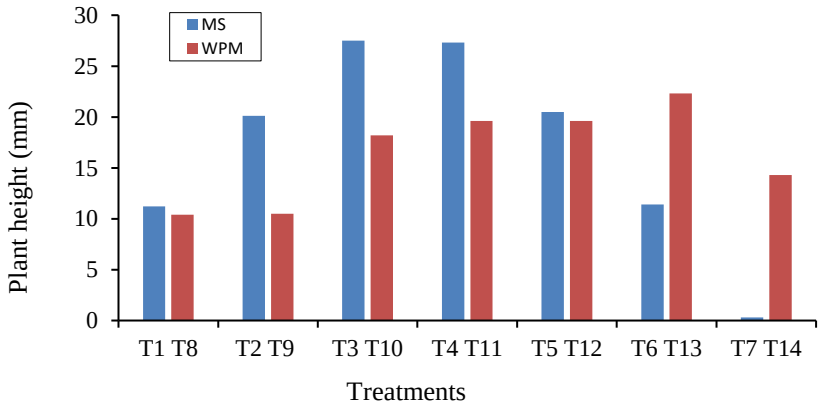


**Figure 4. Plant height 60 days after *in vitro* establishment of *M. domestica* in two culture media added with different doses of growth regulators.**

## Leaf development

The statistical analysis did not report significant differences ($p \geq 0.05$) between treatments with respect to this variable according to the Kruskall-Wallis test for nonparametric data. However, it was observed that T3 (MS with 1.5 mg $L^{-1}$ of BAP) stood out with the best response of leave generation, producing 21.07 leaves on average (Figure 5). A good leaf development increases the possibility of better capturing sunlight, which makes the physiological process of carbohydrate production through photosynthesis more efficient, which will allow their adaptation to the open environment in their acclimatization stage in less time than plants that produce a small number of leaves (Valladares and Niinemets, 2008).

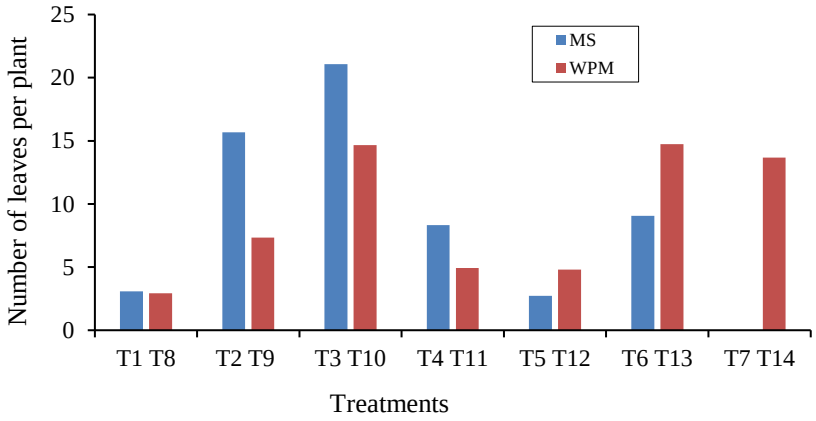


**Figure 5. Number of leaves per plant of *M. domestica* in two culture media added with different doses of growth regulators.**

## Stem diameter

The stem diameter values of the plants obtained at 60 days behaved statistically significantly ($p \geq 0.05$) between the culture media MS and WPM (Table 4). T2 (MS with 0.5 mg $L^{-1}$ of BAP) surpassed the other treatments in this variable, with a mean of 2.5 mm, showing heterogeneity in stem diameter (Figure 6).

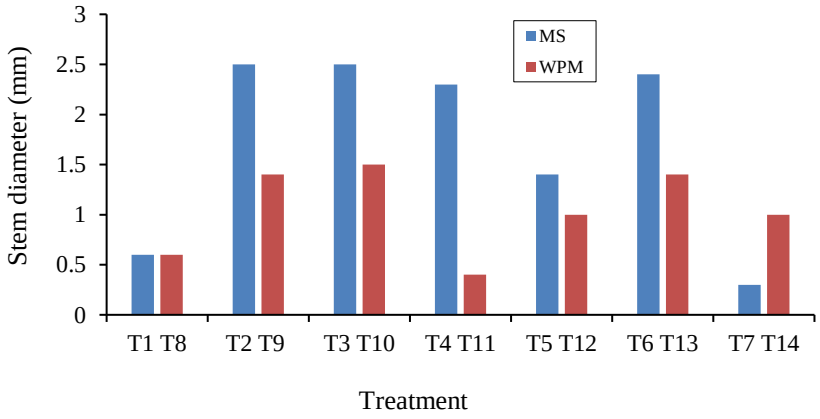


**Figure 6. Stem diameter of *M. domestica* 60 days after *in vitro* establishment in two culture media with different doses of growth regulators.**

## Rooting and root length

At this stage, 100% root generation was achieved in all the treatments evaluated. To assess their response, auxin was combined in greater proportion to the dose of cytokinins (10:1) (Table 2). The effect of the treatments with WPM culture medium stood out from the treatments with MS culture medium at the time of inducing rooting, with the dose of 1.5 mg $L^{-1}$ of IBA and 0.15 mg $L^{-1}$ of KIN (T12) being the one that produced more roots, followed by T11, whose dose was 0.5 mg $L^{-1}$ and 0.05 mg $L^{-1}$ of IBA and KIN, respectively, in the same culture medium (Figure 7). Reducing the nutrient concentration of the MS and WPM media to 50% was fundamental in this morphological response for the species (Hartmann *et al*., 2004; Dalal *et al*., 2006; Gaudie, 2011).

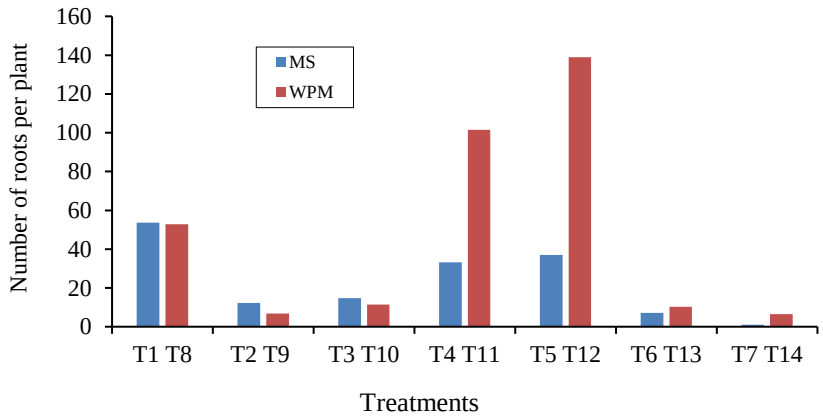


**Figure 7. Number of roots per plant of *M. domestica* produced *in vitro* with two culture media and different doses of growth regulators.**

It is well known that the presence of auxins is necessary for root formation; however, its presence in the culture medium for a long time can inhibit the development of adventitious roots (Dobránszki and Teixeira Da Silva, 2010), a situation that is assumed to have affected the other treatments depending on the type and dose of the growth regulator.

According to the Wilcoxon test, three relevant results can be differentiated from the 14 treatments evaluated, T10 (WPM with 1.5 mg $L^{-1}$ of NAA and 0.15 mg $L^{-1}$ of BAP) showed an average root elongation of 90.47 mm (Figure 8). Likewise, this test showed that T13 (WPM with 0.5 mg $L^{-1}$ of IAA and 0.05 mg $L^{-1}$ of KIN) and T12 (WPM with 1.5 mg $L^{-1}$ of IBA and 0.15 mg $L^{-1}$ of KIN) showed an average length of 68.35 and 63.93 mm, respectively.

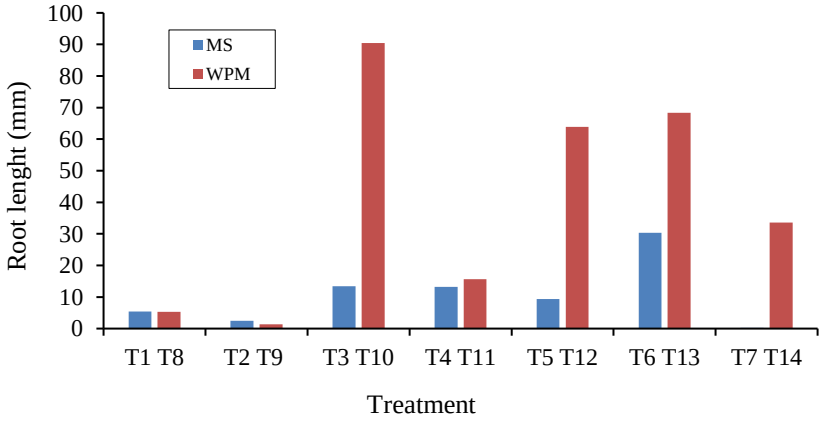


**Figure 8. Root length of *M. domestica* plants produced *in vitro* in two culture media and different doses of growth regulators.**

*Malus domestica* responded heterogeneously to the doses of hormones (auxins) administered (NAA, IBA, IAA), the culture medium supplemented with NAA auxins showed higher levels in root generation than those registered by the treatments with IBA and IAA. This result confirms what was reported by Soni *et al.* (2011), who argue that low concentrations of NAA induce root formation in greater quantity and better development than when explants are exposed to IBA and IAA regulators. According to Druart (1997), the presence of auxins is essential to promote rooting, but in combination with cytokinins, it can enhance root elongation.

In contrast to the above, reports on 'Topaz' apple rootstocks reported by Keresa *et al*. (2012) recorded that the IBA hormone stimulated high root density. On the other hand, Modgil and Thakur (2017) and Modgil *et al*. (2017), state that a rooting greater than 80% can be obtained when using IBA than when using NAA, where this percentage is lower.

Likewise, Amiri and Elahinia (2011) point out that the presence of IBA in the culture medium is essential to root rootstocks of some species of *Malus*; however, this is only possible when the cytokinin level is decreased and the auxin level is increased. However, in this experiment, T13 showed less length than T10 and T12, however, a denser root was observed (Figure 9), which favors the absorption of nutrients in the stage of establishment in substrate for acclimatization. In contrast to the above, Mehta *et al*. (2014) state that between 92 and 98% rooting of MM106 and B9 rootstocks can be obtained in culture medium without the use of growth regulators.

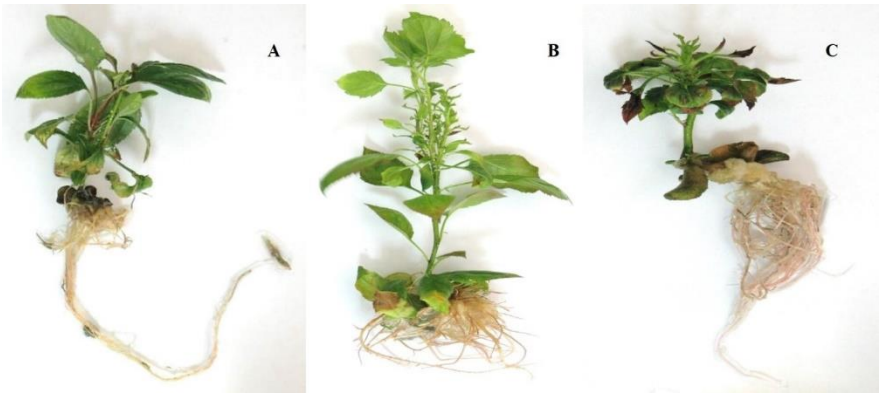


**Figure 9. Rooting response of *M. domestica* plants in WPM culture medium. A) T10 (1.5 mg $L^{-1}$ of NAA and 0.15 mg $L^{-1}$ of BAP); B) T12 (1.5 mg $L^{-1}$ of IBA and 0.15 mg $L^{-1}$ of KIN); C) T13 (0.5 mg $L^{-1}$ of IAA and 0.05 mg $L^{-1}$ of KIN).**

## Conclusions

Growth regulators and the type of culture medium for *in vitro* propagation of *Malus domestica* play an important role in the formation of plant organs. The BAP regulator in MS culture medium showed better results in germination, shoot production and development of the aerial part of the plant. At the rooting stage, the use of the WPM medium in combination with 1.5 mg $L^{-1}$ of IBA and 0.15 mg $L^{-1}$ of KIN had a more favorable effect for root formation; nevertheless, the combination of 1.5 mg $L^{-1}$ of NAA and 0.15 mg $L^{-1}$ of BAP in this same culture medium stimulated root growth significantly than the rest of the treatments. To ensure the success of *in vitro* propagation of *M. domestica*, it is necessary to consider the changes of culture media alternately with the doses of growth regulators recommended here.

In this experiment, it was observed that the alternation of growth regulators and culture media between the different stages of the *in vitro* propagation process of *M. domestica* can be favorable than if a single culture medium and the same doses of regulators are used at all stages. This protocol can be taken as an alternative for conservation and massive propagation of the species, producing plant material that can be used as a rootstock given the adaptability of the plants from which the material was collected.

## Acknowledgements

The authors wish to express their gratitude to Mr. Oscar Villarreal Zamora and to the company Calato SA de CV for the financial support for the acquisition of material and reagents used to develop this research work. Likewise, thanks are expressed to the Juárez University of the State of Durango for providing facilitations, facilities and equipment used, as well as to CONACYT for having granted the maintenance scholarship to the main author of this work, which allowed carrying postgraduate studies in the Institutional Master's Degree in Agricultural and Forestry Sciences.

## Cited literature

Amiri, E. M.; Elahinia, A. 2011. Optimization of medium composition for apple rootstocks. Afr. J. Biotech. 10(18):3594-3601. https://doi.org/10.5897/AJB10.1945.

Azofeifa, A. 2009. Problemas de oxidación y oscurecimiento de explantes cultivados *in vitro*. Agron. Mesoam. 20(1):153-175.

Chaturvedi, H. C.; Sharma, P. L. K.; Agha, B. Q. and Sharma, M. 2004. Production of cloned trees of *Populus deltoides* through *in vitro* regeneration from leaf stern and root explants and their field cultivation. Indian. J. Biotechnol. 3(2):203-208.

Da Câmara M, L.; Da Câmara M., A.; Hanzer, V.; Kalthoff, B.; Weiss, H.; Mattanovich, D.; Regner, F. and Katinger, H. 1991. A new, efficient method using 8 - hydroxyl - quinolinol-sulfate for the initiation and establishment of tissue cultures of apple from adult material. Plant Cell Tissue Organ Cult. 27(2):155-160.

Dalal, A.; Das, B.; Sharma, K.; Mir, A. and Sounduri, S. 2006. *In vitro* cloning of apple (*Malus domestica* Borkh) employing forced shoot tip cultures on M9 rootstock. Indian. J. Biotechnol. 5(4):543-550.

Dobránszki, J. and Teixeira Da Silva, J. A. 2010. Micropropagation of apple a review. Biotechnol. Adv. 28(4):462-488. https://doi.org/10.1016/j.biote chadv.2010.02.008.

Druart, P. 1997. Optimization of culture media for *in vitro* rooting of *Malus domestica* Borkh. cv. Compact Spartan. Biologia Plantarum. 39(1):67-77.

Gaudie, D. 2011. Micropropagation of two apple (*Malus domestica* Borkh) varieties from shoot tip explant. Addis Ababa University. College of Natural Sciences. School of Graguate Studies. Biotechnology Program. Ethiopia. 50 p.

González, C. G.; Villanueva, D. J.; Orona, C. I. y Sánchez, C. I. 2005. Efecto de la lámina de riego en el crecimiento radial de nogal pecanero (*Carya illinoensis* Koch) mediante análisis de imágenes. Agrofaz. 5(2):863-868.

Hartmann, H.; Kaster, D.; Davies, F. and Geneve, R. 2004. Plant propagation: principles and practices. 6th (Ed). Prentice Hall of India Private Limited, New Delhi, India. 770 p.

Hoyos, J.; Perea, C. and Velasco, R. 2008. Evaluation of the effect of different concentrations of phytohormones in micropropagation of dominico hartón plantain (*Musa* AAB Simmonds). Facultad de Ciencias Agropecuarias. 6(2):99-104.

Kepenek, K. and Karoğlu, Z. 2011. The effects of paclobutrazol and daminozide on *in vitro* micropropagation of some apple (*Malus domestica*) cultivars and M9-rootstock. Afr. J. Biotechnol. 10(24):4851-4859. https://doi.org/10.5897/AJB10.1456.

Keresa, S.; Mihovilovic, A.; Baric, M.; Habus Jercic, I.; Sarcevic, H. and Bisko, A. 2012. Efficient axillary shoot proliferation and *in vitro* rooting of apple cv. 'Topaz'. Notulae Botanicae Horti Agrobotanici Cluj-Napoca. 40(1):113-118. https://doi.org/10.15835/nbha4017211.

Levitus, G.; Echenique, V.; Rubinstein, C.; Hopp, E. y Mroginski, L. 2010. Biotecnología y mejoramiento vegetal II. 2ª Edición. Editorial INTA. Instituto Nacional de Tecnología Agropecuaria. Consejo argentino para la información y el desarrollo de la biotecnología. 26-33 pp.

Mc Cown, B. and Lloyd, G. 1981. Woody plant medium (WPM) a revised mineral formulation for micro-culture of woody plant species. HortScience. 16:453.

Mehta, M.; Ram, R. and Bhattacharya, A. 2014. A simple and cost-effective liquid culture system for the micropropagation of two commercially important apple rootstocks. Indian J. Exp. Biol. 52(7):748-754.

Modgil, M.; Parmar, S. and Negi, N. P. 2017. RAPD analysis of long term micropropagated rootstock plants of apple Malling 7. Indian J. Exp. Biol. 55(3):178-183.

Modgil, M. and Thakur, M. 2017. *In vitro* culture of clonal rootstocks of apple for their commercial exploitation. Acta Hortic. 1155:331-335. https://doi.org/10.17660/ActaH ortic.2017. 1155.48.

Montes-Salazar, A. M.; Sepúlveda-Jiménez, G.; Evangelista-Lozano, S. y Rodríguez-Monroy, M. 2016. Estudio preliminar para la propagación *in vitro* de *Cedrus atlantica* por yemas axilares. Rev. Mex. Cienc. Agríc. 7(8):2071-2078.

Murashige, T. and Skoog, F. 1962. A revised medium for growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15(3):473-497.

Murashige, T. 1974. Plant propagation through tissue culture, *Ann*. Rev. Plant Physiol. 25(1):135-160.

FAO. 2014. Organización de las Naciones Unidas para la Agricultura y la Alimentación. Base de datos FAOSTAT. http://www.fao.org/faostat/es/.

Pancaningtyas, S. 2015. Study on the presence and influence of phenolic compounds in callogenesis and somatic embryo development of cocoa (*Theobroma cacao* L.). Pelita Perkebunan. 31(1):14-20. https://doi.org/10.22302/iccri.jur.pelitaperkebunan.v31i1.81.

Rustaei, M.; Nazeri, S.; Ghadimzadeh, M. and Hemmaty, S. 2009. Effect of phloroglucinol, medium type and some component on *in vitro* proliferation of dwarf rootstock of apple (*Malus domestica*). Inter. J. Agric. Biol. 11(2):193-196.

SIAP. 2019. Servicio de Información Agroalimentaria y Pesquera. http://infosiap.siap.gob.mx: 8080/agricola_siap_gobmx/AvanceNacionalSinPrograma.do.

Soni, M.; Thakur, M. and Modgil, M. 2011. *In vitro* multiplication of Merton I. 793-an apple rootstock suitable for replantation. Indian J. Biotechnol. 10(3):362-368.

Tandon, B.; Anand, U.; Alex, B. K.; Kaur, P.; Nandy, S.; Shekhawat, M. S.; Sanyal, R.; Pandey, D. K.; Koshy, E. P. and Dey, A. 2021. Statistical optimization of *in vitro* callus induction of wild and cultivated varieties of *Mucuna pruriens* L. (DC.) using response surface methodology and assessment of L-Dopa biosynthesis. Industrial Crops and Products. 169(1):113626. https://doi.org/10.1016/j.indcrop.2021.113626.

Teixeira da Silva, J. A.; Gulyás, A.; Magyar-Tábori, K.; Min-Rui, W.; Qiao-Chun, W. and Dobránszki, J. 2019. *In vitro* culture of apple and other *Malus* species: recent advances and applications. Planta. 249(4):975-1006. https://doi.org/10.1007/s00425-019-03100-x.

Valladares, F. and Niinements, Ü. 2008. Shade tolerance, a key plant feature of complex nature and consequences. Annual Review of Ecology, Evolution, and Systematics. 39(1):237-257. https://doi.org/10.1146/annurev.ecolsys.39.110707.173506.

Vilchez, J.; Martínez, L.; Álvarez, C.; Albornoz, A.; Albany, N.; Molina, M. y García-Águila, L. 2014. Medio de cultivo y reguladores de crecimiento en la multiplicación *in vitro Psidium guajava* L. Biotecnología Vegetal. 14(1):15-20.

Zhao, X.; Cheng, Z. and Zhang, X. 2008. Cell fate switch during *in vitro* plant organogenesis. J. Integ. Plant Biol. 50(7):816-824. https://doi.org/10.1111/j.1744-7909.2008.00701.x.