



The influence of nanosized zero-valent iron (nZVI) on the micropropagation, antioxidant activity, and phenolic compound content of cherry laurel (*Prunus laurocerasus* L.)

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Abstract

Zero-valent iron nanoparticles (nZVIs) are reducing agents commonly used to purify pollutants in groundwater. However, recent reports have indicated that it also promotes plant growth and development. This study therefore investigated the effects of varying concentrations (12.5, 25, 50, 75, and 100 mg L⁻¹) of nZVI on the micropropagation, total phenolic and flavonoid contents, and antioxidant activity of cherry laurel plants in in vitro tissue culture. The removal of the testa of the seed and the half-cut of the cotyledon were essential for successful germination. Following germination, the optimum 6-benzyladenine (BA) concentration for micropropagation was determined to be 1 mg L⁻¹. Although the highest percentage of shoot tip proliferation (85.15%) was obtained with the inclusion of 75 mg L⁻¹ nZVI in the culture medium, the maximum SFC index value (1.66), together with the highest contents of phenolics and flavonoids were obtained by adding 25 mg L⁻¹ nZVI. This concentration also provided the best values for antioxidant activity. In addition, the maximum amount of chlorogenic acid (39.58 mg analyte g⁻¹ extract), the main phenolic compound in cherry laurel, was also recorded with this concentration. The results revealed that the elevation of the concentration of nZVI in the culture medium led to a reduction in the positive effects on the growth, antioxidant activity and phenolic content of the plants. The optimum nZVI concentration was therefore determined to be 25 mg L⁻¹. The present study has shown that nZVI has the potential to provide encouraging and alternative benefits in in vitro plant tissue culture.

Key message

This is the first in vitro plant study on the impact of nZVI in which optimum concentration of nZVI improved the proliferation rates and phenolic compound production in cherry laurel.

Keywords Cherry laurel · nZVI · Micropropagation · Phenolic compounds

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Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
BA	6-benzyladenine
DMSO	Dimethyl sulfoxide
EDTA-Fe	Ethylenediaminetetraacetate-iron
GA ₃	Gibberellic acid
GAE	Gallic acid equivalent
IBA	Indole-3-butyric acid
LSD	Least significant difference
NPs	Nanoparticles
nZVI	Nanosized zero-valent iron
PVP	Poly-N-vinylpyrrolidone
QE	Quercetin equivalent
ROS	Reactive oxygen species
SFC	Shoot forming capacity
TE	Trolox equivalent
TFC	Total flavonoid content
TPC	Total phenolic content
UAE	Ultrasonic-assisted extraction

Introduction

Cherry laurel (*Laurocerasus officinalis* Roem., syn: *Prunus laurocerasus* L.) is an evergreen tree species that can grow up to 5–15 m high. It has small white blooms and red/purple fruits and grows in damp, shaded habitats. It is found naturally on the Eastern Marmara and Black Sea coasts in Türkiye and used for human consumption, especially in the Eastern Black Sea region. As cherry laurel plants are rich in bioactive substances, the leaves, seeds and fruits of cherry laurel are utilized in conventional medicine to treat a number of diseases, such as stomach ulcers, bronchitis, indigestion, eczema and hemorrhoids (Kolayli et al. 2003). In addition to the antidiabetic effects of seeds (Şenaylı et al. 2012), fresh leaves are used to treat asthma, relieve pain and fever, and relieve cough and dyspepsia (Karataş and Uçar 2018; Küpeli-Akkol et al. 2012). The oil from seeds is also used in the cosmetics industry as well as for therapeutic purposes (Yazıcı et al. 2011).

As natural antioxidants, phenolic compounds play important roles in plant defense mechanisms and constitute a major part of the secondary metabolites in plants. They protect plants from oxidative stress by eradicating reactive oxygen species (ROS) that are produced as a result of biotic and abiotic stress and cause molecular damage (Kolayli et al. 2003). In addition, phenolics are crucial for human health because of their anti-inflammatory and anti-proliferative properties. The antioxidant activity of these compounds in plants, can be determined by subjecting them to a variety of oxidising agents that induce oxidative stress. A common

approach involves the spectrophotometric determination of the reduction reaction of the oxidant by the antioxidant. Among these methods are the DPPH assay, which utilizes the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as the oxidant; the ABTS⁺ assay, employing the 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) radical; the ferric reducing antioxidant power (FRAP) assay, which uses Fe³⁺ as the oxidant; and the cupric reducing antioxidant capacity (CUPRAC) assay, employing Cu²⁺ as the oxidant (Chaves et al. 2020). Cherry laurel is also a valuable resource with its high antioxidant capacity, which is due to the phenolic compounds contained in its leaves, fruits and seeds (Çebi 2018; Yaylaci-Karahalil and Şahin 2011; Kolayli et al. 2003). However, obtaining cherry laurel seedlings via conventional methods is challenging due to the low germination rates of the seeds and the long propagation cycle, making in vitro methods alternative and faster methods. In 2012, Sulusoglu developed an embryo culture protocol for cherry laurel, and in 2013, Sulusoglu and Cavusoglu conducted an in vitro micropropagation study using shoot tip explants. In addition, in vitro propagation of cherry laurel using different explants has also been investigated (Alasania et al. 2021; Georgiana and Adriana 2016). Nevertheless, no study has examined the effectiveness of nanoparticles (NPs) in increasing micropropagation or plant growth in cherry laurel plants.

The use of iron NPs in plant micropropagation has been explored in recent years, with studies showing that they have the potential to improve various stages of the process. For example, iron NPs have been shown to improve nutrient uptake efficiency, diminish the detrimental effects of environmental stresses, and increase overall plant productivity. The beneficial effects of NPs in various applications, including sterilization of explants and removal of contaminants, enhancement of the synthesis of bioactive compounds as elicitors, cryopreservation, genetic transformation, somatic embryogenesis, somaclonal variation, callus induction, and organogenesis, have been demonstrated in previous studies on plant tissue culture, as summarized by Singh et al. (2023). For instance, in vitro application of Fe₃O₄ NPs to flax explants resulted in increased callus size, embryo development and root formation (Kokina et al. 2017). Similarly, the inclusion of iron-doped zinc oxide NPs (Fe-ZnO-NPs) in *F. indica* callus cultures resulted in an increase in biomass and the activation of secondary metabolite production (Khan et al. 2021). Moreover, Fe NPs supplementation in the culture medium of date palm cv. Bartamoda resulted in significant enhancement of the plant's growth parameters (Arafa et al. 2023). In addition, nutrient media supplemented with iron oxide and silver nitrate NPs increased the formation of multiple stems in strawberry plants (Abbas and Abdulhussein 2021). Moreover, iron-based NPs have been found to be

effective elicitors in cell suspension cultures and hairy root cultures. For example, the addition of zinc and iron oxide NPs to *H. perforatum* cell suspension cultures elevated the synthesis of hypericin and hyperforin (Sharafi et al. 2013). Besides, treatment with magnetic Fe₃O₄ NPs enhanced the production of flavonoids, phenolics, anthocyanins, and lignin in *D. kotschy* cell suspension cultures (Taghizadeh et al. 2021). Similarly, the inclusion of iron and zinc oxide NPs in chicory hairy root cultures increased secondary metabolite production (Mohebdini et al. 2017), also rised the production of hyoscyamine and scopolamine in *Hyoscyamus reticulatus* (Moharrami et al. 2017) and of xanthomicrol, cirsimaritin and isokaempferide in *D. kotschy* (Nourozi et al. 2019). Recently, incorporation of 5.6 mg L⁻¹ of iron NPs to the culture medium promoted in vitro rhizome proliferation the hairy roots of *P. vietnamensis* (Huong et al. 2024).

Zero-valent iron NPs (nZVIs), are commonly used to remediate the environment and can used to stimulate plant growth and seed germination as nanofertilizers. The advantage of nZVI, which is the fully reduced form of iron, relies on its higher chemical reactivity, as compared to other iron NPs with different oxidation states, such as Fe₂O₃ (Fe³⁺) and Fe₃O₄ (Fe²⁺/Fe³⁺). In the case of nanofertilizer, the effects of various concentrations of nZVI (0–1000 mg L⁻¹) on the growth of hybrid poplars (*Populus deltoids* × *Populus nigra*) and cattail (*Typha latifolia*) seedlings cultivated in hydroponic media were analyzed. At high concentrations (>200 mg L⁻¹), nZVI appeared to have toxic effects, whereas at low concentrations, it appeared to promote plant growth (Ma et al. 2013). In a separate study, the effects of nZVI on the growth of *Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum* were investigated. It was found to have no significant phytotoxic effect and even increased plant height and biomass (Libralato et al. 2016). Similarly, the application of nZVI to *Arabidopsis thaliana* resulted in a 38% increase in plant biomass compared with that of the control and increased iron content in root and leaf tissue (Yoon et al. 2019). nZVI also has a positive effect on root development and plant growth by increasing water uptake at low concentrations (10–320 µmol L⁻¹) and was found to be more effective than ethylenediaminetetraacetate-iron (EDTA-Fe) solution in peanuts (Li et al. 2015). Furthermore, priming rice seeds with 20 mg L⁻¹ nZVI under greenhouse conditions resulted in the highest activity of root dehydrogenase and antioxidant enzymes. In addition, root and shoot length, biomass and photosynthetic pigment content also increased. However, the application of 160 mg L⁻¹ led to oxidative stress in the seedlings (Guha et al. 2018). In another study, compared with control plants, rice seedlings primed with nZVI and grown under field conditions presented broader leaves, more tillers, and greater biomass, and nZVI was not genotoxic when analyzed via RAPD analysis (Guha et al.

2021). Overall, these studies suggest that nZVI promotes plant growth and development at optimum concentrations, which vary by plant species but generally have negative effects at relatively high concentrations.

Numerous studies have investigated the effects of NPs on in vitro cultured plant explants. However, no in vitro experiments with nZVI on plants are known. The aim of this study was to investigate not only the impact of nZVI at different concentrations on the in vitro proliferation of cherry laurel, but also its effect on antioxidant enzyme activities and the production of phenolic compounds. The results of this study could provide insight into the interaction of nZVI with plants in tissue culture, which could increase its potential applications.

Materials and methods

Chemicals

DMSO: Alfa-Aesar, Cas: 67-68-5, HPLC grade, EtOH: Isolab, Cas: 64-17-5, NaOCl: Sigma-Aldrich, Cat: 42,504-4, Tween 20: Merck, Cas: 9005-64-5, MS: Caisson, Ref: MSP09-50 LT, BA: Sigma, B-3408, IBA: Sigma, I-5386, GA₃: Sigma, G-7645, Sucrose: Caisson, Ref: SO11-1KG, Agar: Duchefa, Cas: 9002-18-0, Methanol: Honeywell, CAS: 67-56-1, LC grade, Gallic acid: Sigma, Cas: 149-91-7, Folin–Ciocalteu reagent: Sigma, Cas: 521-24-4, Na₂CO₃: Merck, Cas: 497-19-8, Quercetin: Sigma 95%, Cas: 117-39-5, NaNO₂: Merck, Cas: 7632-00-0, AlCl₃: Sigma, Cas: 7446-70-0, NaOH: Merck, Cas: 1310-73-2, DPPH: Sigma, Cas:1898-66-4, ABTS: Sigma, Cas:28752-68-3, Neocuproine reagent: Sigma, Cas:484-11-7, Trolox 97%: Sigma, Cas: 53188-07-1, Copper (II) chloride: Sigma, Cas: 7447-39-4, Ammonium acetate: Sigma, Cas: 631-61-8, Glacial acetic acid: Cas: 64-19-7, Sodium acetate trihydrate: Cas: 6131-90-4, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ): Sigma, Cas: 3682-35-7, Hydrochloric acid 37%: Cas: 7647-01-0, FeCl₃.6H₂O: Sigma, Cas: 10025-77-1.

nZVI preparation

The nZVI NPs were synthesized by chemical reduction procedures that utilized FeSO₄ and FeCl₃. Low-molecular-weight poly-N-vinylpyrrolidone (PVP) was used as a stabilizer (Sidorenko et al. 2021). A 50 mg mL⁻¹ stock solution of Fe⁰ NPs was prepared. The dry Fe⁰ NPs were weighed and then dissolved in dimethyl sulfoxide (DMSO) as a solvent, which was subsequently subjected to ultrasonic water bath (Wisd WUC-D06H, Germany; frequency: 50 kHz) treatment at 27 °C for 30 min. The stock solution was kept in the dark at room temperature (25 °C) until use. Due to the

gradual decrease in the homogeneity of the prepared solution over time during storage, the solution was made homogeneous by ultrasonic treatment in a water bath for 10 min prior to each use.

Seed sterilization, germination and micropropagation

The mature cherry laurel fruits used in this study were collected in July 2020 in Kocaeli, Turkey (location: 40° 80'62.7" N, 29°50'53.9" E). For surface sterilization, the exocarp and mesocarp parts of the fruits were first cleaned, and the obtained endocarps were subsequently washed in tap water. The endocarps cracked, and the embryos were extracted. The embryos were initially shaken in 70% v/v EtOH for 2 min, shaken in 20% v/v sodium hypochlorite (NaOCl) (with a few drops of Tween 20 for 25 min, and then rinsed three times with sterilized distilled water for 10 min. After surface sterilization, the testa was removed from the embryo, and half of the cotyledon was carefully cut. These embryos were cultured under aseptic conditions via transfer to MS media (Murashige and Skoog 1962) containing 2 mg L⁻¹ BA, 0.5 mg L⁻¹ indole-3-butyric acid (IBA), 0.2 mg L⁻¹ gibberellic acid (GA₃), 30 g L⁻¹ sucrose and 7 g L⁻¹ agar (Sulusoglu 2012). The cultures were maintained in a growth room at 25 ± 2 °C with a 16-hour photoperiod using cool white fluorescent lamps (PHILIPS, Made in Poland) (36 µmol m⁻² s⁻¹).

To determine the optimum BA concentration for effective *in vitro* micropropagation, shoot tips were removed from *in vitro*-germinated cherry laurel plants after 4 weeks of germination and cultured on MS media with different BA concentrations (1, 2 and 4 mg L⁻¹). After 4 weeks, the most effective BA concentration was determined for proliferation and micropropagation was carried out at this concentration. The propagated microshoots were routinely subcultured to fresh propagation medium at 4-week intervals.

Determination of the effect of nZVI on the proliferation of cherry laurel shoot tips under *in vitro* conditions

All shoot tips utilized in the study were derived from cherry laurel plants that were germinated and propagated *in vitro* as indicated above. *In vitro* shoot tip proliferation studies were conducted by placing a minimum of 10 uniformly excised shoot tips (approximately 3 mm in length) on MS media supplemented with varying concentrations of nZVI (12.5, 25, 50, 75, and 100 mg L⁻¹) and 1 mg L⁻¹ BA in each Petri dish (90 × 15 mm). After 4 weeks of *in vitro* culture, microshoot proliferation (%), average number of microshoots/explant, shoot-forming capacity (SFC; Lambardi et

al. 1993) were determined and average microshoot length was measured. The measured microshoots were then dried in the dark at 30 °C for two days for extraction.

Determination of total phenolic and flavonoid contents, antioxidant enzyme activities and quantitative analysis of phenolic compounds via Ultra high-performance liquid chromatography-orbitrap®-high resolution mass spectrometry (UHPLC-Orbitrap®-HRMS/MS)

Extraction

The microshoots, dried under dark and 30°C conditions, were ground into powder. Cherry laurel powder was extracted at a ratio of 1 g dried plant (approximately 7.5–10 g of fresh plant material that was obtained from 140 to 180 microshoots with 2–6 leaves, 4.5–5.0 mm in length, that were proliferated from shoot tips in control or different concentrations of nZVI containing MS medium with 1 mg L⁻¹ after 4 weeks) per 20 mL⁻¹ methanol using an ultrasonic water bath (frequency: 50 kHz, temperature: 65 °C (± 1 °C) for 15 min) (Wisd WUC-D06H, Germany). The extract obtained via ultrasonic-assisted extraction (UAE) was first passed through a coarse filter and then through a fine filter. Subsequently, it was transferred to a pre-weighed evaporator flask and dried in a vacuum evaporator until the solvent was completely evaporated (BUCHI, Rotavapor® R-300). The weight of the dried extract was calculated by subtracting the tare weight of the evaporator flask from the total weight. The dried extract obtained was re-dissolved in methanol to prepare stock solutions at known concentrations and stored at -20 °C until analysis.

Determination of total phenolic and flavonoid contents in the extracts

The total phenolic content (TPC) of the extracts was assessed via the Folin–Ciocalteu assay (Slinkard and Singleton 1977) with slight modifications. The plant extract solutions were diluted with methanol a concentration of 1 mg mL⁻¹ for analysis. A calibration curve was prepared for gallic acid with standard solutions. In test tubes, 50 µl of the extracts or gallic acid dilutions were mixed with 250 µl of 0.2 N Folin–Ciocalteu reagent. After a three-minute incubation at room temperature, 750 µl of a 7.5% Na₂CO₃ solution was added, followed by vortexing and shaking. The tubes were then kept at room temperature for two hours. The absorbance was measured at 765 nm via a Thermo Scientific™ GENESYS™ 140/150 Vis/UV–Vis spectrophotometer. The total phenolic content was calculated via the calibration curve equation.

$$\text{Absorbance} = 0.0531 \text{ gallic acid } (\mu\text{g}) - 0.0393 \text{ (} R^2 = 0.9986 \text{)}$$

The total flavonoid content (TFC) was determined via a modified aluminum chloride assay described by Barros et al. (2007). A calibration curve was prepared with standard solutions of quercetin. In test tubes, 100 μL of the extracts or quercetin dilutions were mixed with 400 μL of distilled water, followed by the addition of 30 μL of 5% NaNO_2 , 30 μL of 10% AlCl_3 , and 200 μL of 1 M NaOH . The mixture was then brought up to 240 μL with distilled water, vortexed, and allowed to stand at room temperature for 40 min. The absorbances of the reaction mixtures were measured at 515 nm via a Thermo Scientific™ GENESYS™ 140/150 Vis/UV–Vis spectrophotometer. The total flavonoid content was calculated via the calibration curve equation.

$$\text{Absorbance} = 0.0066 \text{ Quercetin } (\mu\text{g}) - 0.0021 \text{ (} R^2 = 0.9964 \text{)}$$

The differences in absorbance between the extract samples and distilled water (blank) were calculated. Each sample was analyzed in triplicate, and the average absorbance was determined. The total phenolic and flavonoid contents of the extracts were quantified as gallic acid and quercetin equivalents (mg g^{-1} extract $\pm$ standard deviation), respectively, via a calibration curve.

Determination of antioxidant activity in the extracts

The free radical scavenging activity of the extracts was evaluated via four different methods: the DPPH radical scavenging assay (Brand-Williams et al. 1995), the ABTS⁺ radical cation scavenging assay (Re et al. 1999), the CUPRAC assay (Apak et al. 2004), and FRAP assay (Benzie and Strain 1996). The antioxidant capacity was measured in Trolox equivalents. Trolox standard solutions of various concentrations were prepared for all the assays, and the plant extract solutions were diluted with methanol to a concentration of 1 mg mL^{-1} . Absorbance measurements were performed via a Thermo Scientific™ GENESYS™ 140/150 Vis/UV–Vis spectrophotometer. The absorbance values of the extracts were corrected by subtracting the values obtained from distilled water (blank). The samples were analyzed in triplicate, and the average absorbance was calculated. The total antioxidant activity of the extracts was expressed as Trolox equivalents (mg g^{-1} extract $\pm$ standard deviation), determined either directly or via a calibration curve.

UHPLC-orbitrap®-HRMS/MS analysis

UHPLC-Orbitrap®-HRMS/MS experiments were performed via an Exactive Plus Orbitrap mass spectrometer equipped with a Phenomenex® Gemini® column (100 mm

$\times$ 2 mm i.d., NX-C18 110 Å 3 μm particle size). Mobile phases A and B consisted of 2% (v/v) glacial acetic acid (prepared in ultrapure water by GFL 2004/Human Power 1) and 99.9% pure methanol (Sigma–Aldrich) for LC–MS, respectively. The total analysis time was set to 20 min. The gradient program was 0–2.00 min 100% A, 2.01–13.00 min 98% B and 2% A, 13.01–15.9 min 98% B and 2% A, and finally 15.9–19.00 min 100%A. The sample injection volume was 20.0 μL , the mobile phase flow rate was 0.3 mL min^{-1} , and the column temperature was set to 30 °C. The Orbitrap HRMS was operated in both positive (Full MS/AIF) and negative (Full MS/AIF) modes using a heated electrospray ionization interface. The ionization interface parameters were set as follows: sheath gas flow rate of 35, auxiliary gas flow rate of 7, spray voltage of 3.5 kV, capillary temperature of 350 °C, auxiliary gas temperature of 350 °C, and S-lens RF level of 50. The mass spectrometer scan range was 60–800 m/z , with a resolution of 17,500; an ACG target of 3×10^6 ; a maximum injection time (IT) of 2 ms; and a collision energy (CE)/step CE of 25 V. For the calibration curves of phenolic compounds via the UHPLC-Orbitrap®-HRMS/MS analysis method, standard concentrations of 10–20–40–60–80–100–200–300–400–500 ng mL^{-1} were prepared and injected three times each. The plant extract solutions were diluted with methanol to a concentration of 1 mg mL^{-1} for all analyses. The solutions were filtered through a 0.22-micron PTFE syringe filter and a 10-mL syringe. Following filtration, 1.5 mL of the solution was transferred into a vial and injected into the device. The device was operated concurrently with TraceFinder 3.2 software (Thermo Scientific) installed on the system computer, and data acquisition and recording were performed via Xcalibur software version 2.1.0.1140 (Dursun et al. 2024).

Statistical analyses

Sterilization and in vitro germination experiments of cherry laurel seeds were conducted utilizing baby food jars and Magenta boxes whereas all proliferation studies of cherry laurel shoot tips, including the determination of the optimal benzyladenine (BA) concentration and assessment of the effects of nZVI, were carried out in Petri dishes. For in vitro proliferation studies, at least 10 shoot tips were utilized for each experiments, and they were repeated at least for five times. The analysis of phenolic and flavonoid contents, as well as the assessment of antioxidant activity, was conducted at least thrice. Nonparametric data (proliferation percentages) were analyzed with the chi-square (χ^2) test. Discrete data (number of microshoots per explant, microshoot length, flavonoid and phenolic contents, and antioxidant activity results) were subjected to analysis of variance (ANOVA) with the least significant difference (LSD) test at

$P \leq 0.05$ for mean comparisons. The graphs were generated using GraphPad Prism 8 software.

Results

Seed sterilization and germination

When sterilized seeds were placed in growth media without removing the testa, some seeds were contaminated due to the presence of the testa. Consequently, in the following sterilization procedures, the testa was removed from the seeds. When the seeds were placed in growth media without being cut, only callus formation was observed, with no visible stem or root formation (Fig. 1a). As a result, one of the most critical factors affecting germination is the horizontal cutting of seeds with a scalpel without damaging the embryo (Fig. 1b, c, d, e). The germination rate of the embryos subjected to these procedures was 85%. The average germination period was approximately 4 weeks (Fig. 1f) (data not shown).

Micropropagation

The optimum BA concentration for cherry laurel micropropagation was determined to be 1 mg L^{-1} , in comparison with the other tested concentrations. Although there was no statistically difference in the average number of microshoots that proliferated per explant, the highest percentage of proliferation (82%), the highest SFC index (1.09), and the longest microshoots were obtained at the 1 mg L^{-1} BA concentration (Fig. 2). Since then, cherry laurel microshoots have been subcultured into MS media supplemented with 1 mg L^{-1} BA every 4 weeks.

Determination of the effect of the nZVI concentration on the proliferation of cherry laurel shoot tips

Compared with the control, all concentrations of nZVI had a positive effect on the proliferation of cherry laurel shoot tips in vitro (Figs. 3 and 4). The highest proliferation rate (85.15%) was obtained with 75 mg L^{-1} nZVI-treated plants,

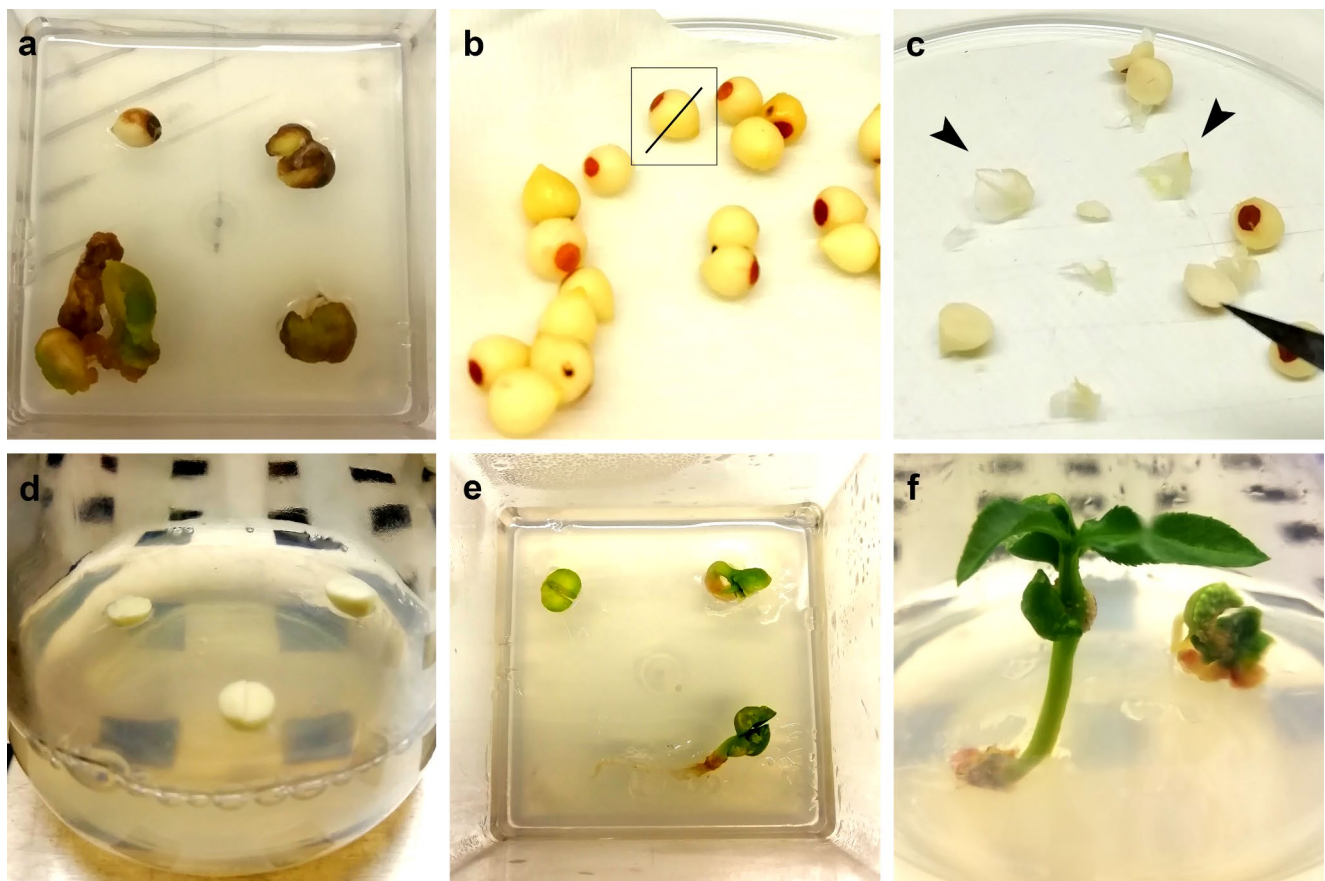


Fig. 1 The germination of seeds in the germination media **a**) Two weeks after sterilized seeds are placed in the germination medium without being cut, **b**) The cut in half place of the seed, **c**) Removal of the testa after the cotyledon is cut, **d**) Bisected testa-free cotyle-

dons containing embryo in the culture medium, **e**) Germination of the embryos after two weeks in the germination medium, **f**) Germinated embryos after 4 weeks

Fig. 2 Effect of BA concentration on the in vitro proliferation of cherry laurel shoot tips after 4 weeks of culture^a. ^aEach trial was performed with at least 50 explants. ^bThe different letters in each column indicate statistically significant differences ($P \leq 0.05$) compared with the chi-square test (χ^2). ^c Different letters in each column indicate statistically significant differences ($P \leq 0.05$) compared with the LSD test. ^d Mean $\pm$ standard error. ^e Shoot-forming capacity (SFC) = percentage of proliferating explants $\times$ average number of shoots proliferated per explant/100 (Lambardi et al. 1993)

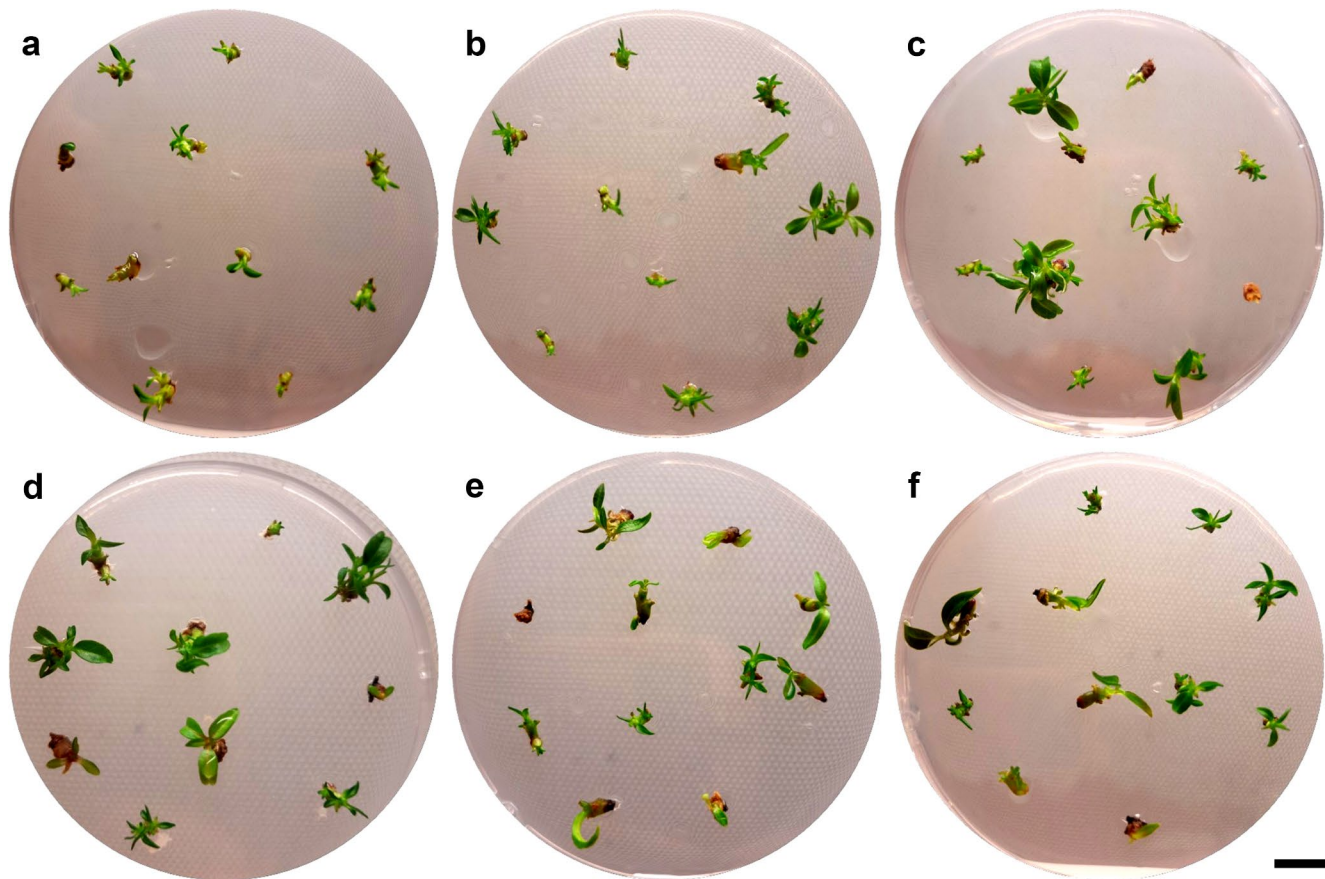
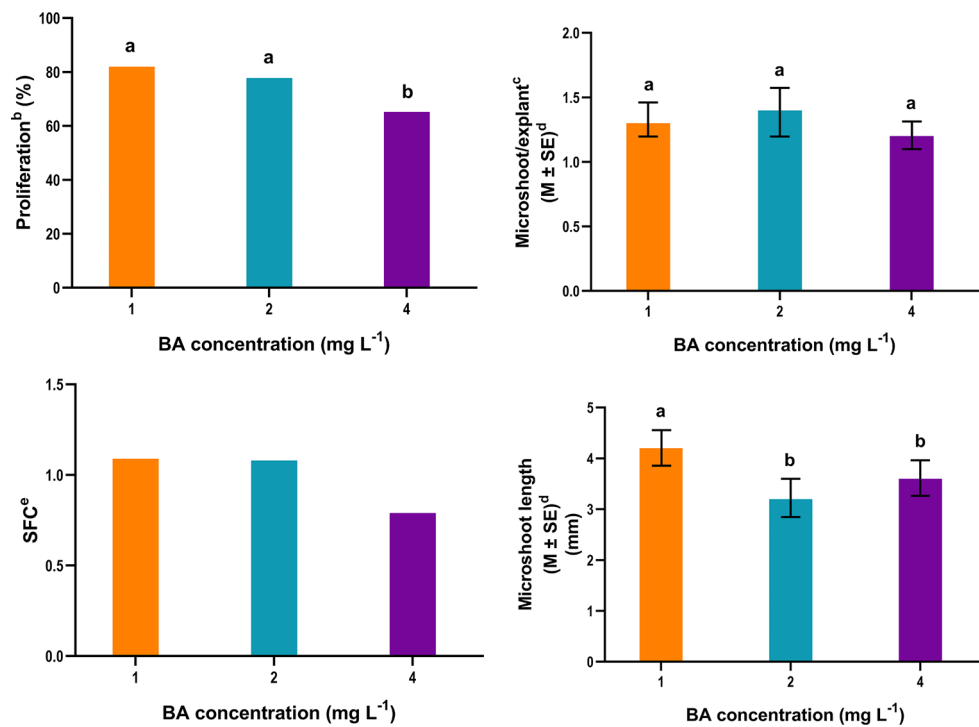


Fig. 3 The effect of nZVI on the in vitro proliferation of cherry laurel shoot tips after 4 weeks of culture All the media used were MS media containing 1 mg L⁻¹ BA **a**) Control, **b**) 12,5 mg L⁻¹ nZVI, **c**) 25 mg L⁻¹ nZVI, **d**) 50 mg L⁻¹ nZVI, **e**) 75 mg L⁻¹ nZVI, and **f**) 100 mg L⁻¹ nZVI. Bar = 1 cm

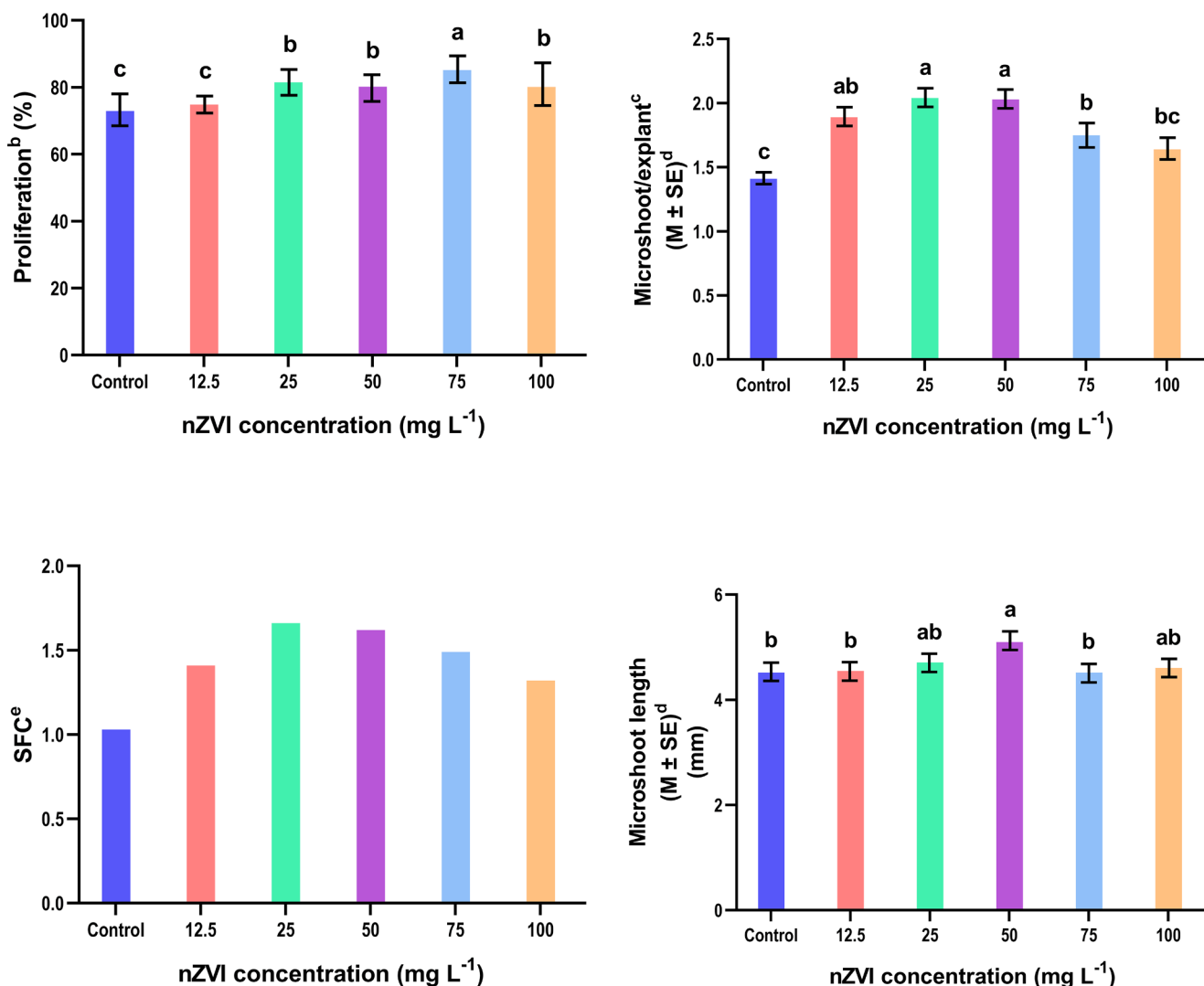


Fig. 4 The effect of nZVI on the in vitro proliferation of cherry laurel shoot tips after 4 weeks of culture^a. ^aEach trial was performed with at least 100 explants. ^bThe different letters in each column indicate statistically significant differences ($P \leq 0.05$) compared with the chi-square test (χ^2). ^cDifferent letters in each column indicate statistically

significant differences ($P \leq 0.05$) compared with the LSD test. ^d Mean $\pm$ standard error. ^e Shoot-forming capacity (SFC) = percentage of proliferating explants $\times$ average number of shoots proliferated per explant/100 (Lambardi et al. 1993)

whereas the lowest value (72.94%) was observed in the control. Compared with that of the control, the mean number of microshoots that proliferated per explant increased in the shoot tips incubated in the culture medium containing nZVI, and the highest number of multiple microshoots (2.04) was achieved in the medium containing 25 mg L⁻¹ nZVI. Accordingly, the addition of 25 mg L⁻¹ nZVI also resulted in the highest SFC index (1.66), while the lowest was calculated for the control (1.03). The shoot tips treated with 50 mg L⁻¹ nZVI presented the greatest average length of microshoots (5.10 mm), which was significantly different from that of the control. In addition, the microshoots cultured on media supplemented with 25 or 50 mg L⁻¹

nZVI had larger and wider leaves than the microshoots in the other groups did (Fig. 3).

Determination of the total phenolic and flavonoid contents and antioxidant enzyme activities of the plant extracts

Since the lowest proliferation results were obtained with 100 mg L⁻¹ nZVI-supplemented medium in comparison with the other tested nZVI concentrations, tests for total phenolic content, flavonoid content and antioxidant activity were performed for 5 groups (control and 12.5, 25, 50, and 75 mg L⁻¹ nZVI), but 100 mg L⁻¹ nZVI was not included in these experiments. The results revealed that 25 mg L⁻¹

Table 1 Results of the total phenolic and flavonoid contents and antioxidant enzyme activities of the plant extracts^a

Experimental groups	TPC (mg GAE g ⁻¹ extract) ^b (M±SE) ^c	TFC (mg QE g ⁻¹ extract) ^b (M±SE) ^c	DPPH (mg TE g ⁻¹ extract) ^b (M±SE) ^c	ABTS (mg TE g ⁻¹ extract) ^b (M±SE) ^c	CUPRAC (mg TE g ⁻¹ extract) ^b (M±SE) ^c	FRAP (mg TE g ⁻¹ extract) ^b (M±SE) ^c
Control	130,80±0,43 ^b	269,85±6,94 ^c	185,87±4,66 ^c	147,30±2,08 ^{ab}	238,49±2,86 ^b	144,66±0,11 ^c
12,5 mg L ⁻¹ nZVI	131,55±1,15 ^b	280,45±5,46 ^b	198,52±2,71 ^b	149,47±2,67 ^a	234,16±6,58 ^{bc}	148,79±0,47 ^b
25 mg L ⁻¹ nZVI	140,09±0,43 ^a	297,12±6,94 ^a	210,68±3,94 ^a	150,07±1,49 ^a	266,95±4,50 ^a	154,93±0,96 ^a
50 mg L ⁻¹ nZVI	124,02±0,43 ^c	244,09±1,52 ^d	170,60±2,89 ^d	137,03±1,90 ^b	225,87±7,67 ^c	127,61±0,99 ^c
75 mg L ⁻¹ nZVI	120,50±2,42 ^d	209,24±8,02 ^e	171,79±1,24 ^d	135,26±1,49 ^b	210,74±5,33 ^d	137,64±0,96 ^d

^aEach trial was performed at least 3 times^bThe different letters in each column indicate statistically significant differences ($P \leq 0.05$) compared with the LSD test^cMean ± standard error**Table 2** The number of phenolics in the plant extracts was determined via UHPLC-Orbitrap®-HRMS/MS (*metabolites significantly different from those in the control plant group)

Compounds (µg analyte g ⁻¹ extract)	nZVI concentrations (mg L ⁻¹)				
	Control	12,5	25	50	75
Salicylic acid	28,15	22,77	22,32	35,98	31,81
Vanillic acid	94,75	58,57	85,97	87,58	73,90
*Caffeic acid	72,67	76,61	86,90	63,12	56,76
*Chlorogenic acid	29681,59	34221,77	39582,97	32731,21	8511,04
Catechin (Cianidanol)-p	3658,11	3010,75	2854,54	2776,16	2878,00
Apigenin 7-glucoside	N/F	N/F	N/F	N/F	N/F
*Rutin	111,57	182,61	183,67	153,45	78,77
Luteolin 7-glucoside	13,68	N/F	N/F	17,14	13,11
*Quercetin	N/F	5,93	6,73	8,62	6,30
*Isoquercitrin	351,51	414,98	528,75	527,76	447,27
Narcissin	33,92	35,28	31,07	69,48	56,14
*Nicotiflorin	N/F	74,81	71,26	75,00	33,08
*Eriodictyol	109,10	147,61	260,67	215,83	95,38
*Kuromanine	84,35	127,83	131,00	115,69	69,69
Phloridzin	13,08	10,73	12,86	10,91	16,31

nZVI yielded the highest values for each parameter tested, whereas the shoot tips treated with 12.5 mg L⁻¹ nZVI presented statistically significant increases in total flavonoid content and DPPH and FRAP activities compared with those of the control group. The other tested nZVI concentrations provided lower results than did the control (Table 1).

The total content of phenolic compounds (mg GAE g⁻¹ extract) in the control was 130.80±0.43. The inclusion of 25 mg L⁻¹ nZVI increased the content to 140.09. Similarly, the total content of flavonoid compounds (mg QE g⁻¹ extract) was also increased with the addition of 25 mg L⁻¹ nZVI (269.85 versus 297.12 in the control and 25 mg L⁻¹ nZVI, respectively). In terms of DPPH antioxidant activity, the positive influence of the addition of 25 mg L⁻¹ nZVI was also detected (185.87 versus 210.68 25 mg Trolox equivalent g⁻¹ extract in the control and 25 mg L⁻¹ nZVI, respectively) in

the control (185.87), whereas it was calculated as 210.68 in the 25 mg L⁻¹ nZVI-treated samples. Likewise, the ABTS⁺ antioxidant activity value of the control was 147.30, while this value was calculated as 150.07 in the 25 mg L⁻¹ nZVI-treated samples. The CUPRAC antioxidant activity value of the control was 238.49; it was calculated as 266.95 in the 25 mg L⁻¹ nZVI. Similarly, the FRAP antioxidant activity value of the control was 144.66; for 25 mg L⁻¹ nZVI, it was calculated as 154.93.

UHPLC-Orbitrap®-HRMS/MS analysis results

Fifteen phenolic compounds were analyzed in the plant extracts via UHPLC-Orbitrap®-HRMS/MS. Table 2 presents the phenolic metabolite contents of all the experimental groups. Similarly, similar to the phenolic content, the flavonoid content and antioxidant activity of 100 mg L⁻¹ nZVI plant extracts were not analyzed. The results revealed that the levels of phenolic metabolites generally decreased in shoot tips treated with 75 mg L⁻¹ nZVI. When the experimental groups were compared, the levels of eight metabolites significantly increased. The caffeic acid content was 72.67 µg analyte g⁻¹ extract in the control group, 76.61 µg analyte g⁻¹ extract in the 12.5 mg L⁻¹ nZVI-treated group, and 86.90 µg analyte g⁻¹ extract in the 25 mg L⁻¹ nZVI-treated group. The chlorogenic acid content was 29681.59 µg analyte g⁻¹ extract in the control plant. It significantly increased in the 12.5, 25 and 50 mg L⁻¹ nZVI-treated plants (34221.77, 39582.97, and 32731.21 µg analyte g⁻¹ extract) but decreased to 8511.04 µg analyte g⁻¹ extract at 75 mg L⁻¹ nZVI. The rutin content was 111.57 µg analyte g⁻¹ extract in the control. It significantly increased in the 12.5, 25 and 50 mg L⁻¹ nZVI-treated samples (182.61, 183.67, and 153.45 µg analyte g⁻¹ extract, respectively) but decreased to 78.77 µg analyte g⁻¹ extract at 75 mg L⁻¹ nZVI. The isoquercitrin metabolite was 351.51 µg analyte g⁻¹ extract in the control. In the 12.5, 25, 50, and 75 mg L⁻¹ nZVI-treated samples (414.98, 528.75, 527.76, and 447.27 µg analyte g⁻¹ extract), an increase in the

isoquercitrin metabolite was also observed. Moreover, the eriodictyol content was $109.10 \mu\text{g analyte g}^{-1}$ extract in the control. It significantly increased with the 12.5, 25, and 50 mg L^{-1} nZVI treatments (147.61 , 260.67 , and $215.83 \mu\text{g analyte g}^{-1}$ extract, respectively) but decreased to $95.38 \mu\text{g analyte g}^{-1}$ extract at 75 mg L^{-1} nZVI. In addition, the kuromanine content was $84.35 \mu\text{g analyte g}^{-1}$ extract in the control. It significantly increased in the 12.5, 25, and 50 mg L^{-1} nZVI treated samples (127.83 , 131.00 , and $115.69 \mu\text{g analyte g}^{-1}$ extract, respectively) but decreased to $69.69 \mu\text{g analyte g}^{-1}$ extract at 75 mg L^{-1} nZVI.

While the control plants did not contain quercetin metabolites, the plants treated with 12.5, 25, 50, or 75 mg L^{-1} nZVI (5.93, 6.73, 8.62, or $6.30 \mu\text{g analyte g}^{-1}$ extract) had similar shoot tips. Similarly, the control did not contain the nicotiflorin metabolite; however, it was detected in the plants treated with 12.5, 25, 50, and 75 mg L^{-1} nZVI (74.81, 71.26, 75.00, and $33.08 \mu\text{g analyte g}^{-1}$ extract, respectively).

Discussion

The cultivation of cherry laurel (*Prunus laurocerasus*), which is a popular ornamental plant around the world, should be expanded because of the presence of medicinally important phenolic compounds. Previous studies have developed an alternative in vitro propagation method that is faster and more effective than traditional methods (Sulusoglu 2012; Sulusoglu and Cavusoglu 2013). In a previous study on embryo culture, the testa was removed, and half of the cotyledons were removed (Sulusoglu 2012). In our study, we once again demonstrated the importance of these stages for successful germination. Nevertheless, no study has investigated the potential for enhancing the efficiency of in vitro propagation cherry laurel. Despite the numerous uses of NPs in tissue culture, including enhancing plant growth and proliferation, there are no studies on the use of nZVI to increase the efficiency of in vitro propagation. Although research on the effects of nZVI on plant growth and metabolism in vitro is lacking, studies conducted in greenhouse environments (both soil and hydroponics) have demonstrated that nZVI promotes plant development and increases biomass. The present study examined the influence of nZVI on cherry laurel shoot tips *in vitro*. The findings showed that these nanoparticles do not have a toxic effect on plant growth and even promote the growth of plants at low concentrations. The results of this study match those reported in the literature with other plants (i.e., hybrid poplars, cattail, rice, and peanut), indicating that low concentrations (with an optimum concentration varying for each plant) of nZVI generally promote plant growth.

Our study revealed that an increase in the nZVI applied to the plant decreased its positive effect on plant growth, leading to a decrease in the number of microshoots per explant and the SFC index. Accordingly, the microshoots treated with 25 mg L^{-1} nZVI had the highest SFC index (1.66), whereas the lowest SFC index was observed with 100 mg L^{-1} nZVI (1.32). Thus, the optimum concentration for micropropagation of cherry laurel was found to be 25 mg L^{-1} nZVI. Similarly, rice seeds nanoprimering with nZVI exhibited increases in root and shoot length, photosynthetic pigment content, and biomass compared to the control plants in 14-day-old seedlings (Guha et al. 2018). The optimum concentration for plant growth and development was found to be 20 mg L^{-1} nZVI and the positive effects of nZVI decreased with elevation of its concentration up to 80 mg L^{-1} , which supports the findings of our study. A follow-up study showed that rice plants grown in the field exhibited broader leaves and increased biomass following nanoprimering treatment (Guha et al. 2021). After 4 weeks, the microshoots growing in the medium containing nZVI were larger and had wider leaves than the control microshoots did (Fig. 3). Consistent with the micropropagation findings, the largest microshoots leaves were those that grew from shoot tips treated with 25 and 50 mg L^{-1} nZVI. Moreover, the majority of studies (Libralato et al. 2016; Yoon et al. 2019) have indicated that plants treated with nZVI have larger leaves and increased biomass. These results could be due to an increase in plasma membrane H⁺-ATPase activity, which resulted in increased leaf area and stomatal aperture with nZVI treatment (Kim et al. 2014). Additionally, enhanced antioxidant properties observed in nZVI-treated plants may contribute to improved stress tolerance and resistance, thereby supporting their growth and development (Guha et al. 2018).

Plant-derived secondary metabolites, including drugs, food additives, and flavors, can serve as sources for industrial production. A variety of biotic and abiotic stress factors can lead to induce oxidative stress in plants, especially the stimulation of the accumulation of phenolic compounds as secondary metabolites. The addition of chemical stimulants mimicking the effects of stress in plants, such as NPs to the culture medium can increase the ability of NPs to bind to receptors on the surface of plant membranes, triggering the exchange of Ca²⁺ ions. This activates NADPH oxidase causes the formation of reactive oxygen species (ROS), known as signaling molecules (Prasad et al. 2024). Accumulation of oxidative ROS causes changes in transcription-level gene expression and increases the synthesis of secondary metabolites. Many studies have reported that iron-based NPs can increase secondary metabolite production (Almagro et al. 2020; Khan et al. 2021; Moharrami et al. 2017; Mohebodini et al. 2017; Nourozi et al. 2019;

Sharafi et al. 2013; Taghizadeh et al. 2021). At the same time, high levels of ROS in plant cells are detrimental, often causing oxidative stress that leads to cellular damage and, ultimately, cell death. Conversely, at low concentrations, ROS play a pivotal role as signaling molecules, promoting critical physiological processes such as cell division, differentiation, and organogenesis. Moreover, they contribute to the regulation of plant hormone levels and the antioxidant enzyme activities, thereby supporting plant growth and adaptation to environmental stimuli (Huang et al. 2019). The results of this study showed that treating plants with nZVI also increased the levels of secondary metabolites. Specifically, shoot tips treated with 25 mg L⁻¹ nZVI presented the highest phenolic and flavonoid contents compared with those of the control group. Similarly, this plant group presented the highest antioxidant activity value. Phenolic compounds are potent antioxidants commonly found in plants. Therefore, it is not surprising that this group of plants has the highest antioxidant value. The treatment of nZVI in plants can regulate antioxidant activities and metabolism. In rice seedlings, nanopriming with 20 mg L⁻¹ nZVI resulted in higher SOD (antioxidant enzyme that reduces O₂^{•-} to H₂O₂) activity and lower proline levels than those in control plants (Guha et al. 2018). Similarly, the application of Fe₂O₃ NPs to peanuts reduced the levels of abscisic acid (ABA) production associated with stress and increased antioxidant enzyme activities compared with those in control plants and promoted plant growth (Rui et al. 2016). In another study, *Gerbera jamesonii* treatment with iron NPs improved both antioxidant enzyme activities (SOD, CAT and APX) and the growth and development (The Vinh et al. 2024). The application of the optimal concentration of Fe₂O₃ NPs on watermelon seedlings enhanced their growth and stimulated antioxidant enzyme activity (Li et al. 2013). According to our findings, increasing the nZVI concentration decreased the antioxidant activity values compared with those of the control group. We conducted four different tests to measure antioxidant activity, and all of them demonstrated that the addition of 25 mg L⁻¹ nZVI to the medium produced the best results. This finding was consistent with our micropropagation results, leading us to conclude that low concentrations of nZVI can reduce plant stress in in vitro environments and promote growth.

In our study, we confirmed that the phenolic and flavonoid contents of the plants increased when the plants were subjected to both spectrophotometric methods and quantitative analysis of different phenolic compounds via UHPLC-Orbitrap®-HRMS/MS. Among the 15 different phenolic compounds analyzed, chlorogenic acid was present in the highest quantities relative to the other compounds. This result confirms that chlorogenic acid is the main phenolic compound in cherry laurel, which is consistent with the findings

of previous studies (Bayrambaş et al. 2019; Karabegović et al. 2014; Yaylaci-Karahalil and Şahin 2011). Karabegović et al. (2014) reported that 28.67 mg g⁻¹ chlorogenic acid was extracted from leaf extracts via ultrasonic-assisted extraction with HPLC. This amount is quite similar to the chlorogenic acid content found in the control plant extracts (29.68 mg g⁻¹ extract) in the present study. Our findings also demonstrated that the amount of chlorogenic acid increased in the plant extracts treated with nZVI but decreased at a concentration of 75 mg L⁻¹. The highest amount was obtained from the plant extract supplemented with 25 mg L⁻¹ nZVI (39.58 mg g⁻¹ extract). As the concentration of nZVI increased, the levels of certain phenolic compounds decreased relative to those of the control, suggesting that the optimum nZVI concentration was 25 mg L⁻¹. Like those of chlorogenic acid, the amounts of caffeic acid, rutin, isoquercitrin, eriodictyol, and kuromanine first increased in comparison with those in the control and then decreased as the applied nZVI concentration increased. Interestingly, the addition of nZVI to the culture medium enabled the production of two new phenolics, quercetin and nicotiflorin, both of which were not detected in the control plant extract but were present in all the plant extracts treated with nZVI. The production of secondary metabolites is known to differ on the basis of the culture and environmental conditions of the plant (Yang et al. 2018). Since the only variable was the inclusion of nZVI in in vitro conditions in the present study, the presence of these two metabolites could be due to the presence of nZVI in the nutrient medium in a threshold manner since the concentration of these metabolites decreased in microshoots treated with the highest tested concentration (75 mg L⁻¹) of nZVI. Moreover, apigenin 7-glucoside was not detected in any of the extracts, in contrast to previous reports that have shown its presence in cherry laurel leaves (Karabegović et al. 2014). This finding also again demonstrates that the secondary metabolites produced by the plant are subject to changes due to the conditions in which it grows.

In this study, the impact of various concentrations of nZVI on the shoot tips of cherry laurel was investigated in vitro. Previous studies have reported the beneficial effects of nZVI on plants under *in vivo* conditions. However, this is the first report examining the impact of nZVI on the in vitro culture of plant explants. The shoot tips treated with 25 mg L⁻¹ nZVI presented the best SFC index and high antioxidant activity. In addition, changes in the amounts of flavonoid and phenolic compounds were observed in the microshoots treated with nZVI, and these changes occurred in relation to concentration. At 25 mg L⁻¹ nZVI, the cherry laurel plants presented the highest concentration (39.58 mg g⁻¹ extract) of chlorogenic acid, which is the major phenolic compound. However, the observed beneficial effects decreased with increasing concentrations of nZVI. Consequently, low

concentrations of nZVI appear to have good potential for enhancing the micropropagation of cherry laurel and the production of secondary metabolites under in vitro conditions due to its higher chemical reactivity that resulted with elevated plant growth and antioxidant activity alongside other metal nanoparticles.

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Data availability The data in this study are available on request from the corresponding author.

Declarations

Competing interests The authors have no relevant financial or nonfinancial interests to disclose.

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