Highly Efficient Verbascoside Production from Olive (*Olea europea* L. cv Cellina di Nardò) *In Vitro* Cell Cultures

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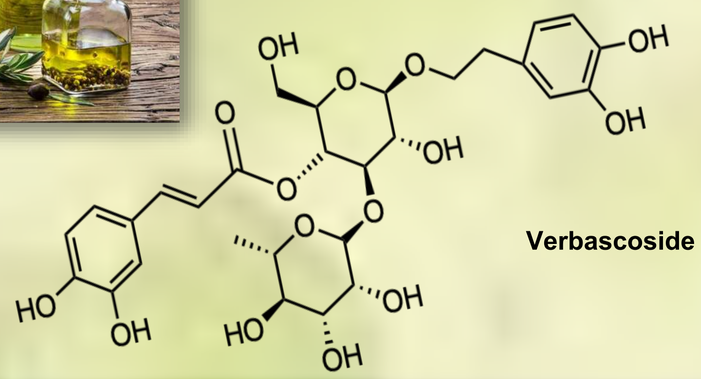
INTRODUCTION

Olive plant tissues, drupes, and olive oil contain several phenolic compounds and terpenoids that are well-known for their highly beneficial effects on human health. Among these polyphenols, olives contain verbascoside (or acteoside), a water-soluble phenylethanoid compound. Verbascoside is structurally characterized by caffeic acid and hydroxyphenylethyl (hydroxytrosol) moieties attached to a *β*-glucopyranose via a glycosidic bond. Verbascoside is renowned for its multiple health benefits, primarily attributed to its antioxidant, antibacterial, antiviral, anti-inflammatory, and antitumor activities. Due to its beneficial health properties, verbascoside is of great industrial interest, resulting in a high economic value.

Plant cell culture technology is considered a sustainable method of producing secondary metabolites, offering several advantages over other strategies, including its potential for scaling up in industrial bioreactors.

Herein, we report the results of establishing a 'Cellina di Nardò" ("CdN") olive *in vitro* cell culture, which efficiently produces verbascoside from an established and actively growing olive cell suspension culture.



Verbascoside

MATERIALS AND METHODS

Establishment and Characterization of "CdN" Olive Cell Suspension Cultures

Micropropagated shoots of "CdN" olive were used as source material for callus induction, which was initiated by leaf fragments. They were placed in Petri dishes containing 10 mL of callus induction medium (CIM), which consisted of MS salts and vitamins, supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar. After autoclaving at 121 °C for 20 min, CIM was enriched with filter-sterilized 2,4-D (9.05 µM) and zeatin (0.91 µM). When an abundant and friable callus had developed, it was used for the initiation of suspension cultures, transferring approximately 1 g fresh weight to 100 mL Erlenmeyer flasks containing 25 mL liquid CIM. After two subcultures, the hormonal composition of the liquid medium (CIM) was progressively reduced by 20-fold (CIM01). The cultures were placed on a rotary shaker (80−100 rpm), in a growth room at 25 °C, in the light (120 µmol m^{-2} s^{-1} provided by Philips TLD/83 white fluorescent light) and were transferred every two weeks by inoculating 5 mL of the "old" suspension culture in 45 mL of liquid CIM01.

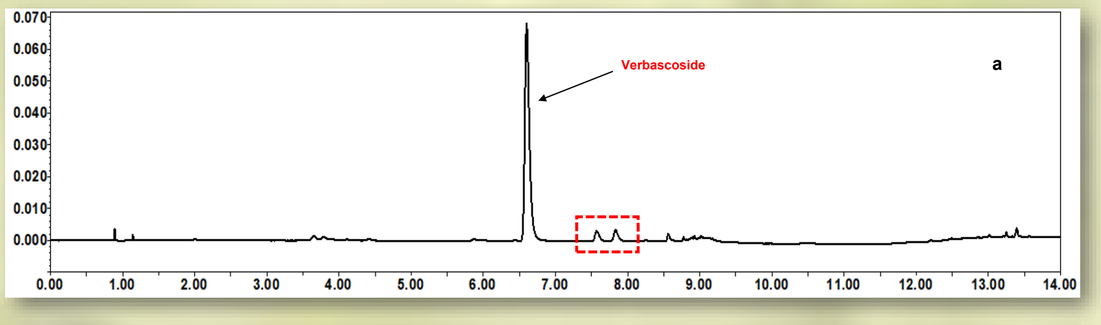
***In vitro* setting of "CdN": field cuttings settled in culture (A); axenic shoots (B); callus cultures (C) and cell suspension cultures (D).**

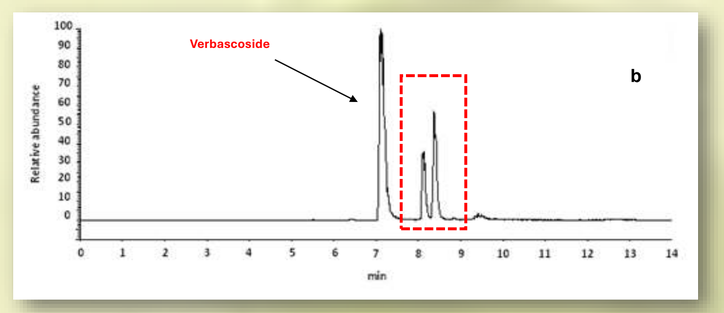
Sample Preparation and Extraction of Phenolic Compounds from "CdN" Olive Cells

Freeze-dried cells were macerated in 80% methanol without stirring. After centrifugation and supernatant collection, the extraction process was repeated on a rotary shaker at room temperature. After the second centrifugation, the supernatants from both extractions were combined, the organic solvent was removed by rotavapor, then the obtained sample was freeze-dried. The dried extract was redissolved in 1 mL of 80% methanol, filtered and diluted (1:100) with the same solvent before UPLC/MS analysis.

RESULTS AND DISCUSSION

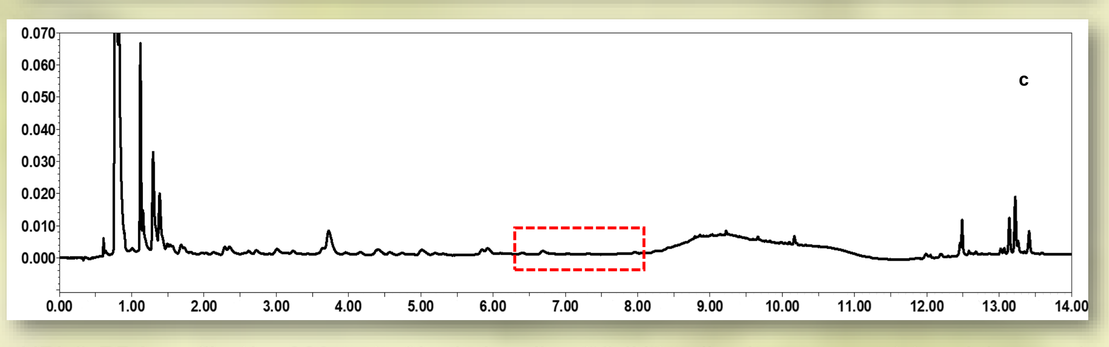
After establishing, olive cell suspension culture growth features and vitality were characterized. Following several growth cycles, the culture showed a sigmoidal growth curve, reaching the maximum growth at 15 days. Moreover, the cells were treated with fluorescein diacetate and observed under fluorescent light. At microscopic observation they showed cells vitality higher than 90%.





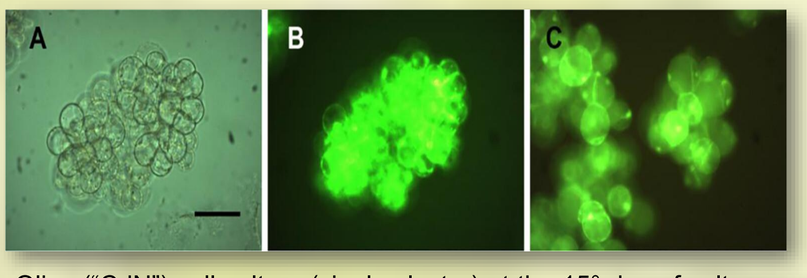
UPLC-ESI-MS profile of olive ("CdN") cell culture extract (m/z = 623.59)

The production of verbascoside increased throughout the growth cycle of the cells, which maintained the ability to continuously produce verbascoside. The verbascoside production reached 102.4 mg/g DW after ten months of repeated subcultivations. The phenolic content of the cell extract was notably high, and interestingly, the antioxidant capacity was found to be extremely high (Table 1).



UPLC-PDA profile (λ = 320 nm) of both the olive ("CdN") cell culture extract (a) and the growing medium (b) at the 15th day of cell culture.

PDA chromatograms of the cells extract show the presence of a predominant compound along with two minor peaks (a). The predominant compound was identified as verbascoside by comparing its retention time, PDA and MS spectra with those of the reference standard (b). The two minor peaks showed UV and MS spectra very similar to that of verbascoside, suggesting the presence of small amounts of two verbascoside isomers in the extract. The chromatographic profile of the culture medium in which the cells were cultivated did not exhibit any corresponding peaks (C).



Olive ("CdN") cell culture (single cluster) at the 15° day of culture. The cells were treated with fluoresceine diacetate and observed in bright (A) and fluorescent (B) and (C) light. Cell nuclei and chloroplasts were easily detected (C).

Table 1

Growth cycle	Verbascoside (mg/g_{DW})	Folin-Ciocalteu Assay ($mg\ GAE/g_{DW}$)	TEAC Assay ($\mu mol\ TE/g_{DW}$)
February 2022	27.8 ± 0.4 c	17.9 ± 0.2 c	115.9 ± 1.8 c
March 2022	45.1 ± 2.2 b	29 ± 1.4 b	188 ± 9.1 b
October 2022	51.5 ± 6.2 b	32.8 ± 3.9 b	214.7 ± 25.8 b
December 2022	102.4 ± 2.4 a	65.9 ± 4.9 a	427.1 ± 12.2 a

CONCLUSION

The olive cell suspensions' consistent ability to produce verbascoside all year round, together with high-level production (up to 10 % DW), is a very promising feature. This suggests the potential for the olive culture to be effectively exploited for the biotechnological production of this bioactive compound.