

Article

Production of Bioactive Metabolites in *Streptomyces coelicolor* Cultivated in the Presence of *Citrus* Seeds

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Abstract

Plant–microbe interactions can modulate the production of bioactive compounds involved in plant growth-promoting activity. This study investigates the metabolic reprogramming of the actinomycete model strain *Streptomyces coelicolor* M145 during co-cultivation with *Citrus aurantium* and *Citrus limon* seeds, used as defined plant-derived chemical inputs, under contrasting nutritional conditions with or without L-tryptophan (Trp) supplementation. Untargeted metabolome profiling revealed medium- and *Citrus* seed-dependent metabolic shifts in co-cultures compared with corresponding *Citrus* seed and *S. coelicolor* single cultures used as controls. Under R2YE production conditions, co-cultivation with *C. limon* resulted in 27 extracellular metabolites, compared with 11 detected in the TSB vegetative medium; similar trends were observed for *C. aurantium*. Multivariate analyses confirmed that growth medium, *Citrus* species, and Trp significantly shaped *S. coelicolor* extracellular metabolic profiles, mainly in quantitative terms. Production conditions, particularly with Trp supplementation, promoted metabolites associated with antimicrobial activity and iron acquisition, whereas vegetative conditions promoted primary metabolism and biotransformation of *Citrus*-derived compounds. Spent medium bioassays on *Solanum lycopersicum* showed that these metabolic differences were correlated with distinct biological responses. Overall, these findings demonstrate that defined plant-derived inputs modulate *S. coelicolor* specialized metabolism in a context-dependent manner, generating metabolomic signatures associated with differential plant growth responses compared to single cultures.

Keywords: *Streptomyces coelicolor*; *Citrus limon*; *Citrus aurantium*; bioactive metabolites; co-culture; HPLC-ESI-QTOF-MS



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1. Introduction

Actinomycetota phylum members of the genus *Streptomyces* are widely recognized for their exceptional capacity to synthesize structurally diverse bioactive (often called secondary or specialized) metabolites with antibiotic, antifungal, immunomodulatory, and plant growth promoting activities [1–3]. Among them, *Streptomyces coelicolor* M145, a derivative of the widely investigated A(3)2 strain [4,5], is a model actinomycete for studying prokaryotic morphological and physiological differentiation, gene expression

regulation [6–8], antibiotic biosynthesis [9,10] and secretome—also including extracellular vesicles and their protein, nucleic acid, and metabolite cargo [11,12].

S. coelicolor's well-characterized genome and regulatory networks make this bacterial strain a suitable system for investigating environmentally induced metabolic reprogramming. Genome sequencing has revealed that *S. coelicolor* harbors a substantially larger repertoire of biosynthetic gene clusters (BGCs) than those expressed under standard laboratory conditions, indicating its potential to synthesize numerous secondary metabolites beyond canonical compounds such as actinorhodin and undecylprodigiosin [5,13,14]. Many of these BGCs remain transcriptionally silent or poorly expressed; consequently, the associated specialized metabolic pathways are referred to as “silent”, and the corresponding compounds as “cryptic” metabolites, which are not detected under conventional cultivation [15]. Indeed, streptomycete secondary metabolism is governed by multilayered regulatory networks involving transcription factors, developmental cues, and environmental signals that modulate the activation of BGCs [16]. The lack of appropriate physiological or environmental stimuli—such as nutrient limitation, interspecies interactions, stress signals, or specific physicochemical conditions—often prevents the activation of these cryptic pathways in standard growth media. Consequently, the metabolic potential of *S. coelicolor* is only partially realized under routine laboratory conditions.

Beyond their established role in antibiotic production, actinomycetota members are increasingly acknowledged as key members of the plant microbiome due to their capacity to interact with host plants and modulate growth and stress responses [17,18]. Actinomycetota members, including streptomycetes, are emerging as Plant Growth Promoting Bacteria (PGPB), contributing to plant fitness by enhancing nutrient availability, producing phytohormones such as indole-3-acetic acid (IAA), and providing protection against pathogens through antimicrobial compound secretion and competitive exclusion [3,19–21]. Understanding how *Streptomyces* responds to plant-derived signals is therefore of significant interest, both for elucidating regulatory mechanisms underlying secondary metabolism and for identifying new bioactive compounds with potential applications in agriculture and biotechnology.

In natural ecosystems, *Streptomyces* spp. are prominent members of the rhizosphere and seed-associated microbiota, where they actively perceive and potentially respond to plant-released metabolites that shape their developmental programs and bioactive metabolite production [22]. Co-culture systems have emerged as powerful tools for activating silent or weakly expressed BGCs in actinomycetes, as interactions with plant tissues or plant-derived compounds can profoundly modulate microbial physiology and trigger metabolic reprogramming [23–25]. Plant metabolites, including coumarins, flavonoids, terpenoids, organic acids, and phenolic compounds can act as chemical cues capable of influencing antibiotic biosynthesis, morphological differentiation, and regulatory networks in *Streptomyces* [26–35]. Seeds of *Citrus* species represent a chemically rich yet experimentally tractable source of such metabolites. In particular, *Citrus limon* and *Citrus aurantium* differ in their relative composition of flavanones and limonoids, providing a comparative system to evaluate how distinct plant-derived chemical profiles influence microbial metabolism [36–38]. These metabolites are naturally encountered by soil- and seed-associated actinomycetes and may function as ecological signals rather than mere antimicrobial agents due to their low abundance levels in the soil [32,38,39]. Therefore, *Citrus* seeds provide a chemically defined system to test the effects of compositionally distinct metabolite pools on *S. coelicolor* metabolic regulation. Unlike rhizosphere-based studies, where multiple variables coexist, this approach enables the investigation of plant-derived signals under controlled conditions. Within this framework, L-tryptophan (Trp) represents a key metabolic hub linking ecological signaling and biotechnological potential, as it is either a precursor of indole compounds, such as indole-3-acetic

acid [40], and a modulator of morphophysiological differentiation and specialized metabolite production in *S. coelicolor* [41,42].

Untargeted metabolomics aims to comprehensively profile cellular metabolites and associated metabolic alterations, and advances in analytical technologies now enable the identification and relative quantification of thousands of metabolites in complex biological samples [43–46]. Among these, liquid chromatography–mass spectrometry (LC-MS) is widely used due to its high sensitivity and versatility for metabolite detection across different chemical classes [44,47].

Accordingly, the objectives of this study are to: (i) characterize the extracellular metabolite profiles of *S. coelicolor* in co-culture with *C. limon* and *C. aurantium* seeds under defined nutritional conditions; (ii) assess how Trp supplementation influences metabolite profiles within these co-culture systems; and (iii) evaluate the biological relevance of the observed metabolomic changes through plant growth bioassays.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions

S. coelicolor seed cultures were prepared by inoculating 10^8 spores into 30 mL of R2YE medium [48] and incubating them for 48 h at 30 °C with shaking at 200 rpm. Biomass was collected by centrifugation ($3000\times g$, 10 min, 4 °C), washed twice with sterile distilled water, and resuspended in 30 mL of sterile water. Aliquots of 500 μ L of this suspension were inoculated into 10 mL of R2YE—a typical production-oriented medium favoring secondary metabolism in streptomycetes—[48] or Tryptic Soy Broth (TSB) (Condalab, Spain, Madrid)—used for supporting vegetative bacterial growth—and incubated for 72 h at 30 °C under agitation at 180 rpm. Seeds of *C. limon* and *C. aurantium* were surface-sterilized following Faddetta et al. (2021) [49]: sterile water (5 min), 70% ethanol (1 min), 2.5% sodium hypochlorite (2 min), 70% ethanol (1 min) (Carlo Erba Reagents, Milan, Italy), followed by repeated rinsing in sterile water.

Co-culture experiments included: *S. coelicolor* alone, *S. coelicolor* with *C. limon* seeds, *C. limon* seeds alone, *S. coelicolor* with *C. aurantium* seeds, and *C. aurantium* seeds alone, each in the absence or presence of L-tryptophan (0.1 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). Three seeds were introduced into each 10 mL culture to obtain a consistent and biologically meaningful seed-to-medium ratio. Considering an average seed weight of approximately 0.20 g for *C. aurantium* and similar *Citrus* species, the co-culture carried a biomass input of 6–7.5% (*w/v*) per culture, which aligns with plant biomass loadings usually employed in microbial fermentations using untreated or pre-treated solid plant materials as the substrate [50–52]. Additionally, using three seeds also reduces variability arising from differences in metabolite content among individual seeds and ensures reproducible exposure of *S. coelicolor* to *Citrus*-derived chemical cues. All experiments were performed in biological triplicates.

2.2. Metabolite Analysis via HPLC-ESI-QTOF-MS

The spent media were analyzed using high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS). Analyses were performed in both positive and negative ion modes using an Agilent 1260 Infinity HPLC system, connected to a reverse-phase Poroshell 120 EC-C18 column [3], with the appropriate Poroshell security guard supplied from Agilent Technologies, Santa Clara, California, USA. Samples derived from three independent biological replicates were analyzed, and each sample was subjected to triplicate technical runs. Mass spectral data were processed using MassHunter Qualitative Analysis B.06.00 (Agilent Technologies, Santa Clara, California, USA) for metabolite annotation. Compounds were assigned based on

accurate mass matching against the METLIN database (https://metlin-nl.scripps.edu/landing_page.php?pgcontent=mainPage, accessed on 3 April 2026), using a mass tolerance of ± 10 ppm. While the majority of metabolites were putatively annotated based on accurate mass matching (Level 2 annotation) [53], the identities of 3-methylindole, L-tryptophan, limonin-17- β -D-glucoside, naringin, hesperidin, streptorubin B, and undecylprodigiosin were additionally confirmed through the analysis of authentic standards analyzed individually using the same HPLC-ESI-QTOF-MS method, based on matching retention times and accurate masses.

Metabolomic data analysis was performed using the web-based platform MetaboAnalyst (version 6.0, <https://www.metaboanalyst.ca/MetaboAnalyst/ModuleView.xhtml>, accessed on 3 April 2026) [54]. The peak intensity table obtained from chromatographic analysis was uploaded as a peak intensity dataset including sample names and corresponding experimental groups. Prior to statistical analysis, data integrity was verified, and missing values were handled using the default MetaboAnalyst procedure based on small value replacement. Analytical reproducibility was evaluated by calculating the coefficient of variation (CV) across technical replicates. Features with a relative standard deviation (RSD) higher than 25% were excluded from further analysis. To reduce systematic variability among samples, data were normalized by sum normalization, followed by log10 transformation to stabilize variance and approximate a normal distribution of metabolite intensities. Subsequently, Pareto scaling was applied to enhance the contribution of medium-intensity metabolites while minimizing the influence of very large variables.

Multivariate statistical analyses were conducted to explore metabolic differences among experimental groups. Principal Component Analysis (PCA) was used as an unsupervised method to visualize clustering patterns and overall variance within the dataset. The statistical significance of group separation was further assessed using PERMANOVA.

Partial Least Squares Discriminant Analysis (PLS-DA) was applied as a supervised method to identify metabolites contributing to the separation between experimental conditions. Model performance was evaluated using the coefficient of determination (R^2) and predictive ability (Q^2), obtained through cross-validation in MetaboAnalyst. The optimal number of components was selected based on the highest Q^2 value to avoid overfitting. Model robustness was further assessed using permutation testing ($n = 1000$), and the observed model performance was compared against randomly generated models. Statistical significance of metabolite differences among groups was evaluated using a one-way analysis of variance (ANOVA), followed by post-hoc multiple comparison tests implemented in MetaboAnalyst.

$$\log_2 \text{FC} = \log_2 \left(\frac{\text{Mean intensity treated}}{\text{Mean intensity non treated}} \right) \quad (1)$$

Positive $\log_2 \text{FC}$ values indicate metabolites enriched in co-culture, whereas negative values indicate metabolites reduced compared to single culture conditions.

2.3. Plant Material

Seeds of tomato (*Solanum lycopersicum*, cv Red Cherry) were surface-sterilized using 70% ethanol for 2 min and then soaked in 2.5% (w/v) sodium hypochlorite solution for 2 min as reported in Faddetta et al. (2023) [3]. Afterward, three rinses in sterile distilled water (5 min each) were carried out. Surface-sterilized seeds were incubated for 45 min into a dilution (1:10 distilled water) of co-culture supernatants. In parallel, control condition experiments were performed using sterile distilled water (sdH_2O). Ten seeds were placed for each replicate and were aseptically transferred to Petri dishes with filter paper (Whatman, Maidstone, UK) soaked in 3 mL of distilled sterile water. Plates were incubated in dark at 25–26 °C for six days for germination and afterwards they were put in a climatic chamber

set for long-day conditions (16/8 h light/dark) at 25–26 °C and a minimum light intensity of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for another six days. The plant responses (root and shoot growth at 12 days after treatment—DAT) to each thesis were analyzed with the one-way ANOVA. Tukey's test ($p < 0.05$) was used to test the significance among different treatments.

3. Results

3.1. Bioactive Metabolite Profiling

3.1.1. Experimental Design and Reproducibility of Untargeted Metabolomic Analysis

To investigate how plant-derived components influence *S. coelicolor* metabolism, a structured experimental design was implemented in which growth medium and Trp supplementation were considered as contextual factors shaping metabolite profiles. The experimental setup included three variables: (i) the presence of *C. limon* or *C. aurantium* seeds; (ii) the growth medium (TSB, supporting vegetative growth, and R2YE, favoring secondary metabolism); and (iii) the absence or presence of Trp supplementation (TSB+Trp and R2YE+Trp). For each condition, co-culture systems were analyzed in comparison with the corresponding controls, defined as the sum of *S. coelicolor* and *Citrus* seed single cultures grown under identical medium and Trp conditions. This design allowed the evaluation of co-culture-specific metabolite profiles while accounting for background contributions from both microbial and plant components (Tables S1–S4). The following comparisons were applied independently to each *Citrus* species:

1. Co-culture in R2YE vs. corresponding single cultures in R2YE;
2. Co-culture in R2YE with Trp vs. corresponding single cultures in R2YE with Trp;
3. Co-culture in TSB vs. corresponding single cultures in TSB;
4. Co-culture in TSB with Trp vs. corresponding single cultures in TSB with Trp.

This experimental framework enabled the identification of metabolite patterns specifically associated with co-culture conditions under defined environmental contexts, rather than direct quantitative comparisons between media or Trp supplementation alone.

In *S. coelicolor*, production of bioactive compounds is strongly influenced by nutritional conditions [55]. Accordingly, R2YE and TSB were selected to represent two contrasting metabolic states, enabling comparison between a production-oriented condition promoting secondary metabolism and a balanced medium supporting growth and biomass accumulation. This framework allowed quantitative assessment of extracellular metabolic complexity under defined plant-derived and nutritional conditions. Overall, these results can highlight the interplay between plant-derived compounds, microbial metabolism, and the modulatory effects of Trp supplementation.

The untargeted metabolomic analysis of *S. coelicolor* and *Citrus* seed single cultures and their co-cultures revealed a diverse set of metabolites—including siderophores, antimicrobials, indole-related compounds, and other specialized metabolites (Tables S1–S4)—which can be grouped into major biochemical classes, namely tryptophan-derived metabolites, *Citrus* limonoids and limonoid glucosides, *Citrus* flavonoids, siderophores, mixed microbial secondary metabolites, and prodiginines (Table 1).

Unless otherwise specified, metabolite assignments correspond to putative annotations based on accurate mass matching (Level 2 annotation), whereas 3-methylindole, L-tryptophan, limonin-17- β -D-glucoside, naringin, hesperidin, streptorubin B, and undecylprodigiosin were experimentally confirmed using authentic standards.

The overall profile reflects the activation of multiple biosynthetic pathways together with contributions from the plant systems.

Table 1. Abundance profiles of metabolites detected in spent media from co-cultures of *S. coelicolor* M145 with *C. limon* or *C. aurantium* seeds, grown in R2YE or TSB media with or without Trp supplementation, relative to single cultures under the same conditions. Metabolites are grouped according to their putative biochemical class or metabolic origin.

Metabolite Class	Metabolite	<i>S. coelicolor</i> and <i>C. limon</i> Co-Culture Abundance Profile ¹				<i>S. coelicolor</i> and <i>C. aurantium</i> Co-Culture Abundance Profile ¹			
		R2YE	R2YE+Trp	TSB	TSB+Trp	R2YE	R2YE+Trp	TSB	TSB+Trp
Tryptophan-derived metabolites	3-Methylindole	↓	↓	↓	↓	↓	↓	↓	↓
	L-Tryptophan	↓	↓	↓	↓	↓	↓	↓	↓
	N-Acetyl-L-tryptophan	=	=	↑	↑	↓	↓	=	↑
<i>Citrus</i> limonoids and limonoid glucosides	Isolimononic acid glucoside	↑	↑	–	–	↑	↑	–	–
	Limonin-17-β-D-glucoside	↓	↓	↓	↓	↓	↓	Lost	↓
	Deacetyl nomilinic acid glucoside	↑	↓	–	–	↑	=	–	–
	Isoobacunoic acid-17-β-D-glucoside	↑	↑	=	↑	↑	↓	=	↑
	Ichangin	↓	↓	–	–	↓	↓	–	Lost
	Nomilinic acid-17-β-D-glucoside	↑	↑	↑	↑	↑	↑	–	–
	Obacunone glucoside	↑	↑	↑	↑	↑	↑	–	–
	Nomilin A-ring lactone	=	↑	↓	↓	=	↓	Appeared	=
	Obacunoic acid	↑	↑	↑	↑	↑	↑	↑	↑
	Limonate A-ring lactone	–	–	–	–	↑	↓	↑	↓
<i>Citrus</i> flavonoids	Naringin	–	–	–	–	=	=	–	–
	Hesperidin	↑	↑	–	–	=	=	–	–
	Melitidin	–	–	–	–	=	=	↑	↑
	Brutieridin	–	–	–	–	=	=	↑	↑
Siderophores	Desferrioxamine B	=	=	–	–	=	=	–	–
	Nocardamin	=	=	–	–	↑	=	–	=
Polyketides, alkaloids and related microbial secondary metabolites	Futalosine	↓	↓	Appeared	Appeared	↓	↓	↑	↑
	Anthranoyllycoctonine	=	=	–	–	=	↑	–	–
	Streptazolin	=	=	=	=	=	↓	=	=
	Clecarmycin C	=	↑	–	–	↓	↓	–	–
	Nanaomycin	↓	↑	↓	↓	Lost	Lost	Lost	Lost
	Germicidin A	=	=	=	=	↓	↑	=	↑
Prodiginines	Germicidin B	=	=	↑	↑	↓	↑	=	↑
	Undecylprodigiosin	Lost	Lost	–	–	↑	↓	–	–

¹ ↑, increased abundance in co-culture compared to the control (i.e., sum of abundances in single cultures); ↓, decreased abundance in co-culture compared to the controls; =, no substantial abundance variation; Appeared, detected only in the co-culture; Lost, detected in at least one single culture but not in co-culture.

The evaluation of analytical reproducibility revealed a wide variability in the initial dataset, with several features exceeding the 25% RSD threshold. After filtering, the retained features showed a markedly reduced dispersion, with CV values confined below the defined threshold, confirming improved analytical reproducibility (Figure S1).

Multivariate statistical analysis was performed to evaluate global metabolic differences among the experimental conditions. PCA, applied as an unsupervised method, revealed a clear separation of samples along PC1 (49.2%), primarily reflecting differences in culture medium composition and *Citrus* seed supplementation. Samples containing *Citrus* seeds were mainly distributed on the negative side of PC1, whereas control conditions clustered on the positive side. R2YE-associated samples further grouped with metabolites linked to secondary metabolism. Replicates clustered closely, confirming good analytical reproducibility (Figure 1).

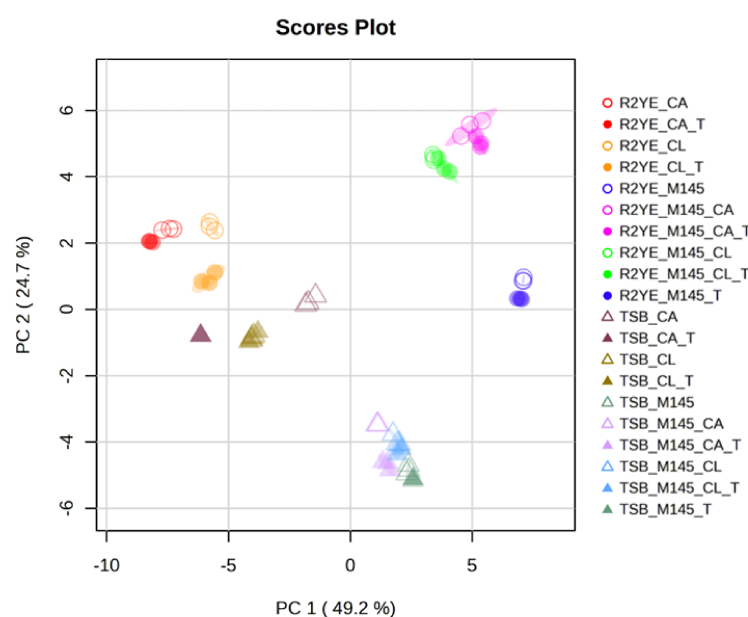


Figure 1. PCA score plot of the metabolomic profiles of *S. coelicolor* grown under different culture conditions. The bacterium was cultivated in two media, TSB and R2YE, in the presence of *C. aurantium* (CA) or *C. limon* (CL) seeds, and with supplementation of L-tryptophan (T). The first two principal components explain 73.9% of the total variance (PC1 = 49.2%, PC2 = 24.7%). Each point represents an individual sample, and colors indicate the different experimental groups as reported in the legend. The label M145 refers to the *S. coelicolor* M145 strain. Suffixes denote specific experimental conditions: CA = *C. aurantium*, CL = *C. limon*, T = L-tryptophan supplementation. Statistical significance of group separation was confirmed by PERMANOVA ($R^2 = 0.99917$, $p = 0.001$).

The PCA biplot highlights the main variables driving sample distribution (Figure 2). Microbial metabolites (e.g., germicidins, desferrioxamine B, nanaomycin) were associated with the positive side of PC1, mainly corresponding to R2YE cultures, whereas *Citrus*-derived compounds (e.g., hesperidin, ichangin, deacetyl nomilinic acid glucoside) were linked to the negative side, particularly in *Citrus*-seed-containing cultures. Consistently, flavonoids and limonoids were primarily associated with *C. aurantium*, contributing to sample separation (Table 1). PC2 (24.7%) further discriminated samples, mainly separating R2YE and TSB conditions. Overall, both medium composition and *Citrus* presence strongly shaped the metabolomic profiles of *S. coelicolor*, with Trp further contributing to metabolic differentiation.

PLS-DA confirmed the separation trends observed in PCA, showing clear clustering of samples according to growth medium and supplementation conditions (Figure 3). R2YE and TSB cultures formed distinct groups, with additional discrimination driven by *Citrus* seeds

and Trp supplementation, highlighting their combined effect on the metabolomic profile of *S. coelicolor*.

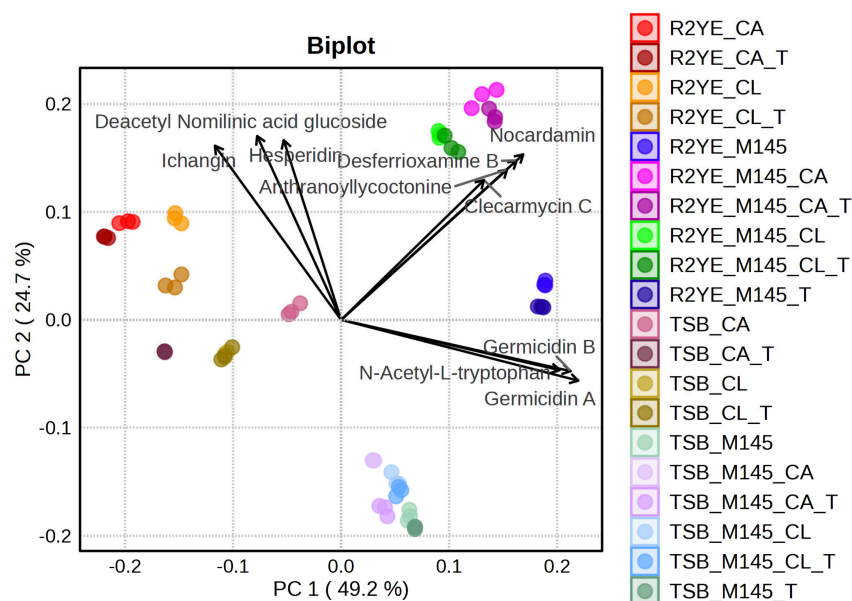


Figure 2. PCA biplot showing sample distribution and metabolites contributing to group separation. Each point represents an individual sample, and colors indicate the different experimental groups as reported in the legend. The label M145 refers to the *S. coelicolor* M145 strain. Suffixes denote specific experimental conditions: CA = *C. aurantium*, CL = *C. limon*, T = L-tryptophan supplementation.

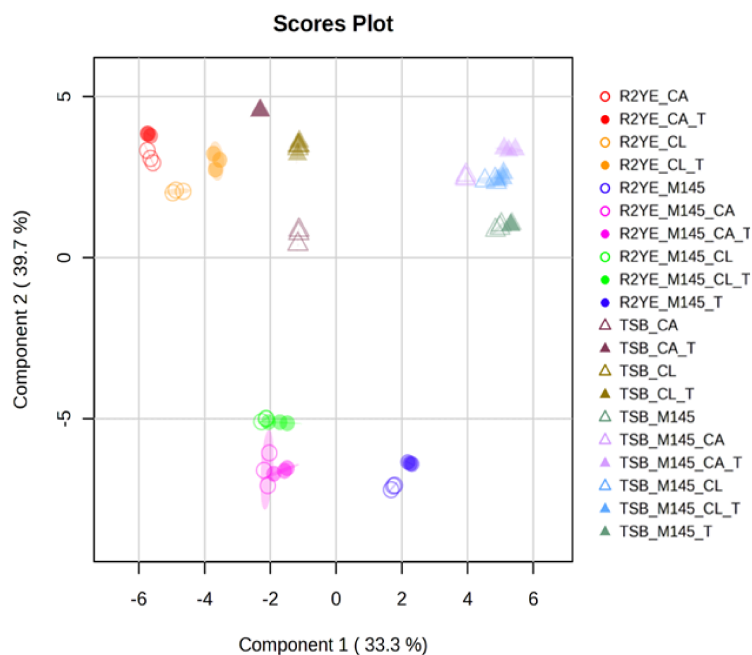


Figure 3. PLS-DA plot. The first two components explain 73% of the variance (Component 1 = 33.3%, Component 2 = 39.7%), highlighting metabolic differences associated with growth medium and supplementation. Each point represents an individual sample, and colors indicate the different experimental groups as reported in the legend. Colored ellipses represent the 95% confidence regions of the different experimental groups. The label M145 refers to the *S. coelicolor* M145 strain. Suffixes denote specific experimental conditions: CA = *C. aurantium*, CL = *C. limon*, T = L-tryptophan supplementation.

Model performance reached optimal R^2 and Q^2 values with five components, with no indication of overfitting (Figure S2A, Table S5). Permutation testing ($n = 1000$) confirmed model robustness ($p < 0.001$; Figure S2B).

Univariate analysis (one-way ANOVA, FDR-adjusted $p < 0.05$) identified key discriminant metabolites (Table S6), including germicidin A, germicidin B, N-acetyl-L-tryptophan, fufalosine, and desferrioxamine B. Tukey's HSD post hoc test confirmed significant differences across conditions, supporting the influence of medium composition, *Citrus* supplementation, and tryptophan on metabolite production.

3.1.2. Metabolites Detected in the Supernatant of *S. coelicolor* M145 Cultures Grown in R2YE or TSB Media in Absence or Presence of Trp

Metabolites detected in the supernatant of *S. coelicolor* M145 cultures included tryptophan-derived compounds, siderophores, and a range of microbial secondary metabolites, as confirmed by their presence in single-culture controls (Tables 1 and S1–S4; Figure S3–S9). Genome mining analysis performed using antiSMASH [56] identified biosynthetic gene clusters corresponding to detected metabolite classes, including siderophores (desferrioxamines), polyketide-derived metabolites (germicidins), and prodiginine compounds (Figure S10). Siderophores, including desferrioxamine B and nocardamin, were detected mainly under R2YE conditions, indicating a medium-dependent occurrence, while they were not detected under TSB conditions. Microbial secondary metabolites, including germicidin A, germicidin B, streptazolin, fufalosine, and anthranoyllycoctonine, were detected across conditions with a medium-dependent presence. A broader diversity of metabolites was observed in R2YE, whereas a more limited set was detected in TSB. Prodiginine-related metabolites, including undecylprodigiosin and streptorubin B, were detected under R2YE conditions, while they were not detected under TSB conditions. These differences corresponded to patterns observed in multivariate analysis, as shown by PCA biplot (Figure 2), where samples grown in R2YE clustered separately from those grown in TSB and were associated with metabolites related to secondary metabolism.

Overall, the metabolite profile of *S. coelicolor* cultures reflected medium-dependent detection patterns, with R2YE supporting a broader occurrence of specialized metabolites. Trp supplementation did not alter the classes of metabolites detected but influenced whether specific metabolites were detected under particular conditions.

3.1.3. Metabolites Detected in the Supernatant of Seeds of *Citrus* Species Incubated in R2YE or TSB Media in Absence or Presence of Trp

Metabolites detected in the supernatant of *C. limon* and *C. aurantium* seed cultures were primarily represented by limonoids, limonoid glucosides, and flavonoids, as confirmed by their occurrence in seed controls (Tables 1 and S1–S4).

Limonoids and their glucosides were widely detected across both *Citrus* species. Compounds such as isolimononic acid glucoside, nomilinic acid-17- β -D-glucoside, obacunone glucoside, and obacunoic acid were consistently detected, indicating their plant origin. In contrast, other compounds, including limonin-17- β -D-glucoside and ichangin, showed variable occurrence across conditions.

Species-specific patterns were observed. Limonoate A-ring lactone was detected exclusively in *C. aurantium*, whereas limonin derivatives were more commonly associated with *C. limon*. Additional limonoids, including deacetyl nomilinic acid glucoside and isoobacunoic acid-17- β -D-glucoside, showed condition-dependent occurrence.

Flavonoids were detected only in *C. aurantium* cultures. Naringin and hesperidin were detected in R2YE conditions, whereas melitidin and brutieridin were detected in TSB conditions, indicating medium-dependent metabolite profiles.

Overall, the metabolites detected in *Citrus* seed cultures were primarily determined by species identity and growth medium, while Trp supplementation influenced the occurrence of specific metabolites without introducing new metabolite classes.

3.1.4. Comparative Metabolomic Profiles in Co-Cultures of *S. coelicolor* and *Citrus* Species Seeds in R2YE or TSB Media in Absence or Presence of Trp

Metabolite detection in co-culture systems revealed a complex composition reflecting the combined contribution of microbial and plant-derived metabolites (Figures 1–3; Table 1).

In co-cultures with *C. limon* seeds, 27 metabolites were detected in R2YE medium using HPLC-ESI-QTOF-MS operating in both ionization modes (Table S1). Among these, 15 metabolites were associated with *S. coelicolor* metabolism and 10 with *Citrus*-derived pathways, while L-tryptophan and 3-methylindole were shared between both components. In TSB medium, the same co-culture system yielded 11 metabolites, mainly detected in negative ion mode, indicating contributions from both microbial and plant-derived compounds but with reduced overall diversity (Table S2). An analogous analytical workflow was applied to *C. aurantium* co-cultures (Figures S6–S9; Tables S3–S4). In this case, the metabolite profile was characterized by the presence of *Citrus*-specific flavonoids, as naringin, hesperidin, melitidin, and brutieridin were exclusively detected in *C. aurantium*-containing samples (Tables 1, S3 and S4).

Tryptophan-derived metabolites, including L-tryptophan, 3-methylindole, and N-acetyl-L-tryptophan, were consistently detected across all co-culture conditions. *Citrus* limonoids and their glucosides were widely represented, together with microbial secondary metabolites such as germicidins, siderophores, and prodiginine-related compounds, indicating the coexistence of multiple metabolic pathways within the system. Within this complex metabolic framework, distinct response patterns emerged across metabolite classes (Table 1). Tryptophan-derived compounds showed consistent negative abundance trends across all conditions, whereas several microbial secondary metabolites (e.g., germicidins and fufalosine) displayed increased abundance specifically in TSB co-cultures, particularly in the presence of Trp. In contrast, *Citrus*-derived metabolites exhibited species-dependent and condition-specific responses. These condition-dependent patterns were further reflected in the multivariate analysis. In accordance with the PCA biplot (Figure 3), co-culture samples clustered according to growth medium and co-culture composition, with separation also observed between *Citrus* species, indicating distinct metabolite profiles under the tested conditions. R2YE-associated samples were separated from TSB samples and were associated with metabolites related to secondary metabolism, whereas TSB samples showed a more compact distribution.

Quantitative differences in metabolite abundance between co-cultures and the corresponding controls are reported as Log₂ fold change values (Figures 4 and 5; Tables S7–S10).

Tryptophan-derived metabolites showed consistent negative Log₂ values across conditions, with L-tryptophan displaying marked reductions, particularly in R2YE (e.g., −2.02 to −4.06 in *C. aurantium* and −3.58 to −5.16 in *C. limon*). In contrast, N-acetyl-L-tryptophan showed condition-dependent responses, with increased values observed in TSB co-culture conditions including Trp supplementation (e.g., up to +0.97). Microbial secondary metabolites exhibited pathway-specific variability. Germicidin A and germicidin B showed increased abundance particularly under TSB co-culture conditions including Trp supplementation (e.g., up to +1.48 and +1.86, respectively), whereas fufalosine displayed negative values in R2YE (down to −1.56) and strong positive increases in TSB (up to +2.71). In contrast, prodiginine-related metabolites showed reduced abundance in co-cultures under Trp-supplemented conditions, with undecylprodigiosin shifting from positive to negative values in *C. aurantium* co-cultures (from +2.66 to −0.91). *Citrus*-derived metabolites showed species-dependent modulation. In *C. aurantium* co-cultures, flavonoids exhibited strong positive Log₂ fold change values under Trp supplementation in TSB conditions (e.g., brutieridin +1.70 to +2.59, melitidin +1.22 to +2.01, and naringin up to +2.75), whereas in *C. limon* co-cultures the most pronounced increases were observed for limonoid-related

compounds such as obacunoic acid (+2.58 to +3.41) and obacunone glucoside (+1.20 to +1.63). Overall, the combined qualitative and quantitative analyses highlight the complex and condition-dependent metabolic profiles of co-culture systems, consistent with the combined contribution of microbial metabolism, plant-derived compounds, growth medium composition, and Trp availability.

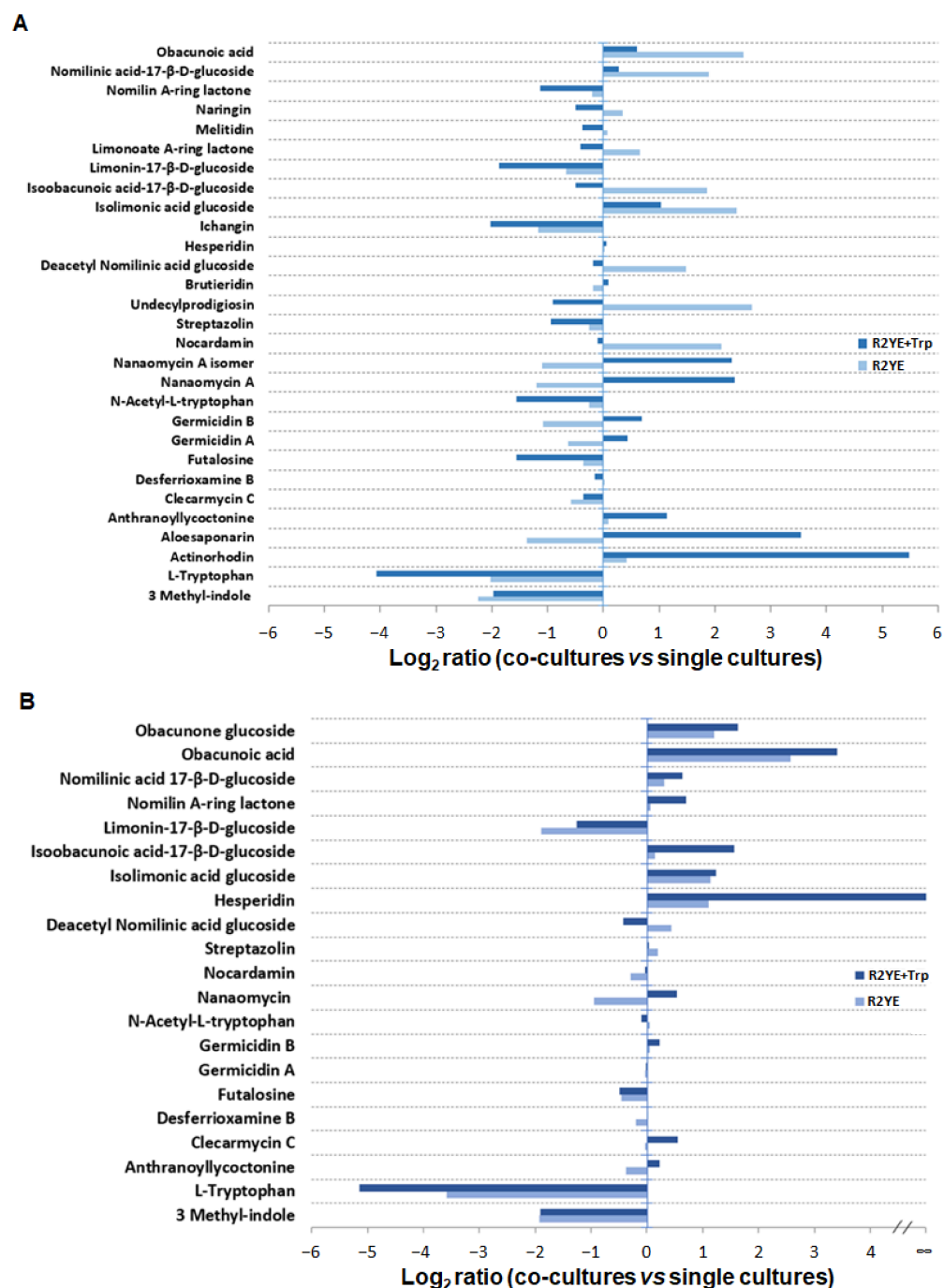


Figure 4. Log₂ ratio of spent medium metabolite levels in *S. coelicolor*–*C. aurantium* (A) and *S. coelicolor*–*C. limon* (B) co-cultures compared with the summed levels from the corresponding single cultures grown in R2YE, without or with L-tryptophan (+Trp) supplementation. Reported values represent mean measurements from biological triplicates, with statistical significance assessed by one-way ANOVA ($p < 0.05$).

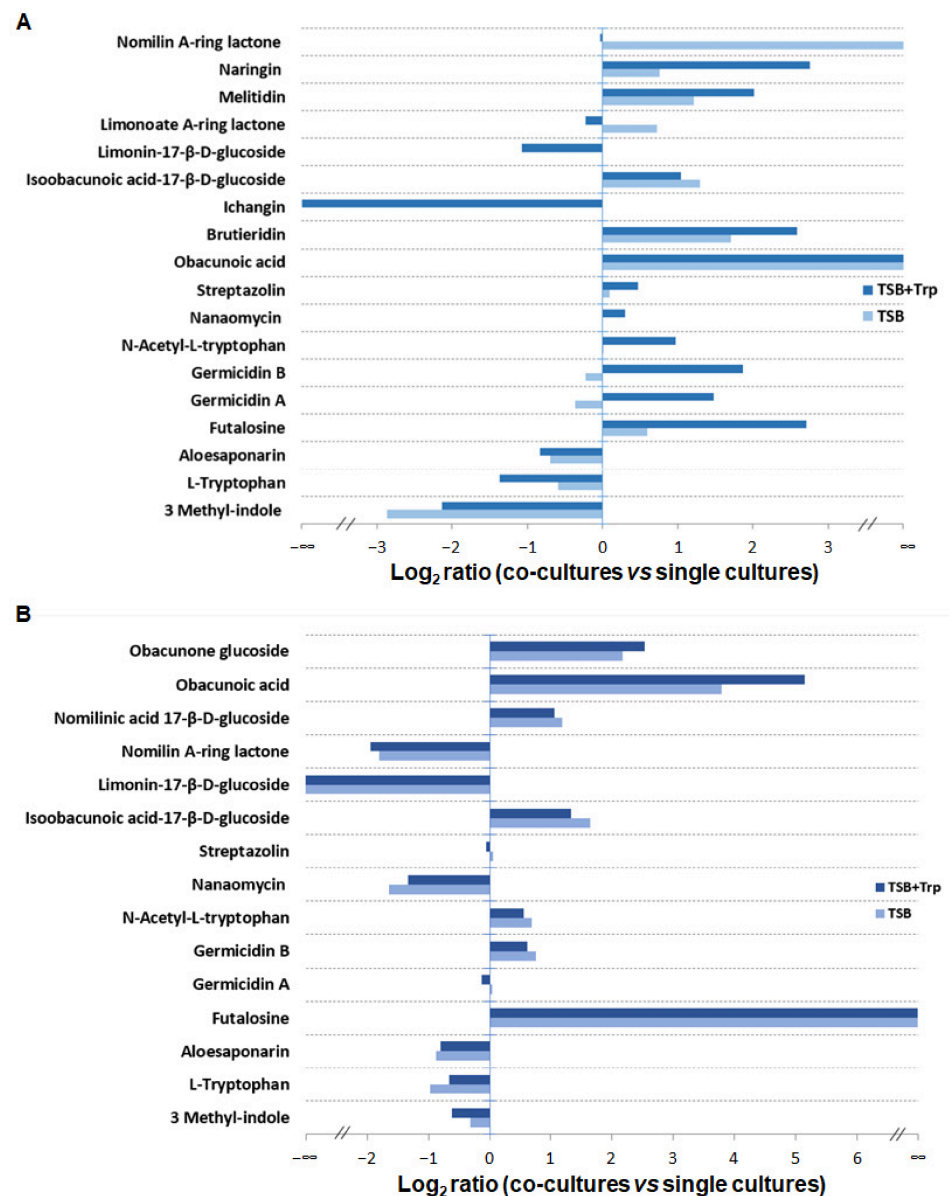


Figure 5. Log₂ ratio of spent medium metabolite levels in *S. coelicolor*–*C. aurantium* (A) and *S. coelicolor*–*C. limon* (B) co-cultures compared with the summed levels from the corresponding single cultures grown in TSB, without or with L-tryptophan (+Trp) supplementation. Reported values represent mean measurements from biological triplicates, with statistical significance assessed by one-way ANOVA ($p < 0.05$).

Hierarchical clustering analysis (Figure 6) revealed distinct groups of metabolites with coordinated abundance patterns across conditions. Microbial-derived metabolites (e.g., germicidins, N-acetyl-L-tryptophan, futalosine) clustered separately from *Citrus*-derived compounds, indicating distinct but interacting metabolic networks. In addition, samples grouped according to growth medium and co-culture composition, indicating condition-dependent metabolomic profiles.

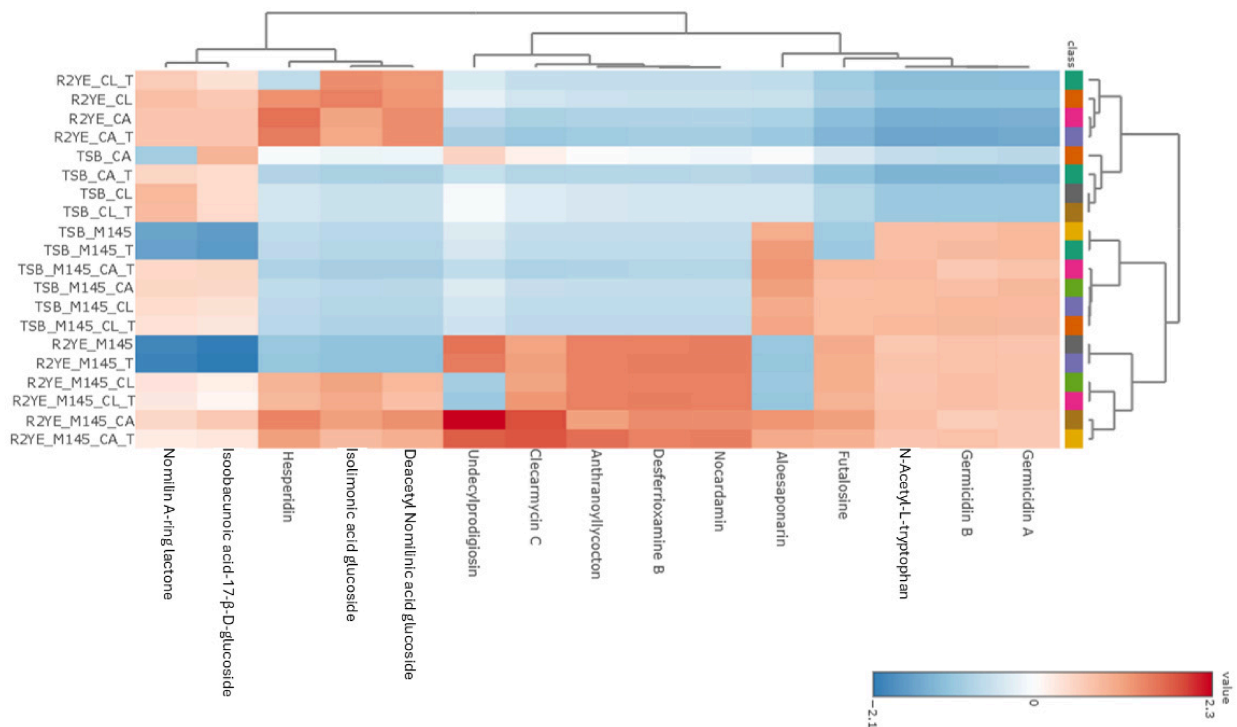


Figure 6. Hierarchical clustering heatmap of metabolite profiles based on Pearson correlation coefficients. Rows represent metabolites and columns represent samples under different culture conditions in R2Y or TSB growth media. CL: *C. limon*. CA: *C. aurantium*. T: L-Tryptophan.

3.2. Effects on Tomato Seedlings Growth

3.2.1. Experimental Overview of Co-Culture Effects on Tomato Seedling Growth

To assess possible emergent biological effects of *S. coelicolor* co-cultivated with *C. aurantium* and *C. limon* seeds in R2YE and TSB media, with or without Trp supplementation, root and shoot development of *S. lycopersicum* seedlings was evaluated through in vitro experiments using spent culture media in a seed imbibition-based assay. Considering the aim of these assays, and given that preliminary experiments showed that uninoculated R2YE and TSB media had effects comparable to distilled water in this assay (Figure S11), the same experimental design adopted for untargeted metabolomic analysis was applied.

Data were analyzed by one-way ANOVA followed by Tukey's HSD test, revealing a strong context-dependent biological response generated by the interaction between streptomycete- and *Citrus* seed-derived compounds under the different nutritional conditions provided by R2YE and TSB.

3.2.2. Impact of *S. coelicolor*–*C. aurantium* Co-Cultures on Tomato Seedling Growth

All treatments resulted in an increase in both root and shoot length of *S. lycopersicum* seedlings compared to sdH₂O used as the control (CT), with responses depending on growth media and Trp supplementation (Figure 7, Table S11). Root growth ranged from 6.97 to 9.22 cm, while shoot length increased from 1.30 cm in the control to values up to 2.29 cm, with generally stronger effects observed for shoot development.

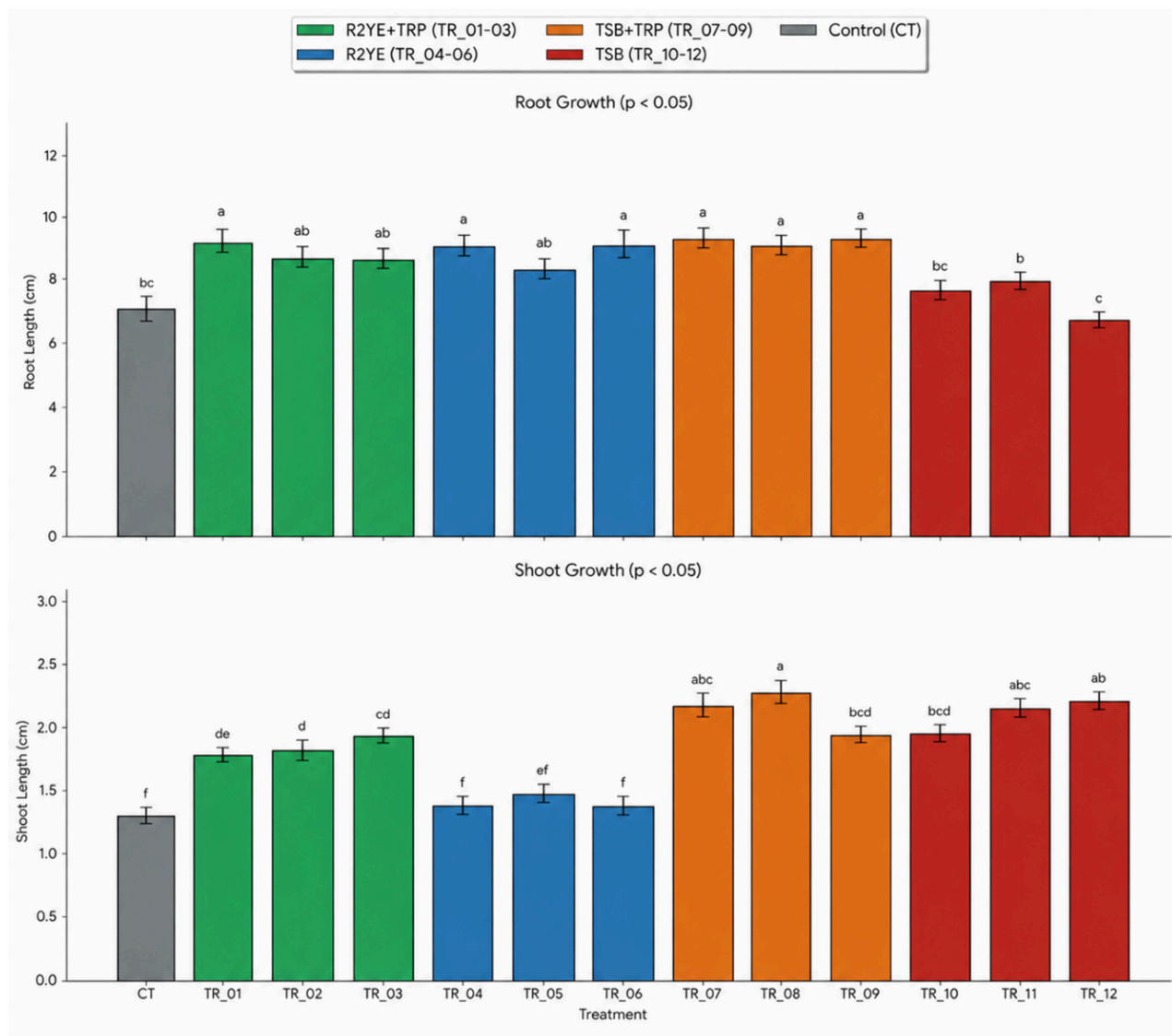


Figure 7. Root and shoot length (cm) of *S. lycopersicum* seedlings exposed to different treatments (TR) performed using spent culture media obtained from *S. coelicolor*–*C. aurantium* seed co-cultures and corresponding single cultures. Treatments (also reported in Table S11) consisted of sdH₂O as the control (CT) or the following spent culture media: TR_01, R2YE+Trp with *Citrus* seeds; TR_02, R2YE+Trp with *Citrus* seeds and *S. coelicolor*; TR_03, R2YE+Trp with *S. coelicolor*; TR_04, R2YE with *Citrus* seeds; TR_05, R2YE with *Citrus* seeds and *S. coelicolor*; TR_06, R2YE with *S. coelicolor*; TR_07, TSB+Trp with *Citrus* seeds; TR_08, TSB+Trp with *Citrus* seeds and *S. coelicolor*; TR_09, TSB+Trp with *S. coelicolor*; TR_10, TSB with *Citrus* seeds; TR_11, TSB with *Citrus* seeds and *S. coelicolor*; TR_12, TSB with *S. coelicolor*. Bars represent mean $\pm$ standard error. Different letters above bars indicate statistically significant differences among treatments ($p \leq 0.05$; post hoc test).

The presence of *Citrus* seeds alone induced condition-dependent responses. In R2YE medium, seed-derived treatments were associated with increased root length (up to +26.6%), while shoot growth remained only slightly affected. In contrast, in TSB medium, seed treatments resulted in a more pronounced increase in shoot length (up to +48.6%), with a comparatively minor effect on root development.

Similarly, *S. coelicolor* alone promoted seedling growth in a medium-dependent manner. In R2YE, treatments using *S. coelicolor* M145 spent media were associated with an increase in root length (up to +28.5%), whereas shoot development remained limited. Conversely,

in TSB, *S. coelicolor* M145 induced a strong increase in shoot length (up to +76.4%), while root growth was unchanged or slightly reduced (−1.8%).

In particular, in TSB medium, root growth was strongly enhanced by all treatments including Trp supplementation, showing increases close to 30% relative to the control. Treatments without Trp supplementation to the growth medium induced smaller root responses, and in the case of *S. coelicolor* alone, a slight reduction in root length was observed. Shoot growth was consistently stimulated in TSB, with increases ranging from 48.6% to 76.4%, the highest values being recorded for the *S. coelicolor* spent medium treatments.

The comparison between co-culture and single-culture treatments revealed variable responses depending on the experimental conditions. In R2YE, co-cultures generally resulted in root growth values that were lower than or comparable to those observed in the corresponding single-culture treatments. In TSB, co-culture treatments showed moderate increases in both root and shoot growth relative to *Citrus* seed single-culture-derived treatments, but values remained within the range observed for single cultures.

Overall, R2YE-derived spent media generally favored root elongation, often accompanied by limited shoot responses, whereas TSB-derived spent media promoted strong shoot stimulation across multiple treatments. Within this framework, tryptophan exerted a modulatory rather than dominant effect on growth responses: although it enhanced growth under specific conditions, particularly in TSB-derived spent media, it did not override the effects associated with seed presence or microbial treatments.

3.2.3. Impact of *S. coelicolor*–*C. limon* Co-Cultures on Tomato Seedling Growth

Unlike *C. aurantium*, *C. limon* seeds frequently generated spent media associated with asymmetric growth responses, particularly under R2YE conditions (Figure 8, Table S12). In the R2YE medium, all treatments promoted root elongation relative to the control, with increases ranging from 0.9% to 25.5%, the strongest response being observed in the presence of *S. coelicolor*. In contrast, shoot growth was consistently reduced across all R2YE treatments (−4.7% to −15.7%), indicating a preferential allocation toward root development under these conditions.

The contribution of *C. limon* seeds in a single culture was moderate and context-dependent. In R2YE, treatments using spent media from *C. limon* seed single cultures resulted in increased root length (up to +15.6%) with limited or negative effects on shoot growth. In TSB, seed treatments showed only minor effects on root development (up to +2.5%), while shoot growth was markedly enhanced (up to +35.7%). A slight increase in shoot growth was observed in the presence of seeds mainly in TSB medium, particularly when combined with tryptophan and *S. coelicolor*.

Similarly, treatments using spent *S. coelicolor* single-culture media showed medium-dependent responses. In R2YE, *S. coelicolor* promoted root elongation (up to +25.5%) but was associated with reduced shoot growth. In TSB, *S. coelicolor* had limited effects on root length but strongly stimulated shoot growth (up to +73.4%), representing the highest response observed.

In TSB medium, root responses were generally limited and variable. Treatments containing tryptophan induced slight reductions or minimal variation in root length (−4.6% to +0.4%), whereas treatments without tryptophan resulted in small positive effects, with a maximum increase of 10.5% in the seed and *S. coelicolor* co-cultures. In contrast, shoot growth was consistently and strongly stimulated across all TSB treatments, with increases ranging from 35.7% to 73.4%.

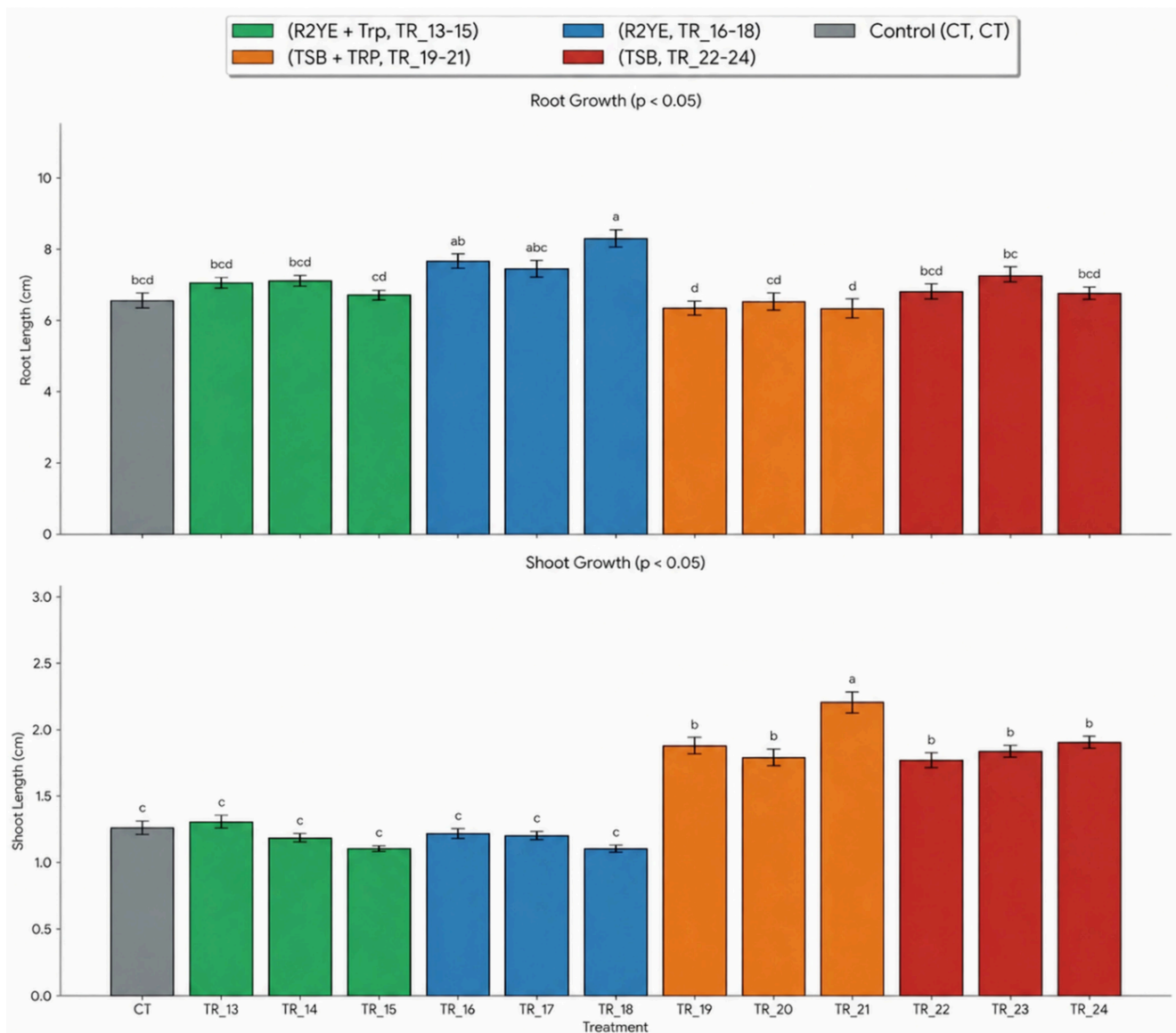


Figure 8. Root and shoot length (cm) of *S. lycopersicum* seedlings exposed to different treatments (TR) performed using spent culture media obtained from *S. coelicolor*–*C. limon* seed co-cultures and corresponding single cultures. Treatments (also reported in Table S12) consisted of sdH₂O as the control (CT) or the following spent culture media: TR_13, R2YE+Trp with *Citrus* seeds; TR_14, R2YE+Trp with *Citrus* seeds and *S. coelicolor*; TR_15, R2YE+Trp with *S. coelicolor*; TR_16, R2YE with *Citrus* seeds; TR_17, R2YE with *Citrus* seeds and *S. coelicolor*; TR_18, R2YE with *S. coelicolor*; TR_19, TSB+Trp with *Citrus* seeds; TR_20, TSB+Trp with *Citrus* seeds and *S. coelicolor*; TR_21, TSB+Trp with *S. coelicolor*; TR_22, TSB with *Citrus* seeds; TR_23, TSB with *Citrus* seeds and *S. coelicolor*; TR_24, TSB with *S. coelicolor*. Bars represent mean $\pm$ standard error. Different letters above bars indicate statistically significant differences among treatments ($p \leq 0.05$; post hoc test).

The comparison between co-culture and single-culture treatments revealed variable patterns. In R2YE, co-cultures were associated with moderate root increases and consistently reduced shoot growth compared to single-culture conditions. In TSB, co-culture treatments resulted in increases in both root and shoot growth relative to seed-only treatments, although values remained within the range observed for *S. coelicolor* alone.

Overall, R2YE-derived spent media promoted root elongation at the expense of shoot development, whereas TSB-derived spent media consistently favored shoot growth across treatments. Within this framework, co-culture responses were primarily quantitative and condition-dependent, with no evidence of systematic synergistic enhancement compared

to single-culture conditions. Co-culture treatments occasionally resulted in enhanced growth responses compared to seed-only conditions, particularly under TSB medium, where both root and shoot development showed moderate increases. However, these improvements were not consistently observed across all conditions and did not exceed the highest responses recorded for *S. coelicolor* single cultures, indicating that co-culture effects are context-dependent and not systematically synergistic. Tryptophan exerted a modulatory effect on these responses without overriding the influence of medium composition or biological components.

4. Discussion

The metabolic response of *S. coelicolor* M145 is strongly influenced by the combined effects of growth medium composition, Trp availability, and the presence of *Citrus*-seed-derived metabolites. These factors determine either the activation or repression of specific secondary metabolic pathways, thus influencing the biological activity of the system.

Metabolite annotations were largely consistent with previously described biosynthetic pathways in *S. coelicolor* and other *Streptomyces* species, as supported by literature data [57–61]. This agreement supports a strong genome–metabolome relationship, in which the detected metabolite classes reflect the intrinsic biosynthetic potential of the strain. In particular, siderophores such as desferrioxamines are consistent with well-characterized iron acquisition strategies in *Streptomyces* [57,58]. Similarly, polyketide-derived metabolites, including prodiginines such as undecylprodigiosin and streptorubin B, are characteristic of *S. coelicolor* secondary metabolism [59]. Their co-occurrence indicates an active and coordinated biosynthetic network. Germicidin-type metabolites are also consistent with known regulatory roles in development and spore germination [60,61], suggesting a broader regulatory function under the tested conditions. Additional compounds, including RK-397, pimprinethine, streptazolin, futasoline, and N-acetyl-L-tryptophan, further illustrate the metabolic versatility of *Streptomyces* [62–66].

In contrast, some annotations are less supported by genome mining data. Compounds such as clemastin C and nanaomycin [67,68] lack corresponding biosynthetic gene clusters in *S. coelicolor* based on antiSMASH analysis [56], suggesting tentative identification based on spectral similarity. A similar consideration applies to anthranoyllycoctonine, typically associated with plant metabolism [69], whose detection in *Streptomyces* systems may reflect structurally related metabolites or uncharacterized biosynthetic potential. These observations highlight the need to distinguish microbial metabolites from matrix-derived compounds in co-culture systems.

The occurrence of 3-methylindole further reflects system complexity, as it has been associated with microbial catabolism of tryptophan or IAA [70], indicating active tryptophan-derived metabolic routes. Overall, these findings suggest that Trp acts as a contextual modulator of the co-culture system rather than a direct driver of specific biosynthetic pathways in *S. coelicolor*.

The growth medium emerged as a major determinant of metabolic output. R2YE, a production-oriented medium, promoted the accumulation of secondary metabolites, including siderophores and polyketide-derived compounds, whereas TSB favored metabolic profiles associated with vegetative growth and the biotransformation of *Citrus*-derived compounds. Consequently, medium composition strongly influenced both primary and secondary metabolism in *S. coelicolor*.

These differences were reflected in *S. lycopersicum* seedling assays, with distinct biological responses under the tested conditions. *S. lycopersicum* was selected as a model system due to its well-characterized genetics, reproducible early development, and sensitivity to plant growth-promoting bacteria and microbially derived metabolites [3,21,71–73]. In

this present study, R2YE-derived supernatants promoted root growth, while TSB-derived ones enhanced shoot development, indicating that root–shoot allocation is shaped by the biochemical composition of metabolically conditioned media. These patterns are consistent with plant responses to microbially derived metabolites [72,74].

These results are consistent with the reported role of Trp as a precursor of auxin biosynthesis in both plants and microbes [75,76], suggesting that the observed responses may reflect metabolite-mediated effects established during incubation rather than direct nutrient input to tomato seedlings. The observed responses align with allelopathic effects of *Citrus* spp. [77,78], while co-incubation with *S. coelicolor* suggests partial transformation of inhibitory compounds.

Citrus seeds acted as chemically active modulators of *S. coelicolor* metabolism in a species-specific manner. Both *C. aurantium* and *C. limon* introduced flavonoids and limonoids, with compositional differences reflected in distinct metabolite profiles and plant responses [79]. Flavanone glycosides (e.g., naringin, hesperidin, brutieridin) characterized *C. aurantium*, whereas limonoids predominated in *C. limon*, consistent with previously reported species-specific patterns and associated biological effects [80,81].

Co-culture conditions generated non-additive responses, with metabolite profiles differing from simple combinations of single cultures. These effects were primarily quantitative and condition-dependent, reflecting modulation of existing metabolic networks. These findings support the ability of *Streptomyces* to biotransform plant-derived compounds [82] likely to act as antimicrobial or signaling molecules, as observed for plant secondary metabolites involved in microbe–plant interactions [77,83].

The integration of metabolomic data with plant bioassays indicates that early seedling development is influenced by complex mixtures of microbial and plant-derived metabolites, reflecting the system-level nature of the interaction. Modulation of siderophore production and specialized metabolites further suggests links between nutrient availability, stress response, and secondary metabolism. Changes in desferrioxamine B and nocardamin, particularly under R2YE and Trp supplementation, support a role for iron-responsive metabolic regulation such as the Ferric uptake regulator (Fur) system, which is also influenced by Trp [84,85]. The PCA biplot further indicated that microbial metabolites involved in iron acquisition and secondary metabolism contribute to the observed metabolic differentiation, suggesting an active microbial response associated with iron limitation or environmental stress [86]. These observations support the hypothesis that *Citrus*-derived compounds may act as environmental modulators of microbial metabolism, although additional targeted studies would be required to clarify the underlying mechanisms involved. Iron limitation is a well-established trigger of siderophore biosynthesis in *Streptomyces* and can also activate antibiotic production, consistent with the observed enhancement of iron-related and secondary metabolic pathways [57,87].

At the same time, *Citrus*-derived metabolites—particularly limonoids and flavonoids such as hesperidin, naringin, and limonin-related glucosides—showed condition-dependent variations, including increases, decreases, and appearance/disappearance patterns, indicating active interaction with microbial metabolism. The modulation of tryptophan-derived compounds further highlights the involvement of amino acid-driven metabolic networks. The decrease in L-tryptophan observed in several co-culture conditions, coupled with the increase in downstream metabolites, is consistent with its metabolic utilization directly or indirectly driving the biosynthesis of specialized compounds. Importantly, these patterns should not be interpreted as the result of a direct comparison between conditions with and without Trp supplementation, but rather as the outcome of co-culture systems analyzed under different environmental contexts, in which Trp acts as a contextual and modulatory factor. These variations, primarily reflected as quantitative changes in metabolite

abundance, support the interpretation that co-culture effects are modulatory rather than indicative of the emergence of entirely new metabolic states, with coordinated responses particularly evident under R2YE+Trp conditions.

Taken together, these observations support a model in which *Citrus*-derived compounds modulate nutrient availability and chemical signaling, while tryptophan contributes as a metabolic input that shapes context-dependent reprogramming of *S. coelicolor* metabolism. Unlike previous co-culture studies focused on microbial partners [33–35,88,89] or plant-associated systems [30,90,91], this study shows that plant-derived chemical inputs from surface-sterilized seeds can significantly influence microbial secondary metabolism under defined conditions. This integrative framework allows a comparative interpretation of metabolomic profiles across complex biological systems and strengthens the evidence for plant–microbe metabolic crosstalk with potential biotechnological applications [90].

Although pooled quality control (QC) samples were not included in the present untargeted metabolomics workflow, data reproducibility was supported through the use of independent biological replicates, technical triplicates, and CV-based filtering. Nevertheless, the inclusion of pooled QC samples in future studies would further strengthen analytical robustness. In this context, interpretations remain correlative, and targeted investigations will be required to establish causal relationships between specific metabolites and biological effects. Therefore, further reductionist approaches will be necessary to disentangle direct and synergistic effects and to identify potentially undetected metabolites.

Collectively, these findings highlight that early plant growth responses are maximized in multicomponent, pre-conditioned systems, in which *Citrus*-derived metabolites, microbial activity, and incubation media interact to shape complex metabolic outputs.

5. Conclusions

This study demonstrates that *S. coelicolor* exhibits distinct quantitative metabolic responses when cultivated in the presence of *C. aurantium* and *C. limon* seeds, with outcomes strongly influenced by the growth medium and tryptophan availability.

R2YE favored the accumulation of secondary metabolite, particularly antibiotic-related compounds, whereas TSB was associated with primary metabolism and the transformation of plant-derived compounds.

L-tryptophan acted as a modulatory factor, influencing pathways associated with indole-derived metabolites, siderophore biosynthesis, and antibiotic production in a context-dependent manner.

Metabolomic analyses revealed that the observed metabolic profile likely results from the interaction between microbial metabolism and *Citrus*-derived bioactive metabolites, highlighting the role of defined plant-derived inputs as modulators of specialized metabolism.

The functional relevance of these interactions was supported by bioassays on *S. lycopersicum*, where plant responses varied depending on the combined effects of *Citrus* seeds, streptomycete activity, nutrient conditions, and tryptophan.

This approach provides a framework for disentangling plant chemical signaling from whole-plant physiology, enabling a global investigation of plant–microbe metabolic interactions. Nevertheless, a reductionist approach, based on the addition of individual metabolites or combinations thereof, could further clarify these effects; however, this would require systematic evaluation of concentration-dependent and temporal dynamics.

From an applied perspective, these findings support the rational design of plant–microbe systems for enhancing the production of bioactive compounds and developing metabolite-driven biostimulants.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation12070321/s1>. Figure S1: Violin plots showing the distribution of coefficient of variation (CV%) across technical replicates before (A) and after (B) filtering (RSD < 25%). The box represents the interquartile range (25–75%), whiskers indicate $1.5 \times$ IQR, and the dot represents the median. The y-axes are scaled independently to improve visualization of the different variability ranges, with a red line at 25% range. Figure S2: Validation of the PLS-DA model. (A) R^2 and Q^2 values as a function of the number of components. (B) Permutation test output. Figure S3: Representative HPLC/ESI/QTOF trace of spent medium from cultures of *S. coelicolor* M145 in R2YE growth medium in presence of *C. limon* (positive ion mode). Figure S4: Representative HPLC/ESI/QTOF trace of spent medium from cultures of *S. coelicolor* M145 in R2YE growth medium in presence of *C. limon* seeds (negative ion mode). Figure S5: Representative HPLC/ESI/QTOF trace of spent medium from cultures of *S. coelicolor* M145 in TSB growth medium in presence of *C. limon* seeds (negative ion mode). Figure S6: Representative HPLC/ESI/QTOF trace of spent medium from cultures of *S. coelicolor* M145 in R2YE growth medium in presence of *C. aurantium* seeds (positive ion mode). Figure S7: Representative HPLC/ESI/QTOF trace of spent medium from cultures of *S. coelicolor* M145 in R2YE growth medium in presence of *C. aurantium* seeds (negative ion mode). Figure S8: Representative HPLC/ESI/QTOF trace of spent medium from cultures of *S. coelicolor* M145 in TSB growth medium in presence of *C. aurantium* seeds (positive ion mode). Figure S9: Representative HPLC/ESI/QTOF trace of spent medium from cultures of *S. coelicolor* M145 in TSB growth medium in presence of *C. aurantium* seeds (negative ion mode). Figure S10: Identification of secondary metabolite regions in the *S. coelicolor* A3(2) genome by AntiSMASH analysis using relaxed strictness. Figure S11: Shoot (left) and root (right) length (cm) in *S. lycopersicum* seedlings exposed to H₂O, R2YE, and TSB. Treatments as defined in the Section 2. Bars represent mean $\pm$ standard error. Different letters above bars indicate statistically significant differences among treatments ($p \leq 0.05$). Different colours were used for the tree treatment and tissue: H₂O grey (black grey for shoot and light grey for root); R2YE cyan (black cyan for shoot and light cyan for root); TSB yellow (black yellow for shoot and light yellow for root). Table S1: Composition of spent media from cultures of *S. coelicolor* M145 in R2YE growth medium in presence of *C. limon* seeds. Table S2: Composition of spent media from cultures of *S. coelicolor* M145 in TSB growth medium in presence of *C. limon* seeds. Table S3: Composition of spent media from cultures of *S. coelicolor* M145 in R2YE growth medium in presence of *C. aurantium* seeds. Table S4: Composition of spent media from cultures of *S. coelicolor* M145 in TSB growth medium in presence of *C. aurantium* seeds. Table S5: Cross-validation parameters obtained from the PLS-DA model using an increasing number of latent components (1–8 components). Classification accuracy, coefficient of determination (R^2), and predictive ability (Q^2) are reported for each model. Table S6: One-way ANOVA results for the selected discriminant metabolites identified across the different experimental conditions. Reported values include F-statistics, p -values, false discovery rate (FDR)-adjusted p -values, and the most biologically relevant pairwise comparisons identified by Tukey’s HSD post hoc test. Table S7: Log₂ fold change values of metabolites detected in *S. coelicolor*-*C. aurantium* seed co-cultures grown in R2YE medium, with and without Trp supplementation, compared with the summed levels of the corresponding single cultures. Table S8: Log₂ fold change values of metabolites detected in *S. coelicolor*-*C. aurantium* seed co-cultures grown in TSB medium, with and without Trp supplementation, compared with the summed levels of the corresponding single cultures. Table S9: Log₂ fold change values of metabolites detected in *S. coelicolor*-*C. limon* seed co-cultures grown in R2YE medium, with and without Trp supplementation, compared with the summed levels of the corresponding single cultures. Table S10: Log₂ fold change values of metabolites detected in *S. coelicolor*-*C. limon* seed co-cultures grown in TSB medium, with and without Trp supplementation, compared with the summed levels of the corresponding single cultures. Table S11: Root and shoot growth responses of *S. lycopersicum* seedlings exposed to treatments using spent cultivation media from *S. coelicolor*-*C. aurantium* seed co-cultures and corresponding single cultures. Values are means. Δ and percentage values indicate absolute and relative changes compared to the control condition plants. Table S12: Root and shoot growth responses of *S. lycopersicum* seedlings exposed to treatments using spent cultivation media from

S. coelicolor–*C. limon* seed co-cultures and corresponding single cultures. Values are means. Δ and percentage values indicate absolute and relative changes compared to the control condition plants.

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

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Abbreviations

BGCs	Biosynthetic Gene Clusters
PGPB	Plant Growth Promoting Bacteria
IAA	Indole3Acetic Acid
HPLC-ESI-QTOF-MS	High-Performance Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry

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