Cyanobacteria Increase the Activity of Enzymes Involved in the Lignin Biosynthesis Pathway in *Thymus vulgaris* L.

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Abstract

The effects of plant inoculation with plant growth-promoting cyanobacteria on the activity of phenylalanine ammonia-lyase (PAL), peroxidase (POD), and cell wall POD enzymes in *Thymus vulgaris* L. were investigated. A greenhouse experiment with five replications was carried out using inoculation of five cyanobacteria suspensions (2.5%) including *Anabaena torulosa* ISB213, *Nostoc calcicola* ISB215, *Nostoc ellipsosporum* ISB217, *Trichormus doliolum* ISB214 and *Oscillatoria* sp. ISB2116 on T. vulgaris plants. After four months, the plants were harvested, and the enzyme levels were measured. The statistical analysis revealed that the treatment with N. *ellipsosporum* ISB217 had the most significant impact on PAL enzyme activity in the treated plants. Compared to the control group (0.13 ± 0.002), the plants treated with T. *doliolum* ISB214 exhibited the highest activity of POD (0.69 ± 0.03) enzyme and cell wall POD (0.53 ± 0.05). Also, the amount of lignin in the treated plants had a significant increase compared to the control. Considering the valuable medicinal properties of the secondary metabolites found in thyme plants and the crucial role of nutritional management in enhancing the production and quality of this medicinal plant. The usage of appropriate growth stimulants has the potential to enhance the biosynthesis of these compounds. This study demonstrated that application of microalgae extract as a completely natural and eco-friendly substance by increasing the enzymes activity related to the synthesis of cell wall materials can boost the resistance of plants against biotic and abiotic stresses.

Keywords: *Thymus vulgaris* L., PAL, POD, Cyanobacteria, *Nostoc*

Introduction

Thymus vulgaris L. is a perennial plant belonging to the Lamiaceae family that has a worldwide distribution. This plant possesses numerous medicinal properties attributed to

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its bioactive compounds. These bioactive compounds contributed to the plant's antimicrobial, antifungal, and antibiotic effects among others. Phenols and flavonoids are valuable secondary metabolites in plants that play a crucial role in plant defense against herbivory and pathogens. These metabolites are synthesized from the phenylpropanoid pathway and phenylalanine in plants (Zaynab et al., 2018). Phenylalanine ammonia-lyase (PAL) serves as a pivotal enzyme in the phenylpropanoid pathway, which is responsible for the synthesis of various plant secondary metabolites. PAL plays a crucial role not only in the production of these metabolites but also in plant development and defense against environmental stresses. By catalyzing the conversion of phenylalanine to cinnamic acid, PAL initiates the phenylpropanoid pathway, which leads to the production of a diverse array of secondary metabolites, including flavonoids, lignin, phytoalexins, and many other compounds. These secondary metabolites contribute to multiple aspects of plant development, such as pigmentation, structural integrity, and defense against pathogens and pests.

Moreover, PAL is involved in the plant's response to environmental stresses. When plants encounter biotic or abiotic stressors, PAL activity can be induced, leading to increased production of secondary metabolites with protective functions. These metabolites can act as antioxidants, antimicrobial agents, or signaling molecules, helping plants withstand adverse conditions and improve their resilience.

Therefore, PAL not only influences the biosynthesis of various secondary metabolites but also plays a crucial role in plant development and adaptation to environmental stresses. (Feduraev et al. 2020). The major products of this pathway include lignin polymers, flavonoids (anthocyanin pigments), isoflavonoids, and phytoalexins (Koch and Smith, 2013). Different types of peroxidase enzymes (PODs) have a functional group called free protoporphyrin IX and oxidize various substrates in the presence of hydrogen peroxide. The number of isoforms of these enzymes in plants can be extremely high, with approximately forty peroxidase isoform genes present in each plant (Mika and Lütjohann, 2003). Group III peroxidases, which were among the first peroxidases to be discovered, are secreted outside the cell or stored in vacuoles. These PODs have various metabolic roles in plants, such as auxin metabolism, cell wall compound interactions, synthesis of phytoalexins, metabolism of reactive oxygen species, and lignin and suberin formation (Kawakawa et al., 2003). Also, these enzymes are involved in scavenging hydrogen peroxide, oxidizing phenolic and non-phenolic compounds, lignin synthesis, and defense against pathogens (Pandey et al., 2017). Lignin is the third most abundant natural polymer in plants, following cellulose and hemicellulose, and provides favorable conditions for coping with stress. The main pathway for the production of monolignols (lignin) is the phenylpropanoid pathway, which starts with the amino acid

phenylalanine and is further converted into monolignols. The monolignols synthesized are transformed into lignin by cell wall-bound peroxidase enzymes (Barros and Dixon, 2020). Soil microorganisms such as cyanobacteria are one of the key factors in the biogeochemical cycle of organic and mineral nutrients and improve soil quality and plant access to nutrients and can be affected by several factors (Gao et al., 2020). Cyanobacteria can affect the physicochemical and biological properties of the soil and improve the growth and fertility of plants by having the ability to fix nitrogen and produce growth stimulating metabolites (Shariatmadari et al., 2022). Through an array of physiological, biochemical, and molecular mechanisms, cyanobacteria improve plant growth and development. Furthermore, cyanobacteria have demonstrated potential in mitigating both biotic and abiotic stress in plants (GR et al., 2021). Ismail et al. (2022) conducted a study on *Zea mays* L. plants exposed to cadmium stress and found that inoculation with cyanobacterial biofertilizer increased the activity of the PAL enzyme and various peroxidase isozymes. Similarly, Prasanna et al. (2012) observed increased levels of peroxidase and PAL enzymes in maize plant hybrids treated with different cyanobacteria. These findings highlight the positive effects of utilizing biofertilizers in alleviating biotic and abiotic stresses by promoting lignification, which acts as a defensive barrier in plants. Building upon this knowledge, the present study aims to investigate the impact of cyanobacterial treatments on PAL and peroxidase enzymes, as well as lignification levels, in thyme plants.

Materials and methods

Isolation and identification of cyanobacteria

soil samples were collected from five distinct stations located in the northern regions of Iran specifically, Mazandaran, Golestan, and West Azarbaijan Provinces following the methodology described by Rangaswamy (1966) and soil culturing techniques. The isolated cyanobacterial colonies were subsequently inoculated onto sterile plates containing BG-11 and BG-11 nitrate-free culture media for purification (Stanier et al., 1971). Taxonomic study and morphometric identification of isolated species were performed after two weeks of incubation at 25 ± 2 °C under a light/dark cycle of 12/12 h with artificial light of 74 mol photons m-2s-1), using light microscopy and it was done according to the method of Komark and Hauer (2013).

Preparation of cyanobacterial suspension

Cyanobacterial suspensions (0.2%) of five isolated and purified species including *Anabaena torulosa* ISB213, *Nostoc calcicola* ISB215, *Nostoc ellipsosporum* ISB217, *Trichormus doliolum* ISB214, and *Oscillatoria* sp. ISB2116 were prepared by homogenizing 0.2 g of purified samples after four weeks of cultivation in 1000 ml of distilled water.

Pot experiment

Seedlings of *T. vulgaris* L. were obtained from the Iran Institute of Medicinal Plants (ACECR) and the voucher specimens code

is SANRU-H1104. After identification the seedlings were deposited in the herbarium of Sari Agricultural Sciences and Natural Resources University. Five seedlings for each treatment were grown in pots, which had five-liter capacity and 20 cm diameter with 31% silt, 40% sand, and 29% clay. The pots were arranged completely randomized with five replicates in standard greenhouse conditions with a photoperiod of 10/14 h, temperature of 25.0 ± 2 °C and relative humidity of 55 ± 5%, and one week before planting and then every two weeks, the pots were inoculated with 100 ml of 0.2% cyanobacterial suspension. Control pots were only watered (100 ml each time).

Enzymes extraction and activity assay

For extraction of PAL, 2 ml of sodium phosphate buffer (200 mM) at pH = 6.2 containing 5% (w/v) PVPP and 2% (v/v) Triton X-100 was added to 0.1 g of leaf tissue and centrifuged for 20 min (15,000 g at the 4 °C). Next, 800 µl of the enzyme extract, 600 µl of Tris buffer (50 µM, pH = 8.8) and 900 µl of phenylalanine (2 µM) were added and the reaction mixture was allowed to proceed for 30 min. Then, by adding 100 µl of HCl (2N), the production of cinnamic acid from phenylalanine was stopped. The final mixture was centrifuged (at 6000 g) for 5 minutes by adding 2 ml of toluene and vortexing (for 30 seconds). Finally, enzyme activity was estimated based on the cinnamic acid standard, assuming an extinction coefficient of 20,000 M^{-1} cm^{-1}, by recording the absorption increase at 290 nm in the toluene phase (Whetten and Sederoff, 1992).

In the next step, 0.1 g of plant leaf was homogenized in a cold mortar with 2 ml of 0.1 M phosphate buffer (pH 6.8). The homogenate was then centrifuged at 13,000 g for 15 minutes (4 °C). The supernatant was used for measuring the activity of soluble POD, while the residue from extraction, after being washed four times with distilled water and adding 2 ml of NaCl (1 M), was used to assay the activity of cell wall peroxidase. For the activity of both POD enzymes, 25 mM phosphate buffer (pH 6.8), 20 mM guaiacol, and 40 mM hydrogen peroxide were added to 10 µl of the enzyme extract (the final volume of the reaction mixture was 3 ml). The estimation of enzyme activity was calculated based on the amount of tetragaiacol produced in the presence of electrons resulting from the decomposition of H_2O_2 by the enzyme in one minute in the kinetic mode of the Shimadzu spectrophotometer (UV-1600). To estimate the activity of the enzyme, the extinction coefficient of tetraguaiacol at 470 nm (26.6 L $mmol^{-1}$ cm^{-1}) was used (Kar and Mishra, 1976).

Lignin measurement

Lignin assay was carried out using floroglucinol as a reagent and one g of plant leaf was washed with 50% methanol then combined with 5 ml of HCl in ethanol (1:1 ratio) and heated in a boiling water bath for 3 h. Next, one ml of the resulting extract was mixed with 100 µl of floroglucinol dissolved in HCl (1M) and kept in darkness for 4 h and then read at 488 nm (Barzegargolchini et al., 2017).

Statistical analysis

SAS Statistical Package (2009) was used for statistical analysis of data. One-way ANOVA statistical analysis was applied to analyze all data and Duncan's multiple range test was used for means comparison. Data analysis charts were drawn using Excel 2016 software.

Results

The results of this study demonstrated that thyme plants inoculated with cyanobacterial suspensions (0.2%) showed an increase in enzyme activity compared to control plants significantly. Assessment of PAL activity in thyme leaves showed that the treatment of *N. ellipsospurom* ISB217 with a 12.5% increase had the greatest effect in treated plants compared to the control. Except for *A. torulosa* ISB213, the rest of the treatments increased PAL enzyme activity (Figure 1).

Based on the results of the effect of cyanobacterial treatments on POD activity, except *Oscillatoria* sp. ISB2116, all treatments had a positive effect. *T. doliolum* ISB214 showed a significant increase in the activity of this enzyme in the treated plants so the activity of this enzyme increased from 0.13 ± 0.002 in the control plants to 0.69 ± 0.03 (multifold increase) in the treated plants. Following that, the treatment of *A. torulosa* ISB213 increased the activity of this enzyme by 69.23% in the treated plants compared to the control (Figure 2).

Similarly, the activity of cell wall POD also had a considerable increase with the treatments of *T. doliolum* ISB214 (from 0.09+0.09 in control plants to 0.54 + 0.09 in treatments) and *A. torulosa* ISB213

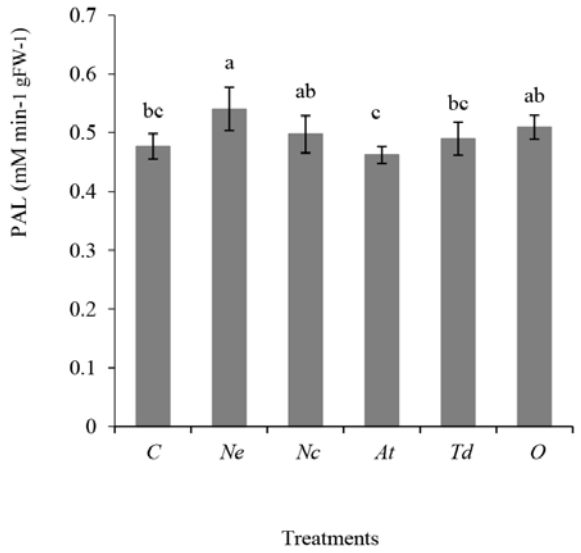

Fig. 1. PAL enzyme activity in thyme plants with 0.2% cyanobacterial suspensions inoculation. C: Control, Ne: *N. ellipsosporum* ISB217, Nc: *N. calcicola* ISB215, At: *A. torulosa* ISB213, Td: *T. doliolum* ISB214, O: *Oscillatoria* sp. ISB216. Bars on each column represent the SE. The same letters are not significantly different at $P < 0.05$

(30.77%) (Figure 3).

The results also showed that the application of cyanobacteria significantly improved the lignin content in the leaves of the treated plants compared to the control. The highest amount of lignin was observed in the treatment of *Oscillatoria* sp. ISB216 and *N. ellipsosporum* ISB217, increased

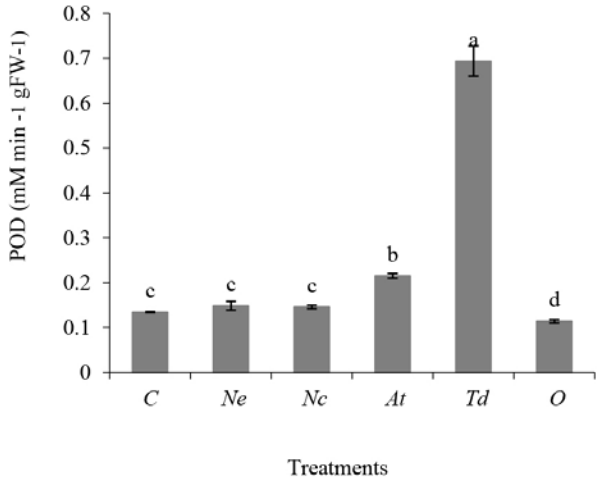

Fig. 2. POD enzyme activity in thyme plants with 0.2% cyanobacterial suspensions inoculation. C: Control, Ne: *N. ellipsosporum* ISB217, Nc: *N. calcicola* ISB215, At: *A. torulosa* ISB213, Td: *T. doliolum* ISB214, O: *Oscillatoria* sp. ISB216. Bars on each column represent the SE. The same letters are not significantly different at P< 0.05

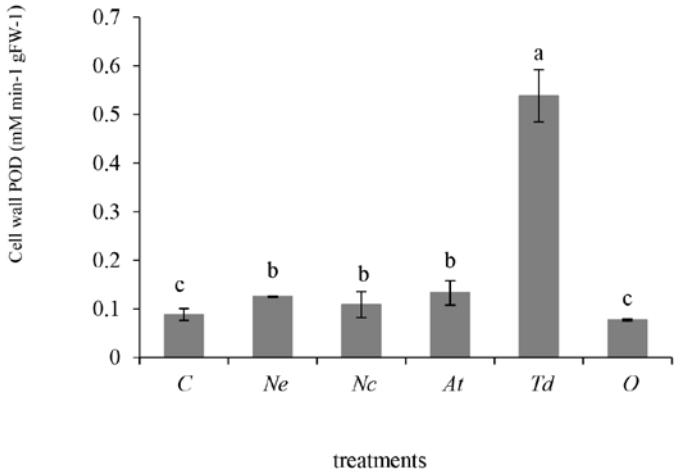

Fig. 3. Cell wall POD enzyme activity in thyme plants with 0.2% cyanobacterial suspensions inoculation. C: Control, Ne: *N. ellipsosporum* ISB217, Nc: *N. calcicola* ISB215, At: *A. torulosa* ISB213, Td: *T. doliolum* ISB214, O: *Oscillatoria* sp. ISB216. Bars on each column represent the SE. The same letters are not significantly different at P < 0.05

Table 1. Percentage changes of enzymes activity and lignin content with cyanobacteria treatment in thyme plant

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	Control	*N. ellipsosporum* ISB217	*N. calcicola* ISB215	*A. torulosa* ISB213	*T. doliolum* ISB214	*Oscillatoria* sp. ISB216
PAL	0.48 ± 0.04	12.5% +	4.17% +	4.17% -	4.17% +	6.25% -
POD	0.13 ± 0.002	15.38% +	15.38 +	69.23% +	430.77% +	15.38% +
Cell wall POD	0.09 ± 0.04	50% +	37.5% +	62.5% +	575% +	0%
Lignin	0.15 ± 0.03	86.67% +	20% +	46.67% +	46.67% +	93% +

+Increase; -Decrease

the content of lignin by 93.33% and 86.67%, respectively. The comparison of percentage changes in lignin content and studied enzymes due to the application of cyanobacteria extract in thyme plant is presented in the Table 1.

Discussion

This study investigated the potential of five cyanobacterial strains as biofertilizers in *T. vulgaris* L. The treatment of this medicinal plant with *T. doliolum* ISB214 and *N. ellipsospurom* ISB217 showed the highest increase in the activity of the investigated enzymes in the leaves of the treated plants. Previous studies showed that cyanobacteria can enhance nutrient uptake, promote growth, and improve crop adaptability under both normal and stress-like salinity conditions (Mutale-joan et al., 2021). Also, the extracts of cyanobacterial strains on several plant species, such as rice, wheat, maize, cotton, etc., have demonstrated the synthesis of signaling metabolites related to tolerance to stress conditions (Singh, 2014). The use of *Nostoc* sp. and *Anabaena* sp. as biofertilizers reduced the oxidative stress and toxicity caused by cadmium in *Zea mays* L. plants under stress and significantly increased the activity of PAL enzyme and peroxidase isoenzymes. The effect of cyanobacteria may be due to the significant increase of enzymatic and non-enzymatic antioxidants in plants under stress (Ismail et al., 2021). Similarly, Prasanna et al. (2012) used the inoculation of three strains of rhizobacteria and three strains of cyanobacteria including two strains of *Anabaena* sp. and *Calothrix* sp. to improve rice plant performance, which increased the activity of PAL enzyme and antioxidant enzymes. In the report presented by Samadi et al. (2015), methyl jasmonate and salicylic acid treatments increased the amount of PAL enzyme and total phenolic and flavonoid content. The study also demonstrated that the amount of phenol was strongly influenced by PAL enzyme activity and biostimulant concentration. Mehrabanjoubani et al. (2019) reported that rice plants fed with extra Fe caused a significant increase in cell wall POD and PAL activity along with an increase in lignin

accumulation in the roots of treated plants. Therefore, by using biocatalysts in optimal concentrations, secondary compounds can be obtained in appropriate amounts.

The results from this study demonstrated that the inoculation of cyanobacteria in *T. vulgaris* L. as a medicinal plant leads to an increase in the activity of enzymes associated with the lignification and interestingly, stimulation of these enzymes results in raised level of lignin content. Consequently, these biofertilizers can be utilized to enhance plant resistance against both biotic and abiotic stresses.

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