Eastern Journal of Agricultural and Biological Sciences (EJABS)
ISSN: 2753-3247
Website: https://qabasjournals.com/index.php/ejabs

In vitro Propagation and influence of Silicon Nanoparticles on Growth of *Thymus serpyllum*

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ARTICLE INFO

Received: November 1, 2022
Accepted: December 5, 2022
Volume: 2
Issue: 4

KEYWORDS

Thymus, Zeatin, SiO_2-NP_s, Stem segment, Micropropagation

ABSTRACT

Thymus serpyllum L. is a perennial shrub, aromatic plant, native to Europe. It is known as Breckland, wild or creeping thyme. The major ingredients essential oils are thymol and carvacrol, which is a phenol monoterpenes derivative with pharmacological properties. The aim of this research was applying a suitable protocol for achieving micropropagation and growth improvement. Stem segments have been cultured on Murashige and Skoog (MS) medium fortified with 6-benzyl adenine (BA) or kinetin (Kin) or with zeatin for induction shoots. shoots of 5 cm have been cultured on half MS medium fortified with various concentrations of indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA) for rooting. The multiply shoot were transferred on MS fortified with SiO_2-NP_S at 0, 50, 100 and 200 mg/ L. The best results of establishment was obtained with 1.0 mg/L BA that gave maximum shoots/ explant and shoot length. In multiplication stage highest shoots/ explant and shoot length were achieved with 1.0 mg/L BA and 1mg/L Kin. The highest percentage of rooting was at 1.0 mg/L IBA or NAA after one month. The maximum number of roots / explant and root length were at 0.5 mg/L IBA. The highest number of shoots / explant was obtained at 200 mg/L SiO_2-NPs, but 100 mg/L SiO_2-NPs gave maximum shoot length, number of roots / plantlet and root length. This study was the first to establish a rapid and reliable protocol for micropropagation of wild thymes; proposing as well the potential of using this biotechnology in rescuing wild Thymus species.

Introduction

Thymus is a perennial evergreen aromatic plant grown in many countries for its therapeutic properties, for example as an antioxidant, antimicrobial, or anti-inflammatory [1-3]. The genus Thymus is represented by 220 species and belongs to the Lamiaceae family. The herb is also known as wild thyme or Banajwain and highly treating several popular ailments in folk as well as in allopathic medicine system due to the presence of essential oils [4].

Thymus serpyllum L. is a perennial shrub, native to the northern and central regions of Europe. It is known as Breckland thyme, wild thyme, or creeping thyme [5]. Many studies on the chemical composition and yields of the essential oils of *Thymus serpyllum* L. bring it to among the world's top ten essential oils and are used as food preservative and in reliving rheumatism, in hair loss treatment. [6-7]. Due to the excellent quality of the essential oil derived from its aerial portions, certain Thymus species have been discovered for medicinal, food and cosmetic uses (8).

Although it was noted [9-10] as dominant components in literature, carvacrol and thymol possess strong cytotoxic activity and are used against proliferating cells in case of cancer. Carvacrol is a major monoterpenoic phenol that is believed to hinder cancer progression. shows a potential antitumor role by inhibiting cell proliferation and preventing metastasis in DEN-induced hepatocellular carcinogenesis. Thymoquinone present in the thymus has antioxidant effects in brain tissue in vivo. Hexane extract of plant showed its anticancerous potential against 6 cell lines [11] Antitumor potential of the plant is also worked out by a number of workers [12-14].

Silicon is the second most abundant element in the earth's crust and plays a vital role in many biogeochemical processes. Silicon reinforce the physical barrier of plants by its deposition on plant cell walls, thus promoting the ability of lodging tolerance and disease resistance [15]. The applications of silicon and SNPs reduce the oxidative stress response by priming defense reactions under biotic and abiotic stresses [16-19]. Moreover, SNPs have a small particle size and easily penetrate the plant cell wall and the organelles [20]. In comparison with bulk materials, NPs have a high surface-area-to volume ratio, thus improving their reactivity and biochemical activity [21-22]. Silicon (Si) does not put under the category of important elements of higher plants, and its essentiality has not been proven yet. It regarded as one of the most beneficial elements for the life of plant [23]. Many studies reported that it is essential for various species of plant such as wheat, rice, maize and bamboo [24]. In wheat grains, SiO2-NPs improved the biomass [20]. In generally, Proper elucidation of plants mechanisms at the level of biochemical, physiological, and molecular nonmaterial's led to the promote plant growth and development [25]. In vitro propagation it is an aseptic technique by which small explants cultured in artificial medium give rise to complete plant directly and used as tool for ex situ conservation. The current research was initiated to assess effects of nanosilicon dioxide on the growth of wild thymus explants in vitro culture

1. Methodology

1.1 Plant material

The plants of *Thymus serpyllum* L. were collected from The Society of Ahmed Urabi in Cairo, Egypt.

1.2 Sterilization of explant

The plant material (stem nodal segments) was collected from open filed with non-destructive approach. Contamination was important factor for the successful culture of the plant, so the collected explants were initially rinsed in water with 2–3 drops of liquid soap with constant stirring for 10 min. The surface sterilization of plants was carried out under laminar air chamber with 20 % clorox (5.25% sodium hypochloride) solution for 10 min, followed by rinsing with autoclaved water to remove all the traces of commercial bleach.

1.3 Establishment stage

Stem nodal segment explants were transferred to MS medium supplemented with 3% (w/v) sucrose and 0.3% (w/v) phytagel and two concentrations of 6- benzyl adenine (BA) of 1 and 2mg/L or kinetin (Kin) of 1 and 2mg/L or 0.1 and 0.2 mg/L zeatin. MS medium without plant growth regulators (PGR) served as a control. The pH of the medium was adjusted to 5.7 ± 0.1 and sterilized in an autoclave at a pressure of 1.06 kg/cm and 121 °C for 15 min. The cultures were incubated at 25 ± 1 °C at a photoperiod of 16/8 h light/darkness under cold white fluorescent tubes of 2500-3000 lux. The mean number and length (cm) of shoots/explant were recorded after one month of culture.

1.4 Multiplication stage

The shoot produced in vitro were transferred to MS medium supplemented with 3% (w/v) sucrose and 0.3% (w/v) phytagel and different concentrations of cytokinins; BA at 1mg / L with 1mg / L kin or 0.1 zeatin or individually,while, MS support without PGR acted as a control. The pH of the medium was adjusted, autoclaved, and the cultures were incubated as mentioned in the shoot induction stage. The number and length (cm) of shoots per explant were recorded after one month of culture. Subculturing was done every one month.

1.5 Rooting and acclimatization stages

The multiplied axillary shoot clusters were transferred for rooting on half-strength MS medium supplemented with one of the following auxin treatments 0.5, 1.0 and 2.0mg/L indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA). Half strength MS medium without PGRs served as a control for rooting induction. The pH was adjusted, the medium was autoclaved, and the cultures were incubated as mentioned in the previous stages. The percentage of rooted shoots (rooting %) and the number and length (cm) of roots/explant were scored after one month of culture on the rooting medium.

Rooted plantlets were removed from the nutrient medium and washed thoroughly with distilled water to remove residues of the medium, then were transferred to plastic pots in a mixture of sand and peat (1: 2 v/v) (Peat moos, PROMIX®). The pots were covered by translucent polythene plastic bags to maintain high humidity and prevent the dissection of the newly transferred plantlets. Transplants were irrigated and the plastic bags were pored (one pore/5 days for 5 weeks) to decrease the humidity and acclimatize the plants to the external atmosphere gradually. After 6 weeks, the plastic bags were removed completely and the plants allowed to grow under open conditions.

2.6. *Silicon Dioxide Nanoparticles synthesis*

The method used in this study was according to that described by Essien et al. (**23**) with some modification. The reagent chemicals used for synthesizing the porous silica network was "sodium meta silicate", Na2SiO3 and HCl (37 %). Na2SiO3 (5g) was dissolved in deionized water (10 ml) and 2 M HCl (26 ml) was added drop wise under stirring using a magnetic stirrer until a gel was formed at 50° C. The gel prepared above was immediately poured into deionized water and washed successively to remove the NaCl formed during the hydrolysis stage of the reaction. No precipitate was found when the liquid from the last filtration was tested using dilute AgNO3 solution to ascertain the complete removal of NaCl from the gel network.

2.6.1.*Thermal Treatment*

After washing, The formed gel was put in an oven to dry at 120° C for 24 hrs. then calcined at 600° C for other 3 hours in a furnace and thereafter milled to form powders.

2.6.2. *UV-Vis spectroscopy*

The Sio_2 nanoparticles were characterized using UV/VIS Spectrophotometer. The scanning range for the samples was 824-979 nm. Millie-Q water was used as a blank reference.

2.6.3. *Effect of SiO2-NPs on Thymus serpyllum growth*

After rooting stage, MS was supplemented with the fresh prepared SiO2-NP_S at 0, 50, 100 and 200 mg/ L. All media were supplemented with 30 g/ L sucrose and 7.5 g agar/ L and autoclaved at 121°C for 15 min. One plant were cultured on 40 ml of solid medium and incubation in culture room. Four weeks later, number of shoot per explant and shoots length, number of root per explant and root length were determination .

Experimental design and statistical analysis

In vitro experiments were subjected to the completely randomized design. At least ten replicates were cultured for each treatment and the experiments were repeated twice. One-way analysis of variance (ANOVA) was used to evaluate significant differences among the mean values of different treatments by the Costat software program for statistical analysis, The mean comparisons were performed by the least significant difference value (LSD) at 5% level of probability according to the method described by Gomez and Gomez [24]

2. Results and Discussion

Stem nodal segments were cultured on MS medium plus different concentrations of cytokinins in establishment and multiplication stage and auxin in rooting stage.

Establishment stage

Data in table (1) show significant effects of cytokinin types and concentration on treatment in all studied traits. The average number of shoot per explant ranged between 2.88 to 5.41 shoot per explant while, average length of shoot values was 0.95 to 2.35 cm. The maximum value was recorded with MS supplemented with 1mg/L BA compared with control MS (without addition of cytokinin). Increasing the concentration of BA led to a decrease in the number and length of axillary shoots. The same trend was noted with kin, Meanwhile increasing the concentration of Zeatin increased the values of studied parameters, as the highest mean number and length of axillary shoots per explant were recorded at the lowest concentration (1mg/L) and were decreased by increasing BA concentration.

This result is in harmony with Al-kowni et al., [28] who reported that the medium supplemented with 1mg/L Kin and 0.3 mg/L Gibberellic acid was optimum for shoot proliferation of *Thymus incanus*. Concerning the effect of type and concentrations cytokinin with WPM medium the best proliferation shoot recorded of *Thymus serpyllum* (Table 2) where significant differences were recorded among treatment. BA concentrations was significantly superior than Kin and Zeatin concentrations for average number of shoots / explants. Increase concentration BA decrease the number of shoots but, it was increased with concentration of Kin and Zeatin. The best value was 10.10 at WPM supplemented with 1mg/L BA. Concerning average of shoot length at concentration of BA and zeatin was increased, but decreased at kin concentration. The best value was 2.51 at WPM supplemented with 1mg/L Kin. In conclusion, for the in vitro establishment of *Thymus serpyllum*, MS medium supplemented with 1mg/L BA was the best medium for stem nodal segment explants. We noted that WPM fortified with BA and Kin shoot tip was necrosis but with Zeatin stem thickness. Same results were obtained by Khajuria et al., [29] who found that the maximum average number of shoots and average length was recorded when MS medium have 0.5 mg/L Kin combine with 2.0 mg/L BA.

Table(1) Effect of cytokinin type and concentrations with MS medium on establishment of thymus serpyllum stem nodal segment

Cytokinin type and Concentration (mg/l)			Average number of shoots/ explant	Average length of shoot (cm)
BA	Kin	Zeatin		
0.0	0.0	0.0	2.88[b]	1.36[b]
1.0	0.0	0.0	5.41[a]	2.35[a]
2.0	0.0	0.0	2.88[b]	1.15[b]
0.0	1.0	0.0	3.69[ab]	1.45[b]
0.0	2.0	0.0	3.25[ab]	1.45[b]
0.0	0.0	0.1	3.24[ab]	0.95[b]
0.0	0.0	0.2	3.75[ab]	0.96[b]

Table(2) Effect of cytokinin type and concentrations with WP medium on establishment of *Thymus serpyllum* stem nodal segment

Cytokinin type and Concentration (mg/l)			Average number of shoots/ explant	Average length of shoot (cm)
BA	Kin	Zeatin		
0.0	0.0	0.0	4.83[c]	2.04[a]
1.0	0.0	0.0	10.10[a]	1.49[a]
2.0	0.0	0.0	9.65[ab]	2.48[a]
0.0	1.0	0.0	5.65[c]	2.51[a]
0.0	2.0	0.0	6.54[bc]	2.30[a]
0.0	0.0	0.1	4.14[c]	1.72[a]
0.0	0.0	0.2	4.97[c]	2.29[a]

These results are in context with Ozudogru et al., [30] in *Thymus vulgari* L. who found that optimum shoot proliferation regeneration rate with 8.6 shoots produced per explant was obtained when semi-solid MS medium was supplemented with 1 mg/L kin and 0.3 mg/L GA3.

Multiplication Stage

According to data in Table 3,and Fig. 2 A. results showed that highly significant deferent between the treatment in all parameter. The best treatment was MS supplemented with 1mg/L BA and Kin. It gave maximum value of average number of shoots per explant and length of shoot (13.25 and 22.18cm, respectively) also, Bakhtiar et al., [31] reported that the highest frequency of shoot multiplication was observed with 2.0 mg/ L BAP combine with 1.0 mg/L NAA in *Thymus persicus*. In addition, Ana-Maria and Ramona [32] found that the highest multiplication rate (5.3 shoots per explant) and the highest shoots length were obtained on the medium without phytohormones in *Thymus vulgar*.

Table(3) Effect of cytokinin concentrations with MS medium on Multiplication of *Thymus serpyllum*

Cytokinin type and Concentration (mg/l)			Average number of shoots/ explant	Average length of shoot (cm)
BA	Kin	Zeatin		
1.0	0.0	0.0	6.25[b]	10. 84[b]
1.0	1.0	0.0	13.25[a]	22.18[a]
1.0	0.0	0.2	12.0[a]	16.25[ab]

Root stage

Table(4) Effect of IBA and NAA concentrations with 1/2 MS medium on root growth of *Thymus serpyllum*

Auxine type and Concentration (mg/ l)		root formation (%)	Average number of roots/ plantlet	Average length of root (cm)	Average length of shoot (cm)
IBA	NAA				
0.0	0.0	0 b	0.0 b	0.00c	0.0 b
0.5	0.0	70 a	10.5a	0.86a	5.4 a
1.0	0.0	30 ab	3.0 b	0.50abc	2.9 a
2.0	0.0	50 a	3.8 b	0.75ab	4.7 a
0.0	0.5	70 a	5.2ab	0.63ab	4.4 a
0.0	1.0	60 a	2.4b	0.32abc	3.8 a
0.0	2.0	50 a	3.2 b	0.25bc	5.1 a

Data in Table 4 reflect the percentage of root formation as affected by treatments. Observations show that the addition of IBA and NAA to the culture media increased percentage of root formation compared with culture medium without IBA and NAA. The highest significant value was recorded with 0.5 mg/L IBA or NAA. Increasing IBA or NAA from 1.0 to 2.0 mg/L, decreased significantly the percentage of root forming (Fig. 2B).

Concerning average number of roots per plantlet and length of root they were decreased with increasing the concentration of auxin. The maximum values were achieved with half MS supplemented with 0.5 mg/L IBA (10.5 and 0.86cm respectively), compared with half MS without auxin. These results are in harmony of the finding of many research, where Ozudogru et al., [30]reported that rooting in *Thymus vulgaris* L. the shoots were easily obtained on semi-solid MS medium that was either hormone-free or supplemented with auxin. Also, Bakhtiar et al., [31] found that the maximum number of rootlets was induced on half-strength MS medium with 0.5 and 1.0 mg/L IBA in *Thymus persicus*. In addition, Alkowni et al., [28] on *Thymus incanus* found that developed shoots easily rooted on media contained 0.01mg/L 2,4 D. while, Ana-Maria and Ramona [32] found that culture on MS medium with mineral salts reduced by half, supplemented with 2 mg/L IBA has been shown to be most effective for in vitro rooting of shoots in *Thymus vulgaris*.

Acclimatization stage

Healthy rooted clusters were successfully transferred to a mixture of 1:1:1 v/v/v sand: peat: perlit for three weeks in a growth chamber, then gradually acclimatized in the greenhouse with a percentage of survival of about 40% after one month (Fig. 2c).

Characterization of silicon nanoparticles

UV-VIS spectroscopy

The UV-VIS spectrum of Sio_2NPs is illustrated in Figure (1). SiO_2-NPs have provided peaks of absorption between 824-979 nm. The UV-VIS spectrum of synthesised SiO_2-NPs gave absorbance peak at 883nm. The particle size ranged from 5 to 2000 nm [33].

Effect of SiO_2-NP_s on *Thymus serpyllum* growth

Results in table (5) inducted that there was significant effects among treatments on all studied traits after one month. average number of shoot per explant ranged between 2.33to 12.17 shoots. The highest value obtained at MS supplemented with 200 mg/L of SiO_2-NP_s. When using MS with 100mg/l SiO_2-NP_s gave the maximum value of average length of shoot, average number of roots/ plantlet and average length of root (2.12,7.5 and 4.06) respectively. These results were in harmony with Reezi et al., [34] who demonstrated that added silicon concentrations about (50–100 mg/ L) in the medium for roses (*Rosa* spp. L.) enhanced their vegetative growth. It was found that Si application improved growth and yield through improving plant water status, modification of ultrastructure of leaf or ganelles, activation of plant defense systems and mitigation of free radicles (35).

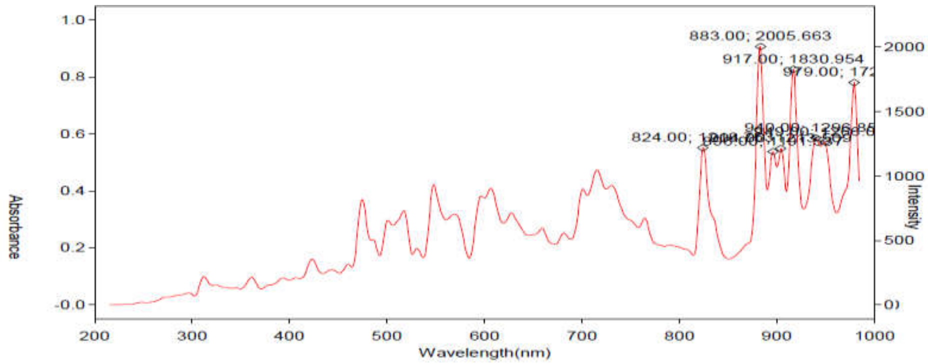

Figure (1) UV/Vis spectrum of the synthesized silicon nanoparticles

Table(5) Effect of SiO_2-NP_s concentrations with MS medium on multiplication and rooting of *Thymus serpyllum*

SiO2-NPs Concentration (mg/l)	Average number of shoots/ explant	Average length of shoot (cm)	Average number of roots / plantlet	Average length of root (cm)
0.0	2.33b	0.31b	1.5b	1.28 b
50	4.33 b	0.42b	4.3ab	3.25 ab
100	7.67 ab	2.12a	7.5a	4.06 a
200	12.17 a	1.68a	8.3a	3.59 a

In the same Studies of Yuvakkumar et al., [36] showed that nanosilicon increased growth factors in maize crop. However, Avestan et al., [37]. reported that added 100 mg/L silicon oxide on MS medium improved the growth of apple rootstock explants.

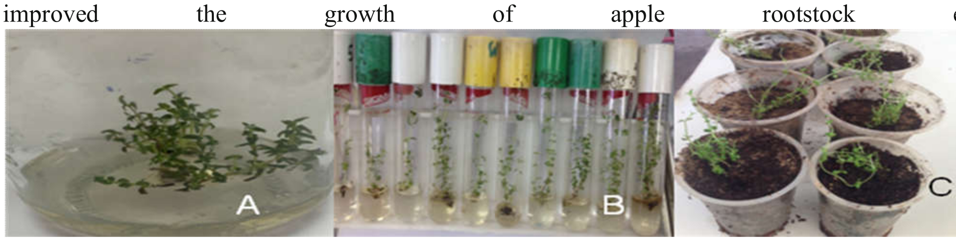

Figure (2). *In vitro* propagation of *Thymus serpyllum*; (a) multiplication of shoots (b) rooted plantlet, and (c) acclimatization of transplants in the greenhouse

Conclusion

Silicon nanoparticles could enhance plant growth and development by increasing the photosynthesis and nutrient absorption rate of plants in adverse environments. Silicon nanoparticles could form silica precipitates on the surface of plants and increase the mechanical force of plants that build the first protective barrier for plants against environmental stressors. precipitates on the surface of plants and increase the mechanical force of plants that build the first protective barrier for plants against environmental stressors.

Funding:: "This research received no external funding"

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