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Zinc (ZnO) and Titanium (TiO_2) 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 (TiO_2) 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 25th August 2024

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Abstract

Salvia rosmarinus is an aromatic, perennial, evergreen shrub belonging to Lamiaceae or mint family that is frequently cultivated as an ornamental and culinary herb. It is a rich source of antioxidants and anti-inflammatory. These plants germinate slowly from seed, and farmers find successful propagation from stem cuttings, layering and division. Nanoparticles effect plant growth during micropropagation, reduce contamination, and serve as an alternative in plant tissue culture. They have unique physicochemical properties like high surface area, high reactivity, tunable pore size and particle morphology, and serve as "magic bullets" to release their content at target site in plant. Our study deals with the effect of Zinc oxide and Titanium dioxide nanoparticles on *in vitro* micropropagation of *Salvia rosmarinus.* Nodal explants cultivated on basic MS medium were supplemented with Zinc Oxide (ZnO) and Titanium Dioxide (TiO_2) nanoparticles in different concentration (20 mg/l, 40 mg/l and 60 mg/l) for a 60-day period. Measurements of number of sprouts, number of nodes, sprout length, number of roots and root length were measured every 10 days. The results showed the highest regeneration percentage, number of nodes and root length in plants grown on MS media (control group) among all plants. Application of 20 mg/l and 60 mg/l of ZnO showed the biggest sprout length and number of nodes respectively as compared to control and treated plants. The application of 20 mg/l of TiO_2 showed greatest number of sprouts among all plants. Overall, ZnO showed their best result at 20 mg/l and 60 mg/l but TiO_2 at 20 mg/l because their increasing concentration showed toxic effects on the plants growth. In future, the nanoparticles application could act as an environmentally friendly technique to show their positive response towards the plants growth through minimizing the microbial growth on growth media.

Key words: Growth parameters, Micropropagation, *Salvia rosmarinus*, TiO_2, ZnO

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List of the abbreviations used in the thesis

ZnO	Zinc Oxide
TiO_2	Titanium Dioxide
DNA	Deoxyribonucleic acid
pH	potential of hydrogen
NPs	Nanoparticles
CNTs	Carbon nanotubes
SiO_2	Silicon Dioxide
UV	Ultraviolet
Zn	Zinc
Ag NPs	Silver nanoparticles
MS	Murashige & Skoog
KOH	potassium hydroxide
CO_2	Carbon dioxide
mg/l	milligrams per liter
RNA	Ribonucleic acid
IBA	Indole-3-butyric acid
ROS	Reactive Oxygen Species

1. Introduction

Salvia rosmarinus Spenn. also known as rosemary, is a plant that belongs to the Lamiaceae family (Malvezzi et al. 2020). It is native to dry scrub and rocky places in the Mediterranean areas of southern Europe to western Asia. It shows its best growth in neutral to acidic soil with moderate fertility. It is a widely recognized aromatic plant that is used for decorative, cooking, medical and ritual purposes (Ribeiro-Santos et al. 2015). It possesses antimicrobial, antioxidant, antiviral and antispasmodic properties (Skocibusic et al. 2006). This plant contains a large number of secondary metabolites such as essential oils, terpenoid compounds, and phenolic derivatives (Arikat et al. 2004). The oil extracted from its aerial parts is consumed in the making of liqueurs and perfumes (Ciani et al. 2000).

Plant tissue culture is a biotechnological technique that was widely used for the plant breeding and micropropagation, transgenic plants regeneration, crops improvement, virus elimination, and healthy plant material preservation (Drew et al. 2018). The optimization of mineral elements in the medium generally improves the differentiation and development of explants (Hameg et al. 2020). The application of nanoparticles in the growth medium has efficiently played a great part in callus formation, organogenesis, somatic embryogenesis, somaclonal variation, genetic transformation, and the secondary metabolites production since the last decades (Kim et al. 2017). Nanoparticles biogenic synthesis has gained a great attention because these particles are suitable, non-toxic, and environmentally friendly (Khan et al. 2019).

Application of nanoparticles is a novel area that improves the growth, yield, and survivability of economic and medicinal plants (Alenezi et al. 2022). The incorporation of nanotechnology in agricultural activities through the application of Zinc Oxide and Titanium Dioxide nanoparticles is revolutionizing the plant micropropagation. These nanoparticles are favorable antimicrobial agents due to their photocatalytic activity, particle size, high penetration and safety for multicellular organisms (Xia et al. 2017). These nanoparticles may be used as bactericidal and fungicidal drugs for sterilization of explants during clonal micropropagation of plants while taking account the possible phytotoxicity of these particles (Zakharovaa & Gusev 2019).

Recent studies had shown that application of ZnO nanoparticles altered the germination and growth of rice by promoting the early plant development (Aghakhani et al. 2018) and is being used to lessen abiotic stress specifically salinity stress (Alharby et al. 2016). TiO_2 nanoparticles are effective for improving wheat growth and their widespread application encompasses agricultural activities (Faraz et al. 2020; Hadi & Abass 2021).

Micropropagation causes microbial contamination that decreases the regenerative ability, callus growth, adventitious shoot growth, and even tissue death of explant (El-Banna et al. 2021). The main objective in the micropropagation of plants by nanoparticles is to improve their growth by enhancing the uptake of nutrients and controlling microbial contaminants (Mokbel et al. 2017). Agroforestry strengthens food security by increasing crop productivity and soil fertility as well as expand income sources. In agroforestry systems, nanotechnology restores soil health by monitoring microbial growth (Zhang et al. 2019) and remediates polluted soil (Mukherjee et al. 2018) to enhance the growth of different crops and their development (Alabdallah et al. 2020). Our study deals with the effect of ZnO and TiO_2 nanoparticles in different concentrations on the *in vitro* micropropagation of *Salvia rosmarinus* by using nodal segment of this plant.

2. Literature review

2.1. *Salvia rosmarinus* Spenn

2.1.1. Taxonomy

Lamiaceae family is one of the largest dicotyledonous plants families that includes almost 236 genera and more than 7100 species (Aghakhani et al. 2018). This family includes a variety of scented herbs and shrubs such as basil, mint, oregano, thyme, and lavender (Musolino et al. 2023). Some genera of this family contain well-known therapeutic and aromatic plants to produce specialized metabolites, like essential oils and various non-volatile constituents with multiple pharmacological activities and several applications in the food industry, cosmetics, and medicines (Pandey et al. 2017).

The genus *Salvia* is the major genus in the Lamiaceae family and comprises of 900 species (Hao & Xiao 2015). These species are known for the broad spectrum of their biological activities that possess antioxidant, anti-inflammatory, antinociceptive, anticancer, antimicrobial, antidiabetic, antiangiogenic, hepatoprotective, cognitive and memory-enhancing properties. The medical activity of genus *Salvia* is associated with important phytochemicals like phenolic acids, terpenoids and flavonoids (Zhumaliyev et al. 2023).

Rosemary is the common name used for *Salvia rosmarinus* Spenn. that belongs to the Lamiaceae family and is an oldest shrub native to Mediterranean. It is a well-known aromatic and ornamental plant (Leporini et al. 2020). The name generally used, *Rosmarinus officinalis* L. is a synonym of the real name, *Salvia rosmarinus* as current evidence had showed that *Rosmarinus* L. are converted in Salvia L. (Drew et al. 2017).

2.1.2. Distribution

It is mainly distributed in the western-central part of the Mediterranean basin, even though it was first found in different sites of eastern part (Morales et al. 2010). It is naturally cultivated along western and southern coasts of Turkey, and is widely grown in France, Italy, Spain, Portugal, and Greece (Malayoglu 2010). It is very common on the seashore and is grown in the sea climate (Sasikumar 2012). It is a xeromorphic plant that flourishes naturally on cliffs, sand and stony regions near the sea across the globe that includes Africa, Europe and Asia (Ribeiro-Santos et al. 2015).

2.1.3. Soil management

Land for rosemary cultivation should be free from weeds, shades, and ploughed two to three times to make the soil favorable for the seedlings. Rosemary grows well in sandy loam soil but soil with high clay content and water lodged area is not suitable. It is cultivated on loamy soil with proper irrigation in an open and sunny position (Ribeiro-Santos et al. 2015). It can be harvested 3-4 times in one year and can last for up to 7 years. It can averagely withstand salt and drought stress (Berdahl & Mckeague 2015). Ideal pH for its growth ranges from 6 to 7.5, that is intolerant of heavy clay and poor drainage (Khongthaw et al. 2023). The acid tolerance of rosemary is a good opportunity in areas affected by soil acidity. Soil acidity is the major land degradation problem caused by high rainfall and leaching, acidic parent material, decaying organic matter and harvesting of high yielding crops (Kochian et al. 2004).

2.1.4. Morphology

Rosemary is an evergreen, rounded shrub with aromatic, small needle-like and grey-green leaves (Ribeiro-Santos et al. 2015). It is a perennial shrub with height of 1 to 2 meters with erect branches. Its leaves are evergreen, 2-4 cm long and 2-5 mm wide, with upperside green, and underside white with small thick woolly hairs (Zigene al. 2023). Leaves are without petiole and flowers are arranged in clusters along the axis. All the leaves blossoms and scented members are aromatic. The flowers are usually white to pink or white and occasionally purple that appears in late spring. Four nut fruit with brown color, firm, round and its egg is small inside the fruit (El-Rajoob et al. 2008). It is characterized by cymose inflorescences, brown, square and woody trunks with tight branches and scratched bark (Begum et al. 2013).

Figure 1. ***Salvia rosmarinus*** **Spenn**

2.1.5. Rosemary Propagation

The Propagation of rosemary can be done by seeds or by using vegetative parts like layering and cutting (Plant production directorate, 2012). Rosemary cuttings used for seedling preparation is top 10-15cm part taken from disease free mother plant not more than one year old. The bottom two thirds of the cuttings are taken from leaves that are inserted in a proper growth media. The cuttings can be prepared in green house and transplanted to the main field after 60-90 days. Rooting hormones help in root formation within 2-4 weeks. Rosemary stems bearing flowers, old woody and very young plants are not suitable for cutting preparation (Mekonnen et al. 2016). Moreover, mass propagation by tissue culture technique is also an alternative means of rosemary propagation (Banjaw et al. 2024).

2.1.6. Chemical composition

The success of *S. rosmarinus* is associated with its phytochemical composition, which consists of bioactive compounds such as polyphenols and phenolic terpenoids, and rich amounts of essential oil (Leporini et al. 2020). Its polyphenols include rosmarinic acid, while its phenolic terpenoids include carnosic acid, carnosol, ursolic acid and caffeic acid (Bai et al. 2010; Borras-Linares et al. 2014). Essential oils are mainly characterized by monoterpene hydrocarbons (39.32-40.70%) and oxygenated monoterpenes (36.08-39.47%). The 1,8-cineole, α-pinene, camphor, and trans-caryophyllene being the best prominent compounds (Leporini et al. 2020).

2.1.7. Antioxidant activity

It is a widely recognized species around the world with the maximum antioxidant activity (Drew et al. 2017). Their powerful antioxidant activity is related with the synergistic actions of several metabolites and the presence of polyphenol, rosmarinic acid, and phenolic diterpenes i.e. carnosol and carnosic acid (Raskovic et al. 2014). It affects the quality of the oil to avoid the production of free radicals and losses of a desirable effective compounds (Santo & Kalidas 2012).

2.1.8. Uses in food and medicine

S.rosmarinus can be used in the preparation of food owing to its flavoring characteristics (Ghorbani & Esmaeilizadeh 2017). It has been used as a flavoring and spicing agent or as ingredients in cosmetics, perfumes, and lotions (Sarkic & Stappen 2018). Aqueous extract of rosemary positively affected the sensory properties of yoghurt i.e. flavour, texture and appearance (Ali et al. 2021). In Ethiopia, fresh leaves of rosemary known as 'Asmerino' or 'YetibseKetel' are utilized as a mean seasoning meat and to add flavors to food in many diners and hotels (Mekonnen & Manahile 2017).

It has been traditionally added in herbal medication (Apostolides et al. 2013) due to its digestive, diuretic, balsamic (Colica et al. 2018) and rubefacient properties (Ferreira 2010). It is also important in the preparation of folk and oral medicine to release renal colic, dysmenorrhea and muscle contractions due to therapeutic properties (Ribeiro-Santos et al. 2015). It is utilized to cure the nervous, cardiovascular, gastrointestinal, genitourinary, menstrual, hepatic, reproductive and respiratory systems diseases (Begum et al. 2013). It is also highly beneficial in the treatment of depression, neurological diseases, obesity and inflammation (Shimira et al. 2022). It also possesses anti-inflammatory, anti-microbial, hypoglycemic, anti-diabetes, antioxidant properties and is able to avoid the neurovegetative diseases (Garcia et al. 2016). It improves the digestion, increases mental and concentration capacity and is fruitful in releasing stress and anxiety during late age (Brindisi et al. 2020).

2.1.9. Role of rosemary in agroforestry

Agroforestry emerges as a sustainable cultivation pattern that finds resonance in farming of rosemary (Seker et al. 2023; Palada et al. 2005). The strategic incorporation of rosemary with compatible tree species adapts biodiversity, restricts soil erosion, and provides a resistant ecosystem for the herb to grow. This symbiotic relationship indicates the relation between agriculture and ecology, representing the principle of sustainability in cultivation practices (Lehmann et al. 2020; Nelson & Shilling 2005).

In addition to industrial and cultural benefits, rosemary also possesses social and environmental advantages. The production of rosemary has become business because it offers employment opportunities. In various studies, the incorporation of rosemary plant strips on farmland recovers the prevailing systems through increasing complex biological interactions. Rosemary cultivation in hilly areas offers a useful system to trap agricultural runoff and dropping soil erosion to avoid the degradation of soil. It has been indicated that rate of yield by the incorporation of rosemary to the farmland could be controlled by rosemary leaf or essential oil yield (Zuazo et al. 2008).

Climate adaptability: Rosemary grows best in areas with an average temperature ranging from 20 to 25^{o}C, receiving average annual rainfall above 500 mm and altitude range from 1500 up to 3000 m above sea level (German et al. 2016). Rosemary grows in dry and warm conditions and is adapted to different soil types in varying climates. Suitable areas for its growth are moderate and sub-tropical regions but temperature below -3 °C hinder growth. It is widely grown in lower mountain forests, dry valleys in Bolivia, rocky hills in Bermuda and European areas. The increased Rosemary's cultivation is related to their regenerative capabilities making it valuable for decorative and medicinal prospective (Khongthaw et al. 2023; Aziz et al. 2022).

Intercropping: Rosemary plant spacing depends on variety, production purpose and management practices. Rosemary can be cultivated either by mono cropping or multiple cropping system owing to its compatibility with other crops. Nigussie et al. (2017) reported the yield and competitive advantage of intercropping rosemary with carrot. Moreover, insertion of onion with 80% a rosemary population density increased yield rate and effectiveness on individual planted crop per unit area specified by greater ratio of land equivalent and relative crowding coefficient (Adafre et al. 2019).

Pest control: The increasing crop biodiversity, such as intercropping, can enhance pests' natural enemies in agroecosystems (Batista et al. 2017). Rosemary is being cultivated as an intercrop to reduce the damage caused by insects in the agricultural and horticultural systems of *Capsicum annuum* (Ben et al. 2017) and *Camellia sinensis* fields (Zhang et al. 2014). Rosemary showed its effectiveness in preventing aphid, thrips, and whitefly attack by acting as repulsive intercrop in the field because rosemary intercropping did not harm the growth of predatory bug *O. sauter* and *E. formosa* on sweet pepper (Li et al. 2021).

2.2. Nanoparticles

Nanoparticles (NPs) are the chemical entities with at least one of their three dimensions lower than 100 nm and have significantly different physical, chemical and biological properties to their macro-sized, and high surface area to volume ratio to facilitate the contact with plant cells (Jaberzadeh et al. 2013). In recent times, the nano-sized or “nanoparticles” have gained a lot of attention due to our still-increasing ability to synthesize and manipulate these materials (Nowacka & Bucheli 2007). The materials used to prepare nanoparticles are metals and their oxides, lipids, emulsions and ceramics. Recently, plants are also generally used in the preparation of nanoparticles due to less toxicity and low cost (Khan et al. 2019).

Nanoparticles are extremely useful in agriculture because they can alleviate the effects of plant diseases and are active components in nano-fertilizers (Liu et al. 2019; Gkanatsiou et al. 2019). They may also be used in the preparation of several sensors, herbicides, phytoimmunity stimulants, nano pesticides and agents to remove pesticide from plants and soil (Shang et al. 2019). Nanoparticles are also used in other fields such as cosmetic, energy, electronic, pharmaceutical, biomedical, catalytic, environmental, and material applications (Nowacka & Bucheli 2007). The natural nanoparticles can improve world health by increasing the human’s immunity with less drug toxicity and high biocompatibility by enhancing the establishment of a pure green environments for all living organisms on the planet (Abada Abasi et al. 2024).

Nanotechnology is the fabrication, characterization and utilization of nanomaterials. Recently, nanotechnology has opened new areas of interest for researchers in different fields including industry, medicine, energy and agriculture (Alabdallah & Alzahrani 2020). Moreover, the use of nanoparticles provides an opportunity for sustainability due to their distinct physiochemical features in the biosystem (El-Badri et al. 2021).

2.2.1. Classification of Nanoparticle

Nanoparticles exist in different forms, and they are classified into three classes due to their composition, properties and potential applications (Abdal Dayem et al. 2017).

Organic Nanoparticle: The nanoparticle in this class are proteins, carbohydrates, lipids, polymers, and organic materials (Pan & Zhong 2016). These NPs are biodegradable and non-toxic with a hollow core and are produced by non-covalent intermolecular forces to make them more labile. Dendrimers, liposomes, micelles, and protein complexes like ferritin are the common examples of organic nanoparticles (Ng & Zheng 2015). These nanoparticles are typically used in the treatment of cancer and in the delivery of targeted drug (Gujrati et al. 2014).

Carbon-based Nanoparticle: This class includes carbon quantum dots, fullerenes, and carbon black nanoparticles. These nanoparticles are used in medicine delivery, storage of energy, photovoltaic appliances, as well as environmental sensor to evaluate microbial ecology because of their exceptional electrical conductivity, electron affinity, high strength, visual and sorption features (Mauter & Elimelech 2008). Carbon nanoparticles and nano-diamonds are examples of complex carbon-based nanoparticles. Their less toxic and biocompatibility properties enable them efficient in drug delivery in addition to tissue engineering (Mochalin et al. 2012).

Inorganic Nanoparticle: These nanoparticles are lacking carbon or organic constituents and usually are metal, ceramic and semiconductor (Nascimento et al. 2018). Certain metal nanoparticles show distinctive thermal, magnetic, and biological properties which make them vital substances in nanodevices for physical, chemical, biological, biomedical and medicinal applications (Khan et al. 2019). Semiconductor materials are different from bulk materials (Dreaden et al. 2012) and are significant in photo catalysis, optical, and electronic engineering (Sun et al. 2000). Ceramic nanoparticles include carbonates, carbides, phosphates, metal, and metal oxides that includes titanium and calcium (Thomas et al. 2015). Their strength and load ability make them valuable in biomedical uses (Moreno-Vega et al. 2012) as well as in catalysis, dye breakdown, photonics, and optoelectronics (Thomas et al. 2015).

2.2.2. Types of nanoparticles in plant applications

2.2.2.1. Nano Pesticides

Pesticides are used to increase and improve the crop yield by protecting plants from plant diseases and insects (Jampilek & Kralova 2017). The use of pesticides is lethal and toxic for the environment with many issues such as the application of pesticides at a high concentration harms the ecosystem, increase bioaccumulation, making the soil infertile, and disrupts the microbiota (Meena et al. 2020). The production of effective and non-toxic pest killer is difficult and costly; however, nanotechnology offers a novel solution (Sasson et al. 2007) with the certain advancement in the various areas of agriculture and food. Nanotechnology finds its application in the protection of plant against pests rather than it expands its application to reduce waste, control plant growth, improve food quality and attain increasing global food production (Jampilek & Kralova 2017). The most common nano-pesticides trials are nano-herbicides, nano-insecticides, nano-nematocides, and nano-fungicides (Mittal et al. 2020).

2.2.2.2. Nano fertilizers

Nano fertilizers are eco-friendly fertilizers with the ability to improve the application fertilizers and minimize the mineral loss particularly phosphorous and nitrogen (Dimkpa & Bindraban 2017). It causes the slow and organised release of nutrients to their targeted place to avoid the environmental and water pollution (Dwivedi et al. 2016). These fertilizers give nutrients to plants and improves their effect even applied in small quantity (Rameshaiah et al. 2015). The nutrients uptake can be enhanced by encapsulating the fertilizers in nano shape that diminishes the loss of nutrient, boost the quality and yield of crop alongwith minimizing the environmental degradation. The foliar spray of nanofertilizers also protect the plants from stress (Tarafdar et al. 2012). Nanoparticulate fertilizers includes some other nanoparticles like CNTs, TiO_2 and SiO_2 to improve the growth of different plants. The mixture of TiO_2 and SiO_2 increases nitrogen fixation, growth, and improve the germination of seed in soybeans (Lu et al. 2002).

2.2.2.3. Nano biosensor

Nanotechnology could increase the activity of any biosensor to find its importance in agriculture. Nano sensors could exhibit their strength in different fields i.e. cultivation and harvesting of crops, pathogen recognition, soil pH and nutrients. Nanoparticles possesses unique surface composition, electrical and thermal features, improved sensitivities and detection making them appropriate for sensing systems (Yao et al. 2014). A new and highly output smart plant sensing method is used in the development of stress tolerance cultivars of plants by increasing the use of restricted resources (nutrients and water) for increasing crop yield. This smart nano sensors is used to optimize the growth of plant and stress can be identified accurately through wireless and optical signals for real-time observation of plant health (Giraldo et al. 2019).

2.2.3. Metal Nanoparticles

2.2.3.1. Zinc oxide (ZnO) nanoparticles

ZnO nanoparticles have high binding energy, refractive index, thermal conductivity, and piezoelectric nature, high absorbance of UV light and antibacterial properties. Their size can vary from a few nanometres to the higher limit size of nanoparticle (100 nm) and a suitable synthesis method is selected to adjust their shape easily. The surface of Zinc oxide nanoparticles is often modified with the inorganic compounds and organic compounds to enhance their stability in colloidal suspension to improve their effects on plants and reduce their potential toxicity (Kołodziejczak-Radzimska et al. 2014). Bare and surface-modified Zinc oxide nanoparticles find their application in the laboratory, greenhouse, and field experiments on different crops because they have UV protective and antimicrobial properties and play their nutritional role by slowly releasing the Zn for plants (Kolencik et al. 2020; Tarafdar et al. 2012).

ZnO NPs can be dissolve easily as compared to other nanoparticle (Bian et al. 2011) that affect plant growth partially due to their nano-specific attributes, and largely by releasing the Zn as an essential element for various functions on the cellular level (Singh et al. 2021). These nanoparticles are very crucial in the growth of plant and provide protection to different plant species against salt stress (Gaafar et al. 2020). These nanoparticles increase the seed germination, antioxidants activity and production of protein (Garcia-Lopez et al. 2018; Salama et al. 2019), photosynthesis and grain yields (Faizan et al. 2018; Hussain et al. 2021), and release of essential minerals (Peralta-Videa et al. 2014).

2.2.3.2. Titanium dioxide (TiO_2) nanoparticles

TiO_2 nanoparticles are semiconductive material that are insoluble having a high refractive index, UV absorption, photocatalytic and antimicrobial features (Macwan et al. 2011). Their size can be varied from a few nanometres up to 100 nm in any direction and their form can be modified during the synthesis in order to get nanorods and spherical nanoparticles (Silva et al. 2013). The surface properties of TiO_2 nanoparticles are often changed to obtain their stability, increase their beneficial effects and reduce their harmlessness. These nanoparticles have a wide range of applications in various areas of human and agriculture (Chen & Mao 2007). Their environmental uses include purification of water, destruction of pollutants, and coating for antimicrobial activity, biosensing and drug supply (Jarosz et al. 2016).

Application of TiO_2 nanoparticles protect the seeds, enhance their germination and plant growth, and control crop diseases (Servinet al. 2015), destroy pesticides and find their remains (Aragay et al. 2012). These nanoparticles also act as functional photocatalyst to enhance the photosynthesis and plant growth at certain wavelengths (Lei et al. 2007). These nanoparticles are also used to enhance growth of root and shoot, and seed yield. These nanoparticles also increase the production of chlorophyll, soluble leaf protein and carotenoids content (Raliya et al. 2015) and increase the usage of various essential nutrients (Tan et al. 2017). The application of these NPs induces stem elongation, 1000 grains weight and yield in barley (Moaveni et al. 2011). Their foliar application also increases the yields of coriander (Khater et al. 2015).

2.2.4. Mechanism of Nanoparticles interaction

NPs are applied to the roots or as a foliar spray to improve growth, chlorophyll content, photosynthesis, nutrient absorption, stress tolerance, crop yield and quality (El-Said et al. 2014). After application of NPs to the plant, NPs firstly stick to the surface of root, then these small sized NPs penetrate to enter into the epidermal layer of cell wall and cell membrane through the porous cell wall of roots. Here, NPs create large pores in the cell wall to help in internalization of bigger sized NPs into the roots. Then these NPs are moved into the adjacent cells via plasmodesmata (Schwab et al. 2016; Su et al. 2019). After passing through the root epidermis and cortex, NPs enters into the endodermis through the porous cell wall of the endodermal cells. Then NP is efficiently uptaken in the Casparian strip that is a confined impregnation of the primary cell wall encircling the endodermal layer in form of a belt (Geldner 2013). After that, NPs enter into the xylem where the efficient uptake of NPs into roots is affected by transpiration rate and is considered an important factor for the efficient delivery of NPs in plants (Spielman-Sun et al. 2020).

In foliar spray, NPs can directly interact with leaf cells through stomata or cuticular pathways (Eichert & Goldbach 2008). NPs upto 50 nm enter plant leaves following the cuticular uptake pathways where composition of protective layer (cuticle) and surface properties of NPs can affect the effectiveness of NPs uptake (Avellan et al. 2019). The cuticular pathway is followed mostly to small NPs while the stomatal pathway is the major pathway for the uptake of foliar NPs in plants (Yu et al. 2013). After crossing the leaf epidermis either stomata or the cuticular pathway, foliar NPs are mainly distributed in plants by phloem transport by two major pathways: firstly directly into intracellular spaces present between mesophyll cells (palisade and spongy mesophyll cells) to the phloem, and secondly from mesophyll cells to the phloem. In this, plasmodesmata between mesophyll and bundle sheath cells could be the main pathway for NP transport from mesophyll cells to bundle sheath cells and then to the phloem (Danila et al. 2016).

2.2.5. Role of nanoparticles in agriculture

Nanotechnology is a promising tool not only in industrial and medical scales but also in agriculture, because some nanomaterials are beneficial for crops that improve their growth, yield, nutritional status and antioxidant defences (Del Buono et al. 2021; Sturikova & Krystofova 2018). NPs have also made an impact on sustainable agriculture as agricultural products produced from nanoparticles are crucial for increasing growth and yield of crops. They also provide favourable alternatives for farmers as compared to conventional chemicals and techniques being extremely small size particles, simplicity of transport, easy handling, long-term storage, high efficiency and nontoxicity. Because of this, nano-based commercialization is gaining popularity worldwide (El-Said et al. 2014).

2.2.6. Enhancing stress tolerance in plants

Nanotechnology has the ability to open novel approaches to cope with abiotic stresses that severely affect the development and growth of plants (Nair & Chung 2014). Nanoparticles are broadly used to protect the plants against abiotic stresses like salinity, drought, heavy metals, flooding and extreme temperatures in plants (Faizan et al. 2021). Application of nanoparticles improve the nutrient uptake, regulate hormone levels, and mitigate stress-induced damage to stimulate plant growth (Hayat et al. 2023).

Application of silver nanoparticles (Ag NPs) improves the wheat growth by enhancing antioxidant activity and reducing oxidative damage under drought stress (El-Saadony et al. 2022). Furthermore, the application of Ag NPs enhanced antioxidant enzyme activities, reduced ROS (reactive oxygen species) levels, and improved nutrient uptake to improve plant growth under salt stress (Al-Khayri et al. 2023). These nanoparticles also help to reduce the ionic imbalance produced by high levels of salt by controlling the intake and accumulation of ions within plant cells (Abasi et al. 2022).

ZnO nanoparticles can promote the activities of antioxidant enzymes such as catalase, superoxide dismutase, and peroxidase in plants to alleviate the damaging effects of reactive oxygen species produced under drought stress (Rehman et al. 2023). ZnO NPs can also improve efficiency of water used in plants through moderating stomatal conductance and transpiration rate to maintain the water balance under drought stress (Seleiman et al. 2023). The application of ZnO stimulate plant growth under salt stress by promoting activities of antioxidant enzyme, reducing ROS levels, and alleviate lipid peroxidation due to higher amount of salt (Abdel Latef et al. 2017).

Application of TiO_2 NPs enable the plants to tolerate drought stress through different mechanisms like photosynthetic efficiency and antioxidant activation. TiO_2 NPs application increase the rate of photosynthesis by improving chlorophyll content that led to better plant growth and development under drought stress (Ramadan et al. 2022). TiO_2 NPs can improve the intake and usage of nutrients in plants growing under saline conditions that help to neutralize the mineral imbalances produced by high concentration of salt (Abdel Latef et al. 2018).

2.3. Plant tissue culture

Plant tissue culture plays an important part in various fields such as agriculture, horticulture, research, and conservation sectors (Bhatia 2015). It is directed towards the growth of plant cells or parts of plants on a nutrient medium under a controlled, sterile and simulated environment. It is an important technique used for embryogenesis, morphogenesis, nutrition, germplasm conservation, genetic manipulation, large-scale clonal propagation, production of pathogen-free plants and useful metabolites (Thorpe 2007). Plant tissue culture is involved in research and commercial seed production ultimately leading to better crop yields, effective management of diseases in addition to the protection of endangered plant species (Wang et al. 2016).

The success of *in vitro* plant culture depends on several factors such as genotype, the type of explants, surface disinfection methods, the culture medium, plant growth regulators, light intensity, photoperiod and temperature (Sivanesan & Park 2015). The composition of the nutrient media strongly effects the morphogenetic potential of the explants. This medium generally consists of macro and micronutrients, amino acids, organic compound, vitamins, carbon sources, hormones, and solidifying agents. The optimization of the mineral elements in the nutrient media improves cell proliferation, organogenesis, somatic embryogenesis, shoot quality and the bioactive compound content in cell and organ cultures (Hameg et al. 2020).

2.3.1. Micropropagation

Micropropagation is a vital technique used for propagation of crops on large scale, medicinal and aromatic plants, trees, and economically important plants (Rout et al. 2000). This method involves aseptic culturing of explants i.e. apical or axillary meristems, or some other organ of the plant grown on chemically defined culture media by keeping the cultures in suitable environmental conditions of temperature, light, and humidity (Xu & Huang 2014). The advantages of micropropagation are the high genetic and sterile quality of the explants for producing plants on large scale in a small space and short time (Kavand et al. 2011). This method involves the following four stages during organogenesis:

- The first stage includes culture initiation in which specific and actively growing part of plant (explants) are selected for culturing on a defined media.
- The second stage includes the response of explant cells to the stimulus of hormones (auxin and cytokinins) that starts the division and development of organs from explant (organogenesis).
- In the third stage, shoots developed from the multiplication stage are elongated and subsequently rooting starts either *in vitro* or *ex vitro*.
- The fourth stage is a crucial stage in the micropropagation because it provides the adaptation to plants growing *in vitro* (Rout et al. 2006).

2.3.2. Role of nanoparticles in plant tissue culture

Nanotechnology techniques applied to propagation methods play an essential role in ensuring sustainable agricultural practices and enhancing plant productivity (Alenezi et al. 2022). Application of nanoparticles led to the successful removal of microbe contaminants from explants and played a positive role in callus induction, organ development, somatic embryogenesis, soma clonal variation, genetic transformation and secondary metabolite production (Kim et al. 2017). In plant tissue culture, the use of nanoparticles improves seed germination, protection, yield and growth of plants (Wang et al. 2017).

Nanoparticles increase the strength and duration of tissue culture media and growth substrates by avoiding the degradation of phenolic compounds (Farrokhzad et al. 2022) and results in healthier and more productive explants (Khan et al. 2016). Nanoparticles are also significant role in controlling the enzymatic browning produced within the plant tissue culture medium (Permadi et al. 2023). Particular nanoparticles like gold and silver, exhibit antioxidant properties that are efficient for scavenging and responding ROS generated during the enzymatic browning process (Araujo et al. 2020). The reduced levels of ROS inhibit the oxidation of phenolic compounds and slow down the browning process (Horison et al. 2022). In plant tissue culture, the application of silver NPs (AgNPs) is widely studied (Sarmast & Salehi 2016).

2.3.3. Role of nanoparticles in micropropagation

Nanoparticles affect biomass production, root growth and shoot growth, biochemical and physiological activities (Siddiqi & Husen 2010). There are a few studies about the effect of NPs on *in vitro* plant growth and production. However, there is a need to investigate the effects of nanoparticles on growth and regeneration of plants. Ag NPs exhibit high antimicrobial activity, high thermal stability and low volatility that could be used in the micropropagation of woody plants. Modifying the culture medium with Ag NPs during multiplication and rooting decreases the pathogen invasion, and also promote the development of the root system and accelerates the growth of the vegetative part of the shoots (Vasyukova et al. 2021).

Phytomediated ZnO NPs application to *in vitro Ochradenus arabicus* promoted shoot growth and resulted in high accumulation of biomass (Al-Qurainy et al. 2021). Application of ZnO NPs in MS media resulted in the emergence of few shoots and wgite thin roots covered by thick root hairs from stem explants of *Brassica nigra* (Zafar et al. 2016). The application ZnO NPs induce callus formation and shoot regeneration in rapeseed (Mousavi & Lahouti 2018). The shoot proliferation rate in date palm was also found to be higher from buds cultured with ZnO NPs on MS medium (Awad et al. 2020). ZnO NPs promoted the shoot and root lengths, and biomass in *Vigna radiata* (Mahajan et al. 2011). Application of ZnO nanoparticles to the Murashige and Skoog medium improves the shoot elongation of the *Phoenix dactylifera* (Awad et al. 2020).

2.3.4. Antimicrobial properties of nanoparticles

Preventing microbial contamination in plant tissue cultures is important for successful micropropagation because epiphytic and endophytic organisms can cause damage to micropropagated plants at each growth stage by reducing multiplication, rooting rates and even death (Leifert & Casselles 2001). Another the most important limiting factors, especially in woody plants is *in vitro* fungal and bacterial contamination. The effective commercial micropropagation depends on various aspects like culture medium, plant growth regulators, explants age and donor plant (Sarmast et al. 2011). Several disinfecting agents are unable to remove contaminants in explants because they affect the organogenesis due to their phytotoxicity (Wang et al.2017). In many studies, surface sterilization of explants with metal and metal oxide nanoparticles decrease the microbial contamination (Sarmast & Salehi 2016). The application of different nanoparticles has been suggested to reduce microbial contamination:

Zinc oxide and Titanium dioxide nanoparticles being antimicrobial agents act as a bactericidal and fungicidal drugs in biotechnology for sterilization of explants during clonal micropropagation of plants due to their photocatalytic activity, particle size, concentration, morphology, and surface modification. The primary mechanisms of their toxicity is generation of ROS that leads to oxidative damage (Zakharovaa & Gusev 2019).

Silver nanoparticles have good ability to remove fungal, bacterial and virus contamination without adversly effecting the plant growth and production (Castellano et al. 2007; Abdi et al. 2008). It exhibits broad-spectrum antimicrobial activity *in vitro* that binds to microbial DNA to prevent bacterial replication, and to the sulfhydryl groups of the metabolic enzymes in the bacterial electron transport chain to cause their inactivation (Slawson et al. 1990).

Gold nanoparticle's antimicrobial activity is inversely proportional to their size, the smaller the size of these nanoparticles, the higher will be antimicrobial activity against a various microbes such as *K. pneumonia, S. aureus, P. vulgaris, E. coli,* and *B. subtilis* (Thangamani & Bhuvaneshwari 2019).

2.3.5. Nanoparticles in plant biotechnology

Advancements in nanotechnology offer a wide range of possibilities for novel applications of nanoparticles in the biotechnology and agricultural sector (Siddiqui et al. 2015). Nanobiotechnology is the outcome of combining the sciences of nanotechnology and biotechnology with specifics purposes (Madkour 2019). NPs extensively used in genetic engineering are magnetic NPs, carbon nanotubes, and DNA nanostructures (Zhao et al. 2017). Each type of NP delivers different genetic material, for example, carbon nanotubes can deliver RNA and DNA (Bates & Kostarelos 2013), while silicon NPs can deliver DNA and proteins, while polymeric NPs can transfer encapsulated RNA, DNA, and proteins into cells (Zhou et al. 2018). But metallic NPs can transfer only DNA as genetic material and these NPs carrying exogenous DNA were delivered to pollen. The plants pollinated through transformed pollen produce transformed seeds, which were further used in the cultivation of transgenic plants (Zhao et al. 2017).

Genome editing: Technology is being used in the growth of plants with better yields and high nutritional content alongwith higher resistance to herbs, insects and diseases. GE tools have certain restriction that includes high time consumption and complex protocols, tissue damage, incorporation of DNA in the host genome, and less efficient in transformation (Ahmar et al. 2021). To overcome these problems, nanotechnology is a modern technique for introducing transgenes into plants through particular delivery systems of gene. Nanoparticle mediated gene delivery is better than conventional methods because it improves the transformation efficiency for both temporal and permanent genetic modifications in different species (Grunewald et al. 2013; Vanhaeren et al. 2016).

Genetically engineered plants: Nanotechnology can also minimize uncertainty and assist to coordinate with the management approaches of agriculture using molecular production methods by serving as an alternative approach to conventional techniques. NPs are also used to facilitate GE through an effective and targeted transfer of plasmids, RNA, and ribonucleoproteins (Mout et al. 2017). These technologies have improved the accuracy of plant breeding in offering new opportunities for gene choice and alteration, also reduce the time to eliminate unwanted genes and enable the breeder to approach essential genes (Perez-de-Luque 2017).

Disease resistant plants: Genetic modification through nanoparticles mediated plant transformation has the ability to promote the disease resistance in plants (McKnight et al. 2003). The introduction of resistance genes in plant cells develop the resistant plants to reduce expenses on the agrochemicals used for disease control (Bouwmeester et al. 2009).

3. Aims of the Thesis

The aim of this thesis is to evaluate the effect of Titanium dioxide (TiO_2) and Zinc oxide (ZnO) nanoparticles on micropropagation of *Salvia rosmarinus* by using nodal segments. Specific objectives is to find the effect of different concentrations of nanoparticles on different growth parameters of *Salvia rosmarinus* grown on MS media.

3.1. Hypothesis

Rosemary is commonly recognized for its antioxidant and antimicrobial characteristics, moreover, rosemary essential oils inhibit the enzymatic browning and microbial growth in culture media. NPs has positive effect on callus induction, somatic embryogenesis, cell suspension culture, and plant sterilization in micropropagation. Therefore, nanoparticles application improves the growth, nutritional status, antioxidant defences and production of secondary metabolite in plants by controlling the microbial growth.

4. Methods

4.1. Plant material

The plant material selected for this study was *Salvia rosmarinus* belonging to Lamiaceae family that was taken 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 through micropropagation before the nanoparticles application. This plant was chosen for its antioxidant, anti-inflammatory and antimicrobial properties, and the ability to reduce neurodegenerative disease (Perry et al. 2000). Moreover, it can serve as a natural pesticide that helps in phytoremediation process (Bozin et al. 2007).

4.2. Micropropagation methodology

4.2.1. Growth medium preparation

For *in vitro* micropropagation of the plants, a basic MS medium (Murashige & Skoog 1962) was used, and the composition of the medium is presented in Table 1. To prepare 1 litre of the MS medium, 100 ml of solution A and 10 ml of each solutions B, C, D, E and V was measured with a graduated cylinder, mixed them in a beaker and then 300 ml of distilled water (H_2O) was added. After this, 100 g of Myo-inositol and 30 g of sucrose were weighed on a weighing balance and added into the solution during stirring. The pH value of the solution was measured and adjusted to 5.7 by using potassium hydroxide (KOH). Subsequently 8 g of agar was weighed and added to a beaker filled with 500 ml of distilled H_2O and stirred. Both solutions were heated in a microwave, mixed together and filled with warm distilled water to the final volume of 1 litre. The warm medium was divided in 40 conical flasks and these conical flasks were closed with aluminium foil for autoclaving.

Table 1. Composition of MS medium (Murashige & Skoog 1962).

Murashige – Skoog Medium			
Storage solutions		**Weight used**	**Volume used (pH 5.7)**
A	NH_4NO_3	165 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 liter of MS medium:

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

4.2.2 Medium sterilization

A physical destruction of microorganisms was done by using an autoclave immediately after the preparation of the medium to ensure that MS medium was not contaminated. The MS medium was autoclaved at the temperature (121°C) and the pressure (105 kPa) for about 20 minutes.

4.2.3. Preparation of aseptic condition

The necessary tools used for micropropagation were sterilized. For this, petri dishes, tweezers, scalpels and pasteur pipettes were wrapped in aluminium foil for sterilization at 160 °C in a dry heat sterilizer for 3 hours. The flowbox used for the micropropagation was cleaned using 70% ethanol while the medium and tools wrapped in foil were placed inside, thereafter they were sterilized with 70% ethanol. Then the UV lamp and fan were switched on for minimum 30 minutes. To maintain aseptic conditions, both hands were sprayed with 70% ethanol whereas the tweezers and scalpel were placed in a flask containing 70% ethanol that were regularly flamed during micropropagation. Earlier to nanoparticle application the manual propipetter was placed in 70% ethanol bath for about 24 hours.

4.2.4. Micropropagation and application of nanoparticles

For the experiment, only nodal segments were used for micropropagation. Nodes with the length of about 1 cm were divided in the flowbox and were grown in basic MS medium. 40 nodal segments were prepared for each treatment and control group. The explants were allowed to grow for 5 days. The growth conditions were 16/8 (day/night cycle) at 25/23 °C temperature with 2500 lx of light intensity. After this, for healthy growth of explants Zinc oxide and titanium dioxide solution in different concentrations was added to MS media using pasteur pipettes. While no solution was applied into the control group after the initial micropropagation.

The medium for nanoparticles was made in the similar way as basic MS medium described in table 1, but it was not heated, and agar was not added into it. After that, the medium was divided into different beakers. Zinc oxide and titanium dioxide were weighed and added into separate medium solutions to achieve the desirable concentrations of 20 mg/l, 40 mg/l and 60 mg/l, as described in table 2. The beakers were sealed with Parafilm M(R) and aluminium foil and then sterilized in the autoclave for 20 minutes.

Table 2. List of concentration of treatments used in the experiment

Treatments	**Nanoparticles (mg/l)**		**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. Data calculation

Plant growth was observed over a period of 60 days. Data was collected after every 10 days. Regeneration percentage, number of sprouts, length of sprouts, number of nodes, number of roots, and length of root all were traced and measured. Measurements were taken through the tube walls using a paper ruler.

4.4. Statistical analysis

The statistical analysis for the comparison of different concentrations of ZnO and TiO_2 nanoparticles (20mg/l, 40mg/l and 60mg/l) was performed by using Student's T-test for each sample. The differences were considered to be statistically significant at ($p<0.05$).

5. Results

5.1. Plant regeneration (%)

The maximum percentage of plant regeneration from explants were seen in plants grown on basic MS medium (control group) as compared to application of ZnO and TiO_2 nanoparticles solution to explants. Application of ZnO nanoparticles in different concentration (20mg/l, 40mg/l and 60mg/l) showed highest regeneration percentage rate in Z1 treatment with 20mg/l of ZnO nanoparticle solution while the lowest regeneration rate was in Z2 treatment with 40mg/l application of ZnO nanoparticle solution to explants. Application of different concentration (20mg/l, 40mg/l and 60mg/l) of TiO_2 application showed best regeneration percentage in T2 treatment with 40mg/l and the less regeneration percentage in T1 treatment with 20mg/l application of TiO_2 nanoparticles solution to explants. ZnO nanoparticle application showed the better percentage rate among all plants as compared to TiO_2 nanoparticles.

The statistical effect of nanoparticles on regeneration percentage was evaluated by student's t-test and the statistically significant differences ($p<0.05$) were found in Z2 (40mg/l), Z3 (60mg/l), T1 (20mg/l), T2 (40mg/l) and T3 (60mg/l) treatments (Figure 2A).

5.2. Number of sprouts per plants

For number of sprouts, TiO_2 nanoparticles application showed best result as compared to plants grown on MS medium and treated with ZnO nanoparticles solution. Application of different concentration (20mg/l, 40mg/l and 60mg/l) of ZnO nanoparticles showed highest number of sprouts in Z3 treated plants with 60mg/l, and almost similar number of sprouts were calculated in Z1 and Z2 treated plants with 20mg/l and 40mg/l application of ZnO nanoparticles solution. Moreover, TiO_2 nanoparticles application in different concentration (20mg/l, 40mg/l and 60mg/l) showed highest number of sprouts in T1 treated plants and lowest in T3 treated plants with 20mg/l and 60mg/l of TiO_2 nanoparticles solution respectively. Overall, there was a reduction in number of sprouts by the application of ZnO nanoparticles.

The effect of nanoparticles on number of sprouts was evaluated by student's t-test and the statistically significant differences ($p<0.05$) were found in Z1-20mg/l, Z2-40mg/l and Z3-60mg/l, and T1-20mg/l and T3-60mg/l treatments (Figure 2B).

5.3. Length of sprouts (mm)

Application of ZnO nanoparticles showed the highest sprout length in plants as compared to those grown on MS medium and treated with TiO_2 nanoparticles. Overall, the plants grown on MS medium i.e. control group showed a reduction in sprout length. The different treatments of ZnO nanoparticles solution i.e. Z1-20mg/l, Z2-40mg/l and Z3-60mg/l exhibited longest sprout length in Z1-20mg/l and shortest in Z3-60mg/l treatments of ZnO nanoparticles. In case of TiO_2 nanoparticles application, their different treatments i.e. T 1-20mg/l, T2-40mg/l and T3-60mg/l exhibited longest sprout length in T1-20mg/l treatment while T2-40mg/l and T3-60mg/l treatments showed the similar sprout length in treated plants. Overall, ZnO nanoparticles application showed better sprout length in all treated plants.

The effect of nanoparticles on number of sprouts was evaluated by student's T-test and the statistically significant differences ($p<0.05$) were found in Z1 (20mg/l), Z2 (40mg/l), Z3 (60mg/l), and T2 (40mg/l) and T3 (60mg/l) treatments (Figure 2C).

5.4. Number of nodes per sprouts

Application of ZnO nanoparticles showed the maximum number of nodes as compared to plants grown on MS medium and treated with TiO_2 nanoparticles. The number of nodes were similar in plants grown on MS medium (control group) and Z1 treatment with the 20mg/l application of ZnO nanoparticles solution. Different treatments (Z1-20mg/l, Z2-40mg/l and Z3-60mg/l) of ZnO nanoparticles revealed highest number of nodes in Z3 treatment with 60mg/l and lowest in Z1 treatment with application of 20mg/l ZnO nanoparticles solution to plants respectively. Moreover, different treatments (T1-20mg/l, T2-40mg/l and T3-60mg/l) of TiO_2 nanoparticles to plants showed greatest number of nodes in T1-20mg/l treatment but smallest in T2-40mg/l and T3-60mg/l treatments respectively. Overall, ZnO nanoparticles application showed best results for number of nodes among all treated plants.

The effect of nanoparticles on number of nodes determined by student's t-test was statistically significantly different ($p<0.05$) in control group, and T1-20mg/l, T2-40mg/l and T3-60mg/l treatments (Figure 2D).

5.5. Number of roots per plants

The greatest number of roots were obtained in plants grown on basic MS medium i.e. control group as compared to plants treated with ZnO and TiO_2 nanoparticles. In application of

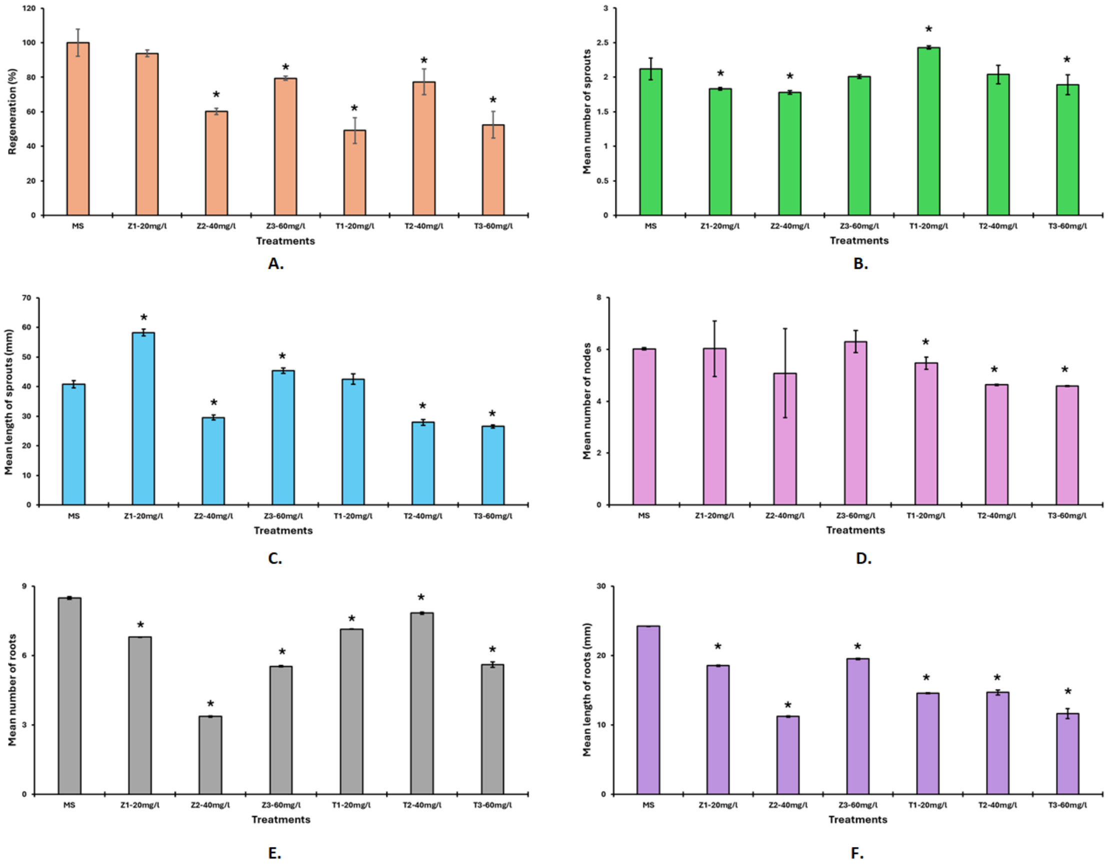
ZnO nanoparticle, their different treatments (Z1-20mg/l, Z2-40mg/l and Z3-60mg/l) showed highest number of roots in Z1-20mg/l and the smallest in Z2-40mg/l treated plants. On the other hand, different treatments (T1-20mg/l, T2-40mg/l and T3-60mg/l) of TiO_2 nanoparticle showed highest number of roots in T2-40mg/l and lowest in T3-60mg/l treated plants. Overall, plants treated with TiO_2 nanoparticles showed better results as compared to ZnO nanoparticle.

The effect of nanoparticles on number of roots was evaluated by student's t-test and the statistically significant differences ($p<0.05$) were found in control group and all treatments (Z1-20mg/l, Z2-40mg/l, Z3-60mg/l, T1-20mg/l, T2-40mg/l and T3-60mg/l) (Figure 2E).

5.6. Length of roots (mm)

The highest length of roots was detected in plants grown on basic MS medium as compared to plants treated with different concentration of ZnO and TiO_2 nanoparticles solution. Application of ZnO in different concentration i.e. 20mg/l, 40mg/l and 60mg/l showed highest root length in Z3 treated plants with 60mg/l and lowest in Z2 treated plants with 40mg/l application of ZnO nanoparticles solution. Furthermore, different concentration i.e. 20mg/l, 40mg/l and 60mg/l of TiO_2 nanoparticles showed almost similar root length in T1 (20mg/l) and T2 (40mg/l) treated plants while it was decreased in T3 (60mg/l) treated plants. Among all treated plants, ZnO application showed greater length of roots as compared to TiO_2 nanoparticles.

The effect of nanoparticles on length of roots was evaluated by student's t-test and the statistically significant differences ($p<0.05$) were evaluated in control group and T1-20mg/l, T2-40mg/l and T3-60mg/l treatments (Figure 2F).



*** indicates significant at 5% significance level.**

Figure 2. Effect of different concentrations of ZnO and TiO_2 nanoparticles on (A) regeneration %, (B) mean number of sprouts, (C) mean length of sprouts, (D) mean number of nodes, (E) mean number of roots and (F) mean length of roots of *Salvia rosmarinus*.

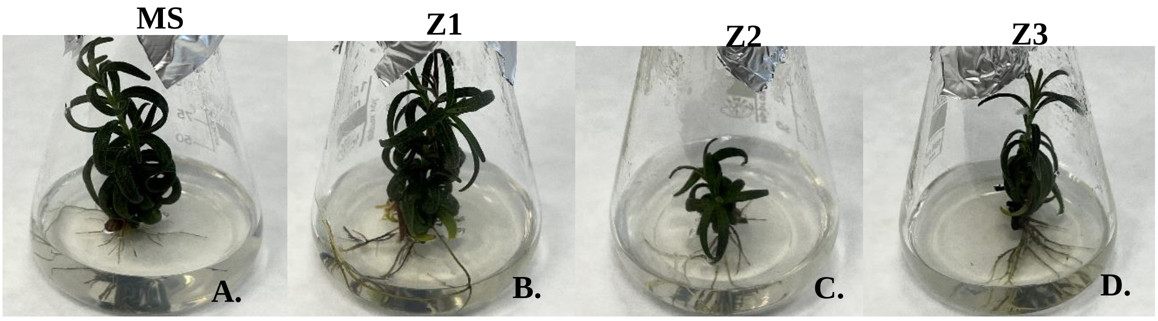


Figure 3. Phenotypic results with ZnO nanoparticles (A) 0mg/l, (B) 20mg/l, (C) 40mg/l and (D) 60mg/l concentrations.

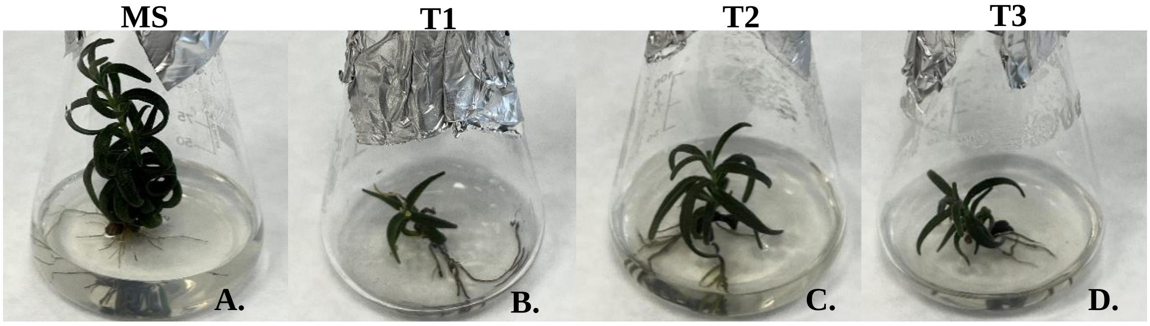


Figure 4. Phenotypic results with TiO_2 nanoparticles (A) 0mg/l, (B) 20mg/l, (C) 40mg/l and (D) 60mg/l concentrations.

6. Discussion

Salvia rosmarinus has economic, social and environmental benefits due to the antimicrobial effects of its chemical components. Micropropagation of rosemary is a source of providing secondary metabolites to enhance the availability and uptake of nutrients in agroforestry systems (Aman & Afrasiab 2014). Nanotechnology is environmentally friendly technique because it uses plants, bacteria and various bio-resources for the synthesis of nanoparticles (Lateef et al. 2016; Khan et al. 2019) and also possess the potential in forestry to improve the quality of plants (Wagay et al. 2023). Application of nanoparticles in the agriculture field is a novel techniques to promote productivity of crop under normal as well as harsh environmental situations (Zulfiqar & Ashraf 2021). Moreover, NPs also control the fungal and bacterial growth to improve the crop production and minimize the crop losses (Rizwana et al. 2022).

The addition of ZnO NPs to tissue culture media can positively influence callus development, rooting, and somatic embryogenesis in various plants (Shafique et al. 2020; Al-Mayahi 2021). Previous studies showed that the application of 10 mg/L of ZnO NPs in the culture media showed higher growth parameters in *Salvia officinalis* (Alenezi et al. 2022). Alizadeh & Dumanoglu (2022) stated that the application of IBA-nZnO showed highest root length in *in vitro* rooting of apple microcuttings with a healthy root system. In our results, application of 60 mg/l of ZnO showed highest root length in rosemary as compared to 20 mg/l and 40 mg/l. ZnO nanoparticles could enter the cell walls and increase the levels of auxin that help to promote cell division (in the presence of cytokinins), cell elongation and mineral absorption (Hanif et al. 2024).

The effectiveness of ZnO NPs for the plants regeneration in *in vitro* cultures reveal their specific properties as an indicator of plant growth and the individual response of plant species (Gharpure & Ankamwar 2020). Excessive amounts of zinc oxide nanoparticles can negatively affect the plants. In previous studies, Thunugunta et al. (2018) investigated that ZnO nanoparticles application inhibited the regeneration capacity of eggplant seeds in the Murashige-Skoog medium with their increasing concentration from 5 to 20 mg/L than control. In our study, regeneration of *Salvia rosmarinus* was also inhibited by increasing the concentration of ZnO from 20 mg/L to 60 mg/L than control. This is due to destructive effect of ZnO nanoparticles on homeostasis, hormonal signalling, transport of heavy metals, and photosynthesis with a reduction in the chlorophyll and carotenoids content (Adhikari et al. 2020).

The behaviour of plants towards the nanoparticles depends on the plant, and the kind and concentration of nanoparticles (Bayat et al. 2022). Previous studies showed that TiO_2 at a concentration of 10 mg/l enhanced the regeneration of wheat seeds to significantly improve the plant growth (Feizi et al. 2012). Chutipaijit & Sutjaritvorakul (2018) stated that *in vitro* micropropagation of rice in the culture media provided with 20 mg/l TiO_2 nanoparticles showed the greatest regeneration rate and 40 mg/l of TiO_2 nanoparticles were the best media for callus induction and regeneration of plant. In our study, application of 40 mg/l of TiO_2 showed highest regeneration percentage from nodal segment. The changing behaviour of regeneration rate is associated with the photocatalytic activity of nanoparticles that vary in size and shape (Ma et al. 2012).

Titanium dioxide nanoparticles affect cell division, cell size, callus induction and hormone rates (Gibberellins and cytokinin) to significantly increase the growth of Rosmarinus *officinalis* plants (Golami et al. 2018). In previous studies, Waani et al. (2021) described that application of TiO_2 nanoparticles at concentrations of 5 mg/l to 150 mg/l significantly promoted the root length of rice seedlings. In our results, the highest and almost similar root length was observed by the application of 20 mg/l and 40 mg/l of TiO_2 nanoparticles. These positive results are related to the improved uptake of the water and nutrient due to nanoparticles application (Dehkourdi & Mosavi 2013).

ZnO nanoparticles act as natural regulator in plants by modulating important physiological parameters, thereby, improves plant growth and development (Faizan et al. 2021). Previously, Hanif et al. (2024) described that foliar spray of optimum concentration of ZnO nanoparticles (50 mgl^{-1}) increased the No. of nodes in tomato and their number was gradually increased as the concentration of nanoparticle increases until 100 mgl^{-1}. In our results, the application of 60 mgl^{-1} ZnO nanoparticles showed highest number of nodes in *Salvia rosmarinus*. This is because the optimum concentration of ZnO nanoparticles improved the levels of plant secondary metabolites and osmolytes to enhanced plant growth parameter (Hanif et al. 2024).

TiO_2 nanoparticles increased CO_2 fixation by enhancing the rubisco activity to improve the growth (Qi et al. 2013). Previous study by Singh & Verma (2022) reported that TiO_2 nanoparticles application improved the sprout growth in canola. In our results, application of 20 mgl^{-1} of TiO_2 nanoparticles showed highest sprout length but it was decreased at 40 mgl^{-1} and 60 mgl^{-1}. The higher amounts of TiO_2 nanoparticles also affect growth of *Oryza sativa* because TiO_2 nanoparticles harm the cell wall and cell membrane of plants (Mirzajani et al. 2013).

NPs always showed positive results on different morphological parameters of plants by increasing the proline synthesis and the activity of different enzymes (Helaly et al. 2014; Khalid et al. 2022). In previous studies, Prasad et al. (2012) described that the application of 1000 mg/ lof ZnO nanoparticles increased sprout growth in peanut (average size ~25 nm). In our study, application of 20 mg/l of ZnO nanoparticles showed highest sprout length as compared to 40 mgl^{-1} and 60 mgl^{-1}. The small size of ZnO nanoparticles allows it to enter into the plant cells to promote the seed growth (Das et al. 2014).

7. Conclusions

In present study, effect of different concentration of Zinc Oxide and Titanium Dioxide nanoparticles on *in vitro* micropropagation of *Salvia rosmarinus* was evaluated and compared. The application of nanoparticles improved the antioxidant properties, photosynthetic efficiency and proline accumulation to provide stability to plants. For micropropagation of *Salvia rosmarinus,* a nodal segment was grown on basic MS media (control group) and by following the different treatment of Zinc oxide (Z1-20mg/l, Z2-40mg/l and Z3-60mg/l) and Titanium dioxide (T1-20mg/l, T2-40mg/l and T3-60mg/l) for 60 days. The growth conditions were day and night cycle (16/8) by keeping a temperature day and night (25/23 °C) with a light intensity (2500 lx). Readings were taken after every 10 days. The results showed highest regeneration percentage in Z1-20mg/l, number of sprouts in Z3-60mg/l, sprout length Z1-20mg/l, number of nodes in Z3-60mg/l, number of roots in Z1-20mg/l and root length in Z3-60mg/l treated plants. In case of Titanium dioxide application, highest regeneration percentage was found in T2-40mg/l, number of sprouts in T1-20mg/l, sprout length T1-20mg/l, number of nodes in T1-20mg/l, number of roots in T2-40mg/l and root length in T1-20mg/l and T2-40mg/l treated plants. Moreover, increasing concentration of nanoparticles decrease the growth of *Salvia rosmarinus* as regeneration percentage, number and length of sprout, number of roots and nodes, and length of nodes were decreased in Z2-40mg/l treatment. Similarly, regeneration percentage was decreased in T1-20mg/l, number and length of sprout, number of roots and nodes, and length of nodes were decreased in T3-60mg/l treatment. The negative effects of nanoparticles inhibit the chlorophyll content, photosynthetic effectiveness and suppresses the plant growth. Nanotechnology is considered a green method due to elimination and control of environmental pollutants to obtain sustainable growth. Hence, ZnO and TiO_2 could act as bactericidal and fungicidal drugs to sterilize the explants during clonal micropropagation of plants on growth media.

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