 doi:10.20944/preprints202203.0118.v1

Communication

Bivariate OSMAC designs expand the secondary metabolite production space in *Corallococcus coralloides*

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Abstract: The scarcely investigated myxobacterium *Corallococcus coralloides* holds a large genome containing many uncharacterized biosynthetic gene clusters (BGCs) that potentially encode the synthesis of entirely new natural products. Despite its promising genomic potential, suitable cultivation conditions have not yet been found to activate the synthesis of new secondary metabolites (SMs). Finding the right cultivation conditions to activate BGCs in the genome remained a major bottleneck and its full biosynthetic potential was so far not retrieved. Here, we therefore applied a bivariate OSMAC approach, using a combination of two elicitor changes at once for activation of BGCs and concomitant SM production by *C. coralloides*. The bivariate OSMAC screening was carried out in 24-well System Duetz-plates, applying univariate and bivariate OSMAC conditions. We combined biotic additives and organic solvents with minimal media and complex growth medium. The success of the method was evaluated by the number of new mass features detected in the respective extracts. We found synergistic effects in bivariate OSMAC designs. The number of new mass features detected in bivariate OSMAC exceeded the sum of new mass features found in the respective univariate OSMAC with only one elicitor. Overall, the bivariate OSMAC screening led to 26 new mass features, which were not detected in the univariate OSMAC design. Hence, the presence of multiple elicitors in the bivariate OSMAC designs successfully activated the biosynthetic potential in *C. coralloides*. We propose the bivariate OSMAC designs with a complex combination of elicitors as a straightforward strategy to robustly expand the SM space of microorganisms with large genomes.

Keywords: myxobacteria; secondary metabolites; multifactorial OSMAC; screening method

1. Introduction

Bacteria are among the most promising sources of new bioactive compounds such as antibiotics. However, only a small percentage of microorganisms has been investigated for the ability to synthesize secondary metabolites. It is estimated that only between 1 and 15% of the microbial world have been cultured until today [1]; hence, the majority of bacteria remains unknown and are referred to as microbial dark matter with unknown biosynthetic potential [2]. In addition, known and sequenced bacteria still show a huge uncharacterized biosynthetic potential in their genomes that has not been related to the production of any secondary metabolites (SMs) yet. These regions are often arranged in biosynthetic gene clusters (BGCs) in the microbial genome [3]. The number of such orphan BGCs without assigned gene product is estimated to be 5 to 10 times higher compared to the number of constitutively active BGCs [4]. A positive correlation between genome size and the number of BGCs has been found [5]. Thus, bacteria with large genomes generally

 doi:10.20944/preprints202203.0118.v1

possess more BGCs and have been frequently reported as a source of new antibiotics. Bacteria with extremely large genomes were found among the gram-positive actinobacteria and the gram-negative proteobacteria. Within the latter phylum, the myxobacteria are well known for their predation of gram-positive bacteria such as *Bacillus* strains through antibiotic compounds [6]. *Bacillus subtilis* is a known prey to *Myxococcus xanthus* and has also developed a defense mechanism by producing bacillaene - a well-known and characterized antibiotic [7]. Myxobacterial SMs with antibiotic activity are rather new and mainly comprise gulmirecins, myxopyronins and corallopyronins [8]. However, further investigations are necessary to fully exploit the entire biosynthetic potential of myxobacteria and actinobacteria, as a rich source of various chemically distinguished SMs [9].

In this study, the myxobacterium *Corallococcus coralloides* DSM 2259 was selected to investigate the SM production space of this strain, which has a large genome and many interesting genomic features. According to Livingstone et al., *Corallococcus* species are potent sources of many new compounds due to their individual predatory activity that require a diversified biochemistry [10]. In our previous study, *C. coralloides* was included in a multitude of OSMAC screening conditions. However, none of the 12 known BGCs and other predicted genomic features were activated using a single elicitor [11]. We assumed that the exposure to single stimuli was not sufficient for the activation of its BGCs. To better exploit this largely unknown biosynthetic potential, we exposed *C. coralloides* to multiple elicitors in a single OSMAC design, providing a multitude of stimuli at once for robust generation of new SM mass features (MFs). In our bivariate OSMAC approach, we tested and combined minimal media and complex media with biotic additives and organic solvents in cultures of *C. coralloides*. Based on the appearance of new MFs of extracted supernatant samples in LC-MS/MS measurements, we compared the efficiency of SM production in bi- and univariate conditions in terms of generated new MFs.

2. Materials and Methods

2.1 Assessment of the biosynthetic potential

The bioinformatics genome analysis was conducted with the online-tool antiSMASH 6.0 bacterial version [12]. The genome data of *C. coralloides* DSM2259 were obtained from NCBI (accession number: CP003389). Detection strictness was set to strict mode. Extra features were kept in default mode (KnownClusterBlast, ActiveSiteFinder and SubClusterBlast: ON, ClusterBlast, ClusterPfamAnalysis and Pfam-based GO term annotation: OFF). The results are listed in Table S1.

2.2 Bivariate OSMAC experiments

24-well System Duetz plates were used in the OSMAC experiments. Each plate contained triplicates (= three wells) of the control group (*C. coralloides* on SP medium: raffinose x 5 H_2O: 1.18 g/L, sucrose: 1.0 g/L, galactose: 1.0 g/L, soluble starch: 5.0 g/L, Bacto® casitone: 2.5 g/L, $MgSO_4$ x 7 H_2O: 0.5 g/L, K_2HPO_4: 0.25 g/L, final pH = 7.4 [13]), one blank well with non-inoculated SP medium (contamination control), duplicates of the univariate factors (media and additives, 4 factors per plate, 8 wells in total) and triplicates of the bivariate setups (4 bivariate setups per plate, 12 wells in total). The plates were used with a liquid volume of 3 mL. The initial OD_{600} was set to 0.1 with precultured *C. coralloides* for inoculation. The inoculated plates were incubated on a rotary shaker with 2.5 cm deflection at 200 rpm at 30 °C for 6 days. The inoculation plans for all plate designs can be found in the supporting information in Figures S1-S3.

The utilized media were: M9 medium (0.25 g/L $MgSO_4$ x 7 H_2O, 0.015 g/L $CaCl_2$ x 2 H_2O, 1 mL/L 1M thiamine-HCl x 2 H_2O, 2.1 g/L D(+) glucose, 20 mg/L L-proline, 100 mL/L salt solution (60 g/L Na_2HPO_4, 30 g/L KH_2PO_4, 10 g/L NH_4Cl, 5 g/L NaCl), pH 7.4), GMS medium (2 g/L $NaNO_3$, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4$ x 7 H_2O, 0.02 g/L $MnSO_4$ x 5 H_2O,

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0.02 g/L $FeSO_4$ x 7 H_2O, 0.02 g/L $CaCl_2$ x 2 H_2O, 5 g/L D(+) glucose, pH 7.0), MD1 medium (3.0 g/L casitone, 0.7 g/L $CaCl_2$ x 2 H_2O, 2.0 g/L $MgSO_4$ x 7 H_2O, 1 mL/L Trace Element Solution (TES) SL4, 0.5 mg/L vitamin B12, pH 7.0 with the following composition of TES SL4: 0.5 g/L EDTA, 0.2 g/L $FeSO_4$ x 7 H_2O, 0.01 g/L $ZnSO_4$ x 7 H_2O, 0.003 g/L $MnCl_2$ x 4 H_2O, 0.03 g/L H_3BO_3, 0.02 g/L $CoCl_2$ x 6 H_2O, 0.001 g/L $CuCl_2$ x 2 H_2O, 0.002 g/L $NiCl_2$ x 6 H_2O, 0.003 g/L Na_2MoO_4 x 2 H_2O) [14,15]. For GMS FeX medium, i.e. GMS medium without iron, the GMS recipe was used, but $FeSO_4$ x 7 H_2O was omitted. A list of chemical suppliers can be found in Table S2. The investigated additives were acetonitrile (ACN, 3% v/v), toluene (Tol, 1% v/v), autoclaved cell pellets (P, 2% v/v) and autoclaved supernatants (Sup, 10% v/v) of *B. amyloliquefaciens* DSM7 (Ba) and *S. griseochromogenes* DSM40499 (Sg).

2.3 Cultivation

C. coralloides was grown on SP medium for inoculation of System Duetz plates for 24 h at 30 °C and 150 rpm. To obtain cell pellets and supernatants as additives, *B. amyloliquefaciens* was grown on NB medium (peptone from meat: 5.0 g/L, meat extract: 3.0 g/L, final pH = 7.0) and *S. griseochromogenes* was grown on GYM medium (glucose 5 x H_2O: 4.4 g/L, yeast extract: 4.0 g/L, malt extract: 10.0 g/L, final pH = 7.2) [16,17]. Both strains were cultivated for 120 h at 30 °C and 150 rpm and autoclaved prior to harvesting via centrifugation.

2.4 Extract and Sample Preparation

Cells were separated from the supernatant by centrifugation of the entire plates at 4700 rpm. The cell-free supernatant of each well was transferred to separate glass Pyrex® tubes for preparation of the metabolite extracts. An equivalent volume of 2 mL of ethyl acetate was added to each tube and extracted in a multirotator (Grant-bio PTR-60, Grant Instruments Ltd, Shepreth, England) for 5 minutes at 50 rpm with a change of direction every 10 seconds for better mixing of the 2-phase-system. This was repeated twice with removal of the organic phase and addition of fresh solvent in between each round. The ethyl acetate extract was collected in separate Pyrex® tubes for each well and transferred to LC glass vials in 1.5 mL portions to evaporate the solvent to dryness in a vacuum centrifuge. For solvent evaporation, the vacuum-high vapor (V-HV)-mode was used at 30 °C for 50 minutes. 40 µL of MS-grade methanol were added to each dry extract and an ultrasound bath was used for 3 minutes to help solubilize the dried extract.

2.5 LC-MS/MS measurement

Metabolite analytics was conducted on an LC-MS, composed of an Agilent Technologies 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, USA) coupled to a Bruker Compact ESI-QTOF-MS System (Bruker, Billerica, USA). ACN (solvent A) and H_2O with 0.1% formic acid (solvent B) were used as solvents with a flow rate of 0.4 mL/min. The injected sample volume was 2 µL. The following HPLC method was employed: 0-10 min 5%-98% solvent A, 10-15 min 98% solvent A isocratic, 15-17 min 98%-5% solvent A, 17-20 min 5% solvent A at a temperature of 40°C with a C18-column (100x2.6mm NucleoShell RP18, Macherey&Nagel, Düren). The Diode Array Detector (DAD) was set to a range of 205 nm to 400 nm. The mass spectrometer, comprising an ESI ion source (nebulizing gas pressure: 4 bar, drying gas flow: 12 L/min, drying temperature: 220 °C, capillary voltage: 4500 V) and a TOF analyzer ($100 < m/z < 850$), was used in positive mode.

2.6 Mass feature detection

A new MF was defined as a unique compound whose production was triggered by the investigated change in culture conditions. They do not originate from media or additives themselves and are not produced under control conditions (SP medium, no additives). New MFs are characterized by an m/z-value and a retention time (t_R) and were identified with the tool MZmine Version 2.35 for all uni- and bivariate extracts [18]. The steps and settings for raw data processing and peak list processing in MZmine can be found in Tables S3 and S4. MFs were also examined regarding their adduct form in the

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Bruker Compass Data Analysis Viewer software (Bruker, Billerica, USA) version 4.4 to identify the exact mass of the corresponding compound. Due to biological variation in the cultures MFs can have different intensities in replicates. For standardization, MFs were only included in the evaluation if their intensity surpassed a signal threshold of 10^5. The compounds' identities were not further characterized in this study. To identify new MFs in univariate screenings, MFs from the control group sample (*C. coralloides* grown on SP medium) and those from background samples (medium, additives) were subtracted from all MFs detected in the univariate sample extracts (Tables S5 and S6). The remaining MFs are new MFs specific to the univariate culture condition change. For bivariate MFs the univariate MFs were also subtracted from all detected MFs in the bivariate sample. Comprehensive lists of all univariate and bivariate new MFs can be taken from Tables S7 and S8. The new MFs of bivariate experiments do not include any of the new MFs of their corresponding univariate experiments.

2.7 Calculation of foldchange

To compare univariate and bivariate experiments we defined an indicator based on how many new MFs were generated in the OSMAC experiments. The indicator was the foldchange in the number of new MFs in bivariate screenings compared to the sum of MFs obtained in univariate experiments (Equation 1)

$$foldchange\ of\ new\ MFs = \frac{\#\ of\ new\ bivariate\ MFs}{\Sigma\ of\ new\ univariate\ MFs} \quad (1)$$

.

3. Results and Discussion

Although a high number of BGCs can be found in the genome of *C. coralloides* (Figure S1), none of the predicted compounds were detected up to now [11]. Thus, the activation of the biosynthetic potential requires a different approach. We chose to combine two stimuli at once to see whether synergistic effects in SM production can be observed and if *C. coralloides*' SM space can be expanded by applying the bivariate OSMAC approach. The measured output of this study is the number and characterization of new mass features (MFs) per bivariate culture condition.

3.1. The biosynthetic potential of C. coralloides DSM2259

C. coralloides DSM2259 was selected for this study, because it is a rather unknown bacterium with a large, fully sequenced genome and many gene clusters that code for natural products- many not even identified yet. So far, three natural products have been isolated from other *C.coralloides* cultures: corallorazines [19], coralmycins [20], and corallopyronin [21]. There are only few studies, which investigated *C. coralloides* DSM2259 and no SM compound has so far been reported for this specific strain despite its fully sequenced and analyzed genome [22]. Most studies of *C. coralloides* have been performed as *in silico* genomics [22–26]. After our first OSMAC study, this is now the second study which investigates the SM production space of *C. coralloides* DSM2259.

We analyzed its genome with antiSMASH bacterial version 6.0.0 (NCBI accession # CP003389) and found that 15.1% of *C. coralloides* genome were related to the biosynthesis of SMs. In total, the analysis yielded 33 BGCs assigned to the biosynthesis of different natural product classes. Figure 1 summarizes the genome analysis. Among them were polyketides (PKS), nonribosomal peptides (NRPS), terpenes, and lanthipeptides. Putative products were assigned to 13 BGCs by antiSMASH but none of them has so far been reported for this strain (Figure 1). A detailed list of the assigned classes and putative SM products is presented in the Table S1. The BGCs assigned to geosmin and a carotenoid display 100% sequence similarity to the entries from the Minimum Information about a

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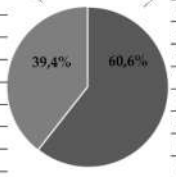
Biosynthetic Gene Cluster (MIBiG) database. Additionally, 83% similarity to the myxochelin A/B BGC and 66% similarity to the myxoprincomide BGC were detected. The other nine assigned BGCs possess less than 40% similarity to the MIBiG database entries. 20 BGCs could not be assigned to any BGCs in the MIBiG database and are, therefore, so-called orphan BGCs [25].

assigned BGCs (13/33)

NP class	Assigned compound	Sequence similarity
Terpenes	Carotenoid	100%
Terpenes	Geosmin	100%
T1PKS-NRPS	Myxochelin A / B	83%
T1PKS-NRPS	Myxoprindomide c-506	66%
NRPS	APE Ec	36%
T3PKS	Alkylpyrone-407	34%
T1PKS	Puwainaphycin A	30%
T1PKS	chloromyxamide	24%
T1PKS-NRPS	Rakicidin A	22%
T1PKS-NRPS	chloromyxamide	13%
Other	Streptobactin	11%
T1PKS-NRPS	BE-43547A1 etc.	10%
RiPP-like	Ficellomycin	5%



orphan BGCs (20/33)

NP class	# of BGCs
PKS	0
NRPS	3
PKS-NRPS	4
Terpenes	2
NRPS-like	0
Lanthipeptides	5
RiPP-like	4
other	2

Figure 1. Classification of all BGCs identified in the genome analysis using antiSMASH 6.0 on the genome of *C. coralloides* DSM2259 and their putative products including sequence similarity.

The large number of orphan BGCs in *C. coralloides* DSM 2259 requires novel strategies to fully discover and characterize its biosynthetic potential.

3.2 Discovery of new secondary metabolite MFs after bivariate OSMAC screening

C. coralloides possesses a large genome of 10.08 Mbp and 33 BGCs coding for potential SMs and accordingly has a large biosynthetic potential [11] (and new genome analysis for this study). However, in our previous study, we found that *C. coralloides* produced only 35 new MFs in response to various OSMAC stimuli [11]. Previous experience suggests that the number of new MFs should be much higher in terms of genome size and the number of BGCs predicted. For example, four other investigated bacteria produced more than 120 new MFs each as response to the applied stimuli. This observation inspired us to modify the OSMAC approach with a bivariate design. For this, we combined two OSMAC-stimuli in one experiment and evaluated the number of new MFs specific for *C. coralloides*. This novel bivariate approach aimed to increase the efficiency of generating new MFs, which serve as a proxy of the number of produced SMs.

As control or reference point, *C. coralloides* was cultivated in SP medium without any additives. Since the new MFs in uni- and bivariate cultivations were defined as not being produced under control conditions, only MFs that were first detected in the uni- or bivariate extracts were termed new MFs. Thus, no up- and downregulation of new MFs was investigated.

Figure 2A shows that between 0 and 7 new MFs were detected in the univariate experiments with additive-containing complex media and cultivations with minimal media. Most cultivation conditions resulted in only a small number up to 2 new MFs, as expected. The use of GMS FeX medium and the addition of pellet from *S. griseochromogenes* (P_{Sg}) alone did not lead to any new MFs. The highest number of new MFs in the univariate screening was detected in the SP medium cultures with added supernatant of *B. amyloliquefaciens*. M9 medium was used in all three experimental set-ups and showed different numbers of new MFs. This might have resulted from differences in inoculation. It is also possible that the MFs were produced but their concentration was beneath the detection limit.

Interestingly, when the supernatant of *B. amyloliquefaciens* was combined with M9 medium, the previously observed MFs were not produced anymore, and instead 13 new

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MFs were detected (Figure 2B). The other combinations of M9 medium and biotic additives also resulted in new MFs. The cultivations in M9 medium with autoclaved cell pellet of *B. amyloliquefaciens* (P_{Ba}) led to 9 new MFs and with *S. griseochromogenes* (P_{Sg}) to 5 new MFs (Figure 2B). The addition of supernatant of *S. griseochromogenes* (Sup_{Sg}) to an M9 medium culture led to 3 new MFs. In literature, biotic additives have been reported to successfully induce or increase antibiotic production [27]. In our study, especially the combination of M9 minimal medium with biotic factors, such as the addition of supernatant or cell pellets seems to be very effective for the synthesis of new MFs indicating the production of new secondary metabolites.

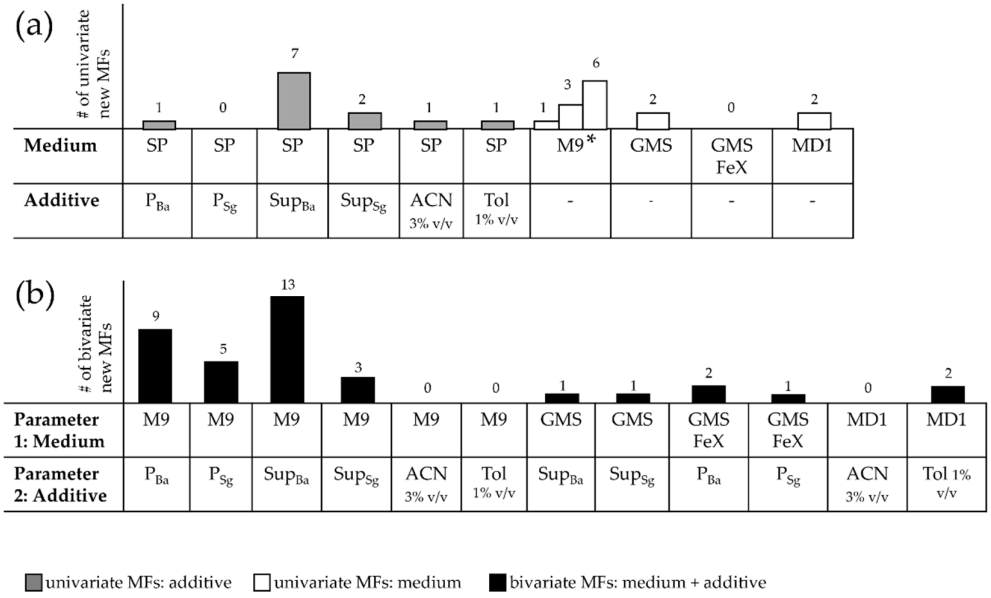

Figure 2. Number of new MFs obtained in univariate (A) and bivariate (B) OSMAC experiments with *C. coralloides* DSM2259 cultivated in SD-plates at 30°C for 6 days. The bars represent the number of reproducible MFs, i.e. MFs present in both duplicates (univariate) or all triplicates (bivariate) with an intensity of at least 10^5, specific for each culture condition. The new MFs detected in bivariate experiments do not contain MFs from univariate experiments, see section *2.6 Mass feature detection*. Abbreviations: P = pellet, Sup = supernatant, Ba = *B. amyloliquefaciens*, Sg = *S. griseochromogenes*, ACN = acetonitrile, Tol =toluene, GMS = glucose, minerals, salts, FeX = without Fe^{3+}, M9 and MD1 are names of minimal media. *M9 medium was used in all three SD-plates. For the evaluation of new bivariate MFs, the respective number of new MFs was used: 1 new MF for the plate with the pellet experiments, 3 new MFs for the plate with the supernatant experiments and 6 new MFs for the solvent experiments.

The use of GMS or GMS FeX medium in combination with the cell pellets and supernatants of *B. amyloliquefaciens* and *S. griseochromogenes* was not as effective as the experiments based on M9 medium. Three out of four setups led to one new MF and the combination of GMS FeX with P_{Ba} generated 2 new MFs. Although both media are minimal media, they differ in their composition (see section *2.2 Bivariate OSMAC Experiments*). GMS possesses less buffering capacity but more Glucose than M9. M9 additionally offers thiamine and proline since it was originally a medium for recombinant *E. coli*. It can thus be hypothesized, that either a buffered system or the additional components support new MF synthesis.

To verify whether non-biotic additives, such as organics solvents are also effective for bivariate screening with *C. coralloides*, we set up combinations of organic solvents and

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MD1 complex medium. In univariate experiments on SP medium one new MF was detected for acetonitrile and toluene each. The addition of the solvents to M9 cultures did not produce any new MFs. When using the complex MD1 medium instead, two new MFs were detected in the cultures supplemented with toluene. In case of the addition of organic solvents, generally only a few new MFs were formed per change at the same time. Therefore, the tested organic solvents are considered a less promising additive in this approach. It has been previously shown for Streptomyces and Bacillus strains that only specific organic compound promote the production of certain antibiotics [28]. Therefore, it can be expected that some tested solvents might not lead to new MFs while others do. The addition of organic solvents seems to be a field where a broader screening is reasonable. It has been reported that an addition of toluene increases the saturated fatty acid content in the bacterial membranes and, thus, leads to increased membrane rigidity [29]. Possibly, an altered membrane composition influenced the secretion of metabolites in *C. coralloides*. Acetonitrile is a toxic small chemical and some acetonitrile-degrading microorganisms have also been reported; among them *Pseudomonas aeruginosa* and *Rhodococcus rhodochrous*. In other living organisms acetonitrile is toxic because of its degradation into hydrogen cyanide and acetaldehyde [30]. Apparently, acetonitrile is not a good choice of secondary metabolite eliciting additive for *C. coralloides*.

We showed that the bivariate OSMAC approach displayed synergistic properties when combining minimal media with autoclaved cell pellets and supernatants. Here, a synergistic effect of the two culture conditions in bivariate screenings was necessary to activate the production of new MFs. In particular, we confirmed that a combination of culture conditions was necessary for generating new MFs, whereas some corresponding univariate designs did not trigger any MFs.

3.3 Condition-specificity of new MFs and efficiency of bivariate culture conditions

To assess the specificity of the conditions that trigger new MFs, we compared which MFs could be triggered by exclusively one or more cultivation conditions. In total, 26 new MFs were detected in the 12 distinct, bivariate culture conditions. 18 of the 26 new MFs, i.e. 69%, were only detected under exactly one bivariate culture condition set (Figure 3, **Error! Reference source not found.**6 and **Error! Reference source not found.**7). Seven MFs (= 27%) were detected in extracts from two different bivariate culture condition sets and one MF was even detected in extracts from three different bivariate culture condition sets. Thus, most MFs were only produced in exactly one specific cultivation condition. The implication for further screenings is that a broad application of different conditions is recommended to stimulate the production of new SMs.

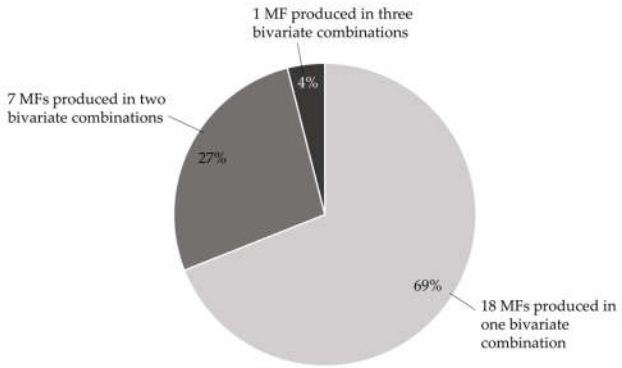

Figure 3. Condition-specificity of new bivariate MFs in *C. coralloides*' OSMAC screenings. Percentage of the 26 new MFs produced under one, two and three bivariate conditions.

Our results are a strong indication that synergistic effects can result from the combination of multiple elicitors in one OSMAC cultivation (Figure 2). To compare the effi-

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ciency of bivariate culture condition for generating new MFs, we calculated the foldchange of the number of new MFs in comparison to the total number of new MFs in the corresponding univariate experiments (Table 1, Eq. 1). A foldchange of one means, that an equal number of bivariate MFs in comparison to the sum of univariate MFs was detected. Values higher than one indicate that more bivariate than univariate MFs were produced. Four out of 12 bivariate screening conditions resulted in such an increased foldchange. Moreover, three out of the four bivariate OSMAC screenings with a foldchange greater than one included biotic additives combined with M9 medium. The highest foldchanges were determined for the combination of M9 medium with cell pellets of *B. amyloliquefaciens* and *S. griseochromogenes*. It can be hypothesized that the combination of these stress factors, particularly starvation in addition to elicitors from other microbes activates multiple BGCs for survival and defense.

Medium	**M9**						**GMS**		**GMS FeX**		**MD1**	
Additive	P_{Ba}	Sup_{Ba}	P_{Sg}	Sup_{Sg}	ACN 3%	Tol 1%	Sup_{Ba}	Sup_{Sg}	P_{Ba}	P_{Sg}	ACN 3%	Tol 1%
Foldchange of new MFs	4	1,2	5	0,6	0	0	0,1	0,25	2	n.a.	0	0,7

Table 1. Foldchange of new MFs of bivariate OSMAC screenings in relation to their new MFs from the combined univariate OSMAC conditions in SD-cultivation plates with *C. coralloides*. Abbreviations: n.a. = foldchange could not be determined, because the number of univariate MFs is zero.

Culture conditions, which include biotic additives, especially cell pellets, increase the chances of finding new MFs in *C. coralloides* leading to novel compounds. The conditions in this small, initial bivariate screening were not able to activate the silent BGCs yet and much biosynthetic potential of *C. coralloides* remains to be uncovered by the investigation of further culture condition sets. Our study successfully demonstrated that bivariate OSMAC designs efficiently generate novel condition-specific MFs that were not produced in their corresponding univariate culture conditions.

4. Conclusions

Inducing SM production in wild types has been challenging due to many silenced regions in the genome under standard laboratory conditions. OSMAC screenings have been established as a simple and untargeted strategy to activate the biosynthetic potential using diverse unspecific stimuli during cultivation of microbes. However, no microbe has so far been fully elucidated for its biosynthetic potential [31]. One of the very promising bacteria for the production of relevant SM is *C. coralloides*. *C. coralloides* is a bacterium with a large genome and high biosynthetic potential. Still, current OSMAC cultivation approaches did not allow for an entire mapping of its feasible SM production space based on the number of BGCs present in its genome. Here, we proposed a new strategy of bivariate OSMAC screenings: exposing *C. coralloides* to combined elicitors and profit from synergistic effects in bivariate OSMAC cultivations. To demonstrate that bivariate OSMAC designs efficiently generate new and condition-specific SMs, we analyzed and compared the generated MFs in univariate and bivariate OSMAC conditions. Our study clearly pointed out an increase in potentially interesting MFs in bivariate OSMAC screenings compared to their corresponding univariate, or one factor at time, approach. The high condition-specificity of new MFs in our bivariate OSMAC designs stresses the importance to use a bivariate approach for further expansion of the SM space in known and novel microbes. Moreover, the presented bivariate OSMAC approach is a first step towards the implementation of multifactorial screenings for the discovery of new natural compounds. The exposure to multiple elicitors opens an almost infinite number of combinations of stimuli to trigger the production of many compounds at the same time. However, this might not be the sole key to success, as shown by the difference in effectiveness of our

bivariate culture conditions. Biotic additives showed better MF generation in comparison to organic solvents. Possibly, these complex elicitors imitate part of the natural habitat. Future screening designs might also consider a combination of elicitors and culture conditions that are likely to be present in natural habitats, to yield an even larger number of diverse MFs. To this end, our bivariate approach and the implementation of multifactorial OSMAC screenings are key to succeed in discovering new MFs, which eventually translate into the discovery of new natural products and, in the long run, activating silent BGCs [32].

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author Contributions: Conceptualization, J.S. and S.L.; methodology, J.S.; software, J.S.; validation, J.S.; formal analysis, A.S. and J.S.; investigation, A.S. and J.S.; resources, S.L.; data curation, A.S. and J.S.; writing—original draft preparation, J.S.; writing—review and editing, G.H., K.R., S.L.; visualization, J.S.; supervision, S.L. and K.R.; project administration, S.L.; funding acquisition, S.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data is contained within the article or Supplementary Material.

Acknowledgments: The authors would like to acknowledge the scientific advice and help with analytics provided by C. Zammarelli.

Conflicts of Interest: The authors declare no conflict of interest.

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