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The effect of methyl jasmonate and TiO_2 nanoparticles on phenolic and terpenoides compounds in peppermint (*Mentha piperita* L.)

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Abstract

Mentha piperita L., as a rich source of phenolic acids and essential oils, is known for its medicinal importance. This study aimed to investigate the effects of different concentrations of methyl jasmonate (MeJA) (0, 0.1, and 0.5 mM) and TiO_2 nanoparticles (TiO_2 NPs) application including 0, and 150 mg L^{-1} on phenolic compounds and essential oil (EO) of *Mentha piperita* L. The results showed that phenolic content (76%), rosmarinic acid content (87%) and caffeic acid content (78%) were improved by simultaneous application of MeJA (0.1 mM) and TiO_2 NPs (150 mg L-1) compared to control. The highest menthol production (44.51%) was belong to exogenous application of 0.1 mM MeJA when TiO_2 NPs application was 150 mg L^{-1}. This research enabled us to introduce an ideal combination of plant growth regulators (MeJA) and TiO_2 NPs application for increasing major components of essential oils in peppermint.

Key words: Caffeic acid, Essential oil, Menthol, Rosmarinic acid.

1. Introduction

Peppermint (*Mentha piperita* L.) is a natural hybrid of water mint (*Mentha aquatic* L.) and spearmint (*Mentha spicata* L.) [1]. Peppermint is effective in improving upper gastrointestinal disorders, irritable bowel syndrome, muscle spasms, and respiratory problems. The antimicrobial and antiviral effects of peppermint EO have been confirmed [2]. The shoots of peppermint contain essential oils, phenolic compounds, flavonoids, fatty acids, vitamins, minerals, and salicylic acid [3]. Peppermint EO is mainly composed of menthol (29-48%), menthon (20-31%), menthofuran (6.8%), and menthyl acetate (3-10%) [4]. Menthol is the most important component of the EO that is biosynthesized and accumulated in the capillaries of the secretory glands on the surface of the epidermis. Peppermint EO also contains compounds such as limonene, 1,8 cineole, isomenthone, isopulegol, pulegone, and carvone [5]. The production of secondary metabolites in plants is mainly related to the environmental stresses and their accumulation incited by elicitors [6]. Among the various elicitors, MeJA is recognized as effective elicitor for inducing secondary metabolite synthesis in plants [7]. MeJA is derived from linoleic acid in the octadecanoid pathway and plays an important role in activating plant defense responses such as biosynthesis of specific secondary metabolites [8]. The results of Malekpour et al. (2015) showed that MeJA increases essential oils in basil. NPs are defined as particles with a size between 1-100 nm [9]. NPs have recently been used in agriculture due to their positive effect on the growth and improve some EO contents [10]. The notable role of TiO_2 NPs on the production of EO has been reported in the plants such as *Mentha piperita* L. (Ahmad et al. 2019), *Salvia officinalis* L. (Ghorbanpour et al. 2015), *Rosmarinus Officinalis* L. (Golami et al. 2018), and *Vetiveria zizanioides* L. (Shabbir et al., 2019). The TiO_2 NPs application significantly increased menthol, menthone and menthyl acetate in *Mentha piperita* L. [10] and thymol and carvacrol in *Thymus vulgaris* [11]. Due to positive effects of MeJA on plant growth and secondary metabolites production, and considering the combined effects of TiO_2 NPs with MeJA has not been studied on peppermint. In this study, the application of MeJA and TiO_2 NPs was evaluated on phenolic compounds and EO of peppermint.

2. Materials and methods

2.1. Plant material and growth condition

Each experimental unit was a plastic pot (22 cm diameter×20 cm height) that was filled with the autoclaved growing mixture (0.11 MPa, 120 °C, 1 h) of cocopeat: vermicompost: perlite: soil (1:1:1:1, v/v). The chemical properties of soil were observed in table 1. The pots were randomly arranged and plants grown at the natural condition with relative humidity ranging from 50 to 70% and average day and night temperatures 32 and 17°C, respectively. The rhizomes were first planted in pots then 30 days- old seedlings were transferred to plastic pots. Each treatment was replicated in three pots and each pot had three seedlings.

Table 1: Chemical analysis of soil

PH	EC (dS/m)	N (%)	P (mg/kg)	K (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)
7.25	1.2	1.4	120	1250	842.8	36.6	62.8

2.2. TiO_2 NPs

TiO_2 NPs were purchased from US Research Nanomaterials (USA). TiO_2 solution was prepared at concentrations of 0 and 150 mg L^{-1} with filtered double–distilled water (DDW). 150 mg L^{-1} (150 mg L^{-1}) was applied on the 30 days-old seedlings as foliar spraying. DDW was used as a control spray treatment. 150 mg L^{-1} was applied at an interval of 7 days (7 times) with the help of a hand sprayer. The working solution was sonicated to avoid aggregation when required.

2.3. MeJA treatment

One month after transplanting of seedlings, plants with different concentrations of 2% ethanol-soluble MeJA (V/V) (0, 0.1, 0.5 mM), separately and with 150 mg L^{-1} TiO_2 NPs were sprayed. Different concentrations of MeJA were applied at an interval of 12 days (4 times) with the help of a hand sprayer. Control plants were also sprayed with water.

2.4. Experimental design and treatments

The experiment was performed with different concentrations of MeJA (0, 0.1, 0.5 mM) and two concentrations of TiO_2 NPs (0, and 150 mg L^{-1}). So, a factorial experiment was conducted based on a completely random design with three replications.

2.5. Determination of total phenolic content (TPC)

TPC of the extracts were quantified using spectrophotometer (Analytic Jena, SPECORD 210, Germany) by literature methods described by Sadasivam and Manickam (2008). Leaf samples (500 mg) were ground along with 5 times volume of 80% ethanol, using mortar and pestle. Centrifugation of the homogenate at 10,000 rpm for 10 min at 4°C was done, saving the supernatant. The supernatant was evaporated to dryness, adding 5 mL of DDW thereafter. Subsequently, 0.5 mL of Folin-Ciocalteau reagent and 2 mL of 20% Na_2CO_3 were added to each tube. The OD of the solution was measured at 650 nm against a reagent blank. Gallic acid was used as a standard by expressing the results with mg Gallic acid equivalents (GAE) g^{-1} of fresh weight.

2.6. Determination of Rosmarinic acid

Dry plant powder (0.1 g) was ground with 10 ml of 80% methanol and placed in a shaker at 70 ° C for 90 minutes. The resulting extracts were filtered through filter paper. The absorption of methanolic extracts at 333 nm was read by spectrophotometer (Analytic Jena, SPECORD 210, Germany). Then, using a standard curve, the concentration of rosmarinic acid was determined [12].

2.7. Determination of Caffeic acid

Samples were ground on ice with 80% methanol at a ratio of 1.5 w/v until a homogeneous solution was obtained. The resulting homogenate was stirred for three hours at 40 ° C and then centrifuged at centrifuge at 45 rpm for 45 minutes and the supernatant was used to measure caffeic acid. Take 1 ml of 80% methanolic extracts and add 1 ml of Arno reagent (containing 10% sodium molybdate and 10% sodium nitrate), 1 ml of sodium hydroxide 1 M, 1 ml of hydrochloric acid 0.1 M was added, then vortexed and the absorbance of the mixture was measured immediately at 490 nm using a spectrophotometer (Analytic Jena, SPECORD 210, Germany). In the control sample, instead of the extract, 1 ml of 80% methanol was added [13].

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed using IBM SPSS Version 23.0 and Duncan's multiple range test was used to determine significant differences at $P < 0.05$. All experiments were carried out in triplicate and expressed as mean ± standard error (SE).

3. Results

3.1. Total phenolic content (TPC)

We observed that TiO_2 NPs application, significantly increased TPC in all levels of MeJA applying. The exogenous applications of MeJA in all concentrations, significantly enhanced TPC in both, with TiO_2 NPs -sprayed and without TiO_2 NPs plants ($P<0.05$). The highest TPC was obtained in plants treated with 0.1 mM MeJA and 150 mg L^{-1} TiO_2 NPs (76.15 % higher than control). Results also showed that, extracts obtained from MeJA -treated plants with lower concentrations, generally possessed higher TPC compared to the plants treated with higher concentrations (Table 2).

3.2. Rosmarinic acid and Caffeic acid

Results in Table 2 show that the highest content of rosmarinic acid (98.654 ± 0.1877 mg. g -1 dry weight) and caffeic acid (68.97 ± 0.344 mg. g^{-1} dry weight) are belong to application of 0.1 mM MeJA and 150 mg L -1 TiO_2 NPs (87% and 78% higher than control (without elicitation) respectively. Overally, exogenous application of MeJA, significantly increased rosmarinic and caffeic acids in both, TiO_2 NPs, treated plants and without.

Table 2: Effect of SA, MeJA and TiO_2 NPs on Biochemical Traits *on Mentha piperita* L. plants

Treatments	TPC (mg g^{-1} F.W)	RA (mg. g^{-1} D.W)	CA (mg. g^{-1} D.W)
Control	13.04±0.0920^{f}	52.51±0.3084^{f}	38.72±0.05291^{e}
150 mg L^{-1} TiO_2 NPs	19.52±0.1384^{d}	69.78±0.5130^{e}	55.84±0.1196^{c}
0.1 mM MeJA	22.24±0.2398^{b}	80.46±0.1225^{c}	58.49±0.06736^{b}
0.5 mM MeJA	18.57±0.1887^{e}	75.51±0.1872^{d}	46.63±0.157072^{d}
0.1 mM MeJA + 150 mg L^{-1} TiO_2 NPs	22.97±0.2769^{a}	98.65±0.1872^{a}	68.97±0.344487^{a}
0.5 mM MeJA + 150 mg L^{-1} TiO_2 NPs	20.82±0.0906^{c}	89.87±0.1872^{b}	58.49±0.123308^{b}

In each column different letters (a–f) mean significant differences at $P \leq 0.05$. Means ± S.D from the three experiments. CA (Caffeic acid) ; RA (Rosmarinic acid); TPC (Total Phenolic content)

3.3. The essential oils content

Thirty-four constituents were identified in the essential oils of peppermint (based on GC–MS analyses). The main constituents of peppermint EO were menthol (24.21-44.51%) as the major component, isomenthone (4.01-12.24%) and 1,8-Cineole (10.61-24.84%). Other major constituents included α-pinene, β-pinene, sabinen, menthyl acetate, Bornyl acetate, Camphene, Caryophyllene, Germacrene D, Humulene, pulegone, menthofuran. The results showed that MeJA, and TiO_2 NPs application regardless of a few cases, increased EO constituents compared to the control (Table 3). Menthol percentage in TiO_2 NPs treated plants with different concentrations of MeJA had a significant increase compared to control. The highest menthol percentage was belong to 0.1 mM MeJA in TiO_2 NPs treated plants (44.51%) which was 83% more than the control (24.21%). Other constituents in essential oils also had significant fluctuation with elicitation compared to controls. The results also showed that menthofuran content considerably decreased with MeJA elicitation but increased with TiO_2 NPs application. Conversely, pulegone content in 0.5 mM MeJA elicited plants was the highest both with TiO_2 NPs application and without. The EO chromatograms for control and 0.1 mM MeJA×150 mg L^{-1} TiO_2 NPs treatments were depicted in Fig 1.

Table 3: Effect of SA, MeJA, and TiO_2 NPs application on percentage composition of *Mentha piperita* essential oils

Concentration (%)								
			0*			150*		
No	**compounds**	**RT**	**0****	**0.1 mM MeJA**	**0.5 mM MeJA**	**0****	**0.1 mM MeJA**	**0.5 mM MeJA**
1	α-Pinene	4.044	1.4	1.38	1.5	0.6	1.76	1.49
2	Comphene	4.35	ND	0.41	0.38	ND	0.51	0.47
3	Sabinen	4.757	0.88	1.3	1.49	0.51	1.48	1.46
4	Nopinen	4.866	2.91	2.97	2.69	ND	ND	2.96
5	β-Pinene	4.873	ND	ND	ND	0.95	3.33	ND
6	p-Menth-8-en-3-ol acetate	5.945	ND	ND	1.04	ND	ND	ND
7	1,8-Cineole	6.006	10.61	24.84	21.96	5.65	20.23	24.41
8	Limonene	6.14	ND	ND	ND	1.05	ND	ND
9	4,7,dimethyl,undecane	6.23	ND	ND	ND	1.3	ND	ND
10	Isomenthone	9.027	4.01	0.34	12.24	4.24	8.01	4.54
11	Menthofuran	9.224	16.73	1.01	5.11	22.71	0.99	2.67
12	Isomenthol	9.394	ND	ND	0.62	2.6	ND	ND
13	Linderol	9.448	7.09	ND	0.76	ND	ND	1.26
14	(-)-Menthol	9.611	24.21	33.29	30.67	39.31	44.51	34.26
15	dl-Menthol	10.12	ND	ND	ND	0.78	ND	ND
16	Pulegone	11.172	8	9.86	10.16	1.19	1.48	10.5
17	Bornyl acetate	12.367	1.07	0.84	0.53	ND	0.75	0.95
18	Menthol, acetate	12.564	0.86	ND	0.4	10.48	ND	ND
19	Menthofurolactone	14.193	ND	ND	1.5	1.94	ND	1.12
20	Dihydrojasmone	14.56	1.18	ND	ND	ND	ND	ND
21	4-(2-Methyl-3-oxocyclohexyl)butanal	15.565	1.91	ND	ND	ND	ND	ND
22	Caryophyllene	15.843	7.22	15.49	4.96	0.91	11.01	8.95
23	Humulene	16.739	ND	0.98	0.34	ND	0.67	0.58
24	5Caranol,(1S,3R,5S,6R)	16.746	3.5	ND	ND	ND	ND	ND
25	Germacrene D	17.37	2.07	3.51	1.22	ND	3.26	2.03
26	Bicyclogermacrene	17.717	ND	ND	ND	ND	ND	ND
27	Elemene isomer	17.723	ND	ND	ND	ND	0.49	ND
28	Mint furanone	18.04	ND	ND	ND	1.29	ND	ND
29	Phenol, 3,5-bismethyl]-2,4,6-trimethyl	18.056	0.87	ND	ND	ND	ND	ND
30	7a-Hydroxymintlactone	19.72	ND	ND	ND	1.11	ND	ND
31	Caryophyllene oxide	19.76	2.34	1.31	0.8	ND	0.48	1.08
32	Cyclobarbital	20.928	0.5	ND	ND	ND	ND	ND
33	(+)-T-Cadinol	21.179	1.31	1.4	0.69	ND	1.04	1.27
34	Di-n-decylsulfone	21.831	0.8	ND	ND	ND	ND	ND

RT= Retention time
0, 150 = 0 and 150 mg L^{-1} TiO_2 NPs
ND= not detected

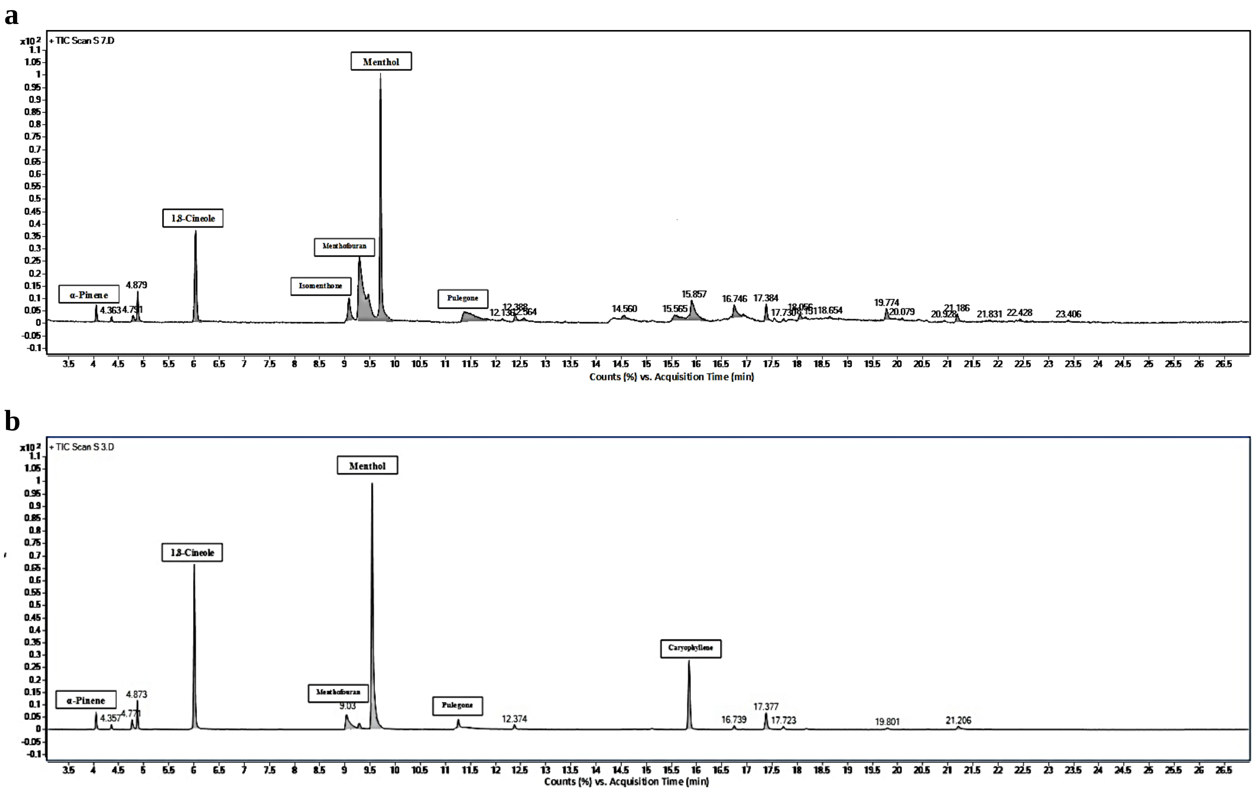

Figure 1*: Mentha piperita* EO chromatograms in (a) control, and (b) 0.1 mM MeJA×150 mg L^{-1} TiO_2 NPs treatments.

4. Discussion

Application of plant growth regulators and TiO_2 NPs are well investigated, but combined effects of TiO_2 NPs with MeJA have not been studied on peppermint. In this study, we found that, MeJA in TiO_2 NPs treated plants had positive effects on secondary metabolite production. The results of this study revealed that phenolic content in TiO_2 NPs treated plants was increased. This is in agreement with the result of Oloumi *et al.* (2015), who stated that, application of nano-sized CuO and ZnO particles in *Glycyrrhiza glabra* seedlings, had been positive effects on secondary metabolite (phenolic and glycyrrhizin) production. It is widely accepted that elicitors bind to receptor proteins in the plasma membrane and increase the biosynthesis of secondary metabolites by activating specific genes and the signal transduction pathway (as part of the defense response) [14]. Tan et al. (2017) were observed an increase in rosmarinic acid contain in the *Dracocephalum moldavica* treated with NPs. Results of this study showed that MeJA elicitation, increase the total phenolic content. The exogenous application of MeJA increases secondary metabolites, including alkaloids, phenolics, and terpenes in aromatic and medicinal plants [15]. MeJA as an important signaling molecule increases phenolic compounds by increasing the activity of PAL, the main enzyme in the synthesis of phenolic compounds [16]. Some reports have shown that MeJA increases the accumulation of phenolic acids.

MeJA enhanced rosmarinic acid and lithospermic acid B in *Salvia miltiorrhiza* hairy root cultures [17, 18]. Also, the application of MeJA in hairy root cultures of *Coleus blumei*, *Coleus forskohlii*, and cell cultures of *Agastache rugosa* Kuntze increased RA accumulation [17, 19]. An increase in RA content has been reported with MeJA-treated cells of

Lithospermum erythrorhizon [20]. MeJA increased rosmarinic acid, caffeic acid, chlorogenic acid, and cinnamic acid contents in *Mentha spicata* [16]. The findings of Yousefian et al. (2020) showed that the application of MeJA increases the relative expression of MsPAL, MsC4H, and Ms4CL genes involve in the phenylpropanoid pathway. Many studies suggest that MeJA may bind to membrane receptors to produce active oxygen and protein kinases [21]. The increase of phenolic compounds and flavonoids using MeJA, indicates the inductive effect of elicitors on the metabolic pathway of phenylpropanoids [22].

In the present study, the content of terpenoids was significantly affected by MeJA, and TiO_2 NPs. Previous reviews show that, TiO_2 NPs increase the photosynthetic capacity of plants by directly affecting chlorophyll and indirect by other factors content resulting in glucose synthesis progress. Glucose is a suitable precursor for the synthesis of essential oils, especially monoterpenes [23]. The application of TiO_2 NPs had positive effect on EO production through increased the expression of enzymes involved in the biosynthesis of monoterpenes [24]. In an experiment, Ahmad et al. (2018), found that increasing menthol concentration could be probably due to an increase in the expression of the enzyme menthone reductase, which reduces menthone to menthol. In general, better plant performance [25], improved photosynthesis [26], higher expression involved in terpenoids biosynthesis [10], and an increase in oil glands density have also been attributed to the application of TiO_2 NPs. The positive effect of TiO_2 NPs on essential oils production has been reported in *Salvia officinalis* [27], *Rosmarinus Officinalis* [28], and *Vetiveria zizanioides* [29]. In a study conducted by Ahmed et al (2018) on *Mentha pipperita*, the amount of menthol, menthone, and menthyl acetate increased within the foliar-applied TiO_2 NPs compared to control. In an experiment, higher oil yield in MeJA treated basil plants attributed to increasing plant growth, improving nutrient uptake and changing in leaf oil gland population and biosynthesis of monoterpenes [30]. In Basil plants, MeJA significantly increased the content of monoterpenes [31]. In another experiments, foliar application of MeJA increased thymol and carvacrol in essential oils obtained from *Thymus daenensis* aerial parts [32] and also had a positive effect on the biosynthesis of peppermint EO [33]. Talebi et al. (2018) reported that the extracts obtained from MeJA-treated plants generally possessed higher percentage of linalool and 1,8-cineole compared to the extract of control plants.

5. Conclusion

Plant elicitation due to its promising role in crop-growing systems, could be an important strategy. This compounds are not only ecologically friendly, but are also cost-effective and reliable. Based on our results, MeJA is effective on improving phenolic compound and important constitutes of EO in TiO_2 NPs treated plants. In this study, the positive effect of TiO_2 NPs on improving phenolic and terpenoides compounds of plant was confirmed. However, conducting a gene expression profile and studying quantitative gene expression under different concentrations of MeJA and TiO_2 NPs could be helpful to better understand the mechanisms involved in monoterpenes biosynthesis increase.

6. Acknowledgments

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