Bioprospecting *Trichoderma*: A Systematic Roadmap to Screen Genomes and Natural Products for Biocontrol Applications

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Natural products derived from microbes are crucial innovations that would help in reaching sustainability development goals worldwide while achieving bioeconomic growth. *Trichoderma* species are well-studied model fungal organisms used for their biocontrol properties with great potential to alleviate the use of agrochemicals in agriculture. However, identifying and characterizing effective natural products in novel species or strains as biological control products remains a meticulous process with many known challenges to be navigated. Integration of recent advancements in various "omics" technologies, next generation biodesign, machine learning, and artificial intelligence approaches could greatly advance bioprospecting goals. Herein, we propose a roadmap for assessing the potential impact of already known or newly discovered *Trichoderma* species for biocontrol applications. By screening publicly available *Trichoderma* genome sequences, we first highlight the prevalence of putative biosynthetic gene clusters and antimicrobial peptides among genomes as an initial step toward predicting which organisms could increase the diversity of natural products. Next, we discuss high-throughput methods for screening organisms to discover and characterize natural products and how these findings impact both fundamental and applied research fields.

Keywords: predictive biology, functional genomics, secondary metabolites, antimicrobials, integrated pest management, plant-microbe interactions, graph theory—graph algorithms, drug discoveries

INTRODUCTION

The discovery and usage of biological controls as management strategies started with astute observations of ecological niches for studying microbial interactions (Dubos, 1939; Dubos and Cattaneo, 1939; van den Bosch et al., 1982; Dias et al., 2012; Barratt et al., 2018). Later came the rise of biological control applications due to a consortium of scientists and industry partners working on multidisciplinary ideas and projects that initially seemed unrelated yet were serving a common goal. Invasive species cost around $120 billion USD yearly in crop yield losses (Pimentel et al., 2005); plant pathogens alone, primarily fungi, result in annual crop financial losses estimated at $23.5 billion USD (including control costs) (Rossman, 2009). Even more alarming

are the pesticide-resistant populations that exist, which have led to the conception of several organizations, e.g., the Fungicide Resistance Action Committee (https://www.frac.info/), and the Insecticide Resistance Action Committee (https://irac-online.org/). However, not all microbes associated with crops are harmful (Stark, 2010). In fact, beneficial microbes have become an integral component of pest management strategies to control pest populations or promote plant health (Meena et al., 2017). Factors that influence the use of beneficial microbes as biological control products are stress-induced environments, nutrient-deficient areas, and known populations of plant pathogens that can be controlled (Hayat et al., 2010; Stark, 2010; Chen et al., 2018; Begum et al., 2019; Kulimushi et al., 2021). In general, the use of biological control products is preferred for many reasons, including the reduction of pesticide use, cost-effectiveness, and its efficacy against a broad range of natural pest and support services (Bale et al., 2008; Benjamin and Wesseler, 2016; Barratt et al., 2018). Yet, biological control product applications face several challenges including invasive species stemming from the fungus used as an active ingredient; increasing crop groups, cultivars and varieties; pest complexes and resistances; incompatibility with pesticides; non-targeted effects, and risk assessment strategies (Bale et al., 2008; Barratt et al., 2018; Köhl et al., 2019). Given the complexity of these challenges, herein we propose a roadmap for bioprospecting microbes, using *Trichoderma* species as our model organisms. The suggested roadmap integrates predictive biology, functional genomics, high-throughput analytics, and next-generation biodesign and genome engineering approaches. Moreover, because newly discovered and characterized biological controls can have various applications, we provide a summary of important advantages and drawbacks that should be considered. Using this framework, we begin to predict which species among the already sequenced *Trichoderma* have unique potential as valuable biocontrol agents or source of natural products.

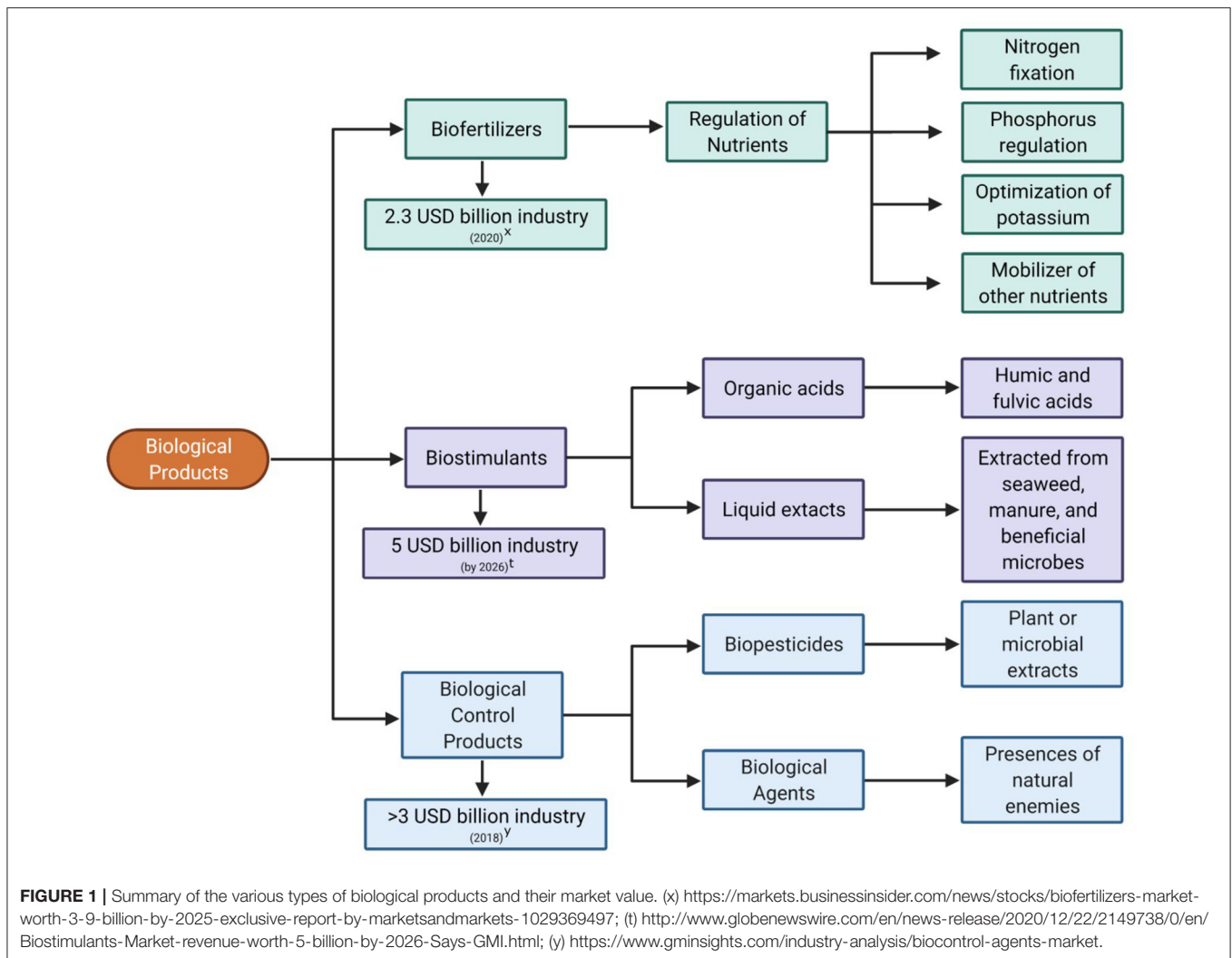## The Growing Market for Biological Products

Biological products including biofertilizers, biostimulants, bioherbicides, and biological control products are a multi-million-dollar industry (Bale et al., 2008; Barratt et al., 2018; van Lenteren et al., 2018) and projected to become a multi-billion-dollar industry in the next few years (**Figure 1**). Currently, there are few studies investigating the impact that *Trichoderma* or its derived natural products have on the biological control market. Although *Trichoderma* species represent 50–60% of the fungal biological control agents (Whipps, 2001; Verma et al., 2007), their potential market value remains uncertain. Natural product–derived drugs represent 25–50% of currently marketed drugs (Kingston, 2011) and have been the source of new drugs for nearly 40 years (Newman and Cragg, 2020). The development of biological control products was predicted to be costly; however, the rate remains reasonable compared with the cost of the synthesis, toxicological evaluation, and marketing of a new pesticide (Bale et al., 2008). The main caveat is that it can take up to 10 years to complete an extensive efficacy and

non-targeted effect studies before releasing a new biological control product (Bale et al., 2008).

Biofertilizers consist of microorganisms that enhance the supply of adequate nutrients to crop plants (Reddy et al., 2020). Biostimulants are any substances/mixtures of natural origin or microorganisms that improve the condition of a crop without causing adverse effects (Rouphael and Colla, 2020). Biological control products comprise both biopesticides and biological control agents. Biopesticides are microbes known to produce antagonistic secreted molecules (i.e., metabolites, peptides, etc.), while biological control agents are microbes used as active ingredients owing to their ability to compete for food or space, their mycoparasitism and antibiosis capacities, or their ability to induce plant defense responses (Contreras-Cornejo et al., 2016). Characterizing the diversity of natural products among microbial protagonists, like *Trichoderma*, will undoubtedly add to the growing market and provide necessary resources required for a sustainable future in agriculture.

Trichoderma As a Ubiquitous Genus Worthy of Exploring for Biocontrol Product Discovery

Trichoderma are asexual, spore-producing, fungicolous ascomycete fungi that are easily isolated, culturable in substrate media, and present in nearly all soils and other diverse habitats (Harman et al., 2004; Harman, 2006; Schmoll and Schuster, 2010; Druzhinina et al., 2011; Kubicek et al., 2019; Sun et al., 2019). *Trichoderma* species are generalists, as they can thrive on resources provided by plants, other fungi, and animals (Kubicek et al., 2019). In some scenarios, many species of *Trichoderma* may act as facultative endophytes (Druzhinina et al., 2011). *Trichoderma* species evolved as versatile biotrophic associates that promote plant health and growth and have shown biocontrol activity against various plant pathogens (Vinale et al., 2008a,b; Lorito et al., 2010). Thus, they have become popular choices as biological control products (Howell, 2003; Benítez et al., 2004; Vinale et al., 2008a,b; Lorito et al., 2010; Kubicek et al., 2011; Kumar and Ashraf, 2017; Mukhopadhyay and Kumar, 2020). Numerous *Trichoderma* species are biological control agents, biofertilizers, and biostimulants, and they produce secondary metabolites with biopesticide activities (Harman et al., 2004; Harman, 2006; Vinale et al., 2008a,b; Lorito et al., 2010; Schmoll and Schuster, 2010; Druzhinina et al., 2011; Mendoza-Mendoza et al., 2018; Kubicek et al., 2019; Thambugala et al., 2020). As of July 2020, there are 375 species with valid names (Cai and Druzhinina, 2021). As of April 2021, there were 453 records of *Trichoderma* species found on Index Fungorum (http://www.indexfungorum.org) and 337 taxonomic species with sequences on the National Center for Biotechnology Information (NCBI), excluding confer (Cf.) names and uncharacterized isolates. However, only a handful of *Trichoderma* species are used for their biocontrol properties, surpassing any other fungal genera used as biological control agents. Currently, 31 fungal genera are used as active ingredients in biological control products, of which 26 belong to the Ascomycota phylum and the rest are basidiomycetes (**Figure 2A**). *Trichoderma* has by far the

FIGURE 1 | Summary of the various types of biological products and their market value. (x) https://markets.businessinsider.com/news/stocks/biofertilizers-market-worth-3-9-billion-by-2025-exclusive-report-by-marketsandmarkets-1029369497; (t) http://www.globenewswire.com/en/news-release/2020/12/22/2149738/0/en/Biostimulants-Market-revenue-worth-5-billion-by-2026-Says-GMI.html; (y) https://www.gminsights.com/industry-analysis/biocontrol-agents-market.
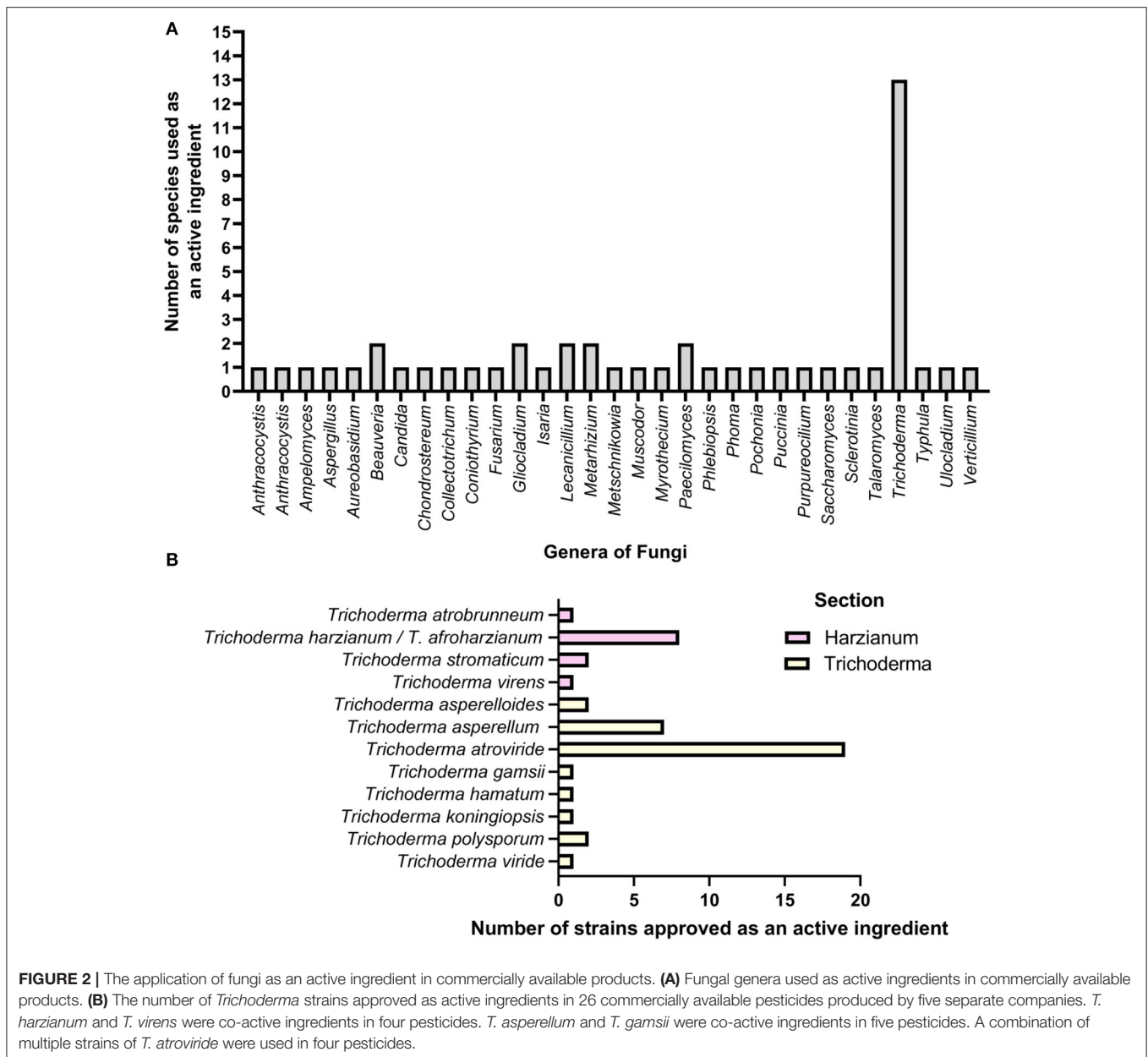
highest number of species used as biocontrol agents, a total of 13 (**Figure 2B**). A similar study showed that ascomycetes have the highest number of bioactivities against plant pathogens, and *Trichoderma* was the most used active ingredient (Thambugala et al., 2020).

Within the species of *Trichoderma*, 30 strains are approved and used in current biocontrol products in Asia (Japan), European Union, North America (U.S.A.), Oceania (Australia and New Zealand), and South America (Brazil and Uruguay), as evidenced by the research conducted for this paper and the results in other literature (Kaewchai et al., 2009; van Lenteren et al., 2018; **Supplementary Table 1**). There are 14 root applications, 1 root and foliar application, and 2 foliar sprays available with *Trichoderma* species as active ingredients known to have antagonistic effects against 21 soil-borne pathogens and 7 foliar pathogens (**Supplementary Table 1**). This list includes only product labels that specifically indicate which *Trichoderma* species and strains are used.

The effects of *Trichoderma* species on other organisms are largely influenced by the production and secretion of metabolites,

which have various established roles. Fungal metabolites have been reported to act either as communication signaling molecules between microorganisms and their hosts, or as defense agents in interactions with neighboring organisms. They were also shown to influence the development of the producing organism and to stimulate or inhibit the biosynthesis of other metabolites (Keller et al., 2005; Pusztahelyi et al., 2015; Macheleidt et al., 2016; Keller, 2019; Rokas et al., 2020). Genes responsible for the biosynthesis of secondary metabolites are often arranged into clusters (Keller, 2019). Those clusters are regulated by environmental signals and by transcriptional and epigenetics modulators (Keller, 2019). Different classes of secondary metabolites reported in fungi are indole alkaloids, non-ribosomal peptides (NRPs), polyketides, shikimic acid-derived compounds, and terpenoids (Keller et al., 2005; Pusztahelyi et al., 2015; Keller, 2019). Although *Trichoderma* is one of the mass producers of secondary metabolites with 23 identified families, classes, or compounds (Reino et al., 2008), and some with genetic accessibility (Schmoll and Schuster, 2010; Cardoza et al., 2011; Mukherjee et al., 2012; Keswani et al., 2014; Contreras-Cornejo et al., 2016; Zeilinger

FIGURE 2 | The application of fungi as an active ingredient in commercially available products. (A) Fungal genera used as active ingredients in commercially available products. (B) The number of *Trichoderma* strains approved as active ingredients in 26 commercially available pesticides produced by five separate companies. *T. harzianum* and *T. virens* were co-active ingredients in four pesticides. *T. asperellum* and *T. gamsii* were co-active ingredients in five pesticides. A combination of multiple strains of *T. atroviride* were used in four pesticides.

et al., 2016; Keller, 2019; Li et al., 2019; Vicente et al., 2020), little is known about the biosynthetic gene clusters responsible for the production of those metabolites. Moreover, the level of diversity among secondary metabolites produced across known *Trichoderma* species is still largely indefinite (Kubicek et al., 2019).

METHODS

To provide a holistic view of metabolites produced by *Trichoderma* species, we summarized data provided by three

recent reviews. An estimated 440 different molecules/non-volatile compounds/metabolites were identified and characterized from *Trichoderma* species (Keswani et al., 2014; Contreras-Cornejo et al., 2016; Li et al., 2019). The activities of those 440 projected compounds, reported in reviews by Keswani et al. (2014), Contreras-Cornejo et al. (2016), and Li et al. (2019), are assembled in **Figure 3**. If a compound has no activity reported, it is categorized as an "unknown function." Compounds that function as biofertilizers or biostimulants are in the category "promoting plant growth or development." Compounds with medical implications, such as being anti-tumor, anti-cancer, and so on, are placed in the "therapeutics" category.
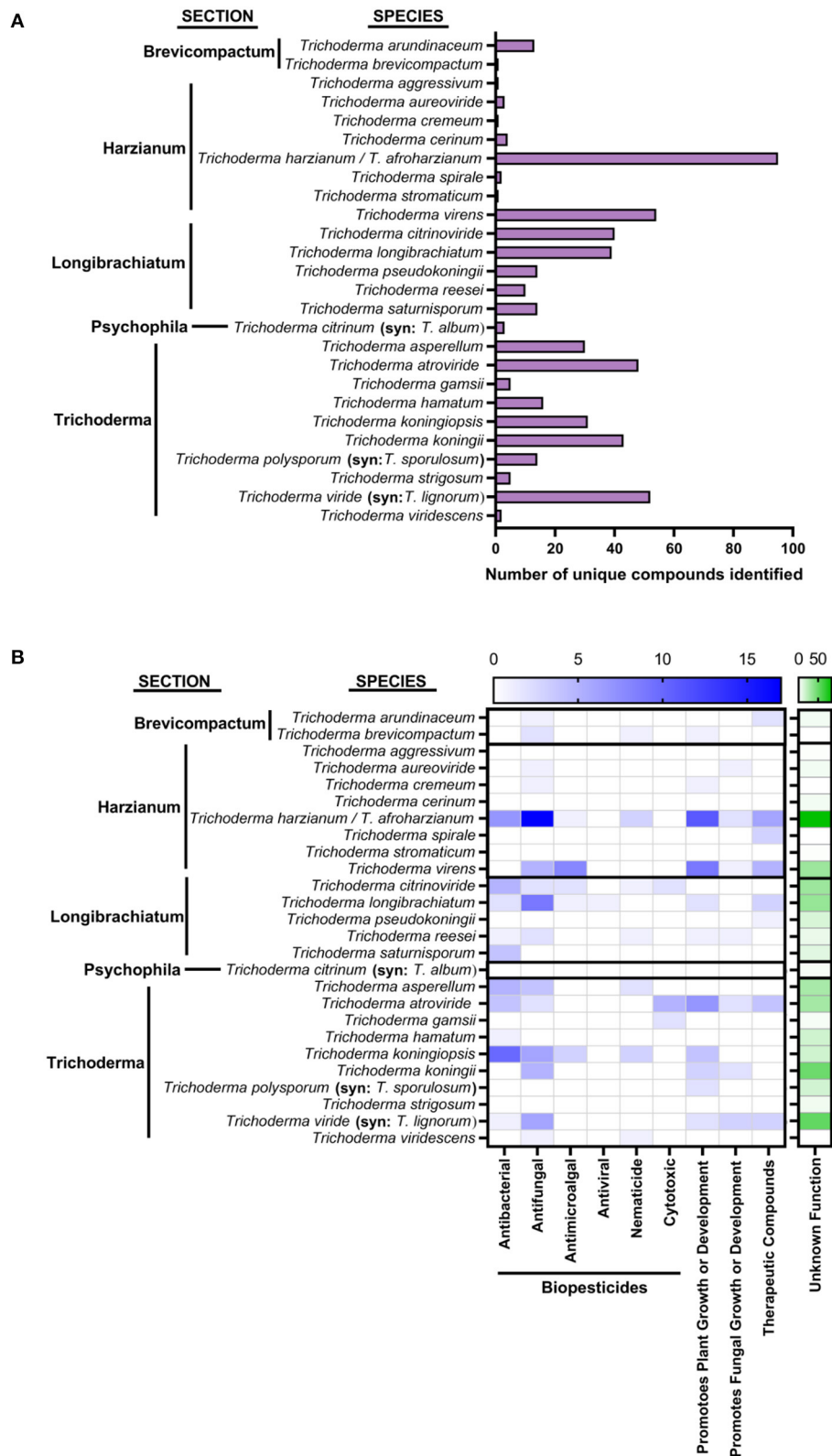
FIGURE 3 | Number of known compounds identified in *Trichoderma* species **(A)** Species of *Trichoderma* organized by section, showing the number of unique compounds identified. **(B)** Their bioactivity refers to metabolites' activities. Metabolites with antibacterial, antifungal, antimicroalgal, antiviral, nematicide, and cytotoxic properties could be used as biopesticides. Metabolites that promote plant growth or development could be used as biofertilizers or biostimulants. Metabolites that promote the fungus' own growth and development have no assigned category. Therapeutic compounds have been identified for their potential use in the medical industry.

Other compounds are categorized based on their functions ascribed in previous reports. Species names were validated based on accepted *Trichoderma* names (Zhu and Zhuang, 2015; Li J. et al., 2018; Cai and Druzhinina, 2021) and Index Fungorum's current nomenclature (http://www.indexfungorum.org/) as of April 2021. The taxonomy of *Trichoderma* is cumbersome and there are multiple discrepancies in the nomenclature at the species level, thus leading to incorrect identifications of strains (Cai and Druzhinina, 2021). *Trichoderma* has infrageneric groups that are divided into sections or groups. Sections were assigned based on previous phylogenies data of *Trichoderma* (Druzhinina and Kubicek, 2005; Zhu and Zhuang, 2015; Li J. et al., 2018; Cai and Druzhinina, 2021). Pachybasium is paraphyletic (Druzhinina and Kubicek, 2005), and whether it is a section or group is indistinct. Therefore, all species belonging to Pachybasium "clade A" or "clade B" were placed in "Pachybasium." Finally, *T. harzianum* strain T22, one of the commonly used active ingredient in commercial products, was determined to be *T. afroharzianum* (Chaverri et al., 2015; Cai and Druzhinina, 2021), the causal agent of *Trichoderma* ear rot on maize (Pfordt et al., 2020). Both species are part of the *T. harzianum* species complex (Chaverri et al., 2015). However, strain T22 still is reported in commercial products as *T. harzianum* or in the literature as *T. harzianum* or *T. afroharzianum*. Without further bioinformatic investigation, it is difficult to know of the reported strains used in commercial product or previous publications (other than strain T22) with biocontrol activity is *T. harzianum* or *T. afroharzianum*, so we used the term "*Trichoderma harzianum*/*T. afroharzianum*" in our analysis for **Figures 2**, **3** and **Supplementary Table 1**. However, for the antiSMASH and amPEPpy results coupled with the graph theoretic analysis, we used the genome of *T. harzianum* strain CBS 226.95 which is the neotype (Rifai, 1969; Druzhinina et al., 2018) and *T. afroharzianum* strain BFE349 (Landeis and Schmidt-Heydt, 2021).

RESULTS

Collectively, 27 described species of *Trichoderma* were reported to produce multiple compounds that were identified and characterized as (1) an enzyme that is part of a pathway to produce a secondary metabolite, (2) a described secondary metabolite, or (3) a compound with an unknown function as shown in **Figure 3**. Multiple species shared several of these compounds. Of these identified metabolites, several have been shown to have antibacterial or antifungal properties both *in vitro* and *in vivo* (Keswani et al., 2014; Contreras-Cornejo et al., 2016; Li et al., 2019); yet, most of these molecules' specific activity remains unknown (Contreras-Cornejo et al., 2016; **Figure 3B**). Many of these compounds are intermediates in the biosynthetic pathways of several *Trichoderma* species secondary metabolites that have agricultural and medical applications. Finally, there are biofumigants with antimicrobial and/or plant growth-promoting properties that are volatile organic compounds, as reviewed by Lee et al. (2016), Li N. et al. (2018), and Guo et al. (2019). However, they are not listed in **Figure 3**.

As shown in **Figure 3A**, the sections Harzianum, Trichoderma, and Longibrachiatum have the most identified compounds as of 2019. Harzianum has the most described functions for these identified compounds. Most of these compounds fall into the category biopesticides (as stated previously, **Figure 3B**). However, to our knowledge, none of these compounds have been used as active ingredients in commercial biopesticides.

Not surprisingly, the top five compounds frequently found, as shown in **Figure 3**, are mostly shared among various *Trichoderma* species (Reino et al., 2008). These are Koninginins, Trichorovins, Trichokonins, 6-Pentyl-2*H*-pyran-2-one, and Trichocaranes. Koninginins have structural similarities to compounds like flavonoids and vitamin E. They can inhibit phospholipase A2 (PLA2) and have been shown to have mycotoxic capabilities (Souza et al., 2008) as well as have antimicrobial properties (Reino et al., 2008). Koninginins have been found in *T. koningii, T. aureoviride, T. harzianum, T. kongingiopsis*, and a brown mutant of *T. viride* exposed to ultraviolet light (Reino et al., 2008). Recently they were identified from *Phomopsis stipata* (Biasetto et al., 2020). Trichorovins is an 11-residue peptaibol, originally described from *T. viride* (Fujita et al., 1994), that forms voltage-dependent and cation-selective ion channels in planar lipid bilayer membranes (Wada et al., 1996). Trichorovins have been found in *Trichoderma longibrachiatum; T. lixii; T. harzianum,* and *T. viride.* Trichokonins were described from *T. koningii* (Huang et al., 1995) and are broad-spectrum antimicrobial peptaibols with bioactivity over a wide pH and temperature range. They have no loss of activity even after autoclaving and are insensitive to proteolytic enzymes (Xiao-Yan et al., 2006). Trichokonins were found in *T. koningii, T. longibrachiatum,* and *T. pseudokoningii.* The category 6-Pentyl-2*H*-pyran-2-one are 2-pyranones, antifungal agents, with phytotoxic activity and antagonistic effects against multiple pathogenic fungi (Reino et al., 2008). This compound gives *Trichoderma* spp. a coconut aroma (Reino et al., 2008). They have been found in *T. atroviride, T. harzianum, T. koningii, T. viride, T. viridescens, T. asperellum,* and peaches (Parker et al., 1997; El-Sayed et al., 2014). Finally, Trichocaranes are metabolites with carotene skeletons that inhibit the growth of etiolated wheat coleoptiles (Macias et al., 2000). They appear to be unique to *T. virens*.

Besides secondary metabolites, antimicrobial peptides (AMPs) are another resource for biological products. AMPs, a cell defense mechanism produced by many organisms, are short and generally positively charged peptides that can directly kill microbial pathogens by modulating the host defense system (Mahlapuu et al., 2016; De Cesare et al., 2020). There has been increased AMP research over the years because of concerns regarding the advent of a "post-antibiotic era" (Mahlapuu et al., 2016). In addition, bacterial resistance to AMPs has been shown to be low or potentially negligible (Spohn et al., 2019). To date, there are more than 3,000 characterized AMPs based on their source, activity, structural characteristics, and amino acid composition (Wang et al., 2016; Huan et al., 2020). Many AMPs interact with membranes, causing cell wall inhibition and nucleic acid binding (De Cesare et al., 2020). Among

other types, *Trichoderma* has a unique class of AMPs called peptaibols that include rare amino acids in their sequences, which provide resistance to the host or pathogen proteases and induce programmed cell death in plant fungal pathogens (Montesinos, 2007; Shi et al., 2012; Arinbasarova et al., 2017; Dotson et al., 2018; De Cesare et al., 2020; Sood et al., 2020). While the discovery of AMPs is not new, recent technological and computational advancements are expected to improve their classification, exploration, and characterization (De Cesare et al., 2020; Huan et al., 2020).

Roadmap

As previously mentioned by Bale et al. (2008), it can be as much as 10 years before a newly discovered biological control agent is released. Therefore, we provide a roadmap to guide researchers with a thorough experimental plan to discover a novel product and implement it into the market. Our roadmap details two starting points, an omics road or biodesign road, to predict and identify putative natural products. Using reference genomes, the omics road queries candidate species for predicted backbone enzymes, putative metabolites, or annotated proteins relevant to biocontrol. Given the dynamic nature of genome expression, computational approaches, like machine-learning or graph theoretical methods, benefit greatly from the addition of functional genomics data (e.g., transcriptomics, proteomics, and metabolomics). In parallel or separately, the challenges of linking predictable gene clusters to their corresponding compounds (Kenshole et al., 2021) is addressed by following the biodesign road to extract putative metabolites, isolate them, and test for bioactivity. Both roads merge at the implementation step, where the metabolite characterized for specific bioactivity can be used as a biological control product. The implementation will determine the compound, its bioactivity, the gene(s) or biosynthetic pathways responsible for its production, and its potential use in a greenhouse or field setting. Collectively, the roadmap provides insightful experimental planning that might allow for faster approval of a novel biological control product into the market (**Figure 10**).

Genomic Prediction Tools Highlight Natural Product Diversity Across *Trichoderma* Species

Advancements in high-throughput sequencing technologies have greatly reduced the cost of genotyping organisms, which has expanded genomic libraries for numerous fungi. With increased fungal genome data now available, advances in computing and bioinformatic algorithms are improving our understanding of fungal biology and evolution (Ma and Fedorova, 2010; Aguilar-Pontes et al., 2014; Grigoriev et al., 2014; Stajich, 2017) and new machine learning-based gene prediction tools are beginning to address the problem of gene function discovery (Chavali and Rhee, 2018; Mahood et al., 2020).

When using genomic prediction tools, it's important to note that several genes, which are often clustered together on the chromosomes, can be required for the biosynthesis of a single compound (Smedsgaard and Nielsen, 2005). Moreover,
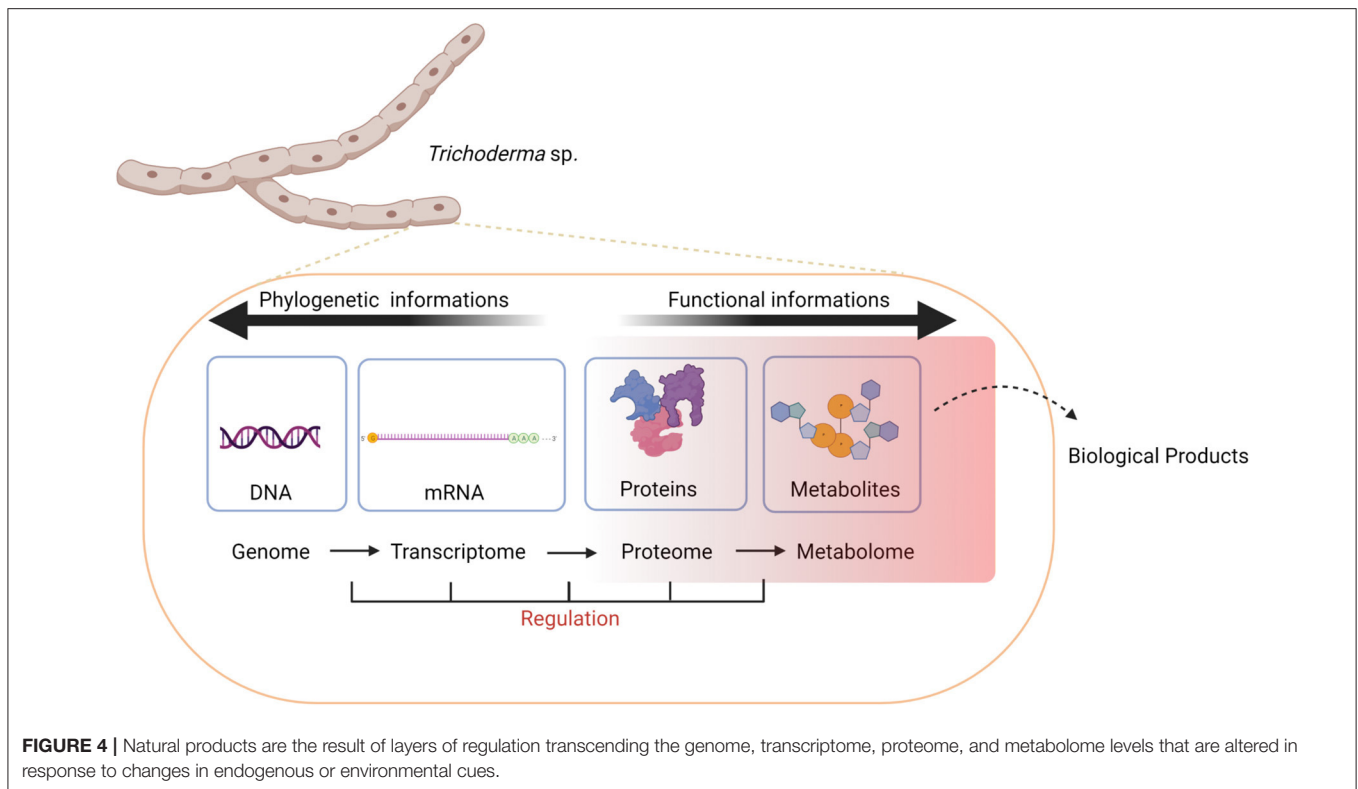
many gene clusters associated with the production of natural products respond to specific stimuli, such as environmental cues, nutrients, signaling compounds, or other stress factors (Hertweck, 2009) to become active (Gupta et al., 2014; Khan et al., 2020) (**Figure 4**). Hence, genomic predictions are often complemented with other omics measurements (e.g., transcriptome and proteome) to better understand what biological conditions are required for production of select natural products.

As of April of 2021, there were 25 species of *Trichoderma* available for download from the NCBI database (ncbi.nlm.nih.gov; **Table 1**). *Trichoderma cyanodichotomus, T. longibrachiatum, T. parareesei,* and *T. reesei* are type specimens; *T. asperellum* and *T. oligosporum* are holotype specimens; and *T. harzianum* is a neotype specimen. It is worth mentioning that the species *T. atrobrunneum* strain ITEM 908 and *T. atroviride* strain IMI 206040 are registered active ingredients in biological products (**Supplementary Table 1**). The rest of the sequenced strains are characterized species with no assigned type.

Herein, we used machine-learning tools to examine the diversity of predicted natural products across available *Trichoderma* species' genomes and graph theory to interpret and qualify the results to highlight organisms having intriguing potential for natural product discovery. Currently, there are several tools available to mine genomic data for the presence of biosynthetic pathways associated with the production of natural product, which are often referred to as secondary metabolites (Fedorova et al., 2012; Chavali and Rhee, 2018), and the origin of specialized molecules like antimicrobial peptides (Xu et al., 2021). Using the widely-used antiSMASH (https://antismash.secondarymetabolites.org/#!/start) tool, we analyzed each publicly available *Trichoderma* genome for genome-wide identification, annotation, and analysis of secondary metabolite biosynthetic gene clusters (BGCs) (Blin et al., 2019). Additionally, each genome was interrogated by the software tool amPEPpy, which uses a random forest classifier to predict putative antimicrobial peptides based on protein sequence characteristics often attributed to antimicrobials (e.g., small open reading frames, positively charged, etc.) (Lawrence et al., 2020).

Secondary Metabolite Exploration Through Genomic Mining and Computational Analysis

Using antiSMASH v5.0, we estimated the total number of backbone enzymes and putative metabolites predicted for each genome, as shown in the heatmap generated in GraphPad Prism v 9.1.0 (221) (**Figure 5**). The backbone enzyme is the first enzyme in the metabolic pathway that catalyze the synthesis of the core structure, which later undergo several modifications by tailoring enzymes leading to the final product. We have organized the heatmap data (**Figure 5**) according to the circular phylogeny (**Figure 6**) produced based on genomes with protein sequences available and based on previous publications for taxonomic resolution within the *Trichoderma* genus for species not included in our phylogenic analysis. However, the

FIGURE 4 | Natural products are the result of layers of regulation transcending the genome, transcriptome, proteome, and metabolome levels that are altered in response to changes in endogenous or environmental cues.

taxonomic resolution for each section within *Trichoderma* is an ongoing investigation (Druzhinina and Kubicek, 2005; Cai and Druzhinina, 2021).

Based on *Trichoderma* comparative genomic screening, the Harzianum section has the highest number of backbone enzymes and putative metabolites predicted in those species. However, in commercial biocontrol products, Trichoderma is better represented than Harzianum, despite having fewer predicted backbone enzymes and putative metabolites (as shown in **Figure 2B**). Type 1 polyketide synthases (T1PKSs) followed by NRP synthetases (NRPSs), were the backbone enzymes most often predicted in *Trichoderma*. NRP is a type of antimicrobial peptide that will be further discussed in this review. This group of metabolites includes peptaibols, which are linear peptides containing between 7 and 20 amino acid residues. In general, polyketides are poorly investigated as biocontrol agents (Daguerre et al., 2017). Specifically, T1PKS have rarely been examined in *Trichoderma* and are proposed to be mostly orthologous groups (Baker et al., 2012). To further investigate these highly anticipated backbone enzymes, machine-learning tools like the DDAP database for screening of biosynthetic T1PKS pathways (https://tylii.github.io/ddap/) can be used. Additionally, NRP discovery and the comprehensive peptaibiotics database can be applied to uncover the identities of these NRPs in *Trichoderma* (Caboche et al., 2008; Stoppacher et al., 2013; Neumann et al., 2015; Flissi et al., 2020). Finally, the data shown in **Figure 5** are comparable to those from a previous review in which polyketides, NRPs, and terpenes were largely represented in the genomes

of *T. reesei, T. atroviride,* and *T. virens* (Zeilinger et al., 2016).

The top identified putative metabolites with sequence matches ≥75% were dimethyl bassianin (23 matches), clavaric acid (20 matches), napththopyrone (16 matches), enniatin (7 matches), and AbT1 and Serinocyclin A/B with 4 matches each. Dimethyl bassianin (PubChem name is Bassianin) is a pyridine that provides yellow pigment (Lagashetti et al., 2019). Clavaric acid is a triterpenoidal inhibitor with antitumor and antionocogenic activities (Jayasuriya et al., 1998). Napththopyrone is an aromatic polyketide with cytotoxic, antitumor, antimicrobial, and tyrosine kinase properties (Bokesch et al., 2010; Lu et al., 2014; Venice et al., 2020). It has been shown to have a moderate ability to inhibit multidrug transporters and is a pigment that protects fungi from a wide range of predators (Bokesch et al., 2010; Lu et al., 2014; Venice et al., 2020). Enniatin is a mycotoxin discovered in *Fusarium.* Enniatin B was reported to have antifungal activity toward *T. afroharzianum* strain T22 (Meca et al., 2010). AbT1 is a precursor of cyclic peptide antibiotic Aureobasidin A from the crucial industrial yeast, *Aureobasidium pullulans* (Slightom et al., 2009). Serinocyclin A/B is a cyclic heptapeptide reported to cause a sublethal locomotory defect in mosquito larvae (Krasnoff et al., 2007). The top five putative metabolites with sequence matches <75% were squalestatin S1 (syn: zaragozic acid, 30 matches), neurosporin A (12 matches), tryptoquialanine (11 matches), depudecin (10 matches), and tricholignan A (8 matches). Squalestatin S1 controls cholesterol biosynthesis. It targets squalene synthases and has a broad spectrum of antifungal properties (Bonsch

TABLE 1 | List of *Trichoderma* species examined in this study.

Species	Strain	Ecology/importance	Strain registered as microbial biological control agent?	References (publications or institutions)	NCBI BioProject number(s)
Trichoderma afroharzianum	BFE349	Identifying secondary metabolite gene clusters	No	Landeis and Schmidt-Heydt, 2021	PRJNA682927
Trichoderma arundinaceum	IBT 40837	Produces terpenoid toxins-Trichothecenes	No	Proctor et al., 2018	PRJNA374046
Trichoderma asperellum	CBS 433.97	Biocontrol agent and plant growth promoter	No	Druzhinina et al., 2018	PRJNA453597, PRJNA207877
Trichoderma atrobrunneum	ITEM 908	Biocontrol agent and registered to produce commercial biopesticides	Yes	Fanelli et al., 2018	PRJNA428936
Trichoderma atroviride	IMI 206040	Mycoparasite of fungal pathogens	Yes	Kubicek et al., 2011	PRJNA264112, PRJNA19867
Trichoderma brevicompactum	IBT 40841	Producer of industrial enzymes and biocontrol agents	No	Proctor et al., 2018	PRJNA374046
Trichoderma citrinoviride	TUCIM 6016	Reference genome	No	Druzhinina et al., 2018	PRJNA453598
Trichoderma cyanodichotomus	TW21990-1	Novel species	No	Zhou et al., 2020	PRJNA598077
Trichoderma erinaceum	CRRI-T2N1	Biocontrol agent and plant growth promoter	No	Swain et al., 2018	PRJNA632694
Trichoderma gamsii	T6085	Suppression of plant diseases	No	Baroncelli et al., 2016	PRJNA342687
Trichoderma guizhouense	NJAU 4742	Mycoparasitism on soil-borne fungi, colonizes roots of plants, shows enhancement of nutrient uptake, promoting plant growth	No	Zhang et al., 2019	PRJNA314460
Trichoderma hamatum	GD12	Biocontrol agent and plant growth promoter	No	Studholme et al., 2013	PRJNA178391
Trichoderma harzianum	CBS 226.95	Biocontrol of plant pathogens	No	Druzhinina et al., 2018	PRJNA207867
Trichoderma koningii	JCM 1883	Cellulase enzyme production	No	Halliwell and Griffin, 1973; RIKEN Center for Life Science Technologies, Division of Genomic Technologies	PRJDB3615
Trichoderma koningiopsis	POS7	Enzyme production	No	Castrillo et al., 2017	PRJNA356137
Trichoderma lentiforme	CFAM-422	Producer of cellulases and hemicellulases	No	Steindorff et al., unpublished[1]	PRJNA473534
Trichoderma lixii	MUT3171	Mycoremediation	No	Venice et al., 2020	PRJNA514353
Trichoderma longibrachiatum	ATCC 18648	Biocontrol agent and nematicide activity	No	Xie et al., 2014	PRJNA207876
Trichoderma oligosporum	CGMCC 3.17527	Biocontrol agent (especially against white nose syndrome in bats)	No	Wang et al., unpublished[2]	PRJNA644467
Trichoderma parareesei	CBS 125925	Ancestor of industrial cellulase and hemicellulase producer *T. reessi*	No	Yang et al., 2015	PRJNA287603
Trichoderma pleuroti	TPhu1	Causes green mold disease of cultivated mushrooms	No	Urbán et al., 2016	PRJNA335988
Trichoderma reesei	QM6a/ATCC 13631	Used for cellulase production	No	Martinez et al., 2008	PRJNA225530, PRJNA15571

(Continued)

TABLE 1 | Continued

Species	Strain	Ecology/importance	Strain registered as microbial biological control agent?	References (publications or institutions)	NCBI BioProject number(s)
Trichoderma virens	IMV 00454	Isolated from Chernobyl Exclusion Zone	No	Singh et al., 2017	PRJNA355122
Trichoderma virens	Gv29-8	Mycoparasite	No	Kubicek et al., 2011	PRJNA264113, PRJNA19983
Trichoderma viride	Tv-1511	Cellulase enzymes for biological transformations, growth-promoting and biocontrol effects	No	Guo et al., 2020	PRJNA543939
Cordyceps militaris	CM01	Pathogen of insects, therapeutic agent	–	Zheng et al., 2011	PRJNA225510, PRJNA41129

[1]*Steindorff, A. S., Formighieri, E. F., Midorikawa, G. E. O., Tamietti, M. S., Ramos, E. Z., Silva, A. S., et al. (unpublished). Genome analysis of cellulolytic fungus Trichoderma lentiforme CFAM-422.*
[2]*Wang, C., Zeng, Z., and Zhuang, W. (unpublished). Comparative molecular evolution of chitinases in Ascomycota with emphasis on mycoparasitism lifestyle.*

et al., 2016; Lebe and Cox, 2019). Neurosporin A is involved in fungal sexual development, provides chemo-resistance to arthropod predation, and demonstrates significant insecticidal activity (Zhao et al., 2017). Tryptoquialanine is an indole alkaloid and a tremorgenic mycotoxin that can elicit intermittent or sustained tremors in vertebrates (Gao et al., 2011). Depudecin is a small linear polyketide known to be a histone deacetylase inhibitor. Moreover, this compound contributes to pathogenesis, has antiprotozoal activity, and provides a more significant fitness benefit (Reynolds et al., 2017). Tricholignan A is a redox-active ortho-hydroquinone that facilitates reductive iron assimilation and plays a potential role in promoting plant growth under iron-deficient conditions (Chen et al., 2019).

A list of the putative metabolites predicted in the genomes of *Trichoderma* species is presented in **Table 2**. A caveat for associating putative or known metabolites with *Trichoderma* species in **Table 2** is that numerous strains have been misidentified at the species level for several reasons: taxonomic identification is missing, non-specific analytical methods were used, species no longer exist in culture, or they do not have DNA sequences available (Nielsen et al., 2005; Degenkolb et al., 2008b; Tijerino et al., 2011; Cai and Druzhinina, 2021). Altogether, this caveat has been problematic for predictive metabolomics in *Trichoderma*. Those putative metabolites, excluding ferrichrome, were described in 31 separate genera, of which 30 belong to the Ascomycota and 1 belongs to the Basidiomycota. Ferrichrome is found in numerous ascomycete and basidiomycete fungi (Haas, 2014). Considering that *Trichoderma* is an ascomycete fungus, one can speculate that these predicted putative metabolites found in other ascomycete fungi could be produced by *Trichoderma* species as well, particularly since they all share a most recent common ancestor (Schoch et al., 2009; Spatafora et al., 2017). Moreover, horizontal gene transfer of secondary metabolite gene clusters has been demonstrated between ascomycete species (Kroken et al., 2003; Patron et al., 2007; Khaldi et al., 2008; Cardoza et al., 2011; Slot and Rokas, 2011; Sieber et al., 2014; Dhillon et al., 2015; Tran et al., 2019; Rokas et al., 2020) and

between ascomycete species and other microbes (Schmitt and Lumbsch, 2009; Lawrence et al., 2011). To investigate these putative metabolites in *Trichoderma*, it should be considered that sometimes antiSMASH does not accurately predict gene cluster borders. Therefore, researchers should consider looking at some neighboring genes when performing knockout or overexpression studies to characterize a biosynthetic gene cluster. Besides antiSMASH, there are other computational programs for secondary metabolite gene mining that are less commonly used, e.g., SMURF and ClusterFinder (Tran et al., 2019). Using other available programs for comparison might be useful to obtain more accurate biosynthetic gene cluster (BGC) border predictions. Finally, inconsistency in the nomenclature of metabolites in different biochemical databases used for genome-scale metabolic screening occurs and should be considered in the experimental design (Pham et al., 2019).

To quantify the uniqueness and predictive diversity of natural products across the *Trichoderma* sections or functional categories of secondary metabolites, we used graph theory as a robust mathematical platform to represent the relationships between or influence of antiSMASH results across the different *Trichoderma* sections. In graph theory analysis, a graph is comprised of nodes and edges (Newman, 2018) and, in this context, the sections and backbone enzymes represent the nodes, and the edges are weighted by the average number of times a category is observed by all the species in a section (**Figure 7A**). We used two different graph-theoretic measures to quantify and rank the importance of sections and backbone enzymes. These are (1) the strength and (2) PageRank of the nodes. The strength of the node is determined by the summation of all the edges from (out-strength) or to (in-strength) a node, representing the same values reported previously in **Figure 5E**. The PageRank measure (Page and Brin, 1998), which is the underlying method used by Google search engine to rank web pages based on the links between web pages, quantifies the relative importance of a node (section or backbone enzyme) based on the connections it has. We rank the sections and the enzymes for being the most influential
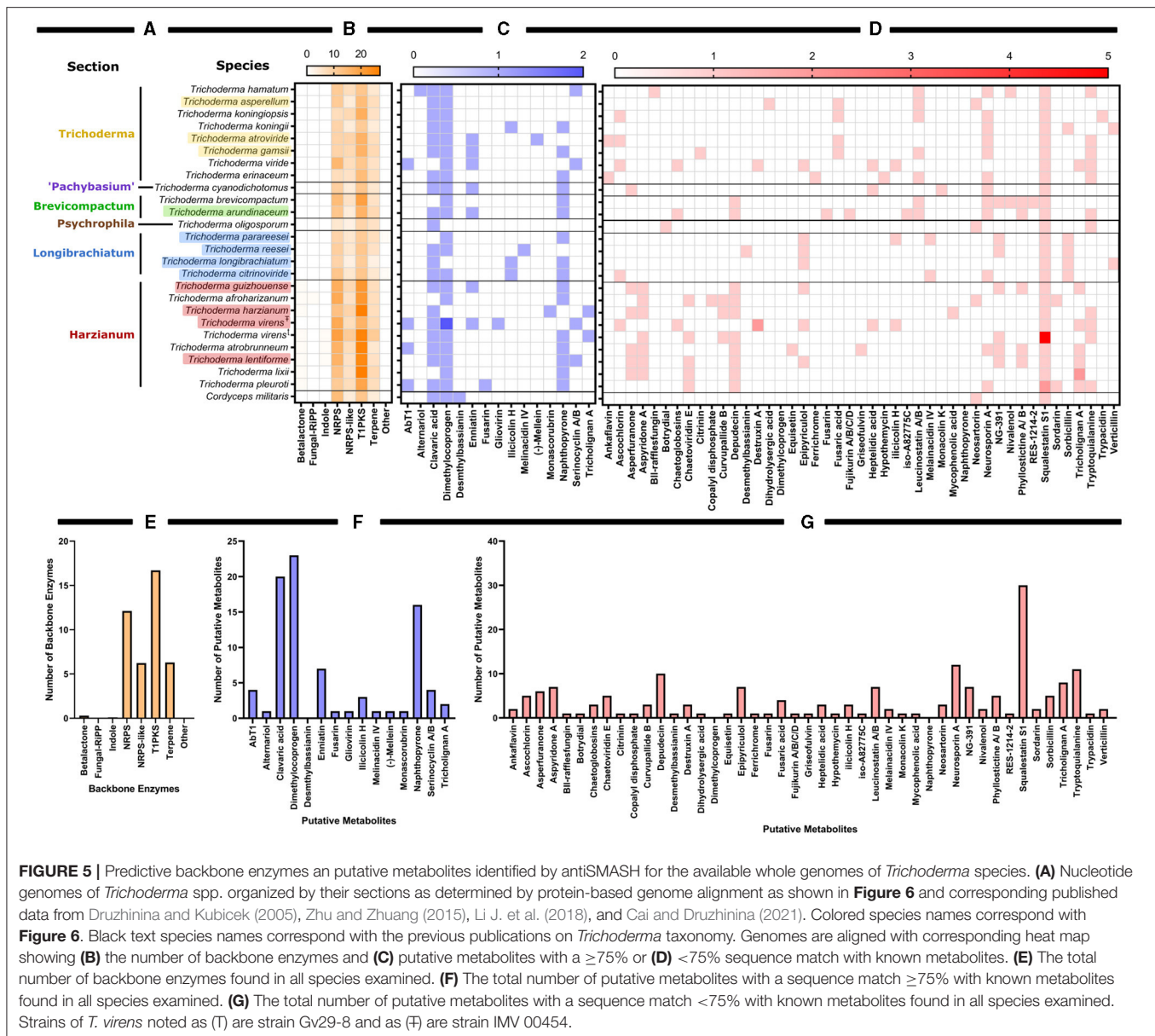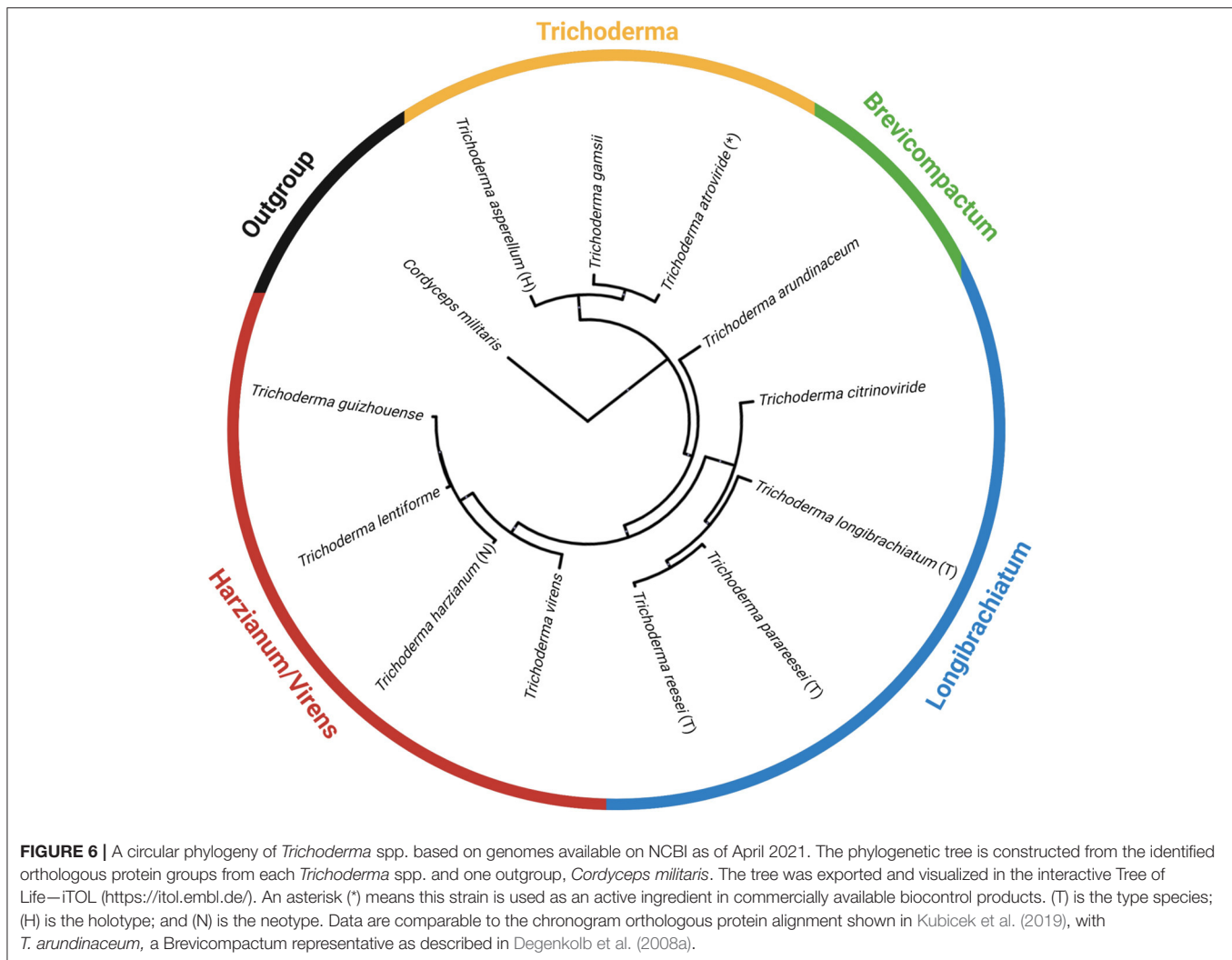
FIGURE 5 | Predictive backbone enzymes an putative metabolites identified by antiSMASH for the available whole genomes of *Trichoderma* species. **(A)** Nucleotide genomes of *Trichoderma* spp. organized by their sections as determined by protein-based genome alignment as shown in **Figure 6** and corresponding published data from Druzhinina and Kubicek (2005), Zhu and Zhuang (2015), Li J. et al. (2018), and Cai and Druzhinina (2021). Colored species names correspond with **Figure 6**. Black text species names correspond with the previous publications on *Trichoderma* taxonomy. Genomes are aligned with corresponding heat map showing **(B)** the number of backbone enzymes and **(C)** putative metabolites with a ≥75% or **(D)** <75% sequence match with known metabolites. **(E)** The total number of backbone enzymes found in all species examined. **(F)** The total number of putative metabolites with a sequence match ≥75% with known metabolites found in all species examined. **(G)** The total number of putative metabolites with a sequence match <75% with known metabolites found in all species examined. Strains of *T. virens* noted as (T) are strain Gv29-8 and as (Ŧ) are strain IMV 00454.

and influenced nodes, respectively, using the directed PageRank measures broadcasting and receiving measures (Grindrod et al., 2011) as shown in **Figures 7B,C**. These values are min-max normalized (between 0 and 1). The above graph-theoretic analysis was performed separately for the interaction among the sections and putative metabolites with ≥75% and <75% matched sequences with known metabolites. We only report the min-max normalized broadcasting and receiving PageRank values for these analyses, as shown in **Figures 7D–G**.

Overall, the graph theory analysis highlighted notable interactions between organisms and secondary metabolite predictions that can assist with selecting organisms or natural products for further analyses. Interestingly, the Harzianum section has the most prevalence of predicted backbone enzymes and putative metabolites compared to all the other sections

examined. This data supports what has been observed in several publications in identifying bioactive compounds (**Figure 3**) and why several biological products use species from this section are used as active ingredients (**Figure 2B**). The Trichoderma section showed a higher prediction of putative metabolites found in their genomes (**Figures 7D,F**), which may explain why species from the Trichoderma section have the most used active ingredient in commercial agricultural products (**Figure 2B**). While the Longibrachiatum section was shown to have a relatively high number of backbone enzymes found in their genomes (**Figure 7B**), species from Longibrachiatum are not listed as active ingredients in commercial agricultural labels (**Figure 2B**). It is interesting, though, that three well-studied species, *T. reesei, T. parareesei,* and *T. longibrachiatum* (**Supplementary Table 1**) are used in other industries. For example, *T. reesei* strain DSM

FIGURE 6 | A circular phylogeny of *Trichoderma* spp. based on genomes available on NCBI as of April 2021. The phylogenetic tree is constructed from the identified orthologous protein groups from each *Trichoderma* spp. and one outgroup, *Cordyceps militaris*. The tree was exported and visualized in the interactive Tree of Life—iTOL (https://itol.embl.de/). An asterisk (*) means this strain is used as an active ingredient in commercially available biocontrol products. (T) is the type species; (H) is the holotype; and (N) is the neotype. Data are comparable to the chronogram orthologous protein alignment shown in Kubicek et al. (2019), with *T. arundinaceum,* a Brevicompactum representative as described in Degenkolb et al. (2008a).

32338 was genetically modified to produce muramidase enzyme used as an additive for chickens and other minor poultry for fattening (Rychen et al., 2018). *Trichoderma reesei* is often genetically manipulated to overexpressed genes responsible for cellulases that are then used in the biotechnology industries (Druzhinina and Kubicek, 2017; Hinterdobler et al., 2021). *Trichoderma reesei* has been reported to sexually reproduce which can provide tools for fast and improved development of strains for industrial use (Seidl et al., 2009). However, there can be a pitfall in releasing these species in the environment as an active ingredient because it has the potential to sexually recombine with a native population. But alternatives like the sympatric species, *T. longibrachiatum,* and *T. parareesei* have a clonal lifestyle (Druzhinina et al., 2008, 2010; Atanasova et al., 2010), therefore could be suitable species to use as biological control products. *Trichoderma parareesei* have been shown to produce cellulases like, *T. reesei,* and have biostimulant activities by increasing seedling lateral root development on tomatoes and produced antagonistic effects against fungal foliar pathogen, *Botrytis cinerea* (Rubio et al., 2014). Several publications show

the effects of *T. longibrachiatum* as a fungicide and nematicide on several crops, yet the efficacy is unknown (Migheli et al., 1998; Rojo et al., 2007; Zhang et al., 2015, 2017, 2018b). Our data suggest that by using genome-mining companioned with computational analysis, there is evidence of several known *Trichoderma* species that have not been fully explored for their use as a biological product in agriculture. This untapped resource could be part of a solution to overcoming pathogen resistance or other issues growers face today, mainly if the environmental conditions and origins of species isolation are known.

Antimicrobial Peptides Exploration Through Genomic Mining and Computational Analysis

To date, there were more than 30 computational methods for AMP prediction and identification as of 2021 (Xu et al., 2021). Of those computational methods, amPEPpy is a user-friendly, open-source, portable, multi-threaded command-line application for predicting AMPs through genome-based screening using a random forest classifier (Lawrence et al., 2020). amPEPpy was validated as predicting AMPs more

TABLE 2 | A descriptive list of putative metabolites predicted from antiSMASH based on screening *Trichoderma* genomes.

Putative metabolite name from antiSMASH result	Type	Function	Characterized in *Trichoderma* species?	Fungal genera where metabolite has been characterized	References
AbT1	NRP	Precursor of the cyclic peptide antibiotic, Aureobasidin A	Not characterized in *Trichoderma*	*Aureobasidium*	Slightom et al., 2009; Wang et al., 2020
Alternariol	Terpene	Antifungal and phytotoxin	Found in *Trichoderma* sp. strain Jing-8, showed to be DPPH-radical-scavenging and growth inhibitory Antibiotic	*Alternaria*	Davis and Stack, 1994; Zhang et al., 2017; Li et al., 2019
Ankaflavin	Polyketide: Iterative Type 1	Yellow pigment, anti-inflammation, anti-oxidation, anti-diabetes, immunomodulation and antitumor agent	Not characterized in *Trichoderma*	*Monascus*	Cheng M. J. et al., 2010
Ascochlorin	Terpene + Polyketide	Antibiotic, inhibitor of mitochondrial cytochrome bc1 complex	Not characterized in *Trichoderma*	*Ascochyta*	Berry et al., 2010
Asperfuranone	Polyketide	Antitumor agent	Not characterized in *Trichoderma*	*Aspergillus*	Wang et al., 2010
Aspyridone A	NRP + Polyketide: Iterative type I	Antifungal	Similar in structure to Harzianopyridone, isolated from *T. harzianum*	*Aspergillus*	Wasil et al., 2013; Shenouda and Cox, 2021
BII-rafflesfungin	NRP	Antifungal	Not characterized in *Trichoderma*	*Phoma*	Sinha et al., 2019
Botrydial	Terpene	Antifungal and phytotoxin	Not characterized in *Trichoderma*, but it regulates expression of trichothecene biosynthesis in *T. arundianceum*	*Botrytis*	Malmierca et al., 2016
Chaetoglobosins	NRP + Polyketide: Iterative type I	Antitumor agent	Not characterized in *Trichoderma* but would not be surprising to be present in *Trichoderma* given the large number of polyketide present. Chaetoglobosins and Chaetoviridin biosynthesis is recently characterized in *Chaetomium globosum*	*Chaetomium*	Daguerre et al., 2017
Chaetoviridin E	Polyketide	Antibiotic and cytotoxic	Not characterized in *Trichoderma* but would not be surprising to be present in *Trichoderma* given the large number of polyketide present. Chaetoglobosins and Chaetoviridin biosynthesis is recently characterized in *Chaetomium globosum*	*Chaetomium*	Daguerre et al., 2017
Citrinin	Polyketide: Iterative Type 1	Antibiotic; mycotoxin	Not well-characterized in *Trichoderma*. *Trichoderma hamatum* has been shown to reduce the amount of citrinin produced by other fungus	*Aspergillus, Monascus, Penicillium*	Abd-Allah and Ezzat, 2005; Doughari, 2015
Clavaric acid	Terpene	Farnesyltransferase inhibitor	Not characterized in *Trichoderma*	*Hypholoma*	Jayasuriya et al., 1998

(Continued)

TABLE 2 | Continued

Putative metabolite name from antiSMASH result	Type	Function	Characterized in *Trichoderma* species?	Fungal genera where metabolite has been characterized	References
Copalyl disphosphate	Terpene	Tanshinone biosynthesis	Previous study predicted *T. afroharzianum* genome to contain BGC necessary for biosynthesis of this terpene molecule	*Phomopsis*	Toyomasu et al., 2007; Landeis and Schmidt-Heydt, 2021
Curvupallide B	NRP + Polyketide	Phytotoxin	Previously predicted to encoded by *T. afroharzianum*	*Curvularia*	Abraham et al., 1995; Landeis and Schmidt-Heydt, 2021
Depudecin	Polyketide: Iterative Type 1	Inhibitor of histone deacetylase, has anti-angiogenic activity	Not characterized in *Trichoderma*	*Alternaria*	Matsumoto et al., 1992
Desmethylbassianin	NRP + Polyketide: Iterative type I	Closely related to tenellin which are cytotoxic	Not characterized in *Trichoderma*	*Beauveria*	McInnes et al., 1974
Destruxin	NRP	Insecticide and antitumor agent	Not characterized in *Trichoderma*	*Metarhizium, Alternaria, Trichothecium, Aschersonia*	Pedras et al., 2002; Golo et al., 2014
Dihydrolysergic acid	Ergot Alkaloid	Vasorelaxant	Not characterized in *Trichoderma*	*Claviceps*	Arnold and Panaccione, 2017
Dimethylcoprogen (=N alpha-dimethylocoprogens)	NRP	Phytotoxin	Not characterized in *Trichoderma*	*Alternaria*	Jalal et al., 1988
Enniatin	NRP	Antibiotic, antihelmintic, antifungal, herbicidal, and insecticidal compound	Not characterized in Trichoderma, could be Enniatins-like genes	*Fusarium*	Prosperini et al., 2017
Epipyriculol	Polyketide	Phytotoxin	Not characterized in *Trichoderma*	*Pyricularia*	Masi et al., 2017
Equisetin	NRP + Polyketide	Antibiotic, cyctotoxic, has inhibitory effect on HIV-1 integrase	Trichosetin (an Equisetin homolog) isolated in a co-culture with *T. harzianum* and *Catharanthus roseus*	*Fusarium*	Singh et al., 1998; Marfori et al., 2002
Ferrichrome	Other	Siderophore	Found in *T. virens*	Majority of fungi can produce	Haas, 2014; Mukherjee et al., 2018
Fujikurin A/B/C/D	Polyketide	Need more characterization	Not characterized in *Trichoderma*	*Fusarium*	Von Bargen et al., 2015
Fusaric acid	Polyketide	Antibiotic	Not characterized in *Trichoderma*	*Fusarium*	Yabuta et al., 1939
Fusarin	NRP + Polyketide	Mycotoxin	Not characterized in *Trichoderma*	*Fusarium*	Niehaus et al., 2013
Gliovirin	NRP	Antibiotic	An antibiotic found in *T. virens* (isolated from the Gv strains) and *T. longibrachiatum*	*Trichoderma virens* (syn: *Gliocladium virens*)[T]	Stipanovic and Howell, 1982; Keswani et al., 2014; Contreras-Cornejo et al., 2016
Griseofulvin	Polyketide: Iterative Type 1	Antifungal	Not characterized in *Trichoderma*	*Penicillium*	Huber, 1967
Heptelidic acid (syn: Koningic acid)	Terpene	Antibiotic and antitumor agent	Antimicrobial found in *T. virens* and *T. viride*	*Chaetomium* and *Trichoderma virens* (syn: *Gliocladium virens*)[T]	Itoh et al., 1980
Hypothemycin	Polyketide	Antifungal and antitumor agent	Not characterized in *Trichoderma*	*Hypomyces*	Wee et al., 2006
Ilicicolin H	NRP + Polyketide	Antimicrobial	Not characterized in *Trichoderma*	*Clonostachys rosea* (syn: *Gliocladium roseum*)[I]	Schroers et al., 1999; Singh et al., 2012;

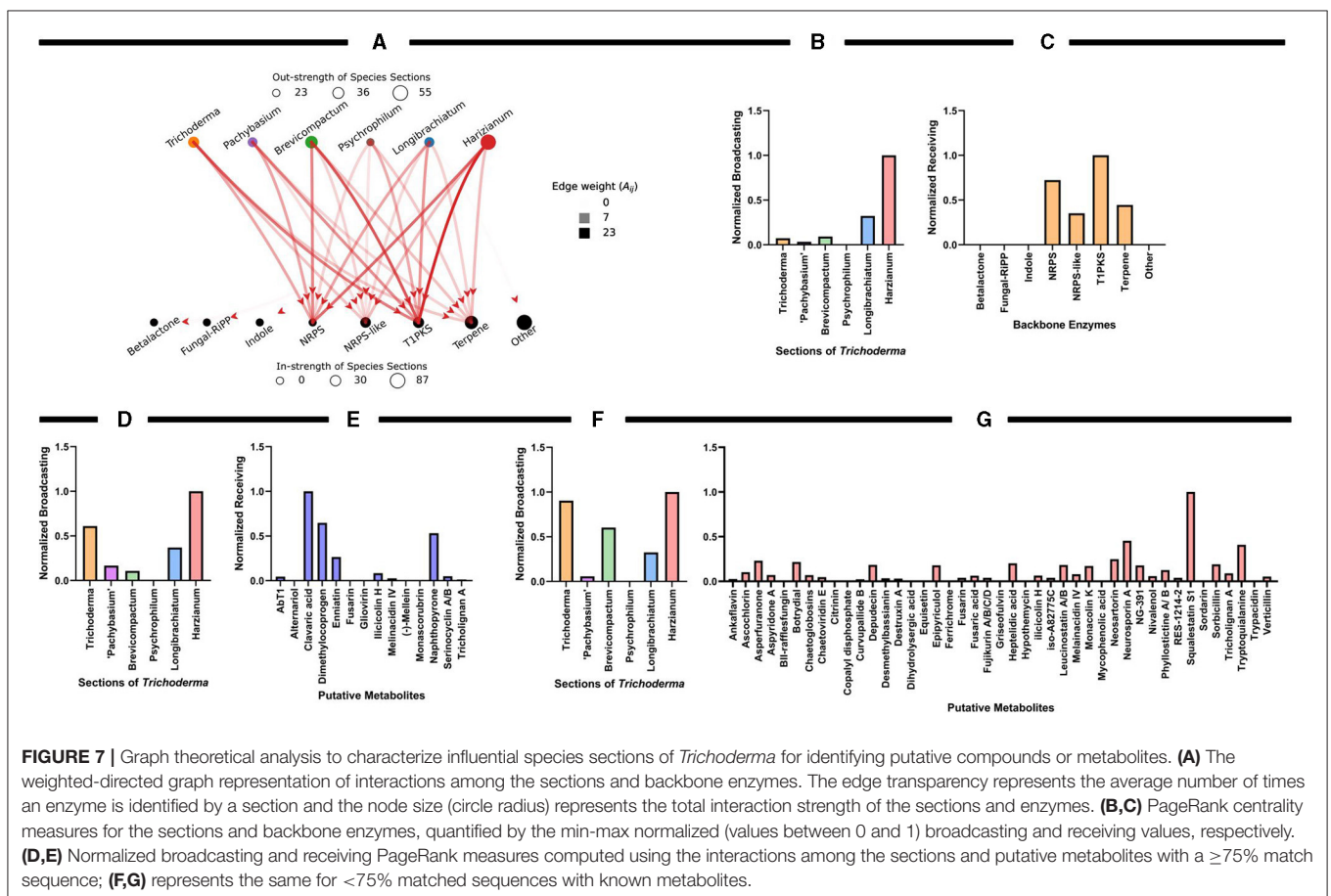*(Continued)*

TABLE 2 | Continued

Putative metabolite name from antiSMASH result	Type	Function	Characterized in *Trichoderma* species?	Fungal genera where metabolite has been characterized	References
ISO-A82775C	Other	Cytotoxicity and antitumor	Not characterized in *Trichoderma*	*Pestalotiopsis*	Suzuki et al., 2017
Leucinostatin A/B	Polyketide	Antibiotic and anti-trypanosomal activity	Not characterized in *Trichoderma*	*Paecilomyces*	Cerrini et al., 1989
Melinacidin IV	Alkaloid	Antimicrobial	Not characterized in *Trichoderma*	*Westerdykella*	Ebead et al., 2012
(-)-Mellein	Polyketide	Neurotoxic effects	Antifungal found in *T. aggressivum*	*Aspergillus*	Moore et al., 1972; Krupke et al., 2003
Monacolin K	Polyketide	Chemical structure similar to lovastatin, a key enzyme in cholesterol lowering drugs	Not characterized in *Trichoderma*	*Aspergillus, Monascus*	Endo et al., 1986
Monascorubrin	Polyketide	Pigment	Not characterized in *Trichoderma*	*Monascus* and *Penicillium*	Woo et al., 2014
Mycophenolic acid	Terpene + Polyketide: Iterative Type I	Antibiotics; immunosuppressant medication	Not characterized in *Trichoderma*	*Penicillium*	Allison et al., 1993
Naphthopyrone	Polyketide	Non-toxic pigment, used for fungal defense; antimicrobial activity; protection against predation	Pigments found in ascomycete fungi, displays cytotoxic activity, predicted to be in *T. lixii*	*Aspergillus, Fusarium, Penicillium*	Xu et al., 2019; Venice et al., 2020
Neosartorin	Polyketide	Yellow pigment	Not characterized in *Trichoderma*	*Neosartorya*	Proksa et al., 1998
Neurosporin A	Polyketide	Insecticide; provide resistance to arthropod predation	Not characterized in *Trichoderma*	*Neurospora*	Zhao et al., 2017
NG-391	NRP + Polyketide	Mycotoxin	Not characterized in *Trichoderma*	*Metarhizium*	Donzelli et al., 2010
Nivalenol	Terpene	Mycotoxin	Nivalenol are trichothecenes and *Trichoderma* produces trichothecenes, mostly in the Brevicompactum section	*Fusarium*	Yamashita et al., 1995; Nielsen et al., 2005; Tijerino et al., 2011; Gutiérrez et al., 2020
Phyllostictine A/B	NRP + Polyketide	Phytotoxin, used as biocontrol herbicide	Not characterized in *Trichoderma*	*Phyllosticta*	Evidente et al., 2008
RES-1214-2	Polyketide	Endothelin antagonists	Not characterized in *Trichoderma*	*Pestalotiopsis*	Ogawa et al., 1995
Serinocyclins A/B	NRP	Sublethal toxic effect	Not characterized in *Trichoderma*	*Metarhizium*	Krasnoff et al., 2007
Sorbicillin	Polyketide	Cytotoxic, antioxidant, antiviral, and antimicrobial activity.	Found in *T. longibrachiatum* and *T. reesei*	*Clonostachys, Emericella, Penicillum, Trichoderma, Verticillium*	Keswani et al., 2014; Meng et al., 2016; Derntl et al., 2017
Sordarin	Polyketide	Antifungal	Predicted to be in *T. harizanum* because it has similar genes clusters, *sdnA* and *sdnO,* in genome	*Sordaria*	Hauser and Sigg, 1971; Baroncelli et al., 2016; Kudo et al., 2016
Squalestatin S1 (syn: Zaragozic acid)	Terpene	Antifungal	Not characterized in *Trichoderma*	*Sporormiella* and *Leptodontium*	Bergstrom et al., 1993; Bills et al., 1994
Tricholignan A	Polyketide: Iterative Type 1	Facilitate reductive iron assimilation in plants	Found in *T. harizanum* to facilitate reductive iron assimilation in plants	*Trichoderma*	Chen et al., 2019

(Continued)

Putative metabolite name from antiSMASH result	Type	Function	Characterized in *Trichoderma* species?	Fungal genera where metabolite has been characterized	References
Trypacidin	Polyketide	Cytotoxic, antibiotic	Not characterized in *Trichoderma*	*Aspergillus*	Gauthier et al., 2012
Tryptoquialanine	NRP	Phytotoxin, insecticide, antifungal	Genes responsible for the biosynthesis of Tryptoquialanine, homologs were found in the genome of *T. reesei*	*Penicillium*	Ariza et al., 2002; Gao et al., 2011; Costa et al., 2019a,b; Costa et al., 2021;
Verticillin	NRP	Cytotoxic and bacteriostatic activities	Not characterized in *Trichoderma*	*Clonostachys rosea* (syn: *Gliocladium roseum*)[I], *Paecilomyces*, *Verticillium*[*]	Schenke et al., 2011; Kim and Movassaghi, 2015

The backbone enzyme type, the function of the predicted metabolite, whether it has been identified in Trichoderma species, and which fungi produce these metabolites are described. Collectively, 31 genera were identified to produce these metabolites (excluding Trichoderma) where 30 genera belong to Ascomycota and one genus belong to Basidiomycota. [T]Gliovirin and Heptelidic acid were initially described in Gliocladium virens which were transferred to Trichoderma. [I]Ilicicolin H and potentially Verticillin A were initially described in Gliocladium roseum which was transferred to Clonostachys rosea. [*]Verticillin A was initially described in Verticillium, however, the genes required to produce this metabolite are not found in Verticillium species (Schenke et al., 2011). Not characterized means the metabolite name has not been identified in Trichoderma.

FIGURE 7 | Graph theoretical analysis to characterize influential species sections of *Trichoderma* for identifying putative compounds or metabolites. **(A)** The weighted-directed graph representation of interactions among the sections and backbone enzymes. The edge transparency represents the average number of times an enzyme is identified by a section and the node size (circle radius) represents the total interaction strength of the sections and enzymes. **(B,C)** PageRank centrality measures for the sections and backbone enzymes, quantified by the min-max normalized (values between 0 and 1) broadcasting and receiving values, respectively. **(D,E)** Normalized broadcasting and receiving PageRank measures computed using the interactions among the sections and putative metabolites with a $\geq$75% match sequence; **(F,G)** represents the same for <75% matched sequences with known metabolites.

accurately than other approaches (Xu et al., 2021). We used amPEPpy to predict promising antimicrobial agents (Lawrence et al., 2020) within the *Trichoderma* genomes. We compared predicted AMPs sequences to sequences available on NCBI as of April 2021 because there are no online tools that cross-link data generated from amPEPpy to numerous known AMPs, as antiSMASH does with secondary metabolites. Accession numbers generated by amPEPpy are found in

Supplementary Table 2 and functional annotations were summarized by major keywords into categories. Each functional annotation categories represent the percentage of amino acid sequences that match the predicted AMP motif: 100, 99.9–95.0, 94.9–90.0, 89.9–80.0, 79.9–70, and 69.9–51.0%. Anything below 50.9% was not considered for further analysis. A heatmap was generated to reflect these results in GraphPad Prism v 9.1.0 (221), indicating the number of times each category was predicted.

Based on *Trichoderma* comparative genomic screening, the Harzianum section has the highest number of predicted AMPs. Interestingly, among all organisms a large percentage of AMPs were annotated as hypothetical proteins, and the large number of observed hypothetical proteins in *T. harzianum, T. virens, T. reesei, T. atroviride,* and *T. asperellum* is intriguing. In some instances, these hypothetical proteins have a high sequence homology between species. In fact, *T. lentiforme* (accession number KAF3073950) and *T. arundinaceum* (accession number RFU77176) were predicted to have a 100% match for an unknown AMP and currently are described as "hypothetical protein" on NCBI.

Although hypothetical proteins dominated the annotations in the NCBI accession keyword search (**Figure 8D**), we listed the top five annotated proteins from each species. Among these, proteins annotated as non-ribosomal peptide synthetases (NRPSs) is a notable annotation because these proteins are known to produce AMPs in bacteria and fungi (Finking and Marahiel, 2004; Felnagle et al., 2008; De Cesare et al., 2020). Apart from this annotation, it was interesting to observed "glycoside hydrolase family" and "cytochrome P450 family" annotations as abundant keyword annotated protein names (**Figure 8C**). Interestingly, glycoside hydrolase family are found in many microbes and plants and are frequently associated with microbe-host interactions (Faure, 2002; Xu et al., 2004; Berlemont and Martiny, 2016; Snarr et al., 2017; Mendoza-Mendoza et al., 2018). Additionally, they have been shown to be required for needed for virulence in oomycetes and fungi triggering pathogen-associated molecular patterns (Mendoza-Mendoza et al., 2018; Tan et al., 2020; Zhang et al., 2021), formation of biofilms in bacteria (Szymańska et al., 2020), as a therapeutic for antibiofilm activity from pathogens across taxonomic kingdoms (Snarr et al., 2017), and in chemical defenses against herbivory (Xu et al., 2004). Glycoside hydrolases have also been shown to be involved in the production of secondary metabolites and peptaibols that have been found in 21 genomes of *Trichoderma* spp. (Fanelli et al., 2018; Mendoza-Mendoza et al., 2018). In a study to target activity-specific genetic markers for biocontrol from genomes of *Trichoderma* spp., Fanelli et al. (2018) compared protein family (Pfam) database, domains associated with stress tolerance and antagonistic activities, and found that 7 out of 15 Pfam domains with specific roles were CAZyme, containing hundreds of CAZymes proteins (Mistry et al., 2021). Two other Pfam domains with specific roles were the ABC transporter region and peptidase, which were also found in the results of this study (Fanelli et al., 2018). Like the glycoside hydrolases, cytochrome P450 superfamily have also been associated with antimicrobials (Geisler et al., 2013) and the production of primary secondary metabolites (Shin et al., 2018).

Like the antiSMASH analysis, we used graph-theoretic analysis to further interrogate the predictions from amPEPpy to quantify the importance (or ranking) of the sections with respect to their probability of having AMP-related genes or keyword protein annotations. Two separate analyses were performed using the average values for the species of each section from the data in **Figures 8A,B**. We measured the min-max normalized broadcasting PageRank of the sections and the receiving PageRank of their probability of having AMP-related genes (**Figures 9A,B**) and, correspondingly, the measures related to the keyword protein annotations (**Figures 9C,D**).

The graph-theoretic analysis suggests that the Brevicompactum section, followed by Harzianum, had the most influence on the data (**Figure 9A**). Interestingly, there were several AMPs identified by amPEPpy that fall into the categories of 94–80% match based on current definitions of AMPs (**Figure 9B**). The graph-theoretic analysis also suggests that section Brevicompactum, closely followed by Longibrachiatum, have the most prevalence of predicted proteins potentially involved in AMP biosynthesis and production compared to all the other sections examined (**Figure 9C**). As illustrated in **Figure 9D**, graph theory results further substantiate the prevalence of "glycoside hydrolase family," "cytochrome P450 family," and "glycosyltransferase."
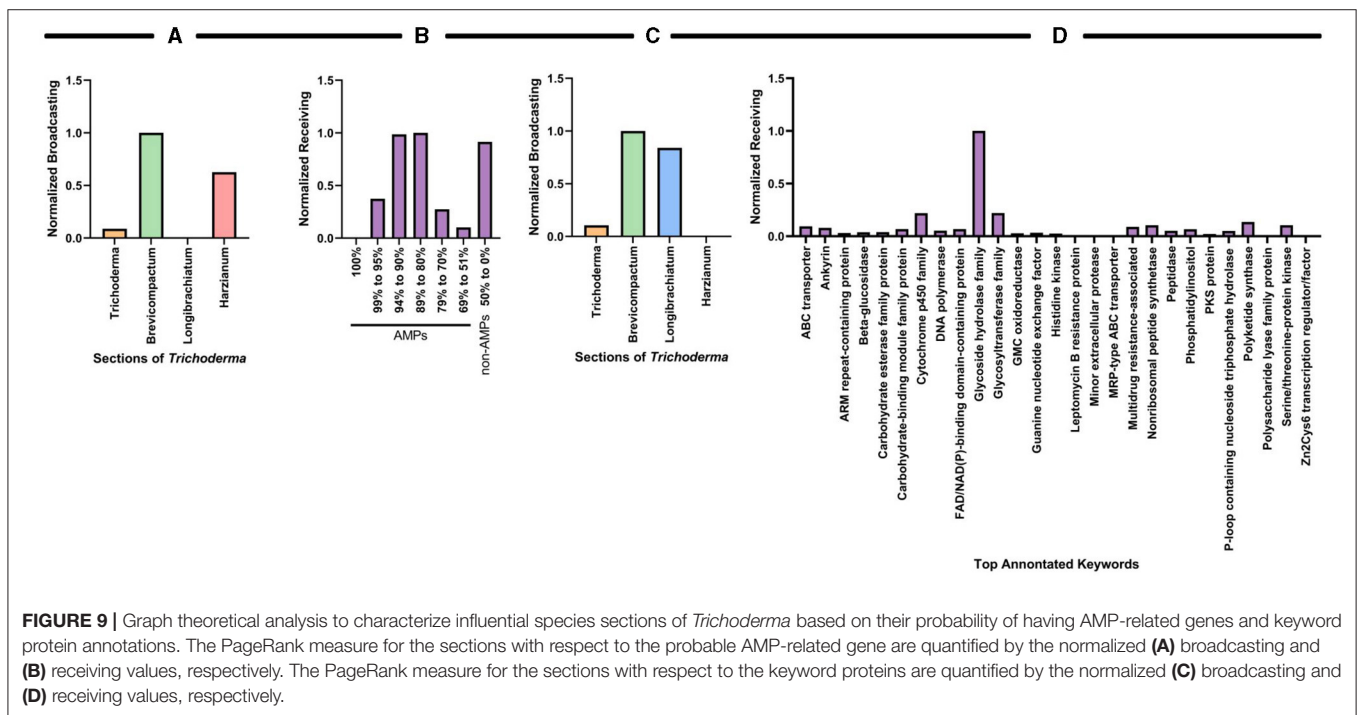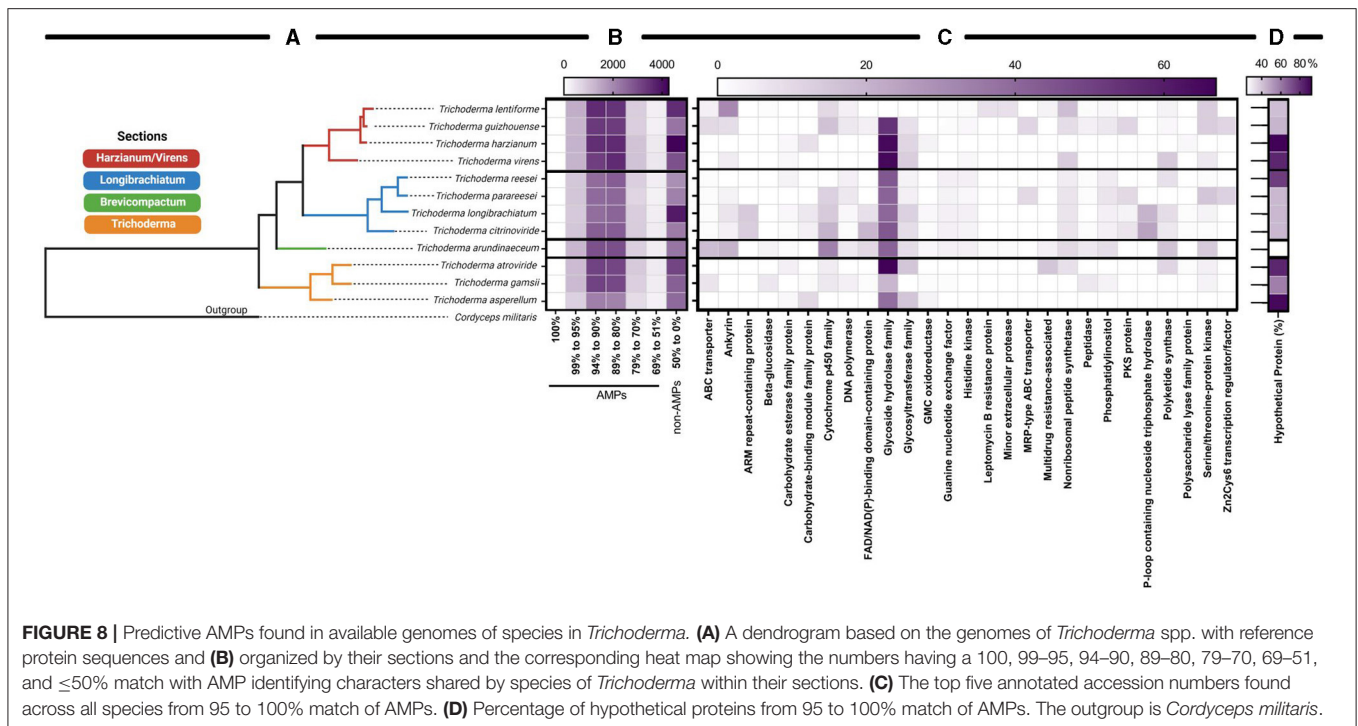
Overall, by using whole-genome sequencing data available on NCBI, we were able to demonstrate the utility of genome prediction tools to identify putative secondary metabolites and AMPs that warrant further interrogation. Next, we describe how these inferred natural products can be experimentally interrogated with or without a priori knowledge.

DISCUSSION

Experimental Framework to Characterize Natural Products in *Trichoderma* Species

In general, competition assays and choice or no-choice assays are often used as a first step for comparing the effects of a fungus on other microbes (Köhl et al., 2019) and would be useful in identifying negative association between an organism and the metabolic substances produced by another microbe (Fravel, 1988; Köhl et al., 2019). It is necessary to also understand how a particular fungus or natural product behaves in greenhouse conditions, which can be used to explore host-microbe interactions (Gibson et al., 1999) and efficacy against pathogens, host range, and environmental conditions before field trials. Next, with appropriate permits in-hand, field trial can be conducted to know how a biological product will perform in natural conditions, that are inherently more complex and less predictable. Often, off-target effects against non-target species, like insects, vertebrates, and weeds, as well as potential toxicity and carcinogenic effects on humans can be examined (Headrick and Goeden, 2001).

Herein, we limit the scope of this entire process to an experimental framework for the rapid discovery and identification of natural product and therefore will not be describing topics related to greenhouse or field trials (**Figure 11**).

FIGURE 8 | Predictive AMPs found in available genomes of species in *Trichoderma*. **(A)** A dendrogram based on the genomes of *Trichoderma* spp. with reference protein sequences and **(B)** organized by their sections and the corresponding heat map showing the numbers having a 100, 99–95, 94–90, 89–80, 79–70, 69–51, and ≤50% match with AMP identifying characters shared by species of *Trichoderma* within their sections. **(C)** The top five annotated accession numbers found across all species from 95 to 100% match of AMPs. **(D)** Percentage of hypothetical proteins from 95 to 100% match of AMPs. The outgroup is *Cordyceps militaris*.

FIGURE 9 | Graph theoretical analysis to characterize influential species sections of *Trichoderma* based on their probability of having AMP-related genes and keyword protein annotations. The PageRank measure for the sections with respect to the probable AMP-related gene are quantified by the normalized **(A)** broadcasting and **(B)** receiving values, respectively. The PageRank measure for the sections with respect to the keyword proteins are quantified by the normalized **(C)** broadcasting and **(D)** receiving values, respectively.

Extraction of Natural Products From *Trichoderma* Species

Most *Trichoderma* isolates have been recovered from soils, from the fruiting bodies of other fungi, or from dead wood colonized by them (Druzhinina et al., 2011, Kubicek et al., 2019). The soil suspension, dilution, and aliquot methods are recommended for isolation of individual colonies. The most efficient substrate medium for the isolation and culture of *Trichoderma* isolates is potato dextrose agar supplemented with chloramphenicol, streptomycin, and rose bengal (Vargas Gil et al., 2009). Following the isolation and growth, the crude extract is collected to separate the desired natural products from the raw materials. The most

common extraction methods are solvent extraction, distillation, pressing and sublimation (Zhang et al., 2018a) (**Figure 12**).

Enrichment, Isolation, and Purification of Natural Products

Trichoderma crude extracts contain a complex mixture of biological molecules, with the bioactive products representing <1% of the crude extract (Odendaal et al., 2011). The low abundance of naturally occurring biological products represents a major bottleneck in their discovery (Hanson and Howell, 2004; McMullin et al., 2017). To selectively isolate low-abundance biological products from the complex mixture, various enrichment strategies have been developed. Size exclusion ultrafiltration strategies, such as molecular weight cutoff spin column filters, represent common methods of enriching the low-molecular-weight fraction of a crude extract (Hanson and Howell, 2004; McMullin et al., 2017). In addition to molecular weight cutoff, gel-based separations, solvent extractions, and size exclusion chromatography are all frequently used for biological product enrichment (Rivera-Chávez et al., 2017; Song et al., 2018; Zhang et al., 2018a).

Although the enrichment step helps to increase the representation of low-abundance biological products, the resultant fraction may still have impurities and diverse biological molecules. Thus, this fraction can be further fractionated based on physicochemical properties (e.g., polarity, hydrophobicity, stability, solubility) to isolate and purify the specific target product with bioactivity (Rivera-Chávez et al., 2017). The fractionation step starts with separation and detection of the natural products *via* chromatographic methods followed by detection methods such as ultraviolet light detectors. Based on a specific retention time or window, natural products can be selectively or broadly collected, lyophilized, and resolubilized in the appropriate solvent and concentration. The detailed technologies and techniques for isolation and purification of natural products from microbes have been extensively reviewed and explained by previous methodology and review publications (Bucar et al., 2013; Joana Gil-Chávez et al., 2013; Gomes et al., 2017; Zhang et al., 2018a; Atanasov et al., 2021).

Bioactivity Assays of Natural Products

There are two primary routes that can be taken to apply the active ingredient of interest: using the whole fungus as the active ingredient or applying the natural product of the fungus, whether biosynthesized by the organism or chemically synthesized *in vitro*. In deciding between using the fungus or using the compound alone, it is useful to consider the mechanism by which it will carry out its effect.

Using the fungus itself, one mode of action is for the beneficial fungus to outcompete the pathogenic organisms for nutrients and space on the plant by colonizing the mutual areas (Ghorbanpour et al., 2018). For this to be effective, the fungus must have strong colonization capabilities and persist long enough to out-compete the pathogenic population. *Trichoderma* excels at root colonization because of its production of compounds such as hydrophobins, which allow its hyphal filaments to attach to a multitude of surfaces,

aiding its ability to survive in the presence of other unwanted organisms (Guzmán-Guzmán et al., 2017). Furthermore, some *Trichoderma* species produce siderophores, which chelate iron, making it unavailable in the environment and potentially inhibiting the growth of competing organisms (Harman et al., 2004). In addition to the competition, *Trichoderma* can use mycoparasitic interactions to recognize and attack the parasite (Błaszczyk et al., 2014). One mechanism *Trichoderma* uses during its attack is to secrete various cell wall–degrading enzymes such as cellulase, pectinase, glucanase, lipase, and protease, which degrade several compounds, including chitin and glucan polysaccharides (Błaszczyk et al., 2014). Initially, lectins are used to recognize the pathogen and the hyphae coil around the pathogen and then penetrate the cell and use the enzymes previously mentioned to degrade the cell wall (Ghorbanpour et al., 2018). In addition to mycoparasitism, these cell wall degrading enzymes are involved in antibiosis, an antagonistic interaction in which *Trichoderma* produces these and other antimicrobial compounds, such as antibiotics and secondary metabolites, that exhibit a lethal effect on the parasitic organism (Błaszczyk et al., 2014); however, mycoparasitism and antibiosis may not be the primary mechanisms of biocontrol (Lorito et al., 2010). Included in this list of antimicrobials are compounds such as trichodermin, herizanolide, peptabiols, and epipolythiodioxopiperazines (Ghorbanpour et al., 2018). Two additional mechanisms include mycovirus-mediated cross-protection and induced systemic resistance. While these are different mechanisms—one is an infection with a non-virulent mycovirus and the other a beneficial organism inducing an immune response—they have similar overarching effects of priming/preparing the plant defense system for infection with pathogens (Ghorbanpour et al., 2018).

The other alternative is to use a natural product of the fungus as the active ingredient, which may allow for a more targeted outcome and circumvent the issues that may come with introducing a non-native organism to a new environment (Butt et al., 2001). These natural products, such as secondary metabolites and antimicrobial peptides, can also assist in the plant's defense mechanisms, function in an antibiotic manner, and compete against pathogens for nutrients such as metals (Keswani et al., 2019).

Once either the compound or the entire fungus is selected, a series of trials is necessary to validate its efficacy. For antimicrobial efficacy, it's important to establish a minimum inhibitory concentration with respect to the pathogenic organism targeted by the biocontrol agent and the commensal organisms that the product will encounter, determining if and how detrimental these are to them. To determine the minimum inhibitory concentration, the reconstituted samples from the fraction collectors in an appropriate solvent can be serially diluted and tested. Alternatively, a micro-titer broth dilution can be carried out (Aboobaker et al., 2019). This relatively easy screening method is a valuable tool, as any potential compounds can be immediately weeded out if they require an extremely high concentration to be successful. Additionally, dual-culture assays can be performed to determine the suppression of pathogenic growth directly (Lahlali and Hijri, 2010). Once initial screening

has taken place, larger-scale experiments such as greenhouse trials can be carried out. During a greenhouse trial, data can be gathered on the effects of inoculating a plant of interest with the biocontrol agent; and preliminary conclusions will be reached regarding the overall health of the plant and any antagonistic effects (Perelló et al., 2003). Upon completion of greenhouse trials, larger-scale field experiments could be done to more closely mimic the conditions biocontrol agents encounter once they are deployed in nature. In these field experiments, larger-scale data, such as any effects on growth, seed production, and pathogen reduction, will be excellent indicators of market viability (Abdel-Fattah et al., 2007). Once the vetting process is completed and the biocontrol agent has been thoroughly assessed, mass production issues must be explored to ensure the product is effective and lucrative, including growth conditions of the fungus, synthetic synthesis of individual compounds vs. extraction from the fungus, formula optimization for prolonged shelf life, and determination of a dispersal method.

Natural Product Identification and Characterization

Following the detection and isolation of fractions with relevant bioactivity, biological product identification can be carried out with chromatography-coupled mass spectrometry (MS) for complex samples or direct injection/infusion for pure or low-complex mixtures. For chromatographic techniques, gas chromatography (GC) and liquid chromatography (LC) represent the common choices. GC is commonly used for volatile and polar small compounds, while LC allows the identification of mid-polar to non-polar metabolites. The choice of technique used can dictate what types of small molecules can be identified and this needs to be made on a case-by-case basis. The biological products predicted using prediction tools like antiSMASH and amPEPpy from the *Trichoderma* genome can help provide information needed to select suitable techniques for biological product profiling.

Alongside the chromatographic techniques, MS represents a key experimental platform that is applied in the discovery, and characterization of natural products. Generally, MS-based approaches used to measure natural products is broadly categorized as metabolomics. Metabolomics, either targeted or untargeted, can be used to identify the natural products in *Trichoderma*. The advantages and disadvantages of these approaches have been reviewed extensively (Roberts et al., 2012; Schrimpe-Rutledge et al., 2016). For the identification, quantification, and structural elucidation of natural products, MS data are searched against the spectral libraries.

A significant challenge in the sensitive identification of biological products *via* MS includes the lack of representative databases or spectral libraries. Thus, the characterization of biological products with the use of *de novo* search strategies (Cheng Q. et al., 2010). *De novo* sequencing algorithms derive biological product structures, molecular formulas, or sequences using only fragmented ionic information from the tandem mass spectra, thus providing the complete profiling of all biological products. For metabolomics, SIRIUS 4 is one of the leading *de novo* software, using deep neural networks for metabolite identification from high-resolution tandem MS data (Dührkop

et al., 2019). It integrates high-resolution isotope pattern analysis and fragmentation trees with structural elucidation to provide a combined and coherent assessment of molecular structures from MS/MS data for large datasets (Dührkop et al., 2019). This tool allows the identification of the molecular formula of a query compound with very high accuracy; no spectral or structural databases are required for this step of the analysis because all theoretically possible molecular formulas are considered, allowing one to overcome the limitations of the current structural databases (Dührkop et al., 2019). For peptide-based biological products, PEAKS is well-established search software that also utilized machine-learning and combines *de novo* sequencing with traditional database searching (Ma et al., 2003; Zhang et al., 2012).

Genetic Engineering

Several transformation systems, relying on effective selectable markers, have been developed to genetically modify different *Trichoderma* species. The most widely used and optimized procedures for doing so are based on protoplasts (Gruber et al., 1990; Li et al., 2017) and *Agrobacterium tumefaciens*-mediated transformation (Zhong et al., 2007; Yang et al., 2011). Other less common genetic approaches are reported to be used in studying *Trichoderma*, such as Biolistics (gene gun) (Te'o et al., 2002), and electroporation (Wanka, 2021). The most investigated species in genetic engineering studies to produce enzymes or metabolites is *T. reesei* (Liu et al., 2015). Genetic transformation systems have also been developed for other *Trichoderma* species, including *T. harzianum* (Goldman et al., 1990; Zeilinger, 2004), *T. viride* (Manczinger et al., 1997), *T. atroviride* (Zeilinger, 2004; Calcáneo-Hernández et al., 2020), and *T. longibrachiatum* (Sánchez-Torres et al., 1994). In recent years, the CRISPR/Cas9-mediated genome editing system has been successfully employed in *T. reesei* and delivered in the format of either plasmid (Liu et al., 2015) or ribonucleoprotein complexes (Hao and Su, 2019). Finally, the transformation methods developed for *Trichoderma* species can be used for overexpression experiments on putative backbone genes or transcription factors as a strategy to increase the production and allow the characterization of specific metabolites produced in low amounts or not produced under standard culture conditions.

Methods to Implement Computational Analysis Into Biological Products for Integrated Pest Management System
Fungi as an Active Ingredient

For numerous biological products, the active ingredient uses reactivated lyophilized cells of the fungus itself. To propose new *Trichoderma* species as active ingredients requires a fundamental understanding of the fungus behavior within an environment. Therefore, the approach should consider the fungus lifecycle, its interactions with other microbes and organisms, its responses to abiotic and biotic stresses, its pathogenicity behavior on cash crops, and its ability to promote the growth of competing weeds that can serve as vectors for other plant pathogens. Regarding *Trichoderma* species, several were shown to promote plant growth under abiotic and biotic stress conditions. Although all

studies reviewed concentrated on how the fungus helped a plant in stress conditions, few or none focused on how stress affected the survivability of the fungus. The benefits of establishing a new species or strain as an active ingredient reside in getting more specific and long-lasting effects, cost-efficient disease management, and higher yields of cash crops. Particularly in *Trichoderma*, there is a strong history of species used as successful active ingredients (**Figure 2**, **Supplementary Table 1**). Another consideration is the effectiveness of a strain within a species or the efficacy of mixing different strains or species of *Trichoderma* and fungicides as formulations and active ingredients.

Importantly, due to the genetic diversity of *Trichoderma* strains, they can be unpredictable in terms of mechanisms and biocontrol improvement in different environmental settings (Benítez et al., 2004) and therefore should not be generalized at the species level. For this reason, companies use a particular strain in their products. Combinations of *Trichoderma* strains or species have been shown to better promote growth of cash crops in agriculture and forestry than a single strain or species (Chirino-Valle et al., 2016; Halifu et al., 2019; Chen et al., 2021). As such, there are several commercially available products with mixed strains or species of *Trichoderma* for better biocontrol, like Bioten® WP, BioWorks® Rootshield® *Plus*⁺ WP; Kiwivax®, Vinevax™ Bio-Dowel, and Vinevax™ Pruning Wound Dressing, to name a few (**Supplementary Table 1**). In addition, there are genetically altered hybrid strains between closely related *Trichoderma* species created by protoplast fusion (Stasz et al., 1988). These mutant strains had shown better biocontrol efficacy by selecting secondary metabolites or enzymes used for antimicrobial activity than individual species (Stasz, 1990; Hanson and Howell, 2002). However, to our knowledge, it is unknown if there are any commercial products or field experiments to test the efficacy of these modified fungal strains. Finally, mixing biological control agents (like *Trichoderma*) with fungicides (or derivatives of natural products) has been shown to reduce disease pressure (Ons et al., 2020). Since biological controls are dependent on environmental conditions, combining a biological product with fungicides can increase efficacy (Ons et al., 2020). However, researchers should examine if the *Trichoderma* strains are susceptible to the fungicide before using them in a mixed application. Moreover, there are few studies investigating biological products durability to control plant pathogens (Bardin et al., 2015), and it is known that there are pathogens resistant to some fungicides (Hahn, 2014; Hollomon, 2015). Therefore, further studies are needed to determine the durability and environmental sustainability by combining both biological products with fungicides.

Some disadvantages to applying active fungal strains in a field is that a fungus could sexually recombine with a native population, introducing unknown ecological consequences, or simply be incapable of surviving in the newly introduced environment. Thus, understanding the fungal lifecycle is critical to designing a new biological product. Fungal clones can expand their populations but still lack adaptability because of short-term survival mechanisms (Drenth et al., 2019). Sexual recombination is suitable for long-term survival and adaptability to a changing environment (Drenth et al., 2019). However, when non-native fungi capable of sexually reproducing are introduced into an environment, they can reproduce with a native population, creating hybridization and potentially having non-target effects. Hybridization has been shown in numerous pathogenic fungi and has led to the decline of several host crops or ornamental plants and targeting of new hosts (Schardl and Craven, 2003; Depotter et al., 2016; Stukenbrock, 2016). Alternatively, hybridization in symbiotic fungi has been beneficial for plant adaptation (particularly in grasses) for nutrient acquisition and stress conditions (Hamilton et al., 2010; Saari and Faeth, 2012; Oberhofer et al., 2014; Jia et al., 2016). Therefore, a screen of potential mating type genes within a species should be considered with the proposition of knocking out the mating genes if possible.

Fungi, in particular *Trichoderma*, have been used as active ingredients in bioherbicides to control weeds; however, there are no reports of their natural products being used (Kremer, 2005; Triolet et al., 2020). There are ongoing investigations to understand the mechanisms involved in controlling weed populations without affecting the crop of interest (Harding and Raizada, 2015). The main limiting factors for using fungi as bioherbicides are their narrow host range, their specific biotic requirements for good efficacy, and their possible non-targeted effects (Kremer, 2005). Therefore, the extraction of compounds possessing herbicide properties from these fungi can be a fruitful endeavor for overcoming these limitations (Kremer, 2005; Harding and Raizada, 2015; Triolet et al., 2020).

In the promising bioactive ingredient from a *Trichoderma* species (**Figure 3**), much bioactivity has been observed in the laboratory. However, the potential for transferring these species from the lab to the field is vague.

Natural Products as an Active Ingredient

Natural products deriving from microbes have also been used as active ingredients (Singh and Yadav, 2020) primarily those released during antibiosis interactions. Antibiosis is the secretion of natural products (e.g., secondary metabolites, AMPs) that have antimicrobial properties in the vicinity of other microbes (Howell, 1998). Secreted molecules might be the key to triggering a response from a host plant or *Trichoderma* itself. For example, the production of peptides or small molecules from a pathogenic fungus and its perception through G protein-coupled receptors might trigger a mycoparasitism response in *Trichoderma* species (Druzhinina et al., 2011). Several classes of molecules secreted by *Trichoderma*, like xylanases, peptaibols, swollenin, and cerato-platanins, have been reported to act as microbe-associated molecular patterns that trigger immunity in plants (Howell, 2003; Druzhinina et al., 2011; Newman et al., 2013). Owing to the difficulty of mass-producing biological products using fungi as active ingredients, and the agrochemical effects on the environment, natural products appear as promising solutions for implementation in integrated pest management (Dayan et al., 2009; Yan et al., 2018; Singh and Yadav, 2020; Triolet et al., 2020). To propose a new natural product as an active ingredient, a fundamental understanding of the

molecule chemistry and behavior within an environment is needed. The main benefits of this approach are that these natural products could be used in combination with existing pesticides with known identities, bioactivities, and effects on plant growth promotion and defenses. An example of a natural product used in pesticides is the quinone outside inhibitor (Q_oI) family of fungicides derived from the group of natural products called "strobilurin" (Balba, 2007; Vincelli, 2012). This group was first isolated from the wood-rotting fungus *Strobilurus tenacellus* (Anke et al., 1977). Q_oI fungicides inhibit mitochondrial responses by binding at the Q_o site of the cytochrome bc1 enzyme complex. Q_oI fungicides are a preventative measure through translaminar movement and

are known to result in plant growth enhancements (Balba, 2007; Vincelli, 2012). Q_oI fungicides pose less risk to human health than alternative pesticides (Balba, 2007; Vincelli, 2012). Unfortunately, there are resistant Q_oI fungicide populations because of disease pressure in growers' fields, creating a need for alternative sources of natural products (https://www.frac.info/). As shown in **Figure 5** and **Table 2**, *Trichoderma citrinoviridie, T. virens,* and *T. viride* all have BGCs with a 50% sequence match, and *T. atroviride* and *T. koningiopsis* both have BGCs with a 37% match with the cluster encoding the putative metabolite Ascochlorin. Berry et al. (2010) have demonstrated that Ascoholorin, isolated from the pathogenic fungus *Ascochyta viciae*, is a cyctochrome bc1 inhibitor acting
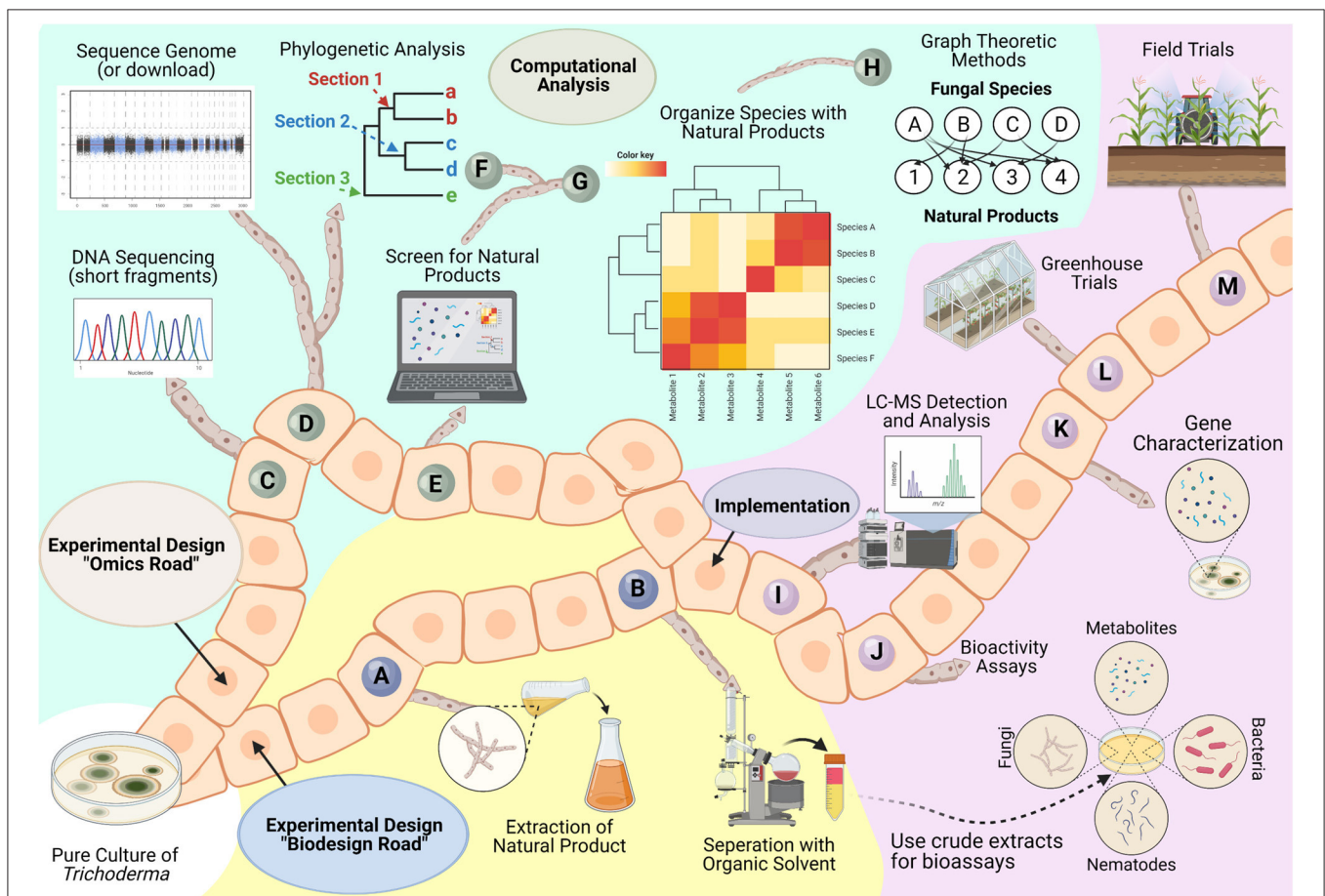
FIGURE 10 | Roadmap for bioprospecting *Trichoderma* for natural products. Isolation of an axenic *Trichoderma* isolate is needed to initiate the roadmap. The biodesign road details the following general steps to extract and experimentally screen bioactive compounds: **(A)** Grow the culture in a liquid broth medium and collect the supernatant by filtration; **(B)** Use an appropriate organic solvent (e.g., ethyl acetate, methanol, etc.) to extract metabolites present in the filtrate. This crude exudate is composed of a mixture of natural products that can be further interrogated to identify targeted bioactivity properties. To predict putative natural products before extensive laboratory studies, then the "omics" road can be followed using the following steps: **(C)** Extract genomic DNA from the isolate and determine the species identity by amplifying and sequencing genetic markers like the internal transcribed spacer region (ITS), elongation factor 1-alpha (*tef1*), and RNA polymerase II subunit (*rbp2*) (Cai and Druzhinina, 2021); **(D)** Sequence reference genome of isolate; **(E)** Predict natural products using tools such as antiSMASH or amPEPpy. With this information, **(F)** comparative genomic analyses can be performed to determine which taxonomic section the target isolate belongs to, and **(G,H)** can be used to relate predictions across organisms to highlight the uniqueness and diversity of putative natural products across available *Trichoderma* by using graph theory. Both roads are led to an implementation step where experimental design aims to **(I)** selectively characterize predicted or isolated compound(s) by liquid or gas chromatography coupled to mass spectrometry, **(J)** evaluate bioactivity for selected compound(s), **(K)** determine the genes and proteins responsible for the production of the compound(s), **(L)** conduct trials to determine its potential usefulness in greenhouse conditions, and **(M)** test the compound effects in field trials.

on both Qi and Qo sites. Currently, Ascoholorin has not been identified or characterized in *Trichoderma* species. However, based on our bioinformatic results, there might possibly be an Ascoholorin-like compound in *Trichoderma* species, which could be investigated as a biopesticide.

To our knowledge, there are minimal caveats for introducing natural products into the environment; yet they might present some unknown non-targeted effects on beneficial microbes, livestock, and humans. Natural products are potentially unstable in extremely cold temperatures; therefore, they may not overwinter in the field, allowing more pathogenic inoculum to increase. They might also become denatured after long exposure to environmental factors, such as ultraviolet radiation.
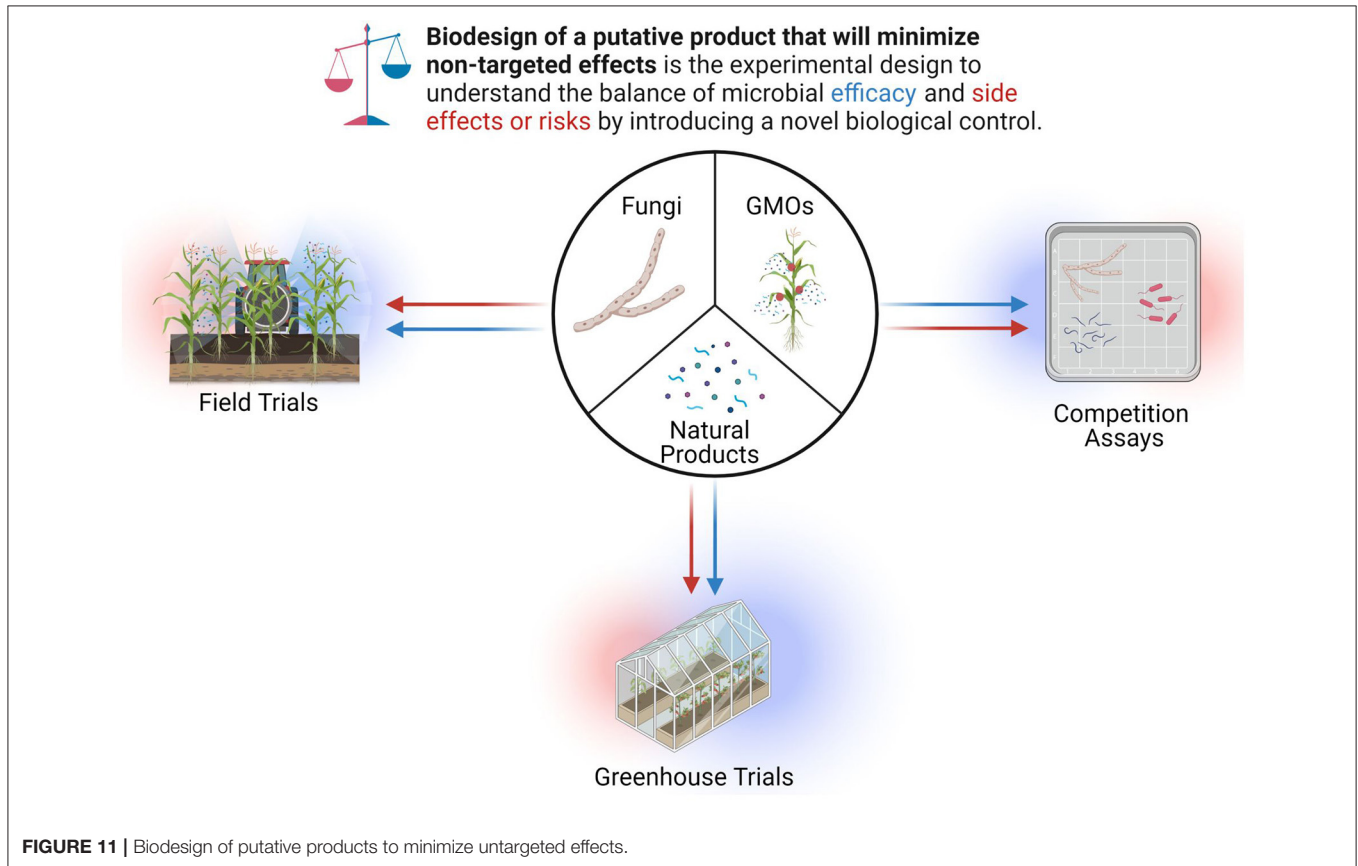
FIGURE 11 | Biodesign of putative products to minimize untargeted effects.
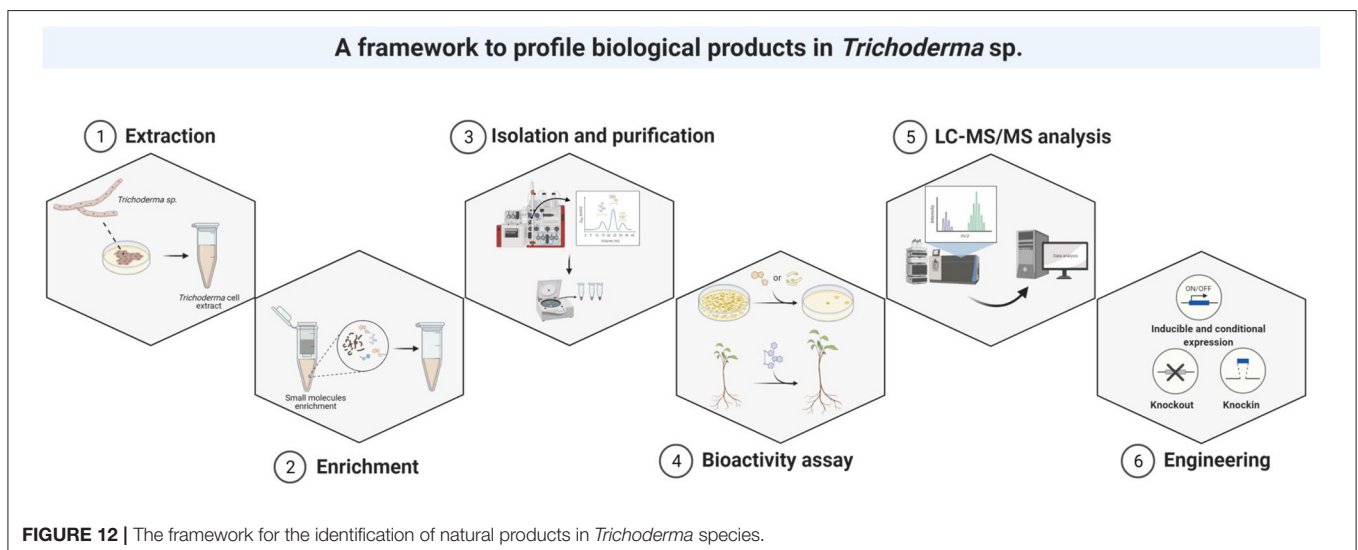
FIGURE 12 | The framework for the identification of natural products in *Trichoderma* species.

Genetically Modified Crops With Biosynthetic Gene Clusters

Genetically modified organisms (GMOs) are engineered to produce a natural product by inserting a gene of interest into the target plant genome. This can enhance a plant's ability to fight against pathogenic insects and resist herbicides. The implementation of GMO crops with natural products derived from microbes started over 30 years ago. Currently, several GMO crops engineered with natural products, such as corn, soybeans, papaya, tomatoes, rice, and cotton, are available. The world's first GMO crop, FLAVR SAVR, was a virus-resistant tomato variety that suppressed the enzyme polygalacturonase, which dissolves cell-wall pectin and causes the fruit to become soft (Bruening and Lyons, 2000). Currently, three GMO crops are commonly used: *Bacillus thuringiensis* corn (Bt corn), Roundup Ready crops, and the Rainbow papaya, all approved by the United States Environmental Protection Agency and the United States Department of Agriculture. Bt corn is a hybrid plant bioengineered with a broad-spectrum insecticide in which the active ingredient is crystal (Cry) protein toxins (Gewin, 2003; Koch et al., 2015). Roundup Ready crops (soybeans, corn, alfalfa, cotton, and sorghum), genetically modified to contain a protein-coding for a glyphosate-insensitive enzyme, can kill competing weeds and reduce the source of inoculum for many plant pathogens. The Rainbow papaya has a gene from a papaya ringspot virus inserted into the fruit to make it resistant to the same virus, using the same logic as vaccination in mammalians.

Previous successes have shown that producing natural biological products directly from GMO crops is a feasible procedure. To our knowledge, there are no reports of inserting BGCs from *Trichoderma* into GMO crops. Thus, this idea may represent a new niche to explore in agriculture. However, caution is essential in creating GMO crops, as there are many rules and regulations to follow depending on the agricultural system for each country.

CONCLUSIONS

The success of any biological product will rely on its efficacy, cost efficiency, stability, and profitability. Our roadmap outlines the multifaceted abilities of *Trichoderma* as the prominent biological control agent. In addition, we demonstrate how to use omics and machine learning to identify new or existing products that can be used in the market and how to extract and implement those products while listing the benefits and caveats (**Figure 13**).

However, efficiently, and effectively translating laboratory observations to field applications should be linked to a purpose tied to a specific market or reason. For example, renewable and sustainable biofuels and bioproducts from plant biomass are a steadily growing industry representing alternative fuel sources to petroleum. Identifying novel or existing *Trichoderma* species or natural products to help promote terrestrial plants for higher biofuel and bioproduct production or the promotion of associated beneficial microbes can improve efforts to advance the use of bioenergy. Experiments have shown that *Trichoderma* species can improve the growth parameters of bioenergy crops like hybrid grasses (Chirino-Valle et al., 2016). In addition, there is a need to understand how using biocontrol products to influence environmental microbiome processes that drive global carbon cycling will impact climate change. Biological control could be a way to biologically mitigate heavy metals, radionuclides, or agrochemicals present in the soil, coastal sediments, freshwater, and subsurfaces.

FIGURE 13 | Considerations for how to introduce biological products from *Trichoderma* into field applications.
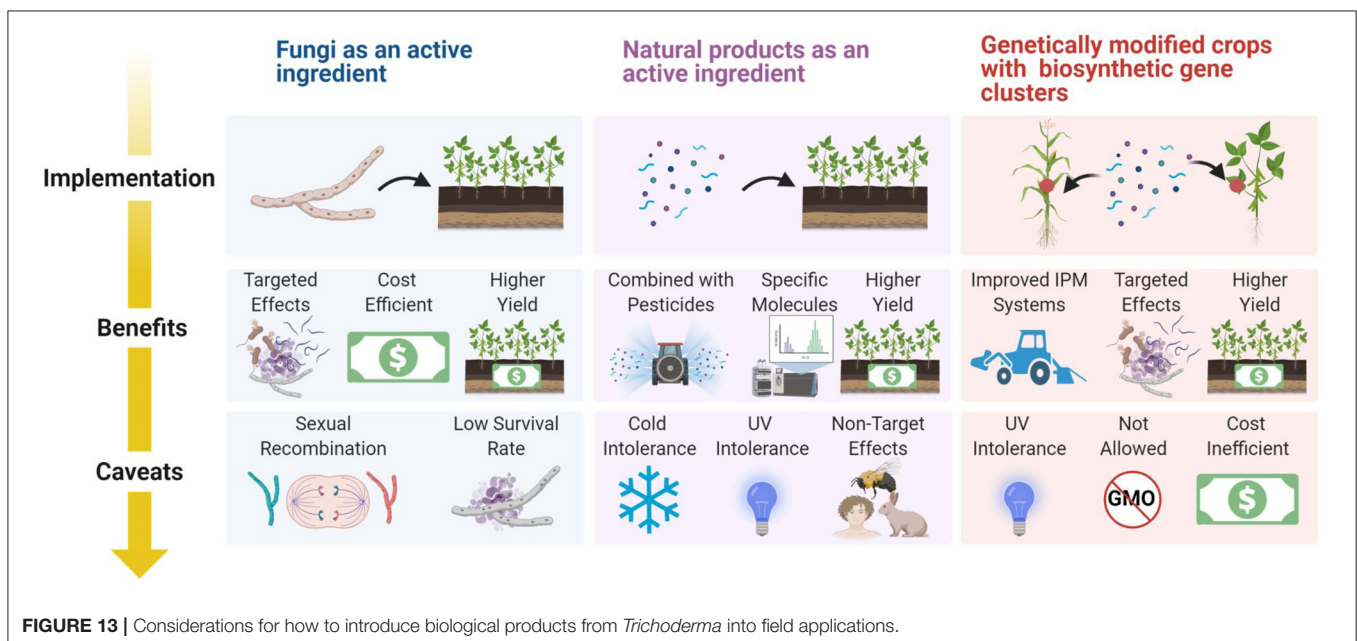
Another example is sustainable agriculture, where the results should address farmers' and industry partners' needs and concerns. Farmers will adopt sustainable programs when there is a short-term economic outcome and if doing so benefits their farms, the environment, or both (Piñeiro et al., 2020). Ultimately, any biological control implementation should follow the goals of integrated pest management—economic viability, environmental safety, and social acceptability. These goals address the needs and concerns of the producer, the seller, and the consumer (Dara, 2019). For some engineered products, like GMOs, an understanding of the geopolitical climate is necessary for developing research and economic plans.

As outlined in this systematic review, new issues will arise as more biological products are developed, marketed, and implemented into agricultural or environmental settings. We have observed that several known compounds have been identified from *Trichoderma* species. However, these results do not translate into active ingredients available on the market. We suggest that the implementation team involved in developing products should be multidisciplinary. For example, for a promising biological product to be marketed, an ideal implementation team should include, but not be limited to, researchers that know the market/economy, chemistry, computational analysis, and the integral dynamics of an agricultural microcosm (e.g., plants, microbes, insects, etc.). Lastly, there are numerous new publications that are made yearly based on novel features from global studies on *Trichoderma* isolates. With this extensive dataset, there is need for additional high-quality genomes and representation of the various sections within *Trichoderma*. Altogether, this comprehensive roadmap can serve as guidance for the future production of biological products from *Trichoderma* and could be used for other fungal systems.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

TR, HS, MS, JL, and PA designed research. TR, HS, MG, JE, MS, JL, and PA performed research and wrote the manuscript. MG conducted graph-theory analysis. JE and TR conducted phylogeny analysis. TR and HS analyzed antiSMASH results. TR and MS analyzed amPEPpy results. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffunb.2021.716511/full#supplementary-material

Supplementary Table 1 | List of products that uses *Trichoderma* strains used as an active ingredient.

Supplementary Table 2 | List of accession numbers and probability to be an AMP for each *Trichoderma* species examined with amPEPpy.

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