Quantitative and qualitative improvement of the content of some medicinal compounds of the pencil tree (*Euphorbia tirucalli* L), using chelate iron fertilizer

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Abstract

Euphorbia tirucalli or pencil tree contains bioactive substances with therapeutic effects. The present study investigated the effect of N20P20K20 fertilizer with a concentration of 3 g/l individually or in combination with chelate iron fertilizer, 0.2 or 0.4 g /80 cm² of soil, on fresh and dry weight, and on terpenoid content of this plant. A total of 3 treatments in four replications were considered in pots for 11 months. Control plants received no fertilizer during this period. The experiment was conducted in a complete randomized block design. Contrary to usual, the growth of the pencil tree did not show a positive response to any of the fertilizers and even the fresh weight of the roots significantly reduced. Although no significant difference was observed in terms of total terpenoid content between treatments, based on the results of gas chromatography and mass spectrometry (GC-MS), 11 bioactive terpenoids with medicinal properties (including antimicrobial and anti-inflammatory properties) were found in considerable amounts in the combined treatment, N20P20K20 and chelate iron fertilizer, 0.2 g/80 cm² of soil. It is noteworthy that out of these eleven compounds, only 3 cases were identified in control plants and only 5 cases in plants obtained from individual N20P20K20 treatment. The results of the present study indicate the positive effect of chelate iron fertilizer on the reorientation of secondary metabolism of *Euphorbia tirucalli* towards the production of valuable medicinal compounds.

Keywords: bioactive, chelate iron, GC-Mass, nitrogen, pot culture, secondary metabolite

Bagheri, F., A. Sateei, M. Ahmadi Golsefidi and M. Ebadi . 2025. 'Quantitative and qualitative improvement of the content of some medicinal compounds of the pencil tree (*Euphorbia tirucalli* L), using chelate iron fertilizer*'. Iranian Journal of Plant Physiology 15* (3), 5647-5656.

Introduction

Plants need the iron more than all other micronutrients. Iron is one of the low-consumption and immobile essential elements for plants(Pirzad and Barin, 2018). Nevertheless, due to the plant's greater need for it compared to other trace elements, some classify iron as a high-consumption element. Iron is important because of its two vital functions(Pooladvand et al., 2012). It is part of the catalytic group of many oxidoreductase enzymes and plays an important role in the synthesis of chlorophyll, or in the synthesis of heme containing proteins (Peyvandi et al., 2011). Deficiency of this element always

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Received: November, 2022
Accepted: March, 2024

causes simultaneous destruction of chlorophyll and destruction of chloroplast structure ((HOSSEINI et al., 2016; Zokaee Khosroshahi et al., 2023). Studies show a positive effect of iron fertilizer on different crops and medicinal plants such as cowpea (Khalaj et al., 2020), sunflower (Mirzapour and Khoshgoftarmanesh, 2008), lettuce (Roosta and Jafari, 2013), thyme (Yadegari and Ghorbani, 2012), anise (Nateghi et al., 2016), and Lemongrass (Hassani Moghadam et al., 2020).

Euphorbia tirucalli belongs to the family Euphorbiaceae and is native to the tropics. This plant has pencil-shaped bushes, which is why it is also called pencil tree (Julius and Damme, 2011). Studies have shown that the ingredients in different parts of this plant have medicinal properties and can be used as medicine after extraction and purification (Mwine and Damme, 2011). It has also been found that bioactive compounds in this plant may have antioxidant, antimicrobial, antifungal, and anti-cancer properties (Mali and Panchal, 2017). This plant contains a significant amount of latex, which has a large amount of sterols and terpenoids (Uchida et al., 2010).

Plants have the ability to produce different types of metabolites. It is estimated that there are over one million metabolites in the entire plant kingdom. Most of these compounds are secondary specialized metabolites that play several physiological and ecological roles in plants. These roles include defense against vegetarians and pathogens, attracting pollinators and grain carriers, as well as taking part in signaling procedures (Yonekura-Sakakibara et al., 2019). Terpenoids or isoprenoids are natural isoprene-based products with essential roles in the metabolism of all organisms. Plants have relatively high amounts of secondary metabolites, and many of them can be considered as terpenoids. The evolution of terpenoids secondary metabolism in plants has been mainly achieved by increase in primary metabolism genes such as cytochrome P450 and terpene synthase gene families in plant genomes (Bergman et al., 2019). The chemical diversity of terpenoids reflects a natural history that is partly created by herbivores' stress or other selective pressures from animals. It seems that these pressures lead to the emergence of different types of terpenoids in plants, with wide applications in medicine and natural products (Gershenzon and Dudareva, 2007). Numerous studies on the genus euphorbia have shown that this genus contains bioactive compounds, especially terpenoids such as diterpenes and triterpenes (Barla et al., 2006; Uchida et al., 2010; Qaisar et al., 2012; Abreu et al., 2014; Yusoff et al., 2017; Duong et al., 2019; Qi et al., 2020). For example, Fernandez-Arche et al. (2010) identified a kind of euphorbia lacteal triterpene called tirucallol and noted that it has potent anti-inflammatory properties.

Despite extensive studies around the world on Euphorbia tirucalli and bioactive compounds derived from different parts of this plant grown in natural habitats, there is no studies on the responses of this plant to application of fertilizers. Therefore, the aim of the present study was to investigate the effect of iron fertilizers on growth parameters and bioactive compounds of this ornamental-medicinal plant.

Materials and Methods

Plants cultivation

Potted planting was done for 11 months from the beginning of August, 2020 to the beginning of July, 2021. Before planting, cuttings of Euphorbia tirucalli were prepared from the greenhouse of the Islamic Azad University, Gorgan Branch, which were left in a dark place for 10 days for their ends to dry in the air. Then, the cuttings were planted in potted soil, irrigated with 40 ml of water every 15 days for 7 months to complete the rooting process. Then, the treatments were performed along with weekly irrigation for another 4 months. The applied soil was a mixture of cocopeat, peat moss, and perlite with equal amounts.

Applied fertilizers

NPK fertilizer (20 20 20) is a nitrogen fertilizer that in addition to nitrogen, phosphorus, and potassium also contains small amounts of some metal elements (magnesium, iron, zinc, manganese, copper, molybdenum). The ferric chelate fertilizer used in this study was the brand name HUM IRON, the main components of which being biologically active, including humic acids

along with iron, zinc, and manganese, in their chelate form. Although some other metal cations accompany this fertilizer, it is known as iron fertilizer in the market.

Experimental treatments

Control and experimental treatments included the following

The control and experimental groups in the study included soil without fertilizer, nitrogen fertilizer treatment N.P.K (20.20.20) without iron fertilizer, and combined treatment of nitrogen and iron (HUM) fertilizers + N.P.K (20.20.20) which is called HUM + 20 in this study.

These treatments were prepared for iron fertilizers in the amount of 0.2 and 0.4 g per 80 cm squares of potting soil surface (C2 and C4, respectively), and for NPK fertilizer, 3 g/l, in the amount of 40 ml, that was applied for each irrigation period during 4 months from the beginning of applying treatments. Irrigation was performed for control with common water. Thus, 3 treatments and one control were prepared in the present research.

Measurement of growth parameters

Four months after the start of the treatments, the plants were harvested for the laboratory assays. Growth parameters including fresh and dry weight of roots and shoots were measured. To determine dry weight, the plant sample, including shoots and roots, was dried at 90 ℃ for 24 hours in an electric oven and then weighed by a digital scale.

Total terpenoid assay

Following Ghorai, et al. (2012), 7 ml of 95% methanol was added to 1 g of fresh plant sample in a mortar. The mortar was then placed in an ice bucket, and the plant material was ground near ice to obtain a homogeneous solution, which was then transferred into test tubes. For centrifugation, the test tubes were placed in 4000 rpm at room temperature for 15 minutes and the supernatants were collected in other test tubes. Three ml of chloroform was poured into another test tube and then 500 µl of the collected supernatant was added to the chloroform. After vortex, the test tubes were left for 3 minutes and then 200 ml of concentrated sulfuric acid was added to each of the test tubes. The test tubes were then cooled in an ice bucket for a maximum of 15 minutes and placed in the dark at room temperature for 1.5 to 2 hours. The supernatant was then drained very slowly and 3 ml of 95% methanol was added to the reddish-brown precipitate. When completely dissolved, the optical densities (ODs) were determined at 538 nm against blank (95% methanol). To prepare a standard sample, 200 µl of linalool solution in methanol of known concentration was added to 1.5 ml of chloroform. The concentration of total terpenoids was calculated in terms of micro equivalent linalool per gram of fresh weight using the standard equation and regarding final extract volume (3 ml).

GS-MS analysis

Measurement of bioactive compounds was also performed using a Shimadzo GC-MS device model TQ-8050, for control, NPK, and HUM+20 (C2) samples after the following steps:

Extraction by Soxhlet method

Five (5) grams of dried plant shoot powder was obtained from a mixture of five separate ground plants shoots (1 gram of the dry mass of each plant's shoot replicate), and was used for extraction process with 450 ml of n-hexane in a Soxhlet apparatus for 24 hours. Thereafter, the device was opened and the extract was rotated by a rotary equipment for further drying. An empty plate was weighed and the extract was transferred into the plate to be dried completely in the open air. Then the plate's weight was measured again for obtaining the dried extract weight.

The dried parts on the plate were then collected, and dissolved in 700 µl of methanol, from which one µl was injected into the GC-MS apparatus.

Statistical Analysis

The experiments were carried out in a complete randomized blocks design with 4 replications for each treatment and each measurement, except

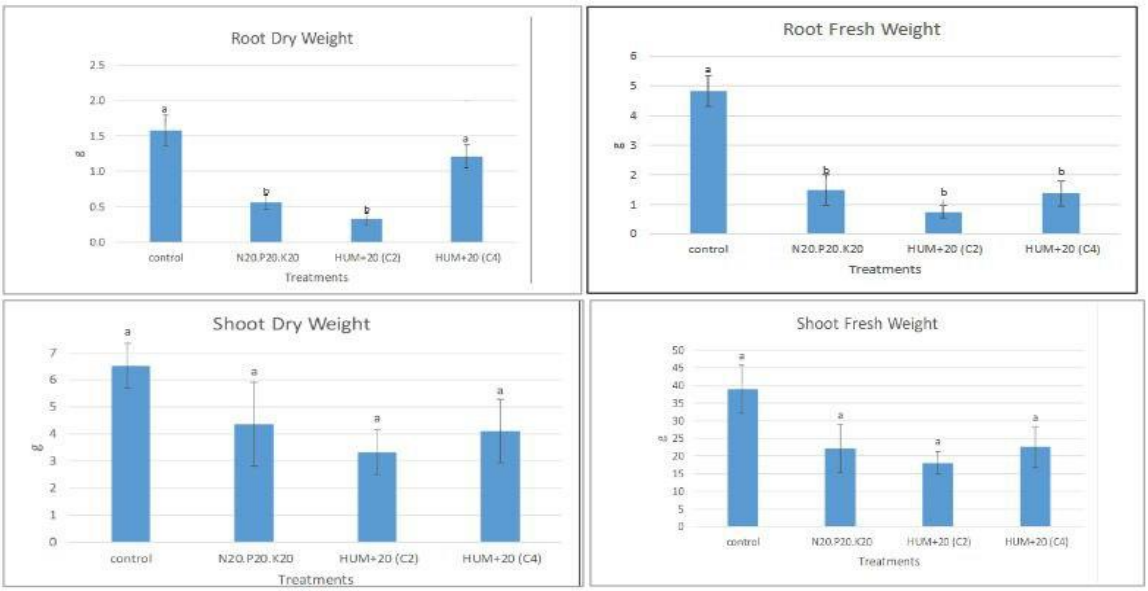


Fig. I. Fresh and dry weights of roots and shoots of *Euphorbia tirucalli* in different treatments; the same letters show no significant difference between groups ($p < 0.05$).

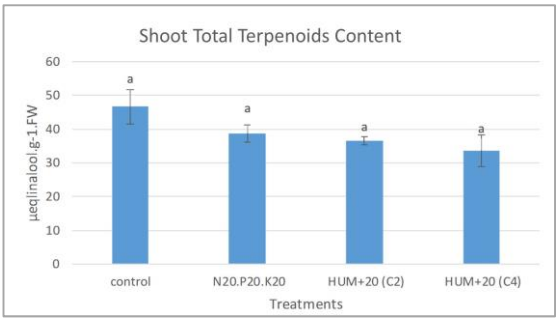


Fig. II. Total terpenoid contents in *Euphorbia tirucalli* as affected by different fertilizer treatments; the same letters show no significant difference between groups ($p < 0.05$).

for GC-MS analysis with a single replication, obtained from a mixture of five plants' pulverized shoots. Significance of treatment effects was assessed using one-way ANOVA and differences between treatments were investigated using Tukey test at $P<0.05$. Statistical measurements were performed using SPSS program (Version 23), and graphs were drawn using Excel software (Microsoft Office, 2016).

Results

The root fresh weight in the control was significantly higher than the treatments ($p < 0.05$), and no significant difference was observed between the treatments (Fig. I). The highest root dry weight was observed in control samples, with no significant difference from HUM+20(C4). Root dry weight in control and HUM+20(C4) was also significantly higher than N20P20K20 and HUM+20(C2), and there was no significant difference between N20P20K20 and HUM+20(C2). On the other hand, fresh weight and dry weight of shoots in the control were apparently higher than the treatments, although the differences were not statistically significant between all of the groups.

Results of total terpenoid content measurements in the aerial parts are shown in Fig. (II). Based on these results, although the apparent highest and lowest values belonged to control and HUM+20(C4) respectively, no significant differences were observed between treatments, or between any of treatments and control ($P < 0.05$).

Suxhelet extracted mass for 5 g of shoot dry weight, obtained from 5 samples in each

treatment or control conditions was 0.174g, 0.150g, and 0.275g for control, N20P20K20, and with N20P20K20 treatment, or control in *Euphorbia tirucalli* (Table 1).

Table 1
Some important GC-MS detectable bioactive compounds in *Euphorbia tirucalli* shoot samples, grown in N20P20K20, or HUM+20(C2) treatments, or in control conditions

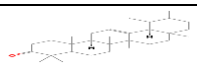
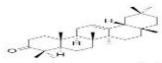
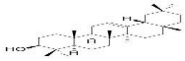
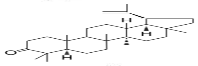
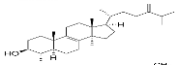
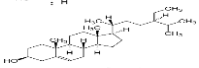
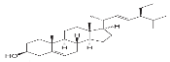
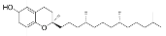
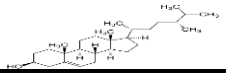
Chemical Name	Chemical Structure	Percentage in GC-MS Detectable Extract (%)		
		HUM+20 (C2)	$N_{20}.P_{20}.K_{20}$	Control
α-Amyrone		14.85	9.58	Not Found
β-Amyrone		14.85	13.84	11.73
β-Amyrin		11.28	6.40	7.81
Lup-20(29)-en-3-one		3.87	3.80	5.98
Tirucallol		16.40	0.20	Not Found
Obtusifoliol		13.65	Not Found	Not Found
β-sitosterol		19.55	Not Found	Not Found
γ-sitosterol		19.55	Not Found	Not Found
Stigmasterol		4.23	Not Found	Not Found
β-tocopherol		2.38	Not Found	Not Found
Campesterol		5.44	Not Found	Not Found

Table 2
Type and properties of terpenoids identified in thyrocal euphorbia under the influence of HUM + 20 (C2) treatment

Chemical Name	Type of Terpenoid	Properties	Reference(s)
α-Amyrone	A triterpene	Anti-inflammatory Anti-bacterial, antifungal activity	de Almeida et al., 2015
β-Amyrone	A triterpene	Anti-inflammatory Anti-bacterial, antifungal activity	de Almeida et al., 2015
β-Amyrin	A triterpene	Anti-inflammatory, Antihyperglycemic and hypolipidemic effects	Almeida Santos et al., 2012 Nogueira et al., 2019
Lup-20(29)-en-3-one	A triterpene	Anti-leukemia activities	Hata et al., 2003
Tirucallol	A triterpene	Anti-inflammatory Anti-microbial activity	Mali and Panchal, 2017
Obtusifoliol	A sterol	Anticancer and decreasing cholesterol level	Kajikawa et al., 2004
β-Sitosterol	A steroid	Antitumor activity	Aleksandrov et al., 2019
γ-Sitosterol	A steroid	Anticancer activity	Sundarraj et al., 2012
Stigmasterol	A steroid	Anticancer activity	Patil and Rajput, 2012
β-Tocopherol	Vitamin E	Antioxidant	Fritsche et al., 2017
Campesterol	A steroid	Anticancer activity	Woyengo et al., 2009

HUM+20(C2), respectively. On the other hand, the results of the study of some GC-MS detectable extracted compounds showed that the HUM + 20 (C2) treatment increased the percentage of some important bioactive compounds in comparison Among the bioactive compounds identified in HUM + 20 (C2) treatment samples, Obtusifoliol, β-sitosterol, γ-sitosterol, Stigmasterol, β-tocopherol, and Campesterol were not present in any of the control treatments and N20P20K20. Also, α-

Amyrone and Tirucallol terpenoids were not present in the control treatment.

Discussion

The results of the present study showed that applying nitrogen fertilizer alone or in combination with chelate iron fertilizer in different concentrations not only did not cause an increase in fresh weight or dry weight of shoot or root in *Euphorbia tirucalli*, but also lead to significant decrease in root system growth. These findings do not correspond to the results of the study of Mirzashahi et al. (2016), who claimed that the use of iron fertilizer improves soybean growth indices. They also explained that the positive effect of iron fertilizer on growth indices in plants could be due to the role of iron compounds in reactions related to cell division and growth. Our findings are not also in agreement with the results of Abbas et al. (2009) who reported wheat growth improvement by using NPK along with iron fertilizer. Iron is an essential nutrient for plant growth and development that plays a very important role in chlorophyll synthesis, thylakoid synthesis, and chloroplast development (Buchanan et al., 2000). Therefore, when a plant encounters iron deficiency, its hindered growth is expected. Sateei (2022) observed that negative effects of iron fertilizers on *Euphorbia tirucalli* could be due to the presence of zinc in these fertilizers and not necessarily the presence of iron itself. On the other hand, low needs of *E. tirucalli* as a desert originated plant to iron (and other macro-micronutrients), might not be overlooked. This probable low requirement, in turn, may cause negative growth responses in higher concentrations of fertilizers (Tripathi, et al., 2015).

On the other hand, the addition of iron fertilizer did not cause a significant change in the amount of terpenoids compared with the control or NPK treatments. However, very few studies have been conducted on the role of iron fertilizers on the level of secondary metabolites in plants, and it is not yet clear exactly what role iron can play in the contents of secondary metabolites. In one of the most recent published studies Bustamante et al. (2020) emphasized that the effect of fertilizer on production of terpenoids in plants prominently depends on two elements: nitrogen and phosphorus. These researchers suggested that nitrogen is an essential element for protein synthesis and terpene synthase activity, and that phosphorus is needed for the synthesis of terpenoid precursors, ATP, and NADPH, as well as for terpenoid synthesis. Access to higher concentrations of nutrients can lead to higher rates of carbon stabilization, protein synthesis, and enzymatic activity that in turn can lead to the production of more terpenoids and other secondary metabolites (Litvak et al., 1996). But based on the hypothesis that carbon is in balance with nutrients, and growth is in balance with differentiation, higher access to nutrients can lead to more plant growth instead of increasing carbon-based secondary compounds (Bustamante et al., 2020).

In addition to increasing plant access to iron through fertilization with iron-containing fertilizers, even in the case of nitrogen and phosphorus, some studies have not found a clear relationship between the concentration of these nutrients and the content of terpenoids (King et al., 2004; Blanch et al. al., 2009).

The results of the GC-MS extractable compounds analysis of Euphorbia tirucalli under HUM + 20 (C2) treatment in the present work (Table1) indicate the emergence or increase in the percentage of some bioactive compounds with different medicinal properties. This finding, beside prominently more Soxhlet extracted mass related to this treatment, may lead us more confidently to accept HUM+20(C2) as an effective treatment to increase the contents of at least 11 valuable medicinal compounds in *E. tirucalli*. Information on these bioactive compounds (which basically include terpenoids such as sterols, steroids, vitamin E, etc.) and their properties (e.g., anti-inflammatory, anti-cancer, antioxidant, etc.) is provided in Table 2. It seems that although NPK treatment, can reorient the metabolism of terpenoids in E. tirucalli, this reorientation is more prominent, qualitatively and quantitative, when NPK is combined with iron chelate fertilizer.

Conclusion

The use of NPK fertilizer alone or in combination with iron chelate significantly reduced the growth

of the root system of Euphorbia tirucalli, compared to the control samples, but did not cause a significant change in the fresh and dry weight of the shoot. Also, fertilizer treatment did not cause a significant difference in terpenoid content of the entire shoot. However, the results of GC-MS showed that the content of 11 bioactive compounds (sterols or other terpenoids) with medicinal properties increased in HUM + 20 (C2) treatment, which indicates the effectiveness of this fertilizer to increase the beneficial bioactive compounds in this plant by changing in the terpenoid metabolism pathways.

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