ELUCIDATION OF BIOCHEMICAL DEFENCE MECHANISMS IN WHEAT (*TRITICUM AESTIVUM* L.) AGAINST ROOT-LESION NEMATODE (*PRATYLENCHUS THORNEI*)

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ABSTRACT

Pratylenchus thornei is an economically damaging root-lesion nematode that has a worldwide distribution. It is one of the major threats for wheat production in Australia and is particularly damaging in the northern grains region of the country. This nematode causes nutrient deficiency and water stress in wheat, which results in yield loss. Recent studies suggest that resistance in wheat occurs post penetration of the nematodes into the roots. Little is known about the biomolecules responsible for providing defence against *P. thornei* in wheat. In this thesis, histopathology, comparative enzyme profiling and metabolomics studies were conducted to elucidate the potential defence mechanisms in wheat against *P. thornei* infestation. Mainly, two sources of resistance against *P. thornei* were used in this study for different experiments (i) GS50a and its derived lines (ii) synthetic hexaploid CPI133872 and its derived lines. These were compared with susceptible wheat genotypes that were parents of the resistant derivatives.

Histopathological analysis was performed on one moderately resistant wheat genotype (QT8343; a GS50a derived line) and two susceptible wheat genotypes (Gatcher and Janz) to understand critical time points for reduced nematode reproduction in moderately resistant wheat cultivars. The significantly reduced nematode numbers were recorded inside QT8343 at 4 to 12 weeks post nematode inoculation (PNI). Clear differences were observed in both *P. thornei* nematode numbers and egg depositions at 8 weeks post nematode inoculation (PNI), with significantly ($P \leq 0.05$) fewer nematodes and eggs inside the roots of the moderately resistant genotype (QT8343) compared with the susceptible wheat genotypes (Gatcher and Janz). The results have suggested that 8 weeks PNI could be a critical time point for changes in nematode reproductions inside resistant wheat genotypes. No effect of nematode inoculation was found on total protein content, cell-wall bound phenolics and lignin, plant height, shoot and root biomass of moderately resistant and susceptible wheat genotypes, in this histopathological study.

The time point 8 weeks PNI was selected for further biochemical profiling of the wheat roots, namely, total phenol estimation, estimation of phenol oxidase activities and detailed metabolic profiling. The effects of total phenol and phenol oxidases in wheat

defence against *P. thornei* were evaluated in 21 wheat genotypes ranging in susceptibility and resistance to *P. thornei*. Polyphenol oxidase (PPO) and peroxidase (POD) enzyme assays were optimised as there was no standardised protocol to test multiple samples at a time using a microplate reader. Higher constitutive levels of total phenols were found in resistant synthetic hexaploid wheats CPI133872 (576 µg gallic acid equivalent (GAE)/g root) and CPI133859 (518 µg GAE/g root) at 8 weeks PNI, compared with moderately resistant and susceptible bread wheat genotypes (192 to 390 µg GAE/g root). The activity of PPO was induced in response to *P. thornei* in resistant (CPI133872) and moderately resistant bread wheat genotypes (GS50a and its derivate QT8343), becoming maximal at 4 weeks PNI. The activity of POD was similarly induced in response to *P. thornei* in CPI133872 at 6 weeks PNI. Different genetic sources of resistance to *P. thornei* showed diverse defence mechanisms and differences in timing of responses. The results have suggested both higher levels of total phenol and phenol oxidases could be responsible for superior resistance in the synthetic hexaploid CPI133872. In contrast, although total phenol contents in moderately resistant GS50a and its derived lines were comparable to susceptible wheat genotypes (Gatcher and Janz), the oxidised phenolic molecules due to higher level of phenol oxidases in GS50a and its derived lines than in Gatcher and Janz could be responsible for providing defence against *P. thornei*.

Metabolomic profiling was performed with resistant (QT16528; an advanced breeding lines derived from the synthetic hexaploid CPI133872) and susceptible wheat genotypes (including Janz) to understand the role of wheat metabolites in resistance and susceptibility to *P. thornei*. Detailed untargeted metabolic profiling using high performance liquid chromatography (HPLC) mass spectrometry (MS) was performed on the wheat roots at 8 weeks PNI. The majority of metabolites potentially responsible for resistance in QT16258 were found to be constitutively expressed. Gossypetin-8-glucosides, desoxypeganine, and hirsutine metabolites which were significantly ($P \leq$ 0.01) higher in concentration in QT16258 than Janz, could potentially act as acetyl choline esterase inhibitors of *P. thornei* to damage neural connections and restrict nematode motility inside QT16258 root tissue. Significantly expressed flavonoid

metabolites such as quercetin-3,4'-O-di-beta-glucoside, myricetin-xyloside in QT16258 could have important roles in reducing *P. thornei* reproduction and egg deposition. Resistance in QT16258 could also be due to increased deposition of cutin, suberin and wax on the root cell walls to impede penetration of *P. thornei* and its movement inside the root. Some metabolites occurring at higher concentrations in susceptible Janz, including indole acetic acid and vanillin acetate conjugates could be attractants for *P. thornei* and phenolics, including coniferyl alcohol could be part of a hypersensitive browning reaction resulting from *P. thornei* invasion.

These findings suggest that phenolics in the presence of phenol oxidases can have important roles in wheat defence against *P. thornei*. Eight weeks post nematode inoculation is a critical time point for detailed biochemical studies as there were highly significant ($P \leq 0.05$) differences both in egg deposition and nematode numbers inside roots of resistant wheat genotypes. The defence in wheat against *P. thornei* is mostly constitutive and several biomolecules including metabolites and enzymes are likely to be acting together. Understanding the biochemical defence mechanisms in wheat against *P. thornei* could lead to novel nematode management tools to minimise plant damage and consequent loss in wheat yield from this nematode species.

CERTIFICATION OF THESIS

This Thesis is the work of Md Motiur Rahaman except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

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STATEMENT OF CONTRIBUTION

1. **Rahaman MM,** Zwart RS, Seneweera S & Thompson JP (2021) Comparative differential reproduction rate of *Pratylenchus thornei* and histopathology in moderately resistant and susceptible wheat genotypes over time. (Prepared for submission to *Plant Pathology;* Q1; Impact Factor: 2.169; SNIP: 1.218) **(Chapter 2)**

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ABBREVIATIONS

APB	Amino-ethyl-diphenyl borinate
BCA	Bicinchonic acid
BSA	Bovine serum albumin
FA	Fatty acid
FDR	False discovery rate
GSEA	Gene set enrichment analysis
HMDB	Human metabolome database
HPLC	High performance liquid chromatography
In	Inoculated
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidyl ethanolamine
MG	Monoacylglycerol
ms	Milli seconds
MS	Mass spectrometry
MS2	MS-MS
PCA	Principal components analysis
PG	Phloroglucinol

PLASMA	Plant specialised metabolome annotation
PLS-DA	Partial least square discriminant analysis
PNI	Post nematode inoculation
POD	Peroxidase
PPO	Polyphenol oxidase
QQQ	Triple staged quadrupole
QTL	Quantitative trait loci
Q-TOF	Quadrupole time of flight
RLN	Root-lesion nematode
RT	Retention time
SWATH-MS	Sequential window acquisition of all theoretical mass spectra
Un	Uninoculated
VIP	Variable importance in projection

CHAPTER 1

REVIEW OF LITERATURE

This review of literature covers the general discussion of the literature relevant to the PhD topic “Elucidation of biochemical defence mechanisms in wheat against root-lesion nematode *Pratylenchus thornei*”. Research questions have been presented after a thorough review of the literature and critical discussions on this topic. The chapter has been formatted according to the journal style of “Australasian Plant Pathology”.

1. Introduction

Nematodes are eel-like microfauna belonging to the phylum Nematoda (Lambert and Bekal 2002; Hodda 2011), Nematodes do not possess a circulatory or respiratory system or a skeleton, the functions of which are performed by their unsegmented body wall or cuticle . Nematodes exist in terrestrial as well as marine habitats in most ecological niches on the planet, and to date, around 25,000 species have been identified (Dieterich and Sommer 2009). Some soil-borne nematodes are beneficial, contributing to ecosystem health (Burges 2012), while others are harmful to plants. Plant-parasitic nematode species can cause severe damage to different crops and significantly reduce global food production (Bird and Koltai 2000). Plant-parasitic nematodes have a needle like stylet connected to their oesophagus (Lambert and Bekal 2002), which is used to pierce plant tissues, enabling the nematodes to feed thereby damaging normal plant physiology and growth (Gregory 2008; Nyarko and Jones 2016).

There are sedentary plant-parasitic nematodes, which remain in one place throughout their life cycle once they establish a feeding site on the plant roots, and migratory nematodes, which move through plant tissues and can also move out of the root into the soil to invade other plant roots (Lambert and Bekal 2002). Sedentary root nematodes manipulate the machinery of the host plant to provide biotrophic feeding sites within the host tissue, forming macroscopic galls (e.g. root-knot nematodes *Meloidogyne* spp.) or cysts (e.g. cyst nematodes *Heterodera* spp. and *Globodera* spp.) (Gheysen and Fenoll 2002). Migratory nematodes can be ectoparasitic, remaining outside the plant root and feeding on superficial tissue (e.g. stunt nematodes *Tylenchorhynchus* spp.), or endoparasitic feeding on the root cortex and migrating as they feed, leaving behind cavities (e.g. root-lesion nematodes (RLN) *Pratylenchus* spp.). Collectively, sedentary and migratory parasitic nematodes lead to substantial losses in the yield of different crops, equivalent to >100 billion USD per year globally (Nicol et al. 2011; Jones et al. 2013).

Root-lesion nematodes (*Pratylenchus* spp.) are the third most economically damaging group of plant-parasitic nematodes after root-knot and cyst nematodes (Jones et al. 2013). There are over 60 characterised *Pratylenchus* species that cause significant crop

losses globally (Castillo and Volvas 2007; Jones and Nyarko 2014). The most important of these species are *Pratylenchus thornei,* Sher and Allen 1953, *P. neglectus* Rensch 1924*, P. penetrans* Cobb 1917, *P. crenatus* Loof 1960*, P. zeae* Graham, 1951, *P. vulnus,* Allen and Jensen 1951 and *P. coffeae* Goodey 1951*,* due to their wide host range, which included the most economically important agricultural crops globally (Castillo and Vovlas 2007).

Wheat (*Triticum aestivum* L.) is one of the crops seriously affected by *P. thornei* infestation (Smiley and Nicol 2009). Wheat is one of the key staple food and cereal cash crops grown around the world (Briggle and Curtis 1987). It has been estimated that more than 20% of calories consumed by the world's population come from wheat. The current global population is 7.7 billion, and it is expected to reach 9.7 billion by 2050 (Cassman et al. 2003; Fischer et al. 2014). Reduction of the damage caused by *P. thornei* in wheat by effective management strategies could increase wheat productivity in Australia and globally to a considerable extent, thus improving global food security in the future. *Pratylenchus thornei* can also cause significant damage in other commercial grain crops, such as barley (*Hordeum vulgare* L.), mungbean (*Vigna radiate* L. Wilczek), and chickpea (*Cicer arietinum* L.) (Thompson, 1987; Thompson and Haak, 1997; Castillo et al. 2008; Nicol et al. 2011; Fanning et al. 2020).

Like other migratory endoparasitic nematodes, *P. thornei* feeds in the root cortex and causes water (Whish et al. 2014) and nutrient stress (Thompson et al. 2012a) in wheat. The above-ground symptoms of *P. thornei* in susceptible wheat are reduced number of tillers, stunted growth and leaf chlorosis, which can be confused with symptoms of water and nutrient deficiency in the soil. The below-ground symptoms of susceptible wheat are brown or black coloured lesions in the roots (Castillo and Vovlas 2007). The most effective strategy for sustainable management of *P. thornei* is the development and use of resistant wheat genotypes (Thompson et al. 1999, 2008). Inheritance of *P. thornei* resistance in wheat is polygenic and additive (Zwart et al. 2004). Several quantitative loci (QTL) for resistance in different wheat germplasms were identified against *P. thornei*. These sources were used to incorporate resistance into high yielding wheat lines

in breeding programs. Nevertheless, there are no wheat genotypes available to date that can completely stop reproduction of *P. thornei* (Thompson et al. 2020).

Furthermore, limited studies have explored the mechanisms of resistance in wheat against *P. thornei* and more investigation into mechanisms could improve the development of resistant cultivars (Linsell et al. 2014a). Linsell et al. (2014a) provided evidence that resistance to *P. thornei* occurs in wheat roots after penetration of *P. thornei,* as equal numbers of *P. thornei* penetrated roots of both susceptible and resistant wheat genotypes up to 16 days post nematode inoculation. *Pratylenchus thornei* migration and juvenile maturation were supressed and egg deposition and hatching were inhibited post-penetration in a resistant synthetic hexaploid wheat derivative cv. Sokoll.

The biochemical interactions of different host plant species with root-lesion nematode species, *P. coffeae*, *P. penetrans*, *P. zeae* and *P. neglectus* have been studied to a greater extent than wheat – *P. thornei* interactions (Ohri and Pannu 2010). Biomolecules responsible for providing defence against *Pratylenchus* spp. include plant metabolites, pathogenesis-related proteins (such as cellulase, glucanase, peroxidase, polyphenol oxidase) and cell wall polymers (Desmedt et al. 2020).

In this chapter, the literature on the significance of *P. thornei* as a cause of loss of wheat yield is discussed. This is followed by a review of literature contributing to our understanding of defence mechanisms in wheat against *P. thornei* with respect to changes in wheat tissue in response to *P. thornei* infestation, and potential biochemical defence mechanisms in wheat. In conclusion, the gaps in the literature are identified and the research questions addressed in this thesis are presented.

2. *Pratylenchus thornei* infestation in wheat

2.1 Wheat yield loss due to Pratylenchus thornei *infestation*

In accordance with the definitions provided by Cook and Evans (1987), resistance is the capability of a plant genotype to inhibit nematode reproduction inside the roots. Tolerance is the ability of a genotype to grow and yield well when attacked by nematodes compared with other more intolerant genotypes (Cook and Evans 1987). Resistant wheat genotypes retard the build-up of *P. thornei* during the growing season, resulting in less root damage and greater grain yield than for susceptible genotypes in *P. thornei* infested fields (Thompson et al. 2020). In contrast, wheat genotypes that have tolerance, but are susceptible to *P. thornei* can tolerate damage caused by the nematodes and yield well, despite allowing considerable reproduction of nematodes inside the roots (Cook and Evans 1987).

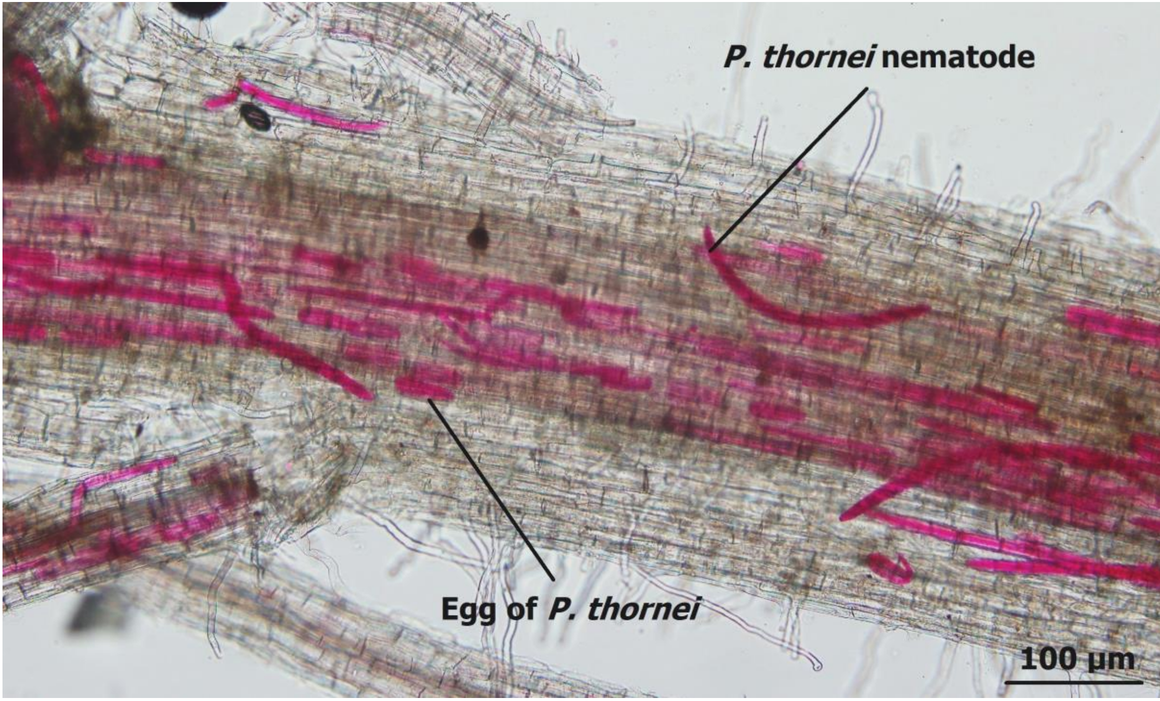

Figure 1 Severe nematode infestation in a susceptible wheat (cv. Petrie) root at 16 weeks after *Pratylenchus thornei* inoculation. Nematodes and eggs are stained pink using acid fuschin staining. Scale bar 100µm. Photo Source: Md Motiur Rahaman

The magnitude of yield loss that *P. thornei* causes to a wheat crop depends on the initial nematode population density in the soil at sowing and the resistance and tolerance of the wheat variety (Taylor et al. 2000). Due to the severe infestation of *P. thornei* (Figure 1) and damage of the root cortex, intolerant wheat roots are unable to absorb sufficient nutrient and water from the soil. Therefore, the symptoms of *P. thornei* infestation in wheat under field conditions are similar to nutrient and water stresses leading to stunted growth, poor tillering and reduced yield (Thompson et al. 2012a; Whish et al. 2014). Yield loss in wheat due to *P. thornei* infestation has been determined using nematicide and fumigant treated and untreated wheat plots (Reen et al. 2014; Taylor et al. 1999). Results of such experiments in Australia have shown that *P. thornei* population densities increase rapidly and there can be 38–85% yield loss in intolerant/susceptible genotypes (Doyle et al. 1987; Nicol et al. 2011).

2.2. Distribution of Pratylenchus thornei

Pratylenchus thornei is distributed in tropical, sub-tropical and temperate regions around the world (Jatala and Bridge, 1990) and has been reported in most of the top ten wheat producing countries in the world, including, China, India, Russia, USA, France, Canada, Pakistan, Germany, Australia and Ukraine (FAOSTAT 2017). Therefore, damage to wheat crops due to *P. thornei* is not only limited to Australia (Nicol and Ortiz-Monasterio, 2004; Nicol and Rivoal, 2007; Smiley and Nicol, 2009), but is also a major concern for many countries (Table 1). In some instances, the occurrence of *P. thornei* has been reported by states within countries for example India and USA (Table 1).

In countries other than Australia, *P. thornei* has been reported to cause the following yield losses in intolerant wheat genotypes: 37% in Mexico, 50% in USA, and 70% in Israel (Smiley and Nicol 2009). In three states of the USA (Oregon, Idaho and Washington) the estimated dollar value of damage by *P. thornei* to wheat production was 51 million USD (Smiley 2009).

It is also possible that wheat yield losses caused by *P. thornei* infestation occur in other countries, but they have not been detected or as well characterised because the

symptoms of infestation can be mistaken for nutrient and water deficiencies in crop plants (Whish et al. 2014).

Table 1 Distribution of *Pratylenchus thornei* in different parts of the world. The countries in bold are among the top ten wheat-producing countries in the world.

Continent	**Countries where presence of *P. thornei* reported**	**References**
Africa	Algeria, Egypt, Kenya, Libya, Morocco, South Africa, Sudan and Tunisia	Nicol (1996); Ammati (1987); Troccoli et al (1992); CABI (2012); Mokrini et al. (2019)
Asia	**India** (Assam, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Madhya Pradesh, Maharashtra, Manipur, Punjab, Rajasthan, Tamil Nadu and Uttar Pradesh); Iran; Israel; Japan; Jordan; Korea Republic; **Pakistan**; Saudi Arabia; Syria; Tajikistan; and Turkey	Fortuner (1977); Loof (1978); Nicol (1996); Maqbool (1987); Greco et al (1988); CABI (2012)
Australia/Oceania	**Australia**	Baxter and Blake (1967); Fortuner (1977); Nicol (1996); Thompson et al. (2010)
Europe	Belgium; Bulgaria; Croatia; Cyprus; Denmark; **Germany**; Greece; Italy (Mainland Italy and Sicily); Netherlands; Poland; Azores, Portugal; Romania; Slovakia; Slovenia; Spain (Mainland Spain and Canary Islands,); and UK (England and Wales)	Fortuner (1977); CABI (2012); Mokrini et al. (2019)
North America	Mexico; **Canada (**Ontario); **USA (**California, Colorado, Idaho, Maryland, Nebraska, Oregon, Utah, Virginia and Washington**)**	Loof (1978); Nicol et al (2011)
South America	Argentina, Chile and Venezuela	Loof (1978); Nicol et al (2011)

In Australia, there is an estimated 38 million AUD loss in the value of wheat production per annum in the sub-tropical grain region of eastern Australia, and without existing control measures, *P. thornei* infestation has the potential to cause 104 million AUD losses per annum in this region (Murray and Brennan 2009).

2.3 Management of Pratylenchus thornei

There are many challenges in managing *P. thornei* population densities in soil. *Pratylenchus thornei* has a broad host range and it can infest many cereals and pulse crops (Nicol et al. 2011). The resistant wheat varieties available to date can reduce nematode reproduction inside roots and in the surrounding soil, but they do not fully inhibit nematode reproduction. Therefore, *P. thornei* may remain in the soil, even after growth of moderately resistant varieties, to attack subsequent crops. Furthermore, as the soil roots dry during drought periods, *P. thornei* can go into a state of anhydrobiosis to survive adverse environmental conditions and become active again when the soil is re-wet and to invade host plants. Management strategies such as growing resistant and/or tolerant wheat genotypes, crop rotation, and tillage and stubble management practices can be used to reduce *P. thornei* numbers in cropping systems (Thompson et al. 2008). Crop rotation with resistant crops and/or cultivars can be an effective management strategy (Owen et al. 2014). Non-host and resistant crops often used for crop rotation include cotton, linseed, sorghum and canola (Owen et al. 2010, Reen et al., 2014). However, crop rotation does not provide a permanent solution to *P. thornei* infestation as it does not eliminate the nematodes completely. Chemical nematicides are expensive and the most effective ones have been removed from the market due to environmental hazards (Ristaino and Thomas 1997; Sánchez-Moreno et al. 2009). Tillage and stubble management can influence *P. thornei* numbers to some extent but not enough to limit *P. thornei* infestation in wheat (Thompson 1992; Reen et al., 2014). The most promising and effective management strategy of *P. thornei* for wheat is the development of resistant genotypes (Trudgill 1991; Thompson et al. 2020).

3. Resistant wheat sources and parasitism of *Pratylenchus thornei*

3.1. *Sources of wheat resistance to* Pratylenchus thornei

Partial resistance to *P. thornei* has been identified in different wheat sources, including West Asian and North African landraces (Thompson et al. 2009; Thompson and Seymour 2011), Iranian landraces (Sheedy and Thompson, 2009), synthetic hexaploid wheats (Thompson 2008), and tetraploid and diploid relatives of bread wheats (Thompson and Haak 1997; Thompson et al. 1999; Sheedy et al. 2012). A moderately resistant selection, GS50a, from the very susceptible wheat cultivar Gatcher, was discovered in a *P. thornei* infested field in the sub-tropical grain region of eastern Australia (Thompson and Clewett 1986). GS50a has been used as a reference genotype to compare the resistance levels of other wheat genotypes (Thompson et al. 2020). Advanced breeding lines with resistance to *P. thornei* have been produced using GS50a and other sources of resistance mentioned above, crossed with Australian wheat cultivars, providing tolerant and resistant germplasm for plant breeding (Thompson et al. 1999, Thompson et al. 2008, Thompson et al. 2020). The Australian Wheat and Barley Molecular Marker Program (AWBMMP) (http://www.markers.net.au/) has identified quantitative trait loci (QTL) for resistance in sources including GS50a, synthetic hexaploid wheat (CPI133872) and landrace wheats from Iraq and Morocco.

3.2. Quantitative trait loci for Pratylenchus thornei *resistance*

Modern bread wheat is a hexaploid (2n = 42 = 6x) comprised of AABBDD (2n=42=6x) genomes. The progenitors of these genomes were *Triticum uratu,* Tumanian ex Gandilyan (A genome donor), *Aegilops speltoides,* Tausch (B genome donor) and *Aegilop tauschii,* Cosson (D genome donor) (Petersen et al. 2006, El Baidouri et al. 2017). Quantitative trait loci (QTL) mapping approaches have been used in several studies to tag the loci associated with resistance to *P. thornei* in moderately resistant wheat genotypes (Table 2). Three major loci for *P. thornei* resistance, on chromosomes 2BS, 6DS and 6DL, were identified in the mapping population produced from a cross between a *P. thornei* resistant synthetic hexaploid wheat, CPI133872, and a susceptible bread wheat genotype, Janz (Zwart et al. 2005, 2010), and these loci have been

confirmed in several other sources of resistance. However, the role of major genes in the resistance QTL of different wheat genotypes contributing to resistance against *P. thornei* is not completely understood. Such understanding could be fundamental to the elucidation of wheat defence mechanisms against *P. thornei*.

Table 2 *Pratylenchus thornei* resistance and susceptible quantitative trait loci (QTL) identified in different wheat populations.

Population	Chromosomes						References
	1B	**2B**	**3B**	**5B**	**6D**	**7B**	
CPI133872/Janz DHs		2B			6D		Zwart et al. 2005, 2010
W-7984/Opata RILs		2B			6D		Zwart et al. 2006
Morocco426 (AUS13124)/Janz DHs		2B	3B				Schmidt et al. 2005
Morocco426 (AUS13124)/Janz DHs		2B				7B	Thompson et al. 2015b
Iraq43 (AUS4926)/Janz DHs	1B (suscep tibility)		3B				Schmidt et al. 2005
Iraq43 (AUS4926)/Janz DHs					6D	7B	Thompson et al. 2015b
AUS49307.2/Pastor RILs	1B	2B			6D		Toktay et al. 2006
Croc_1/Ae.squarrosa224//Opata x Pastor RILs			3B				Toktay et al. 2006
Sokoll/Krichauff		2B			6D		Linsell et al. 2014b

Major QTL for resistance against *P. thornei* do not necessarily provide resistance against other *Pratylenchus* spp., such as *P. neglectus*. For example, GS50a is resistant to *P. thornei* but susceptible to *P. neglectus*. Therefore, the wheat biochemical defence against *P. thornei* is likely to be different from the biochemical defence and resistance mechanisms to other *Pratylenchus* spp. Studies on the inheritance of *P. thornei* resistance in synthetic hexaploid and landrace wheats revealed the minimum number of effective genes to be in the range of four to six (Thompson et al. 2012b). The

understanding of combinatorial gene transfer from the resistant sources and their pyramidal effect in resistant plants is vital for the development of wheat genotypes with improved *P. thornei* resistance.

Wheat genotypes with QTL for *P. thornei* resistance at different chromosomal locations could possess different biochemical mechanisms against the infestation of *P. thornei,* which could involve various plant metabolites and pathogenesis related proteins. Therefore, understanding defence mechanisms in wheat against *P. thornei* is unlikely to be simple, as different genes can act together to give ultimate defence against the nematode. Therefore, multiple approaches are needed for a comprehensive understanding of defence. In a recent study, some candidate genes for resistance were proposed in QTL regions on chromosomes 2BS and 6DS for resistance to *P. thornei* in the synthetic derived wheat line Sokoll, which included phenyl ammonium lyase (PAL), chalcone synthase, isoflavone reductase (IFR), flavonoid 3′hydroxylases, nucleotide-binding site-leucine-rich repeats (NBS-LRR) proteins, and ribosome inactivating protein (Rahman et al. 2020). Also, the QTL for *P. thornei* resistance on 6DS in the International Triticeae Mapping Initiative (ITMI) population of RILs from the cross of synthetic wheat W-7084/Opata was located close to a ribosome-inactivating protein locus (Zwart et al. 2006).

3.3 *Life cycle of* Pratylenchus thornei

Adult *P. thornei* (female) have a body length of 560–610 µm and a width of 18.1–19.7 µm (Fortuner 1977, Thompson et al. 2017). The dry weight of the adult female *P. thornei* is around 0.023 µg (Thompson 2020 pers comm). As with all other plant parasitic nematodes, *P. thornei* has four juvenile stages (J1-J4) before becoming adults (Castillo and Vovlas 2007). The juvenile stage J1 of *Pratylenchus* spp. forms first inside the nematode egg and then moults into the J2 stage inside the egg before hatching (Roman and Hirchmann 1969; Fosu-Nyarko and Jones 2016). The identification of subsequent life stages of *P. thornei* in a sample is generally done according to size, with each stage increasing in size, and reproductive anatomy. Juvenile stage 4 (J4) can be identified by a cleared area where the vulva will appear (Figure 2) when it moults to the

adult stage (Thompson et al. 2017). Mature *Pratylenchus* adults are capable of laying eggs every three days (Castillo and Vovlas 2007). *Pratylenchus* spp. lay eggs both in the soil and inside plant roots (Jones and Fosu-Nyarko 2014).

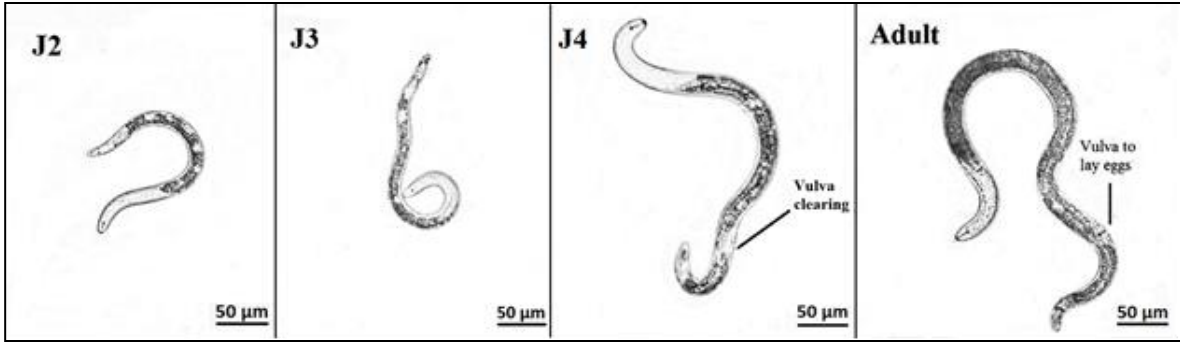

Figure 2 Motile life stages of *Pratylenchus thornei*: three juvenile stages (J2, J3 and J4) and adult stage. Scale bar 50 µm. Photo source: Md Motiur Rahaman

Male nematodes are rarely found in the species *P. thornei* (Fortuner 1977). Female *P. thornei* reproduce by an asexual process called parthenogenesis. *Pratylenchus* spp. can complete its life cycle in 45-65 days (Agrios 2005). There can be three to six generations of *P. thornei* in one complete wheat-cropping season (Larson 1953). The generation times of *Pratylenchus* spp. depend on several parameters including the host plant species, the nematode species, and the soil temperature and moisture (Prasad et al. 1999; Castillo and Vovlas 2007; Jones et al. 2013). *Pratylenchus thornei* reproduces optimally at 20-25°C in wheat (Thompson et al. 2015c). When the soil becomes dry and there is no host to invade, *Pratylenchus* spp. can stay deep in the soil to survive via anhydrobiosis (Glazer and Orion 1983; Dagan 1984; McSorley 2003). Once the soil becomes moist and there is availability of host plants, *Pratylenchus* spp. migrate to the top layer (0-20 cm) of the soil again and invade the host (McSorley 2003).

3.4. Parasitism of Pratylenchus thornei *in wheat*

Understanding *P. thornei* infestation, feeding and reproduction are vital components for executing suitable management strategies to control the nematode populations and improve wheat yield. Nicol et al (2012) sequenced a mixed life-stage population derived

from a single female *P. thornei* using Roche 454 sequencing. A total of 787,275 reads were assembled into 34,312 contigs. Annotation of 3,048 contigs revealed insights into *P. thornei* parasitism. The major annotated transcripts included cell wall degrading enzymes and neuropeptide. There were 57% of contigs that were potentially unique to *P. thornei* parasitism. However, 43% of contigs were found to have similarity with various plant-parasitic nematodes including *P. penetrans, P. coffeae*, *P vulnus, Radopholus similis,* Cobb, 1893*, Heteroderidae, Meloidogynidae and Caenarhabditis elegans* (Nicol et al. 2012). One of the significant differences that migratory nematodes such as *P. thornei* have from the sedentary cyst (*Heteroderidae spp.)* and root knot nematodes (*Meloidogynidae spp.)* is effector molecules. The effector molecules in sedentary parasitic nematodes induce permanent feeding sites, whereas *Pratylenchus* spp migrates as it feeds without having any effector molecule.

All *Pratylenchus* spp. pierce the root tissue using a stylet and secrete hydrolytic enzymes (cellulases, glucanse, and pectate lyase) to damage the root cortical cell walls (Lambert and Bekal, 2002; Nyarko and Jones 2016). Both the piercing of root cell wall with the syringe-like stylet of the nematode and cell wall degrading enzymes, damage the plant cell wall, which paves the entry of the nematode inside the root tissue. *Pratylenchus thornei* feeds on the root cortex and ingests cell cytoplasm, leaving behind cavities as it migrates inside the root, causing characteristic dark brown or black lesions in roots (Castillo and Vovlas, 2007). The root damage caused can also attract secondary pathogens, e.g. bacterial and fungal pathogens that take advantage of the wounding to infect roots and cause further damage (Fosu-Nyarko and Jones, 2016).

3.5. Attraction, penetration, movement and reproduction of Pratylenchus thornei *inside root tissues*

The attraction to and penetration of *Pratylenchus* spp. inside root tissue will vary from species to species. The most studied *Pratylenchus* spp. infestation in host plant is *P. penetrans,* which infests legume and food crops (Nicol et al. 2011). *Pratylenchus penetrans* occurred preferentially inside the main root cortex and the lateral root

branches damaging the root cortex area of alfalfa (*Medicago sativa* L. cv. *Du Puits*) (Townshend et al. 1989). In a previous study on *P. thornei*, no preferential zones for penetration inside wheat roots were found and the nematodes were equally attracted towards susceptible and resistant wheat genotypes up to 16 days after nematode inoculation (Linsell et al. 2014a). Similarly, Castillo et al (1998) also did not find preferential penetration zone for *P. thornei* in chickpea cultivars. *Pratylenchus neglectus* had no preference for wheat roots of susceptible or resistant genotypes for up to 4 weeks (Farsi 1996).

The reproduction of *P. thornei* inside susceptible and resistant wheat genotypes was similar up to 2–8 weeks (Linsell et al. 2014a, Talavera and Vanstone 2001). The reproduction of *P. thornei* was increased exponentially with time after nematode inoculation (Baxter and Blake 1967, 1968; Thompson et al. 2015a). The maximum discrimination in reproduction between susceptible and resistant wheat genotypes was found to be 16 weeks after nematode inoculation under controlled glasshouse conditions (Thompson et al. 2015a).

Reduced nematode numbers inside resistant cultivars over time, in comparison to susceptible genotypes, could be due to unavailability of hatching factors and less egg deposition inside resistant genotypes (Linsell et al. 2014a). Root exudates of various plant species including welsh onion (*Allium fistulosum*) and tall fescue (*Festuca arundinacea*) were found to be hatching inhibitors of plant-parasitic nematodes. However, specific metabolites responsible for hatching inhibition are not well known (Sikder & Vestergård, 2019). Additionally, as the nematodes feed and move inside root cortex, inhibition of nematode movement and restricted availability of nutrition could delay fitness and maturity of *P. thornei* inside roots to delay egg deposition.

Deposition of reduced numbers of eggs inside roots post nematode penetration was found in wheat genotypes resistant to *P. neglectus* (Farsi, 1996) and banana genotypes resistant to *P. goodeyi* (Prasad et al. 1999). There was also evidence of reduced numbers of hatched eggs of *P. zeae* in roots of maize (De Waele et al. 1988) and of *P. penetrans* on effect of root diffusates from maize (*Zea mays* L. cv. Husar), carrot (*Daucus carota*

L. cv. Masei), black salsify (*Scorzonera hispanica* L. cv. Omega) and marigold (*Tagetes* cv. Single Gold) (Pudasaini et al. 2008)

The information on critical time points to understand the changes in *P. thornei* reproduction and egg depositions inside wheat roots is limited. Some investigators suggested that plant sampling could be done in a nematode infested soil and nematode counted per gram of root weight, as the most appropriate way to understand resistance and susceptibility of the genotypes (Trudgill and Phillips 1997). For plant breeding purposes, Thompson et al (2015a) recommended comparing final population densities of *P. thornei* in the roots and surrounding soil after 16 weeks growth of wheat genotypes for maximum discrimination of levels of resistance/susceptibility. *Pratylenchus thornei* has ~6 weeks long life cycle (Larson 1956) and checking reproduction at early time-points might not allow significant discrimination of nematode numbers inside root of susceptible and resistant genotypes. In contrast, sampling at maturity might miss the critical time-points when differences in nematode number inside resistant and susceptible genotype are first apparent. Therefore a study of nematode reproduction over time (1-16 weeks) could shed more light on resistance of genotypes against the nematode.

4. Mechanisms of defence against nematodes

4.1. Physical barriers to infestation and reproduction of nematode

The outer surface of a plant, consisting of primary and secondary cell walls, provides the first line of defence against pathogen infestation. The plant cell wall consists of complex biopolymers (such as lignin, cellulose, hemicellulose, pectin), which provide rigidity (Freeman and Beattie 2008). There is little evidence that plant cell walls provide resistance against *P. thornei* at the preliminary level of penetration and feeding (Linsell et al. 2014a; Talavera 2001). No differences in penetration of the root of resistant and susceptible wheat genotypes were reported up to 16 days *P. thornei* infestation (Linsell

et al. 2014a). It is possible that the nematode has difficulty in penetrating at a later time of infestation, due to changes in root morphology and rigidity induced by infection by *P. thornei*.

Modifications in wheat root morphology to combat nematode infestation include: lignin deposition, callose deposition, suberin and cutin deposition and increases in cell wall bound phenolics. Increases in cell wall biopolymers can increase rigidity of the cell wall, which could prevent nematode movement and digestion of cellulose of the cell wall (Wuyts et al. 2007). Increased lignin deposition was reported in resistant banana genotypes against the migratory parasitic nematode *Radopholus similis,* Cobb, 1893 (Wuyts et al. 2007). Changes in root cell morphology could encourage or discourage a particular life stage (J2, J3, J4 and adult) to increase or decrease in proportion of other life stages. Phenylalanine ammonia lyase (PAL) is a key enzyme of the phenyl propanoid pathway, which produces precursors in the synthesis of polyphenol compounds like lignin and flavonoids in plants. Increased expression of the enzyme PAL and of lignin contents was reported in resistant banana (*Musa* spp.) cultivars upon challenge with *P. coffeae* (Devi et al 2007; Backiyarani et al. 2013). The gene for the enzyme PAL was also proposed as a candidate resistance gene in wheat against *P. thornei* infestation (Rahman et al. 2020). Increases in cell wall bound phenolics and callose deposition could also play an important part in plant defences against RLN (Chen and Kim 2009). Regulation of biosynthesis of the cell wall biopolymer callose has also been proposed as a link to cell signalling (Vatten et al. 2011). Changes in cell wall composition after *P. thornei* infestation in wheat and changes in life stages of the nematode over time have not been studied. A comparative study on cell wall bound phenolics and lignin could improve our understanding of the defence of wheat against *P. thornei*.

4.2 Biochemical responses to nematode infestation and reproduction

Information on the biochemical defensive strategies of wheat plants against *P. thornei* is scarce. With limited information on wheat-RLN interactions available, work on antimicrobial compounds in wheat defence against other pathogens will be discussed in

the following sections. Potential biochemical defence mechanisms in wheat against *P. thornei* will be discussed in the light of studies on other plant-RLN (*P. penetrans, P. coffeae, P. zeae and P. neglectus*) interactions. Previous investigations suggested that several genes could be responsible for providing defence against *P. thornei* in an additive way (Zwart et al. 2004), and the expression of the genes could potentially be both constitutive and inductive.

4.2.1 Constitutive defences

Some biochemicals or biopolymers are always expressed and are present in living plants of a particular genotype, whether or not they encounter a pathogen (Dixon 2001). Such constitutively expressed pre-existing antimicrobial compounds are known as phytoanticipins (Vanetten et al, 1994). These include chemicals like catechins, avenacin and lectins, which can be found in plant cells prior to exposure to pathogens (Bell, 1981). Constitutive biochemicals can be associated with plant cell walls or stored in vacuoles and vesicles of the cells in active or inactive forms (Mansfield, 1983). Antimicrobial compounds, such as glucosinolates, can be found constitutively in plants as inactive forms. Once the cell is damaged by pathogen infestation, enzymes such as myrosinase from the damaged cells activate glucosinolates to produce toxic thiocyanate and isothiocyante, which can inhibit plant pathogens (Mansfield 1983; Holst and Fenwick 2003). For example, the suppressive effect of 2-phenyl ethyl glucosinolate was observed in *Brassica* sp. root tissue against *P. neglectus* (Potter et al. 1998). Constitutive phytoanticipins in the plant may be insufficient to give protection against all pathogens that a plant encounters at a certain time (Vanetten et al, 1994). These consititutive chemicals provide a primary defence before the recognition of pathogens and induction of a cascade of defensive biomolecules.

The defence of plants against several root-lesion nematode species (*P. penetrans*, *P. coffeae, P. zeae and P. neglectus*) was found to be both constitutive and induced after nematode infestation (Baldridge et al. 1998; Soriano et al. 2004a; Soriano et al. 2004b; Devi et al. 2007; Backiyarani et al. 2013; Vaganan et al. 2014). Constitutive

biomolecules that are pre-formed in plants could act immediately on the pathogens. From pathogen recognition to production of defensive molecules may take some time to occur in an induced defence response.

4.2.2 Induced defences

Induced responses are the result of complex defensive pathways. In contrast to constitutive defence, almost all plants also initiate induced biochemical responses upon encounter and recognition of pathogens. Induced defence could be the production of new types of compounds or over expression of existing constitutively expressed compounds. The induced antibiotic compounds in plants in response to pathogenic attack are called phytoalexins (Vanetten et al, 1994). A range of biomolecules can be produced as phytoalexins, which include low molecular weight secondary metabolites. Recognition of the pathogen by the plant is necessary for the initiation of cellular signalling to induce defence response. The first type of recognition of the pathogen by the plant is governed by pathogen associated molecular patterns (PAMPs), which could be cell surface components of pathogens, including parasitic root-lesion nematodes (Sato et al. 2019). Other components that could trigger defence in plants could include secretions of the nematode and damage associated molecular pattern (DAMP).

Salicylate and jasmonate pathways were found to be responsible for defensive signalling in host plants against parasitic nematodes and the start of a cascade of phytoalexin production upon elicitor recognition (Bennett and Wallsgrove 1994; Klessig et al. 1998; Fujimoto et al. 2011; Nahar et al. 2011). There are certain elicitors that initiate the production of the induced phytoalexin compounds in plants. Elicitors that induce phytoalexin production can originate from either the plant pathogen or from necrotic plant tissues (Darvill and Albersheim 1984; Bruce 1989, Ryals et al. 1996; Oliveira et al. 2016). The elicitor induced phytoalexin can act at the site of nematode infestation and/or can cause a systemically induced defence response in plant tissue away from the site of infestation.

Applications of methyl jasmonate and salicylic acids were considered responsible for increased expression of phenyl ammonium lyase in a wheat genotype inoculated with *P. thornei* (Ketabchi et al. 2014). Phenylalanine ammonia lyase is a key enzyme of phenyl propanoid metabolism, which leads to production of a range of secondary metabolites, particularly phenolic compounds including flavonoids.

4.2.3 Secondary metabolites in plant defence

Plants produce two types of metabolites, namely, primary metabolites and secondary metabolites. Primary metabolites are involved in primary metabolic processes of the plant, such as respiration and photosynthesis (including sugars, amino acids, nucleotides, vitamins, organic acids). Secondary metabolites are produced via re-direction of the primary metabolism with the help of key enzymes of secondary metabolism, such as PAL, chalcone synthase (CHS), polyphenol oxidase (PPO), peroxidase (POD) and others (Croteau et al. 2000). Primary metabolites take part in the growth, development and reproduction of the plant, whereas secondary metabolites are not directly involved in these functions (Grotewold 2005). Primary metabolites can also participate in cell signalling to up-regulate or down-regulate the expression of particular genes to promote defences in plants against various pathogens. However, secondary metabolites play more important roles in plant defence mechanisms and adaptations to the environment (Croteau et al. 2000). There are possibly more than 100,000 secondary metabolites in plants, which are generally classified according to their chemical structure (Dixon 2001).

4.2.4. Secondary metabolites in defence against root-lesion nematode

Plant secondary metabolites have been found to have roles in defence against plant parasitic nematodes. The four major classes of secondary metabolites (Kabera et al. 2014) that play vital roles in plant defence against various pathogens are: phenolics, alkaloids, terpenoids and their glycosides. The effects of secondary metabolites on nematode life cycles and reproduction rates have mostly been studied using sedentary

parasitic nematodes. For example, phenolic, terpenoids, and alkaloids classes of metabolites were found to affect *Meloidogyne incognita,* Kofoid & White, 1919 (Sikder & Vestergård, 2019). Also, the isoflavonoid compound glyceolin accumulated inside the roots of resistant but not susceptible genotypes (Kaplan et al., 1980). The terpenoid aldehyde compound gossypol in resistant cotton (*Gossypium hirsutum* L.) root was proposed responsible for resistance to *M. incognita* (Veech and Mcclure 1977). *Meloidogyne incognita* larvae lost motility when gossypol was applied in an *in vitro* analysis with a concentration equivalent to its concentration in cotton roots (125µg/ml). Veech and McClure (1977) suggested that production of gossypol locally at the nematode infected site could be reason for cotton resistance against *M. incognita*.

Among the four major classes of secondary metabolites, phenolic molecules were found to be often associated with disease resistance in plants against root-lesion nematode infestation (Table 3). Most studies on plant–RLN interactions have predominantly investigated *P. penetrans* (Table 3). Increases in mRNA levels for PPO, POD and PAL enzymes were recorded after *P. penetrans* infested alfalfa (*Medicago sativa*) roots (Baldridge et al. 1998). A higher constitutive level of medicarpin (phenolic flavonoids) was found inside alfalafa roots, which had anti nematode activities against *P. penetrans*. The medicarpin compound slowed the motility of *P. penetrans* in an *in vitro* test (Baldridge et al. 1998).

Phenolic molecules have been associated both with defence and with hypersensitive browning reactions in nematode infested root tissues (Giebel, 1982). Phenolic molecules in the presence of phenol oxidases can be oxidised to quinone compounds, which can polymerise to form root browning products. The phenolic compound chlorogenic acid and the enzyme PPO increased in tomato roots after infestation by *P. penetrans* (Hung and Rohde, 1973). Chlorogenic acid will oxidise in presence of PPO to become more toxic compound to the nematode and provide defence in the plant against the nematode.

An increase in coumestans (flavonoids) upon exposure to *P. scribneri* has been reported in lima bean roots (Rich et al. 1977). In the root of resistant lima bean coumestans increased in response to *P. scribneri.* The coumestans compounds isolated from lima bean root reduced the motility of the nematode at a concentration of 5µg/ml in an *in*

vitro test (Rich et al. 1977). A flavone glycoside inhibited *P. neglectus* penetration in oats (Soriano et al. 2004a). In a recent study, benzoxazinoid and glycosides in wheat were proposed to be responsible for defence against *P. neglectus* (Frew et al. 2018) as a decrease in concentration of some of the bezoxazinoid compounds in wheat genotypes Ventura and Yitpi possibly increased the numbers of *P. neglectus* inside roots.

Studies on the interaction of banana plant with *P. coffeae* revealed an increase in the defensive enzymes PPO, POD and PAL after nematode infestation of resistant plant genotypes compared to susceptible genotypes (Devi et al. 2007; Backiyarani et al. 2013; Vaganan et al. 2014).

The expression of induced compounds may differ over time for both susceptible and resistant cultivars (Backiyarani et al. 2013). The contenst of total phenol, peroxidase and β-1, 3-glucanase were induced in higher in concentration the roots of resistant banana cultivars than in susceptible ones. Backiyarani et al (2013) suggested that for *Musa-P.coffeae* interactions, samples should be collected within 8 days of inoculation of the nematode. Increased activities of PPO and β-1, 3-glucanase occurred 2 days after nematode inoculation with most at 6 days after inoculation. However, the total phenol was still induced in higher amount more than 12 days post inoculation (Backiyarani et al. 2013).

Resistant banana cultivars (*Musa* spp.) had higher constitutive levels of PAL and total and soluble phenolics compared to susceptible genotypes. However, resistant cultivars showed a strong induced response when the plants were inoculated with *P. coffeae* and significantly increased contents of PAL and phenolics were recorded being maximal at 7 days post inoculation (Vaganan et al. 2014).

Table 3 Phenolic compounds and phenol oxidases identified in plants inoculated with *Pratylenchus* spp.

Plant species	Root-lesion nematode species	Phenolics and phenol oxidases identified in plant-nematode interactions	References
Tomato (*Solanum lycopersicum*)	*P. penetrans*	Phenolic compound chlorogenic acid and the enzyme polyphenol oxidase	Hung and Rohde (1973)
Lima bean (*Phaseolus lunatus*)	*P. scribneri*	Phenolic compounds coumestrol and psoralidin	Rich et al. (1977)
Lima bean (*Phaseolus lunatus)*	*P. penetrans*	Phenolic compound coumestrol	Veech, (1982)
Kidney bean (*Phaseolus vulgaris*)	*P. penetrans*	Phenolic compound phaseolin	Anderson, (1971)
Bananas (*Musa* spp.)	*P. coffeae*	Polyphenol oxidase (PPO), peroxidase (POD), and phenyl ammonium lyase (PAL)	Devi et al. (2007), Backiyarani et al. (2013), Vaganan et al. (2014)
Alfalfa (*Medicago sativa* L.)	*P. penetrans*	Over expression of the transcripts related to polyphenol oxidase (PPO), peroxidase (POD), and phenyl ammonium lyase (PAL) synthesis	Baldridge et al. (1998)
Brassica spp.	*P. neglectus*	2-phenyl ethyl glucosinolate	Potter et al. (1998)
Oats (*Avena sativa)*	*P. neglectus*	Flavone glycosides	Soriano et al. (2004a)
Wheat (*Triticum aestivium*)	*P. neglectus*	2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (HMBOA-Glc), 2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc)	Frew et al. (2018)

The abundance of total phenol, phenol oxidases and specific metabolites have not been studied in wheat in response to *P. thornei* infestation. Inhibitory metabolites in wheat

could be nematistatic, nematicidal or inhibitory of the reproductive cycle of *P. thornei* over time and this should be investigated.

4.2.5 *Potential antimicrobial or anti-nematode compounds in wheat*

Wheat has a range of different secondary metabolites (Olenichenko et al., 2008), the increased expression of which in resistant wheat genotypes could limit nematode reproduction. Linsell et al. (2014) showed there were effects of selected wheat root extracts and exudates (water-soluble) on *P. thornei* motility, egg hatching and reproduction for selected time points and wheat genotypes. However, specific compounds in root exudates and or root extracts were not identified in that study.

The most studied phenolic compounds in wheat are phenolic hydroxamic acids and bezoxazinoid compounds (Table 4). Hydroxamic acids are a class of weak acids with the general formula R-CONHOH; where R is an organic residue (C_6H_5-). They can be acylated to the alcohols of phenols to form phenolic hydroxamic acid. Different concentrations of hydroxamic acids occured in the shoot and root tissue of wheat (Wu et al. 1999, 2000, 2001). A range of hydroxamic acids were identified in wild relatives (*Triticum* species) of bread wheat (Niemeyer et al. 1992).

Giambanelli et al (2013) showed that the content of secondary metabolites such as lipids, tocols, carotenoids, sterols and phenolic compounds varies between wheat (*Triticum* species) genotypes.

The level of phytosterol in different wheat (*Triticum aestivum*) genotypes varied with changes in 26 genotypes and environmental condition at four different locations in Europe: Martonvásár (Hungary) Enchantillon (France), Woolpit (U.K.) and Choryn (Poland) (Nurmi et al. 2010). Phytosterol has significant antibacterial properties as part of innate immunity in plants (Wang et al. 2012).

Phenolic acids induced by *cis*-jasmonatein wheat root tissues, such as trans-p-coumaric acid, syringic acid, p-hydroxybenzoic acid, vanillic acid, and cis- and trans-ferulic acids, were found to affect development cycles of pests as well as weeds (Moraes et al. 2008).

Table 4 Potential antimicrobial compounds in wheat roots and root exudates

Root/ root exudates	Potential antimicrobial compounds in wheat (phenolic, phenolic hydroxamic acids and hydroxamic acids)	Proposed role in defence	References
Roots and root exudates	Hydroxamic acids	Root exudates tested for allelopathy but the result was negative against wild oats, (*Avena fatua*)	Pérez and Ormenonunez (1991)
Roots	*p*-hydroxybenzoic, vanillic, *cis*-*p*-coumaric, syringic, *cis*-ferulic, *trans*-*p*-coumaric, and *trans*-ferulic acids, respectively. *trans*-Ferulic	Allelopathic to annual ryegrass	Wu et al. (2000)
Roots	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)	Allelochemicals	Wu et al. (2001)
Roots	2-*α*-D-glucopyranosyloxy-4-hydroxy1,4-benzoxazin-3-one (DIBOA-*α*-D-glucoside), 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-*α*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-*α*-D-glucoside), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), benzoxazolin-2-one (BOA), and 6-methoxybenzoxazolin-2-one (MBOA)	Proposed as allelochemicals	Villagrasa et al. (2006)
Cis-jasmonate induced in root tissue	2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), 6-methoxy-benzoxazolin-2-one (MBOA) and 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), and phenolic acids such as trans-p-coumaric acid, syringic acid, p-hydroxybenzoic acid, vanillic acid and cis- and trans-ferulic acid	Proposed induced molecules were possibly capable of reducing developments of pest, disease and weed.	Moraes et al. (2008)
Root exudates	Unknown compounds	Inhibition of *Gaeumannomyces graminis* var. *tritici*	Schalchli et al. (2012)
Roots and root exudates	Unknown compounds	Effects on motility of root-lesion nematode *P. thornei* observed	Linsell et al. (2014)
Roots and shoots	2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (HMBOA-Glc), 2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc)	Potential effect on *P. neglectus*	Frew et al. (2018)

The presence of phenolic metabolites, namely, coumaric acids, ferulic, and syringic acids, were also reported in wheat genotypes in other studies (Guenzi and McCalla 1966, 1967; Lodhi et al. 1987; Salomonsson et al. 1978). These compounds were identified in wheat as different alleleochemicals. Allelochemicals are secondary metabolites in plants, which generally interact with the microbiome. Allelochemicals have been found to

counteract abiotic and biotic stressors (Gross, 2009). However, many allelochemicals can have dual roles in plant development/reproduction as well as in interactions with other organisms. Higher expression of these phenolic acids, benzoxozinoids, hydroxamic acids, sterols and other phytochemicals in resistant wheat sources could have effects on reproduction and infestation of *P. thornei*.

Effects of elicitors on wheat also induced antimicrobial compounds. Deoxynivalenol (DON), from *Fusarium* spp. was responsible for programmed cell death for elicited defensive pathways in wheat (Desmond et al. 2008). The root necrosis following *P. thornei* infestation in wheat roots and up-regulation or down-regulation of certain compounds is worth investigating. Gondor et al (2016) suggested that salicylic acid can induce flavonoid metabolism in wheat (*Triticum aestivum* cv. Mv Emese). Flavonoids were found to be effective in acting against plant parasitic nematodes (Chin et al. 2018).

4.2.6 *Potential mode of action of plant metabolites against plant-parasitic nematodes (sedentary and root-lesion)*

The effects of secondary metabolites on nematode life cycles and reproduction have been mostly studied using sedentary parasitic nematodes. In addition to phenolic metabolites, terpenoids and alkaloids metabolites affected sedentary nematodes such as *M. incognita* (Sikder and Vestergård, 2019). The defence against parasitic nematodes can vary between nematode species and their host plant combinations. The modes of parasitism of migratory root lesion nematode and sedentary nematodes (cyst and root-knot nematode) are different, but both have similar life stages in their cycle.

P. thornei shares 43% of its contigs with other root lesion nematodes and with sedentary nematode species (Nicol et al. 2011). Therefore, there could be common defence mechanisms among *P. thornei* and other migratory nematode species and sedentary species, as well as unique defence properties of resistant wheat genotypes against *P. thornei* infestation. Effects of phenolic metabolites on sedentary and migratory nematodes with respect to motility, mortality and egg hatching have been reported (Rich et al. 1977; Baldridge et al. 1998; González-Pérez and Estévez Braun, 1998; Wuyts et al.

2006). The oxidation of the phenolic molecule chlorogenic acid by induced polyphenol oxidase in tomato roots may decrease motility of *P. penetrans* (Hung and Rohde 1973; Balaji and Mahajan 1977).

Besides phenolic compounds, the steroid compound 20-hydroxyecdysone (20E) that was induced by methyl jasmonate in spinach (*Spinacia oleracea* cv. Avon) reduced *P. neglectus* reproduction (Soriano et al. 2004b). *Pratylenchus neglectus* exposed to 20E, either directly *in vitro* or at concentrations above 4.2×10^{-7} M induced in spinach, suffered abnormal moulting and immobility. Alkaloid metabolites could potentially act as acetyl choline esterase inhibitors (Hillhouse et al. 2004; Selkirk et al. 2005; Konrath et al. 2013) to damage neural connections of plant nematodes and disrupt their motility inside resistant root tissue. Furthermore, isothiocyanate compounds reduced motility of the root-knot nematode *Meloidogyne hapla* (Dahlin and Hallmann 2020). Isothiocyanate compounds can be stored in plant roots as pre-formed consititutive glucosinolates (Potter et al. 1998), which can be hydrolised into thiocyanate and iso thiocyanate in response to plant parasitic nematode infestaion.

Hatching of eggs due to unavailabilty of hatching factors, degradation of egg shells and limitations on feeding inside roots of resitant genotypes might reduce nematode reproduction. Glycoalkaloids, α-chaconine and solanine have been identified as egg hatching factors for cyst nematode (*Globodera rostochinensis*) in the diffusates of potato roots (Devine et al. 1996). Plant chitinases could act specifically on the chitin of nematode eggs to cause damage (Kathiresan and Mehta 2006). A sufficient level of expression of some compounds in plants can be necessary for RLN to complete their life cycle. A reduced level of expansin protein (Le EXPA5) in tomato root was responsible for the incomplete life cycle of the root-knot nematode *Meloidogyne javanica* (Gal et al. 2006).

It is also noteworthy that root metabolites can be attractants to microbes (Castro et al. 1989; Halbrendt 1996; Bertin et al. 2003; Bais et al. 2006; Curtis 2008; Romeo et al. 2012). The presence of the auxin indole acetic acid (IAA) in root exudates can be sensed by parasitic nematodes, attracting them towards the host root tissue (Curtis 2008). Indole acetic acid can bind to the cuticle of *M. incognita* and cause changes in

root cuticle surface signaling that attracts the nematode towards the root thereby promoting infestation. This attraction was proved with both root exudates and *in vitro* application of IAA (Curtis, 2008).

Nematodes can also disable the defence mechanisms of plants by inactivating enzymes (for example, cytochrome P450) that plants produce in defence (Dieterich and Sommer, 2009). The secretions of nematodes and their surface coat are recognized by the host plant, thereby starting a defensive response. Nematodes can change their surface morphology to avoid recognition by the plants. Most phytoalexins act on the cell membranes of microorganisms. Parasitic nematodes can avoid the effects of some phytoalexins by changes in surface coat morphology. Synthetic auxin and kinetin, and tomato root diffusate, change the surface coats of the *Globodera rostochiensis* and *M. incognita*, which could increase their pathogenicity by avoiding the defence of the host plant (De Mendoza et al. 2000; Akhkha et al. 2002). Therefore, versatility of the defence molecules that plants can produce in response to nematode attack can minimize the susceptibility of plants to the nematodes (Ntalli and Caboni, 2012).

The Time Space Effect (TSE) is important for effective defence against RLN (Veech 1982). A susceptible plant may elicit and accumulate defensive molecules at the damaged site over time, but RLNs could escape by migrating away to an uninfected site nearby. Resistant plants can initiate a defence response in the uninfected parts to avoid invasion over time (Ryals et al. 1996). Defensive molecules may be nematostatic or nematicidal, but in either case there is incomplete inhibition of reproduction in the partially resistant wheat genotypes. Compounds released after a certain time may restrict nematode migration and further feeding on cortical tissues. Defensive compounds could play a major role in preventing the completion of the nematode reproduction cycle.

5. Objectives of the study and thesis outline

There are different resistant wheat sources, which limit *P. thornei* nematode reproduction inside roots and the surrounding soil. Two sources of resistance against *P. thornei* were used in this study for different experiments (i) GS50a and its derived line QT8343, and (ii) synthetic hexaploid CPI133872 and its derived line QT16258. These resistant wheat genotypes were compared with susceptible wheat genotypes, Gatcher and Janz, which are parents of the resistant derivatives.

After a thorough literature review it is evident there are limited studies to understand the biochemical defence mechanisms of these resistant wheat sources against *P. thornei* infestation.

The work reported here provides insights to understand the defence mechanisms in wheat against the *P. thornei* nematodes. The following research gaps in knowledge of biochemical defence in wheat against *P. thornei* were identified and are addressed in this thesis using selected wheat genotypes for different experimentation.

Knowledge gap 1: Information on critical time points during plant growth to understand differential changes in *P. thornei* reproduction and egg deposition inside the roots of resistant and susceptible genotypes is not well understood.

Chapter 2 is focused on understanding changes in the reproduction and life stages of *P. thornei* in the roots of resistant and susceptible wheat genotypes over time of plant growth and after nematode inoculation. The aim of the study was to understand the critical/early time points of the changes in nematode reproduction and life stages. The changes of total protein contents in the contrasting wheat genotypes QT8343 (resistant) and Gatcher and Janz (susceptible) were also determined to assess changes in total protein content with nematode infestation.

Knowledge gap 2: There is a lack of knowledge on the role of phenolic compounds and oxidative enzymes PPO and POD in the defence responses in wheat against *P. thornei*. Understanding the constitutive or induced expression of total phenolic compounds and

oxidative enzymes in resistant and susceptible wheat genotypes will provide important insights into possible resistance mechanisms in wheat roots against *P. thornei*.

Chapter 3 of this thesis focuses on two genetically different sources of *P. thornei* resistance, CPI133872 and GS50a, and susceptible wheat genotypes (Janz and Gatcher). It aims to provide insights into possible resistance mechanisms in wheat against *P. thornei* by investigating constitutive and induced levels of (i) total phenols, (ii) PPO activity, and (iii) POD activity, at different time points after nematode inoculation. The relationship between total phenol levels and resistance levels is further explored with a larger number of wheat genotypes, ranging from resistant to very susceptible to *P. thornei*.

Knowledge gap 3: The relative abundance of specific metabolites in resistant and susceptible wheat genotypes and their possible roles in plant defence against *P. thornei* are currently unknown.

In Chapter 4 of this thesis, untargeted liquid chromatography mass spectrometry (LC-MS) was performed to comprehensively profile the largest possible array of metabolites in the roots of a *P. thornei*-resistant (QT16528) and a *P. thornei*-susceptible wheat genotype (Janz) after growth with or without inoculation with *P. thornei.* Based on the results from Chapter 3, where the *P. thornei* resistant genotype CPI133872 was found to have significantly higher total phenol levels than the susceptible wheat genotypes, an adapted advanced breeding line derived from CPI133872 (QT16528) was selected for the metabolomics study. A set of in-house authentic chemical standards was used to identify significantly expressed metabolites in resistant and susceptible genotypes in response to *P. thornei* challenge. The differential expression profile of metabolites will provide insight into potential mechanisms for plant defence against *P. thornei* and support the discovery of biomarkers for resistance.

Chapter 5 of this thesis provides a general discussion of the potential biochemical defence mechanisms in wheat against *P. thornei* and discusses prospects for future investigations.

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CHAPTER 2

COMPARATIVE DIFFERENTIAL REPRODUCTION RATE OF *PRATYLENCHUS THORNEI* AND HISTOPATHOLOGY IN MODERATELY RESISTANT AND SUSCEPTIBLE WHEAT GENOTYPES OVER TIME

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This study was conducted to understand the differential reproduction rate of *P. thornei* inside the roots of moderately resistant and susceptible wheat genotypes over time (1 to 12 weeks). The Whitehead tray method for nematode extraction and acid fuchsin staining of roots were used to determine the presence of nematodes and their eggs inside wheat root tissues. The rate of *P. thornei* reproduction, number of eggs deposited and proportion of life stages were observed in the root tissues of susceptible and moderately resistant wheat genotypes over time. The results of this study informed the critical/early time points of the changes in nematode reproduction and life stages for comparative enzyme profiling and detailed metabolomics analysis.

[Supplementary material associated with this chapter is attached in Appendix A.]

Title

Comparative differential reproduction rate of *Pratylenchus thornei* and histopathology in moderately resistant and susceptible wheat genotypes over time

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Abstract

The Australian wheat industry faces significant yield loss from the root-lesion nematode *Pratylenchus thornei*. The life stage dynamics of *P. thornei* were evaluated in wheat genotypes ranging in resistance/susceptibility, namely, QT8343 (moderately resistant), Janz (susceptible to very susceptible) and Gatcher (susceptible to very susceptible). Adult and J4 life stages preferentially entered the roots compared with J2 and J3 for all three wheat genotypes at 1 and 2 weeks post nematode inoculation (PNI). However, by 4 weeks there were significantly more ($P \leq 0.05$) J2 and J3 in the roots of Gatcher and Janz than in QT8343. The numbers of juvenile (J2, J3 and J4) and adult *P. thornei*, expressed both per gram of root and per whole plant root system, were significantly fewer for QT8343 than for Gatcher and Janz at 4, 8 and 12 weeks PNI. Eggs were observed in the roots of Gatcher and Janz at 2 weeks and in QT8343 at 4 weeks. From 4

weeks onwards, there were fewer eggs inside the roots of QT8343 than Gatcher or Janz. For all genotypes, the proportion of juvenile life stages increased over time whereas the proportion of adult life stages decreased inside the roots over time, up to 12 weeks PNI. In histopathological studies, no effects of nematode inoculation were found on total cell wall bound phenolics and lignin or on total protein content of the roots. The study indicated that 8 weeks post *P. thornei* inoculation was a suitable time point for biochemical studies of defence mechanisms against *P. thornei* in wheat genotypes.

Keywords: Wheat, *Pratylenchus thornei*, Nematode egg deposition, Nematode life stages, Protein estimation

1. Introduction

In the subtropical grain region of eastern Australia, wheat yield loss due to *P. thornei* has been estimated at 38 million AUD per year, and with potential loss of 104 million AUD if management strategies are not used (Murray & Brennan, 2009). Some wheat genotypes express partial resistance to *P. thornei,* and are capable of limiting nematode reproduction inside the plant roots, thereby withstanding nematode damage to a considerable extent (Thompson et al., 2008). Partial resistance against *P. thornei* has been identified in several sources of wheat germplasm, including the Australian bread wheat selection GS50a, West Asian and North African wheat landraces including Iranian wheat landraces, and synthetic hexaploid wheats (reviewed by Thompson et al. 2008). Australian pre-breeding and breeding programs are working towards incorporating this partial resistance into elite high yielding wheat cultivars. However, to date no full resistance against *P. thornei* has been identified that completely prevents nematode reproduction.

Pratylenchus thornei is secretes cell wall degrading enzymes through its syringe-like stylet that enable the nematodes to penetrate and invade wheat root tissues.. Wheat genotypes susceptible to *P. thornei* suffer serious damage due to cavities formed in the root cortical tissue as a result of nematode penetration and feeding (Castillo & Vovlas,

2007). This damage of the cortical cells can hamper absorption of sufficient water and nutrients from the soil for optimum plant growth (Thompson et al., 2012a; Whish et al., 2014). Wheat genotypes susceptible to *P. thornei* exhibit above-ground symptoms of reduced tillering and yellowing of lower leaves and suffer from grain yield loss (Thompson et al. 2012a).

There is a lack of knowledge of the physiological responses of wheat roots at the cellular level against the penetration and feeding of *P. thornei*. Necrosis of root cells and *P. thornei* reproduction were studied in susceptible wheat genotypes (Baxter & Blake, 1967, Baxter & Blake, 1968) over time. These previous studies indicated few *P. thornei* had invaded the wheat roots at 2 weeks (Baxter & Blake 1967). Baxter and Blake (1968) found that *P. thornei* eggs were deposited singly or in groups along the track of female *P. thornei* migration inside roots. The nematode numbers inside susceptible wheat roots increased exponentially and were followed up to 40 days (Baxter & Blake, 1968). However, information on critical time points to understand the changes in *P. thornei* life stages and egg depositions inside wheat roots between resistant and susceptible wheat genotypes are not well understood.

The defence mechanisms of resistant wheat plants are likely to act after root penetration by *P. thornei* (Linsell et al. 2014), and may initiate early after penetration with the cumulative effects compounding over time to result in lower reproduction rates and nematode population densities as the life cycles proceed. According to a previous study, statistically significant differences between wheat genotypes in final population densities of *P. thornei* were detectable at 8 weeks after nematode inoculation (Thompson et al., 2015a). Due to the exponential increase in nematode population densities over time, the difference in population densities of *P. thornei* in susceptible (Gatcher) and resistant (GS50a) genotypes were maximal at 16 weeks post nematode inoculation (PNI), under controlled glasshouse conditions, and this time was adopted as best practice for screening wheat genotypes for resistance to *P. thornei* for plant breeding purposes (Thompson et al. 2015a). However, the reproduction rate of *P. thornei* also depends on

environmental conditions with the optimum temperature for *P. thornei* reproduction in wheat being in the range of 20 to 25°C (Thompson et al., 2015b).

Reduced motility and lower egg deposition inside the *P. thornei* resistant wheat genotype Sokoll, occurred in plants grown on an agar medium up to 10 days (Linsell et al. 2014). Furthermore, Linsell et al. (2014) suggested that maturity of *P. thornei* and egg laying could be delayed or fewer eggs could be deposited, which could account for reduced reproduction in the resistant wheat genotype Sokoll over time (up to 45 days) in comparison with the susceptible genotype Krichauff. The life cycle of *Pratylenchus* spp. is completed in approximately six weeks under favourable environmental conditions and has four distinct vermiform life stages including three juvenile stages J2, J3, J4) and adults. The J1 stage develops and moults to J2 inside the egg (Castillo and Vovlas 2007). There could be physical barriers in resistant wheat genotypes that establish over time to limit movement and further penetration of *P. thornei* inside wheat roots. Phenolic molecules have been found to be effective defensive compounds against *Pratylenchus* spp. (Chitwood, 2002). Phenolic compounds in the presence of phenol oxidases can be converted into lignin to provide cell wall rigidity (Matern & Kneusel, 1988). The enzymes polyphenol oxidase (PPO) and peroxidase (POD) were increased in resistant wheat genotypes as defence against *P. thornei* (Rahaman et al., 2020). The PPO and POD activity were found to be higher in concentration and induced by *P. thornei* inoculation in resistant wheat genotypes CPI133872, GS50a and QT8343, with maximal values at 4 weeks PNI compared to susceptible wheat genotypes, Gatcher and Janz (Rahaman et al. 2020). Additionally, changes in enzyme profiles including phenol oxidases (PPO and POD) and pathogenesis related proteins can change the total protein expression in wheat genotypes resistant to stress (Devi et al., 2017).

Rahman et al. (2020) included phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) among potential candidate resistance genes in wheat acting against *P. thornei*. These two are key enzymes for phenyl propanoid biosynthesis and flavonoid biosynthesis, which produce a range of phenolic molecules. In addition to increases in total soluble phenolics there could be differences in cell wall bound phenolics and their

distribution in root tissues of resistant wheat genotypes compared to susceptible genotypes. Deposition of lignin and flavonoids in the cortex of resistant wheat roots could increase rigidity and provide defence against *P. thornei*. Phloroglucinol (PG) can bind to cinnamaldehyde (phenolics) of lignin components (Adler, 1977) and colour lignin deposition facilitating visualisation in root tissues. Similarly, 2 aminoethyldiphenyl borinate can bind to cell wall bound flavonoids to give yellow green fluorescence (Peer et al., 2001).

The present study focuses on understanding the comparative changes in the reproduction and life stages of *P. thornei,* including eggs, within the same root systems, for resistant and susceptible wheat genotypes over time. The aims of this study were to monitor the changes in nematode numbers and egg deposition over time from 1 to 12 weeks PNI in *P. thornei* resistant (QT8343) and susceptible wheat genotypes (Gatcher and Janz) by (i) counting the total number of *P. thornei*, (ii) counting the numbers of each *P. thornei* vermiform life stage J2, J3, J4 and adults, and (iii) recording the numbers of nematode eggs. Furthermore, indicators of changes to cell wall components were investigated by comparing *P. thornei* inoculated and uninoculated wheat roots through (i) visualisation of lignin deposition, (ii) visualisation of total phenols, and (ii) estimation of the total protein content in wheat roots.

2. Materials and methods

2.1 Nematode inoculum

Pratylenchus thornei used in this study was originally isolated from soil collected from Formartin (latitude 27.46°S, longitude 151.43°E), Queensland, Australia. Nematode inoculum for the experiments was prepared from an open pot culture maintained on the susceptible wheat cultivar Petrie for 16 weeks. Nematodes were extracted from the soil and roots of the pot culture using the Whitehead Tray method (Whitehead and

Hemming, 1965). Total numbers of *P. thornei* per mL and its composition in the four life stages (J2, J3, J4 and adults) were recorded.

2.2. Plant materials

The three wheat genotypes used in this study were (i) QT8343, rated resistant to moderately resistant (R-MR) to *P. thornei*, and (ii) Janz and (iii) Gatcher, both rated susceptible to very susceptible (S-VS) to *P. thornei* (Thompson et al. 2020). Wheat genotype QT8343 is an advanced breeding line derived from GS50a (GS50a/3*Cunningham//Janz), where GS50a is a *P. thornei* tolerant and resistant selection from Gatcher (Thompson et al., 2008).

2.3. Surface sterilisation of seeds

Wheat seeds were surface sterilised by immersing them in 70% ethanol for 5 min followed by 2.5 % sodium hypochlorite solution for 15 min. Then they were rinsed with sterile Milli–Q® water five times. The seeds were imbibed by soaking in sterile Milli–Q® water for 12 h, then placed on moist sterile filter paper inside petri plates and allowed to germinate for 48 h.

2.4. Plant growth conditions

2.4.1. Experiment 1: *Pratylenchus thornei* reproduction over time on wheat grown in soil

QT8343, Janz and Gatcher were grown in pots in the presence or absence of *P. thornei,* with destructive sampling at five time points (1, 2, 4, 8 and 12 weeks PNI (Figure S1). The treatments were replicated three times for each time point and arranged in a completely randomized design produced using R-software version 3.5.1.

Soil for the pot experiment was collected from Formartin, Australia and steam:air pasteurised at 85°C for 45 min prior to use (Thompson 1990). The initial soil moisture content was determined by drying 100 g of pasteurised soil at 105°C inside a forced draught oven for 48 h. The experiment was conducted in a controlled environment growth cabinet (Bioline, Perceival Scientific Inc,). Light intensity was maintained at 400 µmole/m^2 S with a photoperiod of 13 h light and 11 h dark. Temperature was maintained at 22°C ± 2°C throughout the experiment and monitored every 30 min by an iButton (Thermochron®) positioned at 3 cm depth in the soil inside a pot.

Two-day old surface sterilised and pre-germinated seedlings were transplanted into 15 cm square pots holding 330 g (dry soil weight equivalent) of pasteurised soil. Three seedlings were planted in each pot. Five days after planting (i.e. 7 day old seedlings), 2,000 *P. thornei* in 10 mL water were inoculated in a 30 mm deep hole alongside each seedling. Seedlings in control pots were similarly inoculated with 10 mL water without nematodes. The plants were carefully top watered on alternate days in order to maintain 56 % moisture content, equivalent to pF2 in this soil (Thompson & Haak, 1997). The term pF 2 is the log to base 10 of 100 cm water suction. For field capacity (56% moisture in soil) that are equivalent are 10 kilopascals or 0.1 bars (both equivalent to 100 cm suction or pF 2) (Thompson et al. 2017).

Plant roots were collected from the treatments at the various time-points by washing carefully under running tap water to remove soil particles, with extra caution taken to minimise damage to the roots while washing. The roots were then washed with sterile Milli-Q® water and blotted three times with fresh paper tissues. The roots were weighed and divided into three subsamples of equal weight for the following: (i) extraction of live nematodes directly from the roots using the Whitehead tray method, (ii) staining of nematodes and eggs inside the root samples using acid fuchsin, and (iii) estimation of total protein content. Nematode extraction and acid fuchsin staining were performed within 48 h of root harvesting. Small subsamples of roots of each genotype, both inoculated and uninoculated with *P. thornei*, from 8 weeks PNI were also used for root

cross sectioning for phloroglucinol (PG) and 2 amino-ethyl-di phenyl borinate (2-APB) staining.

The average height of the three plants in each pot was recorded at 8 and 12 weeks. Fresh and dry biomass (oven dried at 80 °C for 48 h) of the shoot were recorded at each time point. The mean and standard error were calculated from three replicates per treatment.

2.4.2. Experiment 2: *Pratylenchus thornei* reproduction over time in wheat grown on agar under gnotobiotic conditions

Wheat seeds were surface sterilised as described above and transferred aseptically into 1L Schott bottles (DURAN® GLS 80) containing 100 mL of semi-solid tap water agar (0.3%). QT8343, Janz and Gatcher were grown in the presence and absence of *P. thornei* for sampling at 1, 5 and 10 days post nematode inoculation (PNI). The treatments were replicated three times for each time point and arranged in a completely randomized design produced using R-software version 3.5.1 (R Core Team, 2017).

Two experiments (Experiment 2a and Experiment 2b) were conducted in parallel. In Experiment 2a, *P. thornei* infestation was monitored in the three wheat genotypes at 1, 5 and 10 days post nematode inoculation. In experiment 2b the three wheat genotypes were grown to check cell wall bound phenolics and lignin at 1, 5 and 10 days post nematode inoculation.

Four surface sterilised and germinated seedlings were placed on 100 mL sterile tap water agar inside each bottle. A total of 6,000 *P. thornei* (1,500/seedling) in 12 ml water suspension were inoculated per bottle. For control treatments, 12 ml sterile water without nematodes was added per bottle. The roots from Experiment 2a were stained with acid fuschsin. Wheat genotypes grown on the agar for 1, 5 and 10 days after transplantation (Experiment 2b) were used for cross sectioning and PG and 2-APB staining. Ten day old root exudates were collected from the agar medium after plant growth (Experiment 2b). Sterile Milli-Q water (50 ml) was added to the agar inside the

bottle. The suspension was mixed well with a glass rod and kept at 4°C overnight. Then the water suspension was filtered through Whatman No. 1 filter paper in a funnel (Figure S2) and the filtrate collected. The root exudates in aqueous solutions were freeze dried and stored at –20°C until used for total protein estimation.

2.5. Nematode extraction and recording of life stages

Pratylenchus thornei were extracted from roots using a modified Whitehead tray method (Whitehead and Hemming, 1965). The roots were cut into 1-2 cm pieces and put on tissue supported on a plastic mesh inside a plastic tray with 1L water just covering the roots. After 2 days incubation at 22°C the nematodes were sieved using a 20-µm aperture sieve and nematodes were collected in a 30 mL sample container. Tap water (1 L) was added to the tray to incubate for another 5 days for further extraction of nematodes from the roots. After extraction, the roots were oven dried at 80°C for 48 h. The nematodes from the samples collected at 2 days and 7 days were counted in life stage categories (Thompson et al. 2017) using a 1-mL Peters nematode counting chamber under an Olympus BX53 optical microscope (Olympus, Tokyo, Japan). The nematode numbers were expressed per gram of dry root weight and per root system.

2.6. Acid fuchsin staining of nematodes and eggs inside root tissue

The root samples were stained with 0.1% acid fuchsin (Sigma Aldrich) in a 30 mL stain tube as described in Rahaman et al. (2020). The presence of vermiform nematodes and eggs were recorded in stained whole root systems at 1, 2 and 4 weeks PNI and in 10 randomly selected root pieces of 35 mm length mounted on microscope slides at 8 and 12 weeks PNI,. The roots were observed under an optical microscope (BX53, Olympus, Tokyo, Japan) in both bright field and differential interference contrast (DIC) modes at magnification of x200. The presence of the stained nematodes and numbers of eggs were recorded in each replicate of the individual genotypes. Images of the stained *P. thornei*

inside roots were obtained with a digital camera (DP26) using cellSens Olympus imaging software version 1.9.

2.7. Lignin staining of wheat roots

The *P. thornei* inoculated and uninoculated wheat roots were cross-sectioned and stained with phloroglucinol (PG) (Sigma-Aldrich) (Speer, 1987) to visualise induced lignification of wheat roots. Randomly selected roots were hand sectioned with razor blades. The thinnest sections were selected for staining and microscopic analysis for all the genotypes. These assays were repeated three times on the wheat roots grown on agar, sand and soil respectively. Moreover, the assay were done in more than one roots of a biological replicate. Cross sections (~10 uniform pieces) from the randomly selected roots were immersed in 1% PG (prepared in 92% ethanol). The samples were left for 3 min and then transferred to a solution of 25% hydrochloric acid (HCl). Once a red colour appeared within the section, one drop of a solution prepared with glycerol, HCl, lactic acid and PG-ethanol (50:40:7:3) was added. A cover slip was placed on top of the stained cross section. The stained root samples were observed under a fluorescence microscope under bright field mode (Olympus BX 53) (Lanoue et al., 2010).

2.8. Phenol staining of wheat roots

An aqueous solution (0.25%) of 2-amino ethyl diphenyl borinate (2-APB) (Sigma-Aldrich) was prepared (Lanoue et al., 2010). Cross sections (10 uniform pieces) from the randomly selected roots were placed on a microscope slide in 2-APB solution,and covered with a cover slip The sections were examined for root necrosis after *P. thornei* infection. The phenol staining in the root cell wall was observed under bright field illumination as yellow green fluorescence with excitation filter FITC (460-490 nm) and cutting filter (515 nm). The SIS Cell software was used to obtain photomicrographs with a digital camera mounted on the microscope (Olympus BX53).

2.9. Total protein content of wheat roots

Total protein content in wheat roots at 2 and 8 weeks PNI was determined by the bicinchonic acid (BCA) method (Smith et al., 1985). This method was followed using the Pierce microplate BCA protein assay kit (Thermofisher Scientific®) according to the manufacturer's instructions. Subsamples of 15 mg freeze dried wheat root were ground in a mortar and pestle with liquid nitrogen. Total protein was extracted from the wheat root powder in sodium phosphate (NaH_2PO_4 and Na_2HPO_4) buffer (pH 7). Three technical replicates were used for the blank control, the standard bovine serum albumin (BSA) dilutions (serially diluted, 0 to 2000 µg/mL) to establish a standard curve, and for each biological replicate of the wheat root extracts. Nine microliters of each technical replicate of the root samples and the standards were pipetted to the center of the microplate well. Then 4 µl of compatibility solution was added into each well. The plate was covered with aluminum foil and shaken on a plate shaker at medium speed for 1 min to mix the solution well. Next the plate was incubated at 37°C for 15 min, then 260 µl of BCA reagent was added to each well. The plate was covered again and the solution was mixed on a plate shaker for 1 min. The plate was incubated at 37°C for 30 min and then removed from the incubator and allowed to cool at room temperature for 5 min. Absorbance of the blank control, BSA standards, and wheat root protein extracts at 562 nm was recorded on a plate reader (Fluostar Omega, BMG Labtech). The average absorbance of the standard control was subtracted from BSA standards and wheat root proteins. A standard curve was prepared relating absorbance values at 562 nm to the different concentrations (µg/mL) of BSA standard (Supplementary Fig. S6).

2.10. Statistical Analyses

Data were analysed using Genstat ® for windows™ (VSN International, 2015). Analysis of variance (ANOVA) was performed to check the significance of F-values for factors of genotypes, nematode inoculation and time and their interactions. Least significant differences (LSD) at $P \leq 0.05$ were determined. Prior to ANOVA the data sets were

checked for normal distribution and log transformed (ln(x+1), where x is nematode count) for the data sets that were not normally distributed. Linear regression was used to relate ln(total *P. thornei*/root system) based on individual replicates to time after nematode inoculation for the three wheat genotypes in Experiment 1.

3. Results

3.1. Total *Pratylenchus thornei* population densities over time

At the early sampling times of 1 and 2 weeks PNI, the number of *P. thornei* in the roots of Janz and Gatcher were not significantly different ($P \geq 0.05$) from QT8343, whether expressed either as mean number per g of root (Fig 1a) (1,316 to 1,611 *P. thornei*/g root in Gatcher; 1,127 to 1,463 *P. thornei*/g root in Janz; 900 to 1,064 *P. thornei*/g root in QT8343) or number per whole root system (Fig. 1b). There were marked increases in the number of *P. thornei* in the roots of Gatcher and Janz between 2 and 4 weeks and again between 8 and 12 weeks (Fig 1a, b). For QT8343, there were only gradual changes in *P. thornei*/g root (Fig. 1a) over time, with a continuous gradual increase in the number of *P. thornei*/root system (Fig 1b). The first significant differences ($P \leq 0.05$) were recorded at 4 weeks PNI between the susceptible genotypes (4,880 and 5,090 *P. thornei* per g of dry root for Gatcher and Janz, respectively) and the moderately resistant genotype (555 *P. thornei* per g of dry root for QT8343), with a nine-fold difference in *P. thornei* population densities. The final number of *P. thornei* at 12 weeks PNI for Gatcher (51,590 *P. thornei*/g of dry root) was more than two-fold that of Janz (19,480 *P. thornei*/g of dry root) and more than 35-fold that of QT8343 (1,440 *P. thornei*/ g of dry root). There were highly significant linear relationships between P. *thornei* per root system, expressed on a log scale (ln(x+1)), and time after inoculation (Fig 1c), indicating exponential growth of *P. thornei* population densities in all three genotypes. The rate coefficient was greatest for Gatcher (0.4499) followed by Janz (0.3791) and least for

QT8343 (0.1719), with the predicted number of *P. thornei*/root system after 12 weeks being 14,180 for Gatcher, 6,960 for Janz, and 560 for QT8343.

3.2. Numbers of *P. thornei* life stages over time

Most of the increase in population density of *P. thornei* over the 12 week period after inoculation ,as measured by extraction using the Whitehead tray method, was accounted for by increases in the early generations of J2 (Fig 2a, b) and J3 (Fig 2c, d), with a lesser contribution by J4 (Fig 2e, f) and least by the adult life stage (Fig 2g, h). The initial inoculum was a mixture of 18% J2, 33% J3, 21% J4 and 28% adult life stages. At 1 week PNI the percentages of life stages in the wheat roots were: for Gatcher 6% J2, 15% J3, 26% J4 and 50% adult; for Janz 16% J2, 13% J3, 29% J4 and 41% adult; and for QT8343 12% J2, 23% J3, 25% J4 and 39% adult (Supplementary Figure S3). These values indicate that the adult and J4 life stages preferentially entered the roots of all three wheat genotypes. However, by 4 weeks there was a significant ($P \leq 0.05$) increase in the numbers of J2 (Fig 2a, b) and J3 (Fig 2c, d) in the roots of Gatcher and Janz with lesser increases in the numbers of J4 (Fig 2e, f) and adults (Fig 2g, h). This resulted in a change toward higher percentages of J2 and J3 life stages and lower percentages of J4 and adults in the roots of Gatcher (30% J2, 39% J3, 17% J4 and 12% adult), and Janz (36% J2, 35% J3, 16% J4 and 13% adult), compared to QT8343 (9% J2, 32% J3, 26% J4 and 30% adult) (Supplementary Figure S3). Finally at 12 weeks after inoculation, there had been further increases in the numbers of J2 (Fig 2a, b) and J3 (Fig 2c, d) in the roots of Gatcher and Janz in comparison with QT8343. There were similar but lesser trends in the numbers of J4 (Fig 2e, f) and adult (Fig 2g, h) life stages. This resulted in further changes in the composition of the populations toward higher percentages of J2 and J3 life stages and lower percentages of J4 and adult life stages in the roots of Gatcher (49% J2, 35% J3, 11% J4 and 5 % adult) and Janz (53% J2, 27% J3, 13% J4 and 7% adult), and to a lesser change in QT8343 (26% J2, 33% J3, 24% J4 and 16% adult).

3.3. Visualisation of nematodes and eggs inside wheat roots

Vermiform nematodes stained by acid fuschsin were observed inside the roots of all wheat genotypes from 1 week PNI. Eggs of *P. thornei* were first observed at 2 weeks PNI and onward for susceptible genotypes (Janz and Gatcher) and only from 4 weeks PNI onwards for the resistant genotype (QT8343). Eggs were frequently found inside the roots of susceptible genotypes (both Janz and Gatcher) at 8 weeks PNI (Figure 3) and 12 weeks PNI (Figure 4). Large numbers of eggs were found inside the roots of Gatcher (Fig 3b) and Janz (Fig 3d) compared to few numbers inside the roots of QT8343 (Fig. 3f). At 12 weeks, more eggs and clumped nematodes were found inside the roots of Gatcher than inside Janz. Necrosis of the root cortex was confirmed in the acid fuchsin stained roots of Gatcher and Janz at 8 weeks PNI (Fig. 5). No necrosis was observed in the roots of QT8343.

The nematodes were also found inside the roots of Gatcher and Janz grown on agar medium (Experiment 2a) with eggs observed at 10 days PNI (Supplementary Figure S5 a and b). However, the infection rate was lower in comparison to the roots collected from the soil experiment. No eggs were observed in the roots of QT8343 at 10 days in this experiment.

3.4. Lignin staining of wheat roots

No differences in cell wall lignification visualised by phloroglucinol (PG) staining of the cinnamaldehyde of lignin were observed between uninoculated and inoculated treatments of any of the genotypes at any of the time points studied (8 weeks PNI for Experiment 1; and 1, 5 or 10 days PNI for Experiment 2). Lignification was observed in the vascular cylinder, but not in the root cortex, for all wheat genotypes in the presence and absence of *P. thornei* infestation over time. The deep red PG staining of the cinnamaldehyde of lignin in the vascular cylinder was relatively more intense in

QT8343 (Fig. 6d) than in Gatcher and Janz (Fig. 6 b, c) at all time points. The intensity of the red staining was deeper at 8 weeks PNI for all the genotypes with or without inoculation with *P. thornei,* compared to the younger roots at 1, 5 and 10 days. (data not shown).

3.5. Phenol staining of wheat roots

Necrosis of the root cortex was rarely found with 2-amino ethyl-di phenyl borinate (2-APB) staining of the randomly selected pieces of root cross sections in Gatcher. Furthermore, the relatively higher intense yellow green fluorescence due to the presence of cell wall bound phenolics was not found in any genotypes and their treatments both in Experiment 1 and Experiment 2 (Figure 7). Similar to lignin staining, the intense yellow green fluorescence was found in the vascular cylinder containing xylem and phloem tissue with no considerable differences among genotypes and treatments both in agar medium at 1, 5 and 10 days and soil medium at 8 weeks PNI. There were no noteworthy differences in the intensity of yellow green fluorescence in the root cortex area among any genotypes and treatments at any time points.

3.6. Total protein content of wheat roots

The protein content was expressed as bovine serum albumin equivalent (BSAE), according to the standard curve of BSA (Supplementary Figure S5). No effect of *P. thornei* inoculation on the total protein content of susceptible (Gatcher, Janz) and moderately resistant (QT8343) wheat genotypes was found at 2 weeks PNI or 8 weeks PNI (Fig. 8). However, at 2 weeks the mean total protein content in QT8343 (uninoculated; 31.0 mg, inoculated, 28.8 mg) was found to be significantly ($P \leq 0.05$) higher than in Janz (uninoculated; 21.7 mg, inoculated, 23.0 mg) but not in Gatcher (uninoculated; 26.2 mg, inoculated, 25.4 mg). At 8 weeks there was no significant

difference ($P \leq 0.05$) in average protein contents of the three wheat genotypes (data not shown).

Protein content was not found in the detectable range in root exudates of all the genotypes collected from the agar medium at 10 days (data not shown).

3.7. Shoot, root biomass and plant height

P. thornei inoculation did not affect plant height of any genotypes in Experiment 1 (data not shown). Additionally, no effects of *P. thornei* inoculation on differences in shoot and root weight of uninoculated and inoculated wheat genotypes were found at the different time points (1 to 12 weeks PNI) (Supplementary Figure S8a, b and c) except for fresh root weight of Gatcher inoculated (8.26 g/per pot) and uninoculated (10.26 g/per pot) treatments at 8 weeks PNI and Janz inoculated (8.50 g/per pot) and uninoculated (10.33 g/per pot) at 12 weeks PNI. Both fresh and dry shoot biomass was of similar value for all the genotypes at different time points of the study (shoot dry weights, Gatcher, 0.12-7.76 g/per pot; Janz, 0.11-7.46 g/per pot; QT8343, 0.15-8.16 g/ per pot) (Supplementary Figure S6 a and b).

4. Discussion

Results of our study on the dynamics of *P. thornei* life stages in the roots of soil-grown wheat indicated that the mature adult and J4 stages of *P. thornei* preferentially penetrated the roots, compared with the immature J2 and J3 stages. There was no significant difference in nematode numbers between the susceptible wheat genotypes Gatcher and Janz and the resistant genotype QT8343 in this penetration phase. Similarly, in a previous study a higher percentage of adult rather than J2 *P. thornei* penetrated wheat roots up to 8 days PNI in sand culture, with no difference between the

resistant genotype Sokoll and the susceptible genotype Krichauff (Linsell et al. 2014). Our observations of roots stained with acid fuchsin showed the presence of some eggs in the root cortex of the susceptible genotypes Gatcher and Janz first at 2 weeks PNI, and of the moderately resistant genotype QT8343 first at 4 weeks PNI, but at significantly lower numbers than in the susceptible genotypes. It is apparent that the resistance mechanisms of QT8343 do not act through preventing penetration of its roots by *P. thornei,* but by delaying egg production by adults and maturation of J4 life stages that have already entered the root cortex.

At 4 weeks PNI, there was a substantially increased number of total *P. thornei* in the roots of Gatcher and Janz, which was mainly composed of J2 and J3 life stages. For *P. thornei* in the root system of QT8343, there was a small decrease at 4 weeks PNI in the number per g root, but a small increase in the number per whole root system. For QT8343 at 4 weeks PNI, there was a much lower proportion of J2 out of the total life stages compared with the susceptible genotypes Gatcher and Janz. Thus it is apparent that the greater fecundity of *P. thornei* in the roots of these susceptible genotypes than in QT8343, was expressed in the greater numbers of the early life stage juveniles J2 and J3 by 4 weeks PNI.

The total population of *P. thornei* in the root system was effectively modelled by an exponential growth curve over the time period up to 12 weeks PNI, with the rate coefficient for Gatcher and Janz being 2.6 and 2.2 times respectively that of QT8343. Population densities of *P. thornei* also followed an exponential growth curve in the root system of the susceptible wheat genotype Gabo up to 40 days PNI (Baxter and Blake 1968), and in the roots and root-zone soil of several wheat genotypes up to 16 weeks PNI (Thompson et al. 2015a).

As the inheritance of resistance in wheat genotypes is polygenic and additive (Thompson and Seymour, 2011), several biomolecules could act together to reduce nematode reproduction at 4, 8 and 12 weeks and egg deposition at 8 and 12 weeks. Our study suggests that 8 weeks PNI could be a good time point for understanding plant mechanisms controlling nematode reproduction. At that time differences in numbers of

both vermiform nematodes and eggs inside susceptible and resistant wheat genotypes were distinct and statistically significant ($P \leq 0.05$). Different classes of metabolites including flavonoids, terpenoids and alkaloids could be responsible for reduced nematode numbers (Wuyts et al., 2006, Sikder & Vestergård, 2019) in resistant wheat genotypes. Rahaman et al. (2020) suggested that constitutive levels of total phenol and induced phenol oxidases were responsible for reduced nematode numbers in synthetic hexaploid wheats (CPI133872 and CPI133859) at 2 to 8 weeks PNI. Furthermore, Rahaman et al. (2020) showed that the total phenol content of QT8343 was similar to Gatcher and Janz. While there might be no significant differences in total phenolic contents, differences in specific phenolic molecules might play an important role in providing defence against *P. thornei*. The relative abundance of specific metabolites that are possibly responsible for defence against *P. thornei* inside roots of resistant wheat genotypes has not yet been examined in detail and should be pursued in future studies. A detailed metabolic study at 8 weeks PNI might shed light on metabolites responsible for reduced nematode numbers and delayed egg deposition inside resistant wheat roots.

No statistically significant effects of *P. thornei* inoculation were found in total protein content of roots. Despite this, there could be changes in specific proteins such as enzymes that were not reflected in changes in total protein content. Enzymes such as chitinase could act on the chitin of *P. thornei* egg shells to damage the viability of the eggs (Kathiresan & Mehta, 2006). Effect of enzymes and or hatching factors on eggs were not studied in this work. However, degradation or damage of eggs due to enzyme activities could be a reason for fewer egg numbers inside the roots of resistant wheat genotypes. Moreover, reduced hatching of the available eggs due to unavailability of hatching factors could also be a reason for the cumulative decrease in nematode numbers (Linsell et al., 2014, De Waele, 1988, Pudasaini et al., 2008). As with phenolic molecules, there could be specific pathogenesis related proteins including enzymes responsible for reduced *P. thornei* numbers inside resistant wheat roots but not reflected in differences in total protein content. Moreover, as the total protein in root exudates was found to be insufficient for estimation, amino acid estimation and gas chromatography (GC) - mass spectrometry (MS) analysis of the root exudates could provide more

information on the role of root exudates in wheat against *P. thornei* infestation in future studies.

It is possible that if an invading microorganism is able to overcome the physical barriers in plant tissues and penetrate, further rigidity of cell walls could be induced at nearby unaffected sites of the plant to prevent further penetration (Malinovsky et al., 2014). To explore this possibility in the wheat-*P. thornei* interaction, lignin and cell wall bound phenolics were also stained with 2-amio ethyl phenyl borinate (2-APB) and phloroglucinol (PG), respectively. However, no significant differences between susceptible and moderately resistant genotypes were found at early time points up to 10 days PNI and also later at 8 weeks PNI. The comparatively intense lignin staining of the vascular cylinder in the resistant wheat genotype QT8343 in this study, compared to that in susceptible genotypes Gatcher and Janz, could be part of a general defence mechanism for the protection of the vascular cylinder expressed constitutively in this genotype. Wuyts et al (2007) found *Radopholus similis* induced intense staining of lignin in the vascular cylinder of banana genotypes. Although more peroxidase and polyphenol oxidase were significantly induced in QT8343 at 4 weeks PNI than in susceptible Gatcher and Janz, the total phenol contents were comparable at 2 to 8 weeks PNI in our previous study (Rahaman et al. 2020). However, oxidised phenolic compounds could play important roles in the defence against *P. thornei* in the presence of the phenol oxidases (Hung & Rohde, 1973).

Understanding the combined effects of all lignin components (cinnamaldehyde, confieryl alchol and sinapyl alcohol) could be more informative as PG only stains cinnamaldehyde in root tissues. Therefore, estimation of total lignin content by thioglycolic acid (Lange et al., 1995) is recommended in future studies. The present study has been focused on comparative fluorescence intensity due to the presence of cell wall bound phenolics in the root cross section. However, control root cross sectioning without 2-APB stain, should also be considered to check auto-fluorescence of the root cross sections and investigated in future studies with more wheat genotypes of different sources and levels of resistance to *P. thornei* infestation. Inoculation of the nematode at

different times during plant growth could further improve understanding of both the penetration strategy of the nematode and changes in constitutive and induced cell wall rigidity over time and their relation with growth stage of particular wheat genotypes. The physical damage of the root including browning and necrosis due to nematode infestation were not studied in this work. The damage of the root cortex after nematode infestation over time both in susceptible and resistant wheat genotypes should be investigated in future studies along with the population density in the damaged and undamaged root tissue parts.

The root has three dimensional architecture and some layers cannot be visualised using 2D photomicrography. A three dimensional image of the nematode infested root or imaging of the root's different layers could shed more light on the activity of *P. thornei* inside the wheat root over the time of plant growth and inoculation. Temporal and spatial factors that influence wheat root-*P. thornei* interactions could be investigated in future studies with confocal and two photon microscopy.

In conclusion, this study of wheat –*P. thornei* interactions has provided comprehensive understanding on the reproductions of *P. thornei* inside the root tissues over time, under the growing conditions used here . The less proportion of juveniles inside resistant wheat genotypes indicated that there are possibly less eggs hatching into juveniles, which was supported by less egg deposition inside resistant wheat root tissues over time. The cumulative effect of both reduced nematode reproduction and egg deposition were highly significant ($P \leq 0.05$) at 8 weeks PNI and onwards. Therefore 8 weeks PNI could be a critical time point in glasshouse condition at which to understand the wheat defence mechanism against *P. thornei* infestation.

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Conflict of interest: The authors declare no conflict of interest

List of Figures

Figure 1. Total number of *Pratylenchus thornei* (a) per g root and (b) per root system of wheat genotypes Janz (red), Gatcher (blue) and QT8343 (green) at 1 to 12 weeks after inoculation, and (c) regression relationships between total *P. thornei* per root sysem and time after inoculation :

Gatcher $Y = 0.4499X + 4.161, R^2 = 0.92, P < 0.0001, df = 13$

Janz $Y = 0.3701X + 4.407, R^2 = 0.83, P < 0.0001, df = 13$

QT8343 $Y = 0.1719X + 4.2701, R^2 = 0.79, P < 0.0001, df = 13$

Where Y is ln (total *P. thornei* / root system+1) and X is time after inoculation (weeks).

Points are plotted as $\log_e$ transformed values on left Y axis with back transformed values on right Y axis. Bar makers represent lsd (*P*= 0.05) in (a) and (b), and ±SEM in (c)

Figure 2. Population densities of four life stages (juvenile J2, J3, J4 and adult) of *P. thornei* per g root and per root system of three wheat genotypes Janz (red), Gatcher (blue) and QT8343 (green) from 1 to 12 weeks post nematode inoculation (a) J2/g roots (b) J2/root system (c) J3/g roots (d) J3/root system (d) J4/g roots (e) J 4/root system (f) adults/g roots (g) adults/root system. Graphs are presented with the mean transformed values displayed on left vertical axis and appropriate l.s.d. bar, and equivalent back transformed values on the right vertical axis. Bar marker = lsd (*P*=0.05).

Figure 3. Acid fuchsin stained *Pratylenchus thornei* vermiform stages and eggs inside susceptible (Janz and Gatcher) and moderately resistant (QT8343) wheat roots at 8 weeks post nematode inoculation. A, B, Gatcher; C, D, Janz; E, F; QT8343. Scale bar 100 µm (a-f)

Figure 4. Number of *P. thornei* eggs present in the roots of wheat cultivars Gatcher, Janz and QT8343 (a) eggs per root system averaged over 2 and 4 week time points after inoculation, (b) eggs per 35 cm length of root averaged over 8 and 12 week time points. Bar markers represent lsd at P =0.05. BTM means back transformed means (from log (x +1) transformation).

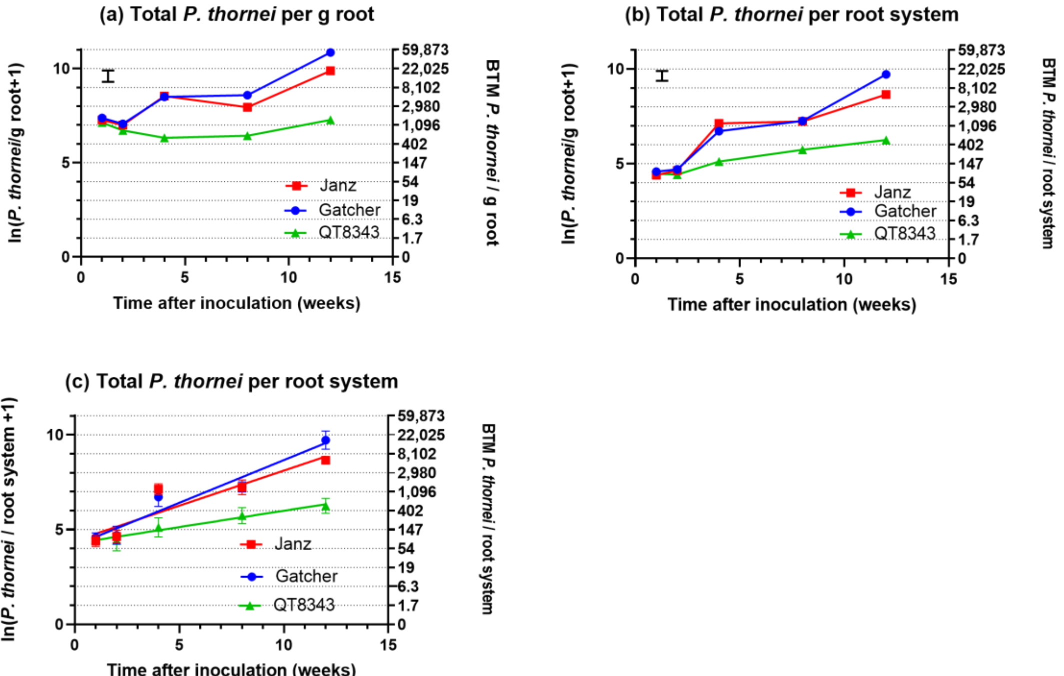
Figure 5. *Pratylenchus thornei* and eggs after acid fuschsin staining (a) (with a magnified view of egg on the right side (b) attached to damaged root cortex of Gatcher at 8 weeks PNI.

Figure 6. Phloroglucinol (PG) staining for lignin deposition of uninoculated treatments of wheat root cross sections at 10 days of growth on agar a) anatomy of a wheat root cross section (unstained) (b) Gatcher (c) Janz (d) QT8343. Scale bar (a-c) 50 µm.

Figure 7. Root cross section of wheat genotypes a) Gatcher, (b) Janz and (c) QT8343, stained for phenolics with 2 amino-ethyl-di phenyl borinate (2 APB) and examined under a compound microscope with bright field (BF) (left image) and Fluorescein isothiocyanate (FITC) filter (right images) for uninoculated control samples (top images) and *P. thornei* inoculated images (bottom images), respectively, at 8 weeks post nematode inoculation

Figure 8. Protein content of roots of wheat genotypes Gatcher, Janz and QT8343 inoculated with *P. thornei* (+) or not (-) at (a) 2 weeks, bar marker= lsd ($P \leq 0.05$) =5.44. The protein content is expressed as bovine serum albumin equivalent (BSAE). Solid colours are the uninoculated treatments whereas the patterns are inoculated treatments. N=3, value =mean

5. **Images, graphs and tables**



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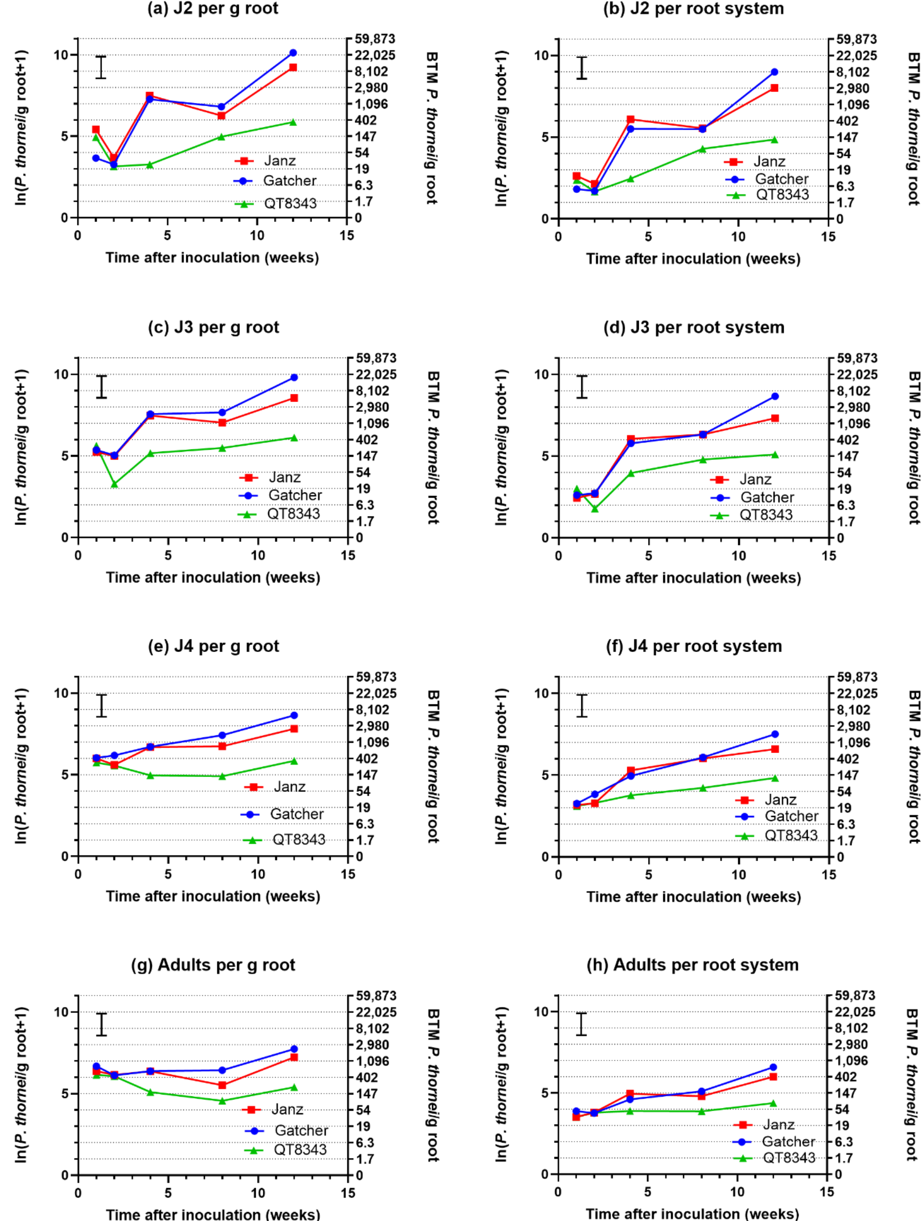

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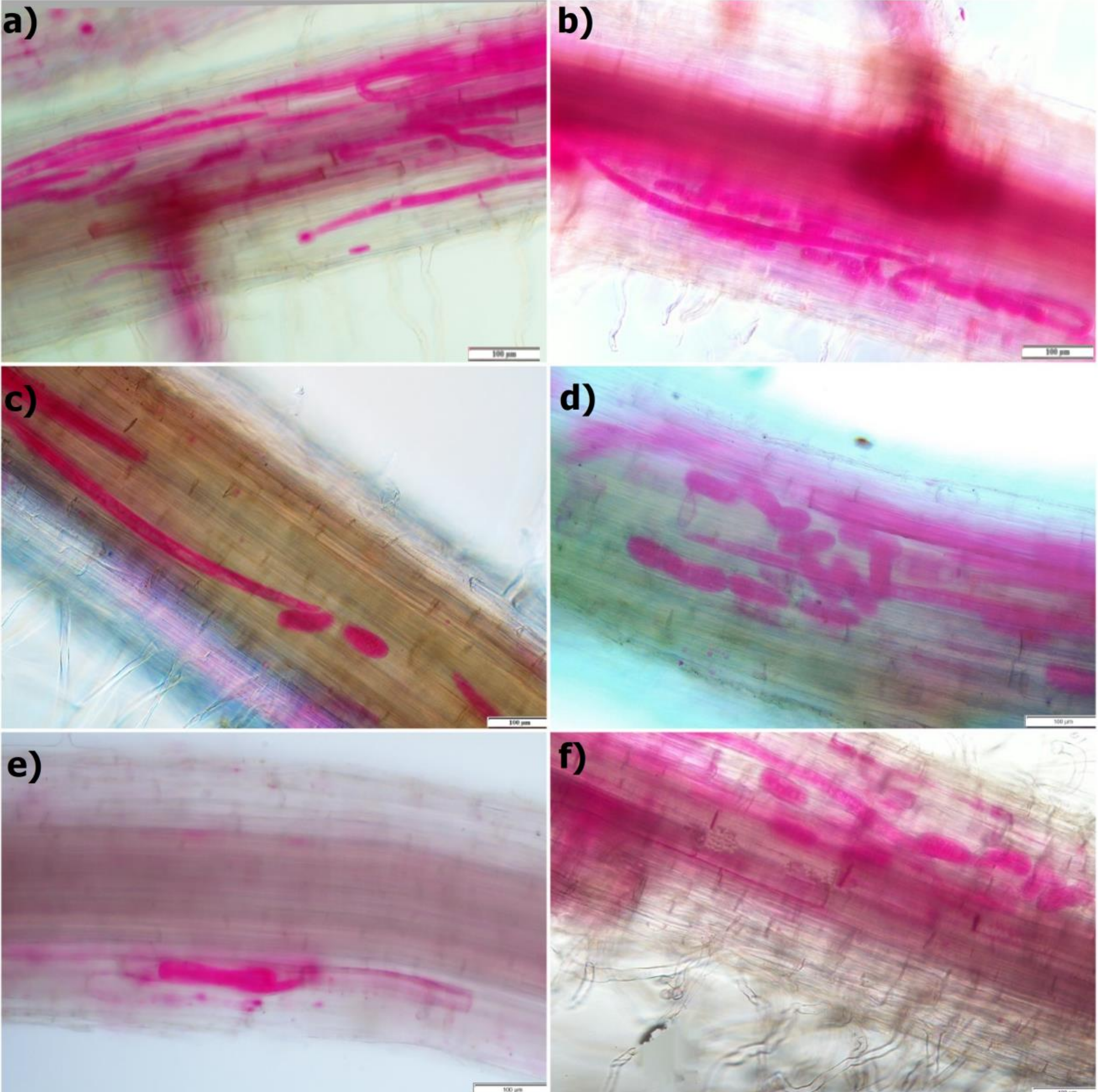

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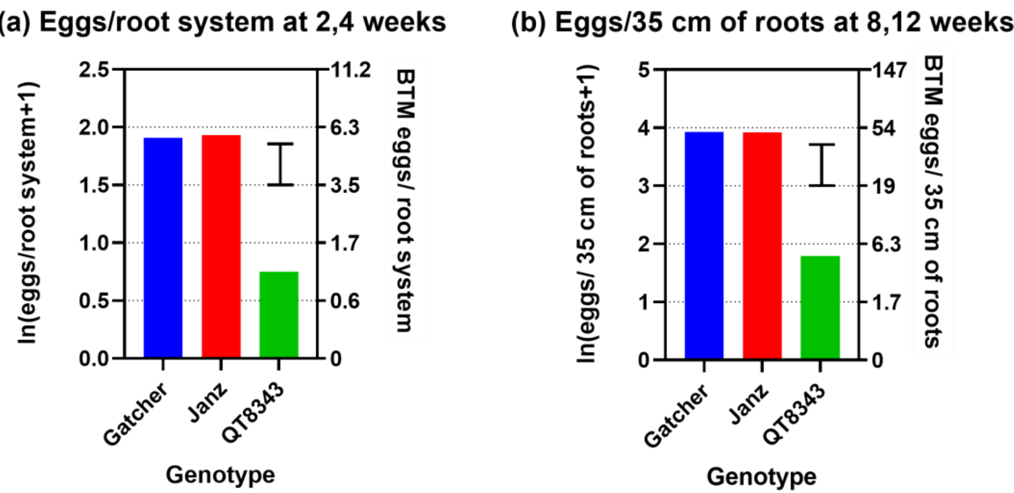

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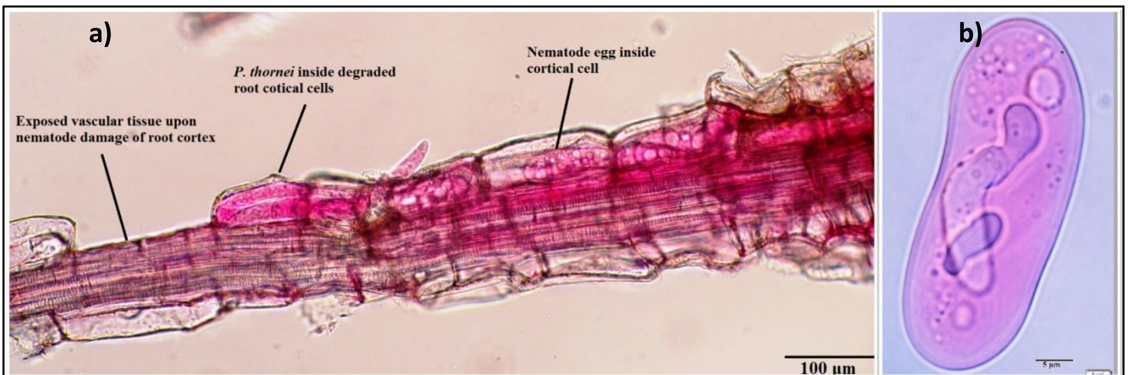

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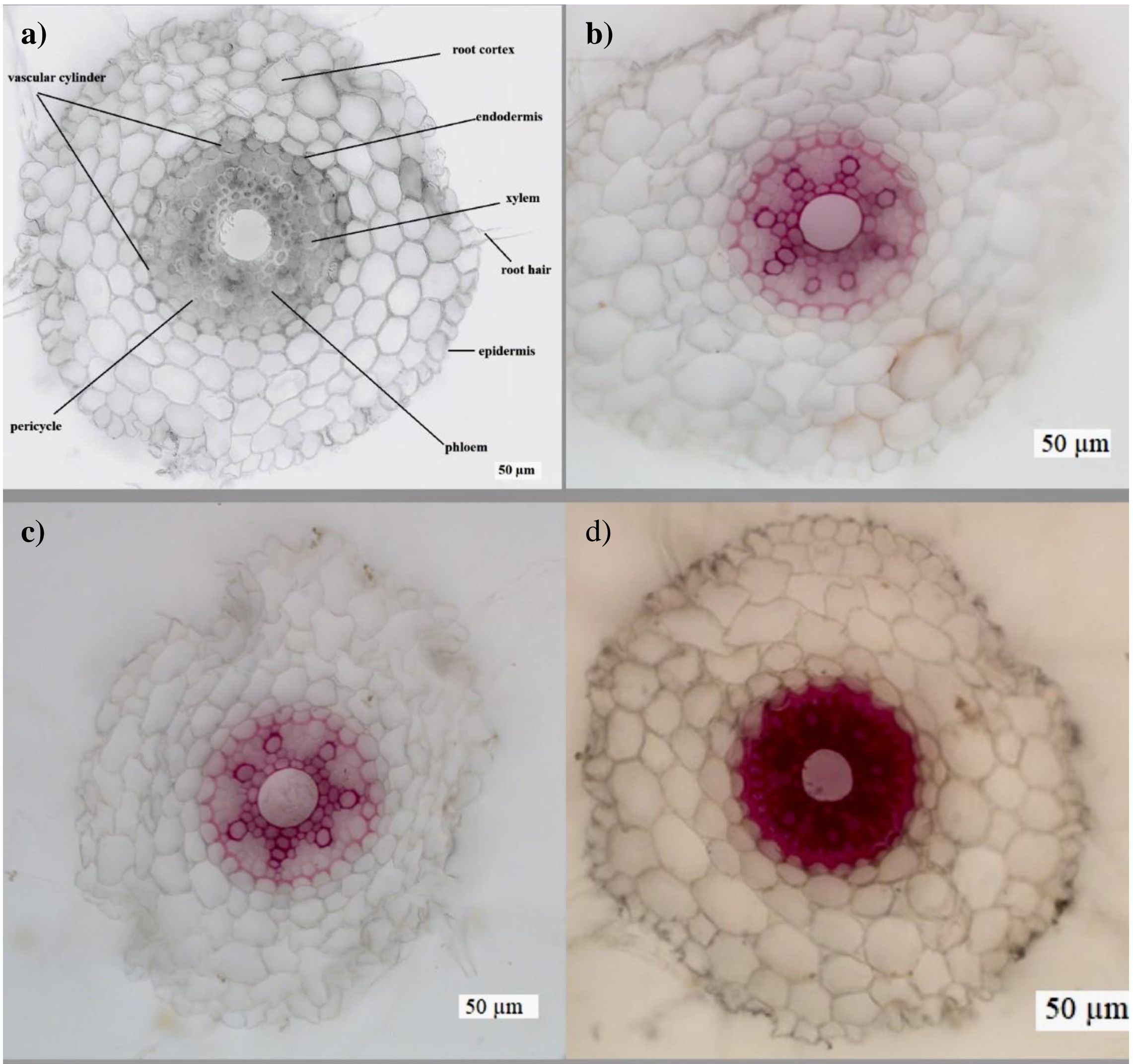

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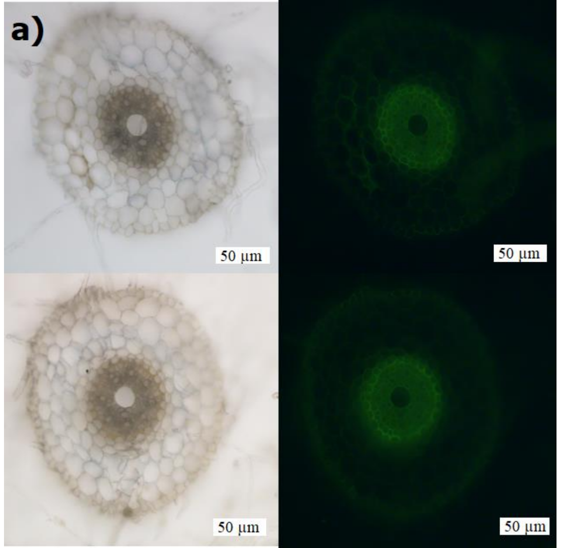

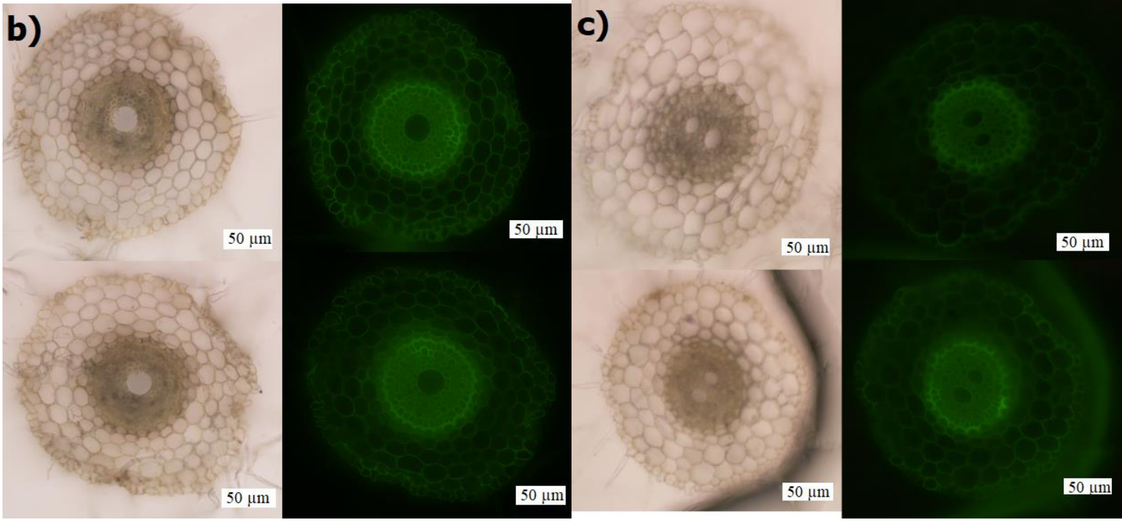

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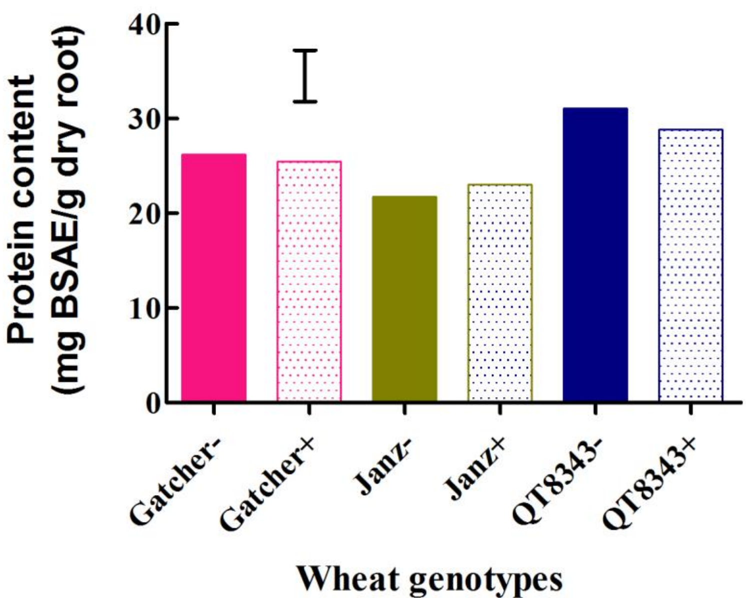

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CHAPTER 3

CONSTITUTIVE AND INDUCED EXPRESSION OF TOTAL PHENOL AND PHENOL OXIDASES IN WHEAT GENOTYPES RANGING IN RESISTANCE/SUSCEPTIBILITY TO THE ROOT-LESION NEMATODE *PRATYLENCHUS THORNEI*

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Rahaman MM, Zwart RS & Thompson JP (2020) Constitutive and induced expression of total phenol and phenol oxidases in wheat genotypes ranging in resistance/susceptibility to the root-lesion nematode *Pratylenchus thornei*. *Plants* 9, 1-17. (Q1; Impact Factor: 2.762; SNIP: 1.273) doi:10.3390/plants9040485

In this study, wheat genotypes ranging from resistant to very susceptible to *P. thornei* were used to investigate the level of total phenols and phenol oxidases, polyphenol oxidase (PPO), and peroxidase (POD) expressed in root tissues when grown in the presence and absence of *P. thornei* over time (2 to 8 weeks). Different wheat resistance sources, namely, GS50a and derived lines and synthetic hexaploid CPI133872 and derived lines, were used for the study. The methods for PPO and POD assays were optimised for microplate reader to test multiple sample at a time under the same experimental condition. Nematodes were extracted at 10 weeks both from the wheat root and soil and the proportion of life stages were recorded. Findings from this study provide insights into the possible role of total phenol and phenol oxidases in wheat genotypes both from the aspects of resistance and susceptibility to *P. thornei*.

[Supplementary materials associated with this article are attached in Appendix B]

Article

Constitutive and Induced Expression of Total Phenol and Phenol Oxidases in Wheat Genotypes Ranging in Resistance/Susceptibility to the Root-Lesion Nematode *Pratylenchus thornei*

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Abstract: Plant-derived phenolic compounds contribute to the defense against various pathogens, including root-lesion nematodes (*Pratylenchus* spp.). However, there are no reports on the role of phenolic compounds in wheat (*Triticum aestivum*) against *Pratylenchus thornei*. In this study, wheat genotypes ranging from resistant to very susceptible to *P. thornei* were used to investigate the level of total phenols and phenol oxidases, polyphenol oxidase (PPO), and peroxidase (POD) expressed in root tissues when grown in the presence and absence of *P. thornei* over time (2–8 weeks). Higher constitutive levels of total phenols were found in resistant synthetic hexaploid wheats CPI133872 (576 μg gallic acid equivalent (GAE)/g root) and CPI133859 (518 μg GAE/g root) at 8 weeks after sowing, compared with moderately resistant and susceptible genotypes (192 to 390 μg GAE/g root). The activity of PPO was induced in resistant (CPI133872) and moderately resistant (GS50a and its derivate QT8343) genotypes, becoming maximal at 4 weeks after *P. thornei* inoculation. The activity of POD was induced in CPI133872 at 6 weeks after *P. thornei* inoculation. Different genetic sources of resistance to *P. thornei* showed diverse defense mechanisms and differences in timing responses. The combined effects of total phenols and oxidative enzymes could be important for defense against *P. thornei* in some resistant wheat genotypes.

Keywords: *Pratylenchus thornei*; wheat; resistance; defense response; plant phenols; polyphenol oxidase; peroxidase; microplate reader for enzyme assay

1. Introduction

The root-lesion nematode *Pratylenchus thornei* is a plant-parasitic nematode that causes yield loss in wheat (*Triticum aestivum*) in many countries [1]. It is one of the major threats to wheat production in the subtropical grain region of eastern Australia [2]. *Pratylenchus* spp. are migratory endoparasites that secrete cell wall degrading enzymes, such as cellulase, glucanase, and pectate lyase, and, together with stylet thrusting and body movement, they penetrate the epidermis of root cells to feed and migrate within the plant root cortex [3]. The feeding, migration, and multiplication of nematodes damages the root system, resulting in the poor uptake of water and nutrients by the plant, which in turn results in yield loss [4,5]. The life cycle (egg, J2, J3, J4, and adult) of *Pratylenchus* is completed within 45 to 60 days [3,6,7], resulting in exponential multiplication of the nematode population densities within the growing season of a susceptible wheat crop [8].

The most effective management strategy for *P. thornei* is the use of resistant wheat cultivars [9,10]. Resistant host plants retard nematode reproduction in roots, reducing the nematode population densities in the soil to attack subsequent crops [9]. No wheat genotype completely prevents the

reproduction of *P. thornei*. Hexaploid wheat genotypes ($2n = 6x = 42$) with high levels of resistance to *P. thornei* have been identified, including GS50a, a selection from the susceptible wheat cultivar, Gatcher [11], West Asian and North African wheat landraces [12], Iranian wheat landraces [13], and synthetic hexaploid wheat genotypes [10,14,15]. Furthermore, resistance to *P. thornei* has also been identified in tetraploid ($2n = 4x = 28$, *Triticum turgidium* subsp. *durum* (AABB genomes) and diploid ($2n = 2x = 14$, *Aegilops tauschii* (DD genome) and *Triticum urata* (A^mA^m genome) genome donors of hexaploid wheat [16,17]. Zwart et al. found that the inheritance of resistance in wheat is additive and polygenic [18]. Several quantitative trait loci (QTL) linked to *P. thornei* resistance have been identified in the aforementioned germplasm [14,15,19,20]. However, investigations into the mechanisms of resistance conferred by the quantitative trait loci (QTL) in wheat against *P. thornei* are limited.

The first insights into the biochemical resistance mechanisms in wheat to *P. thornei* suggest that defense responses occur post-penetration of the roots because both resistant and susceptible genotypes were penetrated by approximately similar numbers of nematodes [21]. However, *P. thornei* reproduction rate was found to be significantly less in moderately resistant genotypes than in susceptible genotypes at 16 weeks post-nematode inoculation (PNI) [8]. Thus, upon successful penetration by *P. thornei*, resistance mechanisms of the wheat plant come into play preventing the reproduction and/or feeding of the nematodes. Defense mechanisms activated in the plant root system could include changes in cellular morphology [22] or the production of biochemical compounds such as primary and secondary metabolites, pathogenesis-related proteins such as chitinases, β-1,3 glucanases, peroxidases, and/or lipid transfer proteins [23–25]. These changes in defense can be constitutive, such as pre-existing physical barriers or phytoanticipins, or induced phytoalexins that are activated following nematode penetration. Linsell et al. proposed that the defense in resistant wheat against *P. thornei* is constitutive [21]. However, the constitutively expressed biochemical molecules responsible for the defense were not identified in that study.

Phenols are widespread secondary metabolites in plants that have been identified as important compounds in the defense of plants to pathogens [26,27], including defense against certain root-lesion nematode species, namely *P. penetrans, P. coffeae,* and *P. zeae* [28–32]. Phenols are mainly produced through the shikimic acid–phenylpropanoid pathway and range from simple phenols, such as cinnamic acid, to complex phenol polymers such as lignin [33]. Matern and Kneusel proposed that phenol is a rapidly synthesized biomolecule after microbial infection, which can be polymerized by oxidative enzymes into cell walls as lignin [34]. Phenols also help to neutralize reactive oxygen species (ROS) that are produced in plants as an immediate defense against pathogenic attack [35]. The reduction in elevated levels of ROS is important for healthy plant cells after preliminary defense. In addition, the migration of nematodes inside the plant root system can cause root cells to produce free phenols, which react with the plant oxidative enzymes polyphenol oxidase (PPO) and peroxidase (POD) to form toxic quinone [27]. Phenols are also known to be involved in plant pigmentation, growth, signaling molecules, and reproduction [33,34,36].

Increases in phenolic compounds and activity of PPO and POD enzymes have been reported to be both constitutive and induced defense mechanisms in plants against root-lesion nematode infection [35]. Studies on the interaction of banana cultivars with *P. coffeae* showed higher levels of defensive enzymes PPO and POD in both inoculated and non-inoculated treatments of resistant cultivars in comparison to susceptible cultivars, indicating that these enzymes are part of the constitutive defenses of plants [30,36,37]. On the other hand, the amount of the phenolic compound chlorogenic acid and PPO increased significantly in resistant tomato root infected by *P. penetrans*, indicating an induced defense response [38].

There is a lack of knowledge on the role of phenolic compounds and oxidative enzymes PPO and POD in the defense responses in wheat against *P. thornei*. Understanding the constitutive or induced expression of total phenolic compounds in resistant and susceptible wheat genotypes will provide important insights into a possible resistance mechanism of wheat against *P. thornei*. This study focuses on two genetically different sources of *P. thornei* resistance, CPI133872 and GS50a [18], and

susceptible wheat genotypes (Janz and Gatcher), and aims to gain insights into possible resistance mechanisms in wheat against *P. thornei* by investigating constitutive and induced levels of (i) total phenols, (ii) PPO activity, and (iii) POD activity, at different time points after nematode inoculation. The relationship between total phenol levels and resistance level is further explored in a larger number of wheat genotypes, ranging from resistant to very susceptible to *P. thornei*.

2. Results

2.1. Experiment 1: Accumulation of Total Phenols Over Time

The concentration of total phenols in the roots of both resistant and susceptible genotypes increased over time from 2–8 weeks post-nematode inoculation (PNI), with non-inoculated treatments decreasing at 6 weeks before increasing again by 8 weeks (Figure 1). The resistant genotype CPI133872 had significantly higher ($p < 0.05$) concentrations of total phenols than GS50a and QT8343 (a derivate of GS50a) and susceptible genotypes (Janz and Gatcher) for both inoculated and non-inoculated treatments at all time points. The concentration of total phenols for non-inoculated CPI133872 ranged from 506.3 µg gallic acid equivalent (GAE)/g root at 2 weeks to 802.2 µg GAE/g root at 8 weeks. Similarly, the concentration of total phenols for inoculated CPI133872 ranged from 493.0 µg GAE/g root at 2 weeks to 725.4 µg GAE/g root at 8 weeks. Contrastingly, all other genotypes ranged from 279.0–403.4 µg GAE/g root at 2 weeks to 451.8–628.9 µg GAE/g root at 8 weeks.

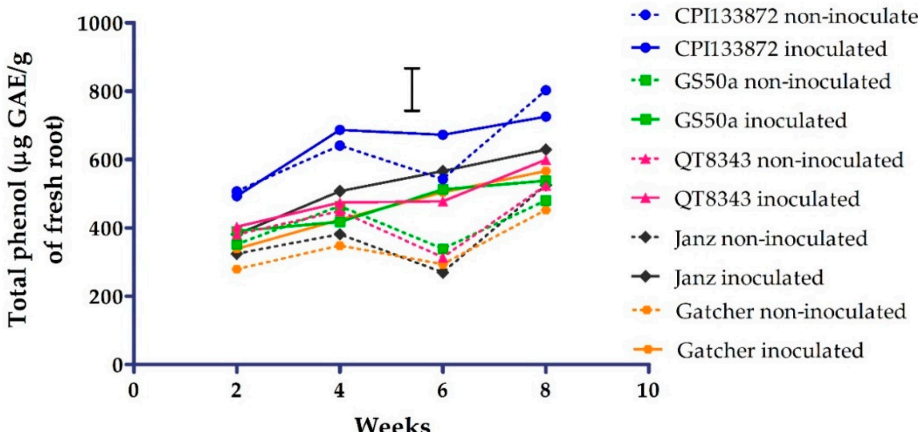

Figure 1. Total phenols (µg gallic acid equivalent (GAE)/g of fresh root) from 2–8 weeks in the roots of *Pratylenchus thornei* inoculated (solid line) and non-inoculated (dotted line) resistant wheat genotypes—CPI133872 (blue), GS50a (green), and QT8343 (red)—and susceptible wheat genotypes—Janz (black) and Gatcher (orange). The values are the means of three replicates. The bar marker indicates the least significant differences (LSD) = 154 ($p = 0.05$) for the interaction genotype* *P. thornei* * time.

2.2. Experiment 2: Constitutive Levels of Total Phenols in Gnotobiotic Conditions

The high level of total phenols in non-inoculated resistant genotype CPI133872 was confirmed in an assay conducted under gnotobiotic conditions. Total phenols in non-inoculated treatments for CPI133872 (719 µg GAE/g root) were significantly higher ($p < 0.05$) than all other non-inoculated genotypes, which ranged from 377 µg GAE/g for susceptible genotype Gatcher to 435 µg GAE/g root for moderately resistant genotype QT8343 (Figure 2).

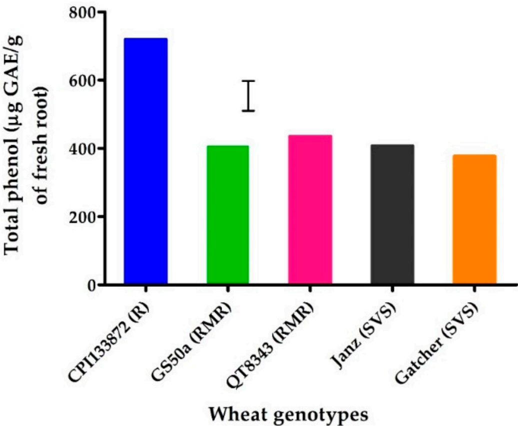

Figure 2. Total phenols (µg gallic acid equivalent (GAE)/g of fresh root) at 3 weeks in the roots of resistant wheat genotypes—CPI133872 (blue), GS50a (green), and QT8343 (red)—and susceptible wheat genotypes—Janz (black) and Gatcher (orange)—grown on agar in gnotobiotic conditions. The values are the means of three replicates. The bar marker indicates LSD = 84 (p = 0.05). The *Pratylenchus thornei* resistance rating of each wheat genotype is shown in parenthesis—R = resistant, MR = moderately resistant, S = susceptible, and VS = very susceptible.

2.3. Experiment 3: Total Phenols in Wheat Genotypes Ranging in Resistance/Susceptibility to P. thornei

The evaluation of the concentration of total phenols in 21 wheat genotypes ranging from resistant to very susceptible at 8 weeks PNI, and including genotypes with several different sources of *P. thornei* resistance, revealed a significantly higher ($p < 0.05$) level of total phenols in the two synthetic hexaploid wheat genotypes, CPI133872 (576 µg GAE/g root) and CPI133859 (518 µg GAE/g root), compared to all other wheat genotypes for both inoculated and non-inoculated treatments (Figure 3).

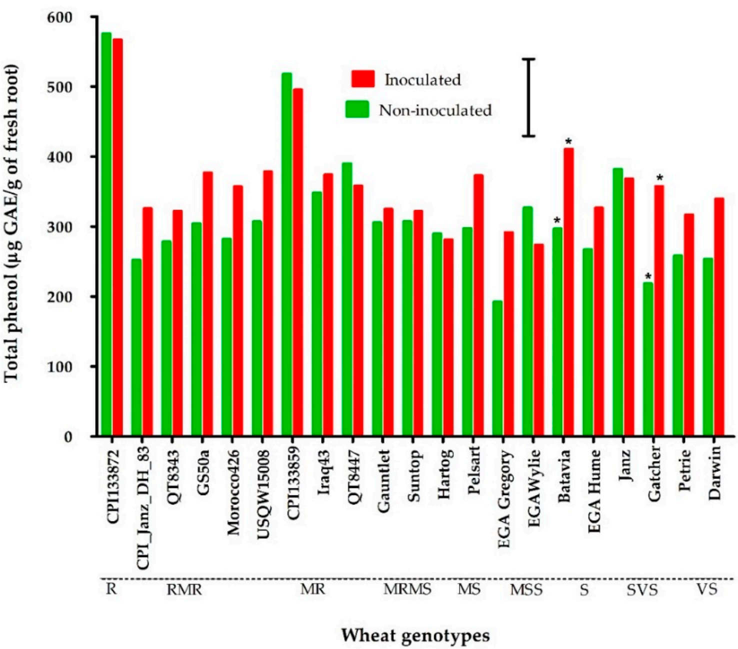

Figure 3. Total phenols (µg gallic acid equivalent (GAE)/g of fresh root) at 8 weeks in the roots of *Pratylenchus thornei* inoculated (red) and non-inoculated (green) wheat genotypes ranging in resistance and susceptibility to *Pratylenchus thornei*. The values are the means of three replications. * represent a statistically significant difference ($p < 0.05$) between inoculated and non-inoculated treatments of a genotype. The bar marker indicates LSD = 110 (p = 0.05) for the interaction genotype* *P. thornei*. The resistance rating of each wheat genotype is shown along the X-axis.

All other non-inoculated treatments of moderately resistant genotypes, namely GS50a and its derivatives QT8343 and QT8447, landraces originating from the Middle East (Morocco426 and Iraq43), and breeding lines derived from CPI133872 (CPI133872_Janz_DH083 and USQW15008) did not accumulate significantly different levels of total phenols (252–390 µg GAE/g root) than the susceptible genotypes (192–382 µg GAE/g root). The same trend was seen for inoculated treatments, with all sources of resistance (other than CPI133872 and CPI133859) not significantly different ($p < 0.05$) in the level of total phenols than the susceptible genotypes, with levels of total phenols ranging from 274–411 µg GAE/g root (Figure 3). In general, the concentration of total phenols for each genotype did not differ significantly between inoculated and non-inoculated treatments, except for two susceptible genotypes, Gatcher and Batavia, where the inoculated treatment was significantly higher ($p < 0.05$) in total phenols than the non-inoculated treatment: Gatcher (219 µg GAE/g root for non-inoculated and 358 µg GAE/g root for inoculated) and Batavia (297 µg GAE/g root for non-inoculated and 411 µg GAE/g root for inoculated).

2.4. Polyphenol Oxidase Enzyme Activity

Polyphenol oxidase enzyme activity in the roots of resistant and susceptible wheat genotypes varied significantly ($p < 0.05$) over time from 2–8 weeks PNI. At 2 weeks PNI, PPO activity was significantly ($p < 0.05$) higher in only the CPI133872 inoculated treatment (108 tyrosinase equivalent (TE)/0.1 g root) compared with the CPI133872 non-inoculated treatment (55 TE/0.1 g root). For all other genotypes at 2 weeks there were no significant differences ($p < 0.05$) between inoculated and non-inoculated treatments. The activity of PPO increased in inoculated and non-inoculated treatments for all genotypes at 4 weeks and then decreased by 6 weeks and continued to decrease further by 8 weeks (Figure 4). At 4 weeks PNI, the inoculated treatments of the resistant genotype CPI133872 and moderately resistant genotypes GS50a and QT8343 were significantly ($p < 0.05$) higher in PPO activity (103–135 TE/0.1 g root) than the non-inoculated treatments of these genotypes (66–94 TE/0.1 g root). Contrastingly, for susceptible genotypes, no significant differences were found between inoculated (69–85 TE/0.1 g root) and non-inoculated treatments (52–70 TE/0.1 g root). For the susceptible genotype Janz, there were no significant differences between inoculated and non-inoculated treatments at any time point from 2–8 weeks PNI. For the susceptible genotype, Gatcher, the only significant difference ($p < 0.05$) between treatments was recorded at 6 weeks PNI, between inoculated (86 TE/0.1 g root, the highest PPO activity level recorded for this genotype) and non-inoculated treatments (50 TE/ 0.1 g root) (Figure 4).

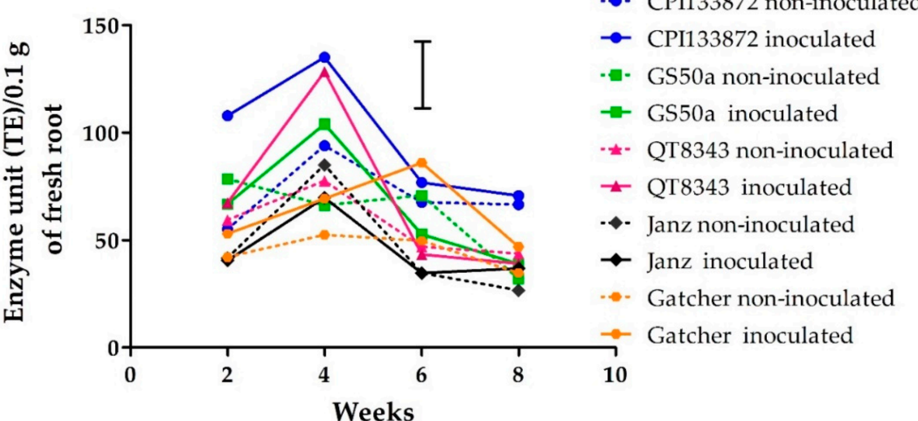

Figure 4. Polyphenol oxidase activity (tyrosinase equivalent (TE)/0.1 g fresh root) from 2–8 weeks in the roots of *Pratylenchus thornei* inoculated (solid line) and non-inoculated (dotted line) resistant wheat genotypes—CPI133872 (blue), GS50a (green), and QT8343 (red)—and susceptible wheat genotypes—Janz (black) and Gatcher (orange). The values are the means of three replicates. The bar marker indicates LSD = 33 ($p = 0.05$) for the interaction genotype* *P. thornei* * time.

2.5. Peroxidase Enzyme Activity

Peroxidase enzyme activity varied significantly ($p < 0.05$) over time between inoculated and non-inoculated treatments for only the resistant genotype CPI133872. The activity of POD increased between 2 and 4 weeks for both inoculated and non-inoculated treatments of all genotypes and then decreased at 6 and 8 weeks, for all genotypes except CPI133872 (Figure 5). The highest levels of POD enzyme activity were recorded at 4 weeks for non-inoculated (59 horse radish peroxidase equivalent (HRPE)/0.1 g root) and inoculated treatments (54 HRPE/0.1 g root) of CPI133872. At 6 weeks, the inoculated treatment of CPI133872 maintained high levels of POD activity (51 HRPE/0.1 g root), whereas the non-inoculated treatment of CPI133872 decreased significantly ($p < 0.05$) (31 HRPE/0.1 g root) (Figure 5).

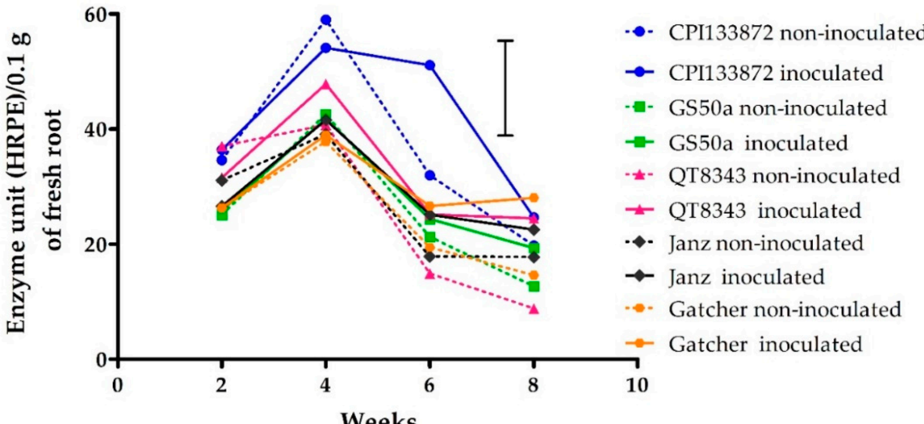

Figure 5. Peroxidase activity (horse radish peroxidase equivalent (HRPE)/0.1 g fresh root) from 2–8 weeks in the roots *Pratylenchus thornei* of inoculated (solid line) and non-inoculated (dotted line) resistant wheat genotypes—CPI133872 (blue), GS50a (green), and QT8343 (red)—and susceptible wheat genotypes—Janz (black) and Gatcher (orange). The values are the mean of three replicates. The bar marker is LSD = 17 ($p = 0.05$) for the interaction genotype* *P. thornei* * time.

2.6. Nematode Quantification

The presence of *P. thornei* inside the root tissue was confirmed in all inoculated treatments by staining and microscopic observation. Nematodes were also extracted at 10 weeks PNI. The total number of *P. thornei*/g of dry root were greatest in Gatcher followed by Janz, QT8343, CPI133872, and GS50a. Similar results were obtained for *P. thornei*/kg of soil and roots (Table 1). All nematode life stages (J2, J3, J4 and adults) were observed present in both the stained root samples and the extracts of soil samples of for all genotypes at 10 weeks PNI.

Table 1. Total *Pratylenchus thornei* numbers (natural log transformed mean value) in different wheat genotypes at 10 weeks post-nematode inoculation.

Genotype	ln (*P. thornei*/g of Dry Root + 1) (Back Transformed Mean))	ln (*P. thornei*/kg of Soil and Root + 1) (Back Transformed Mean)
CPI133872	7.859 ^{b} (2588)	8.295 ^{b} (4004)
GS50a	7.783 ^{b} (2399)	8.536 ^{b} (5095)
QT8343	8.074 ^{ab} (3210)	8.733 ^{b} (6204)
Janz	8.472 ^{ab} (4779)	9.285 ^{ab} (10775)
Gatcher	8.997 ^{a} (8079)	10.018 ^{a} (22426)

Means followed by the same letter in a column are not significantly different by LSD ($p < 0.05$).

There was no substantial difference in the proportion of life stages among Gatcher, Janz, and QT8343 at 10 weeks PNI. However, some differences in the proportion of life stages per gram of dry root were recorded in Gatcher compared to GS50a and CPI133872 (Figure 6). No contamination of *P. thornei* in non-inoculated treatments was recorded.

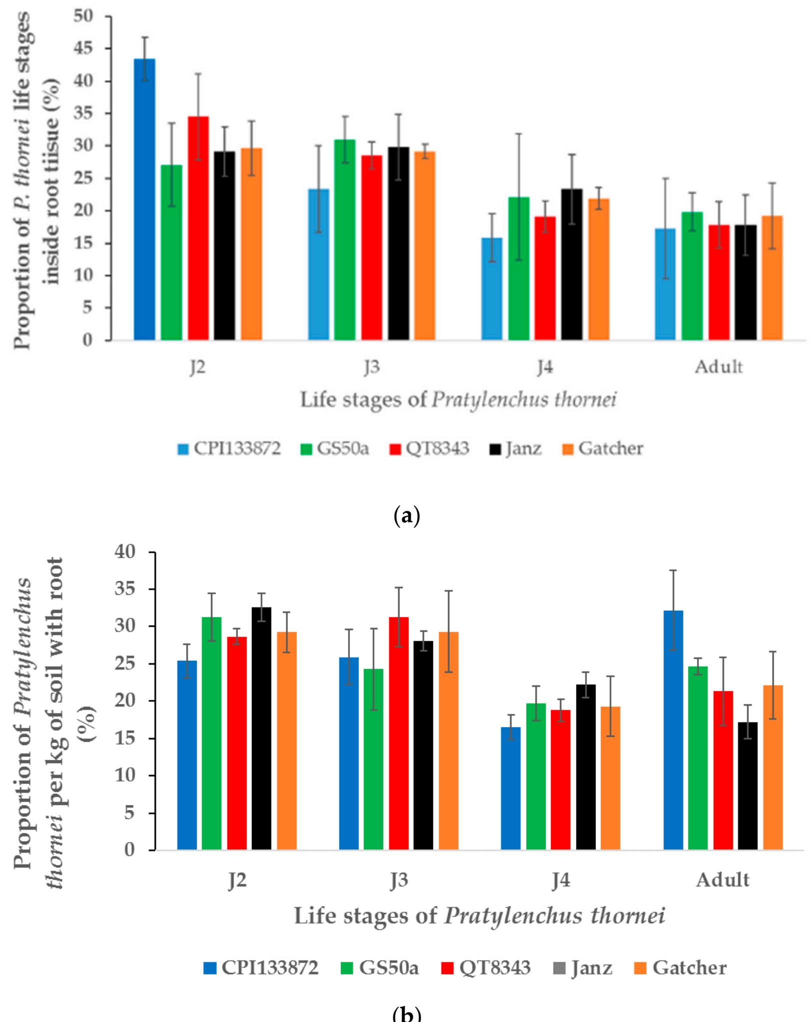

Figure 6. Proportion of *Pratylenchus thornei* life stages (**a**) inside roots of different wheat genotypes and (**b**) per kg of soil and root. The values are the mean ± standard error.

2.7. Determination of Dry Shoot Weight and Fresh Root Weight

No significant differences ($p < 0.05$) were observed in the mean dry shoot weights (Figure S1) and fresh root weights (Figure S2) of inoculated and non-inoculated treatments for any of the wheat genotypes.

3. Discussion

This is the first study to investigate the defensive role of total phenol content and phenol oxidative enzymes (PPO and POD) in wheat against *P. thornei*. In this study, high constitutive total phenol content in the resistant synthetic hexaploid wheat line CPI133872 was confirmed in three independent experiments. Similarly, high constitutive expression of total phenols was found in another resistant synthetic hexaploid wheat line CPI133859. However, moderately resistant genotypes derived from CPI133872, namely CPI133872_Janz_DH83 (a double haploid line from the cross between CPI133872 and Janz) and its derivative USQW15008 (an advanced breeding line from CPI133872_Janz_DH83)

did not have higher levels of total phenols than susceptible genotypes at 8 weeks. A similar trend was found in the moderately resistant genotypes GS50a and GS50a-derived lines QT8343 and QT8447, as well as moderately resistant landrace wheats Iraq43 and Morocco426, in which the phenol content was not significantly different from the susceptible genotypes. These results suggest that phenolic compounds may play a role in contributing to *P. thornei* resistance in the synthetic hexaploid lines but not in the other sources of *P. thornei* resistance evaluated in this study.

The synthetic hexaploid wheats CPI133872 and CPI133859 were developed by the International Maize and Wheat Improvement Center (CIMMYT) and share a common durum wheat parent (AABB genome) but different *A. tauschii* parents (DD genome) [10,39]. Studies on the inheritance of *P. thornei* resistance in these two synthetic hexaploid wheats revealed the minimum number of effective genes to be in the range of four to six genes [39], with CPI133872 and CPI133859 sharing at least one common resistance gene [18]. Resistance in GS50a has been determined to be genetically different from that of CPI133872 and CPI133859 [11,18]. High constitutive total phenol content could be an inheritable component of resistance that was not transferred or alternatively was suppressed when CPI133872 was crossed with Janz.

The significant ($p < 0.05$) decline of total phenols in non-inoculated treatments at 6 weeks in Experiment 1 may be due to the changes in plant developmental stage [40]. During this period, wheat is, generally, at the booting and heading stage depending on the genotype [41]. Iannucci et al. also reported that the concentration of phenolic compounds in the rhizosphere soil of wild oat (*Avena fatua*) varied with the age of the plant [42]. A sharp decline in phenolic compounds has been reported in that study during stem elongation and early booting stage [42]. However, the total phenol content at 6 weeks PNI in inoculated treatments for both resistant and susceptible genotypes did not decline, contrary to the non-inoculated treatments. This suggests that *P. thornei* infestation might induce changes in phenylpropanoid pathways to keep the phenol production at an increased level irrespective of the changes in the developmental stage of wheat genotypes. Rahman et al. recently identified key enzymes of phenylpropanoids, such as phenyl ammonium lyase and chalcone synthase, as candidate genes in QTL regions on chromosomes 2BS and 6DS for resistance to *P. thornei* in the synthetic derived wheat line Sokoll [43]. Initial QTL mapping studies reported the co-location of resistance to *P. thornei* in Sokoll on chromosomes 2BS and 6DS with QTL for resistance to *P. thornei* in CPI133872 [14,19]. However, more recent mapping studies have identified kompetitive allele specific PCR (KASP) markers that select for the CPI133872-derived resistance, but do not select for Sokoll-derived resistance, suggesting that *P. thornei* resistance in these synthetic hexaploid wheat genotypes is controlled by different genes [44] and therefore with possibly different resistance mechanisms.

Both constitutive and induced phenolic compounds have been previously reported to be responsible for defense in host plants against various root-lesion nematodes [35–38]. Backiyarani et al. reported that total phenol in non-inoculated resistant banana genotypes was higher than in the non-inoculated susceptible genotype, suggesting potential constitutive biochemical defensive against *P. coffeae* infestation [30]. Constitutive phenols were also responsible for the inhibition of burrowing nematode *Radopholus similis* infection in banana cultivars [45]. The amount of the phenolic compound chlorogenic acid and PPO increased significantly in the roots of resistant tomato infected by *P. penetrans*, indicating an induced defense response [46].

Increases in the levels of phenol oxidative enzymes in plants wounded and/or infected by pathogens is well documented. As key enzymes acting on the phenylpropanoid pathway, PPO and POD could play important roles in plant resistance to nematodes in several ways; (a) reaction with plant phenolics to form toxic quinone, (b) the production of reactive oxygen species that can act on pathogens or initiate defensive gene expression, (c) reduction in the availability of cellular protein to plant pathogens, (d) crosslinking phenolic compounds into lignin and other cell wall polymers to strengthen cell walls as a physical barrier to pathogens, and possible formation of brown melanin polymer crosslinking phenols in presence of cellular proteins and amino acids [47–50]. In our study, levels of PPO and POD were higher in CPI133872, GS50a, and QT8343 at 4 weeks PNI than in the

susceptible genotypes Janz and Gatcher. Combined effects of oxidative enzymes and total phenols at 2 and 4 weeks PNI could be a key factor for defense in *P. thornei* resistant and moderately resistant wheat genotypes. Higher levels of phenols, PPO and POD, at 2 and 4 weeks PNI could be a factor contributing to the superior resistance of CPI133872 to *P. thornei*.

Comparative enzyme assays of PPO and POD in the roots of different wheat genotypes were performed using a microplate reader to measure absorbance. Microplate readers have not been commonly used previously for enzyme assays of plant extracts [51]. The concentration of substrates and enzyme extracts was optimized to obtain results in the linear range of enzyme kinetics, and the results were expressed according to standard enzyme activities instead of the international unit (IU) of enzyme activities. A major benefit of using a microplate reader in comparison to cuvette assays is that multiple samples can be analyzed at the same time, which is required for analyzing plant samples with a high number of treatments and biological replicates. Moreover, due to the speed and reproducibility of the procedure, multiple assays can be performed under the same controlled conditions.

Resistance in wheat against *P. thornei* occurs post nematode penetration of the wheat root [21,52]. Significant differences in *P. thornei* numbers between resistant and susceptible wheat genotypes can be reliably found at later times (16 weeks PNI) of plant growth, in which time several life cycles of the nematodes contribute to the exponential increase in nematode numbers [8]. The time points and the tissue location of total phenolic compounds could be very important factors for providing defense against *P. thornei*. Lignin biosynthesis is linked with phenylpropanoid metabolism, which leads to total phenol expression [53]. Higher contents of the oxidative enzymes polyphenol oxidase and peroxidase in moderately resistant genotypes at 2–4 weeks PNI could enhance the polymerization of the phenol monomer monolignol to form lignin [29,54]. The conversion of monolignol into lignin not only can give rigidity to cell walls to retard nematode movement and penetration but it can also protect plant cellulose from nematode degradation [55]. Increases in the total phenols and lignin content of plant cell walls were identified as induced defense responses in resistant banana cultivars on infection by the burrowing nematode *Radopholus similis* [56]. The estimation of lignin in roots of different wheat genotypes is required to provide insights into the relationship between total phenols and lignin content over time in *P. thornei* resistant wheat genotypes.

The rapid accumulation of phenolic compounds at the infection sites of plants is a well-documented initial defense response of plants to pathogen infection. Increase in total phenolic compounds at infection sites in susceptible genotypes could be a part of a hypersensitive reaction rather than providing defense in other uninfected parts of the roots. Relatively lower PPO and POD activity in susceptible wheat genotypes might also be responsible for the ineffectiveness of the increased total phenol as a defense mechanism in susceptible genotypes. Hung et al. proposed that oxidized phenol potentially acts against the root-knot and root-lesion nematodes, not the phenol itself [46].

Nematode extraction and root staining confirmed that there was infection in the wheat roots in all inoculated samples. The numbers of *P. thornei* were significantly greater in susceptible genotypes (Gatcher and Janz) than in the resistant (CPI133872) and moderately resistant (GS50a, QT8343) genotypes when extracted at 10 weeks. The compound effect of nematode reproduction at different rates inside different wheat genotypes magnify differences at 16 weeks of plant growth [8]. The *P. thornei* inoculum used in the experiments contained mixed life stages in approximately equal proportions. Interestingly, soil plus roots from the resistant genotype CPI133872 contained a higher proportion of adult *P. thornei* than soil from other genotypes at 10 weeks PNI. Flavonoids have been shown to reduce the hatching of nematode eggs [57], suggesting that phenolic compounds could affect the proportion of nematode life stages. Furthermore, isoflavonoids are phenolic compounds that have been causally linked to the reduced motility of *P. scribneri* in resistant lima beans [58] and *P. penetrans* in lucerne [38]. The resistance of CPI133872 could result in overall fewer numbers of new juveniles, or the juveniles and adults could leave the roots due to unfavorable conditions inside resistant root tissues. Further detailed histopathology observations of the *P. thornei*–wheat interaction over time would be valuable in understanding the effect of host resistance on the nematode life stages.

Knowledge of the specific types of phenolic compounds and other secondary metabolites in resistant and susceptible genotypes at specific times of *P. thornei* infestation in future studies could further improve our understanding of the defense mechanisms. Moreover, further investigations into larger numbers of wheat lines derived from the synthetic hexaploids CPI133872 and CPI133859 would evaluate the predictive value of total phenol, or specific phenolic compounds, as well as phenol oxidative enzymes, as metabolic biomarkers [59,60] for predicting *P. thornei* resistance phenotypes. A holistic metabolomics approach is recommended for future studies.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

4.1.1. Experiment 1: The Accumulation of Total Phenols and Oxidative Enzymes (PPO and POD) Over Time

Two susceptible wheat genotypes (Gatcher and Janz) and three resistant to moderately resistant wheat genotypes (CPI133872, GS50a, and QT8343) were grown as a glasshouse experiment at the Leslie Research Facility (27°32′02″ S 151°56′09″ E), Toowoomba, Australia, in August to October. Treatments were replicated three times in a factorial randomized block design with five genotypes, two nematode treatments (inoculated and non-inoculated), and five harvest time points (2, 4, 6, 8, and 10 weeks post-nematode inoculation (PNI)).

A self-mulching black vertisolic soil was collected from Formartin, Australia (27°22′12″ S 151°25′53″ E), and pasteurized in an air: steam stream at 85 °C for 45 min [61]. The soil moisture content was determined by drying 100 g of steam-pasteurized soil at 105 °C for 48 h. Slow-release fertilizer Osmocote® Plus Trace Elements (Scott Australia, Baulkham Hills, Australia) was used at the rate of 1 g per pot of 330 g soil (oven-dried equivalent). The pots were placed on glasshouse benches fitted with a continuous bottom watering system with water tension maintained at 2 cm by a capillary system using bidim® matting (Geofabrics Australasia, Brisbane, Australia) [62]. The inoculated treatments were separated from the non-inoculated treatments by a 5-cm gap between the capillary mats to avoid cross-contamination. Under-bench heating was used to maintain soil temperature at 22 ± 4 °C, the optimum temperature for *P. thornei* reproduction [63]. Soil temperature was monitored throughout the experiment using iButtons (Thermochron®, Baulkham Hills, Australia) placed in arbitrarily selected pots at 3 cm of depth in the soil.

Two seeds were sown on a base layer of soil (80% of the total soil volume). Nematodes were added in a 10-mL liquid suspension to inoculated treatments, at the rate of 3300 *P. thornei* (J2: 23.5%, J3: 30%, J4: 16.5% adult 30%) equivalent to 10 nematodes/g of oven-dried soil per pot (70 mm square, 150 mm height). The seed and nematodes were covered with the remaining 20% soil [62]. Ten days after sowing, plants were thinned to one seedling per pot with a scalpel blade, retaining roots inside the pots. The roots of each genotype were assessed for total phenol content and oxidative enzymes (PPO and POD) at 2, 4, 6, and 8 weeks PNI. A final time-point at 10 weeks was used to assess nematode reproduction in the roots and soil for each genotype.

4.1.2. Experiment 2: Constitutive Levels of Total Phenols in Wheat Roots under Gnotobiotic Conditions

Wheat seeds of five genotypes (Gatcher, Janz, GS50a, QT8343 and CPI133872), were grown without *P. thornei* inoculation in a controlled environment growth cabinet (Bioline, Percival Scientific, IA, USA) for 3 weeks. The seeds were surface sterilized according to Wu et al. with slight modification [64]. The seeds were covered in 70% ethanol for 5 min followed by diluted bleach solution (NaOCl) (2.5%) for 15 min. The seeds were then rinsed with sterile Milli–Q® (Merck, Darmstadt, Germany) reverse osmosis water five times. The seeds were imbibed by soaking in sterile Milli–Q® water for 12 h, then placed on autoclaved petri plates containing moistened sterile filter paper and allowed to germinate for 48 h. Two-day old surface-sterilized and pre-germinated seedlings were transferred aseptically into Schott bottles (DURAN® GLS 80, DWK Life Sciences, Wertheim, Germany) containing 200 mL of

0.3% autoclaved tap water agar [64]. Four seedlings were placed on the agar per bottle. The lids were screwed on loosely and then wrapped with parafilm. The experiment was arranged in a completely randomized design with three replicate bottles for each genotype.

Light intensity inside the growth cabinet was maintained at 400 μ mole/m^2 with a photoperiod of 13 h light and 11 h dark. The intensity of the light was measured with a photosynthetically active radiation (PAR) light meter (Li-250A, Li-COR Bio-Sciences, Lincoln, NE, USA). The light wavelength was in the PAR range of 400 to 700 nm. The temperature was maintained at 22 ± 2 °C throughout the experiment.

4.1.3. Experiment 3: Total Phenols in Wheat Genotypes Ranging in Resistance/Susceptibility to *P. thornei*

Twenty-one wheat genotypes were selected to cover a range of resistance/susceptibility to *P. thornei* [65] (Table 2). The wheat genotypes were grown using the same glasshouse conditions as described for Experiment 1. Treatments were replicated three times in a split plot design with plus or minus *P. thornei* inoculation randomized to the main plots and wheat genotypes randomized in the sub-plots in a row : column design. The plants were grown in the glasshouse for 8 weeks from July to September.

Table 2. Origin and resistance rating to *Pratylenchus thornei* for wheat genotypes.

Genotype	Origin	Rating [65]
CPI133872	Synthetic hexaploid	R
CPI133872_Janz DH083	CPI133872 derived line	R-MR
QT8343	GS50a derived line	R-MR
GS50a	Selection from Gatcher	R-MR
Morocco 426	Landrace	R-MR
USQW15008	CPI133872_Janz_DH083 derived line	MR
CPI133859	Synthetic hexaploid	MR
Iraq 43	Landrace	MR
QT8447	GS50a derived line	MR
Gauntlet	Cultivar	MR-MS
Suntop	Cultivar	MR-MS
Hartog	Cultivar	MS
Pelsart	Cultivar	MS
Gregory	Cultivar	MS-S
Wylie	Cultivar	MS-S
Batavia	Cultivar	S
Hume	Cultivar	S
Janz	Cultivar	S-VS
Gatcher	Cultivar	S-VS
Petrie	Cultivar	VS
Darwin	Cultivar	VS

R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, VS = very susceptible.

4.2. Sample Collection and Storage

The plant roots from the pot experiments were washed under running tap water to remove the soil, taking care to minimize damage to the roots. The roots were then washed with sterile Milli–Q® (Merck, Darmstadt, Germany) reverse osmosis water and then blotted dry with tissue paper. The plant roots from the Schott bottles were removed from the agar and blotted dry gently with tissue paper. The fresh root weight of each plant was recorded. Small pieces (4 to 6 cm) of the roots (300 mg) from Experiments 1 and 3 were sampled for acid fuchsin staining, to check nematode infection in the roots of the collected samples. The remaining roots were placed inside 50-mL screwcap falcon tubes and immediately frozen with liquid nitrogen. Frozen roots were stored at −80 °C until analyses for total phenols and oxidative enzymes were undertaken. Whole root and soil from the last time point

(10 weeks) from Experiment 1 was stored at 4 °C and later used for nematode extraction. The plant tops were oven dried for 48 h at 80 °C to determine dry weight.

4.3. Estimation of Total Phenols

The total phenol content of the roots was determined by the Folin–Ciocalteu method [66] with slight modifications. A sample of the root material (0.1 g fresh weight) from each treatment, previously ground and homogenized in liquid nitrogen with a chilled mortar and pestle, was transferred to a fresh microcentrifuge tube. Two milliliters of 95% methanol was added to the finely ground root powder. The root powder in methanol was transferred into a 2 mL microcentrifuge tube and incubated at room temperature for 48 h. The samples were then centrifuged (Centrifuge 5424 R, Eppendorf, Hamburg, Germany) at 13,000 g for 5 min and the supernatant containing the root extract was transferred to a fresh microcentrifuge tube. One hundred microliters of root extract was mixed with 200 µL of 0.2 N Folin–Ciocalteu reagents, a mixture of phosphomolybdate and phosphotungstate (Sigma Aldrich, St Louis, MO, USA). Blank tubes were prepared using 100 µL of 95% methanol in place of the root extract. The reaction tubes were thoroughly mixed by vortexing and then incubated for 5 min at room temperature. Eight hundred microliters of sodium carbonate solution (700 mM) was added to the reaction mixture and further incubated for 2 h at room temperature in the dark. The tubes were centrifuged at 3000 g for 30 s to remove any suspended particles. Two hundred microliters of the supernatant from each sample were transferred to a 96-well microplate (Costar®, Thermo Fisher Scientific, Waltham, MA, USA). The absorbance was measured at 765 nm using a microplate reader (Fluostar Omega, BMG Labtech, Mornington, Australia). The total phenol content was determined by comparison with a standard curve for gallic acid (Sigma Aldrich, St Louis, MO, USA) in the range 0 to 200 µg/mL (Figure S3). The samples were prepared in triplicate and the best-fitted results were used for the calculation of total phenols in the root samples under the same conditions and using the same stock of reagents. Three technical replicates for each of three biological replicates were analyzed and mean values expressed as µg of gallic acid equivalent (GAE)/g of fresh root.

4.4. Preparation of Enzyme Extracts and Optimization of Protocol for Microplate Reader

Assays for PPO and POD were performed with root materials collected from Experiment 1. The concentrations of substrates for both PPO and POD were optimized prior to performing the enzyme assays with the wheat root extracts. Enzyme kinetics were tested with commercially purchased tyrosinase (Sigma Aldrich, St Louis, MO, USA, ≥1000 unit/mg of solid) with pyrocatechol as substrate for PPO activity (Figure S4) and horseradish peroxidase (Sigma Aldrich, St Louis, MO, USA, ≥250 unit/mg of solid) with guaicol as substrate in the presence of hydrogen peroxide for POD activity (Figure S5) to prepare standard curves. A regression equation in the linear range from the standard curve was used to calculate enzyme activity in the wheat root tissue samples [67]. Different concentrations of wheat root extracts were tested to find the linear range within the value of standard curve. The amount of PPO in wheat root extracts was expressed as tyrosinse equivalent (TE)/0.1 g fresh root. The amount of POD was expressed as horseradish peroxidase equivalent (HRPE)/0.1 g fresh root, respectively.

A microplate reader was used to test multiple samples at a time according to Siguemoto and Gut [51] with modification. The protocol was optimized for a microplate reader to calculate PPO activities in multiple wheat samples at a time. Enzymes were extracted from 100 mg of root powder in 0.05 M sodium phosphate buffer (NaH_2PO_4 and Na_2HPO_4) at pH 7. Root materials were ground in liquid nitrogen with a chilled mortar and pestle. The root powder was homogenized with 2 mL of phosphate buffer and kept at 4 °C overnight. The homogenate solution was centrifuged at 15,000 rpm for 15 min at 4 °C [68]. The supernatants were transferred to fresh 2 mL microcentrifuge tubes and kept at 4 °C until used for the enzyme assay. One hundred microliter of the supernatant was diluted to 1 mL with phosphate buffer. This supernatant was used as the enzyme extract. Enzyme assays were completed within 2 h of extraction. A multichannel pipette was used to pipette buffer and enzyme

extracts followed by the respective substrates of both PPO and POD assays. The assays were done in 96 well microplates (Costar®, Thermo Fisher Scientific, Waltham, MA, USA).

4.5. Polyphenol Oxidase Enzyme Assay

For the PPO enzyme assay, 33 µL of the enzyme extract was added to 100 µL of sodium phosphate buffer (pH 7). The solution mixture was kept at 25 °C for 15 min to equilibrate the temperature. Freshly prepared 0.05 M pyrocatechol (Sigma Aldrich, St Louis, MO, USA) solution was also left to equilibrate at 25 °C for 15 min. After equilibration, 67 µL of pyrocatechol was added to the buffer and the enzyme. The absorbance was taken every 30 s for 3 min at 420 nm wavelength using a microplate reader (Fluostar Omega, BMG Labtech, Mornington, Australia). A blank was set with phosphate buffer and pyrocatechol without the addition of enzymes. The mean enzyme activity for each root sample was calculated from absorbance values at 30 and 60 s using the regression equations relating enzyme activity to absorbance at those respective times (Figure S4).

4.6. Peroxidase Enzyme Assay

The peroxidase enzyme assay was performed with the same enzyme extracts as used for the PPO assay. Forty-two microliters of enzyme extract was added to 125 µL of buffer. The solutions were kept at 25 °C for 15 min to equilibrate along with freshly prepared 10 mM guiaiacol (Sigma-Aldrich, St Louis, MO, USA) and 6.4 mM hydrogen peroxide (Sigma Aldrich, St Louis, MO, USA). Then, 17 µL of equilibrated guaiacol was added to the buffer enzyme mixture followed by 17 µL of hydrogen peroxide. The absorbance of the resultant mixture was recorded at 470 nm every 30 s for 3 min. The blank was prepared with phosphate buffer and pyrocatechol without the addition of enzymes. The absorbance values at 30 and 60 s were used for the calculation of enzyme activity as per the regression equation of the standard curve at those respective times (Figure S5).

4.7. Nematode Quantification

Pratylenchus thornei were extracted from the roots and soil of the samples collected at the 10-week time point in Experiment 1, using the Whitehead tray method [69]. The roots were separated from soil, washed, and chopped into smaller pieces (1 to 3 cm) and placed on a Whitehead tray for extraction at 22 °C. The remainder of the soil samples were put on another Whitehead tray separately to the roots. The root and soil samples were incubated at 22 °C with 1 L water per tray for 7 d and 4 d, respectively, to allow the nematodes to migrate from the roots and soil into the water. A 20-µm aperture sieve was used to collect the nematodes in 28-mL sample tubes. Following nematode extraction, the root samples were oven dried at 80 °C for 48 h and the dry weight of the roots was recorded. The extracted nematodes were counted using a 1-mL Peters nematode counting chamber (Chalex Corporation, Park City, USA) under an Olympus BX53 compound microscope (Olympus, Tokyo, Japan). The nematode numbers were expressed per g of dry root and per kg of soil, respectively.

Root samples from Experiments 1 and 2 were stained with 0.1% acid fuchsin according to a method modified from Bybd et al. [70]. The roots were chopped and placed inside a stain tube [71] and 10 mL of acid fuchsin stain (0.1% *w*/*v*, prepared in 90% lactic acid solution) were added to each tube. The roots in acid fuchsin solution were heated in a boiling water bath for 2 min, washed in tap water, and then transferred to a sample tube containing 90% lactic acid solution. Three drops of 8 N HCl were added per tube to aid destaining of the root tissues. The stained nematodes in the roots were observed under an Olympus BX53 compound microscope (Olympus, Tokyo, Japan) in both bright field and differential interference contrast (DIC) modes.

4.8. Statistical Analysis

Experimental designs and data analysis were performed using R-software version 3.5.1 [72] and Genstat® for Windows™ [73]. Analysis of variance (ANOVA) was conducted for total phenol content, PPO activity, and POD activity. The significance of the differences in total phenols, PPO activity, and

POD activity was tested among genotypes and the inoculation treatments at a 5% significance level using the least significant differences (LSD).

5. Conclusions

Our study has revealed high constitutive levels of total phenols in the synthetic hexaploids CPI133872 and CPI133859. The activity of PPO was induced in resistant (CPI133872) and moderately resistant (GS50a and derivate QT8343) genotypes and was maximal at 4 weeks after *P. thornei* inoculation. The activity of POD was induced in CPI133872 at 6 weeks after *P. thornei* inoculation. Different genetic sources of resistance to *P. thornei* showed diverse defense mechanisms and differences in the timing of responses. The combined effects of total phenols and oxidative enzymes could be important for defense against *P. thornei* in some resistant wheat genotypes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/9/4/485/s1, Figure S1: Dry shoot weight (g) of *Pratylenchus thornei* inoculated and non-inoculated treatments of five wheat cultivars over the time points (2–10 weeks) Figure S2. Fresh root weight of inoculated and non-inoculated treatment of five wheat genotypes over the time points (2–8 weeks). Figure S3. Standard curve of gallic acid for total phenols estimation in wheat root samples. Figure S4 and Figure S5: Standard curves of tyrosinase and horseradish peroxidase in linear range of concentration at different time (30–180 s) of enzyme assay progression.

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CHAPTER 4

METABOLOMIC PROFILING OF WHEAT GENOTYPES RESISTANT AND SUSCEPTIBLE TO ROOT-LESION NEMATODE *Pratylenchus thornei*

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No previous studies have investigated the abundance of metabolites in susceptible and resistant wheat genotypes in response to *P. thornei* infestation. In this chapter, metabolomic profiling was performed with a resistant (CPI133872 derived line, QT16258) and a susceptible wheat (Janz) to understand the role of wheat metabolites in resistance and susceptibility to *P. thornei*. CPI133872 was found to have a higher constitutive level of total phenol in our previous study (Chapter 3) and 8 weeks PNI was found to be a critical time point to investigate defensive biochemicals in resistant wheat genotypes (Chapter 2). A detailed untargeted metabolic profiling was performed at 8 weeks PNI of the root tissue of the two wheat genotypes either inoculated or not with *P. thornei*. Untargeted liquid chromatography mass spectrometry analysis (LC-MS) of the wheat root samples was performed for separation and identification of potential metabolites providing resistance against *P. thornei*. The potential roles of significant metabolites that were higher in expression in the resistant wheat genotype were discussed. The significant metabolites which were higher in expression in susceptible Janz were also discussed in respect to susceptibility to the nematodes and hypersensitive browning reactions.

[Supplementary data associated with this article are attached in Appendix C]

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Metabolomic profiling of wheat genotypes resistant and susceptible to root-lesion nematode *Pratylenchus thornei*

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Abstract

Key message **Metabolic profiling of *Pratylenchus thornei* resistant and susceptible wheat genotypes indicates that fatty acid, glycerolipid and flavonoid classes of metabolites constitutively expressed in resistant wheat roots could reduce nematode reproduction.**

Abstract The root-lesion nematode *Pratylenchus thornei* reduces wheat production in many parts of the world. In this study the metabolic profiles of two wheat genotypes 'QT16258' (moderately resistant) and 'Janz' (susceptible) were compared at 8 weeks post inoculation with or without *P. thornei*. We performed untargeted liquid chromatography mass spectrometry analysis (LC–MS) of the wheat root samples. A total of 11,704 MS features were identified, out of which 765 MS features were annotated using in-house chemical standards. Principal components analysis (PCA) and partial least square discriminant analysis (PLS-DA) indicated dissimilarity of the metabolome between *P. thornei* resistant and susceptible genotypes. Two-way analysis of variance indicated that metabolic differences were mainly constitutive rather than induced by inoculation with *P. thornei*. Eighty-four annotated metabolites were significantly ($p \leq 0.01$) higher in relative concentration in 'QT16258' than 'Janz' and belonged to the following classes of metabolites: flavonoids, fatty acids, glycerolipids, alkaloids, tannins, nucleotides, steroid glycosides and terpenoids. Eighty-five annotated metabolites were significantly ($p \leq 0.01$) higher in relative concentration in 'Janz' than 'QT16258' and belonged to the following classes of metabolites: amino acids, sugars, flavonoids and alkaloids. Several metabolites at higher concentration in 'QT16258', including quercetin-3,4'-O-di-beta-glucoside (flavonoid), linoleic acid (fatty acid), lysophosphatidylethanolamine (glycerolipid), hirsutine (alkaloid), 1-methylsulfinylbutenyl-isothiocyanate (glucosinolate), could potentially strengthen the root cell walls to inhibit nematode penetration and/or reduce nematode motility. Some metabolites at higher concentrations in susceptible 'Janz', including phenolics, coniferyl alcohol and indole acetic acid conjugates, could be nematode attractants as well as part of a hypersensitive browning reaction to nematode invasion.

Keywords Liquid-chromatography · Mass spectrometry · Metabolome · Wheat · *Pratylenchus thornei* · Plant metabolites

Abbreviations

ANOVA	Analysis of variance
PBQC	Pooled biological quality control
FA	Fatty acid
FDR	False discovery rate
GSEA	Gene set enrichment analysis
HMDB	Human metabolome database
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
In	Inoculated
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MGMG	Monogalactosyl monoacylglycerol
ms	Milli seconds
MS	Mass spectrometry
MS2	MS–MS

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PCA	Principal components analysis
PLA	Phospholipase A
PLASMA	Plant specialized metabolome annotation
PLS-DA	Partial least squares discriminant analysis
PNI	Post nematode inoculation
QQQ	Triple staged quadrupole
QTL	Quantitative trait loci
Q-TOF	Quadrupole time of flight
RLN	Root-lesion nematode
RT	Retention time
SWATH-MS	Sequential window acquisition of all theoretical mass spectra
Un	Uninoculated
VIP	Variable importance in projection

Introduction

Wheat (*Triticum aestivum*) is cultivated as a staple cereal crop throughout the world. The root-lesion nematode *Pratylenchus thornei* is a biotic stressor that causes significant reductions in wheat yield and is widely distributed in many parts of the world, including USA, Mexico, Israel, Spain, India, Italy, Iran, Algeria, Tunisia, Morocco, Syria, Iraq, Turkey, Pakistan, the former Yugoslavia (Serbia, Croatia and Slovenia), Canada and Australia (Nicol and Rivoal 2007; Smiley and Nicol 2009). Damage to root tissue due to infestation by *P. thornei* causes water stress and nutrient deficiency in wheat plants (Thompson et al. 2012a; Whish et al. 2014). Symptoms of *P. thornei* infestation include stunted growth and reduced tillering in susceptible wheat genotypes, resulting in substantial grain yield losses of 38 to 85% (Doyle et al. 1987; Nicol et al. 2011).

The continued search for novel sources of resistance and incorporating those resistance genes into high yielding genotypes are required to cope with future demand for wheat grain and food security (Cassman et al. 2003). Sources of resistance to *P. thornei* have been identified in wild and related wheat species, synthetic hexaploid wheats and wheat landraces (Thompson and Haak 1997; Thompson 2008; Sheedy and Thompson 2009; Thompson et al. 2009; Thompson and Seymour 2011; Sheedy et al. 2012). While multiple quantitative trait loci (QTL) linked to genomic regions associated with resistance to *P. thornei* have been discovered (Schmidt et al. 2005; Zwart et al. 2006, 2010; Linsell et al. 2014a, b), limited studies have explored the mechanisms of resistance or the biochemical compounds that are responsible for defense against *P. thornei* in wheat.

Inheritance of *P. thornei* resistance in wheat is polygenic and additive (Zwart et al. 2004; Thompson et al. 2012b). Therefore, numerous biomolecules could be responsible for contributing to defense against *P. thornei*, including plant metabolites, pathogenesis related proteins (cellulase, glucanase, peroxidase, polyphenol oxidase) and/or changes in cell wall polymers. There are different types of plant metabolites, which are responsible for plant defense against biotic and abiotic stresses. The main classes of plant metabolites which play parts in plant defenses are phenolics, glycosides, terpenoids, hormones and alkaloids (Grotewold 2005). There are also various subclasses of phenolic compounds that have a common phenol ring (C_6H_5OH) in their structure. The phenol subclasses include flavonoids, phenolic acids, stilbenes and lignans (Dai and Mumper 2010). Flavonoids can be further classified into sub-classes anthocyanins, flavanones, chalcones, flavones, flavonols and isoflavonoids (Panche et al. 2016). Flavonoids have major roles in plant defense against biotic and abiotic stresses as well as growth, reproduction and pigmentation (Lev-Yadun and Gould 2008; Panche et al. 2016). Different classes of plant metabolites reported to exhibit nematicidal properties against *Pratylenchus* spp. (Ohri and Pannu 2010; Chin et al. 2018) in various plant species include flavonoids, terpenoids, alkaloids (Wuyts et al. 2006; Soriano et al. 2004a) and steroidal hormones (Soriano et al. 2004b).

Phenolic metabolites have been reported to decrease egg hatching and motility, and increase mortality of *Pratylenchus* spp. (Rich et al. 1977; Baldridge et al. 1998; Wuyts et al. 2006). The nematicidal effect of 2-phenyl ethyl glucosinolate was observed in *Brassica* spp. root and leaf tissue against *Pratylenchus neglectus* (Potter et al. 1998). Benzoxazinoid glycosides in wheat were proposed as defensive chemicals against *P. neglectus* (Frew et al. 2018). A flavone glycoside has been found to inhibit *P. neglectus* penetration in oats (Soriano et al. 2004a). Beside phenolic compounds, the steroid compound 20-hydroxyecdysone (20E), which was induced by methyl jasmonate in spinach (*Spinaciaoleracea* cv. Avon), reduced *P. neglectus* reproduction (Soriano et al. 2004b). *Pratylenchus neglectus* exposed to 20E, either directly in vitro or induced in spinach at concentrations above 4.2×10^{-7} M, suffered abnormal moulting, immobility, reduced infection, impaired development and death.

Resistance to *P. thornei* occurs in wheat roots after penetration, as the roots of susceptible and resistant wheat genotypes had equal numbers of *P. thornei* penetrate up to 16 days post nematode inoculation (PNI) (Linsell et al. 2014a, b). *Pratylenchus thornei* has five life stages, namely the juvenile stages J1 (that occurs inside the egg), J2, J3, J4 and the adult stage (Thompson et al. 2017). *Pratylenchus thornei* migration and maturation were supressed and egg deposition and hatching were inhibited post penetration in a resistant synthetic hexaploid wheat derivative 'Sokoll' compared with the moderately susceptible wheat genotype 'Krichauff' (Linsell et al. 2014a, b). Biochemical defense against *P. thornei* in 'Sokoll' was thought to be constitutive in nature (Linsell et al. 2014a, b), however, the biochemical compounds were not identified. Recently, it was suggested

that plant metabolites in resistant wheat genotypes could play an important role in defense against *P. thornei* (Rahaman et al. 2020; Rahman et al. 2020). High levels of constitutive total phenols were found in the roots of synthetic hexaploid wheat lines 'CPI133872' and 'CPI133859', both resistant to *P. thornei* (Rahaman et al. 2020). Candidate genes for resistance involved in the synthesis of plant metabolites (phenylalanine ammonia-lyase, chalcone synthase, isoflavone reductase-like protein, flavonoid 3′-hydroxylases) were reported in 'Sokoll' (Rahman et al. 2020). However, the relative abundance of specific metabolites in resistant and susceptible wheat genotypes and their possible roles in plant defense against *P. thornei* are currently unknown.

In this investigation, untargeted liquid chromatography mass spectrometry (LC–MS) was performed to comprehensively profile the largest possible array of metabolites in the roots of a *P. thornei*-resistant and a *P. thornei*-susceptible wheat genotype after growth with or without inoculation with *P. thornei*. The differential metabolite profiles provide insight into potential mechanisms for plant defense against *P. thornei* and support the discovery of biomarkers for resistance.

Materials and methods

Plant materials

Two wheat genotypes, 'QT16258' (moderately resistant) and 'Janz' (susceptible) were used in *P. thornei* time course and metabolomic profiling experiments. 'QT16258' is an advanced breeding line developed by the University of Southern Queensland, Toowoomba, Australia (J. Sheedy, pers. comm.). 'QT16258' is derived from a doubled haploid line from the 'CPI133872'/'Janz' mapping population (Zwart et al. 2005) that was further crossed with Australian bread wheat cultivars 'Wylie' and 'Gregory'. The resistance to *P. thornei* in 'QT16258' originates from the resistant synthetic hexaploid wheat 'CPI133872'. 'CPI133872' is a relatively unadapted synthetic hexaploid and it was preferable for this study to have its resistance introgressed into agronomically adapted 'Cook' type germplasm for comparison with the susceptible widely adapted Australian wheat 'Janz'. 'Janz', 'Wylie' and 'Gregory' are all 'Cook' type wheats and 'QT16258' has three doses of these in its pedigree as well as the introgressed *P. thornei* resistance from 'CPI133872' (J. Sheedy, pers. comm.).

Nematode culture

The culture of *P. thornei* used was originally isolated from soil collected from Formartin (latitude 27.46°S, longitude 151.43°E), Queensland, Australia and maintained by open pot culture on wheat (Thompson et al. 2020). Inoculum of *P. thornei* for the experiments was obtained from open pot cultures on the very susceptible wheat 'Petrie', grown for 16 weeks in a pasteurized vertisol. The nematodes were extracted from the roots and soil from the cultures using the Whitehead tray method (Whitehead and Hemming 1965), and collected as a water suspension using a 20-μm aperture sieve. Nematode life stages were counted using a 1-mL Peters nematode counting chamber (Chalex Corporation) under an Olympus BX53 compound microscope. The percentage of life stages of the inoculum were J2, 21.6%; J3, 34.3%; J4, 23.3%; adult, 20.8%.

Pratylenchus thornei time course experimental design

To monitor nematode reproduction over time, 'QT16258' and 'Janz' were grown in the presence of *P. thornei*, with destructive sampling at 6, 8 and 16 weeks PNI. The treatments were replicated four times for each time point, and arranged in a completely randomized design produced using R-software version 3.5.1 (R Core Team 2017).

Metabolomic profiling experimental design

'QT16258' and 'Janz' were grown for a single time point (8 weeks PNI). The experiment consisted of four treatments: (i) 'QT16258' inoculated with *P. thornei*; (ii) 'QT16258' uninoculated; (iii) 'Janz' inoculated with *P. thornei*; and (iv) 'Janz' uninoculated. One replicate consisted of one plant per pot. A total of nine replicates per treatment were grown in a completely randomized block design produced using R-software version 3.5.1 (R Core Team 2017). Three replicates were allocated for initial optimization and validation of the reverse phase high performance liquid chromatography (HPLC)-mass spectrometry (MS) protocol. The six remaining replicates were used for untargeted metabolic profiling using the optimized HPLC–MS protocol.

Plant growth conditions

Washed coarse sand was autoclaved twice at 121 °C for 30 min. A self-mulching vertisolic soil, collected from Formartin, Australia was pasteurized in air:steam at 85 °C for 45 min (Thompson 1990). The sand and soil was mixed in a ratio of 1:2 (v/v) as used by Wuyts et al. (2007). The final moisture content of the sand and soil mixture was determined by oven drying at 105 °C. Slow-release fertilizer Osmocote® Plus Trace Elements (Scott Australia) was mixed in at the rate of 2 g per 550 g (dry weight equivalent) of sand-soil mixture per pot.

The wheat seeds were surface sterilized using 70% ethanol for 5 min, followed by diluted bleach solution (2.5%

NaOCl) for 15 min, and kept in a sterile Petri plate for 72 h according to Wu et al. (2000) with slight modification as mentioned in Rahaman et al. (2020). Three-day old, germinated seedlings were transplanted into individual pots containing 550 g (oven dry weight equivalent) of sterilized sand-soil mixture. After five days, 16,500 *P. thornei* of mixed life stages were added to each pot in a suspension of 30 mL water, by making a 30 mm deep hole in the soil close to the seedlings. Plastic saucers (140 mm) were placed underneath individual pots. The pots were watered every alternate day to water holding capacity using Milli Q® water (Thompson and Haak 1997). The same batch of *P. thornei* inoculum and the same wheat seed lots were used for both the resistance time course and the metabolomics experiments.

Plants for the metabolomics experiment were grown in parallel with the resistance time course experiment in the same controlled environment growth cabinet (Bioline, Percival Scientific Inc.). Light intensity inside the growth cabinet was maintained at 400 µmol m^{-2} s^{-1} with 13 h light and 11 h dark. The light wavelength was in the photosynthetically active radiation range (400–700 nm). The temperature was maintained at 22 ± 2 °C throughout the experiments and monitored using an iButton (Thermochron®) positioned in an arbitrarily selected experimental pot.

Plant growth parameters

The growth parameters of the wheat plants were recorded at the time of sample collection for each experiment including plant height (cm), number of tillers, number of spikes, Zadoks score (Zadoks et al. 1974) and shoot dry weight. Plant tops were dried at 80 °C for 48 h to determine the shoot dry weight.

Sample collection and storage

For the resistance time course experiment, at 6, 8 and 16 weeks PNI, the plant tops were removed and the roots and soil were stored in the pots at 4 °C until further processing. The roots from each pot were washed thoroughly on a 250-µm aperture sieve under running tap water to remove the soil and recover the roots. Two thirds of the washed roots were sub-sampled for nematode extraction and one third for acid fuchsin staining.

The root samples from the metabolomic profiling experiment were harvested at 8 weeks PNI. For this, the plant roots were washed free of the soil-sand mixture under running tap water, then rinsed with sterile Milli–Q® water and blotted dry with tissue paper, taking care to minimize damage to the roots. Plant tops were then removed and the roots were placed in 50 mL screw cap tubes, which were frozen immediately with liquid nitrogen and stored at − 80 °C, until the LC–MS analyses were undertaken.

In preparation for LC–MS analysis, the root samples were removed from the − 80 °C freezer and immediately freeze-dried (Alpha 2–4 LDplus, Martin Christ Gefrietrocknungsanlagen GmbH) for 72 h, the time when test samples had reached constant weight.

Pratylenchus thornei resistance time course experiment

Nematode quantification

For the time course experiment, *P. thornei* were extracted from the root sub-samples using a modified Whitehead tray method (Whitehead and Hemming 1965). The root samples for nematode extractions were incubated at 22 °C with 1 L water per sample for 7 days, to allow the nematodes to migrate from the roots into the water. A 20-µm aperture sieve was used to collect the nematodes in ~ 15 mL of water in 28 mL sample tubes. After nematode extraction, the roots were oven dried at 80 °C for 48 h and dry weights were recorded. The extracted nematodes were counted in life stage categories as previously described (Thompson et al. 2017) using a 1-mL Peters nematode counting chamber (Chalex Corporation) under an Olympus BX53 compound microscope. The numbers of each life stage and total nematodes were expressed per gram of dry root weight. The remaining root sub-samples were stained with acid fuchsin following a modified method of Bybd et al. (1983) as described in Rahaman et al. (2020). The stained roots were observed under an Olympus BX53 compound microscope at magnification of × 200, and the presence of eggs and nematodes were recorded. Images of the stained *P. thornei* inside roots were obtained with a digital camera (DP26) using CellSens Olympus imaging software version 1.9.

Statistical analysis of resistance time course experiment

Genstat® for Windows™ (VSN International 2014) and R software version 3.5.1 (R Core Team 2017) were used for statistical analysis of plant growth parameters and extracted nematode numbers by applying analysis of variance (ANOVA) and least significant difference test (at 5% significance level). The nematode data were log transformed ($\ln(x+1)$), where x equals *P. thornei* per g of dry root) to satisfy the assumptions of normality and homogeneity of residual variances.

Metabolomic profiling experiment

Metabolite extraction

All chemicals used in the study were HPLC and MS grade. Acetonitrile (LC–MS grade) was obtained from Merck

(Australia), and all other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA). The water used was Milli Q® (Merck, Darmstadt, Germany) grade.

Aliquots of 30 mg freeze-dried root tissue were weighed into cryomill tubes (Precellys 24, Bertin Technologies). Then 500 µL methanol solution (100%) and 20 µL internal standard ($^{13}C_6$-Sorbitol/$^{13}C^{15}N$-Valine) were added to each tube. Samples were homogenized using a cryomill (Precellys 24, Bertin Technologies), operating at − 10 °C and programmed to run three repeated shaking steps for 45 s intervals at 6400 rpm, with a 30 s pause in between (6400–3 × 45 × 30). The tubes were vortexed, and shaken at 800 rpm for 15 min at 20 °C on a thermomixer (Eppendorf, Hamburg, Germany), and then centrifuged (Heraeus Fresco 17, Thermo Fisher Scientific) at 13,000 rpm for 15 min. The supernatant was transferred to a new 1.5 mL micro centrifuge tube. The pellet was used for a second extraction by adding another 500 µL aliquot of methanol solution. The tubes were vortexed and centrifuged at 13,000 rpm for 15 min and the supernatant was combined with the first extract. The combined extracts were vortexed and 0.2 mL aliquots were transferred to HPLC vials for the LC–MS analysis.

The HPLC–MS methods were optimized using two pooled samples (three biological replicates of both genotypes pooled together) of uninoculated treatments and inoculated treatments in separate pools. These were validated using in-house standards, prior to analysis of the final 24 samples (six biological replicates of four treatments). A small aliquot of each biological replicate was taken and the 24 samples in the study set were mixed together to form a pooled biological quality control (PBQC) mixture. The PBQC was used to check the quality and reproducibility of the HPLC–MS method by producing 11 PBQC samples, which were run for the duration of the analytical study in random order along with the individual biological replicates.

Liquid chromatography mass spectrometry (LC–MS) conditions

Untargeted metabolomic profiling was performed with the Metabolomics Australia platform (Metabolomics Australia, University of Melbourne, Victoria) using HPLC–MS. The methodology used in the study was adapted from Tsugawa et al. (2019). The analysis was performed using a liquid chromatography (triple) quadrupole time of flight mass spectrometer (Sciex 6600, AB Sciex, Framingham, MA, USA). The mass spectrometer was fitted with a Duo (positive/negative) electrospray ionization source coupled to a HPLC system (Agilent Technologies, Santa Clara, CA, USA). The HPLC system comprised a thermostated auto sampler, vacuum degasser, binary pump and column oven. The liquid chromatographic separations of the metabolites were achieved using an acquity bridged ethyl hybrid C18 column (1.7 µm, 2.1 mm × 100 mm, Waters, Rydalmere NSW, Australia). The column temperature was maintained at 40 °C. Sample injection volume to the HPLC system was 10 µL. Two solvents were used as the mobile phase for the chromatographic separation of the metabolites from the wheat root extracts; mobile phase A (water including 0.1% formic acid) and mobile phase B (acetonitrile including 0.1% formic acid). The mobile phases were run through a gradient program using the two solvents according to the settings: 99.5% mobile phase A/0.5% mobile phase B at 0 min; 99.5% mobile phase A/0.5% mobile phase B at 0.1 min; 20% mobile phase A/80% mobile phase B at 10 min; 0.5% mobile phase A/99.5% mobile phase B at 10.1 min; 0.5% mobile phase A/99.5% mobile phase B at 12.0 min; 99.5% mobile phase A/0.5% mobile phase B at 12.1 min; 99.5% mobile phase A/0.5% mobile phase B at 15.0 min. Different flow rates were used for the gradient elution of the metabolites in the wheat root extracts. The flow rate was 0.3 mL min^{-1} at 0 min, 0.3 mL min^{-1} at 10 min; 0.4 mL min^{-1} at 10.1 min; 0.4 mL min^{-1} at 14.4 min and 0.3 mL min^{-1} at 14.5 min.

The mass spectrometry was set at positive and then negative ion modes with the data collection range of *m/z* 70–1700 time of flight-mass spectrometry (TOF–MS). The other settings of the instrument were as follows; curtain gas: 25 psi; gas 1: 20 psi; gas 2: 15 psi; ion spray voltage 5 kV; source temperature: 450 °C; accumulation time 25 ms. Sequential window acquisition of all theoretical (SWATH) MS2 (MS–MS) spectra were acquired in 25 m/*z* increments from 100 to 1000 m/*z* (35 isolation windows), and accumulation time of 25 ms. The collision energy for each window was set to 30 with a spread of 10. The final MS features obtained in the separation, elution and detection, were characterized by mass-to-charge ratio (*m/z* ratio) and retention time (RT). The peaks were normalized according to internal standards ($^{13}C_6$-Sorbitol/$^{13}C^{15}N$-Valine) and the weight of the sample analyzed.

Data processing and statistical analyses

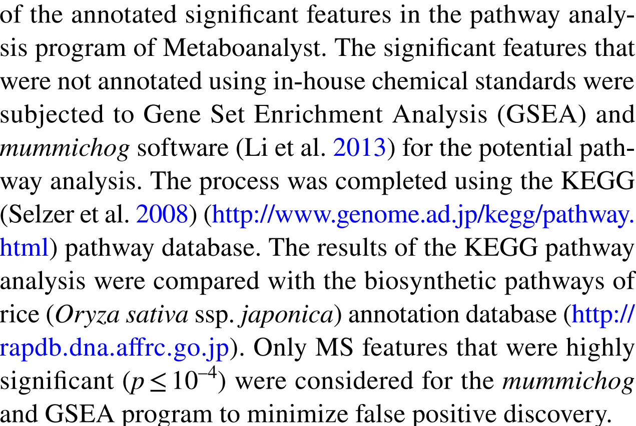
Mass spectrometry features were obtained using the algorithm of MS-DIAL (http://prime.psc.riken.jp) and peak intensity tables were prepared (Tsugawa et al. 2015). Statistical analysis was performed using the web based metabolomics tool, Metaboanalyst 4.0 (https://www.metaboanalyst.ca/), designed at University of Alberta (Edmonton, Alberta, Canada) (Chong et al. 2019). The data were filtered (25% for 500–1000 MS features, 40% for over 1000 MS features) to remove MS features with very small values or near constant values across the data set, or of low repeatabilty based on QC samples. The retained data were log transformed and auto scaled prior to univariate and multivariate statistical analysis. Principal components analysis (PCA) and partial

least square discriminant analysis (PLS-DA) were performed for visualizing interrelations between genotypes and *P. thornei* inoculation treatments. The top 20 metabolites with higher abundance on most positive and negative values of PC1 and PC2 axes were obtained from the PCA loading files. The PLS-DA model was tested using permutation testing and cross validated.

Analysis of variance (ANOVA) was performed on the four groups of treatments; 'QT16258' uninoculated, 'QT16258' inoculated, 'Janz' uninoculated and 'Janz' inoculated. Univariate ANOVA was used to find significant features that differed on the basis of genotype by analysing 'QT16258' and 'Janz' with inoculated and uninoculated treatments grouped for each genotype. Fisher's protected LSD tests ($p \leq 0.01$) were performed to find significant features in the four groups of treatments. A two-way ANOVA was conducted using two factor analysis in Metaboanalyst 4.0 to determine which significant features may be induced by inoculation or expressed constitutively. Two-tailed Student's *t* tests were performed using the following treatment comparisions:'QT16258' uninoculated and 'QT16258' inoculated, 'Janz' uninoculated and 'Janz' inoculated, 'QT16258' uninoculated and 'Janz' uninoculated, 'QT16258' inoculated and 'Janz' inoculated. Volcano plots were produced for groups of different treatments to visualize the *p* value (≤ 0.05) and fold change (≥ 2) value. The most significant features among different treatments were tabulated using fold change analysis, *p*-value and t-score. Results of both ANOVA and t-tests were used for potential pathway analysis. A heat map was produced using annotated metabolites identified by two-way ANOVA ($p \leq 0.001$), by clustering of the genotypes and their features using Pearson correlation coefficient as similarity measure, and the Ward metric for the clustering algorithm.

The MS features were mostly annotated using an in-house library, which was prepared based on authentic chemical standards (DeHaven et al. 2012; De Souza et al. 2019), and searched on the database Plant Specialized Metabolome Annotation (PLaSMA) (http://plasma.riken.jp/). The annotation was completed based on RT and fragmentation pattern (MS2) of the MS features, and also based on accurate mass only (W/O). The annotated metabolites were categorized based on three confidence levels of identification: level 1 (confirmed compound structure), level 2 (probable compound structure), and level 4 (unequivocal molecular formula) (Schymanski et al. 2014). The unannotated 20 most significant ($p \leq 10^{-5}$) MS features based on one-way ANOVA and post hoc analysis for both 'QT16258' and 'Janz' were putatively annotated using publicly available databases for metabolite identification: Metlin (https://metlin.scripps.edu), Human Metabolome Database 4.0 (HMDB) (https://hmdb.ca/) and Mass Bank (https://massbank.eu/).

Pathway analyses were conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) ID and HMDB ID



of the annotated significant features in the pathway analysis program of Metaboanalyst. The significant features that were not annotated using in-house chemical standards were subjected to Gene Set Enrichment Analysis (GSEA) and *mummichog* software (Li et al. 2013) for the potential pathway analysis. The process was completed using the KEGG (Selzer et al. 2008) (http://www.genome.ad.jp/kegg/pathway.html) pathway database. The results of the KEGG pathway analysis were compared with the biosynthetic pathways of rice (*Oryza sativa* ssp. *japonica*) annotation database (http://rapdb.dna.affrc.go.jp). Only MS features that were highly significant ($p \leq 10^{-4}$) were considered for the *mummichog* and GSEA program to minimize false positive discovery.

Results

Plant growth parameters

At 8 weeks PNI for the metabolomic profiling experiment, 'QT16258' was at the pre-booting stage (Zadoks scale 33–39), while 'Janz' was at the spike stage (Zadoks scale 50–58). Inoculation with *P. thornei* did not significantly ($p > 0.05$) affect root biomass, shoot biomass, number of tillers and plant height in 'QT16258' and 'Janz' compared to the uninoculated treatments (data not shown). The shoot dry biomass was similar at 8 weeks in both 'QT16258' and 'Janz'. However, the shoot dry biomass was significantly ($p \leq 0.05$) higher in 'QT16258' (15.38 g) than 'Janz' (10.3 g) at 16 weeks (Fig. S1) PNI. Both the genotypes reached maturity at 16 weeks PNI (Zadoks scale 87–92).

Pratylenchus thornei time course

The presence of nematodes inside root samples harvested at 6, 8 and 16 weeks PNI confirmed infection of *P. thornei* in wheat genotypes 'QT16258' and 'Janz'. The numbers of *P. thornei*/g of dry root were significantly ($p \leq 0.05$) lower in 'QT16258' than in 'Janz' at all time points (Table 1). However, there was no significant difference in the proportion

Table 1 Total numbers of *Pratylenchus thornei* (natural log transformed mean value ln (*P. thornei*/g of dry root + 1)) and back transformed mean value (shown in parenthesis) in two wheat genotypes ('QT16258' and 'Janz') at 6, 8 and 16 weeks post-nematode inoculation

Genotype	Nematode count ln (*P. thornei* /g dry root + 1)		
	6 weeks	8 weeks	16 weeks
'QT16258'	8.30^{b} (4024)	7.94^{b} (2807)	7.00^{b} (1097)
'Janz'	9.46^{a} (12,836)	8.89^{a} (7259)	7.99^{a} (2951)

Different letters indicate significant difference ($p < 0.05$) between genotypes at that time point

of life stages (J2, J3, J4 and adult) inside the roots of 'Janz' and 'QT16258' at each time point (Fig. 1). Acid fuchsin staining further confirmed the presence of nematodes in the roots of inoculated samples (Fig. S2). Eggs and nematodes were found inside roots of both genotypes at all time points. Nematodes were observed less frequently inside roots of 'QT16258' than 'Janz' confirming the trend that was observed with the nematode extraction data at all time points (Fig. 1).

Metabolomic profiling experiment

Optimization and quality controls

The resolution of ion chromatogram peaks in both positive (Fig. S3) and negative ion mode for the optimization study was found to be appropriate to proceed with the liquid chromatography-mass spectrometry (LC–MS) analysis of individual samples. The PCA of the pooled inoculated and uninoculated samples in the optimization study showed separation of scores between the two treatments on PC1 and PC2 (Fig. S4). In the PCA plot of all samples used in the study (n = 24), PBQC replicates were found to be clustered together tightly, validating the reproducibility of the data (Fig. S5).

Extraction of mass features and chemometric analysis

Liquid chromatography mass spectrometry (LC–MS) analysis detected 6,541 MS features in positive ion mode and 5163 MS features in negative ion mode. A data matrix consisting of a total of 11,704 MS features for the 24 samples (composed of two wheat genotypes × two *P. thornei* treatments (inoculated and uninoculated) × six biological replications) was processed for statistical analyses. Out of the 11,704 MS features, 765 were identified and annotated by in-house chemical standards. There were 90 annotated metabolites that were common in both positive and negative ion mode. Data filtering in Metaboanalyst 4.0 removed non-informative MS features (25% filtering for 500–1000 MS features, ≥ 40% for over 1000 MS features) on the basis of very small values, or near constant values across the data set, or low repeatabilty based on QC samples (Table 2). Due to server limitations (< 5000 MS features), further processing of the filtered data set resulted in 4997 MS (out of 11,704 MS) features for statistical analyses. The data filtering of 765

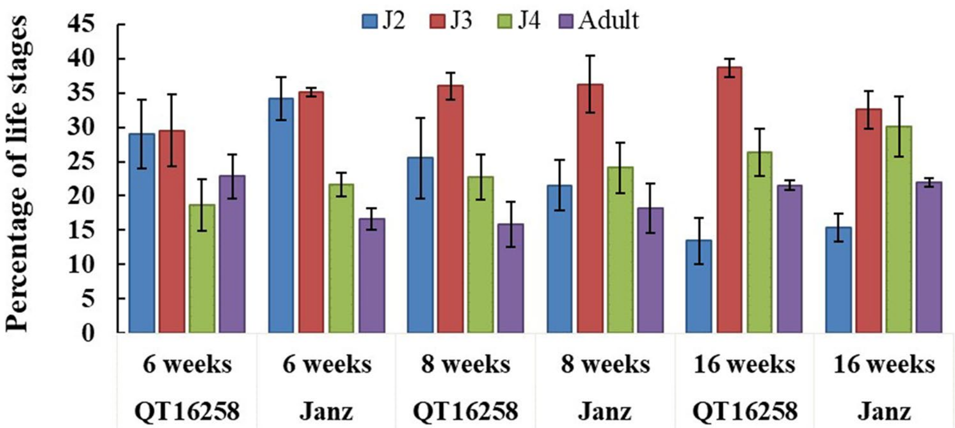


Fig. 1 Mean percentage of juvenile life stages (J2, J3, J4) and adult *Pratylenchus thornei* inside moderately resistant ('QT16258') and susceptible ('Janz') wheat roots at 6, 8 and 16 weeks post nematode inoculation. Error bars represent standard error of mean (n = 4)

Table 2 Percentage variance accounted for by the first two components (PC1 and PC2) from principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) (C1 and C2) based on 24 biological samples for different data sets of mass spectrometry (MS) features

Data set	Total MS features	MS features after data filtering	PCA*		PLS-DA*	
			PC1 (%)	PC2 (%)	C1 (%)	C2 (%)
Positive ion mode	6541	3924	21.1	13.1	20.8	8.3
Negative ion mode	5163	3097	20.9	14.5	20.6	5.7
Combined ion mode (Total)	11,704	4997	21.6	13.5	21.3	7.0
Combined ion mode (annotated metabolites only)	765	573	23.7	13.8	23.5	7.6

*PCA and PLS-DA analyses exclude the data of pooled biological quality control (PBQC) samples

annotated metabolites retained 573 annotated metabolites for statisical analysis (Table 2).

Analysis of PCA and PLS-DA plots showed that the metabolite profiles of the 'QT16258' and 'Janz' genotypes and their inoculation treatments differed, based on the MS features and annotated metabolites. The concentration of a metabolite varied among genotypes and treatments to account for the separation in chemometric PCA. The percentages of variance explained by the first two principal components were similar when data sets for the two different ion modes, combined ion mode or only annotated features were analysed (Table 2). For the annotated data set, PC1 and PC2 accounted for 23.7% and 13.8% of the variance, respectively (Fig. 2). The PCA plots for the data set of annotated metabolites only, and total data set of MS features, are shown in Fig. 2 and Fig. S5, respectively. The 20 metabolites responsible for maximum separation in PC1 and PC2 axes, in respect to their PCA loadings, are given in Tables 3 and 4 respectively. Metabolites at the most positive side of the PC1 axis represent the uninoculated and inoculated treatments of the resistant genotype 'QT16258' and predominantly belonged to the flavonoid class of metabolites (Table 3).

Metabolites at the most negative side of the PC1 axis (Fig. 2) represent uninoculated and inoculated treatments of susceptible genotype 'Janz', and predominantly belonged to the amino acid/amino acid derivatives class of metabolites. Metabolites on the positive and negative side on the PC2 axis represented inoculated treatments of both 'Janz' and 'QT16258'. However, metabolites at the most negative side of PC2 were more responsible for separation in inoculated treatments of 'QT16258' than inoculated treatments of 'Janz' (Fig. 2), and predominantly belonged to the phenylpropanoids excluding flavonoids class of metabolites. Whereas in the most positive direction of PC2 axis, the predominant class of metabolites was flavonoids (Table 4).

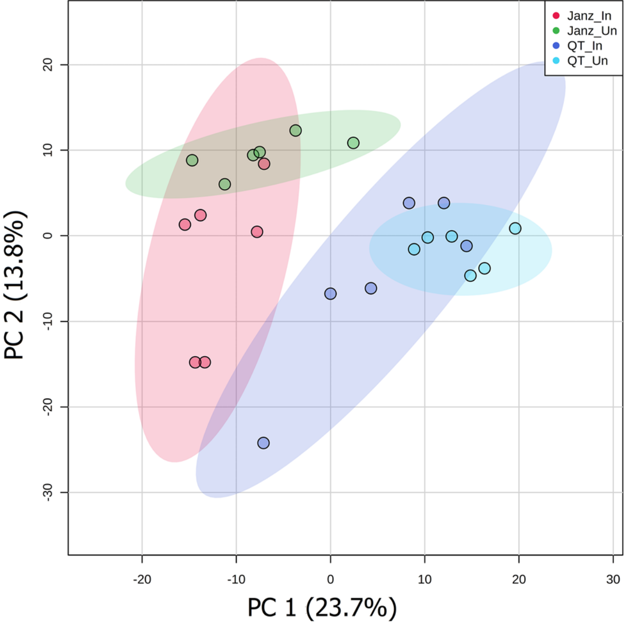


Fig. 2 Principal components analysis (PCA) of combined ion modes (positive and negative) based on LC–MS metabolomic profiles of 573 metabolites annotated by in-house chemical standards among wheat genotypes 'Janz' and 'QT16258', and their treatments (inoculated and uninoculated with *Pratylenchus thornei*), each treatment combination with six biological replications. The percentage variance accounted for in PC1 and PC2 is indicated in parentheses on the respective axes

Component 1 of the PLS-DA plot accounted for 23.5%, wheareas component 2 accounted for 7.6% of the total variance amongst treatments (Fig. 3). Variable importance of projection (VIP) scores were determined for the metabolites mostly responsible for dissimilarity between genotypes, and their inoculation treatments, in the PLS-DA model. Fifteen of the 20 most important (based on VIP score) metabolites in the PLS-DA model had a high relative concentration in 'QT16258', and low relative concentration in 'Janz' (Fig. S6 and Table S1). Of these 15 metabolites, the predominant class of metabolites was flavonoids.

Only three metabolites (all flavonoids) were at highest concentration in the 'QT16258'inoculated treatment (quercetin-3, 4'-O-di-beta-glucoside and two other flavonoid glycosides (Flavonol base 4O, 1MeO, O-Hex-Hex and Flavonol base 3O, O-dHex, O-Hex-Hex). The five metabolites with highest concentration in 'Janz' belonged to numerous metabolite sub-classes: one amino acid derivative, one terpenoid, one benzenoid, and two unclassified metabolites having annotation with molecular formula but not confirmed compound name. Most of the important features identified by the PLS-DA (Fig. S6) were also found in the top 20 loadings of PCA analysis in positive direction of PC1 axis (Table 3, column 1), and predominantly belonged to the flavonoids class of metabolites. Important features from the PLS-DA model on the basis of their VIP scores were also identified by analysis of the filtered data set of MS features (4997 MS features with both annotated and unannotated metabolites), in which a few annotated metabolites were also recorded in the top 20 PLS-DA important features based on VIP score (Data not shown).

Analysis of variance, Student's t-test, fold change and clustering analysis

Univariate ANOVA and post hoc analysis of 'QT16258' and 'Janz' (both inoculated and uninoculated) identified significantly ($p \leq 0.01$) different MS features based on genotype (Table 5). There were 450 MS features that were in significantly ($p \leq 0.01$) higher concentrations in 'QT16258' (both inoculated and uninoculated) than in 'Janz'. On the other hand, there were 697 MS features that were in significantly

Table 3 Twenty annotated metabolites (excluding unclassified metabolites), with highest positive loadings and highest negative loadings along principal component 1 (PC1) from the principal components analysis of annotated mass spectrometry (MS) features

Metabolite[a] on PC1 axis most positive)	m/z_Retention time	Annotation level[b]	Class of metabolites	Metabolite[a] (on PC1 axis most negative)	m/z_Retention time	Annotation level[b]	Class of metabolites
Flavanone base + 3O + C-Hex pos	435.1318_ 0.97	2	Flavonoid	Feruloyl agmatine isomer of 1607 pos	307.1787_3.5	2	Amino acid derivative (KEGG: C18325)
Flavone base + 3O + C-Pen + C-Pen pos	535.1514_1.51	2	Flavonoid	Pentose + Proline neg	274.0936_5.41	2	Amino acid derivative
Quercetin-3,4'-O-di-beta-glucoside neg	627.1562_3.99	2	Flavonoid	4-Acetamidobutanoate neg	144.0664_2.14	1	Amino acid derivative (KEGG: C02946)
Flavone base + 3O, + 2MeO, + O-guaiacylglycerol pos	527.1527_6.29	2	Flavonoid	D-Pantothenic acid neg	218.106_2.71	1	Vitamin/Amino acid derivative (KEGG: C00864)
Biochanin-7-O-glucoside neg	447.1301_8.3	2	Flavonoid	Homogentisate pos	169.0483_7.11	1	Amino acid derivatives (KEGG: C00544)
Flavonol base + 3O, + O-hex, + O-Hex-Hex neg	611.1622_3.95	2	Flavonoid	N-Acetyl-DL-Methionine neg	190.0519_3.2	1	Amino acid derivative
Flavonol base + 4O + 1MeO + O-Hex-Hex neg	657.162_4.13	2	Flavonoid	Acetyl leucine isomer of 164 pos	172.0951_3.95	1	Amino acid derivative (KEGG: C02710)
Flavan-3-ols + 3O pos	257.0825_0.86	2	Flavonoid	Tryptophan pos	203.0801_3.68	1	Amino acid (KEGG: C00078)
Flavone base + 3O + O-Hex-Pen neg	563.139_3.85	2	Flavonoid	Phenylacetyl aspartic acid neg	250.0748_4.39	2	Amino acid derivative
Flavonol base + 4O + O-dHex + O-Hex-dHex pos	755.1957_6.9	2	Flavonoid	N-Acetyl-D-Tryptophan neg	245.0933_4.64	1	Amino acid derivative
Fatty acid 18:3 (+ 1O) neg	277.2188_9.85	2	Fatty acid	N-Acetyl-L-Phenylalanine neg	206.0845_4.4	1	Amino acid derivative (KEGG: C03519)
Lysophosphatidylethanolamine 18:1 neg	480.3081_10.42	2	Lipid	N-Methyl-D-Aspartic acid pos	148.0579_0.96	1	Amino acid derivative (KEGG: C12269)
Lysophosphatidylethanolamine 18:2 neg	476.2755_10.7	2	Lipid	N-Acetyl-L-Leucine neg	172.1007_4	1	Amino acid derivative
Azelaic acid neg	187.0971_4.9	1	Fatty acid (KEGG: C08261)	Hexose + C13H21O2 pos	389.2224_10.93	2	Sugar (KEGG: C00984)
Tubotaiwine neg	325.1917_7.2	2	Alkaloid	Disaccharides 2Methyl-Hex-Pen neg	385.1316_5.44	2	Sugar
Corynanthine neg	399.1952_6.81	2	Alkaloid	Sinapic acid_b pos	225.0724_5.33	2	Phenylpropanoid (KEGG: C00482)
Hydroxyferulic acid neg	209.0448_3.01	1	Phenylpropanoid	Chalcone base + 3O + 1MeO + 1Prenyl pos	353.1397_4.63	2	Phenylpropanoid
Diferuloyl glycerol pos	443.1302_6.45	2	Phenylpropanoid	Isoflavone base + 4C + 1Prenyl pos	355.1184_8.13	2	Flavonoid
1-Methylsulfinylbutenyl isothiocyante neg	176.0129_4.03	2	Isothiocyanate/glucosinolate	Malvidin-3-O-glucoside pos	494.1435_3.67	2	Anthocyanin /Flavonoid
Glutamyl-S-methyl cysteine pos	263.0623_0.87	2	Amino acid derivative	Mitragynine pos	443.2162_4.26	2	Alkaloid

[a] pos means metabolites detected in positive ion mode wheareas neg means metabolites detected in negative ion mode from high performance liquid chromatography mass spectrometry

[b] Annotation levels are given according to Schymanski et al. (2014)

higher concentrations ($p \leq 0.01$) in 'Janz' (both inoculated and uninoculated) than in 'QT16258'.

The annotations of the MS features were done based on three levels of confidence, based on in-house chemical standards and searched on the PLaSMA database, as provided in Supplementary Table S2. Out of the 573 annotated metabolites, 169 were significant based on univariate analysis of variance ($p \leq 0.01$), with 84 significantly higher in 'QT16258' (mean of uninoculated and inoculated) and 85 significantly higher in 'Janz' (mean of uninoculated and inoculated) (Table S2). Less effect of nematode inoculation was recorded on the significant features of both the genotypes in univariate analysis of variance ($p \leq 0.01$). The difference between uninoculated and inoculated treatments within a genotype was found mostly due to higher relative concentration in uninoculated genotypes than the inoculated genotype. Of the 84 metabolites with significantly higher concentration of metabolites in 'QT16258', four were in higher concentration in the inoculated treatment, 60 were not significantly different between the treatments, whereas 20 were significantly ($p \leq 0.01$) higher in the uninoculated treatment.

Furthermore, volcano plots were produced to visualize the significant ($p \leq 0.05$) pattern of fold changes in relative concentration of metabolites between pairs of genotypes and treatments (Fig. S7). The variations in metabolites were more significant between genotypes than between the uninoculated and inoculated treatments of a genotype (Table S3 and Table S4). A full list of constitutively expressed ('QT16258' uninoculated compared with 'Janz' uninoculated) resistance related metabolites with their fold change value based on volcano plot analysis is given in Supplementary Table S5.

A two-way ANOVA with a false discovery rate (FDR) adjusted p-value ≤ 0.05, further indicated that there was more dissimilarity in metabolite abundance in 'QT16258' and 'Janz' without nematode inoculation (Fig. S8). The inoculation had less or no effect on the overall significant metabolic profile between the two genotypes as shown in the Venn diagram (Fig. 4). The concentrations of 1561 ($1551 + 8 + 2$) metabolites were significantly different between the genotypes, 18 ($8 + 8 + 2$) metabolites significantly different between treatments (inoculation with *P. thornei*) and three ($1 + 2$) metabolites were significanlty influenced by the interaction of genotypes and treatments (Fig. 4). The concentrations of ten ($8 + 2$) metabolites were simultaneously affected by genotypes and treatments, and two metabolites were simultaneously affected by both genotypes and treatments and their interactions, whereas 3,429 metabolites were not significantly affected.

Putative annotations of those 18 metabolites affected by treatments are provided in Supplementary Table S6, and the full report of two-way ANOVA is provided in Supplementary Table S7. Based on two-way ANOVA, out of 573 annotated metabolites 197 were significantly different ($p \leq 0.05$) in concentration between genotypes, with no significant differences between inoculation treatments or the interaction between genotypes and inoculation treatment, whereas 376 annotated metabolites were not significantly affected (Fig. S9).

Patterns of significant (adjusted p-value ≤ 0.001) up or down regulation of annotated metabolites based on two-way ANOVA ($n = 54$) are presented in a heat map (Fig. 5). Metabolites were up and down regulated in both 'Janz' and 'QT16258' and their treatments. Flavonoids, glycerolipids, fatty acids and alkaloids were predominantly higher in concentration in 'QT16258', and lower in concentration in 'Janz' (Table 6). Of 27 metabolites significantly ($p \leq 0.001$) higher in 'QT16258' than in 'Janz', most of them have been reported in the literature to be associated with antimicrobial or plant defense roles including anti-nematode activities (Table 6).

A dendrogram based on the dissimilarity of the metabolic profile of individual replicates of the genotypes and their treatments revealed three groups. Both Group 1 and 2 had a mix of inoculated and uninoculated samples. Group 1 included 10 samples from 'Janz', Group 2 included 10 samples from 'QT16258', while Group 3 included all inoculated samples, two from 'Janz' and two from 'QT16258' (Fig. S10). Similar dendrograms were obtained from both positive and negative ion mode MS features sets, the combined data set, and annotated metabolites only (data not shown). Distinction between inoculated and uninoculated treatments of the genotypes was not found in the dendrograms. Separation of the replicates was mostly due to genotype difference.

The classes of metabolites significantly different ($p \leq 0.01$, based on univariate ANOVA) abundance in 'QT16258' and 'Janz' are presented in Fig. 6. Overall, the metabolite class with the highest number of metabolites that were significantly different between genotypes was flavonoids. There were 14 flavonoid compounds that were relatively higher in concentration in 'QT16258' than in 'Janz', and 15 flavonoid compounds in higher concentration in 'Janz' than in 'QT16258'. However, the flavonoid compounds in a higher concentration in 'QT16528' had up to nine-fold change in values compared with those in higher concentration in 'Janz', which had up to only three-fold change in concentration between genotypes, based on volcano plot analysis (Table S5). There were 21 glycerolipid and fatty acid molecules that were in significantly higher concentration ($p \leq 0.01$) in 'QT16258' than in 'Janz'. In addition to flavonoids, glycerolipids and fatty acids, other classes of metabolites that were relatively higher in number in 'QT16258' included steroid glycosides, tannins, nucleotides and terpenoids. The clear difference in relative

Table 4 Twenty metabolites (excluding unclassified metabolites), with highest negative loadings and highest positive loadings along principal component 2 (PC2) from the principal components analysis of annotated mass spectrometry (MS) features

Metabolites[a] (on PC2 axis most positive)	m/z_ Retention time	Annotation level[b]	Class of metabolites	Metabolites[a] (on PC2 axis most negative)	m/z_ Retention time	Annotation level[b]	Class of metabolites
Flavone base + 3O + 1MeO + 1Prenyl pos	367.1141_6.7	2	Flavonoid	Calycanthine pos	347.2329_5.06	2	Alkaloid (KEGG:C10573)
Isosakuranetin-7-O-rutinoside pos	639.1974_4.81	2	Flavonoid	Harmine pos	211.0881_6.08	2	Alkaloid (KEGG:C06538)
Pterocarpan base + 1O, + 1MeO pos	271.0957_4.91	2	Flavonoid	Hirsutine pos	369.2251_6.5	2	Alkaloid
Eriodictyol-7-O-neohesperidoside pos	597.1762_4.01	2	Flavonoid	Hirsuteine pos	365.1862_4.25	2	Alkaloid
Biflavonoid-flavone base + 3MeO and flavone base + 3MeO pos	605.1426_4.62	2	Flavonoid	7-Hydroxymitragynine pos	415.2299_6	2	Alkaloid
Dihydrohesperetin-7-O-neohesperidoside neg	613.2131_4.22	2	Flavonoid (KEGG: C09806)	Coumaroyl quinic acid isomer of 759, 760 neg	337.0891_3.34	2	Phenyl propanoid
Flavone base + 4O + 1Prenyl_b neg	355.117_5.84	2	Flavonoid	Feruloyl allylamine pos	234.1147_4.41	2	Phenyl propanoids/isoflavonoid
Flavone base + 3O + 1MeO + C-Hex-Hex neg	623.1552_5.56	2	Flavonoid	Sinapoyl + C6H9O5 pos	369.1195_3.5	2	Phenyl propanoid
3,4-Dimethoxycinnamic acid_neg	207.0664_6.66	2	Phenyl propanoid	Coumaric acid isomer of 189, 194 pos	147.0441_2.93	2	Phenyl propanoid (KEGG: C05838)
Coniferyl alcohol + O-Hex neg	387.1288_2.39	2	Phenyl propanoid/monolignol	Coumarin base + 1O, + 1MeO + O-Hex_a neg	399.0906_4.61	2	Phenyl propanoid
Tryptamine neg	159.0961_3.1	1	Amino acid derivatives (KEGG: C00398)	Feruloyl quinic acid isomer of 887, 888_c neg	367.1035_3.53	2	Phenyl propanoid
N-Fructosyl phenylalanine pos	328.1349_8.82	2	Amino acid derivatives	Caffeoyl putrescin isomer of 1059 pos	251.1399_2.79	2	Phenyl propanoid
Benzoic acid + 2O + O-Pen neg	285.0603_3.65	2	Aromatic carboxylic acid derivative/Benzenoid	Coumaroyl putrescin pos	235.1455_2.93	2	Phenyl propanoid
Vanillin acetate neg	195.0632_5.84	2	Phenol ester, Benzenoid	Salicylate neg	137.022_5.02	1	Aromatic acid
Pumiloside pos	513.1942_3.72	2	Alkaloid	Quercetin 3-O-2''-O-6'''-O-p-coumaroyl-b-D-glucopyranosyl-a-L-rhamnopyranoside neg	757.19_3.57	2	Flavonoid
Carboline metabolite (C26H26N2O8) pos	495.1849_3.69	2	Alkaloid	Oleanane -4H + 2O pos	441.3773_5.89	2	Terpenoid
trans-piceid neg	389.1214_3.65	2	Lipid, stilbene (KEGG: C10275)	Ginsenoside F1_b pos	683.4434_11.69	2	Terpenoid
4-Hydroxyphenylacetate pos	153.0531_5.57	1	Benzenoid (KEGG: C00642)	2',4'-Dihydroxyacetophenone neg	151.0434_4.72	1	Alkyl-phenyl ketones (KEGG: C03663)
Quinoxalinedione + 2Methyl + C5H11O4 pos	325.1363_3.59	2	Benzenoid	Petroselinic acid neg	281.2487_11.75	1	Fatty acids (KEGG:C08363)
Lariciresinol_a neg	359.1508_4.39	2	Biosynthesis of various secondary metabolites (KEGG: C10646	Isopropylmalic acid neg	175.06_3.52	2	Fatty acid

[a] pos means metabolites detected in positive ion mode wheareas neg means metabolites detected in negative ion mode from high performance liquid chromatography mass spectrometry

[b] Annotation levels are given according to Schymanski et al. (2014)

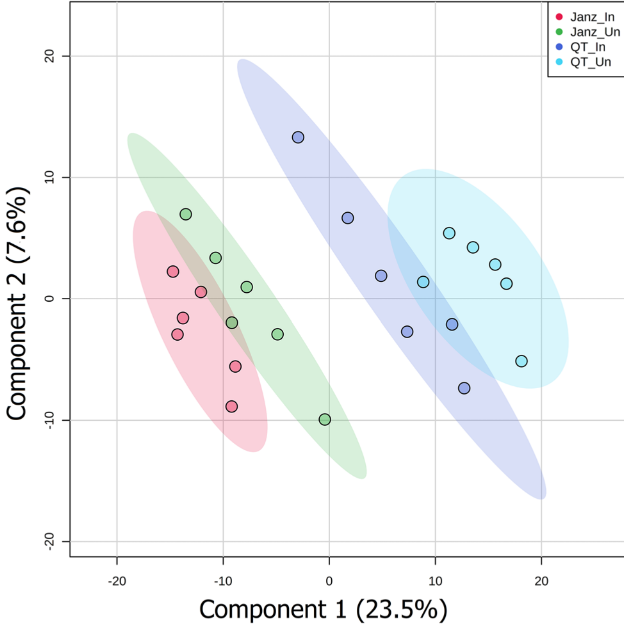


Fig. 3 Partial least square discriminant analysis (PLS-DA) model of combined ion modes (positive and negative) based on LC–MS metabolomic profiles of 573 metabolites annotated by in-house chemical standards among wheat genotypes 'Janz' and 'QT16258', and their treatments (inoculated and uninoculated with *Pratylenchus thornei*), each treatment combination with six biological replications. The percentage variance accounted for by each component is indicated in parentheses on each axis

concentration of some significant metabolites can be seen in the box plots in Figs. 7 and 8 for'QT16258' and 'Janz' respectively.

The metabolites that were found in higher abundance in 'QT16258' inoculated than in 'QT16258' uninoculated and 'Janz' treatments, on the basis of univariate analysis without FDR adjusted *p* values (≤ 0.01), include gossyptein-8-glucosides (flavonoid glycosides), flavonol base 5O, O-hex (flavonoid glycosides), benzoic acid 1O, O-hex (phenolics glycosides), and $C_{20}H_{22}O_6$ (matairesinol) (lignan class of phenolics). Likewise, metabolites that were in higher abundance in inoculated than in uninoculated 'Janz' and 'QT16258' treatments were petroselinic acid (fatty acid), hexose (isomer of $C_{13}H_{19}O$) (sugar), S8-8S hexoside (sugar), gelsemine (alkaloid), 1-isothiocyanato-4-methylsulfinyl-butane, remerine (alkaloid), and $C_5H_5N_5$(adenine) (nucleotide).

The MS features that were expressed in higher concentrations in 'QT16258' were also annotated (putative) manually using public databases (Table 7). Similar to the analyses of the dataset of metabolites annotated by in-house standards, the predominant classes of metabolites identified by manual annotation were glycerolipids, fatty acids and flavonoids. The MS features that were in higher abundance in 'Janz' than in 'QT16258' were annotated with public databases as well (Table S8). The classes of metabolites include flavonoids, terpenoids, alkaloids, sugars and hormones. These top 20 putatively annotated MS features with highly significant probabilities ($p \leq 10^{-5}$) were mostly expressed constitutively in 'Janz' with less or no effect of inoculation. However, a very few synthetic compounds were also recorded in putative annotation for significant MS features both in 'QT16258' and 'Janz' respectively.

Pathway predictions

Biosynthetic pathway analysis was used to identify pathways in both genotypes that may significantly contribute to defense responses and/or susceptibility to *P. thornei*. The biosynthetic pathways predicted by *mummichog* and gene set enrichment analysis (GSEA) programs corroborated the pathways for compounds annotated by in-house chemical standards and annotation searches in public metabolomics databases. The pathways found to be changed in 'QT16258' are mostly related to plant defense mechanisms, including linoleic and linolenic acid metabolism, stilbenoid, diarylheptanoid and gingerol biosynthesis, fatty acid and unsaturated fatty acid biosynthesis, flavonoid biosynthesis and some compounds related to amino acid metabolism (Table S9). The pathways identified in 'Janz' are potentially involved in growth, reproduction and defense, including amino acids metabolism, caffeine metabolism, terpenoid, phenylalanine metabolism, ascorbate and aldarate metabolism, and zeatin and riboflavin biosynthesis (Table S10).

Table 5 Significant mass spectrometry (MS) features in 'QT16258' and 'Janz' (from both inoculated and uninoculated treatments of each genotype) identified by univariate analysis of variance and post hoc analysis ($p \leq 0.01$, p value not false discovery rate adjusted)

Data set	Total MS features	MS features after data filtering*	Number of significant MS features		
			Total	'QT16258' > 'Janz'	'Janz' > 'QT16258'
Positive ion mode	6541	3924	838 (12.8%)	293 (4.5%)	545 (8.3%)
Negative ion mode	5163	3097	734 (14.2%)	267 (5.2%)	467 (9.0%)
Combined ion modes (Total)	11,704	4997	1,147 (9.8%)	450 (3.8%)	697 (6.0%)
Combined ion mode (Annotated metabolites only)	765	573	169 (22.1%)	84 (10.9%)	85 (11.1%)

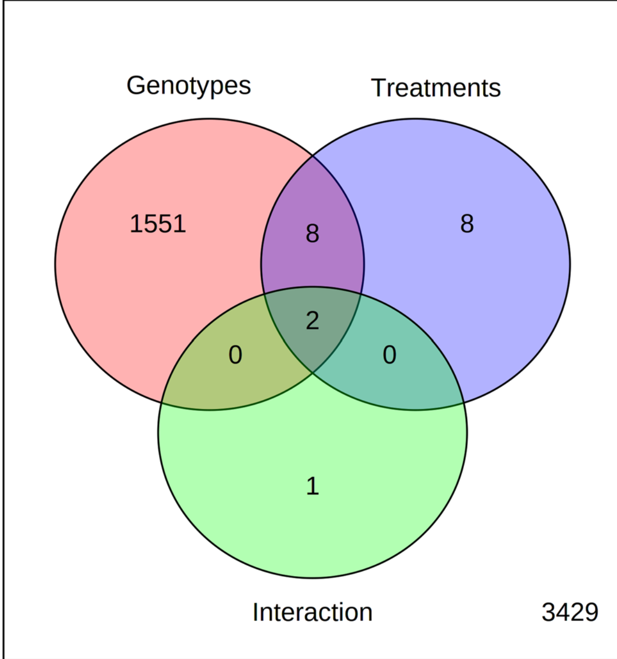


Fig. 4 Venn diagrams from two-way analysis of variance (ANOVA) of total 4997 metabolites, indicating numbers of metabolites significantly different (adjusted p value ≤ 0.05) for genotypes ('QT16258 and 'Janz'), and for inoculation treatments (with or without *Pratylenchus thornei*), and their interaction. Number of metabolites with no significant differences are indicated in right bottom corner

Common pathways that were significantly altered in both genotypes include arginine and proline metabolism, alpha-linolenic acid and purine metabolism. The pathway for changes in the outer layer of the cell wall (cutin, suberin and wax biosynthesis) was potentially unique in 'QT16258' and absent in 'Janz' (Table S11).

Discussion

This is the first study to investigate variations in the metabolomic profiles of resistant and susceptible wheat cultivars inoculated with the root-lesion nematode *P. thornei*. An untargeted approach was utilized in this study to comprehensively profile the largest possible array of metabolites in the wheat genotypes at 8 weeks PNI. This time point was chosen for the metabolic profiling study as distinctive differences in nematode numbers and in egg deposition inside the roots of *P. thornei* resistant and susceptible wheat genotypes were found at 8 weeks PNI in our previous study (Rahaman et al. 2019). This was confirmed by the parallel *P. thornei* time course experiment where nematodes and eggs were found to be significantly less in number inside the roots of 'QT16258' than in 'Janz' at 8 weeks PNI.

Differences in MS feature/metabolite abundance were found to be both constitutive and induced in both the genotypes by univariate ANOVA and volcano plot analysis. However, the metabolites that exhibited the most significant features and largest fold change value were constitutively expressed. Furthermore, very few or no interactions between genotypes and nematode treatments were found in two-way ANOVA of metabolites, which implies the defense against *P. thornei* is most likely constitutive in nature in 'QT16258'. This supports the findings of Rahaman et al. (2020) that constitutively expressed total phenol could be providing defense against *P. thornei* in the synthetic hexaploid wheat 'CPI133872', the wheat line from which the resistance in 'QT16528' is inherited. Similarly, Linsell et al. (2014a, b) proposed that defense against *P. thornei* in the wheat genotype 'Sokoll' is constitutive in nature. The comprehensive approach of metabolomic analyses has sensitively and robustly shown that defense responses in 'QT16258', when challenged by *P. thornei*, are predominantly constitutive.

Altogether 84 confirmed annotated metabolites were significantly ($p \leq 0.01$) higher in concentration in 'QT16258' than in 'Janz'. In general, these metabolites predominantly belonged to the classes glycerolipids, fatty acids and flavonoids, and many have been reported in the literature to have roles in plant defense against fungi and nematodes. Lipid and fatty acid compounds can initiate pathogen recognition and the expression of defense compounds, such as pathogenesis related (PR) proteins and cell wall lipids and polymers (Jung et al. 2009; Rolland et al. 2002; Walley et al. 2013). The small phospholipids, glycerolipids and long chain fatty acids could have important roles in initiating defense pathways in 'QT16258' as well as in plant growth and reproduction (Martin 1998; Okazaki and Saito 2014). Phospholipids, such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and glycerolipids, such as monogalactosyl monoacylglycerol (MGMG), in the presence of phospholipase A (PLA) enzymes, synthesize long chain fatty acids such as alpha-linolenic acid (18:3), which can produce jasmonic acids downstream of the jasmonic acid biosynthesis pathway (Lim et al. 2017). Jasmonic acid signaling is important for the interaction of plants with pathogens as well as growth and development. Although jasmonic acid and its derivative methyl jasmonate induce systemic acquired resistance in plants, the increased level of jasmonic acids can also sustain constitutive defense in plants, including activation of phenylpropanoid metabolism (Chen et al. 2006). Phenylpropanoid metabolism in plants produces a range of metabolites. Fatty acids (18:1, 18:2 and 18:3) were previously reported as part of constitutive defense in barley against the pathogenic fungus *Fusarium*

Fig. 5 Heat map showing differences in concentrations among genotypes (‘Janz’ and ‘QT16258’) either uninoculated or inoculated with *Praylenchus thornei* treatments for significant ($p \leq 0.001$) annotated metabolites, identified via two-way analysis of variance from LC–MS in the combined positive and negative ion modes. The scale bar represents normalized intensity of metabolites. Full names of some abbreviated metabolite names in the heatmap are; 4-Hydroxyphenyl ac = 4-hydroxy phenyl acetate, Dihydroxymandelic = Dihydroxymandelic acid, Indole 3 acetyl L = Indole 3 acetyl L glutamic acid, 1-Methylsulfinylbutenyl = 1-Methylsulfinylbutenyl isothiocyante, Quercetin-3, 4’-O-di- = Quercetin-3, 4’-O-di-beta-glucoside, LPE = lysophosphatidylethanolamine, FA = fatty acid. A full list is given in Table 6. R = replicate

graminearum (Kumaraswamy et al. 2011; Bollina et al. 2011), and could also provide constitutive defense against *P. thornei*. Additionally, small lipophilic molecules have previously been reported as inhibitory compounds against root-knot nematodes *Meloidogyne incognita* and *M. graminicola* in tomato and rice roots (Dutta et al. 2012).

Acetylcholine is an important neurotransmitter in nematodes to control movement and motility, the function of which depends on the enzyme acetylcholine esterase (Selkirk et al. 2005). Gossypetin-8-glucosides, desoxypeganine, and hirsutine metabolites could potentially act as acetylcholine esterase inhibitors (Hillhouse et al. 2004; Selkirk et al. 2005; Konrath et al., 2013) of *P. thornei* to damage neural connections and its motility inside ‘QT16258’ root tissue. Acetylcholine esterase inhibition is also the mode of action of powerful synthetic nematicides such as aldicarb (Opperman and Chang 1990). Moreover, isothiocyanate compounds reduced motility of the root-knot nematode *M. hapla* (Dahlin and

Table 6 Most significant ($p \leq 0.01$) compounds based on two-way ANOVA annotated by in-house chemical standards at higher concentration in 'QT16258' than in 'Janz' (n = 54), their class of metabolite, and putative defense role. This is a subset of all significant metabolites including putatively annotated significant mass spectrometry (MS) features

Metabolites[a] (annotated by standard)	Molecular formula	m/z_ Retention time	Annotation level[b]	Class of metabolite	Possible role in plant from the literature	ID (KEGG/ HMDB/ PubChem)[c]
QT16258 > Janz ($p \leq 0.001$)						
FA 18:2 (1O) neg (Linoleic acid)	$C_{18}H_{32}O_2$	293.2127_10.34	2	Fatty acid	Part of constitutive defense and cell signalling in plants (Kumaraswamy et al. 2011; Bollina et al. 2011)	5,280,450
Isohernandezine neg	$C_{39}H_{44}N_2O_7$	653.322_6.97	2	Tannin	Unknown	7,098,682
7-Hydroxymitragynine pos	$C_{23}H_{30}N_2O_5$	415.2299_6	2	Indole alkaloid	Unknown	44,301,524
Hirsutine pos	$C_{22}H_{28}N_2O_3$	369.2251_6.5	2	Indole alkaloid	Antimicrobial, inhibitor of acetyl choline esterase(Konrath et al. 2013)	3,037,884
Isorhamnetin-3-O-galactoside-6''-rhamnoside neg	$C_{28}H_{32}O_{16}$	625.1761_	2	Flavonoid O-glycoside	Quercetin derivative, strong antimicrobial	44,259,338
FA 18:3(1O)_b neg (alpha linoleic acid)	$C_{18}H_{30}O_2$	295.2281_10.16	2	Fatty acid	Part of constitutive defense and cell signalling in plants (Kumaraswamy et al. 2011; Bollina et al. 2011)	NA
FA 9:2 (1O) neg	$C_9H_{14}O_3$	169.0876_7.12	2	Fatty acid	Part of constitutive defense and cell signalling in plants (Kumaraswamy et al. 2011; Bollina et al. 2011)	NA
Tubotaiwine neg	$C_{20}H_{24}N_2O_2$	325.1917_7.2	2	Alkaloid	Antimicrobial/part of plant growth and regulation	13,783,720
Flavonol base + 3O + O-dHex + O-Hex-Hex neg	$C_{33}H_{40}O_{20}$	757.2208_4.19	2	Flavonoid O-glycoside	Antimicrobial/part of plant growth and regulation	NA
Strictosamide_a neg	$C_{26}H_{30}N_2O_8$	499.2064_10.59	2	Monoterpene indole alkaloid	Antimicrobial (antiviral, antibacterial)	10,345,799
Desoxypeganine neg	$C_{11}H_{12}N_2$	173.1122_5.23	2	Quinazolines alakloid	Choline esterase inhibitor	442,894
LPE 16:0 neg	$C_{21}H_{44}NO_7P$	452.2701_10.39	2	Lipid	Part of cell membrane and possible anti nematode compounds (Martin 1998; Okazaki and Saito, 2014)	53,480,922
Deoxyvasicinone neg	$C_{11}H_{10}N_2O$	187.0917_0.87	2	Quinazoline alkaloid	Ovipoisition, deterrent, feedant, fungicidal, antibacterial	68,261
Flavanone base + 3O + C-Hex pos	$C_{21}H_{22}O_{10}$	435.1338_0.97	2	Flavonoid O-glycoside	Antimicrobial/part of plant growth and regulation	23,502,663
S-Lactate neg	$C_3H_5O_3$	89.0232_0.97	1	Primary metabolite	Unknown	C00186
Fumaric acid neg	$C_4H_4O_4$	115.0021_0.97	2	Primary metabolite	Storage/ Maintain cellular pH and turgor pressure	C00122
Inosine 5'-monophosphate neg	$C_{10}H_{13}N_4O_8P$	347.0375_1.15	1	Glycoside	Cell signaling molecule to initiate defense response, regulation of cell growth	C00130
Xanthosine 5'-monophosphate neg	$C_{10}H_{13}N_4O_9P$	363.0352_1.19	1	Purine	Regulation of cell growth	C00655
Glutamyl-S-methylcysteine pos	$C_9H_{16}N_2O_5S$	263.0623_0.87	2	Amino acid derivative	Nutrient, seed development	13,894,650
1-Methylsulfinylbutenyl isothiocyante neg	C_6H_9NS	176.0129_4.03	2	Glucosinolate/Isothiocyanate	Antimicrobial, potential anti- nematode compound (Dahlin & Hallmann, 2020)	5,368,086

Table 6 (continued)

Metabolites[a] (annotated by standard)	Molecular formula	m/z_ Retention time	Annotation level[b]	Class of metabolite	Possible role in plant from the literature	ID (KEGG/ HMDB/ PubChem)[c]
LPE 18:1 pos	$C_{23}H_{46}NO_7P$	480.3065_9.14	2	Lipid	Part of cell membrane and possible anti nematode compounds (Martin 1998; Okazaki and Saito, 2014)	53,480,924
LPE 18:3 pos	$C_{23}H_{42}NO_7P$	476.2751_9.23	2	Lipid	Part of cell membrane and possible anti nematode compounds (Martin 1998; Okazaki and Saito, 2014)	HMDB0011478
3,4-Dihydroxybenzoate neg	$C_7H_6O_4$	153.0188_2.77	1	Polyphenolic compounds	Antimicrobial	C00230
Myricetin-3-O-xyloside neg	$C_{20}H_{18}O_{12}$	451.087_5.55	2	Flavonoid	Antimicrobial, potential anti- nematode compound (Wuyts et al. 2006; Chin et al. 2018; Kirwa et al. 2018)	44,259,440
Flavonol base + 4O + 1MeO + O-Hex-Hex neg	$C_{28}H_{32}O_{18}$	657.162_4.13	2	Flavonoid O-glycoside	Antimicrobial/part of plant growth and regulation	NA
Coumarin + 1O + 1MeO + O-Hex-Hex pos	$C_{22}H_{28}O_{14}$	515.1419_0.89	2	Coumarin and derivatives	Nematicidal and plant growth hormones and growth regulators in order to rheostat the respiration, photosynthesis, as well as protection against infection	NA
Quercetin-3,4'-O-di-beta-glucoside neg	$C_{27}H_{30}O_{17}$	627.1562_3.99	2	Flavonoid O-glycoside	Antimicrobial/ strong radical scavenger, potential anti- nematode compound (Wuyts et al. 2006; Chin et al. 2018; Kirwa et al. 2018)	22,630,387
Putative compounds based on molecular formula QT16258 > Janz ($p \leq 0.001$)						
C23H42NO7P neg (: lysophosphatidylethanolamine)	$C_{23}H_{42}NO_7P$	474.2629_9.73	4	Lipid	Antimicrobial	HMDB0011479
C18H26O2 neg (19-Nortestosterone)	$C_{18}H_{26}O_2$	275.2025_9.55	4	Synthetic steroid	NA	9904
C34H40N4O8 neg (DIOX-H)	$C_{34}H_{40}N_4O_8$	633.2968_9.48	4	Unclassified	Unknown	4,437,560
Janz > QT16258 ($p \leq 0.001$)						
Indole-3-acetyl-L-glutamic acid pos	$C_{15}H_{16}N_2O_5$	303.1081_2.54	2	Plant hormone conjugate	Possible nematode attractant (Curtis, 2008), growth and reproduction	HMDB0038665
Coniferyl alcohol + O-Hex neg	$C_{10}H_{12}O_3$, O-Hex	387.1288_2.39	2	Lignin precursor	Role in browning/Lignin biosynthesis (García et al. 2017)	NA
Vanillin acetate neg	$C_{10}H_{10}O_4$	195.0632_5.84	2	Phenolic ester	Possible nematode attractant (Fleming et al. 2017)	61,229
Flavone base + 3O + 1MeO + C-Hex-Hex neg	$C_{21}H_{20}O_{10}$	623.1552_5.56	2	Flavonoid O-glycoside	Antimicrobial/part of plant growth and regulation	HMDB0037451
Isoflavone base + 1O + 2MeO + O-HexC7H12NO neg (afrormosin-7-O-glucoside)	$C_{23}H_{24}O_{10}$	586.2372_5.73	2	Isoflavonoid o-glycoside	Antimicrobial/part of plant growth and regulation	12,444,947

Table 6 (continued)

Metabolites[a] (annotated by standard)	Molecular formula	m/z_ Retention time	Annotation level[b]	Class of metabolite	Possible role in plant from the literature	ID (KEGG/ HMDB/ PubChem)[c]
Benzyl alcohol Hex-Hex pos (Benzyl 2-O-b-D-glucopyranosyl-b-D-glucopyranoside)	$C_{19}H_{28}O_{11}$	477.1573_7.51	2	Alkyl benzyl hexoside	Unknown	73,157,713
4-Hydroxyphenylacetate pos	$C_8H_7O_3^-$	153.0531_5.57	1	Benzenoid	Electron carriers, antioxidants, attractants, and defense compounds	4,693,933
3,4-Dimethoxycinnamic acid_a neg	$C_{11}H_{12}O_4$	207.0664_6.66	2	Plant phenolic	Key metabolites of phenylpropanoid metabolism	HMDB0034315
Feruloyl agmatine isomer of 1607 pos	$C_{15}H_{22}N_4O_3$	307.1787_3.5	2	Amino acid derivative	Nutritional components	46,173,376
Dihydroxymandelic acid neg	$C_8H_8O_5$	183.0341_0.93	1	Phenolic	Could play part in pigmentation	C05580
Geniposide neg	$C_{17}H_{24}O_{10}$	433.1375_2.54	2	Glycoside	Antimicrobial	107,848
N-Acetyl-D-Tryptophan neg	$C_{13}H_{14}N_2O_3$	245.0933_4.64	1	Amino acid derivative	Precursors for synthesis of the hormone auxin, phytoalexins, glucosinolates, and both indole- and anthranilate-derived alkaloids	C03137
Tryptophan pos	$C_{11}H_{12}N_2O_2$	203.0801_3.68	1	Amino acid	Nutrients	C00078
Silychrystin pos	$C_{25}H_{22}O_{10}$	481.1142_3.6	2	2-arylbenzofuran flavonoid	Unknown	4,481,797
Chalcone base + 3O + 1MeO + 1Prenyl pos (Licoagrochalcone C)	$C_{21}H_{22}O_5$	353.1397_4.63	2	Flavonoid precursor	Starting materials for a diverse set of metabolites (flavonoids), antimicrobial, flower pigment	5,318,990
Paeoniflorin pos	$C_{23}H_{28}O_{11}$	479.1551_3.82	2	Monoterpene glycoside		442,534
Putative compounds based on molecular formula Janz > QT16258 ($p \leq 0.001$)						
C28H34O14 neg (Isosakuranetin-7-O-rutinoside)	$C_{28}H_{34}O_{14}$	595.1918_2.65	4	Flavonoid O-glycoside	Antimicrobial	16,760,075
C17H24O11_a neg (Secoxyloganin)	$C_{17}H_{24}O_{11}$	403.1242_2.36	4	Secoiridoid glycoside	An antioxidant and an anti-allergic agent	162,868
C29H34O17 neg (Monoxerutin)	$C_{29}H_{34}O_{17}$	653.1723_5.54	4	Flavonoid	Antimicrobial	9,852,585
C14H21NO8 neg (Zinolol)	$C_{14}H_{21}NO_8$	332.1243_3.8	4	Sugar derivative	Unknown	16,216,016
C11H10N2O2 neg (Tolimidone)	$C_{11}H_{10}N_2O_2$	203.0814_4.64	4	Synthetic	Irritant	39,065
C20H20O7 neg (Sinensetin)	$C_{20}H_{20}O_7$	371.111_6.47	4	7-o-methylated flavonoids	Unknown	HMDB0036633
C17H28O16 neg	$C_{17}H_{28}O_{16}$	489.1393_5.27	4	Unknown	Unknown	77,714,590
C17H30O10 neg (cis-3-Hexenyl b-primeveroside)	$C_{17}H_{30}O_{10}$	393.1751_ 5.81	4	Lipid	Lipid transport	HMDB0031690

[a] pos means metabolites detected in positive ion mode, whereas neg means metabolites detected in negative ion mode by mass spectrometry

[b] Annotation levels are given according to Schymanski et al. (2014)

[c] The numbers following "HMDB" indicate a HMDB ID, numbers followed by "C" indicate a KEGG ID and the numbers only indicate a Pubchem ID for detailed information of the corresponding annotated metabolite.

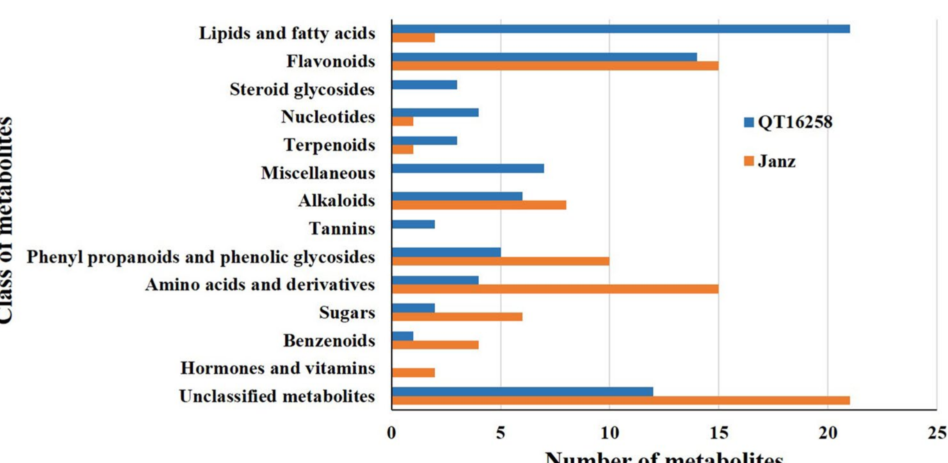


Fig. 6 Number of metabolites in each class significantly ($p \leq 0.01$) expressed in higher concentration in 'QT16258' (blue) and 'Janz' (orange), based on univariate analysis of variance of annotated metabolites (using authentic chemical standards), considering both the significant metabolites in uninoculated and inoculated treatments together for the respective genotype

Hallmann 2020), and 1-methylsulfinylbutenyl isothiocyante was present in a higher in concentration in 'QT16258' than in 'Janz'. It is noteworthy that compounds such as gossypetin-8-glucosides and matairesinol were greater in inoculated 'QT16258' than in uninoculated 'QT16258' (based on univariate analysis) and might have an induced defense role. Matairesinol is a lignan compound, induced in response to biotic and abiotic stresses in plants (Bagniewska-Zadworna et al. 2014).

The roles of several other significant metabolites in reducing nematode reproduction in resistant wheat genotypes are worth consideration. Gallic acid and the unsaturated fatty acids linoleic acid and lauric acid, which were in higher concentrations in 'QT16258' than in 'Janz', were previously reported to have nematicidal activity in vitro against the cyst nematode *Heterodera zeae* (Faizi et al. 2011). Furthermore, significantly expressed flavonoid metabolites in 'QT16258' might have important roles in reducing *P. thornei* reproduction. For example, quercetin and myricetin reduced egg hatching and nematode mobility of both sedentary (*M. incognita*) and migratory (*Radopholus similis*) nematodes (Wuyts et al. 2006; Chin et al. 2018; Kirwa et al. 2018). Flavone glycosides inhibited *P. neglectus* penetration in oats (Soriano et al. 2004a). However, the effects of particular flavonoids could differ from pathogen to pathogen (Tabashnik 1987; Wuyts et al. 2006). With respect to the mode of action, flavonoids could inactivate the enzymes of pathogens by chelating metal ions necessary for their function (Treutter 2005; Skadhauge et al. 1997).

Importantly, more than one class of metabolites could act together for the defense mechanism in resistant wheat genotypes. The glutathione pathway, which was significantly upregulated in 'QT16258', could remove harmful reactive oxygen species, and also initiate a cascade of defense responses (Ogawa, 2005) in 'QT16258'. Resistance in 'QT16258' could also be due to increased deposition of cutin, suberin and wax on the root cell walls to impede penetration of *P. thornei* and its movement inside the root. Many long chain fatty acid molecules were significantly more abundant in 'QT16258' both from the annotation based on in-house chemical standards, public databases and *mummichog* pathway analysis. Fatty acid molecules with carbon chain C18, such as FA 18:1, FA 18:3, FA 18:3_a, FA 18:3_b, FA 18:2, FA 18:4, play a vital role in the biosynthesis of cutin, suberin and wax (Nawrath 2002). Histopathology studies are required to provide evidence of changes in the deposition of cell wall components in *P. thornei* resistant and susceptible wheat genotypes.

The non-proteogenic amino acid ornithine was in higher abundance in 'QT16258' than in 'Janz', and could provide tolerance of drought and water limited conditions, which wheat plants experience as a consequence of *P. thornei* infestation. External application of L-ornithine to sugar beet improved the protein profile, total soluble sugars and amino acids, which was associated with increased drought tolerance of treated plants compared to control plants (Hussein et al. 2019).

Metabolites like coniferyl alcohol, which was significantly higher in abundance in 'Janz' could be responsible for root browning, which is a symptom of *Pratylenchus* spp. infection in roots (Castillo and Vovlas 2007). However the degree of root browning was not specifically investigated in this study. Root browning is a result of localized cell death and is associated with a hypersensitive response to nematode infection in resistant plants (Fosu-Nyarko and Jones 2016), but also occurs in susceptible plants less frequently (Sato et al. 2019). Coniferyl alcohol and its sugar conjugates have a role in browning of fresh cut lettuce (*Lactuca sativa*) due to oxidation and polymerization reactions (García et al. 2017). Some phenolic and flavonoid compounds such as 3,4-dimethoxycinnamic acid and flavonoid glycosides were also significantly increased in 'Janz'. Flavonoids also play a part in pigmentation, growth and reproduction (Liu et al.

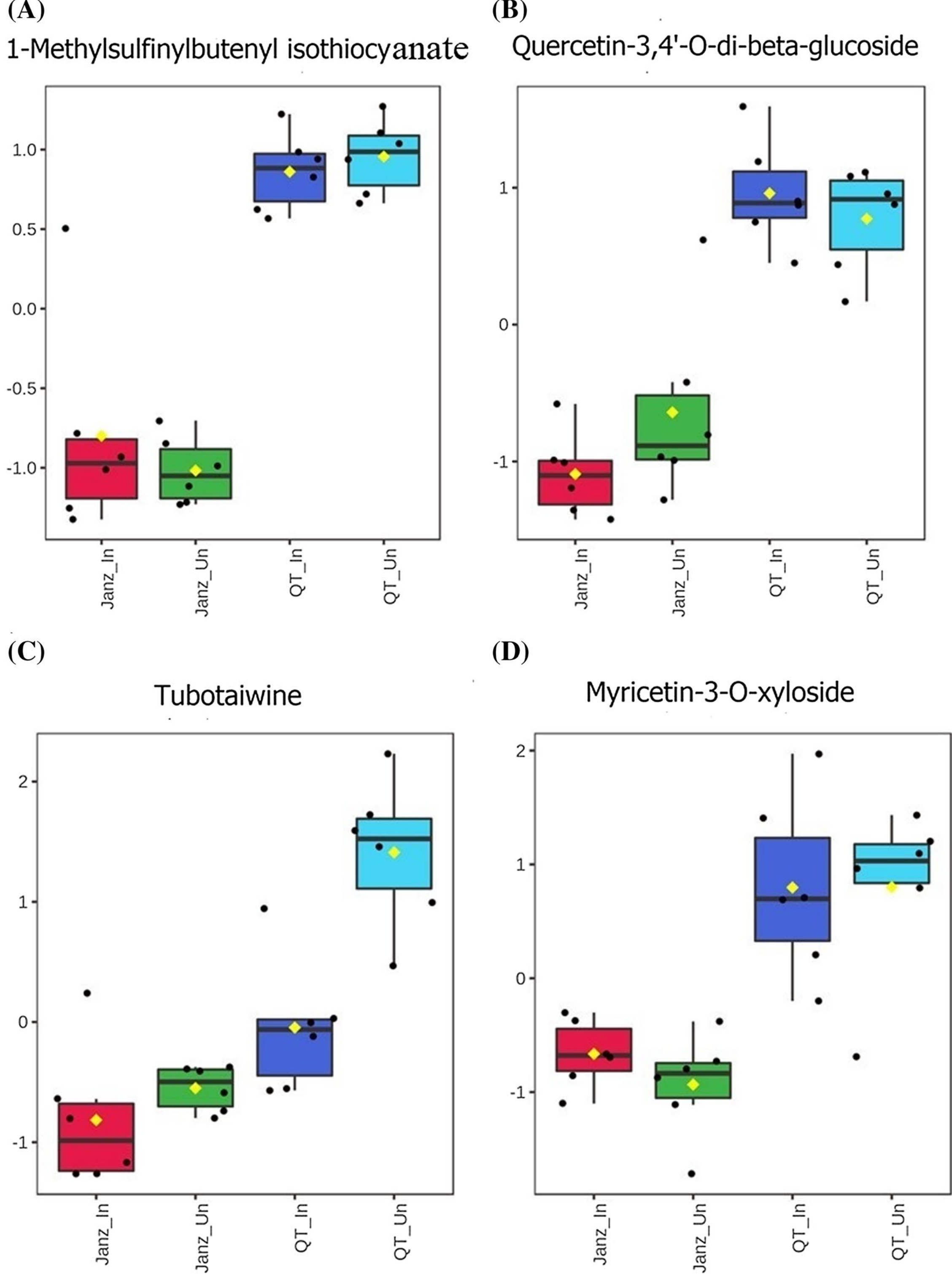


Fig. 7 Box plots of four metabolites **a** 1-Methylsulfinylbutenyl isothiocyanate (Rt: 4.03 m/z: 176.0129), **b** Quercetin-3,4'-O-di-beta-glucoside (Rt: 3.99 m/z: 627.1562), **c** Tubotaiwine (Rt: 7.2 m/z: 325.1917), **d** Myricetin-3-O-xyloside (Rt: 5.55 m/z: 451.087), with a significantly ($p \leq 0.01$) higher concentration in 'QT16258' inoculated (QT_In: dark blue) and uninoculated (QT_Un: light blue) treatments, than in 'Janz' inoculated (Janz_In: red) and uninoculated (Janz_Un: green) treatments. Relative concentration of the metabolites is shown along the y-axis as log2 normalized value

Fig. 8 Box plots of four metabolites **a** Coniferyl alcohol + O-Hex (Rt: 2.39 m/z: 387.1288), **b** Indole-3-acetyl-L-glutamic acid (Rt: 2.95 m/z: 305.1183), **c** Dihydroxymandelic acid (Rt: 0.93 m/z: 183.0341), **d** Vanillin acetate (Rt: 5.84 m/z: 195.0632), with a significantly ($p \leq 0.01$) higher concentration in 'Janz' treatments than in 'QT16258'. Relative concentration of the metabolites are shown along the y-axis with log2-normalized value. The x-axis represents different genotypes and their inoculated and uninoculated treatments respectively. QT_In: dark blue = QT16258 inoculated, QT_Un: light blue = QT16258 uninoculated, 'Janz'_In: red = Janz inoculated, 'Janz'_Un: green = 'Janz' uninoculated

Table 7 Putative annotation of the 20 most significant mass spectrometry (MS) features based on univariate ANOVA (top 20) in 'QT16258' (uninoculated and inoculated) > 'Janz' (uninoculated and inoculated). Different letters indicate significant difference ($p \leq 10^{-5}$) between treatments; a > b > c > d

m/z	Retention time (mins)	Ion mode[a]	Annotation (putative)	Molecular formula	ID (KEGG/HMDB/PubChem)[b]	Class of metabolite	Possible role in plant reported in literature	Significant differences			
								QT16258 (Un)	QT16258 (In)	Janz(Un)	Janz (In)
								QT16258 > Janz			
601.1759	3.24	pos	Podorhizol beta-D-glucoside	$C_{28}H_{34}O_{13}$	C10876	Phenylpropanoid	Rigidity to cell wall	a	a	b	b
407.2395	7.62	Pos	(4,5-Dihydroxy-3,4-bis(hydroxymethyl)-4a,8,8-trimethyl-1,4,4a,5,6,7,8,8a-octahydro-1-naphthalenyl hexanoate	$C_{21}H_{36}O_6$	45,359,477	Fatty acid	Cell signaling	a	a	b	b
597.1895	0.94	Pos	Chalconaringenin 2',4'-di-O-glucoside	$C_{27}H_{32}O_{15}$	C06561	Flavonoid lipid/Flavonoid	Antimicrobial/radical scavengers	a	a	b	b
191.036	4.05	neg	8-Hydroxy-7-methoxy-2H-1-benzopyran-2-one (8-hydroxy-7-methoxy-coumarin)	$C_{10}H_8O_4$	HMDB0030692	Phenylpropanoid	Antimicrobial	a	a	b	b
633.1507	6.79	neg	Catechin 3-O-rutinoside	$C_{27}H_{34}O_{15}$	44,257,079	Flavonoid glycosides	Antimicrobial	a	a	b	b
577.1697	3.27	neg	Deoxyloganin tetraacetate	$C_{25}H_{34}O_{13}$	C11646	lipid	Cell signaling/Lipid transport	a	a	b	b
176.0126	4.55	neg	2-Aminomuconate semialdehyde;	$C_6H_7NO_3$	C03824	Amino acid derivatives	Nutients	a	a	b	b
596.2191	3.24	pos	Acrimarine J	$C_{35}H_{33}NO_8$	HMDB40386	Alkaloids	Nutrients	a	a	b	b
152.9907	10.59	neg	No match					a	a	b	b
585.2313	4.46	neg	Aspecioside	$C_{29}H_{42}O_{10}$	C08850	Terpenoids (steroid glycoside)	Unknown	a	a	b	b
195.1378	10.14	neg	4-Hydroxy-6-dodecenoic acid lactone	$C_{12}H_{20}O_2$	HMDB0032331	Lactones	Food additive	a	a	b	b
479.1998	6.95	neg	Acevaltrate	$C_{24}H_{32}O_{10}$	C16752	Fatty esters (monoterpenoids)		a	a	b	b
275.0241	1.19	neg	Dihydroferulic acid 4-O-sulfate	$C_{10}H_{12}O_7S$	HMDB0041724	Phenylpropanoids	Nutrients	a	a	b	b
882.8223	0.74	pos	No match					a	a	b	b
321.0847	3.25	pos	7-Methylxanthosine	$C_{11}H_{15}N_4O_6$	C16352	Purine alkaloids	Part of caffeine metabolism	a	a	b	b
155.1078	7.52	Pos	(2E)-2,6-Dimethyl-2,5-heptadienoic acid	$C_9H_{14}O_2$	HMDB0035135	Lipid	Cell signaling	a	b	c	c
137.1336	10.12	Pos	(S)-alpha-Phellandrene	$C_{10}H_{16}$	C11391	Lipid	Cell signaling/ Lipid transport	a	b	c	c

Table 7 (continued)

m/z	Retention time (mins)	Ion mode[a]	Annotation (putative)	Molecular formula	ID (KEGG/HMDB/PubChem)[b]	Class of metabolite	Possible role in plant reported in literature	Significant differences QT16258 (Un)	QT16258 (In)	Janz(Un)	Janz (In)
135.1169	10.27	Pos	p-Mentha-1,3,8-triene	$C_{10}H_{14}$	HMDB0037013	Lipid	Cell signaling/Lipid transport	a	b	c	c
95.0849	0.12	Pos	1-Methyl-1,3-cyclohexadiene	C_7H_{10}	HMDB0031532	Unsaturated hydrocarbons	Cell signaling/Lipid transport	a	b	c	c
155.106	6.76	Pos	Boschnialactone	$C_9H_{14}O_2$	HMDB0038697	Fatty acid	Cell signaling	a	b	c	d
395.066	1.06	pos	Quercetin 4'-isobutyrate	$C_{19}H_{16}O_8$	44,259,328	Flavonoid	Antimicrobial/radical scavengers	a	a	b	c
343.2115	5.48	neg	3-Methylbutyl 3-oxobutanoate	$C_9H_{16}O_3$	HMDB0036396	Beta keto acids and derivative	Unknown	b	a	c	c

[a] pos means metabolites detected in positive ion mode whereas neg means metabolites detected in negative ion mode by mass spectrometry

[b] The number following "HMDB" is the HMDB ID, the numbers following "C" is the KEGG ID and the plain number without suffix is Pubchem ID in the table for detailed information of the corresponding annotated metabolite

2018), and could take part in browning of roots and hypersensitive response cell death (Sato et al. 2019).

Compounds such as petroselinic acid, gelsemine, 1-isothiocyanato-4-methylsulfinyl-butane, and remerine were found as induced compounds in inoculated 'Janz' (based on univariate analysis). These compounds were reported as antimicrobial compounds and have a possible role in plant defense (Vaughn and Berhow 2005; Chirumbolo 2011). The time and location of the metabolites could also be a critical factor whether a plant succumbs to nematode infestation or withstands it (Veech 1982).

Additionally, some of the metabolites could act as nematode attractants. The metabolite indole-3-acetic acid (IAA), which was significantly ($p \leq 0.01$) greater in 'Janz' than in 'QT16258', has been reported as a potential attractant for *M. incognita* (Curtis 2008). IAA can bind to the cuticle of *M. incognita* and change signaling in the root cuticle surface that attracts the nematode towards the root thereby promoting infestation. This attraction was proved with both root exudates and in vitro application of IAA (Curtis 2008). Furthermore, the amino acid arginine and phenolic aldehyde vanillin conjugates, which were higher in concentration in 'Janz' than in 'QT16258', were reported as attractants of *M. incognita* in vitro (Fleming et al. 2017). Interestingly, the abundance of these potential attractants was not significantly different between the uninoculated and inoculated 'Janz' treatments, and therefore must be considered constitutive. Further investigation is required to confirm if 'Janz' constitutively expresses these compounds as root exudates that attract *P. thornei*.

Some variation in the metabolite profiles of the 'QT16258' and 'Janz'are likely to be also due to differences in the growth stages of the genotypes at the time of sample collection. 'Janz' grew quickly in comparison to 'QT16258'. The significant MS features found in 'Janz' could be due to growth and reproduction processes of the plant. Zeatin biosynthesis is one of the potential pathways that was significantly changed in 'Janz' compared with 'QT16258', and is part of the cytokinin group of compounds, which are phytohormones (Schafer et al. 2015). Zeatin was a potential attractant to root-knot nematode *M. incognita* (Kirwa et al. 2018). Other metabolites in significantly higher concentrations in 'Janz', including tryptophan and other amino acid derivatives and pantothenic acid, are involved in plant growth and development (Raman and Rathinasabapathi 2004; Tzin and Galili 2010). Tryptophan also has a role in plants as the precursor of IAA (auxin hormones). The phytohormones IAA and zeatin could also play important roles in growth and development in 'Janz'. Higher levels of IAA and zeatin can promote lateral root development (Tian et al. 2014). Furthermore, cis-zeatin is a hormone in plant roots that increases in response to phosphate starvation to increase the length of roots and root hairs to promote phosphorous

absorption from the soil (Silva-Navas et al. 2019). The metabolites that were significantly different in 'Janz' in the present study were mostly part of the following biosynthetic pathways: tropane, piperidine and pyridine alkaloid biosynthesis, purine metabolism, and tyrosine and tryptophan biosynthesis, which have been reported in wheat during post anthesis stages (Thomason et al. 2018). Many amino acids biosynthetic pathways were significantly changed in both the genotypes. Arginine was the common amino acid metabolic pathway that was significantly changed for both genotypes, but predominantly in 'Janz'. The predominant changes in various amino acid profiles in 'Janz' could be due to the heading and grain filling stage of the genotype (Pan et al. 2006). The increased amino acid biosynthesis could be utilized for increased protein synthesis contributing to the growth and nitrogen accumulation in grain (Causin 1996; Pan et al. 2006) of wheat genotypes.

Identification of metabolites from the 4,997 MS features in this study is important for biological interpretation of the data. In this study, both an in-house chemical library prepared with authentic standards and public metabolomics databases were used to annotate MS features and understand their potential biological function. The annotation results based on the authentic in-house chemical library corroborated putative annotation of the most significant features in both the genotypes with similar classes of metabolites indicated. However, there is limitation of annotation within public databases (Scalbert et al. 2009), due to variations in LC–MS instrumentation and procedures among laboratories. There is the possibility of misidentification of some of the metabolites during annotation, as there could be more than one compound with the same molecular mass. Therefore, further investigations using a targeted approach with selected relevant MS features could be used to confirm the presence of those metabolites in the wheat roots. A few synthetic compounds were also recorded as part of the manual annotation. Therefore, the manually annotated metabolites should be considered carefully and validated in follow-up studies. Subsequent targeted metabolomics studies of the significant molecular features in the moderately resistant wheat genotype 'QT16258' could substantiate the metabolite fingerprinting and further support the results of pathway analysis of this study. Transcriptomics and proteomics studies will be very important to understand the genes related to the metabolites and the abundance of enzymes over-expressed or down-regulated in particular metabolic and defense pathways in the wheat genotypes to support these findings.

The functional characterisation of these constitutive compounds through in vitro assays is required for confirmation of their specific role in *P. thornei* resistance. This study provides the first stage towards discovery of biomarkers for resistance against *P. thornei* in future. For example, Willett et al. (2020) proposed from an untargeted metabolomics study that pipecolic acid could play an important and major role in reducing the population density of sting nematodes (*Belonolaimus longicaudatus*) in tolerant Bermuda grass (*Cynodon transvaalensis*), and could be used as a biomarker for plant breeding programs. Considering that the defense molecules identified in this study are expressed constitutively, plants could be phenotyped for resistance to *P. thornei* using metabolites associated with resistance as biomarkers, without even the need for challenging the plants with nematodes. It could also be important to investigate whether these metabolites are present in high concentrations only in the plant roots or whether they are also present in high concentrations in leaf tissue, which is easier to collect for analysis than root tissue.

Conclusions

In this study, we showed that there is a difference in the abundance of metabolites in resistant and susceptible wheat genotypes at 8 weeks PNI. Metabolic profiling of *P. thornei* resistant and susceptible wheat genotypes indicates that fatty acid, lipid and flavonoid classes of metabolites in resistant wheat roots could reduce nematode reproduction**.** The defense against *P. thornei* is constitutive in the resistant wheat genotype 'QT16258'. A range of small and long chain fatty acids and glycerolipid molecules in 'QT16258' could initiate a cascade of defense signaling, including jasmonic acid mediated production of plant metabolites and pathogenesis related proteins. The abundance of fatty acids and glycerolipids could also strengthen the cell walls by deposition of cutin, suberin and wax to inhibit nematode penetration in the root. The relatively higher abundances of some flavonoids and alkaloids could play important roles in inhibiting nematode reproduction inside the roots of 'QT16258'. Increased levels of phytohormones and amino acids were identified in 'Janz' in this study, which can be associated with development of root tissues and involvement in plant growth and reproduction. The specific flavonoids and phenolics identified in 'Janz', such as coniferyl alcohol could be part of a hypersensitive browning reaction rather than providing defense against the nematode. Whereas IAA and vanillin acetate conjugates could attract *P. thornei* in 'Janz' and promote infestation in the roots constitutively. Future targeted metabolomics approaches could shed more light on the role of these metabolites in defense against *P. thornei* in wheat. In vitro tests of effects of the identified metabolites on the nematodes are also recommended for future studies to understand potential modes of action and to validate these findings.

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Authors contribution All authors contributed to the study conception and design. Sample preparation, data collection and analysis was performed by MR, and TR, MR, RZ and JT contributed to interpretation of data. The first draft of the manuscript was written by MR and all authors reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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CHAPTER 5

GENERAL DISCUSSION, CONCLUSION AND FUTURE PROSPECTS

1. General Discussion

Infestation of wheat roots by the root-lesion nematode *Pratylenchus thornei* causes significant economic loss in different parts of the world (Nicol et al., 1999; Castillo & Vovlas, 2007; Smiley & Nicol., 2009). Some wheat genotypes express partial resistance to *P. thornei* limiting to various extents nematode reproduction inside their roots, root damage, yield loss and the number of nematodes carried over in the soil (Thompson et al., 1999; Thompson et al., 2008). The aim of this PhD study was to identify defensive biomolecules and their potential modes of action and pathways in providing defence mechanisms in resistant to moderately resistant wheat genotypes against *P. thornei* infestation in comparison with susceptible wheat genotypes.

The inheritance of resistance to *P. thornei* in wheat has been found to be polygenic and additive (Zwart et al., 2004; Thompson & Seymour, 2011; Thompson et al., 2012). Therefore, this PhD study was performed considering more than one type of biomolecule as potential components of defence including proteins, particularly enzymes, and metabolites.

The life stage dynamics and histopathology study was important to understand the critical time points where biomolecules including metabolites can act to reduce nematode reproduction in resistant wheat genotypes (Chapter 2). A range of time points (1 to 12 weeks) was used initially for this PhD work to understand the reproduction of *P. thornei* and changes in life stages inside root tissues. This study has revealed that although the differences in nematode reproduction between resistant and susceptible wheat genotypes can be found at 4 weeks and onwards (4 to 12 weeks), the distinct

differences in both nematode numbers and deposited eggs inside roots occur at 8 weeks PNI. Understanding the critical time points has created a foundation for detailed biochemical profiling, namely polyphenol oxidase profiling, total phenol estimation and untargeted metabolic profiling, in plants grown and inoculated under controlled environmental conditions. Previously, there was no standardised protocol to study comparative peroxidase and polyphenol oxidase enzyme assays of multiple samples in a timely manner under the same experimental conditions. The enzyme assay protocols were optimised in this PhD work for a microplate assay to check comparatives enzyme activities in resistant and susceptible wheat genotypes in response to *P. thornei* infestation (Chapter 3). In addition to 8 weeks PNI, other time points were also considered for the assay and the results have been confirmed in more than one experiment with wheat grown both in soil and agar systems.

The highest expression of total phenol was recorded at 8 weeks PNI for all genotypes and significantly ($P \leq 0.05$) higher concentrations of total phenol were found in the most resistant synthetic hexaploid wheats (CPI133872 and CPI133859) along with increased activity of polyphenol oxidases (PPO and POD). However, the total phenol content in moderately resistant GS50a and its derived lines (QT8343) was comparable to susceptible genotypes (such as Gatcher and Janz) at all time points (2 to 8 weeks). From studies on inheritance of resistance, the estimated minimum numbers of genes required for effective resistance to *P. thornei* in wheat is four to six (Thompson et al., 2012). The genetic resistance source in GS50a is different from that of synthetic hexaploids (Zwart et al., 2004). CPI133872 and CPI133859 share at least one common resistance gene that is not present in GS50a.

It is noteworthy that there was significantly higher activity of PPO in both synthetic hexaploid wheats and GS50a and their resistant derived lines compared to the susceptible genotypes (such as Gatcher, Janz). Thus, oxidised phenolic molecules could provide defence in GS50a and its derived lines, even though they had comparable total phenolic contents to susceptible genotypes. Phenolics in the presence of PPO and POD can be converted into lignin to give cell wall rigidity in resistant wheat genotypes. The lower levels of PPO and POD in susceptible wheat genotypes could be the reason for the

ineffectiveness of total phenol in defence against *P. thornei*. Phenolics could also be involved in different pathways in susceptible wheat genotypes and could be converted into root browning products rather than providing defence via lignin formation or toxic oxidised phenolic metabolites.

Moreover, there could be specific phenolic metabolites that play major roles in defence against *P. thornei*. The concentrations of specific metabolites that contribute to the total phenol contents were studied to understand their roles in defence in one of the resistant derivatives of CPI133872, namely QT16258. An untargeted approach was followed to record the largest array of metabolites with significantly different levels of abundance between a resistant wheat genotype (QT16258) and a susceptible genotype (Janz) (Chapter 4). The metabolic profiling revealed more than one type of secondary metabolite could be responsible for providing defence in QT16258. Flavonoids (such as quercetin-3,4'-O-di-beta-glucoside and myricetin-xyloside) were found in high concentrations in QT16258 and have been reported to be responsible for reduced egg deposition in other plant-nematode interactions. Alkaloids (such as hirsutine) were also found in high concentrations in QT16258 and have been reported to be responsible for reduced nematode motility. Additionally, lipid components of compounds such as cutin, suberin and wax that were found in high concentration in QT16258, have been reported to be responsible for increased rigidity of cell walls to minimise penetration of the nematode.

A summary of the thesis with the key findings of each experimental chapter (Chapter 2 to Chapter 4) is shown in Figure 5.1.

Chapter 2

Comparative differential reproduction rate of *Pratylenchus thornei* and histopathology in moderately resistant and susceptible wheat genotypes over time

- Significant (P<0.05) differences in *P. thornei* numbers with higher numbers inside roots of susceptible wheat genotypes than moderately resistant wheat genotypes at 4 to 12 weeks PNI.
- Clear difference in egg deposition with higher egg numbers inside susceptible wheat genotypes at 8 weeks PNI.
- In addition to less egg deposition and delayed egg deposition, inhibition of juvenile maturity could also be a reason for less nematode numbers inside resistant wheat root at 4 to 12 weeks PNI.
- No effect of nematode inoculation was found on total protein content, cell wall bound phenolics and lignin contents.
- No effects of nematode inoculation was found on plant height, shoot and root biomass of moderately resistant and susceptible wheat genotypes grown under controlled glasshouse conditions with plentiful supply of water and nutrients.
- Eight weeks PNI could be critical for detailed biochemical studies to understand the defence mechanism.

Chapter 3

Constitutive and induced expression of total phenol and phenol oxidases in wheat genotypes ranging in resistance/susceptibility to the root-lesion nematode *Pratylenchus thornei*

- The combined effects of total phenols and oxidative enzymes could be important for defence against *P. thornei* in some resistant wheat genotypes.
- Greater total phenol was constitutive in resistant CPI133872 and CPI133859, whereas phenol oxidases POD and PPO were inducible in resistant and moderately resistant wheat genotypes.
- Effect of both phenol oxidases and total phenol could be responsible for providing defence against *P. thornei* in some of the resistant wheat genotypes.
- Total phenol in susceptible wheat genotypes could be responsible for browning reaction rather than providing defence.

Chapter 4

Metabolomic profiling of wheat genotypes resistant and susceptible to root-lesion nematode *Pratylenchus thornei*

- Chemo-metric analysis indicated dissimilarity of the metabolome between *P. thornei* resistant and susceptible genotypes at 8 weeks PNI.
- The metabolic defence in moderately resistant wheat genotypes against *P. thornei* is mostly constitutive.
- Metabolites in resistant wheat genotype (QT16258) might be responsible for reduced egg deposition as well as nematode motility inside root tissue and might strengthen the root cell wall to inhibit nematode penetration.
- Significantly higher concentrations of metabolites in susceptible Janz, including indole acetic acid conjugates could act as *P. thornei* attractants and phenolics could be part of a hypersensitive browning reaction.

Figure 5.1. Outline of the thesis with key messages of each chapter

The research questions posed with the outcomes of this thesis are summarised below:

A. How do the defence biochemicals expressed in the roots of resistant wheat genotypes act against *P. thornei*? Are they constitutive or inducible?

Most defence compounds against *P. thornei* in the wheat genotypes investigated were found to be constitutively expressed, in respect to expression of total phenol and the expression of the different classes of metabolites such as flavonoids, alkaloids, and lipids. However, the phenol oxidases polyphenol oxidase and peroxidase were induced over time in moderately resistant wheat genotypes and attained maximal concentration in the roots at 4 weeks PNI. Additionally, the biomolecules potentially responsible for attracting nematodes, such as vanillin acetate and indole acetic acid conjugates were found to be expressed constitutively in the susceptible wheat genotype. Therefore, mechanisms contributing to the resistance and susceptibility of wheat genotypes appear constitutive in nature. However, the combined effect of both constitutive and inducible defence compounds might be responsible for providing overall defence against *P. thornei*.

B. Are there any changes in wheat root morphology and cell wall composition upon *P. thornei* infestation?

In this study, there was no effect of *P. thornei* inoculation on the cell wall bound lignin and phenol surrounding the root cortex area of the resistant (QT8343) and susceptible (Gatcher and Janz) wheat genotypes. However, the vascular cylinder in QT8343 stained more intensely for lignin than in Janz and Gatcher. The lignin present in the vascular cylinder could be part of general plant defence by strengthening the xylem for better water transport. Moreover, plant roots have three dimensional architecture and the different layers cannot be visualised using 2D photomicrographs. Three dimensional images of the nematode infested root or image of the root by different layers could shed more light on the activity of *P. thornei* inside wheat roots over the time. Temporal and

spatial interactions of wheat root-*P. thornei* should be investigated in future studies with confocal and two photon microscopy along with the staining techniques.

C. What are the molecules responsible for plant defence and what are their possible roles in limiting the reproduction of *P. thornei* in resistant wheat genotypes? What are the molecules responsible for susceptibility in susceptible wheat genotypes?

Different classes of metabolites are potentially involved in resistance or susceptibility of wheat genotypes to *P. thornei* infestation. The flavonoid class of metabolites could be responsible for reduced egg deposition, the alkaloid class of metabolites could be responsible for reduced motility, whereas the lipid class of metabolites could be responsible for providing cell wall rigidity in the resistant wheat genotype QT16258. The pathways that were significantly ($P \leq 0.05$) changed in QT16258 compared to Janz include flavonoid biosynthesis, linoleic acid metabolism, linolenic acid metabolism, stilbenoid, diaryl heptanoid and gingerol biosynthesis, fatty acid and unsaturated fatty acid biosynthesis, and cutin, suberin and wax biosynthesis. Most of these pathways are related to plant defence against pathogens. Furthermore, the enhanced oxidation of phenolic metabolites in the presence of PPO and POD might produce more toxic compounds inside moderately resistant wheat roots to act against the nematodes.

Some metabolites at higher concentrations in susceptible Janz, including the phenolic coniferyl alcohol, could be part of a hypersensitive browning reaction to nematode invasion; and indole acetic acid and vanillin acetate conjugates could be nematode attractants. The pathways which were significantly ($P \leq 0.05$) changed in Janz compared to QT16258 included amino acids metabolism, caffeine metabolism, terpenoid metabolism, phenyl alanine metabolism, ascorbate and aldarate metabolism, and zeatin and riboflavin biosynthesis. Most of these pathways are related to growth and reproductions in plants. Zeatin was previously found related to attraction of parasitic nematodes towards the host root tissue (Kirwa et al., 2018).

2. Conclusion and Future Prospects

There are different sources of wheat resistance against *P. thornei* infestation. However, information on the resistance mechanisms of those sources is limited. The work described in this thesis has contributed to elucidation of wheat defence mechanisms against *P. thornei* infestation. Two wheat resistance sources and their derived lines were used in this work, namely, GS50a and synthetic hexaploid CPI133872. CPI133872 responded in a relatively more comprehensive way to provide defence against *P. thornei*. Therefore, the advanced breeding line QT16258, which is a CPI133872 derived line, was used for detailed untargeted metabolic profiling. Untargeted metabolic profiling revealed highly significant differences in metabolite abundance in the resistant (QT16258) and susceptible (Janz) wheat genotypes in roots as part of defence and susceptibility to *P. thornei*. Possible modes of action of the identified metabolites have been supported by the anti-nematode activities of those metabolites reported in the literature.

The present study could aid in understanding biomarkers of resistance against *P. thornei* in the wheat genotypes studied (CPI133872 and QT16258). Analysis of the metabolic profiles of the parental resistant genotype and/or other genotypes from the CP1133872/ Janz mapping population having differing tolerance and or resistance levels, will be important for identification of candidate metabolic features and potential biomarkers in future studies. A comparative study on differentially expressed genes (DEGs) at 8 weeks PNI, and other time points, could provide an overview of defence, and identify the genes responsible for reprogramming secondary metabolites for increased expression of defence in resistant wheat sources.

Detailed biochemical studies of other sources of *P. thornei* resistance should be considered to understand the importance of resistance traits in each source. Since several QTL have been linked to *P. thornei* resistance, an integration of the genes in different QTL with the candidate biomarker for resistance against *P. thornei* should be pursued. Understanding key resistance traits in different resistant wheat sources is important for effective and efficient breeding and introgressing different sources of resistance into one breeding line with combined mechanisms of resistance.

Counts of egg deposition in wheat genotypes with established resistance/susceptibility ratings (Thompson et al., 2020) should be pursued in future studies. Such experiments should be done with an increased number of replicates as the amount of root tissue at early time points of plant growth (0 to 4 weeks) is small. In addition, the shells of nematode eggs contain chitin compounds, which could be damaged by the enzyme chitinase in the roots of resistant wheat sources causing eggs to degrade and reducing hatching. A comprehensive and comparative study on egg degrading enzymes such as chitinase over time is recommended for future studies.

Several metabolites identified in this study, including fatty acids, lipids and sugars can initiate a cascade of defence responses including biosynthesis of pathogenesis related proteins (Rolland et al., 2002; Jung et al., 2009; Walley et al., 2013). Therefore, the roles of these molecules in cell signalling should be assayed. Untargeted proteomics profiling could improve our understanding and is recommended. The findings of the research presented in this thesis should be validated with *in vitro* assays to confirm the mode of action of the identified metabolites in resistant wheat genotypes in defence against *P. thornei*. Comprehensive bioassays of flavonoids, such as quercetin and myricetin, and alkaloids such as hirustine, could provide insights into changes in egg deposition and motility of vermiform life stages of *P. thornei* inside the roots of resistant compared to susceptible wheats.

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APPENDIX A

ELECTRONIC SUPPLEMENTARY INFORMATION OF CHAPTER 2

Article title

Comparative differential reproduction rate of *Pratylenchus thornei* and histopathology in moderately resistant and susceptible wheat genotypes over time

Journal

Plant Pathology (Wiley-Blackwell Publishing, UK)

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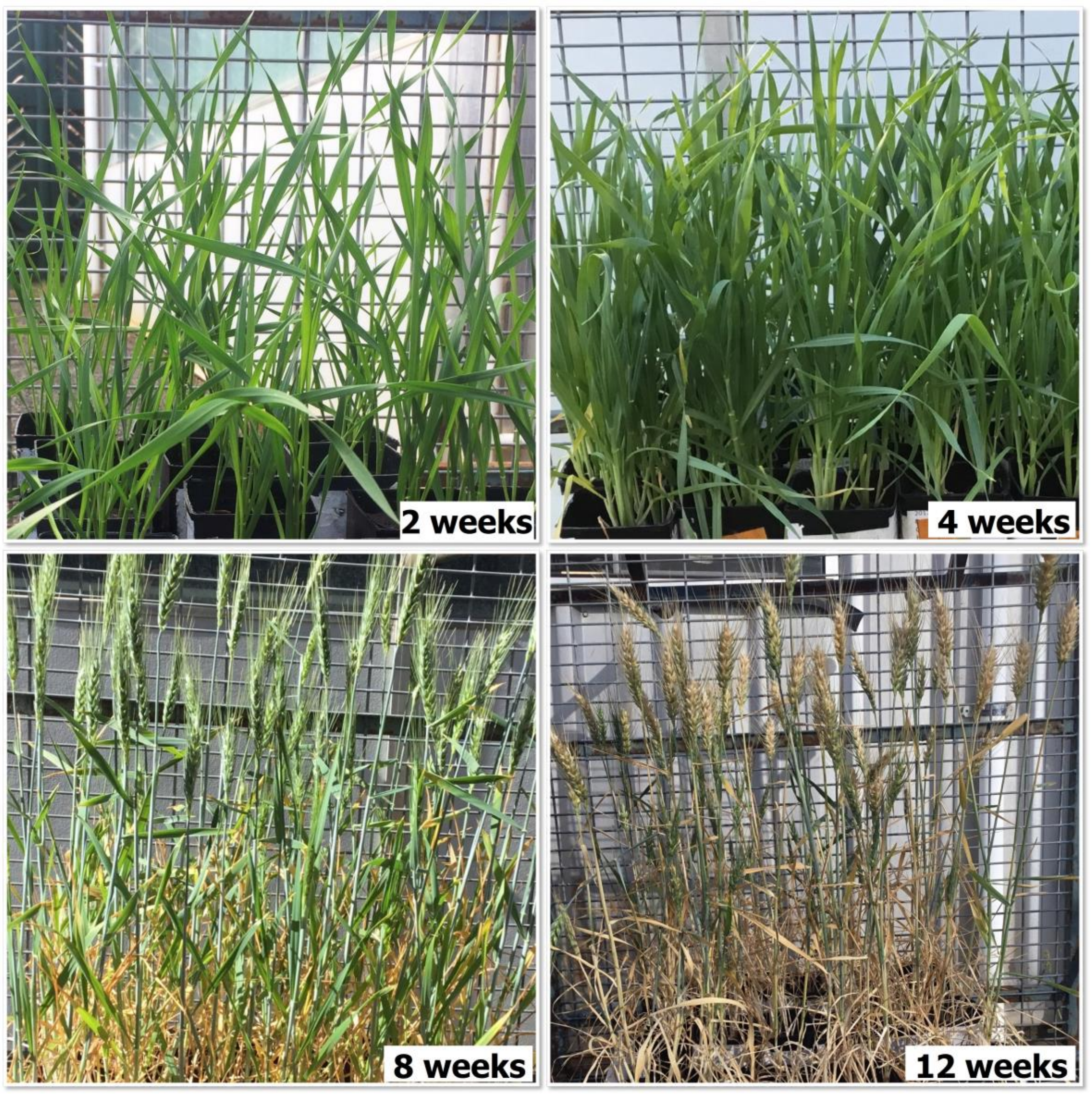
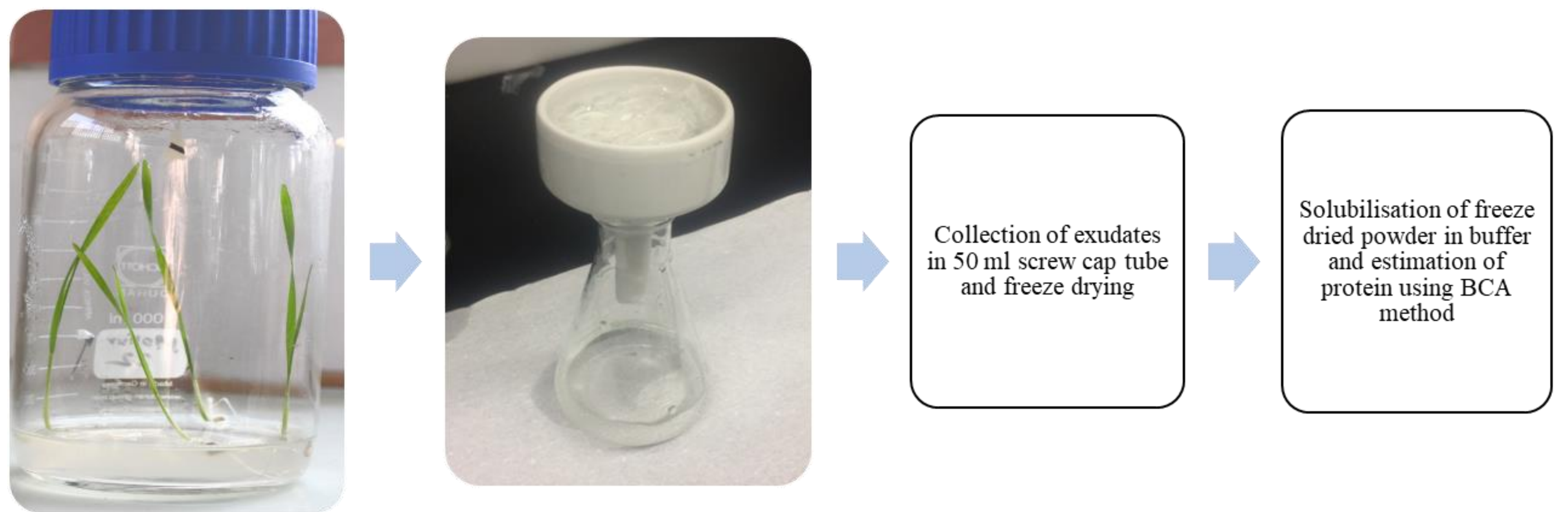
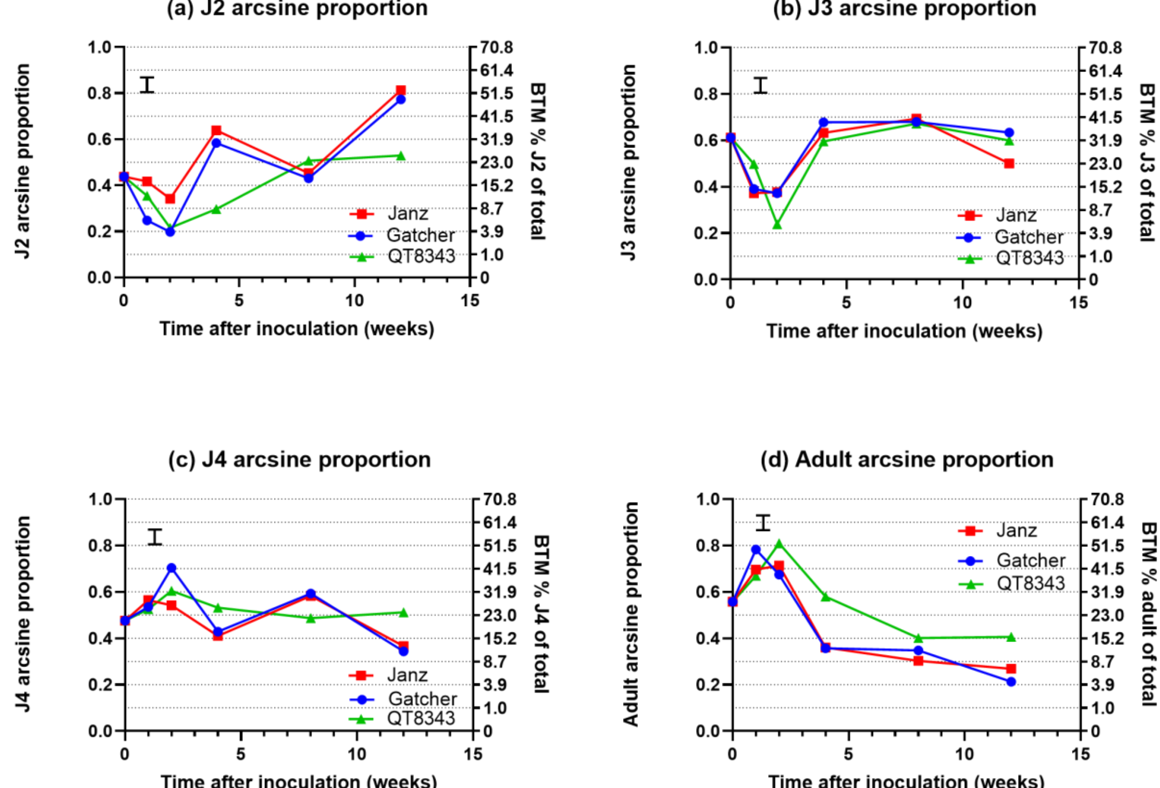


Supplementary Figure S1. Wheat growth at different time points. The pictures were taken immediately before the root sample collection respective time points.

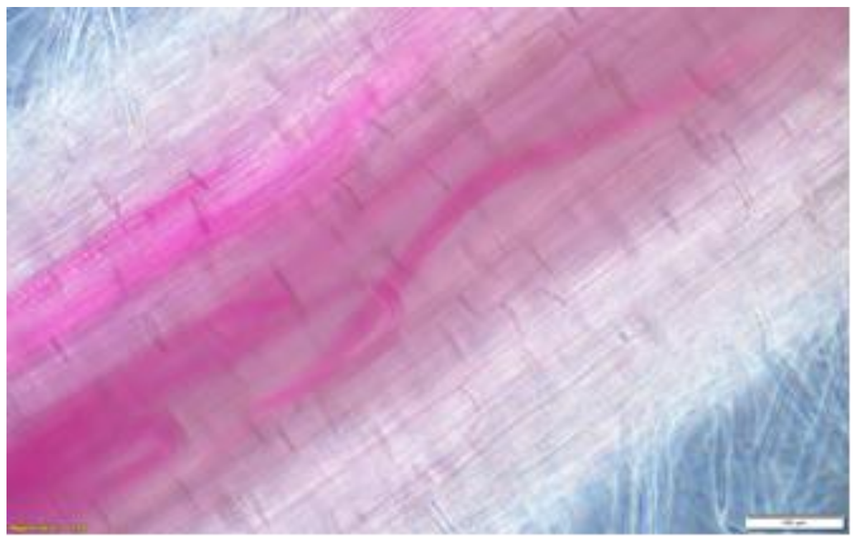


Supplementary Figure S2. Collection of root exudates from water agar medium (0.3%) at 10 days of seedling growth.

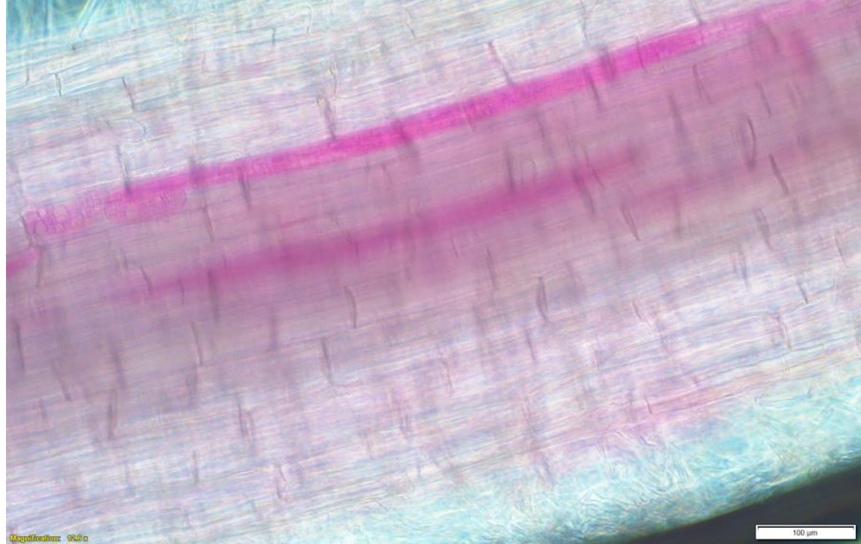


Supplementary Figure S3. Proportion of various life stage out of total *Pratylenchus thornei* (a) J2 (b) J3 (c) J4 and (f) adults in the roots of wheat genotypes Janz (red), Gatcher (blue) and QT8343 (green) at various times after inoculation. Points are plotted for the arcsine proportion transformed mean values on the left vertical axis with back transformed percentages on the right vertical axis. Bar marker = lsd (P=0.05).

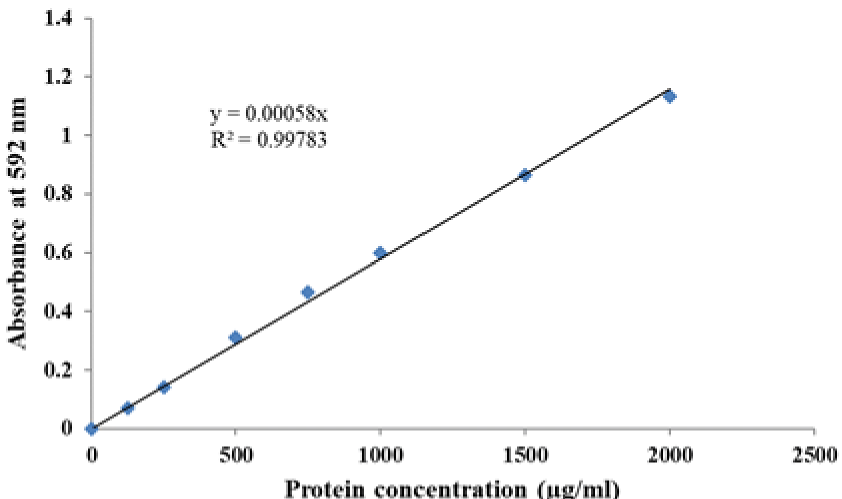
a)



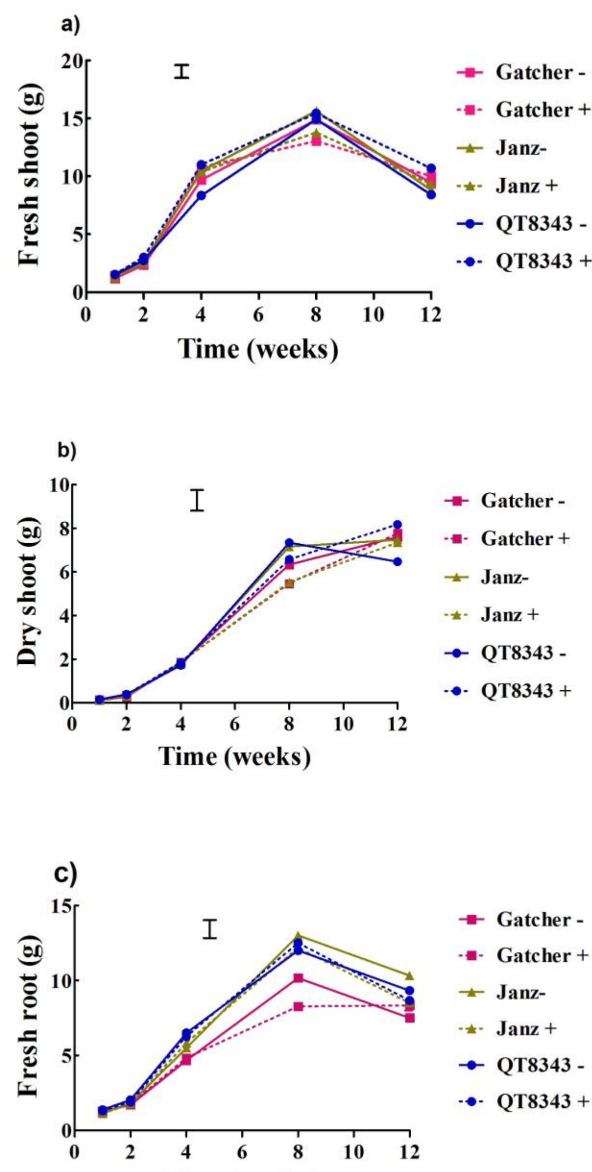
b)



Supplementary Figure S4. *Pratylenchus thornei* nematode in acid fuchsin stained root of Gatcher grown on water agar medium a) many nematodes clumped (left) b) nematodes with egg. Scale bar (a-b) 100 µm.



Supplementary Figure S5. Standard curve relating absorbance at 592 nm to protein concentration as bovine serum albumin (BSA) determined by the bicinchonic acid method for protein estimation in wheat roots.



Supplementary Figure S6. Plant growth status per pot (3 plants) of Gatcher (pink), Janz (green) and QT8343 (blue) over the different time points (1-12 weeks PNI), '-' indicates wheat genotypes were *P. thornei* uninoculated whereas '+' indicates the wheat genotypes were *P. thornei* inoculated (a) fresh shoot biomass, bar marker = lsd ($P \leq 0.05$) =1.291; (b) dry shoot biomass, bar marker = lsd ($P \leq 0.05$) =0.991; (c) fresh root weight, bar marker = lsd ($P \leq 0.05$) =0.939.

APPENDIX B

ELECTRONIC SUPPLEMENTARY INFORMATION OF CHAPTER 3

Article title

Constitutive and induced expression of total phenol and phenol oxidases in wheat genotypes ranging in resistance/susceptibility to the root-lesion nematode *Pratylenchus thornei*

Journal

Plants (*MDPI, Basel, Switzerland*)

Authors

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Supplementary Material

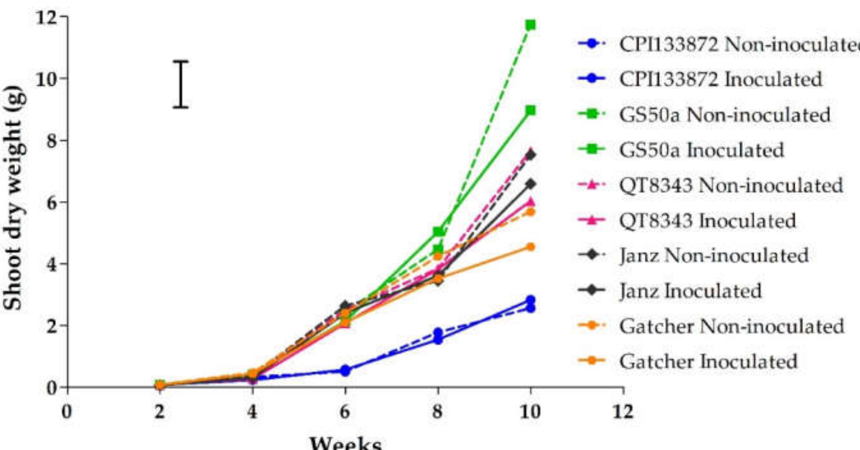

Figure S1. Dry shoot weight (g) of *Pratylenchus thornei* inoculated and non-inoculated treatments of five wheat cultivars over the time points (2–10 weeks). Values are the mean of three replicates. Bar marker indicates LSD = 1.72 ($p = 0.05$), for the interaction genotype* *P. thornei* * time.

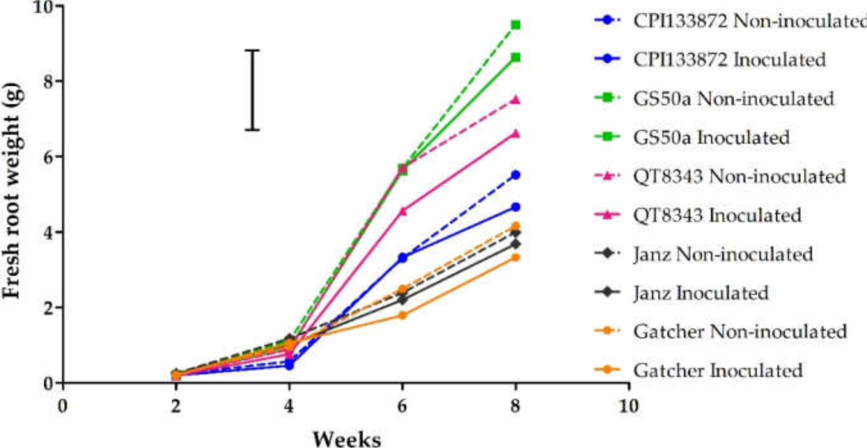

Figure S2. Fresh root weight of inoculated and non-inoculated treatment of five wheat genotypes over the time points (2–8 weeks). Values are the mean of three replicates. Bar marker indicates the least significant differences LSD =2.17 ($p = 0.05$), for the interaction genotype* *P. thornei* * time.

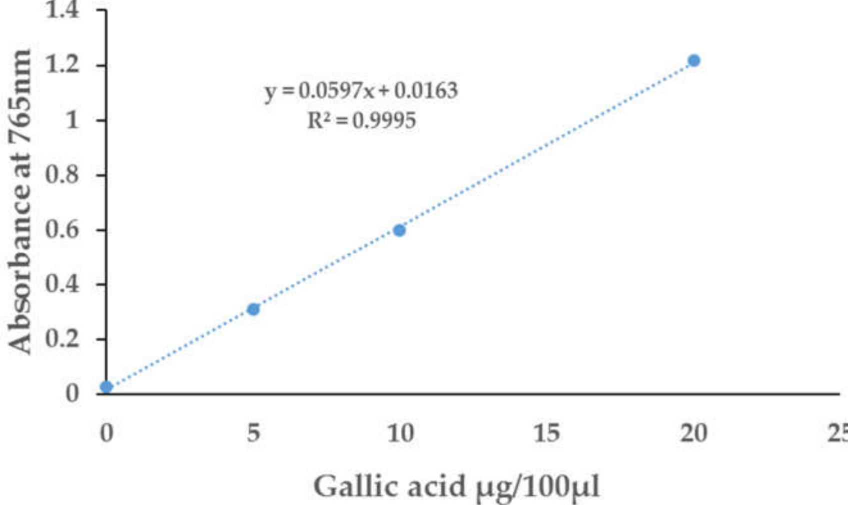

Figure S3. Standard curve of gallic acid for total phenols estimation in wheat root samples.

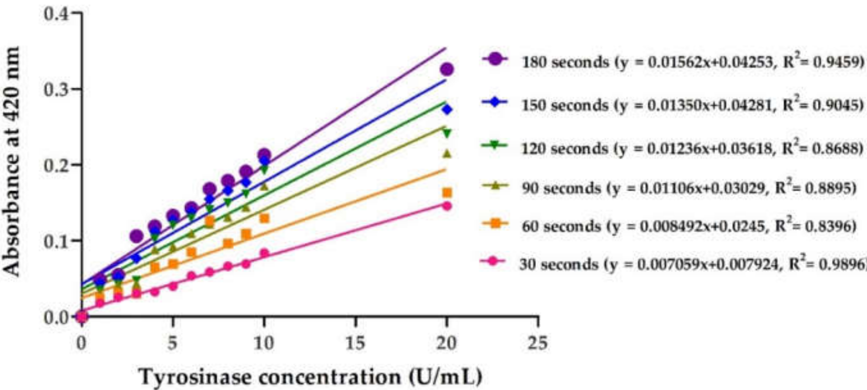

Figure S4. Standard curve of tyrosinase in linear range of concentration at different times (30–180 Seconds) of the enzyme assay progression reacting with pyrocatechol (50 mM) substrate.

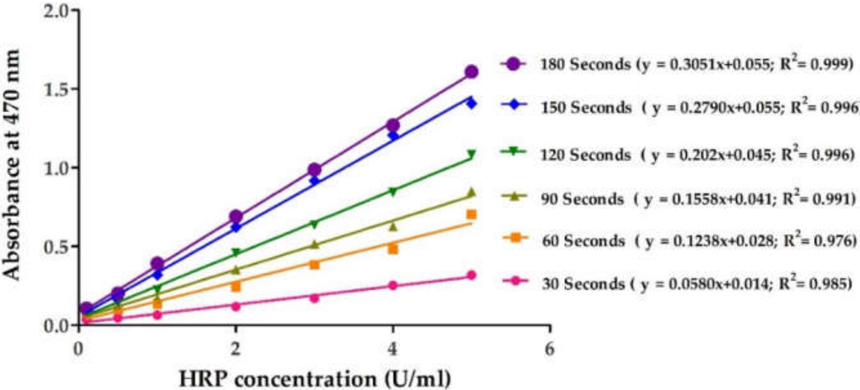

Figure S5. Standard curve of horseradish peroxidase (HRP) in linear range of concentration at different times (30–180 seconds) of the enzyme assay progression reacting with guaiacol (10 mM) and hydrogen peroxide (6.4 mM) substrate.

APPENDIX C

Electronic supplementary information

Article title

Metabolomic profiling of wheat genotypes resistant and susceptible to root-lesion nematode *Pratylenchus thornei*

Journal

Plant Molecular Biology (Springer, Netherland)

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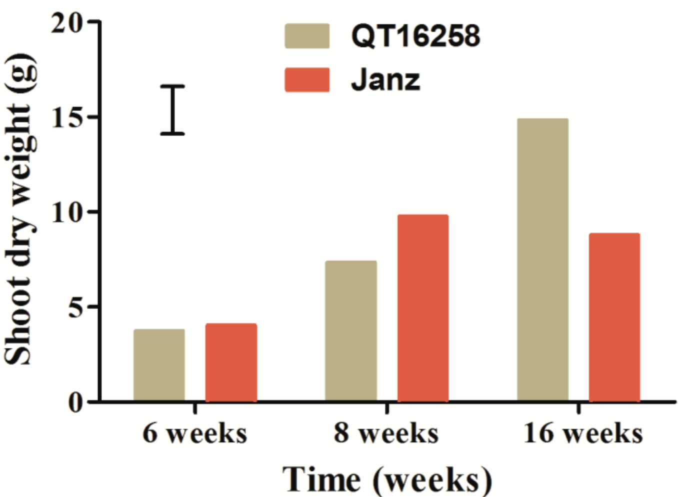
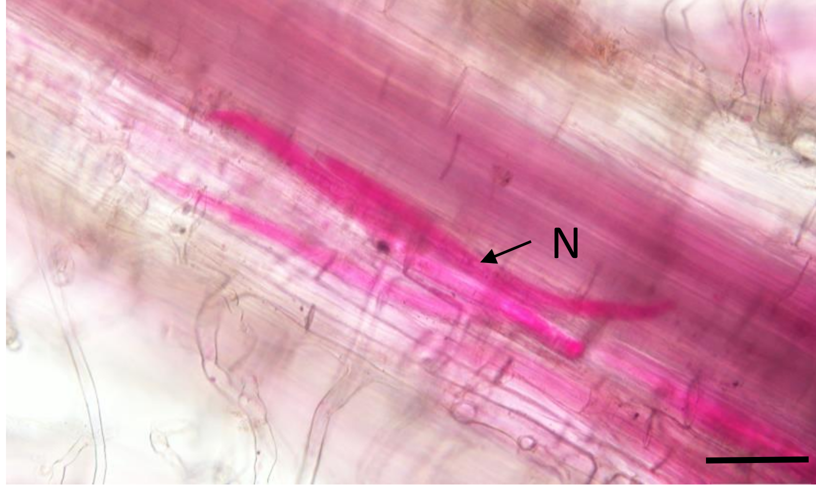
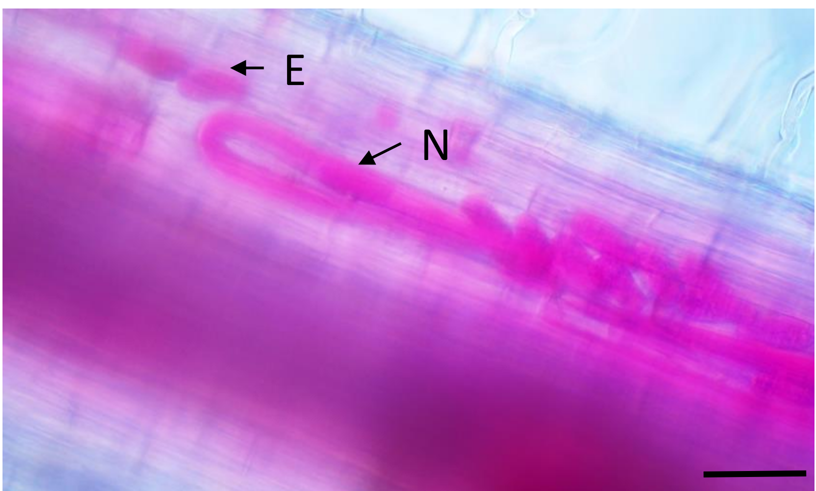


Supplementary Fig. S1 Mean shoot dry weight of 'QT16258' and 'Janz' in resistance time course experiment over 6 to 16 weeks (n=4). Bar marker is lsd ($p<0.05$)= 2.474.

(a)

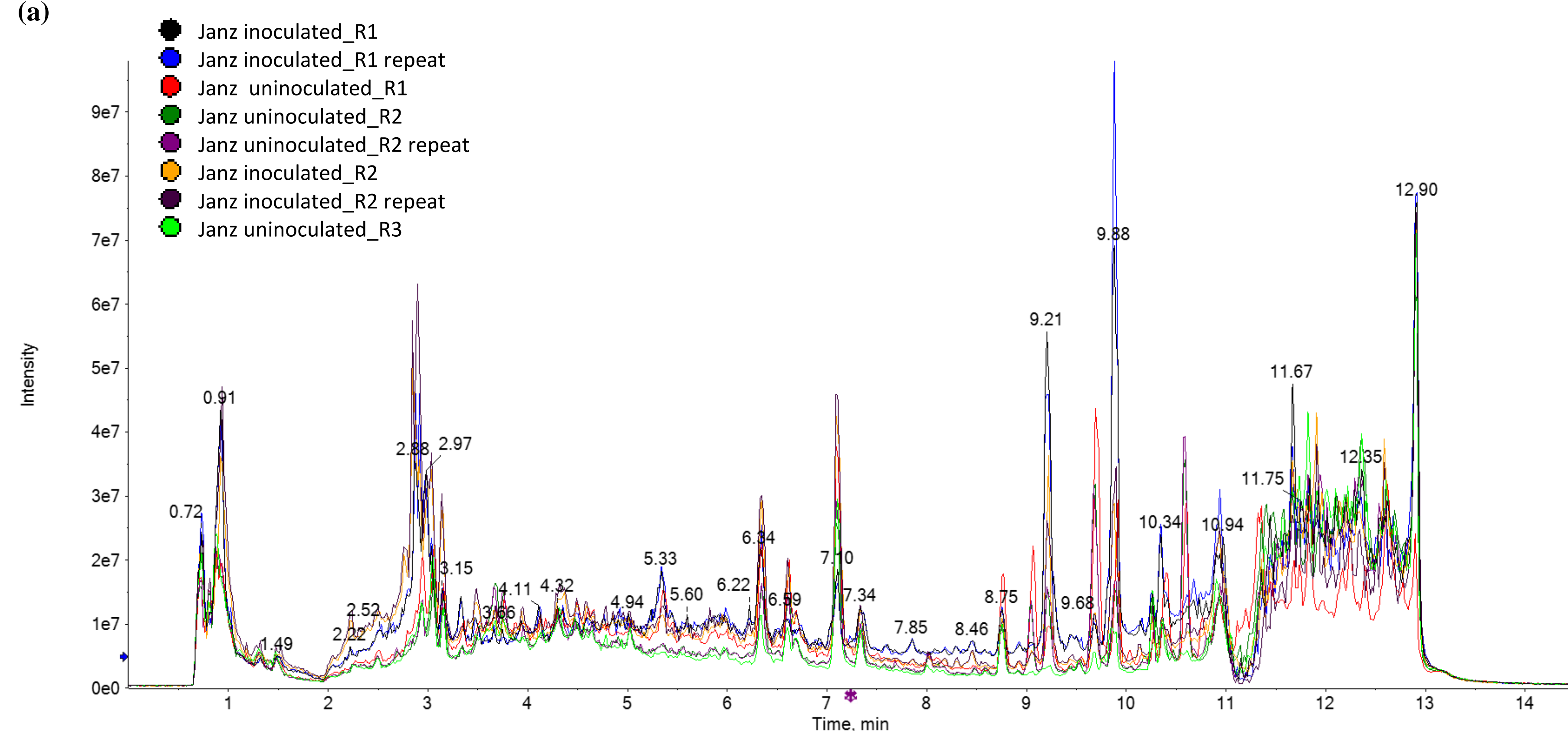


(b)

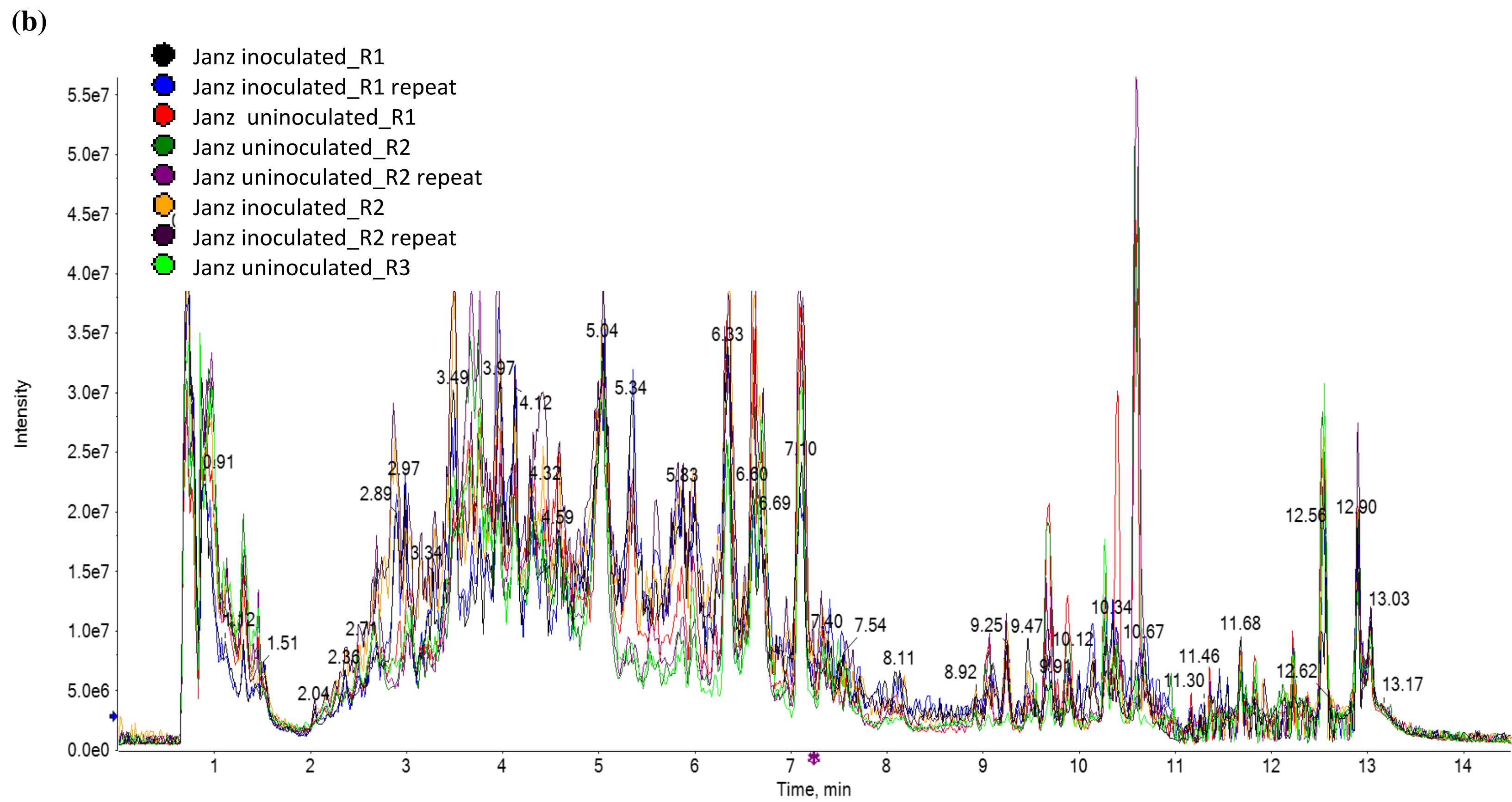


Supplementary Fig. S2 Acid fuchsin stained *Pratylenchus thornei* nematodes (N) and eggs (E) inside roots at 8 weeks of (a) 'QT16258' and (b) 'Janz'. Scale bar = 100 µm**.**

(a)

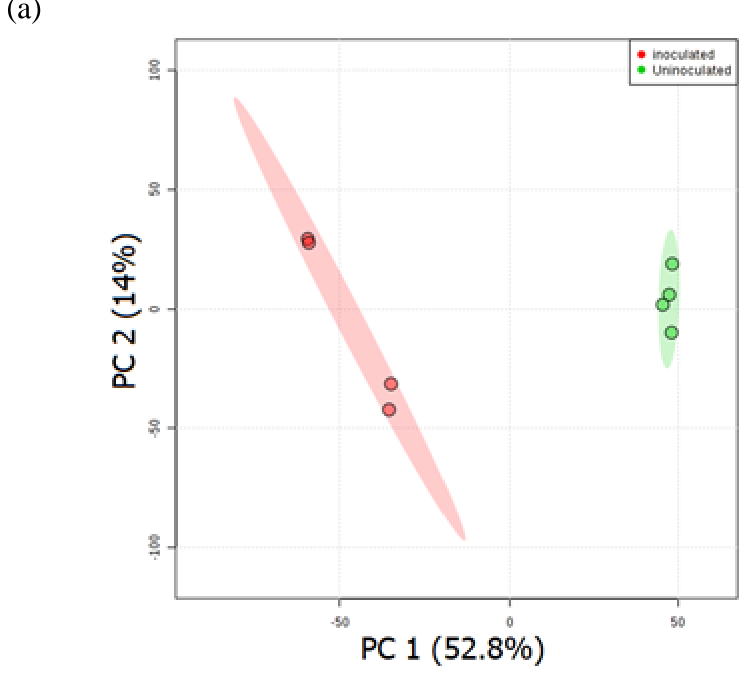


(b)

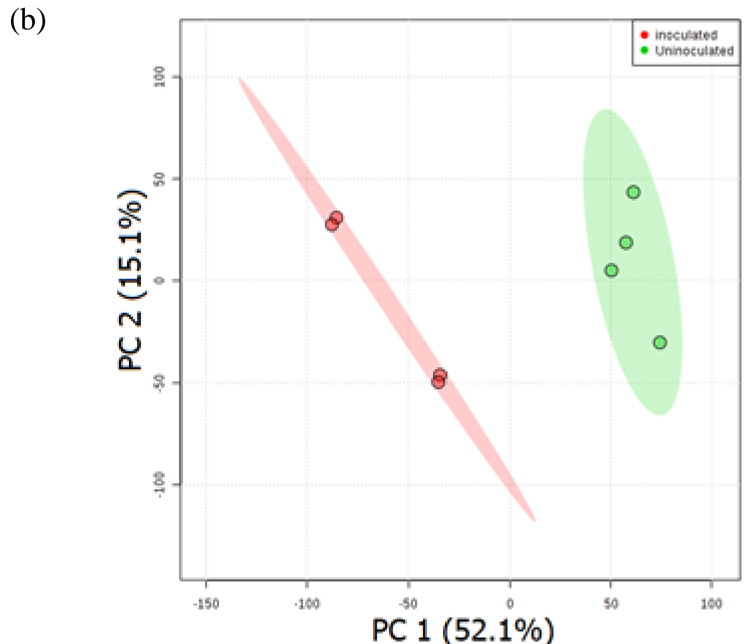


Supplementary Fig. S3 Total Ion Chromatogram (TIC) for the root extracts from biological replicates (R1, R2 and R3) and technical replicates (repeat) of 'Janz' inoculated and uninoculated samples used for optimisation of mass spectrometry in (a) positive ion mode and (b) negative ion mode.

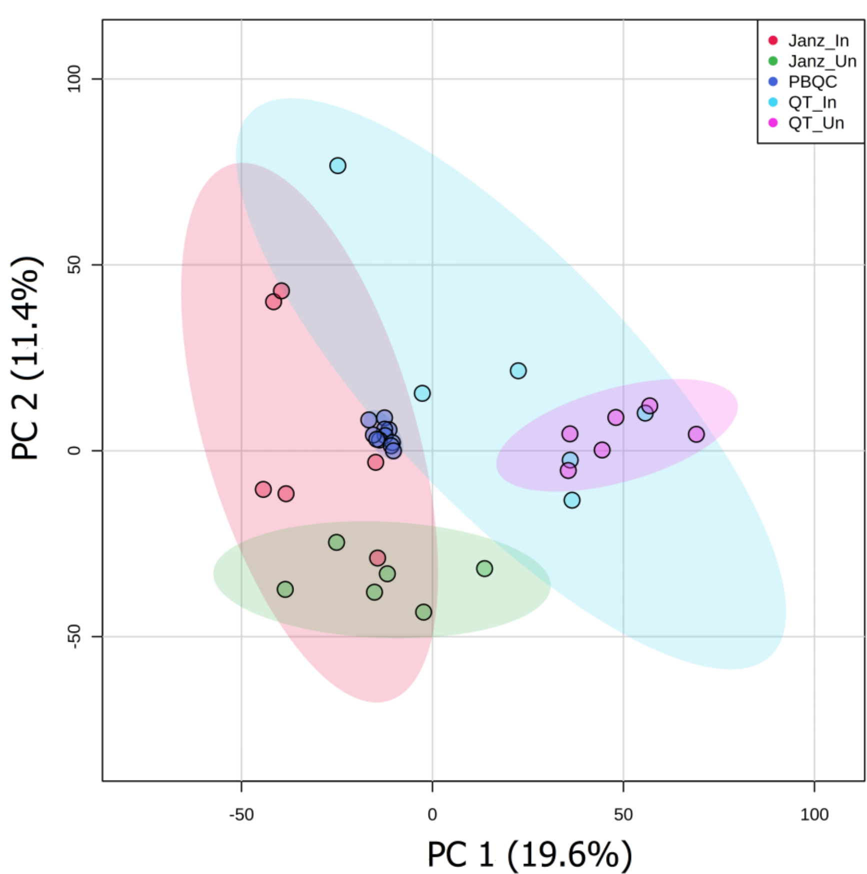
(a)



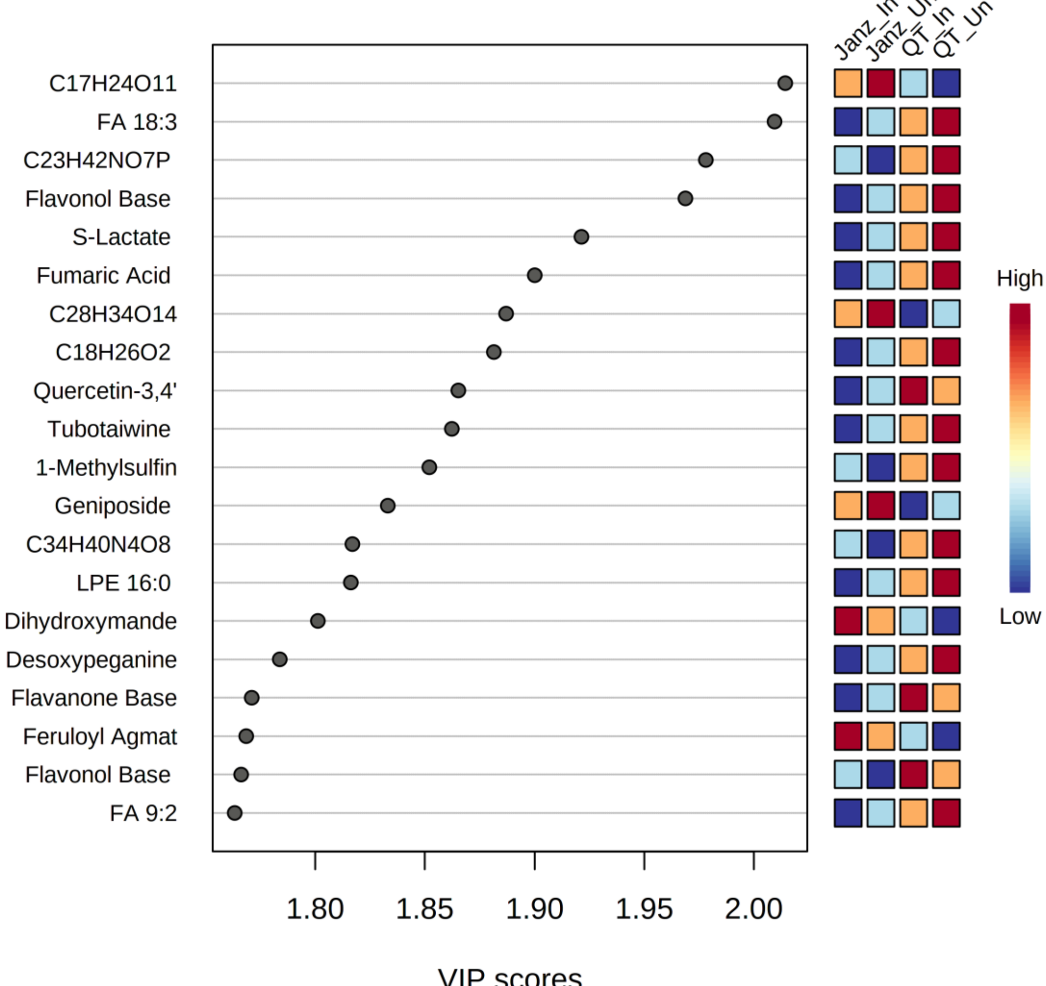
(b)



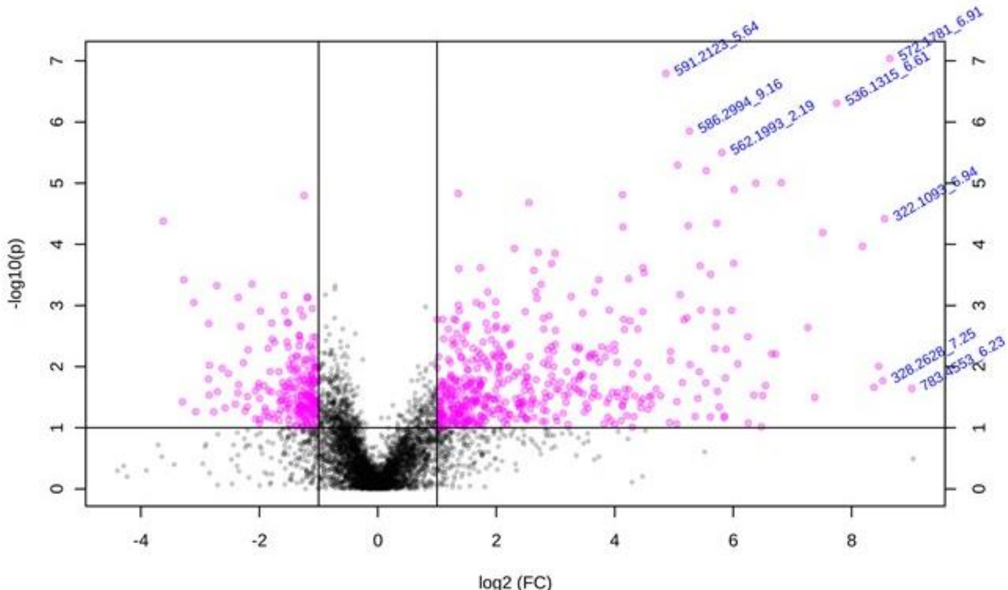
Supplementary Fig. S4 Principal Components Analysis (PCA) of the pooled samples used for the optimization study in (a) positive ion mode and (b) negative ion mode.



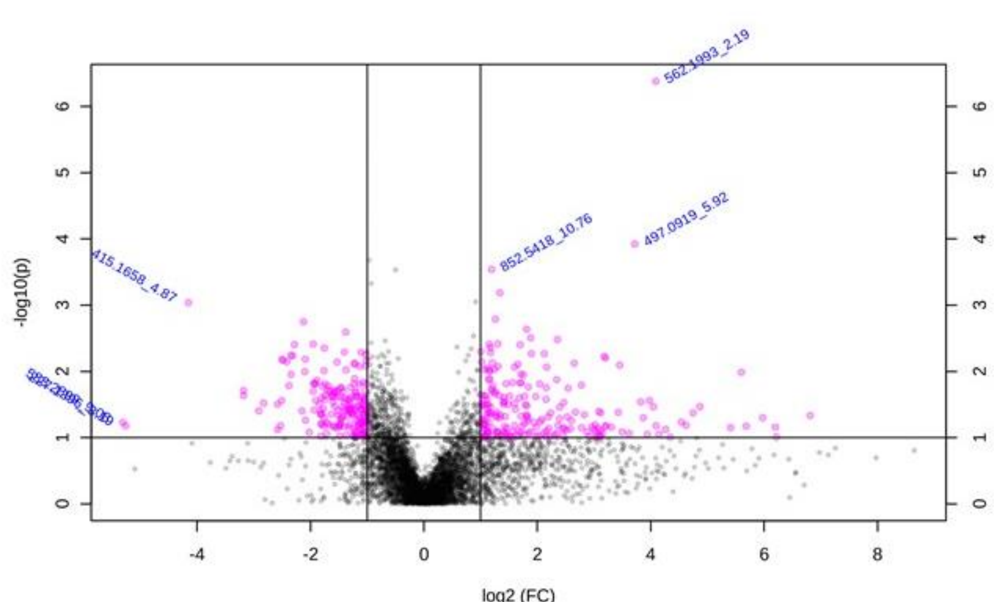
Supplementary Fig. S5 Principal Components Analysis (PCA) of all samples (n = 24) used in the study (‘Janz’ inoculated (red), ‘Janz’ uninoculated (green), ‘QT16528’ inoculated (light blue), ‘QT16528’ uninoculated (pink)) and 11 pooled biological quality control (PBQC) samples (dark blue), comprising 4,997 MS features in combined positive and negative ion modes.



Supplementary Fig. S6 Top 20 annotated metabolites based on highest variable importance projection (VIP) score in component 1 of partial least squares discriminant analysis (PLS-DA). The heat map on the right side shows relative concentration of the metabolites. Abbreviated names are: Quercetin-3, 4'= Quercetin-3, 4'-O-di-beta-glucoside; Dihydroxymande = Dihydroxymandelic acid; 1-Methylsulfin = 1-Methylsulfinylbutenyl isothiocyante; Flavonol base = Flavonol base 4O, 1MeO, O-Hex-Hex (Rt: 4.13 m/z: 657.162), and Flavonol base 3O, O-dHex, O-Hex-Hex (Rt: 4.09 m/z: 595.1685), FA= fatty acid, LPE= Lysophosphatidylethanolamine. First numeral in FA 18:3, LPE 16:0, FA 9:2 indicates total carbon numbers in the compound, whereas second numeral indicate number of double bonds in the compound.

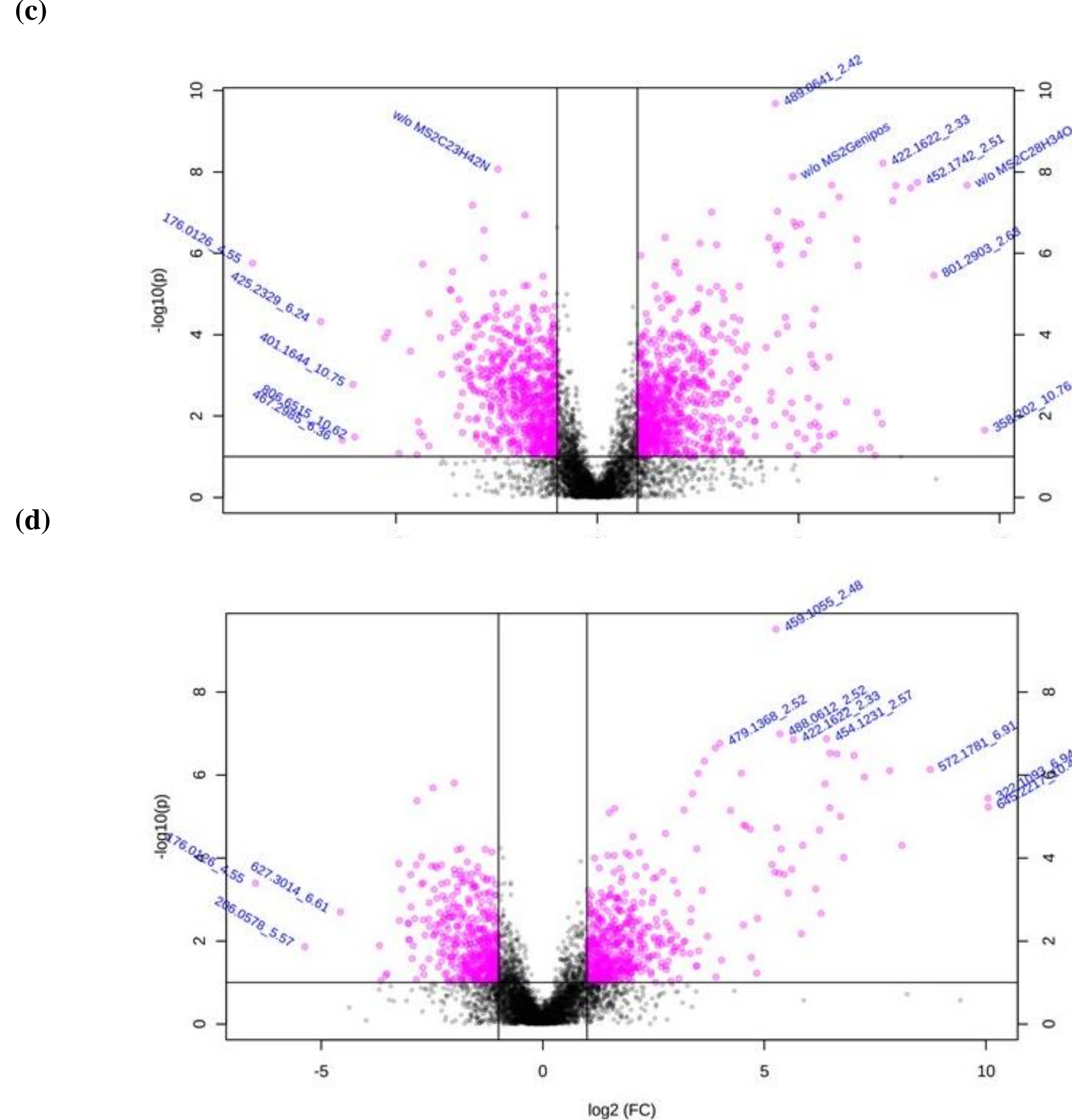


(b)

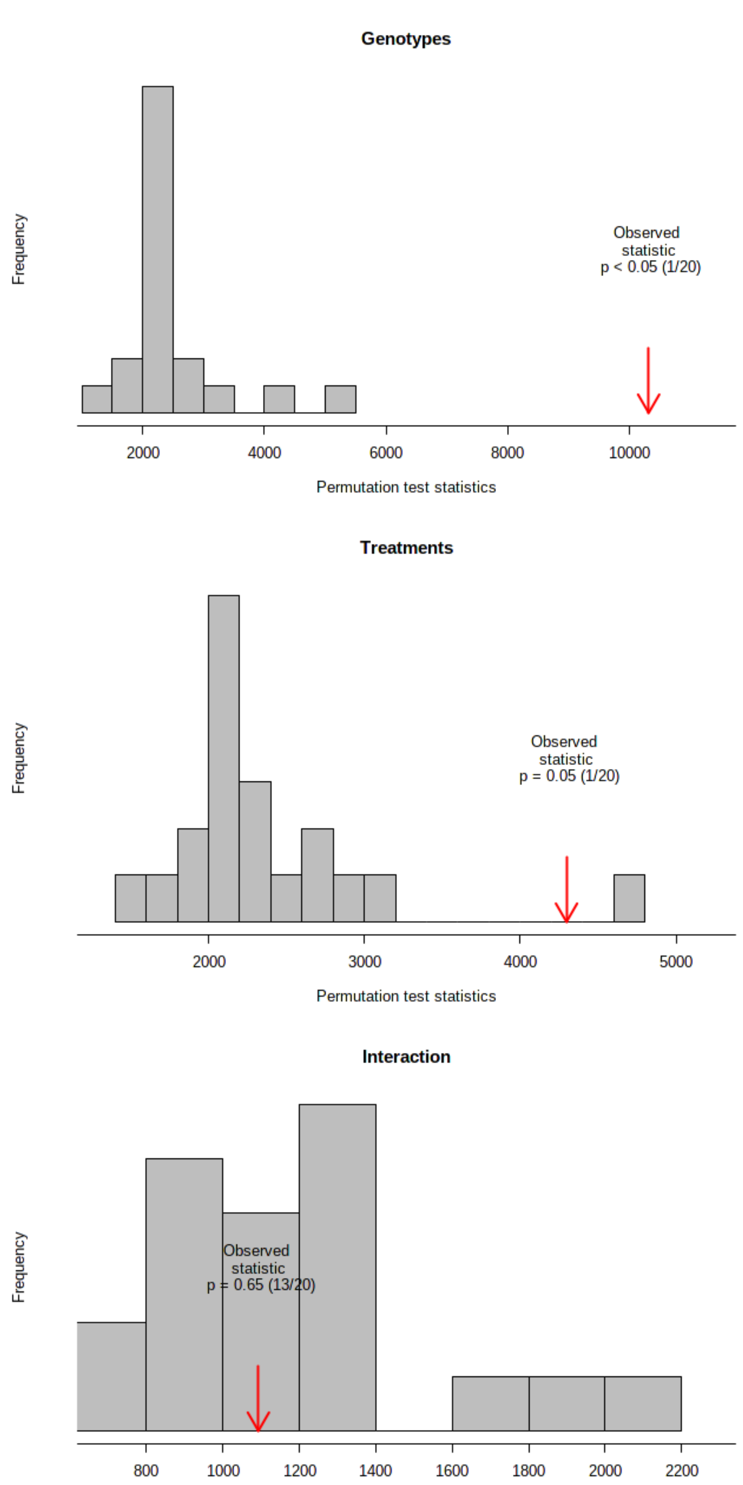


(c)

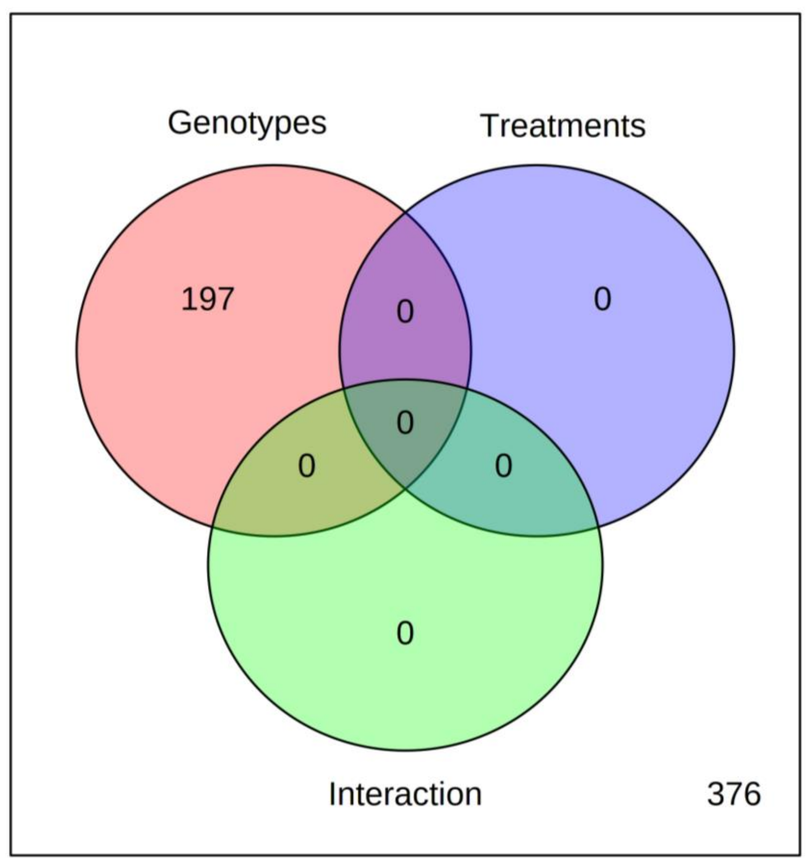
(d)



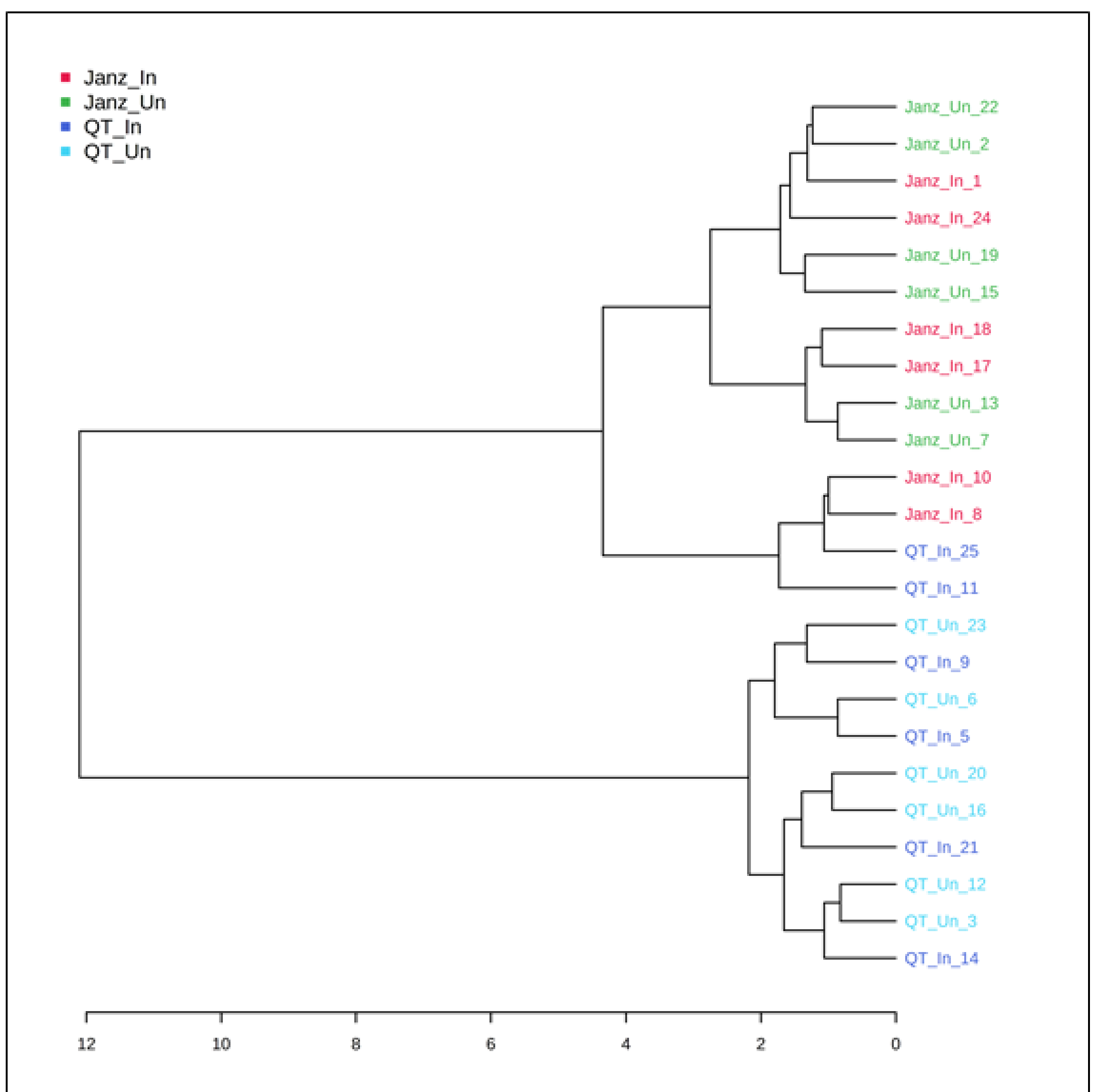
Supplementary Fig. S7. Volcano plots of four different treatments compared with each other respectively (a) 'Janz' uninoculated compared to 'Janz' inoculated (b) 'QT16258' uninoculated compared to 'QT16258' inoculated (c) 'QT16258' uninoculated compared to 'Janz' uninoculated (d) QT inoculated compared to 'Janz' inoculated in combined ion modes. The x-axis of individual graphs represents the magnitude of fold change in log2 scale, whereas the y-axis represents statistical significance in –log10 scale. The points highlighted in pink are the significant compounds selected based on the default *p* value threshold (0.05) and fold change value greater than 1.5. Points with a fold-change value less than 1.5, and *p* value >0.05 are shown in gray.



Supplementary Fig. S8 The results of model validation through permutations in two-way analysis of variance of wheat genotypes and treatments using 4,997 mass spectrometry (MS) features (post data filtering).



Supplementary Fig. S9 Venn diagrams from two-way analysis of variance (ANOVA) of 573 annotated metabolites metabolites indicating numbers of metabolites significantly different (adjusted p value ≤ 0.05) for genotypes ('QT16258 and 'Janz'), inoculation treatments (with or without *Pratylenchus thornei*) and their interaction. Number of metabolites with no significant differences is indicated in right bottom corner.



Supplementary Fig. S10 Dendrogram from cluster analysis based on 4,997 mass spectrometry features (post data filtering) in combined ion mode (positive ion mode and negative ion mode) of six biological replicates of 'Janz' and 'QT16258' wheat genotypes either uninoculated (Un) or inoculated (In) with *Pratylenchus thornei*. Distance measure Pearson and clustering algorithm Ward were used for the presentation of the dendrogram.

Supplementary Table S1 Partial least square discriminant analysis (PLS-DA) variable importance in projection (VIP) score of annotated metabolites ; VIP score of component 1 were considered for presentation of top 20 metabolites; Pos means metabolites detected in positive ion mode wheareas Neg means metabolites detected in negative ion mode for mass spectrometry

Metabolites	Component 1 (VIP Score)	Component 2 (VIP Score)
C17H24O11_A Neg	2.0143	1.9054
FA 1831O_B Neg	2.0094	1.9431
C23H42NO7P Neg	1.9781	1.8628
Flavonol Base 3O, O-Dhex, O-Hex-Hex Neg	1.9688	1.8628
S-Lactate Neg	1.9214	1.8112
Fumaric acid Neg	1.9001	1.7922
C28H34O14 Neg	1.887	1.798
C18H26O2 Neg	1.8815	1.8131
Quercetin-3,4'-O-di-beta-glucoside Neg	1.8653	1.77
Tubotaiwine Neg	1.8623	1.7778
1-Methylsulfinylbutenyl Isothiocyante Neg	1.852	1.7461
Geniposide Neg	1.8331	1.7481
C34H40N4O8 Neg	1.8169	1.7355
LPE 160 Neg	1.8162	1.7137
Dihydroxymandelic acid Neg	1.8012	1.7342
Desoxypeganine Neg	1.7838	1.6797
Flavanone Base 3O, C-Hex Pos	1.771	1.6698
Feruloyl Agmatine Isomer Of 1607 Pos	1.7685	1.6767
Flavonol Base 4O, 1Meo, O-Hex-Hex Neg	1.7662	1.6715
FA 921O Neg	1.7633	1.6604
Strictosamide_A Neg	1.7437	1.6425
Coumarin 1O 1Meo, O-Hex-Hex Pos	1.7391	1.6672
Mitragynine Pos	1.7316	1.6388
FA 182O Neg	1.7288	1.6818
Deoxyvasicinone Neg	1.7218	1.6249
FA 1802OSo4 Pos	1.7157	1.6166
D-Pantothenic acid Neg	1.7152	1.6165
FA 1831O Pos	1.7064	1.6185
Amino-Nitro-Toluene Pos	1.7049	1.6064
Inosine 5'-Monophosphate Neg	1.7032	1.7049
C20H20O7 Neg	1.6958	1.6948
Silychrystin Pos	1.6947	1.6028
FA 1841O Pos	1.6837	1.5949
Isoflavone Base 4C, 1Prenyl Pos	1.6742	1.5773
Corynanthine Neg	1.6726	1.5753
Flavone Base 3O, C-Pen, C-Pen Pos	1.6688	1.5728
N-Acetyl-Dl-Methionine Neg	1.6645	1.5674
LPE 181 Pos	1.6556	1.5675
Isorhamnetin-3-O-Galactoside-6''-Rhamnoside Neg	1.6549	1.6112

Glutamyl-S-Methylcysteine Pos	1.6541	1.5657
MGMG 183 Pos	1.6521	1.5593
C12H18O3 Neg	1.6495	1.5543
Tryptophan Pos	1.6447	1.5488
Disaccharides 2Methyl-Hex-Pen Neg	1.6312	1.5399
Azelaic acid Neg	1.6258	1.531
Flavan-3-Ols 3O Pos	1.6148	1.5208
Chalcone Base 3O, 1Meo, 1Prenyl Pos	1.6127	1.5217
C14H21NO8 Neg	1.6084	1.5184
N-Fructosyl Tyrosine Pos	1.6068	1.5137
Glutamyl-S-C3H5-Cysteine Sulfoxide Neg	1.606	1.5123
LPE 181 Neg	1.6049	1.5267
3,4-Dihydroxybenzoate Neg	1.5943	1.5754
LPC 160 Neg	1.5921	1.513
Myricetin-3-O-Xyloside Neg	1.5878	1.5476
D-Fructose Pos	1.578	1.5211
Phenylacetic acid Neg	1.577	1.5057
N-Acetyl-D-Tryptophan Neg	1.5764	1.4844
4-Acetamidobutanoate Neg	1.5663	1.5077
Indole-3-Acetyl-L-Glutamic acid Pos	1.5613	1.5716
Vanillin Acetate Neg	1.5551	1.474
Licoagroside B Not Validated Neg	1.5477	1.4808
Strictosidine_A Neg	1.5447	1.4635
Flavone Base 3O, 2Meo, O-Guaiacylglycerol Pos	1.5388	1.4527
Gelsemine Neg	1.5388	1.451
Linoleate Neg	1.5321	1.4439
Hirsutine Pos	1.5288	1.456
MGMG 182 Neg	1.5277	1.4517
FA 1831O_A Neg	1.5265	1.498
Methoxybenzenediol O-Hex Pos	1.5207	1.4321
N-Fructosyl Isoleucylglutamate Neg	1.5182	1.4647
LPC 182 Neg	1.5181	1.4301
Paeoniflorin Pos	1.5119	1.4379
Isohernandezine Neg	1.5028	1.4163
Lauric acid Pos	1.5004	1.4445
Caffeoyl Quinic acid Isomer Of 831, 832, 834 Pos	1.4976	1.4212
Etoposide Neg	1.4943	1.4825
MGMG 183-A Neg	1.4934	1.4543
Xanthosine 5'-Monophosphate Neg	1.4817	1.4011
N-Methyl-D-Aspartic acid Pos	1.4805	1.4051
Gallic acid Hexoside Neg	1.4785	1.4252
Valylphenylalanine Pos	1.477	1.4385
C13H18O2 Neg	1.4726	1.4081
Flavone Base 3O, O-Hex-Pen Neg	1.4659	1.3974
Epigallocatechin-3-Gallate Neg	1.4647	1.4207
Glutamyltyrosine Pos	1.4644	1.3798

D-Tryptophan Neg	1.4616	1.3834
Desoxypeganine Pos	1.4572	1.4252
MGMG 183-B Neg	1.4481	1.425
Citrate Neg	1.4448	1.3746
Gelsemine Pos	1.4435	1.3673
C29H34O17 Neg	1.4392	1.3555
5'-Methylthioadenosine Pos	1.439	1.3628
D-Tryptophan Pos	1.4291	1.3607
S420P318/F12 Neg	1.4268	1.346
Hexose C13H21O2 Pos	1.4256	1.3462
1-Isothiocyanato-4-Methylsulfinyl-Butane Neg	1.4231	1.3468
C7H11NO3 Neg	1.414	1.4117
Harmalol Pos	1.4127	1.3302
LPE 182 Neg	1.4067	1.3766
7-Hydroxymitragynine Pos	1.4055	1.3297
Phenylalanylvaline Pos	1.4051	1.3456
S-Malate Neg	1.3966	1.3351
Indole-3-Acetyl-L-Isoleucine Pos	1.395	1.3584
LPE 183 Pos	1.3864	1.3106
C25H26O5 Neg	1.3852	1.3076
C21H36O10 Neg	1.3824	1.3028
Alpha-Hederin_B Pos	1.3814	1.3021
Flavone Base 3O 1Meo 1Prenyl Neg	1.3721	1.2945
Dgmg 183_B Neg	1.371	1.3564
Malvidin-3-O-Glucoside Pos	1.3651	1.3177
C11H10N2O2 Neg	1.3611	1.282
Aconitic acid Not Validated, Isomer Of 273 Pos	1.3576	1.298
Flavone Base 3O, C-Pen-Hex Pos	1.3471	1.2778
Homogentisate Pos	1.3471	1.3829
C17H28O16 Neg	1.341	1.2674
Flavonol Base 4O, 1Meo, O-Hex, O-Hex, O-Hex Pos	1.3404	1.4137
S419P317/F12 Pos	1.334	1.2581
4-Hydroxyphenylacetate Pos	1.3338	1.2597
Flavone Base 3O, O-Hexa Neg	1.3301	1.2528
C16H21NO4 Neg	1.326	1.2899
Benzoic acid 2O, O-Hex Neg	1.3229	1.2539
Feruloyl O-Methyldehydrodopamine Pos	1.32	1.3088
4-Deoxyphloridzin Pos	1.3124	1.2557
Flavone Base 3O, 1Meo, C-Hex-Hex Neg	1.3116	1.2388
Adenine Pos	1.3097	1.2339
Hydroxyferulic acid Neg	1.3094	1.2953
C10H11NO3 Neg	1.3089	1.2325
Hesperetin-7-O-Rutinoside Neg	1.3005	1.3374
Flavonol Base 4O, O-Hex-Dhex-Pen Neg	1.2981	1.2267
Isoflavone Base 1O, 2Meo, O-HexC7H12NO Neg	1.2946	1.259

5S-5-Carboxystrictosidine Neg	1.2912	1.2722
LPE 160 Pos	1.291	1.2335
Flavone Base 4O, 1Prenyl_B Neg	1.2888	1.2588
Dgmg 183_A Neg	1.2875	1.2675
3-Hydroxycinnamic acid Neg	1.286	1.2173
Acetylleucine Isomer Of 164 Pos	1.2852	1.2906
Remerine Pos	1.2768	1.2024
Diferuloyl Glycerol Pos	1.2743	1.2606
Formyl-L-Methionyl Peptide Pos	1.2727	1.2088
Alpha-Hederin_A Pos	1.2712	1.2176
Dicaffeoyl Quinic acid Neg	1.268	1.1963
L-Tryptophan Neg	1.2665	1.2221
FA 1832O Neg	1.2651	1.3026
L-Ornithine Pos	1.2535	1.2037
812O Fatty Acyl Hexoside Pos	1.253	1.1879
Biochanin-7-O-Glucoside Neg	1.2434	1.2279
Flavonol Base 4O, O-Dhex, O-Hex-Dhex Pos	1.2431	1.3237
2-Amino-2-Methylpropanoate Neg	1.2419	1.1714
Sinapic acid_B Pos	1.2365	1.3236
7-O-Methylquercetin-3-O-Galactoside-6"-Rhamnoside Pos	1.2364	1.1871
N-Fructosyl Glutamylphenylalanine Neg	1.2309	1.1712
Isoflavone Base 2O 1Meo 1Prenyl Pos	1.2287	1.1571
Harmalol Neg	1.2272	1.1711
Allysine Not Validated Neg	1.2219	1.2886
N-Acetyl-L-Leucine Neg	1.2216	1.2293
Putrescine Pos	1.2203	1.193
Indole-3-Acetyl-L-Phenylalanine Neg	1.2174	1.1648
Phenylacetylaspartic acid Neg	1.2061	1.2278
Glutamyl-S-C3H5-Cysteine Sulfoxide Pos	1.2036	1.1395
N-Acetyl-L-Phenylalanine Neg	1.2016	1.2051
Tetrasaccharides Hex-Hex-Hex-Hex Neg	1.1996	1.1565
N-Acetylserotonin Neg	1.1975	1.1799
Chelidonine Pos	1.1876	1.1833
Spirostane -2H, 1O, O-Pen-Dhex Neg	1.1784	1.1185
FA 911O Pos	1.1781	1.1926
Glutamyl-S-Allylcysteine Pos	1.1749	1.1207
Phenyl-Butyryl-Glutamine Pos	1.1704	1.2162
Vincanidine Neg	1.1656	1.1661
3,4-Dimethoxycinnamic acid_A Neg	1.1644	1.1083
S-5'-Adenosyl-L-Homocysteine Pos	1.1642	1.1175
Pentose Proline Neg	1.1589	1.1833
Tanegoside Not Validated Pos	1.1579	1.1065
Benzyl Alcohol Hex-Pen Neg	1.1567	1.1575
Hirsuteine Neg	1.1562	1.1169
C28H38O13 Neg	1.1543	1.0874
Indole-3-Acetyl-L-Alanine Neg	1.1494	1.1544
DIBOA O-Hex-Hex Neg	1.1481	1.1308

3,5-Dimethoxycinnamic acid Pos	1.138	1.1499
Epicatechin Neg	1.1301	1.0922
Tryptophan Neg	1.1295	1.0718
Chlorogenoquinone Not Validated Pos	1.126	1.0776
Uridine-5-Monophosphate Neg	1.1245	1.0589
Apodanthoside Not Validated Neg	1.1237	1.0666
Schizandrin Neg	1.1229	1.0578
Ginkgolide B Pos	1.1199	1.064
Flavone Base 4O, C-Dehydro-Dhex-Dhex Pos	1.1186	1.0748
Ginsenoside Rh2 S-Form Pos	1.1139	1.0544
D-Saccharic acid Pos	1.1135	1.0686
FA 1811O Neg	1.1114	1.0858
Benzyl Alcohol Hex-Hex Pos	1.1108	1.0462
Coumaroyl Agmatine Isomer Of 1297 Pos	1.1061	1.0884
7-Methylsulfenylheptyl Isothiocyanate Neg	1.1058	1.0475
Isoleucylaspartate Neg	1.1055	1.0472
Ginsenoside F1_B Pos	1.1039	1.0395
Licodione Base 3O, 2Prenyl Pos	1.1004	1.0895
Flavone Base 4O, 1Prenyl Pos	1.0924	1.0317
1-Naphthylamine Pos	1.0909	1.112
Convolidine Pos	1.0899	1.0263
Chalcone Neg	1.0868	1.0234
Cholesteryl Acetate Pos	1.0855	1.0357
Flavonol Base 2Meo Pos	1.0855	1.0414
Isomajdine Neg	1.0845	1.179
C17H30O10 Neg	1.0828	1.1037
Chlorogenic acid Neg	1.0774	1.0152
Flavone Base 3O, 2Meo, O-Malonylhex, O-Guaiacylglycerol Neg	1.076	1.0133
Isoleucine Pos	1.0726	1.0292
Thiourea Neg	1.0675	1.0185
S-Malate Pos	1.0616	1.0713
L-Methionine Sulfoximine Neg	1.0598	1.0357
2R-2-Hydroxy-2-Phenethylglucosinolate Pos	1.0553	1.0005
Formononetin-7-O-Glucoside_A Neg	1.0468	0.99472
Podophyllotoxin Neg	1.0442	1.0487
Phloretin C-Hex, C-Hex Pos	1.0438	1.0897
C12H22O9 Neg	1.0427	1.1954
Tricaffeoyl Quinic acid Pos	1.0271	1.0472
Gossypetin 8-Glucoside Neg	1.025	1.0404
Sinapic acid Neg	1.024	1.061
Dimboa O-Hex-Hex Neg	1.0235	1.0937
L-Arginine Pos	1.0214	0.97894
Tryptamine Neg	1.0133	1.0037
Palmitate Neg	1.0105	0.95151
Dihydroresveratrol Pos	1.0059	0.96153
Eriodictyol-7-O-Neohesperidoside Pos	1.0022	0.96611
1244O Fatty Acyl Hexoside Pos	0.99651	1.2226

FA 1852O Pos	0.99524	1.0334
C26H32O11 Neg	0.98836	1.0096
3,5-Dimethoxycinnamic acid_B Neg	0.98411	0.99817
Flavone Base 2O, 2Meo, C-Hex Neg	0.98343	0.92616
Pyruvate Neg	0.98081	0.92551
Flavone Base 3O, 1Meo, O-Hexa-Hexa Pos	0.97356	0.91742
L-Isoleucine Pos	0.9728	0.91715
Camptothecin Neg	0.97046	0.91939
C11H18N2O5S2 Neg	0.96858	0.91208
3,4-Dihydroxyphenylacetate Pos	0.96339	1.0105
Hydroxypyridin C5H10NO Or 6-Hydroxypseudooxynicotine NOt Validated Pos	0.96258	0.92598
Hexose C13H19O Isomer Of 1062 Neg	0.95756	0.92393
Secoisolariciresinol_C Neg	0.95419	0.90429
Dihydrohesperetin-7-O-Neohesperidoside Pos	0.95365	0.96329
C28H35NO11 Neg	0.95165	0.90241
Flavanone Base 4O, 2Prenyl Neg	0.94788	0.9017
Tryptophanol Not Validated Pos	0.94742	0.91597
N-Fructosyl Gamma-Glutamyl-S-Methylcysteine Pos	0.9464	0.89307
C17H24O11_B Neg	0.94606	0.92319
Etoposide Pos	0.94499	1.0179
3-Hydroxybenzaldehyde Neg	0.94415	0.90718
C26H34N2O16 Neg	0.93945	0.90213
3,4,5-Trihydroxystilbene Pos	0.93931	0.88576
C40H36O10 Neg	0.93801	0.88825
Hexose C13H21O2 Isomer Of 1145 Neg	0.93801	0.94698
Quercetin-4'-O-Glucoside Neg	0.93657	0.90331
13C15N Valine Is Pos	0.92909	0.94653
Coniferyl Alcohol O-Hex Neg	0.92767	0.88634
Flavone Base 3O, 1Meo, 1Prenyl Neg	0.92761	1.0445
Flavone Base 3O, C-Hex-Coumaroylhex Pos	0.92715	0.87893
C21H20O4 Neg	0.92604	1.0277
L-Serine Pos	0.92516	0.87573
Malonylhexcer T180 Pos	0.92268	0.92895
Isoflavanone Base 3O, 1Prenyl Pos	0.92161	0.95014
5S-5-Carboxystrictosidine_A Pos	0.92158	0.87955
Uridine 5'-Diphosphoglucose Neg	0.91608	0.8788
Glutamyl-S-C8H17O-Cysteinylglycine_B Pos	0.91506	0.90326
S-2-CarboxypropylGlutathione Neg	0.9107	0.9565
LPC 183 Pos	0.90871	0.87098
Ginsenoside Compound K Neg	0.9073	0.85499
FA 1841O Neg	0.90557	0.96364
Deoxyadenosine Pos	0.90323	1.1196
C20H22O6 Neg	0.90219	0.85874
Senecionine Pos	0.89814	0.84573
Captopril Pos	0.89412	0.8744
Robinin Pos	0.8919	0.84004

C21H24O10 Neg	0.8891	0.90675
S,S--Tetrandrine Neg	0.88853	0.86857
4-Methoxycinnamic acid Pos	0.88824	0.87399
N-Fructosyl Gamma-Glutamyl-S-1-PropenylCysteine Sulfoxide Pos	0.88456	0.84645
Feruloyl Lactate Pos	0.87889	0.8287
Hdmboa O-Hex Pos	0.87476	0.82829
C22H22O8 Neg	0.87171	0.89177
L-Alanine Pos	0.86537	0.83471
Kaempferol-3-O-Glucuronoside Pos	0.86349	0.8193
Smiglaside C Not Validated Pos	0.86184	1.0135
Geniposide Pos	0.86098	0.85227
Isofraxidin Neg	0.85483	0.86532
L-Methionine Pos	0.85298	0.80383
Allicin 2H Not Validated, Isomer Of 327 Neg	0.85124	0.85953
C9H16Os3 Neg	0.84898	0.80868
N-Fructoryl Cysteinylalanine C2H5S Neg	0.84603	0.81554
Anthraquinone Base 1O, Meoh, 1Meo, O-Hex-Pen Pos	0.84472	1.017
C9H18Os3 Neg	0.84434	0.79578
Diferuloyl Putrescine Neg	0.84308	0.89041
L-Pipecolic acid Pos	0.84285	0.80698
Glutamyl-S-C8H17O-Cysteinylglycine_A Pos	0.84201	0.8143
4-Methylpentyl Glucosinolate Pos	0.84139	0.86244
Strictosamide Pos	0.84088	0.79431
Amygdalin Neg	0.83743	0.80286
Diboa O-Hex Neg	0.83626	0.78866
Flavonol Base 5O, O-Hex Neg	0.83565	0.7948
Sakuranetin Neg	0.83243	1.2483
Benzoic acid 2O, O-Pen Neg	0.8265	0.78219
Silychrystin_A Neg	0.82187	0.85326
Hexcer T180 Pos	0.81983	0.7858
Isoreserpin Pos	0.81981	0.92562
Theophylline Neg	0.8196	0.97475
Citric acid Not Validated, Isomer Of 228 Neg	0.81384	0.83173
Procyanidin B2 Pos	0.81363	0.96548
Boldine Pos	0.81137	0.77017
Justicidin G Neg	0.81102	0.76531
Indole-3-Acetyl-L-Phenylalanine Pos	0.81078	0.85025
Flavone Base 3O, 2Meo, O-Hexa-Hexa Pos	0.80626	1.0974
Methoxycinnamic acid Pos	0.80304	0.77386
Allicin Not Validated Neg	0.79734	0.76714
FA 1842O Neg	0.79414	0.74779
Diferuloyl Putrescine Pos	0.79022	0.99189
Coumaroyl Hexoside Isomer Of 690, 691 Neg	0.78985	0.78544
D--Trehalose Neg	0.78841	0.7424
DisaccharideHex-Hex Pos	0.78639	0.8982
Deoxyloganic acid Not Validated Pos	0.78617	1.1393

S418P316/F12 Pos	0.786	0.77505
C20H28O11 Neg	0.78498	1.0396
C24H20O11 Neg	0.78321	0.75928
Phenylalanylisoleucine Isomer Of 1328 Pos	0.78277	0.75715
Lariciresinol Pos	0.77483	0.81337
Coumaroyl Hexoside Isomer Of 690, 692 Neg	0.77428	0.88801
Flavonol Base 3O, O-Hex-Hex Neg	0.77206	0.77328
L-Aspartate Pos	0.76576	0.7361
Carboline Metabolite C26H26N2O8 Pos	0.75988	0.76379
Glutamyltyrosine Neg	0.75694	0.72309
Dicaffeoyl Quinic acid Pos	0.75361	0.78139
Solanidine Base O-Hex-Dhex Pos	0.7526	0.71155
4-Coumarate Neg	0.75035	0.72934
Laudanosine_C Neg	0.75012	0.7257
Enterolactone Neg	0.74896	0.71203
S8-8S Hexoside Neg	0.74653	1.1135
Hdmboa O-Hex_B Neg	0.74087	0.75266
C19H28O10 Neg	0.74033	0.78659
Sempervirine Pos	0.73965	0.69803
Triacetyl Resveratrol Neg	0.73304	0.7338
Isoflavone Base 2O, O-Acetylhex Neg	0.73292	0.69089
Furostane Base -2H O-Hex Neg	0.72889	0.74917
C16H20O10 Neg	0.72686	0.68964
S,S--Tetrandrine Pos	0.72505	0.7541
Eleutheroside E Neg	0.72499	0.84387
Coumaroyl Putrescin Pos	0.71793	0.67637
3,4-Dihydroxyphenyl Glycol Neg	0.71672	0.70288
Flavanone Base 4O, 1Prenyl Pos	0.70822	0.67519
2-Methoxycinnamic acid Pos	0.70399	0.97545
Biflavonoid-Flavone Base 3Meo And Flavone Base 3Meo Pos	0.69038	0.65449
Arctigenin Neg	0.68715	0.65721
Hmboa O-Hex Neg	0.68642	0.64646
Rosmarinic acid Not Validated Neg	0.68142	0.83461
Loganin Pos	0.67564	0.66523
Norleucine Neg	0.67272	0.63489
Dehydrophytosphingosine Not Validated - 2H Pos	0.67244	0.64331
Ferulate Neg	0.67237	0.63349
Sinapic acid_A Pos	0.66789	0.64603
Pyroglutamic acid Not Validated, Isomer Of 89 Pos	0.66351	0.63508
Caffeine Pos	0.66269	0.8122
Coumaric acid Isomer Of 189, 194 Pos	0.66152	0.62453
Benzoic acid 2O, O-Hex Pos	0.65805	0.727
Dimboa O-Hex Pos	0.65391	0.63398
L-Histidine Neg	0.65348	0.82207
C21H32O10 Neg	0.65119	0.64498

Sakuranetin Pos	0.64224	0.82273
Chalcone Base 2O, 1Meo, 1Prenyl Or Licochalcone A Not Validated Neg	0.64184	0.61649
Flavone Base 3O, 2Meo, O-Hex Neg	0.64055	0.89555
CycloLeucylprolyl Pos	0.63872	0.846
Cephaeline Neg	0.63436	0.67169
Indole-3-Acetyl-L-Leucine Pos	0.63337	0.60687
Lariciresinol_B Neg	0.62546	0.68374
Pseudocopsinine Neg	0.6253	0.68378
Sanguinarine Pos	0.62376	0.60882
LPE 182 Pos	0.61534	0.83828
L-Isoleucine Neg	0.6138	0.91115
N-Fructosyl Alliin Pos	0.60325	0.57462
Flavone Base 3O, 2Meo, O-Malonylhex, O-Guaiacylglycerol Pos	0.60089	0.97704
Phytosphingosine Not Validated, Isomer Of 1697 Pos	0.60032	1.3466
Phytosphingosine Not Validated, Isomer Of 1696 Pos	0.59776	0.6318
2'-Deoxycytidine 5'-Monophosphate Neg	0.59532	0.92992
Flavone Base 3O, 1Meo, O-Hexa-Feruloylhexa Pos	0.59344	0.5619
C30H22O11 Neg	0.5901	0.7586
Piperlongumine Pos	0.57993	0.5637
Hydroxyphenylethanol Pen Pos	0.57936	0.6206
Theobromine Neg	0.57816	0.61412
Quercetin-3-O-Vicianoside Pos	0.5743	0.58922
D--Galactosamine Pos	0.57012	0.58101
L-Aspartate Neg	0.56538	0.53239
C24H44O25S Neg	0.56535	0.55877
Benzyl Alcohol Hex-Hex Neg	0.56383	0.54115
Feruloyl Quinic acid Neg	0.56371	0.53665
Phillyrin Neg	0.56282	0.57007
Dihydrohesperetin-7-O-Neohesperidoside Neg	0.56129	0.53295
Sempervirine_A Neg	0.55872	0.52725
Malonyltryptophan Pos	0.55758	0.84083
Flavone Base 4O, O-Hexa-Hexa Pos	0.55178	0.63475
Hirsuteine Pos	0.54916	0.52877
Glycolate Neg	0.54596	0.75355
Isoflavone Base 2O, O-Hex Pos	0.5438	0.51355
Malvidin-3,5-Di-O-Glucoside Neg	0.54361	0.56236
7-Methylsulfenylheptyl Isothiocyanate Pos	0.54253	0.62588
Chalcone Base 3O, 1Meo, 1Prenyl Neg	0.53984	0.62431
Quercetin 3-O-2"-O-6"'-O-P-Coumaroyl-B-D-Glucopyranosyl-A-L-Rhamnopyranoside Neg	0.5394	0.59061
C15H19No5 Neg	0.53871	0.57782
Pentose Proline Pos	0.53517	0.51069
C12H23NO7S Neg	0.52865	0.49808

Secoisolariciresinol_A Neg	0.52781	0.79812
Quercetin-3-O-Xyloside Neg	0.52776	0.53409
N-Fructosyl Phenylalanine Pos	0.52441	0.53808
Flavonol Base 2O, 1Meo Neg	0.52433	0.5162
Aminobenzoyl-Glutamate Neg	0.51948	0.68898
N-Fructosyl Isoleucine Pos	0.51793	0.63055
C15H26O10 Neg	0.5161	0.53487
Acetosyringone Pos	0.5158	0.54829
Hexosyl Lpe 182 Neg	0.51463	0.51206
Flavone Base 3O, C-Hex-Feruloylhex Neg	0.51421	0.59252
Hdmboa O-Hex_A Neg	0.51185	0.49423
C26H36N2O8 Neg	0.50503	0.89143
Vanillic acid O-Sulfonatehex Neg	0.50203	0.49695
Isosakuranetin-7-O-Rutinoside Neg	0.50011	0.55747
Diosmetin-7-O-Rutinoside Pos	0.4936	0.56315
Flavone Base 3O, O-Hexa, C-Hex, C-Hex Pos	0.49328	0.55826
Isopropylmalic acid Neg	0.49321	0.56673
Coumarin Base 1O, 1Meo, O-Hex_A Neg	0.48961	0.55124
Medicagenic acid Base O-Hex Pos	0.48702	0.46283
Voacristine Pos	0.48073	0.48771
Caffeoyl Putrescin Isomer Of 1059 Pos	0.47905	0.52178
Flavone Base 3O, 1Meo, C-Hex-Feruloylhex Pos	0.47903	0.47535
L-Asparagine Neg	0.47432	0.46504
Acacetin-7-O-Rutinoside Neg	0.47047	0.45604
Trans-Piceid Neg	0.46492	0.74995
2-Acetamido-2-Deoxy-Beta-D-Glucosylamine Pos	0.46463	1.2653
Coumarin 1O Neg	0.46293	0.436
Biotin Neg	0.46162	0.83642
4-Hydroxybenzoate Pos	0.45672	0.43387
C17H14O6 Neg	0.45602	0.44966
Matairesinol Neg	0.45548	0.60878
Gluconic acid Neg	0.45039	0.50729
Remerine Neg	0.448	0.90731
C23H26N2O5 Neg	0.44719	0.42136
Thymidine Pos	0.43551	0.51665
N-Fructosyl Gamma-Glutamyl-S-Methylcysteine Neg	0.43343	0.57547
Glutathione Not Validated Pos	0.43092	0.71585
Justicidin G Pos	0.42697	0.40414
C20H22O8 Neg	0.42597	0.50561
Captopril Neg	0.42399	0.39933
Caffeoyl Putrescin Isomer Of 390 Neg	0.42314	0.43844
Ginsenoside F3_A Pos	0.42053	0.60543
D-Alanine Neg	0.41882	0.39786

Flavone Base 3O, 2Meo, O-Hex, O-Guaiacylglycerol Pos	0.41637	0.77037
L-Phenylalanine Pos	0.41593	0.40403
Hdmboa Not Validated, Isomer Of 871 Pos	0.41548	0.47343
5S-5-Carboxystrictosidine_B Pos	0.41295	0.54297
Hdmboa Not Validated, Isomer Of 868 Pos	0.41096	0.51282
Tanegoside Not Validated Neg	0.41013	0.3879
Aconitic acid Not Validated Neg	0.4087	0.40267
LPC 181 Neg	0.40509	0.80673
Succinic acid Not Validated Neg	0.40207	0.65236
Flavin Adenine Dinucleotide Neg	0.39724	0.37767
Tetrahydroalstonine Neg	0.39145	0.65958
Arbutin Pos	0.38994	0.36771
Solanidine Base O-Hex-Hex Pos	0.38971	0.43159
Pentasaccharides Hex-Hex-Hex-Hex-Hex Neg	0.38952	0.39345
C7H12O6 Neg	0.3873	0.38309
Pentose-Hexose C5H9 Neg	0.38034	0.75525
G8-O-4Fa Sulfate Pos	0.37732	0.35581
N-Acetyl-D-Tryptophan Pos	0.37666	0.54973
Indole-3-Acetyl-L-Glutamic acid Neg	0.37636	0.35954
Coumaroyl Agmatine Isomer Of 1297 Neg	0.37542	0.40172
Lyalosidic acid Neg	0.3711	0.58948
Arabinose Pos	0.36437	0.4407
Carboline Base 4H, Carboxylic acid Pos	0.36399	0.377
Licodione Base 2Prenyl Neg	0.3633	0.61248
Iso-Gamma-Fagarine Neg	0.36178	0.34121
Biflavonoid-Flavone Base 3O And Flavone Base 3O 1Prenyl Pos	0.35063	0.34299
Arbutin Neg	0.34388	0.35491
4-Quinolinecarboxylic acid Pos	0.33889	0.41631
L-Tyrosine Pos	0.33555	0.4043
Flavonol Base 3O O-Hex-Dhex Neg	0.32002	0.66718
Alpha-D-Glucose 1-Phosphate Neg	0.30913	0.30933
Glutathione Pos	0.30677	0.41841
Myricetin-3-O-Galactoside Pos	0.30571	0.69763
Aboa Pos	0.305	0.4249
8-Methylsulfinyloctyl Isothiocyanate Pos	0.30248	0.52363
Coumarin Base 1O, 1Meo, O-Hex_B Neg	0.29843	0.35908
L-Phenylalanine Neg	0.29509	0.28011
Malonylhexcer T180 Neg	0.29399	0.49675
Flavone Base 3O, C-Hex-Feruloylhex Pos	0.29369	0.47037
L-Asparagine Pos	0.29192	0.36194
Formononetin-7-O-Glucoside_B Neg	0.29098	0.59734
1344O Fatty Acyl Hexoside Neg	0.28708	0.27092
Isosakuranetin-7-O-Rutinoside Pos	0.28616	0.33969
Ellipticine Pos	0.27568	0.49687

Coumaroyl Quinic acid Isomer Of 759, 760 Neg	0.26884	0.45357
Tetrasaccharides Hex-Hex-Hex-Hex Pos	0.26523	0.52203
Matairesinol Pos	0.2614	0.37863
Tyrosine Not Validated Neg	0.26095	0.34037
Hexsose C5H10O3, 1Phosphate Pos	0.25629	0.24658
Dehydrophytosphingosine Not Validated, Isomer Of 1679 Pos	0.25552	0.25189
Alpha,Beta-Dihydroresveratrol Neg	0.25145	0.62223
C14H22O10 Neg	0.24856	0.46514
Formononetin-7-O-Glucoside Pos	0.24831	0.55768
Trans-Piceid Pos	0.24378	0.48943
Dodecyl Sulfate Neg	0.23929	0.23134
Formyl-L-Methionyl Peptide Neg	0.23859	0.90801
Flavone Base 3O, C-Pen-Feruloylhex Pos	0.23792	0.35532
L-Tyrosine Neg	0.23417	0.24249
Alanylleucine Isomer Of 675 Pos	0.23189	0.26364
Trans-Cinnamic acid Pos	0.23077	0.44104
Glutamylglycine Pos	0.22997	0.87032
Ononin Neg	0.22927	0.28442
Dihydroxyfumaric acid Neg	0.22694	0.49036
FA 1822O Neg	0.22652	0.21768
C20H34O9 Neg	0.22451	0.71719
Phillyrin Pos	0.22077	0.46858
Isodityrosine Not Validated Pos	0.21487	0.35714
Benzoic acid 1O, 2Meo, O-Hex Neg	0.21413	0.74438
Ginsenoside F1 Neg	0.21406	0.20718
Phenylglycine Pos	0.20856	0.35418
Bergenin Pos	0.20756	0.1998
Flavone Base 3O, 1Meo, C-Hex-Feruloylhex Neg	0.20102	0.7761
Salicylate Neg	0.19702	0.35258
Podophyllotoxin Pos	0.19452	0.24536
Smiglaside C Not Validated Neg	0.18992	0.49871
Okanin-4'-O-Glucoside Pos	0.18764	0.21119
Eriodictyol-7-O-Neohesperidoside Neg	0.18292	0.35001
Phenylalanine Pos	0.18177	0.19258
Glutamylphenylalanine Neg	0.17981	0.42803
Dicaffeoyl Coumaroyl Spermidine Pos	0.17313	0.17763
Feruloyl Quinic acid Isomer Of 886, 887_A Neg	0.17038	0.17023
Flavone Base 3O 1Meo 1Prenyl Pos	0.16818	0.56827
2',4'-Dihydroxyacetophenone Neg	0.16553	0.22279
Smiglaside C Not Validated 1Acetyl Pos	0.16354	0.39056
Benzoic acid 1O, O-Hex Neg	0.15085	0.16992
C15H20O13S Neg	0.15037	0.28298
O-Acetylsolasodine Neg	0.1424	0.5067
Isoflavone Base 2O, O-Hex Neg	0.14061	0.55865

MGMG 20 Neg	0.14011	0.23525
L-Histidine Pos	0.13634	0.2264
9-Nitro-20S-Camptothecin Pos	0.12455	0.34777
Adenosine Neg	0.1191	0.52307
Eriodictyol-7-O-Glucoside Neg	0.11792	0.39561
Flavanone Base 3O, 1Meo Pos	0.11647	0.54102
DIBOA Pos	0.11215	0.10823
3',5'-Cyclic Amp Neg	0.1111	0.46552
Thiopurine S-Methylether Neg	0.10813	0.17696
LPC 160 Pos	0.10153	0.80713
Camptothecin-Oh Neg	0.09828	1.0502
Petroselinic acid Neg	0.086611	0.3198
Flavonol Base 3O, O-Hex, O-HexC6H9O4 Pos	0.081856	0.27608
Robinin Neg	0.080662	0.52835
Ginsenoside F3_B Pos	0.077197	0.15975
Ginsenoside F1_A Pos	0.073672	0.16997
L-Glutamine Neg	0.066706	0.063815
Sinapoyl C6H9O5 Pos	0.065012	0.29205
Trans-Cinnamic acid Neg	0.064542	0.24878
Guanosine Not Validated Pos	0.062472	0.10672
Lariciresinol_A Neg	0.061673	0.82735
L-Valine Pos	0.055594	0.053487
Pterocarpan Base 1O, 1Meo Pos	0.0535	0.061937
Feruloyl Quinic acid Isomer Of 886, 888_B Neg	0.049575	0.055251
Magnolol Neg	0.047885	0.2284
Paeoniflorin Neg	0.04633	0.089882
Anthranoyllycoctonine Neg	0.042876	0.36139
Inosine Neg	0.04105	0.50642
C7H8N2S Neg	0.040698	0.78316
Feruloyl Quinic Acid Isomer Of 887, 888_C Neg	0.040606	0.28113
C5H5N5 Neg	0.032004	0.26381
2,4-Dihydroxypteridine Pos	0.027698	0.15542
Deoxypumiloside Pos	0.026731	0.084733
N-Fructosyl S-2-CarboxypropylGlutathione_B Neg	0.025631	0.30647
Malvidin-3,5-Di-O-Glucoside Pos	0.017295	0.32925
Coniferyl Aldehyde O-Hex Neg	0.01294	0.14029
Quinoxalinedione 2Methyl C5H11O4 Pos	0.0082094	0.76413
Flavonol Base 5O, O-Hex, O-Hex Pos	0.0015546	0.53213
Feruloyl Dehydrotyramine Isomer Of 1654 Pos	0.0014886	0.26123

Supplementary Table S2 All significant annotated metabolites based on univariate ANOVA ($p \leq 0.01$) with post hoc analysis; For metabolite names, annotation type is indicated by w/o for accurate mass, and MS2 for MS-MS fragmentation pattern; Pos means metabolites detected in positive ion mode whereas neg means metabolites detected in negative ion mode by mass spectrometry. Annotation levels are given according to Schymanski et al. (2014).

Metabolite	*p* value	Confidence level of annotation	Significant expression type (average)
w/o MS2 C28H34O14 neg	1.09E-12	Level 4	Janz > QT16258
w/o MS2 C17H24O11_a neg	8.08E-12	Level 4	Janz > QT16258
w/o MS2 Geniposide neg	1.37E-10	Level 2	Janz > QT16258
w/o MS2 1-Methylsulfinylbutenyl isothiocyante neg	5.72E-09	Level 2	QT16258 >Janz
w/o MS2 C23H42NO7P neg	6.81E-09	Level 4	QT16258 >Janz
w/o MS2 C34H40N4O8 neg	1.34E-07	Level 4	QT16258 >Janz
w/o MS2 Quercetin-3,4'-O-di-beta-glucoside neg	1.59E-07	Level 2	QT16258 >Janz
w/o MS2 FA 1831O_b neg	1.91E-07	Level 2	QT16258 >Janz
w/o MS2 Flavonol base 4O, 1MeO, O-Hex-Hex neg	8.85E-07	Level 2	QT16258 >Janz
Dihydroxymandelic acid neg	9.88E-07	Level 1	Janz > QT16258
w/o MS2 Tubotaiwine neg	1.18E-06	Level 2	QT16258 >Janz
w/o MS2 Flavone base 3O, 1MeO, C-Hex-Hex neg	1.72E-06	Level 2	Janz > QT16258
w/o MS2 C29H34O17 neg	1.96E-06	Level 4	Janz > QT16258
S-LACTATE neg	2.24E-06	Level 1	QT16258 >Janz
w/o MS2 Flavonol base 3O, O-dHex, O-Hex-Hex neg	2.76E-06	Level 2	QT16258 >Janz
w/o MS2 C18H26O2 neg	3.28E-06	Level 4	QT16258 >Janz
w/o MS2 Fumaric acid not validated neg	6.80E-06	Level 2	QT16258 >Janz
w/o MS2 Vanillin acetate neg	7.58E-06	Level 2	Janz > QT16258

w/o MS2 3,4-Dimethoxycinnamic acid_a neg	1.19E-05	Level 2	Janz > QT16258
w/o MS2 Indole-3-acetyl-L-glutamic acid pos	1.38E-05	Level 2	Janz > QT16258
w/o MS2 FA 182O neg	2.05E-05	Level 2	QT16258 >Janz
w/o MS2 Flavanone base 3O, C-Hex pos	2.06E-05	Level 2	QT16258 >Janz
w/o MS2 Tryptophan pos	2.09E-05	Level 2	Janz > QT16258
w/o MS2 Feruloyl agmatine isomer of 1607 pos	2.88E-05	Level 2	Janz > QT16258
w/o MS2 Isohernandezine neg	3.10E-05	Level 2	QT16258 >Janz
w/o MS2 C14H21NO8 neg	3.21E-05	Level 4	Janz > QT16258
w/o MS2 Myricetin-3-O-xyloside neg	4.46E-05	Level 2	QT16258 >Janz
w/o MS2 Coniferyl alcohol O-Hex neg	5.00E-05	Level 2	Janz > QT16258
w/o MS2 Silychrystin pos	6.59E-05	Level 2	Janz > QT16258
w/o MS2 FA 921O neg	7.40E-05	Level 2	QT16258 >Janz
4-HYDROXYPHENYLACETATE pos	8.09E-05	Level 1	Janz > QT16258
w/o MS2 LPE 181 pos	9.20E-05	Level 2	QT16258 >Janz
w/o MS2 Desoxypeganine neg	1.02E-04	Level 2	QT16258 >Janz
w/o MS2 LPE 160 neg	1.11E-04	Level 2	QT16258 >Janz
w/o MS2 LPE 183 pos	1.21E-04	Level 2	QT16258 >Janz
w/o MS2 Coumarin 1O 1MeO, O-Hex-Hex pos	1.31E-04	Level 2	QT16258 >Janz
w/o MS2 FA 1841O pos	1.40E-04	Level 2	QT16258 >Janz
w/o MS2 Benzyl alcohol Hex-Hex pos	1.50E-04	Level 2	Janz > QT16258
w/o MS2 Hirsutine pos	1.60E-04	Level 2	QT16258 >Janz
XANTHOSINE 5'-MONOPHOSPHATE neg	1.69E-04	Level 1	QT16258 >Janz
3,4-DIHYDROXYBENZOATE neg	1.79E-04	Level 1	QT16258 >Janz
D-PANTOTHENIC ACID neg	1.89E-04	Level 1	Janz > QT16258
N-ACETYL-D-TRYPTOPHAN neg	1.98E-04	Level 1	Janz > QT16258
INOSINE 5'-MONOPHOSPHATE neg	2.08E-04	Level 1	QT16258 >Janz
w/o MS2 Quercetin-4'-O-glucoside neg	2.18E-04	Level 2	QT16258 >Janz
w/o MS2 Disaccharides 2Methyl-Hex-Pen neg	2.27E-04	Level 2	Janz > QT16258

w/o MS2 Isoflavone base 1O, 2MeO, O-HexC7H12NO neg	2.37E-04	Level 2	Janz > QT16258
w/o MS2 Glutamyl-S-methylcysteine pos	2.47E-04	Level 2	QT16258 >Janz
w/o MS2 C17H30O10 neg	2.56E-04	Level 4	Janz > QT16258
w/o MS2 Deoxyvasicinone neg	2.66E-04	Level 2	QT16258 >Janz
w/o MS2 C11H10N2O2 neg	2.76E-04	Level 4	Janz > QT16258
N-ACETYL-DL-METHIONINE neg	2.85E-04	Level 1	Janz > QT16258
w/o MS2 Paeoniflorin pos	2.95E-04	Level 2	Janz > QT16258
w/o MS2 Mitragynine pos	3.05E-04	Level 2	Janz > QT16258
w/o MS2 C20H20O7 neg	3.14E-04	Level 4	Janz > QT16258
w/o MS2 N-Fructosyl tyrosine pos	3.24E-04	Level 2	QT16258 >Janz
w/o MS2 Chalcone base 3O, 1MeO, 1Prenyl pos	3.33E-04	Level 2	Janz > QT16258
w/o MS2 C17H28O16 neg	3.43E-04	Level 4	Janz > QT16258
w/o MS2 Amino-nitro-toluene pos	3.53E-04	Level 2	QT16258 >Janz
w/o MS2 Gossypetin 8-glucoside neg	3.62E-04	Level 2	QT16258 >Janz
w/o MS2 7-Hydroxymitragynine pos	3.72E-04	Level 2	QT16258 >Janz
w/o MS2 Flavone base 4O, 1Prenyl_b neg	3.82E-04	Level 2	Janz > QT16258
w/o MS2 FA 1802OSO4 pos	3.91E-04	Level 2	QT16258 >Janz
PETROSELINIC ACID neg	4.01E-04	Level 1	Janz > QT16258
N-METHYL-D-ASPARTIC ACID pos	4.11E-04	Level 1	Janz > QT16258
w/o MS2 Isoflavone base 4C, 1Prenyl pos	4.20E-04	Level 2	Janz > QT16258
w/o MS2 FA 1831O pos	4.30E-04	Level 2	QT16258 >Janz
w/o MS2 C11H16O3 neg	4.40E-04	Level 4	QT16258 >Janz
w/o MS2 Corynanthine neg	4.49E-04	Level 2	QT16258 >Janz
w/o MS2 Isoleucylleucine pos	4.59E-04	Level 2	QT16258 >Janz
w/o MS2 Benzoic acid 2O, O-Pen neg	4.69E-04	Level 2	Janz > QT16258
w/o MS2 Gelsemine neg	4.78E-04	Level 2	Janz > QT16258
w/o MS2 MGMG 183 pos	4.88E-04	Level 2	QT16258 >Janz
w/o MS2 alpha-Hederin_b pos	4.98E-04	Level 2	QT16258 >Janz

PHENYLACETIC ACID neg	5.07E-04	Level 1	Janz > QT16258
AZELAIC ACID neg	5.17E-04	Level 1	QT16258 >Janz
w/o MS2 Flavone base 3O, C-Pen, C-Pen pos	5.27E-04	Level 2	QT16258 >Janz
w/o MS2 Benzoic acid 2O, O-Hex neg	5.36E-04	Level 2	QT16258 >Janz
w/o MS2 C28H38O13 neg	5.46E-04	Level 4	QT16258 >Janz
w/o MS2 S8-8S hexoside neg	5.56E-04	Level 2	Janz > QT16258
w/o MS2 5S-5-carboxystrictosidine neg	5.65E-04	Level 2	QT16258 >Janz
w/o MS2 Licoagroside B not validated neg	5.75E-04	Level 2	Janz > QT16258
w/o MS2 MGMG 183-a neg	5.85E-04	Level 2	QT16258 >Janz
w/o MS2 C12H18O3 neg	5.94E-04	Level 4	QT16258 >Janz
w/o MS2 Flavan-3-ols 3O pos	6.04E-04	Level 2	QT16258 >Janz
w/o MS2 Ginsenoside F1_b pos	6.14E-04	Level 2	QT16258 >Janz
w/o MS2 Gallic acid hexoside neg	6.23E-04	Level 2	QT16258 >Janz
D-FRUCTOSE pos	6.33E-04	Level 1	QT16258 >Janz
w/o MS2 LPE 181 neg	6.43E-04	Level 2	QT16258 >Janz
w/o MS2 FA 1831O_a neg	6.52E-04	Level 2	QT16258 >Janz
w/o MS2 Isofraxidin neg	6.62E-04	Level 2	Janz > QT16258
w/o MS2 Ginsenoside Rh2 S-FORM pos	6.72E-04	Level 2	QT16258 >Janz
w/o MS2 Gelsemine pos	6.81E-04	Level 2	Janz > QT16258
w/o MS2 Caffeoyl quinic acid isomer of 831, 832, 834 pos	6.91E-04	Level 2	QT16258 >Janz
w/o MS2 LPC 160 neg	7.00E-04	Level 2	QT16258 >Janz
w/o MS2 Biflavonoid-flavone base 3MeO and flavone base 3MeO pos	7.10E-04	Level 2	Janz > QT16258
D-TRYPTOPHAN pos	7.20E-04	Level 1	Janz > QT16258
w/o MS2 Benzyl alcohol Hex-Pen neg	7.29E-04	Level 2	Janz > QT16258
w/o MS2 S420P318/F12 neg	7.39E-04	Level 4	QT16258 >Janz
w/o MS2 Flavone base 3O, 2MeO, O-MalonylHex, O-guaiacylglycerol pos	7.49E-04	Level 2	Janz > QT16258
D-SACCHARIC ACID pos	7.58E-04	Level 1	Janz > QT16258
w/o MS2 Flavonol base 5O, O-Hex neg	7.68E-04	Level 2	QT16258 >Janz

w/o MS2 Etoposide neg	7.78E-04	Level 2	Janz > QT16258
w/o MS2 Epigallocatechin-3-gallate neg	7.87E-04	Level 2	QT16258 >Janz
L-ORNITHINE pos	7.97E-04	Level 1	QT16258 >Janz
D-TRYPTOPHAN neg	8.07E-04	Level 1	Janz > QT16258
w/o MS2 Benzyl alcohol Hex-Hex neg	8.16E-04	Level 2	Janz > QT16258
w/o MS2 Flavone base 3O, 2MeO, O-guaiacylglycerol pos	8.26E-04	Level 2	QT16258 >Janz
w/o MS2 $C_{15}H_{10}O_3$ neg	8.36E-04	Level 4	Janz > QT16258
4-ACETAMIDOBUTANOATE neg	8.45E-04	Level 1	Janz > QT16258
w/o MS2 $C_{16}H_{21}NO_4$ neg	8.55E-04	Level 4	Janz > QT16258
w/o MS2 Harmalol neg	8.65E-04	Level 2	Janz > QT16258
w/o MS2 MGMG 183-b neg	8.74E-04	Level 2	QT16258 >Janz
w/o MS2 $C_{15}H_{26}O_{10}$ neg	8.84E-04	Level 4	Janz > QT16258
L-TRYPTOPHAN neg	8.94E-04	Level 1	Janz > QT16258
w/o MS2 Flavone base 3O 1MeO 1Prenyl neg	9.03E-04	Level 2	Janz > QT16258
w/o MS2 Harmalol pos	9.13E-04	Level 2	Janz > QT16258
w/o MS2 trans-piceid neg	9.23E-04	Level 2	Janz > QT16258
w/o MS2 $C_{25}H_{26}O_5$ neg	9.32E-04	Level 4	Janz > QT16258
GUANOSINE pos	9.42E-04	Level 1	QT16258 >Janz
S-5'-ADENOSYL-L-HOMOCYSTEINE pos	9.52E-04	Level 1	Janz > QT16258
w/o MS2 1-Isothiocyanato-4-methylsulfinyl-butane neg	9.61E-04	Level 2	Janz > QT16258
w/o MS2 Strictosidine_a neg	9.71E-04	Level 2	QT16258 >Janz
w/o MS2 N-Fructosyl isoleucylglutamate neg	9.81E-04	Level 2	Janz > QT16258
w/o MS2 MGMG 182 neg	9.90E-04	Level 2	QT16258 >Janz
w/o MS2 $C_{13}H_{18}O_2$ neg	1.00E-03	Level 4	QT16258 >Janz
w/o MS2 Remerine pos	1.01E-03	Level 2	Janz > QT16258
w/o MS2 Etoposide pos	1.02E-03	Level 2	Janz > QT16258
LAURIC ACID pos	1.03E-03	Level 1	QT16258 >Janz
THEOBROMINE neg	1.04E-03	Level 1	Janz > QT16258

w/o MS2 Malvidin-3-O-glucoside pos	1.05E-03	Level 2	Janz > QT16258
w/o MS2 Methoxybenzenediol O-Hex pos	1.06E-03	Level 2	QT16258 >Janz
w/o MS2 Furostane base -2H O-Hex neg	1.07E-03	Level 2	Janz > QT16258
LINOLEATE neg	1.08E-03	Level 2	QT16258 >Janz
w/o MS2 LPC 182 neg	1.09E-03	Level 2	QT16258 >Janz
w/o MS2 Aconitic acid not validated, isomer of 273 pos	1.10E-03	Level 2	QT16258 >Janz
w/o MS2 Tricaffeoyl quinic acid pos	1.11E-03	Level 2	QT16258 >Janz
w/o MS2 Valylphenylalanine pos	1.12E-03	Level 2	Janz > QT16258
w/o MS2 Glutamyltyrosine pos	1.13E-03	Level 2	QT16258 >Janz
w/o MS2 Ginkgolide B pos	1.14E-03	Level 2	QT16258 >Janz
w/o MS2 LPE 182 neg	1.14E-03	Level 2	QT16258 >Janz
w/o MS2 Flavonol base 4O, O-Hex-dHex-Pen neg	1.15E-03	Level 2	QT16258 >Janz
HOMOGENTISATE pos	1.16E-03	Level 1	Janz > QT16258
w/o MS2 C21H36O10 neg	1.17E-03	Level 4	Janz > QT16258
w/o MS2 Eriodictyol-7-O-glucoside neg	1.18E-03	Level 2	Janz > QT16258
2-AMINO-2-METHYLPROPANOATE neg	1.19E-03	Level 2	Janz > QT16258
w/o MS2 Flavone base 3O, O-Hex-Pen neg	1.20E-03	Level 2	QT16258 >Janz
w/o MS2 Benzoic acid 1O, O-Hex neg	1.21E-03	Level 2	QT16258 >Janz
w/o MS2 C12H22O9 neg	1.22E-03	Level 4	Janz > QT16258
w/o MS2 Ginsenoside Rg5 neg	1.23E-03	Level 2	QT16258 >Janz
w/o MS2 4-Deoxyphloridzin pos	1.24E-03	Level 2	Janz > QT16258
w/o MS2 alpha-Hederin_a pos	1.25E-03	Level 2	QT16258 >Janz
w/o MS2 Isoreserpin pos	1.26E-03	Level 2	Janz > QT16258
w/o MS2 Hexose C13H21O2 pos	1.27E-03	Level 2	Janz > QT16258
THIOUREA neg	1.28E-03	Level 1	Janz > QT16258
S-MALATE pos	1.29E-03	Level 1	QT16258 >Janz
w/o MS2 812O fatty acyl hexoside neg	1.30E-03	Level 2	Janz > QT16258
w/o MS2 Isoflavone base 2O 1MeO 1Prenyl pos	1.31E-03	Level 2	Janz > QT16258

w/o MS2 Hexose C13H19O isomer of 1062 neg	1.32E-03	Level 2	Janz > QT16258
w/o MS2 Phenylalanylvaline pos	1.33E-03	Level 2	Janz > QT16258
w/o MS2 C5H5N5 neg	1.34E-03	Level 4	Janz > QT16258
w/o MS2 Formononetin-7-O-glucoside_a neg	1.35E-03	Level 2	Janz > QT16258
w/o MS2 C20H22O6 neg	1.36E-03	Level 4	QT16258 >Janz
w/o MS2 Methoxycinnamic acid pos	1.37E-03	Level 2	Janz > QT16258
5'-METHYLTHIOADENOSINE pos	1.38E-03	Level 1	QT16258 >Janz
w/o MS2 Desoxypeganine pos	1.39E-03	Level 2	QT16258 >Janz
w/o MS2 Isoflavone base 2O, O-AcetylHex neg	1.40E-03	Level 2	Janz > QT16258
w/o MS2 C7H11NO3 neg	1.41E-03	Level 4	Janz > QT16258
w/o MS2 Eriodictyol-7-O-neohesperidoside pos	1.42E-03	Level 2	Janz > QT16258

Supplementary Table S3 Volcano plot analysis based on $p \leq 0.05$ and fold-change (>2.00) of 573 annotated metabolites in combined ion modes (positive and negative).

Comparison type	Treatments	Number of upregulated metabolites	Number of down regulated metabolites
Within genotype	Janz Uninoculated	20	31
	Janz Inoculated	31	20
Within genotype	QT16258 Uninoculated	11	16
	QT16258 Inoculated	16	11
Between genotypes	QT16258 uninoculated	74	92
	Janz uninoculated	92	74
Between genotypes	QT16258 inoculated	68	37
	Janz inoculated	37	68

Supplementary Table S4 Volcano plot analysis based on $p \leq 0.05$ and fold-change (>2.00) of 4,997 mass spectrometry (MS) features in combined ion modes (positive and negative).

Comparison type	Treatments	Number of upregulated metabolites /MS features	Number of down regulated metabolites /MS features
Within genotype	Janz Uninoculated	174	362
	Janz Inoculated	362	174
Within genotype	QT16258 Uninoculated	125	105
	QT16258 Inoculated	105	125
Between genotypes	QT16258 uninoculated	597	654
	Janz uninoculated	654	597
Between genotypes	QT16258 inoculated	335	403
	Janz inoculated	403	335

Supplementary Table S5 Constitutively expressed metabolites from comparing 'QT16258' uninoculated with 'Janz' uninoculated in volcano plot analysis at $p \leq 0.05$ and Fold change (FC) >2.00; pos means metabolites detected in positive ion mode wheareas neg means metabolites detected in negative ion mode by mass spectrometry

Metabolite	Expression type	Raw.*p* value	Fold change
1-Methylsulfinylbutenyl isothiocyante neg	QT16258>Janz	2.93E-08	111.6869
Desoxypeganine neg	QT16258>Janz	0.0000791	33.2524
Xanthosine 5'-Monophosphate neg	QT16258>Janz	0.020746	18.2047
Tubotaiwine neg	QT16258>Janz	0.0000214	11.5576
Isoleucylaspartate neg	QT16258>Janz	0.018503	11.2095
Isoleucylleucine pos	QT16258>Janz	0.0094289	11.1550
C34H40N4O8 neg	QT16258>Janz	0.00000751	11.1161
Myricetin-3-O-xyloside neg	QT16258>Janz	0.00074771	9.9800
C11H16O3 neg	QT16258>Janz	0.0032391	9.6145
Hirsutine pos	QT16258>Janz	0.00020923	9.3084
Gallic acid hexoside neg	QT16258>Janz	0.00019463	9.0245
Benzoic acid 2O, O-Hex neg	QT16258>Janz	0.010941	8.1077
Quercetin-3,4'-O-di-beta-glucoside neg	QT16258>Janz	5.7992E-06	8.1057
Azelaic acid neg	QT16258>Janz	0.0054291	7.9764
Alpha-hederin_b pos	QT16258>Janz	0.0053223	7.6799
FA 182O neg	QT16258>Janz	0.00027081	7.5092
Chalcone neg	QT16258>Janz	0.075429	7.3708
Lauric acid pos	QT16258>Janz	0.012973	6.9560
S420P318/F12 neg	QT16258>Janz	0.0000634	6.6260
7-Hydroxymitragynine pos	QT16258>Janz	0.0015854	6.6181
FA 921O neg	QT16258>Janz	0.0016731	6.5462
Isohernandezine neg	QT16258>Janz	0.00021036	6.4931
FA 1822O neg	QT16258>Janz	0.034702	6.3776

Flavonol base 4O, 1MeO, O-Hex-Hex neg	QT16258>Janz	0.00042684	6.1900
FA 911O pos	QT16258>Janz	0.0027275	5.6776
Guanosine pos	QT16258>Janz	0.0092163	5.4756
C23H42NO7P neg	QT16258>Janz	5.21E-08	5.4445
LPE 182 neg	QT16258>Janz	0.00011056	5.4124
alpha-Hederin_a pos	QT16258>Janz	0.0083804	4.8494
Quercetin-4'-O-glucoside neg	QT16258>Janz	0.0041933	4.7851
Chlorogenoquinone Not validated pos	QT16258>Janz	0.011288	4.3251
D-Fructose pos	QT16258>Janz	0.0031219	4.3223
Epigallocatechin-3-gallate neg	QT16258>Janz	0.0049912	4.2686
C12H18O3 neg	QT16258>Janz	0.0025609	4.2308
Petroselinic acid neg	QT16258>Janz	0.0064585	4.0700
Ginsenoside F1_b pos	QT16258>Janz	0.0012495	4.0442
Secoisolariciresinol_c neg	QT16258>Janz	0.036714	4.0225
N-Fructosyl gamma-glutamyl-S-methylcysteine pos	QT16258>Janz	0.017742	3.8803
3,4-Dihydroxybenzoate neg	QT16258>Janz	0.014111	3.8802
Tricaffeoyl quinic acid pos	QT16258>Janz	0.0054843	3.8436
N-Fructosyl tyrosine pos	QT16258>Janz	0.0069579	3.5613
FA 1831O_b neg	QT16258>Janz	0.0000402	3.2682
Glutamyl-S-methylcysteine pos	QT16258>Janz	0.0016528	3.2348
Flavone base 3O, O-Hex-Pen neg	QT16258>Janz	0.0018789	3.2036
Coumarin 1O 1MeO, O-Hex-Hex pos	QT16258>Janz	0.01003	3.1180
Inosine 5'-monophosphate neg	QT16258>Janz	0.0000826	3.0597
Amygdalin neg	QT16258>Janz	0.09869	3.0540
Eleutheroside E neg	QT16258>Janz	0.059469	3.0522
Flavone base 3O, C-Hex-feruloyl Hex neg	QT16258>Janz	0.051281	2.9767
C28H38O13 neg	QT16258>Janz	0.0010308	2.9548
C5H5N5 neg	QT16258>Janz	0.018477	2.9118

Flavanone base 3O, C-Hex pos	QT16258>Janz	0.03602	2.8043
Amino-nitro-toluene pos	QT16258>Janz	0.013395	2.7522
DIMBOA O-Hex-Hex neg	QT16258>Janz	0.087321	2.7458
Flavonol base 3O, O-dHex, O-Hex-Hex neg	QT16258>Janz	0.00061	2.7419
Flavonol base 5O, O-Hex neg	QT16258>Janz	0.068691	2.6966
Ginsenoside Rh2 S-FORM pos	QT16258>Janz	0.00094599	2.6575
5S-5-carboxystrictosidine_a pos	QT16258>Janz	0.0016725	2.6316
Corynanthine neg	QT16258>Janz	0.00081027	2.5632
MGMG 183-a neg	QT16258>Janz	0.0031967	2.4895
Caffeoyl quinic acid isomer of 831, 832, 834 pos	QT16258>Janz	0.050505	2.4629
FA 1841O pos	QT16258>Janz	0.0031126	2.4617
LPE 183 pos	QT16258>Janz	0.026312	2.4097
9-Nitro-20S-camptothecin pos	QT16258>Janz	0.096885	2.3886
FA 1831O pos	QT16258>Janz	0.0093832	2.3739
MGMG 183 pos	QT16258>Janz	0.0010092	2.3634
Quercetin 3-O-2"-O-6"'-O-p-coumaroyl-b-D-glucopyranosyl-a-L-rha	QT16258>Janz	0.011021	2.3480
FA 1831O_a neg	QT16258>Janz	0.0017665	2.3451
C18H26O2 neg	QT16258>Janz	0.00022034	2.3088
5'-Methylthioadenosine pos	QT16258>Janz	0.0037802	2.2940
Fumaric acid neg	QT16258>Janz	0.0014557	2.2666
Flavan-3-ols 3O pos	QT16258>Janz	0.04192	2.2661
2R-2-Hydroxy-2-phenethylglucosinolate pos	QT16258>Janz	0.05715	2.2612
MGMG 182 neg	QT16258>Janz	0.0064005	2.2583
Strictosidine_a neg	QT16258>Janz	0.085908	2.2200
Citric acid Not validated, isomer of 228 neg	QT16258>Janz	0.011207	2.2176
7-O-Methylquercetin-3-O-galactoside-6"-rhamnoside pos	QT16258>Janz	0.099069	2.2034
ADENINE pos	QT16258>Janz	0.01773	2.1111
LPE 160 neg	QT16258>Janz	0.00069677	2.1025

MGMG 183-b neg	QT16258>Janz	0.0040975	2.0942
Spirostane -2H, 1O, O-Pen-dHex neg	QT16258>Janz	0.06422	2.0922
Furostane base -2H 1O, O-Hex, O-Pen-dHex pos	QT16258>Janz	0.050812	2.0792
LPC 181 neg	QT16258>Janz	0.0123	2.0635
N-Fructosyl glutamylphenylalanine neg	QT16258>Janz	0.010604	2.0523
Flavonol base 4O, O-dHex, O-Hex-dHex pos	QT16258>Janz	0.035415	2.0303
HDMBOA not validated, isomer of 868 pos	QT16258>Janz	0.0076375	2.0252
Flavone base 3O, C-Pen-Hex pos	QT16258>Janz	0.015224	2.0141
C28H34O14 neg	Janz>QT16258	2.54E-08	605.14
Laudanosine_c neg	Janz>QT16258	0.096779	180.97
Licoagroside B not validated neg	Janz>QT16258	0.014322	131.93
Convolidine pos	Janz>QT16258	0.088896	112.7
C22H22O8 neg	Janz>QT16258	0.0052163	75.342
Etoposide neg	Janz>QT16258	0.00047039	41.531
Geniposide neg	Janz>QT16258	1.05E-08	29.199
Feruloyl agmatine isomer of 1607 pos	Janz>QT16258	0.00011215	22.097
C17H24O11_a neg	Janz>QT16258	0.000000605	21.517
Gelsemine neg	Janz>QT16258	0.012852	14.627
N-Fructosyl isoleucylglutamate neg	Janz>QT16258	0.026736	14.405
4-ACETAMIDOBUTANOATE neg	Janz>QT16258	0.0054712	10.998
3,4-Dimethoxycinnamic acid_a neg	Janz>QT16258	0.0000423	10.47
Indole-3-acetyl-L-isoleucine pos	Janz>QT16258	0.036666	10.293
Dihydroxymandelic acid neg	Janz>QT16258	0.00083297	10.133
C16H21NO4 neg	Janz>QT16258	0.0075471	8.986
Vanillin acetate neg	Janz>QT16258	0.0000348	8.6076
C29H34O17 neg	Janz>QT16258	0.000024	8.4761
Isoreserpin pos	Janz>QT16258	0.0037482	8.0775
Gelsemine pos	Janz>QT16258	0.099538	7.1001

Deoxyloganic acid Not validated pos	Janz>QT16258	0.075475	6.7528
Benzyl alcohol Hex-Hex pos	Janz>QT16258	0.000057	6.6801
Mitragynine pos	Janz>QT16258	0.00028778	6.6204
L-SERINE pos	Janz>QT16258	0.0030853	6.4738
Indole-3-acetyl-L-glutamic acid pos	Janz>QT16258	0.00013264	6.0761
C20H20O7 neg	Janz>QT16258	0.0024281	6.0689
C14H21NO8 neg	Janz>QT16258	0.0014935	5.819
LPE 160 pos	Janz>QT16258	0.0041433	5.2278
Disaccharides 2Methyl-Hex-Pen neg	Janz>QT16258	0.0035063	5.1736
Phenylalanylvaline pos	Janz>QT16258	0.0062388	4.821
4-Hydroxyphenylacetate pos	Janz>QT16258	0.00013696	4.6598
Hexose C13H21O2 pos	Janz>QT16258	0.016838	4.6365
Indole-3-acetyl-L-phenylalanine neg	Janz>QT16258	0.044335	4.4077
Tetrahydroalstonine neg	Janz>QT16258	0.094545	4.3997
Sinapic acid_b pos	Janz>QT16258	0.0088901	4.1032
Isomajdine neg	Janz>QT16258	0.0076161	4.0834
L-Alanine pos	Janz>QT16258	0.048011	4.0703
Formononetin-7-O-glucoside_a neg	Janz>QT16258	0.020465	3.858
L-Methionine pos	Janz>QT16258	0.014359	3.6483
N-Acetyl-DL-methionine neg	Janz>QT16258	0.00036539	3.6296
C25H26O5 neg	Janz>QT16258	0.0004315	3.6109
C17H28O16 neg	Janz>QT16258	0.0034182	3.5995
Isoflavone base 1O, 2MeO, O-HexC7H12NO neg	Janz>QT16258	0.0011455	3.5995
Tryptophan pos	Janz>QT16258	0.00047293	3.5281
Flavone base 4O, 1Prenyl_b neg	Janz>QT16258	0.0013911	3.3969
Licodione base 3O, 2Prenyl pos	Janz>QT16258	0.0011164	3.3921
Indole-3-acetyl-L-alanine neg	Janz>QT16258	0.065805	3.3782
2-Amino-2-Methylpropanoate neg	Janz>QT16258	0.018703	3.3778

N-Methyl-D-Aspartic acid pos	Janz>QT16258	0.0024635	3.3761
C17H30O10 neg	Janz>QT16258	0.0016407	3.3715
Isoflavone base 2O, O-AcetylHex neg	Janz>QT16258	0.0015111	3.3088
D-Saccharic acid pos	Janz>QT16258	0.0057328	3.3077
3-Hydroxycinnamic acid neg	Janz>QT16258	0.062765	3.28
Valylphenylalanine pos	Janz>QT16258	0.010295	3.2766
Isoflavone base 2O 1MeO 1Prenyl pos	Janz>QT16258	0.020505	3.237
Tryptophan neg	Janz>QT16258	0.06971	3.2365
Captopril pos	Janz>QT16258	0.051235	3.1512
Phenyl-butyryl-glutamine pos	Janz>QT16258	0.04285	3.1179
Silychrystin_a neg	Janz>QT16258	0.010472	3.069
Flavanone base 4O, 1Prenyl pos	Janz>QT16258	0.0013233	3.0171
D-Tryptophan neg	Janz>QT16258	0.0216	3.0113
Flavone base 3O, 1MeO, C-Hex-Hex neg	Janz>QT16258	0.000862	3.0052
Malvidin-3-O-glucoside pos	Janz>QT16258	0.017411	2.9703
812O fatty acyl hexoside neg	Janz>QT16258	0.03634	2.9249
Isofraxidin neg	Janz>QT16258	0.00079969	2.9074
Paeoniflorin pos	Janz>QT16258	0.0000399	2.8623
N-Acetyl-D-tryptophan neg	Janz>QT16258	0.00000824	2.8379
Dihydroresveratrol pos	Janz>QT16258	0.036177	2.765
Coniferyl alcohol O-Hex neg	Janz>QT16258	0.00000085	2.6953
7-Methylsulfenylheptyl isothiocyanate neg	Janz>QT16258	0.025472	2.6841
Isoflavone base 4C, 1Prenyl pos	Janz>QT16258	0.0070993	2.6732
L-Tryptophan neg	Janz>QT16258	0.010711	2.61
Homogentisate pos	Janz>QT16258	0.0023031	2.5929
Flavone base 4O, 1Prenyl pos	Janz>QT16258	0.040247	2.557
4-Deoxyphloridzin pos	Janz>QT16258	0.001472	2.5541
N-Fructosyl phenylalanine pos	Janz>QT16258	0.0098681	2.4955

Phenylacetic acid neg	Janz>QT16258	0.0050644	2.4899
Putrescine pos	Janz>QT16258	0.062315	2.4611
D-Pantothenic acid neg	Janz>QT16258	0.0016441	2.4536
Benzoic acid 2O, O-Pen neg	Janz>QT16258	0.00078463	2.4106
Dihydrohesperetin-7-O-neohesperidoside pos	Janz>QT16258	0.028705	2.3992
Flavone base 3O, 1MeO, O-HexA-feruloyl-HexA pos	Janz>QT16258	0.027389	2.3931
3,5-Dimethoxycinnamic acid_b neg	Janz>QT16258	0.0018468	2.3501
Biflavonoid-flavone base 3MeO and flavone base 3MeO pos	Janz>QT16258	0.0063848	2.3335
D-Tryptophan pos	Janz>QT16258	0.0069924	2.3313
1-Isothiocyanato-4-methylsulfinyl-butane neg	Janz>QT16258	0.058972	2.316
C11H10N2O2 neg	Janz>QT16258	0.0000542	2.309
HMBOA O-Hex neg	Janz>QT16258	0.054396	2.2939
Furostane base -2H O-Hex neg	Janz>QT16258	0.0031391	2.2488
Trans-cinnamic acid neg	Janz>QT16258	0.012897	2.2369
Eriodictyol-7-O-neohesperidoside pos	Janz>QT16258	0.00065408	2.2218
Tryptamine neg	Janz>QT16258	0.021795	2.1614
Dihydrohesperetin-7-O-neohesperidoside neg	Janz>QT16258	0.0083781	2.1509
C30H22O11 neg	Janz>QT16258	0.00039422	2.1148
Benzyl alcohol Hex-Hex neg	Janz>QT16258	0.0048632	2.1126
Flavonol base 3O O-Hex-dHex neg	Janz>QT16258	0.088405	2.0852
DIMBOA O-Hex pos	Janz>QT16258	0.042737	2.083
Justicidin G neg	Janz>QT16258	0.0034837	2.0819
Glutamyltyrosine neg	Janz>QT16258	0.033836	2.0693
Flavone base 3O, 2MeO, O-malonylHex, O-guaiacylglycerol pos	Janz>QT16258	0.050717	2.0661
Harmalol pos	Janz>QT16258	0.00019086	2.0596
C24H20O11 neg	Janz>QT16258	0.043723	2.0493
D--Trehalose neg	Janz>QT16258	0.0043875	2.0393
ABOA pos	Janz>QT16258	0.041617	2.0239

C21H36O10 neg	Janz>QT16258	0.049869	2.0023
Flavone base 3O 1MeO 1Prenyl neg	Janz>QT16258	0.0096805	2.0004

SupplementaryTable S6 Putative annotation of 18 mass spectrometry (MS) features (identified by mass-to-charge ratio (m/z)) significant in *Pratylenchus thornei* inoculated (In) treatments of 'QT16528' and 'Janz' (based on two-way ANOVA, false discovery rate adjusted $p \leq 0.05$).

Mass spectrometry features	Ion mode	*p* value (adjusted)	Putative annotation	Class of metabolites
562.1993	positive	4.71E-08	no match with plant metabolites	
497.0919	negative	5.66E-06	Cyanidin 3-O-beta-D-glucoside	Anthocyanin
572.1781	positive	4.57E-05	Jadomycin B	Type II polyketide
451.1204	positive	0.0021095	Steppogenin 4'-O-beta-D-glucoside	Flavonoids
115.0527	positive	4.33E-03	2,5-Dioxopiperazine	Amino acid derrivative
446.17	positive	5.54E-03	Narceine	Alkaloids
160.0762	positive	0.0055391	Indole-3-acetaldehyde (indole derrivative)	Tryptophan metabolism/hormones
293.2106	negative	0.013217	(15Z)-12-Oxophyto-10,15-dienoic acid	Lipid
698.4399	positive	0.013217	2-Acetamido-2-deoxy-?-D-glucopyranosyl)oxy]-16-hydroxyolean-12-en-28-oic acid	Terpenoids (saponins)
209.081	negative	0.03805	D-Arginine	Amino acid

S8-8S hexoside	negative	0.03805	S8-8S hexoside	Glycosides
383.1355	negative	0.039182	Macrozamin	Glycosides
645.2217	positive	0.039592	4'-O-Methylneobavaisoflavone 7-O-(2"-p-coumaroylglucoside)	Flavonoids
852.5418	positive	0.039592	Phosphatidylcholine	Lipid
640.205	positive	0.042436	Labriformin	Terpenoids
602.3009	positive	0.042436	no match with plant metabolites	
623.3369	negative	0.042436	Spongipregnoloside	Lipids/steroids
667.3869	negative	0.042782	28-Glucopyranosyl-3-methyloleanolic acid	Terpenoids (saponins)

interaction

Supplementary Table S7 Two-way ANOVA between all annotated and unannotated mass spectrometry features for genotypes 'QT16528' and 'Janz'; treatments 'Uninoculated and inoculated with *Pratylenchus thornei* ; and the interaction between genotypes and treatments ; pos means metabolites detected in positive ion mode wheareas neg means metabolites detected in negative ion mode by mass spectrometry

Metabolites	Genotypes(adj.p)	Treatments(adj.p)	Interaction(adj.p)
562.1993_2.19 pos	0.047154	4.71E-08	0.79649
497.0919_5.92 neg	0.1134	5.66E-06	0.76649
572.1781_6.91 pos	8.14E-07	4.57E-05	6.71E-05
451.1204_3.78 pos	0.77418	0.0021095	0.76649
115.0527_2.22 pos	0.013173	0.0043282	0.95223
446.17_3.78 pos	0.0010352	0.0055391	0.86194
160.0762_2.24 pos	0.062856	0.0055391	0.88398
293.2106_8.45 neg	2.99E-06	0.013217	0.92495
698.4399_10.85 pos	0.58625	0.013217	0.80702
209.081_3.71 neg	0.13616	0.03805	0.87533
S8-8S hexoside neg	0.80518	0.03805	0.90065
383.1355_3.81 neg	0.013848	0.039182	0.82326
645.2217_10.41 pos	6.53E-08	0.039592	0.0055165
852.5418_10.76 pos	0.30306	0.039592	0.98395
640.205_4.69 pos	0.0094655	0.042436	0.98892
602.3009_6.09 pos	0.032475	0.042436	0.86466
623.3369_9.17 neg	0.12609	0.042436	0.76649
667.3869_10.82 neg	0.0077997	0.042782	0.86466
178.1066_2.26 pos	0.01801	0.052626	0.95102
453.2655_11 neg	0.040916	0.052626	0.97578
171.1019_8.97 neg	8.79E-05	0.06907	0.88398
441.1493_2.64 pos	0.012308	0.06907	0.92417
77.0919_1.04 pos	0.028019	0.06907	0.97578
Tubotaiwine neg	0.00010203	0.085393	0.76649
201.1089_7.57 neg	0.0016151	0.085393	0.79649
409.253_10.98 neg	0.018543	0.085393	0.87709
293.2078_8.72 neg	0.013848	0.090813	0.80702
613.514_10.95 pos	0.0017148	0.092981	0.9826
235.1437_10.91 pos	0.041876	0.094327	0.94168
171.1036_8.43 neg	7.03E-05	0.10184	0.8743
775.2306_3.36 neg	0.0091738	0.10184	0.86562
322.1103_9.24 pos	1.01E-05	0.10279	0.86562
171.1018_6.82 neg	0.0004712	0.10279	0.82138
293.2096_7.54 pos	0.00084311	0.10279	0.72981
378.0273_1.04 pos	0.0011775	0.10279	0.82138
327.2104_7.59 neg	0.0022315	0.10279	0.76649
295.226_8.68 pos	0.041511	0.10279	0.80702
324.1231_8.48 pos	2.11E-06	0.10488	0.69172

171.1001_6.08 neg	0.00013998	0.10488	0.80702
171.1038_10.18 neg	0.0014397	0.10488	0.92962
295.2288_10.43 pos	0.0018218	0.10488	0.92417
329.2265_6.82 neg	0.0022573	0.10488	0.88398
509.1658_3.55 pos	0.0023627	0.10488	0.90065
293.2101_9.54 pos	0.0065086	0.10488	0.99745
N-Fructosyl tyrosine p	0.015414	0.10488	0.86058
144.0798_4.95 pos	0.029979	0.10488	0.96473
Gelsemine pos	0.033259	0.10488	0.99442
76.0886_0.98 pos	0.03547	0.10488	0.95219
663.9874_5.11 neg	0.0058636	0.10641	0.98907
365.231_10.98 neg	0.0083319	0.10641	0.94383
592.2043_4.23 pos	0.018627	0.10641	0.94168
613.1733_3.63 neg	0.024146	0.10641	0.90791
211.1333_6.8 neg	0.0035394	0.1083	0.90791
220.9338_0.79 pos	0.017972	0.1083	0.94383
507.1501_3.57 neg	4.97E-06	0.10903	0.82138
351.1325_4.32 pos	0.0031672	0.10903	0.95219
397.132_10.27 neg	0.015826	0.10903	0.84969
Gelsemine neg	0.011446	0.11045	0.88398
FA 1831O_b neg	2.10E-05	0.11411	0.80702
212.9874_3.06 neg	3.83E-05	0.11411	0.95
210.9939_0.97 neg	0.00033293	0.11411	0.86821
699.3173_6.52 neg	0.00033293	0.11411	0.84586
326.2303_5.98 pos	0.0014397	0.11411	0.92417
379.036_2.96 neg	0.01331	0.11411	0.82326
455.2135_2.43 pos	0.026302	0.11411	0.79649
397.1245_9.59 neg	0.033277	0.11751	0.9416
399.1228_2.29 pos	0.039501	0.11751	0.94383
155.1079_9.09 pos	0.004209	0.11784	0.90065
343.1028_7.06 pos	0.0085504	0.11913	0.95531
404.0981_0.95 neg	0.00087618	0.12178	0.95434
711.2062_3.53 pos	0.013453	0.12178	0.95661
204.1024_4.96 pos	0.020187	0.12213	0.98907
155.106_6.76 pos	3.11E-05	0.12793	0.90791
313.2305_8.81 neg	0.0012998	0.12793	0.76649
304.1224_5.12 neg	0.0393	0.12993	0.94168
356.9628_5.12 neg	0.0077665	0.13285	0.96156
796.6196_11.74 neg	0.018156	0.13285	0.22452
499.3285_10.41 pos	0.0024787	0.13343	0.90791
306.1139_9.24 pos	5.06E-06	0.13403	0.86857
693.5148_10.9 pos	0.00011653	0.13403	0.98021
305.1062_4.15 neg	0.018938	0.13403	0.80702
151.1118_10.43 pos	8.33E-05	0.13577	0.9826
508.1489_3.54 neg	8.76E-05	0.13577	0.99578
295.2243_6.76 pos	0.0014851	0.13577	0.84586
337.1071_6.05 neg	0.0016607	0.13577	0.95216
177.0536_4.9 neg	0.0034264	0.13577	0.98021

277.2136_8.73 pos	0.0042648	0.13577	0.80222
337.2736_10.46 pos	0.040313	0.13577	0.97317
291.2301_10.92 pos	0.025101	0.13609	0.95216
656.916_5.06 neg	0.030603	0.13647	0.98866
277.216_8.35 pos	8.03E-05	0.13766	0.82138
291.1907_8.2 neg	0.001302	0.13766	0.82274
MGMG 183 pos	0.010496	0.13766	0.99749
171.1009_9.47 pos	0.014786	0.13766	0.80702
229.1371_6.83 neg	0.035803	0.13766	0.79649
522.3537_10.57 pos	0.030458	0.13875	0.76649
S-Lactate neg	3.63E-05	0.14399	0.82326
327.2175_6.46 neg	0.0011572	0.14477	0.90065
136.0615_2.08 pos	0.0032231	0.14477	0.80702
254.097_5.07 pos	0.0038384	0.14477	0.84969
781.2861_6.62 neg	0.019644	0.14477	0.97317
847.2715_6.21 pos	0.022355	0.14477	0.98432
1-Isothiocyanato-4-met	0.03215	0.14477	0.95048
425.0538_4.81 neg	8.86E-05	0.14533	0.90791
307.1837_8.46 neg	0.0046951	0.14533	0.76649
442.1635_8.47 pos	0.027875	0.14533	0.82021
245.2275_10.41 pos	0.0046062	0.14909	0.91343
529.1465_2.73 neg	0.0019119	0.15064	0.77718
555.1489_4.75 pos	0.0028289	0.15064	0.98569
195.1378_6.76 pos	0.0030938	0.15064	0.86665
275.9941_1.15 pos	0.01214	0.15064	0.95216
176.0126_4.55 neg	2.72E-08	0.15124	0.94794
338.14_9.23 pos	7.45E-06	0.15124	0.84969
Flavonol base 3O, O-d	2.42E-05	0.15124	0.97317
324.1206_6.98 pos	4.99E-05	0.15124	0.76649
277.2178_9.49 pos	0.00012494	0.15124	0.82138
N-Acetyl-DL-methionii	0.0014397	0.15124	0.88398
Quercetin-4'-O-glucosi	0.0032231	0.15124	0.95102
650.4401_11.44 pos	0.0060618	0.15124	0.89669
209.0598_7.12 pos	0.011311	0.15124	0.84586
392.134_4.98 pos	0.019139	0.15124	0.97578
569.0944_4.93 neg	0.022109	0.15124	0.85422
396.0351_1.17 pos	0.029579	0.15124	0.96158
308.127_9.92 pos	0.035039	0.15124	0.84969
335.2184_8.49 pos	0.00027493	0.15282	0.90791
465.1043_4.14 neg	0.018621	0.15282	0.99578
397.295_10.96 pos	0.035707	0.15282	0.97578
241.1065_10.93 pos	0.010147	0.15326	0.86478
210.0852_0.86 pos	0.011562	0.15446	0.98852
338.0789_5.6 neg	0.00011057	0.15598	0.90791
181.0629_7.74 pos	0.0034672	0.15621	0.90138
747.2494_4.37 neg	0.0036975	0.15621	0.97578
495.2944_10.4 pos	0.005294	0.15621	0.85422
605.1966_6.07 pos	0.0065585	0.15621	0.95216

378.9132_0.82 neg	0.024266	0.15621	0.97578
C21H36O10 neg	0.041006	0.15621	0.97317
131.0483_7.4 pos	0.03538	0.15931	0.94383
229.1535_2.19 pos	0.043086	0.15931	0.92911
620.3304_8.13 pos	0.0028289	0.15959	0.8592
Mitragynine pos	0.0015471	0.16038	0.99876
197.1168_4.52 pos	0.013322	0.16038	0.82326
325.1039_6.04 neg	0.047367	0.16038	0.97409
209.0559_7.74 pos	0.0016647	0.16217	0.89669
321.0296_2.57 neg	0.0031521	0.16367	0.97578
95.0495_3.79 pos	0.019412	0.16367	0.9826
615.2016_4.72 neg	0.0016141	0.16403	0.92962
353.1039_5.58 neg	0.00025564	0.16422	0.88671
135.1152_10.94 pos	0.0076717	0.16493	0.97034
529.2998_10.44 neg	0.010347	0.16493	0.95661
280.0962_4.63 pos	0.024651	0.16493	0.97578
171.0993_6.76 pos	0.00093312	0.16536	0.84969
471.1485_2.67 pos	0.046512	0.16536	0.94134
222.0756_2.58 neg	0.0053283	0.16793	0.89392
357.2986_10.3 pos	0.0068966	0.16841	0.82326
393.0506_2.71 neg	4.24E-09	0.16934	0.86065
677.2106_5.03 pos	1.51E-07	0.17163	0.92417
Flavone base 3O, 1Me	1.13E-05	0.17163	0.84969
293.2081_9.54 neg	2.80E-05	0.17163	0.82326
279.231_10.77 pos	8.59E-05	0.17163	0.76649
3,4-Dimethoxycinnami	9.86E-05	0.17163	0.86466
167.0348_3.34 neg	0.000115	0.17163	0.82326
268.1028_2.06 pos	0.00046514	0.17163	0.82138
243.2086_10.96 pos	0.00054478	0.17163	0.97578
Disaccharides 2-methyl	0.0016522	0.17163	0.82326
443.1208_0.98 pos	0.0025343	0.17163	0.88398
637.1815_0.95 neg	0.0034303	0.17163	0.99637
357.0743_3.48 neg	0.0062619	0.17163	0.92911
589.2546_5.93 pos	0.0065086	0.17163	0.97578
288.9211_0.8 pos	0.014421	0.17163	0.97578
248.9603_0.85 neg	0.020629	0.17163	0.94383
501.215_3.91 pos	0.020818	0.17163	0.99413
374.124_4.99 pos	0.025731	0.17163	0.98395
783.3099_6.59 pos	0.042742	0.17163	0.9826
164.995_6.51 neg	0.045172	0.17163	0.95
467.3586_9.99 pos	0.045431	0.17163	0.80702
566.1487_0.91 neg	0.0040211	0.1721	0.9826
261.1217_5.95 pos	0.013195	0.17257	0.97578
841.2629_1.07 pos	0.014412	0.17257	0.95216
409.1748_5.22 pos	0.0073899	0.1734	0.98866
393.2627_10.32 pos	0.00033228	0.17404	0.94383
679.3751_10.52 neg	0.011623	0.17523	0.97578
D-Pantothenic acid neg	0.00093759	0.17558	0.94168

213.148_6.76 pos	0.0048549	0.17558	0.85422
225.0088_3.31 neg	0.006444	0.17558	0.93572
306.0042_1.16 pos	0.0058709	0.17622	0.82326
322.1294_4.88 neg	0.015114	0.17622	0.99413
384.9151_0.74 pos	0.017285	0.17622	0.99865
596.839_0.77 pos	0.048612	0.17622	0.99774
559.2423_5.91 pos	0.035285	0.17654	0.97578
397.1967_2.37 pos	0.00058673	0.17673	0.76649
229.1392_6.44 neg	0.013344	0.17798	0.88398
582.4556_7.74 pos	0.00726	0.1784	0.98866
721.3242_4.07 neg	0.031789	0.17845	0.97578
127.0393_1.33 pos	0.048815	0.17884	0.99637
239.2348_10.28 pos	0.037959	0.17897	0.99578
591.3929_11.68 neg	0.023601	0.1791	0.88398
371.1066_3.96 pos	0.0020695	0.18017	0.94168
739.2815_4.52 neg	0.023614	0.18017	0.90065
338.0846_2.56 neg	0.025496	0.18017	0.91343
277.1745_10.89 neg	0.028752	0.18017	0.95661
545.2234_4.35 neg	0.036363	0.18017	0.89874
221.0661_3.68 neg	0.046356	0.18017	0.90301
279.2264_10.45 neg	0.0092538	0.18197	0.94383
149.1314_9.49 pos	0.0003677	0.18238	0.84969
645.1459_4.67 pos	0.016749	0.18494	0.82326
226.9672_0.82 neg	0.036491	0.18494	0.95773
637.3785_10.28 pos	0.038792	0.18494	0.99407
657.2035_3.09 neg	0.039339	0.18494	0.88398
251.106_4.67 pos	0.040508	0.18666	0.95219
761.3126_6.36 pos	0.0025722	0.18683	0.98866
853.3167_6.4 pos	0.014492	0.18683	0.98281
315.0739_2.67 neg	0.0001832	0.18707	0.82326
611.2342_5.93 pos	0.0056003	0.18767	0.94383
504.3443_10.58 pos	0.0028658	0.18923	0.70088
797.3214_5.94 pos	0.0051118	0.18923	0.97578
898.8006_0.75 pos	0.0093681	0.18923	0.97578
Flavonol base 5O, O-H	0.012027	0.18923	0.97578
137.025_3.35 neg	0.045926	0.18923	0.98432
360.1495_4.63 neg	0.01868	0.18968	0.94383
784.3527_4.03 pos	0.028359	0.19092	0.98852
523.3002_10.96 pos	0.024039	0.19221	0.97578
426.9009_0.74 pos	0.0037828	0.19274	0.98833
538.4281_7.71 pos	0.011586	0.19274	0.99749
Gossypetin 8-glucoside	0.0024227	0.19348	0.94579
357.1312_5.01 pos	0.02839	0.19348	0.82326
171.1035_5.65 neg	0.00031631	0.19479	0.97578
179.0287_5.39 pos	0.0056003	0.19479	0.90065
216.0898_3.8 neg	0.049456	0.19591	0.96156
275.0241_1.19 neg	9.79E-06	0.19732	0.98866
621.2084_3.91 neg	0.048401	0.19847	0.97578

491.1548_5.01 pos	0.016639	0.19912	0.82326
356.9071_0.79 pos	0.039344	0.19912	0.89473
611.3012_5.54 neg	1.36E-05	0.19939	0.84969
195.1398_6.41 pos	0.00033379	0.20289	0.82326
429.1211_0.98 neg	0.032506	0.20289	0.99457
728.2014_0.9 neg	0.00049547	0.20317	0.99413
313.0908_3.52 pos	0.0019472	0.20327	0.98569
425.0576_5.07 pos	0.013271	0.20327	0.89669
853.3143_7.01 pos	0.034501	0.20392	0.99151
198.9407_0.74 pos	0.018657	0.20411	0.99749
253.0913_10.61 neg	0.039151	0.20411	0.99749
437.1544_4.47 neg	0.043086	0.20411	0.99745
215.0671_4.31 pos	0.018543	0.20526	0.99745
D-FRUCTOSE pos	0.0093844	0.20587	0.84969
551.3496_10.94 pos	0.041327	0.20587	0.97578
255.0494_2.75 neg	1.41E-05	0.20808	0.82326
167.0701_2.36 pos	0.00078802	0.20814	0.88842
562.3137_9.31 neg	0.014622	0.20859	0.84969
Biflavonoid-flavone ba	0.005481	0.20922	0.94383
325.0935_8.13 pos	0.013637	0.20922	0.95773
251.0936_4.31 pos	0.040963	0.20922	0.82326
515.3206_10.62 neg	0.028603	0.21024	0.99745
Fumaric acid neg	7.30E-05	0.21222	0.89669
567.1707_1.04 neg	0.012745	0.21291	0.98907
171.101_8.87 pos	0.0072109	0.21438	0.82138
720.5204_11.38 pos	0.013195	0.21472	0.96684
720.1396_1.4 pos	0.016997	0.21472	0.95102
541.1818_5.97 pos	0.0073899	0.21509	0.8467
441.3192_10.94 pos	0.036188	0.21573	0.99749
427.2682_6.43 pos	0.00019761	0.21579	0.95216
630.8789_0.74 neg	0.014834	0.21579	0.95219
Strictosidine_a neg	0.019203	0.21579	0.98126
MGMG 182 neg	0.020458	0.21579	0.99749
263.236_10.58 pos	0.022109	0.21579	0.98866
316.9478_0.83 neg	0.035558	0.21846	0.95219
601.1759_3.24 pos	5.01E-08	0.21852	0.97317
545.2911_7.22 pos	0.0086839	0.21852	0.90065
327.1806_9.85 neg	0.014786	0.21852	0.76649
557.2244_6.43 pos	0.027831	0.22095	0.97578
560.2461_5.85 pos	0.012532	0.22119	0.90138
681.2416_4.59 neg	0.00091382	0.22183	0.88685
597.286_10.43 neg	0.0022355	0.22183	0.98395
186.091_4.99 pos	0.016579	0.22183	0.99454
672.413_10.28 pos	0.045596	0.22183	0.99549
278.219_6.81 pos	0.00066814	0.2226	0.98866
163.0606_10.32 pos	0.015133	0.2226	0.92539
202.181_0.91 pos	0.023024	0.2226	0.99637
670.5076_7.77 pos	0.0050547	0.2227	0.97578

626.4814_7.71 pos	0.0044846	0.22301	0.98866
355.2809_10.58 pos	0.031646	0.22374	0.98866
899.8467_5.11 neg	0.041581	0.22374	0.95216
335.2206_9.45 pos	0.013794	0.22445	0.98845
351.2126_6.41 pos	0.00025564	0.22593	0.9278
727.2689_6.07 pos	0.0089336	0.22593	0.99637
134.0186_1.07 neg	0.012368	0.22593	0.98866
482.3581_10.61 pos	0.027803	0.22607	0.92417
546.3987_10.94 pos	0.043086	0.22607	0.97578
398.9055_0.75 pos	0.0083995	0.22691	0.90065
280.238_10.6 neg	0.025459	0.22691	0.99413
347.1842_5.34 neg	0.033894	0.227	0.82138
516.8986_0.74 neg	0.012209	0.22827	0.94544
458.2652_9.24 pos	0.0017354	0.22864	0.98111
494.4042_7.69 pos	0.0093063	0.22922	0.98907
Coniferyl alcohol O-H	0.00049547	0.23052	0.79649
443.0588_3.68 neg	0.000129	0.23169	0.95219
714.5354_7.73 pos	0.0044784	0.23185	0.98852
583.2173_6.15 neg	0.00026454	0.23198	0.80702
461.2272_3.44 pos	0.0011418	0.2349	0.98852
224.0699_7.08 pos	0.0047699	0.23528	0.91371
819.356_5.78 pos	0.042878	0.23606	0.94383
225.0086_3.88 neg	0.00030589	0.23632	0.97578
345.0514_2.44 neg	0.0036975	0.23632	0.97578
331.1184_4.71 neg	0.023814	0.23632	0.84586
306.2761_9.88 pos	0.027247	0.23632	0.86466
531.2085_6 pos	0.045493	0.23632	0.95
559.2415_5.23 pos	0.024379	0.23644	0.95661
267.0873_6.73 neg	0.026855	0.23684	0.76649
146.0453_0.9 neg	0.00073201	0.23744	0.82326
FA 1831O pos	0.0032432	0.23744	0.95216
312.9988_0.75 pos	0.025545	0.23744	0.89392
393.0505_3.57 neg	5.71E-10	0.2386	0.82138
288.084_5.04 pos	0.0044448	0.2386	0.85422
455.1862_5.49 neg	0.015931	0.2386	0.88503
N-Methyl-D-aspartic ac	0.0028964	0.2394	0.80702
196.0719_5.1 pos	0.0011569	0.24026	0.97578
313.2743_10.3 pos	0.0021786	0.24097	0.97578
317.2089_4.37 pos	0.0063315	0.24312	0.82326
445.1458_6.63 pos	0.021071	0.24577	0.88671
393.263_9.43 pos	0.00017734	0.24665	0.82138
890.2559_0.96 neg	0.0023938	0.24719	0.99578
319.2245_10.11 pos	4.25E-05	0.24797	0.84969
603.1827_6.41 pos	0.0063287	0.24838	0.84389
196.0795_6.46 pos	0.019203	0.24869	0.98907
590.4279_10.92 pos	0.048612	0.25027	0.97578
FA 1802OSO4 pos	0.0023938	0.25254	0.84969
471.0186_4.23 neg	0.0028027	0.25254	0.97578

416.2462_7.22 pos	0.014914	0.25311	0.90065
603.2839_6.16 neg	0.016117	0.25727	0.95207
500.3109_9.27 pos	0.016212	0.25767	0.95434
543.2736_7.24 neg	0.0079239	0.25784	0.82138
Deoxyvasicinone neg	0.0019409	0.25826	0.94072
569.2273_5.96 neg	0.044017	0.2592	0.86562
240.1003_6.53 pos	0.03025	0.26096	0.84969
195.1378_10.14 neg	1.37E-05	0.26231	0.76649
171.104_9.57 neg	0.00057009	0.26364	0.97578
485.1069_4.69 pos	0.043031	0.26364	0.82138
495.261_5.93 neg	0.040226	0.2643	0.97317
325.1848_8.52 neg	0.0089844	0.26636	0.82274
336.2398_3.33 pos	0.01266	0.26677	0.90065
255.2337_11.03 neg	0.048815	0.26905	0.94168
279.2313_10.12 pos	3.83E-05	0.26926	0.82274
632.471_11.38 pos	0.031056	0.27041	0.98907
477.1399_3.48 neg	4.99E-05	0.27181	0.88398
637.1824_1.3 neg	0.0032541	0.27228	0.97578
756.2776_0.95 pos	0.041186	0.27363	0.94168
152.0567_4 pos	8.33E-05	0.2737	0.90065
569.1995_5.4 neg	0.020506	0.27375	0.98907
798.2532_1.07 neg	0.04218	0.27451	0.99454
373.1288_5.52 neg	0.0007533	0.27527	0.97578
333.1312_4.64 pos	0.028605	0.27527	0.86562
343.1016_3.89 pos	0.037959	0.27527	0.98907
569.0935_4.45 neg	1.41E-05	0.27535	0.88503
593.2601_9.66 neg	0.039561	0.27535	0.93522
665.2476_3.92 pos	0.011364	0.27563	0.89392
800.2787_1.07 pos	0.030602	0.27615	0.92962
309.2011_8.92 neg	0.039876	0.2787	0.82138
261.2219_8.99 pos	0.0001665	0.27896	0.90065
HexCer t180 pos	0.048633	0.27945	0.97317
767.2402_4.16 neg	0.0084862	0.28108	0.82274
142.0512_2 neg	0.039293	0.28125	0.99749
321.2054_10.97 neg	0.043171	0.28125	0.98833
539.2876_6.39 neg	0.018175	0.28182	0.97317
466.3731_7.62 pos	0.016575	0.28463	0.97578
329.2239_8.02 neg	0.011704	0.28612	0.86748
266.0689_3.19 neg	0.035205	0.28612	0.98432
848.2498_0.92 pos	0.009785	0.28656	0.90791
559.4856_10.95 pos	0.022747	0.28661	0.97578
225.0675_9.63 pos	0.031972	0.28709	0.8895
607.264_5.25 pos	0.0026353	0.28804	0.97317
426.6281_1.21 pos	0.027787	0.2884	0.98833
Hexose C13H21O2 po	0.017285	0.29057	0.95216
311.2875_10.79 neg	0.046356	0.29057	0.80702
445.1277_3.31 neg	5.65E-05	0.29444	0.95102
96.9605_3.61 neg	0.00011035	0.29444	0.98866

622.4038_10.79 pos	0.017394	0.29444	0.95434
424.8923_0.8 pos	0.018686	0.29444	0.98128
417.1526_6.2 pos	0.019173	0.29496	0.82138
C17H30O10 neg	0.0007533	0.29806	0.95102
676.4955_11.36 pos	0.039782	0.29806	0.99749
191.0687_6.11 pos	0.00016493	0.29945	0.86201
509.13_3.19 neg	1.01E-05	0.2998	0.84969
801.2903_2.63 pos	1.13E-11	0.3047	0.97578
413.2954_10.59 pos	0.012454	0.30547	0.97578
469.1765_5.96 neg	0.045593	0.30676	0.93626
537.1369_4.78 pos	0.00063967	0.30804	0.90791
219.1765_10.82 neg	0.032205	0.30828	0.95102
597.1895_0.94 pos	5.32E-06	0.31027	0.95048
343.2115_5.48 neg	1.08E-05	0.31027	0.33271
226.0121_3.27 neg	0.00023293	0.31027	0.95661
231.0376_7.14 pos	0.0010526	0.31027	0.84969
403.2821_11.21 neg	0.0059993	0.31027	0.89392
294.1133_8.32 pos	0.028457	0.31027	0.78816
278.9089_0.82 neg	0.0044946	0.31036	0.89392
136.0615_0.95 pos	0.0073219	0.31036	0.76649
353.2296_6.79 pos	0.016432	0.31036	0.95102
453.1573_5.34 pos	0.019629	0.31036	0.97578
541.1702_4.17 pos	0.0017621	0.3115	0.80702
393.1167_4 pos	3.11E-05	0.31235	0.97578
95.0849_10.12 pos	1.58E-05	0.31346	0.80702
605.4724_11.84 pos	0.0065585	0.31579	0.82138
895.276_4.65 neg	0.00023293	0.31587	0.79649
121.0257_3.31 pos	0.040508	0.31665	0.84617
816.2903_4.1 pos	0.0014397	0.31731	0.82138
359.1482_4.03 pos	0.0035306	0.31731	0.95
560.885_0.81 neg	0.008288	0.31731	0.89392
319.1221_3.81 neg	0.030754	0.31731	0.90065
440.2_4.1 pos	0.043254	0.31754	0.94954
457.1327_2.51 pos	3.43E-09	0.3211	0.95531
414.2309_6.84 pos	0.00053838	0.32117	0.99749
218.1139_5.35 pos	0.027787	0.32166	0.97578
127.0387_7.12 pos	0.012579	0.32346	0.87279
321.0847_3.25 pos	1.01E-05	0.32351	0.97578
459.2234_5.15 pos	0.00087796	0.32479	0.90065
510.1035_5.37 pos	0.011407	0.32479	0.97578
472.2079_3.45 pos	0.0010648	0.32609	0.82326
435.137_1.26 pos	0.014554	0.32675	0.95661
336.261_10.64 pos	0.005934	0.3283	0.80702
LPE 182 neg	0.027831	0.3283	0.82326
429.1519_4.94 pos	0.024957	0.32893	0.82138
607.2109_5.6 pos	0.036176	0.32896	0.94383
455.2167_6.21 neg	0.045004	0.32932	0.98847
762.4861_10.86 pos	0.030172	0.32957	0.97578

Flavanone base 3O, C-	0.00024577	0.33044	0.82138
208.0757_8.68 pos	0.049964	0.33137	0.99826
C15H26O10 neg	0.013787	0.33261	0.92911
317.1035_3.65 neg	8.05E-05	0.3333	0.82326
Benzyl alcohol Hex-H(	0.00057009	0.3333	0.82138
583.2196_5.76 neg	0.0022946	0.33523	0.94755
339.1215_5.65 pos	0.0089519	0.33561	0.82138
403.2288_10.93 pos	0.022452	0.33703	0.99578
432.2148_10.69 neg	7.34E-05	0.33785	0.80702
306.0773_4.11 neg	0.015425	0.33802	0.76649
637.3932_10.96 neg	0.025883	0.33802	0.80702
413.0819_6.63 pos	0.040381	0.33819	0.95219
443.1668_5.83 pos	0.000129	0.33936	0.8336
135.1173_4.46 pos	0.0050256	0.33936	0.76649
187.0491_8.15 pos	0.019453	0.3418	0.9782
403.0921_3.68 neg	0.027074	0.34251	0.92172
856.54_11.78 neg	0.027253	0.34252	0.95216
85.0281_1.48 pos	0.021649	0.344	0.98281
133.1009_9.28 pos	0.0066027	0.34485	0.95661
617.2613_6.63 neg	0.013344	0.34485	0.98868
Vanillic acid O-sulfona	0.037984	0.34485	0.97578
510.3996_7.65 pos	0.049929	0.34916	0.95219
379.0935_10.58 neg	0.025611	0.34978	0.93273
354.2743_10.43 pos	0.031533	0.3509	0.76649
C7H11NO3 neg	0.015338	0.35218	0.9416
323.0914_6.09 pos	0.0044679	0.35243	0.97317
710.4614_10.82 pos	0.0016141	0.3534	0.78816
407.2395_7.62 pos	6.86E-06	0.35386	0.95
541.8762_0.8 pos	0.0059993	0.35455	0.88842
177.0559_2.31 neg	0.0075733	0.35471	0.95
585.2277_6.38 neg	0.011191	0.35471	0.99578
413.1251_10.56 pos	0.048979	0.35471	0.90238
399.1079_6.44 pos	1.12E-05	0.35516	0.82138
443.2263_6.14 neg	0.0010842	0.35516	0.95531
Furostane base -2H O-:	0.0066072	0.35668	0.87533
Malvidin-3-O-glucoside	0.014384	0.35668	0.84586
319.1166_3.65 pos	0.011104	0.35953	0.76649
C12H18O3 neg	0.0029729	0.36117	0.98379
302.1977_2.53 pos	0.0028964	0.3615	0.90065
133.0155_4.83 neg	0.016212	0.36294	0.90065
579.1847_6.43 neg	0.00025594	0.36313	0.86562
197.081_2.23 pos	0.00078802	0.36329	0.82274
423.0625_3.88 neg	2.26E-05	0.3652	0.97852
583.2168_5.44 pos	0.00016416	0.36523	0.90065
351.1302_4.86 pos	0.011533	0.36523	0.88671
LPE 160 neg	0.00046744	0.36891	0.95216
480.2791_3.96 pos	0.0019802	0.37045	0.85841
785.3257_5.25 pos	0.021571	0.37082	0.90065

787.1624_1.08 pos	0.026469	0.37129	0.88502
Isoflavone base 4C, 1P	0.0011971	0.37146	0.97578
335.258_10.5 pos	0.0023699	0.37146	0.98866
811.3121_5.74 neg	0.0039542	0.37146	0.89392
731.3002_6.28 pos	0.037593	0.37146	0.97578
C28H38O13 neg	0.0057012	0.37149	0.94755
609.191_0.99 neg	0.0058598	0.37352	0.94383
509.1452_1.5 pos	0.039078	0.37509	0.98845
751.2295_8.13 pos	0.013988	0.37742	0.87322
857.3035_6.83 pos	0.04861	0.37767	0.92608
411.1896_5.37 pos	0.030681	0.37886	0.98907
489.0641_2.42 pos	8.38E-08	0.38097	0.84586
146.0585_4.62 pos	0.00055541	0.38149	0.97578
315.0724_2.18 neg	0.00031621	0.38176	0.91127
LPC 183 pos	0.041771	0.38176	0.98539
291.071_0.92 pos	0.035216	0.38255	0.99749
245.0918_4.6 pos	0.00090969	0.38472	0.97578
235.0628_6.66 neg	0.045669	0.38642	0.80702
641.317_5.32 neg	0.00022915	0.38787	0.85946
601.274_8.16 neg	0.0077388	0.38787	0.90791
229.071_7.12 pos	0.0095494	0.38787	0.84586
560.2702_6.54 pos	0.036866	0.38787	0.94383
409.1765_5.4 neg	0.043503	0.3881	0.97578
492.8958_0.81 neg	0.0076314	0.38834	0.95216
273.2211_5.02 pos	0.045552	0.38947	0.80702
803.3647_5.29 neg	0.0017123	0.39012	0.8336
404.1443_5.04 pos	0.002254	0.39097	0.84969
306.2786_10.73 pos	0.0018218	0.39104	0.90791
279.1002_4.66 pos	0.015605	0.39141	0.95048
547.1906_6.05 pos	0.0039936	0.39388	0.95216
720.4373_10.9 pos	0.010496	0.39467	0.97782
735.2457_3.89 pos	0.045077	0.39467	0.88842
556.2379_3.94 pos	0.049575	0.3953	0.92911
210.9915_2.36 neg	0.010496	0.39615	0.94855
149.0601_3.17 pos	0.019909	0.39698	0.97578
577.1688_7.2 neg	0.00050301	0.39786	0.89392
FA 182O neg	0.00047275	0.39939	0.76649
179.026_5.44 neg	0.0002292	0.39944	0.82138
664.4062_11.06 neg	0.021717	0.39944	0.99413
615.2435_5.8 pos	0.0038275	0.40121	0.98907
547.1937_7.45 pos	0.0044402	0.40128	0.97578
337.1058_5.8 pos	0.0015122	0.40129	0.97174
632.8748_0.74 neg	0.0063496	0.40161	0.97578
135.0439_5.4 neg	0.023653	0.40179	0.97578
422.2346_3.8 pos	0.0014113	0.40206	0.89392
777.272_7.3 neg	0.0023508	0.40206	0.89392
589.1885_5.05 pos	0.0021584	0.40416	0.98852
755.2391_4.03 neg	0.019412	0.40604	0.99774

613.2231_6.4 pos	0.00070888	0.40711	0.76649
555.173_3.88 neg	1.14E-05	0.40889	0.98395
351.2504_9.58 pos	3.38E-05	0.40889	0.82274
199.0735_4.65 pos	0.00011653	0.40889	0.92962
Desoxypeganine pos	0.032144	0.40889	0.99637
134.0349_6.63 pos	0.040963	0.40889	0.88398
270.0889_5.09 pos	0.013469	0.40926	0.99892
290.083_4.4 neg	0.016581	0.40926	0.97578
558.2412_5.97 neg	0.046381	0.40926	0.95661
786.2816_4.13 pos	0.0050035	0.40933	0.89473
517.1537_3.56 neg	0.045174	0.40933	0.76649
352.8969_0.75 pos	0.010552	0.41224	0.89392
313.2729_10.91 pos	0.029079	0.41248	0.82138
391.2447_9.6 pos	0.00010405	0.4162	0.93522
652.1703_6.62 pos	0.034877	0.4162	0.97578
Putrescine pos	0.03085	0.41681	0.98119
586.4681_10.75 pos	0.00078802	0.41843	0.82326
505.3001_11.03 neg	0.038006	0.41843	0.99865
551.2839_6.45 pos	0.0025829	0.41935	0.82326
517.1859_7.12 pos	0.0075733	0.41973	0.92417
177.0543_3.16 pos	0.0091579	0.4206	0.99637
C23H42NO7P neg	4.69E-08	0.42144	0.76649
507.1548_1.35 pos	0.019888	0.42269	0.89476
646.1541_4.49 neg	0.00029173	0.42475	0.82326
437.1218_6.52 pos	0.041511	0.42475	0.97578
Indole-3-acetyl-L-isoleu	0.013876	0.42571	0.98892
5'-Methylthioadenosine	0.028457	0.42603	0.97317
534.1667_6.58 pos	0.0036975	0.42708	0.97317
555.4315_7.12 pos	0.0089845	0.42708	0.88398
673.2142_4.79 neg	0.035125	0.4273	0.89473
425.2159_4.44 pos	0.039151	0.42905	0.99749
665.1807_3.1 neg	0.00058673	0.4302	0.98847
746.8535_0.74 neg	0.0025354	0.43028	0.93626
449.143_4.88 neg	0.014691	0.43067	0.82326
339.0526_1.12 pos	0.0020514	0.43306	0.93273
273.0843_1.1 pos	0.018756	0.43306	0.82326
457.1368_4.5 pos	0.028752	0.43344	0.82138
554.4246_7.71 pos	0.040278	0.4337	0.97317
78.9591_1.02 neg	0.048163	0.4337	0.82138
849.2694_0.99 pos	7.13E-05	0.43406	0.98803
465.1971_4.93 neg	0.00018432	0.43406	0.84987
722.4622_10.94 pos	0.019813	0.43533	0.94383
329.1392_4.39 pos	0.011091	0.43582	0.92911
348.3629_9.92 pos	0.020389	0.43752	0.84969
544.3322_10.5 pos	0.026809	0.43752	0.84969
319.2251_5.02 pos	0.020196	0.43897	0.82274
72.9924_1.04 neg	0.044939	0.43897	0.96684
148.9458_0.85 neg	0.00071846	0.44033	0.9826

145.0283_3.16 pos	0.013157	0.44095	0.99121
449.2268_8.58 neg	1.58E-05	0.44144	0.90791
527.2075_5.94 neg	0.024039	0.44144	0.95216
486.3912_10.25 pos	0.013445	0.44155	0.96015
225.111_10.49 pos	0.0074275	0.44195	0.90791
445.1671_4.51 pos	0.00079533	0.44455	0.84969
339.1228_4.26 neg	0.0011202	0.44501	0.79649
N-Fructosyl isoleucylgl	0.0063957	0.44501	0.99637
99.9258_11.24 neg	0.023814	0.44501	0.91343
831.2334_7.07 neg	0.029734	0.44885	0.89392
FERULATE neg	0.032796	0.44885	0.94383
455.1121_0.88 pos	0.0011749	0.44917	0.91371
LPE 183 pos	0.00047435	0.44954	0.87624
359.2064_10.94 pos	0.027037	0.45001	0.97578
207.1116_2.35 pos	0.010985	0.45057	0.97578
227.0578_5.03 pos	0.040111	0.45262	0.82138
553.3025_10.54 pos	0.036439	0.45284	0.84586
313.0566_4.01 neg	0.015761	0.45294	0.90274
354.1086_1.09 pos	0.022056	0.45335	0.95219
301.0741_3.82 pos	0.025446	0.45335	0.92417
173.1296_10.16 pos	0.011985	0.4536	0.94383
265.1522_2.5 pos	0.010496	0.45367	0.90791
626.867_0.74 pos	0.00079297	0.45395	0.84586
514.2104_3.06 pos	0.039143	0.45395	0.90791
Citrate neg	0.043086	0.45613	0.92911
439.0755_1.05 neg	0.025692	0.45635	0.92608
149.1315_10.21 pos	7.87E-06	0.45714	0.79649
579.1826_7.18 pos	0.0013181	0.45767	0.92417
242.9237_0.83 pos	0.0044089	0.45767	0.87533
137.0584_2.29 pos	0.0081326	0.45767	0.86562
548.3655_10.64 pos	0.013465	0.45767	0.79649
346.2579_6.38 pos	6.82E-05	0.45817	0.97578
380.1527_2.72 neg	0.03215	0.45952	0.97578
145.0279_7.12 pos	0.004968	0.46126	0.88398
403.0882_3.31 neg	0.021709	0.46319	0.96158
Valylphenylalanine pos	0.0027878	0.46377	0.99637
253.2189_9.52 neg	0.0045266	0.46377	0.89669
497.227_6.91 neg	0.013866	0.46377	0.90065
543.272_6.85 pos	0.0011642	0.46424	0.95102
270.0872_3.86 pos	0.02279	0.46424	0.99637
847.551_10.68 pos	0.043935	0.46631	0.86562
454.1231_2.57 pos	9.47E-11	0.46824	0.97578
321.1915_3.66 pos	0.00044732	0.46835	0.97578
858.8354_0.74 neg	5.67E-05	0.46899	0.79649
593.2658_10.03 neg	0.0038478	0.46899	0.92911
249.0573_3.95 neg	0.0088139	0.46899	0.80702
800.2352_1.45 neg	0.046356	0.46899	0.90065
137.0282_4.31 neg	0.00020797	0.47015	0.97578

176.5115_3.16 pos	0.02875	0.47015	0.98594
371.1474_4.57 pos	0.00535	0.47075	0.84969
Methoxycinnamic acid	0.007028	0.47086	0.97317
279.2316_11.39 neg	0.00052308	0.47117	0.97317
741.2496_5.59 neg	0.023396	0.47141	0.89392
715.1927_4.24 neg	0.018889	0.47215	0.86562
219.025_0.82 pos	0.00063346	0.47294	0.98539
142.0661_4.64 pos	0.014786	0.47294	0.99454
736.2294_6.7 pos	0.021622	0.47294	0.98847
591.2074_4 pos	0.032731	0.47294	0.94755
364.1362_4.96 neg	0.012103	0.47317	0.97578
248.1287_3.16 pos	0.0095517	0.47406	0.98907
117.034_0.93 pos	0.027787	0.47406	0.98907
737.3657_8.02 pos	0.045871	0.4745	0.92417
541.212_3.43 neg	0.0015294	0.47458	0.82326
477.1277_2.33 neg	0.00021429	0.47496	0.87477
281.102_4.34 pos	0.01786	0.47496	0.95216
Benzoic acid 2O, O-He	0.022719	0.47618	0.97317
586.2523_4.02 pos	0.001415	0.47703	0.8635
349.1045_4.77 pos	0.0019642	0.47791	0.95102
471.1829_5.41 neg	0.0037549	0.47791	0.82138
705.2323_3.3 pos	0.0038478	0.47791	0.98569
695.2211_4.59 neg	0.0094763	0.47791	0.98395
220.1183_2.69 pos	0.048633	0.47791	0.97317
605.1966_6.46 pos	0.00071846	0.47929	0.84969
409.253_8.48 pos	0.0027526	0.4807	0.89392
768.8596_0.95 pos	0.045669	0.4807	0.98868
117.0341_3.16 pos	0.027074	0.48178	0.98119
557.237_5.99 neg	0.027138	0.48178	0.97578
880.2847_7.12 pos	0.0078264	0.48395	0.95102
595.3099_6.53 pos	0.0073899	0.48567	0.90065
227.0901_2.35 pos	0.0015247	0.48573	0.90065
3-Hydroxycinnamic aci	0.03051	0.48573	0.99745
409.1702_3.42 neg	0.025396	0.48622	0.95661
677.2066_5.38 pos	5.71E-10	0.48647	0.94072
C13H18O2 neg	0.010977	0.48696	0.82326
759.2421_1.2 pos	0.00099461	0.48815	0.99551
Etoposide neg	0.0048549	0.48815	0.86665
295.2278_9.68 neg	0.0024614	0.48931	0.82138
795.2916_5.58 neg	0.0010796	0.48988	0.82138
835.4624_10.83 neg	0.0020311	0.49325	0.82138
613.8898_0.74 pos	0.00042221	0.49373	0.86665
659.326_4.55 neg	0.037142	0.49456	0.85428
293.006_1.19 pos	9.75E-05	0.49567	0.97317
C17H24O11_a neg	2.31E-10	0.49687	0.90791
495.197_6.45 neg	0.0025658	0.49749	0.76649
527.1525_7.04 pos	0.040963	0.49786	0.89856
407.1311_3.64 neg	0.0015369	0.49791	0.87533

511.2851_9.8 pos	0.0015375	0.4995	0.97578
627.2098_5.86 pos	0.011548	0.4995	0.99249
Methoxybenzenediol C	0.011854	0.4995	0.99121
161.0587_4.04 pos	0.020711	0.4995	0.93191
537.1844_2.13 neg	0.011928	0.50039	0.76649
134.0383_3.16 pos	0.033753	0.50039	0.97578
726.4783_10.86 pos	0.0017231	0.50054	0.82138
600.2297_2.51 pos	2.50E-09	0.5017	0.99637
853.2621_1.37 pos	0.048085	0.50212	0.95219
651.2017_5.89 pos	0.0054612	0.50399	0.90065
386.136_6.09 pos	0.0028784	0.5054	0.82326
597.1782_4.53 neg	0.0033483	0.5054	0.86562
614.1802_7.94 pos	0.0044951	0.5054	0.82138
527.2854_6.58 pos	0.0065585	0.5054	0.76649
760.2401_0.97 pos	0.00016152	0.50567	0.97578
647.1658_4.49 neg	0.0015231	0.5059	0.84238
480.2072_6.88 neg	0.0034771	0.50759	0.86562
293.2126_10.74 neg	0.0054612	0.50759	0.82138
371.1477_4.98 pos	0.010496	0.50759	0.82326
356.0197_2.46 pos	0.011543	0.50759	0.99578
222.0778_3.19 neg	0.011704	0.50901	0.99578
409.0468_2.48 neg	0.00043939	0.50945	0.97317
683.2733_2.76 pos	0.0011775	0.51048	0.90065
851.2625_0.9 pos	0.01019	0.511	0.94168
801.35_6 neg	0.00086088	0.51207	0.82326
631.2448_5.43 neg	0.041006	0.51207	0.82326
395.066_1.06 pos	5.14E-06	0.51239	0.76649
337.1861_3.57 pos	0.00043263	0.51239	0.86466
349.1971_5.35 pos	0.022313	0.51448	0.90065
279.1024_4.2 pos	0.0046686	0.51516	0.76649
597.1956_6.14 neg	0.0018649	0.51543	0.85422
180.0654_3.19 neg	0.0077298	0.51543	0.97578
428.1128_6.67 neg	0.025045	0.51543	0.84969
383.3657_9.69 pos	0.033166	0.51543	0.98907
881.2336_0.93 pos	0.029734	0.51602	0.93791
C28H34O14 neg	3.24E-11	0.5165	0.88398
261.2196_9.8 pos	0.00086411	0.5165	0.90515
334.1413_3.5 pos	0.015435	0.5165	0.86562
533.2266_5.35 pos	0.016581	0.5165	0.9826
666.4329_10.98 pos	0.019801	0.5165	0.85331
383.0906_2.28 pos	0.021307	0.5165	0.99551
353.0923_1.15 pos	0.044805	0.51661	0.92539
169.0504_3.88 pos	0.0078271	0.51813	0.98907
385.1297_5.69 neg	1.03E-05	0.51868	0.90335
381.0772_0.89 pos	0.039151	0.51879	0.82326
226.0697_3.87 pos	0.0014263	0.52045	0.84586
547.19_8.09 pos	0.010496	0.52086	0.96268
506.3227_9.39 pos	0.045077	0.52156	0.97578

496.3365_10.18 pos	0.0032679	0.5216	0.98907
311.1276_4.68 pos	0.00010617	0.52227	0.95588
413.0507_2.29 pos	0.005919	0.52227	0.97578
166.9925_5.1 neg	0.030346	0.52227	0.97578
571.1755_4.62 pos	0.0076296	0.5225	0.97578
459.2486_10.44 pos	0.044752	0.52347	0.92962
269.0803_4.4 pos	0.042853	0.52371	0.97578
Phenylalanylvaline pos	0.0063328	0.52708	0.97578
597.1927_6.36 pos	0.043215	0.52726	0.97578
637.2198_6.16 pos	0.007028	0.52776	0.82326
799.2393_0.95 neg	0.0023627	0.52907	0.89058
675.397_11.31 neg	0.0046221	0.5295	0.84586
459.1115_3.71 neg	0.002571	0.5303	0.98933
443.1369_6.66 neg	0.041281	0.53217	0.82326
388.0966_4.39 neg	0.012729	0.53324	0.96473
518.2501_9.31 neg	0.029477	0.53404	0.84586
427.1373_5.09 pos	0.00046744	0.5362	0.90065
275.1999_10.29 neg	5.50E-05	0.53684	0.82138
277.2161_10.27 pos	8.84E-05	0.53684	0.80702
395.2008_4.48 pos	0.015931	0.5402	0.82326
719.1697_1.27 pos	0.0034846	0.54089	0.83892
Geniposide neg	1.61E-09	0.54277	0.98907
787.2831_5.43 neg	0.00012193	0.54376	0.93398
314.0925_3.42 pos	0.0042086	0.54376	0.99637
759.2504_4.39 pos	0.0077665	0.54376	0.84969
137.0596_4.32 pos	0.00045177	0.54476	0.83892
314.1546_10.6 neg	0.0040211	0.54499	0.80702
597.1974_5.84 neg	0.00033447	0.54738	0.79649
132.0829_4.59 pos	0.042857	0.54738	0.99243
70.9991_0.77 neg	0.00049547	0.54785	0.84969
281.0684_3.87 neg	0.0015782	0.54804	0.89392
219.1013_4.7 pos	0.0031664	0.54813	0.99865
159.1153_10.93 pos	0.015967	0.54855	0.9607
295.1147_3.42 neg	0.041114	0.55011	0.84586
845.2394_7.12 pos	0.010698	0.5503	0.92417
195.001_4.63 neg	0.038617	0.55085	0.92417
306.1173_2.35 neg	0.0023779	0.55282	0.99865
Linoleate neg	0.024039	0.55426	0.99226
317.2071_10.3 pos	0.00099518	0.5552	0.80702
521.2006_4.61 neg	0.0049222	0.5552	0.95531
476.9118_0.81 neg	0.0077665	0.5552	0.95102
311.2554_9.48 pos	0.011153	0.5552	0.94383
312.0744_2.33 neg	0.011189	0.5552	0.85422
679.1993_6.48 neg	0.012049	0.5552	0.98907
505.3441_10.81 pos	0.014066	0.5552	0.95216
231.0524_4.2 neg	0.047865	0.5552	0.82274
353.2658_10.36 pos	0.00065204	0.55522	0.82326
561.1983_4.15 neg	0.020378	0.55522	0.8895

437.2175_2.99 neg	0.048952	0.55522	0.97578
151.1484_4.46 pos	0.0080352	0.55592	0.84586
601.152_5.01 pos	0.011174	0.5565	0.88842
358.1428_4.32 pos	0.0041827	0.5579	0.99749
525.1628_3.92 neg	0.024174	0.55831	0.99749
263.1059_3.79 pos	0.007028	0.55959	0.89506
246.2054_2.27 pos	0.020748	0.56013	0.99637
331.2837_10.97 pos	0.021622	0.56251	0.97578
527.2828_9.83 neg	0.0011775	0.56465	0.94785
752.8599_0.74 pos	0.00059162	0.56476	0.86562
607.2_4.55 neg	0.039344	0.56476	0.99578
502.298_9.12 neg	0.039669	0.56476	0.92417
158.9771_0.76 neg	0.00013803	0.56495	0.82138
514.1901_3.81 pos	0.0032872	0.56528	0.97578
145.1024_9.19 pos	0.0051832	0.5656	0.95219
623.1961_4.38 neg	0.00017907	0.56641	0.85422
289.0147_1.2 neg	0.0010796	0.56641	0.94168
188.0699_4.36 pos	0.0031794	0.56641	0.98907
515.1579_4.87 pos	0.0063287	0.56641	0.98852
705.163_4.75 neg	0.027138	0.56641	0.82138
443.3098_10.78 pos	0.028265	0.56641	0.82138
Flavone base 3O, 2Me	0.044771	0.56641	0.97578
290.1498_3.53 pos	5.19E-05	0.57188	0.92539
538.2301_4.32 pos	0.00057009	0.57188	0.82326
359.1097_6.36 pos	0.0018238	0.57188	0.91711
342.1049_5.49 neg	0.015761	0.57275	0.82138
271.2034_4.42 pos	0.02573	0.57275	0.95219
700.2793_3.81 pos	0.027875	0.57275	0.89058
518.3193_10.37 pos	0.028752	0.57275	0.97578
130.0624_2.78 pos	0.0011263	0.57333	0.97317
639.2402_2.55 pos	4.15E-08	0.57374	0.95219
596.2131_2.59 pos	0.00011326	0.57374	0.98833
256.297_9.01 pos	1.58E-05	0.57756	0.82983
516.1971_3.69 pos	0.00014411	0.57756	0.92417
Homogentisate pos	0.0094316	0.57756	0.90065
553.204_5.67 neg	0.010496	0.57756	0.80702
203.1059_4.78 pos	0.037613	0.57756	0.84987
466.2943_9.87 neg	0.045223	0.57756	0.97578
167.0239_0.91 neg	0.048153	0.57803	0.97578
373.1276_4.62 neg	0.0023627	0.58167	0.97578
881.5187_10.68 pos	0.035921	0.5823	0.94168
D-Saccharic acid pos	0.0032646	0.5827	0.95
333.2003_8.15 pos	0.00058386	0.58366	0.82326
603.1956_6.07 pos	0.0029236	0.58371	0.99576
Flavanone base 4O, 1P	0.047497	0.58399	0.82138
580.2625_4.82 neg	0.020378	0.58444	0.99749
203.052_0.88 pos	0.00039131	0.58504	0.84969
474.266_9.08 neg	0.0055567	0.58569	0.92417

616.1762_7.96 pos	0.0073182	0.58847	0.82326
344.1021_2.21 neg	0.0021893	0.59065	0.97578
Feruloyl agmatine isom	5.19E-05	0.59085	0.94383
307.1754_2.76 pos	0.00020797	0.59085	0.95102
262.9261_0.8 neg	0.00030727	0.59085	0.9826
345.0967_7.12 pos	0.011415	0.59085	0.90515
726.2228_4.25 pos	0.018596	0.59085	0.94383
341.1138_1.3 neg	0.023928	0.59085	0.98866
248.1866_7.86 pos	0.0047098	0.59307	0.90065
549.1856_3.92 neg	0.0093844	0.59307	0.82326
371.1467_6.14 pos	0.031323	0.59307	0.92911
402.1145_4.82 pos	0.0015471	0.59591	0.86562
791.2344_4.12 pos	0.013424	0.5964	0.98907
570.0945_4.39 neg	0.00027769	0.59684	0.97578
213.111_4.11 pos	0.014619	0.59805	0.93626
205.0519_6.29 neg	0.01756	0.59883	0.92417
727.2431_7.64 neg	0.027253	0.59883	0.97578
722.4604_10.66 pos	0.045331	0.59938	0.82326
477.1379_4.65 neg	0.038394	0.60124	0.82138
728.436_10.77 pos	0.039078	0.60124	0.95216
314.1213_1.54 pos	0.01235	0.60188	0.94383
379.1642_5.29 neg	0.029594	0.60263	0.92911
133.0146_3.83 neg	0.00080403	0.603	0.93522
825.2809_4.9 neg	9.84E-05	0.60396	0.82326
515.1263_3.61 neg	1.05E-06	0.60487	0.99749
245.0003_0.87 pos	2.16E-05	0.60487	0.90065
853.4724_11.26 neg	0.003432	0.60487	0.91227
526.3113_9.87 neg	0.026469	0.60487	0.98569
459.1055_2.48 pos	6.53E-13	0.60537	0.82326
509.2757_5.6 neg	0.022168	0.60537	0.92417
751.1763_3.71 neg	0.0093681	0.60543	0.97578
503.1899_3.34 pos	0.00955	0.60553	0.97578
598.212_2.54 neg	2.85E-11	0.60834	0.97034
703.5626_10.93 pos	0.023334	0.60933	0.90065
764.3106_4.78 pos	0.04854	0.60963	0.97578
894.2627_1.03 neg	0.047773	0.6117	0.87322
376.1244_3.33 pos	0.034278	0.6119	0.94383
577.4847_10.93 pos	0.044771	0.61206	0.96684
241.1957_9.53 pos	9.84E-05	0.61269	0.76649
551.207_3.98 pos	0.0056755	0.61269	0.82138
138.0556_0.91 pos	0.00445	0.61361	0.92911
450.2663_9.52 neg	0.0036975	0.61494	0.90791
Flavone base 3O, C-Pe	0.033702	0.61494	0.9826
605.1734_2.45 neg	4.97E-06	0.6157	0.97578
353.9024_0.79 pos	0.012985	0.61622	0.86065
452.3642_10.57 pos	0.027787	0.61649	0.95531
Harmalol pos	0.0028619	0.61816	0.9416
679.4326_10.91 pos	0.010054	0.61937	0.98432

289.0832_4.64 neg	0.00012973	0.61986	0.94383
Chalcone base 3O, 1M	0.00046135	0.61986	0.98907
545.2863_6.24 pos	0.00066411	0.61986	0.90065
LPC 160 neg	0.0047699	0.61986	0.97578
80.0498_10.89 pos	0.32152	0.61986	0.017179
312.3603_10.54 pos	0.0010526	0.62214	0.86058
Glutamyl-S-allylcysteir	0.0033161	0.62214	0.97782
337.2739_9.67 pos	0.023885	0.62214	0.97578
670.2141_3.65 pos	0.0001832	0.62251	0.98432
208.0753_6.55 pos	0.018635	0.62251	0.94134
663.424_10.88 pos	0.029858	0.62251	0.82138
897.1968_0.9 pos	0.0032501	0.62323	0.89438
551.1766_4.47 neg	0.023334	0.62324	0.92911
339.125_4.7 neg	2.42E-06	0.6233	0.90238
213.1775_8.24 pos	0.0029455	0.62355	0.94383
657.3027_6.31 neg	0.010496	0.62444	0.84586
713.2135_4.17 neg	0.032144	0.62444	0.97578
259.2071_9.58 pos	2.35E-05	0.62466	0.82138
Benzoic acid 2O, O-He	0.0026028	0.62516	0.97782
613.2463_4.83 neg	0.012399	0.62548	0.9826
263.1353_3.19 neg	0.019644	0.62548	0.98933
Etoposide pos	0.0070336	0.62568	0.82326
724.8667_0.74 pos	0.0010224	0.62631	0.82326
487.1754_0.96 neg	0.029979	0.62631	0.97317
524.1473_0.84 pos	0.049174	0.62631	0.95216
248.1149_3.19 neg	0.022869	0.62713	0.99745
831.282_6.47 pos	9.88E-05	0.62859	0.91882
209.1178_9.71 neg	0.0053341	0.6296	0.82138
Apodanthoside Not val	0.021734	0.63002	0.88842
583.2155_5.95 pos	0.0010765	0.63015	0.95
357.123_2.31 neg	0.004629	0.63015	0.99749
677.6964_0.88 pos	0.0088587	0.63015	0.94383
C18H26O2 neg	2.59E-05	0.63089	0.97578
565.2037_5.89 pos	0.00023453	0.63089	0.9278
235.0977_3.82 pos	0.049051	0.63089	0.89392
504.3131_9.76 neg	0.021307	0.63151	0.99442
DGMG 183_b neg	0.031045	0.6317	0.94168
823.2743_6.15 neg	0.022747	0.63179	0.97782
239.2362_10.96 pos	0.043215	0.63263	0.9454
650.4392_11.74 pos	0.011971	0.63363	0.98395
4-Hydoxyphenylacetate	0.00027769	0.63371	0.82326
LPE 160 pos	0.012261	0.63371	0.92417
LPE 181 neg	0.0041468	0.63419	0.97782
857.2311_1.41 neg	0.021191	0.63453	0.97578
340.1261_5.68 pos	0.0012804	0.63565	0.86665
323.1259_5.04 pos	0.0020013	0.63659	0.97969
833.2942_5.81 pos	0.0025202	0.63659	0.92417
170.0585_4.61 pos	0.0037768	0.63659	0.99744

807.285_6.26 neg	4.85E-05	0.63667	0.92911
547.1944_7.06 pos	0.00093241	0.63667	0.90065
349.0223_2.31 neg	8.59E-05	0.64115	0.80702
439.1031_4.79 neg	1.13E-08	0.64176	0.95
691.4268_11.55 neg	0.013876	0.64176	0.99513
729.8685_0.75 neg	0.029979	0.64176	0.85946
760.2391_1.31 pos	0.044752	0.64236	0.88398
455.2126_6.64 neg	0.0098754	0.64244	0.98907
565.1654_2.51 neg	3.24E-11	0.64292	0.90065
125.0973_5.09 neg	0.024907	0.6431	0.97578
146.0602_2.77 pos	0.0073899	0.64333	0.98539
759.2365_0.93 pos	5.79E-05	0.64405	0.87322
322.2108_2.64 pos	0.017285	0.64405	0.96644
497.2299_6.51 neg	0.044804	0.64405	0.88398
Flavone base 3O, C-He	0.045596	0.64427	0.94383
737.4833_10.42 pos	0.012532	0.6446	0.82138
Vanillin acetate neg	2.21E-05	0.64478	0.90065
587.2068_4.46 pos	0.01103	0.64478	0.89506
277.2157_11.2 neg	0.024039	0.64548	0.99283
521.1627_5.11 pos	0.01431	0.64585	0.95219
243.209_9.85 pos	0.0016439	0.64623	0.87533
478.3253_10.38 pos	0.0044239	0.64623	0.99637
DIBOA O-Hex neg	0.029979	0.64623	0.99637
581.2023_6.26 neg	0.00018432	0.64866	0.85946
244.9648_0.78 neg	8.84E-05	0.64902	0.83198
807.4747_11.27 neg	0.004751	0.64985	0.94383
739.2279_3.23 neg	0.018027	0.64986	0.98395
590.1924_4.31 pos	0.010496	0.6506	0.9782
804.5322_10.83 pos	0.045514	0.6506	0.92417
580.8583_0.75 pos	0.0057012	0.65086	0.82326
485.379_11.5 pos	0.00087618	0.65098	0.82138
346.1029_0.95 pos	0.0015294	0.65151	0.97578
519.2783_4.8 pos	0.01294	0.65151	0.82326
641.2269_6.26 pos	0.032144	0.65151	0.97578
255.0732_0.9 pos	0.00011753	0.65474	0.82138
205.0856_5.03 pos	0.0039111	0.65474	0.84586
175.0748_5.04 pos	0.002257	0.65527	0.99494
335.0906_7.6 pos	0.00032219	0.65726	0.79649
D-Tryptophan pos	0.0014263	0.65726	0.86821
369.1322_5.61 pos	5.19E-05	0.65906	0.99413
74.0275_3.1 neg	0.0035647	0.65906	0.97578
3,5-Dimethoxycinnami	0.049096	0.65949	0.82138
138.9716_0.74 pos	0.00027769	0.66005	0.82326
589.2026_8.14 pos	0.0012249	0.66005	0.98395
Coumarin 1O 1MeO,	0.00064705	0.66022	0.99637
241.2194_9.44 neg	0.013173	0.66022	0.97578
685.1758_4.24 neg	0.013232	0.66022	0.80702
490.3572_10.61 pos	0.048087	0.66185	0.82326

523.1722_4.21 pos	0.00010246	0.66188	0.98119
282.276_10.36 pos	0.023795	0.66329	0.97578
653.2072_4.36 neg	0.0007533	0.66382	0.84969
547.2321_4.49 neg	0.0037248	0.66447	0.82274
474.2198_1.01 pos	0.012103	0.66447	0.98933
Flavone base 3O, O-H(	0.010286	0.66508	0.97578
601.3116_10.23 pos	0.007943	0.66609	0.94383
351.2155_4.44 neg	0.010496	0.66609	0.95216
Silychrystin pos	0.00012614	0.66686	0.84969
142.0663_3.11 neg	0.0042192	0.66686	0.95219
116.0527_3.1 neg	0.0085504	0.66686	0.9826
659.3641_8.94 pos	0.023024	0.66806	0.90065
261.2243_10.96 pos	9.84E-05	0.6696	0.94134
407.2382_8.92 pos	0.0018218	0.66978	0.82138
831.2552_0.87 pos	0.003347	0.66978	0.97578
247.1038_3.19 neg	0.02093	0.66978	0.99774
572.43_9.93 pos	0.046507	0.67131	0.95236
495.3898_10.92 pos	0.041511	0.67572	0.97578
432.1712_0.95 pos	0.00041107	0.67715	0.95
556.1516_6.83 pos	0.0048028	0.67772	0.84586
619.3207_9.57 neg	0.0011202	0.67775	0.76649
729.2408_5.2 neg	0.013672	0.67775	0.85422
327.2084_8.39 neg	0.032969	0.67775	0.76649
170.0602_2.78 pos	9.84E-05	0.67789	0.97578
849.7695_0.93 pos	0.0025719	0.67789	0.97578
Hesperetin-7-O-rutinos	0.0076228	0.67789	0.85331
624.2257_4.79 pos	0.00038271	0.67803	0.97578
434.2634_9.47 pos	0.0060165	0.67873	0.90065
857.2635_7.43 pos	0.017182	0.67922	0.95531
Isohernandezine neg	0.00019332	0.67952	0.79649
534.2922_10.37 pos	0.023955	0.68004	0.95219
551.1922_7.39 neg	0.0013379	0.68191	0.9826
381.1207_5.87 pos	0.0041189	0.68356	0.90274
435.1042_6.56 neg	0.0056755	0.68356	0.86821
575.1867_7.48 pos	0.0024227	0.68394	0.98395
331.0663_0.87 neg	0.00010047	0.68413	0.90065
745.2466_6.69 neg	0.0041183	0.68413	0.82274
589.1862_4.66 pos	0.00023293	0.68486	0.97578
295.1053_2.4 neg	0.014302	0.68486	0.84586
539.178_4.24 neg	8.23E-05	0.68877	0.97578
707.187_4.28 neg	0.00053838	0.68877	0.94383
332.0974_3.99 pos	0.030106	0.68877	0.97578
260.1879_8.26 pos	0.0039244	0.68947	0.92417
663.1551_4.38 neg	0.002119	0.69013	0.95216
365.1351_4.66 pos	0.0016147	0.6911	0.91343
151.0396_3.85 pos	0.026401	0.69251	0.84969
241.1534_2.05 pos	0.0024226	0.69261	0.88635
S420P318/F12 neg	0.0074275	0.6931	0.82138

520.2667_10.39 neg	0.031491	0.6931	0.9782
537.302_9.8 pos	0.03538	0.6931	0.98845
452.1742_2.51 pos	1.23E-11	0.69378	0.82326
576.1827_3.87 neg	0.00015373	0.69378	0.97578
741.287_3.1 neg	0.012027	0.69378	0.84969
641.4399_10.36 pos	0.011971	0.69462	0.95773
480.4173_9.84 pos	0.014066	0.69462	0.86644
378.0237_3.74 pos	0.027787	0.69462	0.84586
Benzyl alcohol Hex-H(	0.028359	0.69462	0.76649
498.232_6.47 neg	0.032499	0.69462	0.95102
417.1514_5.15 pos	0.043991	0.69462	0.97258
328.1897_10.66 neg	0.02573	0.69487	0.82326
126.9728_0.73 pos	0.00020797	0.69492	0.80702
186.0544_3.09 neg	0.0057993	0.69589	0.97578
Glutamyl-S-methylcyst	0.00093652	0.69612	0.84586
Tryptamine neg	0.0055722	0.69622	0.97317
335.2162_5.31 neg	0.0087655	0.69894	0.85422
503.1886_4.32 pos	0.0065585	0.69903	0.95219
421.1146_3.12 neg	0.00015235	0.70167	0.82326
585.2867_6.95 pos	0.0038275	0.70237	0.84586
290.1494_0.95 pos	0.00033293	0.70272	0.97578
722.4171_8.59 pos	0.00052628	0.7051	0.82326
LPC 182 neg	0.015621	0.7051	0.97317
536.4139_8.24 pos	0.0038628	0.70625	0.95296
475.3036_10.7 pos	0.00036928	0.70707	0.9285
238.0341_1.04 pos	0.015404	0.70707	0.96871
370.1382_5.56 pos	0.0016141	0.70824	0.83198
175.1478_4.46 pos	0.0095533	0.71047	0.82326
540.3047_9.27 pos	0.0044784	0.71081	0.76649
162.0304_3.11 pos	0.0071224	0.71213	0.98847
441.1131_3.61 pos	0.0035647	0.71217	0.97317
479.1998_6.95 neg	7.68E-06	0.71276	0.81223
210.9917_3.08 neg	5.93E-05	0.71293	0.90065
583.215_6.44 pos	7.52E-05	0.71326	0.84969
619.1754_6.6 pos	0.021885	0.71385	0.82326
387.0911_4.3 neg	0.038137	0.71431	0.97409
237.0613_1 neg	0.0012451	0.71545	0.88398
825.2811_4.18 neg	0.0015271	0.71575	0.82138
230.0992_2.31 pos	0.036584	0.71575	0.95
158.0847_2.72 neg	0.002571	0.71689	0.98866
L-Methionine pos	0.031086	0.71689	0.90791
189.0732_2.74 pos	0.00032098	0.71797	0.92417
765.2334_7.28 pos	0.00044732	0.71797	0.96684
431.2179_10.84 neg	1.08E-05	0.71798	0.97578
509.166_4.29 neg	0.0034981	0.71798	0.97578
Thiourea neg	0.0044784	0.71798	0.97317
211.1722_4.46 pos	0.00726	0.71798	0.82138
162.9826_5.46 neg	0.014069	0.71798	0.92608

426.9172_0.76 neg	0.015761	0.71868	0.90065
Ginkgolide B pos	0.026469	0.7193	0.84969
Flavone base 2O, 2Me	0.039876	0.71935	0.98395
DGMG 183_a neg	0.044771	0.71935	0.99587
797.2373_3.69 neg	0.0011331	0.72059	0.9782
144.08_3.05 pos	0.0030835	0.72085	0.97578
301.2156_5.02 pos	0.029014	0.72129	0.82138
357.1319_5.9 pos	0.00022735	0.72192	0.82326
248.1301_3.54 pos	0.00038809	0.72192	0.90065
779.2924_5.58 neg	0.014302	0.72192	0.82326
729.2573_7.11 neg	0.019412	0.723	0.97578
629.1812_4.66 pos	0.048078	0.72345	0.88767
401.1127_4.81 neg	0.00445	0.72438	0.82326
743.2985_3.1 pos	0.0056755	0.72446	0.90065
187.1471_10.27 pos	0.00039946	0.72553	0.82326
750.2311_0.88 pos	8.69E-05	0.72818	0.86821
744.2367_3.8 neg	0.015133	0.72818	0.92911
C17H28O16 neg	0.0006793	0.72863	0.92417
187.4961_3.06 pos	0.0018218	0.7288	0.98907
719.1788_1.01 pos	5.79E-05	0.72935	0.76649
641.1726_4.32 neg	0.01603	0.73153	0.79649
341.1041_5.59 neg	0.0069523	0.73189	0.88398
763.2564_5.79 neg	0.024165	0.73189	0.90065
614.8892_0.74 neg	0.0093681	0.73208	0.80702
477.1091_4.35 pos	0.0042216	0.73233	0.94383
179.1412_10.3 pos	0.013232	0.73502	0.84963
518.166_3.59 pos	0.0030405	0.73538	0.91371
581.2009_6.37 pos	1.82E-05	0.73642	0.82138
421.1331_2.33 neg	0.00016493	0.73642	0.89669
245.1142_2.49 neg	0.0034771	0.73642	0.95
259.2051_8.92 pos	0.014786	0.73642	0.94755
135.1169_10.27 pos	5.19E-05	0.73674	0.76649
461.1281_3.06 neg	0.0035394	0.73674	0.95048
495.1488_3.83 neg	0.0062565	0.7375	0.97578
718.4628_10.55 neg	0.020222	0.73757	0.97578
515.6444_1.57 pos	0.024954	0.73757	0.94755
377.1364_4.71 pos	0.0023052	0.73809	0.97578
845.2454_6.6 pos	0.0010526	0.73821	0.95048
377.1414_11.2 neg	0.0020642	0.74028	0.95219
739.3396_5.97 neg	0.0036472	0.74028	0.84586
335.1264_4.66 pos	0.00022735	0.74051	0.99745
191.0697_4.01 pos	0.00027493	0.74051	0.79649
623.4203_10.85 pos	0.012399	0.74054	0.82326
248.1289_0.93 pos	0.01881	0.74202	0.99578
626.2394_4.7 pos	0.0020981	0.74218	0.99749
193.1581_4.46 pos	0.014135	0.74218	0.80702
424.0657_3.92 neg	0.016575	0.74218	0.88398
487.1251_4.25 pos	0.021649	0.74218	0.95661

545.1968_3.12 neg	0.026772	0.74218	0.97578
353.2687_9.16 pos	0.028603	0.74218	0.9826
520.1994_3.84 pos	0.038538	0.74218	0.97578
233.0818_4.32 pos	0.04367	0.74218	0.82326
651.2045_6.91 neg	0.045514	0.74455	0.95216
517.2626_4.38 neg	0.0071025	0.74653	0.97578
335.257_9.04 pos	0.017521	0.74667	0.97578
490.1746_0.96 pos	0.0017674	0.74769	0.84969
C25H26O5 neg	0.00393	0.74769	0.92417
866.845_0.74 pos	0.019303	0.74769	0.94383
240.9513_0.74 pos	0.020506	0.74769	0.90065
577.1697_3.27 neg	6.07E-07	0.74927	0.94383
479.1535_3.66 neg	0.0016722	0.74927	0.84586
497.3116_8.94 pos	0.017227	0.75033	0.82326
152.9907_10.59 neg	2.11E-06	0.75303	0.82138
557.1652_6.7 pos	0.011413	0.75477	0.82326
Isoflavone base 1O, 2M	0.00084385	0.75484	0.82274
354.9258_0.74 pos	0.0050035	0.75484	0.90065
556.1492_7.12 pos	0.022747	0.75484	0.90515
636.3869_10.84 pos	0.036203	0.75513	0.90065
317.1054_4.11 neg	2.82E-05	0.75581	0.97578
575.3168_9.41 pos	0.0056003	0.75581	0.84969
239.0558_3.87 neg	0.0095494	0.75586	0.9416
455.1092_4.58 neg	0.0092023	0.75598	0.92417
467.0984_7.2 neg	0.041006	0.75598	0.86665
874.745_7.12 pos	0.048171	0.75598	0.94383
537.2623_6.72 neg	0.0074275	0.75751	0.97578
753.2487_7.19 pos	0.03329	0.75751	0.98432
645.8742_0.74 pos	0.00013573	0.75808	0.86562
Tricaffeoyl quinic acid	0.027353	0.75926	0.76649
577.2795_6.44 neg	0.00088739	0.75983	0.82326
369.1343_5.73 neg	0.00058572	0.75993	0.92417
635.3174_8.01 neg	5.72E-05	0.75994	0.97317
405.1392_2.39 neg	0.00011054	0.75994	0.82326
503.1884_4.81 pos	8.50E-06	0.76025	0.98281
691.1857_4.22 pos	0.031715	0.76025	0.92911
619.1857_2.62 pos	9.23E-07	0.7603	0.82326
Myricetin-3-O-xyloside	0.00023733	0.7603	0.95
327.124_4.26 neg	0.017355	0.7603	0.82138
74.0262_2.83 neg	0.018543	0.7603	0.99637
366.1127_0.96 pos	0.044617	0.7603	0.89392
885.2574_7.6 neg	0.045004	0.7603	0.99578
467.3713_11.95 pos	0.014302	0.76128	0.82326
336.9231_0.74 pos	0.0013347	0.76175	0.84586
705.2144_6.73 pos	0.0035647	0.76186	0.90065
519.18_3.17 neg	0.019187	0.76186	0.95661
351.011_1.16 pos	0.011312	0.76302	0.82138
473.0983_2.51 pos	1.23E-08	0.76486	0.97578

532.05_2.54 neg	5.06E-06	0.76521	0.84969
149.0459_2.35 neg	4.15E-08	0.76548	0.86562
C11H10N2O2 neg	0.00042528	0.76808	0.96156
837.4876_11.01 neg	0.01736	0.76914	0.82326
319.2271_5.3 pos	0.046573	0.76991	0.97578
172.9758_1.09 pos	0.0041579	0.77004	0.82138
705.2126_7.34 pos	0.038617	0.7766	0.84969
198.9603_0.76 neg	2.00E-05	0.77685	0.82274
723.2507_4.89 neg	0.00013787	0.77715	0.80702
391.0963_3.2 neg	0.000115	0.77982	0.92962
843.2898_3.74 neg	0.00044732	0.78089	0.90791
791.2307_3.68 pos	0.024685	0.78089	0.9782
Licoagroside B not vali	0.0014009	0.7825	0.99151
466.0644_3.45 neg	0.00081832	0.78403	0.98432
571.1786_5.03 pos	0.022747	0.78403	0.97578
559.2746_4.47 neg	0.00021285	0.78484	0.99774
209.0808_4 pos	0.0030287	0.78605	0.90065
858.5041_10.23 pos	0.021622	0.78605	0.91325
809.3026_6.18 neg	0.00068536	0.78653	0.86065
557.2401_4.8 pos	0.001602	0.78653	0.95531
745.2422_7.83 neg	0.0034423	0.78653	0.97578
439.1395_0.89 pos	0.0052277	0.78706	0.97317
706.215_7.3 pos	0.027109	0.78706	0.86174
Phenyl-butyryl-glutami	0.027831	0.78863	0.98907
C34H40N4O8 neg	6.50E-06	0.78883	0.76649
159.0927_4.64 neg	0.0031822	0.78905	0.96373
323.1266_4.29 pos	0.00024296	0.7894	0.82326
S-5'-Adenosyl-L-Homo	0.0019667	0.78945	0.90065
332.1281_10.64 pos	7.84E-05	0.7896	0.97578
561.34_10.74 pos	0.0039607	0.79092	0.77718
337.274_10.75 pos	0.010006	0.79092	0.97409
625.343_5.58 neg	0.0045266	0.79135	0.94168
614.2276_2.64 pos	2.04E-10	0.79278	0.76649
425.3364_11.72 pos	0.027145	0.79278	0.90335
339.1988_10.89 neg	0.036061	0.79278	0.97409
433.1487_4.24 pos	0.01786	0.79387	0.99637
-Chelidonine pos	0.01827	0.79483	0.90065
188.0712_2.8 pos	0.0027878	0.79564	0.96086
399.2352_7.55 pos	0.0071025	0.79692	0.82326
605.191_5.08 neg	0.023807	0.79701	0.9826
643.3123_4.69 pos	0.0065585	0.7972	0.82138
Glutamyltyrosine neg	0.045735	0.7972	0.97174
423.2755_10.46 pos	0.0020591	0.7973	0.82326
383.2412_6.96 pos	0.00393	0.7977	0.82326
483.365_11.65 pos	0.017427	0.79883	0.77718
551.1898_6.12 neg	0.00049547	0.80155	0.98866
603.3297_10.8 pos	0.00058386	0.80224	0.79649
334.1383_3.76 pos	4.45E-05	0.80445	0.98395

297.0364_3.61 neg	7.87E-06	0.80507	0.95102
567.2782_6.14 pos	0.045004	0.80507	0.90065
578.194_3.81 pos	0.0002292	0.80519	0.95531
465.1998_5.26 neg	0.02176	0.80519	0.90065
409.1848_3.05 pos	2.82E-05	0.8054	0.89392
519.1852_3.79 neg	0.010602	0.8054	0.90754
Flavonol base 4O, O-H	0.013195	0.80578	0.98128
373.2223_4.47 pos	0.0071213	0.80907	0.82138
631.2862_8.22 neg	3.38E-05	0.80915	0.79649
727.241_3.84 neg	0.0012508	0.80915	0.90065
315.1914_9.78 pos	0.00027279	0.80974	0.82326
229.1414_10.83 pos	0.036317	0.81355	0.97578
596.2191_3.24 pos	7.43E-07	0.81372	0.97578
Flavone base 3O, 2Me	0.0044402	0.81388	0.97578
293.0887_2.55 neg	1.16E-10	0.81395	0.76649
607.2031_5.57 neg	0.029075	0.81521	0.86562
206.1036_2.89 pos	0.045496	0.81581	0.99749
730.8671_0.74 neg	0.0056755	0.81671	0.82138
135.1154_9.5 pos	0.00033047	0.81721	0.94383
1-Methylsulfinylbuteny	6.79E-08	0.81786	0.89392
173.1313_10.95 pos	0.007028	0.81786	0.97578
217.1599_10.9 pos	0.016884	0.81786	0.97578
7-Methylsulfenylheptyl	0.0070336	0.8182	0.97578
586.4508_10.44 pos	0.040313	0.81911	0.95216
462.1812_0.97 pos	0.029035	0.81977	0.85422
502.3303_9.92 pos	0.016581	0.82019	0.89263
200.0718_4.57 pos	0.00375	0.82021	0.90791
637.2732_5.06 pos	0.019003	0.82272	0.97034
882.2338_6.73 neg	0.029734	0.82272	0.94789
305.1603_3.57 neg	0.00040487	0.82299	0.94383
163.1502_10.95 pos	0.0076311	0.82309	0.97578
804.2542_6.58 pos	0.040628	0.82309	0.95102
728.8788_0.74 neg	0.0087752	0.82373	0.86562
Quercetin-3,4'-O-di-bet	2.11E-06	0.82378	0.84969
266.163_0.89 pos	0.0082309	0.82378	0.90065
Harmalol neg	0.0014039	0.82483	0.97578
775.2758_4.7 pos	0.0014536	0.82625	0.95661
377.317_10.91 pos	0.01235	0.82668	0.98907
Diferuloyl glycerol pos	0.036359	0.82717	0.98609
463.1463_2.61 neg	0.00011737	0.8275	0.69172
643.4532_11.69 pos	0.013916	0.8275	0.82138
316.2158_3.18 pos	0.023614	0.8275	0.95216
462.1068_6.73 pos	0.036736	0.82798	0.88398
255.0772_4.65 pos	0.033277	0.82806	0.99749
577.2648_10.56 neg	0.00071378	0.82991	0.92216
842.8569_0.74 neg	0.016532	0.82991	0.82326
429.1531_2.65 neg	0.023041	0.82991	0.94383
3,5-Dimethoxycinnami	0.033651	0.82991	0.99578

175.0399_6.43 pos	0.017835	0.83214	0.98907
7-Hydroxymitragynine	0.0019802	0.8322	0.82326
247.0796_6.65 pos	0.010617	0.8322	0.90274
416.1459_6.13 pos	0.0070105	0.83323	0.97578
555.2208_5.71 neg	1.61E-05	0.83492	0.90065
533.1705_0.96 neg	0.023108	0.83511	0.94466
188.0696_3.06 pos	0.0011572	0.83616	0.9782
481.1769_5.99 neg	0.0015771	0.83755	0.76649
253.0934_9.85 neg	0.0072496	0.83755	0.90065
272.9551_0.76 neg	0.0001665	0.83949	0.82138
108.9628_0.74 pos	0.00072799	0.83986	0.82138
184.0718_10.93 pos	0.013344	0.83986	0.98933
477.2233_5.51 neg	0.046736	0.83986	0.97578
577.3343_10.2 pos	0.020117	0.8404	0.80702
488.0612_2.52 pos	6.93E-12	0.8406	0.94383
LPE 181 pos	0.00059882	0.8406	0.85601
527.1561_0.9 pos	0.0068718	0.8406	0.97578
349.1206_6.44 neg	0.006444	0.84101	0.84586
508.1511_3 neg	0.00047435	0.84147	0.69288
795.4412_7.86 neg	0.0014898	0.84276	0.82138
631.2839_7.46 neg	2.52E-05	0.84445	0.82876
555.1921_4.87 neg	0.03538	0.84461	0.82138
633.3964_10.66 pos	0.010633	0.84754	0.92417
559.1627_3.83 neg	6.52E-05	0.84863	0.80702
289.09_7.1 pos	0.035379	0.84875	0.97578
D-Tryptophan neg	0.0012432	0.84933	0.98907
473.3289_11.73 neg	0.003432	0.84933	0.84969
857.221_0.94 neg	0.034117	0.84954	0.97612
Flavonol base 4O, 1M	8.76E-06	0.84971	0.99637
142.0629_3.07 pos	0.0010928	0.84979	0.97853
159.0905_2.79 pos	0.0022605	0.84979	0.93821
FA 1831O_a neg	0.0023779	0.84979	0.84969
152.0118_2.67 neg	0.0020013	0.85008	0.92495
422.1622_2.33 pos	6.73E-12	0.85109	0.82138
Isoflavanone base 3O,	0.013772	0.85109	0.97578
116.0542_2.83 neg	0.02198	0.85118	0.99637
475.2188_4.83 neg	0.038801	0.8514	0.91012
499.1222_3.82 pos	0.023108	0.85255	0.96644
679.2133_0.85 pos	0.037593	0.85255	0.95219
706.2354_3.57 pos	0.039339	0.85255	0.92608
627.3014_6.61 neg	0.0016805	0.8532	0.94383
751.2327_7.61 pos	0.00084022	0.85329	0.98907
457.1695_4.45 neg	0.0024187	0.85329	0.9826
375.1297_2.31 neg	0.0028289	0.85329	0.97578
385.1293_6.18 pos	0.010714	0.85329	0.88635
L-Tryptophan neg	0.0014397	0.85396	0.96156
443.0872_2.34 pos	0.00078696	0.85516	0.94755
595.1833_6.05 neg	0.040628	0.85527	0.95773

476.2716_10.33 pos	0.00085064	0.85552	0.90065
743.2578_5.33 neg	0.0019409	0.85552	0.94168
659.4006_11.51 neg	0.00025564	0.85784	0.82138
581.2018_5.92 neg	5.47E-05	0.85803	0.88842
505.3507_7.47 pos	3.92E-05	0.86033	0.80702
453.2398_6.75 pos	0.00059206	0.86048	0.99637
777.2719_6.57 neg	0.00093687	0.86048	0.92417
193.1581_4.81 pos	0.0031391	0.86048	0.82138
307.0946_5.57 pos	0.034877	0.8611	0.92417
579.2133_5.16 pos	0.0058674	0.86116	0.84969
517.2625_4.83 neg	0.0003819	0.862	0.82138
160.076_3.06 pos	0.0011569	0.862	0.99576
856.5228_10.93 pos	0.00043263	0.86229	0.82326
Sinapic acid_b pos	0.015757	0.86306	0.97782
541.2612_4.8 pos	0.00019761	0.86315	0.85694
511.194_6.01 neg	0.0054612	0.86315	0.80222
C14H21NO8 neg	5.19E-05	0.86394	0.98395
4-Deoxyphloridzin pos	0.0045681	0.86394	0.97578
C12H22O9 neg	0.0067518	0.86394	0.92417
423.33_9.98 pos	0.028572	0.86394	0.95223
402.3592_9.79 pos	0.045172	0.86394	0.99578
599.1442_4.3 neg	7.34E-05	0.86419	0.82326
499.36_11.72 neg	0.0016607	0.86462	0.82138
404.143_5.89 pos	8.84E-05	0.86513	0.82138
400.9863_2.57 neg	0.00072834	0.86513	0.90065
602.1708_4.45 pos	0.037503	0.86619	0.87322
441.0974_0.88 pos	0.00074895	0.86643	0.95102
411.1944_3.73 pos	0.02024	0.86657	0.76649
862.5425_10.75 pos	0.0077955	0.86671	0.76649
651.2411_2.7 neg	0.013066	0.8673	0.8592
289.0366_3.58 neg	0.0022495	0.86775	0.97317
617.1356_3.25 pos	2.37E-05	0.8691	0.84586
623.2105_5.07 pos	0.010979	0.8691	0.92417
434.1181_0.92 pos	0.0015061	0.86917	0.78816
139.0016_1.52 pos	0.043841	0.86964	0.86562
389.2284_9.08 pos	0.00027644	0.87017	0.82326
322.1188_4.66 pos	0.0014397	0.87017	0.95216
867.2384_1.34 pos	0.03303	0.87017	0.98166
579.1857_6.11 neg	0.018027	0.87119	0.94755
205.0977_2.76 pos	0.00023564	0.87312	0.98852
Indole-3-acetyl-L-gluta	3.11E-05	0.87381	0.82138
725.2252_8.51 neg	0.0010505	0.87381	0.99413
Phloretin C-Hex, C-He	0.03734	0.87424	0.99892
C17H24O11_b neg	0.016695	0.8743	0.97578
500.2697_9.73 pos	0.018027	0.87473	0.98281
727.2017_3.75 pos	0.00058608	0.87557	0.87533
382.9256_0.74 pos	0.00058572	0.8758	0.91095
N-Acetyl-D-Tryptophai	0.00032616	0.87584	0.97578

467.3692_11.5 pos	0.0053641	0.87591	0.82138
520.3399_9.92 pos	0.043604	0.87665	0.89337
389.1548_3.74 pos	0.042959	0.87677	0.98907
757.2423_2.64 neg	1.19E-06	0.87721	0.82138
118.0649_2.78 pos	0.0018296	0.87797	0.9782
543.1307_1.29 pos	0.010196	0.87797	0.98907
669.1978_5.57 pos	0.010652	0.87871	0.98395
731.425_10.6 pos	0.011667	0.87965	0.82138
601.2283_4.79 neg	0.0076314	0.88012	0.97317
264.0786_1.14 pos	1.41E-05	0.88013	0.99576
745.2729_5.32 neg	1.87E-06	0.88218	0.82138
324.0983_4.63 neg	0.0016285	0.88218	0.84481
307.0963_6.82 neg	0.0015369	0.88265	0.76649
499.2662_6.77 neg	0.012085	0.88375	0.97335
844.2348_6.63 pos	0.030681	0.88417	0.90065
731.2611_3.05 pos	0.0023225	0.88456	0.8024
551.187_5.91 pos	0.0001138	0.88512	0.84969
450.9062_0.74 pos	0.0049378	0.88512	0.84586
775.44_10.25 neg	0.014019	0.88579	0.98907
Hirsutine pos	0.00049547	0.88728	0.82402
829.2642_6.73 pos	0.00021723	0.88767	0.90515
295.082_3.86 pos	0.00043263	0.88812	0.94383
82.0149_0.73 pos	0.00043263	0.88868	0.94383
735.2332_7.35 pos	0.011593	0.89129	0.86821
479.1559_5.48 pos	0.01756	0.89148	0.95661
557.1608_6.13 pos	3.63E-05	0.89177	0.97578
588.1812_1.38 pos	0.011811	0.89278	0.97578
176.0601_7.43 pos	0.045552	0.89435	0.97782
383.1082_5.22 neg	2.73E-05	0.89446	0.9454
579.2604_2.79 neg	0.00075846	0.89446	0.92495
146.0603_3.11 pos	0.0021027	0.89467	0.99578
753.2343_6.78 pos	0.00297	0.89487	0.996
299.2583_9.4 pos	0.044939	0.89487	0.94383
133.1007_6.64 pos	0.002257	0.89583	0.76649
545.3843_10.89 pos	0.029858	0.89583	0.97578
775.2564_6.95 neg	0.009773	0.89663	0.94785
576.1834_3.39 neg	0.00021723	0.89706	0.97317
382.1154_5.79 pos	0.0075786	0.89718	0.93522
259.132_3.11 neg	0.010496	0.89718	0.98907
Aconitic acid not valida	0.010196	0.89754	0.95434
534.3176_7.8 pos	0.0006456	0.89767	0.95
137.0598_4.66 pos	0.0050107	0.89767	0.94785
278.2192_10.03 neg	0.028457	0.89774	0.94383
185.0704_4.55 pos	0.000474	0.89783	0.90791
428.9185_0.75 neg	0.00052308	0.89783	0.86562
574.1069_4.67 pos	0.0084702	0.89783	0.97578
741.2485_8.74 neg	0.0011128	0.89915	0.95661
553.2786_10 pos	0.0037549	0.89915	0.9988

273.2227_5.28 pos	0.045223	0.90055	0.9826
399.2372_5.94 pos	0.0097625	0.90073	0.84586
749.2181_8.47 pos	0.018331	0.9019	0.97317
170.0605_3.06 pos	0.0019642	0.9026	0.99774
409.0917_6.74 pos	0.036188	0.9026	0.97317
371.1131_8.47 pos	0.0044402	0.90274	0.9826
747.2656_6.39 neg	0.013173	0.90274	0.83892
727.2739_10.45 pos	0.0011263	0.90368	0.82326
255.0887_3.32 neg	0.010496	0.90368	0.82138
585.2338_5.65 neg	0.0017074	0.90524	0.84969
142.0629_2.78 pos	0.0052021	0.90524	0.97578
233.0803_3.9 pos	0.047389	0.90524	0.95773
814.2748_1.05 pos	0.039339	0.90571	0.86562
143.0763_2.76 pos	0.001755	0.90712	0.99774
725.4054_9.08 pos	0.0074275	0.90712	0.94134
191.0716_3.58 pos	0.034877	0.90712	0.99169
283.0478_3.25 neg	0.0243	0.90803	0.98432
617.3049_10.24 neg	9.84E-05	0.9082	0.85946
578.1652_5.57 neg	0.013728	0.90831	0.90515
597.2333_4.87 neg	2.96E-05	0.90887	0.86562
683.2244_0.94 neg	0.025571	0.91021	0.82138
747.3307_6.35 pos	0.010985	0.91035	0.91178
385.3301_10.92 pos	0.00539	0.91154	0.97578
546.1486_3.91 pos	0.014786	0.91154	0.84969
776.2347_1.23 pos	0.0289	0.91172	0.99637
129.017_1.52 pos	0.048612	0.91389	0.90065
387.1421_5.14 pos	0.00011753	0.91417	0.82138
328.1262_4.3 neg	0.00072834	0.91417	0.82326
507.1461_2.54 neg	0.0034303	0.91417	0.82138
678.2142_0.92 pos	1.24E-05	0.91458	0.82326
467.154_4.72 pos	0.0010553	0.91486	0.97578
659.5125_10.88 pos	0.024312	0.91592	0.99413
MGMG 183-a neg	0.0015539	0.91665	0.95661
142.0661_2.82 neg	0.0050107	0.91665	0.97578
382.0731_3.43 pos	0.033259	0.91687	0.95219
823.4681_11.01 neg	0.00068352	0.9173	0.82138
746.1928_3.81 pos	0.0015294	0.9173	0.84704
Gallic acid hexoside ne	0.0034261	0.9173	0.89263
500.9116_0.74 neg	0.00090324	0.91772	0.82274
135.1172_4.8 pos	0.0022788	0.91772	0.82326
429.1501_3.1 neg	0.01103	0.91772	0.88425
Flavonol base 4O, O-d	0.027787	0.91863	0.97578
307.1909_7.7 pos	0.0033251	0.91876	0.95219
576.2011_9.93 neg	0.048466	0.9191	0.92417
637.2225_2.54 neg	9.79E-05	0.92183	0.9826
327.1236_4.69 neg	0.00033778	0.92183	0.97578
707.3941_10.22 pos	0.015195	0.92286	0.90065
565.2032_6.3 pos	0.00038809	0.9229	0.94383

468.3078_9.26 pos	0.0032236	0.9229	0.99578
425.2329_6.24 pos	0.0090618	0.9229	0.72981
128.0496_3.13 pos	0.011189	0.9229	0.90065
661.1478_1.13 pos	0.039218	0.9229	0.97578
369.1343_4.64 neg	0.023639	0.92574	0.9826
582.2063_6.49 neg	0.00018432	0.92584	0.84969
313.0569_3.48 neg	0.018945	0.92585	0.95026
407.3363_10.92 pos	0.03051	0.92585	0.98866
219.1748_9.78 pos	0.00040934	0.92655	0.82138
803.5463_11.52 neg	0.01827	0.92655	0.82326
633.2308_4.85 neg	0.0074256	0.92678	0.90065
493.2163_4.98 pos	0.030603	0.92678	0.98432
507.6515_1.32 pos	0.031559	0.92678	0.97578
460.2019_0.98 pos	0.032958	0.92754	0.99637
543.274_6.51 pos	0.0056755	0.92824	0.99749
685.2004_5.66 neg	0.0047033	0.92951	0.95219
449.1434_5.3 pos	0.0018296	0.92979	0.99413
389.1569_4.97 pos	0.0042758	0.9299	0.88921
159.0904_3.06 pos	0.00089954	0.93081	0.99637
619.1776_7.43 pos	0.043935	0.93081	0.98432
768.7462_1.3 pos	0.001755	0.93109	0.97317
601.299_10.81 neg	0.0023225	0.93161	0.9826
487.3598_10.58 pos	0.030798	0.93161	0.76649
277.2193_9.1 neg	0.012103	0.93247	0.97578
Caffeoyl quinic acid isc	0.0040268	0.93249	0.97853
258.1239_5.99 pos	0.0002747	0.93519	0.96473
184.0729_9.69 pos	0.018974	0.93524	0.97578
Flavonol base 4O, 1Me	0.045391	0.93544	0.9782
118.0649_3.06 pos	0.00047275	0.93632	0.98395
353.2657_10.01 pos	0.00017684	0.93738	0.96673
549.2551_4.46 pos	0.015902	0.93738	0.82138
435.1692_4.08 neg	0.030754	0.93738	0.99576
776.7352_1.28 pos	0.035977	0.93738	0.84586
332.1332_1.54 pos	0.044453	0.93738	0.95661
747.2012_3.79 pos	0.014896	0.94059	0.88398
544.2028_3.9 pos	0.0072955	0.94089	0.86562
245.1402_11.02 pos	0.0032252	0.94125	0.80702
577.1735_2.69 neg	0.0035973	0.94125	0.99749
289.1784_7.68 pos	0.002749	0.94136	0.82326
637.3367_7.48 neg	2.64E-05	0.94164	0.82138
813.5287_11.64 neg	1.55E-05	0.945	0.82138
145.6144_3.06 pos	0.00022522	0.94562	0.97578
339.1065_3.25 pos	0.00078802	0.94578	0.95773
840.2696_1.37 pos	0.0012451	0.94578	0.88398
410.9245_0.74 pos	0.0017621	0.94666	0.9826
861.2509_7.38 neg	0.0093844	0.94757	0.9205
347.183_7.66 pos	0.0044846	0.94794	0.82138
185.0806_3.38 pos	0.00182	0.94811	0.9826

858.529_10.89 pos	0.042155	0.94827	0.97409
768.2465_1.22 pos	0.00032098	0.94976	0.97578
595.0808_3.61 neg	1.17E-06	0.95052	0.98907
86.9928_0.73 pos	0.00022735	0.95052	0.82138
235.1695_9.48 neg	2.19E-05	0.95103	0.82138
582.2046_5.98 neg	8.33E-05	0.95103	0.84969
548.1819_2.71 pos	0.00025564	0.95208	0.94383
228.088_4.37 pos	0.045004	0.95208	0.96473
805.3868_5.76 neg	0.030809	0.95246	0.9782
548.1472_3.77 pos	0.029035	0.95296	0.99749
311.1273_4.31 pos	0.0025202	0.95297	0.97317
572.3757_10.58 pos	0.018945	0.95413	0.93132
553.2289_9.39 pos	0.0039936	0.95508	0.90791
677.15_5.19 neg	0.042878	0.95826	0.82138
691.1862_3.69 pos	0.046531	0.95963	0.97578
585.2313_4.46 neg	4.97E-06	0.96035	0.82274
598.1872_1.09 pos	0.0004726	0.96035	0.94383
339.1216_5 pos	0.043718	0.96035	0.9826
877.4738_11.02 neg	0.0011852	0.96076	0.80707
258.1711_7.8 pos	0.0024977	0.96076	0.97578
156.9626_0.74 pos	0.0016141	0.961	0.90791
559.1789_4.66 pos	0.0003677	0.96188	0.99578
638.8834_0.74 pos	0.00013573	0.96318	0.91711
373.2221_4.8 pos	0.0010796	0.96318	0.82138
649.3387_5.57 pos	0.045552	0.96318	0.99194
216.9323_0.82 neg	0.002195	0.96346	0.97578
321.1126_7.14 pos	0.0001138	0.96374	0.96524
524.9033_0.74 pos	0.0017795	0.96436	0.96524
425.1628_6.65 neg	0.0053817	0.9653	0.82326
Licodione base 3O, 2P	0.013988	0.96541	0.90754
96.9866_0.73 pos	0.0018119	0.96751	0.82326
L-Ornithine pos	0.01532	0.96751	0.82138
479.1368_2.52 neg	4.05E-07	0.96888	0.97317
211.1626_7.8 pos	0.0039936	0.97234	0.97529
581.2379_5.14 pos	0.0075733	0.97234	0.86194
560.1804_4.14 pos	0.0086527	0.97234	0.98847
297.2888_6.57 pos	0.041874	0.97234	0.98907
807.2849_6.73 pos	1.24E-05	0.97302	0.94383
466.0667_4 neg	0.00029173	0.97343	0.97578
111.008_1.52 pos	0.013232	0.97343	0.89473
476.2797_9.93 neg	0.016692	0.97343	0.97578
283.0516_2.47 neg	0.020389	0.97343	0.89392
Isoleucylaspartate neg	0.044251	0.97343	0.97578
515.2968_10.7 pos	0.0059194	0.9735	0.88842
801.2697_7.26 pos	0.024651	0.9735	0.90065
414.9153_0.73 pos	0.0061238	0.97385	0.90065
609.1666_2.43 neg	1.24E-05	0.97387	0.92911
273.0875_4.6 pos	0.0023225	0.97387	0.91371

100.9337_11.22 neg	0.0076314	0.97387	0.92911
Tryptophan pos	5.79E-05	0.97408	0.98907
877.473_10.77 pos	0.011312	0.97474	0.82138
309.1242_5.52 neg	0.022608	0.97645	0.96473
116.0374_4.65 neg	0.0021471	0.97698	0.98833
3,4-Dihydroxybenzoate	0.0009046	0.97786	0.97578
886.2294_7.11 pos	0.036359	0.97786	0.97578
386.932_0.74 neg	0.00042098	0.97793	0.82326
581.3663_8.48 pos	0.015682	0.97793	0.89669
611.2122_6.69 neg	0.0065823	0.98005	0.79649
264.0765_0.88 pos	0.00020797	0.98058	0.97409
Dihydrohesperetin-7-O	0.040508	0.98175	0.92417
653.2046_4.7 neg	0.046382	0.9824	0.98866
191.036_4.05 neg	6.60E-07	0.98274	0.85331
421.2147_7.05 pos	0.00095097	0.98274	0.82274
132.0813_2.77 pos	0.0014397	0.98274	0.95216
585.2039_5.86 pos	0.023795	0.98274	0.82326
Isoflavone base 2O 1M	0.0067545	0.98378	0.95219
262.2222_9.98 pos	0.00068957	0.98598	0.97578
MGMG 183-b neg	0.0042932	0.9862	0.89392
162.0277_4.57 neg	0.030434	0.98636	0.95661
187.0742_5.04 pos	0.010583	0.98679	0.89884
459.2236_4.82 pos	0.04367	0.98815	0.91371
Epigallocatechin-3-gall	0.0047715	0.99117	0.98281
781.1937_1.06 neg	0.0078407	0.99191	0.97578
70.9791_0.73 pos	0.00028251	0.99238	0.80702
882.8223_0.74 pos	1.16E-05	0.99261	0.82138
247.1285_2.49 pos	0.014838	0.9932	0.97578
543.1307_0.89 pos	0.041129	0.99343	0.97578
343.1031_5.31 pos	0.0089816	0.99369	0.97578
436.2826_9.81 neg	0.032408	0.99386	0.95
468.0779_3.98 pos	6.82E-05	0.99391	0.84969
478.291_9.88 pos	0.0067196	0.99481	0.97578
Theobromine neg	0.036584	0.99481	0.76649
296.9435_0.74 pos	0.0035394	0.99524	0.98907
184.0742_10.38 pos	0.005147	0.99565	0.92608
258.0746_0.85 neg	0.03303	0.99565	0.96058
570.2085_5.49 neg	0.037593	0.99565	0.91343
719.2158_6.12 pos	1.26E-08	0.99612	0.97578
387.1425_6.45 pos	2.26E-05	0.9963	0.80702
715.2327_5.34 neg	0.017499	0.99664	0.92911
453.1884_4.7 neg	0.00079182	0.99668	0.84586
623.2036_3.95 neg	0.03813	0.99741	0.99578
599.2115_5.75 neg	0.0003317	0.99823	0.80702

SupplementaryTable S8 Putative annotation of most significant (top 20) mass spectrometry features (identified by mass-to-charge ratio (m/z)) in ‘Janz’ (uninoculated (Un) and inoculated (In)) > ‘QT16258’ (uninoculated (Un) and inoculated (In)).

m/z	Retention time (mins)	Ion mode	Annotation (putative) (Level 3)	Class of metabolite	Possible role in plant reported in literature
459.1055	2.48	Positive	Epigallocatechin 3-O-cinnamate	Tannins	Plant development
422.1622	2.33	Positive	N-Formyldemecolcine	Alkaloids	Unknown
488.0612	2.52	Positive	Penicilloic acid of 5-OH	Amino acid	Unknown
452.1742	2.51	Positive	Flucythrinate	Synthetic pesticide	NA
598.212	2.54	Negative	Clomiphene citrate	Synthetic drug	NA
565.1654	2.51	Negative	Protoaphin aglucone	Phenolic glycosides	Pigmentation
677.2066	5.38	Positive	Citbismine F	Quinolines and derivative	Cell membrane component
393.0505	3.57	Negative	Coumeroic acid	Benzenoids	Unknown
457.1327	2.51	Positive	Cycloartobiloxanthone	Benzenoids	antimicrobial
719.2158	6.12	Positive	Clorobiocin	Benzenoids	Products of streptomyces
439.1031	4.79	Negative	Gardenoside	Terpenoids	antimicrobial

473.0983	2.51	Positive	Eriodictyol 3'-O-glucoside	Flavonoids	Antimicrobial/Growth and reproduction
149.0459	2.35	Negative	D-Ribulose	Sugars	Primary metabolism, photosynthesis
454.1231	2.57	Positive	trans-Zeatin riboside monophosphate	Growth hormone	Growth and reproduction, potential nematode attractant
600.2297	2.51	Positive	No match	NA	NA
293.0887	2.55	Negative	6-Hydroxyl-1,6-dihydropurine ribonucleoside	purine	Growth and reproduction
572.1781	6.91	Positive	Amorphigenin O-glucoside	Iso flavonoids	antimicrobial
502.1853	8.5	Positive	1,3-Dimethoxy-5-(2,4,6 trimethoxyphenoxy)-2-(3,4,5-trimethoxyphenoxy)benzene ;	Benzenoids	Unknown
393.0506	2.71	Negative	Coumeroic acid	Benzenoids	Unknown
562.1993	2.19	Positive	5,7-Dihydroxy-3',4'-dimethoxy-8-(3-hydroxy-3-methylbutyl)-isoflavone 7-glucoside	Iso flavonoids	Antimicrobial/Growth and reproduction

The numbers followed HMDB is HMDB ID, numbers followed by C is KEGG ID and the plain numbers without alphabet is Pubchem ID in the table for detailed information of the corresponding annotated metabolite.

Supplementary Table S9 Pathways significantly changed in ‘QT16258’ (uninoculated and inoculated). The analysis is based on exact KEGG ID and HMDB ID match and performed on a subset of total identified metabolites which were higher in relative abundance and significantly different from 'Janz' (uninoculated and inoculated). The significance tests of the metabolites were performed using one way ANOVA and post hoc analysis.

Pathway	-log10(p)
Linoleic acid metabolism	3.17
Stilbenoid, diaryl heptanoid and gingerol biosynthesis	2.19
Purine metabolism	1.94
Arginine biosynthesis	1.73
Biosynthesis of unsaturated fatty acids	1.55
Glutathione metabolism	1.37
Arginine and proline metabolism	1.33
Phenylpropanoid biosynthesis	1.14
Cysteine and methionine metabolism	0.92
Flavonoid biosynthesis	0.91
Amino sugar and nucleotide sugar metabolism	0.86
Fatty acid biosynthesis	0.78

Supplementary Table S10 Pathways significantly changed in ‘Janz’ (Uninoculated and inoculated). The analysis is based on exact KEGG ID/HMDB ID match and performed on a subset of total identified metabolites which were higher in relative abundance and significantly different to ‘QT16258’ (uninoculated and inoculated). The significance tests of the metabolites were performed using one way ANOVA and post hoc analysis.

Pathways	-log10(p)
Caffeine metabolism	2.16
Phenylalanine metabolism	1.99
Ascorbate and aldarate metabolism	1.61
beta-Alanine metabolism	1.61
Tyrosine metabolism	1.61
Phenylalanine, tyrosine and tryptophan biosynthesis	1.44
Pantothenate and CoA biosynthesis	1.4
Tryptophan metabolism	1.4
Galactose metabolism	1.26
Arginine and proline metabolism	1.23
Glycine, serine and threonine metabolism	1.09
Ubiquinone and other terpenoid-quinone biosynthesis	1.04
Aminoacyl-tRNA biosynthesis	0.83

Supplementary Table S11 Mass spectrometry (MS) peak to pathway analysis using *mummichog* and gene set enrichment analysis (GSEA) based on *p* -value and t-score of unannotated MS features.

Pathway	Changed in
Cutin, suberine and wax biosynthesis	QT16258
Linoleic acid metabolism	QT16258
alpha-Linolenic acid metabolism	QT16258
Monoterpenoid biosynthesis	QT16258
Biosynthesis of unsaturated fatty acids	QT16258
Purine metabolism	Janz
Phosphonate and phosphinate metabolism	Janz
alpha-Linolenic acid metabolism	Janz
Riboflavin metabolism	Janz
Diterpenoid biosynthesis	Janz
Zeatin biosynthesis	Janz
Anthocyanin biosynthesis	Janz
Tropane, piperidine and pyridine alkaloid biosynthesis	Janz

APPENDIX D

CONFERENCE PRESENTATIONS

Poster presentations

1. **<u>Rahaman MM</u>**, Zwart RS, Thompson JP & Seneweera, S (2019) Wheat histopathology and defensive biochemistry against root-lesion nematode (*Pratylenchus thornei*). Australasian Plant Pathology Society Conference, Melbourne, Australia. **pp,** 141, doi:10.13140/rg.2.2.10545.53607

2. **<u>Rahaman MM</u>**, Thompson JP, Zwart RS & Seneweera, S (2017) Elucidation of molecular defence mechanisms in wheat (*Triticum aestivum*) against root-lesion nematode (*Pratylenchus thornei*). Australasian Plant Pathology Society Conference, Brisbane, Australia, **pp,** 144 doi: 10.13140/rg.2.2.15631.07841

Oral presentation

1. **<u>Rahaman MM</u>** (2017) Elucidation of molecular defence mechanisms in wheat (*Triticum aestivum*) against root-lesion nematode (*Pratylenchus thornei*). Nematology workshop, Australasian Plant Pathology Society Conference, Toowoomba, Queensland, Australia.