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ORIGINAL ARTICLE

Improved antioxidant metabolism in shoot cultures of *Ruta graveolens* (L.) in response to methyl jasmonate and abscisic acid

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Abstract

Polyphenolic compounds are an important class of plant secondary metabolites known to exhibit high antioxidant properties. Plant tissue cultures have been an attractive alternate source of such compounds and their biosynthesis can be improved by elicitation. The present study evaluated the effect of phytohormone elicitors viz. methyl jasmonate and abscisic acid on antioxidant components in in vitro shoot cultures of *Ruta graveolens* L., a well explored medicinal plant. Phytohormones were added into the culture medium and the shoot cultures were harvested after 5, 10 and 15 days post elicitation for analyses. Elicitor type and exposure time significantly influenced each analyzed parameter. Highest content of total phenolic compounds (TPC) was recorded in shoots exposed to methyl jasmonate for 5 days (44.33 µg Gallic acid equivalent g^{-1} FW), representing a 4.4- fold increase compared to control. Highest total flavonoid content (TFC) (6.6-fold increase; 1.43 µg Quercetin equivalent g^{-1} FW) and total flavonol (TFL) (2-fold increase; 1.22 mg Rutin equivalent g^{-1} FW) was recorded in shoots treated with abscisic acid for 5 and 15 days, respectively. Phenyl ammonia lyase (PAL) activity was in correspondence with TPC and TFC in shoots treated with both the elicitors. The antioxidant potential as revealed by DPPH and FRAP assay was highest in shoots elicited with abscisic acid (2-fold increase, 10th day) and methyl jasmonate (1.5- fold increase, 5th day), respectively. Results of reverse phase high performance liquid chromatography (RP-HPLC) revealed MJ as the better elicitor for production of phenolics. This study demonstrates the potential of elicitation strategy for enhanced accumulation of polyphenolic compounds in shoot cultures of *R. graveolens*.

Key message

Methyl jasmonate and abscisic acid increased antioxidant metabolite production in shoot cultures of *R. graveolens* without compromising on the growth. Exposure time of MJ and ABA significantly influenced the metabolite synthesis.

Keywords Active principles · Antioxidants · Elicitors · Flavonoids · In vitro · Phenolics

Introduction

Ruta graveolens L. (rue), an evergreen herb or a small shrub belonging to the family Rutaceae is a well explored medicinal plant. It is beneficial for the treatment of neuromuscular problems, gastric disorders, stiff neck, dizziness, rheumatism, eyestrain-induced headache and inflammation (Raghav et al. 2006). These therapeutic properties are attributed to polyphenols and alkaloid secondary metabolites viz., rutin, psoralen, limonene, pinene, coumarins, furanocoumarins and acridone (Elansary et al. 2020).

Phenolics constitute major antioxidant compounds that protect the cells from damaging effects of reactive oxygen species (ROS) generated normally in cells during metabolism. ROS have implications in several health disorders

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and the current knowledge on the relevance of antioxidant compounds in the fight against pathogens, commuter diseases, diabetes, cardiovascular, neurodegenerative diseases like Parkinson's and Alzheimer's disease (Lima et al. 2019) makes them high demand compounds in pharmaceutical and cosmetic industries. Natural antioxidants have attracted much attention because of the increasing awareness about their safety and efficacy. This makes industries heavily dependent on natural sources and plants in particular. The useful compounds are obtained by extraction from the plant that threatens its conservation. Further, secondary metabolites are generally low bulk compounds and therefore the quantity harvested is not sufficient to meet the ever-increasing demand. In vitro plant tissue cultures have proved to be a good alternate and perpetual source for the production of secondary compounds (Chandran et al. 2020). In recent years researchers have focused on the biotechnological production of useful compounds by employing elicitation strategy. In this, biotic and abiotic molecules are added in small concentrations into the growth medium which then attaches to special receptors located on plant cell membranes, enters into the cell and triggers signal transduction cascade which leads to transcriptional reprogramming of genes encoding enzymes of secondary metabolite biosynthesis pathways (Ramirez-Estrada et al. 2016). This strategy has been reported to enhance the yield of secondary metabolites in *Gynura bicolor* (Shimizu et al. 2010), and *Knautia sarajevensis* (Karalija et al. 2020).

Methyl jasmonate (MJ), a naturally occurring fragrant volatile ester form of jasmonic acid, is known to work as signal transducer to stimulate plant defense mechanisms by regulating secondary metabolism (Benevenuto et al. 2019). Given this role of MJ, it has been suggested that MJ could be used as a chemical elicitor (Rubio-Rodríguez et al. 2021). Abscisic acid (ABA) is an isoprenoid phytohormone. It regulates many aspects of plant growth and response to environmental stress. It is reported to induce the expression of genes involved in phenylpropanoid biosynthetic pathway leading to the accumulation of defense metabolites (Gai et al. 2020).

R. graveolens is scarcely available in nature and its cultivation is also little due to lengthy and insufficient propagation methods. Given its immense medicinal value several workers have developed its tissue cultures for conservation and metabolite production (Bohidar et al. 2008). There are many reports on elicitation of coumarins and furanocoumarins in *R. graveolens* (Ahmad et al. 2020). However, as per the literature no systematic studies have been conducted to improve polyphenolic compounds in *R. graveolens* except for the one which demonstrated the role of different light conditions on accumulation of phenolics in shoot cultures (Szopa et al. 2012).

Active compounds of *R. graveolens* L. are primarily concentrated in stem and leaves. Therefore, we established its shoot cultures and attempted to apply an elicitation strategy to improve polyphenol biosynthesis. For this purpose, we used MJ and ABA as elicitors. The aim of the study was to test the efficacy of MJ and ABA on the accumulation of polyphenolic compounds and antioxidant potential in shoot cultures of *R. graveolens*. We also examined the effect of elicitor exposure period as this factor is known to influence the efficiency of the elicitor.

Materials and methods

Plant material and *in vitro* shoot culture

Ruta graveolens L. plants maintained at the Department of Dravyaguna Herbal Garden, School of Ayurveda, D.Y.Patil Deemed To Be University, Navi Mumbai, India were used for the collection of explants. Nodal segments of *R. graveolens* were washed under running tap water for 5 min, surface sterilized with 0.1% mercuric chloride (w/v) for 5 min followed by thorough washing with distilled water and inoculated on culture initiation medium i.e. MS medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose, 0.8% (w/v) agar, 1.0 mg L^{-1} 6-benzyl aminopurine (BAP) and 0.25 mg L^{-1} Indole acetic acid (IAA) (Bohidar et al. 2008). Cultures were incubated at 25 ± 2 °C, under a 16 h photoperiod of light intensity of 45 µMol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. The shoots obtained were multiplied by subculturing thrice at an interval of 21 days on the same fresh medium in culture bottles. The multiplication phase shoot cultures were used as the experimental material (Fig. 1).

Elicitation treatments

A shoot cluster weighing 0.5 g (containing ca. 8 shoots measuring 0.5 cm) was inoculated on culture initiation medium supplemented with: (a) methyl jasmonate (100 µM), (b) abscisic acid (20µM) and (c) no elicitor (control), six replicates each. For each treatment and control, shoots were harvested on 5th, 10th and 15th day of elicitation for growth and biochemical studies. Growth of shoots was determined in terms of fresh weight (FW) (g), number of shoots per cluster and shoot length (cm).

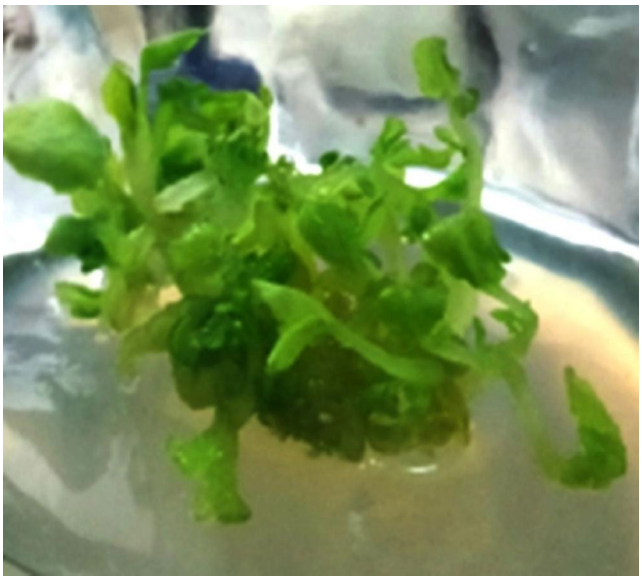

Fig. 1 In vitro shoot culture of *R. graveolens*

Biochemical studies

Extract preparation

For biochemical studies, extracts from shoots of control and elicited cultures were prepared as described by Mihai et al. (2011) with slight modification. A methanol: 0.1% HCl 80:20 (v/v) solution was used as a solvent. The sample to solvent ratio was 1:2. The sample paste was suspended in a conical flask and subjected to ultra-sonication at a frequency of 47 kHz using an ultrasonicator model No. 5.5 L 150 H (Dakshin Ultrasonics, India). The extract obtained was kept overnight at 40 °C in the refrigerator for 12 h. The insoluble material was removed by centrifugation at 4000 x g for 15 min (Superspin R-V/Fa, Plasto crafts, India) and the extracts were concentrated by evaporation using rotavapor (Rotary Vacuuma 'Digital Bath', Superfit Continental Pvt. Ltd. India) and used for biochemical studies as described below:

Total phenolic content

The total phenolic content (TPC) was determined using the Folin Ciocalteu (FC) method described by Supritha and Radha (2018). 0.5 ml of sample extract (1 g L^{-1}) was added to 2.5 ml 10-fold diluted FC reagent and 2 ml 7.5% sodium carbonate (Na_2CO_3). The tubes were kept in dark at room temperature for 30 min followed by measurement of absorbance at 760 nm against a blank UV visible spectrophotometer (UV-1700 Pharma Spec, Shimadzu). The total phenolic content was determined from the standard curve of gallic acid and expressed as mg gallic acid equivalent (GAE) g^{-1} FW.

Total flavonoid content

The aluminium chloride colorimetric assay was used to measure the total flavonoid content (TFC) of the plant extracts (Marinova et al. 2005). 1 ml of the extract (1 g L^{-1}) or standard (quercetin) was added to a 10 ml volumetric flask containing 4 ml of distilled water. 0.3 ml of 5% sodium nitrite ($NaNO_2$) was added to the flask and after five minutes, 0.3 ml of 10% aluminum chloride ($AlCl_3$) was added. The volume was made up to the mark with distilled water. The solution was mixed and allowed to stand for 30 min at room temperature. The absorbance of reaction mixture was measured against the blank at 510 nm using a UV visible spectrophotometer. The total flavonoid content was quantified from the standard curve of quercetin and expressed as mg quercetin equivalents (QE) g^{-1} FW.

Total flavonol assay

The aluminium chloride method described by Akkol et al. (2008) was followed for the estimation of total flavonol (TFL) content. 2 mL of shoot extract (1 g L^{-1}) was mixed with 2 mL (20 g L^{-1}) AlCl3 and 6 mL(50 gL^{-1}) sodium acetate (Akkol et al. 2008) followed by incubation at 20 °C for 2.5 h. The absorbance of the reaction mixture was recorded at 440 nm. Total flavonol content was measured from the standard curve of rutin and expressed as mg rutin equivalents (RE) g^{-1} FW.

Phenylalanine ammonia-lyase (PAL) activity assay

Fresh shoots (1 g) were homogenized in 10 mL Tris-HCl (pH 8.2, 100 mM) buffer containing β-Mercaptoethanol (15 mM). The mixture was centrifuged at 10,000 x g, 4 °C for 15 min and the clear supernatant was used as enzyme extract. The reaction mixture containing enzyme extract (0.5 mL), 16 mM of L-phenylalanine, 50 mM of Tris–HCl buffer (pH 8.9), and 3.6 mM of NaCl was incubated at 30 °C for 60 min and then the reaction was stopped by adding 50 µL of 5 N HCl. The PAL activity was determined by measuring the initial (0 min) and final absorbance (60 min) of the reaction solution at 290 nm (Dong et al. 2010). The amounts of cinnamic acid produced within 1 h per mg of protein were used to express one unit of PAL activity. Total protein estimation was carried out according to Bradford assay (1976) using bovine serum albumin as the standard.

Analysis of antioxidant potential

Antioxidant activity was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (FRSA) and Ferric ion reducing antioxidant power assay (FRAP) assay.

For DPPH-FRSA assay, protocol described by Vijayaraghavan et al. (2013) was followed using butylated hydroxy toluene (BHT) as standard. The ability of extracts to scavenge DPPH radical was assessed and antioxidant activity was expressed as % DPPH inhibition calculated using the formula:

DPPH-FRSA (% inhibition) = Abs of control – Abs of sample/ Abs of control *100.

Where, Abs control is the absorbance of the control (standard) and Abs sample is the absorbance of the test sample (extract).

FRAP assay was performed using the method described by Benzie and Strains (1996). The antioxidant activity based on ferric ion reducing ability was calculated using a standard curve of ascorbic acid at 593 nm. The FRAP result was expressed as mg of ascorbic acid equivalent antioxidant capacity g^{-1} FW (mg AEAC.g^{-1} FW).

HPLC analysis

Quantification of phenolic compounds was performed for shoots from control and those treatments which showed maximum TPC (MJ-5 d and ABA-15 d post elicitation) using HPLC (Waters Model 2487 with UV detector). The stationary phase was C18 column (5µM). The standards of gallic acid, catechol, caffeic acid, ferulic acid, p-coumarin and vanillin were prepared in methanol (1000 ppm) and diluted to 50 ppm. The elution system was composed of 20% methanol, 1% acetic acid and 80% water with a linear gradient scheme and the detector was set at 280 nm (Ranade et al. 2016). For quantification of rutin, the standard was prepared in methanol (1000 ppm) and diluted to 100 ppm. The mobile phase consisted of methanol–water,1:1 (v/v) (pH 2.8 adjusted with phosphoric acid) with isocratic scheme and absorbance was monitored at 360 nm (Kuntić et al. 2007). Methanolic extracts were used for HPLC and the phenolic compounds and rutin were identified on the basis of their retention time on comparing with the standard chromatogram of a mixture of pure phenolic compounds and rutin, respectively. The concentration of compounds was determined from the peak area measurement and was expressed as mg g^{-1}FW.

Statistical analysis

Experiments were conducted in randomized design; results were expressed as mean values (± standard deviation, SD) with three replications. Each treatment was tested for two-way ANOVA assuming no sphericity with 95% confidence interval. Tukey's multiple comparisons test was further applied to validate the difference between means of each treatment. All statistical testing and graphs plots were done using Graph Pad Prism 8.0.1.

Results and discussion

R. graveolens shoot cultures are known to synthesize polyphenolic compounds. The present study examined the effect of exogenous addition of MJ and ABA on accumulation of these metabolites in shoot cultures of *R. graveolens*. Analysis of variance (ANOVA) revealed that elicitor type, its contact time and the interaction between elicitor and contact time significantly influenced all the examined parameters.

Effect of elicitors on shoot growth

ABA is generally considered as a growth retardant (Rai et al. 2011) with few exceptions that suggest its role in promotion of shoot growth. MJ is also known to inhibit growth by suppression of cell proliferation and expansion or disruption of meristematic activity (Kamińska, 2021) and has shown negative effects on biomass accumulation in in vitro cultures of *Gynura bicolor* (Shimizu et al. 2010). Contrastingly, in the present study both the elicitors did not reduce any of the growth parameters but showed a slight increase (1.1-1.3-fold) upon long duration exposure. A maximum increase of 1.3-fold was seen in fresh weight and shoot number on ABA on 15th day post elicitation, compared to control (Table 1). Studies have revealed that growth-inhibiting effects of exogenous ABA can be counteracted by cytokinins present in the medium (Rai et al. 2011) and this might explain the slight increase seen in the present study. There are reports where MJ addition in the medium increased in vitro growth like shoot proliferation in *Musa acuminata* (Mahmood et al. 2012), shoot length in *Ziziphora persica* (Zare-Hassani et al. 2019, plant height in *Artemisia annua* (Alam and Albalawi 2020; Avalbaev et al. 2016) reported the role of MJ in increasing endogenous cytokinins level which affects cell division and growth. The differential effect of exogenous elicitors may arise from modification of synthesis, catabolism, activation, sequestration, transport, or sensitivity to endogenous phytohormones of the same or other type (Gaspar et al. 1996).

Table 1 Effect of elicitors on growth parameters of *in vitro* shoot cultures of *R. graveolens*

Elicitor	Duration of exposure (days)	Fresh weight (g)	Mean length of shoots (cm)	Mean number of shoots
MJ (100 µM)	0	0.52 ± 0.14	0.5 ± 0.94	7.00 ± 0.83
	5	0.65 ± 0.15	0.9** ± 0.54	7.01 ± 0.76
	10	0.9** ± 0.28	1.02* ± 0.47	7.01 ± 0.97
	15	1.3** ± 0.66	1.1 ± 0.87	12.04* [a] ± 0.75
ABA (20 µM)	0	0.52 ± 0.14	0.5 ± 0.94	7.00 ± 0.83
	5	0.67 ± 0.38	1.2*** ± 0.7	8.1 ± 0.64
	10	1.12*** ± 0.29	1.5*** ± 0.30	8.7 ± 0.47
	15	1.45*** ± 0.56	1.5** ± 0.09	13.02** ± 0.29
Control (without elicitor)	0	0.52 ± 0.14	0.5 ± 0.94	7.00 ± 0.83
	5	0.64 ± 0.22	0.6 ± 0.94	7.00 ± 0.83
	10	0.73 ± 0.36	0.8 ± 0.28	7.01 ± 0.45
	15	1.12 ± 0.76	1.2 ± 1.2	10.08 ± 0.66

Values are the means ± SD (n=3),***, ** and * indicates the significant difference between the means of the treatment and the respective control at p<0.001, p<0.01 and p<0.05 respectively, Tukey's test. A shoot cluster (0.5 g) inoculated on medium with and without elicitor comprised of 7 shoots measuring 0.5 cm

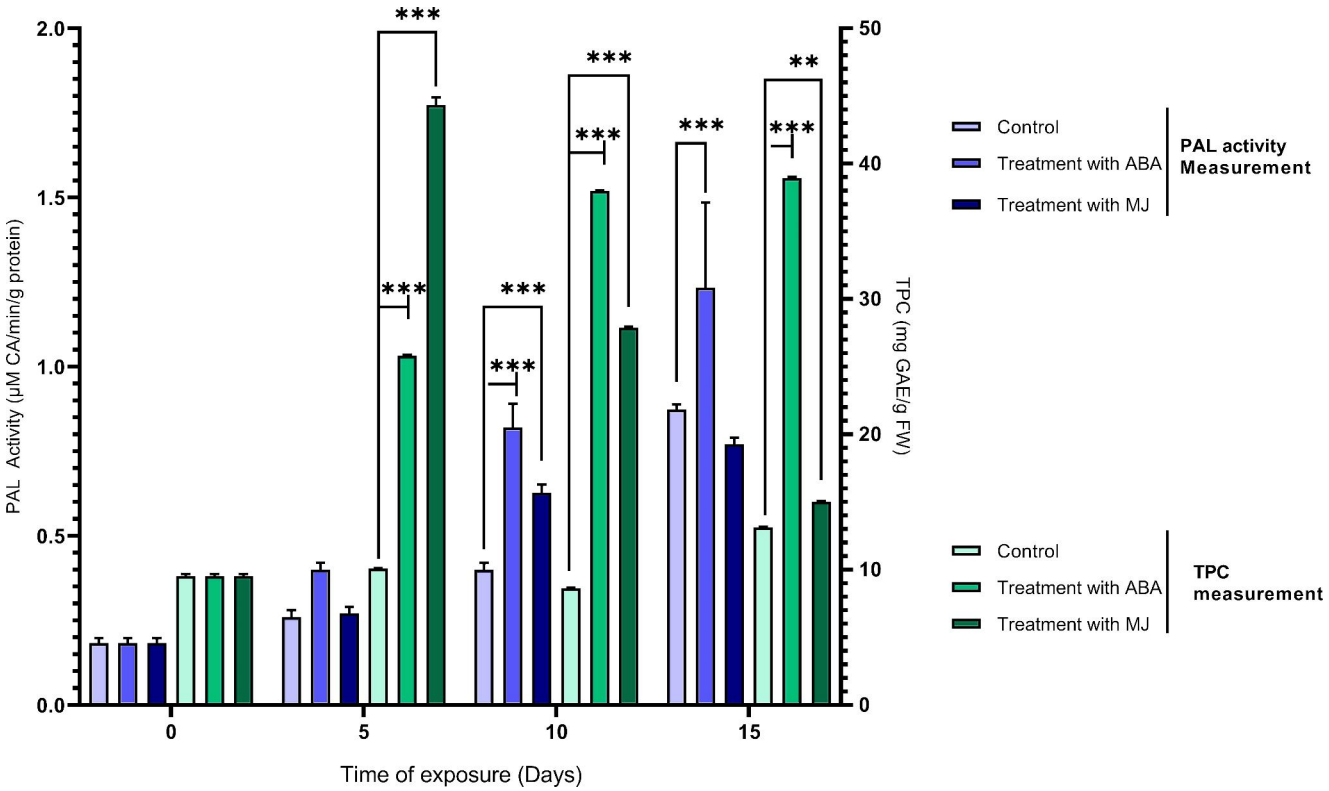

Fig. 2 Effect of elicitors on total polyphenol concentration (TPC) and PAL activity in shoot cultures of *R. graveolens*. Values are means ± standard deviation of six replicates,*** and ** indicates the significant difference at p < 0.001 and p < 0.01, respectively, Tukey's test

Effect of elicitors on total phenolic content (TPC), total flavonoid content (TFC) and total flavonol content (TFL)

TPC, TFC, TFL are the most common markers of antioxidant potential. In the present study, both the elicitors increased TPC compared to control, and the highest value of 44.33 mg GAE g^{-1} FW was obtained on MJ on 5th day post elicitation, representing a 4.4-fold increase over control followed by a 3.5-fold increase on ABA on 10th day post elicitation (Fig. 2). With the increase in exposure time, TPC declined on MJ but remained constant on ABA. Maximum accumulation of phenolics after short exposure of elicitor has been reported in *K. sarajevensis* (Karalija et al. 2020).

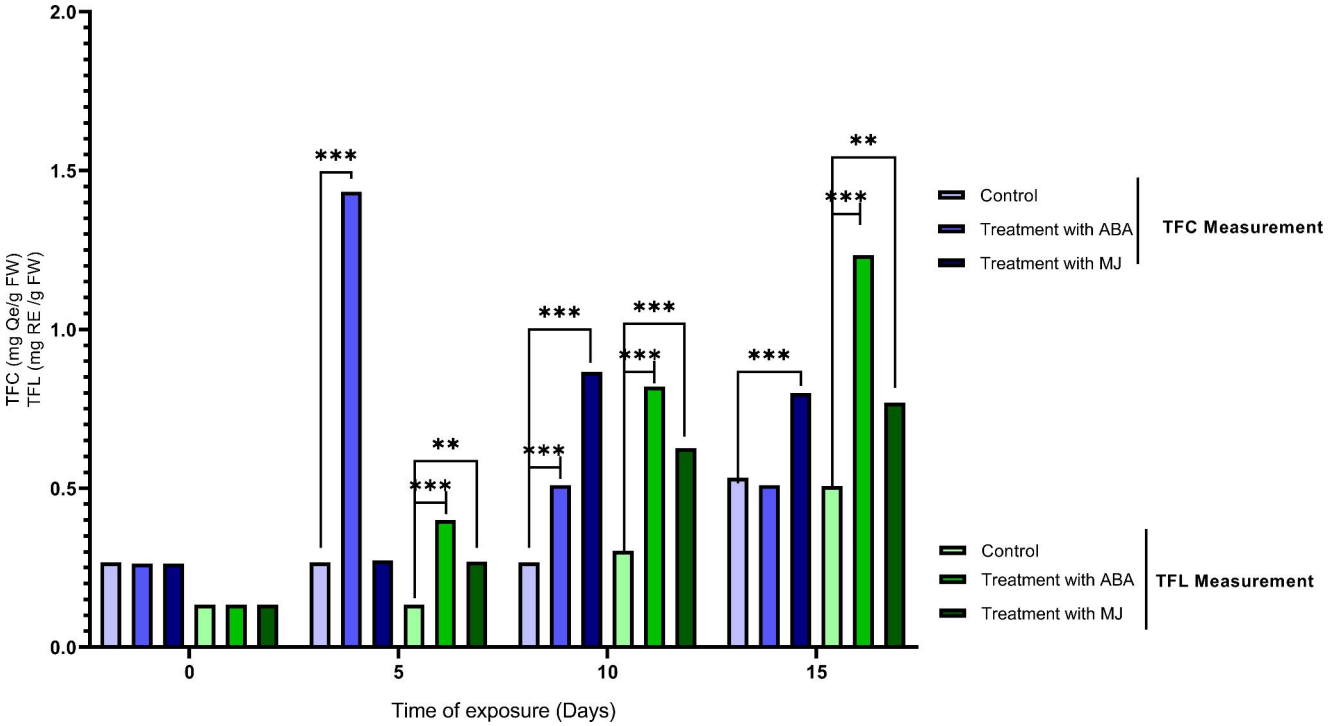

Fig. 3 Effect of elicitors on total flavonoid concentration (TFC) and total flavonol concentration (TFL) in shoot cultures of *R. graveolens*. Values are means ± standard deviation of six replicates,*** and ** indicates the significant difference at $p < 0.001$ and $p < 0.01$, respectively, Tukey's test

On MJ, TFC was similar to control (0.26 mg QE g^{-1} FW) on 5th day post elicitation but increased by 4-fold (0.87 mg QE g^{-1} FW) on 10th day and remained constant thereafter (Fig. 3). This is in agreement with elevated TFC levels observed in in vitro cultures of *Momordica charantia* (Chung et al. 2016) elicited with MJ. TFC increased most by 6-fold (1.43 mg QE g^{-1} FW) over control in shoots treated with ABA for 5 days and it then decreased by 104%with increase in exposure time. According to Piątczak et al. (2016), decline in metabolite content with increased elicitor contact might be the result of either metabolites degradation in situ or its leakage into the medium. Similar to our observations, exogenous application of ABA increased the phenolics and flavonoid contents in Tea (Gai et al. 2020). Recently, the transcriptomic and metabolomic studies revealed that exogenous ABA induced the expression of genes involved in flavonoid biosynthesis (Gai et al. 2020). Authors explained that on ABA, increase in biomass results in nutrient stress which stimulates the synthesis of antioxidant phenolics.

Flavonols are a type of flavonoid compound. Highest TFL (1.22 mg RE g^{-1} FW) was obtained in shoots elicited with ABA for 15 days, representing 2-fold increase compared to control (0.52 mg RE g^{-1} FW). MJ also increased flavonol content by nearly 2-fold on 5th and 10th day post elicitation (Fig. 3). On ABA treatment, TFL increased with increase in exposure time while a reverse trend was seen for TFC. This indicates that it's not flavonol but other flavonoids that contributed to the high TFC on the 5th day but its contribution increased with increase in exposure time. On MJ treatment, both TFC and TFL increased with exposure time. These results suggest that each compound may be differently influenced by a particular elicitor.

Both MJ and ABA are involved in plants' response to stress by regulating the synthesis of defense molecules. They initiate de novo transcription of genes encoding enzymes of the biosynthetic pathways of phenolics and flavonoids (Gai et al. 2020). Jalalpour et al. (2014) showed that increased phenolic metabolism in MJ elicited cells was due to enhancement of endogenous jasmonate levels through induction of lipoxygenase (LOX) activity. It is clear from our results that both elicitors displayed different effects on phenolics and flavonoids accumulation which could be attributed to the difference in the mode of action of the two phytohormones. They may induce different signal transduction pathways ultimately resulting in accumulating metabolites in different concentrations. These concentrations also varied with elicitor contact period which could be because for each biosynthetic pathway the time required for its enzymes to show maximal activity is different (Piątczak et al. 2016).

Table 2 RP-HPLC based quantification of phenolic compounds and rutin in *R. graveolens* L. shoot cultures treated with elicitors

Elicitor	Gallic acid (mg g^{-1})	Catechol (mg g^{-1})	Vanillin (mg g^{-1})	Caffeic acid (mg g^{-1})	p-Coumaric acid (mg g^{-1})	Ferulic acid (mg g^{-1})	Rutin (mg g^{-1})
MJ day 5	39.4	40.1	72.0	27.0	17.4	21.7	NQ
MJ day 15	NQ	NQ	NQ	NQ	NQ	NQ	88.7
ABA day 15	-	-	39.6	14.8	78.4	-	123.8
Control- day 5	-	-	24.1	-	-	-	NQ
Control- day 15	-	-	30.0	-	9.5	-	34.5

MJ: methyl jasmonate,, ABA: abscisic acid, NQ: Not quantified, Quantification was done for the treatments showing highest TPC and TFL and their respective controls

Effect of elicitors on PAL activity

Polyphenols are produced through the phenylpropanoid pathway and this is the most analyzed pathway in elicitation studies. Biosynthesis of phenolics and flavonoids initiates through deamination of L-phenylalanine to trans-cinnamic acid by the enzyme PAL (Sharma et al. 2019). In the present study, shoots treated with MJ and ABA showed 10-fold (2.8 µM CA $min^{-1}.g^{-1}$ protein) and 7.6-fold (2.0 µM CA $min^{-1}.g^{-1}$ protein) increase in PAL activity, respectively compared to control on 5th day post elicitation (Fig. 2). On both elicitors, the activity declined with exposure time, the effect however was more significant on MJ. Increased PAL activity was also in correspondence with increased TPC and TFC in both MJ and ABA treated shoots, except for TFC on MJ on 5th day post elicitation. Increased accumulation of these metabolites is usually associated with increased activity of PAL (Rubio-Rodríguez et al. 2021). Our results agree with the role of this enzyme in antioxidant metabolite synthesis.

RP-HPLC quantification of phenolics and rutin

RP-HPLC was performed for some of the polyphenolics to see their individual contribution to TPC and TFC. It was carried out for only those treatments which showed maximum TPC (5 days MJ treated and 15 days ABA treated shoots). It was observed that MJ induced accumulation of all the analyzed phenolics viz. gallic acid, catechol, caffeic acid, p-coumaric acid, caffeic acid, vanillin and ferulic acid while ABA treated shoots showed the presence of only p-coumaric acid, vanillin and caffeic acid (Table 2, Supplementary information). Control shoots showed the presence of vanillin at the concentration of 24.1 $mg.g^{-1}$ and 30.0 $mg.g^{-1}$ on the 5th and 15th day of incubation, respectively. Its accumulation increased by 198%(72.0 $mg.g^{-1}$) and 32% (39.6 $mg.g^{-1}$) on MJ (5 days) and ABA (15 days), respectively. p-coumaric acid was also detected in control shoots on the 15th day (9.5 $mg.g^{-1}$) and that was increased by 2-fold and 8-fold on MJ and ABA, respectively. The absence of other phenolics in control shoots could be because their synthesis was not within the detectable limits. In the present study, presence of additional phenolics in elicited shoot cultures indicates the role of elicitors in the regulation of their biosynthetic pathways. MJ-mediated elicitation of phenolic compounds has also been reported in in vitro cell cultures of *Vitis vinifera* (Sák et al. 2014).

Rutin was quantified in shoots from control and those treatments which showed maximum TFL (MJ-15 d and ABA-15 d post elicitation). In control shoots, rutin was detected at a concentration of 34.5 $mg.g^{-1}$. Its accumulation increased by 3.5-fold and 2.5-fold in shoot treated for 15 days with ABA and MJ, respectively (Table 2). This increase was in correspondence with TFL indicating substantial contribution of rutin in TFL.

Effect of elicitors on antioxidant activity (DPPH & FRAP)

The protective effect of phenolics and flavonoids are attributed to their antioxidant capacity. It was determined using widely accepted assays that measure different antioxidative ability like free radical scavenging (DPPH assay) and transition metal ion reduction (FRAP assay). In response to both elicitors, shoots showed higher DPPH-FRSA compared to control indicating synthesis of DPPH scavenging antioxidant compounds (Fig. 4). The highest value of DPPH inhibition (82%) was recorded on ABA on 10th day post elicitation which was 2-fold higher than control (42.33%) and it declined to 78% with increased duration of exposure. On MJ, DPPH inhibition was 21.9% which was less than control (36.86%) on 5th day post elicitation but was higher than control on long exposure, representing a 13% increase over control (42.26%).

Antioxidant activity measured by FRAP assay increased and decreased with duration of exposure of ABA and MJ, respectively. The highest FRAP value of 0.4 mg AEAC g^{-1} FW was obtained on MJ on 5th day post elicitation, representing an increase of 1.5-fold followed by ABA treatment for 15 days which showed a 1.7-fold increase over control (Fig. 4).

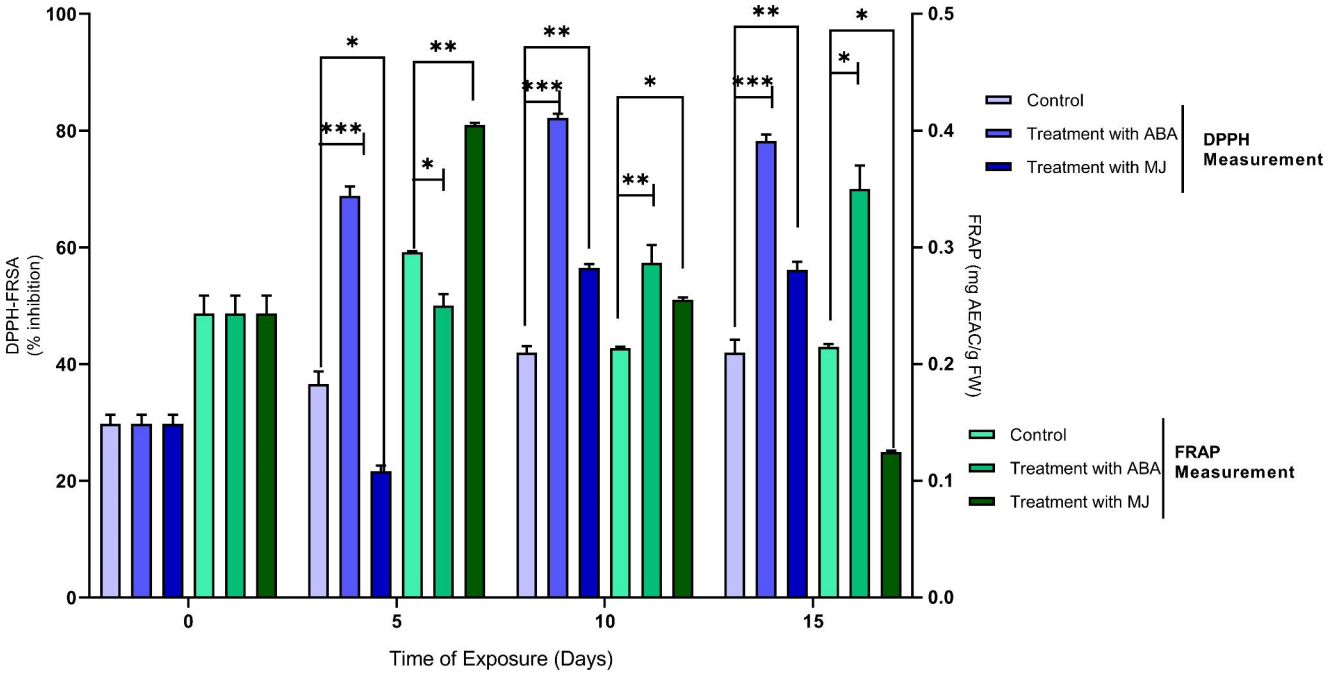

Fig. 4 Effect of elicitors on antioxidant potential; DPPH activity (% inhibition) and FRAP value in shoot cultures of *R. graveolens*. Values are means ± standard deviation of six replicates,*** and ** indicates the significant difference at $p < 0.001$ and $p < 0.01$, respectively, Tukey's test

Wee et al. (2015) also reported a 1.3-fold increase in antioxidant activity (DPPH and FRAP) in MJ elicited-callus cultures of *Sauropus androgynous*. ABA showed high FRAP value (1.2 fold) than control (0.29 mg AEAC g^{-1} FW) on the 10th day of exposure which supports the previously realized fact that ABA can stimulate the expression of antioxidant genes thus enhancing the antioxidant capacity in plants (Choudhary et al. 2012).

On comparing the results of two assays it was found that both DPPH inhibition and FRAP values increased with exposure time of ABA but on MJ the two parameters showed a contrasting trend. Also, the increase in antioxidant capacity measured by DPPH was more than FRAP for both elicitors. This difference could be because the two methods have different principles and mechanisms to measure antioxidant capacity. Comparison of antioxidant capacity and antioxidant compound contents revealed that results of DPPH-FRSA correspond with TPC and TFC in case of ABA and MJ elicitation, respectively. On both ABA and MJ, FRAP values corresponded with TPC but not with TFC indicating more contribution of phenolics than flavonoids in reducing ferric ions. It is known that those antioxidants that react quickly with transition metal ion will react slower or may not react with DPPH due to steric hindrances while some reports have shown that a combination of polyphenolic compounds produced a synergistic effect on DPPH and FRAP (Khorsidi and Nijavan 2006).

Conclusion

Elicitors increased polyphenolics accumulation and antioxidant potential in shoot cultures of *R. graveolens* L. Exposure time of elicitor also played a significant role in metabolite synthesis. Maximum accumulation of TPC and TFC was obtained after short time exposure of MJ and ABA, respectively. Shoots treated with MJ for 5 days showed the presence of all the phenolics estimated by HPLC. Both the elicitors improved metabolite production without compromising on biomass growth. These findings suggest that shoot cultures of *R. graveolens* could be used as a potential source of polyphenolic compounds with antioxidant activity which are in great demand in pharmaceutical, cosmetic and food industries.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-023-02474-x.

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Author Contribution N. Joshi, K. Agarwal contributed in conduct of experiments and interpretation of results. N. Joshi and S. Gosh contributed in interpretation of results, data analysis and preparation of manuscript.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare that they have no conflict of interest.

References

Ahmad TA, Al-Mahdawe MM, Nadir DS (2020) Effect of methyl jasmonate on the production of furanocoumarins in cell suspension cultures of *Ruta graveolens* L. Plant Cell Tiss Organ Cult 143:565–571. https://doi.org/10.1007/s11240-020-01941-z

Akkol EK, Göger F, Kosar M, Baser HC (2008) Phenolic composition and biological activities of *Salvia halophila* and *Salvia virgata* from Turkey. Food Chem 108:942–949. https://doi.org/10.1016/j.foodchem.2007.11.071

Alam P, Albalawi TH (2020) In vitro alteration of artemisinin biosynthesis in *Artemisia annua* L during treatment with methyl jasmonate. Trop J Pharm Res 19:33–37. https://doi.org/10.4314/tjpr.v19i1.5

Avalbaev A, Yuldashev R, Fedorova K, Somov K, Vysotskaya L, Allagulova C, Shakirova F (2016) Exogenous methyl jasmonate regulates cytokinin content by modulating cytokinin oxidase activity in wheat seedlings under salinity. J Plant Physiol 191:101–110. https://doi.org/10.1016/j.jplph.2015.11.013

Benevenuto RF, Seldal T, Hegland SJ et al (2019) Transcriptional profiling of methyl jasmonate-induced defense responses in bilberry (*Vaccinium myrtillus* L.). BMC Plant Biol 19:70. https://doi.org/10.1186/s12870-019-1650-0

Benzie IF, Strain JJ (1999) Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol 299:15–27

Bohidar S, Thirunavoukarasu M, Rao T (2008) Effect of plant regulators on in vitro micropropagation of garden rue *(Ruta graveolens* L.). Int J of Integrative Biol 3(1):36–43

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Chandran H, Meena M, Barupal T, Sharma K (2020) Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. Biotech Rep 26:e00450. https://doi.org/10.1016/j.btre.2020.e00450

Choudhary R, Saroha AE, Swarnkar PL (2012) Effect of abscisic acid and hydrogen peroxide on antioxidant enzymes in Syzygium cumini plant. J Food Sci Technol 49: 649–652. https://doi.org/10.1007/s13197-011-0464-3

Chung IM, Thiruvengadam M, Kaliyaperumal R, Govindasamy R (2016) Elicitation enhanced the production of phenolic compounds and biological activities in hairy root cultures of bitter melon (*Momordica charantia* L.). Brazilian Arch of Bio and Tech 59:e160393

Dong J, Wan G, Liang Z (2010) Accumulation of salicylic acid-induced phenolic compounds and raised activities of secondary metabolic and antioxidative enzymes in *Salvia miltiorrhiza* cell culture. J Biotechnol 148(2–3):99–104. https://doi.org/10.1016/j.jbiotec.2010.05.009

Elansary HO, Szopa A, Kubica P, Ekiert H et al (2020) Polyphenol Content and Biological Activities of *Ruta graveolens* L. and *Artemisia abrotanum* L. in Northern Saudi Arabia. Processes 8:531. https://doi.org/10.3390/pr8050531

Gai Z, Wang Y, Ding Y et al (2020) Exogenous abscisic acid induces the lipid and flavonoid metabolism of tea plants under drought stress. Sci Rep 10:12275. https://doi.org/10.1038/s41598-020-69080-1

Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA (1996) Plant hormones and plant growth regulators in plant tissue culture. In vitro Cell Dev Biol Plant 32:272–289. https://doi.org/10.1007/BF02822700

Jalalpour Z, Shabani L, Afghani L, Sharifi-Tehrani M, Amini, SA (2014) Stimulatory effect of methyl jasmonate and squalestatin on phenolic metabolism through induction of LOX activity in cell suspension culture of yew. Turk J of Biol 38:76–82. https://doi.org/10.3906/biy-1306-91

Kamińska M (2021) Role and activity of jasmonates in plants under in vitro conditions. Plant Cell Tiss Organ Cult 146:425–447. https://doi.org/10.1007/s11240-021-02091-6

Karalija E, Zeljković S, Parić A (2020) Harvest time–related changes in biomass, phenolics and antioxidant potential in *Knautia sarajevensis* shoot cultures after elicitation with salicylic acid and yeast. In Vitro Cell Dev Biol-Plant 56:177–183. https://doi.org/10.1007/s11627-019-10028-0

Khorsidi M, Nijavan AM (2006) The effects of abscisic acid and CaCl2 on the activities of antioxidant enzymes under cold stress in maize seedlings in the dark. Pak J Biol Sci 2006:9:54–59

Kuntić V, Pejić N, Ivković B, Vujić Z, Ilić K, Mićić S, Vukojević V Isocratic RP-HPLC method for rutin determination in solid oral dosage forms. J Pharm Biomed Anal 43:718 – 21. https://doi.org/10.1016/j.jpba.2006.07.019

Lima MC, Paiva C, Sousabc C, Fernandez-Pradad J, Hareld JD, Dubreuild EL (2019) A review of the current evidence of fruit phenolic compounds as potential antimicrobials against pathogenic bacteria. Microbial Pathogen 130:259–270. https://doi.org/10.1016/j.micpath.2019.03.025

Mahmood M, Bidabadi SS, Ghobadi C, Gray DJ (2012) Effect of methyl jasmonate treatments on alleviation of polyethylene glycol-mediated water stress in banana (*Musa acuminata* cv. 'Berangan', AAA) shoot tip cultures. Plant Growth Regul 68:161–169. https://doi.org/10.1007/s10725-012-9702-6

Marinova D, Ribarova F, Atanassova M (2005) Total phenolics and total flavonoids in bulgarian fruits and vegetables. J Univ Chem Technol Metall 40(3):255–260

Mihai R, Cristina S, Helepciuc F, Brezeanu A, Stoian G (2011) Biotic and abiotic elicitors induce biosynthesis and accumulation of resveratrol with antitumoral activity in the long-term *Vitis vinifera* L. callus cultures. Rom Biotechnol Lett 16(6):6683–6689

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Piątczak E, Kuźma Ł, Wysokińska H (2016) The influence of methyl jasmonate and salicylic acid on secondary metabolite production in Rehmannia glutinosa Libosch. Hairy root culture. Acta Biol Cracov Bot 58:57–65. https://doi.org/10.1515/abcsb-2016-0004

Raghav SK, Gupta B, Agrawal C, Goswami K, Das HR (2006) Anti-inflammatory effect of *Ruta graveolens* L. in murine macrophage cells. J Ethnopharmacol 104(1–2):234–239

Rai MK, Shekhawat NS, Harish, Gupta AK et al (2011) The role of abscisic acid in plant tissue culture – a review of recent progress. Plant Cell Tiss and Org Cult 106:179–190

Ramirez-Estrada K, Vidal-Limon H, Hidalgo D, Moyano E, Golenioswki M, Cusidó RM, Palazon J (2016) Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules 21:182

Ranade R, Jain A, Joshi N (2016) Estimation of phenolic compounds by RP-HPLC and antioxidant activity in leaf and stem extracts of

Barleria prionitis L. Int J Pharm Sci Res 7(6):2445–2457. https://doi.org/10.13040/IJPSR.0975-8232.7

Rubio-Rodríguez E, Ileana V, Sepúlveda-García EB et al (2021) Secondary metabolite production and related biosynthetic genes expression in response to methyl jasmonate in *Castilleja tenuiflora* Benth in vitro plants. Plant Cell Tiss and Org cult 144(3):519–532. https://doi.org/10.1007/s11240-020-01975-3

Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng B (2019) Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules 24(13):2452. https://doi.org/10.3390/molecules24132452

Shimizu Y, Maeda K, Kato M et al (2010) Methyl jasmonate induces anthocyanin accumulation in *Gynura bicolor* cultured roots. In Vitro Cell Dev Biol -Plant 46:460–465. https://doi.org/10.1007/s11627-010-9294-7

Sák M, Dokupilová I, Mihálik D, Lakatošová J, Gubišová M, Kraic J (2014) Elicitation phenolic compounds in cell culture of Vitis vinifera L. by Phaeomoniella chlamydospora. Novo Biotechnol et Chim 13(2):162–171

Supritha P, Radha KV (2018) Estimation of phenolic compounds present in the plant extracts using high pressure liquid chromatography, antioxidant properties and its antibacterial activity. Indian J Pharma Edu Res 52(2):321–326

Szopa A, Ekiert H, Szewczyk A et al (2012) Production of bioactive phenolic acids and furanocoumarins in in vitro cultures of *Ruta graveolens* L. and *Ruta graveolens* ssp. divaricata (Tenore) Gams. Under different light conditions. Plant Cell Tiss Organ Cult 110:329–336. https://doi.org/10.1007/s11240-012-0154-5

Vijayaraghavan K, Ali SM, Maruthi R (2013) Studies on phytochemical screening and antioxidant activity of *Chromolaena odorata* and *Annona squamosa*. Internat J of Innov Res in Sci Eng and Technol 2:7315–7732

Wee SL, Yap WSP, Alderson PG, Khoo TJ (2015) Effects of elicitors on in vitro cultures of *Auropus androgynous* (sweet shoot) for sustainable metabolite production and antioxidant capacity improvement. Acta Hortic 1083:145–155

Zare-Hassani E, Motafakkerazad R, Razeghi J, Kosari-Nasab M (2019) The effects of methyl jasmonate and salicylic acid on the production of secondary metabolites in organ culture of *Ziziphora persica*. Plant Cell Tiss Organ Cult 138:437–444. https://doi.org/10.1007/s11240-019-01639-x