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Review

Metabolomic applications for understanding complex tripartite plant-microbes interactions: Strategies and perspectives

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ABSTRACT

Phytopathogens from the *Alternaria* sp., *Fusarium* sp., *Penicillium* sp., and *Pseudomonas* sp. and their toxigenic metabolites - alternariol, fumonisin, citrinin, and coronatine respectively, negatively impact crop yields and sales by eliciting plant diseases and/or causing human and veterinary toxicoses upon the consumption of contaminated food. These phytopathogens and their associated toxins, however, are present and most likely in undetectable concentrations pre-harvest and post-harvest of many major staple crops. Metabolomic approaches have been used extensively for better characterizing and diagnosing human disease, plant disease and, their etiological agents. Their use in agro-industrial research focusing specifically on tripartite (plant - toxicogenic microbe - beneficial microbe) interactions is, however, limited. Since new approaches for eradicating food-borne pathogens, increasing crop productivity and improving agro-international trade are being sought worldwide, the consequent integration of metabolomic approaches and perspectives in crop protection strategies for better understanding plant - toxicogenic microbe - beneficial microbe interaction in tandem is discussed.

1. Introduction

Food shortage and insecurity are still global issues because of the ever-increasing human population, and the corresponding increased demands this places on farming. Many agro-industrial plant-borne fungal and bacterial pathogens (*e.g. Alternaria* sp., *Burkholderia* sp., *Fusarium* sp., *Penicillium* sp., *Pseudomonas* sp., *Rhizoctonia* sp., and *Xanthomonas* sp.), produce bioactive metabolites (alternariol, toxoflavin, fumonisin, citrinin, coronatine, RS-toxin, and albicidin respectively), that compromise the quality and usability of harvested crops, subsequently negatively impacting human and animal health. These phytopathogens are mostly associated with cereal mildews, and fusariosis or smuts/spots [1–4], and subsequently have a major impact on many populations globally, especially those who rely on cereals (like maize, wheat, and barley) as a staple food source (FAOSTAT, 2019). Many of the crop protection strategies and or plant disease management approaches used to eradicate or manage these crop invaders, relies mainly on the use of resistant crop cultivars and or synthetic antimicrobials [5]. More recently, however, the use of safer/environmentally friendly microbe-derived antimicrobials is becoming more popular [6,7]. Despite this, however, none of the strategies have led to the total eradication of these harmful phytopathogens, which regularly re-emerge and remain prevalent in many regions of the developing world [5,8]. The many precautionary measures put in place to prevent pre-harvest plant damage or post-harvest/storage crop loss (*e.g.* sun drying, air drying, and chemical application) by the unwanted toxicogenic microbes are considered insufficient, since regular accounts of both human and livestock poisoning from these toxins still exist [2,2,3,4,9–12].

Reports about the histological, genomic, transcriptomic, and proteomic characteristics of these plant pathogens, the beneficial microbes, and the impact these have on the host plant are readily available [13–15]. The information about the real-time metabolic changes induced by the microbial activities (herein toxicogenic microbe-beneficial microbe) on the host plant and *vice versa*, is however scarce. Considering this, additional information on the metabolic changes induced during tripartite interactions (plant, toxicogenic microbes, and beneficial microbes), hereafter defined as plant-microbes symbiosis, interpreted alone or in combination with previous omics data, would significantly improve our understanding of the interaction. Understanding these interactions could ultimately lead to formulating new crop protection strategies for improving crop quality and yields [16–18]. Beneficial microbes herein are plant microbiota that improve the plant's: (a) resistance to various plant stressors, (b) nutrient acquisition and growth capability, (c) defense response metabolism and or

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(d) produce anti-phytopathogenic biomolecules. Detailed reviews on beneficial microbes and their metabolic products functioning as plant growth promoters and/or phytopathogen biocontrollers (offering plant protection) are available for further reading [6,7,19,20]. Whilst early metabolic data characterizing various model plants (*e.g. Arabidopsis thaliania* and *Medicago truncatula*), phytopathogens (*e.g. Aspergillus flavus*, *Pseudomonas syringae*, and *Botrytis cinerea*) and plant beneficial-microbes (*e.g. Bacillus thuringiensis*, *Penicillium chrysogenum*, and *Pseudomonas fluorescens*) exist, the information was acquired using targeted research approaches. These studies identified/quantified specific phytopathogen virulence biomolecules [12,21–23], elucidated the mechanisms of action of growth-promoting or biocontrol compounds/organisms [24–27], or studied a plant response to a specific phytopathogen/anti-phytopathogenic agent [28–33].

More recently however, untargeted and semi-targeted metabolomic profiling, focused on understanding competitive, amensalistic, commensalistic, and mutualistic associations between plants, phytopathogens and beneficial microbes, are being utilized for these purposes [11,27,34–37]. The metabolomic data on tripartite plant-microbes symbiosis is complex, yet limited, which necessitates this review. This paper focuses only on the bacterial and fungal investigations completed to date on this topic. The paper starts by explaining the various perturbations that can be associated with the aforementioned tripartite interactions, followed by a discussion of the literature describing how metabolomics has contributed to a better understanding of the changes to the phytopathogen metabolome during plant-microbe symbiosis, the metabolomics of beneficial microbes during concurrent host-phytopathogen interactions, and lastly how plant metabolome profiling could contribute to a better understanding of plant-microbes symbiosis.

2. Perturbations associated with a tripartite plant-microbes symbiosis

Studies investigating host plant-microbes interactions as either a one-way (amensalism and commensalism) or a two-way association (mutualism and competition) have been well-reported [38,39]. The application of metabolomics for efficient discrimination of metabolite origins and for understanding roles of all interacting organisms during tripartite plant-microbes symbiosis is, however, scarce. During the cohabitation of a phytopathogen, beneficial organism and the host plant, an alteration in each of their respective metabolomes is expected, due to the influx of foreign biomolecules from all the participants [40]. Specifically, the foreign metabolites originating from the interacting pathogen or beneficial organism, are expected to directly or indirectly alter various biosynthetic pathways of the host plant [17,21]. Invariably, during this tripartite coexistence, an influence (negative or positive) is exerted on the physiology of all the three participants in concert, since antibiosis brings about a disruption in the growth and proliferation of a pathogen, while the virulent pathogen causes a diseased state in the host plant. Considering this, characterizing the altered metabolic state of these interacting tripartite living systems (plant-microbes symbiosis) simultaneously, may assist to better understand the mechanisms and adaptations related to these interacting species.

Furthermore, it should be mentioned, that various teratogenic, carcinogenic, hepatotoxigenic, mutagenic, and neurotoxigenic substances (*e.g.* type B trichothecene, fumonisin, beauvericin, moniliformin, deoxynivalenol, fusaproliferin, patulin, and enniatins) produced by various mycotoxicogenic species (*e.g. Aspergillus*, *Fusarium*, and *Penicillium* sp.), are sometimes masked, or initially in an inactive state, and only become detectable when the plant is used as food for livestock, or during the processing of cereal foods for later human consumption. This subsequently makes these compounds and the changes induced by them during such circumstances difficult to assess or monitor in the infected pre-harvest and post-harvest plants. Herewith, metabolomics also offers a unique opportunity by which phytopathogen-specific metabolic biomarkers or plant disease biomarkers (indicative of infection or early plant disease biomarkers) might be identified in an untargeted manner, and then used to monitor the presence of the phytopathogen and/or the presence/progression of the disease.

Studying in-field plant community dynamics presents a great challenge since free-growing plants interact with a multitude of microbes, and hence determining the exact cause of the perturbations/metabolite source, in a supposedly tripartite symbiosis, can become complicated. Hence an unbiased metabolite separation and or allocation method is required in many plant-microbe metabolomic investigations. Taking this into consideration, William Allwood, et al. [41], proposed a growth phase (time-course dependent) differential filtering and centrifugation dual metabolome profiling procedure, for the determination of metabolite source in more complex tripartite interaction studies, which might be used in free-growing plants. Here, the growth phases of the host plant and the interacting microbes are monitored independently, and the plants and microbes are harvested at different growth phases based on biomass monitoring. Fig. 1 illustrates the complex metabolomic profiles that we propose could be used for simultaneously investigating various

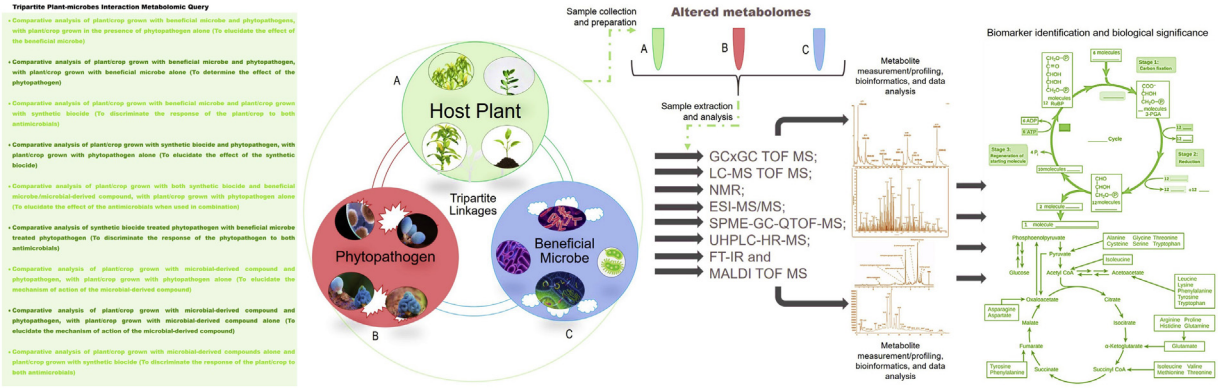

Fig. 1. Example of the complex multi-comparative metabolomic profiling perspectives for tripartite plant-microbe interactions. This comparison should facilitate metabolite source attribution. **Key:** Solid Phase Microextraction: (SPME); QTOF: Quadruple Time of Flight; GC: Gas Chromatography; LC: Liquid Chromatography; HR: High Resolution; MS: Mass spectrometry; UHP: Ultra-High Performance; NMR: Magnetic Resonance, FT-IR: Fourier Transform Infra-Red Spectroscopy, and MALDI TOF MS: matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry analyzer.

tripartite plant-microbes symbiosis. This multi-comparison approach could aid in the understanding of metabolite sources and impacts during the different interactions.

Further considerations when planning a total metabolome profiling study (aimed at evaluating full metabolic signatures) investigating tripartite plant-microbes symbiosis are the strengths and limitations of each metabolomic analytical instrument, in the light of the expected outcomes. Various analytical instruments (*e.g.* headspace solid-phase microextraction gas chromatography (HS-SPME-GC)); ultrahigh-resolution liquid chromatography (UHLC-HR) coupled to time of flight or electrospray ionization mass spectrometry (TOF-MS/ESI-MS), selected by considering compound polarity and dimensionality, analytical sensitivity, resolution, repeatability, and reproducibility, are typically used for untargeted metabolomics. Concise reviews on metabolomic instrumentation, methodology and data analysis used, including the pros and cons of each analytical tool, are available for further reading [42–45].

2.1. Mechanism of activity and roles of specific metabolites in tripartite plant-microbes interactions

Whilst the metabolic interplay within the tripartite plant-microbes linkage is expected to follow previously documented mechansims of plants defense, beneficial microbes mode of action, and phytopathogen virulence mechansism, new metabolite markers identified using untargeted metabolomics techniques, will allow for the identification of novel mechanisms related to the afore mentioned. The question, however, is that, can untargeted metabolomics provide an opportunity to discover novel mechanisms not related to previously document ones? If yes the researchers will thus avoid the inclination to screen for previously documented mechanisms. It is also important to note, however, that the impact of the mechanisms is dependent on several factors, not limited to the physiology of the host plant, the response of the phytopathogen to the plant's defense mechanism, the phytopathogen's virulence mechanism and the biocontrol mechanisms of the beneficial microbes investigated [46].

The exogenous and/or endogenous colonization of plant parts by toxicogenic or beneficial microbe result in the production and accumulation of numerous plant specific metabolites that play significant role in plant growth and health. For instance the colonization of host-plants by beneficial microbe led to the accumulation of various beneficial metabolites in the plant (*e.g.* catalpol, coumestrol, daidzein, gallic acid, myricitin, and tomatidine) which correspondingly led to: a. increased drought and salt tolerance, b. reduced pest damage, c. reduced pathogen proliferation, and d. higher nodulation in the host plant. [47–50]. The reviews by Etalo, et al. [51] and Korenblum and Aharoni [47], provides information on plant metabolome distruptions induced by colonizing beneficial microbes and the significance of the specific metabolites produced due to the pertubations.

Concise reviews on the mechanisms underlining various plant metabolite's (*e.g.* phytohormones, phytoalexins and defensins) role in plant disease resistance are available [52–57]. For example, the mechanism of action of the antifungal defensin NaD1 from *Nicotiana alata*, includes membrane permeabilization of *Fusarium* hyphae, disruption of cell cytoplasm and subsequent induction of reactive oxygen species and cell death [54]. Furthemore, the two classical plant defense responses to phytopathogen attack (systemic acquired resistance (SAR) and induced systemic resistance (ISR)) have also been comprehensively discussed elswhere [58–60].

The virulence mechansims of phytopathogens (*e.g.* their host-plant debilitating mechanisms and antagonistic mechanisms) and the mechanism of action of beneficial microbes (direct and indirect biocontrol mechanisms) have also been adequately documented in existing reviews [46,61–64]. For example, phytotoxins produced as virulent factors for some phytopathogens (*e.g. P. syringae* spp. and *Rhizobium* spp), function by distrupting the metabolism of the host-plant, *via* elevated ethylene production, which in turn reduces plant nodulation and plant biomass. Furthermore, various beneficial microbes have previously been reported to produce rhizobitoxine and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminases, which inhibit the both synthesis and function of the ethylene precursor ACC and ACC synthase enzyme respectively [62,65,66] – a direct biocontrol mechanism. Some beneficial microbes also produce lytic metabolites (*e.g.* cellulases, chitinases, proteases, and -1,3 glucanases), capable of lysing the cell walls of many pathogenic fungi, while others produce siderophores (iron sequesters), preventing the acquisition of iron by such phytopathogens, subsequently inhibiting phytopathogen growth and colonization [62] – an indirect biocontrol mechanism.

The kind of microbial colonizer (phytopathogen or beneficial microbe) and the type of colonization (unilateral or multilateral), impacts the metabolite levels in the host-plant metabolome – and determines to a large extent the mechanisms that will be in play during the tripartite linkages. Often, unilateral or multilateral microbial colonization could lead to an accumulation of specific plant metabolite classes like the alkaloids, benzoxazinoids, isoprenoids, lignans, oxylipins, phenolics, and terpenes. In other situations, depending on the colonization type (unilateral or multilateral) and the colonizers (phytopathogen or beneficial microbe), the metabolite levels in the host-plant metabolome decreases or remains unchanged. According to Rodriguez, et al. [67], the host-plant colonization patterns of phytopathogens and beneficial microbes, and the subsequent metabolite changes may be identical, and the mechanisms by which the plant differentiates the origins of such, still needs to be understood. Untargeted metabolomics shows promise for elucidating such.

3. Phytopathogen metabolome changes during plant-microbes symbiosis

Recently, Azzollini, et al. [68], applied HS-SPME-GC–MS and LC-HRMS metabolomics to investigate the dynamics by which volatile and non-volatile metabolites are altered during fungal cohabitation. The study profiled the metabolome of two grapevine pathogens; *Eutypa lata* and *Botryosphaeria obtusa* (in co-culture), and investigated their individual responses to a commercially available 2-nonanone antifungal metabolite, the origin of which was not specified. Complete inhibition of the two grapevine pathogens was observed after seven days. The other major volatile and non-volatile metabolites identified in the study included decane, a yet unidentified compound from the sesquiterpene class and O-methylmellein.

A proton nuclear magnetic resonance (^{1}H NMR) metabolomics approach was used by Sevastos, et al. [69], to characterize the metabolic disturbances induced in the metabolome of wild type *F. graminearum*, four carbendazim-resistant *F. graminearum* strains, (after treatment with the synthetic fungicide carbendazim) and an untreated *F. graminearum* strain. The authors observed a positive correlation among some metabolite levels (upregulated: L-serine, D-glucose, L-methionine, L-glutamate, L-phenylalanine, pyroglutamate, and citrate; down-regulated: threonine, D-myo-inositol, L-sucrose, and malate) detected in the wild and resistant *F. graminearum* strains, which could be later exploited in biomarker identification/selection for disease detection and monitoring. Prior to that, using LC—MS, Farrés, et al. [70] described the altered metabolome induced by toxic copper (Cu(II)) residues on a laboratory wine yeast strain, *Saccharomyces cerevisiae* (*BY4741*). Significant increases were recorded in the metabolic levels of

trehalose, nicotinate D-ribonucleoside, L-glutamic acid, and nicotinamide D-ribonucleotide with a resultant decrease in the concentration of glutathione. Although the yeast's growth was not affected by sublethal concentrations of Cu(II), higher Cu(II) concentration led to its DNA damage and oxidative stress. Thus affirming the potential negative impact of Cu(II) containing fungicides on the wine yeasts (*e.g. S. cerevisiae*). High level copper residues in grapes may cause slow or stuck fermentation due to distruption of the beneficial *S. cerevisiae* metabolome. These studies summarized in Table 1 show the capacity of metabolomics to characterize the mechanisms associated with the effects of antimicrobial agents on the plant microflora.

4. Beneficial-microbe metabolome changes during plant-microbes symbiosis

Metabolomics of late has been used to characterize the altered metabolic state of various beneficial microbes that positively influence plant viability and growth, and those offering protection from invading phytopathogens (Table 2). An untargeted metabolomics investigation by Danquah, et al. [71], characterized the altered metabolic responses of marine-adapted fungal isolates to phytopathogenic bacteria (*Ralstonia solanacearum* and *P. syringae*), and a fungal (*B. cinerea* and *Magnaporthe oryzae*) challenge. From the mono- and co-culture studies performed for natural product discovery, several known (*e.g.* mitorubrins, 3,4-dihydromitorubrinol acetate, emerimicins, cytochalasins, ophiobolins, ustilaginoidins, eremophilanes, tenuazonic acid and sclerosporins) and novel metabolites (*e.g.* ergone) with proven antimicrobial bioactivity were characterized. The detected novel metabolites suggest the activation of cryptic biosynthetic pathways in the beneficial marine fungal isolates.

Of interest are the glyphosate-induced perturbations in some Pseudomonad's metabolome reported by Aristilde et al. (2017). The metabolome disruptions of four beneficial *Pseudomonas* strains (*P. fluorescens* RA12, *Pseudomonas protegens* Pf-5, *Pseudomonas putida* KT2440, and *P. putida* S12), when grown under different concentrations of succinate and glyphosate (a toxic component of herbicides) were elucidated by ^{13}C-assisted LC–MS metabolomics. Glyphosate negatively affected the growth of *P. putida* S12 and *P. putida* KT2440, while *P. fluorescens* RA12 and *P. protegens* were unperturbed in low or high glyphosate concentrations. The study confirmed that glyphosate targets the aromatic amino acid biosynthetic pathway of the affected organisms. Other major biomarkers identified were tryptophan, phenylalanine, shikimate-3-phosphate, citrulline, thymidine, fumarate, valine, glutamine, and ornithine. Their results suggest that the simultaneous use of glyphosate-containing herbicides might compromise the use of these beneficial microbes in an agricultural setting.

5. Plant metabolome changes during plant-microbes symbiosis

Metabolomic studies investigating various plant responses to either toxicogenic or beneficial microbe perturbations is far more prevalent in the literature compared to the two previously discussed sections (section 3 and 4) (Table 3). A study by Saia, et al. [72], describes one of the earliest attempts to understand tripartite plant-beneficial microbes symbiosis. Using GC-TOF-MS and HILIC-Q-TOFMS (hydrophilic interaction chromatography time-of-flight mass spectrometry), the symbiotic impact of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) consortium on the metabolome of durum wheat (*Triticum durum* Desf.) was determined. By comparing the wheat root metabolite profiles from singular AMF treatments, with

Table 1
Recent metabolomic elucidation of phytopathogen metabolome changes during plant-microbes symbiosis.

Profiled metabolome: interacting phytopathogen	Interactions and study conditions	Significant metabolites identified	Metabolomic analytical resource	References
a. *E. lata* and *B. obtuse*;	a. Phytopathogen (*E. lata* and *B. obtuse*) + beneficial compound (2-nonanone; *in vitro*	a. 2-nonanone and *O*-methylmellein;	a. HS-SPME-GC-MS and LC-HRMS;	a. [68];
b. Wild type *F. graminearum* and carbendazim-resistant *F. graminearum* and	b. Phytopathogen (wild type *F. graminearum* and carbendazim-resistant *F. graminearum*) + fungicide (carbendazim); *in vitro*	b. L-glutamate, pyroglutamate, L-methionine, L-phenylalanine, D-myo-inositol, and L-threonine; and	b. ^{1}H NMR and	b. [69]; and
c. *P. syringae* pathovar *tomato* (*Pst.*)	c. Phytopathogen (*P. syringae* pathovar *tomato*) + *A. thaliana*; *in vitro* growth chamber experiment	c. Unspecified	c. FT-IR	c. [41]

Key: HS-SPME: Headspace-Solid Phase Microextraction; GC: Gas Chromatography; LC: Liquid Chromatography; HR: High Resolution; MS: Mass spectrometry; ^{1}H NMR: Nuclear Magnetic Resonance and FT-IR: Fourier Transform Infra-Red Spectroscopy.

Table 2
Recent applications of metabolomics in elucidating beneficial-microbe metabolome changes during plant-microbes symbiosis.

Profiled metabolome: interacting beneficial microbe	Interactions and study conditions	Significant metabolites identified	Metabolomic analytical resource	References
a. Marine-adapted fungal isolates (*Emericellopsis* sp.; *Hypoxylon* sp.; *Alternaria* sp.; *Acremonium* sp., and *Cosmospora* sp.);	a. Beneficial marine-adapted fungal isolates + phytopathogen (*P. syringae* and *R. solanacearum*); *in vitro*;	a. Mitorubrinol, mitorubrinic acid, cytochalasins, emerimicins, cephalosporins, ophiobolins and zervamicin;	a. UPLC–QTOF–HRMS/MS;	a. [71];
b. *S. cerevisiae*;	b. Beneficial microbe (*S. cerevisiae*) + xenobiotics (copper); *in vitro*;	b. Glutathione, L-Dihydroorotiacid and L-aspartic acid;	b. LC-MS;	b. [70]
c. Pseudomonads (*P. fluorescens* RA12, *P. protegens* Pf-5, *P. putida* KT2440, and *P. putida* S12); and	c. Beneficial microbe (Pseudomonads) + growth inhibitor (glyphosate); *in vitro*; and	c. Tryptophan, phenylalanine, shikimate-3-phosphate, citrulline, thymidine, fumarate, valine, glutamine, and ornithine; and	c. ^{13}C-assisted LC-MS; and	c. [88]; and
d. *Trichoderma asperellum*	d. Beneficial microbe *T. asperellum* + phytopathogen (*Phytophthora capsici*); *in vitro*	d. Viridepyronone, virone, koninginin D, gliotoxin, and acetyltetrahydroxyanthraquinone	d. HPLC and LC-ESI-MS	d. [89]

Key: UHP: Ultra-High Performance; QTOF: Quadruple Time of Flight; LC: Liquid Chromatography; HR: High Resolution; MS: Mass spectrometry; ESI: Electrospray ionization.

Table 3
Recent metabolomic elucidation of plant host metabolome changes during plant-microbes symbiosis.

Profiled metabolome: plant host	Interactions and study conditions	Significant metabolites identified	Metabolomic analytical resource	References
a. Wheat;	a. Wheat+ beneficial microbes (AMF + PGPR); *in planta* field trial;	a. Xilitol, carnitines, D-arabitol, pipecolic acid, 2-oxoglutarate, pyruvate, zymosterol, choline group, ethanolamines, and L-arabinose;	a. GC-TOF-MS and HILIC-Q-TOF-MS;	a. [72];
b. Grape berries;	b. Grape berries + phytopathogen (*B. cinerea*, *Penicillium expansum*, *Aspergillus niger* and *A. carbonarius*); *in vitro* microplate and zip-lock plastic bag assay;	b. 1,5-dimethylnaphthalene, unidentified sesquiterpenes, 2-(4-hexyl-2,5-dioxo-2,5-dihydrofuran-3-yl)acetic acid, m-cresol and γ-nonalactone;	b. SPME-GC-QTOF-MS;	b. [81];
c. Barley, wheat and rice;	c. Barley, wheat and rice + pathogen cassette (*Lr34* resistance gene); *in planta* hydroponic experiment;	c. Gentisic acid O-glucoside, C-glycosylated flavones, isoorientin-7-2″-di-O- glucoside and hordatines;	c. UHPLC-HR-MS;	c. [73];
d. Potato leaf;	d. Potato leaf + Phosphite; *in planta* field trial;	d. Caffeic acid, salicylic acid, and chlorogenic acid;	d. GC-TOF-MS;	d. [90];
e. Apple fruit;	e. Apple fruit + phytopathogen (*P. expansum*); *in vitro* growth chamber experiment;	e. Fructose, malic acid, shikimic acid, ascorbic acid and glutathione;	e. HPLC;	e. [75];
f. *A. thaliana* cell;	f. *A. thaliana* + phytopathogen (*P. syringae)*; *in vitro* growth chamber experiment;	f. Unspecified;	f. FT-IR;	f. [41];
g. Soybean;	g. Soybean + phytopathogen (*R. solani*); *in vitro* growth chamber experiment	g. Coumarins, phytoalexins, and flavonoids;	g. GC-MS	g. [32];
h. Tomato;	h. Tomato + phytopathogen (*B. cinerea* and *P. syringae*) + beneficial compound (hexanoic acid); *in planta* controlled experiment;	h. 1-methyltryptophan;	h. UHPLC-MS/GC-MS;	h. [37];
i. Sugarcane bud setts;	i. Sugar cane bud setts + *Sporisorium scitamineum; in planta* (green-house);	i. Lyxose, glycerate, raffinose, and phenylpropanoid;	i. GC-TOF-MS and LC-ESI-MS/MS;	i. [74];
j. Tomato;	j. Tomato + *Trichoderma* metabolites (6-pentyl-2H-pyran-2-one and harzianic acid); *in vitro* plant growth assay;	j. Alanine, arginine, asparagine, fructose galactose, glucose, glutamine, leucine, methionine, phenylalanine, sucrose, threonine, trigonelline, tyrosine, and valine;	j. HRMAS-NMR; and	j. [76];
k. Citrus leaves; and	k. Citrus seeds + *Candidatus* Liberibacter asiaticus; *in planta* controlled experiment; and	k. Asparagine, choline, glucose, malic acid, maltose, proline, sucrose, threonine, trigonelline, quinic acid, and uridine; and	k. NMR;	k. [77]; and
l. Maize root	l. Wild type (WT) BX regulated maize (*Zea mays* cv.W22) root + BX deficient W22 mutant; *in planta* (green-house)	l. Dihydroxy-7-methoxy-1,4-benzoxazin-3-one and 2,4-dihydroxy-1,4-benzoxazin-3-one	l. UPLC-Q-TOF-MS	l. [78]

Key: GC: Gas Chromatography; HRMAS: High-Resolution Magic-Angle-Spinning; HILIC: Hydrophilic interaction; QTOF: Quadruple Time of Flight; LC: Liquid Chromatography; HR: High Resolution; MS: Mass spectrometry; UHP: Ultra-High Performance; SPME: Solid Phase Microextraction; ESI: Electrospray ionization; and FT-IR: Fourier Transform Infra-Red Spectroscopy.

those from co-culture AMF-PGPR, the authors showed that soil inoculation with AMF, either alone or in combination with PGPR, markedly increased wheat root colonization. They also compared the metabolome of nitrogen-deficient and phosphorus-rich wheat with the aforementioned treatment combinations. Xilitol was a major upregulated metabolite annotated in the study, and depending on the treatment combination, various other compounds including carnitines, D-arabitol, pipecolic acid, 2-oxoglutarate, pyruvate, zymosterol, choline group, ethanolamines, and L-arabinose were upregulated or downregulated. From the study, the authors concluded that only AMF impacted wheat metabolome reprogramming, while the PGPR had no significant additional effect.

Using both GC–MS and LC–MS metabolomics, Bucher, et al. [73], elucidated the changes induced by *Lr34* (a multi-phytopathogen resistance-conferring gene), in field-grown and transgenic green-house cultivated species of barley, wheat, and rice. The plants analysed were with or without rusts and powdery mildew disease. Although most of the secondary metabolites detected in the study were unidentifiable, nine primary metabolites and 16 lipids were disrupted in barley plants. Increased glucose and fructose levels were recorded in the barley plants, while down-regulation of dehydro-ascorbate was observed. Overall, 84 primary metabolites were identified from the extracts of the transgenic plants that included sugars, amino acid derivatives, polyols, organic acids, and several lipid classes. Using an Orbitrap MS (direct infusion) and GC–MS approach, Aliferis, et al. [32], also carried out a time-course monitoring of the response of soybeans to *Rhizoctonia solani* infection. The approach also involved constructing a comprehensive soybean metabolite library, which subsequently accelerated the process of metabolite identification and interpretation of data generated during the study. Biomolecules including coumarins, phytoalexins, and flavonoids, which enhanced soybean's defense traits against biotic stress, were identified in the pool of metabolites detected. What is of additional value, apart from the mechanisms discoveries, is that this study approach can subsequently be adopted in other cereal grain investigations.

A similar GC-TOF-MS and LC-ESI-MS/MS combination approach was used by Schaker, et al. [74], to elucidate the metabolic changes of a seven-month-old (green-house grown) sugarcane bud setts, following artificial treatment with the fungal pathogen *Sporisorium scitamineum*. Quantitative alterations in a subset of 73 metabolites, from the plant metabolome with significant xylose, glycerate, and raffinose upregulation, were identified by GC-TOF-MS. These disruptions were determined to impact the cell wall precursors, amino acid and phenylpropanoid biosynthesis, and other major energy pathways. Furthermore, some rare antifungal-associated biomolecules were identified using the LC-ESI-MS. Most recently,

Žebeljan, et al. [75], investigated the altered metabolome induced by *Penicillum expansum* on apples, with the most prominent changes to be in the ascorbate-glutathione pathway of the fruit, and a subsequent reduction of glutathione and shikimic acid levels as disease severity increased.

A high-resolution magic-angle-spinning nuclear magnetic resonance (HRMAS-NMR) spectroscopy was employed by [76], to determine the metabolome changes in tomato leaves (*Solanum lycopersicum*) due to treatments of tomato seeds with two *Trichoderma* biocontrol metabolites (6-pentyl-2H-pyran-2-one and harzianic acid). Data generated revealed that the tomato leaves metabolome, its seedling fresh weight and seed germination rates were dependent upon the treatment doses of the *Trichoderma* metabolites. Altogether, γ-aminobutyric acid (GABA) and acetylcholine levels in both treatments showed a remarkable increase relative to the control samples. Metabolites with upward modulation in 6-pentyl-2H-pyran-2-one treated samples included arginine, glutamine, leucine, methionine, phenylalanine, sucrose, threonine, trigonelline, tyrosine, and valine, while those with upward modulation in the harzianic acid treatment were alanine, asparagine, galactose, phenylalanine and sucrose. Also relative to the controls the major metabolites with reduced concentration were glucose and fructose. The study corroborated previous claims of the biocontrol properties of *Trichoderma* metabolites.

Another NMR analysis by Padhi, et al. [77], elucidated the root metabolome response of two citrus plant varieties ('Lisbon' lemon (*Citrus limon* L. Burm, f.) and 'Washington Navel' orange (*Citrus sinensis* (L.) Osbeck) to *Candidatus* Liberibacter asiaticus (*C*Las) infection. *C*Las is the causative agent of the citrus greening disease - Huanglongbing (HLB). In general, a significant difference in 27 water-soluble metabolites where reported (asparagine, choline, glucose, malic acid, maltose, proline, sucrose, threonine, trigonelline, quinic acid, and uridine), and all of which were associated with plant defense mechanisms. More specific observations worth mentioning was a significant decrease in the levels of quinic acid and malic acid in the lemon roots only, and some metabolite response overlap in the responses of the two plants to *C*Las. The latter included elevated trigonelline levels, reduced levels of all the sugar metabolites measured, and reduced concentrations of choline, uridine, asparagine, and proline in both species. This study subsequently showed that the management of citrus greening disease (Huanglongbing) might require a varietal treatment or control approach.

The role of the tryptophan-derived heteroaromatic metabolites; benzoxazinoids (BXs), as regulators of plant-microbes interaction, are now becoming better understood. As part of a larger study to correlate the effects of Bx-regulated root metabolites with Bx-dependent rhizosphere microbiota, Cotton, et al. [78] determined, using an untargeted UPLC-Q-TOF-MS metabolomics analysis, the impact of the BXs metabolites on the maize root metabolome. In the study, a comparison was made between the metabolome of wild type (WT) BX regulated maize (*Zea mays* cv.W22) root and a BX deficient W22 mutant (the mutation brought about by inserting transposons at 3 different steps (bx1, bx2 and bx6) of the BX biosynthesis pathway). Data from the analysed crown and primary maize roots extracts indicated that the bx mutations did not significantly affect the growth and development of the maize mutants. Although the levels of the BXs (dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA)) in both the crown and primary roots of the bx1 and bx2 mutants decreased dramatically in comparison with that in the WT, the roots of bx6 mutants, however, showed elevated levels of DIBOA but reduced levels of DIMBOA in comparison with the WT. Whilst the bx1 and bx2 mutations had similar impact on the total BX production, the bx6 mutation, however, had a relatively minor effect on the root BX composition. Overall, the WT and bx roots profiling indicated that the bx1 and bx2 mutations significantly impact the root metabolome and corroborated previous reports of BXs role in metabolic regulation and differentiation of maize roots [78]; and that is, that BXs are involved in the regulation of a vast group of secondary root metabolites [78–80].

In another effort to determine the changes to organoleptic properties of fungal rot grapes, Schueuermann, et al. [81], exposed grape berries to *P. expansum*, *B. cinerea*, *A. niger* or *Aspergillus carbonarius,* and subsequently used an untargeted GC–MS metabolomics approach to identify and quantify the altered volatile metabolites profiles because of the resulting rot. The authors characterized *B. cinerea* incursion to result in the production of 1,5-dimethylnaphthalene and some various sesquiterpenes; *A. niger* (2-(4-hexyl-2,5-dioxo-2,5-dihydrofuran-3-yl) acetic acid), *A. carbonarius* (phenylethyl alcohol and β -damascenone), and *P. expansum* (m-cresol and γ-nonalactone). The study subsequently suggests that the biomarkers can discriminate causative agents of grape bunch rot and can be used in distinguishing between crop phytopathogens causing similar diseases.

Apart from those phytopathogens causing similar plant diseases, efficiently monitoring plant defense responses or susceptibility to pathogenic attack in unrelated phytopathogens (having different life cycles and virulence mechanisms) is also necessary. This should also be considered for beneficial microbes with dissimilar growth patterns and biocontrol mechanisms. Furthermore, in order for a negative or positive symbiosis to occur in a host plant, the phytopathogen or beneficial microbe must efficiently colonize a plant and each tripartite participant (negative colonizer, positive colonizer, and host plant) subsequently elicit an appropriate metabolic response [82]. Considering this, various studies have been performed identifying significant phytopathogen virulence-related metabolites and elucidating their roles and mechanisms in plant pathogenesis [35,83]. The study by Camañes, et al. [37], describes the complexity of assessing the metabolic perturbations of tripartite plant-microbes symbiosis. Using an untargeted UHLC/MS and GC–MS global metabolomics approach, the researchers compared the metabolomics profiles of infected tomato plants, primed tomato plants, and primed plus infected tomato plants. As hypothesized, the metabolic profiles captured from the metabolome of the phytopathogen-infected tomato were different. This correlated with the distinct lifecycle and virulence mechanisms of the phytopathogens investigated. Additionally, 1-methyltryptophan was identified as a unique biomarker associated with the metabolome of the phytopathogen-infected tomato and the hexanoic acid primed tomato metabolome. The study revealed that metabolites elicited by plants in response to biotic and or abiotic perturbations are source dependent.

6. Biomarker application in crop enhancement and protection strategies

Metabolomics offers a unique approach by which phytopathogen-specific metabolic biomarkers or plant disease/defense biomarkers, could be identified using an untargeted approach and then used to monitor phytopathogen infectivity and or disease progression. Furthermore, functional biomolecules associated with the biocontrol organisms, can be isolated or synthesized and used in phytopathogen control, or to evoke anti-phytopathogenic mechanisms in plants. For example, specific biomarkers and or biochemical processes identified during maize-*Fusarium graminearum*-*Bacillus amyloliquefaciens* or soybean-*Rhizoctonia*-*B. amyloliquefaciens* interaction, would most likely contribute to a better understanding of the metabolic regulation of all the interacting living systems, providing valuable insights potentially useful in plant breeding, metabolic bioengineering, and

agrochemistry research. Bio-products like resistant crop cultivars, robust secondary metabolite-producing beneficial microbes, and biofungicides can then be cultivated/cultured/produced and used *in planta.*

Another study by Khan, et al. [84], identified anti-drought stress biomarkers (malonate, leucine, 5-oxo-L-proline, saccharic acid, trans-cinnamate, succinate, and glyceric acid) in the chickpea (*Cicer arietinum* L.) metabolome, when treated with plant growth regulators (salicylic acid and putrescine) and PGPR consortium (*B. thuringiensis*, *Bacillus subtilis*, and *Bacillus megaterium*). Deliberate metabolic reprogramming of the chickpeas targeting the biomarker synthesizing pathways subsequently resulted in drought-tolerant chickpea varieties. Considering the above investigations, metabolomics could also be used to identify various biomarkers in rhizospheric and bulk soil, to assess/monitor plant infection pre-harvest and crop invasion post-harvest, for the purpose of reducing the exposure of livestock and humans to contaminated grains and or farmlands.

7. Concluding remarks

The application of untargeted metabolomics in the fields of pharmacology, chemistry, and clinical medicine, towards the discovery of molecular networks and metabolite interactions/elicitations, biomolecule structures, disease diagnostics, and biomarker identification, is already well established. The application of untargeted metabolomics towards improved crop/plant protection and food security strategies, however, is still relatively new, and the work done to date shows excellent scope, especially for improving our understanding of the overall adaptive physiology of the plant, and that of the interacting microbes [85–87]. A better understanding of the intra- and inter-species microbial interactions occurring at different heterogeneous levels within the plant habitat is imperative. Furthermore, identifying the systemic responses of various crops, to pathogenic stress, and the biocontrol thereof, would enable the crop scientist to identify unique metabolic markers that can be applied toward the early detection of a phytopathogen or its metabolites, in asymptomatic crops [15], as well as towards the development of biofungicides for example, for use during pre-harvest, post-harvest and large scale storage of crops. Novel insights into phytopathogen metabolism using metabolomics would also lead to a better understanding of phytopathogen colonization and pesticide tolerance.

Finally, although tripartite plant-microbes symbiosis metabolomics could be complicated due to the diversity of the associated biomolecules and large data generated, recent improvements to metabolomics methodology (semi-targeted and untargeted), equipment, and chemometrics/bioinformatics have led to faster, easier and more repeatable data acquisition. We foresee an exponentially increased identification and application of metabolite biomarkers in controlled and semi-controlled planting systems in the near future, and if properly integrated into crop protection strategies, food insecurity, and many other challenges farmers face in disease prevalent regions of the globe could be mitigated.

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Declaration of Competing Interest

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

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