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Zinc (ZnO) and Titanium (TiO2) Nanoparticles effect on micropropagation of *Salvia rosmarinus Spenn*

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled "Zinc (ZnO) and Titanium (TiO2) Nanoparticles effect on micropropagation of *Salvia rosmarinus* Spenn**"** independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 24/04/2024

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Bc. Muhammad Waqas Ashraf

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Abstract

This is an interesting study of the convergence between nanotechnology and plant science that investigates how Zinc Oxide (ZnO) and Titanium dioxide propose In vitro propagation *Salvia rosmarinus* Spenn, known as rosemary. The study used nodal explants of rosemary, grown in a simple MS (Murashigue and Skoog, 1962) medium and then subjected to alternative concentration of ZnO or TiO_2 nanoparticles. During a period of 60 days, the experiment monitored and measured several growth parameters namely sprouts emergence length nodal number root development in controlled conditions.

These results of this study are fascinating and important. Lower concentrations of ZnO nanoparticles were seen to promote the length, while higher ones acted negatively on root formation. On the other hand, TiO2 nanoparticles were toxic to root length growth in all tested concentrations. These findings add to the subtlety of understanding how nanoparticles can have an impact on plant growth and that they do not simply depend on concentration but are highly dependent upon type.

This thesis not only contributes to the emerging field of agricultural nanotechnology but also provides potential ways for improving plant productivity and vigour especially economically valuable medicinal plants such as *Salvia rosmarinus* Spenn. The study highlights the possibility of nano-technology application in agriculture carefully implying a future world where nanoparticles could assist to enhance plant growth.

Key words: *In vitro*, Micropropagation, nodal segments, TiO_2 nanoparticles, ZnO nanoparticles,

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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. rosmarinus Spenn - *Salvia rosmarinus* Spenn

TDZ – Thidiazuron

Z – Zeatine

1. Introduction

The incorporation of nanotechnology in agricultural activities, especially through the application of Zinc Oxide ZnO and Titanium Dioxide TiO_2 nano particles is revolutionizing plant micropropagation. This thesis therefore seeks to look at how these nanoparticles affect the micropropagation of *Salvia rosmarinus* Spenn a plant used for medicinal and culinary purposes (Rahimi-Rizi et al. 2023). The central research question is: What impact do ZnO and TiO_2 nanoparticles have on the micropropagation of *Salvia rosmarinus* Spenn, as wellness for increasing agriculture techniques

The use of nanoparticles in plant propagation is a novel area with huge promise as far as improving growth, yield and survivability of plants are concerned. It concerns especially economically and medicinally useful plants such as *Salvia rosmarinus.* Nanotechnology techniques applied to propagation methods play an essential role in ensuring sustainable agricultural practices and enhancing plant productivity (Alenezi et al. 2022).

The positive effect of nanoparticles on the growth of plants has been shown by recent studies. Adhikary et al. (2022) determined that the rice germination and growth was altered by ZnO nano particles, suggesting their participation in promoting early plant development (Adhikary et al. 2018).

According to TiO2 nanoparticles, (Ahmed e al. 2023) referred the positive influences on vetiver a medicinal plant (TiO_2 NPs). This can be aligned to (Faraz et al. 2020; Hadi & Abass, 2019) who stated that TiO_2 nanoparticles are effective for improving growth within wheat plants which then implies their widespread application encompassing agricultural activities (Faraz et al. 2020; Hadi & Abass, 2021).

While numerous investigations have demonstrated the positive outcomes, nanoparticles interacting with plants may be a multi-layered issue. Sun and colleagues, 2020 Consequently, it is crucial to develop a more complete understanding of how different nanoparticle behaviours impact plant developmental dynamics in order to regulate their usage trans-lantally or postharvest for consumers' benefit because the growth process both can be improved by ZnO nanoparticles as well from damages. (Sun et al. 2020)

However, there is a great deficiency of specific studies regarding *Salvia rosmarinus* micropropagation under various ZnO and TiO_2 nanoparticle treatments. This study aims to bridge this gap by analyzing the complex workings of these nanoparticles and *Salvia rosmarinus* at micropropagation level (Alenezi et al. 2022).

This work was made so as to launch an exploratory campaign that synthesizes the effect of ZnO and TiO_2 nanoparticles in micropropagation of *S. rosmarinus* Spenn. With a view to revolutionizing dissemination procedures for plants of therapeutic pragmatic value, this research analyses the impacts and mechanisms studying various aspects of plant nanotechnology as well as sustainable agricultural practices so that it can offer practical insights about its use.

2. Literature Review

2.1 Taxonomy and morphology

2.1.1 Family *Lamiaceae*

Salvia rosmarinus Spenn, called rosemary is part of *Lamiaceae* that encompasses a wide variety of aromatic herbs and shrubs. *Lamiaceae* one of the most prominent families in terms of its impact both on culinary and medicinal realities (Raja 2012). *Lamiaceae* is represented by stems that have square cross-sections, leaf arrangement being opposite to one another, and the leaves are usually aromatic; this second feature prevalent in *Salvia rosmarinus.* This family is characterized by bilabiate flowers and production of essential oil that can be found in aromatherapy, flavouring, as well as natural medicine (Pedersen 2000).

Indeed! Rosemary (*Salvia rosmarinus*) is a member of the Lamiaceae family, which is also known as the mint family. Lamiaceae includes a diverse range of aromatic herbs and shrubs, such as basil, mint, oregano, thyme, and lavender, among others. Rosemary itself is well-known for its culinary uses, medicinal properties, and ornamental value. It's fascinating how within one plant family, there's such a rich assortment of species with various beneficial characteristics!

Lamiaceae consists of over 236 genera and more than seven thousand species, so it is one of the largest plant families in terms number or variety. They are predominant in temperate regions but also prosper in tropical mountainous areas. *Salvia rosmarinus* is one family that includes several well-known herbs such as basil, mint thyme and lavender (Gul et al. 2019). The *Lamiaceae* family of plants is very economically important as these are used for food, pharmacological and cosmetic industries. Most plants are cultivated because of their essential oils which have various benefits ranging from aromatherapy, flavouring and natural medicine. The phytochemical constituents actively involved in their therapeutic properties (Musolino et al. 2023).

Major *Lamiaceae* re-arrangements due to the recent phylogenetic analysis, including those regarding *Salvia rosmarinus*. These studies have helped to clarify the connections between different genera and species in this very heterogeneous family.

Suffice is to say, the *Lamiaceae* offers a very critical setting for understanding *Salvia rosmarinus* Spenn relevance. The family is valuable because of its characteristics, application widespread in different industries and continuing research as a part of taxonomic studies (Bendiksby, M. et al. 2014).

2.1.2 Genus *Salvia* L.

As a genus with wide morphological diversity, it ranges from herbaceous perennials to shrubs. flowers are that of the *Salvia* species, noteworthy for having a well pronounced lower lip and producing essential oil. Such oils have added distinctive aromas and the medicinal properties of these plants (Kintzios 2000). A subspecies of this family is *Salvia rosmarinus* Spenn commonly referred to as rosemary. One of the largest genera in plant kingdom; several species from this genus are aromatic, having broad spectrum with medicinal property (Raja 2012).

Salvia is a cosmopolitan genus comprising more than 900 species that are found throughout the world. It is highly present in the Mediterranean countries, Central and South America as well as regions of Central Asia. The genus is remarkably adaptive to varying environmental conditions and that explains why it spreads in various regions of the world (Wood & Harley 1989).

Culinary, ornamental and therapeutic uses of plants from the *Salvia* are very popular; one can find mention even about *S. rosmarinus*. Many species of *Salvia* are grown for their volatile oils, which have uses in aromatherapy as well as pharmaceuticals and the food industry. It contains some of the popular species like *Salvia officinalis* common sage, and *Salvia sclarea* clary sage (Jash et al. 2016).

Taxonomic revisions recently made based on recent phylogenetic studies have had a significant impact within the *Salvia* genus. These studies have contributed to the clarification of relationships between various species and increased our knowledge regarding evolutionary history of this genus. Such phylogenetic research is responsible for reclassifying rosemary from the *Rosmarinus* genus to *Salvia* (Bendiksby et al. 2011).

2.1.3 *Salvia rosmarinus Spenn*

Salvia rosmarinus Spenn, (Figure 1) also called rosemary is a perennial herb of the *Lamiaceae* family. It is a famous edible and remedial herb known for its needle-shaped leaves with particular fragrance (Leporini et al. 2016)**.** The leaves of Rosemary contain essential oils that are very rich. These oils provide the scent and therapeutic benefits because they have bioactive compounds including 1,8-cineole; alpha pinene and camphor. The oil composition if effected by both environmental conditions as well Geographical area (Flamini et al. 2021).

This is evident in the antioxidant activity of extracts from *Salvia rosmarinus* Spenn. The Phenolic Compounds such as flavonoids and *rosmarinic* acid are responsible for antioxidant activity. Rosemary also effective in removing radicals and oxidative stress by its antioxidant properties (Musolino et al. 2023). For long time this species has been studied for its anti-inflammatory, antibacterial and anticancer properties (Brindisi et al., 2020). This also help in better digestion, increase mental and concentration capacity and very fruitful to release stress and anxiety during late age (Brindisi et al. 2020).

Salvia rosmarinus (rosemary), the plant of Salvia genus of the *Lamiaceae* family, is renowned for its restorative powers. The herbal power of rosemary includes the ability to control infections, anti-oxidative effects, and reduction of inflammation. In this particular case as well as the whole of the Genus from the *Salvia* family, these plants have been widely studied and regarded as the decisive ones to discover their medicinal properties. In the process of further studies their therapeutic benefits had shown to be diverse in nature. Comparing *Salvia rosmarinus* to other medicinal plants in the same genus or family, several key findings emerge: The discovery of healty fats that distinguish Salvia *rosmarinus* from other plants in the same genus or close family, reveal several essential aspects.

Pharmacological effects

For example, *Salvia limbata* is still more a member of the Salvia family which is reported to have certain health benefits. In particular, it has proved helpful by blocking the development of inflammation and alleviation of pain, which make it an alternative candidate for drug development against chronic inflammatory conditions (Karami et al. 2015). It is clearly, the therapeutic value of the genus is confirmed in the case of both, *Salvia rosmarinus* and *Salvia limbata*, with clearly, considerable analgesic and anti-inflammatory properties exhibited in these plants.

Rosemary contains compounds like rosmarinic acid, carnosic acid, and carnosol, which exhibit antioxidant activity. These compounds help neutralize free radicals in the body, reducing oxidative stress and potentially lowering the risk of chronic diseases. Some studies suggest that rosemary extracts possess anti-inflammatory properties, which may help reduce inflammation in the body. This could be beneficial for conditions like arthritis and inflammatory disorders. There is evidence to suggest that rosemary may have positive effects on memory and cognitive function. Inhalation of rosemary essential oil has been associated with improved cognitive performance and mood in some studies. Certain components of rosemary oil, such as cineole and camphor, have demonstrated antimicrobial activity against bacteria and fungi. This could potentially make rosemary useful for combating infections.

Rosemary has traditionally been used to aid digestion and relieve gastrointestinal discomfort. It may help stimulate the production of digestive enzymes and bile, facilitating the digestion process. Some research suggests that rosemary extracts may have anticancer properties, potentially inhibiting the growth of cancer cells and inducing apoptosis (cell death) in certain types of cancer. However, more studies are needed to fully understand this potential effect. Rosemary oil is often used in hair care products due to its purported ability to stimulate hair growth and improve scalp health. It also has antimicrobial properties that may be beneficial for treating certain skin conditions.

Anticancer properties

The *Salvia* which encompassed *Salvia rosmarinus* culturally has long been associated with the different medicinal effects that it may cause, probably anticancer. According to a study that compared different classes of *Salvia*, the genus' broad pharmacological actions which include antioxidant and anti-inflammatory, anti - cancerous are attributed to their rich composition of phenolic acids and flavonoids, which are simply (Yıldırım & Kutlu, 2015; Gaziza et al. 2023).

Salvia rosmarinus, commonly known as rosemary, has been investigated for its potential anticancer properties, particularly due to its rich phytochemical content. Rosemary contains various antioxidants, such as rosmarinic acid, carnosic acid, and carnosol, which help neutralize free radicals and reduce oxidative stress. Oxidative stress can contribute to the development of cancer by damaging DNA and promoting inflammation. By reducing oxidative stress, rosemary may help lower the risk of cancer development. Chronic inflammation is associated with an increased risk of cancer development and progression. Some components of rosemary, such as rosmarinic acid and carnosol, have been shown to possess anti-inflammatory properties, which may help inhibit the inflammatory processes involved in cancer growth.

Studies have suggested that rosemary extracts and essential oils may inhibit the proliferation of cancer cells by interfering with various signalling pathways involved in cell growth and division. For example, carnosic acid found in rosemary has been shown to inhibit the proliferation of colon cancer cells by inducing cell cycle arrest and apoptosis (programmed cell death). Metastasis, the spread of cancer cells from the primary tumour to distant sites in the body, is a major cause of cancer-related mortality. Some research indicates that rosemary extracts may have anti-metastatic effects by inhibiting the migration and invasion of cancer cells. Certain compounds in rosemary may have chemo preventive properties, meaning they can help prevent the initiation or progression of cancer. These compounds may act by blocking the formation of carcinogens, enhancing detoxification processes, or promoting the repair of damaged DNA.

Antioxidant and cytotoxic properties

Against the backdrop of *Salvia urmiensis* and *Salvia hydrangea's* contribution in the extract of antioxidant and cytotoxic, it appears that the genus of *Salvia* can be a great source of natural bioactive compounds (Bahadori & Mirzaei 2015). This data correlates across the genus, where *Salvia rosmarinus* is another of the members which would exhibit antioxidant activities having the potency for its application in medicine.

Rosemary (*Salvia rosmarinus*) has been investigated for its cytotoxic properties, meaning its ability to induce cell death in various types of cancer cells. Several studies have suggested that certain components of rosemary, particularly its essential oils and bioactive compounds, exhibit cytotoxic effects against cancer cells. Apoptosis, or programmed cell death, is a natural process that helps regulate cell growth and eliminate damaged or abnormal cells. Some studies have shown that rosemary extracts and essential oils can induce apoptosis in cancer cells by activating specific pathways that lead to cell death. Components such as carnosic acid and carnosol have been implicated in this process. Rosemary extracts have been found to disrupt the cell cycle progression of cancer cells, leading to cell cycle arrest at specific checkpoints. This prevents cancer cells from dividing and proliferating uncontrollably. By halting the cell cycle, rosemary compounds can inhibit the growth of cancer cells and promote their eventual demise.

Rosemary extracts have been shown to inhibit the proliferation of cancer cells by interfering with processes involved in cell division and growth. Compounds like carnosol and rosmarinic acid may target signalling pathways that regulate cell proliferation, thereby slowing down the growth of cancer cells. Angiogenesis is the process by which new blood vessels are formed to supply nutrients and oxygen to growing tumours. Inhibition of angiogenesis can starve tumours of their blood supply, hindering their growth and metastasis. Some research suggests that rosemary extracts may possess anti-angiogenic properties, which could contribute to their cytotoxic effects against cancer cells. ne advantage of using rosemary for cancer treatment is its potential to selectively target cancer cells while sparing normal cells. Some studies have reported that rosemary extracts exhibit selective cytotoxicity against cancer cells compared to non-cancerous cells, suggesting a favourable therapeutic window for cancer treatment.

Pharmacognostic characteristics

Yet examples have demonstrated distinctions exist in phytochemical content as well as morphological traits among S. evansiana and S. miltiorrhiza, elucidating this diversity within *Salvia*. However, the chemical structure and shape of *S. pratensis* and *S. bertolonii* display marked differences, which are characterized by the fact that they have different essential oil components and leaf shapes (Anačkov et al. 2008). This prompts the recognition of *Salvia* species, including *Salvia rosmarinus*, as integral sources of bioactive compounds which in turn play a crucial role in medical fields.

Rosemary is an evergreen shrub with woody stems and needle-like leaves. The leaves are linear, about 2-4 cm in length, and usually dark green on the upper surface and whitish on the lower surface due to fine hairs. Rosemary produces small, two-lipped, pale blue to white flowers arranged in axillary clusters. The epidermis of rosemary leaves contains elongated cells with thick walls and stomata. Rosemary leaves possess glandular and non-glandular trichomes, which contribute to the plant's aroma and medicinal properties. Cross-sectional examination reveals vascular bundles arranged in a ring with xylem towards the center and phloem towards the periphery. Rosemary contains various phytochemicals that contribute to its medicinal properties.

Rosemary essential oil contains compounds such as α-pinene, camphene, 1,8-cineole, camphor, and borneol, which contribute to its characteristic aroma and pharmacological effects. Rosmarinic acid, carnosic acid, and carnosol are among the major phenolic compounds found in rosemary with antioxidant and anti-inflammatory properties. Determination of moisture content helps ensure the stability and shelf-life of dried rosemary products. These indicate the number of soluble constituents extracted from rosemary using specific solvents, providing information about the plant's chemical composition. Ash content determination helps assess the purity of rosemary by estimating the amount of inorganic material present after incineration.

Ethnopharmacological benefits

The Chinese traditional medicine involves an ethnopharmacological study of medicinal plants from the genus *Salvia*, including *Salvia rosmarinus*, which examines the benefits of plants from the *Lamiaceae* family therapeutically, and consequently emphasizes the role of *Salvia* species including *Salvia rosmarinus* which alleviates health issues (Minhui et al. 2013).

Therefore, Smooth Rosemary exhibits such *Salvia* species as well as the *Lamiaceae* family types of pharmacological properties, which include antioxidant, anti-inflammatory as well as anticancer effects. The wide range capabilities of this herb highlight the importance of the plant in medicinal studies and potentially for producing natural health products with bioactive compounds belonging to it.

Many types of foods use this as spice and useful for meat, bread and vegetables to make them tasty. It also finds its application in manufacturing herbal teas and infusions (Annemer et al. 2021). Research in these modern times has focused on rosemary use for agricultural implementation, particularly its sustainability. This plant can enhance the growth of plants as well aid in making compost for organic farming and gardening (Leporini et al. 2020).

Nanotechnology has simplified the process of growing rosemary and its application in other areas. ZnO nanoparticles have been considered to be an effective method for promoting the size increase, yield positivity and taste enhancement of rosemary tree. Such nanoparticles may allow plants to receive more nutrients and resist stress better (Alenezi et al. 2022).

Figure 1. *Salvia rosmarinus* Spenn. (Musolino et al. 2023)

2.2 Chemical Composition

It is an origin of essential oils, with camphor and 1,8-cineole α -pinene in them makes this plant aromatic like its benefits to health (Flamini et al. 2022; Annemer et al. 2022). However, it contains phenolics such as rosmarinic acid carnosic acid and caffeic acids that are potent antioxidants. It is a stronger antioxidant than because of flavonoids genkwanin luteolin (Brindisi et al. 2020). Camphene and limonene contribute to the herb's well-being, giving it a pleasant smell (Leporini et al. 2020).

2.3 Uses and Importance

Rosemary is commonly applied seasoning for meat, soups and breads particularly in Mediterranean cuisine (Annemer et al. 2018). It is used in traditional medicine for oxidative stress diseases. Accused of increasing cognitive performance and memory. Treating arthritis and muscle pain with anti-inflammatory effects. The essential oils of this plant have been shown to be effective against many pathogens (Shahina et al. 2022). Used in the fragrance and antioxidant properties of creams, lotions or shampoos. Is the object of research because it improves soil quality and plant health, which positively affects ecological farming (Leporini et al. 2020).

Research points to its use in Nano technology evolution for better growth and productivity of plants using some nanoparticles such as zinc oxide, briefly *S. rosmarinus* Spenn is a multi-dimensional species with an extremely wide chemical composition that supports the uses in culinary, medicinal practices also cosmetics and agricultural sectors. The fact that it is located in traditional and modern applications makes its use a resource for health, wellness, and those supporting sustainable practices (Alenezi et al. 2022).

2.4 Ecology

2.4.1 Cultivation

Salvia rosmarinus is a hardy perennial herb with fragrant leaves and much resilience; Spenn's rosemary. It performs well in sandy or loamy soils that are slightly acidic to neutral. This plant came from the Mediterranean region, and it can grow well in hot sunny, dry areas that is why this type of climate patterns are perfect for gardens (Maccioni et al. 2020).

While growing rosemary, the plant requires adequate sunlight not less than six to eight hours per day. It is critical for the development of its oils which are responsible to produce distinct aroma and taste. Once established, it is also drought-tolerant and can therefore be used in water efficient landscaping i.e., xeriscaping. On the other hand, young plants typically need regular watering until their root system is established (Ayoob et al. 2023).

2.4.2 Harvesting and storage

However, when harvesting rosemary's delicate balance is achieved in obtaining a sufficient quantity for immediate use while assuring the plant prospers. Harvesting should occur in the morning after dew has evaporated and before high sun. This time of harvest ensures that the essential oils are retained since they peak up during this period, especially in the leaves (Martin et al. 2023).

For harvesting, cut off the sprigs or branches with a sharp pair of scissors o One should try to prune just above a node leaving the joint, thereby encouraging branching and creating bushiness. For drying, make small bunches of the cut sprigs and hang them upside down in a warm airy dark area. After drying, the leaves can be stripped off from the stems and kept in an airtight container away from light so as to never lose its flavor (Leporini et al. 2020).

2.4.3 Diseases and pests

Rosemary is rather resistant yet not completely disease- and pest free. Fungal diseases such as powdery mildew and root rot are common, in particular when the conditions are humid or if you water too much. These risks can be mitigated by proper drainage and prevented through overhead watering. Other concerns can include pests such as spider mites, aphids and whiteflies. These can be controlled with natural predators, neem oil sprays or insecticidal soaps. The key preventative measures include regular inspections and keeping the circulation of air around plants as low as possible (Aamer et al. 2013; Pieracci et al. 2021).

Powdery Mildew

Powdery mildew is a fungal disease that appears as a white, powdery growth on the leaves of rosemary. It thrives in humid conditions and can weaken the plant if left untreated.

Root Rot

Excessive moisture or poor drainage can lead to root rot, caused by various fungal pathogens. Symptoms include yellowing leaves, wilting, and eventual death of the plant.

Leaf Spot

Several fungal pathogens can cause leaf spot diseases on rosemary. Symptoms include dark spots or lesions on the leaves, which may eventually lead to leaf drop if the infection is severe.

Bacterial blight

Bacterial blight can affect rosemary, causing brown lesions on the leaves and stems. It spreads through contaminated water and can be challenging to control, especially in wet conditions.

Verticillium Wilt

Verticillium wilt is a soil-borne fungal disease that can affect a wide range of plants, including rosemary. It causes wilting, yellowing, and eventual death of the plant by restricting water flow in the vascular system.

Pests

Aphids

Aphids are small, sap-sucking insects that can infest rosemary plants, especially in new growth. They can cause stunted growth, distorted leaves, and the transmission of viral diseases.

Spider Mites

Spider mites are tiny arachnids that feed on the undersides of rosemary leaves, causing stippling, discoloration, and eventually leaf drop if the infestation is severe.

Whiteflies

Whiteflies are small, flying insects that feed on the sap of rosemary plants. Heavy infestations can lead to wilting, yellowing of leaves, and the transmission of viral diseases.

Rosemary beetles

These metallic green beetles feed on the foliage of rosemary plants, causing significant damage to leave and stems if left unchecked.

Rosemary leafhoppers

Leafhoppers are small, wedge-shaped insects that feed on the sap of rosemary plants, causing stippling and yellowing of leaves.

2.4.4 Propagation

Rosemary is often reproduced by taking cuttings, preserves the features of parent clones. It is better to use semi-hardwood cuttings from new growth that has begun hardening. Cuttings should be about 4 inches to 6 inches long and the lower leaves need removed prior placement in a well-drained substrate. Rooting hormone can be used for the development of roots. On the other hand, in vitro propagation techniques having commercial importance are also practiced obtaining genetically uniform and disease-free plantlets (Kostas et al. 2021).

2.5 Micropropagation of medicinal plants of the family *Lamiaceae*

The longstanding use of plants in traditional medicine systems, such as Chinese, Ayurvedic, Unani, and Japanese practices, highlights their critical role in healthcare. These systems, along with more recent ones like Homeopathy and Chiropathy, leverage the physiological effects of plant-derived chemical substances for therapeutic purposes (Shahzad & Parveen 2013). However, the rapid growth of the global population, particularly in developing countries, has led to significant ecological disruptions and the endangerment of numerous plant species (Chebel et al. 1998).

To address the threat of plant extinction, micropropagation, a form of in vitro regeneration, has emerged as a key biotechnological strategy. This technique involves the rapid cloning of plant material using advanced tissue culture methods, producing a large number of plant clones from a single specimen (Shahzad & Parveen 2013).

Minthostachys mollis micropropagation stands as a case in point. An isolation process is carried out during the procedure where the nodal explants are subjected to sterilization and culturing on a half-strength MS medium including different BA and NAA concentrations. This results in the production of numerous auxiliary bumps from every explant after four weeks. After reaching a height of approximately 1.5 cm, these shoots are thereafter transferred to media with decreased levels of NAA and either IBA or IAA in order for root development to occur It has been reported that a combination of 0.05µM NAA and 2.2µM BA in the medium leads to optimal growth for healthy shoots formation (Chebel et al. 1998), reported that 0.5µM NAA is the most effective for root formation, yielding a success rate of 91.6% (Chebel et al. 1998).

In another work by Vasile et al. (2011), Coleus Blumei Benth micro propagation has been studied using binodal mini-top grafting cuttings application. These explants were cultured on a MS medium containing phytohormones such as BA, K and 2IP. The levels of the three hormones were between .5 and 1.5 mg/l. It was found that the regeneration rates were lower using MS medium supplemented by 1.5 mg/l BAP and rhizogenesis appeared to be most effective when a standard basic MS medium with no additives was used (Vasile et al. 2011).

Kaul et al. (2013) developed a protocol for Ajuga bracteosa micropropagation, which is the propagation of sterile plant tissue in MS medium containing sucrose, agar and several PGRs. Afterward, shoots that reached 3-5cm length were transferred to an MS medium with IBA for rooting. In the MS medium supplemented with IAA (2 mg/L) and BA (5 mg/L), 100% shoot regeneration was observed at an average number of 41.4 per culture, After the transfer of these shoots into an MS medium that contained 0.5 mg/l IBA, they developed more than twenty roots per each shoot on average (Kaul et al., 2013)

In micropropagation of *Mentha piperita* L., nodal explants grown on an MS medium amended with various auxin–cytokinins combinations were used. For the regeneration of scions, Zeatin (Z) and IAA at 0.5 mg/l concentrations were found to be most effective while for root formation a mix from BAP and IAA had optimal yield after treatment with 1mg/l concentration (Vasile et al. 2011).

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

The genus *Salvia,* especially *Saliva rosmarinus* formerly known as *Rosmarinus* officinalis has generated much interest in scientific research due to its medicinal value and for ornamental purposes. Sterile culturing of plant tissues through the technique of micropropagation plays a significant role in cloning this species rapidly and efficiently.

Studies by (Annemer et al. 2022; Pieracci et al. 2018), have reported the antimicrobial activities of *Salvia rosmarinus* essential oil with implications to both pharmacological applications as well horticultural use therefore efficient propagation method must be used from this species (Annemer et al. 2022; Pieracci et al. 2019). Martin et al. (2016) demonstrated that extracts from *Salvia rosmarinus* promote a defense response within plants- an indication of the potentiating mechanism for plant resilience under controlled conditions vivo (Martin et al. 2016).

Leporini et al. (2020) as well as (Aamer et al. 2023), both investigating chemical structure and use in health detection, are witnesses to the need for *Salvia rosmarinus* 's in vitro cultivation significance. With this method, you can get these pharmaceutical preparations in a way that is trustworthy. The in vitro cultures of *Salvia rosmarinus* was studies by (Kostas et al. 2022) provide a viable solution to the issue of sustainable utilization of *Salvia rosmarinus* and causation of better understanding of its effects on other plant species.

Ayoob et al. (2023) investigated apportion spacing and nitrogen levels that may impact growth rates and oil content of the Lamiaceae species, Salvia rosmarinus for their valuable use in optimizing culture conditions; in bioreactors that aim to scale up cultures; and to increase production of useful metabolites. This topic has been addressed from a broader perspective in general reviews on the subject by (Jan et al. 2016; Kereša et al. (2018).

Using the highly efficient in vitro propagation technique of Erişen et al. (2020), Meta-Topolin was evaluated; and the genetic stability was assessed. Secondary metabolite profile that could be used in manipulation of other members from this genus (Erisen et al. 2020 & Mascarello et al. 2016). These two works have made great advancements in understanding tissue culture methods of *Salvia spp.* (Erişen et al. 2020; Mascarello et al. 2006).

Further, research by (Bueno et al. 2010; Grzegorczyk and Wysokinska 2008), on in vitro responses of different *Salvia hispanica* L. explants and the liquid shoot culture of *Salvia officinalis* L., serves as a knowledge base enrichment for micropropagation studies with regards, the culture of *Salvia* species in vitro especially from *S. rosmarinus* is an important one due to its pharmacological activities and essential oils it has. The combined results from studies provide a foundation for establishing efficient micropropagation protocols, thus enabling the sustainable sourcing of these economically important plants (Bueno et al. 2010).

2.6 Metal nanoparticles

The application of metal nanoparticles, especially ZnO nanoparticles, in agriculture has shown promising results in enhancing plant growth and resilience.

Abdelaziz et al. (2022) demonstrated the potential of biosynthesized ZnO nanoparticles in controlling Fusarium wilt disease in eggplant (Solanum melongena) and promoting plant growth (Abdelaziz et al. 2022). Likewise, (Adhikary et al. 2022) noted that seed priming with selenium and zinc nanoparticles significantly altered the germination, growth, and yield of directly seeded rice (Oryza sativa L.) (Adhikary et al. 2022).

There are variations in the ability of nanoparticles to promote plant growth among different species. Ahmed et al. (2023) noted a relative impact of foliar application for silicon, titanium, and zinc nanoparticles on the performance of vetiver-a medicinal plant (Ahmed et al. 2023).

The context is stress relieving, (Azmat et al. 2022) explained that ZnO nanoparticles alongside growth-promoting plant rhizobacteria, have the potential to alleviate both heat and drought stress in wheat simultaneously by neutralizing the complementary effects of both stress on germination and development of wheat seedlings.

In this respect, too, the nanoparticles can be applied to in vitro plant culture processes. For example, the use of silica nanoparticles and plant growth regulators by (Kahfri et al. 2023) lead to an enhancement of rootstock in vitro micro-propagation of myrobalan 29C *Prunus cerasifera* L (Khafri et al. 2023).

2.6.1 Zinc oxide (ZnO) nanoparticles

Nowadays there has appeared nanotechnology in agriculture with the use of zinc oxide (ZnO) nanoparticles. To prove the point, (Abdelaziz et al. 2022) presented application of biosynthesized ZnO nanoparticles to control Fusarium wilt disease to eggplant and the plant growth promotion characteristics. Similarly, (Adhikary et al. 2022) reported that seed priming with ZnO resulted in changes of in germination performance, growth and yield of rice (Azmat et al. 2022) commented that combined effects of ZnO nanoparticles and PGPR in wheat plants under heat and drought stress were reported. In addition, (Arafat et al. 2023), remarked that ZnO nanoparticles exhibited positive effects on plant growth and soil quality (Azmat et al., 2022).

And besides the outcomes, we find that ZnO nanoparticles have also been explored in various application areas as well. Perhaps a manure that aids in lowering the chromium stress and in tilting rice plants as a possible one (Prakash et al. 2022). Sawati et al. (2020) examined the environmental impacts of biogenic zinc oxide nanoparticles using the proteomics and transcriptomics approach (Sawati et al. 2015).

2.6.2 Titanium dioxide (TiO2) nanoparticles

The recent years have seen a noticeable rise in the focus on the agro adaptability of TiO2 nano particles. The possible positive effects of these particles on plants are explored with the view to eventually exploit them for applications in agriculture. Faraz et al. (2020) present the literature review about titanium nanoparticles and their application on plant growth, also the usage in agriculture. In this study, Hadi & Abass (2021) examined the influence of the titanium dioxide nanoparticles on the growth of wheat plants that used the nanoparticles as a growth stimulator (Hadi & Abass, 2021).

The role of TiO2 nanoparticles on plants interaction with other components in the entire system was examined by (Shafiq et al. 2022). They also assessed the effects of cadmium resistant microbes, titanium oxide, and zinc nanoparticles as cadmium tolerance enhancers in maize. It was reported that nano-TiO_2 and methyl jasmonate elicitors increased the accumulation of phenolic compounds and antioxidant activity in Salvia tebesana Bunge shake flask cultures, keep in mind, as the authors point out, that one or more such compounds might be phenolic. The TiO_2 nanoparticles, in fact, could be used in boosting secondary molecules production, as the authors emphasize.

In addition, (Nourozi et al. 2021) analysed the induction effect of titanium dioxide nanoparticles on rosmarinic acid biosynthesis gene expression and the production of antitumor compounds in *Dracocephalum kotschyi* transgenic roots to propose a potential approach for increasing medicinal Taken together, these studies reveal the multifaceted and positive impact of TiO_2 nanoparticles in agriculture – from promoting plant growth and yield to increasing resistance to stressors as well as developing metabolites with high commercial value.

Role of ZnO and TiO_2 nanoparticles in plant growth and development

The utilisation of nanoparticles in plant propagation is an emerging field that holds great potential in enhancing the growth, productivity, and resilience of plants.

Improved stress tolerance

Nanoparticles can help plants tolerate various environmental stresses, including drought, salinity, and heavy metal toxicity (Jalil & Ansari 2019). The ZnO and TiO_2 nanoparticles have been explored for their potential to improve stress tolerance in plants like rosemary (Alizadeh-Sani et al. 2018). The ZnO and TiO_2 nanoparticles possess antioxidant properties, enabling them to scavenge ROS such as superoxide radicals and hydrogen peroxide (Ge et al. 2022). By reducing ROS accumulation, nanoparticles help mitigate oxidative stress (Tee et al. 2016), which is a common consequence of various environmental stresses including drought, salinity, and extreme temperatures.

These nanoparticles can stimulate the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). These enzymes play crucial roles in neutralizing ROS and maintaining cellular redox homeostasis, thereby improving the plant's ability to withstand stress conditions. The ZnO and TiO2 nanoparticles can modulate the expression of genes involved in stress responses (Eymard-Vernain et al. 2020). They may upregulate genes associated with stress perception, signal transduction, and stress tolerance mechanisms. This molecular response primes rosemary plants to better cope with environmental challenges. Nanoparticles can enhance the plant's water use efficiency by regulating stomatal conductance and reducing transpiration rates (Al-Selwey et al. 2023).

This is particularly beneficial under drought stress conditions, as it helps plants conserve water and maintain turgor pressure in cells. ZnO and TiO_2 nanoparticles may improve nutrient uptake and utilization efficiency in rosemary, thereby alleviating nutrient stress (Nazari et al. 2023).

They can enhance the availability of essential nutrients in the rhizosphere and facilitate their uptake by plant roots, supporting better growth and stress tolerance. Nanoparticles can also influence the synthesis, metabolism, and signalling of plant hormones involved in stress responses (Tripathi et al. 2022). By modulating hormonal balance, nanoparticles help regulate various stress-related processes such as osmotic adjustment, stomatal closure, and defense gene activation (Al-Khayri et al. 2023).

TiO_2 nanoparticles, in particular, possess photocatalytic properties (Gao & Zhang 2001) that can protect plant cells from photodamage caused by excessive light exposure.

They can scavenge harmful reactive oxygen species generated during photosynthesis, thereby reducing oxidative stress and enhancing stress tolerance.

Enhanced disease resistance

Nanoparticles possess antimicrobial properties that can help plants resist pathogen infections. Silver nanoparticles, copper nanoparticles, and ZnO nanoparticles have been investigated for their ability to inhibit the growth of bacterial, fungal, and viral pathogens, thereby reducing disease incidence and promoting healthier plant growth. Both ZnO and TiO_2 nanoparticles exhibit antimicrobial activity against a wide range of pathogens (Mohammed et al. 2021), including bacteria, fungi, and viruses. When applied to the plant surface or incorporated into growth media, nanoparticles can inhibit the growth and proliferation of pathogens, reducing the risk of disease development in rosemary.

Nanoparticles can stimulate the plant's immune system and induce defense responses against pathogens (Chandra et al. 2015). They may trigger the production of antimicrobial compounds, such as phytoalexins and pathogenesis-related (PR) proteins, which help combat invading pathogens and enhance the plant's resistance to diseases. Nanoparticles can strengthen the cell walls of plant tissues, making them more resistant to penetration by pathogens. By reinforcing physical barriers, nanoparticles help prevent pathogen entry and limit the spread of infection within rosemary plants. Nanoparticles can activate systemic acquired resistance, a defense mechanism in plants that provides broad-spectrum protection against pathogens (Dong et al. 2023).

Through the priming of defense pathways and the accumulation of defense-related compounds, nanoparticles prepare rosemary plants to mount a more robust and effective immune response upon pathogen attack. Nanoparticles can promote the establishment of beneficial microbial symbioses in the rhizosphere, such as mycorrhizal associations and rhizobacterial interactions. These symbiotic relationships enhance plant health and vigour, making rosemary less susceptible to pathogen infections. The ZnO and TiO_2 nanoparticles possess antioxidant properties and can scavenge ROS generated during pathogen attack (Metryka et al. 2021). By reducing oxidative stress, nanoparticles help mitigate the damaging effects of ROS on plant cells and enhance the plant's ability to withstand disease pressure.

Regulation of hormonal pathways

Nanoparticles can modulate hormonal signalling pathways in plants, leading to changes in growth and development (Sonkar et al. 2021). Studies have shown that nanoparticles can influence the synthesis, transport, and perception of plant hormones such as auxins, cytokinins, and gibberellins, which play crucial roles in regulating various physiological processes (Faseela et al. 2024).

Nanoparticles can modulate auxin signalling pathways, which play crucial roles in root development, apical dominance, and vascular tissue differentiation (Yadav et al. 2022). ZnO and TiO_2 nanoparticles may affect the synthesis, transport, and perception of auxins in rosemary (Al-Qudah et al. 2022), influencing processes such as root elongation, lateral root formation, and adventitious root initiation. Nanoparticles may also influence cytokinin metabolism and signalling in rosemary plants (Selvakesavan et al. 2023). Cytokinins are involved in various physiological processes, including shoot growth, leaf senescence, and stress responses (Raines et al. 2016). By modulating cytokinin levels or sensitivity, nanoparticles can affect shoot branching, leaf expansion, and other growth-related traits in rosemary.

The ZnO and TiO_2 nanoparticles could potentially impact gibberellin metabolism and signalling pathways in rosemary (Maity et al. 2022). Gibberellins regulate diverse developmental processes, including seed germination, stem elongation, and flowering (Thomas et al. 2005).

Nanoparticles may influence the synthesis, degradation, or perception of gibberellins, altering plant growth and development accordingly. Nanoparticles may modulate abscisic acid (ABA) responses in rosemary, particularly under stress conditions such as drought or salinity (Lamsaadi et al. 2024). The ABA is a key regulator of plant stress responses, including stomatal closure, osmotic adjustment, and gene expression (Fujita et al. 2011). By affecting ABA synthesis, signalling, or perception, nanoparticles can influence the plant's ability to tolerate environmental stresses. ZnO and TiO_2 nanoparticles might also impact signalling pathways involving jasmonic acid (JA) and salicylic acid (SA), which are involved in plant defense responses against pathogens and herbivores (Almutairi 2019).

By modulating JA and SA levels or signalling, nanoparticles can affect the plant's resistance to biotic stresses, including pathogen infections and insect damage. Nanoparticles may influence the crosstalk between different hormonal pathways in rosemary (Lala 2021). Hormones often interact and regulate each other's synthesis, transport, and signalling, leading to complex hormonal networks that coordinate plant growth, development, and stress responses. Nanoparticles may disrupt or enhance these interactions, resulting in coordinated changes in plant physiology and phenotype.

Enhance the nutrients uptake

Nanoparticles have the potential to enhance the accessibility and absorption of vital nutrients by plants. For instance, the utilisation of TiO2 nanoparticles has been demonstrated to augment the absorption of nitrogen and phosphorus (Zahra et al. 2015). The greater surface area facilitates the absorption and interaction of nutrients, hence improving the accessibility of nutrients in the rhizosphere for uptake by plant roots. It has the power to modify soil characteristics, such as pH and nutrient availability (Dimkpa 2018), thereby producing a more favourable environment for the growth of plants. For instance, studies by (García-Gómez et al. 2020; Bala et al. 2019) have demonstrated that ZnO nanoparticles enhance the accessibility of zinc in soil. Zinc is a crucial micronutrient necessary for the growth and development of plants.

Nanoparticles have the ability to promote the growth and branching of roots, resulting in a more extensive root system that can effectively explore a bigger amount of soil to absorb nutrients. The enhanced root surface area enables plants to more effectively acquire nutrients, even when they are present in low concentrations. It has the ability to engage with nutrient ions in the soil solution, creating complexes or changing their movement (Chen 2018). This interaction can enhance the uptake of nutrients by plant roots by facilitating the movement of nutrients from the soil solution to the root surface. Nanoparticles have the ability to regulate physiological processes associated with the absorption and incorporation of nutrients in plants (Aqeel et al., 2022). For instance, a study by (Zhang et al. 2020) has shown that TiO2 nanoparticles can improve the process of photosynthesis and nitrogen metabolism in plants. This, in turn, can indirectly impact the absorption and utilisation of nutrients.

Nanoparticles have the ability to trigger stress responses in plants, which in turn activate pathways that enhance the absorption and utilisation of nutrients in unfavourable circumstances (Rasheed et al., 2022). For example, a study by (Faizan et al. 2021) demonstrated that ZnO nanoparticles can enhance the activation of genes related to the uptake of nutrients and the ability of plants to withstand stress. The potential of ZnO and TiO2 nanoparticles to improve nutrient uptake in rosemary and other plants has been indicated by these mechanisms (Elsayed et al. 2022). However, to maximise their effects and reduce potential risks, it is crucial to take into account factors such as nanoparticle concentration, exposure duration, and environmental conditions (Nekoukhou et al. 2022).

Stimulated photosynthesis

Applying nanoparticles like ZnO and TiO2 stimulates photosynthesis, leading to enhanced carbon assimilation and thus promoting growth and development. The utilisation of ZnO and TiO2 nanoparticles is a captivating field of study with encouraging implications for the enhancement of plant development and production (Šebesta et al. 2021).

Nanoparticles have the ability to function as light-harvesting antennae, capturing light energy from a wider range of wavelengths, including UV wavelengths that are usually not fully utilised by chlorophyll (Carmeli et al. 2010). The expanded range of light absorption can enhance the total energy available for the process of photosynthesis in plants such as rosemary. TiO2 nanoparticles possess photocatalytic characteristics, which implies that they can facilitate reactions upon exposure to light (Dufour et al., 2015). TiO2 nanoparticles, when exposed to UV radiation, have the ability to produce reactive oxygen species (ROS) that activate photosynthetic activities and improve the absorption of carbon in plants (Liu et al. 2019).

Zinc oxide (ZnO) and titanium dioxide (TiO2) nanoparticles exhibit antioxidant characteristics and have the ability to eliminate reactive oxygen species that are produced during the process of photosynthesis (Thakur et al. 2021). Nanoparticles have the potential to enhance the efficiency of the photosynthetic system and maintain optimal photosynthetic rates in rosemary by reducing oxidative stress and photodamage, as suggested by (Araniti et al. 2020).

It has the ability to affect stomatal conductance, which is the speed at which stomata open and close in order to control gas exchange (Ahmed et al. 2023). By modulating stomatal behaviour, plants can enhance their carbon dioxide intake and reduce water loss through transpiration, leading to improved photosynthetic efficiency (Mony et al. 2022). Nanoparticles can induce the production of chlorophyll, the pigment that is accountable for absorbing light energy in the process of photosynthesis. Rosemary leaves' light-capturing capacity and photosynthetic effectiveness can be improved by increasing their chlorophyll content (Karumannil, et al. 2023).

Additionally, it has the ability to cause alterations in gene expression associated with the process of photosynthesis. Research has demonstrated that ZnO and TiO2 nanoparticles can increase the activity of genes responsible for producing photosynthetic proteins and enzymes involved in carbon fixation (Wang et al. 2017). This leads to enhanced rates of photosynthesis and greater accumulation of biomass in plants.

3. Aims of the Thesis

The aim of this thesis is to determinate the effect of titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles on micropropagation of *Salvia rosmarinus* Spenn. by nodal segments.

3.1 Hypothesis of the study

H1: Nanoparticles of TiO_2 and ZnO have negative impacts on the micropropagation of *Salvia rosmarinus Spenn*. through nodal segments.

H2: Utilization of nanoparticles like ZnO and TiO_2 have positive effects on the micropropagation of *Salvia rosmarinus Spenn*. through nodal segments.

4. Materials and Methods

4.1 Plant material

The main plant species of the study was *Salvia rosmarinus* Spenn, *Lamiaceae* family was obtained. The choice of plant was really good for the research purposes, as it contains different components. It exhibits antioxidant, anti-inflammatory and antimicrobial activities that are some of the main factors in health-related studies. Studies revealed that Rosemary can prevent neurodegenerative disease (Perry et al. 2000) and it can be used in food flavor improver. In the agricultural sector, it can act as a natural pesticide and help with phytoremediation activities. In addition, the chemistry of essential oil Rosemary is interesting for industries like perfumery and cosmetics (Bozin et al. 2007).

4.2 Methodology of micropropagation

4.2.1 Preparation of multiplication medium

For micropropagation of the plants in vitro, a simple MS medium originally established by Murashige and Skoog (1962) was used. The specific composition of this medium is depicted in Table 1. 100 ml of solution A and 10ml each of solutions B, C, D E and V were weighed out in a beaker for the preparation of one litter MS medium. To this, about 300 ml of distilled water (H_2O) was added. Second, 100 g of Precise Myo-inositol and 30 g of Sucrose were added to the solution while stirring. However, the pH of the solution was maintained at 5.7 by adding potassium hydroxide (KOH).

After this, 8 g of agar was weighed and added to a beaker with 500 ml distilled water (H_2O), mixed well. Both solutions were warmly microwaved and mixed. The final volume was made up to 1 litter with warm distilled water (H_2O). The medium, still warm, was divided equally into 80 test tubes each fitted 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

4.2.2 Medium sterilization

In order to ensure that the MS medium was free of any undesirable microorganisms, we took a significant measure. After sterilizing the medium, we autoclaved it immediately. This was done so as to physically destroy any potential microbes that might have been present.

Autoclaving involved heating the MS medium to 121°C under pressure at a value of about 105 kPa for approximately twenty minutes. This process aided in the sterilization of our medium, making it ready for experimentation.

4.2.3 Preparation of aseptic condition

We first sterilized all the necessary tools before initiating on micropropagation process. This involved using aluminium foil to wrap items such as Petri dishes, tweezers, scalpels and Pasteur pipettes that were then subjected to a thorough sterilization in dry heat sterilizer at 160°C for three hours.

In order to keep the flow box where micropropagation occurred completely sterile, we initially disinfected it with 70% ethanol. Then, we put the medium as well as required tools in flow box while they were all still covered with foil. To begin, we disinfected them with 70% ethanol.

In order to achieve total cleanliness, we switched on a UV lamp and fan in the flow box for at least 30 minutes. Before we started working, we sprayed both of our hands with 70% ethanol as a safety measure. Additionally, we placed the tweezers and scalpel in a flask containing 70% ethanol and regularly sterilized them with a flame during the micropropagation process.

As for the manual propipetter, it was immersed in a bath of 70% ethanol for approximately a day before we applied nanoparticles, further ensuring aseptic conditions.

4.2.4 Micropropagation and nanoparticle application

In our experiment, we chose to focus exclusively on micropropagation using nodal segments. Within the controlled environment of the flow box, we carefully divided the sprouts into smaller segments, each measuring around 1 cm in length. These nodal segments were then placed in a basic MS medium. For each of our experimental variations and the control group, we prepared 40 nodal segments.

Next, we allowed the plant explants to grow for a period of 5 days under specific cultivation conditions. This included a daily cycle of 16 hours of light and 8 hours of darkness, with temperatures maintained at 25°C during the day and 23°C at night, all under a consistent light intensity of 2500 lx.

After this growth period, we introduced a solution containing either Zinc oxide (ZnO) or titanium dioxide (TiO2) at varying concentrations (as specified in table 2) to the healthy-looking explants using Pasteur pipettes. It's important to note that we did not apply any solution to the control group after the initial micropropagation.

To prepare the medium for the nanoparticles, we followed a similar procedure to the basic MS medium, as explained earlier (in section 4.2.1). However, we omitted the heating step, and agar was not included. We divided this medium into separate containers and carefully measured and added Zinc oxide (ZnO) or titanium dioxide (TiO2) to achieve the desired concentrations of 20 mg/l, 40 mg/l, and 60 mg/l, as outlined in table 2. These containers were securely sealed with Parafilm M(R) and aluminium foil and then underwent autoclaving for 20 minutes to ensure sterilization.

Table 2. List of variants of the experiment

Variant	**Nanoparticles [mg.l^{-1}]**		**Medium**	**pH**
	ZnO	**TiO2**		
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

4.2.5 Results evaluation

Over a period of 60 days, we closely monitored the growth of plants, collecting data about every ten days. We focused on several aspects: how many sprouts appeared, their lengths, the count of nodes, the number and length of roots, as well as tracking the rate of regeneration and the percentage of plants that developed new side shoots. For measurement purposes, we employed a paper ruler, taking readings through the transparent walls of the tubes holding the plants.

In terms of data analysis, we applied a few statistical techniques. This included the Kruskal-Wallis ANOVA, which is a non-parametric method suitable for comparing various groups, accompanied by multiple comparison methods. To pinpoint differences between specific groups, we also used the Tukey post-hoc test, a reliable method for assessing significant differences between each pair of groups.

5. Results

5.1 Zinc (ZnO) and Titanium (TiO2) Nanoparticles effect on micropropagation of *Salvia rosmarinus* Spenn

5.1.1 Plant regeneration

The results of this experiment indicated that the best outcomes for regeneration were obtained by cultivating nodal explants in conventional MS medium without the addition of nanoparticles (referred to as the Control group). This resulted in a regeneration rate of one hundred percent, as depicted in Figure 2.

Within the ZnO nanoparticle-treated groups, the Z1 version exhibited the highest amount of regeneration. This was due to the fact that the MS medium was added with a ZnO nanoparticle solution at a dosage of 20 mg/l. As a consequence, 92.99% of the nodal explants were able to effectively regenerate.

In the experiment involving TiO2 nanoparticles, the T2 variation, which had a TiO2 content of 40 mg/l, demonstrated the highest regeneration rate, which was approximately 77.36%. The nodal segments that were treated with TiO2 nanoparticles at concentrations of 20 mg/l and 60 mg/l exhibited the lowest regeneration rates.

These nanoparticle concentrations corresponded to regeneration rates of 49.14% in the T1 variation and 52.50% in the T3 variant, as shown in Table 3.

5.1.2 Number of sprouts per plant

The experiment revealed that the highest average sprout count per nodal explant was in the T1 variant, with 2.43 ± 1.00 sprouts. This was closely followed by the control group, which exhibited an average of 2.12 ± 0.70 sprouts. Variants T2 and Z3 also showed comparable results, with mean sprout counts of 2.04 ± 0.78 and 2.01 ± 0.49 respectively. The T3 variant was slightly behind, producing an average of 1.89 ± 1.28 sprouts per explant. On the lower end, variants Z2 and Z1 had the least mean number of sprouts, registering 1.78 ± 0.66 and 1.83 ± 0.46 sprouts respectively, as indicated in Table 3 and also shown graphically in Fig 3.

Statistically, the variance in sprout numbers between all nanoparticle-treated variants and the control group was not significant, indicated by a p-value of 0.1512. This suggests that in this specific experiment, ZnO and TiO2 nanoparticles did not significantly influence the average number of sprouts.

5.1.3 Length of sprouts

In this study, the variant Z1 displayed the longest average sprout length at 58.28 ± 41.58 mm, leading all other groups. In the TiO2 group, the T1 variant showed the highest result, closely followed by the Control group, with lengths of 42.54 ± 32.66 mm and 41.10 ± 28.16 mm respectively. The Z2 variant, another ZnO nanoparticle group, showed a lesser mean sprout length of 29.59 ± 23.23 mm. The lowest sprout lengths were observed in the TiO2 nanoparticle variants, with T2 at 27.92 ± 24.15 mm and T3 at 26.59 ± 26.99 mm, both within a narrow range of 1.33 mm of each other, as detailed inTtable 3.

Regarding growth dynamics, Z1 and Z3 variants exhibited rapid and steady growth, with Z3 initially surpassing Z1 before experiencing a decline in the final measurement. This decrease was attributed to the emergence of new sprouts from the nodal segments, which reduced the average sprout length. A notable increase in growth was seen in the T1 variant, particularly in the last 22 days.

Statistical analysis revealed that Z1 had a significant difference in sprout length compared to the Control group and also differed significantly from the Z2, T2, and T3 variants. This suggests that applying 20 mg/l ZnO nanoparticles positively affects sprout length. In contrast, higher concentrations of ZnO (40 mg/l and 60 mg/l) and all tested concentrations of TiO_2 nanoparticles (20 mg/l, 40 mg/l, and 60 mg/l) showed no significant impact on sprout length Fig 4.

5.1.4 Number of nods per sprout

The highest number of nods per sprout was found in the Z1 variant (6.83 ± 3.61) which was slightly higher than Control (6.02 ± 2.51). In the group with TiO_2 nanoparticles, the T1 variant had the highest mean number of nods at (5.66 ± 3.08). The lowest numbers were obtained from the T2 (4.63 ± 2.18) and T3 (4.58 ± 2.63), correlating with their low mean sprout length (Table 3).

The variants Z1 and Z3 had a steady growth of nod numbers through the 60 days period, with variant Z1 overtaking Z3 at the last 10 days. Correlating with the previous results from the previous capitol 5.1.3

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 ZnO nanoparticles have 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 as shown in Fig 5.

5.1.5 Number of roots per plant

The average number of roots per plant in the Control group was 8.20 ± 4.68. With a score of 7.81 ± 3.69, T2 was the version that had nanoparticles that led to the best result. One type of Z1 in the ZnO group did the best, getting a score of 6.80 ± 4.42. Table 3 shows that the Z2 type had the most trouble with root growth.

Root growth was fastest in the first 34 days for both the Z1 and Z2 versions and the control group. After that, there was only a small rise each day.

Statistical analysis revealed significant differences between the Control group, variation T2, and variant Z2. So, it's safe to assume that 40 mg/l of ZnO nanoparticles stunt root development. In comparison to the Control group, the other groups showed no statistically significant differences. This indicates that root formation is unaffected by ZnO nanoparticles (20 mg/l), 60 mg/l ZnO nanoparticles, and TiO2 nanoparticles Fig 6.

Rooting of *Musa paradisiacal* L. plants was aided by ZnO nanoparticles at concentrations of 100 mg/l and 150 mg/l, as reported by Helaly et al. (2014). On the other hand, the plants' rooting efficiency was lowest in the 50 mg/l ZnO nanoparticle medium. Our previous findings that 40 mg/l ZnO resulted in the fewest roots per plant are comparable to this.

5.1.6 Root length

In the control group, the roots were 23.25 mm long, giving or taking 4.34 mm. In the Z3 form (19.34 ± 6.55), the root length was the longest of the versions that had nanoparticles. Variation T2 in the TiO2 group had the longest length, measuring 14.70 mm ± 4.81. In version Z2, the shortest roots were found to be 11.18 mm ± 6.10, and in form T3, they were 11.92 mm ± 3.38. These results are shown in Table 3. The root growth in the first 21 days was highest in the Control group, followed by types Z3 and Z1. The Control group was particularly affected by this. The other varieties showed more consistent root development. In terms of statistics, there was no change between the Control group and the Z3 variation.

This means that 60 mg/l ZnO has no effect on the length of the roots. The Control group was statistically different from the Z1, Z2, T1, T2, and T3 types. From these results, we can say that 40 mg/l of ZnO nanoparticles and 20 mg/l, 40 mg/l, and 60 mg/l of TiO2 nanoparticles all slow down the growth of roots Fig 7.

Table 3. ZnO and TiO_2 nanoparticles effect on the micropropagation of *Salvia rosmarinus* Spenn from nodal segments.

ZnO (mg/l)	**TiO2 (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	-	-	92.99	1.83 ± 0.46a	58.28 ± 41.58a	6.83 ± 3.61a	6.80 ± 4.42ab	18.55 ± 5.10bc
Z2-40	-	-	61.91	1.78 ± 0.66a	29.59 ± 23.23b	5.08 ± 2.20ab	3.37 ± 4.51b	11.18± 6.10d
Z3-60	-	-	79.46	2.01 ± 0.49a	45.36 ± 39.13ab	6.30 ± 3.55ab	5.54 ± 1.48ab	19.34 ± 6.55ab
-	T1-20	-	49.14	2.43 ± 1.00a	42.54± 32.66ab	5.66 ± 3.08ab	7.15 ± 5.82ab	14.44 ± 8.73cd
-	T2-40	-	77.36	2.04 ± 0.78a	27.92 ± 24.15b	4.63 ± 2.18b	7.81 ± 3.69a	14.70 ± 4.81cd
-	T3-60	-	52.50	1.89 ± 1.28a	26.59 ± 26.99b	4.58 ± 2.63b	5.12 ± 5.62ab	11.92 ± 3.38d
-	-	MS	100	2.12 ± 0.70a	41.10 ± 28.16b	6.02 ± 2.51ab	8.20 ± 4.68a	23.25 ± 4.34a

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

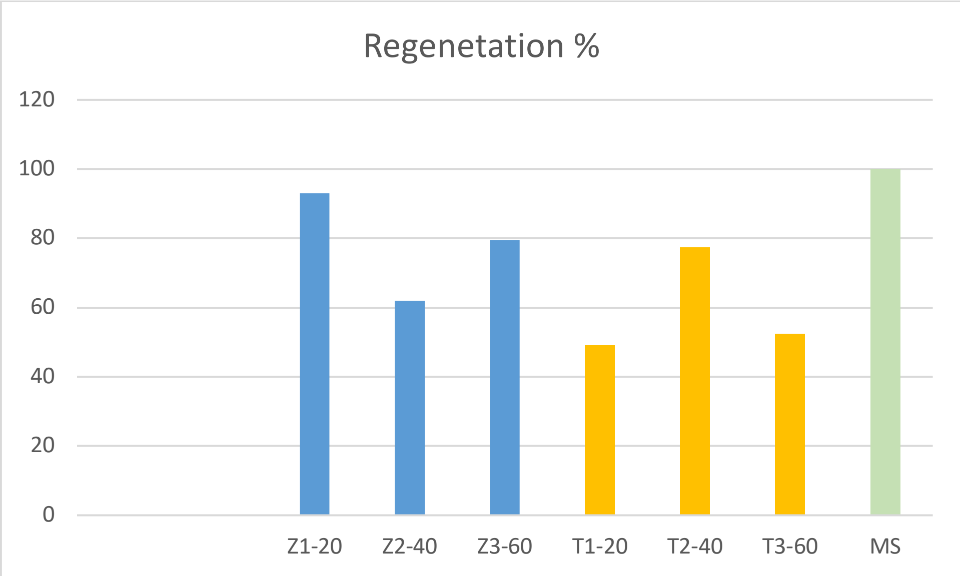

Figure 2. Comparison of the Regeneration % Between different variants

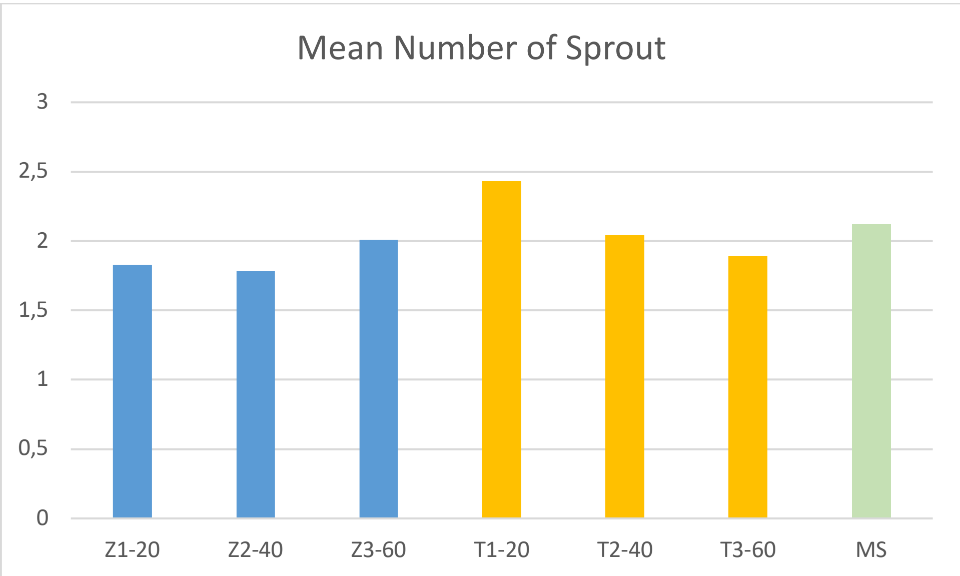

Figure 3. Comparison of the mean number of sprouts between different variants

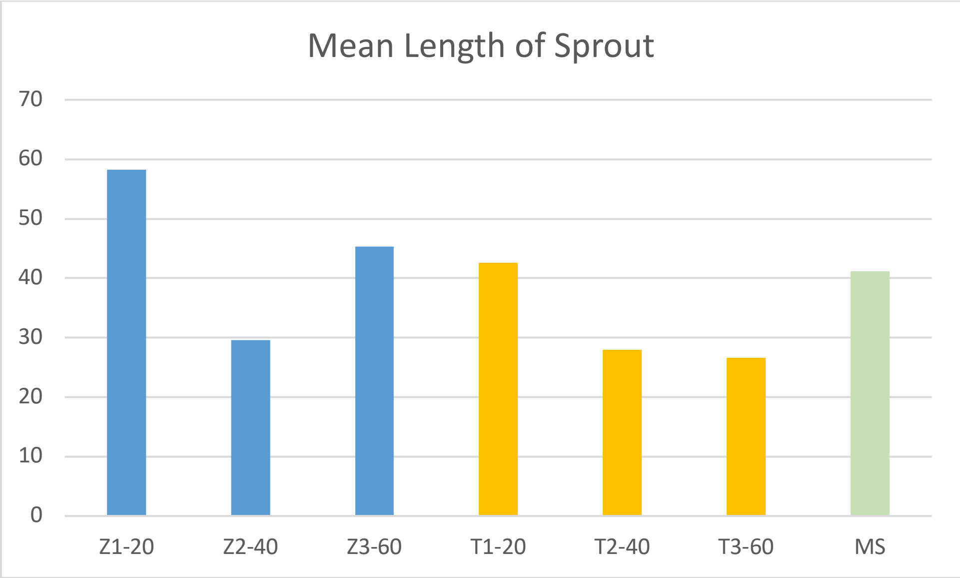

Figure 4. Comparison of the mean length (mm) of sprouts between different variants

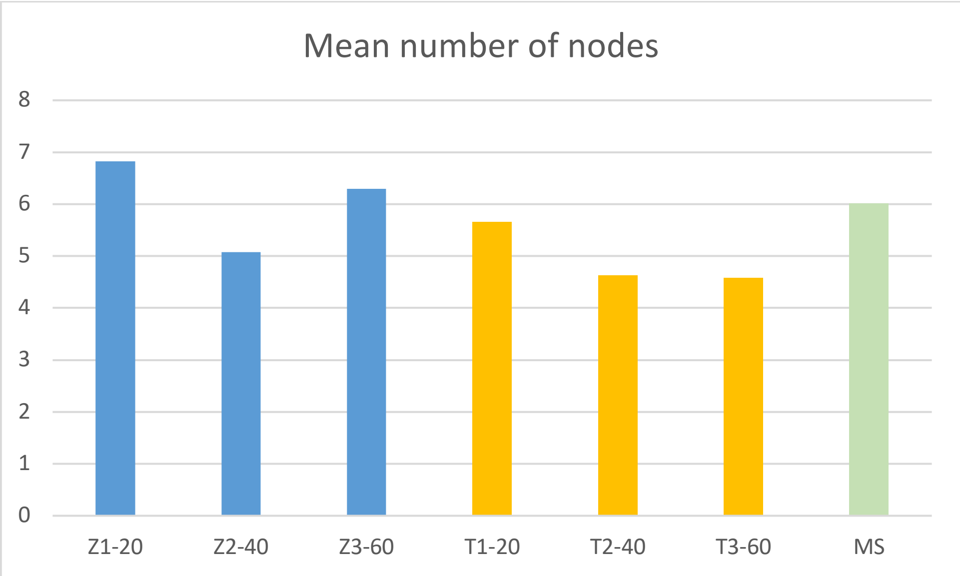

Figure 5. Comparison of the mean number of nodes between different variants

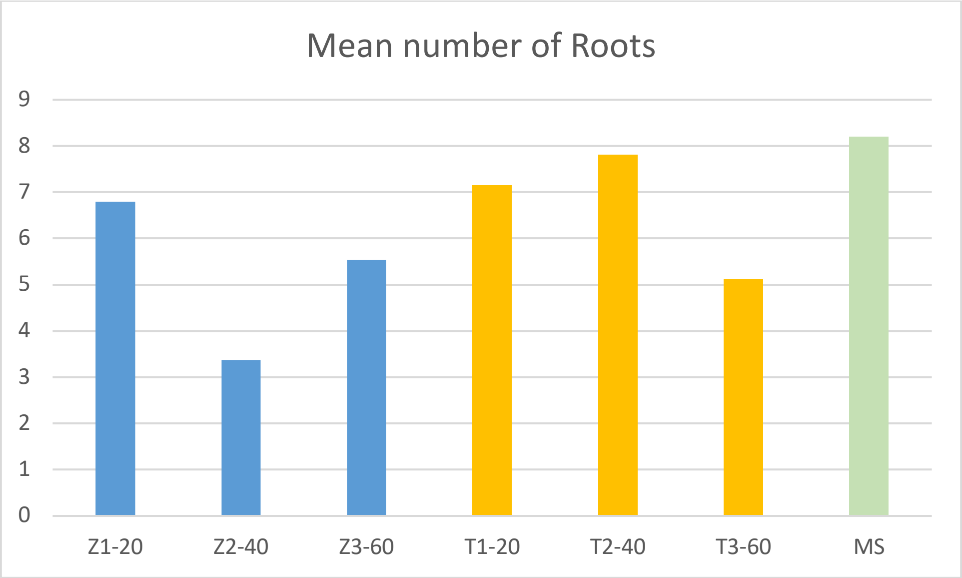

Figure 6. Comparison of the mean number of roots between different variants

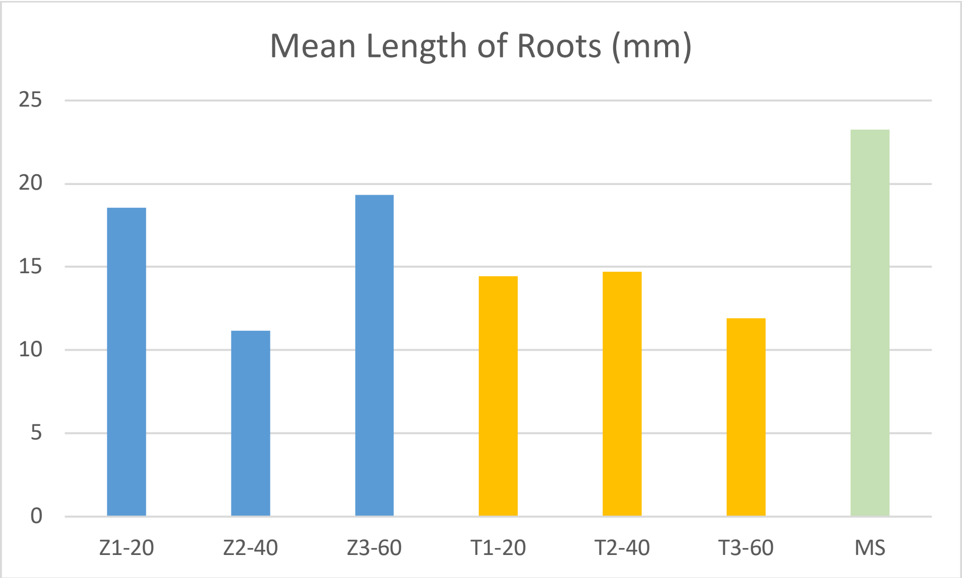

Figure 7. Comparison of the mean length of roots (mm) between different variants

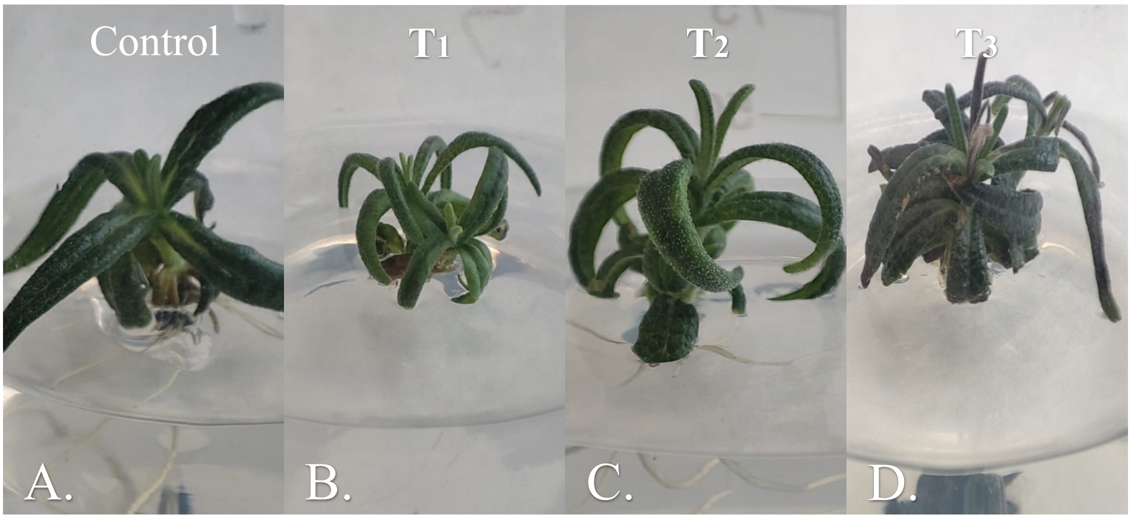

Figure 8: Phenotype results with TiO_2 nanoparticles (A) control plant, (B) 20 mg/l, (C) 40 mg/l, and (D) 60 mg/l concentrations.

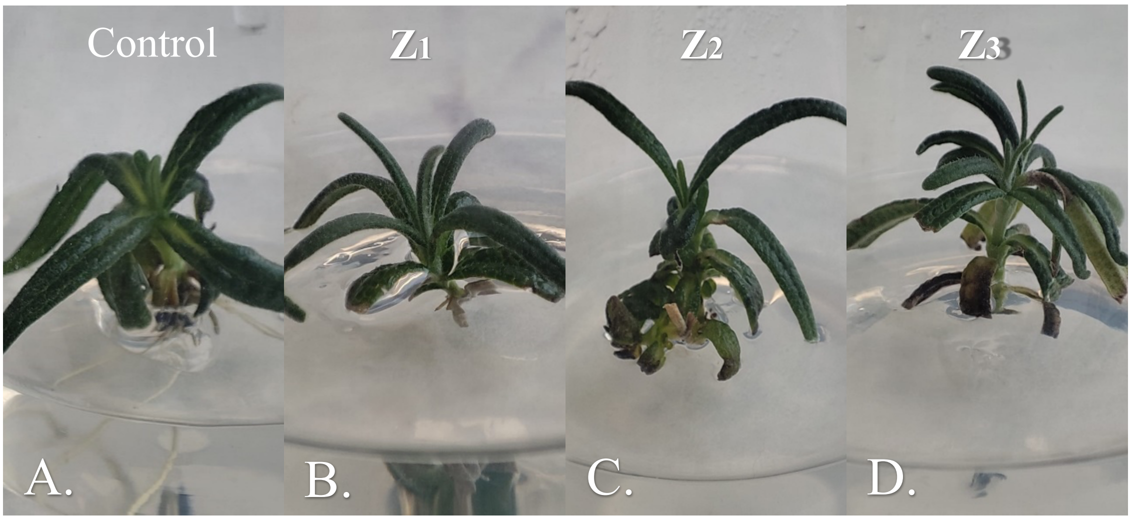

Figure 9: Phenotype results with ZnO nanoparticles (A) control plant, (B) 20 mg/l, (C) 40 mg/l, (D) 60 mg/l concentrations.

6. Discussion

The results of our study demonstrated that the use of nanoparticles enhanced plant growth and development. Specifically, we found that the differences in the percentage of regenerated nodal segments were mainly affected due to the physiological condition and specific characteristics of the parent plant, as well as the size of the nodal explants used. This may be due to the availability and uptake of essential nutrients by plants. For example, TiO_2 nanoparticles have been shown to enhance the uptake of nitrogen and phosphorus (Zahra et al. 2015), leading to increased growth and biomass production in plants. It also has a high surface area-to-volume ratio compared to their bulk counterparts. This increased surface area provides more sites for nutrient adsorption and interactions, enhancing the availability of nutrients in the rhizosphere for uptake by plant roots. It can alter soil properties, such as pH and nutrient availability (Dimkpa 2018), creating a more conducive environment for plant growth. For example, ZnO nanoparticles have been shown to increase the availability of zinc in soil (Bala et al. 2019), which is an essential micronutrient for plant growth and development.

ZnO nanoparticles can stimulate root growth and branching, leading to a larger root system capable of exploring a greater volume of soil for nutrient uptake. This increased root surface area allows plants to access nutrients more efficiently, even at low concentrations. It can interact with nutrient ions in the soil solution, forming complexes or altering their mobility (Chen 2018). This interaction can facilitate the transport of nutrients from the soil solution to the root surface, enhancing their uptake by plant roots. Direct comparisons with previous research on *Salvia rosmarinus* Spenn are not feasible. However, referencing a study by (Helaly et al. 2014) on the application of Zn and ZnO nanoparticles in the in vitro micropropagation of Musa paradisiacal L. It was noted that even at a high concentration of 200 mg/l, these nanoparticles did not significantly hinder plant regeneration.

Another study by (Frazier et al. 2013) revealed that low concentrations of TiO_2 nanoparticles (0.1 and 1.0%) did not impact the germination of *Nicotiana tabacum* L. seeds. In contrast, higher concentrations of these nanoparticles notably reduced the seed germination rate. Comparatively, (Rahmani et al. 2016) found that *Brassica napus* L. seedlings grown in half-strength MS medium with 10 mg/l ZnO nanoparticles showed a positive growth response. It may also be due to nanoparticles can induce stress responses in plants, leading to the activation of mechanisms that improve nutrient uptake and utilization under adverse conditions (Rasheed et al. 2022). The ZnO nanoparticles have been shown to stimulate the expression of genes involved in nutrient acquisition and stress tolerance in plants (Faizan et al. 2021).

However, higher concentrations of 100 mg/l and 1000 mg/l were toxic. This aligns somewhat with our findings, where a 20 mg/l concentration of ZnO exhibited beneficial effects. According to (Aqeel et al. 2022) nanoparticles can modulate physiological processes related to nutrient uptake and assimilation in plants. The TiO_2 nanoparticles have been reported to enhance nitrogen metabolism in plants (Zhang et al. 2020), which can indirectly influence nutrient uptake and utilization leading seeding germination percentage. The potential for ZnO and TiO_2 nanoparticles to enhance nutrient uptake in rosemary and other plant species (Elsayed et al. 2022), it's essential to consider factors such as nanoparticle concentration, exposure duration, and environmental conditions to optimize their effects and minimize potential risks (Nekoukhou et al. 2022).

Our results showed significant changes in root growth of the plants. It may be due to the addition of ZnO and TiO_2 nanoparticles can potentially play a role in promoting root growth in rosemary through various mechanisms (Kralova & Jampilek 2021). Nanoparticles can improve nutrient availability in the rhizosphere, promoting root growth by providing essential nutrients in a more accessible form (Wang et al. 2022). The ZnO and TiO_2 nanoparticles may enhance the solubility and mobility of nutrients in the soil, facilitating their uptake by rosemary roots and stimulating root elongation (Saleh et al. 2021). Furthermore, it can stimulate the proliferation of lateral roots and root hairs (Milewska-Hendel et al. 2022), leading to a denser and more extensive root system in rosemary. The ZnO and TiO_2 nanoparticles can influence root morphology by regulating the expression of genes involved in root development (Landa et al. 2012).

Studies have shown that nanoparticles can promote primary root elongation (Wang et al. 2012) and lateral root formation, resulting in a more robust and well-developed root system in plants like rosemary. Both these can enhance the water-holding capacity of soil and improve water uptake by plant roots (Nassaj-Bokharaei et al. 2021; Zaib et al. 2023). By facilitating water absorption, nanoparticles help alleviate drought stress and promote healthy root growth in rosemary, particularly under water-limited conditions.

In contrast, A study by (Feizi et al. 2011) observed significant positive impacts of TiO2 nanoparticles on the shoot and seedling length of Wheat (*Triticum aestivum* L.) at 2 and 10 ppm concentrations, differing from our findings where TiO_2 nanoparticles did not demonstrate a significant effect. The ZnO and TiO_2 nanoparticles can mitigate the adverse effects of environmental stresses such as salinity, heavy metal toxicity, and soil compaction on root growth (Rajput et al. 2021). By scavenging ROS and enhancing antioxidant defence mechanisms, nanoparticles help protect root cells from oxidative damage and maintain root viability under stress conditions. Nanoparticles can interact with soil microorganisms and promote beneficial microbial symbiosis in the rhizosphere (Mahawar & Prasanna 2018).

By enhancing mycorrhizal colonization and nitrogen-fixing bacterial associations, nanoparticles improve nutrient availability and promote root growth in rosemary through enhanced nutrient uptake and mineralization processes. Despite the lack of statistical significance, a minor downward trend in the average number of sprouts was noted in the T1, T2, and T3 variants with rising concentrations of TiO_2 nanoparticles. This observation was consistent with findings from (Frazier et al. 2013), where a reduction in shoot growth was noted in *Nicotiana tabacum* L. seeds treated with TiO_2 nanoparticles. The stimulation of photosynthesis due to the application of nanoparticles such as ZnO and TiO_2 results in increased carbon assimilation, which contributes to improved shoot growth and development. The application of ZnO and TiO_2 nanoparticles is an intriguing area of research with promising implications for plant growth and productivity (Šebesta et al. 2021).

According to (Carmeli et al. 2010) nanoparticles can act as light-harvesting antennae, absorbing light energy across a broader spectrum, including UV wavelengths that are typically underutilized by chlorophyll. This extended light absorption range can increase the overall energy available for photosynthesis in plants like rosemary. It can influence stomatal conductance, the rate at which stomata open and close to regulate gas exchange (Ahmed et al. 2023). Modulating stomatal behaviour can optimize carbon dioxide uptake while minimizing water loss through transpiration, thereby improving photosynthetic efficiency in plants (Mony et al. 2022). Similarly, the study by (Helaly et al. 2014) on *Musa paradisiacal* L. indicated an increase in the number of shoots with escalating concentrations of ZnO and Zn nanoparticles up to 100 mg/l, but a diminished increase at 200 mg/l. Such an effect, however, was not evident in the current study.

7. Conclusion

This study is among the initial research endeavours to examine the in vitro propagation outcomes of nodal sections of S. rosmarinus utilizing nanoscale ZnO and TiO_2 particles. The objective of this work was to investigate the feasibility of in vitro propagation of Salvia rosmarinus Spenn. using nodal explants treated with ZnO and TiO_2 nanoparticles.

This indicates that TiO_2 nanoparticles at concentrations of 20 mg/l, 40 mg/l, and 60 mg/l were unable to significantly affect the growth of plants, along with a reduction in the root length of the plants. On the other hand, ZnO nanoparticles at a concentration of 20 mg/l increased the height of the plant while simultaneously decreasing the root length. The presence of 40 mg/l of ZnO nanoparticles had a detrimental impact on the plants. This led to the development of the plants in a negative manner, which in turn resulted in a suppressed root system and significantly shorter roots.

At a higher concentration of 60 mg/l the ZnO nanoparticles did not appear to have any impact on the growth of the plants. The addition of ZnO and TiO_2 nanoparticles also had an impact on germination. Seeds germination declined with increasing amounts of TiO_2 nanoparticles. Similarly, other parameters, such as number of sprouts and length of sprouts, regeneration percentages were also significantly affected. The findings of this study are captivating and hold great significance. Higher concentrations of ZnO nanoparticles were shown to negatively impact on root formation, while lower amounts were found to enhance root length. However, TiO_2 nanoparticles exhibited toxicity towards root development at all investigated concentrations.

Thus, this study confirm that negative results are obtained in comparison to the control group which indicate the rejection of our hypothesis I.

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