Methyl Jasmonate Induces Oxidative/Nitrosative Stress and the Accumulation of Antioxidant Metabolites in Phoenix Dactylifera L.

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Research Article

Abstract

Objectives

The present study aimed at exploring the eliciting effects of increasing concentrations (50, 100, and 200 μM) of MeJA. We cultivated actively proliferating buds of *Phoenix dactylifera* L. cv. Barhee in a temporary immersion system and we monitored the bioactive compounds accumulation after 7 days of culture.

Methods

Total phenolic (TPC) and flavonoid (TFC) contents were determined by high-performance liquid chromatography (HPLC), Fourier-transform infrared (FTIR), and radical scavenging activity using DPPH and ABTS assays. We also explored the activity of phenylpropanoid pathway enzymes, namely phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL) and polyphenol oxidase (PPO).

Results

Our results revealed that high MeJA concentrations induced an increase in the PAL, TAL and PPO activities. Several stress markers such as Hydrogen peroxide (H_2O_2), Nitric oxide (NO), Malondialdehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT) and Guaiacol peroxidase (GPOD) were also found to rise. Beside, MeJA caused subsequent elevations in the amount of catechin, 4-Hydroxybenzoic acid, caffeic acid and *p*-Coumaric acid and antioxidant capacity with the lowest DPPH and ABTS IC50 values. Microscopic observations using fluorescence probes such as fluorescein diacetate (FDA) and acridine orange/ethidium bromide demonstrated that the supplementation of MeJA to culture media induced cells death in a dose dependent manner.

Conclusion

The findings suggest that *in vitro* cultures elicited by MeJA of *Phoenix dactylifera* L. have the capacity to produce secondary metabolites.

1. Introduction

Date palm (*Phoenix dactylifera* L.) has been widely cultivated in the Middle East and North Africa; it represents one of the most successful tree species in Tunisia, dating back to the first century BCE (Gros-Balthazard et al. 2021). To further improve is key staple crop, noticeable progress has been made in plant regeneration through *in vitro* organogenesis and somatic embryogenesis (Fki et al. 2011). The production of bioactive compounds in plant cell, tissue, and organ cultures has been a widely studied area in plant biotechnology processes (Efferth 2019). Such organic molecules are studied particularly thanks to their antioxidant properties. The micropropagation of plants in a temporary immersion system constitutes an

efficient alternative for the production of secondary metabolites for pharmaceutical use (Othmani et al. 2017). The major advantages of plant tissue cultures, when compared to traditional whole plant cultivation, reside in their ability to provide a permanent, sustainable, economical and viable production of secondary metabolites, independently of the geo-climatic conditions, i.e, under a precisely controlled microenvironment (Efferth 2019).

Several biotechnological strategies have been applied to enhance *in vitro* productivity of secondary metabolites. To date, elicitation, i.e. the exposure of *in vitro* cultures to biotic and abiotic elicitors, has proved to be a useful tool to enhance the synthesis/accumulation of secondary metabolites, or the induction of novel bioactive compounds (Thakur et al. 2019). Abiotic elicitors are of a non-biological origin (Veersham 2004) and they are classified into physical, chemical, and hormonal elicitor groups. Changes in media compositions are frequently used to provide chemical and physical stresses, such as salts, heavy metals, inorganic and synthetic compounds. Biotic elicitors are derived from biological sources: they are generated by living organisms, like signaling or surface component from fungi, bacteria, viruses and herbivore infections (Venugopalan and Srivastava 2015). Complex preparations such as yeast extract can be used as biotic elicitors as well (Thakur et al. 2019). Elicitors treatments generally induce physiological changes (Sachadyn-Król and Agriopoulou 2020). Indeed, they are able to cause alterations in morphogenesis, photosynthesis, hormonal state, ion transport, biochemical acclimatization, including antioxidative metabolism responses, and gene expression (Baenas et al. 2014). Different culture forms (cell, callus, root, hairy root, entire plant, shoots, and seedling) can be treated by exposure to elicitor (Venugopalan and Srivastava 2015).

Jasmonic acid (JA), methyl jasmonate (MeJA) and salicylic acid (SA) are plant growth regulators that play crucial roles in regulating defense signaling networks directed towards pathogens or herbivorous insects (Per et al. 2018). MeJA is commonly employed as a chemical elicitor with view to increasing the production of phenolic compounds and other secondary metabolites in cells, calluses and tissue cultures of various plants (Ho et al. 2018). Moreover, adding exogenous MeJA to vitro cultures was found to prompt the production of reactive oxygen species (ROS) and to activate defense response through changes in the activity of the antioxidant enzymes. MeJA also stimulates molecular signal transduction and the regulation of gene expression, thus leading to the accumulation of secondary metabolites (HO et al. 2018).

Presumably, no prior research has been conducted with respect to the impact of MeJA application on *in vitro* cultures of *Phoenix dactylifera* L. grown in a temporary immersion bioreactors. Both the nature of the produced phytochemicals as well as the mechanisms of secondary metabolites production under similar conditions remain unidentified. In the present study, we investigated the effects of MeJA application at various concentrations on the morphology of *Phoenix dactylifera* L. buds. We measured phytochemical responses to MeJA treatments including total flavonoids and phenolics levels, as well as tyrosine ammonia-lyase (TAL), L-phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO) and Guaiacol peroxidase (GPOD) activities in bud tissues exposed to various concentrations of MeJA in temporary immersion system.

2. Materials And Methods

1. 1. Plant Material And Elicitor Treatments

Phoenix dactylifera L. bud clusters have been routinely maintained *in vitro* through subcultures and transferred every 6–8 weeks into multiplication media, as previously reported in our research (Fki et al. 2011). In this study, a temporary immersion bioreactor container (RITA™, Cirad, France) with two separate parts was employed. Elicitation experiments were performed in triplicate. All of the aseptic manipulations were carried out under a laminar airflow cabinet. To this end, 20 g fresh weight of growing buds were transferred onto the upper part of the RITA bioreactor containing 200 ml of sterile fresh Murashige and Skoog (MS) liquid medium in the lower part to which various concentrations of Methyl jasmonate (MeJA) were added. MeJA (Sigma-Aldrich) solutions were freshly prepared on the day of application at the following concentrations (0 (Control), 50, 100 and 200 µM), according to Udomsin et al. 2020. The cultures were maintained at 26 ± 2°C under 16/8 h (light/dark) photoperiod. The pumped air traveled down to the medium storage tank through a small pipe.

The pressure that builds up in the media storage tank forces the nutrient solution up to the culture chamber for 30 minutes during the immersion phase. The *in vitro* culture samples were harvested after 7 days of temporary immersion cultivation. For each of the four MeJA concentrations tested, three independent experiments were conducted with triplicate samples of bud explants (n = 12). Changes in buds morphology and viability were recorded based on visual inspection and fluorescent microscopy after staining. Plant materials from tissue cultures were harvested, weighed and aliquots were stored at – 80°C until final extraction for further biochemical assays. Furthermore, other freshly harvested buds were oven dried and used for phytochemical analysis.

2.2. Estimation of cell viability in in vitro cultures: fluorescein diacetate (FDA), acridine orange/ethidium bromide (AO/EB) and DCFH-DA staining using fluorescent microscopy

Browning in plant tissue culture indicates the release of colored compounds from the upper part of the RITA to the medium during growth or subdivision of the explant, in such a way that both the media and explants progressively change into brown. The viability of tissues was estimated after staining with fluorescein diacetate (FDA) according to Cai et al. (2020) and Jones and Saxena (2013). To this aim, tow milligrams of fluorescein diacetate were dissolved in 1 ml acetone. Then, the buds were stained with a reaction mixture, containing FDA solution (60 µl/ml) in liquid culture medium. The blend was left to incubate for 10 min at 25°C and was subsequently washed three times with ultrapure water. Fluorescence staining with AO/EB was employed to estimate cell death (Rybaczek et al., 2015). We identified four types of cells based on the fluorescence emission: (1) Viable cells look green, (2) early apoptotic cells appear green yellow to yellow, (3) late apoptotic cells range from yellow-orange to brilliant orange whereas (4) necrotic cells appear as dark orange to bright red. The production of intracellular reactive oxygen species (ROS) was measured through 2', 7'- dichlorofluorescin diacetate (H_2-DCFDA) oxidation. After treatment with MeJA, buds were incubated with 20 µM H_2-DCFDA at 37°C for 1 hour in darkness then washed twice

with PBS. Buds stained with FDA, AO/EB and H$_2$-DCFDA were visualized with a fluorescence microscope (B-383FL OPTIKA®, Italy). Data was quantitatively analyzed based on ImageJ v1.53K software.

2. 3. Biochemical Assays

2. 3. 1. Measurement of cellular ROS, Hydrogen peroxide (H$_2$O$_2$), nitrite formation (NO), lipid peroxidation and total thiols

A 2', 7'-dichlorofluorescein diacetate (H$_2$-DCFDA) fluorescence probe was used for the monitoring of ROS formation in *Phoenix dactylifera* L. The probe is transformed by intracellular esterase and H$_2$O$_2$ into the polar, fluorescent 2', 7' -dichlorofluorescein (DCF) according to Li (2019). DCF intensity was assessed in a microplate-reader at excitation and emission wavelengths of 488 and 525 nm, respectively, *via* a CFX96 (Bio-Rad) fluorescence plate reader. The level is expressed as relative fluorescence Unit (RFU) per mg of protein (RFU/ mg of protein).

Hydrogen peroxide (H$_2$O$_2$) concentration was measured in bud extract by ferrous oxidation-xylenol orange (FOX) method adapted to microtiter plates according to Gay and Gebicki (2000). Absorbance was read at 570 nm using H$_2$O$_2$ as a standard and findings were presented as µmoles g^{-1} FW.

Nitric oxide (NO) production was measured by a spectrophotometer at 540 nm according to Antoniou et al. (2018) using the Griess reagent. NaNO$_2$ was used as a standard and results were expressed as µmoles g^{-1} FW.

The level of lipid peroxidation was determined by assessing thiobarbituric acid reactive substances (TBARS) with reference to malondialdehyde (MDA) - a known marker of oxidative stress - (Kuk et al., 2003). Values were calculated using 1, 1, 3, 3- tetraethoxypropane as a standard at 532 nm and expressed as nmoles g^{-1} FW.

Total thiols (TSH) content was measured by spectrophotometer at 405 nm using the 5 – 5'-dithiobis-2-nitrobenzoic acid (DTNB) according to the method of Anderson (1985). Glutathione was used as a standard and results were expressed as µM g^{-1} FW.

2. 3. 2. Enzymatic Assays

Catalase (CAT) activity (U min^{-1} mg^{-1} protein) was determined by spectrophotometry at 240 nm according to Aebi (1984).

Superoxide dismutase (SOD) activity (U min^{-1} mg^{-1} protein) was determined by the inhibition on nitroblue tetrazolium (NBT) reduction spectrophotometrically at 560 nm using Giannopolitis and Ries method (1977).

Guaiacol peroxidase (GPOD) activity ($U \, min^{-1} \, mg^{-1}$ protein) was determined through the rate of guaiacol oxidation at 436 nm, according to Pütter's method (1974).

Phenylalanine ammonia-lyase (PAL) activity ($U \, min^{-1} \, mg^{-1}$ protein) was defined as the variation of 0.01 in the assay medium by spectrophotometry at 290 nm based on Assis et al. method (2001).

Tyrosine ammonia-lyase (TAL) activity ($U \, min^{-1} \, mg^{-1}$ protein) which is able to generate 1.0 µmol *p*-coumaric acid per min at 320 nm was calculated according to Wang et al. method (2006).

Polyphenol oxidase (PPO) activity ($U \, min^{-1} \, mg^{-1}$ protein) was estimated according to the method of Soliva et al. (2001) by measuring absorbance at 410 nm.

Protein concentrations in the extracts were quantified by a BCA protein assay kit (Pierce™) using bovine serum albumin as a standard.

2. 4. Phytochemical Assays

Dried buds were ground into fine powder, then extracted with 70% aqueous ethanol (0.1g of extract per 5 mL of extraction solvent) through ultra-sonication for 30 min at room temperature (Ultrasonic bath, Velleman). After centrifuging the mixture at 10,000 g for 10 minutes at 4°C, the supernatants were filtered through a 0.22 µm filter membrane and stored at 4°C. The filtrates were used to analyse TPC, TFC, DPPH, ABTS, HPLC and FTIR.

2. 4. 1. Total Phenolic Content (Tpc)

The ethanolic extracts of TPC were quantified by the Folin Denis method, using gallic acid as standard (Ascacio-Valdés et al. 2014). 90 µL of Folin-Ciocalteu reagent (10%) were added in each well containing 20 µL of the samples in 96 well plates and kept without disturbing for another 5 minutes. Then sodium carbonate (90 µL) was added from a 6% stock solution and then incubated during 90 min at room temperature. Gallic acid (GA and ethanol (20 µL) were used as positive and negative control, respectively. The absorbance was measured at 725 nm using a Metertech M965 microplate spectrophotometer. Total phenolic concentrations were reported as milligrams of GA equivalent (GAE /g DW).

2. 4. 2. Total Flavonoids Content (Tfc)

The ethanolic extracts of TFC were calculated in line with Zhishen et al. (1999). A total 10 µL of Aluminum chloride (10%) and CH_3COOK (1M) was blended with 20 µL of the sample, then, diluted with 160 µL of H_2O and incubated during 30 min. The absorbance was measured at 415 nm with Metertech M965 microplate spectrophotometer. TFC values were calculated using a quercetin (QE) standard curve and represented as mg QE equivalents g^{-1}DW.

2. 4. 3. Antioxidant Capacity

DPPH (1, 1-Diphenyl-2-picrylhydrazyl), an artificial stabilized free radical, was used to determine antioxidant capacity, according to Brand-Williams et al. (1995).

The ABTS$^{\cdot+}$ (2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) scavenging assay was performed according to Re et al. (1999).

2. 4. 4. Measurement Phenolic And Flavonoid Compounds By High Performance Liquid Chromatography (Hplc) Analysis

Phenolic and flavonoid compounds were quantified in accordance with the protocol of Zeb (2015) using Agilent 1200 Series column high performance liquid chromatography (HPLC) system fitted with Diode-Array Detector (DAD) run at 280 nm wavelength. The mobile phase solution was 0.1% H_3PO_4 (Movable Phase A), acetonitrile (Movable Phase B), and Flow-Type Gradient Solution. For each sample, three injections (10 µl) were provided..

2. 4. 5. Fourier-transform Infrared (Ftir) Analysis

Fourier transform infrared spectra (FTIR) of each sample were measured twice in a range of wave numbers $500-4000$ cm^{-1} with a resolution of 4 cm^{-1} using FTIR spectroscope (Spectrum Two™, Perkin Elmer, USA).

2. 5. Statistical Analysis

All experiments were perfomed in triplicate and the results were presented as mean ± standard deviations (SD). The data for TPC and TFC are presented as the mean of three replicates. Linear regression analysis was employed to determine the IC50 values of DPPH and ABTS tests. One-way analysis of variance (ANOVA) was done for all treatments to study if the differences in morphological parameters and content of secondary metabolites between vitro cultures were stimulated by the presence of elicitor. Pearson test was adopted to correlate between variables. *Post hoc* testing for the ANOVAs was conducted using Tukey test with $p < 0.05$ being considered statistically significant. Our analysis was achieved using GraphPad Prism version 9 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

3. 1. Estimation of viability

We found significant morphologic and viability changes in date palm *in vitro* cultures after MeJA treatments induced by the generation and oxidation of phenolic compounds. Browning of buds resulted in poor growth, eventually leading to cell death. As shown in Fig. 1A, significant browning differences ($p < 0.05$) of *Phoenix dactylifera* L. buds cultured on MS based media supplied with varying concentrations of MeJA revealed an increase in tissue browning following a dose-dependent manner. Cell viability was assessed using FDA (Fig. 1B) and double staining with acridine orange/ethidium bromide (AO/EB) with view to distinguishing living/dead cells (Fig. 1C). Viable cells were stained in green with FDA and EB, and non-viable cells were indicated by orange to red fluorescence from AO. As shown in Fig. 1B and C, Control buds displayed green fluorescence (stained by FDA and EB), indicating high viability under growth conditions. MeJA was found to cause severe damage to bud cultures, and the extent of such damage increased with MeJA concentrations.

3. 2. Changes in cellular ROS, Hydrogen peroxide (H_2O_2), nitrite formation (NO), lipid peroxidation and total thiols

The cellular generation of ROS and Hydrogen peroxide (H_2O_2), nitrite formation (NO), lipid peroxidation (MDA) and protein thiols in *Phoenix dactylifera* L. cultivated buds are shown in Table 1. A remarkable increase in MDA ($p < 0.001$), NO ($p < 0.05$), ROS ($p < 0.05$) and H_2O_2 ($p < 0.01$) levels was observed in buds treated with MeJA as compared to the Control group. However, MeJA treatment led to a significant decrease in total thiols ($p < 0.01$) levels in the *Phoenix dactylifera* L. buds compared with the control group.

Table 1 The effect of increasing MeJa concentrations on oxidative stress markers : lipid peroxidation (MDA), hydrogen peroxide (H_2O_2), reactive oxygen species (ROS), nitrite formation (NO_2^-) and total thiol (TSH) in vitro-culture extracts of *Phoenix dactylifera* L.

MeJA concentration (µM)	MDA (nmoles g^{-1} FW)	H_2O_2 (µmoles g^{-1} FW)	ROS (RFU mg^{-1}rotein)	NO_2^- (µM g^{-1} FW)	TSH (µmoles g^{-1} FW)
0	33.64 ± 2.49	5.58 ± 0.17	400.7 ± 20.43	11.28 ± 3.28	148.8 ± 7.24
50	83.80 ± 8.87[***]	12.20 ± 0.44[**]	482.5 ± 41.32[*]	36.07 ± 9.13[*]	100.0 ± 10.08[**]
100	142.90 ± 18.86[***]	15.75 ± 0.88[**]	578.3 ± 68.0[**]	91.26 ± 11.5[**]	80.89 ± 11.9[***]
200	207.40 ± 17.53[***]	20.55 ± 2.87[**]	802.5 ± 97.20[***]	162.3 ± 21.50[***]	39.56 ± 5.44[***]

The values are expressed as means ± standard deviation (SD) of three replicates. Asterisks denote signifcant changes:*$p<0.05$; **$p<0.01$; ***$p<0.001$compared 50 µM; 100 µM and 200 µM groups vs.

control groupbased on Tukey's test.

3. 3. Effects Of Meja On Antioxidant Enzyme Activities

In the present study, the effects of MeJA treatment led to a significant increase ($p < 0.05$) in the activity of SOD, CAT and GPOD activities in *Phoenix dactylifera* L. buds when compared with the Control group (Table 2).

Table 2: The effect of increasing MeJa concentrations on antioxidant enzyme activities in *Phoenix dactylifera* L cultured buds.

MeJA concentration (µM)	Enzyme activities		
	SOD (U min^{-1} mg^{-1}protein)	CAT (U min^{-1} mg^{-1}protein)	GPOD (U min^{-1} mg^{-1}protein)
0	2.12 ± 0.44	5.30 ± 0.59	11.66 ± 1.16
50	6.61 ± 0.45[*]	7.78 ± 0.45[*]	20.24 ± 0.82[*]
100	9.83 ± 1.05[**]	15.78 ± 1.16[**]	29.03 ± 0.70[***]
200	12.60 ± 0.37[***]	23.46 ± 1.28[***]	45.18 ± 3.68[***]

The values are expressed as means ± standard deviation (SD) of three replicates. Asterisks denote significant changes:*p<0.05; **p<0.01;

3. 4. Effects Of Meja On The Phenylpropanoid Pathway Enzymes And Antioxidant Capacities

Changes related to the specific activity of phenylpropanoid pathway enzymes including polyphenol oxidase (PPO), tyrosine ammonia-lyase (TAL) and phenylalanine ammonia-lyase (PAL) are shown in Table 3. The activity of these enzymes was found to be noticeably higher ($p < 0.05$) in a dose dependent manner in buds treated with MeJA when compared to the control group. Likewise, TPC and TFC changed in a linear fashion in response to the increasing concentrations of MeJA (Table 4). The free radical scavenging activity of ethanolic extracts was assessed *via* DPPH˙ and ABTS˙$^+$ assays. Results showed that the extracts obtained from buds treated with MeJA exhibited a remarkable rise in antioxidant activity with a lower value of IC50 in a dose-dependent manner, indicating a significant radical scavenging effect (Table 4).

Table 3: The effect of increasing MeJA concentrations on phenylalanine ammonia-lyase (PAL), Tyrosine ammonia-lyase (TAL) and polyphenol oxidase (PPO) specific activityin vitro-cultured *Phoenix dactylifera*L. buds.

MeJA concentration (μM)	Enzyme activity		
	PAL ($U \min^{-1} mg^{-1}$ protein))	TAL ($U \min^{-1} mg^{-1}$ protein)	PPO ($U \min^{-1} mg^{-1}$ protein)
0	2.44 ± 0.47	1.43 ± 0.18	4.69 ± 0.75
50	4.43 ± 0.16[*]	3.33 ± 0.48[**]	7.56 ± 1.02[**]
100	6.99 ± 1.35[***]	4.46 ± 0.35[***]	10.69 ± 0.51[***]
200	8.91 ± 0.94[***]	6.23 ± 0.93[***]	14.48 ± 0.34[***]

The values are expressed as means ± standard deviation (SD) of three replicates. Asterisks denote signifcant changes:*$p<0.05$; **$p<0.01$; ***$p<0.001$compared 50 μM; 100 μM and 200 μM groups vs. control groupbased on Tukey's test.

Table 4: Comparison of total phenolics (TPC), flavonoids (TFC) contents, DPPH and ABTS scavenging activity in vitro-cultures of *Phoenix dactylifera* treated with increasing concentrations of MeJa.

MeJa concentration (μM)	Secondarymetabolites		IC50 inhibition (μg mL^{-1})	
	TPC (mg GAE g^{-1} DW)	TFC (mg QE g^{-1} DW)	DPPH	ABTS
0	131.6 ± 11.58	25.44 ± 1.17	135.60 ± 2.05	363.90 ± 4.15
50	194.92 ± 8.74[***]	38.33 ± 1.66[***]	116.30 ± 3.70[*]	142.5±16.50[***]
100	244.13 ± 15.11[***]	34.16 ± 0.88[***]	75.85 ± 8.00[***]	106.9 ± 6.80[***]
200	268.38 ± 11.51[***]	44.44 ± 2.54[***]	114.51 ± 7.05[**]	90.2 ± 9.21[***]

The values are expressed as means ± standard deviation (SD) of three replicates. Asterisks denote signifcant changes (Tukey's test,*p<0.05; **p<0.01; ***p<0.001 50 μM; 100 μM and 200 μM groups vs. control group based on Tukey's test.

3. 5. Fourier Transform Infrared (FTIR) spectroscopy and HPLC

Fourier Transform Infrared (FTIR) spectroscopy is a powerful, versatile and non-destructive analytical technique used for chemical characterization of diverse compounds and can provide structural information on molecular features of a large range of molecules from plant extracts. Figure 2A represents

the comparative FTIR spectra (4000 to 500 cm^{-1}) of the ethanolic extracts from *Phoenix dactylifera* L. bud cultures. Similarly to previous reports, area (1800 – 700 cm^{-1}), spectral signals showed 6 specific wavenumber ranges, namely 1600, 1500, 1450, 1170, 1040, and 830 cm^{-1}, which were identified in both phenolic acid (coumaric acid) and flavonoid families (catechin) (Abbas et al. 2017). Our findings did not reveal any noteworthy difference as to the characteristic absorption bands obtained from ethanolic extracts (Fig. 2B): spectral signals were found to be identical between untreated and MeJA-treated buds. However, the spectra produced by MeJA-treated buds showed slight differences in terms of band intensity. In view of these results, an HPLC analysis was achieved in order to gather more information regarding to the extracts compositions. Indeed, Fig. 3 displays representative HPLC chromatograms of the four ethanolic extracts showing catechin, 4-Hydroxybenzoic acid, caffeic acid and *p*-Coumaric acid identified signals. The analysis of chromatography profiles revealed that MeJA supplementation induced higher concentrations of major phenolic compounds in MeJA-treated buds.

4. Discussion

Plant cell cultures can be considered as a renewable resource of active principles from plants. Research focused on the establishment of undifferentiated and differentiated plant material (callus and cell suspension cultures, organogenic cultures) for the production of high-value secondary metabolites. Such molecules are either difficult to synthesize through chemical processes, or generated in small quantities in all plants (Kolewe et al. 2008). *In vitro*-maintained material is potentially able to provide a durable and reliable source of natural products. Recent studies suggest that elicitation and precursor feeding can play a key role (Ho et al. 2020). The first response to elicitors usually starts at the cellular level. Plant cell membranes are fitted with specific receptors that bind to signalling molecules and can trigger processes mediated by enzymatic pathways (Naik and Al-Khayri 2016). The most widely produced phytochemicals deriving from secondary metabolism are phenolics. Such compounds are produced in plants through the phenylpropanoid, pentose-phosphate and shikimate pathways (Balasundram et al. 2006). Flavonoids comprise a varied group of low-molecular-weight phenolic compounds. To date, *ca*.8000 flavonoid molecules have been identified, and the list is continuously expanding (Cheynier 2012).

In the present study, the impact of increasing MeJA concentrations on secondary metabolites profile was explored, and remarkable ($p < 0.05$) changes in both phenolics and flavonoids contents were detected. The level of total phenolic and flavonoid compounds in buds grown at 200 µM MeJA concentrations was found to be higher than those with 50 µM and 100 µM MeJA concentrations and showed a 2-fold increase when compared to control (Table 4). Our findings underscored previous research which demonstrated that MeJA elicitation stimulated the accumulation of secondary metabolites particularly alkaloids (2-hydroxy 4-methoxy benzaldehyde), in both *Salvia* and *Hemidesmus* species cultivated *in vitro* (Nandy et al. 2021; Pesaraklua et al. 2021). In plants, phenolic compounds and flavonoids are known to mitigate the damages of oxidative stress and act as free radical scavengers (Abbasi et al. 2011). For the purpose of this work, we estimated the radical scavenging potential in ethanolic extracts through DPPH and ABTS assays. Our results showed that MeJA-elicited callus have a higher capacity for free radicals

than non-elicited tissue in a dose-dependent manner (Table 4). These findings were confirmed by the subsequent analysis of FTIR spectra and HPLC profiles (Fig. 2 and Fig. 3). Recently, a combination of HPLC and FTIR methods was used to evaluate and discriminate metabolites (Joshi 2012). Indeed, FTIR spectroscopy is recommended as a rapid and reliable tool for the exploration of fingerprints and the prediction of medicinal plant compositions. Such profiles are used to assess both the quality and authenticity of potential sources (Kwon et al. 2014). The FTIR results obtained in the present study highlighted the existence of absorption signals for a specific wavenumber range (1800–700 cm^{-1}). Results showed slight differences in terms of band intensity. Such findings provided crucial qualitative information about the effect of MeJA on the elicitation of antioxidant metabolites. Our results are congruent with prior research thus, illustrating exposure to various concentrations of salicylic acid (SA) as an elicitor, enhanced TPC, TFC, antioxidant potential and the generation of catechin, caffeic acid, kaempferol, and apigenin in callus culture of *Phoenix dactylifera* L. (Al-Khayri and Naik 2020). Such an increase in the synthesis of phenol and flavonoids can be related to the higher activities of phenylpropanoid pathway enzymes, such as polyphenol oxidase (PPO), tyrosine ammonia-lyase (TAL) and phenylalanine ammonia-lyase (PAL). In this pathway, the synthesized phenolics and the flavonoids are able to trigger the main enzymes leading to the accumulation of bioactive compounds (Manivannan et al. 2016). To confirm this hypothesis, we also explored the activity of such enzymes. Our study demonstrates that MeJA was able to alter the activity of the phenylpropanoid pathway enzymes by enhancing PAL, TAL and PPO activities. Our results are in accordance with previous findings of Bouissil et al. (2020). This latter confirmed that, for date palm, elicitation can be evidenced through the activity of phenylalanine ammonia-lyase (PAL) and the rise in phenolic level identified in cultivated roots. Recent studies suggest that oxidative stress is likely to play a key role in MeJA-induced generation of secondary metabolites in plant tissue cultures (Ho et al. 2020). In the present work, the exposure of *in vitro* cultures to MeJA through temporary immersion resulted in a remarkale increase in lipid peroxidation, hydrogen peroxide (H_2O_2), nitrite formation, ROS and protein oxidation (as indicated by the significant decrease in thiol levels). This result suggests that MeJA was able to activate the formation of free radicals in *Phoenix dactylifera* L. buds (Table 1, Fig. 1D). However, MeJA used at 100 and 200 µM concentrations for 7 days induced toxicity in bud cells as evidenced by an elevation of ROS generation, browning and death of cells and a decrease of cellular viability (FDA assay) which was supported by morphological examinations (Fig. 1). In fact, browning is among the most recalcitrant problems in plant tissue culture. It has deleterious effects on explants and may lead to decrease regenerative ability, poor growth and is likely to cause cell/tissue/plant death. Browning is primarily trigged by phenolic compounds accumulation in both tissues and culture media. Eventually, tissue culture techniques mostly consist in wounding the material to prompt isolate explants and culture them in potentially stressful environments; such conditions often elicit the generation and release of phenolic compounds. Thus, this natural defense response induce of toxic accumulation constituent in which eventually harm or kill plant cells and tissues. Our results are in conformity with previous research (Belchí-Navarro et al. 2019), which evidenced the production of ROS, H_2O_2 and nitric oxide occuring in grapevine cells after 24 h of treatment with 100 µM of MeJA. During morphogenesis, the antioxidative enzymes (SOD, CAT and GPOD) play a pivotal role in shielding plant cells from the injurious effects of free radicals (Suzuki and Mittler 2006). Being at the

front the first line of defense, SOD in charge of catalyzing the conversion of O^-_2 into H_2O_2 and O_2; it thus protects plant cells against superoxide induced damage. Furthermore, the H_2O_2 is decomposed by GPOD through the oxidation of co-substrates such as phenols and other antioxidant molecule (Meloni et al. 2003). In the present study, MeJA treatments were found to induce an increase in antioxidant enzymes actvities such as CAT, SOD and GPOD (Table 2). Since MeJA excess provokes an abiotic stress for plants, it was argued that the activities of GPOD, CAT and SOD are potentially affected in buds cultured under these conditions. Our results are clearly in line with the idea that the concentration of MeJA in the growth medium have an impact on antioxidant enzyme activities in *Phoenix dactylifera* L. buds (Ho et al. 2020). A partial correlation analysis was done in order to find the link between all stress markers under study namely (H_2O_2, NO_2^- and ROS), phenylpropanoid pathway enzymes (PAL, TAL and PPO) and accumulated TPC and TFC. From Fig. 4, we noticed high correlation coefficients between stress markers and the specific activity of PAL, TAL and PPO enzymes. Correlation analysis confirmed that, regardless of elicitor concentrations, PAL, TAL and PPO activities showed high positive correlations with stress markers, with high correlation coefficient values more than 0.9. These findings show that, when buds are elicited, H_2O_2 is released leading to the stimulation of antioxidant and phenylpropanoid pathways activating enzymes for the plant cell defense and production of TPC and TFC, respectively.

Our results clearly confirm, for the first time, that MeJA can act a potential elicitor to promote the biosynthesis of pharmaceutically active molecules including phenolic compounds and flavonoids in *Phoenix dactylifera* L.

Declarations

Author contributions

Funding

Conflict of Interest

The authors declare no competing interests.

Acknowledgments

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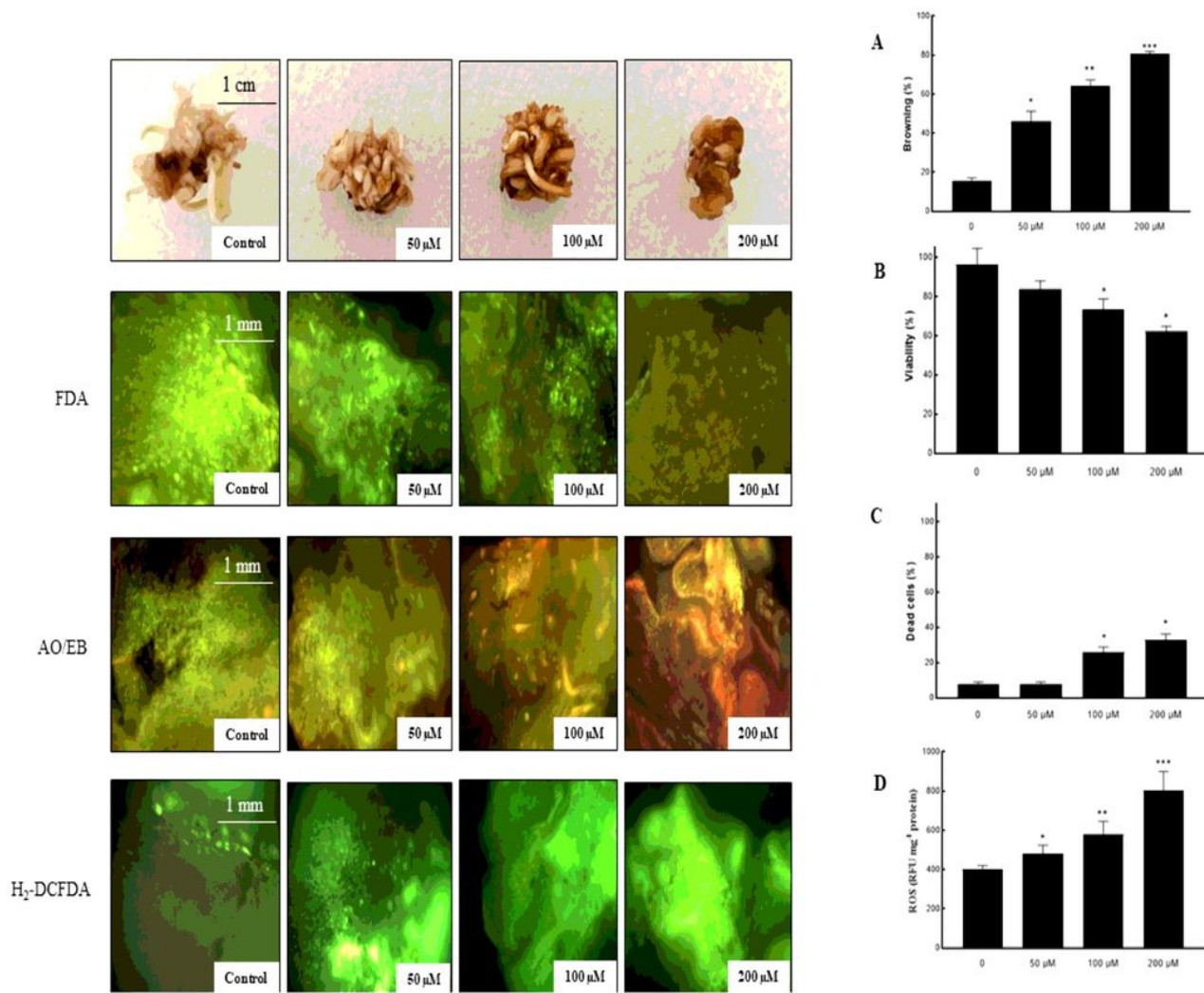
Figures

Figure 1

In vivo buds staining with fluorescein diacetate (FDA), acridine orange/ethidium bromide (AO/EB) and2', 7'- dichloro fluorescin diacetate (H$_2$-DCFDA). (A): Buds browning at a macroscopic level, B-C-D: Fluorescence microscopy analysis of buds stained with FDA (B), with AO/EB (C), and H$_2$-DCFDA (D). Quantitative analysis wasexpressed as means ± standard deviation (SD) of five replicates by using the basic functions of ImageJ v1.53K software.
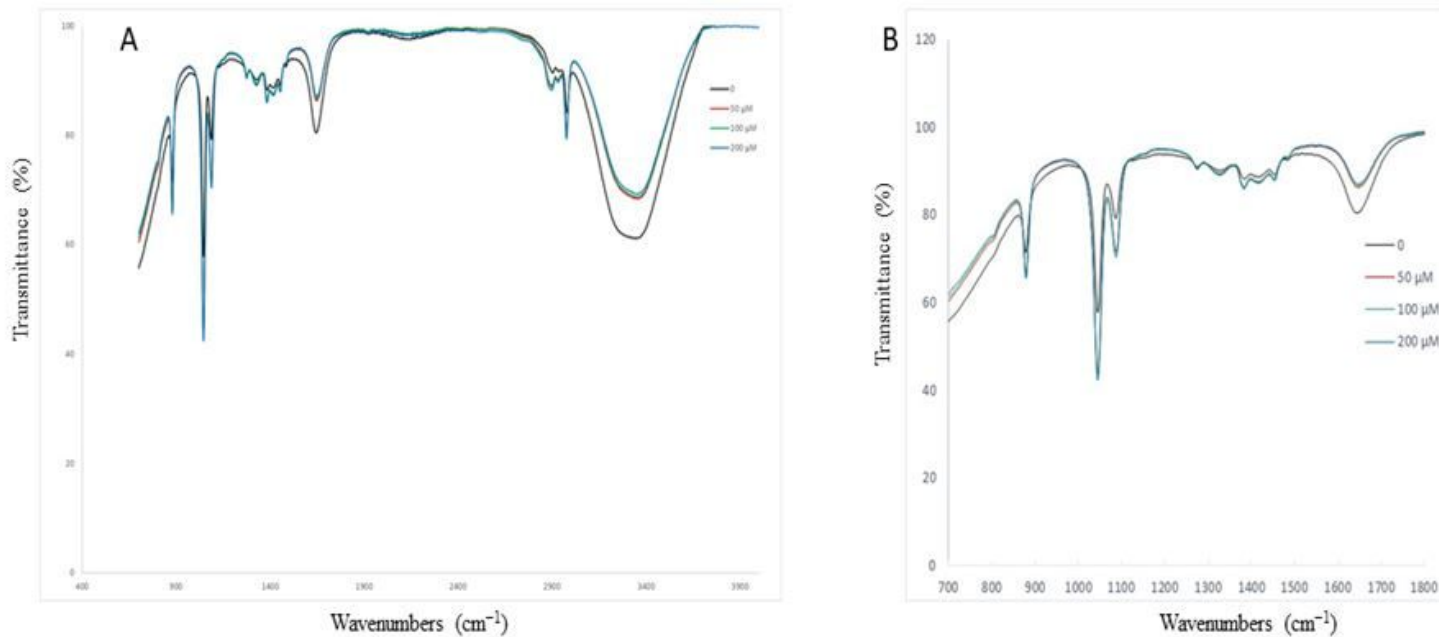
Figure 2

Infrared spectrum of ethanolic extracts in the 4000−500 cm^{-1}(A) and 1800−700 cm^{-1} (B) ranges of Graphs show results from control buds and material exposed to 50, 100 and 200 µM MeJA concentrations.

Image not available with this version

Figure 3

HPLC-UV profiles (280 nm) of ethanolic extracts from *Phoenix dactylifera* L. buds showing catechin, 4-Hydroxybenzoic acid, caffeic acid and *p*-Coumaric acid identified signals.
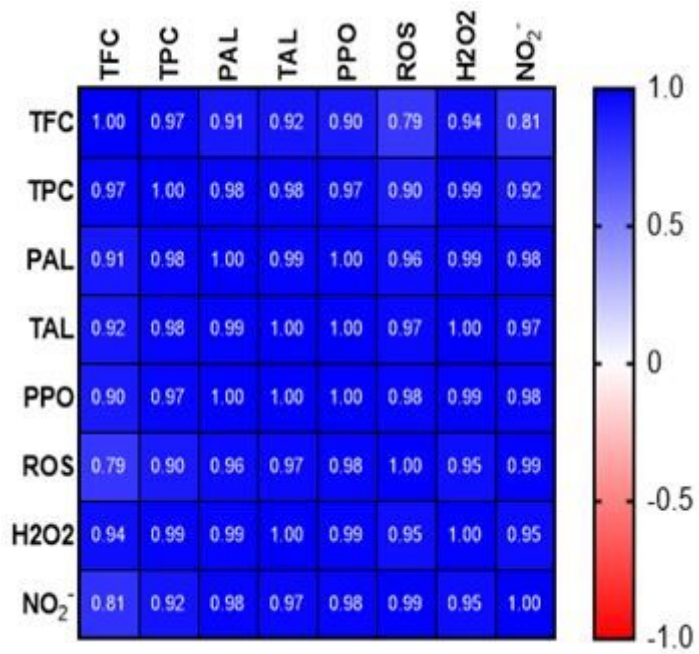
Figure 4

Correlation between activities of phenyl propanoid triggering enzymes (PAL, TAL and PPO), TPC, TFC and various stress markers (ROS, H_2O_2, NO).