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Zinc and titanium nanoparticles effects on Micropropagation *in vitro* from nodal segments of *Monarda didyma L.*

Bc. Thesis

Author: Luis Gustavo Illescas Cienfuegos
Supervisor: doc. Dr. Ing. Eloy Fernández Cusimamani

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DECLARATION

I, Luis Gustavo Illescas Cienfuegos, hereby declare that this thesis, submitted in partial fulfilment of requirements for the bachelor degree in Faculty of Tropical AgriSciences of the Czech University of Life Sciences Prague, is wholly my own work written exclusively with the use of the quoted sources.

In Prague, 2017

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Luis Gustavo Illescas Cienfuegos

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ABSTRACT

Monarda didyma L. (Bee balm) is a medicinal herb from the southern United States, with a wide of effects against several diseases such as colic, diarrhea, cough and asthma. During the last years, its natural oils have been used for the production of different anticancer substances used in different treatments, because of this reason the need to improve their agronomic cultivation techniques has increased.

Nowadays the use of metallic nanoparticles in domestic and industrial products has greatly intensified, and a trend has been generated to investigate the effects they cause on ecosystems, plants, animals and microorganisms. Among the most commonly used are zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles, which had demonstrated positive or toxic effects in different previous studies on cereals, legumes and other medicinal herbs.

Beebalm reacts favorably to *in vitro* culture techniques, for this reason, this study focused on the effects of zinc and titanium nanoparticles within *in vitro* micropropagation from nodal segments in cultivation media with concentrations of 20, 40 and 60 milligrams per liter and measurements every 10 days for 1 month.

The results obtained during this research have demonstrated that the use of zinc oxide nanoparticles has positive effects in the number of regenerated nods, roots and sprouts (length and number). It was concluded that ZnO nanoparticles in concentrations of 40 and 60 mg/l supports *in vitro* regeneration from nodal segments of *Monarda didyma L.* Conversely, TiO_2 nanoparticles showed to have negative effects that retard the *in vitro* regeneration of sprouts and roots from nodal segments of *Monarda didyma L.*

This was the first research made to the observe the effects of zinc and titanium nanoparticles on *in vitro* regeneration for nodal segments on individuals from Genus *Monarda,* family *Lamiaceae*. The conclusive results suggested that further and more dedicated studies should be made on this line of investigation with higher concentrations or with mutual application of nanoparticles and phytohormones.

Key words: In vitro, Lamiaceae, Micropropagation, Monarda, nanoparticles, nodal segments, regeneration, Titanium dioxide, Zinc oxide.

RESUMEN

Monarda didyma L. es una hierba medicinal proveniente del sur de Estados Unidos, con una gran variedad de efectos contra enfermedades como cólicos, diarrea, tos y asma. Durante los últimos años se han utilizado sus aceites naturales para la producción de diferentes sustancias anticancerígenas utilizadas en diferentes tratamientos, y es debido a esto, que se ha incrementado la necesidad de mejorar sus técnicas agronómicas de cultivación.

En la actualidad el uso de nanopartículas metálicas en los productos domésticos e industriales se ha intensificado grandemente, y se han generado una tendencia de investigar los efectos que causan en los ecosistemas, plantas, animales y microorganismos. Entre las más utilizadas se encuentran las nanopartículas de óxido de zinc y dióxido de titanio, las cuales han demostrado tener efectos positivos o tóxicos en diferentes experimentos realizados en cereales, legumbres y otras hierbas medicinales.

Teniendo en cuenta que Monarda didyma L. reacciona favorablemente a las técnicas de cultivo *in vitro*, este estudio estuvo enfocado en los efectos que producen las nanopartículas de zinc y titanio en la micropropagación *in vitro* desde segmentos nodales en medio de cultivo con concentraciones de 20, 40 y 60 miligramos por litro y mediciones cada 10 días durante 1 mes.

Los resultados obtenidos durante esta investigación han demostrado que la utilización de nanopartículas de óxido de zinc tiene efectos positivos en el número de nodos, raíces y tallos regenerados (longitud y cantidad). Se concluyó que las nanopartículas de ZnO en concentraciones de 40 y 60 mg/l soportan la regeneración *in vitro* desde segmentos nodales de *Monarda didyma L*. De forma contraria, las nanopartículas de dióxido de titanio demostraron tener efectos negativos que retardan la regeneración *in vitro* de tallos y raíces desde segmentos nodales de *Monarda didyma L.*

Este estudio fue el primero en hacerse sobre los efectos de las nanopartículas de zinc y titanio en la regeneración *in vitro* desde segmentos nodales en individuos del genero Monarda, de la familia Lamiaceae. Los resultados finales sugieren la recomendación de realizar futuros estudios más detallados en esta línea de investigación, utilizando concentraciones más altas o con aplicación simultanea de nanopartículas y fitohormonas.

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1 INTRODUCTION

Crimson beebalm (*Monarda didyma L.*) is a medicinal perennial herb from the eastern region of the USA with curatives properties against several illnesses as colic, diarrhea, cough, asthma (Ghayur *et al*., 2012) and possible anticancer effects (Sovova *et al*., 2015).

*M. didyma´*s propagation in the crops fields is generally vegetative by the division of root clumps in spring before new growth begins. Generative propagation by seeds is unusual, its use is mainly for breeding procedures (Horáčková a Domkářová, 2003). Micropropagation is also a form of vegetative propagation mainly used for genetical or agricultural studies and preservation of genetic material.

Nanotechnology and nanoparticles production and innovation also initiated a wide interest on their possible uses and applications in agriculture and food production. During the last years, the investigations and studies about nanotechnology development focus on their utilities in the field of electronics development, energy sources, new medicinal procedures and possible direct and side effects on life sciences. In 2010, Bio Nano Electronics Research Center in Japan published a study, which concluded that NPs might facilitate the development of genetically modified crops, plant protecting chemicals and precision farming techniques (Nair *et al.,* 2010).

Nowadays is frequent to find different metal nanoparticles (NP) as a component in home and industrial products with a daily use all around the world. NPs release to the environment is increasing the importance of agricultural studies on its effects on important crops, all types of medicinal plants, fruit trees, textiles plans and other life forms. This current NPs impact is accelerating and requires a closer review of their effects on specific botanical families, genus and species.

Some studies about NPs effects have been realized on different individual species from *Poaceae, Solanaceae* and *Salviniaceae* families. In 2008 and 2009 experiments to study the effects of silver nanoparticles on *Zea mays L.* showed an improvement on quantitative yields (Berahmand *et al.,* 2012). In 2013, an evaluation of exposed *Salvinia natans* to zinc

oxide nanoparticles resulted on stressed and underdeveloped plants (Hu *et al.,* 2013), also another study on how Titanium dioxide nanoparticles affects the growth of *Nicotiana tabacum* concluded its negative impact on the development of 3-week-old tobacco seedlings (Frazier *et al.,* 2013).

Due to the limited existence of metal nanoparticles effects studies on medicinal plants from any family or specifically other *Lamiaceae* species available, this investigation is aimed on the study of the Zinc oxide (ZnO) and Titanium dioxide (TiO_2) NPs possible effects on micropropagation *in vitro* from nodal segments of Crimson beebalm (*Monarda didyma)* and is also focused to provide a closer observation of the NPs effects on the natural processes of growth and *in vitro* regeneration.

2 LITERATURE REVIEW

2.1.1 Bee-Balm *(Monarda didyma*)

Monarda didyma pertains to the botanical mint or *Lamiaceae* family and is also known as Oswego-Tea, bergamot or Crimson beebalm. Is a medicinal perennial herb, part of the genus *Monarda* with about others 30 ornamentals, medicinal and aromatic species originated in the USA, Canada and Mexico.

Monarda genus are plants resistant to unfavorable conditions preferring open spaces (Selaru, 2007) with porous, moist, humid and rich in nutrients soils, and average pH from 5,5 to 7 (Ardelean and Mohan, 2011).

2.1.2 General description

Monarda didyma develops an average height around 100-130 cm from the base with pale green, ovate-lanceolate, petiolate, 7-15 cm long leaves; the rear side from young list on *in vitro* conditions displayed mild dark purple color (Figure 1). Leaves are used to prepare a tea to treat digestive disorders, or can be added and used to season salads (Ciurusniuc and Robu, 2012).

Flowers are brilliant red, tubular, with 2 widely divergent lips. Long stamens protrude beneath the upper lip. Individual flowers are each slightly more than an inch long, surrounded by purplish or reddish bracts and borne in a showy round cluster (Thieme, 2012). *Monarda didyma* grows in moist woods and thickets, stream banks from south Michigan along the mountains to Georgia (Thieme, 2012).

2.1.3 Origin and history

Beebalm is native to eastern North America from Maine to Ohio and south to northern Georgia (Figure 2). The common name Oswego tea refers to the Oswego native Americans living near the city of Oswego in the upper side of New York who taught early white settlers how to make an herbal tea from the plants leaves. Its other common name

bergamont is derived from its fragrance that is alike to the fragrance of the bergamont orange.

The genus name *Monarda* is in recognition of Nicolas Monardes, a Spanish physician, who authored an early herbal that introduced Europe to many of the plants from North America. The species name *didyma* translates from the Latin meaning "in pairs" or "twins" referring to the stamens occurring in pairs.

2.1.4 Medicinal properties

According to H. Kalamouni, Bee-Balm herb has diuretic, antifebrile, carminative, expectorant, rubefaciante, incentive, antiseptic (Kalamouni, 2010), antioxidant and anti-inflammatory properties (Grosso *et al.,* 2009). Recent studies have shown that its essential oils have phenolic compounds with effective inhibitory effects against the development of several fungi species (Mickiene *et al.,* 2014), which makes it an interesting source for further studies on the agronomical field.

Another recent investigation explained that *Monarda didyma* essential oils produced and stored in glandular trichomes on the surface of leaves, flowers and other parts are rich in Thymoquinone (Sovova *et al.,* 2015) a crystalline yellow substance with anticancer effects which selectively targets certain kind of tumor cells, at the same time can also protect healthy tissues during chemotherapy (Schneider-Stock, 2014) producing also neuroprotective effects with influence against forebrain ischemia and Alzheimer (Grosso *et al.,* 2009). Thymoquinone also generates relaxing activities on smooth and cardiac muscle with crescent use in health disorders against several illnesses like colic, diarrhea, cough and asthma (Ghayur *et al.,* 2012).

Figure 1. *Monarda didyma L.* scheme.

Author: Polina Lavrinchuk (2017)

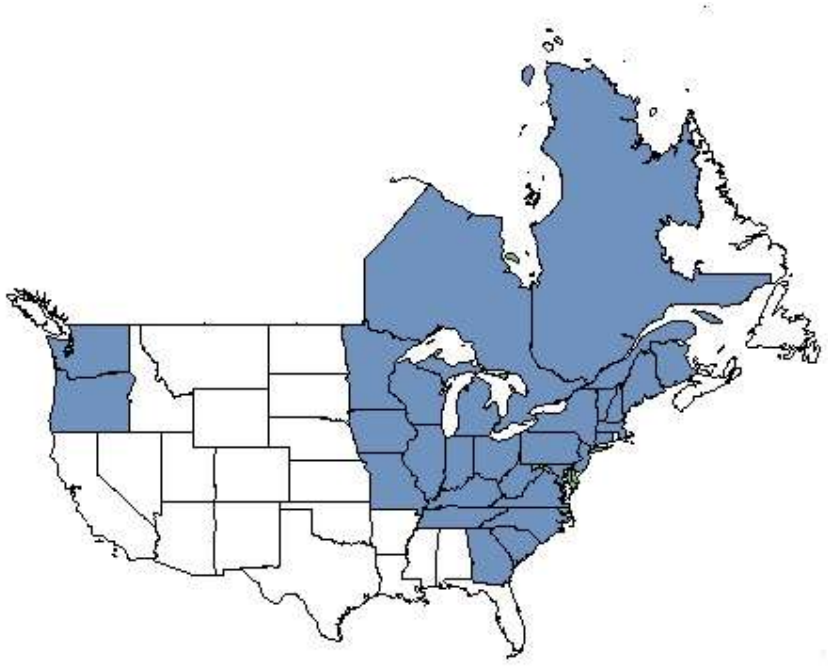

Figure 2. *Monarda didyma* native location in the USA map.
Source: Forest Service, United States Department of Agriculture.

2.2 Metal nanoparticles

Nanoparticles (nano-scale particles = NSPs) are described as materials with at least one of their dimensions being less than a few hundred nanometers, these means being small enough to fall within the nanometric scale generally between 1 and 100 nanometers (Ashgar *et al.,*2012). NSP can drastically modify their physical-chemical properties compared to the bulk material due to their unique diverse nanostructures and properties, such as photocatalysis, electrical conductivity and chemical reactivity (Nel *et al.,* 2006). Engineered nanoparticles (NPs) are applied in a diverse range of industries (electronics, optics, food production, textiles, medical devices, cosmetics, water treatment technology, fuel cells, catalysts, and biosensors) (Ghodake *et al.*, 2011), considerable attention is paid to fullerenes (C60), carbon nanotubes, quantum dots and metal oxides such as TiO_2, ZnO, Fe2O3, Fe3O4, CuO, CeO2, SiO2 and Al2O3 (Menard *et al.,* 2011).

The release of nanoparticles into the environment, including from wastewater treatment plants, is expected to increase in the future. Therefore, it is important to

understand the potential effects on plants, animal and microorganisms. NPs can be more toxic than larger particles of the same composition because of their large specific surface area and unique catalytic properties (Liu *et al.,* 2011).

Technological innovations and nanotechnology occupies a prominent position in transforming agriculture and food production. The development of NPs could open new applications in plant biotechnology and agriculture techniques. Currently, the main thrust of research in nanotechnology focuses on applications in the field of electronics, energy, medicine and life sciences. Experiences gained from these fields facilitate the development of genetically modified crops, plant protecting chemicals and precision farming techniques (Nair et al., 2010) also their possible phytotoxic effects on the environment.

2.2.1 Zinc oxide NPS (ZnO)

Zinc (Zn) belongs to the microbiogenic elements or micronutrients indispensable for plants life, also is one of the essential nutrients required for plant growth. However, is needed in very small amounts, and is an important component of various enzymes that are responsible for driving many metabolic reactions in all crops, such as chlorophyll synthesis, carbohydrate formation and important component of IAA phytohormone (Indole-3-acetic acid) which regulates the plants growth (Pandey *et al*., 2010).

ZnO NPs (particle size<100nm) are one of the most widely used nanoparticles being released in the environment (Borzouyan *et al.,* 2014), are eco-friendly and bio-friendly material recognized as a safe substance by the United States Food and Drug Administration (Taheri *et al.,* 2015).

Studies about the possible ZnO NPs effects on several species have been realized during the last years and have shown different results. Negative effect had been seen on *Arabidopsis thaliana* seedling, both on growth and plant morphology (Landa *et al.,* 2012). Neutral effects or no significant differences had been followed on the seed germination of *Brassica pekinensis* (Xiang *et al.,* 2015) and plant growth of *Salvinia natans* (Hu *et al.,* 2014). Positive effects and responses have been proved on the level of IAA in roots and sprouds

and on growth rate of *Cicer arietinum* (Pandey *et al.,* 2010), and on leaves area indexes, improved growth and yield in mineral poor soils of Corn (*Zea mays*) (Taheri *et al.,* 2015).

Generally, ZnO NPs effects on plants improved growth; leaf area and leaf dry weight. Low concentration in MS medium have not toxic effects, all the same in higher concentrations over 50 ppm could increase toxic effects on growth and plant development (Dastjerdi *et al.,* 2014).

2.2.2 Titanium dioxide NPs (TiO_2)

TiO_2 is a naturally occurring mineral that can exist in three crystalline forms, generally known as rutile, anatase, and brookite (Reyes-Coronado *et al.,* 2008). TiO_2 nanoparticles are widely used in antibacterial soups, cleaning air sprays, skin care products, cosmetics and for decomposing organic matter in wastewater. The element titanium is also found in ilmenite ($FeTiO_3$) and other minerals and ores, processing of which can be produce TiO_2 (Menard *et al.,* 2011). Commercial production of nano-TiO_2 between 2006 and 2010 has been estimated at 5000 metric tons per year, more than 10 000 metric tons per year between 2011 and 2014 and approximately 2.5million metric tons by 2025 (Robichaud *et al.*, 2009).

Completed investigations on *Salvia officinalis* from *Lamiaceae* family and spinach *(Spinacia oleracea)* (Zheng *et al.,* 2005) with maximum concentration of 200 to 500 mg/l TiO_2 NPs have showed increased results on essential oils content, plant growth, chlorophyll formation and photosynthesis promotion (Ghorbanpour, 2015). Other results indicate that TiO_2 NPs treatments in proper concentrations accelerates the germination of the wheat (*Triticum aestivum*) seeds and increases its vigor (Feizi *et al.,* 2012).

Recently, TiO_2 nanoparticles (NPs) have been found to change the dry weight, chlorophyll synthesis, and some characteristics. However, NPs can cause also a variety of negative effects on metabolic processes. Studies found evidence as reduction and alteration in seed germination in *Vicia narbonensis* and *Zea mays* (Ruffini *et al.,* 2011) also decrease in average root length of tobacco seedlings as TiO_2 NPs concentration increased (Frazier *et al.,* 2014).

Titanium dioxide NPs application improves the plant development for some species, on the other hand, also shows negative effects. Their effects on specific genus and species must be further studied. This may cause the limitation of TiO_2 NPs extensive use in agriculture and *in vitro* cultivation.

2.3 Tissue culture

The entire Tissue culture concept is based on the plant cell's ability to differentiate into other cells type and recreated from one single cell an entire organism corps. Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants (Hussain *et al.*, 2012). These techniques have been developed since 1902 when Haberlandt proposed the concept of *in vitro* culture. Nowadays several endangered and rare species have been successfully grown and multiplied by Micropropagation and Tissue culture processes, which has proven a high coefficient of regenerated tissues from a low quantity of initial plants and needed space.

The used media in plant cell and tissue culture most include, towards a successful plant regeneration and development, the necessary elements such as C (Carbon), H (Hydrogen), O (Oxygen) along with macroelements N (Nitrogen), P (Phosphorus), K (Potassium), Ca (Calcium), Mg (Magnesium), S (Sulphur) and microelements Fe (Iron), Mn (Manganese), Zn (Zinc), B (Boron), Cu (Cupper) and Mo (Molybdenum). For this investigation, we will used the Murashige and Skoog developed MS medium (1962).

in vitro Tissue culture is been used towards mass production of plant derived substances in the fields of medicine and research, also for cultivation of comestible plants out of season or in areas with different climates. All these benefits represent for a long number of scientific areas an out looking way into the future and a unique role in sustainable and competitive agriculture and forestry improvement (Saad & Elshahed, 2012).

2.4 The use of nanoparticles during *in vitro* micropropagation

2.4.1 NPs application on food plants

Some studied had been realized on *Zea Mays*, *Solanum lycopersicum*, *Vicia narbonensis* and *Lycopersicon esculentum* during 2011 and 2016 about the effects of silver, zinc and titanium NPs on the *in vitro* micropropagation along with other studies about the production improvement, germination impacts, metabolic activity and mitosis.

The article about effects of Zinc oxide nanoparticles on growth parameters of corn published on 2015 acknowledged that ZnO NPs increased the shoot dry matter and leaf area indexes by 63.8% and 69.7% respectively and concluded its positive effects on *Zea mays* growth in mineral poor soils (Taheri *et al*., 2015). Other study for TiO_2 effects on seed germination from *Vicia narbonensis* and *Zea mays* showed delayed germination progression for the first 24 hours in both materials and evidenced a TiO_2 NPs induced genotoxic effect for both species (Ruffini *et al.,* 2011).

Impact on the germination and metabolic activity of *Solanum lycopersicum* was also observed in 2015 by the University of Allahabad. NPs were applied to tomato seeds to evaluate their effect on germination and metabolic activities of the plant. After the experiments a positive response was found about ZnO NPs at low concentration in comparison to NPs at higher concentration (Singh *et al.,* 2016).

2.4.2 NPs application on medicinal plants from *Lamiaceae* family

There is a reduced number of studies on NPs effects on medicinal plants from the Lamiaceae family. The latest article was done in 2015 by Mansour Ghorbanpour from the Indian Society for Plant Physiology, in which was evaluated the impact of Titanium dioxide NPs in different concentration from 10 till 1000 mg/l on every physiological activity from *Salvia officinalis*. This investigation showed the best results in concentrations of 100 and 200 mg/l (Ghorbanpour, 2015).

3 AIMS OF THE THESIS

The principal aim of this investigation was determinate the effect of the Titanium dioxide (TiO_2) and Zinc oxide (ZnO) on the *in vitro* Micropropagation process from nodal segments of Bee Balm *(Monarda Didyma L.*).

The aim was stablished on the following hypothesis:

H1: Zinc oxide (ZnO) and Titanium dioxide (TiO_2) nanoparticles might have positive effects and improve the *in vitro* micropropagation from nodal segments of *Monarda didyma L.*

H2: Zinc oxide (ZnO) and Titanium dioxide (TiO_2) nanoparticles might have negative effects and be toxic for the *in vitro* micropropagation from nodal segments of *Monarda didyma L*.

4 MATERIALS AND METHODS

The cultivation and *in vitro* micropropagation process occurred between 2015-2016 in the Laboratory of Plants Tissue Culture, Department of Crop Science and Agroforestry, Faculty of Tropical and Subtropical Agriculture of the Czech University of Life Science.

4.1 Plant material

For the investigation *Monarda didyma* samples, maintained in *in vitro* conditions were obtained from the life tissue collection of the Laboratory of Plants Tissue Culture from the Faculty of Tropical AgriScience. The investigation initiated after the successful multiplication of life material on medium (Murashige and Skooge, 1962) without any growth regulators.

4.1.1 Other material

All laboratory equipment such as tweezers, pipettes, beakers, petri dishes, test tubes, chemicals, pH meters, scalpels, parafilm, among others were also provided by the Laboratory of Plants Tissue Culture. Among with heavy equipment such as autoclaves, hot air sterilizer, laminar boxes, culture rooms, incubators and a flow cytometer.

4.2 Methods

4.2.1 Cultivation medium preparation

The selected media for this investigation was Murashige-Skoog media (1962), which was prepared in quantities of 500 ml according to the laboratory protocol (table 1) with an average resulting amount of 50 test tubes with an approximate content of 10 ml of MS each.

First of all, stock solutions A, B, C, D, E, V along with Myo-nositol and Sucrose were measured and mixed (see table 1) inside a Beaker (250 ml). These solutions contain all basic macro and microelements, iron, carbon and vitamins sources required for plants growth

and development. The correct pH was an important aspect to prepare a suitable media, for this reason pH measurement was made during 20 minuts. In case lower pH was drip KOH and in case of higher pH was drip Ascorbic acid till the right pH of 5.7.

The process continued with a second Beaker (250 ml) used to dilute agar in distilled water towards to mix with the first substance. Both beakers and distillated water were heated in microwave around 7 and a half minutes, this was a special step to work with liquid agar to obtain the final media. Finally, MS was poured into test tubes in approximated quantity of 10 ml.

4.2.2 Cultivation medium sterilization

MS was sterilized inside autoclave for 20 minutes at high-pressure of 1 atm and saturated steam at 121 °C, towards to eliminate all possible contamination during cultivation. Then MS was stored for one or two days at room temperature for agar to curdle and finish MS preparation process.

4.2.3 Laboratory instruments sterilization

All the necessary tools (petri dishes, scalpels and tweezers) were wrapped in aluminum foil and then placed into the hot air sterilizer for 3 hours at 160 ° C. The following step was the sterilization of the work area (low flow box) and sterilized wrapped instruments by alcohol (70%). Every tool was given inside the Flow box and once again sterilized during 30 minutes by UV radiation, towards the elimination of all possible fungi or bacterial contamination.

Table 1: Preparation of stock solution for media Murashige - Skoog (1962)

Media Murashige - Skoog			pH 5.7
Stock solution to 1 liter dest. H_20		batch size to 1 liter of stock solution	to 1 liter measure
A	NH4NO3	16.5 g	100 ml
	KNO3	19 g	
	CaCl2.2H2O	4.4 g	
	MgSO4.7H2O	3.7 g	
	KH2PO4	1.7 g	
B	H3BO3	620 mg	10 ml
	MnSO4.4H20 (H2O)	2.23 g (1.69 g)	
	ZnSO4.4H20(7H2O)	860 mg (1.06 g)	
C	KI	83 mg	10 ml
	Na2MoO4.4H2O	25 mg	
D	CuSO4.4H2O	2.5 mg	10 ml
	CoCl2.6H2O	2.5 mg	
E	Na2EDTA	3.72 g	10 ml
	FeSO4.7H2O	2.78 g	
V	Nicotinic Acid vitamin	50 mg	10 ml
	Pyridoxin (B6)	50 mg	
	Thiamin (B1)	10 mg	
	Glyoin (Amino acid)	200 mg	
	Myo-inositol	100 mg	
	Sacharosa	30 g	
	Agar	8 g	

Source: Author (2016)

4.3 Micropropagation procedure by nodal segments

After all sterilization procedures described in point 4.2.2 and 4.2.1 the *in vitro* micropropagation started inside the work area (low flow box) with the carefully extraction of the plant material from the test tubes and treated in the Petri dishes. Every *M. didyma* sprout was reduced into several nods (figure 2) around one centimeter long with no leaves or half its normal size (in the case of long leaves). From each original material sample were sought around 6-7 nods, this means that for each variant were used around 4 material samples.

The nods were settled into the prepared test tubes with fresh MS and then were hermetically closed with lids and parafilm to avoid any possible contamination (figure 3). A regular visual control was made every five days and in the case of any contamination the samples were removed from the cultivation room.

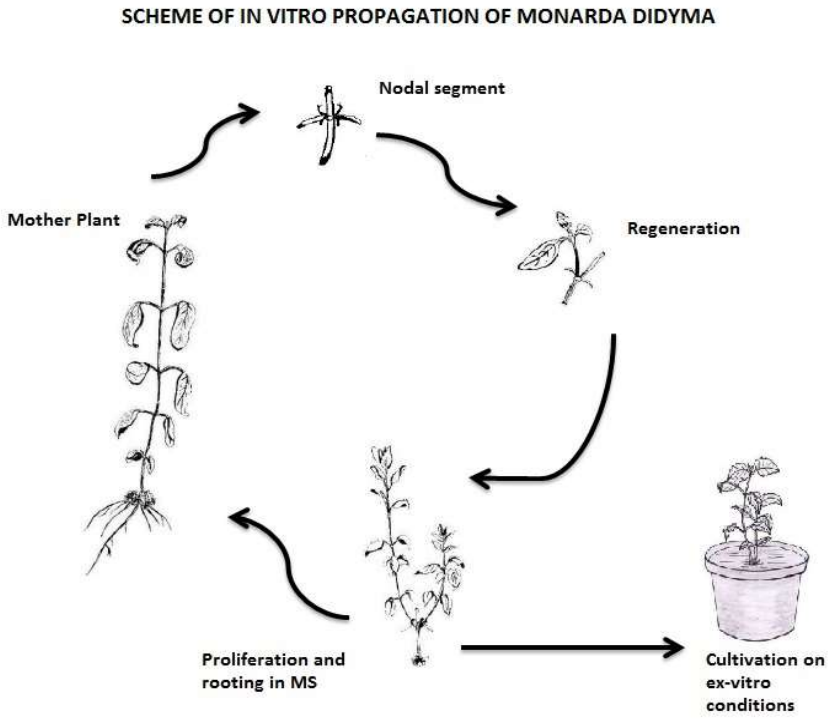

Figure 3. Scheme of *in vitro* propagation procedure of Crimson beebalm (*Monarda didyma L.*)
Source: Author (2017)

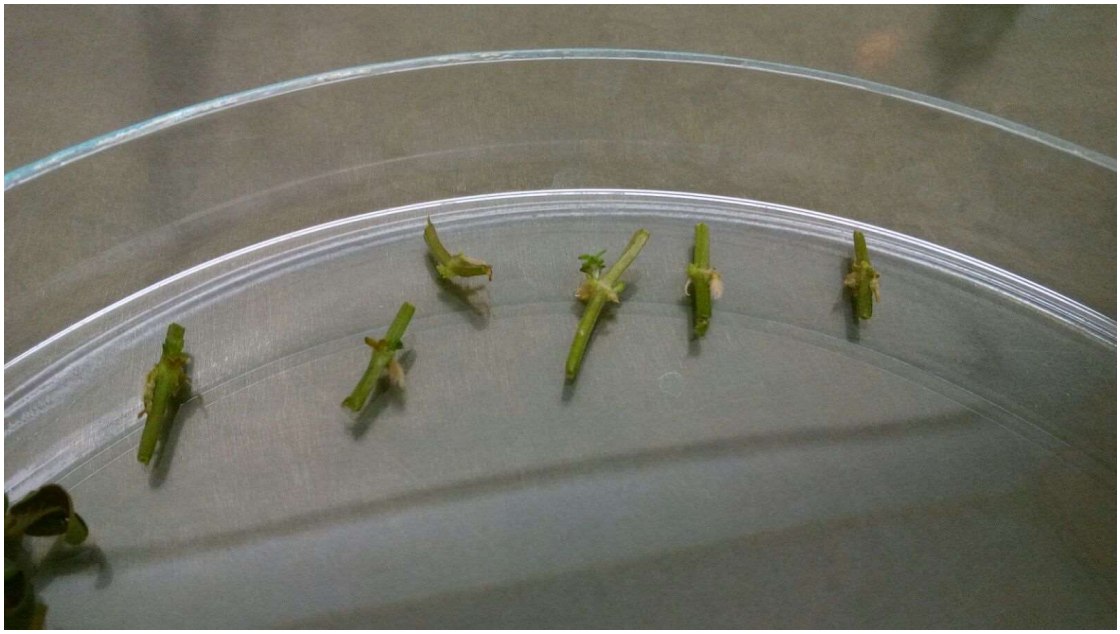

Figure 4. Nodal segments from mother plant.
Source: Author (2016)

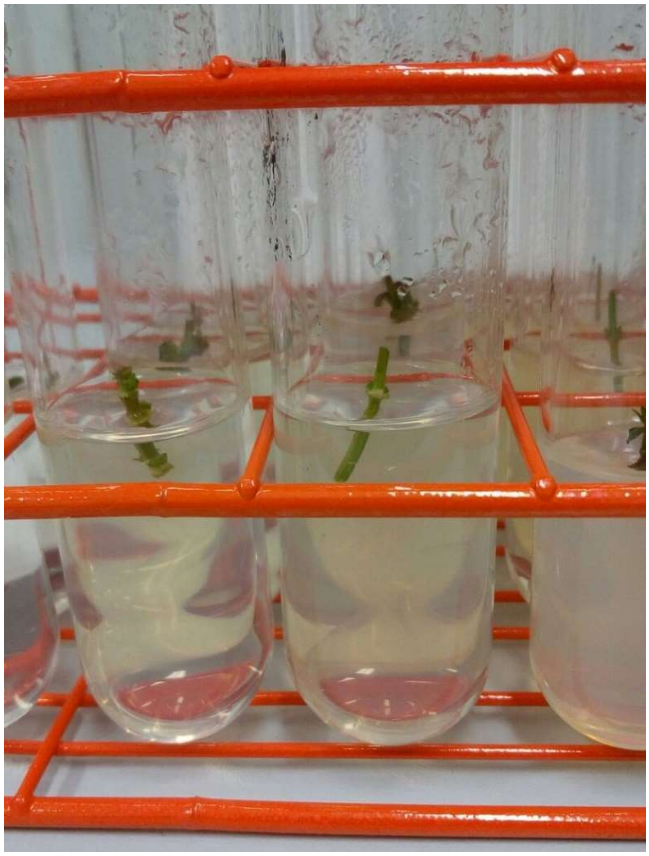

Figure 5. Cultivation of nodal segments on MS with added NPs.
Source: Author (2016)

4.4 NPs effects on micropropagation of Crimson beebalm (*Monarda didyma L.)*

This part of the procedure started after the successful multiplication of the original plant material. It was considered very important to have enough material in case of failure or contamination during the investigation experiments.

Nodal segments were multiplicated on MS with added nanoparticles of Zinc oxide (ZnO) and Titanium dioxide (TiO_2) in different concentrations (Table 2). Three Z variants with MS and Zinc oxide, three T variants with MS and Titanium dioxide and one CONTROL variant with only MS as control. For each variant were utilized 20 nodal segments.

Table 2: Specific variants of cultivation media with NPs concentration for *in vitro* micropropagation of *Monarda didyma L*.

VARIANT	NPs	CONCENTRATION	MEDIUM
CONTROL	--	--	MS
Z1	ZnO	20 mg/l	MS
Z2	ZnO	40 mg/l	MS
Z3	ZnO	60 mg/l	MS
T1	TiO2	20 mg/l	MS
T2	TiO2	40 mg/l	MS
T3	TiO2	60 mg/l	MS

Source: Author (2016)

4.5 NPs application procedure

4.5.1 NPs solution preparation

Nanoparticles preparation was made as the Beaker 1 explained on the section 4.2 (Medium preparation), the same steps as for MS but without agar, with the inclusion of the NPs on the correct concentration for each variant explained on the table 2. The most important aspect was to reach the correct pH of 5.7.

4.5.2 NPs solution sterilization

The NPs solution was also properly sealed and sterilized inside autoclave (same characteristics described in point 4.2.2), and the cooling process was at room temperature during 1.5 hours. In the case of NPs solution, the storage was inside the refrigerator, towards the correct conservation of it chemicals and physical characteristics.

Then in order to avoid any contamination was sterilized once again by UV radiation and sprayed with alcohol (70%).

4.5.3 NPs application

After the third day of the Micropropagation (section 4.2), the NPs solution was added in approximated amount of 2-3 ml to each test tube and then each sample was numbered and sealed with laboratory parafilm.

4.6 Method of measurement

Measurement was made three times for each variant on the 10th, 20th and 30th day after the NPs solution application explained on section 4.5.3 by milimetric rule of 20 centimeters. The main measured parts were number and length of sprouts, number of regenerated nods and roots.

4.7 Statistical evaluation of the collected data

All statistical analyses were performed using ANOVA to compare groups and Tukey test in case of homogeneity of variance. For all tests, differences were considered significant at $P<0.05$. Descriptive statistics were also calculated.

Figure 6. Variant K (control) in the second week from multiplication.
Source: Author (2016)

Figure 7. Zinc variants after 30 days from multiplication.
Source: Author (2016)

5 RESULTS AND DISCUSSION

5.1 Zinc oxide effects on Micropropagation of *Monarda didyma L.*

5.1.1 Regeneration percentage

Control variant (K) with regeneration percentage of 80% showed successful regeneration of 36 new sprouts from 16 nodal segments, very comparable results were observed on variant Z2 (40 ZnO mg/l) with 24 regenerated sprouts from 16 nodal segments (Table 3).

The results for variant Z3 (60 ZnO mg/l) indicated the smallest regeneration percentage with only 55% new sprouts. The lowest average length was obtained for variant Z1 (20 ZnO mg/l) compared with the other variants, although its regeneration percentage was compatible with variants Z2 and control (K) with 29 new sprouts from 16 nodal segments (Table 3).

Was not possible to compare these results with previous studies on Lamiaceae species, anyhow in experiments with *Solanum lycopersicum* was observed a positive response about ZnO NPs at low concentration in comparison to NPs at concentration higher than 100 mg/l (Singh *et al.,* 2016).

5.1.2 Average length and number of new nods per sprout

During the first 10 days from NPs application Z2 and Z3 variants presented a more accelerated sprouts regeneration against control (K), and total opposite reaction was observed for variant Z1 which had the slowest growth between all variants (Figure 10). After the second measure the regeneration started to be slower, is important to mention that control (K) showed a regular and constant growth and regeneration ratio.

Variant Z3 showed the highest results for nodal regeneration with 5.67 regenerated nods per sprout and the samples with the longest length, but with the lowest regeneration percentage, this variant indicated that the concentration of 60 ml/l of Zinc

oxide NPs added to MS media has conflicting effects on *M. didyma L.* regeneration and growth development within *in vitro* micropropagation against control variant with only 15 total regenerated sprouts.

The lowest results were obtained for variant Z1 with an average number of regenerated nods of 3.2, average length per sprout of 4,87 cm, underdeveloped plant with truncated growth, a low average length and with the problematic that some samples withered before the last measure (Figure 10). According to the results comparison was concluded that the concentration of 20 ml/l Zinc oxide NPs had a significant statistical difference against the other variants and caused retardation, along with possible toxic effects for the regeneration and growth development within *in vitro* micropropagation of *M. didyma L.*

The best results with an average nodal regeneration of 5.21 regenerated nods per sprout and 9.42 cm of length were observed for variant Z2. This variant indicated that concentration of 40 ml/l Zinc oxide NPs added to MS media improved *M. didyma L.* regeneration and growth development within *in vitro* micropropagation against variants control K, Z3 and Z1 (Table 3).

In other studies, among the results were observed similar positives effects for ZnO NPs as for growth rate of *Cicer arietinum* (Pandey *et al.,* 2010) and for leaves area indexes in mineral poor soils of Corn (*Zea mays*) (Taheri *et al.,* 2015). Unfortunately, it was not possible to compare with other individuals from genus *Monarda* or *Lamiaceae* family.

5.1.3 Length and number of regenerated roots

Against control (K) all variants (Z1, Z2, Z3) presented retarded roots regeneration, lower that TiO_2 results (Figure 11). After the second measure the regeneration showed a higher retardation against control (K), is possible to explain that this was growth at a lower regeneration ratio for roots.

The average number of regenerated roots was in every variant higher against the result of 3.5 for control variant K. The best root regeneration was observed on variant Z2 with a

result of 9.47, the average length was not significantly different between the other variants (Figure 11).

For variant Z1 the average length was significantly smaller (2.62 cm) against the results for variants K, Z2 and Z3. The concentration of 20 ZnO ml/l slowed down the roots regeneration within *in vitro* micropropagation for *M. didyma L.* Which shows the possible need to add phytohormones to the MS media, in order to improve the regeneration process and reach a complete and faster process (Table 3).

Any other results from previous studies about roots regeneration were not found to make a proper comparison, but it was concluded that in general ZnO NPs at low concentration have positive effects and improve plant development (Singh et al., 2016).

Table 3. Zinc oxide variants. ANOVA, Multiple comparissons by Tuker ($p<0.05$)

VARIANT	NPs CONCENTRATION (mg/l)	REGENERATION PERCENTAGE (%)	LENGTH FOR ONE SPROUT (cm)	NUMBER OF NODS PER SPROUT	NUMBER OF REGENERATED ROOTS	LENGTH OF ONE ROOT (cm)
CONTROL	--	80	9.09 ± 5.13^{b}	3.42 ± 1.79^{cd}	3.5 ± 2.23^{bcd}	5.21 ± 5.72^{b}
Z1	20	80	4.87 ± 10.06^{acd}	3.20 ± 1.31^{cd}	8.8 ± 23.07^{a}	2.62 ± 2.06^{acd}
Z2	40	80	9.42 ± 11.73^{b}	5.21 ± 1.22^{ab}	9.47 ± 13.98^{a}	4.28 ± 2.13^{b}
Z3	60	55	9.53 ± 11.42^{b}	5.67 ± 1.81^{ab}	8.3 ± 20.46^{a}	5.44 ± 6.24^{b}

Source: Author (2017)

Figure 8. Comparison between Zinc oxide NPs variants and Control
Source: Author (2017)

Figure 9. Comparison between Titanium dioxide NPs variants and Control.
Source: Author (2017)

5.2 Titanium dioxide effects on Micropropagation of Monarda didyma L.

5.2.1 Regeneration percentage

Titanium dioxide variants showed deformation on the samples during the micropropagation procedure such as underdeveloped or abnormally bigger leaves or roots, and higher amount of withered plants.

The highest regeneration percentage was observed for variant T2 with 85% and 27 regenerated sprouts from 17 successful samples. Control variant (K) and T1 indicated equal regeneration with 80%, although variant T1 had only 23 new sprouts compared against control variant (K) with 36 new sprouts from 16 nodal segments. And the lowest percentage was observed for variant T3 with 75% (Figure 11).

These results were consistent with other studies for TiO_2 effects on seed germination from *Vicia narbonensis* and *Zea mays* in witches was concluded an induced genotoxic effect for both species (Ruffini *et al.,* 2011).

5.2.2 Average length and number of new nods per sprout

After the first 10 days from NPs application T1, T2 and T3 variants presented a deaccelerated but constant sprouts regeneration against control (K), only Z1 showed lower results (Figure 10). It is important to mention that control (K) showed a regular and constant growth and regeneration ratio. There results were in concordance with other study for TiO_2 effects on seed germination from *Vicia narbonensis* and *Zea mays,* which showed delayed germination progression in both materials and evidenced a TiO_2 NPs induced genotoxic effect for both species (Ruffini *et al.,* 2011).

Variants T1, T2 and T3 showed higher nodal regeneration compared with the control variant (K), however these differences were not statistically significant. The average length per sprout for all the variants was lower against the 9.09 cm for control variant (K) (Figure 10). With only 5.76 cm, 7.53 cm and 7.24 cm respectively, along with the underdeveloped plants with low average length and nods regeneration proved that concentrations of TiO_2

nanoparticles add to MS caused toxicity and has negative effects on the *M. didyma L.* regeneration and growth development within *in vitro* micropropagation (Table 4).

Other researches made with TiO_2 NPS treatments in proper concentrations demonstrated a total different result that indicated an accelerated germination of the wheat (*Triticum aestivum*) seeds and increases its vigor (Feizi *et al.,* 2012). The toxic effects of these NPs might not be the same in other families different of *Lamiaceae* species.

5.2.3 Length and number of regenerated roots

Comparing against control (K) all variants (T1, T2, T3) presented during the first measure a slightly higher roots regeneration that ZnO results (Figure 11). After the second measure the regeneration showed a higher acceleration against control (K), it was also observed that the number of roots increased but the average length was lower that for Zn variants and control (K).

Roots regeneration and the number of new roots were not significantly different between variants control (K), T1 and T3. The best and statistical highest results were obtained for variant T2 with an average length of 9.35 cm per root (Figure 11), but compared with the other results was concluded that there was not any statistical importance between them or against Zinc oxide variants.

Any Titanium dioxide concentration caused retardation and negative results, and not only for roots regeneration, but also for every parameter compared during this study. According to these observations, was concluded that Titanium dioxide NPs have toxic effects within *in vitro* micropropagation for *M. didyma L* (Table 4).

Completed investigations on Salvia officinalis from Lamiaceae family and spinach (Spinacia oleracea) (Zheng et al., 2005) with maximum concentration of 200 to 500 TiO_2 mg/l NPs have showed increased results on essential oils content, plant growth, chlorophyll formation and photosynthesis promotion (Ghorbanpour, 2015). It is possible that the toxicity caused on Monarda didyma L. is cause only for lower concentrations, future studies should be made to reach a better conclusion about higher concentrations.

Table 4. Titanium dioxide variants. ANOVA, Multiple comparissons by Tuker (p<0.05)

VARIANT	NPs CONCENTRATION (mg/l)	REGENERATION PERCENTAGE (%)	LENGTH FOR ONE SPROUT (cm)	NUMBER OF NODS PER SPROUT	NUMBER OF REGENERATED ROOTS	LENGTH OF ONE ROOT (cm)
CONTROL	--	80	9.09±5.13 ^{ab}	3.42±1.79^{a}	3.5±2.28^{c}	5.28±5.27^{a}
T1	20	80	5.76±8.61^{a}	3.59±2.06^{a}	4.3±2.23^{c}	4.21±29.65^{a}
T2	40	85	7.53±11.03^{a}	3.5±0.9^{a}	7.17±6.70^{abd}	9.35±136.93^{a}
T3	60	75	7.24±14.14^{a}	3.9±2.3^{a}	4.62±8.42^{c}	6.17±25.05^{a}

Source: Author (2017)

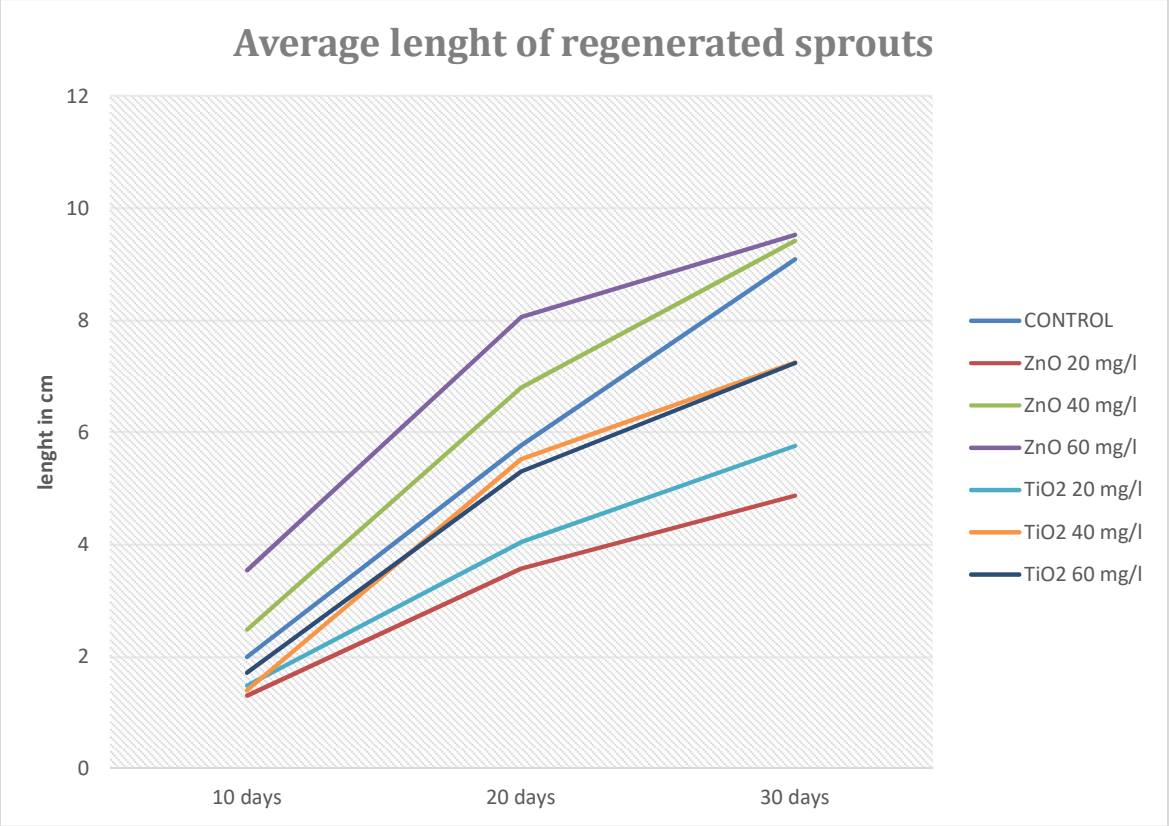

Figure 10. Comparison between the average length of regenerated sprouts during 30 days.

Source: Author (2017)

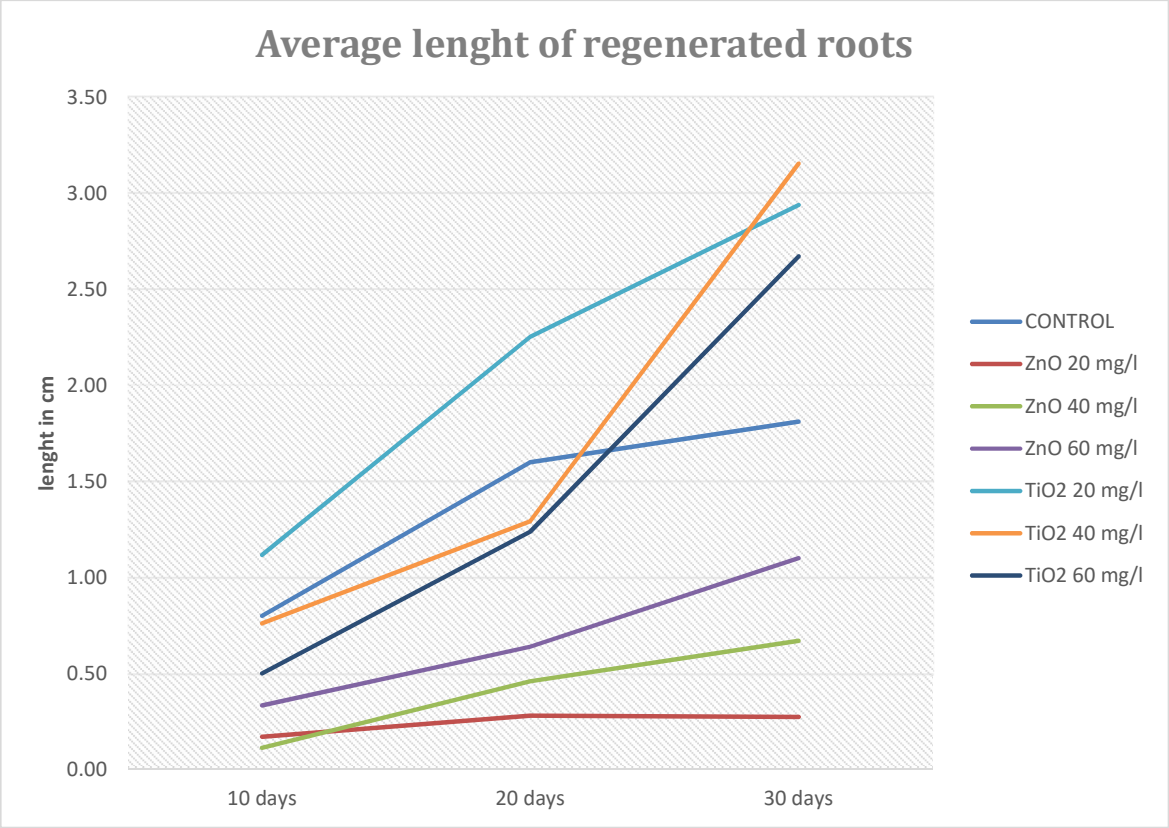

Figure 11. Comparison between the average length of regenerated roots during 30 days.

Source: Author (2017)

6 CONCLUSION

Monarda didyma L. is a highly important herb in terms of medicinal improvement based on its several positive effects against several illnesses, and especially due to its anticancer properties. Also, the closest observation on the effects caused by metal nanoparticles on the *Lamiaceae* individuals provided this investigation with a significant importance in the field of its possible uses in agricultural techniques.

According to the results obtained during this experiment on Zinc oxide and Titanium dioxide nanoparticles effects on Micropropagation *in vitro* from nodal segments of *Monarda didyma L.* the following conclusions were reached:

- Zinc oxide nanoparticles in concentrations of 40 and 60 mg/l has positive effects and improves the regeneration of nods, roots and sprouts from nodal segments within *in vitro* Micropropagation of *Monarda didyma L*.

- Zinc oxide nanoparticles in a concentration of 20 ml/l and Titanium dioxide nanoparticles in concentrations of 20, 40 and 60 mg/l causes toxic effects and inhibits the regeneration of nods, roots and sprouts from nodal segments within *in vitro* Micropropagation of *Monarda didyma L.*

- Zinc oxide nanoparticles have positive effects and improve regeneration of *Monarda didyma L.* within *in vitro* Micropropagation, contrary to titanium dioxide nanoparticles.

This is the first investigation based on zinc and titanium nanoparticles effects on micropropagation *in vitro* from nodal segments of *Monarda didyma L.* and is recommended to continue with further studies on the effects of zinc oxide nanoparticles in higher concentrations added with phytohormones, aiming the possible application of its effects in the cultivation of *Monarda didyma L.* and other medicinal individuals from *Lamiaceae* family.

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