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Response of Rosemary (*Rosmarinus officinalis*) Plants to Riboflavin Foliar Application: Improvement in Growth, Nutrient Content, Antioxidant Enzyme System, Essential Oil Production, and Its Antioxidant Potential

Hebatallah Aly#, Fatma A. Gharib, Eman Z. Ahmed
Botany Department, Faculty of Science, Helwan University, Cairo 11795, Egypt.

ROSEMARY (*Rosmarinus officinalis*) is a valuable aromatic herb native to the Mediterranean region. This study investigates the effects of foliar application of riboflavin on rosemary plants during two seasons in 2021. The first cutting season was in summer (August), and the second was in winter (December). Foliar application of riboflavin at different concentrations (25, 50, and 100mg L^{-1}) increased all growth attributes at two cuts by increasing nutrients, photosynthetic pigments, and photosynthetic activity. Riboflavin at 50mg L^{-1} gave the highest mean values of all the measured growth attributes at both the first and second cuts. It also increased total photosynthetic pigments by 38%. Moreover, nutritional value, essential oil production, and overall antioxidant properties were positively enhanced in riboflavin-treated plants. Additionally, it showed the highest values of both element content and uptake. It increased oil yield (L fed^{-1}) by 116% and 160% at the 1st and 2nd cuts, respectively, with the highest antioxidant potential (IC_{50} = 9.93µL mL^{-1}) at the second cut. Furthermore, riboflavin at 50 mg L^{-1} enhanced the activities of antioxidant enzymes catalase and peroxidase. In contrast, the activities of oxidative enzymes including ascorbic acid oxidase, indole-3-acetic acid oxidase, polyphenol oxidase, and ribonuclease decreased. Furthermore, plants treated with 50mg L^{-1} riboflavin increased the RNA and DNA content. These findings confirm the efficiency of the use of riboflavin at 50mg L^{-1} to improve the growth of rosemary plants with high nutritional content and enhance the antioxidant activity of their essential oil.

Keywords: Antioxidant enzymes, Essential oils, Riboflavin, *Rosmarinus officinalis*, Vegetative growth.

Introduction

Rosemary (*Rosmarinus officinalis*) is a small, evergreen aromatic herb from the *Lamiaceae* family. The plant is native to the Mediterranean region and widely cultivated with year-round availability in Egypt. In addition, rosemary is very valuable economically for its therapeutic and ornamental properties (Nieto et al., 2018; Oliveira et al., 2019).

Rosemary is one of the highly beneficial spices within the *Lamiaceae* family. It is a natural and safe antioxidant alternative to synthetic chemicals. Hence, this plant is utilized as a preservative and antioxidant, particularly in the food industry (Karakol & Kapi, 2021).

The extracts and essential oils of rosemary herb contain various phytochemicals that have pharmacological activities (Aziz et al., 2022), including antioxidant (Habtemariam, 2023), antimicrobial (Hendel et al., 2016; Megateli & Krea, 2018), anticancer (Moore at al., 2016; Huang et al., 2023), and anti-inflammatory (Gonçalves et al., 2022). Besides, rosemary essential oil (EO) has hepatoprotective potential (Rašković et al., 2014), and it improves blood circulation and memory, and activates the immune system (Ahmed & Babakir-Mina, 2020). Rosemary constituents have also

#Corresponding author email: Hebatallah.aly181@science.helwan.edu.eg; hebatallah_aly@yahoo.com
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been used in cosmetics and healthcare products (González-Minero et al., 2020).

These protective effects are mostly related to the phenolic composition and content (Moreno et al., 2006; Nabavi et al., 2015). Treatment, soil, and collection time influence plant growth, yield, chemical composition, and quality of rosemary oil and extract (Lakušić et al., 2013; Gharib et al., 2016a; Mwithiga et al., 2022).

Both the oil and extract from rosemary leaves possess antioxidant properties comparable to known antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, ascorbic acid, and α-tocopherol, but without the cytotoxic and carcinogenic risks associated with synthetic antioxidants (Wang et al., 2008; Rašković et al., 2014; Hendel et al., 2016).

Recently, the application of vitamins to enhance plant growth, quality, and production has received great attention. Riboflavin (vitamin B2) is a water-soluble vitamin. It is an abiotic elicitor that enhances the production of antimicrobial compounds in plants, thereby increasing their resistance to diseases. Furthermore, riboflavin plays a crucial role in various metabolic processes, promoting overall plant growth and development (Olfat et al., 2022). The active forms of riboflavin are flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), synthesized from riboflavin-by-riboflavin kinase (Suwannasom et al., 2020). Both FMN and FAD are two cofactors that play an essential role in the physiological processes of plants, maintaining normal metabolism and cellular functions, as they are involved in various enzymatic reactions, including electron transfer and redox reactions, which are crucial for energy production and the synthesis of important molecules such as DNA and proteins. Additionally, FMN and FAD act as antioxidants, protecting cells from oxidative damage and promoting overall growth (Hu & Guo, 2023). These cofactors serve as essential parts for enzymes that scavenge ROS, like glutathione reductase and NADPH-thiol reductase, and they also function as cofactors for many enzymes that produce reactive oxygen species (ROS), including NADPH oxidase, polyamine oxidase, glycolate oxidase, and fatty acyl CoA dehydrogenase (Sandoval et al., 2008). Riboflavin, also known as a photosensitizer, stimulates the synthesis of ROS (Huang et al., 2006; Deng et al., 2014; Hauvermale & Steber, 2020). Flavoenzymes catalyze the final step in the biosynthesis of heme and chlorophyll in plants (Eggers et al., 2021). Currently, there is a scarcity of research on how riboflavin is metabolized and transported in plants (Lynch & Roje, 2022; Hu & Guo, 2023).

Riboflavin is one of the B-group vitamins that improved the uptake of zinc by clusterbean plants more than mustard plants (Gopalarao et al., 1987). Applying riboflavin at a moderate concentration (50mg L^{-1}) plus micronutrients effectively boosted the growth, oil yield, and crop productivity of flax cv. Giza 5 (El-Shahawy et al., 2008).

Under normal, non-stress conditions, overexpression of the riboflavin-binding protein in transgenic (REAT11) *Arabidopsis thaliana* promotes the transition to flowering, vegetative growth, and photosynthetic capacity compared to the wild type WT Col-0 plant due to having 1.5 times more riboflavin (Deng & Dong, 2013). Previous studies demonstrated that exogenous riboflavin treatment could improve plant disease resistance through the induction of defensive responses instead of the direct inhibition of pathogen growth (Mahmoud et al., 2020). It stimulates plant defense reactions such as the expression of defense-related enzymes including polyphenol oxidase, phenylalanine ammonialyase, and peroxidase, the accumulation of higher levels of phenolics, lignin, and flavonoids (Li et al., 2012), as well as the expression of numerous defense response genes. In addition, riboflavin is essential in both peroxidation and anti-oxidation processes, stimulating ROS synthesis, particularly H_2O_2 and O^{-}_2, during an oxidative burst, which are important signaling molecules that trigger the activation of the plant defense response. Riboflavin application enhanced pathogen resistance in grapevine against downy mildew (Boubakri et al., 2013) and against the fungus *Rhizoctonia solani,* which causes sheath blight in rice (Taheri & Tarighi, 2010). In addition to the role of riboflavin as an antioxidant that protects plants from biotic stress, riboflavin also acts as an effective antioxidant against abiotic stress. For example, riboflavin enhanced drought tolerance in *Hibiscus sabdariffa* salinized seedlings (Azooz, 2009) and tobacco plants (Deng et al., 2014) regulating osmotic and ionic balance.

The present study aims to investigate the effects of foliar application of riboflavin at different concentrations (0, 25, 50, and 100mg L^{-1}) on rosemary plants during two cutting seasons.

Therefore, the main objectives are as follows: (1) measuring overall plant growth in terms of growth parameters, photosynthetic pigments and activity, and essential oil yield; (2) determining the leaf content of nutrients, total carbohydrates, total soluble sugars, and proteins; (3) evaluating the antioxidant capacity of rosemary essential oil.; and (4) assessing the changes in the activities of various antioxidant and oxidative enzymes to evaluate the overall antioxidant properties of rosemary plants under different riboflavin treatments.

Materials and Methods

Plant material

Uniform stem cuttings of rosemary were provided by the Medicinal and Aromatic Plants Research Branch, Horticulture Research Institute, Agriculture Ministry.

The experiment design

A pot experiment was carried out at the Experimental Farm of Helwan University, Cairo, Egypt, from February 2021 to December 2021. Rosemary was grown in pots that were 45cm in diameter and had 16kg of clay loam soil in them. In each pot, two cuttings were planted. The pots were separated into four groups. Riboflavin stock solution (1g/L) was prepared by dissolving 1g of riboflavin in a minimum amount (a few drops) of sodium hydroxide solution (100mM), then completed to 1L by distilled water. Distilled water is used to prepare the different riboflavin treatments from the stock solution.

Fresh solutions of different concentrations of riboflavin (25, 50, and 100mg L^{-1}) were sprayed on the leaves of each group twice, and each group was irrigated with water.

Spraying was applied 75 days post-transplantation, and a second application was done a week later. Treatments were repeated after two months from the initial cut. Distilled water was used to spray the control plants, and the spraying solution volume was kept high enough to completely cover the plant's leaves until it began to drip. There were four treatments, each with four replications, and three plants for each replicate. Pots were organized in a completely randomized block design. From the rosemary plants, two cuts were taken: the first in August (summer), six months after transplanting, and the second four months later (in winter, December). Each pot was fertilized with one gram of ammonium nitrate (33.5% N), two grams of calcium superphosphate (15.5% P_2O_5), and one gram of potassium sulfate (48% K_2O). After planting, these fertilizers were applied twice, 60 and 75 days apart, and once more after the 1st cut. Plant irrigation was performed regularly according to the weather to maintain the soil's moisture content at a field-capacity level.

Analysis of soil

Digestion of soil samples followed McGrath & Cegarra (1992) and evaluation was based on Jackson (1973).

Growth parameters

The aerial parts of rosemary plants (leaves and stems) were harvested by cutting the plants 10 cm above the soil surface at the full blooming stage. Growth attributes were recorded for two cuts: plant height (in cm), branches' number for each plant, and fresh (FW) and dry (DW) weight of herb (g $plant^{-1}$). Plant samples were air-dried until a constant weight was reached. Fresh representative samples were obtained to demonstrate essential oil content and some metabolic activities for each treatment.

Isolation of essential oil

The amount of essential oil in each treated plant during the two cuts was determined by using hydro-distillation method. Fresh rosemary samples from different treatments were hydro-distilled for three hours in a Clevenger apparatus. For different treatments, the oil yield per plant was calculated. After filtration, the resulting oil was dried using anhydrous Na_2SO_4 and then kept in sealed dark glass vials at 3–4 °C.

The content and activity (Hill reaction) of photosynthetic pigments

The concentration of photosynthetic pigments, including chlorophyll (a and b), and carotenoid, was measured in fresh rosemary leaves according to the procedure described by Metzner et al. (1965), and the results were expressed as mg g^{-1} DW equivalent. The method described by Aronoff (1946) and Osman et al. (1982) was used to calculate the photosynthetic activity of isolated chloroplasts. The concentration of reduced potassium ferricyanide ($K_3Fe(CN)_6$), an electron acceptor, was demonstrated using the standard curve and expressed as µmol ferricyanide g chlorophyll $^{-1}$ sec^{-1} (Arnon & Shavit, 1963).

Macro and micro elements

Air-dried leaves from the second cut were

analyzed for their levels of nitrogen, phosphorus, potassium, calcium, magnesium, manganese, iron, and sodium. Following wet digestion of leaf samples, total N content was calculated using the AOAC-modified Micro-Kjeldahl method (1980). The vanadate molybdate method was used to determine P levels (Jackson, 1973). Flame photometer method using atomic absorption spectrometer (ASS Vario 6) was used to determine K and Na concentrations (Williams & Twine, 1960). An inductively coupled plasma (ICP-MS) spectrometry model, Jobin Yvon-Ultima 2, was used to determine Ca, Mg, Mn, and Fe.

Antioxidant activity of rosemary essential oil

The 2,2-diphenyl–l-picrylhydrazyl (DPPH) radical scavenging assay (Brand-Williams et al., 1995) was used to measure the antioxidant activity of rosemary EO from the second cut as affected by different riboflavin treatments. Rosemary EO at different concentrations (1, 5, 10, 25, and 50µL mL^{-1}) was diluted five times with a DPPH methanolic solution. A methanolic solution of DPPH (0.4mM) was used as a blank. The samples were stored in the dark at room temperature. Reduction in free radicals was measured spectrophotometrically at 517 on a UV-Vis spectrophotometer (Jenway 6405) at the end of the reaction (30 minutes). Butylated hydroxytoluene (BHT) is used as a standard reference. All samples were analyzed in triplicate. To calculate the percentage inhibition of the DPPH radical, we utilized the following formula:

$$\% \text{ Inhibition} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

The sample concentration that provides 50% inhibition (IC_{50}) was detected from the graph by plotting inhibition percentage against oil concentration. The radical scavenging activity was identified based on the IC_{50} value.

Antioxidants and oxidative enzymes

Preparation of enzyme extract

Enzyme extract was prepared using fresh rosemary leaves from different treatments at the second cut. According to Kar & Mishra (1976), 20mL of cold Na/K phosphate buffer (0.1M; pH 6.8) was mixed with 1g of fresh tissues. Following filteration, the mixture was centrifuged at 4°C and 6000rpm for 10min. The supernatant was diluted to a known volume and spectrophotometrically assayed for the activities of the following enzymes.

Assay of antioxidant enzymes' activity: catalase and peroxidase

Catalase (CAT) (EC 1.11.1.6)

CAT was measured using Góth's (1991) procedure with a slight modification. Two mL of H_2O_2 were added to 0.4mL of the enzyme extract (65mM H_2O_2 in Na/KP, pH 7.4) to start the reaction. After 4min of incubation at 25°C, the reaction was stopped by adding 1 ml ammonium molybdate (4g L^{-1}). The residual H_2O_2 was monitored spectrophotometrically at 405nm using a Jenway spectrophotometer (6405 UV/Vis model).

Peroxidase (POX) (EC 1.11.1.7)

POX activity was demonstrated following Yamane et al. (1999) modified method. 0.2mL of enzyme extract was mixed with 5.8 ml of an assay mixture made of 4.4mL of 10mM potassium phosphate buffer, 0.4mL of H_2O_2, and 1mL of guaiacol. The changes in the absorbance at 436 nm from 30sec to 3min revealed the enzyme's activity.

Assay of oxidative enzymes' activities

Polyphenol oxidase (PPO) (EC 1.14.18.1)

PPO activity was measured using Mayer & Harel's (1979) modified assay. One mL of enzyme extract was mixed with three mL of buffered catechol solution (0.01M catechol, freshly prepared in 0.1M phosphate buffer, pH 6.0). The enzyme activity was measured using a Jenway 6405 UV/Vis spectrophotometer at 495nm, with readings taken every 30sec for 5min.

Ascorbate oxidase (AO) (EC 1.10.3.3)

The AO assay was determined according to Maxwell & Bateman's (1967) technique. In a clean quartz cuvette, 3mL of the assay mixture, consisting of 1mL of 0.2M phosphate buffer (pH 6.2), 0.2mL of 1mM ascorbic acid, 1.7mL of d.H_2O, and 0.2mL of crude enzyme extract, were combined. The AO activity was calculated by measuring the optical densities at 265nm at 30sec intervals for up to 3min via a Jenway 6405 UV/Vis spectrophotometer.

IAA oxidase (IAAO) (EC 1.2.3.7)

IAA activity was detected using Gordon & Weber's (1951) modified technique. About 0.2mL enzyme extract was mixed with a 1.8 assay mixture composed of 0.2mL of IAA (2mM), 0.2mL of $MnCl_2$ (1mM), 0.2mL of 2-4 dichlorophenol (1mM), and 1.2mL of potassium phosphate buffer (pH 6.5). IAA was added to start the reaction; then, as a control, 1mL of the reaction mixture was added to 2 ml of Salkowski's reagent (0.5M ferric chloride ($FeCl_3$) and 35% perchloric acid

($HClO_4$) in a 1:50 ratio). The remaining reaction mixture (1mL) was incubated for an additional hour in the dark at 30°C, and 2mL of Salkowski reagent were added, followed by further incubation for half an hour at 35°C to complete the reaction. Salkowski's reagent forms a pink-colored complex with IAA and the developed color was monitored spectrophotometrically (Jenway 6405 UV/Vis) at 562nm.

Different enzyme activities were expressed as variations in optical density (OD) g^{-1} DW equivalent hr^{-1}.

Nucleic acids and ribonuclease activity

Nucleic acids from fresh rosemary leaves were extracted according to Marmur (1961). DNA and RNA were quantitatively determined applying the methods adopted by Dische & Schwartz (1954) and Ashwell (1957), respectively. Ribonuclease activity was detected by recording the change in optical density (OD) of mg FW hr^{-1} at 260nm according to Malik & Singh (1980). The resultes were calculated and expressed as OD mg^{-1} DW equivalent hr^{-1}.

Total carbohydrates, total soluble sugars, and proteins

Total carbohydrates and total soluble sugars

Total carbohydrates and soluble sugars were both measured in the dried leaf samples at the second cut using the anthrone assay as described by Umbreit et al. (1959). One hundred milligrams of leaf powder were ground in five milliliters of 70% ethanol to determine the total soluble sugars. Followed by 20min of centrifugation at 4000rpm. After that, distilled water was used to dilute the supernatant to 15mL. Three mL of the sample is mixed with 6 ml of anthrone solution (2g of 95% H_2SO_4 L^{-1}) and kept on a boiling water bath for 3min. Using a spectrophotometer (Jenway 6300), the developed color was measured at 620nm after cooling. Measurements were performed in triplicate for each treatment. The content of total soluble sugars in the samples was calculated using a standard curve of glucose. 45mg of powdered leaves were mixed with 15mL of 1N H_2SO_4 and hydrolyzed in digestion tubes (85–90°C) for 8h to determine the amount of total carbohydrates. Complete the mixture to 45mL by distilled water. Then the total carbohydrates were measured by using the previously described anthrone assay (Umbreit et al., 1959).

Total soluble protein

Dry powdered leaves (100mg) were ground in 5mL of 70% ethanol, and the supernatant was centrifuged at 4000rpm for 20min before being diluted to a volume of 15mL with distilled water. The total soluble protein level was determined using the method of Lowry et al. (1951). 5mL of a fresh mixed solution (50:1 v/v) of 0.5% copper sulfate in 1% sodium tartrate and 2% sodium carbonate in 0.4% sodium hydroxide were homogenized with 1 ml of plant extract. Before adding 0.5mL of Folin, the mixture was left for 10min. After 30min, a spectrophotometer (Jenway 6300) was used to detect the mixture's optical density at 750nm. Three replicates were measured for each treatment. The protein level in the samples was identified via a standard curve made from bovine serum albumin.

Statistical analysis

The experiments were carried out in a completely randomized design. All samples were analyzed in triplicate. The data was analyzed using IBM Statistical Product and Service Solutions, SPSS software Version 26 for Windows (George & Mallery, 2019). Duncan's multiple comparison test was used to detect the statistical significance between means and analysis of variance (ANOVA) at $P < 0.05$. The results were represented as mean value ± standard error (SE).

Results

Analysis of soil

Based on the physical and chemical properties of the soil used in the experiment, the soil type is clay-loamy in texture and slightly alkaline. In Table 1, additional soil characteristics are listed.

Growth parameters

The results presented in Fig. 1 show the effect of foliar application of riboflavin at 25, 50, and 100mg L^{-1} on the rosemary**'s** growth attributes, including plant height, branch number per plant, and fresh and dry leaf and stem weights at two cuts. Foliar application of riboflavin promoted all growth attributes when comparing the corresponding untreated control plants to the treated plants at two cuts. Application of riboflavin at 50mg L^{-1} significantly increased all the growth parameters, giving the highest mean values of plant height (31.5, 24cm), the number of branches (20.63, 35.75), stem fresh weight (8.80, 19.83g $plant^{-1}$) and dry weight (4.35, 11.25g $plant^{-1}$), and leaf fresh weight (39.42, 62.59) and dry weight (14.76, 24.84g $plant^{-1}$) at the first and second cuts, respectively.

TABLE 1. Physical and chemical characteristics of the soil samples from the experimental farm (Faculty of Science, Helwan University) used for the cultivation of rosemary plants in the pot experiment

Physical Properties							Cations and anions (mg/kg)									
Clay (%)	Silt (%)	Fine sand (%)	Coarse sand (%)	Soil texture	pH	EC (dS/m)	SP	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	HCO_3^-	CO_3^-	SO_4^{--}	$CaCO_3$ %	Organic matter (%)
52.96	28.04	13.14	5.86	Clay loamy	7.8	7.26	43	19.19	0.66	37.40	26.24	26.50	2.28	0.0	54.72	5.86	1.05

EC = Electrical conductivity; SP = Saturation percent

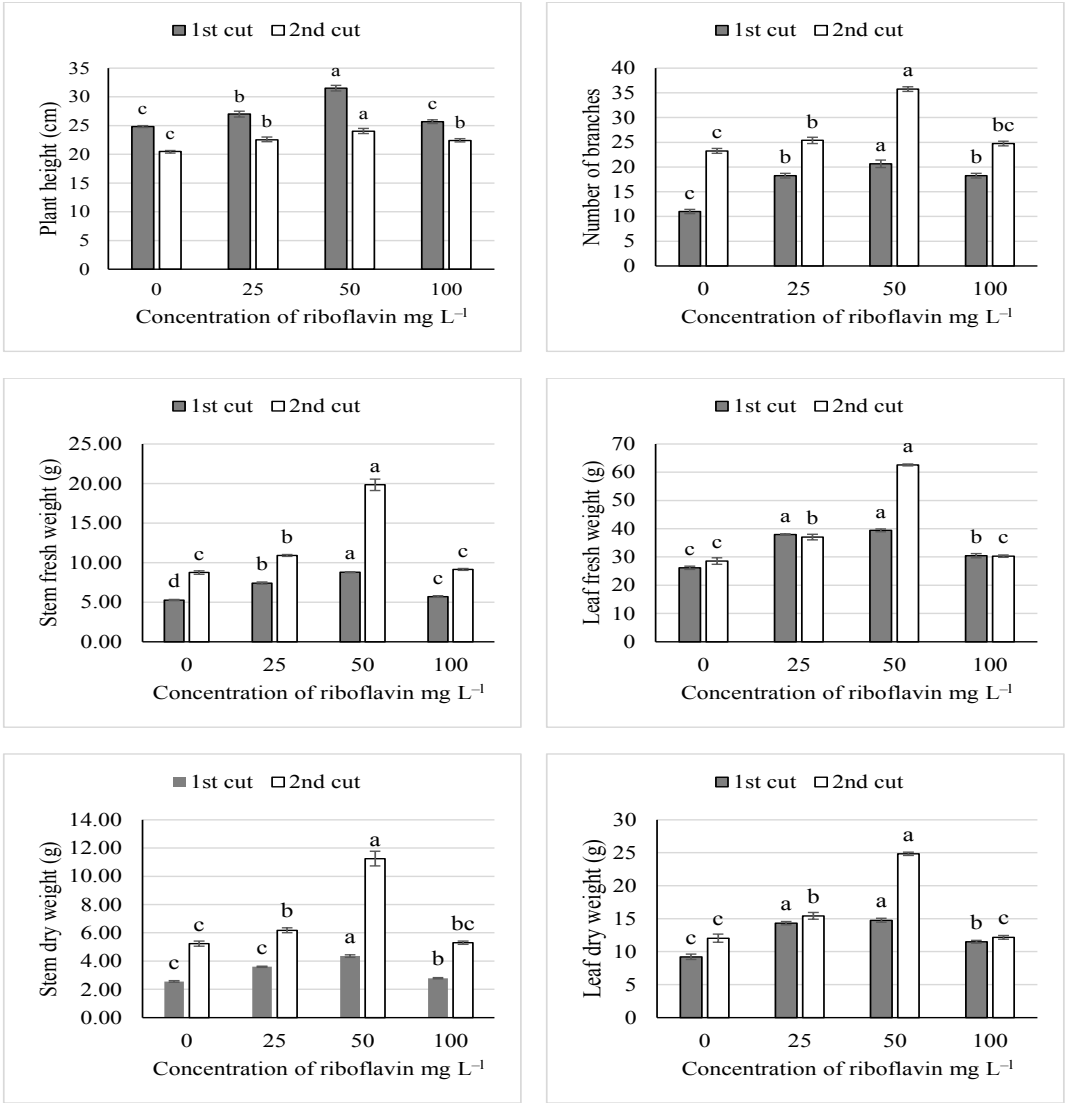

Fig. 1. Effect of foliar application of riboflavin at 0, 25, 50, and 100mg L^{-1} on the vegetative growth of rosemary (*R. officinalis*) plants at the 1st and 2nd cuts (6 and 10 months from transplanting, respectively) [Each result is the mean of 10 replicates. Different letters indicate significant differences between treatments (Duncan test, P ≤ 0.05). Vertical bars represent ± SE]

Photosynthetic activity and pigments

Applying of any concentration of riboflavin to the rosemary leaves increased photosynthetic activity and the content of different photosynthetic pigments (chlorophylls a, b, and carotenoids) and consequently total photosynthetic pigments more than control in the leaves of rosemary plants under study at the second cut (Table 2). However, the most effective concentration was riboflavin at 50mg L^{-1}. This concentration showed the highest levels of chlorophyll a (8.96 mg g^{-1} DW equivalent), chlorophyll b (2.96mg g^{-1} DW equivalent), and carotenoids (8.41mg g^{-1} DW equivalent). As a result, there was a significant increase in the pigment content (20.33mg g^{-1} DW equivalent) and photosynthetic activity (1.34µmol ferricyanide g^{-1} chlorophyll sec^{-1}) compared to the control sample.

Macro- and micro elements

Despite the applied treatment, foliar application of riboflavin increased the content and uptake (mg $plant^{-1}$) of N, P, K, Mg, Mn, Fe, and Ca in the dried leaves of rosemary plants. However, the only exception was the Na content, which decreased and its uptake was affected differentially when comparing the corresponding untreated control plants to the treated plants at the second cut (Table 3). The most effective treatment was riboflavin at 50mg L^{-1}. In most cases, riboflavin applied at 50mg L^{-1} showed the highest values of both element content and uptake. At this treatment, the N concentration (2.13%) was the highest macronutrient, followed by P (0.324%). The micronutrients with the highest concentration were Ca (50.97ppm), Mg (6.41ppm), and Fe (3.67ppm). Consequently, riboflavin 50mg L^{-1} treatment showed the highest percentage change in the leaf nutrient content of riboflavin-treated rosemary plants (Fig. 2).

Total carbohydrates, soluble sugars, and proteins

The data in Fig. 3 shows significant enhancements in the content of total carbohydrates, soluble sugars, and soluble proteins in plants at the second cut upon treatment with riboflavin. The data indicated that in the 2nd cut, foliar application of riboflavin at 50mg L^{-1} obtained maximum values of total carbohydrates (252.68mg g^{-1} DW), soluble sugars (55.68mg g^{-1} DW), and soluble proteins (328.55mg g^{-1} DW, whereas the minimum values were found in the control plants' leaves.

Oil content

The data illustrated in Fig. 4 demonstrate that essential oil percent and yield per feddan based on the dry weight of rosemary plants significantly increased upon the application of riboflavin compared to the control sample. In general, the oil yield in plants from the second cut was higher compared to the first cut. Plants treated with 50mg L^{-1} riboflavin showed the highest values of oil content (0.27, 0.29mL/100 g) and yield (7.17, 11.9L $feddan^{-1}$) at the first and second cuts, respectively.

TABLE 2. Riboflavin–induced changes in photosynthetic activity (µmol ferricyanide g^{-1} chlorophyll sec^{-1}) and pigments (mg g^{-1} DW equivalent) in the leaves of rosemary (*Rosmarinus officinalis*) plants at the second cut (10 months from transplanting) [Values are means of triplicate ± standard error. Different letters indicate significant differences between treatments (Duncan test, P ≤ 0.05)]

Riboflavin concentration mg L^{-1}	Photosynthetic activity (µmol ferricyanide g^{-1} chlorophyll sec^{-1})	Photosynthetic pigments (mg g^{-1} DW equivalent)				
		Chl. a	Chl. b	Chl. a+b	Carotenoids	Total pigments
0.0 (control)	0.90 ± 0.00 b	7.12 ± 0.07 d	2.07 ± 0.01 c	9.18 ± 0.06 c	5.54 ± 0.03 d	14.73 ± 0.06 c
25	1.23 ± 0.07 a	7.80 ± 0.01 b	2.18 ± 0.02 b	9.98 ± 0.01 b	5.91 ± 0.04 c	15.89 ± 0.04 b
50	1.34 ± 0.09 a	8.96 ± 0.01 a	2.96 ± 0.02 a	11.92 ± 0.02 a	8.41 ± 0.07 a	20.33 ± 0.09 a
100	1.31 ± 0.06 a	7.68 ± 0.02 c	2.19 ± 0.02 b	9.87 ± 0.03 b	6.10 ± 0.06 b	15.98 ± 0.09 b
LSD at 5%	0.334	**0.123**	0.113	0.687	0.193	1.167

TABLE 3. The effect of riboflavin foliar spray at 0, 25, 50, and 100mg L^{-1} on the macronutrient and micronutrient content and uptake of air-dried rosemary (*Rosmarinus officinalis*) plant leaf tissue at the second cut (10 months from transplanting)

Riboflavin concentration (mg L^{-1})	Macronutrients content (%)			Micronutrients content (ppm)				
	N	P	K	Mn	Fe	Mg	Ca	Na
0.0	1.90	0.287	0.81	0.132	1.64	4.99	46.62	23.24
25	1.97	0.291	0.88	0.154	2.67	5.88	47.43	18.95
50	2.13	0.324	1.34	0.166	3.67	6.41	50.97	13.00
100	2.06	0.316	0.98	0.159	2.74	6.26	50.94	15.09
	Macronutrients uptake (mg $plant^{-1}$)			Micronutrients uptake (mg $plant^{-1}$)				
0.0	327.94	49.54	139.81	0.23	2.83	8.61	80.47	40.11
25	344.16	50.84	153.74	0.27	4.66	10.27	82.86	33.11
50	768.72	116.93	483.61	0.60	13.25	23.13	183.95	46.92
100	445.17	68.29	211.78	0.34	5.92	13.53	110.08	32.61

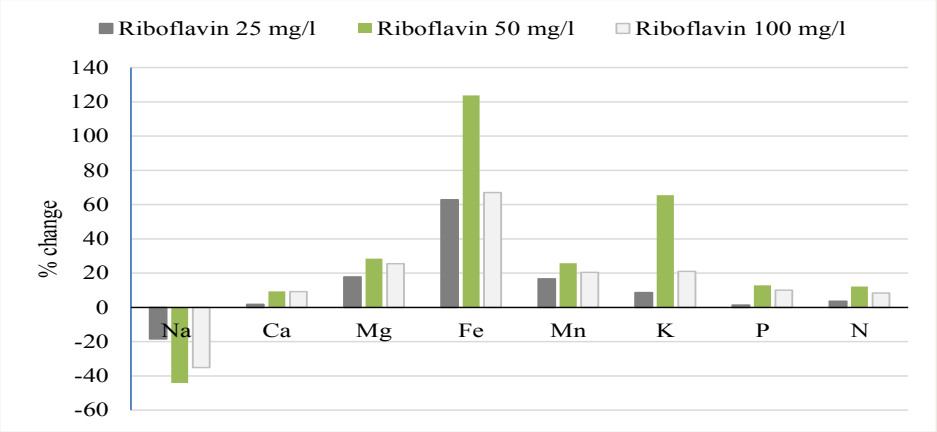

Fig. 2. Percent change in micro (N, P, K) and macro (Mn, Fe, Mg, Ca, and, Na) nutrients in leaves of rosemary-treated plants as influenced by riboflavin foliar application at 0, 25, 50, and 100mg L^{-1} at the second cut (10 months from transplanting)

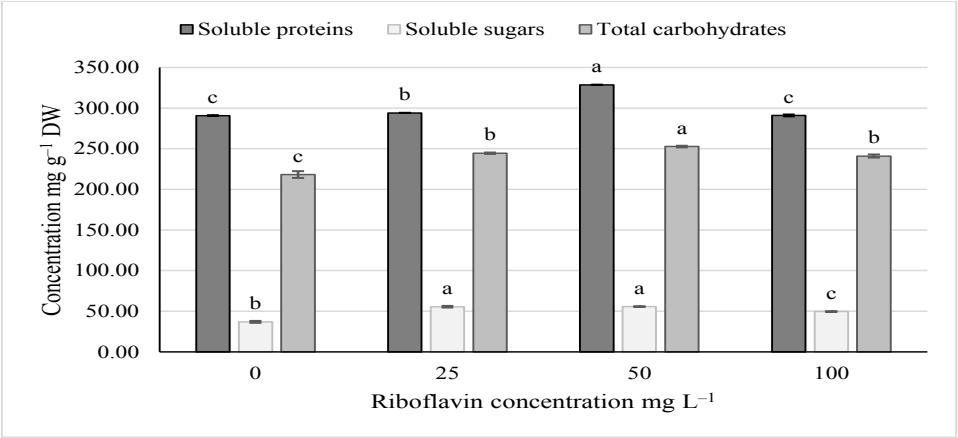

Fig. 3. Effect of foliar application of riboflavin at 0, 25, 50, and 100mg L^{-1} on the contents of total carbohydrate, soluble sugars, and proteins (mg g^{-1} DW) of rosemary (*R. officinalis*) plants at the 2nd cut (10 months from transplanting) [Each result is a mean of three replicates. Different letters indicate significant differences between treatments (Duncan test, $P \leq 0.05$). Vertical bars represent ± SE]

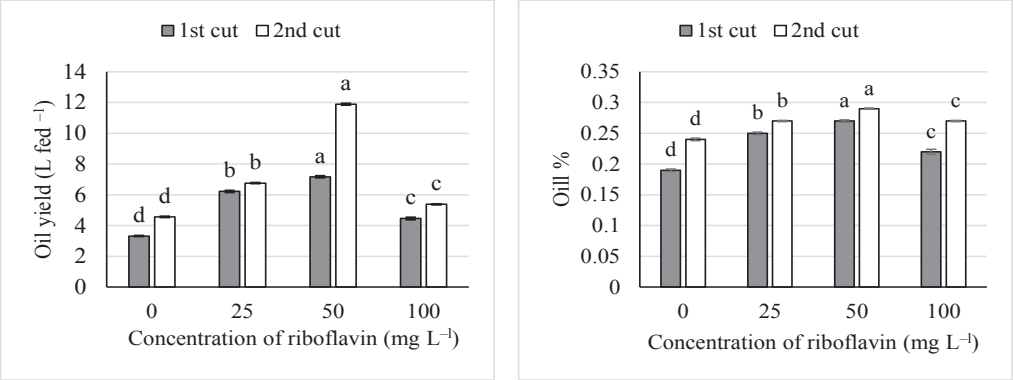

Fig. 4. Effect of riboflavin foliar application at 0, 25, 50, and 100mg L^{-1} on oil percentage (mL $100g^{-1}$) and oil yield (L fed^{-1}, Feddan (fed)=4200 m^2) content of rosemary (*R. officinalis*) plants at the 1st and 2nd cuts (6 and 10 months from transplanting, respectively) [Each result is a mean of three replicates. Different letters indicate significant differences between treatments (Duncan test, P ≤ 0.05). Vertical bars represent ± SE]

Antioxidant activity of the essential oil (EO)

The antioxidant activity of EOs from the second cut was measured using DPPH radical scavenging, as shown in Fig. 5. The results indicated that all tested EOs exhibited antioxidant potential, irrespective of the applied riboflavin concentration. Higher radical scavenging activity was observed in EOs from plants treated with riboflavin. The EO extracted from the leaves sprayed with 50mg L^{-1} of riboflavin had the highest antioxidant potential (IC_{50} = 9.93µL mL^{-1}), followed by the EO extracted from the leaves sprayed with 25 mg of riboflavin (IC_{50} = 14.9µL mL^{-1}), compared to the standard reference BHT (IC_{50}= 19.45µg/mL). The EO obtained from the control sample had the lowest antioxidant potential with IC_{50}= 30.48µL mL^{-1}.

Changes in enzymes' activity

Antioxidant and oxidative enzymes' activity

The data in Fig. 6 demonstrate that riboflavin foliar application at 25, 50, and 100mg L^{-1}, significantly increased the activity level of the antioxidant enzymes catalase (CAT) and peroxidase (POX). Low concentrations of riboflavin (25 and 50mg L^{-1}) significantly increased the activity of CAT and POX enzymes, whereas the high concentration of 100mg L^{-1} decreased it. The treatment with riboflavin at 50mg L^{-1} caused the highest activity of the CAT and POX enzymes, while it decreased the activity of the oxidative enzymes ascorbic acid oxidase (AO), polyphenol oxidase (PPO), indole-3-acetic acid oxidase (IAAO), and ribonuclease (RNase) in the rosemary plants' leaves from the 2nd cut.

Nucleic acid (RNA and DNA) content

The data presented in Fig. 7 showed that riboflavin foliar application stimulated a higher accumulation of nucleic acids (RNA, DNA, and RNA+DNA) compared to the control sample. Application of riboflavin at concentrations of 25 and 50mg L^{-1} enhanced the accumulation of nucleic acids, whereas riboflavin at 100mg L^{-1} decreased it. Plants treated with riboflavin at 50mg L^{-1} accumulated the highest amounts of RNA and DNA.

Discussion

Rosemary is an aromatic herb; its extract and oil are widely used due to their safe and eco-friendly properties (Xylia et al., 2022; Anuradha & Bharadvaja, 2023). Furthermore, it is a valuable source of various bioactive phytochemicals (Miljanović et al., 2023), and it exhibits antimicrobial, antitumor, and antioxidant characteristics (Alhaithloul et al., 2023; Habtemariam, 2023; Huang et al., 2023). Riboflavin (vitamin B2) is a precursor for the flavo-coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which constitute important redox cofactors in plants (Fischer & Bacher, 2011). These cofactors are required for different physiological processes such as DNA repair, fatty acid oxidation, photosynthesis, photoreception, mitochondrial electron transport, metabolism of various cofactors, and many secondary metabolites' biosynthesis (Deng & Dong, 2013; Eggers et al., 2021; Olfat et al., 2022, Hu & Guo, 2023). Moreover, flavoenzymes catalyze the final step in the biosynthesis of heme and chlorophyll in plants (Eggers et al., 2021).

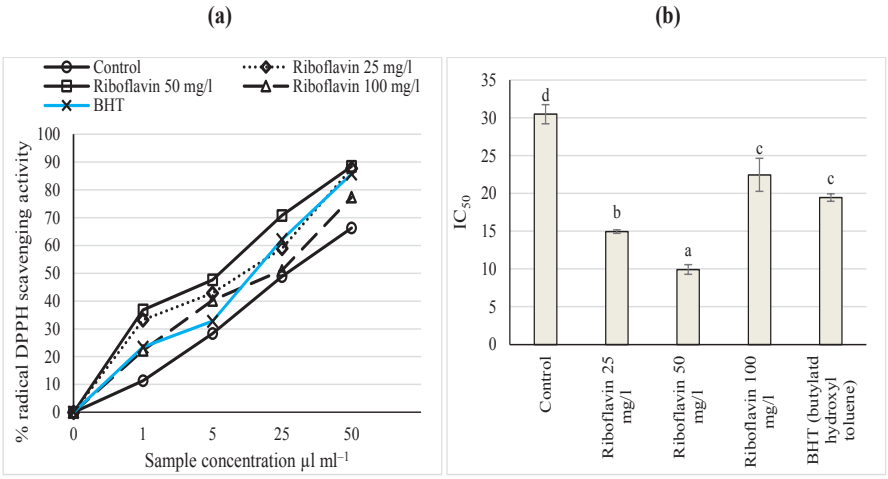

Fig. 5. Free radical scavenging activity of rosemary essential oil at different concentrations (1, 5, 10, 25, and 50µL mL^{-1}) using the DPPH (2,2-diphenyl–l-picryl-hydrazyl-hydrate) assay (a) and IC_{50} values (b), as affected by riboflavin foliar application at 0, 25, 50, and 100mg L^{-1} [Butylated hydroxytoluene (BHT) is used as a standard reference. Each result is the mean of three replicates. Different letters indicate significant differences between treatments (Duncan test, P ≤ 0.05). Vertical bars represent ± SE]

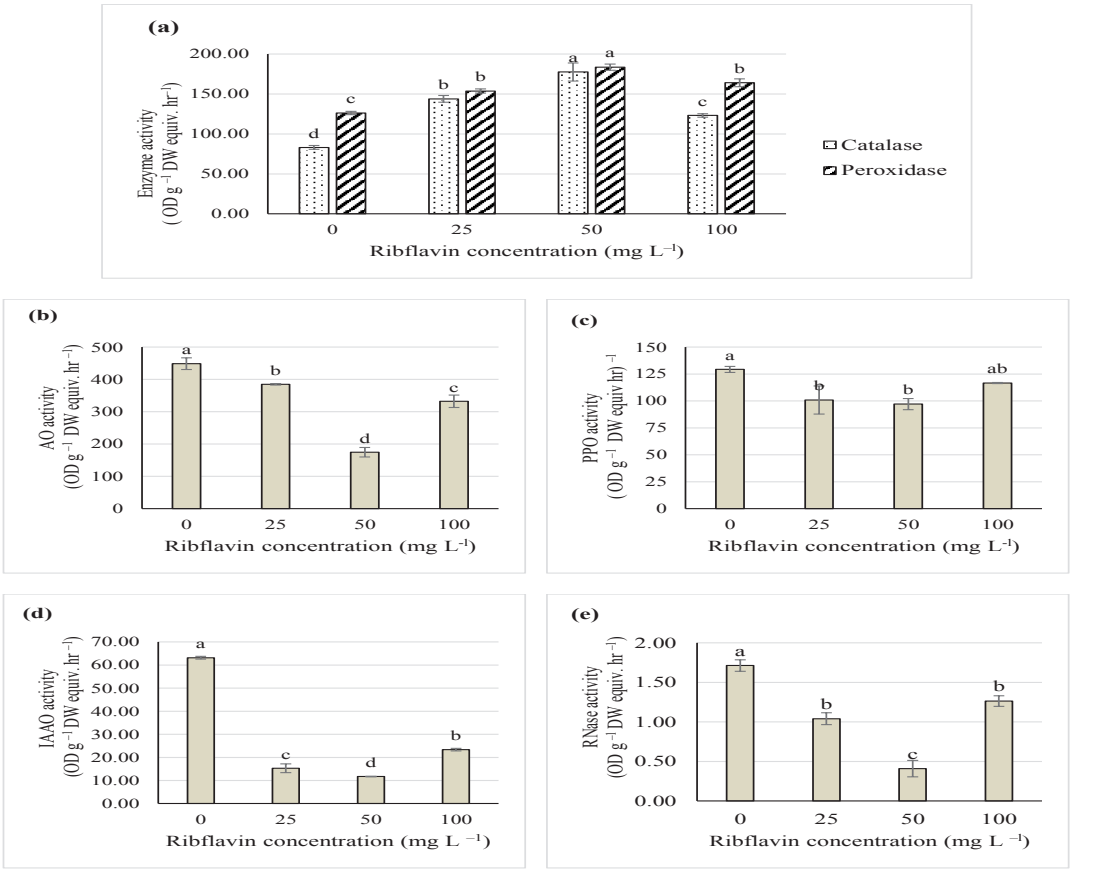

Fig. 6. Effect of riboflavin (0.0, 25, 50, and 100mg L^{-1}) foliar application on the activities of the antioxidant enzymes: CAT, catalase, and POX, peroxidase (a); and the oxidative enzymes: AO, ascorbic acid oxidase (b), PPO, polyphenol oxidase (c), IAAO, indole-3-acetic acid oxidase (d), and RNase, ribonuclease (e), in leaves of rosemary plants from the 2nd cut (10 months from transplanting) [Each result is the mean of three replicates. The activities of different enzymes were expressed as changes in optical density (OD) g^{-1} DW equivalent h^{-1}. Different letters indicate significant differences between treatments (Duncan test, P ≤ 0.05). Vertical bars represent ± SE]

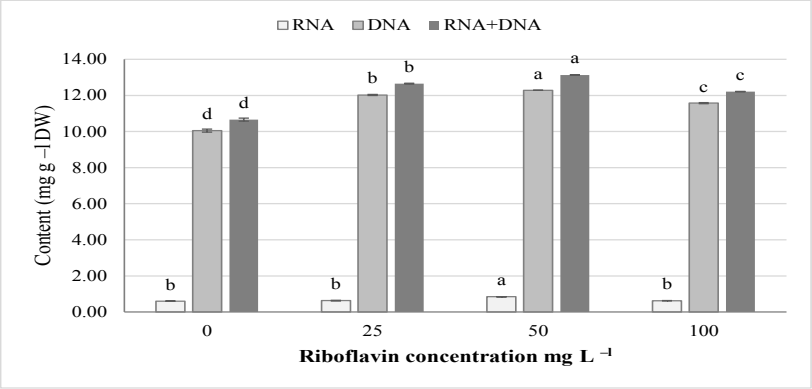

Fig. 7. Effect of riboflavin (0.0, 25, 50, and 100mg L^{-1}) foliar application on nucleic acid (RNA and DNA) content, in leaves of rosemary plants from the 2nd cut [Each result is the mean of three replicates. Different letters indicate significant differences between treatments (Duncan test, P ≤ 0.05). Vertical bars represent ± SE]

This study demonstrated that riboflavin foliar application, especially at 50mg L^{-1}, significantly increased vegetative growth (plant height, plant's number of branches, and FW and DW of leaves and stems), photosynthetic activity, pigment content (Chl. a, Chl. b, and carotenoids), and total photosynthetic pigments, as well as increased uptake of nutrients, compared to respective controls at the 1st and 2nd cutting. Such enhanced plant development in response to riboflavin application might be attributed to the riboflavin-induced increase in the production of photosynthetic pigment and carbon assimilation (Palacios et al., 2014; Hu et al., 2021). Furthermore, riboflavin plays a crucial role in chloroplast development (Hu et al., 2021). A previous study proved that applying a moderate concentration of riboflavin (50mg L^{-1}) plus micronutrients effectively increased the growth and crop productivity of flax cv. Giza 5 (El-Shahawy et al., 2008). Riboflavin at 90 mg L^{-1} improved the vegetative growth and photosynthetic pigment content of geranium (El-Lethy et. al., 2011). Riboflavin-treated cluster bean and mustard plants showed significantly higher zinc uptake (Gopalarao et al., 1987). Additionally, under drought-free conditions, low and moderate (4 and 20µM) levels of riboflavin increased chlorophyll levels in tobacco plants (Deng et al., 2014). Pea (*Pisum sativum*) chloroplasts synthesized FAD from externally added riboflavin (Sandoval et al., 2008). Compared to the wild type, a 1.5-fold increase in total riboflavin level in transgenic (REAT11) *Arabidopsis thaliana* promoted vegetative growth, net photosynthetic capacity, and the transition to the flowering stage (Deng & Dong, 2013). Riboflavin is the precursor of FMN and FAD, which are involved in carotenoid biosynthesis (Eggers et al., 2021). This may explain the higher content of carotenoid observed in the riboflavin-treated plants compared to the control plants. In addition to light harvesting, carotenoids are crucial for the photoprotection of the photosystems (Xu et al., 2020).

In this study, regarding the percent change in the leaf nutrient content of riboflavin-treated rosemary plants, Fe recorded the highest % increase (123.78%) compared to the untreated plants. The content of leaf Fe is positively correlated with chlorophyll content (Xiao et al., 2021). Previous studies have reported that iron (Fe) deficiency inhibits chlorophyll biosynthesis and the development of chloroplasts, leading to chlorosis in plants (Guo et al., 2020; Li et al., 2021). Moreover, riboflavin treatment (50mg L^{-1}) caused the highest content of micro- and macronutrients, which play crucial roles in various physiological processes of plants. Macronutrients like N and P are essential for plant growth and development, and micronutrients like Ca, Mg, Fe, and Mn are involved in enzyme activation and photosynthesis (Tariq et al., 2023).

The results of the present study showed that both total carbohydrates and total soluble sugars had the highest levels in plants treated with 50 mg L^{-1} riboflavin. This is probably due to an increase in the efficiency of photosynthesis as a result of increased photosynthetic activity, pigment content (Chl. a, Chl. b, and carotenoids), and total photosynthetic pigments (Gharib et al., 2016b).

The data revealed that riboflavin significantly increased essential oil percent and yield at both cuts. Oil yield (liter per feddan) increased by 116% and 160% at the 1st and 2nd cuts, respectively, as

influenced by riboflavin application at 50mg L^{-1} in rosemary relative to the control sample. The development of plants and the production of secondary metabolites can be influenced by both biotic and abiotic elicitors (Thakur et al., 2019; Bisht et al., 2023). The current study suggests that the foliar application of riboflavin, which functions as an abiotic elicitor according to Olfat et al. (2022), has the capacity to increase the production and accumulation of essential oils, as previously shown by El-Lethy et al. (2011). Besides, the increased oil content at both the 1st and 2nd cuts could be due to better vegetative growth, nutrient uptake, photosynthetic rate, and final biomass (Sifola & Barbieri, 2006; Ardalani et al., 2017), or it could be due to changes in the total volume of oil glands in the leaves (King et al., 2006).

The antioxidant capacity of rosemary EOs from the second cut was measured with DPPH radical scavenging. The results were expressed as IC_{50} (the concentration that provides 50% inhibition), so a lower IC_{50} value means a higher antioxidant activity. The current study revealed that all tested EOs had antioxidant potential, regardless of the riboflavin concentration used. Higher radical scavenging activity was observed in EOs from plants treated with riboflavin which had lower IC_{50} values ranging from 9.93 to 22.45µL mL^{-1} than the control plants, which exhibited the highest IC_{50} of 30.48µL mL^{-1}. The EO extracted from leaves sprayed with 50mg L^{-1} of riboflavin had the highest antioxidant potential (IC_{50} = 9.93µL mL^{-1}) compared to the standard reference BHT (IC_{50} = 19.45µg mL^{-1}). This means that 1µL EO from plants treated with 50mg L^{-1} riboflavin equals 1.9µg BHT. Previous studies have also shown that rosemary EO exhibited a stronger antioxidant activity than the synthetic antioxidant BHT (Wang et al., 2008; Rašković et al., 2014). The antioxidant potentiality of rosemary essential oil varies depending on the essential oil composition (Rašković et al., 2014). It was enhanced by both major and minor compounds (Wang et al., 2008). Therefore the significant effect of riboflavin on the antioxidant activity of rosemary EO probably correlated with enhanced accumulation of constituents with great antioxidant potentiality. The antioxidant potentiality of rosemary EO is linked to the oxygenated monoterpenes, particularly monoterpenoid ketones, which have potent antioxidant properties (Rašković et al., 2014; Takayama et al., 2016). Riboflavin induces the accumulation of phenolic and flavonoid compounds (Taheri & Tarighi, 2011; Li et al., 2012). Total phenolic compounds in rosemary EO were found to be correlated with its antioxidant activity (Barakat & Ghazal, 2016).

Reactive oxygen species (ROS) are normal plant cellular metabolism products (Mansoor et al., 2022). They are well-known second messengers, playing crucial roles in a number of cellular activities, such as stimulating tolerance to abiotic and biotic stresses. The imbalance between the production of ROS and their scavenging may cause oxidative damage to tissues. Plants counteract the effect of ROS by producing non-enzymatic and enzymatic antioxidants (Mansoor et al., 2022; Kesawat et al., 2023).

Riboflavin is a photosensitizer with a dual function in ROS metabolism (Hauvermale & Steber, 2020). While riboflavin derivates are components of many enzymes generating ROS, like glycolate oxidase and NADPH-dependent oxidase, they also play an important role as cofactors for ROS-scavenging enzymes like NADPH-thiol reductase and glutathione reductase (Sandoval et al., 2008).

The present study illustrated that the activities of the antioxidant enzymes catalase (CAT) and peroxidase (POX) were higher in riboflavin-treated plants compared to control plants. The application of riboflavin at a concentration of 50mg L^{-1} resulted in a significant increase in the activity of CAT and POX by approximately 113.6% and 45.6%, respectively, as compared to the control sample. This increase in CAT and POX activity may contribute to a reduction in cellular damage caused by reactive oxygen species. Our results align with Deng et al.'s (2014) findings, who reported that under drought-free conditions, low or moderate riboflavin levels increased the antioxidant enzyme activities of CAT, GR, APX, and SOD in tobacco leaves. Moreover, treatment with riboflavin at 100 ppm stimulates the activity of antioxidant enzymes (CAT, POX, APX, and GR) in salinized *Hibiscus sabdariffa* seedlings (Azooz, 2009).

While riboflavin increased the activities of the antioxidant enzymes, it decreased the oxidative enzymes, including ascorbic acid oxidase (AO), polyphenol oxidase (PPO), indole-3-acetic acid oxidase (IAAO), and ribonuclease (RNase), in the leaves of rosemary plants from the second cut. The decrease in the activity of oxidative enzymes (AO

and PPO) in plants treated with 50mg L^{-1} riboflavin suggests a corresponding increase in the content of ascorbic acid and phenolics. According to Zhang et al. (2023), strawberries treated with riboflavin and stored at 4 °C showed a decrease in the activity of the PPO enzyme with an increase in the levels of total phenolics and ascorbic acid as compared to untreated strawberries. These non-enzymatic compounds have antioxidant properties and help reduce the negative effects of ROS (Akram et al., 2017; Dumanović et al., 2021). The decrease in oxidative enzymes suggests a potential protective mechanism against oxidative stress in the leaves of rosemary plants. Additionally, riboflavin is believed to play a crucial role in regulating the balance between antioxidant and oxidative enzymes in rosemary plants. These findings suggest that riboflavin treatment enhances the antioxidant defense system in plants (Zhang et al., 2023).

Riboflavin is a natural photosensitizer; when subjected to ultraviolet irradiation, it has the potential to function as a pro-oxidant. Hence, riboflavin can generate ROS when exposed to UV light, which can cause oxidative damage to cells and biomolecules (Olfat et al., 2022).

In the present study, the high riboflavin concentration (100mg L^{-1}) inhibited antioxidant enzyme activity CAT and POX, while activity was enhanced at low riboflavin concentrations (25 and 50mg L^{-1}). According to Deng et al. (2014), this may be because riboflavin, in particular at high concentrations, increases the accumulation of ROS to toxic levels, which can impair the antioxidant defense system. Moreover, plants treated with 100mg L^{-1} riboflavin increased the levels of the oxidative enzymes PPO and AO, which points to a corresponding decrease in the non-enzymatic antioxidants, namely ascorbic acid and phenolic compounds. Low levels of both enzymatic and non-enzymatic antioxidants and a high content of ROS can result in an imbalance between antioxidant and oxidative processes. This leads to increased susceptibility to oxidative stress, which causes damage to cellular components such as DNA, proteins, and lipids. Additionally, the accumulation of ROS can also disrupt normal physiological processes in plants, affecting overall plant growth and development (Khan et al., 2023). This may be the explanation for the low efficacy of riboflavin at 100mg L^{-1} in comparison to the lower concentrations of 25 and 50mg L^{-1}.

RNase are RNA-degrading enzymes that regulate RNA content (Gho et al., 2022). Both ribonucleases and nucleases are involved in abiotic stress responses by regulating RNA catabolism and ROS accumulation (Zheng et al., 2014; Diaz-Baena et al., 2020)

Among the riboflavin treatments, 25mg L^{-1} showed the lowest RNase activity, followed by 50mg L^{-1}. This suggested a possible induction of RNase by ROS levels. It was found that RNase enhances the expression of genes encoding ROS-scavenging enzymes, which effectively regulate ROS homeostasis (Gho et al., 2022). In the Arabidopsis *rns2-2* mutant (lack of rRNA degradation by RNS2) shows elevated ROS levels decreasing the expression and leads to an imbalance in cellular homeostasis (Morriss et al., 2017).

In this study, the application of riboflavin at 50mg L^{-1} enhanced the accumulation of RNA, DNA and soluble protein content. Increased DNA values are correlated with higher protein synthesis to sustain active growth and development (Dhillon, 1988). In *Avicennia marina* trees, RNA:DNA ratio was significantly correlated with growth rates (Reef et al., 2010). Besides, the present study found out that riboflavin enhanced the plant content of micro- and macronutrients. These nutrients are critical in plant development and various metabolic processes (Tariq et al., 2023). In riboflavin-treated plants, the macronutrient with the highest concentration was nitrogen, an essential element in protein and DNA synthesis (Wan et al., 2023).

Conclusion

In conclusion, rosemary is a rich source of essential oil that possesses a strong antioxidant capacity. Moreover, it contains several non-enzymatic antioxidants and essential nutrients and minerals. Consequently, researchers always focus on improving it through safe and inexpensive treatments. Riboflavin's application improved the physiological performance of rosemary as measured by dry matter yield, photosynthetic activity and pigment production, nutrient uptake, essential oil percentage and yield, and the antioxidant enzyme system. The foliar application of riboflavin at low concentrations up to 50mg L^{-1} was more effective, whereas higher concentrations of riboflavin did not show any additional benefits. The application of riboflavin at low concentrations

could be a promising strategy to enhance plant growth and development while minimizing the detrimental effects of ROS accumulation. The most promising riboflavin treatment was 50mg L^{-1}, which gave the highest growth rate for rosemary plants. Therefore, treatment with riboflavin at 50mg L^{-1} is suggested for producing rosemary plants. Generally, riboflavin can improve the antioxidant activities of rosemary oil, making it a good candidate for both pharmaceutical plant-based products and functional foods.

Conflict of interest: The authors declare that there is no conflict of interest.

Authors' contributions: Fatma Abd El Lateef Gharib designed the research work. Experimental work was done by Eman Zakaria Ahmed. Hebatallah Aly analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Data and materials availability: All data generated or analyzed during this study are included in this article and are available from the corresponding author upon reasonable request.

Ethics approval: Not applicable.

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استجابة نباتات إكليل الجبل (*Rosmarinus officinalis*) للتطبيق الورقي للريبوفلافين: تحسين النمو، ومحتوى المغذيات، ونظام الإنزيمات المضادة للأكسدة، ومحصول الزيت العطري، ونشاطه المضاد للأكسدة

هبة الله علي، فاطمة غريب، ايمان زكريا

قسم النبات والميكروبيولوجي - كلية العلوم - جامعة حلوان – القاهرة – مصر

هدفت الدراسة الحالية إلى توضيح تأثير الرش الورقي للريبوفلافين على نباتات إكليل الجبل (الروزماري) خلال موسمين في عام 2021. كان موسم القطف الأول في الصيف (أغسطس) ، والثاني في الشتاء (ديسمبر). في هذه الدراسة ، أدى التطبيق الورقي للريبوفلافين (25، 50، 100 مجم/لتر) إلى زيادة معنوية في معدلات النمو خلال القطفتين والتي تناسبت مع الزيادات في محتوى العناصر المعدنية الغذائية، خضاب البناء الضوئي، ونشاط التمثيل الضوئي. وقد أعطى الريبوفلافين عند 50 مجم/لتر أعلى قيم لمتوسطات طول النبات، عدد الأفرع، الوزن الطازج والجاف لكلا من الساق والأوراق، في القطفتين الأولى والثانية. كما أدى إلى زيادة خضاب البناء الضوئي الكلية بنسبة 38٪ و بالإضافة إلى ذلك أظهر أعلى قيم لكلاً من محتوى العناصر وامتصاصها. سجلت النباتات المعالجة بـ 50 مجم/لتر من الريبوفلافين أعلي قيم للكربوهيدرات الكلية والسكريات الذائبة. كان للريبوفلافين تأثيره الايجابي علي انتاجية الزيت ونشاطه المضاد للأكسدة والذي بلغ أقصاه في النباتات المعالجة بالريبوفلافين 50 مجم/ لتر حيث زاد محصول الزيت (لتر/فدان) بنسبة 116٪ و 160٪ في القطفتين الأولي والثانية على التوالي ، مع أعلى نشاط مضاد للأكسدة (IC_{50} = 9.93 ميكرولتر/مل) في القطفة الثانية. علاوة على ذلك ، عزز الريبوفلافين عند 50 مجم لتر من نشاط إنزيمات مضادات الأكسدة (الكتاليز والبيروكسيديز) وعلى العكس من ذلك ، انخفضت أنشطة الإنزيمات المؤكسدة. بالاضافة الي ذلك ، قد أظهرت النباتات المعالجة بـ 50 ملجم من الريبوفلافين محتويات عالية من الحمض النووي. لقد خلصت النتائج إلى استخدام الريبوفلافين بنسبة 50 ملجم/ لتر هو الأكثر فاعلية في إنتاج نباتات الروزماري، ذات معدلات نمو عالية وانتاجية عالية من الزيت المتميز بفاعلية قوية مضادة للأكسدة.