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Chemical Profile of Cell Cultures of *Kalanchoë gastonis-bonnieri* Transformed by *Agrobacterium rhizogenes*

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Abstract: *Kalanchoë gastonis-bonnieri* Raym.-Hamet & Perrier is a plant used for medicinal purposes in the treatment of several ailments. The aim of this study was to analyze the chemical profile of extracts from *K. gastonis-bonnieri* embryogenic calli, generated from genetically transformed roots by *Agrobacterium rhizogenes*. Putative transformants were verified by PCR. Hydroalcoholic extracts were obtained and the chemical profile was analyzed by LC-ESI-MS/MS. Root formation was obtained from 80% of infected seedlings. Fifteen root lines were isolated, and two lines showed prominent longitudinal growth and profuse branching in the B5 semi-solid medium. In all lines, the formation of nodules and later embryogenic callus was observed. Putative transgenic root lines were cultivated in free-plant growth regulators B5 medium. In the two selected lines, the PCR amplification of *rol*A, *rol*B, *rolC*, *rol*D, and *aux*1 genes was detected. The extract of embryogenic calli showed 60 chemical compounds tentatively identified, such as ferulic acid, quinic acid, neobaisoflavone, and malic acid, among others, and the chemical profile was different in comparison to wild-type extracts. This is the first study reporting the analysis of the chemical profile of hairy root extracts derived from *Kalanchoë gastonis-bonnieri*. This work displays the great potential for obtaining chemical compounds of pharmacological importance from hairy roots and facilitates the identification of new useful drugs against human chronic-degenerative diseases.

Keywords: embryogenic calli; genetic transformation; plant tissue culture; secondary metabolism

Citation: Barrera Núñez, M.G.; Bueno, M.; Molina-Montiel, M.Á.; Reyes-Vaquero, L.; Ibáñez, E.; Del Villar-Martínez, A.A. Chemical Profile of Cell Cultures of *Kalanchoë gastonis-bonnieri* Transformed by *Agrobacterium rhizogenes*. *Agronomy* **2024**, *14*, 189. https://doi.org/10.3390/agronomy14010189

Academic Editors: Justyna Lema-Rumińska, Danuta Kulpa and Alina Trejgell

Received: 20 December 2023
Revised: 8 January 2024
Accepted: 11 January 2024
Published: 15 January 2024

1. Introduction

The main drawback of obtaining bioactive compounds from plants is the variation in the accumulation of secondary metabolites due to development, plant growth cycles, and diversity of environmental conditions [1]. The biosynthesis and accumulation of chemical compounds of interest in highly specialized tissues occurs at specific stages of development [2]. The hairy roots induction through *Agrobacterium rhizogenes* infection is a biotechnological alternative to obtain specific secondary metabolites in vitro cultures free of plant growth regulators [3]. *A. rhizogenes* is a Gram-negative bacterium of the Rhizobiaceae family that induces hairy roots disease by infecting higher plants and inserting root *loci* (*rol* genes) from the root-inducing plasmid (Ri) into the plant genome [4]. From hairy roots, induction of embryogenic calli and regeneration of whole plants has been observed in species such as *Hypericum perforatum* [5], *Tylophora indica* [6], *Gentiana utriculosa* [7], and *Pentalinon andrieuxii* [8].

It has been reported that hairy roots synthesize chemical compounds that are not detected in wild plants [9]. Furthermore, some authors have reported that both shoots and

transgenic plants from hairy roots accumulate higher levels of metabolites in comparison to wild plants. Tusevski et al. (2014) [5], reported the accumulation of naphthodiatrons and specific phenolic compounds in transgenic shoots of *Hypericum perforatum* from hairy roots. Vinterhalter et al. (2019) [10], reported a higher accumulation of xanthones in transgenic plants of *Gentiana utriculosa* regenerated by somatic embryogenesis derived from hairy roots. Hiebert-Giesbrecht et al. (2021) [8], reported the accumulation of terpenoids in leaf of transgenic plants, obtained from hairy roots, compared to wild *Pentalinon andrieuxii*.

The species of the genus *Kalanchoë* (Crassulaceae) are succulent plants, used in traditional medicine to treat several health conditions such as gastric ulcers, asthma, infections, tumors, and blood glucose regulation [11]. Additionally, it is an important ornamental plant. Various studies have been developed around the *Kalanchoe* genus, which provide knowledge on human health [12]. The importance of these plants is found in the diversity of chemical compounds that accumulate which represent the interest in the medicine industry, and as an ornamental plant due to the diversity of the colors and leaf shapes, these characteristics contribute to the economic importance of the *Kalanchoe* genus [13]. In any case, agronomic management for plant production is extremely important because chemical compounds accumulate at different parts of the plant and in response to environmental factors. The production of plants in controlled environments represents an option for maintaining germplasm and keeping plant diversity with all its benefits [14,15]. The application of biotechnological tools to develop technologies that can provide interesting metabolites and generate new plant materials with specific characteristics represents the option to make the most of natural resources. Thirukkumaran et al. [16], reported the application of technologies allowing the production of transgenic plants without selectable marker genes and described that marker-free transgenic *K. blossfeldiana* could be produced using *ipt*-type MAT vector system carrying the chimeric *ipt*. The transformation of *Kalanchoe pinnata* by *Agrobacterium tumefaciens* with ZsGreen1 by Cho et al. [17] selected optimum succulent species for future genetic transformation efforts and the development of an efficient transformation method using a novel fluorescent gene, was accomplished. This method achieves new cultivars of succulents with eye-catching colors or patterns in the leaves and flowers. The potential to develop new cultivars with predictable traits in a reduced period is a great advantage of the genetic transformation approach.

Kalanchoë gastonis-bonnieri Raym.-Hamet & H. Perrier is used in traditional Latin American medicine as a contraceptive and in the treatment of genital and urinary infections, diabetes, kidney infections, gastric ulcers, leishmaniasis, and cancer [18–21]. Few studies have reported the chemical profile of *K. gastonis-bonnieri* [19,21,22]. The aim of this study was to analyze the chemical profile of extracts from the embryogenic calli of *Kalanchoë gastonis-bonnieri* generated from hairy roots.

2. Materials and Methods

2.1. Plant Material

Kalanchoë gastonis-bonnieri was collected in Centro de Desarrollo de Productos Bióticos-IPN, Yautepec, Morelos, México (18°49′53″ N, 99°05′37.40″ W at 1064 m.a.s.l.). The in vitro plants were obtained from vegetative shoots (size: 2–3 cm) that grew from meristematic tissue, at the tip of acuminated adult plant leaves from wild-growing plants that were gently washed with tap water, then with a Tween 20 (Sigma Company, St. Louis, MO, USA) solution (1%) for 1 min, ethanol (70%) for 2 min and NaOCl (0.5%) during 17 min, and rinsed 3 times with sterile distilled water between each solution [23]. Vegetative shoots were cultivated on semi-solid MS (Sigma Company, St. Louis, MO, USA) medium [24], to which a sterile medium was added with 30 g/L sucrose (Sigma Company, St. Louis, MO, USA) and 3 g/L Phytagel (Sigma Company, St. Louis, MO, USA) and the pH was adjusted to 5.8 before autoclaving at 125 °C for 15 min. In glass Gerber-type containers with 20 mL of medium, cultures were incubated in a growth chamber at 25 ± 2 °C with a photoperiod of 16 h light/8 h dark, at 30 μmol/m^2s provided by cool white fluorescent tubes, for 35 d.

2.2. Induction of Transformed Roots

The *A. rhizogenes* strain A4 was used and cultured in YMB medium (2.8 g/L) with 3 g/L of phytagel (Sigma-Aldrich®, St. Louis, MO, USA) and incubated at 29 °C [25] for 3 days. Subsequently, it was kept at 4 °C and reseeded every 30 d. In vitro seedlings of *K. gastonis-bonnieri* were inoculated in the internodal zone by a longitudinal scalpel wound with the *A. rhizogenes* strain A4 and incubated in semi-solid MS medium [24] free of growth regulators added with 30 g/L of sucrose (Sigma Company, St. Louis, MO, USA), and 2.6 g/L of Phytagel (Phytotech, St. Lenexa, KS, USA) [26]. The transformation frequency was determined [27]. The bacterium was eliminated from the plant culture with cefotaxime (Phytotech, St. Lenexa, KS, USA) according to Tavassoli and Safipour-Afshar [28], and the cultures were maintained in semisolid MS medium [24] with cefotaxime for 30 days with subcultures every 7 days to eliminate *A. rhizogenes* residues. The bacteria-free cultures were transferred to a B5 liquid medium for subsequent analyses. Root segments were individualized and transferred to semi-solid B5 medium [29] phytohormones free, supplemented with 30 g/L sucrose, 2 g/L polyvinylpyrrolidone, and 2.6 g/L phytagel (Sigma Company, St. Louis, MO, USA).

Finally, after 55 days, the selected lines were transferred to liquid B5 medium, and the cultures were maintained at the above-mentioned conditions at 100 rpm and sub-cultured every 30 days.

2.3. Morphological Description of In Vitro Cultures

The analysis of the culture development was carried out as previously described [30]. The specific growth rate (μ) and the doubling time (T2) were determined as follows:

$$\mu = ln\,(X - X0/t - t0) \times 100 \qquad T2 = ln2/(\mu)$$

were, X: Final dry weight, $X0$: Initial dry weight, t: Final time, $t0$: Initial time. The plant material morphology was observed in a stereoscopic microscope (Nikon, SMZ-1500, Tokyo, Japan), coupled to a PC (Data image, DS33, Tokio, Japan) with a video camera, controller, and integrated interface. The plant material was disaggregated, and the samples were kept hydrated with B5 liquid culture medium. The samples were displayed in triplicate [31].

2.4. DNA Extraction and PCR Analysis

Genomic DNA from plant material was extracted using the method of Doyle and Doyle [32]. Plasmid DNA of *A. rhizogenes* was extracted with Wizard® Plus SV Minipreps DNA Purification System kit (Promega Corporation, A1460 Madison, WI, USA), following the manufacturer's protocol. DNA from the *A. rhizogenes* A4 strain was used as a positive control, DNA from wild-type *K. gastonis-bonnieri* plants was used as a negative control, and sterile distilled H_2O was used as a negative control, to develop the polymerase chain reaction (PCR). The amplification was carried out in a thermal cycler (Applied Biosystems, Gene Amp PCR Systems 9700, Waltham, MA, USA), using specific primers according to reports in each case. *rolA*: 5′-CGTTGTCGGAATGGCCCAGACC-3′ and 3′-CGTAGGTCTGAATATTCCGGTCC-5′ to amplify a 248 bp fragment, *rolB*: 5′-ACTATAGCAAACCCCTCCTGC-3′ and 3′-TTCAGGTTTACTGCAGCAGGC-5′, to amplify a 652 bp fragment [33], *rolC*: 5′-TGTGACAAGCAGCGATGAGC-3′ and 3′-GATTGCAAACTTGCACTCGC-5′ to amplify a 487 bp fragment, *rolD*: 5′-CCTTACGAATTCTCTTAGCGGCACC-3′ and 3′-GAGGTACACTGGACTGAATCTGCAC-5′ to amplify a 477 bp fragment [34] and *aux*1: 5′-CCAAGCTTGTCAGAAAACTTCAGGG-3′ and 3′-CCGGATCCAATACCCAGCGCTTT-5′ to amplify a 815 bp fragment [35]. DNA electrophoresis was performed in a 1.5% agarose gel at 95V for 60 min. The visualization of the amplified fragments was mixed with a SYBR ®Green (Lonza, Hayward, CA, USA) solution. Electrophoresis was analyzed in a photo-documenter (ChemiDoc™ MP Imaging System BIO-RAD, 170-01402, Hercules, CA, USA).

2.5. Ultrasound Assisted Extraction (UAE)

Metabolites were extracted in an ultrasound bath Branson (Branson Ultrasonics™, 2510R-MTH, Brookfield, CT, USA) with automatic control of time and temperature and ultrasound frequency of 40 kHz. A total of 50 mg of dry and ground biomass were mixed in 2 mL of ethanol (80%, v/v), and were sonicated at 40 ± 5 °C for 30 min. Samples were centrifuged at 3500 rpm for 5 min. The supernatants were recovered and filtered through cellulose membranes (0.22 µm) (MILLEX® GS). Samples were dryness at 25 ± 2 °C and the dried extracts were stored at 4 °C until analyzed [36].

2.6. Chemical Characterization of Kalanchoë Gastonis-Bonnieri Extracts

The chemical profile of extracts was obtained by LC-ESI-MS/MS. Samples were solubilized in 500 µL of MeOH, HPLC grade (Sigma-Aldrich®) and filtered through nylon membrane, (0.45 µm, Agilent Technologies, Santa Clara, CA, USA). The mobile phase for gradient elution consists of two solvents: solvent A (0.1% formic acid (FA) Sigma-Aldrich® in H_2O) and solvent B (0.1% FA in CH_3CN/MeOH (1:1; v/v) Sigma-Aldrich®. The linear gradient profile was as follows: 95% A (5 min), 95–90% A (10 min), 90–50% A (55 min), 50–95% A (65 min), and 95% A (70 min). The injection volume was 10 µL. The flow rate (0.6 mL/min) was split 1:1 before the MS interface. Electrospray ionization analysis (ESI) was performed using a micrOTOF-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in negative ion mode with a capillary potential of 2.5 kV, gas temperature of 180 °C, drying gas flow of 6 L/min, and nebulizer gas pressure of 1.0 Bar. Detection was performed at 50–3000 m/z.

The tentative identification of compounds was based on the comparison of the MS fragmentation profile obtained by the analytical equipment, with the mass spectra of MassBank of North America (https://mona.fiehnlab.ucdavis.edu accessed on 15 July 2021) and Competitive Fragmentation Modeling for Metabolite Identification (http://cfmid3.wishartlab.com accessed on 30 July 2021).

2.7. Statistical Analysis

Relative abundance data of tentatively identified metabolites in *K. gastonis-bonnieri* extracts were analyzed by clustered color mapping, using a Pearson distance measurement mean bond clustering method, using the Heatmapper software (http://www.heatmapper.ca/, accessed on 15 September 2021).

3. Results

3.1. Morphology of the Kgb1 and Kgb2 Cultures and Molecular Analysis

K. gastonis-bonnieri seedlings were obtained from in vitro cultures. The infection with *A. rhizogenes A4* strain was accomplished and the root formation at the infection site was observed after 15 d with a transformation efficiency of 80%. Fifteen root lines were individualized in a semi-solid B5 medium, most of the lines were characterized by slow growth and poor branching, and the formation of cell aggregates was observed 30 d after isolation from the initial explant. In vitro cultures *of K. gastonis-bonnieri* were successfully initiated from aseptically vegetative shoots isolated from wild-growing plants. The in vitro shoots were subjected to *Agrobacterium*-mediated transformation (Figure 1). Figure 1a shows an uninfected explant (negative control), demonstrating that mechanical injury did not result in root formation. In Figure 1b–d, the response of different infected seedlings and the development of hairy roots that emerge from the infection site with plagiotropic growth is shown; Figure 1b shows abundant proliferation of hairy roots, while in Figure 1c, d explants with few roots were obtained; Figure 1d shows callus formation and few roots scarcely hairy developed from the infection site.

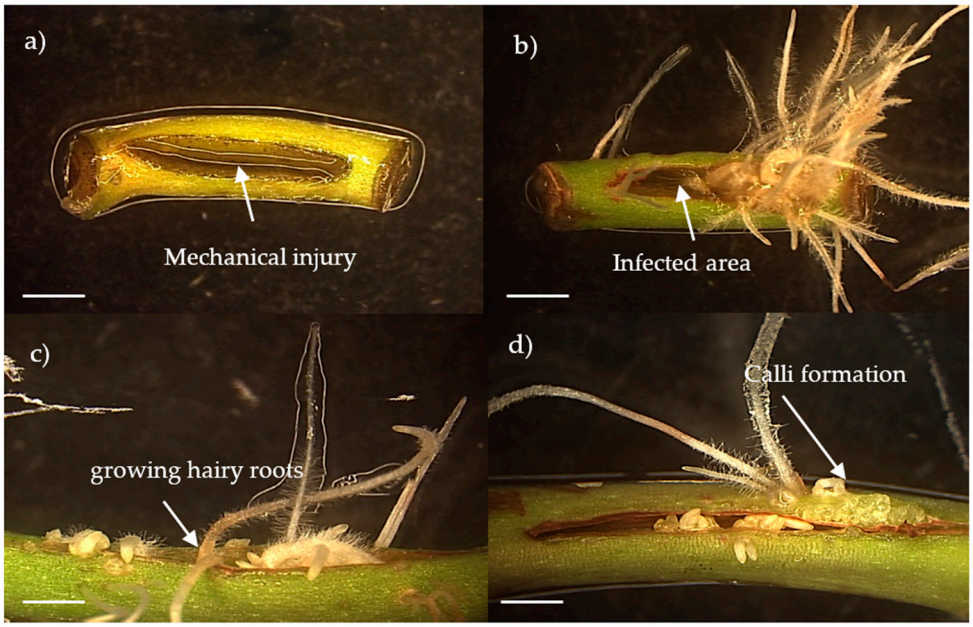

Figure 1. Hairy roots induction and appearance of embryogenic calli of *Kalanchoë gastonis-bonnieri*. in the internodal segment 15 days after infection. (**a**) Control: explant cut out, (**b**–**d**) Infected internodal segments. Reference bar = 1 mm.

A total of 15 lines were individualized and 2 lines, *Kgb*1 and *Kgb*2, were selected due to accelerated growth and abundant secondary roots. Figure 2 shows the follow-up of the development of *Kgb*1 and *Kgb*2 cell cultures at 9 d (Figure 2a), 18 d (Figure 2b), and 25 days (Figure 2c) of the subculture. The images were captured 90 days after remaining in liquid B5 medium. The asynchronous growth in different stages of somatic embryogenesis was observed. The globular (GB), torpedo (T), heart-shaped (H), and embryo (SE), structures were identified. Embryogenic calli cultures were morphologically heterogeneous, with asynchronous development, meaning that the growth of dedifferentiated cells, the cell differentiation, and the development of embryos occur at uncoordinated times, hence the cellular aggregates in *Kgb*-1 as *Kgb*-2 show different stages of embryogenesis through the 25-days of subculture. Figure 2d shows the initial embryogenic aggregates, which were observed since the roots were isolated from the infected explant and remained in a liquid medium. These structures were observed in all stages of culture. The lines currently remain stable with the same characteristics.

Figure 3 shows the amplified fragments of the *rol*A, *rol*B, *rol*C, *rol*D, and *aux*1 genes from the DNA of *Kgb*1 and *Kgb*2 lines; none of the analyzed genes was amplified from *K. gastonis-bonnieri* wild-type plants. These results suggest that the *Kgb*1 and *Kgb*2 lines were induced in the infection mediated by *A. rhizogenes* A4 strain. In this work, it is suggested that both TL-DNA and TR-DNA of *A. rhizogenes* A4 strain were inserted into *Kgb*1 and *Kgb*2 genomes. It has been reported that the response of a plant species to genetic transformation by *A. rhizogenes* is in the function of the integration and combined expression of *rol*A, *rol*B, *rol*C, and *rol*D genes [31,32].

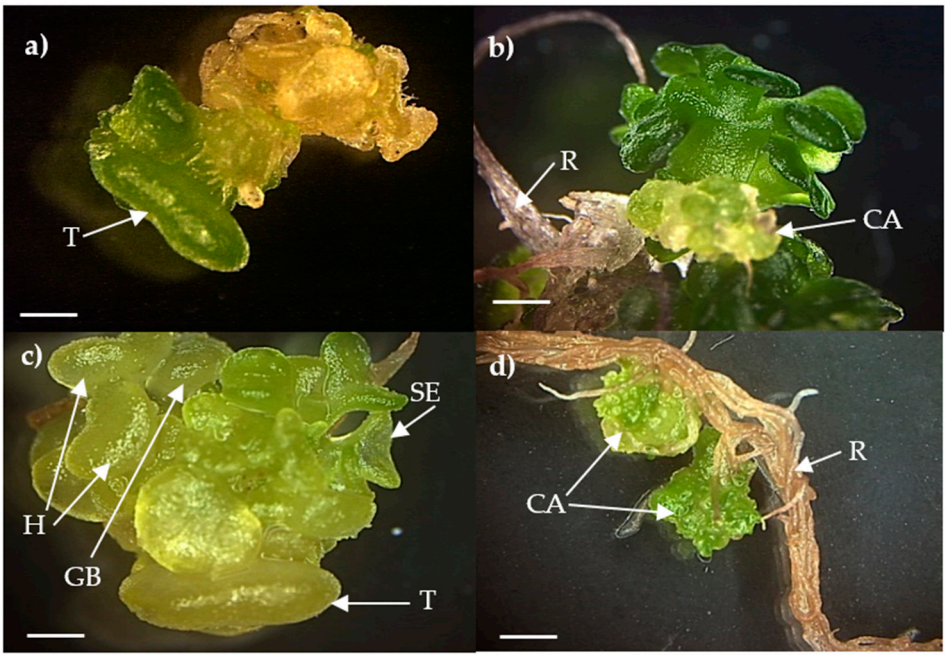

Figure 2. Transgenic embryogenic calli of *Kalanchoë gastonis-bonnieri*, *Kgb*1 and *Kgb*2. (**a**) 9, (**b**) 18, (**c**) 25 days of a subculture in a liquid B5 medium, (**d**) embryogenic aggregates, (CA) calli, (H) heart-shaped, (T) torpedo, (SE) somatic embryo, (R) transgenic root, and (GB) globular-shape. Reference bar = 1 mm.

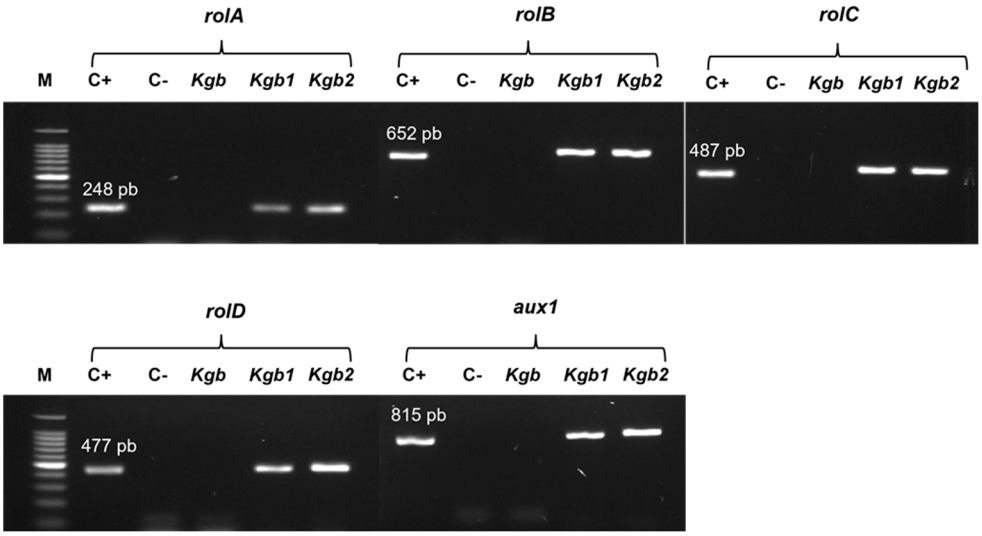

Figure 3. Amplification of *rol*A, *rol*B, rolC, *rol*D, and *aux*1 genes of *A. rhizogenes* from genomic DNA of embryogenic calli (*Kgb*1 and *Kgb*2 lines). (M) molecular weight marker 1Kb; (C+) DNA from *A. rhizogenes* A4 strain (positive control); (C−) water (negative control); (*Kgb*) DNA from wild-type *K. gastonis-bonnieri* (negative control); (*Kgb*1 and *Kgb*2) embryogenic callus lines.

3.2. Analysis of the Chemical Profile of Kgb1 and Kgb2 Extracts

Figure 4 shows changes during the cell growth of *Kgb*1 and *Kgb*2 lines. The stages were defined as follows: stage (I) 1–10 days of culture, as an adaptation period, and changes in cell growth were observed, stage (II) 11–21 d, increase accelerated biomass with doubling time for *Kgb*1 (*T2*) = 3.31 d and cell growth speed (μ) = 0.20 d^{-1}; while for *Kgb*2, *T2* = 4.15 d and μ = 0.16 d^{-1}, stage (III) 21–25 d, a decrease in biomass growth was observed, for *Kgb*1 the *T2* = 20.3 d and μ = 0.03 d^{-1}; finally, for *Kgb*2 *T2* = 48.5 d, and μ = 0.01 d^{-1}; stage (IV) 25–35 d, considerable decrease in cell growth was observed in both lines. The chemical

profile of *Kgb*1 and *Kgb*2 was analyzed at 9, 18 and 25 days of culture, which were selected taking quantity biomass as a selection criteria.

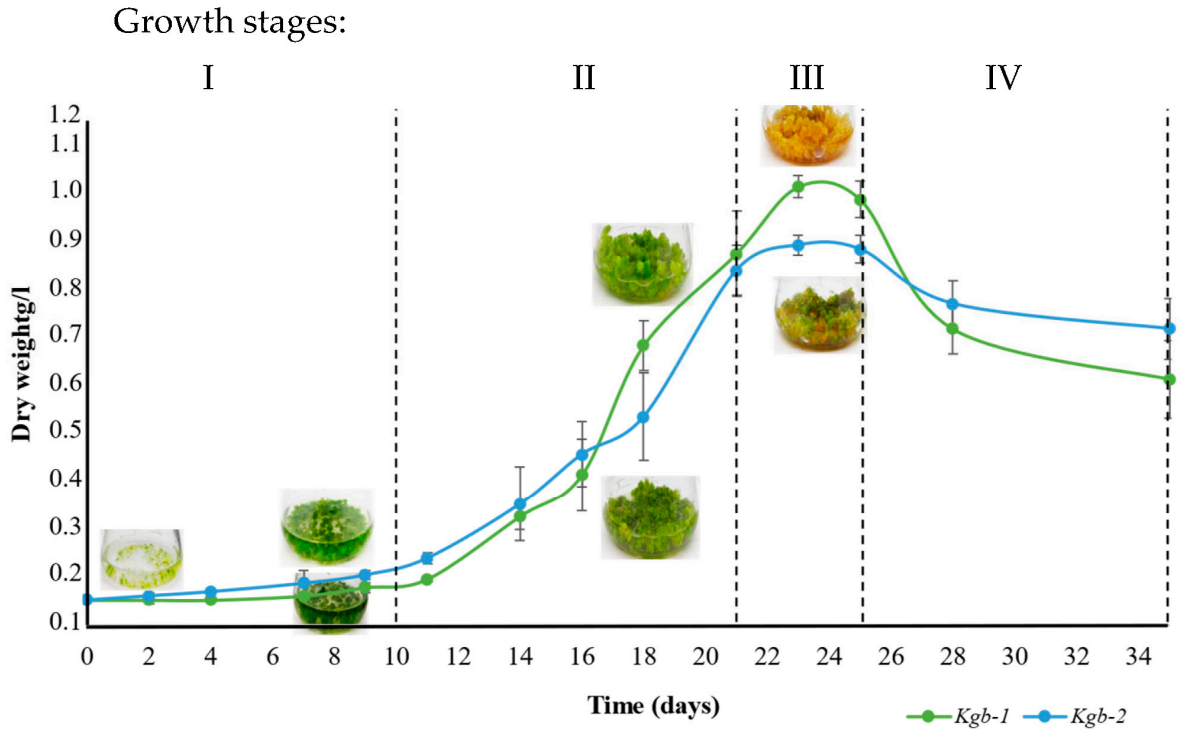

Figure 4. Changes during the cell growth of *Kgb*1 and *Kgb*2 lines in liquid B5 medium. The dotted line indicates the transition between growth stages.

Table 1 shows 60 tentatively identified metabolites in the extracts of *Kgb*1, *Kgb*2 lines at 9, 18, 25 daysof culture, and the wild plant of *K. gastonis-bonnieri*; among them, 18 flavonoids, 11 fatty acids, 5 coumarins, 4 phenolic acids, 3 phenolic compounds, 3 terpenes, 4 carboxylic acids, 1 alkaloid, 2 amino acids, 1 carbohydrate and 8 compounds grouped as others, based on chemical structure. The highest number of flavonoids was identified in the wild type, while fatty acids and carbohydrate were mainly identified in *Kgb*1 and *Kgb*2 lines.

The metabolites were identified according to LC-ESI-MS/MS parameters: retention time, match factor values database, molecular formula, and monoisotopic mass. The tentative identification of compounds was based on the comparison of the MS fragmentation profile obtained by the analytical equipment, with the mass spectra of MassBank of North America and Competitive Fragmentation Modeling for Metabolite Identification.

The *Kgb*1 and *Kgb*2 lines showed a differential chemical profile compared to the wild plant. Particularly, in *Kgb*1 and *Kgb*2 extracts the following chemical compounds were detected: ribose-1-arsenate (0.7), 3-(4-hydroxy-3,5-dimethoxyphenyl)-1-(2,4,6-trihydroxy-3-methoxyphenyl)propane-1,2-dione (RT = 0.9), 2-(1,3-dihydroxy4-oxocyclohex-2-en-1-yl)-5-hydroxy-3,6,7-trimethoxy-4H-chromen-4-one (RT = 1.0), sarmentosin epoxide (RT = 1.1), 4-hydroxycoumarin (RT = 1.4), sarmentosin epoxide (RT = 1.1) D-glutamine (RT = 1.8), 1, 6-dihydroxy-3,7-dimethoxy-2-(3-methyl-2-butenyl)-8-(3-hydroxy-3-methyl-1E-butenyl)-xanthone (RT = 5.1), verbasoside (RT = 5.4), 4-coumaric acid (RT = 5.9), ferulic acid (6.0), hallactone B (RT = 6.3), 6,7,3′,4′-tetrahydroxyflavanone (RT = 6.6), epiafzelechin (2R,3R)(-) (RT = 6.9), naringenin (RT = 7.0), 3-hydroxytetradecanedioic acid (RT = 7.6), (9S,10E,12S,13S)-9,12,13-trihydroxy-10-octadecenoic acid (RT = 8.4), 13-HOTrE (RT = 11.3), ipecoside (RT = 12.3), altamisic acid (12.4), a-linolenic acid (RT = 12.8), linoleic acid (RT = 13.3), and heneicosanoic acid (RT = 14.9), p-coumaraldehyde (RT = 15.2). Although, neobavaisoflavone, malic acid, heneicosanoic acid, heliannuol A, 3-(4-hydroxy-3,5-dimethoxyphenyl)-1-(2,4,6-trihydroxy-3-methoxyphenyl)propane-1,2-dione,sarmentosin epoxide, were major compounds in the *Kgb*1 and *Kgb*2 cell lines, in comparison to wild plant.

Table 1. Tentatively identified compounds in *Kgb*1 and *Kgb*2 cell lines extracts of *Kalanchoe gastonis-bonnieri* (- means no detected, while +, ++ and +++ refer to different peak intensity).

RT (min)	Tentative Identification	Match Factor	Monoisotopic Mass	Main Fragments (*m*/*z*)	Parent Ion [M-H]-	*Kgb*1			*Kgb*2			Wild Type Plants	
						Days of Culture						Leaves	Roots
						9	18	25	9	18	25		
	Flavonoids												
5.9	3,7-Dihydroxy-3′,4′-dimethoxyflavone	79	314.0863	637.1386 638.1423 653.1331	313.0684	-	-	-	-	-	-	+	-
6.1	Syringetin-3-O-glucoside	78	508.1272	477.1005 508.1177 535.2139	507.1111	-	-	-	-	-	-	+	-
6.2	Quercetin-3-O-pentosyl (1-2) acetilpentosida	80	608.1295	327.0844 623.1247 623.2683	607.1259	-	-	-	-	-	-	+	-
6.2	3,4-dimethoxy-myricetin-3-O-dideoxyhexosyl(1-2)-dideoxyhexoside	80	638.1445	521.2012 623.1227 638.1445	637.1383	-	-	-	-	-	-	+	-
6.3	Guaijaverin	79	434.0762	434.0762 491.0744 519.2203	433.0762	-	-	-	-	-	-	+	-
6.5	Apigenin-6-C-glucoside-7-O-glucoside	90	594.1500	461.1064 506.0978 594.1500	593.1466	-	-	-	-	-	-	+	-
6.6	6,7,3′,4′-Tetrahydroxyflavanone	86	288.0149	146.9666 288.1511 309.1295	287.1465	-	-	+	-	-	-	-	-
6.7	Kaempferol-3-O-arabinoside	95	418.0852	418.0852 491.1174 607.2728	417.0791	-	-	-	-	-	-	+	+
6.9	Epiafzelechin (2R,3R)(-)	80	274.1671	187.0955 289.1635 607.2757	273.1671	-	-	+	-	-	-	-	-

Table 1. *Cont.*

RT (min)	Tentative Identification	Match Factor	Monoisotopic Mass	Main Fragments (*m*/*z*)	Parent Ion [M-H]-	*Kgb*1			*Kgb*2			Wild Type Plants	
						Days of Culture						Leaves	Roots
						9	18	25	9	18	25		
7.0	Naringenin	80	272.1627	112.9843 289.1627 607.2727	271.1528	-	-	+	-	-	-	-	-
7.1	Kaempferide 3-glucuronide	95	476.0914	597.2469 607.2738 608.2776	475.0873	-	-	-	-	-	-	-	+
7.2	Diosmine	89	608.1664	475.0857 608.1683 643.1410	607.1641	-	-	-	-	-	-	+	-
7.7	Quercetin	90	302.0368	146.9683 157.0095 302.0331	301.0317	-	-	-	-	-	-	+	+
8.2	2′,7-Dihydroxy-4′-methoxy-8-prenylflavan 2′,7-diglucoside	83	664.2705	653.2342 680.2472 707.2874	663.2626	-	-	-	-	-	-	+	-
8.6	Fisetin	80	286.0392	112.9833 286.0392 315.0477	285.0360	-	-	-	-	-	-	+	+
10.5	Kaempferol-7-neohesperidoside	93	594.2728	197.9606 201.0350 594.2728	593.2716	+	-	-	+	-	-	+	-
11.1	Quercetin-3-O-vicianoside	94	596.2922	197.9612 596.2922 723.3794	595.2873	+	+	-	+	-	-	-	-
11.9	Neobavaisoflavone	90	322.1733	322.1762 406.1516 421.0987	321.1724	+	+	-	-	+++	-	-	+
	Fatty acids												
6.3	Deacetoxy (7)-7-Oxokhivorinic acid	78	520.2216	509.1912 520.2216 536.2101	519.2210	-	-	-	-	-	-	+	-

Table 1. *Cont.*

RT (min)	Tentative Identification	Match Factor	Monoisotopic Mass	Main Fragments (*m*/*z*)	Parent Ion [M-H]-	*Kgb*1			*Kgb*2			Wild Type Plants	
						Days of Culture						Leaves	Roots
						9	18	25	9	18	25		
7.6	3-Hydroxytetradecanedioic acid	83	274.171	146.9682 173.9999 274.1710	273.1676	-	-	+	-	-	-	-	-
8.4	(9S,10E,12S,13S)-9,12,13-Trihydroxy-10-octadecenoic acid	81	330.2366	174.0007 201.0377 330.2366	329.2320	-	+	-	-	-	-	-	-
8.7	Corchorifatty acid F	83	328.2277	201.0351 263.1309 328.2158	327.2141	+	+	+	+	+	+	+	-
9.2	(Z)-9,12,13-trihydroxyoctadec-15-enoic acid	80	330.9998	157.0100 330.2365 397.2198	329.2300	+	++	+	+	+	+	+	-
11.3	13-HOTrE	81	294.2131	275.2000 294.2131 613.9943	293.2095	+	+	+	+	-	+	-	-
11.7	Vernolic acid	83	296.2289	116.9269 296.2289 363.2136	295.2255	+	+	+	+	+	+	+	-
12.4	Altamisic acid	80		112.9851 135.9701 309.1725	279.1628	-	-	-	-	-	+	-	-
12.8	α-Linolenic acid	80	278.7812	135.9699 278.2192 400.2108	277.2160	+	-	-	-	-	+	-	-
13.3	Linoleic acid	80	280.2351	278.7270 280.2351 325.1854	279.2322	+	-	-	-	-	-	-	-
14.9	Heneicosanoic acid	90	326.1831	326.1873 339.2004 340.2043	325.1829	+++	-	-	-	+++	++	-	-

Table 1. *Cont.*

RT (min)	Tentative Identification	Match Factor	Monoisotopic Mass	Main Fragments (*m*/*z*)	Parent Ion [M-H]-	*Kgb*1			*Kgb*2			Wild Type Plants	
						Days of Culture						Leaves	Roots
						9	18	25	9	18	25		
	Coumarins												
1.4	4-Hydroxycoumarin	83	162.0495	117.0193 128.0353 292.1378	161.0451	+	-	-	+	-	-	-	-
4.9	3,4,5-Trihydroxy-6-{[2-oxo-6-(3-oxobutyl)-2H-chromen-7-yl]oxy}oxane-2-carboxylate	85	408.0981	341.0851 408.0981 443.0710	407.0945	-	-	-	-	-	-	+	-
6.3	Hallactone B	80	440.1090	112.9848 174.0002 440.1090	439.1031	-	-	-	+	-	-	-	-
11.4	Rugosal A	87	266.1482	134.8628 135.9684 817.1497	265.1446	-	-	+	+	-	+	++	+++
11.8	Corylifol A	85	390.2340	321.1705 390.2340 411.2125	389.2295	-	-	-	-	-	-	+	-
	Phenolic acids												
5.4	Caffeic acid	93	180.0365	135.0425 180.0365 755.1999	179.0325	-	-	-	-	-	-	+	+
5.7	1-Caffeoyl-4-deoxyquinic acid	90	338.0910	191.0536 338.0910 359.0706	337.0887	-	-	-	-	-	-	+	-
5.9	4-Coumaric acid	81	164.0467	164.0467 279.0519 475.1830	163.0378	+	-	+	+	-	+	-	-
6.0	Ferulic acid	86	194.0537	194.0537 309.0612 311.1114	193.0486	+	-	++	+	-	++	-	-
	Phenolic compounds												

Table 1. *Cont.*

RT (min)	Tentative Identification	Match Factor	Monoisotopic Mass	Main Fragments (*m*/*z*)	Parent Ion [M-H]-	*Kgb*1			*Kgb*2			Wild Type Plants	
						Days of Culture						Leaves	Roots
						9	18	25	9	18	25		
5.2	Syringate	81	198.0453	198.0453 313.0536 431.1863	197.0428	-	-	-	-	-	-	+	-
5.5	Verbasoside	83	462.1709	415.1603 451.1389 462.1709	461.1640	+	-	+	+	-	+	-	-
15.2	*p*-Coumaraldehyde	81	147.8759	178.8796 220.9484 231.9439	146.9643	+	-	-	-	+	+	-	-
	Terpenes												
6.4	Kanokoside D	89	624.2753	577.2622 613.2428 624.2753	623.2694	-	-	-	+	-	-	-	+
10.3	Heliannuol A	94	250.1536	248.9578 250.1536 251.1476	249.1497	+	++	++	+	+++	+++	++	++
12.3	Ipecoside	80	565.3214	116.9282 554.2898 581.3077	564.3191	+	-	-	-	-	-	-	-
	Carboxylic acids												
1.0	Malic acid	80	134.0215	128.0336 341.1061 377.0831	133.0119	+++	+++	+++	-	-	+++	++	+
1.0	DL-Pyroglutamic acid	80	129.0345	290.0858 310.0687 403.1352	128.0336	-	+	-	+	-	-	-	-
1.2	Citrate	90	192.0301	111.0072 128.0334 173.0081	191.0175	-	-	-	-	-	-	+	-
1.3	D-(-)-Quinic acid	91	192.0579	157.0341 377.0813 379.0805	191.0520	-	-	-	-	++	-	+++	-

Table 1. *Cont.*

RT (min)	Tentative Identification	Match Factor	Monoisotopic Mass	Main Fragments (*m*/*z*)	Parent Ion [M-H]-	*Kgb*1			*Kgb*2			Wild Type Plants	
						Days of Culture						Leaves	Roots
						9	18	25	9	18	25		
	Alkaloids												
6.1	Voacristine	80	384.0108	384.0108 481.1331 563.2139	383.0066	-	-	-	-	-	-	-	+
	Amino acids												
0.8	D-Glutamine	87	146.0324	179.0556 215.0324 307.1131	145.0598	+	-	-	+	-	-	-	-
4.3	Tryptophan	82	204.0841	204.0841 261.0370 271.0673	203.0800	-	-	-	-	-	-	+	-
	Carbohydrate												
1.1	Sarmentosin epoxide	90	291.0908	133.0134 200.0561 632.2048	290.0859	+++	++	++	+++	++	++	-	-
	Others												
0.7	Ribose-1-arsenate	89	273.9598	158.9785 273.9598 274.9575	272.9565	++	-	-	+	-	-	-	-
0.9	3-(4-hydroxy-3,5-dimethoxyphenyl)-1-(2,4,6-trihydroxy-3-methoxyphenyl)propane-1,2-dione	90	378.0889	341.1077 379.0830 404.1046	377.0854	-	+++	-	-	-	-	-	-
1.0	2-(1,3-dihydroxy-4-oxocyclohex-2-en-1-yl)-5-hydroxy-3,6,7-trimethoxy-4H-chromen-4-one	79	378.0822	341.1083 404.1043 470.1521	377.0846	+	-	-	-	-	-	-	-
4.4	Cusparine	82	307.1179	112.9835 296.0879 350.1422	306.1163	-	-	-	-	-	-	+	-

Table 1. *Cont.*

RT (min)	Tentative Identification	Match Factor	Monoisotopic Mass	Main Fragments (*m*/*z*)	Parent Ion [M-H]-	*Kgb*1			*Kgb*2			Wild Type Plants	
						Days of Culture						Leaves	Roots
						9	18	25	9	18	25		
5.1	1,6-Dihydroxy-3,7-dimethoxy-2-(3-methyl-2-butenyl)-8-(3-hydroxy-3-methyl-1E-butenyl)-xanthone	86	440.1819	393.1746 429.1495 440.1819	439.1785	-	-	+	+	-	-	-	-
5.8	1-(2H-1,3-benzodioxol-5-yl)-2-[2,6-dimethoxy-4-(prop-2-en-1-yl)phenoxy]propyl benzoate	83	476.1831	429.1758 476.1831 521.1996	475.1800	-	-	-	-	-	-	+	-
8.5	3-(1,2-dihydroxypropyl)-1,6,8-trihydroxyanthracene-9,10-dione	83	330.2334	285.0364 286.0402 330.2334	329.2298	+	-	+	+	-	+	+	-
8.7	Isocyclocalamin	85	502.2151	491.1836 493.1828 518.2009	501.2109	-	-	-	-	-	-	-	+

Figure 5 shows the comparison of the relative abundance of the identified metabolites; the lowest concentration in red light, while the highest in green light. According to the row dendrogram, it is observed that the relative abundance of the compounds is different between extracts of the transformed lines *Kgb*1, *Kgb*2, leaf, and wild type root extracts. Column dendrogram allows for visualizing the formation of two main groups: on the left the extracts obtained from *Kgb*1 of 9, 18, and 25 days of culture and *Kgb*2 of 9 and 25 days, and on the right side the leaf and wild root extracts. Subgroups are formed between the *Kgb*1 and *Kgb*2 lines, showing the chemical compound diversity between the different culture days analyzed. Regarding the relative abundance of 4-coumaric acid, ferulic acid, verbasoside, and 3-(1,2-dihydroxypropyl)-1,6,8-trihydroxyanthracene-9,10-dione. They were similar at 9 and 25 days between *Kgb*1 and *Kgb*2, while the wild type of leaf and root extracts were similar in relation to the abundance of rugosal A, fisetin, quercetin, kaempferol-3-O-arabonoside, and y caffeic acid. Dendrogram also shows the identification of specific compounds in *Kgb*1 and *Kgb*2 extracts in the different culture periods. In the *Kgb*1-d9 extract, particular compounds were identified as ipecoside, linoleic acid, 2-(1,3-dihydroxy-4-oxocyclohex-2-en-1-yl)-5-hydroxy-3,6,7-trimethoxy-4H-chromen-4-one, and linolenic acid. In the *Kgb*1-d18 extract, specific compounds were identified as 3-(4-hydroxy-3,5-dimethoxyphenyl)-1-(2,4,6-trihydroxy-3-methoxyphenyl) propane-1,2-dione, 9,12,13-trihydroxy-10-octadecenoic acid, pyroglutamic acid, quercetin-3-O-vicianoside, and y 13-HOTrE, while in the *Kgb*1-d25 extract, the specific identified compounds were epiafzelechin (2R,3R), 6,7,3′,4′-tetrahydroxyflavanone, and naringenin y 3-hydroxytetradecanedioic acid.

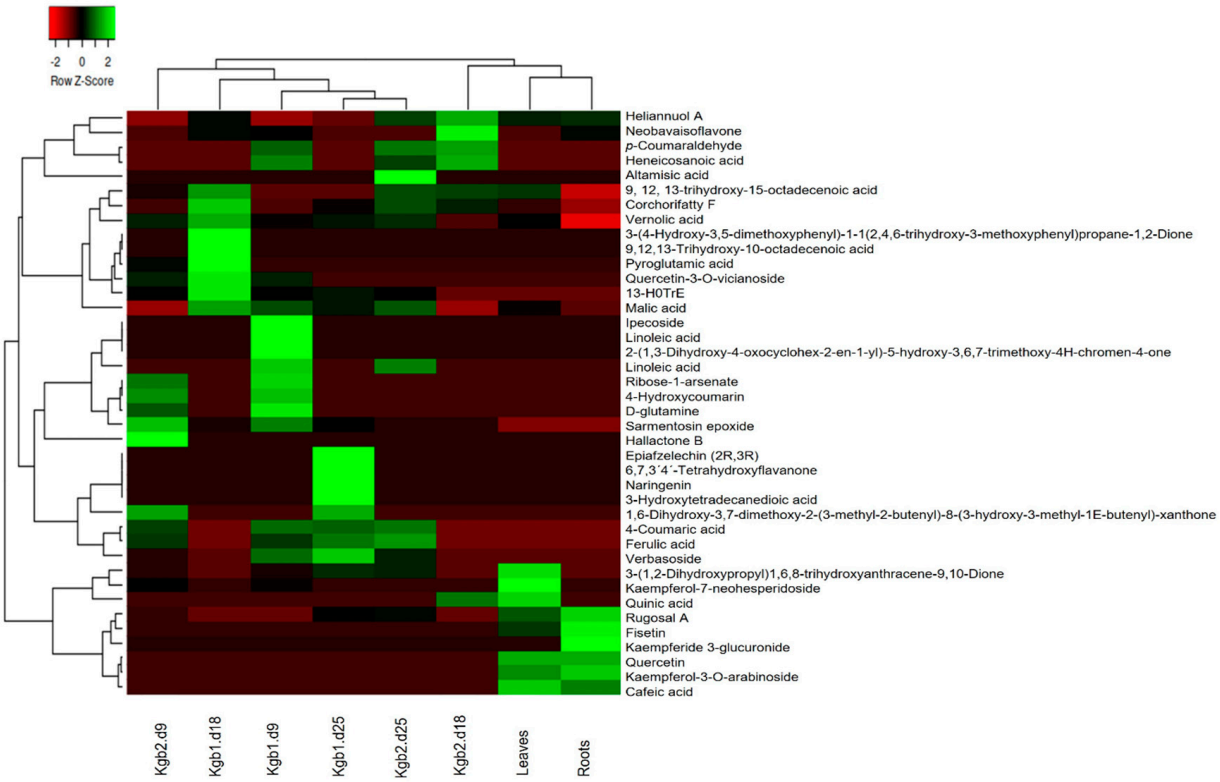

Figure 5. Heat map showing the relative abundance of the compounds tentatively identified in extracts of *Kgb*1, *Kgb*2, wild leaves and roots of *Kalanchoë gastonis-bonnieri*. Color code: light green (highest relative abundance); light red (lower relative abundance).

Likewise, specific compounds were observed in the leaf and/or root extracts: 3-(1,2-dihydroxypropyl)-1,6,8-trihydroxyanthracene-9,10-dione, kaempferol-7-neohesperidoside, rugosal A, fisetin kaempferide 3-glucuronide, quercetin, kaempferol-3-O-arabinoside, and caffeic acid. Similar relative compound abundance was observed for *Kgb*1 and *Kgb*2 extracts at 9 days of culture: ribose-1-arsenate, 4-hydroxycoumarin, D-glutamine, sarmentosin

epoxide, 4-coumaric acid, and ferulic acid; at 25 days of culture: 4-coumaric acid, ferulic acid, and verbasoside.

4. Discussion

Some data on transformation efficiency have been reported: 72% in leaf explants of *Solanum erianthum* D. Don. [36]; 65% in shoot explants and 60 in leaf explants of *Althaea officinalis* [28]; 56% in leaf explants and 29% in seedlings' inter nodal explants of *Salvia bulleyana* [37]. According to this information, the transformation efficiency of *A. rhizogenes* strain A4 is diverse; in this work, A4 strain was capable of infecting *K. gastonis-bonnieri* tissue which led to genetically modified embryogenic calli. Also, Chaudhuri et al. [38] and Tavassoli and Safipour-Afshar [28] reported that transformation efficiency was obtained as a function of culture conditions, *Agrobacterium*-host interaction, age, and explant type. It has also been associated with actively dividing cells showing higher transformation rates.

In this work, 15 lines were individualized, and 2 lines, *Kgb*1 and *Kgb*2, were selected, due to accelerated growth and abundant secondary roots. *Kgb*1 and *Kgb*2 showed spontaneous callus formation. Vinterhalter et al. [10] reported calli and somatic embryo formation at 35 d of culture, from roots induced with the *A. rhizogenes* A4M70GUS strain, in *Gentiana utriculosa*. Hiebert-Giesbrecht et al. (2021) [8] reported in *Pentalinon andrieuxii* the formation of embryogenic callus 6 months after obtaining hairy roots with the *A. rhizogenes ATCC*15834 strain in leaves and hypocotyls. Results obtained in this work represent the possibility of whole plant regeneration; moreover, is an interesting tool to carry out advanced studies on the secondary metabolism of *K. gastonis-bonnieri* transgenic culture. The response of *K. gastonis-bonnieri* agrees with previous reports on the spontaneous formation of embryogenic calli from hairy roots obtained by *Agrobacterium* infection, on different plant species such as *Gentiana utriculosa* with the *A. rhizogenes* A4M70GUS strain [1,10], *Pentalinon andrieuxii* with the *A. rhizogenes ATCC*15834 strain [8]; all these strains carrying the same agropine-like *Ri* plasmid [39]. It has been suggested that abnormal morphological features of hairy roots can be a result of the combined participation of *rol* genes in plant cells since each gene might be associated with specific phenotypic alterations in *Kalanchoë* species; in addition, the response of each plant species against infection by *A. rhizogenes* is diverse [38]. In this work, it is suggested that both TL-DNA and TR-DNA of *A. rhizogenes* A4 strain were inserted into *Kgb*1 and *Kgb*2 genomes. It has been reported that the response of a plant species to genetic transformation by *A. rhizogenes* is in function of the integration and combined expression of *rol*A, *rol*B, *rol*C, and *rol*D genes [40,41].

The observed difference in the chemical profile of the transformed embryogenic calli extracts is possibly due to asynchronous growth in the different culture periods [42]. There is wide variability in the compounds reported in *K. gastonis-bonnieri*, which could be attributed to the analyzed plant material that includes the plant development stage, the collection season, and extraction conditions, even among the transformed cell lines analyzed in this work.

*Kgb*1 and *Kgb*2, at 9 days of culture, showed a greater number of detected compounds, in contrast to culture analyzed at 18 and 25 days, which could be related to the adaptation of subculture towards the beginning of a new cycle [43]; a lower number of compounds were detected at 18 days of culture which could be attributed to accelerated cell growth [43,44], therefore the cell metabolism is redirected toward multiplication and cell growth. Finally, at 25 days of culture, the increase of some compounds was observed, which could be attributed to the accumulation of compounds related to the embryogenic process. The accumulation and changes in metabolites detected are related to different stages of development and growth in the in vitro culture, which is also observed at different developmental stages of wild plants [45–47] as a part of plant development or in response to epigenetic factors.

The extracts of *Kgb*1 and *Kgb*2 lines and leaves showed corchorifatty F acid, a compound identified in rice and other cereals, with antibacterial activity [48]. In *Kgb*1 and *Kgb*2 extracts, sarmentosine epoxide was detected, which has shown antihepatotoxic activity [49]. In this work, malic acid, caffeic acid, rugosal A, campferol 3-O-arabinoside, quercetin,

fisetin, and heliannuol were detected in leaves and roots of *K. gastonis bonnieri* which have not been previously reported in wild plants.

Interestingly, hairy root cultures synthesize compounds that have not been detected in wild plants [9]. Furthermore, some authors have reported that shoots and transgenic plants obtained from hairy roots accumulate specific metabolites compared to the wild plant. Tusevski et al. [5], reported an accumulation of naphthodiatrons and specific phenolic compounds in transgenic shoots obtained of hairy roots from *Hypericum perforatum*. Vinterhalter et al. [7] reported xanthones in transgenic *Gentiana utriculosa* plants regenerated by somatic embryogenesis from hairy roots. Hiebert-Giesbrecht et al. [8] reported terpenoids accumulation in leaf extracts of transgenic plants, which were obtained from hairy roots of *Pentalinon andrieuxii*.

The phenotypic changes showed in transformed cultures, could be due to (a) position and (b) number of copies of the T-DNA inserted in the genome of the host cell, (c) the regulation of gene expression, and (d) protein biosynthesis encoded by *rol* genes in the plant cell, among others [7,8].

The biological activity of some of compounds identified in this work has been reported, quinic and malic acid inhibit the growth of *S. aureus* and *P. aeruginosa* [50]; the neobavaisoflavone is a compound that has antioxidant, anti-inflammatory, and anticancer properties [51]; ferulic acid has a wide variety of effects, especially on oxidative stress, inflammation, vascular endothelial injury, fibrosis, apoptosis, and platelet aggregation [52]. The compounds detected in *Kgb*1 and *Kgb*2 suggest the effect of genetic transformation through the differential biosynthesis of chemical compounds. It has been reported that highly specialized plant organs such as roots and leaves are needed for the biosynthesis of phytochemicals [26]; therefore, *Kgb*1 and *Kgb*2 do not develop highly specialized organs, which could explain the limited accumulation of flavonoids.

5. Conclusions

This is the first report of the chemical profile of transformed cell cultures of *K. gastonis-bonnieri*. Specific compounds that have not been reported in *K. gastonis-bonnieri* wild-type plants were identified. In vitro, culture of genetically transformed embryogenic calli could be an alternative to produce metabolites of commercial interest in the pharmacological industry as treatment against various diseases, such as cancer. In addition, obtaining transgenic *K. gastonis-bonnieri* plants from embryogenic callus could be an opportunity in the ornamental industry since genetic modification could produce plants with different phenotype.

Author Contributions: M.G.B.N.: Conceptualization, Investigation, Methodology, Formal analysis, Writing (Revision—original and final draft. M.B.: Formal analysis, Writing (Revision—original and final draft). M.Á.M.-M.: Methodology, Formal analysis, Revision final draft. L.R.-V.: data curation. E.I.: Conceptualization, Methodology, Investigation, Methodology, Revision—final draft. A.A.D.V.-M.: Conceptualization, Investigation, Methodology, Writing (Revision—original and final draft), Formal analysis, Project administration, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Instituto Politécnico Nacional, México (IPN/SIP 20220810, 20231185).

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: MGBN (744161) acknowledge study grant from CONACYT.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Chung, I.-M.; Rekha, K.; Rajakumar, G.; Thiruvengadam, M. Production of glucosinolates, phenolic compounds and associated gene expression profiles of hairy root cultures in turnip (*Brassica rapa* ssp. *rapa*). *3 Biotech* **2016**, *6*, 175. [CrossRef] [PubMed]
2. Li, Y.; Kong, D.; Fu, Y.; Sussman, M.R.; Wu, H. The effect of developmental and environmental factors on secondary metabolites in medicinal plants. *Plant Physiol. Biochem.* **2020**, *148*, 80–89. [CrossRef]
3. Abhyankar, G.; Suprasanna, P.; Pandey, B.N.; Mishra, K.P.; Rao, K.V.; Reddy, V.D. Hairy root extract of *Phyllanthus amarus* induces apoptotic cell death in human breast cancer cells. *Innov. Food Sci. Emerg. Technol.* **2010**, *11*, 526–532. [CrossRef]
4. Rekha, K.; Thiruvengadam, M. Secondary metabolite production in transgenic hairy root cultures of cucurbits. In *Transgenesis and Secondary Metabolism*; Reference Series in, Phytochemistry; Jha, S., Ed.; Springer: Cham, Switzerland, 2017. [CrossRef]
5. Tusevski, O.; Stanoeva, J.P.; Stefova, M.; Pavokovic, D.; Simic, S.G. Identification and quantification of phenolic compounds in *Hypericum perforatum* L. transgenic shoots. *Acta Physiol. Plant* **2014**, *36*, 2555–2569. [CrossRef]
6. Roychowdhury, D.; Halder, M.; Jha, S. Agrobacterium rhizogenes-mediated transformation in medicinal plants: Genetic stability in long-term culture. In *Transgenesis and Secondary Metabolism*; Reference Series in, Phytochemistry; Jha, S., Ed.; Springer: Cham, Switzerland, 2017. [CrossRef]
7. Vinterhalter, B.; Savić, J.; Zdravković-Korać, S.; Banjac, N.; Vinterhalter, D.; Krstić-Milošević, D. *Agrobacterium rhizogenes*-mediated transformation of *Gentiana utriculosa* L. and xanthones decussatin-1-*O*-primeveroside and decussatin accumulation in hairy roots and somatic embryo-derived transgenic plants. *Ind. Crops Prod.* **2019**, *130*, 216–229. [CrossRef]
8. Hiebert-Giesbrecht, M.R.; Avilés-Berzunza, E.; Godoy-Hernández, G.; Peña-Rodriguez, L.M. Genetic transformation of the tropical vine *Pentalinon andrieuxii* (Apocynaceae) via *Agrobacterium rhizogenes* produces plants with an increased capacity of terpenoid production. *In Vitro Cell Dev. Biol-Plant* **2021**, *57*, 21–29. [CrossRef]
9. Huang, S.H.; Vishwakarma, R.K.; Lee, T.T. Establishment of hairy root lines and analysis of iridoids and secoiridoids in the medicinal plant *Gentiana scabra*. *Bot. Stud.* **2014**, *55*, 17. [CrossRef] [PubMed]
10. Vinterhalter, B.; Krstić-Milošević, D.; Janković, T.; Pljevljakusic, D.; Ninković, S.; Smigocki, A.; Vinterhalter, D. *Gentiana dinarica* Beck. hairy root cultures and evaluation of factors affecting growth and xanthone production. *Plant Cell Tiss. Organ. Cult.* **2015**, *121*, 667–679. [CrossRef]
11. Stefanowicz-Hajduk, J.; Asztemborska, M.; Krauze-Baranowska, M.; Godlewska, S.; Gucwa, M.; Moniuszko-Szajwaj, B.; Stochmal, A.; Ochocka, J.R. Identification of flavonoids and bufadienolides and cytotoxic effects of *Kalanchoë daigremontiana* extracts on human cancer cell lines. *Planta Med.* **2020**, *86*, 239–246. [CrossRef]
12. Hernández-Caballero, M.E.; Sierra-Ramírez, J.A.; Villalobos Valencia, R.; Seseña-Méndez, E. Potential of *Kalanchoe pinnata* as a Cancer Treatment Adjuvant and an Epigenetic Regulator. *Molecules* **2022**, *27*, 6425. [CrossRef]
13. Christensen, B.; Sriskandarajah, S.; Serek MMüller, R. Transformation of *Kalanchoe blossfeldiana* with *rol*-genes is useful in molecular breeding towards compact growth. *Plant Cell Rep.* **2008**, *27*, 1485–1495. [CrossRef]
14. Fkiara, A.; Barba-Espín, G.; Bahij, R.; Müller, R.; Christensen, L.P.; Lütken, H. Elicitation of Flavonoids in *Kalanchoe pinnata* by *Agrobacterium rhizogenes*-mediated Transformation and UV-B Radiation. In *Medicinal Plants: Biodiversity, Sustainable Utilization and Conservation*; Khasim, S., Long, C., Thammasiri, K., Lütken, H., Eds.; Springer: Cham, Switzerland, 2020; pp. 395–403. [CrossRef]
15. Ramasamy, M.; Dominguez, M.M.; Irigoyen, S.; Padilla, C.S.; Mandadi, K.K. *Rhizobium rhizogenes*-mediated hairy root induction and plant regeneration for bioengineering citrus. *Plant Biotechnol. J.* **2023**, *21*, 1728–1730. [CrossRef]
16. Thirukkumaran, G.; Khan, R.S.; Chin, D.P.; Nakamura, I.; Mii, M. Isopentenyl transferase gene expression offers the positive selection of marker-free transgenic plant of *Kalanchoe blossfeldiana*. *Plant Cell Tiss. Organ. Cult.* **2009**, *97*, 237–242. [CrossRef]
17. Cho, K.H.; Vieira, A.E.; Kim, J.; Clark, D.G.; Colquhoun, T.A. Transformation of Kalanchoe pinnata by Agrobacterium tumefaciens with ZsGreen1. *Plant Cell Tissue Organ. Cult.* **2021**, *146*, 401–407. [CrossRef]
18. Yukes, J.; Balick, M. *Dominican Medicinal Plants: A Guide for Health Care Providers*, 2nd ed.; New York Botanical Garden: Bronx, NY, USA, 2010; pp. 7–14.
19. Costa, S.S.; Corrêa, M.F.P.; Casanova, L.M. A new triglycosyl flavonoid isolated from leaf juice of *Kalanchoë gastonis-bonnieri* (Crassulaceae). *Nat. Prod. Commun.* **2015**, *10*, 433–436. [CrossRef] [PubMed]
20. Abdalla, S.L.; Costa, S.S.; Gioso, M.A.; Casanova, L.M.; Coutinho, M.A.S.; Silva, M.F.A.; Botelho, M.C.D.S.N.; Díaz, R.S.G. Efficacy of a *Kalanchoë gastonis-bonnieri* extract to control bacterial biofilms and dental calculus in dogs. *Pesqui. Vet. Bras.* **2017**, *37*, 859–865. [CrossRef]
21. Palumbo, A.; Casanova, L.M.; Corrêa, M.F.P.; Da Costa, N.M.; Nasciutti, L.E.; Costa, S.S. Potential therapeutic effects of underground parts of *Kalanchoë gastonis-bonnieri* on benign prostatic hyperplasia. *Evid.-Based Complement. Altern. Med.* **2019**, *2019*, 6340757. [CrossRef]
22. Siems, K.; Jas, G.; Arriaga-Giner, F.J.; Wollenweber, E.; Dörr, M. On the chemical nature of epicuticular waxes in some succulent *Kalanchoë* and *Senecio* species. *Z. Naturforsch.* **1995**, *C 50*, 451–454. [CrossRef]
23. Vanegas, P.E.; Cruz-Hernández, A.; Valverde, M.E.; Paredes-López, O. Plant regeneration via organogenesis in marigold. *PCTOC* **2002**, *69*, 279–283. [CrossRef]
24. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **1962**, *15*, 472–497. [CrossRef]
25. Hooykas, P.J.J.; Klapwijk, P.M.; Nuti, M.P.; Schilperoort, R.A.; Rörsch, A. Transfer of the *Agrobacterium tumefaciens* TI plasmid to avirulent Agrobacteria and to Rhizobium *ex planta*. *J. Gen. Microbiol.* **1977**, *98*, 477–484. [CrossRef]

26. Sharifi, S.; Sattari, T.N.; Zebarjadi, A.; Majd, A.; Ghasempour, H. The influence of Agrobacterium rhizogenes on induction of hairy roots and ß-carboline alkaloids production in *Tribulus terrestris* L. *Physiol. Mol. Biol. Plants* **2014**, *20*, 69–80. [CrossRef]
27. Torres-García, B.E.; Morales-Domínguez, J.F.; Fraire-Velázquez, S.; Pérez-Molphe-Balch, E. Generación de cultivos de raíces transformadas de la planta medicinal *Bidens odorata* Cav (Compositae) y análisis fitoquímico preliminar. *Polibotánica* **2018**, *46*, 241–257. [CrossRef]
28. Tavassoli, P.; Safipour-Afshar, A. Influence of different *Agrobacterium rhizogenes* strains on hairy root induction and analysis of phenolic and flavonoid compounds in marshmallow (*Althaea officinalis* L.). *3 Biotech* **2018**, *8*, 351. [CrossRef] [PubMed]
29. Gamborg, O.L.; Miller, R.A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **1968**, *50*, 151–158. [CrossRef]
30. Kieran, P.; MacLoughlin, P.; Malone, D. Plant cell suspension cultures: Someengineering considerations. *J. Biotechnol.* **1997**, *59*, 39–52. [CrossRef]
31. Urquiza-López, A.; Álvarez-Rivera, G.; Ballesteros-Vivas, D.; Cifuentes, A.; Del Villar-Martínez, A.A. Metabolite profiling of rosemary cell lines with antiproliferative potential against human HT-29 colon cancer cells. *Plant Foods Hum. Nutr.* **2021**, *76*, 319–325. [CrossRef] [PubMed]
32. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.
33. Petrova, M.; Zayova, E.; Vlahova, M. Induction of hairy roots in *Arnica montana* L. by *Agrobacterium rhizogenes*. *Cent. Eur. J. Biol.* **2013**, *8*, 470–479. [CrossRef]
34. Savić, J.; Nikolić, R.; Banjac, N.; Zdravković-Korać, S.; Stupar, S.; Cingel, A.; Ćosić, T.; Raspor, M.; Smigocki, A.; Ninković, S. Beneficial implications of sugar beet proteinase inhibitor BvSTI on plant architecture and salt stress tolerance in *Lotus corniculatus* L. *J. Plant Physiol.* **2019**, *243*, 153055. [CrossRef]
35. Rana, M.M.; Han, Z.-X.; Song, D.-P.; Liu, G.-F.; Li, D.-X.; Wan, X.-C.; Karthikeyan, A.; Wei, S. Effect of medium supplements on *Agrobacterium rhizogenes* mediated hairy root induction from the callus tissues of *Camellia sinensis* var. *sinensis*. *Int. J. Mol. Sci.* **2016**, *17*, 1132. [CrossRef] [PubMed]
36. Sarkar, J.; Misra, A.; Banerjee, N. Genetic transfection, hairy root induction and solasodine accumulation in elicited hairy root clone of *Solanum erianthum* D. *Don. J. Biotechnol.* **2020**, *323*, 238–245. [CrossRef]
37. Wojciechowska, M.; Owczarek, A.; Kiss, A.K.; Grąbkowska, R.; Olszewska, M.A.; Grzegorczyk-Karolak, I. Establishment of hairy root cultures of *Salvia bulleyana* Diels for production of polyphenolic compounds. *J. Biotechnol.* **2020**, *318*, 10–19. [CrossRef]
38. Chaudhuri, K.N.; Ghosh, B.; Tepfer, D.; Jha, S. Spontaneous plant regeneration in transformed roots and calli from *Tylophora indica*: Changes in morphological phenotype and tylophorine accumulation associated with transformation by *Agrobacterium Rhizogenes*. *Plant Cell Rep.* **2006**, *25*, 1059–1066. [CrossRef]
39. Bahramnejad, B.; Naji, M.; Bose, R.; Jha, S. A critical review on use of *Agrobacterium rhizogenes* and their associated binary vectors for plant transformation. *Biotechnol. Adv.* **2019**, *37*, 107405. [CrossRef] [PubMed]
40. Ozyigit, I.I.; Dogan, I.; Artam Tarhan, E. Agrobacterium rhizogenes-mediated transformation and its biotechnological applications in crops. In *Crop improvement*; Springer: Boston, MA, USA, 2013; pp. 1–48.
41. Sarkar, S.; Ghosh, I.; Roychowdhury, D.; Jha, S. The effects of *rol* genes of *Agrobacterium rhizogenes* on morphogenesis and secondary metabolite accumulation in medicinal plants. In *Biotechnological Approaches for Medicinal and Aromatic Plants*; Kumar, N., Ed.; Springer: Singapore, 2018. [CrossRef]
42. Rattan, S.; Kumar, D.; Warghat, A.R. Growth kinetics, metabolite yield, and expression analysis of biosynthetic pathway genes in friable callus cell lines of *Rhodiola imbricata* (Edgew). *Plant Cell Tiss. Organ Cult.* **2021**, *146*, 149–160. [CrossRef]
43. Chiavegatto, R.B.; Castro, A.H.F.; Marçal, M.G.; Padua, M.S.; Alves, E.; Techio, V.H. Cell Viability, Mitotic Indexand Callus Morphology of *Byrsonima verbascifolia (Malpighiaceae)*. *TropicalPlant Biol.* **2015**, *8*, 87–97. [CrossRef]
44. Sathish, S.; Venkatesh, R.; Safia, N.; Sathishkumar, R. Studies on growth dynamics ofembryogenic cell suspension cultures of commercially important Indica rice cultivars ASD16 and *Pusa basmati*. *3 Biotech* **2018**, *8*, 1–9. [CrossRef]
45. Pan, Y.; Li, L.; Xiao, S.; Chen, Z.; Sarsaiya, S.; Zhang, S.; ShangGuan, Y.; Liu, H.; Xu, D. Callus growth kinetics and accumulation of secondary metabolites of *Bletilla striata* Rchb.f. using a callus suspension culture. *PLoS ONE* **2020**, *15*, e0220084. [CrossRef] [PubMed]
46. Partap, M.; Kumar, P.; Ashrita; Kumar, P.; Kumar, D.; Warghat, A.R. Growth kinetics, metabolites production and expression profiling of picrosides biosynthetic pathway genes in friable callus culture of *Picrorhiza kurroa* Royle ex Benth. *Appl. Biochem. Biotechnol.* **2020**, *192*, 1298–1317. [CrossRef] [PubMed]
47. Wahyuni, D.K.; Rahayu, S.; Zaidan, A.H.; Ekasari, W.; Prasongsuk, S.; Purnobasuki, H. Growth, secondary metabolite production, and in vitro antiplasmodial activity of *Sonchus arvensis* L. callus under dolomite [$CaMg(CO_3)_2$] treatment. *PLoS ONE* **2021**, *16*, e0254804. [CrossRef] [PubMed]
48. Ang, H.; Mak, K.; Lum, M. Liquid chromatography mass spectrometry based high-throughput, unbiased profiling of upland and lowland rice varieties cultivated in Sabah. *Trans. Sci. Technol.* **2020**, *7*, 137–146.
49. Nahrstedt, A.; Walther, A.; Wray, V. Sarmentosin epoxide, a new cyanogenic compound from *Sedum cepaea*. *Phytochemistry* **1982**, *21*, 107–110. [CrossRef]
50. Adamczak, A.; Ożarowski, M.; Karpiński, T.M. Antibacterial activity of some flavonoids and organic acids widely distributed in plants. *J. Clin. Med.* **2020**, *9*, 109. [CrossRef] [PubMed]

51. Maszczyk, M.; Rzepka, Z.; Rok, J.; Beberok, A.; Wrześniok, D. Neobavaisoflavone may modulate the activity of topoisomerase inhibitors towards U-87 MG cells: An in vitro study. *Molecules* **2021**, *26*, 4516. [CrossRef]
52. Li, D.; Rui, Y.-X.; Guo, S.-D.; Luan, F.; Liu, R.; Zeng, N. Ferulic acid: A review of its pharmacology, pharmacokinetics and derivatives. *Life Sci.* **2021**, *284*, 119921. [CrossRef] [PubMed]