Arcopilus aureus MaC7A as a New Source of Resveratrol: Assessment of Amino Acid Precursors, Volatiles, and Fungal Enzymes for Boosting Resveratrol Production in Batch Cultures

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Abstract: The chemical factors that regulate the synthesis of resveratrol (RV) in filamentous fungi are still unknown. This work reports on the RV production by Arcopilus aureus MaC7A under controlled conditions and the effect of amino acid precursors (PHE and TYR), monoterpenes (limonone, camphor, citral, thymol, menthol), and mixtures of hydrolytic enzymes (Glanucanex) as elicitors for boosting fungal RV. Batch cultures with variable concentrations of PHE and TYR (50–500 mg L⁻¹) stimulated RV production from 127.9 ± 4.6 to 221.8 ± 5.2 mg L⁻¹ in basic cultures developed in PDB (pH 7) added with 10 g L⁻¹ peptone at 30 °C. Maximum levels of RV and biomass were maintained during days 6–8 under these conditions, whereas a dramatic RV decrease was observed from days 10–12 without any loss of biomass. Among the tested volatiles, citral (50 mg L⁻¹) enhanced RV production until 187.8 ± 2.2 mg L⁻¹ in basic cultures, but better results were obtained with Glucanex (100 mg L⁻¹; 198.3 ± 7.6 mg L⁻¹ RV). Optimized batch cultures containing TYR (200 mg L⁻¹), citral (50 mg L⁻¹), thymol (50 mg L⁻¹), and Glucanex (100 mg L⁻¹) produced up to 237.6 ± 4.7 mg L⁻¹ of RV. Our results suggest that low concentrations of volatiles and mixtures of isoenzymes with β-1, 3 glucanase activity increase the biosynthesis of fungal RV produced by A. aureus MaC7A in batch cultures.

Keywords: Arcopilus aureus; resveratrol; batch cultures; elicitors

1. Introduction

Resveratrol (3,4',5-trihydroxystilbene) is a potent antioxidant with known nutraceutical activity that has an evident impact in basic and applied fields. The most relevant activities of this phenolic compound are related to its capacity to regulate tumor progression and anti-ageing effects [1]. Due to these properties, resveratrol (RV) is currently used...
as an additive for functional foods and diverse dietary supplements. Recently, it has been reported that mixtures of RV and indomethacin could be used as a viable pharmacological therapy to ameliorate the effects of SARS-CoV-2, also known as COVID-19 [2]. RV is also tagged as a phytoalexin that protects against phytopathogenic microorganisms and represents an important regulator for the initiation of HR-related cell death [3]. Due to the relative simple biosynthesis of RV, diverse biotechnological approaches have been carried out to achieve its scaled production. The natural synthesis of RV involves the transformation of phenylalanine or tyrosine to generate \( p \)-coumaric acid by two biosynthetic steps. Subsequently, \( p \)-coumaric acid is converted to \( p \)-coumaroyl-CoA, which is finally transformed into trans-RV by stilbene synthases (STS), which are apparently confined to the plant kingdom [1]. Thus far, several orthologs involved in RV biosynthesis have been identified from vascular and non-vascular plants, and they have been successfully used for metabolic engineering approaches. To date, few orthologs of STS have been inserted into plant models with the aim of creating new plant foods with added value [4–6]. These new plant foods included the insertion and heterologous expression of STS from *Vitis vinifera* and *Arachis hypogaea*. Simultaneously, several strategies of synthetic biology have been applied to microorganisms used in fermentation processes to scale the production of this compound, which is poorly accumulated inside of plant tissues [7]. To the best of our knowledge, about 20 RV-producing strains of *Saccaromyces cerevisiae* and *Escherichia coli* have emerged as new platforms for scaling RV accumulation [7,8]. Interestingly, some of those platforms produced grams of RV from basic carbon sources such as glucose, fructose, or ethanol [9,10].

The bioprospection of wild filamentous fungi resulted in the discovery of endophytic species able to produce RV [11–14]. Undoubtedly, this finding represents a new and promising area to be addressed, because RV had been considered to be a phytoalexin exclusively produced by plant species until now [3]. However, a recent biochemical screening performed on endophytic fungi isolated from grapes reported at least 13 filamentous fungi able to biosynthesize and excrete RV into the culture media [11–14]. Interestingly, *Arcopilus aureus*, *Fusarium equiseti*, and *Xylaria psidii*, which are considered to be pathogenic organisms that colonize several plant species, were included in the list. The effect of epige netic modulators under batch fermentation revealed that these fungi are able to produce around 50 mg L\(^{-1}\) of RV.

The role of RV in fungi is completely ignored; however, it is known that plants produce it as a defense mechanism against fungal attack [3]. This fact may suggest that RV could exert allelopathic activity against fungal competitors. Volatiles and lysing enzymes are visualized as chemical defenses, which generate several types of stress in such competitors [3]. Considering this fact, this work was focused on the study of RV production under the pressure of plant volatiles and fungal enzymes under batch fermentation. These tests were performed in order to identify elicitors able to stimulate the biosynthesis of fungal RV.

2. Materials and Methods

2.1. Isolation and Identification of *Arcopilus aureus* MaC7A

A wild strain of *Arcopilus aureus* MaC7A was isolated from a mixture of phytopathogens and endophytic fungi found in the green stalks of grape cultures from Santa María de Analco at San Salvador el Verde, Puebla, México (19°15′53″ N; 98°30′55″ W; 2412 masl). The strain was routinely maintained in PDA or in PDB media for further experiments (Figure 1). The morphological characteristics were compared with those previously reported by Lee et al. [15]. The molecular identification was performed using total DNA extracted from the conidia, conidiophores, and mycelium by the CTAB method [16]. The amplification was carried out using the primers ITS5 (5′-GGAAGTAAAGTCTGAAACAAGG-3′) and ITS4 (5′-TCCTCCTGCTTTGATATGC-3′) with 0.90 U of GoTaqVR DNA polymerase (Promega, Madison, WI, USA). Initial denaturation took place at 95 °C for 3 min, followed by 35 cycles of 94 °C denaturation for 40 s, 50 °C annealing for 60 s, and 72 °C extension for 1 min [17]. Finally, the amplicons were sequenced with the cycle sequenc-
ing kit BigDye™ Terminator v3.1 (Applied Biosystems™) in a 3130 sequencer (Applied Biosystems™). The sequence obtained was deposited in the nucleotide databank of the National Center for Genetic Engineering and Biotechnology.

Figure 1. Morphological features of Arcopilus aureus MaC7A. (A) A. aureus in PDA medium. (B) The presence of globular/ellipsoid ascomata. (C) Ascomata showing lateral hyaline hyphae. (D) Terminal brown hyphae showing a coiled shape. (E) Vegetative mycelium. (F) Somas showing tabicated and hyaline hyphae.

2.2. Chemicals

Limonene, citral (geranial and neral mixture), camphor, menthol, thymol, L-phenylalanine, L-tyrosine, Glucanex powder (lysing enzymes from Trichoderma harzianum), NaOH, HCl, resazurin, 9-fluorenylmethyl-chloroformate, and trans-resveratrol were all obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). PDA (4 g L⁻¹ potato extract, 20 g L⁻¹ glucose, agar 15 g L⁻¹) and PDB (4 g L⁻¹ potato extract, 20 g L⁻¹ glucose) media were from Bioxon™, and peptone was from Difco™. Solvents for RV extraction and for the HPLC tests were from J.T. Baker.

2.3. Fungal Viability and Effect of pH and Temperature on Resveratrol Production

The effects of limonene, β-pinene, camphor, menthol, thymol, and Glucanex on the standard growth of Arcopilus aureus were investigated in order to determine their toxic concentrations (minimum inhibitory concentration; MIC). Dose–response curves of the volatiles and Glucanex (10–500 mg L⁻¹) were prepared using resazurin as an indicator of cell viability in 96-well plates at 30 °C for 48 h [18]. PDB was used as the medium of reaction in a final volume of 0.3 mL [18]. Absorbance was recorded at 595 nm in a microplate reader (Powcam). To determine the standard growth in liquid medium, batch cultures of A. aureus were prepared from fresh mycelium discs (10 mm diameter) extracted from 5-day-old PDA cultures. The discs were incubated directly in PDB with 10 g L⁻¹ of peptone at 30 °C and gently shaking (100 rpm). The generation of both RV and biomass were determined every 24 h for 12 days. The effect of pH (4–10) on the ability of A. aureus to produce RV was determined by adjusting the PDB medium with NaOH (0.1N) and HCl (0.1N) and the effect of temperature was studied by adjusting the incubator shaker by 10–40 °C. RV levels were estimated from batch cultures at 7 days. Each treatment was replicated ten times (n = 10).

2.4. Feeding Experiments with Amino Acid Precursors of Resveratrol

Dose–response curves of PHE (50–500 mg L⁻¹) and TYR (50–500 mg L⁻¹) were added into 250 mL glass flasks containing 100 mL of PDB medium previously inoculated with A. aureus and 10 g L⁻¹ peptone, which was reported as the preferred nitrogen source of
this fungus [14]. The batch cultures were kept at 30 °C with slight shaking (100 rpm), and the measurement of the biomass and RV were performed at day 7 post-treatment. The amino acids were previously emulsified in 80% ethanol prior to dissolving them in the PDB medium. The mycelium was extracted, dried under N\textsubscript{2} stream for 10 min, and weighed to estimate the fresh weight [14]. For the kinetics experiments, 200 mg L\textsuperscript{-1} of precursors were used, and RV production was monitored every 24 h for 12 days. Each time of sampling was replicated ten times (n = 10).

2.5. Feeding Experiments with Volatiles and Glucanex

Dose–response curves of limonene, citral, camphor, menthol, thymol (10–50 mg L\textsuperscript{-1}), and Glucanex (15–100 mg L\textsuperscript{-1}) were performed in cultures of 100 mL of PDB medium (pH 7) inoculated with \textit{A. aureus} and 10 g L\textsuperscript{-1} peptone as a nitrogen source [14]. The reactions were performed in 250 mL glass flasks at 100 rpm and 30 °C. The volatiles were previously emulsified in 50% ethanol prior dissolving them in the PDB medium, whereas Glucanex was initially dissolved in a sodium phosphate buffer at pH 7.4. The biomass was extracted and weighed as already reported [14]. RV production was monitored every 24 h for 12 days. Each time of sampling was replicated ten times (n = 10).

2.6. Optimized Batch Cultures

Batch cultures (1 L) were prepared from discs of mycelium (2 cm diameter) extracted from 5-day-old PDA cultures of and incubated directly in glass flasks (1 L) containing 500 mL PDB medium (pH 7; 20 g glucose L\textsuperscript{-1}), 10 g L\textsuperscript{-1} peptone, and 200 mg L\textsuperscript{-1} TYR. Citral (50 mg L\textsuperscript{-1}), thymol (50 mg L\textsuperscript{-1}), and Glucanex (100 mg L\textsuperscript{-1}) were added in separated reactions, which were kept in the dark at 30 °C with slight shaking (100 rpm). Batch parameters were monitored every 24 h for 12 days. The levels of glucose in the studied samples were measured by HPLC (Hewlett Packard 1050 system coupled with a 1047A refractive index detector) using an ORH-801 column under the analytical conditions described by Lefebvre et al. [19]. The levels of TYR were measured by HPLC using 9-fluorenylmethyl-chloroformate as a derivatization agent under the conditions previously reported by Fabiani et al. [20].

2.7. Extraction and Analysis of Resveratrol

The identification and quantification of RV was basically performed in accordance with a previous work [1]. Mycelium was harvested from samples by centrifugation at 3000 × g for 5 min and the free medium was extracted three times with an equivalent volume of diethyl ether. The organic extracts were dried in a centrifuge concentrator (RVC 2-25 CD) for 20 min and resuspended in 1 mL ethanol for HPLC analysis in a Dionex 3000 coupled with a diode array detector. Samples were kept at 4 °C in the dark to avoid oxidative degradation and the isomerization of \textit{trans}-RV to \textit{cis}-RV. HPLC run conditions were those described by Glavnik et al. [21] using a Hypersil ODS C\textsubscript{18} column (5 µm, 250 × 4.6 mm, 5 µm) at 306 nm.

2.8. Statistical Analysis

ANOVA-Tukey tests were carried out with SPSS version 17.0.2 to determine statistically significant differences among the treatments at \( p < 0.05 \).

3. Results

3.1. Morphological, Microscopic and Molecular Features of \textit{Arcopilus aereus} MaC7A

The wild strain of \textit{A. aureus} showed fast radial growth in PDA cultures (0.3275 cm day\textsuperscript{-1} at 25 °C). After ten days, the maximum growth observed was 3.45 cm day\textsuperscript{-1} at 25 °C. The morphology of this fungus in PDA medium showed colonies with white-yellow mycelium, which turned the white PDA medium to purple at the second day post-inoculation (Figure 1A).
The microbiological features of this strain were the presence of globular/ellipsoid ascomata associated with the surface of the colony (Figure 1B). These structures showed an evident amount of lateral hyaline hyphae (Figure 1B, C). The terminal hyphae had a clear coiled shape (Figure 1D). These contained immature ascomata with no lateral hyphae (Figure 1D). The presence of vegetative mycelium was also observed (Figure 1E). The hyphae generated from the soma were tabicated and hyaline (Figure 1F).

The partial sequence of the 18S ribosomal RNA gene obtained by ITS markers confirmed the homology of this fungus with several strains of *A. aureus* deposited at the NCBI nucleotide database (HQ607894.1, MN305798.1, MN588146.1). The sequence was validated and deposited at same data bank with the accession number MW318995.1, as well as being attached as supplementary information (Supplementary Sequence S1).

3.2. Standard batch Cultures of *Arcopilus aureus* MaC7A

The production of RV (Figure 2A, B) and the normal growth of *A. aureus* MaC7A were triggered at the fourth day under the controlled conditions using PDB medium (pH 7) at 30 °C and gently shaking (100 rpm) (Figure 2C). A direct proportional relationship was observed between the biomass and the production of RV until days 6–7. However, after day 9, the endogenous levels of RV decreased dramatically, whereas no statistically significant differences were observed in biomass accumulation from day 6 to day 12. A clear asymptotic tendency was observed for biomass accumulation after day 6. Maximum average levels of RV were calculated around 127.9 ± 4.6 mg L\(^{-1}\) at day 7, and the maximum biomass accumulation was around 195.2 ± 1.3 g fresh weight L\(^{-1}\) at day 8.

Table 1. MIC for volatiles and Glucanex on *Arcopilus aureus* MaC7A.

| Agent    | MIC (mg L\(^{-1}\)) * |
|----------|------------------------|
| Limonene | 267.7 ± 0.54 b         |
| Citral   | 130.82 ± 0.68 d        |
| Camphor  | 389.30 ± 0.79 a        |
| Menthol  | 92.19 ± 0.41 e         |
| Thymol   | 261.8 ± 1.32 b         |
| Glucanex | 201.5 ± 0.59 c         |

* MIC (mean ± SD; n = 10) with different letters indicate statistically significant differences (\(p < 0.05\)).

These data were operational for defining further concentrations able to stress *A. aureus* without affecting cell viability. The pressure of pH and temperature on *A. aureus* revealed that the production of RV had a maximum peak at pH 7 and 30 °C (Figure 3).

Figure 2. Determination of RV in batch cultures of *Arcopilus aureus* MaC7A. (A) RP–HPLC chromatogram of pure RV. (B) RP–HPLC chromatogram of the RV detected in batch cultures of *A. aureus* MaC7A. (C) Kinetics for the production of RV versus biomass formation at 30 °C and pH 7 in cultures of *A. aureus* MaC7A. * Indicates days with statically significant differences (\(p < 0.05\)) for RV and biomass production compared with that of day 7. Error bars indicate SD (n = 10).
3.3. Susceptibility of *A. aureus* MaC7A to Volatiles and Glucanex

*A. aureus* showed a high tolerance to almost all the volatiles assayed except for menthol and citral, which showed MIC values lower than 150 mg L\(^{-1}\) (Table 1). The inhibitory properties of limonene were similar to that of thymol, whereas camphor was less effective at decreasing the viability of *A. aureus*. Interestingly, the lysing enzymes from *Trichoderma harzianum* exerted inhibitory properties at around 200 mg L\(^{-1}\).

Table 1. MIC for volatiles and Glucanex on *Arcopilus aereus* MaC7A.

| Agent   | MIC (mg L\(^{-1}\)) * |
|---------|-----------------------|
| Limonene| 267.7 ± 0.54 \(^{b}\) |
| Citral  | 130.82 ± 0.68 \(^{d}\) |
| Camphor | 389.30 ± 0.79 \(^{a}\) |
| Menthol | 92.19 ± 0.41 \(^{c}\) |
| Thymol  | 261.8 ± 1.32 \(^{b}\) |
| Glucanex| 201.5 ± 0.59 \(^{c}\) |

* MIC (mean ± SD; n = 10) with different letters indicate statistically significant differences (\(p < 0.05\)).

These data were operational for defining further concentrations able to stress *A. aureus* without affecting cell viability. The pressure of pH and temperature on *A. aureus* revealed that the production of RV had a maximum peak at pH 7 and 30 °C (Figure 3).

![Figure 3](image-url)  
**Figure 3.** Effect of pH (A) and temperature (B) on the growth of *A. aureus* MaC7A and its RV production. * Indicates statically significant differences (\(p < 0.05\)) for RV production and biomass compared with that of pH 7 and 30 °C. Error bars indicate SD (n = 10).

Interestingly, both the growth and RV production of *A. aureus* were significantly affected (\(p < 0.05\)) at extreme pH values. A similar tendency was noted for temperatures of 10, 15, 35, and 40 °C, at which low levels of RV were detected. Both biomass and RV production were equally affected under these conditions.

3.4. Resveratrol Production in Feed Batch Cultures with Amino Acidid Precursors

The addition of PHE did not produce a significant change in the amount of biomass in batch cultures in comparison with the control group (205.5 ± 2.5 g L\(^{-1}\)) (Figure 4A). However, the concentration of RV was clearly enhanced (up to 207.8 ± 1.4 mg L\(^{-1}\)) by the addition of 100–500 mg L\(^{-1}\) PHE. Interestingly, no statistically significant differences were observed in the production of RV between the 200 and 500 mg L\(^{-1}\) PHE cultures. On the contrary, the batch cultures containing 500 mg L\(^{-1}\) PHE showed a slight decrease in RV compared with those containing 200 and 300 mg L\(^{-1}\).

The addition of TYR at 100, 200, and 300 mg L\(^{-1}\) to the cultures of *A. aureus* showed evident differences in the accumulation of biomass (238.2 ± 3.6 g L\(^{-1}\)) (Figure 4B). The assessment of the same concentrations of TYR produced higher RV levels (221.8 ± 5.2 mg L\(^{-1}\)) than those obtained with PHE. No statistically significant differences were observed in
RV accumulation between 200 and 300 mg L$^{-1}$ TYR. As for PHE treatment, a substantial decrease was perceived for the highest concentration assayed (500 mg L$^{-1}$).

Figure 4. Production of RV and biomass by the addition of different concentrations of PHE (A) and TYR (B) to batch cultures of *Arcopilus aureus* MaC7A analyzed at 7 days post-treatment. * Indicates statically significant differences (p < 0.05) for RV production compared with the control group (0 mg L$^{-1}$). Means with different letters indicate statistically significant differences among the biomass and amino acid concentrations (p < 0.05). Error bars represent SD (n = 10).

Kinetic analyses of batch cultures containing optimal concentrations of these amino acids (200 mg L$^{-1}$) showed that the highest RV accumulation was maintained from day 6 to day 8 (Figure 5). Nevertheless, RV levels were dramatically decreased after day 10 without a significant loss of biomass.

3.5. Resveratrol Production under the Pressure of Volatiles and Glucanex

Dose–response curves with limonene revealed that this monoterpene was unable to cause any significant change in the biomass of *A. aureus* and its RV production (Figure 6A), at least at the assayed concentrations. Unlike limonene, citral induced an evident change in RV production (187.8 ± 2.2 mg L$^{-1}$) and a significant increase in biomass was detected at 50 mg L$^{-1}$ (255.9 ± 6.4 g L$^{-1}$) (Figure 6B). As for limonene, cultures containing camphor did not produce significant changes in either biomass or RV production (Figure 6C). On the contrary, the addition of thymol (30–50 mg L$^{-1}$) resulted in a significant increase in RV (162.7 ± 4.1 mg L$^{-1}$) and biomass (237.1 ± 5.3 g L$^{-1}$) (Figure 6D). A deleterious effect was observed in cultures enriched with menthol in which concentrations of 20–50 mg L$^{-1}$ produced a significant decrease in biomass (142.5 ± 1.9 mg L$^{-1}$) and RV accumulation (80.2 ± 2.7 mg L$^{-1}$) (Figure 6E). All the concentrations assayed for Glucanex enhanced RV production (up to 198.3 ± 7.6 mg L$^{-1}$); however, only the concentrations of 30–100 g L$^{-1}$ generated a significant increase in biomass (Figure 6F).
Figure 5. Effects of 200 mg L\(^{-1}\) PHE (A) and 200 mg L\(^{-1}\) TYR (B) on the growth of *Arcopilus aureus* MaC7A and its RV production in batch cultures. * Indicates statically significant differences \((p < 0.05)\) for RV production and biomass compared with that of day 6. Error bars represent SD \((n = 10)\).

Figure 6. Production of RV and biomass by the addition of different concentrations \((0–50 \text{ mg L}^{-1})\) of limonene (A), citral (B), camphor (C), thymol (D), menthol (E) and Glucanex (F) \((0–100 \text{ mg L}^{-1})\) in batch cultures of *A. aureus* MaC7A analyzed at 7 days post-treatment. * Indicates statically significant differences \((p < 0.05)\) for RV production compared with the control group \((0 \text{ mg L}^{-1})\). Means with different letters indicate statically significant differences among the biomass and volatile concentrations \((p < 0.05)\). Error bars represent SD \((n = 10)\).
3.6. Resveratrol Production in Optimized Batch Cultures

The production of RV under batch cultures revealed that citral and Glucanex produced up to 225.1 ± 3.5 and 237.6 ± 4.7 mg L\(^{-1}\) RV, respectively (Figure 7A,B). Batch cultures with thymol only produced 176.9 ± 3.5 mg L\(^{-1}\) RV. An inverse relationship was observed between the consumption of TYR and the production of RV. The maximum peak of RV production was observed at day 9 for the cultures containing citral and thymol. The consumption of TYR at day 9 was completed in the cultures containing citral and thymol, whereas for those containing Glucanex, the maximum peak of RV production was observed at day 9. Nevertheless, the consumption of TYR was completed at day 8 in batch cultures containing Glucanex (Figure 7C). A dramatic decrease in RV levels was observed at day 12 in all the studied batch cultures.

![Kinetics of optimized batch cultures of A. aureus MaC7A.](image)

**Figure 7.** Kinetics of optimized batch cultures of *A. aureus* MaC7A. (A) Batch cultures containing 50 mg L\(^{-1}\) citral. (B) Batch cultures containing 50 mg L\(^{-1}\) thymol. (C) Batch cultures containing 100 mg L\(^{-1}\) Glucanex. Each point from the kinetics was assayed ten times (n = 10).

The content of glucose was depleted at day 10, whereas the maximum biomass accumulation was observed from day 7 to day 12. Batch cultures treated with thymol accumulated a similar amount of biomass (229 ± 4.2 g L\(^{-1}\)) to the cultures treated with...
citral and Glucanex. For all the studied cultures, an inverse relationship was observed between the consumption of TYR and the production of RV.

4. Discussion

Endophytic fungi represent a new source for the obtainment of RV under controlled conditions [11–14]. A previous work reported on the production of A. aureus isolated in India, as well as some interesting conditions involved in the production of RV [14]. In the present work, we report the same species isolated in a different geographical region, which shows similar but not identical characteristics to those of the strain isolated previously [14]. The same species was treated with potential elicitors that stimulated RV biosynthesis. According to our results, differences in the biosynthetic capacity of A. aureus MaC7A were observed. Under standard conditions (PDB medium, pH 7.0, 10 g L\(^{-1}\) peptone, 30 °C), the production of RV was around 127.9 ± 4.6 mg L\(^{-1}\) at day 7, and the maximum biomass accumulation was around 195.2 ± 1.5 g fresh weight L\(^{-1}\) at day 8. The maximum accumulation of biomass and RV differed to that observed for the A. aureus isolated in India [14]. This fact strongly suggests a different capacity of A. aureus MaC7A to produce RV. Nevertheless, the optimal temperature and pH were the same as those reported previously for the A. aureus isolated in India [14]. Unlike previous studies, the amount of biomass was relatively constant during days 10–12 under our experimental conditions [14]. It is known that the degradation of biomass does not occur spontaneously. Such loss is produced gradually after the stationary phase in all microorganisms because of the lack of nutrients, which accelerates programmed cell death. The fact that the biomass of A. aureus MaC7A remained relatively constant during days 6–12 indicates that not all of the cells were functional at these times. Optimized batch cultures confirmed that glucose levels decreased gradually and were almost undetectable at day 8. Reasonably, the metabolism of the fungus should be focused on the maintenance of basic functions to assure survival instead of biosynthesizing RV at these unfavorable times.

Since peptone contains variable amounts of bioavailable PHE and TYR, which are generally low or negligible (per 100 g product), the addition of amino acid precursors to the batch cultures was investigated. Dose–response curves with PHE and TYR revealed that concentrations of 200–500 mg L\(^{-1}\) generated an increase in RV accumulation, but the highest concentration of both amino acids (500 mg L\(^{-1}\)) did not produce a substantial increase of RV compared with a concentration of 200 mg L\(^{-1}\). Considering this evidence, further experimentation was performed with 200 mg L\(^{-1}\) as the optimal concentration of amino acid precursors, which was able to increase 60% of the amount of RV compared with that from standard batch cultures. Interestingly, the addition of TYR produced about 12 mg more RV (221.8 ± 5.2 mg L\(^{-1}\)) than PHE (207.8 ± 1.4 mg L\(^{-1}\)). These differences may originate from the solubility and bioavailability of TYR in aqueous media, and probably due to the amphipathic properties of TYR [22]. According to these data, A. aureus MaC7A metabolized both precursors to produce RV, and TYR was more effectively converted into RV. Kinetics of batch cultures containing 200 mg mL\(^{-1}\) demonstrated that the maximum production of RV was maintained from day 6 to day 8 post-treatment. Interestingly, the amount of biomass was more abundant in the batch cultures treated with TYR (236 ± 3.7 g L\(^{-1}\)).

As for plant species, fungi also produce secondary metabolites, which play a crucial ecological role as defensive chemicals [23]. The biosynthesis of these metabolites may be stimulated under the pressure of distinct elicitors, which are sensed by other microbial competitors [24]. It is known that plant volatiles exert diverse changes in the physiology of fungi, including a chemical counter-response [25]. Thus, volatiles may be used as elicitors to stimulate a chemical response in fungi [25]. The volatiles assayed in this study were selected on the basis of their fungistatic or fungicide activity, which can generate physiological stress at low concentrations. These substances were also chosen because of their commercial availability [26]. According to our results, the concentrations of these substances assayed (lower than MIC) probably exerted a kind of pressure on the metabolism of A. aureus,
which was reflected in the accumulation of biomass and RV. According to our data, the assayed concentrations of elicitors did not affect cell viability. To the best of our knowledge, this is the first work showing the effect of volatiles on the production of RV. Limonene and camphor did not produce a statistically significant increase in RV, whereas menthol caused a dramatic reduction in RV concentration. This result may be correlated with the strong antifungal activity of menthol, which was observed in the broth microdilution tests (Table 1). Due to the latter fact, these volatiles were discarded in further experimentation. On the contrary, citral (a mix of isomers) and thymol stimulated the production of RV, with levels of 187.8 ± 2.2 mg L−1 and 162.7 ± 4.1 mg L−1, respectively. According to these results, citral and thymol were able to increase RV production by around 40%, and the biomass by around 25% in comparison with the control batch cultures. Glucanex, which is a mixture of β-1,3 glucanases (with optimal activity at pH 7), produced the best accumulation of RV (198.3 ± 7.6 mg L−1; >50%), with significant biomass accumulation (230.6 ± 3.8 g L−1; >20%). This evidence suggested that the biosynthesis of RV in A. aureus is stimulated by enzymes with antagonist activity produced by fungal competitors [27]. The induction of biomass by Glucanex, thymol, and citral may be produced as an alternative defense of A. aureus to ameliorate the stress caused by these substances. Previous studies suggest that some volatiles and specific enzymes are able to increase fungal biomass [24].

The best variables were reassessed in batch cultures with optimal temperatures, pH, amino acid precursors, and the three more effective elicitors for RV production. Glucose levels decreased gradually and were negligible at day 10, whereas biomass increased within days 7–12. This fact strongly suggests that the carbon source was used for both the accumulation of biomass and the production of RV. The consumption of TYR was completed at day 9 for cultures containing both volatiles and at day 8 for those containing Glucanex, suggesting that the hydrolytic enzymes of Glucanex stimulate the use of TYR for conversion into RV. This fact was endorsed by the highest accumulation of RV in these cultures. The same results demonstrate that the production of RV in A. aureus can be efficiently enhanced by the combination of volatiles and isoenzymes with β-1,3 glucanase activity to generate 225–237.6 mg L−1 RV, which represents almost twofold higher RV concentration than that reported in a previous work [14]. A. aureus MaC7A produced 0.23 g L−1 RV, which is a better amount than that reported by recombinant yeasts that produce RV from p-coumaric acid (0.04 g L−1 RV) [1].

5. Conclusions

Arcopilus aureus MaC7A produced RV under controlled conditions. PHE and TYR stimulated the production of RV up to 127.9 ± 4.6–221.8 ± 5.2 mg L−1, whereas citral and Glucanex were the best elicitors, and enhanced RV production by up to 180 mg L−1. Optimized batch cultures containing TYR, citral, thymol (50 mg L−1), and Glucanex (100 mg L−1) produced up to 237.6 ± 4.7 mg L−1. Our results strongly suggest that the biosynthesis of fungal RV can be enhanced by specific monoterpenes and isoenzymes with β-1,3 glucanase activity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11043833/s1, Sequence S1: Partial ITS sequence of A. aureus MaC7A accession MW318995.1.

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