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Original Article

Physiochemical Properties and Leaf Nutrients of *Satureja mutica* Fisch. & C.A.Mey. Treated with Cattle Manure at Different Plant Densities under Dryland Farming System

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Abstract

The management of plant arrangement and organic fertilizer is an effective practice in agricultural systems particularly in dryland farming condition. The split plot experiment was conducted to evaluate the effect of cattle manure and plant density on essential oil (EO) content, EO yield, photosynthesis contents, leaf nutrients, enzyme activities of *Satureja mutica* Fisch. & C.A.Mey. based on completely randomized block design (CRBD) with three replications during 2018 and 2019. The plants were treated with cattle manure (30 tones ha^{-1}) under high plant density (HPD, 80000 plant ha^{-1}), medium plant density (MPD, 40000 plant ha^{-1}), and low plant density (LPD, 26666 plant ha^{-1}) in dryland farming system. The results showed that the highest EO content (2.2%) and EO yield (10.9 kg ha^{-1}) were observed in the second-year plants with manure application at HPD. Under both HPD and MPD, chlorophyll (Chl) a+b was greater compared to LPD. The higher concentrations of magnesium (Mg) and phosphorous (P) were observed in second-year plants treated with manure application at MPD in comparison with LPD/HPD. Compared to LPD, increased potassium (K) and calcium (Ca) contents were observed at HPD and MPD. The activity of antioxidant enzymes at LPD was significantly higher than other treatments. The use of cattle manure and HPD could increase EO production, but MPD is suggested in improving leaf nutrients of *S. mutica*.

Keywords: Essential Oil, Enzyme Activity, Leaf Nutrients, Organic Fertilizer, Planting Arrangement.

Introduction

The genus *Satureja* (Lamiaceae family) contains more than 200 species of herbs and shrubs, which are widely distributed in the Mediterranean regions [1]. Iran includes 16 species with nine endemic species, which are mainly distributed in rocky mountainous areas [2]. *Satureja mutica* Fisch. & C.A.Mey. is a perennial and relatively woody plant with 30-50 cm in height and a many flowering branches. It naturally grows in Turkmenistan and Iran, mainly in north of Iran on calcareous soils and in temperate climate [3,4]. *S. mutica* is a strongly aromatic plant species with major phytochemical compounds viz. phenols, carvacrol, thymol, and flavonoids [5-7]. Its traditional application has been reported to treat the muscle pain, diarrhea, cramps, and indigestion [8,9].

The appropriate use of organic fertilizers as the main component of soil improvement is the main purpose of food and agriculture organization [10]. In arid and semiarid areas like most parts of Iran the soil suffers nutrients and water availability [11]. Organic and bio fertilizer leads to the increased fertility of soils [12]. *S. mutica* due to the medicinal properties and wide distribution has a big challenge in semiarid area [13]. Generally, organic fertilizer such as manure can improve the plant productivity with rarely environmental concerns [14,15]. Today, it is recognized that application of cattle manure as sail amendment in plant production is useful with no environmental concerns [9,15,16].

Organic fertilizers are widely used to improve the agricultural production [15,16]. Although the improvement of EO quality and quantity, and antioxidant capacity with different organic and chemical fertilizers in

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medicinal plants have been well documented [17-21], there is little information regarding the cattle manure on plant yield and EO production in medicinal and aromatic plants (MAPs). Recently, MAPs have widely applied for their high capacity in neutralizing the toxic free radicals [22]. Hence, attempts in investigation of natural antioxidants in MAPs have been increased [23].

Plant density is a predominant management factors affecting the plant growth, adjusting the capacity to capture radiation, water and nutrients [24, 25]. Change in plant density possesses different microclimate for plant, resulting the various soil moisture, soil microorganism, canopy temperature etc. Regarding the environmental, geographical and edaphic factors, the plants adopt their optimum plant density [26]. Crop spatial pattern is the agronomical important factor that can influence grain yield and crop competitiveness against weeds and environmental stress [27, 28]. The spatial distribution of crops with changing in leaf area index (LAI) significantly influences the sun light rate and soil moisture, which finally determines corresponding weeds and soil microorganisms [29].

Recently, the excessive use of chemical fertilizers in agricultural practices has increased environmental concerns. Organic fertilizers as a substitute of chemical inputs and plant density as the effective agricultural practice can improve plant production. Although cattle manure is a main alternative of chemical fertilizers in Iran, there is little knowledge about its impact on medicinal plants particularly *Satureja* spp under dryland farming system. Therefore, we assessed the effects of organic fertilizers (cattle manure) and plant density on essential oil (EO) content and yield, chlorophyll content, antioxidant activity, and, leaf minerals of *S.mutica* at dryland farming condition.

Material and Methods

Plant Material and Growth Condition

S.mutica seeds were obtained from Research Institutes of Forests and Rangelands (RIFR), Iran. The seeds were sown in the plastic trays filled with perlite and cocopeat (1:1 volumes) at the greenhouse in RIFR. After 60 days, the seedlings were transplanted on the open field in Damavand experimental station (52º 20″ E and 35º 42″ N), Tehran province, during April 2017. The mean annual temperature was 11 °C, which it's minimum and maximum occurred in January–February (-23 °C) and July–August (36 °C), respectively. The mean annual rainfall was 332 mm. The soil and organic fertilizer properties of the case study are presented in Table 1. The study was conducted as split plot in a randomized complete block design (RCBD) with three replications under dryland farming condition during 2018 and 2019. The main plot was fertilizer application in two levels as non- manure application and 30 t ha^{-1} cattle manure, and also sub plot was plant density in three levels *viz*. high plant density (HPD, 80000 plant ha^{-1}), medium plant density (MPD, 40000 plant ha^{-1}), and low plant density (LPD, 26666 plant ha^{-1}). The spacing between rows was 50 cm and within rows were 25, 50, and 75 cm for HPD, MPD, and LPD, respectively. Cattle manure was mixed the soil before transplanting. During the experiment, no pesticide and chemical fertilizers were used and we manually controlled the weeds. In both years, plants were harvested at flowering stage above the soil surface at late September.

EO Content

EO content of the aerial parts was quantified using the method described by the European Pharmacopoeia for oil production [30]. Briefly, 100 g of dried aerial plant parts were subjected to hydro-distillation for 3 hours using a Clevenger-type apparatus.

Chlorophyll (Chl) Determination

The contents of Chl and Chl b were extracted according to Arnon (1949). 200 mg of fresh samples were homogenized in 8 ml 80% acetone. After that, the mixture was centrifuged at 4 °C for 15 min (3000 rpm). Supernatants were used for analyzing chlorophyll content. Absorbance was determined at 645 and 663 nm by the spectrophotometer.

Enzyme Essay

The extraction procedures were carried out at 0–4 °C. Fresh samples (0.5 g) were homogenized in a Heidolph, Diax 900 homogenizer in 5 ml 100 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA-Na2 and 0.5 mM ascorbate. Because ascorbate peroxidase (APX) is labile in the absence of ascorbate, 0.5 mM ascorbate was included for the extraction of this enzyme with the above procedure. The homogenized shoot samples were centrifuged at 10,000 g for 5 min.

Table 1 The soil characteristics of the experimental field

Year	PH	EC (dS m^{-1})	OC (%)	N (%)	P (mg/kg)	K (mg/kg)	Sand (%)	Silt (%)	Clay (%)
Fertilizer	8.1	0.87	1.25	0.97	18	272	22	44	32
Manure	8.2	16.4	0.39	2.1	1.1	1299	-	-	-

Treatment Details

The supernatant was used as a crude enzyme extract. All colorimetric measurements were made at room temperature in a Shimadzu UV/VIS 1201 spectrophotometer.
Superoxide dismutase (SOD) activity was determined as described by Beauchamp and Fridovich (1971). The reaction mixture consisted of 1.17 × 10−6 M riboflavin, 0.1 M methionine, 2 × 10−5 M KCN and 5.6 × 10−5 M nitroblue tetrazolium salt (NBT), which was dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8). 1 ml of enzyme extract was enriched with 3 ml of the reaction. Illumination was started to initiate the reaction at 30 °C for 60 min. The blanks were identical solutions that were kept under dark condition. The absorbance was determined at 560 nm with the spectrophotometer against the blank.
Catalase (CAT) activity was measured according to Chandlee and Scandalios (1948) method applied to measure CAT activity. Briefly, 0.5 g of leaf samples was homogenized in a prechilled pestle and mortar with 5 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was centrifuged at 4 °C for 15 min at 13000 rpm. The supernatant was used for enzyme assay as the extract. The final mixture consisted of 400 μl of 15 mM H2O2, 3.5 ml of 50 mM potassium phosphate buffer (pH 7.0), and 40 μl of enzyme extract. The H2O2 decomposition was followed by the reduction in absorbance at 240 nm.
Ascorbate peroxidase (APX) activity was calculated by following the decrease of ascorbate and measuring the change in absorbance at 290 nm for 1 min in 2 ml of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA-Na2, 0.5 mM ascorbic acid, 0.1 mM H2O2 and 50 μl of crude enzyme extract [31]. The activity was calculated from the extinction coefficient (2.8 mM−1 cm−1) for the ascorbate.

Leaf Mineral Concentrations

The dry ash method was applied to assay the minerals in aerial parts. The samples were dried in an oven at 70 °C. To obtain the white ash, 1g dried aerial samples was transferred into ceramic vessels and slowly subjected to 500°C in the oven. The obtained ash was cooled in room temperature, which each sample was subsequently changed with 20 mL 1N HCl, and put in the sand bath for 30 min. The samples were elutriated in a 100 mL volumetric balloon [32]. Potassium (K) and was measured by flame photometer (Model 410, Corning, Halstead, UK) in the volumetric balloons [32]. Phosphorous (P) was measured by yellow method with vanadate-molybdate [33]. P concentration was determined at 430 nm using the spectrophotometer (Shimadzu, UV3100). Calcium (Ca) and magnesium (Mg) concentration were determined by atomic absorption spectroscopy [32].

Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) and using the SAS software package for Windows (SAS, version 9.3, SAS Institute, Cary, NC). Duncan's multiple range tests showed the comparison of mean values. The data were statistically investigated at 5% probability level.

Results

EO Content and EO Yield

The EO content and yield are presented in figure 1. EO content increased with progressing in plant density and manure application. The EO content in plants at HPD under manure application was significantly higher compared to other experimental treatments. There was no significant difference between the two experimental years on EO content, but EO yield in the second year was greater than the first year. The highest EO yield was observed in the second-year plants at HPD when manure was used (10.9 kg ha^{-1}). Plants cultivated at LPD represented the lower EO content and yield compared to MPD and HPD (Figure 1).

Enzyme Activity

The activity of antioxidant enzymes are presented in table 2. The APX activity rannged from 42.3 U mg^{-1} protien (HPD with manure application in the first year) to 53.7 U mg^{-1} protien (HPD with manure application in the second year). The plants experencing LPD and HPD had higher APX activity compared to LPD. In addiotion, cattle manure increased enzyme activity. In the second-year plants under HPD, manure increased APX activity by 18% compard to control. The highest CAT activity was observed at HPD with manure application in the second year to be 8.23 U mg^{-1} protien. SOD varied from 30 U mg^{-1} protien (HPD withouth manure in the first year) to 35.7 U mg^{-1} protien (HPD with manure application in the second year) (Table 2).

Chl Content

Figure 2 showed the changes of Chl content under plant density and cattle manure. The ratio of Chl a to b in LPD was significantly higher compared to HPD and MPD. The plants experiencing cattle manure had no significant different with control. Chl a + b in plants under HPD and MPD was higher than LPD. We observed the higher Chl content in the second -year plants treated with cattle manure at MPD and HPD. In total Chl did not show the remarkable changes of biochemical status of *S. mutica* under plant density and manure application (Fig. 2).

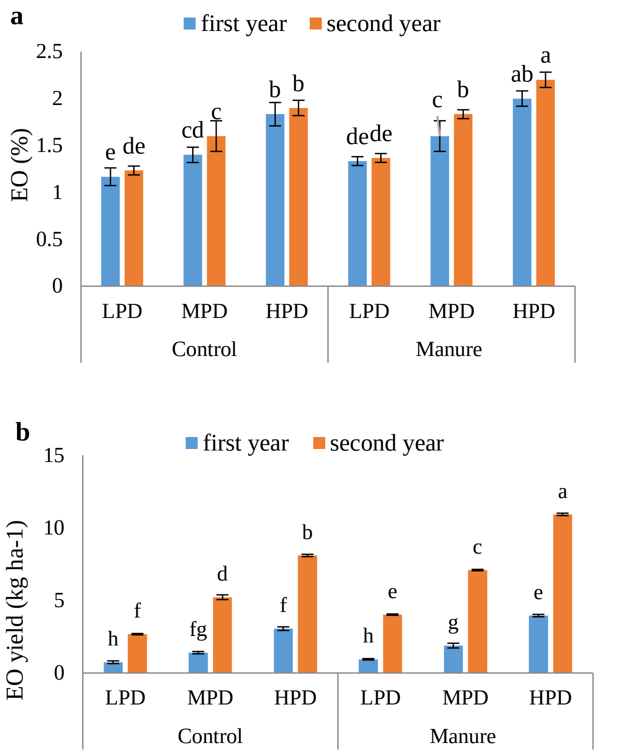

Fig. 1 The essential oil (EO) content (a) and yeild (b) of *S. mutica* Fisch. & C.A.Mey. under plant density and cattle manure. Values are means ± standard deviation (SD) of three replications (n=3). Different letters show statistically significant differences among treatments at *P*≤0.05.

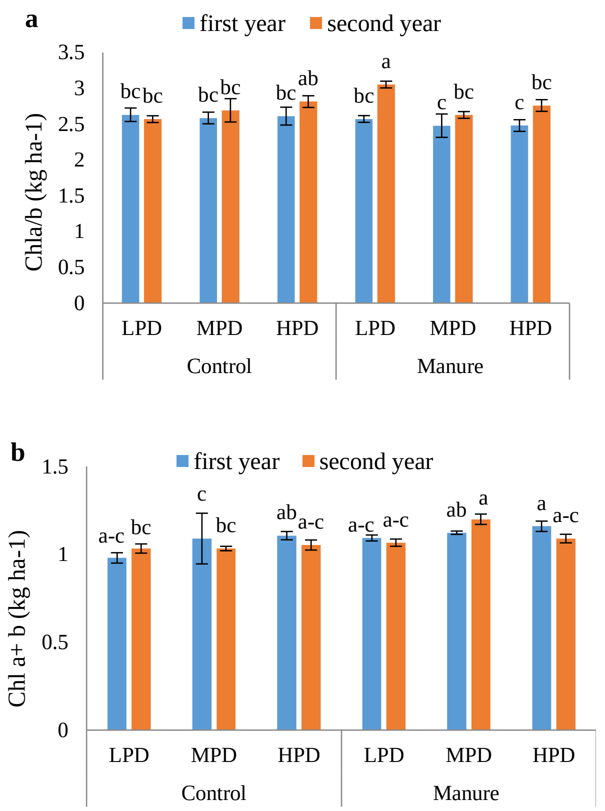

Fig. 2 The chlorophyl (Chl) contetn and ratio in leveas of *S. mutica* Fisch. & C.A.Mey. under plant density and cattle manure. Values are means ± standard deviation (SD) of three replications (n=3). Different letters show statistically significant differences among treatments at *P*≤0.05.

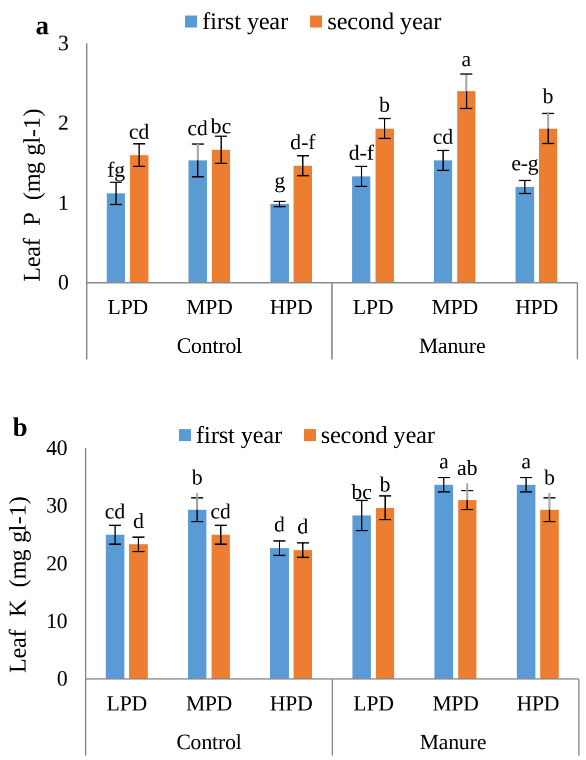

Fig. 3 The Pgospherous (P) and potasium (K) content in leveas of *S. mutica* Fisch. & C.A.Mey. oil (EO) under plant density and cattle manure. Values are means ± standard deviation (SD) of three replications (n=3). Different letters show statistically significant differences among treatments at *P*≤0.05.

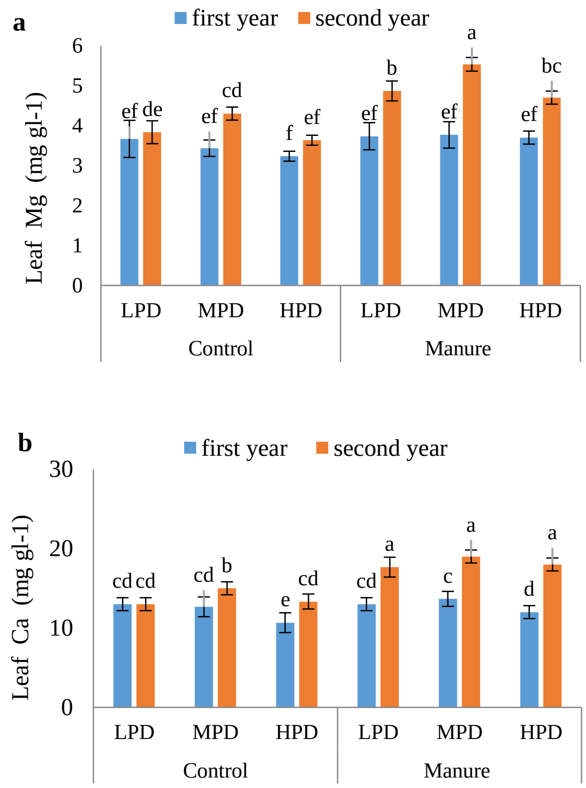

Fig. 4 The mangesium (Mg) and calacium (Ca) (K) content in leaves of *S. mutica* Fisch. & C.A.Mey. under plant density and cattle manure. Values are means ± standard deviation (SD) of three replications (n=3). Different letters show statistically significant differences among treatments at *P*≤0.05.

Table 2 The enzyme activity of *S. mutica* Fisch. & C.A.Mey. under plant density and cattle manure. Values are means ± s Table and deviation (SD) of three replications (n=3). Different letters represent statistically significant differences among treatments at *P*≤0.05.

Year	Organic Fertilizer	Plant density	APX (U mg^{-1} protein)	CAT (U mg^{-1} protein)	SOD (U mg^{-1} protein)
2018	Control	Low	46.0±0.81 de	7.00±0.45 bc	33.7±1.25 a-c
		Medium	44.7±1.23 e-g	6.53±0.12 cd	33.0±0.83 b-d
		High	42.0±0.82 fg	7.17±0.30 b	30.0±0.81 e
	Manure	Low	51.3±3.09 a-c	7.20±0.29 b	35.0±0.82 ab
		Medium	44.3±0.96 e-g	7.97±0.16 a	34.3±2.04 a-c
		High	41.3±1.25 g	8.23±0.24 a	32.0±0.85 c-e
2019	Control	Low	49.3±2.05 b-d	6.27±0.12 d	32.7±0.95 b-d
		Medium	48.0±0.81 c-e	6.33±0.44 d	30.7±1.25 de
		High	45.3±1.24 ef	5.93±0.28 d	32.3±1.22 c-e
	Manure	Low	53.0±0.85 ab	6.47±0.16 c	35.7±0.93 a
		Medium	53.3±1.69 a	6.50±0.17 cd	35.0±0.87 ab
		High	53.7±1.24 a	6.03±0.21 d	33.7±1.34 a-c

Leaf Minerals

The leaf minerals are represented in figures 3 and 4. Leaf P and increased at MPD but decreased at LPD and HPD. In addition, the plants improved P uptake when manure was used. The highest leaf P content (2.4 mg g^{-1}) was observed in second-year plants at MPD with manure application. Hoevever, the first-year plants under LPD without organic fertilizer showed the lowest leaf P contetn. The significant change in K uptake was beloged to plant density. The MPD and manure addition led to increased K uptake. Leaf Mg under MPD and manure application was higher compared to other experimental treatments. In first-year plants under manure application, 14 and 18% increases of leaf Mg were observed at MPD compared to LPD and HPD, respectively. For Ca concentration, cattle manure was responsible for its variations. In second-year plants, manure increased leaf Ca content by 30 and 35% at MPD and HPD, respectively, compared to control.

Discussion

Increased EO yield was observed in the plants treated with manure application compared to control. Previous works have reported the improved EO content and yield in some medicinal plants when manure was used as an organic fertilizer [9,15,34,35]. Manure ameliorate the physico-chemical attributes of soil through upgrading the useful microorganism in rhizosphere. It promotes the physiological and biochemical pathways involved in plant growth and productivity [36]. Under dray farming system, manure due to its potential in absorbing and maintaining the available water has a significant role on plant growth and development through releasing the essential nutrients sustainability [9]. The main reason of increased EO yield at HPD is due to enhanced dry weight of plants at HPD particularly in the second-year plants. The interaction of various parameters such as plant ontogeny, site, photosynthesis rate, photoperiodic modulation, moisture, salinity, and temperature can affect EO production. Plant density affects sunlight rate and canopy temperature, which in turn alter the soil moisture. It influences the nutrient uptake, physiological and biochemical processes in plants such as photosynthesis and EO production pathways [9].

The leaf nutrients had different responses to cattle manure and plant density. Manure mostly increased all studied elements. Manure can improve physico-chemical properties of soil such as soil texture, porosity, pH, and EC properties, which results in increased ion concentrations in soil and subsequently in plants [37]. With manure application, microbial activities and mineralization of nutrients were increased, which results in enhanced nutrients concentration. Enhanced nutrients concentrations with manure application have been reported in spinach [38] and tomato [39]. Plant density significantly affects the soil moisture, which results in change of nutrient concentration in soils and plants. In the present study, MPD and HPD was selected as a suitable practice to meet the optimum K, P, Ca, and Mg concentration. According to our results on plant yield, the main effect of MPD and HPD is improvement of nutrients uptake through maintaining soil moisture, thereby, this increased shoot ions contents. The lower amount of some nutrients at HPD compared to MPD can be related to higher competition among the plants for obtaining more elements at HPD.

Photosynthesis content increased at MPD. There are different theories on plant density to photosynthesis and growth of plants. Some investigators believe dense plant density may cause more lodging, less light penetration in the crop canopy and reduced photosynthetic efficiency that resulted in low plant growth [40]. In some documents, we can observe the main reduction at wide canopies. It can be due to the fact that at LPD, the sunlight easily reduces soil moisture thereby, decreases nutrients uptake by plant. Soratto *et al*. [40] reported

HPD decreased growth of bean plants. Similarly, Yao *et al.* [41] showed that with change in planting density, cotton plants start to improve photosynthetic capacity by increasing photosynthetic N use efficiency and by adjusting leaf area per area. MPD and partially HPD could be that optimum planting patterns due to highlight interception and utilization and better spatial dissemination of leaf N to the carbon assimilation apparatus in leaves. Furthermore, higher photosynthesis pigment (figure 2) under MPD and HPD in the second year is due to improvement in leaf gas exchange, total leaf area, and leaf area index, which finally lead to improvement in plant growth [42].

The antioxidant enzyme activity in LPD was singingly higher than HPD and MPD (table 2). Under LPD the sunlight rate is very harsh and makes a stress condition for plants. Under stress conditions, these are a collection of antioxidants that act to suppress or prevent the formation of free radicals or reactive species in cells. They are efficient in neutralizing any molecule with the potential of developing into a free radical or any free radical with the ability to induce the production of other radicals. SOD and CAT breakdown hydrogen peroxides and hydroperoxides to harmless molecules (H_2O_2/alcohol and O_2) [43]. According the results obtained, we concluded that the use of cattle manure and HPD can result in higher EO production, but MPD is suggested in improving leaf nutrients of *S. mutica* under dry farming system.

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