Efficiency of seed vigor tests in estimating *Melissa officinalis* L. seedling emergence in soil and the effects of iron oxide nanoparticles on the seedling's physiological properties

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Abstract

This study was conducted on *Melissa officinalis* (lemon balm) to compare the effects of seed vigor tests, under laboratory conditions, on seedling emergence from soil (greenhouse) and to study the effects of foliar application of iron oxide nanoparticles (ION) on growth variables and physiological performance of *Melissa officinalis* seedlings. Seed vigor tests were conducted on seeds of three sizes (large, medium, and small) in the laboratory. These tests included standard germination, accelerated aging, cold, Hiltner, and electrical conductivity (EC). Seeds of three sizes were planted in two different soil types, namely loam and clay loam, in a greenhouse. At the two-node stage, foliar applications of iron oxide nanoparticles (control, 15, and 30 ppm) were also made. The Hiltner test best predicted seedling emergence form the soil, whereas the other tests showed no significant predictive power. The findings indicated that the combined treatment of large seeds plus clay loam soil plus application of 30 ppm ION produced the highest seedling height, chlorophyll a and chlorophyll b levels, soluble sugars content, essential oil percentage, and gas exchange, while the combined treatment of small seed + loam soil + no foliar ION application resulted in the lowest levels of these variables. Loam soil with small seeds and no foliar ION spray increased proline concentration and antioxidant enzyme activity. Results of soil texture analysis and Hiltner seed vigor test were found crucial for farmers who cultivate *Melissa officinalis* L. Finally, ION foliar spraying is suggested for better physiological performance and yield of this plant.

Keywords: Hiltner test, nano fertilizer, plant nutrition, seed vigor, soil texture

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Introduction

Medicinal plants are those that have at least one therapeutically useful active component in one or more of their organs. These components serve as basis for production of plant-based chemical-pharmaceuticals. Due to their wide biological and pharmacological activities, higher safety margins, and cheaper production costs, medicines produced from these plants are in demand as

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primary health care components in both developed and developing nations (Yudharaj et al., 2016). Agriculture is the most significant economic activity in rural areas of Iran. The strategy of planting and producing medicinal plants and processing the resulting crops is a technique to lessen the impact on natural vegetation, diversify source of income, and potentially double earnings in villages. To accomplish this goal, it may be crucial to allocate rainfed fields, low-yield lands, or national lands for the cultivation of medicinal plants near villages. Reduction of agricultural water use, preservation of water and soil, creation of jobs, reduction of migration to cities, stimulation of the rural economy, provision of medicinal plants required by pharmaceutical companies, provision of trade, increase in non-oil exports, development of the rural ecotourism sector, and protection of the genetic resources of these plants are some advantages of these measures (Tarhani, 2015).

Melissa officinalis L., sometimes known as lemon balm is a dicotyledon that is a member of the mint family (Lamiaceae). The Mediterranean and northern parts of Iran have the highest abundance of this herbaceous, perennial plant. Its dried leaves include 11.8% polyphenolic constituents and 0.134% citral, along with 11.34% cinnaminic chemicals. Chemical screening also revealed 2.2% methyl heptenone, 14.4% citronellal, 2.7% linalool, 47.5% geranial, 6-7% isogeraniol, geranyl acetate 10%, and 11% caryophyllene oxide along with alpha-pinene, cispara-Meth2-en-7-ol, nerol acetate, IR-alpha-pinene, carane, verbenol, verbenone, menthol, germanicol, longifolene, himachala 2, 4 -diene, and andropholide. Lemon balm is well known for having a variety of medical benefits for ailments like digestive disturbance, oxidative stress, and neurological diseases (Awad et al., 2009).

Most plants are propagated from seed, which makes seed quality assurance a top priority in contemporary seed science and a requirement for producing good yields across all crop plant species. High quality seeds must be used in order to provide a healthy crop yield because seed germination is one of the most crucial plant phenological stages that is influenced by seed vigor. The effects of seed production, harvesting, and storage conditions combine to affect seed quality. Considering that seeds with high germination ability in the laboratory do not necessarily have sufficient germination in the field and produce fewer seedlings, determining seed vigor by appropriate and specific tests for a plant and cultivation conditions is crucial (Hassan and Hassan, 2018).

Seed quality has many aspects including seed germination, seed health, seed purity, seed size, genetic characteristics, seed vigor, and storability. Normally, physiological activity of seeds is determined by a standard germination test. But the standard germination test often estimates seed germination percentage to be greater than is seen under field conditions, because in the standard germination test, natural seedling production is measured under favorable conditions (Zhang and Ghanem, 2018). Determining seed vigor through appropriate and specific tests for a plant and set if cultivation conditions is of particular importance because seeds with high germination ability in the laboratory may not necessarily have sufficient germination in the field and produce fewer seedlings (Hassan and Hassan, 2018). Seed germination, health, purity, size, genetic features, vigor, and storage ability are just a few of the numerous factors that make up seed quality. Typically, the standard germination test is used to assess the physiological quality of seeds. However, because natural seedling production is assessed in a controlled environment, the common germination test frequently overestimates the seed germination percentage compared to field circumstances (Zhang and Ghanem, 2018).

There are numerous seed vigor tests available, but only a small number of them are accepted by seed experts and seed testing organizations. Several studies on seed vigor tests have been published, including standard germination tests for *Vicia sativa* (Tao et al., 2022), accelerated aging test for rice (Wang et al., 2022) and *Salvia officinalis* (Eisvand and Farajollahi, 2017), electrical conductivity test for endemic species (Marin et al., 2018), and cold test for maize (Jha and Mohamed, 2023). Contrary to tests, which are often carried out in a laboratory environment in petri dishes, seed germination takes place in the soil matrix of

a farm, where a range of stresses are often present. Under field conditions, seedling emergence and establishment occur in a range of climatic conditions that are often undesirable. For this reason, seed vigor tests have been developed to provide an appropriate prediction of emergence and seedling deployment for planting under different climatic conditions. Moisture and soil density are important factors affecting germination percentage, germination rate and plant establishment at the field level. Rapid germination is important because it enables the seedling to emerge from the soil before specific conditions lead to the formation of a crust following rainfall or irrigation. In addition to moisture, soil physical resistance also affects germination and initial seedling establishment. As soil physical resistance increases, seed germination decreases. Physical resistance of soil is affected by seed planting depth and soil compaction (Chimungu et al., 2015). Soil density and texture affect porosity, air permeability, rooting ability, nutrient flow and biological activity of soil (Descalzi et al., 2018).

Developing crops can often be iron deficient. Despite the typically high soil iron content, much of this element is fixed as Fe^{3+}, especially at high pH levels, and cannot be absorbed. It is thought that iron shortage in agricultural soils can be remedied by adding ION to the soil via fertilizer or spray applications. In most cases, leaf applied nutrients serve as a supplement to soil fertilizer in order to meet the plant's nutritional needs (Mimmo et al., 2014). Researchers found that foliar application of iron in nano form has positive and significant effects on vegetative and reproductive traits for *Vicia faba* when compared to iron chelate in sand soil conditions in their study of the effects of foliar application of different sources of iron improve morpho-physiological traits and nutritional quality of plants such as lemon balm (Mahmoud et al., 2022).

The aim of this study was to obtain answers to the following questions. First, can the use of seed vigor testing techniques and determination of convergence of laboratory and greenhouse results be effective in identifying the quality of seeds of lemon balm for the farmer? Second, whether seed quality, soil texture, and foliar application of iron nano-oxide have different effects on growth and physiological and antioxidant traits. The final question was whether the difference in seed size and micronutrient leaf nutrition can modulate morphophysiological indices of *Melissa officinalis* under various soil texture conditions.

Materials and Methods

This study was carried out in 2022 to evaluate the seed vigor of the medicinal plant *Melissa officinalis* in laboratory and the greenhouse conditions of Plant Production Engineering and Genetics Department, Faculty of Agriculture, Lorestan University, Iran. Medicinal Plants Research Center in Khorramabad provided 90% viable and 98% pure lemon balm seeds.

Laboratory survey

In the experimental phase, seeds were split into three groups according to their appearance and 1000-seed weight, namely large (1000-seed weight = 0.8 g), medium (1000-seed weight = 0.5 g), and small (1000-seed weight = 0.3 g). A scale with a 0.001-gram precision was utilized for these groupings. The moisture contents of seed samples were then randomly evaluated to prevent the impact of moisture on seed weight. Seeds were subjected to a variety of vigor tests, including normal germination, accelerated aging, cold test, Hiltner, and electrical conductivity tests. The association between the outcomes of each test and the rate of emergence in pot soil was assessed using the T-test. Also, Pearson correlation was used to examine the connection between the electrical conductivity test and the seedling emergence percentage in potted soil.

Vigor tests

Standard germination test

Petri dishes of 9 cm diameter and 1.5 cm height were disinfected with 15% sodium hypochlorite for the standard germination test. Eight replications (400 seeds) were used in total, with 50 seeds sown on filter paper in each Petri dish. Seeds were kept in a germinator at a constant temperature of 25 ± 2 ℃, 8 hours of darkness, 16 hours of light, and 70% relative humidity.

According to ISTA guidelines (ISTA, 2009), normal seedlings with their usual roots, shoots, colors, and sizes as well as abnormally germinated seeds were counted.

Accelerated aging test

Eight (8) replications of 50 seeds were utilized for this test. Seeds were exposed to a temperature of 40 ± 1 ℃ and 100% relative humidity for 24 h (Hampton and Tekrony, 1995). After the seeds underwent accelerated age, they were kept in a germinator for 10 days at a constant temperature of 25 ± 2 ℃, 8 h of darkness, 16 h of light, and 70% relative humidity. Following the criteria of the ISTA (ISTA, 2009) criteria for abnormally germinated seeds, natural seedling count with roots, shoots, and natural color and size was carried out (Fig. I).

Cold test

Eight of the 50-seed replications were put in plastic containers with wet paper for seven days at 8 ℃. The percentage of germination and seed vigor were then assessed after being placed in a germinator at 25 ± 2 ℃ (Fig. I).

Hiltner test

Brick grit weighing 1100 grams and having diameters between 2 and 3 mm was autoclave sterilized; then, 250 ml of distilled water was poured to the brick grits to moisten them. Next, a three-cm layer of brick grits was spread out on the bottom of each container, and the seeds were then covered with a one-cm-thick layer of brick grits (Fig. I). A total of 400 seeds were used in 8 replications (50 seeds in each replication) for this test. The containers holding seeds were put in a germinator at 20 ± 2 ℃ for 14 days, at which timey, normal and abnormal seedlings were counted (Hampton and Tekrony, 1995).

Electrical conductivity test

First, 250 mL distilled water was poured in a 500 mL container and stored at 20 ℃ for 24 h to achieve equilibrium temperature. Then, 8 samples of 50 seeds from each seed mass were carefully weighed and placed in each container containing distilled water. Each container was covered with an aluminum cap and placed at 20 ± 1 ℃ for 24 h. Afterwards, the seeds were removed and the solution was slowly shaken for about 5 minutes and the electrical conductivity of the solutions was determined with an EC meter. The electrical

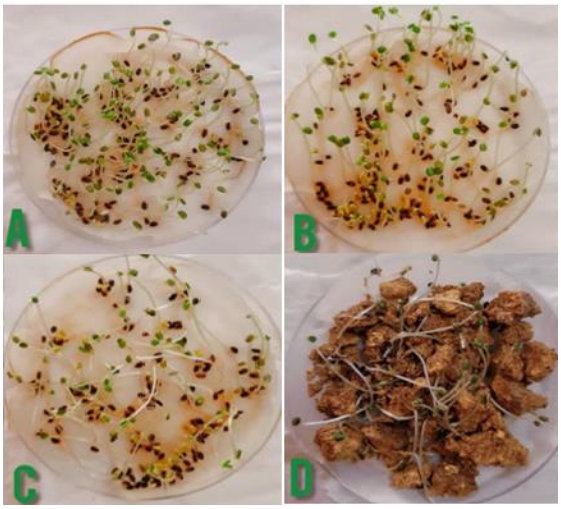

Fig. I. Results of seed vigor tests; A: Accelerated aging test, B: Standard germination test, C: Cold test, and D: Hiltner test.

conductivity of the distilled water was measured at 20 ℃, and the electrical conductivity per gram of seed weight of each experimental unit was calculated from the following equation (Hampton and Tekrony, 1995).

$$EC=ECs/W$$

EC: electrical conductivity, ECs: EC given by the EC meter, W: seed weight in grams.

Greenhouse experiment

The pot experiment was carried out as a factorial experiment based on a randomized complete block design with 3 replications in a greenhouse with a temperature range of 24-34°C and a relative humidity range of 60-80%. The studied factors included soil texture characteristics at two levels (clay loam, and loam), seed size at three levels (large, medium and small) and supplying the plant with ION at three levels: control, application of 15 or 30 ppm solutions. ION characteristics were Fe_2O_3 hematite, density of 24.5 g cm^{-3}, molecular mass 159.69 g mol^{-1}, pH=5-7, particle size 20-40 nm, purity of 98%, specific surface area 40-60 m^2 g^{-1} and melting point 1539 °C, made by Nano Research company, USA. Planting was carried out in the greenhouse in mid-November 2022. A total

Table 1
Physico-chemical analysis of soils

Soil texture	Sand-Silt-Clay	pH	C (%)	N (%)	P (ppm)	K (ppm)	Fe (ppm)
Clay loam	21- 40.5 -38.5	7.40	1.00	0.033	7.02	338	3.1
Loam	35.2 - 42 - 22.8	7.8	0.083	0.07	18	234	2.9

of 54 pots each with a cross sectional area of 30 cm and 50 cm in height were used. Soil was passed through a 5 mm sieve. The pots were filled with soil (Table 1) and pebbles were used at the end of the pot to easily remove excess water from the bottom of the pot. All the pots were filled with the same amount of soil (using scales). Ten lemon balm seeds were planted in each pot at a depth of 1-1.3 cm. ION foliar application was carried out at two nodes of stem by hand spraying.

Seedling emergence percentage

The pots were assessed daily and number of emerged seedlings were counted. Seedling emergence percentage and speed of seedling emergence were calculated using the obtained daily data.

Gas exchange and photosynthesis

Leaf gas exchanges was monitored using a portable infrared gas analyzer (IRGA—LC pro+; ADC Bio Scientific, the UK) five days following ION spraying. The stomatal conductance (g_s) and net photosynthesis rate (P_N) were measured on a sunny day, between 9:00 and 10:00 a.m., using the central leaflet of the first leaf completely expanded from the apex with a photosynthetic flux density (PPFD) of 1200 (µ mol m^{-2} s^{-1}, ambient CO_2 concentration (~ 400 µ mol mol^{-1}), ambient air temperature 22 ℃, and an air flow rate 0.3 mol m^{-2} s^{-1}).

Antioxidant enzyme

Antioxidant enzymes and soluble proteins were extracted from leaf tissues according to the method of (Badawi et al., 2004). The extraction mixture was prepared by homogenizing 500 mg of fresh plant material in 5 mL of extraction buffer, which consisted of 50 mM phosphate buffer (pH 7.6), 1.0 mM ascorbate, and 1.0 mM EDTA. Samples were centrifuged at (14,000 g for 4 mins. at 3 ℃), and the supernatant was collected. Quantification of the total soluble protein levels was performed using the method described by Bradford (1976). Absorbance was measured at 595 nm, using bovine albumin as a standard.

Catalase (CAT) assay

To assay CAT (EC 1.11.1.6), 0.2 mL of the supernatant and 1.8 mL of a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide were mixed, and the absorbance was measured at 240 nm (Havir and McHale, 1987). The CAT activity was expressed in units mg^{-1} protein.

Proline and soluble sugars content

To determine the proline content, 0.5 g of fresh leaves were homogenized with 5 mL of 95% ethanol. The upper phase of the filtrate was separated and its sediments were washed with 5 mL of 70% ethanol twice, and its upper phase was added to the previous upper compartment. The mixture was centrifuged at 3500 g for 10 min at 4 ℃, the supernatant was recovered, and an ethanol extract was kept in a refrigerator at 4 ℃ (Paquin and Lechasseur, 1979). One mL of the alcoholic extract was diluted with 10 mL of distilled water and 5 mL of ninhydrin (0.125 g ninhydrin, 2 mL of 6 mM NH_3PO_4, 3 mL of glacial acetic acid). Then, 5 mL of glacial acetic acid was added, and the mixture was placed in a boiling water bath for 45 min at 100 ℃. The reaction was stopped by placing the test tubes in cold water. The samples were rigorously mixed with 10 mL benzene. The light absorption of the benzene phase was estimated at 515 nm using a PD-303 model spectrophotometer. The proline concentration was determined using a standard curve. Free proline content was expressed as µmol g^{-1} DW of leaves (Irigoyen et al., 1992). To measure the content of soluble sugars,

0.5 g of dry leaves was homogenized with 5 mL of 95% ethanol. One-tenth mL of alcoholic extract, preserved in a refrigerator, was mixed with 3 mL anthrone (150 mg anthrone and 100 mL of 72% sulphuric acid, W/W). The samples were placed in a boiling water bath for 10 min. The light absorption of the samples was estimated at 625 nm using a PD-303 model spectrophotometer. Contents of soluble sugar were determined using glucose standard and expressed as mg g^{-1} DW of leaves.

Photosynthetic pigment assay (chlorophyll a and b)

After extraction of leaves (0.2 g) in 0.5 mL acetone (3% v/v) and then centrifuging (10000 rpm, 10 min), the absorptions of the supernatants were recorded at 645 nm (Chl b) and 663 nm (Chl a) by UV–Vis spectrophotometry (UV-1800 Shimadzu, Japan). The obtained absorptions were converted to the pigment values (Lichtenthaler, 1987).

Essential oil content

Branches were harvested and air dried in the shade. Then, 10 g of the dry branches were crushed and ground into powder to determine the amount of essential oil content by water distillation using a Clevenger device (model EMO500/C). The amount of essential oil (in percent) was also calculated after desiccating the water with dry sodium sulfate (Kapoor et al., 2002).

Data Analysis

Minitab 16 was used to conduct T-tests and drawing graphs. SAS software (Version 9:00 TS Level 00M0) was used for ANOVA. The means were compared by LSD test at 5% probability level.

Results

Seedling emergence percentage

The highest percentage of emergence per pot was observed for clay loam soil and the use of large size seeds (83.07%); the lowest percentage of emergence (77.17%) was observed for loamy soil and the use of small seeds (Fig. II).

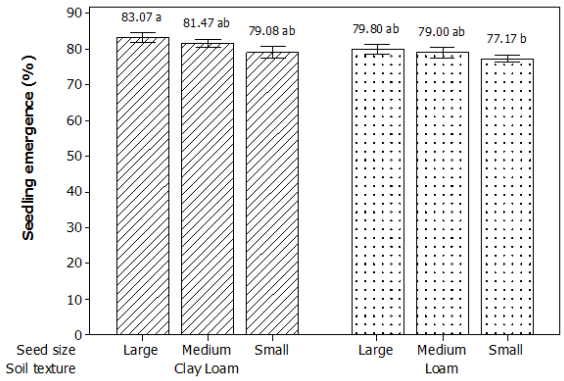

Fig. II. Effects of soil type and seed size on seedling emergence of *M. officinalis* L; bars indicate standard error. Means with at least one common letter are not significantly different from each other according to LSD Test ($p \leq 0.05$).

Plant height

Based on the results of the comparison of the average of triple effects, the highest plant height was obtained in the clay loam soil substrate with the application of 30 ppm iron nano-oxide (50.43 cm) in planting conditions. Minimum plant height was observed for loamy soil and using smaller seeds, and control nutrient conditions (no spraying) (Table 2).

Chlorophyll a

Based on comparisons of the means of triple effects, the highest chlorophyll a content was obtained for clay loam soil, using large size seeds and application of 30 ppm iron nano-oxide (3.91 µg.g FW^{-1}). Also, the lowest chlorophyll a content was observed for loamy soil culture conditions, using smaller seeds, and under control nutrient conditions (no foliar application of iron nano-oxide) (Table 2).

Chlorophyll b

Based on the results of the comparison of the means, the highest chlorophyll b content was obtained for clay loam soil, using large size seeds and application of 30 ppm iron nano-oxide (2.25 µg g FW^{-1}). Also, the lowest chlorophyll b content was for loamy soil culture conditions, using smaller seeds, and under control nutrient conditions (no foliar application of iron nano-oxide) (Table 2).

Stomatal conductance (g_s)

Comparison of the means of triple effects showed that maximum stomatal conductance resulted from culture in clay loam soil, using large size seeds and application of 30 ppm iron nano-oxide (2.64 mol H2O m^{-2} s^{-1}). Minimum stomatal conductance occurred for loamy soil, use of smaller seeds, and under control nutritional conditions (no spraying) (Table 2).

Net photosynthesis rate (P_N)

Based on the comparison of the means, the highest net photosynthesis rate in pots was occurred for clay loam soil, the use of large seeds, and application of 30 ppm iron nano-oxide (7.70 µmol CO_2 m^{-2} s^{-1}). Also, the lowest net photosynthesis rate was the result of culture of smaller seeds in loam soil and under control nutritional conditions (Table 2).

Table 2. Effect of seed size and ION foliar on plant height and some physiological traits *M. officinalis* L. under clay loam and loam soil conditions.

Soil texture	Seed size	ION nutrition treatments	Plant height (Cm)	Chl a (µg.g FW^{-1})	Chl b (µg.g FW^{-1})	g_s (mol H_2O m^{-2} s^{-1})	P_N (µmol CO_2 m^{-2} s^{-1})
Clay-loam	Large	Control	38.01 ± 1.59^{f}	2.06 ± 0.11^{ef}	1.41 ± 0.09^{eg}	1.42 ± 0.16^{ce}	6.25 ± 0.10^{fg}
		FS (15 ppm)	48.25 ± 1.78^{ab}	3.70 ± 0.10^{a}	2.25 ± 0.10^{a}	2.60 ± 0.11^{a}	7.44 ± 0.08^{ab}
		FS (30 ppm)	50.43 ± 0.85^{a}	3.91 ± 0.12^{a}	2.25 ± 0.12^{a}	2.64 ± 0.07^{a}	7.7 ± 0.09^{a}
	Medium	Control	37.41 ± 2.26^{f}	2.02 ± 0.08^{ef}	1.39 ± 0.12^{eg}	1.41 ± 0.10^{ce}	5.94 ± 0.10^{gh}
		FS (15 ppm)	45.87 ± 1.14^{bc}	3.01 ± 0.23^{b}	2.00 ± 0.07^{ab}	1.74 ± 0.14^{bc}	7.26 ± 0.11^{bc}
		FS (30 ppm)	47.43 ± 1.26^{ac}	3.08 ± 0.06^{b}	2.00 ± 0.02^{ab}	1.79 ± 0.11^{b}	7.39 ± 0.07^{ab}
	Small	Control	26.18 ± 0.73^{g}	1.24 ± 0.14^{g}	1.24 ± 0.17^{g}	1.16 ± 0.09^{ef}	5.30 ± 0.21^{i}
		FS (15 ppm)	41.26 ± 1.23^{df}	2.61 ± 0.10^{cd}	1.51 ± 0.06^{eg}	1.48 ± 0.05^{be}	6.54 ± 0.11^{ef}
		FS (30 ppm)	43.98 ± 1.25^{ce}	2.92 ± 0.13^{bc}	1.66 ± 0.07^{ce}	1.68 ± 0.14^{bd}	6.94 ± 0.11^{cd}
Loam	Large	Control	26.85 ± 1.53^{g}	1.76 ± 0.09^{f}	1.35 ± 0.20^{fg}	1.38 ± 0.08^{de}	5.91 ± 0.09^{h}
		FS (15 ppm)	41.35 ± 1.53^{df}	2.85 ± 0.18^{bc}	1.53 ± 0.15^{dg}	1.50 ± 0.10^{be}	6.68 ± 0.19^{de}
		FS (30 ppm)	45.10 ± 1.96^{bd}	2.99 ± 0.26^{bc}	1.96 ± 0.08^{ac}	1.72 ± 0.14^{bd}	7.24 ± 0.12^{bc}
	Medium	Control	26.35 ± 1.81^{g}	1.74 ± 0.09^{f}	1.28 ± 0.07^{g}	1.19 ± 0.10^{ef}	5.76 ± 0.07^{h}
		FS (15 ppm)	40.85 ± 1.26^{ef}	2.36 ± 0.10^{de}	1.45 ± 0.09^{eg}	1.45 ± 0.22^{be}	6.38 ± 0.07^{ef}
		FS (30 ppm)	44.18 ± 0.44^{be}	2.93 ± 0.13^{bc}	1.82 ± 0.12^{bd}	1.70 ± 0.19^{bd}	7.03 ± 0.16^{c}
	Small	Control	25.85 ± 1.76^{g}	1.11 ± 0.15^{g}	1.24 ± 0.18^{g}	1.01 ± 0.07^{f}	5.29 ± 0.16^{i}
		FS (15 ppm)	38.51 ± 1.01^{f}	2.06 ± 0.07^{ef}	1.43 ± 0.13^{eg}	1.42 ± 0.14^{ce}	6.28 ± 0.22^{fg}
		FS (30 ppm)	41.58 ± 1.32^{df}	2.88 ± 0.06^{bc}	1.60 ± 0.16^{df}	1.62 ± 0.17^{bd}	6.94 ± 0.10^{cd}

Table 2. Continued

Soil texture	Seed size	ION nutrition treatments	Proline (µmolg^{-1}FW)	Soluble sugars (mg g^{-1}FW)	Essential oil yield (%)	CAT (U mg^{-1} protein)
Clay-Loam	Large	Control	2.78 ± 0.08^{bc}	1.64 ± 0.13^{ce}	4.87 ± 0.53^{fh}	9.41 ± 0.36^{f}
		FS (15 ppm)	1.20 ± 0.04^{j}	2.69 ± 0.17^{a}	7.47 ± 0.24^{b}	4.34 ± 0.37^{l}
		FS (30 ppm)	0.77 ± 0.04^{k}	2.71 ± 0.11^{a}	8.91 ± 0.15^{a}	3.82 ± 0.76^{m}
	Medium	Control	2.89 ± 0.06^{ab}	1.64 ± 0.18^{ce}	4.81 ± 0.36^{fh}	9.80 ± 0.22^{e}
		FS (15 ppm)	1.72 ± 0.07^{hi}	2.45 ± 0.12^{a}	6.67 ± 0.35^{bd}	6.47 ± 0.23^{k}
		FS (30 ppm)	1.56 ± 0.05^{i}	2.61 ± 0.14^{a}	7.18 ± 0.38^{bc}	6.52 ± 0.37^{k}
	Small	Control	3.15 ± 0.08^{a}	1.56 ± 0.17^{de}	3.72 ± 0.33^{h}	11.74 ± 0.24^{b}
		FS (15 ppm)	2.40 ± 0.07^{df}	1.72 ± 0.08^{ce}	5.00 ± 0.37^{eg}	8.81 ± 0.01^{gh}
		FS (30 ppm)	2.16 ± 0.06^{fg}	1.91 ± 0.10^{cd}	5.49 ± 0.29^{df}	7.70 ± 0.21^{i}
Loam	Large	Control	3.09 ± 0.05^{a}	1.59 ± 0.15^{de}	4.64 ± 0.09^{fh}	10.33 ± 0.10^{d}
		FS (15 ppm)	2.31 ± 0.11^{df}	1.77 ± 0.22^{ce}	5.13 ± 0.44^{ef}	8.58 ± 0.05^{h}
		FS (30 ppm)	1.92 ± 0.06^{gh}	2.34 ± 0.08^{ab}	6.50 ± 0.26^{bd}	6.67± 0.28^{jk}
	Medium	Control	3.13 ± 0.04^{a}	1.57 ± 0.16^{de}	3.81 ± 0.22^{gh}	11.06 ± 0.14^{c}
		FS (15 ppm)	2.48 ± 0.11^{de}	1.71 ± 0.05^{ce}	4.92 ± 0.30^{eh}	9.00 ± 0.04^{g}
		FS (30 ppm)	1.96 ± 0.13^{gh}	2.03 ± 0.15^{bc}	6.15 ± 0.30^{ce}	6.95 ± 1.38^{j}
	Small	Control	3.16 ± 0.04^{a}	1.45 ± 0.09^{e}	2.45 ± 0.16^{i}	16.60 ± 0.66^{a}
		FS (15 ppm)	2.57 ± 0.25^{cd}	1.68 ± 0.15^{ce}	4.91 ± 0.40^{fh}	9.37 ± 0.28^{f}
		FS (30 ppm)	2.27 ± 0.03^{ef}	1.80 ± 0.06^{ce}	5.48 ± 0.24^{df}	8.76 ± 0.26^{gh}

* Means in each column with at least one same letter are not significantly different from each other according to LSD Test ($p \leq 0.05$). Values described corresponding to means from three replications ± standard errors (FS: foliar spraying)

Proline

Based on comparison of the mean effects of the three factors of the study, the lowest proline content was obtained in clay loam soil, using large seeds, and application of 30 ppm iron nano-oxide solution of 0.77 ($molg^{-1}$ FW). Also, the highest amount of proline resulted from cultivation in loamy soil, using smaller seeds, and under control nutrient conditions (no spray application) (Table 2).

Soluble sugars

Based on comparison of the average effect of three factors, the highest soluble sugars content was 2.71 mg g^{-1} FW in clay loam soil, using large seeds, and application of 30 ppm iron nano-oxide solution. On the other hand, the lowest soluble sugars content was the result of culture in loamy soil, using smaller seeds, and under control nutritional condition (no spraying) (Table 2).

Essential oil

Based on comparison of the mean effects of the three factors of the study, the highest percentage of essential oil (8.91%) resulted from culture in clay loam soil, using large seeds, and with application of 30 ppm iron nano-oxide. The lowest essential oil content occurred with culture in loamy soil, using smaller seeds and under control nutritional conditions (no spraying) (Table 2).

Catalase (CAT)

Based on the results of the comparison of the average of the three factors of the lowest catalase enzyme level occurred for clay loamy soil, the use of large size seeds, and application of 30 ppm of iron Nano-oxide (3.82 U mg^{-1} protein) was obtained. Also, the highest amount of catalase enzyme resulted from the loam soil, using seeds with other small and control nutrient conditions (no spraying) factor combination (Table 2).

Vigor tests and seedling emergence percentage in pot soil

The relationship between the results of vigor tests as germination percentage and seedling emergence percent from pot soil was investigated using a T-test (Fig. III). Also, the relationship between electrical conductivity measurement as dS m^{-1} and seedling emergence in pot soil was evaluated by Pearson correlation coefficient. The results showed that among the vigor tests used in this study, only the Hiltner test could accurately and significantly estimate seedling emergence in soil (Table III). As the figure shows, applying a cold test with lower temperature or duration and an accelerated aging test with longer time may make it possible to estimate the emergence of *M. officinalis* L. seedlings in soil, although more research is required for this. Furthermore, there was a negative, albeit non-significant, relationship between EC test and seedling emergence in soil (r=-0.29, p=0.38).

Table 3. T-test probability level of vigor test results with seedling emergence in soil.

Vigor Tests	P value
Hiltner Test	0.188^{ns}
Cold Test	0.024^{*}
Standard Germination Test	0.003**
Accelerating Aging Test	0.000**

ns, *, and ** indicate non-significant, significant at 0.05, andsignficant at 0.001 probability levels, respectively.

Discussion

To make economical use of medicinal plants in the agricultural production fields and to use the potential of such natural resources, proper research on the cultivation of these plants is essential. Considered in the present study were the effects of seed size and soil type on germination and emergence of *M. officinalis* and the effect of these factors on some physiological characteristics of seedlings. In addition, the best test for seedling emergence in soil was determined amongst several common seed vigor tests.

Foliar application of Fe is important to meet plant needs of Fe as a key microelement, which is difficult to absorb in most soils, often due to calcareous conditions (Elemike et al., 2019). By determining the final germination percentage of a seed mass and the percentage of seedlings, it is possible to understand the potential of seed germination and emergence and establishment of

seedlings in the field (Powell et al., 1984). Seed germination is one of the most important phenological stages of crop plants and plants in general; it can have a major effect in determining the production rate of each crop and is one of the most important criteria for plant growth, which is influenced by seed strength. The higher the quality and vigor of seeds, the higher the percentage germination (Farhoudi et al., 2020). Seed size is an important factor affecting uniform emergence and establishment of crop plants. Seed size depends on the processes that take place at the time of seed filling. Seed size and environmental conditions can interact with rapid seedling growth. Seed size not only plays a role in the ability to germinate and emerge at deeper planting depths, but also leads to higher seed tolerance for germination under conditions of high soil density (Benvenuti and Mazzoncini, 2021).

In the present study, decrease in the seed size affected plant quality in two ways. First, the percentage of seedlings appearing in pots decreased. Poor seedling emergence from small seeds, which have lower growth than normal seedlings, use less environmental facilities such as light, moisture, and soil nutrients, and are more sensitive to adverse conditions. Therefore, the optimal quality of lemon balm seedlings grown from smaller seeds was significantly lower than those produced from larger seeds.

One of the goals of foliar application of iron oxide nanoparticles in this experiment was to improve the robustness of weak seedlings produced from smaller seeds. Foliar feeding of iron oxide nanoparticles to seedlings from smaller seeds improved physiological quality of *M. officinalis* seedlings to some extent, but its effect on medium size seeds was greater than smaller seeds. The results showed that with increasing iron content,

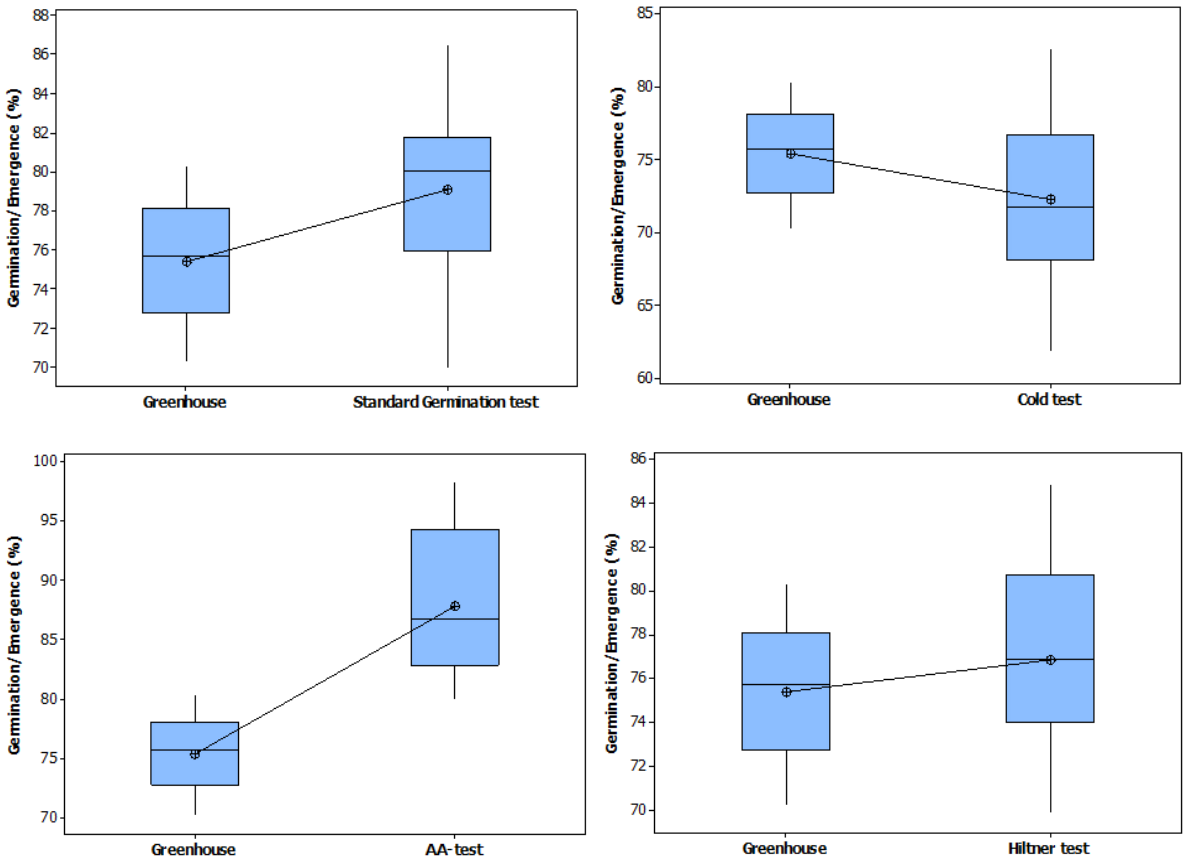

Fig. III. Box plot of the relationship between vigor tests results and seedling emergence percentage in soil (greenhouse) according to T-test. AA-test: accelerated aging test.

most of the growth characteristics increased compared to the control. Considering that the soil is mostly calcareous in the agricultural and garden lands of Iran, the use of nano-iron fertilizer, due to its light and small particles and high responsiveness, can be considered as an appropriate solution across a range of environments. Due to the inactivation of high levels of Fe in some calcareous soils and the reduction of iron absorption in other soils, iron deficiency symptoms such as calcium-induced chlorosis are observed. There are various methods to eliminate iron deficiency such as the use of acidic modified materials, iron chelates, organic compounds, and foliar application of elements (Morales et al., 1998). Foliar feeding (foliar application) and iron chelators are two general methods of application. Iron compounds are the best solution to remove iron chlorosis in all soils, especially in alkaline soils, and can cure the most severe iron-related nutritional problems in plants (Rodríguez-Lucena et al., 2010). Foliar spray addition of elements such as iron, boron, manganese, and copper is more suitable than their application through soil because when nutrients are added to the soil, they adsorb to soil particles and then have less access to the root environment. By the foliar application method, the elements are directly available to the shoots of the plant (Wissuwa et al., 2008). The role of iron in photosynthesis is due to its participation in oxidation and reduction reactions in chloroplast, in which iron participates in electron donor-receptor groups. By increasing the amount of photosynthesis in order to prevent over-accumulation of photosynthetic materials in chloroplasts, the transfer of these substances to active growth tissues is increased and this stimulates overall plant growth (Marschner et al., 2011).

The effect of foliar application of levels of iron oxide nanoparticles on physiological characteristics and growth of *Lallemantia iberica* showed that iron oxide nanoparticles significantly increased physiological characteristics such as chlorophyll and carotenoid contents, and growth variables except leaf number. However, there was no significant effect on proline levels (Javanmard et al., 2022). Mitigating the effect of environmental stresses by micronutrients can be attributed to the activity of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase in plant cells and reduction of toxicity of reactive oxygen species ROS, which modulates stress conditions (Lipiec et al., 2013). Research on the effect of drought stress and foliar application of iron oxide nanoparticles on quantitative and qualitative traits of sesame seeds suggested that foliar application of iron oxide nanoparticles, especially at 15% concentration, is recommended to reduce the harmful effects of drought stress and improve quantitative and qualitative traits of sesame (Karamian Hasan Abadi et al., 2021).

Emergence is the first and most sensitive stage of plant growth and development because the early stages of plant growth including germination, growth and seedling establishment play an important role in the subsequent development of plants. The main point of seed planting is to place seeds at a certain distance apart and depth in the seedbed. Smooth and uniform planting depth provides a better cultivation area for each seed, this is crucial for the timing of the uniformity of emergence, germination rate, and later for crop development and productivity (Batlla and Benech-Arnold, 2007). A major problem for seedlings is the strength of soil resistance, which changes continuously depending on the physical and mechanical properties of the soil. Soil texture, moisture content, compactness, strength, and other physical properties of soil, which are the sources of variability in seed position at planting time (Gonzalez-Andujar et al., 2016). For this reason, comparative evaluation of *M. officinalis* seeding conditions in two different soil textures showed that germination and establishment of healthy plants in clay loam soils were greater than loamy soils. In explaining this phenomenon, it can be stated that in clay loam soil, the clay content in the heavier soil texture was higher than loamy. Heavier soils have higher electrical conductivity due to higher ratios of clay and silt. Such soils have higher water contents and more favorable conditions for rapid seed germination (ISTA, 2009); the distribution of aggregate size in seedbed affects germination, development, and productivity of crops (Baskin and Baskin, 2004).

Poor soil porosity, low oxygen permeability, and poor seed cover cause hypoxic conditions in the soil matrix around the seeds. Such hypoxia leads to an increase in fermentation-related volatile metabolites such as acetaldehyde, methanol, and acetone, which play known toxic roles in the seed germination process (Holm, 1972). Germination tests are used as a qualitative index in evaluation of seeds and seedlings obtained from them. But it cannot be considered as a major indicator of seed quality in the field; the seed is enclosed by cultivation in soil in special environmental conditions that differ significantly from ideal laboratory conditions. It is important to know in such conditions which seed vigor test has the greatest relationship to field conditions. In this case, by identifying the correct test, it is possible to do more accurate testing prior to planting to select seeds that have better capabilities regarding emergence and establishment on the farm. Therefore, in this study, in addition to evaluating and grading the potency and vigor of lemon balm seeds using seed vigor assay tests, the relationship between these tests with the rate of seedling emergence and green cover under pot conditions was investigated in order to predict seedling status, in pots. The results of this study showed that the Hiltner test was able to predict the percentage and rate of emergence of lemon balm seedlings. The cold test was somewhat consistent with pot results, but standard germination, accelerated aging, and electrical conductivity tests lacked this capability. Therefore, we can suggest the Hiltner test to predict the emergence status of *M. officinalis* in pots.

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References

Awad, R., A. Muhammad, T. Durst, V. L. Trudeau, and J. T. Arnason. 2009. Bioassay-guided fractionation of lemon balm (Melissa officinalis L.) using an in vitro measure of GABA transaminase activity. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*. 23:1075-1081.

Badawi, G.H., Y. Yamauchi, E. Shimada, R. Sasaki, N. Kawano, and K. Tanaka. 2004. Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts. *Plant Science.* 166:919-928.

Baskin, C.C., and J. M. Baskin. 2004. Germinating seeds of wildflowers, an ecological perspective. *Hort Technology.* 14:467-473.

Batlla, D., and R. L. Benech-Arnold. 2007. Predicting changes in dormancy level in weed seed soil banks: implications for weed management. *Crop Protection*. 26:189-197.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry.* 72:248-254.

Benvenuti, S., and M. Mazzoncini. 2021. "Active" Weed Seed Bank: Soil Texture and Seed Weight as Key Factors of Burial-Depth Inhibition. *Agronomy*. 11:210.

Chimungu, J.G., K. W. Loades, and J. P. Lynch. 2015. Root anatomical phenes predict root penetration ability and biomechanical properties in maize (*Zea mays*). *Journal of Experimental Botany*. 66:3151-3162.

Descalzi, C., O. Balocchi, I. López, P. Kemp, and J. Dörner. 2018. Different soil structure and water conditions affect the growing response of *Lolium perenne* L. and *Bromus valdivianus* Phil. growing alone or in mixture. *Journal of Soil Science and Plant Nutrition.* 18:617-635.

Eisvand, H. R., and Z. Farajollahi. 2017. Study of seed storability and seed physiological quality of two ecotypes of sage (*Salvia officinalis*) using AA test. *Journal of Plant Production Research*. 24:147-151.

Elemike, E.E., I. M. Uzoh, D. C. Onwudiwe, and O. O. Babalola. 2019. The role of nanotechnology in the fortification of plant nutrients and

improvement of crop production. *Applied Sciences.* 9:499.

Farhoudi, R., A. Modhej, and M. Motamedi. 2020. Evalution of *Arctium lappa* seed dormancy breaking methods. *Iranian Journal of Seed Sciences and Research*. 7:505-517.

Gonzalez-Andujar, J.L., G. R. Chantre, C. Morvillo, A. M. Blanco, and F. Forcella. 2016. Predicting field weed emergence with empirical models and soft computing techniques. *Weed Research*. 56:415-423.

Hassan, F. A., and M. M. Hassan. 2018. The Use of Seed Vigour Tests for Predicting Field Emergence. *International Journal of Applied Science*. 1:p93-p93.

Hampton, J.G., and D. M. Tekrony. 1995. Handbook of vigour test methods. The International Seed Testing Association, Zurich (Switzerland).

Havir, E.A., and N. A. McHale. 1987. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiology.* 84:450-455.

Holm, R. E. 1972. Volatile metabolites controlling germination in buried weed seeds. *Plant Physiology*. 50:293-297.

Irigoyen, J., D. Einerich, and M. Sánchez-Díaz. 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologia Plantarum*. 84:55-60.

ISTA. 2009. International Rules for Seed Testing. *Seed Science and Technology.* (Supplement), 27:1-3.

Javanmard, A., M. Ashrafi, M. R. Morshedloo, M. A. Machiani, and F. Rasouli Maggi. 2022. Optimizing phytochemical and physiological characteristics of Balangu (*Lallemantia iberica*) by foliar application of chitosan nanoparticles and Myco-Root inoculation under water supply restrictions. *Horticulturae*. 8:695.

Jha, Y., and H. I. Mohamed. 2023. Inoculation with Lysinibacillus fusiformis strain YJ4 and Lysinibacillus sphaericus strain YJ5 alleviates the effects of cold stress in maize plants. *Gesunde Pflanzen*. 75:77-95.

Kapoor, R., B. Giri, and K. G. Mukerji. 2002. Mycorrhization of coriander (*Coriandrum sativum* L.) to enhance the concentration and quality of essential oil. *Journal of the Science of Food and Agriculture*. 82(4):339-342

Karamian Hasan Abadi, Z., H. R. Eisvand, M. Daneshvar, and O. Akbarpour. 2021. Study the effect of drought stress and iron oxide nanoparticle foliar application on quantitative and qualitative traits of sesame (*Sesamum indicum* L.). *Environmental Stresses in Crop Sciences.* 14:375-386.

Lichtenthaler, H. K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology.* 148:350-382. https://doi.org/10.1016/0076-6879(87)48036-1.

Lipiec, J., C. Doussan, A. Nosalewicz, and K. Kondracka. 2013. Effect of drought and heat stresses on plant growth and yield: a review. *International Agrophysics.* 27.

Mahmoud, A.W.M., A. A. Ayad, H. S. Abdel-Aziz, L. L. Williams, R. M. El-Shazoly, A. Abdel-Wahab, and E. A. Abdeldaym. 2022. Foliar application of different iron sources improves morpho-physiological traits and nutritional quality of broad bean grown in sandy soil. *Plants.* 11:2599.

Marin, M., G. Laverack, A. A. Powell, and S. Matthews. 2018. Potential of the electrical conductivity of seed soak water and early counts of radicle emergence to assess seed quality in some native species. *Seed Science and Technology*. 46:71-86.

Mimmo, T., D. Del Buono, R. Terzano, N. Tomasi, G. Vigani, C. Crecchio, R. Pinton, G. Zocchi, and S. Cesco. 2014. Rhizospheric organic compounds in the soil–microorganism–plant system: their role in iron availability. *European Journal of Soil Science.* 65:629-649.

Morales, F., R. Grasa, A. Abadía, and J. Abadía. 1998. Iron chlorosis paradox in fruit trees. *Journal of Plant Nutrition.* 21:815-825.

Marschner, P., D. Crowley, and Z. Rengel. 2011. Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis–model and research methods. *Soil Biology and Biochemistry*. 43:883-894.

Paquin, R., and P. Lechasseur. 1979. Studies of a method for the determination of free proline content in plant extracts. *Canadian Journal of Botany*.

Powell, A., S. Matthews, and M. D. A. Oliveira. 1984. Seed quality in grain legumes.https://pascalfrancis.inist.fr/vibad/index.php?action=search&lang=en&terms=%22POWELL%2C+A.+A%22&index=au

Yudharaj, P., M. Shankar, R. Sowjanya, B. Sireesha, E. A. Naik, and R. J. Priyadarshini. 2016. Importance and uses of medicinal plants–An overview. *International Journal of Preclinical and Pharmaceutical Research.* 7:67-73.

Zhang, J., and B. Ghanem. 2018. ISTA-Net: Interpretable optimization-inspired deep network for image compressive sensing. *Proceedings of the IEEE Conference on Computer Vision and Pattern Recognition.* 1828-1837.