



Full Length Article

Effects of Sorbitol Osmotic Stress on Biochemical Attributes and Induction of Secondary Metabolites in *Rosmarinus officinalis* Callus Culture

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Abstract

This research aimed to evaluate the effects of sorbitol-induced osmotic stress on some biochemical properties and secondary phytometabolite production in Rosemary callus cultures. The primary (protein, free amino acids, proline and carbohydrates) and secondary metabolites profiles of callus tissues were measured to evaluate the various metabolic changes in response to the sorbitol levels (30, 60, 90, and 120 g L⁻¹) under the influence of thidiazuron (TDZ). The findings highlighted dose-dependent increases in osmoprotective solutes, such as proline and carbohydrates, and specific changes in the protein and amino acid profiles under moderate stress. Conditional GC–MS analysis indicated that potent biosynthetic activations of precursor fatty acids/methyl fatty acid esters, silane derivatives, and terpenoids accompanied the higher levels of sorbitol. Both pathways displayed type-specific metabolic reprogramming in response to osmotic pressure. Co-modulation of sorbitol in conjunction with TDZ exhibited a potent boosting effect on the callus growth and further stimulated the production of bioactive compounds. Thus, our findings validated osmotic stress as a feasible *in vitro* tool for altering primary and secondary metabolism, providing a regulated pathway for increasing the quantity and variety of bioactive natural products from medicinal plants used as herbal medicaments.

Keywords: GC-MS profiling; *In vitro* elicitation; Osmotic stress; Secondary metabolites; Sorbitol; Thidiazuron (TDZ)

Introduction

Rosemary (*Rosmarinus officinalis* L.) is an aromatic, evergreen shrub in the Lamiaceae family, primarily recognized for its needle-like leaves and culinary/medicinal uses. It is an important medicinal herb and an interesting model system for studying the effects of osmotic stress on secondary metabolism (Macedo *et al.* 2020). Rosemary is one of the most widely used culinary and medicinal herbs with high antioxidant, antimicrobial, and anti-inflammatory activity. These effects are attributed to the presence of bioactive phytochemicals, such as flavonoids, terpenes and phenolic acids (Aziz *et al.* 2022). Production of these compounds is often regulated by mechanisms that respond to environmental stimuli. For example, water stress alters a plant's biochemical status. Osmotic stress induced during tissue culture by applying stress agents, such as sorbitol, triggers the production of some bioactive compounds (Mawia *et al.* 2020). The scaling of bioactive properties in rosemary rheology that co-regulate yields of high-value secondary metabolites with those that potentiate

bioactivity is key to driving improved crop yields and bioactivity in economically important species such as rosemary.

The diversity of secondary metabolites, driven by environmental signals and elicitation strategies, also exerts genetic regulation in rosemary (Alvi *et al.* 2019). Previous studies have shown that abiotic stressors, such as osmotic stress, drought, salinity, and temperature shifts, induce both primary adaptive and secondary metabolic responses (Abdullah *et al.* 2023; Mishra *et al.* 2025). It has recently been reported that stresses such as drought, salinity, and osmotic stress can greatly enhance the biosynthesis of essential oils and phenolic compounds in Lamiaceae species by activating the corresponding biosynthetic pathways (Mansinhos *et al.* 2024). Such responses differ among species. For example, salinity stress has been shown to markedly elevate camphor, borneol, linalool, and verbenone levels, as well as regulate other terpenoid constituents of rosemary (Chetouani *et al.* 2019). These responses are related to a more general plant strategy to maintain cellular equilibrium and enhance defence during stress.

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Notably, tissue culture techniques, especially callus culture, can serve as a potential substitute for conventional field planting, as they provide enhanced control to modify and increase the accumulation of specialized metabolites of interest (Sidik *et al.* 2024). Moreover, abiotic/biotic elicitors can be applied *in vitro* systems, where plant genetic uniformity can be maintained, making these systems excellent platforms for basic studies of plant physiology and for the commercial production of target metabolites (Jasim and Habeeb 2024). Sugar alcohols, like sorbitol, are one of the important abiotic elicitors. Mawia *et al.* (2020) described how sorbitol triggers osmotic stress in cultured cells, activating water-deficit signaling without the ionic toxicity often associated with salt stress. Sorbitol in culture media triggers complex physiological responses including upregulation of antioxidant enzymes (SOD, CAT, POD), modulation of osmoprotectant accumulation (*e.g.*, proline, some amino acids) and activation of secondary metabolite pathways. For instance, in cultured cells of yarrow (*Achillea* spp.). Sorbitol at different concentrations (5–50 g L⁻¹) resulted in the increase of camphor and total dry weight in a dose-dependent manner. This showed that the growth can be enhanced along with the desired targeted metabolite (Açıkgöz *et al.* 2023).

Plant growth regulators (PGRs) are the most crucial substances for *in vitro* plant morphogenesis and metabolic regulation. Thidiazuron (TDZ), a highly effective urea-derived cytokinin analogue, acts as a dual-function cytokinin, mimicking both cytokinin and auxin activity. It stimulates cell division, shoot proliferation, and somatic embryogenesis, impacting metabolite profiles (Erland *et al.* 2020). Thidiazuron has been extensively used in callus culture protocols for rosemary, and bioactivity studies of TDZ-cultured callus have complemented abiotic elicitors in promoting biochemical traits (Aman and Afrasiab 2014; El-zefzafy and Dawoud 2016).

While water stress has been studied across a wide variety of plants, the key biochemical response of rosemary to osmotic stress has not been thoroughly investigated, particularly regarding the effects of sorbitol and Thidiazuron. This study aimed to bridge these gaps by evaluating the effects of TDZ and sorbitol on the biochemical properties of rosemary callus and their ability to induce secondary metabolite production.

Materials and Methods

Experimental details

This study was conducted in the Plant Tissue Culture Laboratory of the College of Agriculture at the University of Basrah, Iraq, for the period from 1st November 2024 to 15th June 2025. In this study, pre-growing rosemary plant callus was used in MS medium containing BA at 3.0 mg L⁻¹, obtained by culturing leaf pieces (Fig. 1). The explants were washed several times with soap and water to remove dust, then with sterile distilled water. Subsequently, the surface sterilization



Fig. 1: Callus produced from growing leaf pieces of the rosemary plant on MS medium supplied with BA at a concentration of 3.0 mg L⁻¹ and auxin NAA at a concentration of 0.2 mg L⁻¹

process was performed by placing the items directly into a 70% ethyl alcohol solution for 5 min. They were washed several times with distilled, sterile water then placed in a sterilization solution containing sodium hypochlorite (active ingredient concentration 1.05%) at 20% v/v, with the addition of 2-3 drops of Tween-20 diffuser. For each 100 mL of solution, it was stirred continuously for 15-20 min, then removed from the sterilization solution and washed several times with distilled, sterile water to remove the harmful effects of the sterilizing agent. This process was done using the Laminar flow air cabinet, and the sample was then placed in a sterile glass container containing sterile distilled water to prevent it from drying out until the transplant was carried out.

Linguistic medium was used, which consisted of MS salts (Murashige and Skoog 1962) and organic materials, Sucrose (30 g L⁻¹), Orthophosphate Sodium (200 mg L⁻¹), Adinine Sulfate (80 mg L⁻¹), Myoinositol (100 mg L⁻¹), Polyvinyl pyrrolidone (PVP) (1 mg L⁻¹), vitamins and glycine (1 mg L⁻¹), then adding Naphthaleneacetic acid (NAA) (0.2 mg L⁻¹) and 6-benzyladenine (BA) (3.0 mg L⁻¹). The pH of the medium was adjusted to 5.7-5.8, then agar at a concentration of 5 g L⁻¹ was added, and the medium was heated to 90°C; it was then distributed in cultivation pipes with dimensions of 2.5 x 18 cm. The nozzles were of pipes blocked with medical cotton and sealed with aluminum sheets. Then the pipes were sterilized in an autoclave at 1.04 kg cm⁻² pressure and 121°C for 20 min and kept in the incubator until the explant was planted.

Effect of sorbitol on biochemical properties and the induction of secondary metabolic compounds in rosemary callus

The callus produced from the medium supplemented with BA was divided into small pieces, and implanted on media containing different concentrations of sorbitol (sugar alcohols) (30, 60, 90 and 120 g L⁻¹) with the thidiazuron at a

constant concentration of 0.5 mg L⁻¹. The transplanting was carried out in a laminar-flow air cabinet, and the tabletop surfaces and tools used in cultivation were all sterilized with 70% ethyl alcohol and diluted chlorine solution. The basal and intermediate pieces of young leaves were planted at the rate of one piece in each test tube. Ten repetitions of one treatment per explant for induction and propagation of callus, and the plants were incubated in the growth room at a temperature of 25 ± 2°C in the dark for four weeks. The tissue analysis was carried out for total proteins, amino acids, free proline and carbohydrates.

Effect of sorbitol on the induction of active compounds of the rosemary plant

Concentrations of sorbitol (30, 60, 90 and 120 g L⁻¹) were added, along with thidiazuron at a concentration of 0.5 mg L⁻¹, in the presence of MS salts and nutrient intermediate components mentioned above. The resulting callus was collected and dried to extract secondary metabolites.

Calculation of osmotic potential (Ψ_s) of sorbitol solutions at different concentrations

To calculate the osmotic potential (Ψ_s) of sorbitol solutions at different concentrations (30, 60, 90, and 120 g L⁻¹), you can use the formula: $\Psi_s = -iCRT$ Where, i = van 't Hoff factor (for sorbitol, $i = 1$ since it does not dissociate in solution), C = concentration of the solute in moles per liter (mol L⁻¹), R = universal gas constant (0.0831 L bar K⁻¹ mol⁻¹), T = temperature in Kelvin (298 K). Sorbitol has a molar mass of 182.17 g mol⁻¹. First, convert the concentrations from grams per liter to moles per liter:

$$\text{Concentration} = \frac{\text{grams per liter}}{\text{molar mass}} \quad (1)$$

Assuming a temperature of 25°C (298°K), substitute into the osmotic potential equation and calculate. Ψ_s For each concentration, Summary of Osmotic Potential Levels: 30 g L⁻¹ ≈ -4.06 bar, 60 g L⁻¹ ≈ -8.12 bar, 90 g L⁻¹ ≈ -12.18 bar, 120 g L⁻¹ ≈ -16.24 bar.

Extraction and quantitative and qualitative evaluation of active substances

The samples of the alcoholic extract of rosemary callus were prepared using the alcohol extraction method, and the active compounds were estimated using a Shimadzu GC-MS 2010 plus gas chromatography device. For this purpose, the resulting callus was dried at 40°C for 24 h, then ground to a powder. Then, 1.5 g of the powder was added to 7.5 mL of 96% pure ethyl alcohol, and the mixture was vibrated for 24 h. The solution was filtered through filter paper in the pickers, then placed in the oven at 40°C for 24 h to convert it into a powder. The powder was then dissolved in 3 mL of pure ethyl alcohol. Then, the active ingredients in the GC-

MS device were quantified using the gas chromatographic separator at the South Oil Company laboratories.

Statistical Analysis

The study experiments were designed using a randomized complete block design (RCBD). The results of the study were analyzed using SPSS analysis of variance with 10 replicates, and the means of the coefficients were compared using the Duncan test at $P < 0.01$ level.

Results

Biochemical properties of rosemary callus

The results showed significant differences in total soluble protein across the different treatments (Table 1; Fig. 2). Sorbitol at 30 g L⁻¹ and 60 g L⁻¹ (-4.06 and -8.12 bars, respectively) had the highest protein content. Protein levels decreased with increasing sorbitol concentration, with yields of 0.15 μg g⁻¹ at 90 g L⁻¹ (-12.18 bar) and 0.12 μg g⁻¹ at 120 g L⁻¹ (-16.24 bar). The concentrations of sorbitol at 30 and 60 g L⁻¹ increased the free amino acids content. The maximum values of 30 and 60 g L⁻¹ (0.44 and 0.45 mg g⁻¹, respectively) were higher than those at the other concentrations (90 and 120 g L⁻¹). Accumulation of free Proline showed a straight linear positive correlation with an increase in the concentrations of sorbitol. The minimum level corresponded to 30 g L⁻¹ (0.46 μg g⁻¹), increasing gradually to 0.52 μg g⁻¹ at 60 g L⁻¹, then to 0.59 μg g⁻¹ at 90 g L⁻¹ and reaching a maximum at 120 g L⁻¹ (0.62 μg g⁻¹). The carbohydrate differed only slightly among the 30 g L⁻¹ treatment (0.13 mg g⁻¹), whereas it reached a significantly higher content (1.20 mg g⁻¹) in the 60 g L⁻¹ treatment. Highest concentrations (90 and 120 g L⁻¹) yielded significantly greater carbohydrate contents (2.59 and 2.62 mg g⁻¹, respectively; 20-fold higher than the lowest treatment).

Secondary metabolite production analysis by GC-MS

The gas chromatography-mass spectrometry analysis for sorbitol concentrations at 30 g L⁻¹ identified 11 compounds, with 4-methyl-2,4-bis (p-hydroxyphenyl)pent-1-ene, 4-Methyl-2,4-bis (p-hydroxyphenyl) pent-1-ene derivative representing the highest percentage (9.02%) (Table 2; Fig. 3). Other notable compounds included tert-butyl dimethylsilyl 4-((2, 2, 3, 3, 4, 4, 4-heptafluorobutanoyl)oxy)-3-methylbenzoate (4.07%) and hexadecanoic acid methyl ester (3.93%).

The metabolite fingerprints obtained from gas chromatography-mass spectrometry analysis for sorbitol concentrations at 60 g L⁻¹. 60 g L⁻¹ (-8.12 bar) of sorbitol treatment produced 11 compounds, with 13-Octadecenoic acid methyl ester showing the highest abundance (11.37%), followed by n-hexadecanoic acid (8.33%) and 1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester

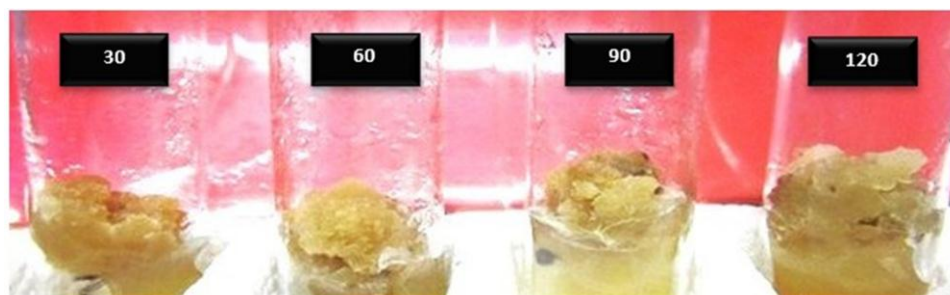
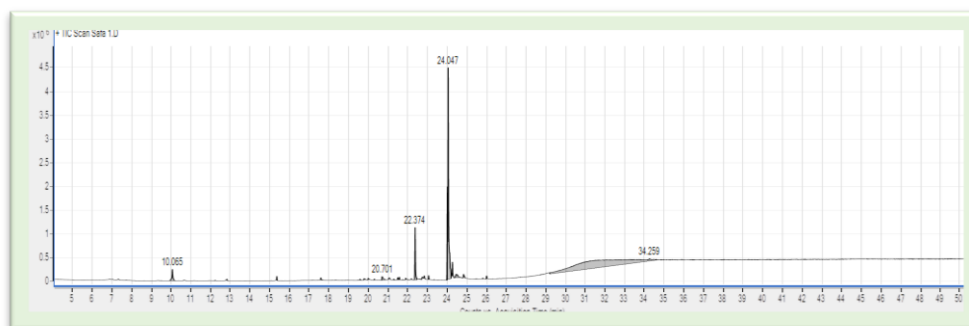
Table 1: Effect of different concentrations of sorbitol with thidiazuron on biochemical properties of rosemary plant callus

Treatment of Sorbitol (g L ⁻¹)	Total soluble proteins (μg g ⁻¹)	Free amino acids (mg g ⁻¹)	Free proline (μg g ⁻¹)	Total soluble carbohydrate (mg g ⁻¹)
30	0.17 ± 0.03b	0.44 ± 0.06a	0.46 ± 0.04b	0.13 ± 0.02c
60	0.18 ± 0.04a	0.45 ± 0.08a	0.52 ± 0.09a	1.20 ± 0.06b
90	0.15 ± 0.07c	0.44 ± 0.04b	0.59 ± 0.06a	2.59 ± 0.06a
120	0.12 ± 0.09d	0.43 ± 0.07c	0.62 ± 0.05a	2.62 ± 0.09a

Means in each column followed by different letters are significantly different ± standard deviation (SD) according to the Duncan test

Table 2: The effect of sorbitol at a concentration of 30 g L⁻¹ (-4.06 bar) on the production of secondary compounds from callus of rosemary plant extract analyzed by GC-MS

Compounds	Retention time (min)	Peak area (%)
Octane, 3,5-dimethyl-	10.066	1.643746089
Cyclohexasiloxane, dodecamethyl-	15.361	0.307894825
Octanal, 2-(phenylmethylene)-	20.703	0.490698093
Hexadecanoic acid, methyl ester	22.373	3.934902734
n-Hexadecanoic acid	22.736	0.32994097
Isonipecotic acid, N-allyloxycarbonyl-, undecyl ester	30.748	0.328487355
4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene,	31.077	9.016633688
4-Methyl-2H-pyran	31.195	1.185006312
tert-Butyldimethylsilyl 4-((2,2,3,3,4,4,4-heptafluorobutanoyl)oxy)-3-methylbenzoate	32.059	4.071088453
Tetramethyl 1-tert-butyl-1,6-dihydro-6-methoxy-2,3,4,5-pyridinetetracarboxylate	32.767	0.728227325
Silane, diethyldecyloxyoctyloxy-	41.767	2.080698611

**Fig. 2:** The effect of different concentrations of sorbitol (g L⁻¹) with the presence of TDZ at a fixed concentration of 0.5 mg L⁻¹ on the biochemical characteristics and the induction of secondary metabolism compounds in the callus of the rosemary plant**Fig. 3:** Chromatogram presentation of the effect of sorbitol at a concentration of 30 g L⁻¹ on the production of secondary compounds from the callus of rosemary plants extract, analyzed by GC-MS

(4.42%) (Table 3; Fig. 4). The metabolite fingerprints obtained from gas chromatography-mass spectrometry analysis for sorbitol concentrations at 90 g L⁻¹, which gave eight compounds, with Silane, diethyldecyloxyoctyloxy- showing the highest percentage (9.02%), followed by n-Hexadecanoic acid (3.93%) and 3-(1'-pyrrolidinyl)-2-butanone (1.64%) (Table 4; Fig. 5).

The metabolite fingerprints obtained from gas chromatography-mass spectrometry analysis for sorbitol at 120 g L⁻¹, which yielded 15 compounds, with Silane and diethyldecyloxyoctyloxy- reaching their peak concentrations (16.20%). Tetracosane represented 9.24%, and hexadecanoic acid methyl ester contributed 3.03% (Table 5; Fig. 6).

Table 3: The effect of sorbitol at a concentration of 60 g L⁻¹ (-8.12 bar) on the production of secondary compounds from the callus of rosemary plants extract, analyzed by GC-MS

Compounds	Retention indicate (min)	Peak area (%)
Octane, 3,5-dimethyl-	10.035	1.269540764
D-Fructose, 1,3,6-trideoxy-3,6-epithio-	17.152	2.368915688
2,4-Di-tert-butylphenol	17.911	0.586945903
Octyl-.beta.-D-glucopyranoside	19.537	0.546261115
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	21.892	4.417080582
Hexadecanoic acid, methyl ester	22.365	2.530475164
n-Hexadecanoic acid	22.75	8.325320477
9,12-Octadecadienoic acid, methyl ester	24	3.6158982
13-Octadecenoic acid, methyl ester	24.044	11.36724923
Methyl stearate	24.257	0.647519703
Vanadium, bis[(1,2,3,3a,7a-.eta.)-1H-inden-1-yl]-	31.204	2.095924227

Table 4: The effect of sorbitol at a concentration of 90 g L⁻¹ (-12.18 bar) on the production of secondary compounds from callus of rosemary plants extract, analyzed by GC-MS

Compounds	Retention time (min)	Peak area (%)
3-(1'-pyrrolidinyl)-2-butanone	10.066	1.643746089
Octane, 2,2,6-trimethyl-	15.361	0.307894825
Hexadecanoic acid, methyl ester	20.703	0.490698093
n-Hexadecanoic acid	22.373	3.934902734
1-Pentene, 4,4-dimethyl-1,3-diphenyl-1-(trimethylsilyloxy)-	22.736	0.32994097
.beta.-Sitosterol acetate	30.748	0.328487355
Silane, diethyldecyloxyoctyloxy-	31.077	9.016633688
Morphinan-14-ol, 6-azido-4,5-epoxy-3-methoxy-17-methyl-, (5.alpha.,6.beta.)-	31.195	1.185006312

Table 5: The effect of sorbitol at a concentration of 120 g L⁻¹ (-16.24 bar) on the production of secondary compounds from the callus of rosemary plants extract, analyzed by GC-MS

Compounds	Retention time (min)	Peak area (%)
1-Dodecanol	21.848	0.29459982
Hexadecanoic acid, methyl ester	22.366	3.02991512
n-Hexadecanoic acid	22.716	0.59606928
Tetracosane	25.727	9.24433745
Eicosanoic acid, methyl ester	25.994	0.22875432
Benzamide, N-(3-methylphenyl)-4-butyl-	31.885	2.82009095
Iopydone	33.365	0.30832549
Succinic acid, 2-methylpent-3-yl pentafluorophenyl ester	33.412	0.29063114
Dihydrotachysterol	34.266	2.58359465
2-Propanone, 1,1,1,3,3,3-hexafluoro-	34.276	0.90914037
Pipecolic acid, N-propargyloxycarbonyl-, tridecyl ester	36.632	1.32931574
4-Methyl-1-nitropyrazole	37.684	0.39945965
5-Methyl-4-hexene-1-yl acetate	39.208	2.20925329
Silane, diethyldecyloxyoctyloxy-	40.481	16.2024532
3-Butene-1,2-diol, 1-(2-furanyl)-	42.743	0.19664825

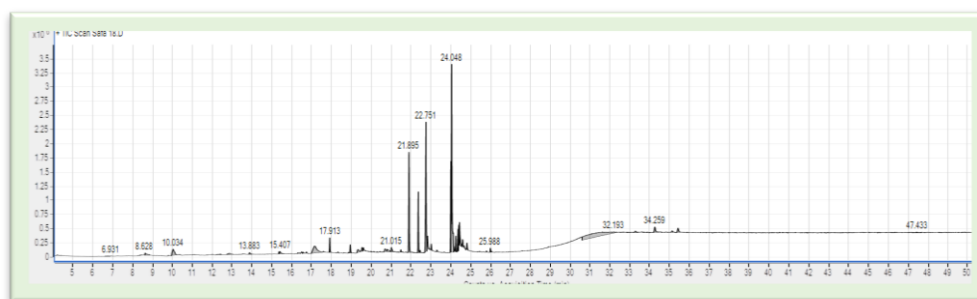
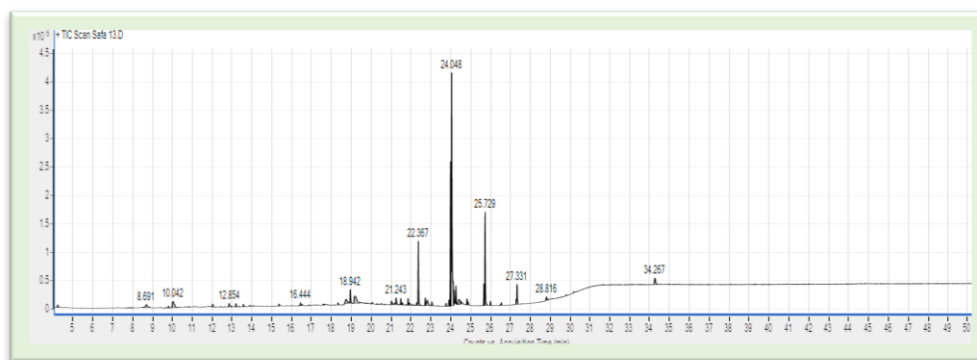
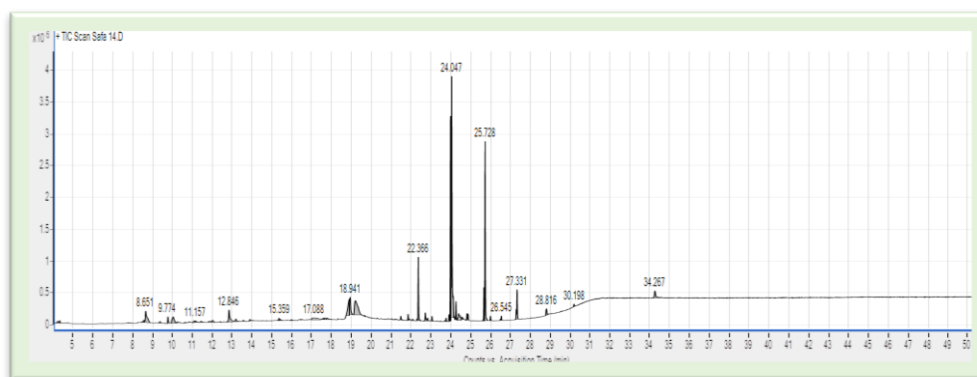
**Fig. 4:** Chromatogram presentation of the effect of sorbitol at a concentration of 60 g L⁻¹ on the production of secondary compounds from the callus of rosemary plants extract analyzed by GC-MS

Table 6: The effect of sorbitol on the production of secondary compounds from callus of rosemary plants growing in MS culture medium

Treatment of Sorbitol (g L ⁻¹)	Hexadecanoic acid, methyl ester	Silane complex, diethyldecyloxyoctyloxy
30	0.21 ± 0.06d	2.08 ± 0.04d
60	2.53 ± 0.04c	2.84 ± 0.03c
90	3.34 ± 0.04b	4.44 ± 0.04b
120	3.93 ± 0.01a	16.20 ± 0.05a

Means in each column followed by different letters are significantly different ± standard deviation (SD) according to the Duncan test

**Fig. 5:** Chromatogram presentation of the effect of sorbitol at a concentration of 90 g L⁻¹ on the production of secondary compounds from the callus of rosemary plants extract, analyzed by GC-MS**Fig. 6:** Chromatogram presentation of the effect of sorbitol at a concentration of 120 g L⁻¹ on the production of secondary compounds from the callus of rosemary plants extract, analyzed by GC-MS

Concentration-dependent responses of key compounds

The effect of sorbitol on the production of secondary compounds from the callus of rosemary plants growing in MS culture medium (Table 6). Two compounds showed particularly strong concentration-dependent responses. Hexadecanoic acid methyl ester, the fatty acid methyl ester, showed progressive increases with sorbitol concentration: 0.21% (30 g L⁻¹), 2.53% (60 g L⁻¹), 3.34% (90 g L⁻¹) and 3.93% (120 g L⁻¹), representing an 18-fold increase from the lowest to the highest treatment. The silane complex, a diethyldecyloxyoctyloxy compound, demonstrated the most dramatic response, increasing from 2.08% at 30 g L⁻¹ to 16.20% at 120 g L⁻¹, representing nearly an 8-fold enhancement. The intermediate concentrations showed 2.84% (60 g L⁻¹) and 4.44% (90 g L⁻¹).

Discussion

Sorbitol-induced osmotic stress is a major contributor to primary metabolic adaptation and stimulation of secondary metabolite biosynthesis in callus cultures of the medicinal plant rosemary. Results confirmed and expanded previous observations of strong regulation of both primary (*e.g.*, osmolyte accumulation; Petrova *et al.* 2024) and secondary (*e.g.*, phenolic and terpenoid) metabolic pathways applicable in plant tissue cultures through abiotic elicitation in non-ionic bond-like sorbitol (Li *et al.* 2023).

Consistent with the classical paradigm of osmotic adjustment, proline and soluble carbohydrates accumulate in a concentration-dependent manner in response to sorbitol treatment. Under water deficit, osmoprotectants like proline

are necessary to maintain cell turgor and protect cellular macromolecules, consistent with Laftouhi *et al.* (2024). Effective antioxidant- and quality-related traits of proline and sugars in rosemary grown in greenhouse trials with water stress are of great importance. Likewise, rosemary callus exhibited sorbitol responses similar to those previously reported in other medicinal species; *i.e.*, the yield of metabolites and the dry weight of these cell cultures increased in response to graded osmotic elicitation, indicating that a metabolic switch toward a more tolerant and synthetic state occurs (Ghanati *et al.* 2014).

GC-MS profiling from our work showed a substantial increase in a wide range of secondary metabolites, including fatty acid methyl esters (hexadecanoic acid methyl ester in particular), silane-derived compounds, and terpenoids, at higher sorbitol concentrations. These metabolic enrichments are consistent with those reported by Laftouhi *et al.* (2024), who also observed a reproducible upregulation of these metabolites in rosemary callus cultures subjected to osmotic stress. In addition, this behaviour is not specific to rosemary, as broad reviews underline the similar generalization (though variability exists per family) that Lamiaceae consistently upregulate the phenylpropanoid, terpenoid, and flavonoid pathways in response to diverse abiotic stresses, yielding both an increased level and diversity of phytochemicals (Mansinhos *et al.* 2024).

A major mechanistic insight from this work is that sorbitol acts both as a drought mimic, establishing signaling for water deficit/osmolyte accumulation in the absence of ionic toxicity, and as a metabolic reprogrammer, promoting the upregulation of many secondary metabolite biosynthesis pathways. Interestingly, sorbitol at 12.18 and 16.24 bar (90–120 g L⁻¹) not only enhanced the accumulation of target compounds known to be produced *via* sorbitol biosynthetic pathways but also promoted the formation of new, low-abundance (minor) metabolites with bioactivity. These results emphasize the malleability of rosemary metabolome and demonstrate that *in vitro* elicitation is a robust platform for metabolic engineering. This effect may have been facilitated by the use of the synthetic cytokinin–urea analogue thidiazuron, which mimics both auxin and cytokinin (Touati *et al.* 2015). This may enhance callus expansion and influence the expression of developmental and metabolic genes. Thidiazuron functions through the phenylpropanoid and subsequent pathways for secondary metabolites and induces robust downstream expression, allowing exploitable upregulation of measurable compounds, including rosmarinic acid, essential oils, and additional phenolics (Pourebadi *et al.* 2015). The correlation between thidiazuron and osmotic stress, as seen here and in other studies, potentially indicates an elaborate signalling pathway underlying the plant cell response system that may be exploited to produce desired bioactives.

Tissue culture platforms provide excellent conditions in a well-defined, controlled environment, thus representing a particular range within the experimental biotechnological

plant cell manipulation methods with homogeneous or mixed abiotic and biotic elicitors to enhance phytochemical profiles. Thus, in this context, *in vitro* systems offer high reproducibility and genetic homogeneity, enabling precise control of stress conditions compared to open-field cultivation. Results showing the effectiveness of sorbitol as an elicitor before the action of those growth regulators justify the need to scale and integrate them at pharmaceutical and commercial levels, with special regard to climate-threatened Mediterranean medicinal and aromatic crops.

Conclusion

The ramifications of exposure to osmotic stress, as seen from this molecular perspective, are that it is not merely harmful but rather a highly powerful developmental and metabolic signal. It is known to be used intentionally to enhance the yield and chemical diversity of important secondary metabolites in rosemary callus cultures. This low-cost osmolyte-driven primary metabolism and second-pathway flux also greatly benefits the medium, accelerating metabolic engineering progress towards more sophisticated metabolic design, which in turn fuels sustainable high-value phytochemistry production.

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Author Contributions

Conceptualization: SMHA and NAA. Methodology: AJH and AAS. Formal analysis: SMHA and NAA. Data curation: AJH and AAS. Visualization: SMHA and NAA. Writing and original draft preparation: AJH and AAS. Writing, review and editing: SMHA and NAA. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

Authors declare no conflict of interest.

Data Availability

The data that made the findings of this research are available from the corresponding author upon reasonable request.

Ethics Approval

Not applicable to this paper.

Funding Source

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