Bioconversion of antifungal viridin to phytotoxin viridiol by environmental non-viridin producing microorganisms

Gilles-Alex Pakora, Stéphane Mann, Daouda Kone, Didier Buisson

► To cite this version:

Gilles-Alex Pakora, Stéphane Mann, Daouda Kone, Didier Buisson. Bioconversion of antifungal viridin to phytotoxin viridiol by environmental non-viridin producing microorganisms. *Bioorganic Chemistry*, 2021, 112, pp.104959. 10.1016/j.bioorg.2021.104959 . hal-03263988

HAL Id: hal-03263988

<https://hal.science/hal-03263988>

Submitted on 26 Nov 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Bioconversion of antifungal viridin to phytotoxin viridiol by environmental non-viridin producing microorganisms

Gilles-Alex Pakora ^{a,b,*}, Stéphane Mann ^a, Daouda Kone ^c and Didier Buisson ^{a,*}

^aLaboratoire Molécules de Communication et Adaptation des Micro-organismes (MCAM), Muséum national d'Histoire naturelle, CNRS ; CP54, 57 rue Cuvier, 75005 Paris, France

^bLaboratoire de Pharmacodynamie Biochimique, UFR Biosciences, Université Félix Houphouët Boigny d'Abidjan (UFHB), 22 BP 582 Abidjan 22, Côte d'Ivoire

^cLaboratoire de Physiologie Végétale, UFR Biosciences, Université Félix Houphouët-Boigny d'Abidjan (UFHB), 22 BP 582 Abidjan 22, Côte d'Ivoire

*** Correspondence:**

Gilles-Alex Pakora

pakoragillesalex@yahoo.fr

Didier Buisson

didier.buisson@mnhn.fr

Abstract

Biotransformation of viridin, an antifungal produced by biocontrol agent, with non-viridin producing microorganisms is studied. The results show that some environmental non-targeted microorganisms are able to reduce it in the known phytotoxin viridiol, and its 3-epimer. Consequently, this reduction, which happens in some cases by detoxification mechanism, could be disastrous for the plant in a biocontrol of plant disease. However, a process fermentation/biotransformation could be an efficient approach for the preparation of this phytotoxin.

Keywords: Biotransformation, Viridin, Detoxification, Reduction, *Pichia*, *Rhodococcus*, *Mucor*

1. Introduction

Biological control is a strategy of pests and disease management using another organism. It's particularly developed in the control of plant diseases. It provides an alternative to the use of chemicals and it receives a good public acceptance as a natural method. Microorganisms are biological control agent acting with different mechanisms, such as antibiosis, mycoparasitism, production of cell wall-degrading enzymes, competition for nutrients and/or space and induced resistance in plant [1,2]. Several mechanisms could be combined in the control disease, which is achieved to antagonize pathogens. *Trichoderma* species are used as biological control agent of several destructive plant pathogens and are commercialized as biological fungicide [3].

Microbial secondary metabolites play a crucial role in the fight against plant diseases [4]. Viridin (**1**), gliovirin and gliotoxin are the most known metabolites involved in the antibiosis. Viridin (**1**), which first described in 1945 [5] is a steroidal antibiotic produced by *Gliocladium* and *T. virens*. Strains are used against damping-off of bean (*Rhizoctonia solani*) [6], of cucumber (*R solani*, *Pythium ultimum*, *Meloidogyne incognita*) [7], cotton seedling disease [8] and gray mold of chickpea (*Botritis cinerea*) [9]. We demonstrated that *T. virens* T7, a biocontrol agent of cocoa black pod diseases, produced gliovirin and viridin **1** [10]. Gamma-ray induced mutants of *Trichoderma* with better antagonistic effect against phytopathogen than the wild-type have been generated. They showed a greatly increasing production of secondary metabolites as viridin [11].

Viridin (**1**) could be reduced to viridiol (**2**) (Fig. 1) by viridin-producing strains [12]. Viridiol (**2**) is less active against phytopathogens but it is a phytotoxin [13,14]. The production of this herbicidal molecule by the microorganisms restricts their use as biological control agent. Studies have been performed to avoid the production of viridiol (**2**). It was found that production of viridiol (**2**) by *G. virens* could be inhibited when culture medium contains cytochrome P-450 inhibitors [15]. Another efficient approach was obtained by producing mutants of *T. virens* which were deficient for the production of viridiol (**2**) [16].

Any molecule that ends up in the environment can undergo biotic and abiotic transformations to give new molecules with new biological activities. It is in this area of chemical ecology that we are interested in microbial biotransformation of chemicals and natural molecules. For example, concerning the former, we have shown that environmental biotransformation of drugs that can lead to the formation of more ecotoxic metabolites [17,18]. For the second, we have shown that juglone, a phytotoxic naphtoquinone produced by the walnut, is reduced by an

endophytic fungus to give isosclerone [19]. We present here a study concerning the production of viridin (**1**) and its microbial metabolism by environmental non-producing microorganisms. The aim of this study is to evaluate their ability to transform viridin (**1**) and to study the relationship between metabolism and detoxifying power.

2. Materials and Methods

2.1. General

TLC was performed on pre-coated silica gel plates (Silice 60 F₂₅₄ Merck, Germany). 1D and 2D NMR spectra were recorded in CDCl₃ and CD₃OD with Bruker 400 and 600 spectrometers using TMS as internal standard. The chemical shifts were given in δ (ppm). HR-ESI-MS and fragmentation MS spectra were obtained with an Applied Biosystem QSTAR Pulsar I mass spectrometer. Analytical HPLC analyses were performed on GILSON system with pump 305, pump 306, gradient dynamic mixer 811B and autoinjector 234. The system was controlled and the results were analyzed by Unipoint Gilson software. The column (Hypersil, C18 5 μ m (250x4.6)) was eluted (1 mL/min) with a gradient solvent system: solvent A (H₂O-TFA 1%) 60% and solvent B (CH₃CN/H₂O-TFA 1% (50/50)) 40% isocratic for 3 min followed by gradient to 60% solvent B in 10 min and held at 60% solvent B for 5 min.

Preparative HPLC were performed on a Agilent system equipped with XDB column (150 x 21.2 mm) using gradient solvent system: solvent A (H₂O-TFA 0.05% /CH₃CN (95/5)) 85% and solvent B (CH₃CN/H₂O-TFA 0.05% (95/5)) 15% isocratic for 2 min. followed to gradient to 25% solvent B in 30 min., flow 10 mL/min. For analytical and preparative HPLC, the detection wavelengths were 230 and 250 nm. Solvent were HPLC grade and use as such.

2.2. Microorganisms.

Trichoderma virens was provided to us by the National Center for Agricultural Research in Côte d'Ivoire [20] and maintained on PDA at 4°C. Other microorganisms were stored at 4°C in medium containing Bacto peptone 5 g/L, yeast extract 5 g/L, malt extract 5 g/L, glucose 20 g/L and agar 20g/L.

2.3. Preparation of viridin (**1**) and viridiol (**2**).

Conidia of *T. virens* grown on PDA for 5 days were collected by sterile deionized water (1.5×10^8 conidia/mL). Fifteen Roux flasks containing 250 mL PDA were inoculated with 2 mL of conidial suspension and the cultures were incubated for 7 days at 25°C in dark. Solid media fermentation and mycelia were recovered and extracted with ethyl acetate 3 times with magnetic stirring. The organic layer was evaporated to obtain 4.79 g of crude extract.

2.3.1. Viridin (1)

This extract is triturated and washed three times with MeOH (3 mL) to give viridin (1) (82 mg) as a pale greenish powder. HR-ESI-MS m/z $[M+H]^+$ 353,1018 (calc. for $C_{20}H_{16}O_6 + H^+$: 353,1025). 1H -NMR ($CDCl_3$, 400 MHz): δ 8.64 (1H, *d*, $J = 8.2$ Hz, H-11), 8.28 (1H, *s*, H-20), 7.95 (1H, *d*, $J = 8.2$ Hz, H-12), 4.26 (1H, *dd*, $J = 5.0$ Hz, 10.6 Hz, H-1), 3.87 (1H, *d*, $J = 5.0$ Hz, H-2), 3.73 (3H, *s*, H-19) 3.61-3.85 (2H, *m*, H-15), 2.72 (2H, *m*, H-16), 1.66 (3H, *s*, H-18), ^{13}C - NMR ($CDCl_3$, 100 MHz): δ 206.3 (C-17), 186.9 (C-3), 173.2 (C-7), 157.9 (C-14), 156.4 (C-10), 149.3 (C-20), 147.0 (C-6), 142.9 (C-5), 137.2 (C-8), 129.8 (C-13), 127.8 (C-11), 127.6 (C-12), 121.6 (C- 4), 83.5 (C-2), 73.2 (C-1), 60.9 (C-19), 41.9 (C-9), 36.3 (C-16), 29.7 (C-15), 28.4 (C-18),

2.3.2. Viridiol (2)

Methanol phases were pulled and after evaporation, the extract was chromatographed on LH 20 and SiO_2 ($CH_2Cl_2/MeOH$) to obtain viridiol (2) (7 mg) as a yellow oil. HR-ESI-MS m/z $[M+H]^+$ 355,1190 (calc. for $C_{20}H_{18}O_6 + H^+$: 355,1182). 1H -NMR ($CDCl_3$, 400 MHz): δ 8.28 (1H, *d*, $J = 8.1$ Hz, H-11), 7.96 (1H, *s*, H-20), 7.81 (1H, *d*, $J = 8.1$ Hz, H-12), 5.15 (1H, *dd*, $J = 3.4$ Hz 1.0 Hz, H-3), 4.34 (1H, *d*, $J = 3.78$ Hz, H-1), 3.87 (1H, *dd*, $J = 6.2$ Hz 4.6 Hz, H-2), 3.80-3.86 (1H, *m*, H-15a), 3.67-3.73 (1H, *m*, H-15b), 3.73 (3H, *s*, H-19) 2.70-2.76 (2H, *m*, H-16), 1.73 (3H, *s*, H-18), ^{13}C - NMR ($CDCl_3$, 100 MHz): δ 206.7 (C-17), 173.7 (C-7), 158.7 (C-10), 158.1 (C-14), 145.8 (C-6), 145.6 (C-20), 142.4 (C-5), 137.0 (C-8), 129.9 (C-13), 127.4 (C-12), 127.3 (C-11), 122.1 (C- 4), 81.7 (C-2), 71.8 (C-1), 61.7 (C-3), 60.8 (C-19), 42.4 (C-9), 36.5 (C-16), 30.5 (C-18), 28.5 (C-15).

2.3.3. Stability of viridin (1)

The effect of pH on viridin stability was studied in citrate buffer media (pH 3 to 8) at 0.5 unit intervals during 48h.

2.4. Biotransformations

2.4.1. Screening of actives strains

Screening experiments were carried out in resting cells conditions. Bacteria and fungi (Table S1) were tested using conventional method and yeasts using our method based on combinatory library of microorganisms (CLM). Cultures were conducted in Erlenmeyer flasks (50 mL) filled with YMS culture medium (20 mL) containing (in g/L): yeast extract 4, malt extract 10, soybean peptone 5 and glucose 16. Microorganisms were grown at 26°C on rotary shaker (160 rpm). After 48 hours, biomasses were recovered by filtration for filamentous fungi and centrifugation for bacteria and yeasts. Biomasses of bacteria and fungi were suspended in 10 mL of citrate buffer (0.1M, pH5) and biomasses of yeasts were suspended in 5 mL and four mixtures were prepared by placing 2 mL of each suspensions as described in Table S2. Viridin (**1**) was added (finale concentration 0.1 mg/mL) in solution of DMSO (10 µL). Incubations were incubated in same conditions. Controls without compound **1** and non-inoculated flasks containing compound **1** were conducted in same conditions.

Experiments at different concentrations of viridin were conducted in the same manner. Every day, samples (500 µL) were withdrawn, for HPLC analysis and then extracted by EtOAc for TLC and MS.

2.4.2. Reduction of viridin (**1**) by *Rhodococcus globerolus*

Culture of *Rhodococcus globerolus* were conducted in 500 mL Erlenmeyer flasks containing 200 mL of YMS inoculated with 48h-old pre-culture. *R. globerolus* cells were taken up in 40 mL of citrate buffer at pH 5 and viridin (20 mg dissolved in DMSO) was added in the flask to be incubated for 48 hours. The procedure and conditions were as in screening experiments. The residue (31 mg) obtained was purified by preparative HPLC to give **2** (1.1 mg) and **3** (1 mg).

Viridiol (**2**): ¹H-RMN (600 MHz, CD₃OD): δ 8.55 (1H, *d*, *J* = 8.1 Hz, H-11), 7.91 (1H, *d*, *J* = 1.0 Hz, H-20), 7.88 (1H, *d*, *J* = 8.1 Hz, H-12), 4.99 (1H, *dd*, *J* = 4.52 1.0 Hz, H-3), 4.30 (1H, *d*, *J* = 3.78 Hz, H-1), 3.79 (1H, *dd*, *J* = 4.52Hz 3.78 Hz, H-2), 3.70-3.80 (H, *m*, H-15a), 3.72 (3H, *s*, OMe), 3.60-3.70 (H, *m*, H-15b), 2.70-2.76 (2H, *m*, H-16), 1.75 (3H, *s*, Me). ¹³C-RMN (150 MHz, CD₃OD) : δ 208.8 (C-17), 173.4 (C-7), 161.6 (C-10), 159.1(C-14), 147.5 (C-20), 146.6 (C-5), 146.3 (C-6), 137.7 (C-8), 130.9 (C-13), 128.0 (C-11), 127.3 (C-12), 125.4 (C-4), 86.5 (C-2), 73.8 (C-1), 64.9 (C-3) , 61.9 (C-19), 43.6 (C-9), 37.2 (C-16), 32.3 (C-18), 29.0 (C-15).

epi-Viridiol **3**: ¹H-RMN (600 MHz, CD₃OD): δ 8.60 (1H, *d*, *J* = 8.2 Hz, H-11), 7.93 (1H, *d*, *J* = 1.2 Hz, H-20), 7.90 (1H, *d*, *J* = 8.2 Hz, H-12), 5.07 (1H, *dd*, *J* = 3.0 Hz 1.2 Hz, H-3), 4.13 (1H, *d*, *J* = 5.1 Hz, H-1), 3.75-3.82 (1H, *m*, H-15a), 3.70-3.75 (1H, *m*, H-15b), 3.68 (1H, *dd*, *J* = 5.1 Hz 3.0 Hz, H-2), 3.66 (3H, *s*, OMe), 2.70-2.74 (2H, *m*, H-16), 1.59 (3H, *s*, Me). ¹³C-RMN

(150 MHz, CD₃OD) : δ 208.8 (C-17), 173.4 (C-7), 159.6 (C-10), 159.1 (C-14), 147.1 (C-20), 146.3 (C-5), 146.3 (C-6), 137.4 (C-8), 131.2 (C-13), 129.1 (C-11), 126.9 (C-12), 125.5 (C-4), 89.1 (C-2), 72.3 (C-1), 66.0 (C-3), 59.8 (C-19), 43.4 (C-9), 36.7 (C-16), 29.0 (C-15), 28.6 (C-18).

2.5. Evaluation of growth inhibiting activity

Cells suspensions of *P. anomala* and *M. racemosus* in glycerol (15%) were obtained from agar slant and spread on the PDA plates. Cells suspension of *R. globerolus* were obtained from 24h-hold culture in LB liquid medium and spread on LB agar medium (yeast extract 5g/L; NaCl 10g/L; agar 20 g/L) using a swab. After one hour, antibiogram paper disks (6 mm diameter) treated with viridin (**1**) or viridiol (**2**) (ten microliters of DMSO solutions of were transferred onto the agar plates (in triplicates). Ten microliters of DMSO were applied to control disks. Plates were incubated at 26°C and 37°C for bacteria, and inhibition zone were daily measured as the formula $S=\pi\times R^2$.

3. Results

T. virens T7, isolated in Cote d'Ivoire, has been used and an optimization of viridin (**1**) production was performed by culturing the microorganism in different conditions. Viridin (**1**) and viridiol (**2**) (Fig. 1) were isolated from an ethyl acetate extract of agar and biomass prepared by culture on solid medium (PDA) in the dark at 25°C. Viridin (**1**) was obtained by precipitation in methanol and viridiol (**2**) by chromatography as described in Materials and Methods. Structures of products were established by comparison with literature. Natural viridin (**1**) which is a α -methoxy ketone can undergo an epimerization at C-2 position to give 2-epi-viridin. [21] In order to study its stability, we incubated **1** in buffer solutions (pH 3.0-8.0). Its isomerization was observed in pH > 5.5. In buffer pH $\leq$ 5.5, viridin (**1**) is stable and no formation of 2-epi-viridin was observed.

Fig. 1. Structures of viridin (**1**) and viridiol (**2**)

It seemed interesting to study the biotransformation of viridin (**1**) by various microorganisms because its fate in the environment, and in particular its biotic transformation products have never been investigated. Since viridin (**1**) is known to be fungistatic and showed low antibacterial activity [22], it was possible to conduct this study under resting cell conditions. In fact, under these conditions the microorganisms are grown in good conditions and the biomass is set to incubate in the presence of the molecule studied. Forty-five microorganisms from a broad range of environment, including bacteria (22 strains), fungi and oomycetes (11 strains) and yeasts (12 strains), have been screened for their ability to biotransform viridin (**1**). While bacteria and fungi have been tested individually, the screening of yeasts was made on mixtures of strains according to our method based on combinatory library of microorganisms (CLM) [23]. Thus, the twelve yeasts were tested in four incubations. Incubation media (37) were analyzed after 48 hours incubation time. The data analysis of TLC showed that fifteen microorganisms were able to transform viridin (**1**) (Table 1). In some cases, the main product had the same R_f value (R_f 0.38, CHCl₃/acetone 7:3) than viridiol (**2**) obtained by fermentation with *T. virens*. LC/MS and NMR data confirmed the structure of viridiol (**2**) (Table S3, Fig S1).

	Genus	Number of tested strains	Number of active strains
Bacteria (22)	<i>Bacillus</i>	4	1
	<i>Nocardia</i>	1	1
	<i>Pseudomonas</i>	3	0
	<i>Rhodococcus</i>	14	9
Oomycetes (3)	<i>Phytophthora</i>	3	0
Fungi (8)	<i>Aspergillus</i>	2	0
	<i>Botrytis</i>	1	1
	<i>Fusarium</i>	1	0
	<i>Mucor</i>	4	3
Yeasts (12)	<i>Cryptococcus</i>	1	0
	<i>Dipodascus</i>	1	0
	<i>Kloeckera</i>	1	0
	<i>Kluyveromyces</i>	2	0
	<i>Pichia</i>	4	1
	<i>Rhodospiridium</i>	2	0
	<i>Yarrowia</i>	1	0

Table 1: Active microorganisms in biotransformation of viridin (**1**)

Half of the tested bacteria were able to transform viridin (**1**), e.g. *Bacillus cereus*, *Nocardia coralina* and some *Rhodococcus* species. Among them, *R. globerolus* caught our

attention because it gave two metabolites. The eleven tested fungi belong to the genus *Aspergillus* (2), *Botrytis* (1), *Fusarium* (1), *Phytophthora* (3) and *Mucor* (4). Three *Mucor* species were able to transform this compound, and one of them, *M. racemosus*, is a plant pathogen (tomatoes[24]). While *Fusarium oxysporum* and *Phytophthora* spp, were not able to transform viridin (**1**) under the study conditions, analysis of the *Botrytis cinerea* incubation medium showed the presence of two new metabolites whose identification requires further investigations. Since a biotransformation was observed for a single mixture of yeasts (pool 2) it was possible to conclude that only one strain is active and to identify it quickly. Indeed, it is the strain *P. anomala*, it was the only one that was present exclusively in this mixture (table S1).

The ability to reduce viridin (**1**) by *M. racemosus*, *P. anomala* and *R. globerolus* had been investigated using concentrations of 0.5 and 1 mg/mL. The kinetic study of reduction of viridin (**1**) by the fungi and the yeast showed the fast disappearance of substrate from the incubation medium because of its absorption into cells. Then, viridiol (**2**) as main biotransformation product was slowly released in incubation medium. In order to carry out a kinetic study, it is necessary to extract viridin (**1**) from the cells by methanol treatment and sonication before HPLC analysis. *M. racemosus* reduced viridin (**1**) at 0.5 mg/ml in 40 hours, and *P. anomala* was less active, since the reduction in more than 48 hours. For both microorganisms, no formation of viridiol (**2**) was observed during incubation at 1 mg/ml. Thus, their ability to reduce viridin (**1**) to give viridiol (**2**) is concentration-dependent.

The biotransformation of viridin (**1**) by *R. globerolus* was even faster, there was only 19% viridin (**1**) left after 4 hours of incubation. TLC profiles and HPLC chromatograms of experiment conducted at 0.5 mg/mL showed the presence of two metabolites whose formation kinetics are presented in Fig. 2. One of them had the same R_f value and retention time (8 min.) than the viridiol (**2**). The second metabolite **3**, with greater R_f (0.48) and retention time (10 min.) was a isomer of **2** with molecular ion at m/z 355.119 for $[M + H]^+$ and formula C₂₀H₁₈O₆. Other peaks observed in its electrospray ionization (ESI) mass spectrum were 336 ($M^+ - 18$), 308 ($M^+ - 46$) and 280 ($M^+ - 74$). This fragmentation pattern was similar to that described for viridiol **2** [12,25]. The ¹H- and ¹³C-NMR spectra of **3** were also very similar to those of **2**, indicating that a reduction of the 3-oxo group in viridin (**1**) had occurred, and not at the C-7 and C-17 carbonyls which appear in the carbon spectrum of **3** at 173.3 ppm and 208.8 ppm, respectively (Table S3, Fig S2 and S3).

Fig. 2. Conversion of viridin (**1**) (X) (0.5 g/l) to viridiol **2** (■) and 3-epi-viridiol **3** (▲) by *R. globerolus* over the incubation time.

In the NOESY spectrum of **3**, a correlation between H-3 and H-18 allowed to determine the α -stereochemistry of the hydroxy group at C-3. Another correlation of H-19 with H-18 confirmed that viridin (**1**) did not undergo epimerization under the pH incubation conditions. Therefore, this second metabolite **3** possesses a 3S-configuration, and was named 3-epi-viridiol. On the other hand, enantiomeric synthesis of both epimers, **2** and **3**, have been described [26].

Fig. 3. Select COSY, HMBC and NOESY correlations

In summary, reduction of viridin (**1**) by *R. globerolus* occurs only in one carbonyl group, but with low stereoselectivity, giving two metabolites without affecting the conjugated double bonds of the molecule (Fig. 4).

Fig. 4. Biotransformation of viridin (1) by *R. globerolus*

As viridin (1) is not active against bacteria, biotransformation cannot be therefore considered as a detoxification mechanism in these organisms. On the contrary, since viridin (1) has antifungal activity, we can suggest such a role in other microorganisms. Thus, we investigated the relationship between the reducing capacity of two selected microorganisms and biological activity of viridin (1) and viridiol (2). However, we also tested their activity against *R. globerolus* as we have shown that this bacterium is capable of reducing 1. Both molecules were inactive.

The antifungal activity of viridin (1) and viridiol (2) was evaluated using paper disk assays and results are given as inhibition areas in Table 2 (*M. racemosus*) and Table 3 (*P. anomala*). In both cases, we observed an inhibition of the growth by viridin (1) but viridiol (2) was totally inactive. However, we pointed out that the inhibition areas decreased during the incubation in particular with fungus. For example, 25 µg of viridin (1) produced an inhibition area of 132 mm² after 24 hours incubation, which decreased to 78 mm² after 48 hours and finally, no inhibition was detected after 72 hours.

Time (h)	Viridin (1) (µg)					Viridiol 2 (µg)
	3.12	6.25	12.5	25	50	50
24	0	50.2	78.5	132.6	314	0
48	0	0	50	78	176	0
72	0	0	0	0	78	0

Table 2: Effect of viridin (1) and viridiol (2) at different concentrations on mycelial growth of *M. racemosus* given in areas of inhibition zones (mm²).

Although viridin (1) was more active against *P. anomala*, the same phenomenon was observed, however the inhibition areas have not totally disappeared after 96 hours. As we checked the stability of the viridin (1) on a PDA culture medium (pH <5.5) we can conclude that the two microorganisms are able to detoxify 1, if its concentration is not too high.

Time (h)	Viridin (1) (μg)		Viridiol (2) (μg)
	5	10	10
48h	113	176	0
72h	50	78	0

Table 3: Effect of viridin (**1**) and viridiol (**2**) at different concentrations on growth of *P. anomala* given in areas of inhibition zones (mm^2).

4. Discussion

Many specialized metabolites produced by plants or microorganisms are defense molecules. Their biosynthesis may be constitutive or stress-induced [27]. In response and to develop, the target organisms must detoxify them by biotransformation. The two main mechanisms for detoxifying xenobiotics, whether natural or not, involve a variety of reactions that aim either to increase their hydrophilia, generally by conjugation [28], or to deactivate them. This, are well described for phytoalexins [29] and mycotoxins [30]. These biotransformations may occur as part of the use of microorganisms for the bio-control of phytopathogens by antibiosis. Detoxification reactions can be carried out by the target microorganisms but also by other endophyte or epiphytic microorganisms. In this case the implementation of biocontrol is more delicate because it is dependent on the absence of these microorganisms. We tested a range of environmental microorganisms and among them, bacteria of the genus *Rhodococcus* which are known to be active in bioconversions [31] and in bioremediation [32]. These endemic bacteria have been found to thrive in soil, water, and eukaryotic organisms.

Viridiol (**2**) which was obtained as main product during all biotransformation was the result of a highly regio-selectivity since viridin (**1**) is a tri-ketone. Two carbonyl groups are adjacent to a benzene nucleus, one forming an indanone framework and the other a naphthalene framework. These two ketones are not reduced during viridin biotransformation. On the contrary, the third carbonyl group of **1**, which is adjacent to methoxy group was reduced to form viridiol (**2**). Our results were consistent with the reduction of viridin (**1**) as detoxification mechanism and showed that non-viridin producing microorganisms are able to transform **1** to **2**. These microorganisms can be non-sensitive (bacteria) or sensitive (yeast and fungi) to viridin (**1**). The reduction of viridin (**1**) by microorganisms present in environment could reduce the effectiveness of biocontrol in two ways.

The first is the transformation of the active substance in a derivative without antifungal activity, which is released in environment. Then, phytopathogen fungi could benefit to this inactivation. The reduction of viridin (**1**) by *P. anomla*, a non-Saccharomyces wine yeast [33], is interesting regarding the biocontrol of *Botrytis cinerea*, responsible of botrytis bunch rot in grape, by *Trichoderma* sp. Indeed, we determined activity of viridin (**1**) against *B. cinerea* and found that spore germination is strongly prevented by viridin (**1**) with MIC 20 ± 2 $\mu\text{g/mL}$. However, despite this good activity, *T. virens* is not used as BCA against grey mould. [34]. Results from biotransformation experiments leading to inactivation provide insights into the structural elements essential to the activity and may assist in the design of new control agents. [35] In the case of viridin (**1**), as the reduction of ketone leads to a total loss of antifungal activity, it is necessary to design molecules for which this reduction is disadvantaged.

The second is that viridiol (**2**) is a phytotoxin with necrotic activity [14] which could have disastrous effects against the treated plant. On the contrary, it is through its herbicidal activity that *T. virens* has begun to be used in the biocontrol against *Setaria viridis* and *Amaranthus retroflexus* [36,37]. However, there is a need to increase the concentration of viridiol (**2**) to ensure reliable performance [3] and the reduction of viridin (**1**) by non-viridin producing microorganisms could be an interesting approach. Indeed, an effective viridiol (**2**) concentration could be obtained by combining the viridin production by *T. virens* and the reduction thereof by a non-viridin producing microorganism. In addition, it can be noted that these products are currently sought for biological studies, in particular such as candidate for PI3K inhibition-based chemotherapy, and several total syntheses are reported [26,38]. It can be highlighted that their preparation by coupling fermentation/biotransformation does not use numerous highly toxic or otherwise problematic reagents contrary to the syntheses described [39].

5. Conclusion

We conducted biotransformation experiments to study the action of microorganisms (45 strains) on viridin (**1**), a molecule produced in large quantities by *Trichoderma virens*, a bio-control agent. The screenings carried out led to the selection of 15 microorganisms capable of transforming viridin (**1**) into viridiol (**2**), a phytotoxin. Among these 15 microorganisms, *R. globerolus*, a bacterium was able to generate in addition to natural viridiol (**2**), its unknown epimer 3-epi-viridiol (**3**). Our results show that the conversion of viridin (**1**) into viridiol (**2**) is not exclusive of viridin-producing fungi. *R. globerolus*, *M. racemosus*, *P. anomala* are able to

reduce viridin (**1**) to viridiol (**2**). These results challenge us on the causes of this conversion of viridin (**1**), it is believed to be the action of a detoxification by *M. racemosus*, *P. anomala*. Finally, our work shows that it is possible to produce viridiol (**2**) for an herbicide use.

Declaration of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

The authors wish to thank L. Dubost for mass spectra, A. Deville for NMR spectra.

Appendix. Supplementary material

Supplementary data of this article can be found at...

References

1. K. K. Pal; B. S. Gardener, Biological control of plant pathogens, The Plant Health Instructor (2006). <https://doi.org/10.1094/PHI-A-2006-1117-02>
2. P. A. O'Brien, Biological control of plant diseases, Australasian Plant Pathol. 46 (2017), 293-304. <https://doi.org/10.1007/s13313-017-0481-4>
3. M. Verma; S. K. Brar; R. D. Tyagi; R. Y. Surampalli; J. R. Valero, Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control, Biochem. Engineering J. 37 (2007), 1-20. <https://doi.org/10.1016/J.Bej.2007.05.012>
4. X. Liu; S. Li, Fungal secondary metabolites in biological control of crop pests. in: Z. An (Ed.), Handbook of industrial mycology, Mycology Series, Vol. 22, Marcel Dekker Inc., New York, 2005, pp 723-747
5. P. W. Brian; J. C. McGowan, Viridin. A highly fungistatic substance produced by *Trichoderma viride*, Nature (London, U. K.) 156 (1945), 144-5. <https://doi.org/10.1038/156144a0>

6. S. C. Dubey; R. Bhavani; B. Singh, Integration of soil application and seed treatment formulations of *Trichoderma* species for management of wet root rot of mungbean caused by *Rhizoctonia solani*, Pest Manag Sci. 67 (2011), 1163-1168. <https://doi.org/10.1002/Ps.2168>
7. D. P. Roberts; S. M. Lohrke; S. L. F. Meyer; J. S. Buyer; J. H. Bowers; C. J. Baker; W. Li; J. T. de Souza; J. A. Lewis; S. Chung, Biocontrol agents applied individually and in combination for suppression of soilborne diseases of cucumber, Crop Prot. 24 (2005), 141-155. <https://doi.org/10.1016/J.Cropro.2004.07.004>
8. C. R. Howell, Biological-control of Pythium damping-off of cotton with seed-coating preparations of *Gliocladium virens*, Phytopathology 81 (1991), 738-741. <https://doi.org/10.1094/Phyto-81-738>
9. M. R. Khan; M. A. Anwer; S. Shahid, Management of gray mold of chickpea, *Botrytis cinerea* with bacterial and fungal biopesticides using different modes of inoculation and application, Biol. Control 57 (2011), 13-23. <https://doi.org/10.1016/J.Biocontrol.2011.01.004>
10. G. A. Pakora; J. Mpika; D. Kone; M. Ducamp; I. Kebe; B. Nay; D. Buisson, Inhibition of *Phytophthora* species, agents of cocoa black pod disease, by secondary metabolites of *Trichoderma* species, Environ. Sci. Pollut. Res. 25 (2018), 29901-29909. <https://doi.org/10.1007/s11356-017-0283-9>
11. W. M. Haggag; H. A. A. Mohamed, Enhancement of antifungal metabolite production from Gamma-ray induced mutants of some *Trichoderma* species for control onion white rot disease, Plant Pathol. Bull. 11 (2002), 45-56.
12. R. W. Jones; J. G. Hancock, Conversion of viridin to viridiol by viridin-producing fungi, Can. J. Microbiol. 33 (1987), 963-966. <https://doi.org/10.1139/m87-169>
13. R. W. Jones; W. T. Lanini; J. G. Hancock, Plant-growth response to the phytotoxin viridiol produced by the fungus *Gliocladium virens*, Weed Sci. 36 (1988), 683-687. <https://doi.org/10.1017/S0043174500075627>
14. P. F. Andersson; S. B. K. Johansson; J. Stenlid; A. Broberg, Isolation, identification and necrotic activity of viridiol from *Chalara fraxinea*, the fungus responsible for dieback of ash, Forest Pathol. 40 (2010), 43-46. <https://doi.org/10.1111/J.1439-0329.2009.00605.X>
15. C. R. Howell; R. D. Stipanovic Suppression of *Gliocladium virens* phytotoxin production with steroid synthesis inhibitors and use of *G. virens* cultures as biocontrol agents. US5268173A, 1993.
16. C. R. Howell; Viridiol deficient mutant strains of the biocontrol agent *Trichoderma virens*, process of making and using as biocontrol agent. US005882915, 1999.
17. C. Laurence; M. Rivard; T. Martens; C. Morin; D. Buisson; S. Bourcier; M. Sablier; M. A. Oturan, Anticipating the fate and impact of organic environmental contaminants: A new approach applied to the pharmaceutical furosemide, Chemosphere 113 (2014), 193-199. <https://doi.org/10.1016/J.Chemosphere.2014.05.036>
18. H. Olvera-Vargas; S. Leroy; M. Rivard; N. Oturan; M. Oturan; D. Buisson, Microbial biotransformation of furosemide for environmental risk assessment: identification of metabolites and toxicological evaluation, Environ. Sci. Pollut. Res. 23 (2016), 22691-22700. <https://doi.org/10.1007/s11356-016-7398-2>
19. S. Prado; D. Buisson; I. Ndoeye; M. Vallet; B. Nay, One-step enantioselective synthesis of (4S)-isosclerone through biotransformation of juglone by an endophytic fungus, Tetrahedron Lett. 54 (2013), 1189-1191. <https://doi.org/10.1016/J.Tetlet.2012.12.038>

20. J. Mpika; I. B. Kebe; A. E. Issali; F. K. N'Guessan; S. Druzhinina; M. Komon-Zelazowska; C. P. Kubicek; S. Ake, Antagonist potential of *Trichoderma* indigenous isolates for biological control of *Phytophthora palmivora* the causative agent of black pod disease on cocoa (*Theobroma cacao* L.) in Cote d'Ivoire, *African J. Biotechnol.* 8 (2009), 5280-5293.
21. J. F. Grove; J. S. Moffatt; E. B. Vischer, Viridin Part I. Isolation and characterisation, *J. Chem. Soc.* (1965), 3803-3811. <https://doi.org/10.1039/Jr9650003803>
22. J. R. Hanson, The viridin family of steroidal antibiotics, *Nat. Prod. Rep.* 12 (1995), 381-384. <https://doi.org/10.1039/NP9951200381>
23. Y. Fromentin; P. Grellier; J. D. Wansi; M. C. Lallemand; D. Buisson, Yeast-mediated xanthone synthesis through oxidative intramolecular cyclization, *Org. Lett.* 14 (2012), 5054-5057. <https://doi.org/10.1021/ol302283p>
24. J.-H. Kwon; S.-B. Hong, Soft rot of yomato caused by *Mucor racemosus* in Korea, *Mycobiology* 33 (2005), 240-242. <https://doi.org/10.4489/MYCO.2005.33.4.240>
25. P. Wipf; A. D. Kerekes, Structure reassignment of the fungal metabolite TAEMC161 as the phytotoxin viridiol, *J. Nat. Prod.* 66 (2003), 716-718. <https://doi.org/10.1021/Np0300277>
26. M. Del Bel; A. R. Abela; J. D. Ng; C. A. Guerrero, Enantioselective chemical syntheses of the furanosteroids (–)-viridin and (–)-viridiol, *J. Am. Chem. Soc.* 139 (2017), 6819-6822. <https://doi.org/10.1021/jacs.7b02829>
27. D. V. Hans; W. M. John; A. B. John; E. F. Edward, Two classes of plant antibiotics: phytoalexins versus Phytoanticipins, *The Plant Cell* 6 (1994), 1191-1191-1192. <https://doi.org/10.2307/3869817>
28. T. P. Fill; H. F. Pallini; Z. U. Din; I. D. Jurberg; J. V. da Silva; E. Rodrigues-Filho, Conjugation of antifungal benzoic acid derivatives as a path for detoxification in *Penicillium brasilianum*, an endophyte from *Melia azedarach*, *Bioorg. Chem.* 81 (2018), 367-372. <https://doi.org/10.1016/j.bioorg.2018.08.038>
29. M. S. C. Pedras; P. W. K. Ahiaonu, Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi, *Phytochemistry* 66 (2005), 391-411. <https://doi.org/10.1016/j.phytochem.2004.12.032>
30. S. P. McCormick, Microbial detoxification of mycotoxins, *J. Chem. Ecol.* 39 (2013), 907-18. <https://doi.org/10.1007/s10886-013-0321-0>
31. A. M. Warhurst; C. A. Fewson, Biotransformations catalyzed by the genus *Rhodococcus*, *Critical Reviews Biotechnol.* 14 (1994), 29-73. <https://doi.org/10.3109/07388559409079833>
32. Kuyukina M.S.; I.B. Ivshina, Application of *Rhodococcus* in bioremediation of contaminated environments, in: H.M. Alvarez (Ed.), *Biology of Rhodococcus*. Microbiology Monographs vol. 16, Springer-Verlag, Berlin, Heidelberg, 2010, pp 231-262. https://doi.org/10.1007/978-3-642-12937-7_9.
33. V. Renouf; O. Claisse; A. Lonvaud-Funel, Inventory and monitoring of wine microbial consortia, *Appl. Microbiol. Biotechnol.* 75 (2007), 149-164. <https://doi.org/10.1007/s00253-006-0798-3>
34. P. A. G. Elmer; T. Reglinski, Biosuppression of *Botrytis cinerea* in grapes, *Plant Pathol.* 55 (2006), 155-177. <https://doi.org/10.1111/J.1365-3059.2006.01348.X>
35. M. Daoubi; R. Hernandez-Galan; A. Benharref; I. G. Collado, Screening study of lead compounds for natural product-based fungicides: antifungal activity and biotransformation of

- 6 α ,7 α -dihydroxy- β -himachalene by *Botrytis cinerea*, J. Agric. Food Chem. 53 (2005), 6673-6677. <https://doi.org/10.1021/jf050697d>
36. C. M. Hutchinson, *Trichoderma virens*-inoculated composted chicken manure for biological weed control, Biol. Control 16 (1999), 217-222. <https://doi.org/10.1006/Bcon.1999.0759>
37. F. M. G. Heraux; S. G. Hallett; K. G. Ragothama; S. C. Weller, Composted chicken manure as a medium for the production and delivery of *Trichoderma virens* for weed control, HortScience 40 (2005), 1394-1397.
38. Y. Ji; Z. Xin; H. He; S. Gao, Total synthesis of viridin and viridiol, J. Am. Chem. Soc. 141 (2019), 16208-16212. <https://doi.org/10.1021/jacs.9b08577>
39. J. Kühlborn; J. Groß; T. Opatz, Making natural products from renewable feedstocks: back to the roots?, Nat. Prod. Rep. 37 (2020), 380-424. <https://doi.org/10.1039/C9NP00040B>