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ORIGINAL ARTICLE

Yeast extract elicitation enhances growth and metabolite production in *Limonium algarvense* callus cultures

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Abstract

Limonium algarvense Erben, a medicinal halophyte, holds significant pharmacological promise due to its rich bioactive compound repertoire. This study aimed to establish robust callus cultures as a sustainable, in vitro model for studying the plant's metabolic responses, particularly focusing on synthesising and accumulating primary and secondary metabolites under various elicitation treatments. Callus cultures were initiated from leaf explants on Murashige and Skoog's medium supplemented with 1 mg/L picloram for 4 weeks. Afterwards, callus cultures were subjected to two elicitor treatments, including salicylic acid–SA and yeast extract–YE at 50 and 100 mg/L for four weeks. Water extracts were assessed for their shifts in primary (total soluble sugars and proteins, and proline), and secondary metabolism (total phenolics, flavonoids, and condensed tannins). In addition, a detailed metabolic profiling was conducted using high-performance liquid chromatography with electrospray ionisation mass spectrometry (HPLC–ESI–MS/MS). Elicitation induced significant shifts in the metabolite synthesis of elicited cultures compared to controls. While YE50 markedly increased the callus yield, the total levels of phenolics, flavonoids condensed tannins and total soluble proteins, the SA50 led to the highest increase in proline content. Metabolomic analysis identified 10 metabolites, including 4-hydroxyphenyllactic acid, hydroxybenzoic acid, riboflavin (Vitamin B2), and dihydroferulic acid methyl ester 4-O-sulfate, that were increased in the YE50 elicitation treatment. This suggests that elicitation can effectively enhance the biosynthesis of primary and secondary metabolites in *L. algarvense* callus cultures, offering great potential for nutritional and medicinal applications.

Key Message

Yeast extract elicitation significantly enhances the growth and metabolite synthesis, including proteins and phenolic compounds, in *L. Algarvense* callus cultures, thereby improving its medicinal value.

Keywords Sea lavender · Salt-tolerant plant · Halophyte · Plant tissue culture

Introduction

Limonium algarvense Erben, a species within the Plumbaginaceae family, is endemic to the Iberian-Mauritanian region. Its range extends from the Algarve coast to the Lusitanian-Andalusian Province, including the Balearic Islands and the coast of Morocco (Erben 1993; Fennane and Ibn-Tattou 1999). This perennial rosette plant is known for its resilience in saline and arid environments, being found in the well-drained upper zones of sandy salt marshes flooded by seawater during high tide (Erben 1993; Costa et al. 2014). Traditionally, *Limonium* spp. has been utilized for its medicinal properties, which include antimicrobial, anti-inflammatory, and antioxidant activities (Mendes and Silva 2018). Particularly, *L. algarvense* has been reported for its significant biological activities, including antioxidant, anti-inflammatory, neuroprotective, anti-diabetic, and anti-melanogenic, contributing to the plant's potential as a source of natural therapeutic agents (Rodrigues et al. 2015, 2016, 2019, 2020, 2021; Pereira et al. 2024; Lescano et al. 2024).

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Moreover, these studies have shown that the major bioactive metabolites include phenolic acids such as gallic, gentisic, and salicylic acids, and various flavonoids like catechins, quercetin, rutin, luteolin, myricetin and apigenin derivatives (Rodrigues et al. 2015, 2016, 2019, 2020, 2021; Pereira et al. 2024; Lescano et al. 2024).

The primary sources for *L. algarvense* involve wild collection and greenhouse cultivation. However, wild harvesting can lead to over-exploitation and habitat degradation, posing significant challenges to environmental sustainability (Hazrati et al. 2023). This species is classified as "Near Threatened," and is close to meeting the criteria for "Vulnerable," due to a continued decline in population, reduced extent of occurrence, and area of occupation (Carapeto et al. 2020). In turn, conventional cultivation is often influenced by climatic conditions and soil quality, impacting the consistency and bioactive compound yield (Rempelos et al. 2023). Tissue culture, including callus culture, offers a promising alternative to traditional plant production systems for the consistent and sustainable production of bioactive compounds. Callus cultures, which are masses of undifferentiated cells induced from plant tissues under controlled conditions, can be used to produce large quantities of plant metabolites independent of external environmental factors. This method ensures a stable and controllable environment, leading to uniformity in the production of desired compounds (Chandran et al. 2020; Ozyigit et al. 2023). Moreover, tissue culture techniques can facilitate the rapid multiplication of plants, conservation of genetic resources, and production of secondary metabolites on a commercial scale. This is particularly valuable for industrial sectors focusing on pharmaceuticals, nutraceuticals, and cosmetics, where high-quality and consistent bioactive compounds are crucial (Chandran et al. 2020; Ozyigit et al. 2023).

Elicitation is a strategy used in plant tissue culture to enhance the production of secondary metabolites. Elicitors are substances that stimulate the plant's defense mechanisms, leading to an increased synthesis of bioactive compounds. Elicitors can be biotic, such as microbial extracts and polysaccharides, or abiotic, such as heavy metals, UV light, and chemical compounds (Jalota et al. 2024). For example, as an abiotic elicitor, salicylic acid acts as a phenolic phytohormone that can regulate plants' immune system, as well as their growth and development (Ali et al. 2024). It can also influence the expression of genes encoding key enzymes for secondary metabolism, contributing to the increase in the production of bioactive compounds such as phenolic acids, flavonoids and tannins (Ali et al. 2024). Whereas, as a biotic elicitor, yeast extract is an abundant source of nutrients, vitamins, amino acids and growth factors essential for plant cells. It can stimulate metabolic responses and transduction signals, thereby catalysing the synthesis of proteins and other bioactive metabolites (Halder et al. 2019).

The application of elicitation techniques has been shown to significantly boost the levels of secondary metabolites in various plant species. This approach can lead to the discovery of novel compounds and improved yields of existing ones, making it highly relevant for industries that are always seeking innovations and novel bioactive substances (Jalota et al. 2024). These techniques have been applied to various medicinal plants, including *Ginkgo biloba*, *Panax ginseng*, *Taxus baccata,* and *Vitis vinifera*. However, despite the existence of several reports on the establishment of callus cultures within the Plumbaginaceae family, including *Armeria maritima* (Gourguillon et al. 2018), *Limonium sinuatum* (Igawa et al. 2002), and *Plumbago zeylanica* (Sivanesan and Jeong 2009), none have investigated the production or elicitation of bioactive metabolites. However, the success of these methods for other medicinal plants indicates the potential for similar approaches to be applied to *L. algarvense*. Thus, this study aimed to establish callus cultures from *L. algarvense* and evaluate their growth and development and assess the impact of biotic and abiotic elicitation on the production of primary and secondary metabolites. This seeks to deepen understanding of the metabolic pathways triggered by elicitation and their potential to enhance the medicinal properties of *L. algarvense*.

Material and methods

Explants disinfection and establishment

L. algarvense seeds were germinated in greenhouse conditions until the seedlings presented 4 leaves in the form of rosette, with approximately 2 weeks after germination. Then, shoot tips were excised from the seedlings to be used as explants, washed under running tap water, immersed in tap water with commercial soap for 15 min, and repeatedly rinsed with distilled water. Explants were surface sterilized by immersion in 0.1% mercury (II) chloride ($HgCl_2$) for 5 min. Then, explants were rinsed 3 times with sterile distilled water (Khadka et al. 2019). The explants were placed in individual glass tubes (20 × 2.0 cm) containing 15 mL of MS basal medium (Murashige and Skoog 1962) free of plant growth regulators (PGRs), with 2% sucrose and 0.8% agar, and maintained under the temperature of 25 ± 2 °C, with a 16/8h light/dark photoperiod provided by LED light (2700 Kelvin) at 3000 lx of light intensity. All media were adjusted to pH 5.8 before autoclaving at 121 °C for 20 min. After 4 weeks, the disinfected explants that showed signs of growth and development of new leaves kept growing for a further 4 weeks, before being used for callus induction.

Callus culture and growth curve

All rosette leaves from 8-week-old plantlets were excised and transferred to Murashige and Skoog medium (MS) (Murashige and Skoog 1962) containing 30 g/L sucrose, 1% agar, supplemented with 1 mg/L picloram (Igawa et al. 2002). Leaves were then cultured in the dark at 25 ± 1 °C, for 4 weeks. The callus cultures were sub-cultured every 4 weeks. To determine the growth curve, approximately 1 g was weighed in Petri dishes with the same medium (in triplicate) and the biomass weight was monitored to determine the fresh weight, every week for 5 weeks. The specific growth rate (μ) was calculated using the formula μ = (ln X2—ln X1) / (t2—t1), where X1 and X2 are the cell concentrations at times t1 and t2, respectively, and ln represents the natural logarithm.

Elicitation of callus culture

For this experiment, approximately 1 g of four-week-old callus were transferred to Petri dishes containing Murashige and Skoog (MS) culture medium supplemented with 2% sucrose, 0.8% agar, and 1 mg/L picloram, and two concentrations of salicylic acid (50 and 100 μM) and yeast extract (50 and 100 mg/L). After 4 weeks of culture in the dark at 25 ± 2°C, the experiment was stopped, and the calli collected, weighted, frozen and freeze-dried. All media were adjusted to pH 5.7–5.8 prior autoclaving at 121 °C for 20 min. All treatments were conducted in triplicate.

Extraction

The freeze-dried callus was ground with a pestle and mortar, and separately mixed with distilled water (1:40, w/v), and extracted in an ultrasound bath for 30 min. The extracts were filtered (Whatman No. 4), frozen and freeze-dried for 3 days (Lescano et al. 2024). The dried extracts were then dissolved in distilled water at a concentration of 10 mg/mL and stored at −20 °C until analysis for their primary and secondary metabolite contents.

Primary metabolism

Proline content

The proline content was determined on the extracts at the concentration of 10 mg/mL, using the method previously detailed in Rodrigues et al. (2024). Samples (500 μL) were mixed with 1 mL of a 1% ninhydrin reagent solution prepared in 60% acetic acid, and incubated for 30 min in a water bath at 100 °C. Then, the mixtures were ice-cooled, and the absorbance was measured at 520 nm on a microplate reader (200 μL/well). A proline calibration curve ranging from 200 to 1.56 μg/mL was used to calculate the proline content ($y = 0.0081x + 0.1203$; $r^2 = 0.959$).

Total soluble protein content

Extracts (5 μL) were mixed with 250 μL of Bradford reagent, and incubated for 30 min at RT. The absorbance of the mixture was measured at 595 nm using a microplate reader (Rodrigues et al. 2024). The protein content was then calculated by comparing the absorbance values with a bovine serum albumin (BSA) calibration curve, which ranged from 1 to 0.002 mg/mL ($y = 0.1871x + 0.4133$; $r^2 = 0.9724$).

Total soluble sugar

Samples (100 μL) were mixed with 100 μL of distilled water, 200 μL of 9% phenol solution, and 1 mL of 96% sulfuric acid. The mixture was then incubated at RT for 30 min, and the absorbance of the samples was measured at 490 nm wavelength using a microplate reader (200 μL/well) (Rodrigues et al. 2024). To calculate the total soluble sugar content, a glucose calibration curve ranging from 1 to 0.002 mg/mL was used for comparison ($y = 1.918x + 0.0077$; $r^2 = 0.9509$).

Secondary metabolism

Determination of total phenolic content (TPC)

The TPC was determined using the Folin – Ciocalteu reagent (F–C) following the method previously described (Rodrigues et al. 2015). The extracts (5 μL at 10 mg/mL) were mixed with a tenfold diluted F–C in distilled water (100 μL) and allowed to stand at RT for 10 min in the dark. Subsequently, 100 μL of sodium carbonate solution (75 g/L in distilled water) was added, and the samples were incubated for 90 min at RT in the absence of light. The absorbance of the samples was measured at 725 nm. A calibration curve was constructed with standard solutions of gallic acid (GA) ranging from 0.003 to 1 mg/mL ($y = 1.431x + 0.0064$; $r2 = 0.9848$). The results were expressed as GA equivalents in milligrams per gram of dried extract (mg GAE/g, DW).

Determination of total flavonoid content (TFC)

The TFC of the extracts was estimated using the aluminium chloride ($AlCl_3$) method in 96-well microplates (Rodrigues et al. 2015). Thirty μL of each extract at 10 mg/mL was mixed with 50 μL of aluminium chloride (2%, w/v, in 98% ethanol), and incubated for 10 min at RT. The absorbance was measured at 420 nm. A calibration curve was prepared using quercetin concentrations ranging from 0.003 to 1 mg/

mL (y = 2.9569x + 0.0204; r2 = 0.9959). The results were expressed in milligrams of quercetin equivalents per gram of dry sample (mg QE/g, DW).

Determination of total condensed tannins content (CTC)

The CTC of the extracts was determined using the 4-dimethylaminocinnamaldehyde- hydrochloric acid DMACA–HCl method as depicted in Rodrigues et al. (2015). Briefly, 10 µL of the extracts at a concentration of 10 mg/mL was mixed with 200 µL of a methanol solution containing 1% (w/v) DMACA and 100 µL of hydrochloric acid (37%, v/v). Following 15 min of incubation, the absorbance was measured at 640 nm. A calibration curve was constructed using catechin concentrations ranging from 0.003 to 1 mg/mL (y = 0.6258x + 0.1146; r2 = 0.9914). The results were expressed as milligrams of catechin equivalents per gram of dried extract (mg CE/g, DW).

Liquid chromatography-mass spectrometry

Two milligrams of the samples were dissolved in 2 ml of ethanol (96%, HPLC grade). All extracts were filtered using 0.22 µm PTFE syringe filters before injection. The injected volume was 5 µL in all cases. Separation of the secondary metabolites was performed using a Dionex Ultimate 3000RS (Thermo Scientific) UHPLC system. The compounds were separated on a Phenomenex Kinetex XB-C18 column (100 mm × 2.1 mm i.d., 2.6 µm) maintained at 25 °C (± 1 °C). Water (eluent A) and methanol (eluent B), both acidified with 0.1% formic acid, were used as the mobile phases. The flow rate was set to 200 µL/min. Gradient elution was performed as follows: from 0 to 3 min, 95% of mobile phase A was used; from 3 to 43 min, a linear gradient to 0% A was applied; from 43 to 61 min, 0% A was maintained; from 61 to 62 min, a linear gradient back to 95% A was applied; and from 62 to 70 min, 95% A was maintained. The UHPLC system was connected to a Thermo Q Exactive Orbitrap hybrid mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Scientific). MS data were collected in both positive (4.0 kV electrospray voltage) and negative (3.8 kV electrospray voltage) ionization modes in separate runs. The mass spectrometer was operated with a resolution of 70,000 for full scans and 35,000 for fragmentation scans. The collision energy was set to 30 NCE, and the scan range was from 100 to 1500 m/z. Calibration of the mass spectrometer was performed using Pierce LTQ ESI Positive Ion Calibration Solution and Pierce LTQ ESI Negative Ion Calibration Solution (both from Thermo Scientific).

Raw data files were analyzed using Trace Finder 3.1 (Thermo Scientific) software. The secondary metabolites were identified based on our previously published works and various online databases, including Metlin, the MassBank of North America, and mzCloud. Identification criteria included exact molecular mass, isotopic patterns, characteristic fragment ions, and retention times. In all cases, the difference between the measured and calculated monoisotopic molecular masses was less than 5 ppm. The relative amounts of metabolites were determined by comparing the peak areas of each compound, providing a semi-quantitative analysis of their abundance across treatments.

Statistical analyses

The results were expressed as the mean ± standard error of the mean (SEM), and the experiments were conducted at least in triplicate. Significant differences were assessed by ANOVA and Student's t-test. *P* values lower than 0.05 were considered significant. All the statistical analyses were done using the XLSTAT statistical platform for Microsoft Excel (version 2013, Microsoft Corporation).

Results and discussion

Establishment of callus culture and growth-curve

Plant callus cultures offer a sustainable approach to producing valuable plant metabolites, including phenolics, proteins, and sugars. This method provides an effective alternative to alleviate pressure on natural habitats (Sood 2021; Wawrosch and Zotchev 2021). While the costs of in vitro culture technology are currently higher than those of traditional agricultural methods, these techniques demonstrate significant potential for future economic and environmental benefits, particularly in the conservation of endangered plant species, such as *L. algarvense* (Espinosa et al. 2018).

In this study, the callus induction was successfully achieved from *L. algarvense* leaf explants treated with 1 mg/L of picloram, with a 100% induction rate observed after four weeks of incubation (data not shown). The application of picloram, a synthetic auxin, has proven highly effective in inducing and establishing callus cultures in various plant species, including *Limonium*. Picloram stimulates cell division and dedifferentiation, resulting in the development of unorganized, proliferative callus tissue (Gantait and Mahanta 2021). For instance, friable callus in *Limonium sinuatum* was successfully induced from leaf segments cultured on half-strength Murashige and Skoog (MS) medium supplemented with 1.0 mg/L picloram, demonstrating its efficacy in initiating callus formation in *Limonium* species (Igawa et al. 2002). This achievement underscores picloram's potential as a valuable tool for advancing research in tissue culture and biotechnology. The successful establishment of *Limonium* callus cultures

provides a robust platform for further applications, such as metabolite production and conservation efforts, solidifying its role in the sustainable utilization of this genus.

Moreover, monitoring cell cultures is essential for identifying distinct growth phases, allowing for the strategic planning of harvest timing to meet specific production goals. Following this, the growth curve of the callus culture was then determined by measuring the variation in fresh biomass weight over a 36-day period (Fig. 1). The lag phase, typically associated with adaptation, was not evident, indicating a rapid initiation of cell division and callus proliferation. The exponential growth phase extended over 28 days and was characterized by a specific growth rate of 0.04 g/day and a doubling time of 16.43 days. After day 28, the culture transitioned to the stationary phase, during which growth plateaued and was maintained until the final observation on day 36.

The growth curve observed for *Limonium* callus cultures in this study aligns with the typical sigmoid pattern commonly reported in in vitro plant cell systems. Comparisons with *Armeria maritima* (Plumbaginaceae family) further validate these findings, as *A. maritima* cells cultured in MS medium supplemented with 4.5 µM 2,4-D and 0.93 µM KIN displayed exponential growth from day 3 to day 14, followed by a stationary phase and a subsequent decline by day 24 (Gourguillon et al. 2018). The growth dynamics of *Limonium* callus cultures differ from suspension cultures due to differences in cell organization and environment. Callus cultures consist of loosely organized cells on solid media, where spatial variation in nutrient and hormone availability occurs, while suspension cultures involve cells in liquid media with uniform exposure to nutrients and growth regulators. These differences, along with species-specific traits, can affect growth rates, metabolic activity, and secondary metabolite accumulation (Galáz-Ávalos et al. 2012).

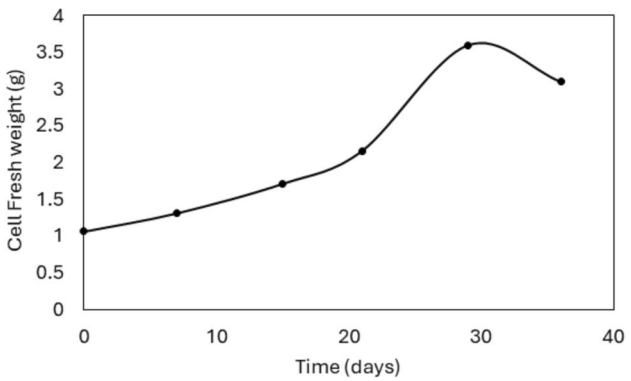

Fig. 1 Growth curve of *L. algarvense* callus in MS medium containing 30 g/L sucrose, 1% agar and 1 mg/L picloram

Effects of elicitation on callus growth and metabolite accumulation

Elicitation is a well-established method for enhancing the production of bioactive metabolites in plant tissue cultures. Elicitors, including biotic or abiotic molecules, mimic natural stress conditions to activate plant defense mechanisms and stimulate the synthesis of secondary metabolites (Halder et al. 2019). Common elicitors such as yeast extract (YE) and salicylic acid (SA) are widely used to boost biomass growth and metabolite production. However, optimizing the selection of elicitors and their dosages is crucial to maximizing yield (Wawrosch and Zotchev 2021). Therefeore, the callus cultures were subjected to elicitation treatments using biotic (yeast extract) and abiotic (salicylic acid) agents at two different concentrations for 28 days (Fig. 2 and Fig. 3). The callus exhibited a color change from white to brown, but no visible morphological differences were observed between treatments throughout the experiment. Biomass growth, measured as fresh weight, varied among the treatments. The YE50 treatment (4.88 g) resulted in the highest biomass and was the only treatment to show a statistically significant increase (105%) compared to the control (2.38 g, $p < 0.05$). In contrast, all other treatments exhibited similar growth to the control (ranging from 3.13 g to 2.28 g).

Yeast extract, a biotic elicitor, is rich in nutrients, vitamins, amino acids, and growth factors essential for cultured cells. It acts as a stimulatory agent, triggering metabolic responses and signal transduction pathways, thereby promoting the synthesis of proteins, nucleic acids, and bioactive metabolites of biotechnological interest (Halder et al. 2019). In addition, stressors, including yeast extract, often elicit biostimulant and elicitor responses, enhancing plant growth and metabolite production. However, excessive stress can induce toxicity, reflecting a biphasic dose–response known as hormesis. This concept describes low-dose stimulation or beneficial effects and high-dose inhibitory or toxic effects (Mattson 2008; Godínez-Mendoza et al. 2023), as observed when the concentration of YE increases.

In addition, the elicitation process had a pronounced impact on the production of primary and secondary metabolites in *L. algarvense* callus cultures (Tables 1 and 2). Proline content increased significantly, particularly in SA50 (9.91 mg/g DW, a 126% increase) and YE100 (6.33 mg/g DW, a 44% increase) compared to the control (4.38 mg/g DW). Total soluble protein exhibited a substantial rise in YE50 (30.04 mg/g DW, a 137% increase) compared to the control (12.67 mg/g DW), while total soluble sugars showed a reduction in YE50 (0.39 mg/g DW, a 33% decrease) relative to the control (0.58 mg/g DW). Among secondary metabolites, YE50 treatment nearly doubled TPC to 25.60 mg GA/g DW (control: 14.10 mg GA/g DW) and increased TFC more than 2.5 times to 12.51 mg QCT/g DW (control:

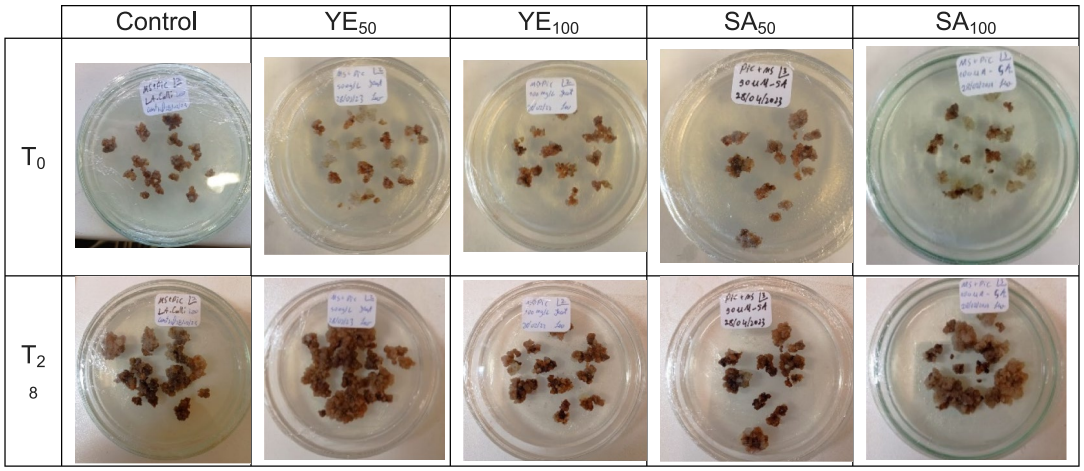

Fig. 2 Aspect of *L. algarvense* callus cultures before and after being subjected to an elicitation treatment for 28 days. YE50: 50 mg/L yeast extract; YE100: 100 mg/L yeast extract; SA50: 50 µM salicylic acid; and SA100: 100 µM salicylic acid

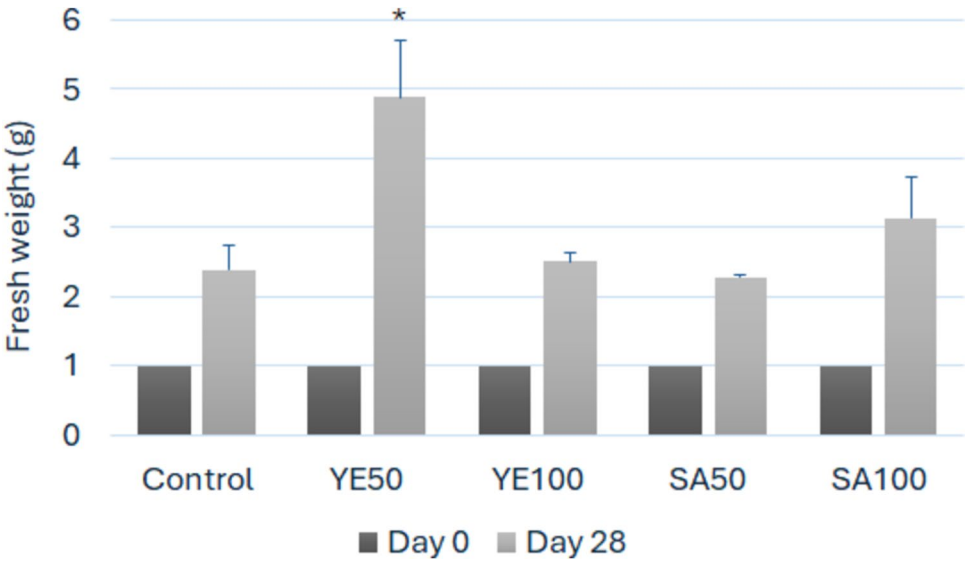

Fig. 3 Growth of *L. algarvense* callus cultures before and after being subjected to an elicitation treatment for 28 days. YE50: 50 mg/L yeast extract; YE100: 100 mg/L yeast extract; SA50: 50 µM salicylic acid; and SA100: 100 µM salicylic acid**.** Columns marked by an asterisk (*) significantly differ from day 0 ($p < 0.05$; student T-test)

Table 1 Total contents of proline, soluble proteins and sugars of *L. algarvense* callus extracts subjected to different elicitation conditions. YE50: 50 mg/L yeast extract; YE100: 100 mg/L yeast extract; SA50: 50 µM salicylic acid; and SA100: 100 µM salicylic acid

Treatments	Proline content (mg/g dw)	Total soluble protein (mg/g dw)	Total soluble sugar (mg/g dw)
Control	4.38 ± 0.08^{bc}	12.67 ± 1.59^{bc}	0.58 ± 0.01^{b}
YE_{50}	5.03 ± 0.44^{b}	30.04 ± 2.76^{a}	0.39 ± 0.02^{c}
YE_{100}	6.33 ± 0.16^{b}	16.94 ± 1.03^{b}	0.66 ± 0.03^{a}
SA_{50}	9.91 ± 0.27^{a}	10.33 ± 1.25^{c}	0.55 ± 0.03^{b}
SA_{100}	3.70 ± 0.05^{c}	8.79 ± 0.79^{d}	0.59 ± 0.02^{b}

Values represent the mean ± standard error of the mean (SEM) of six independent experiments (n = 6)

Values followed by different letters denote significant differences by Tukey's multiple comparisons test ($p < 0.05$)

Table 2 Total contents of phenolics, flavonoids and condensed tannins of *L. algarvense* callus extracts subjected to different elicitation conditions. YE50: 50 mg/L yeast extract; YE100: 100 mg/L yeast extract; SA50: 50 µM salicylic acid; and SA100: 100 µM salicylic acid

Treatments	Total phenolics (mg/g dw)	Total flavonoids (mg/g dw)	Total condensed tannins (mg/g dw)
Control	14.10 ± 0.21^{b}	4.74 ± 0.13^{d}	1.65 ± 0.16^{c}
Ye_{50}	25.60 ± 0.67^{a}	12.51 ± 0.15^{a}	7.84 ± 0.32^{a}
Ye_{100}	13.90 ± 0.58^{b}	9.65 ± 0.30^{b}	2.35 ± 0.16^{b}
Sa_{50}	14.60 ± 0.43^{b}	2.56 ± 0.09^{c}	2.25 ± 0.11^{b}
Sa_{100}	10.70 ± 0.21^{c}	2.54 ± 0.09^{c}	0.64 ± 0.20^{d}

Values represent the mean ± standard error of the mean (SEM) of six independent experiments (n = 6)

Values followed by different letters denote significant differences by Tukey's multiple comparisons test ($p < 0.05$)

4.74 mg QCT/g DW). It also yielded the highest TCT (7.84 mg CTN/g DW, control: 1.65 mg CTN/g DW). In contrast, SA100 significantly reduced TPC, TFC, and TCT levels to 10.70, 2.54, and 0.64 mg/g DW, respectively, demonstrating a suppressive effect on secondary metabolite accumulation. These findings underscore the selective and contrasting effects of elicitation treatments on *L. algarvense* metabolic profiles.

The results highlight the differential effects of elicitation treatments on the metabolic profiles of *L. algarvense* callus cultures, revealing significant insights into their selective modulation of primary and secondary metabolites. Notably, the distinct metabolic responses induced by the elicitors are evident in the changes in proline and protein levels. Under SA50 treatment, protein levels decreased, likely due to a salicylic acid-induced metabolic shift. SA triggers stress-related pathways, redirecting resources toward the synthesis of stress-associated metabolites like proline, which play critical roles in osmotic adjustment and stress tolerance. This reallocation of resources may result in reduced protein synthesis. In contrast, YE50 treatment significantly increased protein levels, reflecting the growth-promoting effects of yeast extract. YE acts as a nutrient-rich bio-stimulant, enhancing metabolic activity and supporting both biomass growth and protein production. These results underscore the contrasting roles of the elicitors: SA primarily activates stress-induced metabolic pathways, while YE promotes growth and protein synthesis through enhanced metabolic activity (Dar et al. 2016; Halder et al. 2019; Sharma and Dubey 2019). YE50 stands out for its ability to significantly enhance secondary metabolite production, nearly doubling total phenolics and more than tripling flavonoids, alongside a substantial increase in condensed tannins. This indicates a robust activation of the phenylpropanoid pathway, likely driven by the elicitor's role in inducing stress and metabolic redirection (Woch et al. 2023). In contrast, the suppressive effects of SA100 on secondary metabolite accumulation underscore a potential dose-dependent inhibitory mechanism, possibly tied to toxicity, feedback inhibition, or hormesis (Mattson 2008; Godínez-Mendoza et al. 2023).

The redirection of sugar metabolism toward secondary metabolite synthesis demonstrates the close link between primary and secondary metabolic pathways. This shift reflects an adaptive strategy, where resources like carbon skeletons and energy, usually allocated for growth, are redirected to produce secondary metabolites in response to stress. In the YE50 treatment, which showed the highest growth rate, the decline in sugar content suggests that as carbohydrate reserves decrease, callus cultures prioritize phenolic compound synthesis. This response likely acts as a protective mechanism, with phenolics reducing stress and enhancing the plant's defense, ensuring survival even with limited energy resources (Bharti et al. 2023).

In addition, a detailed phytochemical profile of *L. algarvense* calli extracts was obtained through metabolomic analysis using UHPLC-ESI-HRMS (Table 3). A total of 20 compounds were identified, primarily belonging to carboxylic acid chemical families. Among these, 10 metabolites were consistently detected across all treatments, including gluconic acid (1), pyridoxine (2), 2-hydroxyglutaric acid (3), citric acid (4), adipic acid (6), hydroxybenzoic acid (8), *p*-coumaroylglucose-O-sulfate (11), 6-O-(*p*-coumaroyl)−1-O-glyceroylglucose (14), *p*-coumaric acid (15), and dihydroferulic acid methyl ester 4-O-sulfate or its isomer (16). Notably, several metabolites exhibited marked increases in their relative abundance under elicitation treatments, as determined by their peak areas in the UHPLC-ESI–MS/MS analysis. For example, 4-hydroxyphenyllactic acid (7), hydroxybenzoic acid (8), riboflavin (13), dihydroferulic acid methyl ester 4-O-sulfate or its isomer (16), and pinellic acid (20) were elevated in callus cultures treated with yeast extract compared to the control culture. Conversely, hydroxybenzoic acid O-hexoside (10) was uniquely identified in SA-treated samples.

Among the detected compounds, only citric acid, hydroxybenzoic acid, and coumaric acid isomers have been previously reported in the leaves and flowers of *L. algarvense* (Rodrigues et al. 2021). Additionally, uralenneoside was identified earlier in extracts from the leaves of *Limonium spathulatum* (Youssef et al., 2022). All other molecules identified in this study are reported here for the first time in this species or genus, highlighting the potential novelty and uniqueness of callus cultures chemical profile. These differences may be due to the distinct metabolic pathways activated in callus cultures compared to whole plants. Callus cultures often experience stress conditions, such as the lack of organized tissue differentiation and exposure to artificial

Table 3 UHPLC-ESI–MS/MS identification of metabolites present in *L. algarvense* callus extracts subjected to different elicitation conditions. YE50: 50 mg/L yeast extract; YE100: 100 mg/L yeast extract; SA50: 50 µM salicylic acid; and SA100: 100 µM salicylic acid

ID	Putative name	Rt (min)	Control	YE_{50}	YE_{100}	SA_{50}	SA_{100}
1	Gluconic acid	2,03	+ + +	+ + +	+	+ +	+
2	Pyridoxine	2,37	+ +	+	+ + +	+ +	+ +
3	2-Hydroxyglutaric acid	2,65	+	+	+ + +	+	+
4	Citric acid	2,94	+	+	+ + +	+ + +	+
5	Glutaconic acid	3,03	+	+	+ + +	-	+ +
6	Adipic acid	11,83	+	+	+	+	+ + +
7	4-Hydroxyphenyllactic acid	13,36	+	+ + +	+ + +	-	+
8	Hydroxybenzoic acid	13,86	+ +	+ + +	+	+ +	+ +
9	Uralenneoside	14,78	-	+	+	+ + +	+ + +
10	Hydroxybenzoic acid O-hexoside	15,38	-	-	-	+	+ + +
11	p-Coumaroylglucose-O-sulfate	16,90	+	+	+ + +	+	+
12	Indole-3-acetic acid	19,76	+	+	+ + +	-	+
13 [1]	Riboflavin	20,51	-	+ + +	+ +	+	+
14	6-O-(p-Coumaroyl)−1-O-glyceroylglucose	20,60	+	+	+	+	+ + +
15 [1]	p-Coumaric acid	20,72	+	+	+	+ + +	+
16	Dihydroferulic acid methyl ester 4-O-sulfate or isomer	20,77	+	+ + +	+	+ +	+
17	Indole-3-lactic acid	21,25	+	+ +	+ + +	-	+ + +
18	Indole-3-carbaldehyde	21,46	+ +	+	+ + +	-	+
19	Malyngic acid or isomer	33,65	+	+ +	+	-	+ + +
20	Pinellic acid or isomer	34,85	+	+ + +	+ + +	-	+ + +

[1]Confirmed by standard

Compound occurrences were distinguished between -, +, + + or + + + for nil, low, medium or high abundance, respectively

growth media, which can lead to the production of unique secondary metabolites not typically found in intact plants (Neumann et al. 2020). Furthermore, the hormonal treatments used to induce and maintain callus cultures can alter metabolic activity, resulting in the synthesis of compounds that differ from those produced in naturally grown plants (Neumann et al. 2020). This highlights the influence of growth conditions and tissue organization on the chemical profiles of plant-derived samples.

The identified molecules present in higher concentrations in YE-elicited *L. algarvense* callus cultures demonstrate significant potential for various applications in health, cosmetics, and therapeutics. 4-Hydroxyphenyllactic acid, with its strong antioxidant and hepatoprotective properties, could be valuable in developing liver-support supplements and antioxidant-rich formulations (Domitrovic and Potocnjak 2016; Zhao et al. 2018). Hydroxybenzoic acid, known for its antioxidant and low-toxicity profile, along with estrogenic activity, holds promise in skincare products and hormonal balance supplements (Gong and Zha 2022; Nguyeb et al. 2022). Riboflavin (Vitamin B2), with its broad applications as an anti-inflammatory, antioxidant, and cosmetic agent, is widely utilized in food, skin conditioning, and hair dye industries, further enhancing the relevance of its production in elicited cultures (Suwannasom et al. 2020; Elgharably et al. 2022). Lastly, Dihydroferulic acid methyl ester 4-O-sulfate or its isomer, identified for its antioxidant, chemotherapeutic potential, and antithrombotic properties, opens possibilities for use in pharmaceutical applications targeting cancer therapies and cardiovascular health (Kumar and Pruthi 2014). These bioactive compounds, produced in enhanced quantities under YE elicitation, demonstrate the significant biotechnological potential of elicited callus cultures for generating high-value metabolites for diverse industries.

Conclusions

This study successfully established *Limonium algarvense* callus cultures as a reliable and sustainable system for exploring and enhancing plant metabolite production. Callus induction, achieved with picloram at 1 mg/L, provided a robust platform for elicitation experiments, showcasing a high induction rate and consistent growth patterns. Biotic and abiotic elicitation strategies significantly optimized both primary and secondary metabolite synthesis. Yeast extract at 50 mg/L (YE50) was particularly effective, boosting biomass growth and the accumulation of key metabolites such as proteins, proline, phenolics, flavonoids, and condensed

tannins. Phytochemical profiling identified a diverse range of bioactive compounds, including 4-hydroxyphenyllactic acid, hydroxybenzoic acid, riboflavin (Vitamin B2), and dihydroferulic acid methyl ester 4-O-sulfate, known for their potent antioxidant and health-promoting properties. These findings underscore the metabolic flexibility of *L. algarvense* callus cultures under elicitation, offering a sustainable source of high-value compounds with potential applications in health, cosmetics, and nutraceuticals. This study provides a solid framework for leveraging plant cell culture systems for metabolite production, contributing to the conservation of *L. algarvense* and fostering innovative biotechnological advancements.

Author contributions Leonardo Lescano: Methodology, Investigation, Visualisation, Data Curation, Writing – Original Draft; Zoltán Cziáky: Methodology, Investigation, Validation, Formal Analysis; Luísa Custódio: Project Administration, Funding Acquisition; Maria João Rodrigues: Conceptualization, Methodology, Investigation, Visualisation, Validation, Data Curation, Supervision, Writing – Review & Editing, Funding Acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability Data will be made available on request.

Declarations

Conflict interest The authors have no relevant financial or non-financial interests to disclose.

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