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Synthesis and accumulation of secondary metabolites by cell cultures of *Zanthoxylum zanthoxyloides* Lam. (Zepernik and Timler): Quantification of hesperidin and chelerythrine

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ABSTRACT: Investigations have been carried out with the aim of producing secondary metabolites *in vitro* by cell cultures of *Zanthoxylum zanthoxyloides*. Cell dedifferentiation from explants was induced in the presence of 2,4-dichlorophenoxyacetic acid or 1-naphthaleneacetic acid in combination with benzyladenine. HPLC analyzes revealed the uneven accumulation, in diversity and quantity, of a number of secondary metabolites in these cell cultures. These secondary metabolites are not synthesized by all cell strains with the exception of hesperidin which is present everywhere. Quantification of hesperidin and chelerythrine showed that 2,4-dichlorophenoxyacetic acid inhibits their synthesis unlike 1-naphthaleneacetic acid and light which seem to stimulate it. This study shows that it is possible to produce secondary compounds *in vitro* by cell cultures of *Zanthoxylum zanthoxyloides*.

KEYWORDS: *Zanthoxylum zanthoxyloides*, cell culture, secondary metabolites, hesperidin, chelerythrine.

1 INTRODUCTION

Plants produce various secondary compounds that affect their relationship with their environment. Man has therefore taken advantage of plant genetic resources by making great use of secondary metabolites for food, therapeutic or cosmetic purposes. The global market for herbal medicines and plant-derived products will grow from $29.4 billion in 2017 to approximately $39.6 billion in 2022 with a compound annual growth rate (CAGR) of 6.1% for the period 2017- 2022 as reported by a BCC Research. Global herbal medicine market revenue are expected to reach US$550 billions by 2030, as stated by INSIGHTSLICE [1].

Plant biomolecules are still relevant as a mode of primary health care for about 85% of the world's population [2]. They remain a primary resource for the discovery of new drugs, with approximately 80% of synthetic products used in pharmacy derived from them [3]. According to Capell *et al*. [4], while the search for anti-SARS-CoV-2 drugs is ongoing, the plant kingdom has emerged as an avenue for the search for anti-SARS-CoV-2 drugs. In the current traditional setup, plant extracts were used to treat different diseases including SARS-CoV-2.

Zanthoxylum zanthoxyloides is a ligneous species accumulating biomolecules [5], [6] used in the treatment of blood cancers (leukaemia, lymphoma) in association with conventional chemotherapies [7], in parasitology [8] and in the treatment of sickle cell anemia [9]. Unfortunately, the cultivation of most medicinal plants is not efficient enough to guarantee a sustainable supply. Indeed *Z. zanthoxyloides* is increasingly used in the preparation of improved phytomedicines, [10], [11]. Factors intrinsic to the species, such as the low germination rate [12] and the impossibility of natural cuttings, make its propagation difficult by traditional methods of propagation. We have already reported in our works, the possibility of multiplying in vitro by vegetative way this

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medicinal ligneous plant [13] and production of chelerythrine and skimmiamine from it's hairy roots [14]. Faced with this situation, methods derived from plant biotechnology, including cell cultures, potentially constitute an alternative for the supply of secondary metabolites of medicinal interest. Indeed, this approach has been used in several species to produce secondary metabolites [15]. The studies have shown the influence of several factors, in particular UV-B and UV-C rays [16] on the biosynthesis of secondary metabolites in an artificial environment. Plant cell cultures over wild plants have the advantage of being fast growing, stable and scalable production systems, independent of various environmental variations, reproducible, and capable of synthesizing new metabolites that are not synthesized normally not in plants [17, [18].

In the present study, we report secondary metabolites, specially hesperidine and chelerythrine accumulaion in cell culture from *Z. zanthoxiloides*.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PLANT MATERIALS

Calluses initiation tests are carried out on different organs of vitroplants from seedlings or in the multiplication phase. These explants are composed of whole leaves six weeks old, cotyledonary nodes two weeks and hypocotyls two weeks after *in vitro* germination.

2.1.2 HPLC MATERIALS

The HPLC apparatus is a Waters system equipped with a gradient pump (Waters 600 controller, Milford, MA), an autosampler equipped with a sample cooling system (Waters 717 plus) and a diode array detector (Waters 996) recording absorbances from 200 nm to 400 nm. Empower2 software from Waters is used for peripheral control, data acquisition and processing. The compounds are separated using a 3 µm column (250×4 mm, Multospher 120 RP18HP; CS-Service, Langerwehe, Germany) at a temperature of 21°C. The mobile phase consists of an aqueous solution of phosphoric acid 0.1% (w/v) (eluent A) and acetonitrile (eluent B) pumped at 0.5 ml min^{-1}.

2.2 METHODS

2.2.1 CALLUSES INITIATION AND MAINTENANCE OF CELL CULTURES

Calluses were initiated from leaf explants taken from 8-week-old vitroplants or from hypocotyl or cotyledonary node explants from 4-week-old seedlings. Initiation was performed in the dark on Murashige and Skoog (MS) culture medium containing 3% (w/v) sucrose and solidified with 0.8% (w/v) agar-agar and combinations of regulators of growth as follow:

- 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) + 1 mg l^{-1} 6-benzyladenine (6-BA);
- 1 mg l^{-1} 1-naphtalenacetic acid (1-NAA) + 1 mg l^{-1} 6-benzyladenine (6-BA).

The initiation and growth of primary calluses takes about six weeks. The calluses, after their initiation and their growth, were subcultured on solid media identical to the initiation media. Cell suspensions were initiated after the transfer of primary calluses in liquid media having the same compositions as those used for initiation. Subcultures were performed periodically every 3 weeks for cell suspensions and 8 weeks for calluses. A control line, derived from a callus initiated from a hypocotyl, was isolated by culture on solid then liquid MS medium without growth regulator. The culture of the cell suspensions was carried out on a horizontal orbital shaker at a speed of 120 rpm in the dark at 25°C. The characteristics of the cell strains and the culture conditions are presented in Table 1.

Table 1. Origin of cell strains and initiation conditions

Explant	Strain annotation	Medium	Light condition	Growth Regulator
Hypocotyl	A0	solid	darkness	Control medium
	A1	solid	Light	2,4-D 1mg.L^{-1} + BA 1 mg.L^{-1}
	A2	solid	Light	2,4-D 1mg.L^{-1} + BA 1 mg.L^{-1}
	A3	liquid	Light	2,4-D 1mg.L^{-1} + BA 1 mg.L^{-1}
	A4	liquid	darkness	2,4-D 1mg.L^{-1} + BA 1 mg.L^{-1}
Leaf	D1	solid	Light	2,4-D 1mg.L^{-1} + BA 1 mg.L^{-1}
	D2	solid	darkness	2,4-D 1mg.L^{-1} + BA 1 mg.L^{-1}
Cotyledonary node	X1	liquid	Light	1-NAA 1mg.L^{-1} + BA 1 mg.L^{-1}
	X2	liquid	darkness	1-NAA 1mg.L^{-1} + BA 1 mg.L^{-1}
Hypocotyl	Z1	liquid	Light	1-NAA 1mg.L^{-1} + BA 1 mg.L^{-1}
	Z2	liquid	darkness	1-NAA 1mg.L^{-1} + BA 1 mg.L^{-1}

2.2.2 CULTIVATION CONDITIONS

In order to study the *in vitro* production of secondary metabolites, cells taken from cell suspensions of 3-week-old cultures and 8-week-old calluses were analyzed. The influence of light on the production of biomolecules by cells has been considered. To do this, the cultures were placed either in total darkness or in light under an irradiance of 120 µE m^{-2} s^{-1} and a photoperiod of 16 hours of light per day.

2.2.3 PREPARATION OF CRUDE EXTRACTS FOR HPLC

The calluses were taken directly from the culture medium. The cells were harvested from the cell suspensions by filtration through a fine sieve. Cell clusters were collected in tubes. The samples were frozen for 4 hours at -20°C then lyophilized for 72 hours in the dark. The lyophilizates were finely ground in a mortar. The powders thus obtained were stored at -20°C. Methanol was used as the extraction solvent. To 25 mg ± 0.1 mg of each sample was added 1 ml of methanol in an Eppendorf tube. The sample was then placed on a vortex for 1 hour, then centrifuged at 15,000g for 15 minutes. The methanolic extract represented by the supernatant is recovered in a new Eppendorf tube and stored at -20°C.

2.2.4 PARTIAL IDENTIFICATION OF THE METABOLITES

The UV spectra obtained for each peak in the different extracts are compared with those of the molecules identified by Chaaib [6]. The elution order of the compounds was also taken into account in order to control the relevance of the partial identification of the compounds. Thus, it was possible to make assumptions as to the type of molecules eluted from the different samples. Among the molecules, only chelerythrine and hespiridine available to us have been rigorously quantified.

2.2.5 QUANTIFICATION OF HESPERIDIN AND CHELERYTHRINE AND ITS DERIVATIVES

Methanolic stock solutions of 2.48 10^{-3} mM chelerythrine and 1.97 10^{-5} mM hesperidin were diluted to form a concentration range. These solutions were used to establish a standard curve to quantify each of these molecules by spectrophotometry

3 RESULTS

3.1 MOLECULES IDENTIFIED

Partial identification by comparison of retention times, elution order and spectra has made it possible to propose ten molecules: Nor-chelerythrine (R) or Avicine (U); Hesperidine; 6-hydroxy-chelerythrine (T) or dihydro-chelerythrine (N) (1); 6-hydroxy-chelerythrine (T) or dihydro-chelerythrine (N) (2); Chelerythrine (Q); Skimmiamine; Pellitorin (I1); N-isobutyl- (2E,4Z) -octa-2,4-dienamide (J); Sesamine (G) and Pellitorine (I2) (Table 2). These compounds are not present homogeneously in the cell strains. On the other hand, all cell strains produce hesperidine. In general, light does not seem to have a significant influence on the accumulation of these compounds.

Table 2. Secondary metabolites identified in the cell cultures (RT: Retention Time, +detected, - not detected)

RT (min)	Compound	A0	A1	A2	A3	A4	D1	D2	X1	X2	Z1	Z2
24,21	Nor-chelerythrine (R) or Avicine (U)	+	+	-	-	-	+	-	+	+	+	+
32,43	Hesperidine	+	+	+	+	+	+	+	+	+	+	+
33,73	6-hydroxy-chelerythrine (T) or dihydro-chelerythrine (N) (1)	-	-	-	-	-	-	-	-	-	+	+
36,9	6-hydroxy-chelerythrine (T) or dihydro-chelerythrine (N) (2)	-	-	-	-	-	-	-	-	-	-	+
38,18	Chelerythrine (Q)	+	-	+	-	-	-	+	+	+	+	+
40,6	Skimmiamine	+	-	-	+	-	-	-	+	+	+	+
55,55	Pellitorine (I1)	-	-	-	-	-	-	-	-	+	-	-
56,46	N-isobutyl- (2E,4Z) -octa-2,4-dienamide (J)	+	+	+	+	-	+	+	+	-	-	-
61,35	Sesamine (G)	-	-	-	-	-	-	-	-	+	-	+
65,91	Pellitorine (I2)	-	-	-	-	-	-	-	+	+	-	+

3.2 HESPERIDINE AND CHELERYTHRINE QUANTIFICATION IN CELL CULTURES

The standard curves obtained from commercial hesperidine and chelerythrine solutions (Fig. 1, Fig. 2) made it possible to determine the concentrations of those tow molecules in the extracts (Table 3). The hesperidine, a flavonoid, and chelerythrine (Q), a benzophenanthridic alkaloid, contents are calculated and expressed in µmol g^{-1} of dry weight (µmol g^{-1} dw) of the cells.

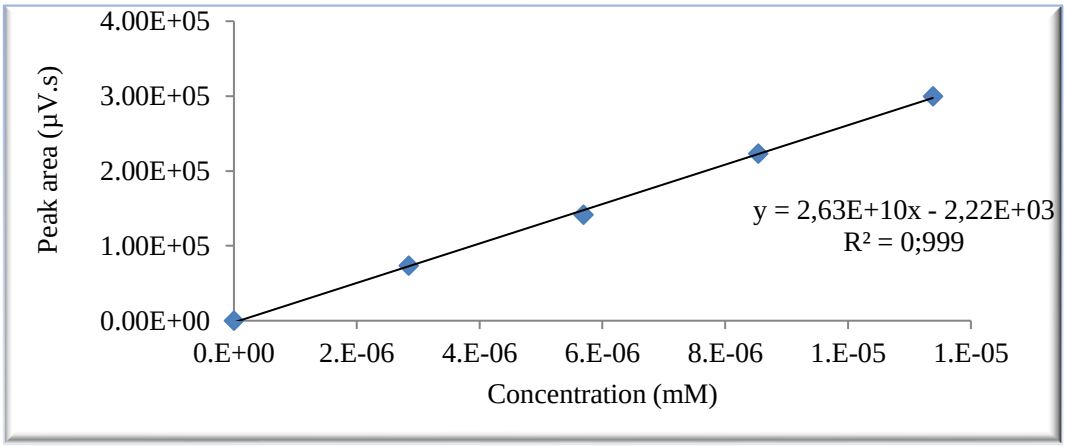

Fig. 1. Calibration curve of hesperidine obtained from the standard

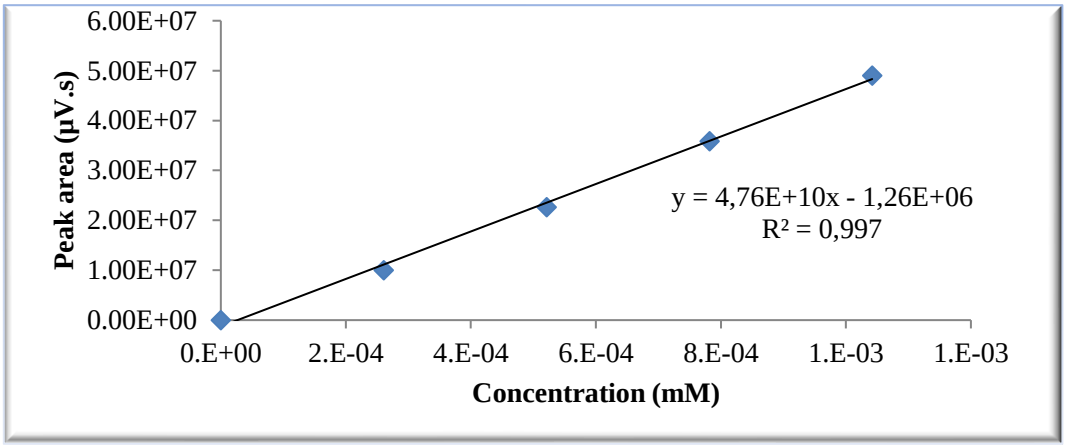

Fig. 2. Calibration curve of chelerythrine obtained from the standard

Table 3. Hesperidine and Chelerythrine (Q) contents of the cells

Explant	Cell Strain	Content (µmol g^{-1} dw)	
		Hesperidine	Chelerythrine
Hypocotyl	A0	2.2	2.3
	A1	3,8	0
	A2	2.9	0
	A3	2.08	0
	A4	1.2	0.45
Leaf	D1	3.3	0
	D2	2.0	0.1
Cotyledonary node	X1	7.3	21
	X2	3.14	0.7
Hypocotyle	Z1	5.39	23.7
	Z2	3.16	1.8

The results (Table 3) show that hesperidin is produced by all cell strains. These contents vary between 1.2 µmol g^{-1} dw and 7.3 µmol g^{-1} dw. The X1 and Z1 accumulate respectively 7.3 and 5.39 µmol g^{-1} dw of hesperidin. The hesperidin concentrations, which are lower in the dark, are 3.14 µmol g^{-1} dw and 3.16 µmol g^{-1} dw respectively in strain X2 and strain Z2. In the dark, the concentrations of hesperidin do not differ too much depending on the auxin used. In general, hesperidin concentrations are higher in extracts from cells cultured in light. The concentration of hesperidin in the calluses of strain A3 is 2.08 µmol g^{-1} dw and that of the same strain cultured under the same conditions but in the absence of growth regulators is 2.2 µmol g^{-1} dw. No significant difference is therefore observed.

Chelerythrine is not produced by all cell strains. In strain A, the control (A0) synthesizes chelerythrine (2.26 µmol g^{-1} dw) while the other strains synthesize very little or almost no chelerythrine in the presence of 2,4-D and 6-BA. In the presence of growth regulators and in the dark, the chelerythrine content reaches 1.76 µmol g^{-1} dw in the Z2 culture whereas it is 0.45 µmol g^{-1} dw in the A4 strain. Only cells cultured in the presence of 1-NAA and 6-BA accumulate chelerythrine in the presence of light. Contents of 20.92 µmol g^{-1} dw and 23.7 µmol g^{-1} dw of chelerythrine were recorded respectively in the X1 culture and the Z1 culture.

The synthesis of hesperidine and chelerythrine by cultured cells of *Z. zanthoxyloides* is stimulated by light and also by 1-NAA associated with 6-BA. The growth regulator 2,4-D in combination with 6-BA does not promote the synthesis of hesperidin and chelerythrine.

4 DISCUSSION

The origin of the dedifferentiated explants does not seem to have an influence on the production of secondary metabolites, on the other hand, light seems to stimulate it. 2,4-D used in association with 6-BA inhibits the synthesis of biomolecules whereas ANA in association with 6-BA would stimulate it. Cell lines do not show the same chemical profiles but hesperidine is detected in all lines. Compounds R or U (TR 24.21 min), Hesperidine (TR 32.43 min), N or T (TR 33.73 min and 36.9 min), Q (TR 38.18 min), of skimmiamine (TR 40.6 min), I (TR 55.55 min), J (TR 56.46 min), G (TR 61.35 min) and I (TR 65.91 min) are identified in the cell cultures. Some compounds present in the whole plant are not found in cell cultures [6].

Our results are in agreement with those of Couillerot *et al.* [12] who showed that cell cultures of *Z. zanthoxyloides* are capable of synthesizing secondary metabolites such as chelerythrine and skimmiamine *in vitro*. The significant reduction in skimmiamine accumulation that we observed after removal of growth regulators confirms the results of Couillerot *et al.* [12] who report a reduction by a factor of 3.5. Thus, the synthesis of secondary metabolites seems to depend on phytohormones, in particular auxins and exogenous cytokinines in this situation. 2,4-D is a synthetic auxin that is not degradable in plant cells. Maintaining cells on a medium containing this auxin could lead to total dedifferentiation following an excessively high auxin level. This hypothesis is validated by the work of Carpin *et al*. [19] who report stimulation of ajmalicine synthesis by suppression of 2,4-D. It is also known that dedifferentiated cells synthesize very little or no secondary metabolites and that production can decline and stop during subcultures [20], [21]. Indeed, the bibliography shows that the synthesis of secondary metabolites in cell lines varies very often, probably due to the metabolic heterogeneity of cells during divisions [22].

The undifferentiated state does not allow the expression of most genes, including those of the biosynthetic pathways of secondary metabolites. These realities explain the absence of several secondary metabolites in our cell cultures of *Z. zanthoxyloides*. For example, the phenylalanine ammonia-lyase (PAL), a key enzyme in primary and secondary metabolism, is activated by phytochrome (Pr and Pfr) and is therefore indirectly dependent on light [23], [24], [25], [26]. Some enzymes of biosynthetic pathways are located in chloroplasts. We believe that the stimulation of plastid differentiation into chloroplasts and the activation of PAL *via* phytochrome by light could explain the favorable action of light on the accumulation of secondary metabolites by cell cultures of *Z. zanthoxyloides*. In addition, the favorable effect of light in general and UV in particular as an abiotic stress on the synthesis of secondary metabolites is well documented today [16].

5 CONCLUSION

In order to produce secondary metabolites in vitro, a few dedifferentiated cell lines have been initiated. Secondary metabolites were detectable in all lines. It is therefore possible to obtain in vitro some secondary metabolites by cell cultures of Z. zanthoxyloides. Overall, light seems to activate the synthesis of secondary metabolites. By comparing the results of the study of the influence of growth regulators, we came to the conclusion that 2,4D would be an inhibitor of the accumulation of secondary compounds in Z. zanthoxyloides, as reported for other plant species. With the use of elicitors or precursors and the optimization of culture conditions, it would be possible to maximize this bioproduction.

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