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Short communication

Phenolic compound production by *Larrea divaricata* Cav. plant cell cultures and effect of precursor feeding

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ABSTRACT

This paper summarizes progress made in using *Larrea divaricata* Cav. cell cultures for the production of the cytotoxic lignan nordihydroguaiaretic acid (NDGA) and the phenylpropanoids *p*-coumaric acid, ferulic acid and sinapyl alcohol. In order to improve the biomass formation and production of these phenolic compounds, four precursors (L-phenylalanine, cinnamic acid, ferulic acid, and sinapic acid) were fed to *L. divaricata* cell cultures. Feeding L-phenylalanine (0.5, 1 and 3 mM) resulted in an increase of NDGA of up to 301.35 ± 1.19, 285.23 ± 28.44 and 190.53 ± 19.50 μg/g DW. The addition of 0.5 μM cinnamic acid enhanced the cell culture growth, but not the NDGA production. When cinnamic acid (1 and 1.5 μM), ferulic acid (0.1, 0.5 and 1 mM) and sinapic acid (0.1, 0.5 and 1 mM) were added, the media became too toxic for the cells, and the production of NDGA and the phenylpropanoids was suppressed. The content of *p*-coumaric acid in the medium supplemented with 3 mM L-phenylalanine increased from 47.43 ± 9.01 to 1157.28 ± 47.79 μg/g DW, whereas the sinapyl alcohol content was not affected by any of the precursors tested, presenting similar values to the control medium.

1. Introduction

Larrea divaricata Cav. (Zygophyllacea) "jarilla" is a perennial woody shrub widely used in American Indian traditional medicine to treat various ailments including digestive disorders, rheumatism, arthritis, venereal diseases and sores [1–4]. In Argentina *L. divaricata* is distributed in the phytogeographical province of the Monte from Salta to Chubut, although in some regions it is scarce because of intensive collection by "jarilleros" (firewood gatherers), who are causing an irrecoverable level of deforestation [5,6].

The resin that covers the leaves of *L. divaricata* yields flavonoid aglycones, as well as several lignans, notably the antioxidant NDGA. Some glycosylated flavonoids, sapogenins, essential oils and waxes have also been identified [6].

Lignans in the narrow sense are derived from two moieties of hydroxycinnamoyl alcohol derivatives, e.g. caffeoyl alcohol, coniferyl alcohol or syringyl alcohol, and are coupled by the 8 and 8′ C-atoms of the side chains [7–9].

The regiospecifically 8–8′ linked lignan nordihydroguaiaretic acid (NDGA) (4,4′-(2,3-dimethylbutane-1,4-diyl)dibenzene-1,2-diol), a prevalent and biologically active compound present in *L. divaricata* leaves, is considered to be a powerful inhibitor of lipoxygenase enzymes, which play an important role in cardiac diseases, asthma, arteriosclerosis, viral infections and cancer [10–14]. Terameprocol, a tetra-*O*-methyl derivative of nordihydroguaiaretic acid, is currently in Phase I/II clinical trials as an anticancer agent [15].

The growing potential of lignans in clinical use has led to the consideration of various alternative systems for their production. A survey of the plant cell culture literature shows that lignans occur in many plant species but only in low concentration, e.g. podophyllotoxin: 0.51 mg/g DW in *Podophyllum hexandrum* [16], 1.1 mg/g DW in *Callitri drummondi* [17], 0.03–2.6 mg/g DW in *Linum album* [18,19]; methoxypodophyllotoxin: 0.08–5.4 mg/g in *L. album* [18,19], 2.01 mg/g DW in *Linum nodiflorum* [20]; mataireinol: 2.24 mg/g DW in *Forsythia* × *intermedia* [21]; pinoresinol: 0.86 mg/g DW in *Forsythia* × *intermedia* [21]; justicidin B: 1.8–6.7 mg/g DW in *Linum austriacum* [22].

Based on knowledge of biosynthetic pathways, the synthesis of plant secondary metabolites can be enhanced by supplementing the culture media with precursors or intermediates. Attempts to induce or increase the yield of the desired product by the exogenous supply of such organic compounds have been effective in many cases [23–25].

The aim of the present work was to establish cell suspension cultures of *L. divaricata* as a potential alternative source of NDGA and other important phenolic compounds, and to explore the potential of these cultures in feeding experiments. The precursors used in this study were intermediates from the phenylpropanoid pathway: L-phenylalanine, cinnamic acid, ferulic acid, and sinapic acid.

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To our knowledge, this is the first report on *Larrea divaricata* Cav. cell cultures and feeding experiments.

2. Materials and methods

2.1. Chemicals and standards

NDGA, L-phenylalanine, *p*-coumaric acid, cinnamic acid, ferulic acid, caffeic acid, sinapic acid, sinapyl alcohol, coniferyl alcohol, quercetin and kaemferol aglycones were purchased from Sigma–Aldrich (St. Louis, MO, USA). The solvent used for the extraction of samples was analytical grade (ethanol and diethyl ether) while those used for the HPLC analysis were HPLC grade (acetonitrile, phosphoric acid, Milli Q distilled water and methanol). All were purchased from Merck Co. (Darmstadt, Germany).

2.2. Plant material

Wild plants of *L. divaricata* Cav. were collected at Santa María de Punilla, "Sierras de Córdoba", located at 45 km north of Córdoba city, Argentina. This region has a temperate Mediterranean climate, with warm summers and dry winters. The annual mean temperature is 16 °C (the mean maximum and minimum temperatures being 34 °C in January and 6 °C in July, respectively), with an average annual rainfall of 550 mm. The location's average altitude is 700 m above sea level [4,26]. A voucher specimen was deposited in the International Herbarium of the National University of Río Cuarto, Argentina (Accesses information RIOC 501).

2.3. Plant cell culture and culture conditions

An efficient method for callus induction is described by Palacio et al. [27]. Callus tissues of *L. divaricata* were established from wild leaves, which were surface disinfected by immersion in 2% copper sulfate solution for 15 min, followed by immersion in ethanol solution 70% for 10 min, and sodium hypochlorite solution 1.5% for 15 min, and finally rinsed three times with sterile distilled water. The explants were placed on Murashige and Skoog (MS) medium [28] supplemented with 9 μM of 2,4-dicholorophenoxyacetic acid (2,4-D) and, 5 μM of N^6-benzyladenine (BA). The pH media was adjusted to 5.6 and solidified using 0.8% plant agar. The calli obtained from the explants were grown at 25 (±2) °C under a 16/8 h light/dark photoperiod by using cool-white fluorescent light (45 μmol m^{-2} s^{-1}). Calli were subcultured every 4 weeks on the same medium. After subculturing for four months, cell suspension cultures were initiated by transferring the calli to a liquid medium (with the same culture conditions), using an inoculation rate of 1/10, and cultured on a rotary shaker (120 rpm) at the same temperature and day/night regime condition as described above for 15 days.

2.4. Experimental design and precursor feeding

For cell suspension culture inoculation, 2 g of cells was aseptically transferred to 20 ml of sterilized medium. L-Phenylalanine, cinnamic acid, ferulic acid, and sinapic acid were dissolved in ethanol and added to the cultures through a membrane filter (0.22 μm) to give final concentrations of 0.5, 1 and 3 mM for L-phenylalanine, 0.5, 1 and 1.5 μM for cinnamic acid, and 0.1, 0.5 and 1 mM for ferulic acid and sinapic acid. These precursors were added to the cell suspension on the first day of the culture cycle. Control experiments were run concurrently in which either no supplements were added to the culture medium or only ethanol was added (final concentration of 0.025%). Cell cultures were subsequently collected to determine the biomass and phenolic compounds by vacuum filtration at defined times. The results presented in this paper have been summarized from three independent experiments. All determinations were performed in triplicate.

2.5. Bioconversion

The amount of substrate converted was calculated from the maximal difference in NDGA content between the control and the substrate-fed cultures. The bioconversion percentage was calculated as follows: substrate converted (mM)/initial substrate concentration (mM) × 100%.

2.6. Analysis

2.6.1. Biomass determination

Cells were collected, separated from the media by filtration, washed with distilled water to remove residual medium, and then filtered under vacuum to obtain fresh pellets, which were immediately frozen in liquid nitrogen. Dry weight (DW) was determined from lyophilized fresh material, expressed as g of DW, and stored at −80 °C until use.

2.6.2. Measurement of cell viability

Cell viability was estimated with fluorescein diacetate (FDA) staining as described by Duncan and Widholm [29]. At least 500 cells were counted and cell viability was presented as the percentage of living cells in the total number of cells counted.

2.6.3. Extraction procedures and determination

The dried cell biomasses were finely ground and the powdered cell material was extracted with ethanol by maceration in a shaker at 120 rpm for 24 h/dark at room temperature. Ethanol extracts were filtered and concentrated under reduced pressure. The cell-free media were partitioned three times with diethyl ether (30 ml each time) and the organic phases were collected and evaporated under reduced pressure. The residues were re-dissolved in methanol (1 ml), filtered through a membrane filter (0.45 μm pore size) and analysed by HPLC. The assays were performed using a Waters™ 2690 HPLC system (Waters, Milford, MA) with a computer controlled system containing Millenium software. Separations were carried out on a reversed phase column Phenomenex Luna 5μ C18 (2) 100 Å (4.6 mm × 250 mm) fitted with a SecurityGuard™ precolumn. The mobile phase consisted of solvent A (water/1% phosphoric acid)/solvent B (acetonitrile/1% phosphoric acid), in linear gradient starting from 70% to 50% of solvent B in 10 min, 50% to 40% of solvent B in 5 min, 40% to 30% of solvent B in 10 min, 30% to 70% of solvent B in 5 min, at a constant solvent flow of 1 ml/min. The eluent was monitored with a multichannel photodiode array detector (Waters 996) at 265 nm for coniferyl alcohol, at 272 nm for cinnamic acid, at 275 nm for sinapyl alcohol, at 281 nm for NDGA, at 310 nm for *p*-coumaric acid, at 325 nm for ferulic acid and caffeic acid, at 328 for sinapic acid, and at 370 nm for quercetin and kaemferol aglycones. The metabolites were identified using the external standard method and quantified from their calibration curves. All experiments were repeated three times and the quantitative determination was performed in triplicate and the results are presented as mean values ± SD.

3. Results and discussion

3.1. Kinetics of biomass formation and phenolic compound production by L. divaricata cell cultures

L. divaricata cell cultures in MS medium containing 2,4-D and BA (9:5 μM) exhibited biomass formation with a lag growth phase of approximately 5 and 7 days for fresh and dry weight, respectively, followed by an exponential phase until day 9 and with a subsequent stationary phase until the end of culture cycle (15 days) (Fig. 1A).

Analysis of the cell biomass and cell-free medium clearly showed that these homogeneous cell cultures had the ability to produce four interesting intracellular phenolic compounds: the lignan NDGA, and phenylpropanoid pathway intermediates: *p*-coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid), ferulic acid ((E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid) and sinapyl alcohol (4-hydroxy-3,5-dimethoxycinnamyl alcohol).

Cinnamic acid, caffeic acid, sinapic acid, coniferyl alcohol, quercetin or kaemferol aglycones were not detected in this system.

The kinetics of phenolic compound production by the cell culture are presented in Fig. 1B. NDGA content began to increase rapidly during the early lag growth phase, reaching a maximum value at day 4 (115.72 ± 8.39 μg/g DW). During the exponential growth phase, the NDGA yield gradually decreased until the stationary phase, when it remained fairly constant. This result suggested that NDGA decreased after 4 days of culturing because of the enzymes of secondary metabolism probably did not work at their maximum rates, because the concentration of precursors, cosubstrates and other necessary intracellular compound would be low.

In contrast, sinapyl alcohol began to accumulate in the late lag growth phase of cell culture, the highest production (70.55 ± 1.40 μg/g DW) being reached at day 5. Contents of *p*-coumaric acid and ferulic acid were first determined in the exponential growth phase. The maximal level of *p*-coumaric acid (50.41 ± 15.99 μg/g DW) was reached at day 7 of culture, and of ferulic acid (57.09 ± 4.52 and 58.98 ± 3.41 μg/g DW) at days 7 and 11.

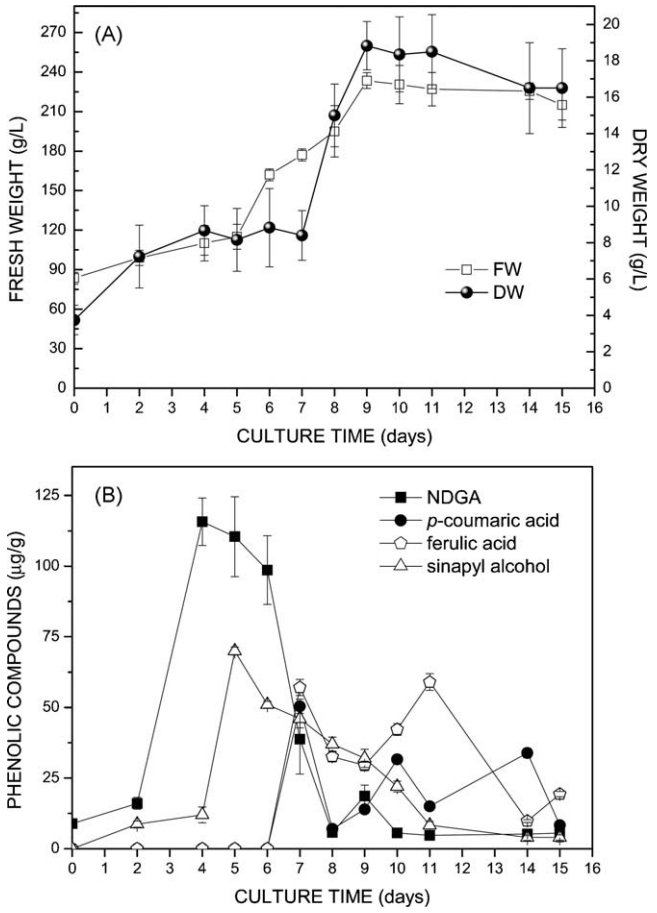

Fig. 1. Kinetics of biomass formation (A) (fresh and dry weight) and phenolic compound production (B) by *L. divaricata* cell cultures on MS medium containing 2,4-D and BA (9:5 µM). Data represent average values from three separate experiments ±SD.

None of the targeted compounds was detected in the cell-free medium throughout the process.

3.2. Effects of precursors on the kinetics of biomass formation and phenolic compound production

3.2.1. Effects of L-phenylalanine feeding

Three concentrations of L-phenylalanine were assayed: 0.5, 1 and 3 mM. Addition of ethanol had no effect on dry weight accumulation. The maximum dry weight in the control medium and ethanol-supplemented medium was reached at day 9, being 18.83 ± 1.33 and 18.01 ± 1.27 g/L, respectively. The NDGA, *p*-coumaric acid, ferulic acid and sinapyl alcohol contents were not affected by the addition of ethanol to cell suspension cultures, which were similar to those of the unsupplemented medium.

The biomass formation kinetics resulting from L-phenylalanine feeding experiments are shown in Fig. 2A. These data indicate that after feeding L-phenylalanine at a concentration of 0.5 and 1 mM, cell cultures grew at a rate comparable with non-fed cultures, but with a difference in the time course of the lag growth phase. The maximum dry biomass (18.27 ± 1.93 and 17.76 ± 2.44 g/L, respectively) reached at day 9 of cell culture was quite similar to that of non-fed cultures. However, compared with the control, a low rate of growth was observed in the cells cultured in presence of 3 mM L-phenylalanine. Our findings are consistent with the result of Edhahiro et al. [30], who examined the effect of L-phenylalanine on anthocyanin accumulation in strawberry cells, and postulated that there was a strong inhibition of cell

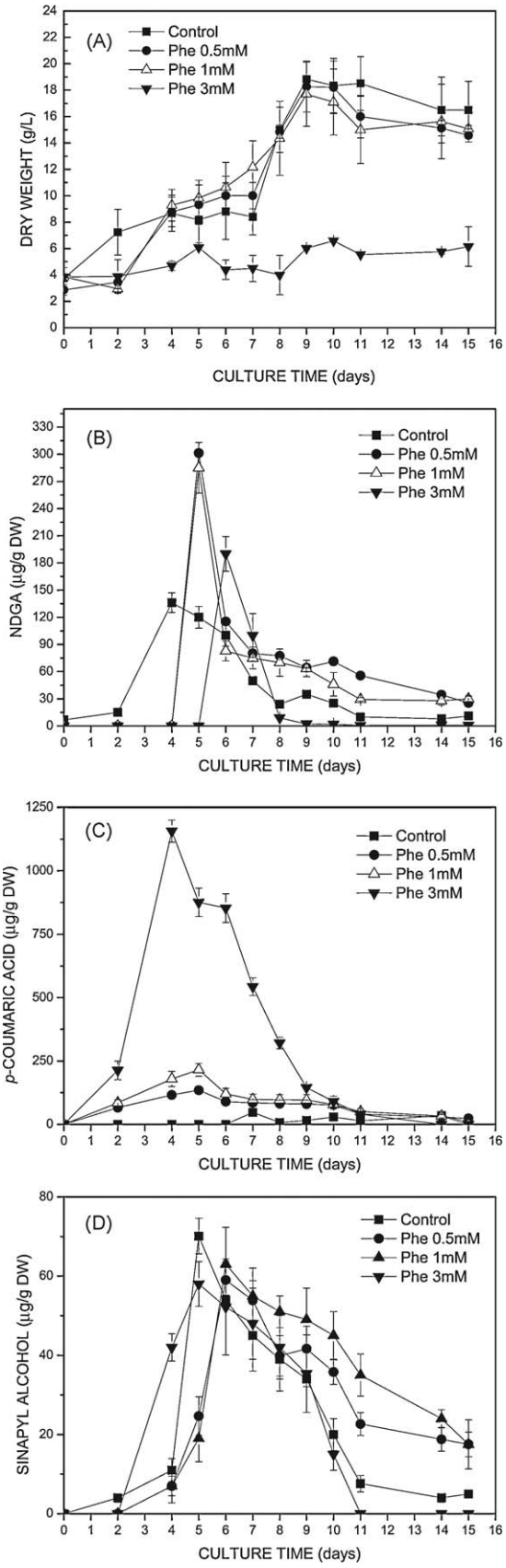

Fig. 2. Kinetics of biomass formation (A) and phenolic compound production: NDGA (B), *p*-coumaric acid (C), sinapyl alcohol (D) by *L. divaricata* cell cultures using control medium with 0.5, 1 and 3 mM of L-phenylalanine. Data represent average values from three separate experiments ±SD.

growth (50% of the cell population) at a high concentration of L-Phe.

In the L-phenylalanine-fed cultures, intracellular phenolic compounds (NDGA, *p*-coumaric acid and sinapyl alcohol) were identified, presenting distinct differences in the kinetics of production (Fig. 2B–D). Fig. 3B shows that NDGA content was stimulated in the media containing L-phenylalanine, reaching maximum values of 301.35 ± 1.19, 285.23 ± 28.44 and 190.53 ± 19.50 μg/g DW at the respective concentrations of 0.5, 1 and 3 mM, which were 2.2-, 2.1- and 1.4-fold higher than in the control. Exogenously applied L-phenylalanine enhances production of secondary metabolites in a variety of plant species, e.g. in *Saussurea medusa* cell suspension cultures it stimulated the production of flavonoids [31], and increased taxol production in *Taxus chinensis* [32].

The higher NDGA biosynthetic capacity observed in L-phenylalanine-fed cultures could be explained by a probable metabolization of this precursor into phenylpropanoid pathway intermediates affording NDGA. Despite the growing potential of clinical uses for NDGA and its derivatives, its biosynthetic pathway in plants remains unknown. Based on phytochemical studies, a provisional pathway has been proposed, with the amino acid L-phenylalanine as the departure point from primary metabolism generating, through the phenylpropanoid metabolic pathway, an allylphenol such as *p*-anol to afford NDGA [33,34].

Therefore, the sinapyl alcohol identified in the cell cultures might be required as one of the two monolignol radicals coupling to afford the lignan formation (NDGA) in *L. divaricata*.

On the other hand, as shown in Fig. 2C, a concentration of 3 mM L-phenylalanine in the media noticeably increased the production of *p*-coumaric acid, which peaked at day 4 of the culture (1157.28 ± 47.79 μg/g DW). At the lower concentrations of this precursor (0.5 and 1 mM) and in untreated cells, the intracellular *p*-coumaric acid content was kept at a relatively low level (9.1- 5.3- and 24.4-fold, respectively).

Sinapyl alcohol production was not affected by changing the L-phenylalanine concentration (Fig. 2D).

3.2.2. Effects of cinnamic acid feeding

The effects of different concentrations of cinnamic acid (0.5, 1 and 1.5 μM) on biomass formation and phenolic contents in the *L. divaricata* cell culture were measured (Fig. 3). The lowest cinnamic acid concentration (0.5 μM) resulted in a similar biomass formation to that of the untreated cells (Fig. 3A). The other concentrations proved to be toxic for the cells, suppressing NDGA, *p*-coumaric, and sinapyl alcohol production.

Cultures fed with 0.5 μM cinnamic acid were tested for NDGA, *p*-coumaric acid, ferulic acid and sinapyl alcohol content. At this concentration, NDGA content decreased, the maximum values being 3.7-fold lower than in untreated cultures (Fig. 3B). Regarding the phenylpropanoid phenolic compounds, the *p*-coumaric acid content was enhanced (4.4-fold higher than in the control) (Fig. 3C), while the sinapyl alcohol content was not improved (Fig. 3D).

L-Phenylalanine and cinnamic acid-fed cell cultures of *L. divaricata* did not have the ability to produce the metabolite ferulic acid in contrast with those under control conditions.

No other targeted intracellular phenolic compounds (cinnamic acid, caffeic acid, sinapic acid, coniferyl alcohol, quercetin and kaemferol aglycones) were found in the L-phenylalanine and cinnamic acid-fed cultures of *L. divaricata.*

3.2.3. Effects of ferulic acid and sinapic acid feeding

None of the administered concentrations of these precursors increased the biomass, resulting in a lack of compounds for testing.

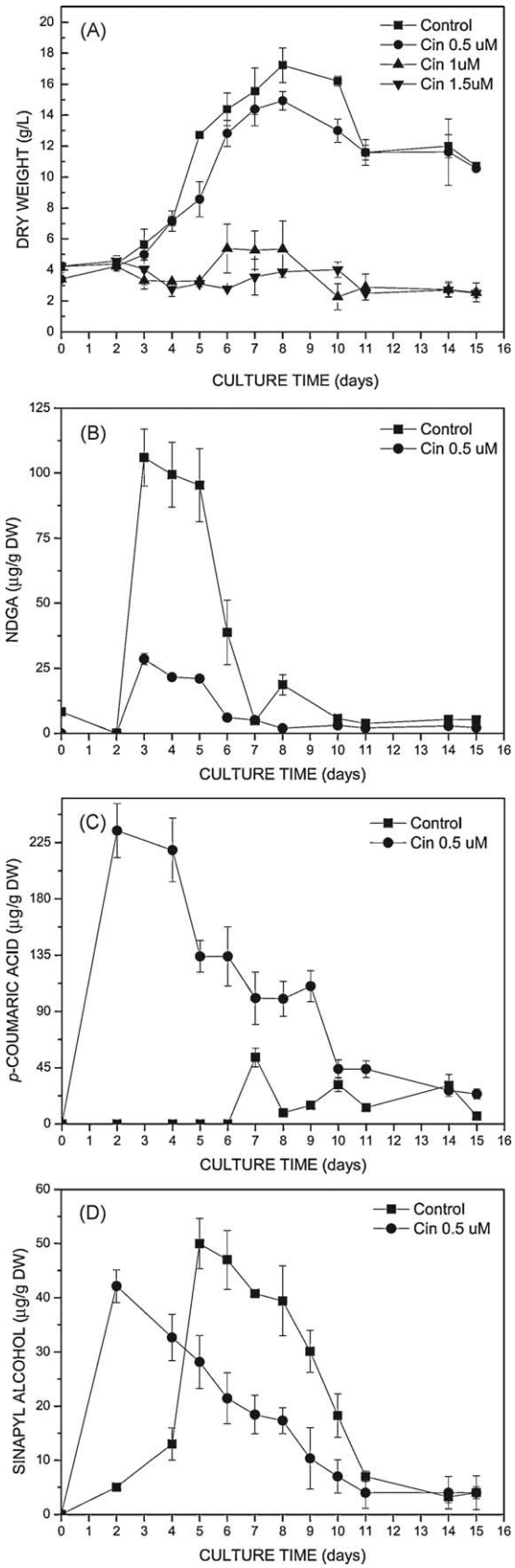

Fig. 3. Kinetics of biomass formation (A) and phenolic compound production: NDGA (B), *p*-coumaric acid (C), sinapyl alcohol (D) by *L. divaricata* cell cultures using control medium with 0.5, 1 and 1.5 μM of cinnamic acid. Data represent average values from three separate experiments ±SD.

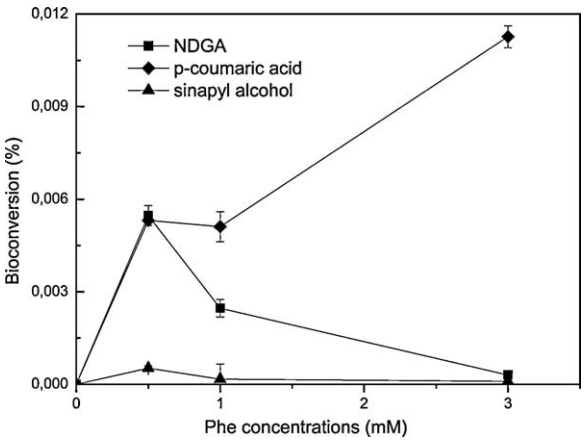

Fig. 4. Relationship between L-phenylalanine concentrations and the bioconversion percentage of NDGA, *p*-coumaric acid and sinapyl alcohol after treating *L. divaricata* cell cultures.

3.3. Bioconversion

Transformation of L-phenylalanine into *p*-coumaric acid, sinapyl alcohol and NDGA was observed in cell suspension cultures of *L. divaricata*. The concentration of 0.5 mM L-phenylalanine was the most effective on the biotransformation into NDGA, resulting in the highest level of bioconversion (Fig. 4). There was a positive relation between the percentage of bioconversion into *p*-coumaric acid and the increase in concentration of L-phenylalanine, with the optimal substrate concentration tested being 3 mM. In contrast, the percentage of bioconversion into sinapyl alcohol decreased with the increase of L-phenylalanine concentrations.

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