Methyl jasmonate induces oxidative/nitrosative stress and the accumulation of antioxidant metabolites in *Phoenix dactylifera* L.

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Abstract

**Objectives** The present study aimed to explore the eliciting effects of increasing concentrations (50, 100, and 200 µM) of methyl jasmonate (MeJA). We cultivated actively proliferating buds of *Phoenix dactylifera* L. cv. Barhee in a temporary immersion system and we monitored the bioactive compound 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 MeJA treatment induced oxidative stress, and at the same time increased the activity of related defense enzymes in a dose-dependent manner. Exogenous application of MeJA at 200 µM increased ROS (two fold), hydrogen peroxide (3.7 fold), nitric oxide (14 fold), MDA (6.3 fold), superoxide dismutase (5.9 fold), catalase (4.4 fold) and guaiacol peroxidase (3.87 fold). Furthermore, the results demonstrated that 200 µM MeJA treatment enhanced the activities of PAL (3.65 fold), TAL (4.35 fold), PPO (threefold) and increased TPC (twofold) and TFC (1.75 fold) contents in buds cultures higher than the control. HPLC analysis showed that buds cultures exposed to 200 µM MeJA accumulated maximum amount of catechin (11 fold), 4-hydroxybenzoic acid (1.48 fold), caffeic acid (2.5 fold) and *p*-coumaric acid (1.76 fold) and demonstrate antioxidant capacity with the lowest DPPH (114.5 µg ml⁻¹) and ABTS (90.2 µg ml⁻¹) IC50 values on day 7 of culture as compared to the control.
The MeJA in the culture medium directly reduced cell viability in a dose dependent manner up to 35% with the highest concentration.

**Conclusion** The results of this study has revealed, for the first time, that MeJA offers a promising potential for the production of phenolic compound in *Phoenix dactylifera* L. buds.

**Keywords** Methyl Jasmonate · *Phoenix dactylifera* L. · Phenolic · Temporay immersion system

**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). The application of innovative tools with a multidisciplinary approach to address issues of in vitro plant regeneration for wider applications in crop improvement, commercial applications, and secondary metabolites has been a largely investigated (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. Nowadays, it is well known that the cultivation through Temporary Immersion System (TIS) has considerable advantages both for the in vitro mass production of plants and for that secondary metabolites. Bioreactor technology provides uninterrupted production of biomass and phenolic compounds with reduced cost and time. In comparison with culturing on semi-solid media, larger containers can be used, and transfer times can be reduced to avoid intensive manual handling. The optimization of the culture medium (nutrients, carbohydrates, growth regulators) and the monitoring of different parameters such as immersion time, temperature, light quality, and aeration, can contribute to producing large amounts of biomass to ensure the stability of bioactive compounds and high yield. Moreover, changing the immersion program could be used to manipulate the metabolic processes of in vitro plants, depending on the primary goal of micropropagation or accumulation of specific secondary metabolites (De Carlo et al. 2021). 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 treatment generally induces physiological changes (Sachadyń-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). Particular interest has been given to secondary metabolites of date palm, their health-benefit and potential use in the booming industries of functional foods and nutraceuticals (Gantait et al. 2018). Naik and Al-Khayri (2017) used a bioreactor technique to accumulate catechin, caffeic acid, apigenin, syringic acid, luteolin, and kaempferol from date palm embryonic callus. They also evaluated the effects of various elicitors such as pectin, yeast extract, salicylic acid, CdCl₂, and AgNO₃ on total phenol and flavonoid contents, antioxidant activity, and catechin, caffeic in cell suspension culture of the date palm (Naik and Al-Khayri 2020). Many of such phenolic and flavonoid compounds, including quercetin, kaempferol, apigenin, and caffeic acid, have antioxidant and...
anti-platelet potential, and hence may protect against cardiovascular diseases (CVDs) through various mechanisms such as by decreasing oxidative stress and inhibiting blood platelet activation. 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 in 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 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.

**Materials and methods**

**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 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 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 min 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.

*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, two 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 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. Intracellular reactive oxygen species (ROS) was measured through 2′, 7′-dichlorofluorescein diacetate (H$_2$-DCFDA) oxidation. After treatment with MeJA, buds were incubated with 20 μM H$_2$-DCFDA at 37 °C for 1 h 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 v1.53 K software.

Biochemical assays

**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$^{-1}$ 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 nmole per g 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.

**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 of nitroblue tetrazolium (NBT) reduction spectrophotometrically at 560 nm using Giannopolites 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 Putter’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 Beaudoin-Eagan and Thorpe (1985) method.

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.

**Phytochemical assays**

Dried buds were ground into fine powder, then extracted with 70% aqueous ethanol (0.1 g 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 min at 4 °C, the supernatants were filtered through a 0.22 µm filter membrane and stored at 4 °C. The filtrates were used to analyze TPC, TFC, DPPH, ABTS, HPLC and FTIR.

**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 min. 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. Absorbance was measured at 725 nm using Metertech M965 microplate spectrophotometer. Total phenolic concentrations were reported as milligrams of GA equivalent (GAE g⁻¹ DW).

**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 CH₃COOK (1 M) was blended with 20 µL of the sample, then, diluted with 160 µL of H₂O 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.

**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⁺ (2, 2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) scavenging assay was performed according to Re et al. (1999).

*Measurement phenolic and flavonoid compounds by high performance liquid chromatography (HPLC) analysis*

The quantitative analyses of phenolic and flavonoid compounds in date palm buds extracts were made by high performance liquid chromatography (Agilent 1260 Infinity quaternary LC, Germany). Phenolic and flavonoid compounds were separated on a C18 column (4.6 mm × 250 mm) and then analyzed using a Shimadzu SPD6AUV detector measuring the optical density at 280 nm during 50 min. The mobile phase was a mixture of A and B solutions: (A) 70% acetonitrile in water and (B) 0.1% phosphoric acid in water with the percentage by volume of (A) solution varying linearly along the time as follows: from 10 to 25% for the first 25 min then from 25 to 80% up to 35 min and finally from 80 to 100% up to 50 min. The column temperature was maintained at 40 °C and the mobile flow rate was fixed at 0.6 mL/min. Catechin, 4-hydroxybenzoic acid, caffeic acid and p-coumaric acid were identified and quantified using external standards and calibration curves.

*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).

*Statistical analysis*

All experiments were performed in triplicate and the results were presented as mean ± standard deviations (SD). 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 in 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 results were analyzed using GraphPad Prism.
version 9 for Windows, GraphPad Software (San Diego, CA, USA).

**Results**

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.

Changes in cellular ROS, hydrogen peroxide ($\text{H}_2\text{O}_2$), nitrite formation (NO), lipid peroxidation and total thiols

The cellular generation of ROS and hydrogen peroxide ($\text{H}_2\text{O}_2$), nitrite formation (NO), lipid

![Fig. 1 In vivo buds staining with fluorescein diacetate (FDA), acridine orange/ethidium bromide (AO/EB) and 2', 7'-dichlorofluorescin 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 was expressed as means ± standard deviation (SD) of five replicates by using the basic functions of ImageJ v1.53 K software](image-url)
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 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 control group.

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 SOD, CAT and GPOD activities in Phoenix dactylifera L. buds when compared with Control group (Table 2).

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).

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, HPLC analysis was undertaken in order to gather more information on extracts compositions. Indeed, Fig. 3 A and supplementary material displays representative HPLC chromatograms of the four ethanolic extracts showing catechin, 4-hydroxybenzoic acid, caffeic acid and \(p\)-coumaric acid identified signals.

### Table 1

| MeJA concentration (µM) | MDA (nmole g\(^{-1}\) FW) | \(H_2O_2\) (µmole g\(^{-1}\) FW) | ROS (RFU mg\(^{-1}\) protein) | \(NO_2^–\) (µM g\(^{-1}\) FW) | TSH (µmole 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

* denote significant changes: \(*p < 0.05\); \(**p < 0.01\); \(**p < 0.001\) compared 50 µM; 100 µM and 200 µM groups vs. control group based on Tukey’s test.
Buds cultures exposed to 200 µM MeJA accumulated maximum amount of catechin (11 fold), 4-hydroxybenzoic acid (1.48 fold), caffeic acid (2.5 fold) and p-coumaric acid (1.76 fold) as compared to the control (Fig. 3 B and supplementary material).

Relationship between stress markers, phenylpropanoid triggering enzymes and phenolic accumulations

Correlation analysis was carried out to establish the relationship between all investigated stress markers, phenylpropanoid triggering enzymes and accumulated phenolic compounds. We observed high correlation coefficients (0.9) between stress markers and activities of PAL and TAL, regardless of elicitor concentrations (Fig. 4). These results indicate that, when buds are elicited, ROS, NO and H₂O₂ are generated leading to the stimulation of antioxidant enzymes (CAT, SOD and GPOD) and PAL, PPO and TAL, Buds cultures exposed to 200 µM MeJA accumulated maximum amount of catechin (11 fold), 4-hydroxybenzoic acid (1.48 fold), caffeic acid (2.5 fold) and p-coumaric acid (1.76 fold) as compared to the control (Fig. 3 B and supplementary material).

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enzymes involved in the phenylpropanoid biosynthetic pathway, for buds defense and biosynthesis of defense phenolic compounds, respectively.

Discussion

Plant cell cultures can be considered as a renewable resource of bioactive molecules. 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 suggested 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...
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 has been 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 twofold 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). Optimization of MeJA concentration, the growth stage and exposure time of cultures are critical factors to induce secondary metabolites (Ho et al. 2020). Elicitation with 100 µM MeJA improved silymarin pigment accumulation in cell culture of Silybum marimum after 3 days of treatment (Sánchez-Sampedro et al. 2005).

In another report, elicitation with 4 µM MeJA during two weeks resulted in a 6.5-fold enhancement of solasodine content (9.33 mg g\(^{-1}\) DW) higher than un-elicited hairy root cultures of Solanum trilobatum. In addition, increased withanolide derivatives (14-fold) was accumulated in 40-day-old hairy roots elicited with 15 µM MeJA for 4 h exposure time. In other studies on P. multiflorum, the phenolic compound in adventitious root culture reached to twofold higher than the control after 7-days treatment with 50 µM MeJA, whereas 3.4-fold higher was observed in hairy roots after 5-day of exposure time (Ho et al. 2018). Treatment of cultures with 100 µM MeJA for 6 days has been reported to influence the accumulation of both phenolic and tanshinones in Salvia miltiorrhiza hairy root cultures.

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 buds have a higher capacity for free radicals than un-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 highlight the existence of absorption signals for a specific wavenumber range (1800–700 cm\(^{-1}\)). Results show slight differences in terms of band intensity. Such findings provide 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). Accumulation of phenolic compounds is due to the up-regulation of the biosynthesis of phenylpropanoid enzymes such as polyphenol...
oxidase (PPO), tyrosine ammonia-lyase (TAL) and phenylalanine ammonia-lyase (PAL) which is in turn dependent on the modulation of transcript levels of genes encoding biosynthetic enzymes under MeJA stress. The main reason for this MeJA-induced accumulation of phenolic compounds is the modulation of phenylpropanoid biosynthetic pathway. MeJA regulates many key genes encoding main enzymes of phenylpropanoid pathway, which results in stimulated biosynthesis of phenolic compounds. Flavonoids are also known for their scavenging ability of H$_2$O$_2$ and are considered to play a crucial role in the phenolic/ascorbate-peroxidase cycle (Manivannan et al. 2016). To confirm this hypothesis, we also explored the activity of PAL, TAL and PPO. Our study demonstrates that MeJA is able to enhance their activities. Our results are in accordance with previous findings of Bouissil et al. (2020). This latter confirmed that elicitation, for date palm, can be evidenced through the activity of phenylalanine ammonia-lyase (PAL) and the rise in phenolic level identified in cultivated roots. Recent studies have suggested 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 has remarkably increased in lipid peroxidation, hydrogen peroxide (H$_2$O$_2$), nitrite formation, ROS and protein oxidation as indicated by the significant decrease in thiol levels. In such antioxidant reactions, thiols undergo one-electron oxidation with the formation of thiyl radicals. However, protective and repairing ability of thiols depends not only on their capacity to detoxify free radicals but also on the chemical reaction and reactivity of the formed thiyl radical. Moreover, rapid and beneficial removal of free radicals induce imbalance in the status of antioxidant reaction, which enhances correcting capacity. Harmful thiyl radicals can produce peroxidative damage therefore, they must directly undergo regeneration to thiols (Waldek 2002).

This result suggests that MeJA is 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 elevated ROS, browning and of cell death 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 in regenerative ability, poor growth and cell/tissue/plant death. Browning is primarily triggered 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. However, this strategy also led to a significant reduction in cell viability and biomass production, thus showing the limits of this approach. In such a context, the use of sequential batch culture could solve this biomass and viability issue by first creating biomass and then proceeding to a permeation treatment to release the produced secondary metabolites. Our results are in conformity with previous research (Belchí-Navarro et al. 2019), which evidenced the production of ROS, H$_2$O$_2$ and nitric oxide 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$_2$O$_2$ and O$_2$, protects plant cells against superoxide induced damage. Furthermore, the H$_2$O$_2$ 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 increase antioxidant enzymes activities 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 MeJA concentration in the growth medium has an impact on antioxidant enzyme activities in Phoenix dactylifera L. buds (Ho et al. 2020). A correlation analysis was done in order to find the link between all stress markers under study namely (H$_2$O$_2$, NO$_2^{−}$ and ROS), phenylpropanoid pathway enzymes (PAL, TAL and PPO) and accumulated TPC and TFC. As shown in Fig. 4, we notice high correlation coefficients between stress markers and the specific activity of PAL, TAL and PPO enzymes. Correlation analysis confirms that, regardless of elicitor concentration, PAL, TAL and
PPO activities show high positive correlations (more than 0.9) with stress markers. These findings demonstrate that, when buds are elicited, \( \text{H}_2\text{O}_2 \) 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. Correlation between oxidative stress and MeJA is very complex. It was proposed that MeJA at a definite concentration can directly modify superoxide dismutase (SOD) structure that stimulates its activity, thus MeJA stimulated both antioxidative and pro-oxidative activity (Ho et al. 2020). 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.

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**Author contributions** Amal Ben Romdhane, Lotfi Fki and Yassine Chtourou conceived and designed the research. Amal Ben Romdhane, Mohamed Maalej, Yassine Chtourou and Lotfi Fki conducted experiments, analyzed the data and drafted the manuscript. Haifa Sebii performed HPLC. Emma Baklouti, Ameni Nasri, Riadh Drira, Mohamed Maalej, Noureddine Drira and Alain Rival contributed to experiments related to the establishment of cell cultures. All authors read and approved the final manuscript.

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**Declarations**

**Conflict of interest** The authors declare no competing interests.

**References**

Abbas O, Compère G, Larondelle Y, Pompeu D, Rogez H, Bae-ten V (2017) Phenolic compound explorer: a mid-infrared spectroscopy database. Vib Spectrosc 92:111–118. https://doi.org/10.1016/j.vibspect.2017.05.008

Abassi BH, Khan M, Guo B, Bokhari SA, Khan MA (2011) Efficient regeneration and antioxidative enzyme activities in *Brassica rapa* var. *turnip*. Plant Cell Tissue Organ Cult 105:337–344. https://doi.org/10.1007/s11240-010-9872-8

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Al-Khayri JM, Naik PM (2020) Elicitor-induced production of biomass and pharmaceutical phenolic compounds in cell suspension culture of date palm (*Phoenix dactylifera* L.). Molecules 25:46–69. https://doi.org/10.3390/molecules25204669

Anderson ME (1985) Determination of glutathione and glutathione disulfide biological samples. Meth Enzymol 113:548–555. https://doi.org/10.1016/S0076-6879(85)30739-9

Antoniou C, Savvides A, Georgiadou EC, Fotopoulos V (2018) Spectrophotometric quantification of reactive oxygen, nitrogen and sulfur species in plant samples. In: Alca’zar R, Tiburcio AF (eds) Methods in Molecular Biology. Springer, New York, pp 155–161. https://doi.org/10.1007/978-1-4939-7398-9_16

Ascacio-Valdés J, Buenrostro J, De La Cruz R, Sepúlveda L, Aguilera A, Prado A, Contreras JC, Rodríguez R, Aguilar CN (2014) Fungal biodegradation of pome-granate ella-gitamins. J Basic Microbiol 54:28–34. https://doi.org/10.1002/jobm.201200278

Assis JS, Maldonado R, Munoz T, Escrubano MI, Merodio C (2001) Effect of high carbon dioxide concentration on PAL activity and phenolic contents in ripening cherimoya fruit. Postharvest Biol Technol 23:33–39. https://doi.org/10.1016/S0925-5214(01)00100-4

Baenas N, García-Viguera C, Moreno DA (2014) Elicitation: a tool for enriching the bioactive composition of foods. Molecules 19(9):13541–13563. https://doi.org/10.3390/molecules190913541

Balasundram N, Sundram K, Samman S (2006) Phenolic compounds in plants and agri-industrial by-products: antioxidant activity, occurrence, and potential uses. Food Chem 99:191–203. https://doi.org/10.1016/j.foodchem.2005.07.042

Beaudoin-Eagan LD, Thorpe TA (1985) Tyrosine and phenylalanine ammonia lyase activities during shoot initiation in tobacco callus cultures. Plant Physiol 78(3):438–441

Belchi-Navarro S, Abellan RM, Pedreño MA, Almagro L (2019) Production and localization of hydrogen peroxide and nitric oxide in grapevine cells elicited with cyclodextrins and methyl jasmonate. J Plant Physiol 237:80–86. https://doi.org/10.1016/j.jplph.2019.03.013

Bouissil S, Alaoui-Talibi ZE, Pierre G, Rchid H, Michaud P, Delattre C, El Medafar C (2020) Fucoidans of moroccan brown seaweed as elicitors of natural defenses in date palm roots. Mar Drugs 18(12):596. https://doi.org/10.3390/md18120596

Brand-Williams W, Culivier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT—Food Sci Technol 28:25–30. https://doi.org/10.1016/S0023-6438(95)80008-5

Cai X, Wei H, Liu C, Ren X, Thi LT, Jeong BR (2020) Synergistic Effect of NaCl pretreatment and PVP on browning suppression and callus induction from petal explants of *Paonia Lactiflora* pall ‘Festival Maxima.’ Plants 9(3):346. https://doi.org/10.3390/plants9030346

Cheynier V (2012) Phenolic compounds: from plants to foods. Phytochem Rev 11:153–177. https://doi.org/10.1007/s11101-012-9242-8

De Carlo A, Tarraf W, Lambardi M, Benelli C (2021) Temporary immersion system for production of biomass and
bioactive compounds from medicinal plants. Agronomy 11(12):2414. https://doi.org/10.3390/agronomy11122414

Effert H (2019) Biotechnology applications of plant callus cultures. Engineering 5:50–59. https://doi.org/10.1016/j.eng.2018.11.006

Fki L, Bouazziz N, Kriaa W, Benjemaa-Masmoudi R, Gargouri-Bouzid R, Rival A, Drira N (2011) Multiple bud cultures of ‘Barhee’ date palm (Phoenix dactylifera L.) and physiological status of regenerated plants. J Plant Physiol 168(14):1694–1700. https://doi.org/10.1016/j.jplph.2011.03.013

Gantait S, El-Dawayati MM, Panigrahi J, Labrooy C, Verma SK (2018) The retrospect and prospect of the applications of biotechnology in Phoenix dactylifera L. Appl Microbiol Biotecnol 19:8229–8259. https://doi.org/10.1007/s00253-018-9232-x

Gay CA, Gebicki JM (2000) A critical evaluation of the effect of sorbitol on the ferric-xylene orange hydroperoxide assay. Anal Biochem 284(2):217–220. https://doi.org/10.1006/abio.2000.4696

Giannopilotes CN, Ries SK (1977) Superoxide dismutase occurrence in higher plants. Plant Physiol 59(2):309–314. https://doi.org/10.1104/pp.59.2.309

Gros-Balthazard M, Flowers JM, Hazzouri KM, Ferrand S, Aberlec F, Salion S, Purugganan MD (2021) The genomes of ancient date palms germinated from 2,000 y old seeds. Proc Natl Acad Sci USA 118(19):e2025337118. https://doi.org/10.1073/pnas.2025337118

Ho TT, Lee JD, Ahn MS, Kim SW, Park SY (2018) Enhanced production of phenolic compounds in hairy root cultures of Polygonum multiflorum and its metabolite discrimination using HPLC and FT-IR methods. Appl Microbiol Biotecnol 102:9563–9575

Hos T-T, Murthy BN, Park SY (2020) Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. Int J Mol Sci 21(3):716. https://doi.org/10.3390/ijms21030716

Jones AMP, Saxena PK (2013) Inhibition of phenylpropanoid biosynthesis in Artemisia annua L: a novel approach to reduce oxidative browning in plant tissue culture. PLoS ONE 8(10):e76802. https://doi.org/10.1371/journal.pone.0076802

Joshi DD (2012) FTIR spectroscopy: herbal drugs and fingerprint prints. In: Joshi DD (ed) Herbal drugs and fingerprints prints. Springer, India, pp 121–146. https://doi.org/10.1007/978-81-322-0804-4_7

Kolewe ME, Gaurav V, Roberts SC (2008) Pharmacologically active natural product synthesis and supply via plant cell culture technology. Mol Pharm 5(2):245–256

Kuk YI, Shin JS, Burgos NR, Hwang TE, Han O, Cho BH, Jung S, Guh JO (2003) Antioxidative enzymes offer protection from chilling damage in rice plants. Crop Sci 43(6):2109–2117. https://doi.org/10.2135/cropssci2003.2109

Kwon YK, Ahn MS, Park JS, Liu JR, In DS, Min BW, Kim SW (2014) Discrimination of cultivation ages and cultivars of ginseng leaves using Fourier transform infrared spectroscopy combined with multivariate analysis. J Ginseng Res 38(1):52–58

Li ZG (2019) Measurement of signaling molecules calcium ion, reactive sulfur species, reactive carbonyl species, reactive nitrogen species, and reactive oxygen species in plants. In: Khan MR, Reddy PS, Ferrante A, Khan NA (eds) Plant signaling molecules: role and regulation under stressful environments. Elsevier, Cambridge, pp 83–103. https://doi.org/10.1016/B978-0-12-186451-8.00005-8

Manivannan A, Soundararajan P, Park YG, Jeong BR (2016) Chemical elicitor-induced modulation of antioxidant metabolism and enhancement of secondary metabolite accumulation in cell suspension cultures of Scrophularia kakudensis Franch. Int J Mo Sci 17(3):399. https://doi.org/10.3390/ijms17030399

Meloni DA, Oliva MA, Martinez CA, Cambraia J (2003) Phytosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ Exp Bot 49:69–76. https://doi.org/10.1016/S0098-8742(02)00058-8

Naik PM, Al-Khayri JM (2017) Extraction and estimation of secondary metabolites from date palm cell suspension cultures. Methods Mol Biol 1637:319–332. https://doi.org/10.1007/978-1-4939-7156-5_26

Naik PM, Al-Khayri JM (2020) Elicitor-induced production of biomass and pharmaceutical phenolic compounds in cell suspension culture of date palm (Phoenix dactylifera L.) extraction and estimation of secondary metabolites from date palm cell suspension cultures. Molecules 25:4669. https://doi.org/10.3390/molecules25204669

Nandy S, Hazra AK, Pandey DK, Ray P, Dey A (2021) Elicitor-induced production of industrially promising vanillin type aromatic compound 2-hydroxy 4-methoxy benzaldehyde (MBAID) yield in the in-vitro raised medicinal crop Hemidesmus indicus (L) R. Br. by methyl jasmonate and salicylic acid. Ind Crops Prod 164:113–375. https://doi.org/10.1016/j.indcrop.2021.113375

Othmani A, Bayoudh C, Sellemi A, Drira N (2017) Temporary immersion system for date palm micropropagation. Methods Mol Biol 1637:239–249. https://doi.org/10.1007/978-1-4939-7156-5_20

Per TS, Khan MIR, Anjum NA, Masood A, Hussain SJ, Khan NA (2018) Jasmonates in plants under abiotic stresses: crosstalk with other phytohormones matters. Environ Exp Bot 145:104–120. https://doi.org/10.1016/j.envexpbot.2017.11.004

Pesaraklua A, Radjabiana A, Salamib SA (2021) Methyl jasmonate and Ag+ as effective elicitors for enhancement of phenolic acids contents in Salvia officinalis and Salvia verticillata, as two traditional medicinal plants. S Afr J Bot 141:105–115. https://doi.org/10.1016/j.sajb.2021.04.032

Putter J (1974) Peroxidase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie, Weinhan, pp 685–690

Re R, Pellegrini N, Protegge A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Rybaczek D, Musiałek MW, Balcerczyk A (2015) Caffeine-induced premature chromosome condensation results in the apoptosis-like programmed cell death in root
meristems of *Vicia faba*. PLoS ONE 10(11):e0142307. https://doi.org/10.1371/journal.pone.0142307

Sachadyn-Król M, Agriopoulou S (2020) Ozonation as a method of abiotic elicitation improving the health-promoting properties of plant products—a review. Molecules 25(10):2416. https://doi.org/10.3390/molecules25102416

Sánchez-Sampedro MA, Fernández-Tárrago J, Corchete P (2005) Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn. Journal Biotechnol 119(1):60–69

Soliva RC, Elez P, Sebastian M, Martin O (2001) Evaluation of browning effect on avocado puree preserved by combined methods. Innov Food Sci Emerg 1:261–268

Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol Plant. https://doi.org/10.1111/j.0031-9317.2005.00582.x

Thakur M, Bhattacharya S, Khosla PK, Puri S (2019) Improving production of plant secondary metabolites through biotic and abiotic elicitation. J Appl Res Med Aromat Plants 12:1–12. https://doi.org/10.1016/j.jarmap.2018.11.004

Udomsin O, Yusakul G, Kittisripanya T, Juengvatanatkul T, Patalun W (2020) The deoxymireostrol and isoflavonoid production and their elicitation of cell suspension cultures of *Pueraria candollei* var. *mirifica*: from shake flask to bioreactor. Appl Biochem Biotechnol 190:57–72. https://doi.org/10.1007/s12010-019-03094-y

Veersham C (2004) Elicitation: medicinal plant biotechnology. CBS Publisher, India, pp 270–293

Venugopalan A, Srivastava S (2015) Endophytes as in vitro production platforms of high value plant secondary metabolites. Biotechnol Adv 33:873–887. https://doi.org/10.1016/j.biotechadv.2015.07.004

Włodek L (2002) Beneficial and harmful effects of thiols. Polish J Pharmacol 54(3):215–223

Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64:555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

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