CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforesty

Zinc (ZnO) and titanium (TiO2) nanoparticles effect on micropropagation of *Satureja Montana* L.

Bachelor Thesis

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Supervisor: doc. Dr. Ing. Eloy Fernández Cusimamani

Author: Mohamed Noman

Declaration:

I declare that this bachelor thesis titled “Zinc (ZnO) and titanium (TiO2) nanoparticles effect on micropropagation of Satureja Montana L.” is my original work and all the the literature used is cited in references. I agree for this work to be placed in the library of CULS Prague and be accessible for research purposes.

In Prague, 21.04.2017

……..

Mohamed Noman

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Abstract

Satureja montana L. commonly known as winter savory is an aromatic perennial herb used as a spice in food preparation and as a component in remedies in traditional medicine due to its essential oils. Several biological properties of its essential oils have been confirmed in recent studies such as antioxidant, anitmicrobal, and antiviral.

Nano-scale particles with dimensions between 1 and 100 nm have been widely employed in different fields such as energy, transportation, cosmetics and agriculture due to their unique physico-chemical properties.

This study deals with the effects of zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles in different concentrations on the in vitro propagation of *Satureja montana* L.

Nodal explants were cultivated on basic MS medium for 5 days and then supplemented with either zinc oxide (ZnO) or titanium dioxide (TiO2) nanoparticles in 20 mg/l, 40 mg/l and 60 mg/l. The nodal explants were cultivated for a 60-day period. The cultivation conditions were 16/8 day night cycle and a temperature of 25/23 °C day/night with a light intensity of 2500 lx. Measurements were taken every 10 days of number of sprouts, number of nods, sprout length, number of roots and root length.

The results show that zinc oxide nanoparticles in 20 mg/l concentrations positively affected sprout length (55.5 mm ± 39.6) however they slightly decreased the length of roots. In 40 mg/l ZnO nanoparticles inhibited root formation (3.21 ± 4.29) and length (10.64 mm ± 5.81), while 60 mg ZnO didn't have any effect on growth of nodal explants. TiO_2 nanoparticles exhibited toxic effects on root length growth.

Keywords: Lamiaceae, In Vitro, Micropropagation, Nanoparticles, Nodal segments, Satureja Montana, TiO_2, ZnO

Contents

List of figures:

List of tables:

List of Abbreviations

2,4-D - 2,4-Dichlorophenoxyacetic acid

2iP - N6-(2-izopentenil)adenine

ANOVA - Analysis of Variance

BA - 6-benzyladenine

CULS - Czech university of life sciences

IAA - Indole- 3-acetic acid

IBA - Indole-3-butyric acid

K - 6-furfurilaminopurine

LPTC - Laboratory of Plant Tissue Cultures

MS - Murashige and Skoog medium (1962)

NAA - Naphthaleneacetic acid

S. hortensis L.- *Satureja hortensis* L

S. montana L.- *Satureja montana* L

Subsp. - Subspecies

TDZ – Thidiazuron

WPM - Woody plant medium

Z – Zeatine

1 introduction

Satureja montana L. (winter savory) Is a perennial and hardy dwarf shrub which is native to Ukraine, North Africa, South Europe and Turkey (Small, 1997). It is an aromatic plant which has been traditionally utilized as a food preservative and a spice in the Balkan region. It is commonly used ingredient in folk medicine, Savory honey for example is used as a remedy for the treatment of bronchitis. It is also utilized as a stimulant, choleretic digestive and antiseptic for gastrointestinal tract, it is even used as a treatment for premature ejaculation (Mihajilov-Krstev et al, 2013). The essential oils obtained from this plant are used in the making of liqueurs and perfumes, and have a potential interest in pharmacology for its antimicrobial effects (Ciani et al,. 2000). Additional biological properties of the essential oils are an antioxidant, antiviral and antispasmodic (Škočibušić et al., 2006).

In recent times particles in the nano-sized range or “nanoparticles” have generated a lot of attention and concern because of our still increasing ability to synthesize and manipulate these materials. Today, nanoparticles are used in many different fields such as cosmetic, energy, electronic, pharmaceutical, biomedical, catalyc, environmental and material applications (Nowacka and Bucheli, 2007). As a cause of their extensive use in consumer products it is likely that nanoparticles will spread into terrestrial, aquatic and atmosphere environments, where their interactions and behaviour is mostly unknown. Consequently, living organisms, mainly the ones who interact strongly with their immediate environment are assumed to be the most affected from the exposition to the nanoparticles. To better understand the potential adverse effects nanoparticles have on the environment, a great attention has been placed on this topic by the scientific community and in recent reports the ecotoxicity of nanoparticles as well as their chemistry have been summarized (Castiglione and Cremonini, 2009).

There have been investigations into the effect of nanoparticles on several plant species, nanoparticles have been shown to have positive and negative effects (Haghighi

and da Silva, 2014). As a result of the lack of studies on the effect of metal nanoparticles on *S. montana* L., this study dealt with the effect of titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles in different concentrations on *in vitro* micropropagation using nodal segments of said plant.

2 Literature review

2.1 Taxonomy and morphology

2.1.1 Family *Lamiaceae*

The *Lamiaceae* family consists of 236 genera and about 7173 species, they can be found in most places of the word, but are absent from the coldest regions of high latitude or altitude (Kubitzki and Kadereit, 2004). They are mostly concentrated in the Mediterranean region (Singh 2004). A distinct characteristic of the family is the glands which contain terpene found in several of its members. Its thanks to these glands that they have an aromatic odor and some of its members are used in folk medicine and food preparation in many parts of the world (Shahidi and Ho, 2000).

They are perennial or annual herbs, shrubs, subshrubs or trees. They can be aromatic or not and are rarely climbers. The stem is erect to prostate and often takes a quadrangular shape, in some cases it forms stolons or either slender or large rhizomes. In most members of this family an *indumentum* of glandular and non-glandular trichomes can be found, simple and branched, usually hair-like, in some rare cases scale-like, typically multicellular uniseriate, either stellate or dendroid, at times glad-tipped, most often large-headed subsessile glands are present (Kubitzki and Kadereit, 2004).

Economically important members of the *Lamiaceae* family are peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), thyme (*Thymus vulgaris*), pot marjoram (*Origanum vulgare*), sweet basil 'niazbo' (*Ocimum basilicum*), and sage (*Salvia officinalis*) which are used in cooking and flavouring. The family also includes rosemary (*Rosmarinus officinalis*) and lavender (*Lavandula angustifolia*) which are a source of popular perfumes. Basil (Ocimum sanctum) which is considered sacred in India. Other members are used as common ornaments such as sage (*Salvia*), *Molucella*, *Coleus, Clerodendrum* and horsemint (*Monarda*). A few species of the genus *Stachys* have tubes which are edible (Singh, 2004). The subfamily *Viticoideae* contains many species that are forest trees and some of them such as many species of *Vitex* and *Gmelina* have

considerable commercial value thanks to their timber. *Tectona grandis* (*Incertae sedis*) which is known commercially as teak also falls under this category and is one of the premier timber trees (Kubitzki and Kadereit, 2004) which is known for its durable and hard wood and is broadly cultivated in Myanmar and India (Singh, 2004).

2.1.2 Genus *Satureja* L.

Pliny a Roman writer was the first to name the *Satureja* L. genus. The name comes form the Latin word "*satureia*" which means "herb of satyrs", for this reason its cultivation was banned in monasteries (Tepe and Cilkiz, 2016). The genus consists of around 200 species worldwide, composed predominantly of aromatic herbs and shrubs, widely found in the Mediterranean region, the Middle East, Northern Africa, Canary Islands, Turkey, Iran and boreal America (Saeidnia et al., 2015; Tepe and Cilkiz, 2016). They inhabit sunny, arid, rocky and stony regions (Serrano et al.,2011). There is confusion over the *Satureja* L. genus generic boundaries (Harley and Paucar, 2000) as a result of this its delimitation from the general such as *Calamintha* and *Micromeria* requires further study (Small, 1997). The common name savory is used for around 30 species of this genus, among which summer savory (*Satureja hortensis* L.) and winter savory (*Satureja montana* L.) are the most cultivated (Saeidnia et al., 2015).

Plants belonging to this genus are used as flavouring compounds in food, cosmetic and pharmaceutical industries as a consequence of their simple cultivation characteristics and sweetness (Tepe and Cilkiz, 2016). The *Satureja* species, have been known for their healing attributes and have been used in folk medicine to treat many different ailments, like muscle pains, indigestion, cramps, diarrhoea, nausea and infectious diseases. There have also been confirmation of their antimicrobial activity against a large range of multidrug resistant microbes. This is thanks to secondary metabolites such as steroids, tannins, flavonoids and essential oils that are present in the species of this genus (Bezić et al., 2009).

Members of the *Satureja* genus typically have glandular hairs that secrete and produce essential oils. It has been proposed to use the content of these essential oils in conjunction with the anatomical structure of the glandular apparatus (the adjoined epidermal cells and glandular cells) as components for the recognition of separate *Satureja* group and thus be useful in solving the difficult and complex taxonomical problems of the group (Bezić et al., 2009).

2.1.3 *Satureja monatana* L.

Satureja montana L. (figure 1) commonly known as winter savory is a perennial, hardy, dwarf shrub. In normal cases its height is between 15 to 40 cm, however in some cases it can reach as tall as 70 cm (Small, 1997). It is woody at the base, branched and forms a compact bush (Stuart, 1979). Essential oils are secreted and produced by gldaular trichomes that cover the surface of the leaves (Dodoš et al, 2015). These leaves are sessile, entire, oblong-linear or oblanceolate (Stuart, 1979) up to 26 mm long and 6.5 mm wide, pointed at the edge pubescent, glandular punctate with distinct midrib. In the leaf axils grows short leafy twigs which are carrying flowers. Flowers in whorls of 2-3, the calyx is tubular (*tubus calycis*), 4.5 to 6.5 mm long, calyx lobes are lanceolate, unequally long, hairy, corolla is 7,5-12 mm long, white, with pink tinge or violet (Gutzerová, 2013). Their flowering spikes are terminal and appear in the early summer to the early autumn (Stuart, 1979). Spots on flowers are common in bloom, the upper labellum (3.5-4 mm) is shorter than the trilobed lower labellum (4.5 mm), there are four stamens and their fruit is *nucula* (Gutzerová, 2013).

The plant is native to the Ukraine, Europe, Turkey and Northern Africa (Small, 1997) where it mostly inhabits rocky limestone habitats that shield it from the Mediterranean coasts strong winds for example abandoned farmlands, rocky pastures and meadows, semi-stable scree slopes. Its habitat rises to 1,200 MASL from the sea level and it can be found even at higher altitudes in some coastal mountains (Dodoš et al., 2015).

Figure 1. *Satureja montana* L. (Belsinger and Tucker, 2016)

Great variability in the morphology of *S. montana* L. even in the same population is a characterization of the species. This is a cause of confusion from the chorological and taxonomic perspectives (Hajdari et al., 2016). The variability is much more prominent between population that are found in more distant and different areas. Populations which are pure are very rare. The most noticeable differences are manifested in the dimensions, hairiness and the form of the leaves, also in the dimensions and form of the corolla, calyx and calyx teeth. As a result of this variability, *S. montana* L. is represented by the following subspecies (Kustrak et al., 1996):

1. subsp. Montana, which is found in central Dinarides both continental and littoral are (Kustrak et al., 1996). This variant is the one most commonly used in cultivation (Small, 1997).

2. subsp. *variegata* (Host) P.W. Ball which occupies the most northwest ares of the species habitat. This subspecies is endemic to the northwest of Croatia and Slovenia and the northeast of Italy. It also inhabits Bosnia and Hercegovina, specifically near the cities of Livno and Bihac (Kustrak et al., 1996).

3. subsp. *psidica* (Wettst.) Silic. This variant is found in the southwest parts of the species habitat (Kustrak et al., 1996).

4. subsp. *illyrica* (Host) Nym., which is found in the Balkan area (Small, 1997).

5. subsp. taurica (Velen.) P. W. Ball, which is native to the Crimean peninsula where it is undergoing experimental cultivation because of its essential oil rich in thymol (Small, 1997).

2.2 Chemical composition

Per the available literature, the chemical composition of *S. montana* L. essential oils differed according to the localities the specimens were collected from and their ontogenetic stages (Mihajilov et al., 2013). This shows the presence of a various different chemotypes (Hajdari et al., 2016).

In addition to producing essential oil, *S. montana* L. produces other active compounds such as hydroxycinnamic and rosmarinic acid (Stahl-Biskup, 1998; Chizzola, 2003). Aromatic monoterpenes are the principal chemical compounds of the essential oil and are the ones causing its characteristic taste and odor (Chizzola, 2003). The phenolic compound which is typical for *S. montana* L. is carvacol and it's the prevalent chemotype which occurs in Italy and the former Yugoslavia (Dudaš et al., 2013).

Satureja montana subsp. *montana*, which is the prevailing *S. montana* L., has traces of to 68 % carvacrol, to 61 % thymol, to 47 % para-cymene, to 23 % γ-terpinene

and to 20 % eucalyptol, which provides a thyme/oregano-like odor (tucker and DeBaggio, 2009).

The populations in the Balkan area include a para-cymene type which has, between 15-48 % para-cymene and 5-21 % borneol, a trace of trans-sabinene hydrate to 21 % and to 14 % γ-terpinene. There is also a trans-sabinene hydrate type, a linalool type, a borneol type, a p-cymen-8-ol type. There is also a carvacrol/thymol type which has between 5-52 % carvacrol, 3-45 % thymol and 4-26 % para-cymene (tucker and DeBaggio, 2009)

2.3 Uses and Importance

The flavours of winter savory (*S. montana* L.) and summer savory (*S. hortensis* L.) are similar to each other, however *S. montana* L. is thought of as inferior by many. S. montana L. flavour was described by Richardson (1991) as being close to the taste of thyme or sage, and its well suited to heavy dishes like stews, soups and baked beans. Its flowering tops and leaves get added into stuffings, egg dishes, poultry, meats, pizza, fish and salads, in some cases it is used a part of bouquet garni in soups (Small, 1997). It is generally a popular herb in the Mediterranean countries because of its frequent use in South European cuisine. In the Dalmatia region of Croatia it is often used as a spice and as a traditional medicinal plant (Škočibušić et al., 2006). In France *S. montana* L. is cultivated for its essential oil which is used as a flavouring condiment for sauces, sausages, soups, canned and prepared meats, table sauces and perfumes it has also been commonly applied to trout and in Belgium and the Netherlands is used for marinating and pickling. In Italy the dried leaves are used in vermouths and bitters. The leaves can also be brewed and drunk as tea (Small, 1997).

Saureja montana L. is used to treat several aliments, for example it is used to expel gas from the alimentary canal, as a laxative, appetizer, stimulant, sedative, to vermifuge and to nic, in traditional folk medicine. Some people consider it an aphrodisiac other consider it an anaphrodisiac (Small, 1997). Traditionally it has also

been used to treat different disorders such as male sexual dysfunction, there was even a patent registered concerning the use of the plant extracts in the treatment of premature ejaculation (Zavatti et al., 2011).

S. montana L. biologically active constituents are credited for its positive effects on the health of humans. The essential oils of the plant have several biological properties such as antioxidant, antiviral, antidiarrhoeal and antispasmodic (Škočibušić et al., 2006). Serrano et al. (2011) observed that the these essential oils and ethanol extracts of the plant include compounds which have antimicrobial properties, which can potentially be utilized in the extension of shelf life and preservation of raw and processed foods as antimicrobial agents.

S. montana L. is not as economically important as its annual cousin *S. hortensis* L. However in Germany and North America it is popular. The words foremost importer of the herb is the United States, which imports around 80 to 90 tons of the herb every year. *S. montana* L. is also the most commonly found type in Morocco, Spain, central Europe and the former Yugoslavia. The major exporters of the herb are France and the former Yugoslavia (Small, 1997)

2.4 Ecology

2.4.1 Cultivation

Satureja montana L. sufficiently in poor soils. While it has been collected in a large range of soil types in Europe, in the nature, it favours well drained and light soils in its cultivation. It is recommended by Barash (1993) sandy, light and well drained soils with only the minimum of organic matter. It has been reported that *S. montana* L. tolerance of pH is between the 6.5 to 7.3 range, some say 6.5 to 7.0. It has been observed by Pellecuer (1973, 1985) that it can be found growing naturally on soils in Europe as acidic as 5.5 to as basic as 9.0, however it is most abundant in the range between 7.5 to 8.5 (Small, 1997).

Its growth occurs between the temperature of 6 and 27 °C, with the optimal temperature being 13 °C. It can grow under a slight shade however it favours sunny locations with plenty of sun. If the soil is overly rich or moist the spices can undergo winter kill as per Barash (1993) (Small, 1997). With the intention of finding climatic conditions range under which a plant could grow, James A. Duke collected what is called "ecosystematic data" on various of plants from different sites around the globe. Duke's study found that *Satureja montana* L. grows in a range of places that receive as little as 29 inches (736.6 mm) of rain annually to places that receive 68.1 inches (1729.74 mm) of rain annually, with the mean precipitation rate being 42.5 inches (1079.5 mm) of rain (Tucker and Debaggio, 2009).

In order to maintain good growth of *S. montana* L., it is necessary to remove the dead wood, to space the plant 30 cm apart and to divide and replant it regularly every 2 to 3 years. It is also recommended to clip the plants back to around 15 cm in height in the fall. It can overwinter outside up to zone 4. To provide winter protection mulch with straws or leaves (Small and Deutsh, 2001).

It is possible to cultivate *Satureja montana* L. as a house plant provided it gets 5 hours of direct sunlight at the minimum every day. Mature plants can be cut to half their height and left to recover outside for 2 to 3 weeks before being brought inside the house. Indoors the soil should be left to become moderately dry between each watering, the plant can be fertilized by a half strength all-purpose fertilizer each 3 or 4 weeks (Small, 1997).

2.4.2 Harvesting and storage

Satureja montana L. is harvested just before its flowering and then using dehydrators it is dried for the commercial market. Home gardeners are able to harvest the herb at any time, however only the tender tip growth should be picked, the herb can then be used frozen, fresh or dried (Small, 1997). To dry S. montana, a cut is made at the stem 20 cm from the plants base. The herb is then hung in a shady and cool area in

an upside-down position (Small and Deutsh, 2001). In the post harvest period the processing techniques such as the drying type used, the applied temperature abd the distillation technique have a significant influence on the aromatic plants quality (Dudaš et al., 2013).

2.4.3 Diseases and pests

S. montana L. is under normal condition pest-free, however it is prone to root rot and winterkill if the soil is overly rich or moist (Small and Deutsh, 2001).

In the north of Italy, near the city of Albenga a blight was found on 3 % of the 500 pottet 2 month old plants. A fungus which had the morphological characteristics of *Rhizoctonia solani* was isolated from the plants. The isolates which were obtained from the affected plants were successfully anastomosed with *Rhizoctonia solani* isolate AG 1 (ATCC 58946) (Garibaldi, 2012).

2.4.4 Propagation

It is possible to generatively propagate *S. montana* L. by seeds althought the germination is uncertain and the time is slow. Vegetative propagation is can be achieved in several way such as dividing already existing plants in the spring, rooting the softwood cuttings in the summer or layering the branches between the late spring to the early summer (Small and Deutsh, 2001).

2.5 Micropropagation of medicinal plants of the family *Lamiaceae*

For thousands of years plants have been used as a source of medicine, their medical value lie in chemical substances that produce physiological reaction in the human body. Natural medicines extracted from plants have a tremendous potential as a new and effective way of dealing with ailments of the modern day. Among the oldest of systems that utilize parts of plants or its whole for treatment of various illnesses is

traditional medicine such as Chinese, Unani, Ayurvedic, Japanese and recently Homeopathy and chiropathy that is about 200 years old (Shahzad and Parveen, 2013).

As a consequence of the increasing world population particularly in the developing world, ecosystems and natural growing sites are getting destroyed and a number of plants have become threatened with extinction (Chebel et al., 1998). To resolve the problem of plant decimation, *in vitro* regeneration or micropropagation is a biotechnological tool that can potentially provide this solution. Micropropagation is the rapid multiplication of stock plant material producing a vast number of clones, this is done with the use of modern plant tissue culture methods (Shahzad and Parveen, 2013).

For the micropropagation of *Minthostachys mollis,* the nodal explants which were extracted under aseptic conditions were cultivated on a half-strength MS (Murashige and Skoog, 1962) medium, supplemented with 6-benzyladenine (BA), and/or naphthaleneacetic acid (NAA) in various concentrations. The number of auxillary shoots regenerated from each nodal explant was recorded after 4 weeks. All the shoots with the height of up to 1.5 cm were transferred to a half-strength MS medium containing 0.05 NAA, 0.05 or 0.5 µM indole-3-butyric acid (IBA), or 0.06 or 0.6 µM indole-3-acetic acid (IAA), this was done for rooting. After a period of 4 weeks the number of roots per shoot as well as rooting percentage were evaluated. It was found that media containing 0.05 µM NAA and 2.2 µM BA produced the optimal number of healthy shoots, higher concentrations resulted in greater hyperhydricity and less extension. 0.5 NAA caused the best root formation and the largest rooting percentage of 91.6 % (Chebel et al., 1998).

In a study Vasile et al., (2011) tested quick methods for the micropropagation of *Coleus Blumei* Benth species using binodal mini-top grafting cuttings which were cultivated on an MS medium supplemented with several phytohormones. BA, 6-furfurilaminopurine (K) and n6-(2-izopentenil)adenine (2IP) in 0.5 mg/l, 1.0 mg/l and 1.5 mg/l concentrations for the induction of rhizogenesis. Per the results the largest regeneration percentage was obtained in MS medium supplimented with 1.5 mg/l BAP

(92.5 % ± 5.8) and the rhizogenesis process was most intense in the case of basic MS medium without any supplements (Vasile et al., 2011).

In the experiment of Kaul et al. (2013) to create a protocol for the micropropagation of *Ajuga bracteosa.* Aseptic tissue culture of the plant were acquired after which the explants were cut using a sterile blade and cultivated on MS medium with 3 % sucrose, 0.63 % agar and supplemented with plant growth regulators in different concentrations and either alone or in combination. For rooting shoots which were able to regenerate to 3-5 cm in length were transferred to a MS medium with IBA. The explants grown on MS medium which was supplemented with IAA (2 mg/L) and BA (5 mg/L) could regenerate 100 % of shoots with an average of 41.4 shoots and length of 8.4 cm per culture. When the excised *in vitro* shoots were transferred to MS medium with IBA in 0.5 mg/l concentration they produced 20 roots per shoot of an average length of 20.2 cm in 100 % of the cultures. Of the three used explants, petiolem leaf and root, the leaves displayed the quickest response and the were followed by petiole while the root explants were the slowest.

In order to evaluate the effects of growth regulators and culture media on the shoot proliferation and growing in *in vitro* conditions of the plant *Cunila galioides* Benth, their axillary buds were used. The best multiplication rate was found in MS medium supplemented with 8.8 µM of BA**.** The best rooting conditions were obtained in MS medium supplemented with IBA in 0.5 to 2.5 µM concentration. The plants exhibited normal development after they were successfully transferred to *in vivo* conditions (Fracaro and Echeverrigaray, 2000).

Mentha piperita L. was cultivated from nodal explants on a basic MS medium, supplimented with various combinations of auxins and cytokinins. The best regeneration rate of scions (23.9 ± 0.576) was obtained on a MS medium with Zeatin (Z) and IAA in 0.5 mg/l concentrations. The best composition for root formation out of the tested varieties proved to be the one with BAP and IAA in 1 mg/l concentration producing 10.2

roots per explant, and for the stimulation of growth in length it was Z and iAA in 0.5 mg/l concentration (Vasile et al. 2011).

2.5.1 *In vitro* micropropagation of genus *Satureja* L.

In a study Mozafari et al., (2015) debeloped an *in vitro* propagation protocol for *Satureja avromanica* using leaf disk and shoot tip explants. The nodal and leaf explants acquired from wild plants were cultivated on MS and Woody plant medium (WPM) and the media were supplemented with BA, BAP and thidiazuron (TDZ) (0, 0.1, 0.5, 1, 1.5, 2, 5 and 10 mg/l) alone or by application of BA and TDZ (0, 2, 5 and 10 mg/l) in combination with IBA and 2,4-D (0, 0.1, 0.5 and 1 mg/l), respectively. The results showed that the highest mean shoot number (6.21) was from explants cultivated on MS medium with 2 mg/l BA. As for shoot length, the best results were from MS medium with 20 mg/l TDZ and MS medium with mg/l BA with shoot lengths at (82 and 4.39 cm, respectively) after a period of 6 weeks of cultivation. Increasing the concentration of the three tested cytokinins led to an increase in regenerated shoot number and an improvement in the response frequency of the explants. WPM medium with 0.1 mg/l IBA wa found to be suitable for rooting of regenerated shoots. In conclusion the application of BA. BAP and TDZ was found to enhance direct shoot regeneration of *Satureja avromanica* while the treatment media supplemented with a combination of IBA and BA as well as 2,4-D and TDZ in the majority of plants resulted in callogenesis.

2.6 Metal nanoparticles

Nano-scale particles or “nanoparticles” are atomic or molecular aggregates with at least one dimension between 1 and 100 nm, they can significantly alter their physico-chemical properties when compared to the bulk material (Castiglione and Cremonini, 2009) These materials are widely employed in cosmetics, antibacterials, air cleaning and skin care products, energy, transportation and even agriculture due to their unique physico-chemical properties. Because of their ability to penetrate cell membranes faster and easier their application as fertilizer in agriculture has great potential (Haghighi and da Silva, 2014) They enter into plant cells through either endocytosis or non-endocytic penetration. They are capable of being taken by the roots and transported to the shoots using the plants vascular system. Their impact on the metabolism of the plant depends on many variables such as the surface chemistry, concentration and hydrodynamic size, also their inhibitory effect on growth varies vastly according to the type of plants and nanoparticles used (Rahmani et al., 2016).

2.6.1 Zinc oxide (ZnO) nanoparticles

In a study Rahmani et al., (2016) observed the effects of ZnO and CuO nanoparticles on the growth of *Brassica napus* L.. The seeds were cultivated on a half-strength MS medium supplemented with either ZnO or CuO nanoparticles in 10, 100 and 1000 mg/l concentration. Results found that the nanoparticles induced the growth responses of the seed at the concentration of 10 mg/l. However the higher concentrations of 100 and 1000 mg/l decreased root and shoot elongation and the dry weights from these results it was concluded that in low concentrations ZnO and CuO nanoparticles are beneficial while in higher concentrations they resulted in toxic responses.

2.6.2 Titanium dioxide (TiO_2) nanoparticles

Titanium dioxide (TiO2) is a commonly used whitening agent in food production, cosmetics, medicines, sunscreens, paints, cosmetics and pharmaceuticals. As a result of its frequent use in industry and daily lives of people, a lot of residues are released into the environment. This caused the TiO_2 nanoparticles to be considered an emerging environmental contaminant (Frazier et al., 2014).

In a study Feizi et al., (2012) observed the effects of bulk and nanosized TiO_2 in (1, 2, 10, 100, and 500 ppm) concentrations on the germination of Wheat (*Triticum aestivum* L. var. Pishtaz) seeds. From the results, it was found that among the germination indices, only the mean germination times were affected by the treatments. The highest and lowest mean germination times (1.35 and 0.89 days) were found in control and TiO_2 nanoparticles in 10 ppm concentration. Both bulk and nanosized TiO_2 had significant effects on the shoot length, seedling length and dry root matter. Applying TiO_2 nanoparticles in 2 and 10 ppm concentrations yielded higher shoot and seedling lengths than the Control or bulk TiO_2 at 2 and 10 ppm concentrations. From this it was concluded that TiO_2 nanoparticles in suitable concentrations promoted the germination of seeds in contrast to bulk TiO_2, however in high concentrations it had an inhibitory effects on wheat.

3 Aims of the Thesis

The aim of this thesis is to determinate the effect of titanium dioxide (TiO2) and zinc oxide (ZnO) nanoparticles on micropropagation of *Satureja montana* L. by nodal segments.

4. Materials and Methods

4.1 Plant material

The plant material chosen as the basis of this study was *Satureja Montana* L. which belongs in the *Lamiaceae* family. The plant was chosen for its medicinal uses in folk medicine and its essential oils which has biological properties such as antimicrobial, antiviral, antioxidant, antispasmodic and antidiarrhoeal (Škočibušić et al., 2006). The plant material was obtained from the plant tissue collection maintained in *in vitro* conditions at the Laboratory of Plant Tissue Cultures (Faculty of Tropical AgriScience), CULS Prague. The plant material was multiplied using micropropagation before starting the experiment.

4.3 Methodology of micropropagation

4.3.1 Preparation of multiplication medium

For the *in vitro* micropropagation of the plants a basic MS medium (Murashige and Skoog, 1962) was used, composition of the medium is shown in Table 1. A litre of the MS medium was prepared by measuring 100 ml of solution A and 10 ml of each of the solutions B, C, D, E and V using a graduated cylinder and mixing them in a beaker after which about 300 ml of distilled water (H_2O) was added. 100 g of *Myo*-inositol and 30 g of Sucrose were weighted on a scale and added into the solution while stirring. The pH value of the solution was measured and adjusted using potassium hydroxide (KOH) to 5.7. Subsequently 8 g of agar was weighted and added to a beaker filled with 500 ml of distilled water (H_2O) and stirred. Both solutions were heated in a microwave then mixed together and filled to the final volume of 1 litre with warm distilled water (H_2O). The still warm medium was divided among 80 test tubes then the tubes were closed with a plastic lid and autoclaved.

Table 1: Components of MS medium **(**Murashige and Skoog, 1962).

Medium Murashige – Skoog			
Storage solutions		Weight to 1 litre of distilled water	Need for 1 litre of MS Medium (pH 5.7)
A	NH_4NO_3	16,5 g	100 ml
	KNO_3	19 g	
	$CaCl_2$	3,3 g	
	$MgSO_4 . 7H_2O$	3,7 g	
	KH_2PO_4	1,7 g	
B	H_3BO_3	620 mg	10 ml
	$MnSO_4 . 4H_2O$ (H_2O)	2,23 g (1,69 g)	
	$ZnSO_4 . 4H_2O$ ($7H_2O$)	860 mg (1,06 g)	
C	KI	83 mg	10 ml
	$Na_2MoO_4 . 4H_2O$	25 mg	
D	$CuSO_4 . 5H_2O$	2,5 mg	10 ml
	$CoCl_2 . 6H_2O$	2,5 mg	
E	Na_2EDTA	3,72 g	10 ml
	$FeSO_4 . 7H_2O$	2,78 g	
V	Nicotinic acid	50 mg	10 ml
	Pyridoxine (B6)	50 mg	
	Thiamine (B1)	10 mg	
	Glycine (amino acid)	200 mg	

Direct weight for 1 litre of MS medium:

- *Myo*-inositol – 100 mg
- Sucrose – 30 g
- Agar – 8 g

4.3.2 Medium sterilization

To ensure that MS medium was not contaminated, a physical destruction of microorganisms was performed using an autoclave immediately after preparation of the medium as the final step.

The MS medium was autoclaved at the temperature of 121 °C and the pressure of 105kPa for the duration of around 20 minutes.

4.3.2 Preparation of aseptic condition

The necessary tools for micropropagation were all prepared for sterilization. Petri dishes, tweezers, scalpels and pasteur pipettes were wrapped in aluminium foil and put to sterilize for 3 hours at 160 °C in a dry heat sterilizer. The flowbox in which the micropropagation was carried, was cleaned using 70% ethanol then the medium and the needed tools were placed inside while still wrapped in foil after they were sprayed with 70% ethanol. Next the UV lamp and fan were turned on for a minimum of 30 minutes. To maintain aseptic conditions, before beginning to work both hands were sprayed with 70% ethanol, the tweezers and scalpel were placed in a flask containing 70% ethanol and were regularly flamed during micropropagation. Before nanoparticle application the manual propipetter was placed in 70% ethanol bath for a about day.

4.3.3 Micropropagation and nanoparticle application

For the experiment, only micropropagation by nodal segments was used. Sprouts were divided in the flowbox into nods with the length of about 1 cm and gown in basic MS medium. 40 nodal segments were prepared for each different variant and control. The plant explants were left to grow for 5 days. The cultivation conditions were 16/8 day night cycle and a temperature of 25/23 °C day/night with a light intensity of 2500 lx. After which, into healthy looking explants a solution with Zinc oxide (ZnO) or titanium dioxide (TiO_2) in different concentrations was added (table 2) using pasteur pipettes. No solution was applied into the control variant after the initial micropropagation.

The medium for nanoparticles was prepared in the same way as basic MS medium explained in capitol 4.3.1, however it was not heated and agar was not added. Afterwards the medium was divided into different beakers. Zinc oxide (ZnO) or titanium dioxide (TiO_2) were weighted and added into separate medium solutions to achieve the desirable concentrations of 20 mg/l, 40 mg/l and 60 mg/l, see table 2. The beakers were sealed with Parafilm M(R) and aluminium foil and sterilized in the autoclave for 20 minutes.

Table 2. List of variants of the experiment

Variant	**Nanoparticles [mg.l^{-1}]**		**Medium**	**pH**
	ZnO	**TiO_2**		
Control	-	-	MS	5.7
Z1	20	-	MS	5.7
Z2	40	-	MS	5.7
Z3	60	-	MS	5.7
T1	-	20	MS	5.7
T2	-	40	MS	5.7
T3	-	60	MS	5.7

4.3.4 Results evaluation

Plant growth was observed through a 60 day period. Data was obtained around every 10 days. The Number of sprouts, lengths of sprouts, number of nods, number of roots, length of roots furthermore regeneration percentage and percent of plants that have formed offshoots, all were traced and measured. Measurements were taken through the tube walls using a paper ruler.

The data were processed using Kruskal-Wallis ANOA, Multiple comparisons and Tukey post-hoc test.

5. Results and discussion

5.1 Zinc oxide (ZnO) and Titanium dioxide (TiO_2) effects in the micropropagation of *Satureja Montana* L.

5.1.1 Plant regeneration

The best results of plant regeneration were obtained from nodal explants grown on basic MS medium without nanoparticles added (Control group), where 100 % of nodal explants were able to regenerate. Best regeneration from among the ZiO_2 variants was in Z1 in which had a 20 mg/l ZnO nanoparticle solution added to the MS medium, where 88.57 % of the nodal explants regenerated. Among the TiO_2 variants T3 (TiO_2 60 mg/l) had the best regeneration at 73.68 %. The worst regeneration rate was obtained from nodal segments in which TiO_2 nanoparticles were applied in 20 mg/l and 60 mg/l concentrations, variant T1 46.8 % and variant T3 50 % (Table 3).

It was concluded that the difference in regeneration percentage of nodal segments is most likely due to the condition and characteristics of the mother plant and the size of nodal explants.

It was not possible to compare the results with previous studies on *Satureja* L. However in a study by Helaly et al., (2014) on Zn and ZnO nanoparticles within *in vitro* micropropagation of *Musa paradisiacal* L. it was concluded that there were no marked negative effects on plant regeneration even at concentration of 200 mg/l.

It was found that low concentrations of TiO_2 nanoparticles (0.1 and 1.0 %) did not affect seed germination in *Nicotiana tabacum* L. however at higher concentrations had a significant effect, decreasing the germination rate of seeds. (Frazier et al., 2013).

5.1.2 Number of sprouts per plant

The largest mean number of sprouts was found in variant T1 which formed (2.31 ± 0.94) sprouts per one nodal explant. Followed by the Control group in which the mean number of sprouts was (2.02 ± 0.67). Similar results were yielded by variants T2 with (1.94 ± 0.74) and variant Z3 with (1.92 ± 0.46) mean number of sprouts. Close to the previous two variants, the T3 variant formed (1.8 ± 1.22) sprouts per nodal explant. The smallest mean number of sprouts were found in variants Z2 at (1.69 ± 0.63) and Z1 at (1.74 ± 0.44) (Table 3).

The difference between results of all the variants with nanoparticles and Control were not statistically significant, with a p value of 0.1512. This mean ZnO nanoparticles and TiO_2 nanoparticles did not have an effect on the mean number of sprouts within this experiment.

Even thought there was no statistical difference between variants a small decrease in the mean number of sprouts can be observed (Table 3) in the T1, T2 and T3 variants with the increase in the concentration of TiO_2 nanoparticles, this aligns with the study of Frazier et al,. (2013) which found that TiO_2 nanoparticles caused a decrease in shoots in case of *Nicotiana tabacum* L. seeds.

In *Musa paradisiacal* L the number of shoots was increased with an increase in ZnO and Zn nanoparticles concentration up to 100 mg/l, less increase was found at 200 mg/l (Helaly et al., 2014). This was not observed in this study.

5.1.3 Length of sprouts

The variant Z1 had the highest mean length of sprouts of all variants at (55.5 mm ± 39.6). Variant T1, which had the highest result from among the TiO_2 group, and Control had results close to each other, T1 was at (40.52 mm ± 31.1) and Control was at (39.14 mm ± 26.82). Variant Z2 was again lesser than the other two ZnO variants with a mean sprout length of (28.17 mm ± 22.12). The worst results were detected in variants with added TiO_2 nanoparticles, variants T2 at (26.59 mm ± 22.81) and variant T3 (25.32 mm ± 25.72), both of which were in a 1.27 mm range of each other (Table 3).

Variants Z1 and Z3 showed a fast-steady growth, Z3 showed a slightly larger growth than variant Z1 until experiencing a drop in the last measurement. The drop was caused by the regeneration of new sprouts from the nodal segments which brought the mean length down. Variant T1 experienced a sharp growth in the last 22 days (Figure 2).

The variant Z1 showed a statistical difference between it and the Control group, it also showed a significant statistical difference between it and variants Z2, T2 and T3. Differences between other variants with nanoparticles and Control was found to be statistically insignificant. From this we denote that the application of 20 mg/l ZnO nanoparticles on nodal explants has a positive effect on the length of sprouts. We also conclude that the application of ZnO nanoparticles in 40 mg/l and 60 mg/l concentrations didn't influence sprout length and so did the application of TiO_2 nanoparticles in 20 mg/l, 40 mg/l and 60 mg/l concentrations.

In a study Rahmani et al., (2016) found that *Brassica napus* L. seedling when grown on ½ strength MS medium supplemented with 10 mg/l ZnO nanoparticles. The nanoparticles induced a growth response at that concentration. While higher concentrations of 100 mg/l and 1000 mg/l had a toxic effect. This is somehow consistent with our results, in which the concentration 20 mg/l ZnO showed positive effect.

Feizi et al., (2011) observed that TiO_2 nanoparticles had a significant effect on shoot length and seedling length of Wheat (*Triticum aestivum* L.). The results from 2 and 10 ppm concentrations of TiO_2 nanoparticles were better than the Control group.
These results conflict with the results obtained from our experiment in which the application of TiO_2 nanoparticles didn't have an effect.

5.1.4 Number of nods per sprout

The highest number of nods per sprout was found in the Z1 variant (6.5 ± 3.44) which was slightly higher than Control (5.73 ± 2.39). In the group with TiO_2 nanoparticles, the T1 variant had the highest mean number of nods at (5.39 ± 2.93). The lowest numbers were again obtained from the T1 (4.41 ± 2.08) and T3 (4.36 ± 2.5), correlating with their low mean sprout length (Table 3).

The variants Z1 and Z3 had a steady growth of od numbers through the 60 day period, with variant Z1 overtaking Z3 at the last 10 days. Correlating with the previus results from the previous capitol 5.1.3 (Figure 3).

There was no statistical difference between all the variants with nanoparticles compared to Control. However the Z1 variant was statistically different from both variants T1 and T2. From this we conclude that the application of 20 mg/l of ZiO nanoparticles has a better effect than applying TiO_2 nanoparticle in 40 mg/l and 60 mg/l nanoparticles. We also conclude that the application of TiO_2 and ZnO nanoparticles doesn't have an effect of nod formations relative to the Control group.

5.1.5 Number of roots per plant

The highest number of roots per plant was found in the Control group (7.81 ± 4.46). Among the variants with nanoparticles T2 yielded the best result (7.44 ± 3.51). In the ZiO group, variant Z1 had the highest results at (6.48 ± 4.21). The worst root formation was observed in the Z2 variant (Table 3)

For the variants Z1, Z2 and the control group, root formation was most prominent in the first 34 days, after this period only a slight increase in the number was achieved. The T1 variant had a significant increase in root number in the last 10 days (Figure 4).

A statistical difference was found between the Control group, variant T2 and the Z2 variant. This suggest that 40 mg/l ZiO nanoparticles have a negative effect on root formation. There was no statistical difference between the other variants and the Control group, from this we deduce that ZiO nanoparticles in 20 mg/l, 60 mg/l concentrations and TiO_2 nanoparticles do not have an effect on root formation

Helaly et al., (2014) found that in *Musa paradisiacal* L. ZnO nanoparticles in 100 mg/l and 150 mg/l concentrations had a positive effect on plant rooting however the minimum rooting was on medium containing ZnO nanoparticles at 50 mg/l. This is similar to our result where 40 mg/l ZnO had the minimum number of roots per plant.

5.1.6 Root length

The control group had the longest roots at (22.14 mm ± 4.13). Among the variants with nanoparticles the Z3 variant had the highest root length (18.42 ± 6.24). In the TiO_2 group the highest length was in variant T2 at (14 mm ± 4.58). The smallest roots were found in variant Z2 (10.64 mm ± 5.81) a similar number was in variant T3 (11.35 ± 3.22) (Table 3).

In the variants Z3, Z1 and the Control group most root growth was in the first 21 days, this was most predominant in the Control group. The other variants had a steadier root growth (Figure 5).

There was no statistical difference between the Control group and Z3 variant. This suggest that 60 mg/l ZnO doesn't have an effect on root length. However there was a statistical difference found between the variants Z1, Z2, T1, T2, T3 and the Control group. From these results we deduce that ZnO nanoparticles in 20 mg/l, 40 mg/l and TiO_2 nanoparticles in 20 mg/l, 40 mg/l, 60 mg/l concentrations inhibit the elongation of roots.

In the study Helaly et al., (2014) found the lowest root lengths of *Musa paradisiacal* L. to be in medium with 50 mg/l. However higher concentrations of 100 mg/l had a significant positive effect. This is similar to our results.

Frazier et al,. (2013) observed that the average root length of *Nicotiana tabacum* L. seeds was decreased as TiO_2 nanoparticles concentrations increased. This is consistent with our results.

Table 3. ZnO and TiO_2 nanoparticles effect on the micropropagation of *S.montana* L from nodal segments.

ZnO (mg/l)	TiO_2 (mg/l)	Control	Regeneration (%)	Mean Number of sprouts	Mean length of sprouts (mm)	Mean number of nods	Mean number of roots	Mean lenght of roots (mm)
				p = 0.1512	p = 1.1593E-06	p = 0.0004	p = 0.0055	p = 1.9895E-13
Z1-20	-	-	88.57	1.74 ± 0.44^{a}	55.5 ± 39.6^{a}	6.5 ± 3.44^{a}	6.48 ± 4.21^{ab}	17.67 ± 4.86^{bc}
Z2-40	-	-	58.97	1.69 ± 0.63^{a}	28.17 ± 22.12^{b}	4.84 ± 2.09^{ab}	3.21 ± 4.29^{b}	10.64 ± 5.81^{d}
Z3-60	-	-	75.67	1.92 ± 0.46^{a}	43.2 ± 37.26^{ab}	6 ± 3.38^{ab}	5.28 ± 1.41^{ab}	18.42 ± 6.24^{ab}
-	T1-20	-	46.8	2.31 ± 0.94^{a}	40.52 ± 31.1^{ab}	5.39 ± 2.93^{ab}	6.81 ± 5.54^{ab}	13.75 ± 8.31^{cd}
-	T2-40	-	73.68	1.94 ± 0.74^{a}	26.59 ± 22.81^{b}	4.41 ± 2.08^{b}	7.44 ± 3.51^{a}	14 ± 4.58^{cd}
-	T3-60	-	50	1.8 ± 1.22^{a}	25.32 ± 25.72^{b}	4.36 ± 2.5^{b}	4.88 ± 5.35^{ab}	11.35 ± 3.22^{d}
-	-	MS	100	2.02 ± 0.67^{a}	39.14 ± 26.82^{b}	5.73 ± 2.39^{ab}	7.81 ± 4.46^{a}	22.14 ± 4.13^{a}

Kruskal-Wallis ANOVA, Multiple Comparisons ($p < 0,05$)

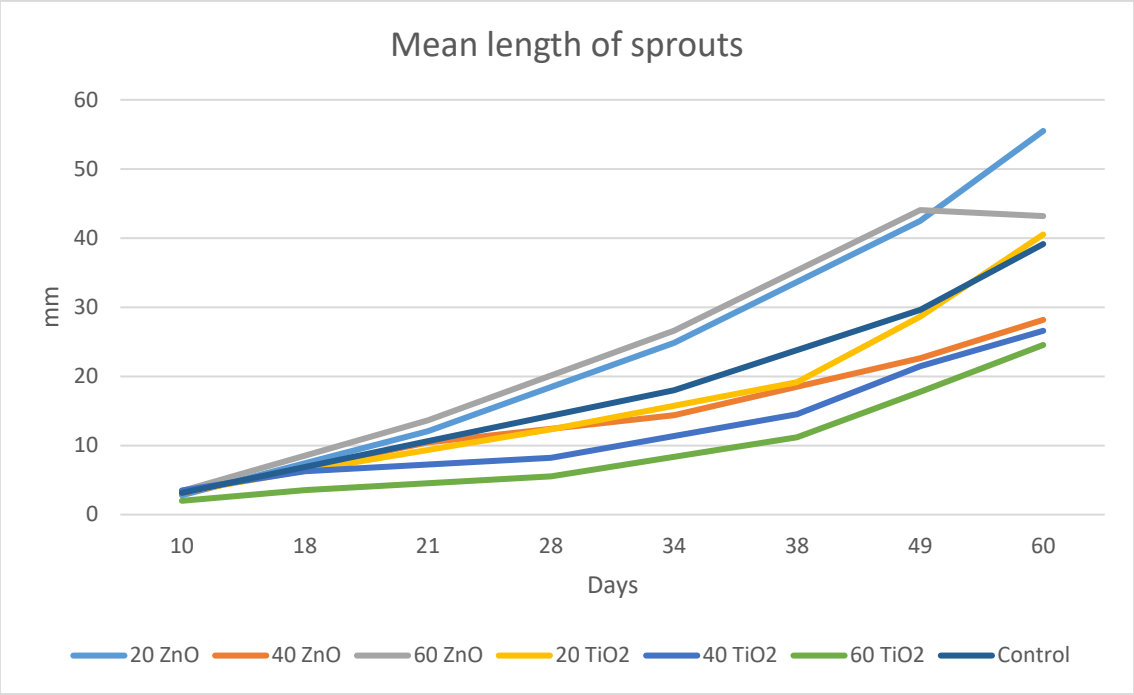

Figure 2. Comparison of the mean length of sprouts in a period of 60 days between different variants.

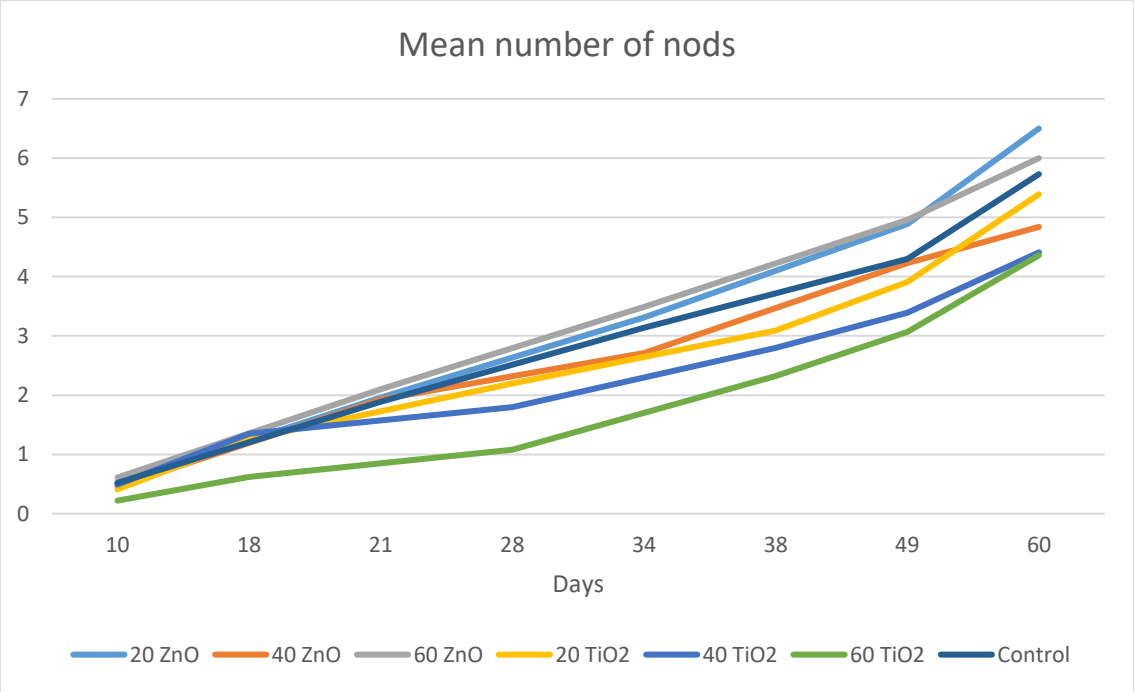

Figure 3. Comparison of the mean number of nods on one sprout in a period of 60 days between different variants.

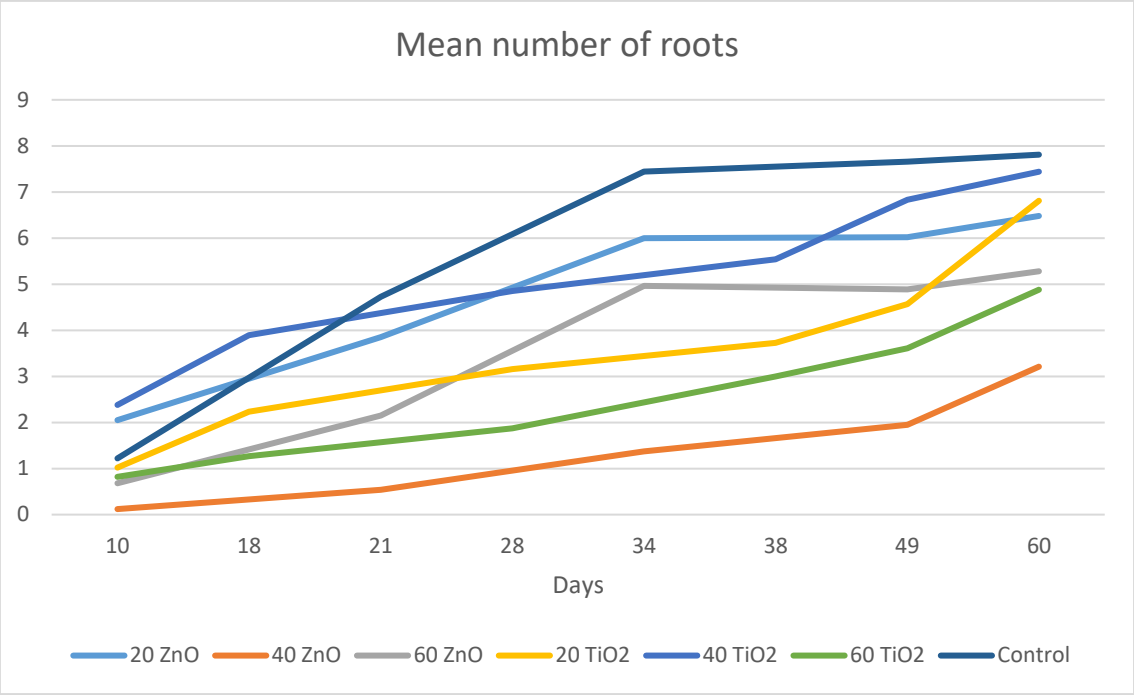

Figure 4. Comparison of the mean number of roots formed in a period of 60 days between different variants.

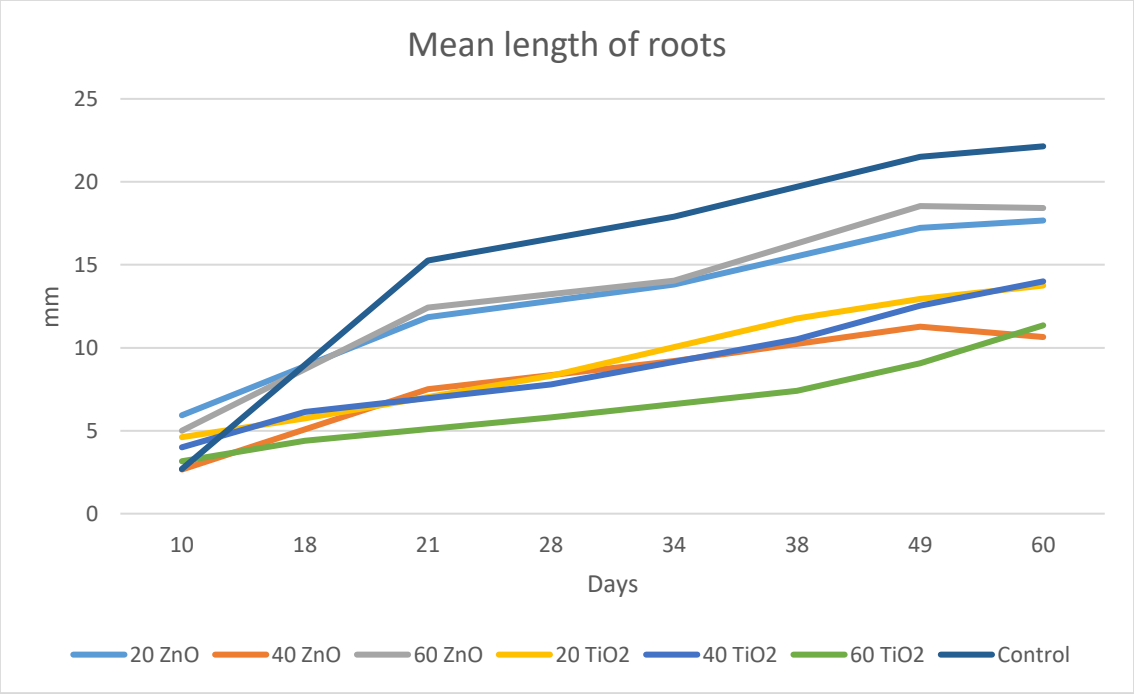

Figure 5. Comparison of the mean length of the longest roots between different variants.

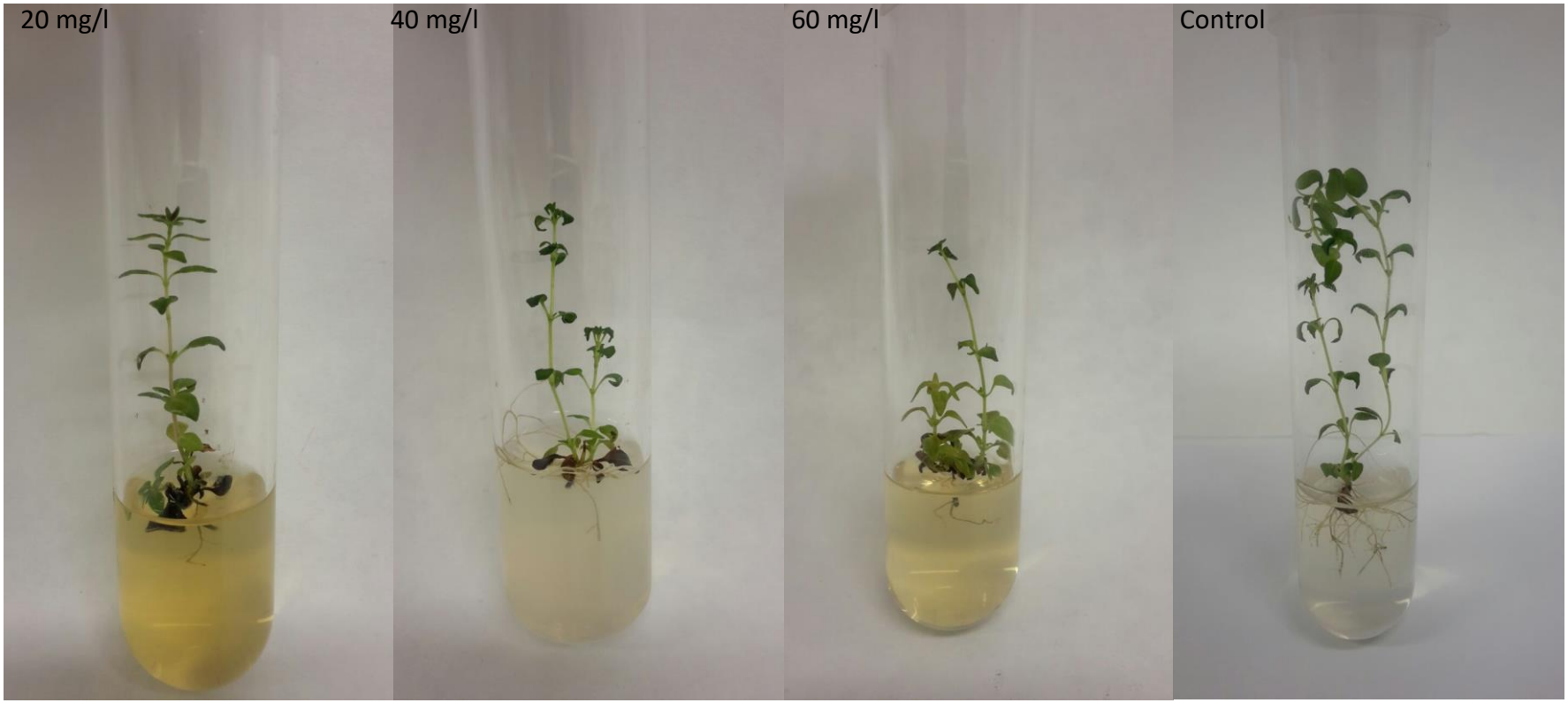

Figure 6: Plants supplemented with TiO_2 nanoparticles in 20 mg/l, 40 mg/l, 60 mg/l concentrations and Control

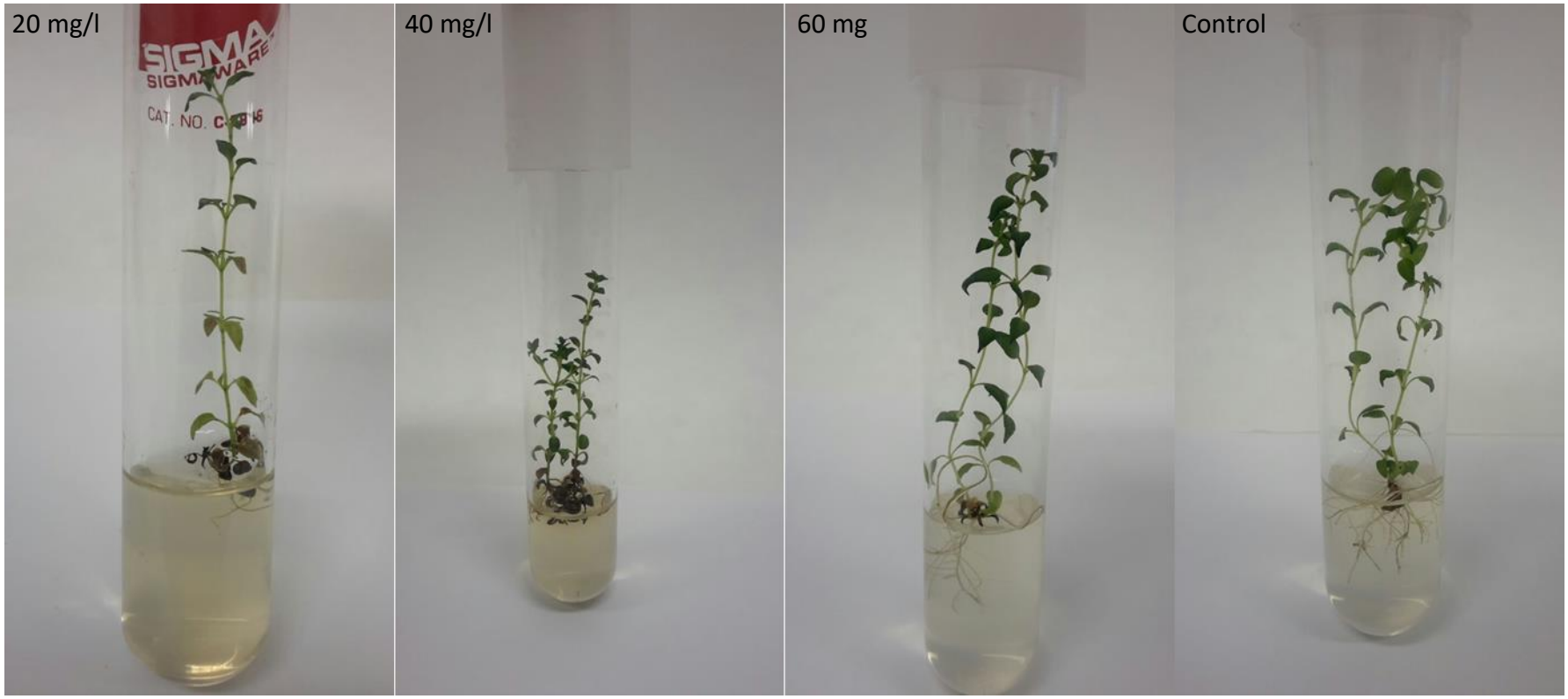

Figure 7: Plants supplemented with ZnO nanoparticles in 20 mg/l, 40 mg/l, 60 mg/l concentrations and Control

6 Conclusion

The results from this study on the effect of zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles on the *in vitro* micropropagation from nodal explants of *Satureja montana* L. showed the following:

Titanium dioxide nanoparticles in 20 mg/l, 40 mg/l and 60 mg/l did not have a significant effect in plant growth, however it showed a toxic effect on root length which increased with nanoparticle concentration increase.

Zinc oxide nanoparticles in 20 mg/l concentration positively affected plant length however slightly decreased root length. However, ZnO in 40 mg/l concentration had a negative impact on the plants by inhibiting root formation and root length. ZnO in higher concentrations (60 mg/l) did not have an effect on plant growth.

This was the first study dealing with metal nanoparticles ZnO and TiO_2 effects on the in vitro micropropagation of *S. montana* using nodal segments.

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