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Comparison of Visual and Normalized Difference Vegetation Index (NDVI) Assessments to Predict the Yield Tolerance of Wheat Genotypes to Root-Lesion Nematode *Pratylenchus thornei*

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Abstract: Wheat breeding programs have selected genotypes that are tolerant to the root-lesion nematode *Pratylenchus thornei* by measuring grain yield in field plots on infested sites. However, quicker methods are desirable to increase the capacity to assess more breeding lines for tolerance without harvesting grain. Two field experiments, time of sowing 1 (TOS1) and time of sowing 2 (TOS2), were conducted in the subtropical grain region of eastern Australia each year for eight years (sixteen experiments total) to characterize 396 wheat genotypes for tolerance when grown on high population densities of *P. thornei*. For each experiment, up to two visual tolerance ratings (TRs) and two normalized difference vegetation index (NDVI) readings were recorded using a Greenseeker™ during crop growth, and grain yield was obtained at crop maturity. The results showed that both TR and NDVI were predictive of tolerance based on the grain yield of the wheat genotypes. Generally, higher genetic correlations between grain yield and each vegetative assessment method were obtained with TOS2 than with TOS1 each year. The vegetative methods for assessing *P. thornei* tolerance proved to be valuable surrogates when grain yield was unreliable for germplasms that were agronomically unadapted to the regional environment. Our study established that at high population densities of *P. thornei* only, NDVI is a high-throughput phenotypic measurement of tolerance that can be used to screen a range of genetically diverse genotypes.

Keywords: NDVI; normalized difference vegetation index; wheat; tolerance; *P. thornei*; MET analysis

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1. Introduction

A constraint to global wheat (*Triticum aestivum*) production is the presence of the root-lesion nematodes *Pratylenchus thornei* and *P. neglectus* in broadacre cropping soils [1–5]. In the subtropical grain region of eastern Australia, located in southern and central Queensland and northern New South Wales, *P. thornei* is the dominant species [4]. *Pratylenchus thornei* parasitizes the root cortex of susceptible plants [6] and can complete its lifecycle in ~45 days [7]. When population densities of *P. thornei* in soil exceed economic damage thresholds, yield loss is a likely consequence of root damage that reduces extraction of soil water and nutrients by the plant [8,9]. However, the degree of yield loss is relative to the population densities of *P. thornei*, the tolerance of the wheat genotype, and the environment [10]. Thus, the threshold for economic damage needs to be considered at the agro-ecological or regional level, rather than nationally [8,10]. In the subtropical grain region of eastern Australia, the minimum population density that causes yield loss of intolerant genotypes has been estimated at 1000 *P. thornei*/kg soil in the whole soil profile up to 90 cm depth [11] or 2000 *P. thornei*/kg soil occurring in any soil layer [4]. The visible damage, caused by parasitism of the plant roots by *P. thornei*, firstly expresses vegetatively as chlorotic leaves, then as a decreased tiller and spike number [8], leading to grain yield losses of up to 60% [12]. Between the years of 2011 and 2019, the average wheat yield in Queensland was 1.7 t/ha [13]. This damage caused to plants of intolerant genotypes can be

detected 50 to 70 days after sowing [9,14]. However, tolerant genotypes can maintain grain yield, despite being grown in soil infested by *P. thornei* [15,16].

Several methods, or experimental approaches, have been developed to measure the level of tolerance of a genotype. Chemical amendments, including fumigants and nematicides, have been applied to the soil to generate population density gradients within the field [8,17,18]. Non-chemical approaches that use different levels of plant resistance have also been used to generate population density gradients of *P. thornei* [10,19]. Recently, Thompson et al. [12] described a method that determined a *P. thornei* tolerance index derived from a multi-environment trial (MET) analysis of multiple wheat field experiments grown on high population densities of *P. thornei* alone that correlated well with tolerance measured by methods that used population density gradients. This method accurately ranked and predicted the tolerance of wheat genotypes by grain yield to *P. thornei* in independent field experiments. Translation of that data into a 1-to-9 ordinal scale is used to characterize wheat genotypes in growers' sowing guides [20].

The experimental approaches described above are valuable for research purposes and for extension of genotype reactions to the grains industry. However, limitations of these methods include their inadequate capacity to screen thousands of breeding lines rapidly and their dependency on the measurement of biomass [11] or harvested grain yield [12]. Although grain yield is the most important trait selected by plant breeders [21], growing plants to maturity and harvesting grain increase costs and limit the number of plots that can be physically handled. To this end, developing a reliable tolerance test that is not constrained by cost or time, and is accurate and efficient, would assist breeding programs to phenotype more genotypes [22–26].

Visual assessment has been the pioneering method for phenotyping many different plant traits and will continue to be an important method for many physiological [27] and pathology-based plant traits [28]. Visual assessment of plant disease is both quick and non-destructive, with changes in plant appearance reflecting the altered physiology of the plant. Visual assessments are often 'convenient' indicators that provide enough information to screen for desired traits [27]. However, visual ratings can be prone to subjectivity related to the operator's level of experience or perception [28–30].

The first visual tolerance rating (TR) protocol for assessing wheat genotypes for tolerance to *P. thornei* was developed by Thompson et al. [31] on a one-to-six scale and was subsequently modified to a one-to-nine scale. This system scores plants or plots on a single ordinal scale based on their overall appearance, as influenced by lower leaf yellowing, tiller number, biomass, and leaf canopy [31]. This system has been used to screen ~2000 individual lines per year to select *P. thornei*-tolerant genotypes from diverse germplasm collections, and/or to phenotype genotypes within breeding populations [31].

Assessment by NDVI provides a non-destructive and objective measure of the greenness of the plant canopy by the reflectance of visible red and near-infrared light [32]. It is widely used and is considered one of the most useful vegetation indices for plant research [14,22,33–35]. For wheat, NDVI has been used successfully to score for yellow leaf spot caused by *Pyrenophora tritici-repentis* [36] and to predict the tolerance of adapted genotypes when grown on low and high population densities of *P. thornei* [14]. Reynolds et al. [27] described NDVI as having high precision and high throughput for canopy traits such as crop emergence, early vigor, and light interception. In this study, our objective was to develop a high-throughput method to determine the tolerance of genetically diverse genotypes grown on high population densities of *P. thornei* in multi-environment trials (METs). For this purpose, 396 wheat genotypes were tested in 16 experiments conducted over eight years. Unlike the 36 regionally adapted genotypes grown on both low and high population densities [14], these 396 genotypes were grown on high populations exclusively. These genotypes comprised (i) advanced breeding lines, (ii) pre-breeding lines, and commercial genotypes available in (iii) Queensland or (iv) other Australian states. This study identified a suitable sowing time to maximize the effectiveness of visual and NDVI methods for predicting tolerance in grain yield. In addition, a geno-

type from each of the nine tolerance groups that was predictive of tolerance assessed by grain yield, TR, and NDVI was identified. The successful application of such an approach can benefit wheat breeding programs that are constrained by resources (land area, cost, and time) and circumvent the need for a two-year experiment to establish low and high population densities of *P. thornei.*

2. Materials and Methods

2.1. General Field Site Management and Soil Characterization

The field site of 20 ha is located at 27.464° S, 151.426° E within a commercial farming enterprise near Formartin, ~50 km WNW of Toowoomba, Queensland, Australia. This site is mid-latitude of the subtropical grain region of eastern Australia wherein ~60% of the precipitation falls during the summer months [37], replenishing stored soil water for winter cropping. Crops are rainfed, relying on both in-crop rainfall and stored soil water [38]. The soil type is a self-mulching black Vertosol [39] of the Waco series [40] and is characterized by high clay content (68% at 0–15 cm grading to 72% clay at 90–120 cm depth). Typically, the soil type has a deep rooting depth with high plant available water capacity (PAWC) (288 mm to 180 cm depth) [9].

The field site is managed as four 5 ha strips (~60 m wide) in a four-year rotation. The rotation is (i) grain sorghum (*Sorghum bicolor*), (ii) weed-free fallow of ~14 months for soil water accumulation and weed control, (iii) a susceptible wheat genotype grown to increase *P. thornei* population densities, (iv) the experimental plots to assess genotype tolerance, and (v) weed-free fallow of ~11 month before sorghum re-cropping. During the periods of fallow and grain sorghum (resistant to *P. thornei*), the population densities of *P. thornei* following the plots of different wheat genotypes decline to relatively low levels [41] then build up on the first wheat crop to provide relatively evenly damaging population densities for the experiments in the following year. Thompson et al. [31] reported that using this crop rotation builds up the population density of *P. thornei* to damaging levels, without building up the ectoparasitic nematode *Merlinius brevidens* or fungi that cause other diseases like crown rot (*Fusarium pseudograminearum*) or common rot root (*Bipolaris sorokiniana*). Weeds are controlled with herbicides, along with strategic tillage if required [42].

2.2. Pratylenchus Thornei and Other Nematodes

The field site has high population densities of the root-lesion nematode *Pratylenchus thornei* co-occurring with low population densities of the ecto-parasitic stunt nematode *Merlinius brevidens*, as well as non-plant parasitic nematode species.

Each year, the initial population density of *P. thornei* for the 16 high-population-density experiments was determined by soil sampling at up to twelve different positions in a grid pattern across the 5 ha experimental strip after the application of nitrogen fertilizer but prior to sowing the wheat experiments. The population density of *P. thornei* in this strip had been built up by growing a susceptible wheat genotype in the preceding year. At each position, two 43 mm diameter tubes were pushed to 90 cm depth by a custom-built experimental hydraulic corer mounted on a vehicle. Each core was divided into depth layers (for the experiments from 2011 to 2015, these were 0–15, 15–30, 30–60, and 60–90 cm, and from 2016 to 2019, they were 0–30, 30–60, and 60–90 cm). Dividing the soil core into the increments as described here assists with the mixing of the samples during processing and is more accurate than mixing the whole 90 cm as a single sample. In each year, the cores at each sampling position were bulked at each depth, sealed in plastic (PVC) bags, and stored at 4 °C until further processing.

Soil samples were broken manually into <10 mm aggregates, mixed manually, and a 150 g subsample was taken for nematode extraction and a 100 g subsample was taken for soil moisture determination. Live nematodes were extracted by a modified Whitehead tray method [43] based on the principles of the Baermann method [44]. For this, the 150 g subsample of soil was spread evenly across facial tissues on a plastic mesh (33 × 22 cm) wet with 1 L of water in a plastic tray (45 × 30 × 13 cm) and incubated for 48 h at 22 °C. The

nematodes extracted were concentrated on a 200 mm diameter sieve with a pore aperture of 20 µm into ~10 to 15 mL water collected in a sample vial. Nematodes were identified as *P. thornei* [6], *M. brevidens* [45], or non-plant parasitic nematodes (absence of a stylet) and enumerated microscopically at 40× and 100× magnification in a gridded counting slide (1 mL Peters slide, Chalex Corporation, Portland, Oregon) on an Olympus BX50 compound microscope (Olympus Tokyo).

Soil gravimetric water content (GWC) was determined by drying the 100 g subsample in a forced draught oven at 105 °C for 48 h. The plant available water (PAW) for 0–90 cm was calculated using the GWC, the bulk density (BD), and crop lower limit (CLL) for each depth interval, as described previously [8]. Initial population densities were expressed as nematodes/kg of oven-dried soil equivalent (0–90 cm).

2.3. General Management of All Wheat Field Experiments

Nitrogen (N) fertilizer was applied as granular urea (46% N) drilled into the soil at 50 mm depth, supplying approximately 100 kg N/ha, at 1–2 months prior to wheat sowing. Granular Starter Z (Incitec Pivot, Southbank, Melbourne, Australia) was drilled at 40 kg/ha beside the seed at sowing to supply 8 kg P/ha and 1 kg Zn/ha. Wheat seeding rates were adjusted according to thousand-seed weight and germination percentage of each genotype seed lot to sow 100 viable seeds/m^2. All experimental plots were sown using a tractor-mounted cone seeder; wheat seeds were drilled into moist soil, which was firmed around the seed by trailing press wheels. The plots were 8 m in length and sown on 2 m plot centers. The distance between neighboring plots was 50 cm. Plots in eleven of the experiments consisted of seven drill rows, 25 cm apart, and in five experiments, there were five drill rows, 36 cm apart. In-crop weeds were controlled by registered selective herbicides applied at label rates when required. Foliar wheat diseases were controlled by registered fungicides at label rates when required.

2.4. In-Crop Normalized Difference Vegetation Index (NDVI) and Visual Tolerance Rating Assessments

The average normalized difference vegetation index (NDVI) for a field plot was determined from ~40 readings along the length of the plot by positioning the sensing head of the Greenseeker™ (Model 505, N-Tech Industries, Dalton, GA, USA) over the middle wheat row of each plot while walking at ~80 m/min. The Greenseeker™ is an active sensor that emits its own light during operation and measures the ratio of absorbed and reflected visible red and near-infrared light to calculate NDVI [46], with vegetative NDVI values ranging from 0 to 1. The higher this value, the greater the amount of green biomass that is being measured, and the higher tolerance of the genotype to *P. thornei* [14]. Each plot had either one (NDVI1) or two (NDVI2) readings taken at separate dates during the season.

The tolerance rating system with the respective symptoms is given in Table 1. Each plot had either one (TR1) or two (TR2) visual tolerance rating (TR) assessments taken on separate dates during the season. Usually, TR and NDVI were assessed on the same day.

Table 1. The scoring system used for visual assessment (tolerance rating, TR) of wheat genotypes for *Pratylenchus thornei* tolerance (modified by J. Sheedy from Thompson et al. [31]).

Score	Description	Category
1	Whole plant chlorotic, stunted, and possibly purple. Limited leaf development. May produce a single head.	Very intolerant (VI)
2	Very severe lower leaf chlorosis and necrosis. Reduced tillering. Leaf canopy dramatically reduced.	Intolerant–very intolerant (I–VI)
3	Severe lower leaf chlorosis and necrosis. Markedly reduced tillering. Leaf canopy markedly reduced.	Intolerant (I)
4	Obvious lower leaf chlorosis and necrosis. Reduced tillering. Leaf canopy reduced.	Moderately intolerant–intolerant (MI–I)
5	Moderate lower leaf chlorosis. Leaf canopy does not fill inter-row gap.	Moderately intolerant (MI)

Table 1. *Cont.*

Score	Description	Category
6	Some lower leaf chlorosis. Leaf canopy virtually fills inter-row gap.	Moderately tolerant–moderately intolerant (MT–MI))
7	Minor lower leaf chlorosis. Leaf canopy virtually fills inter-row gap.	Moderately tolerant (MT)
8	Virtually no visible symptoms. Leaf canopy fully covers inter-row gap.	Tolerant–moderately tolerant (T–MT)
9	No visible symptoms. Leaf canopy fully covers inter-row gap.	Tolerant (T)

2.5. Three Experiments with High and Low Initial Population Densities of Pratylenchus thornei

Three field experiments, each evaluating 36 wheat genotypes for tolerance, were grown on both low and high population densities of *P. thornei* during 2013 and 2015 [14]. A two-year strip plot design experiment was deployed, in which two wheat genotypes were sown, namely, QT8343 (resistant to *P. thornei*) and Kennedy (susceptible to *P. thornei*), in the first year to set up low and high population densities of *P. thornei*, respectively. In the second year, three replicates of the 36 wheat genotypes were sown on both these low and high population densities. The population densities of *P. thornei* in the soil (0–90 cm) were determined by taking two cores from each of 18 plots with both high (after Kennedy) and low (after QT8343) population density in each of the three replicate blocks close to sowing the second-year experiment. The mean population densities of *P. thornei* for the low and high treatments were determined by the methods described in Section 2.2.

Each plot of wheat was scored twice during the growing season when symptoms of intolerance were most evident in the intolerant check genotypes. In this study, an experienced tolerance assessor (ETA) had >15 years of experience (ETA) of using the TR protocol, while an inexperienced tolerance assessor (ITA) had <2 years of experience (ITA).

The Agricultural Production Systems Simulator (APSIM) [47] was used to predict the mean Zadoks growth stage (Z) [48] from 19 genotypes for both assessment times for each experiment. For Experiment 1, at 78 and 92 days after sowing (DAS), the mean growth stage was flag leaf emergence (Z37) and ear emergence (Z54), respectively. For Experiments 2 and 3, at 78 and 99 DAS, the mean growth stage was sixth node (Z36) and ear emergence (Z59), respectively.

2.6. Wheat Experiments Grown on High Population Densities of Pratylenchus thornei

2.6.1. Experimental Design and Conduct

There were 16 field experiments conducted between 2011 and 2019 that were grown only on high population densities of *P. thornei* at Formartin. To produce a high population density of *P. thornei* for the experimental year, a susceptible wheat genotype was grown the previous year, as described in Section 2.1.

These field experiments comprised three replicates of ~100 wheat genotypes as treatments in a randomized row/column design, and included check genotypes representing levels of tolerance established in previous research [8]. These check genotypes and other test genotypes occurring in more than one experiment provided concurrency necessary for successful MET analysis. Generally, in each year, two experiments with similar sets of wheat genotypes (treatments) were sown, with each sowing date separated by approximately three weeks. In this study, the first time of sowing (TOS1) experiment was aimed to be sown in the last week of May, and the second time of sowing (TOS2) experiment was aimed to be sown in the third week of June. However, the actual sowing dates reflected when topsoil moisture was suitable for plant establishment. The growth of the wheat in each plot was visually scored by an experienced tolerance assessor (ETA) either once or twice during the growing season (Table 2) when the symptoms of intolerance (as described in Table 1) were most evident in the intolerant check genotypes. Usually, the NDVI was determined on the same day as the TR. Dates of soil sampling, sowing, and NDVI and TR assessments for each experiment are shown in Table 2.

Table 2. Dates of soil sampling, sowing, and two times of canopy assessment by normalized difference vegetation index (NDVI1 and NDVI2) or visual trial ratings (TR1 and TR2) and other agronomic information relevant to the 16 field experiments conducted between 2011 and 2019 at Formartin, Queensland.

Experiment [a]	Soil Sampling Date	Sowing Date	NDVI1	NDVI2	TR1	TR2
11TOS1	12-Apr-11	25-Jun-11	13-Sep-11	19-Sep-11		19-Sep-11
11TOS2	12-Apr-11	11-Jul-11		19-Sep-11	13-Sep-11	19-Sep-11
12TOS1	17-May-12	30-May-12	30-Aug-12		30-Aug-12	
12TOS2	17-May-12	21-Jun-12	26-Sep-12	18-Oct-12	25-Sep-12	18-Oct-12
13TOS1	09-May-13	04-Jun-13		20-Sep-13	05-Sep-13	20-Sep-13
13TOS2	09-May-13	20-Jun-13	05-Sep-13	25-Sep-13	05-Sep-13	25-Sep-13
14TOS1	20-May-14	28-May-14	17-Sep-14		17-Sep-14	
14TOS2	20-May-14	25-Aug-14	17-Sep-14		17-Sep-14	
15TOS1	15-May-15	29-May-15	02-Sep-15	23-Sep-15	02-Sep-15	23-Sep-15
15TOS2	15-May-15	16-Jun-15	23-Sep-15	08-Oct-15	23-Sep-15	08-Oct-15
16TOS1	16-May-16	04-Jul-16	07-Sep-16		07-Sep-16	
16TOS2	16-May-16	14-Jul-16	07-Sep-16	28-Sep-16	07-Sep-16	
17TOS1	15-May-17	09-Jun-17	18-Sep-17		18-Sep-17	
17TOS2	15-May-17	12-Jul-17			20-Oct-17	
19TOS1	06-Jun-19	13-Jun-19	21-Aug-19	09-Sep-19	09-Sep-19	20-Sep-19
19TOS2	06-Jun-19	28-Jun-19			09-Sep-19	20-Sep-19

[a] Year: yy; TOS1: time of sowing 1; TOS2: time of sowing 2.

2.6.2. Cumulative Thermal Time (CTT), Rainfall, and Assessment Timings

Data on daily maximum and minimum temperatures were acquired from the Queensland Government station located at Dalby (Dalby Post Office, weather station ID: 41023; 37 km NNW from the experimental site) [49]. Cumulative thermal time (CTT) was calculated as the sum of the average daily temperatures from sowing to the respective assessment time. The base temperature for wheat growth was taken as 0 °C [50]. Rainfall data were collected on-site.

Days after sowing (DASs), cumulative thermal times (CTTs in °Cd) and cumulative rainfall (CR in mm) in relation to assessments in each experiment are shown in Table 3.

Table 3. Days after sowing (DASs), cumulative thermal time (CTT in °Cd), cumulative rainfall from sowing (CR in mm) until each assessment activity (normalized difference vegetation index, NDVI1 and NDVI2; visual rating, TR1 and TR2), and CTT and CR from sowing to 31 October for each of the 16 experiments grown between 2011 and 2019. Cumulative rainfall (CR in mm) between soil sampling and sowing is also provided.

Experiment [a]	Sowing [b]	NDVI1			NDVI2			TR1			TR2			Seasons end [d]		
	CR	DAS	CTT	CR [c]	DAS	CTT	CR [c]	DAS	CTT	CR [c]	DAS	CTT	CR [c]	DAS	CTT	CR [c]
11TOS1	65	80	991	56	86	1088	56				86	1088	56	128	1861	199
11TOS2	65				70	909	56	64	811	56	70	909	56	112	1681	199
12TOS1	12	92	1128	91				92	1128	91				154	2259	130
12TOS2	48	97	1283	54	119	1702	94	96	1262	54	119	1726	94	132	1980	94
13TOS1	37				108	1548	102	93	1263	85	108	1548	102	149	2414	120
13TOS2	50	77	1045	72	97	1423	89	77	1045	72	97	1423	89	133	2196	107
14TOS1	0	112	1477	27				112	1477	27				156	2386	45
14TOS2	30	23	364	3				23	364	3				67	1273	15
15TOS1	0	96	1235	24	117	1570	33	96	1235	24	117	1570	33	155	2330	67
15TOS2	0	99	1322	33	114	1589	36	99	1322	29	114	1589	36	137	2082	67
16TOS1	8	65	913	71				65	913	71				119	1870	181
16TOS2	12	55	783	56	76	1134	128	55	783	56				109	1740	166
17TOS1	0	101	1429	21				101	1429	21				144	2375	83
17TOS2	12							100	1672	57				111	1908	70

Table 3. *Cont.*

Experiment [a]	Sowing [b] CR	NDVI1 DAS	NDVI1 CTT	NDVI1 CR [c]	NDVI2 DAS	NDVI2 CTT	NDVI2 CR [c]	TR1 DAS	TR1 CTT	TR1 CR [c]	TR2 DAS	TR2 CTT	TR2 CR [c]	Seasons end [d] DAS	Seasons end [d] CTT	Seasons end [d] CR [c]
19TOS1	0	69	900	0	88	1186	5	88	1186	5	99	1371	5	140	2248	14
19TOS2	0							73	998	5	84	1183	5	125	2060	14

[a] Year: yy; TOS1: time of sowing 1; TOS2: time of sowing 2. [b] Cumulative rainfall (mm) between soil sampling and sowing of the experiments. [c] Cumulative rainfall (mm) from sowing of the experiment to the assessment activity. [d] Seasons end (31 October).

2.6.3. Wheat Genotypes Assessed

Altogether, 430 different genotypes were assessed over the 16 experiments conducted over 8 years, of which 396 were common wheat (*Triticum aestivum*) and 34 were durum wheat (*Triticum turgidum* ssp. *durum*). Out of the common wheat genotypes, 159 were released as named wheat genotypes available to growers for commercial grain production in Australia, 151 were advanced breeding lines, and 79 were pre-breeding lines. Commercial genotypes of wheat were delineated into three groups as suitable or not for commercial grain production in Queensland by reviewing grower guides and extension material published by breeding companies, respective state government agencies, and the Grains Research and Development Corporation (GRDC). The first group contained genotypes that were adapted and recommended for production in Queensland (QComm; n = 60). The second group contained genotypes that were recommended for production in other regions of Australia (AComm; n = 86). The third group contained genotypes that were of very slow maturity, i.e., facultative types that required shorter periods of vernalization (ASlow; n = 13). Advanced breeding lines developed for Queensland (QABLs; n = 50) and other advanced breeding lines with no known production area (AABLs; n = 101) were also evaluated. The Queensland pre-breeding lines (QPBLs; n = 79) were a group of genotypes that were developed by hybridizing a synthetic wheat hexaploid parent (CPI133872) that is resistant and tolerant to *P. thornei* and *Pratylenchus neglectus* with commercial genotypes. Seven winter genotypes that required longer vernalization did not produce grain and were omitted from this analysis.

2.6.4. Determination of Grain Yield

Approximately one month prior to crop maturity, plots were trimmed by a tractor-mounted slasher to approximately 6 m length in preparation for mechanical harvest of grain with a combine harvester. The grain harvested from each plot was weighed, and a 100 g subsample was dried at 105 °C for 48 h, and then reweighed to determine grain moisture, calculated as the weight of lost moisture on drying as a percentage of the undried sample weight. In this study, grain yield results are presented as kg/ha, at 12% grain moisture equivalent for all genotypes.

2.7. Statistical Analyses

2.7.1. Statistical Analysis of Normalized Difference Vegetation Index (NDVI), Tolerance Ratings by an Experienced (ETA) and Inexperienced Tolerance Assessors (ITA), and Grain Yield for the Three Experiments with Low and High Initial Population Densities of *Pratylenchus thornei*

A linear mixed model was used to analyze grain yield, NDVI, and visual ratings for the three experiments with two *P. thornei* densities. The environment term encompassed the Year 1 genotypes, the experiment, and the measurements and was fitted as a fixed effect. For each genotype, the best linear unbiased prediction (BLUP) was calculated for the NDVI, tolerance rating, and grain yield, and a residual maximum likelihood was used to estimate the respective variance parameters [51]. AS-Reml R [52] in the R software [53] was used for these analyses. Genetic correlations between (1) grain yield, (2) NDVI, and the tolerance ratings by (3) ETA and (4) ITA were generated from these BLUPs.

2.7.2. Statistical Analysis of Initial *Pratylenchus thornei* Population Densities and Plant Available Water (PAW) for the 16 Wheat Experiments

Yearly pre-sowing data to 90 cm soil profile depth for population density of *P. thornei* transformed by loge(x+1) and PAW were subjected to one-way analysis of variance, followed by Bonferroni's post hoc test for multiple comparisons at $p \leq 0.05$ using Genstat for Windows 22nd Edition [54].

2.7.3. Statistical Analysis of Normalized Difference Vegetation Index (NDVI), Trial Ratings, and Grain Yield for the 16 Wheat Experiments

Yield and the different instances of NDVI and tolerance rating measured on each experiment were analyzed together via a multi-trait, multi-environment trial analysis, using a linear mixed model framework. The analysis approach was like that outlined in Dreccer et al. [55], whereby the combinations of trait, assessment time, and experiment were combined to define a new factor labeled "TraitTimeExp", with 61 unique levels. Fixed terms in the model included TraitTimeExp, crop type, and their interaction. Random terms to capture the interaction between genotype and TraitTimeExp were fitted separately for each crop type. Random terms describing the experimental design structure of each experiment were included separately for each TraitTimeExp combination. Furthermore, terms to account for global and extraneous spatial variation were included separately for each TraitTimeExp, following the methods of [56]. The residual covariance between traits measured at different times of assessment was estimated separately for each experiment using an unstructured covariance matrix.

A factor analytic (FA) model was fitted to the genotype by TraitTimeExp interaction effects for the common wheat genotypes, enabling the parsimonious estimation of heterogenous genetic correlation (covariance) between all pairs of TraitTimeExps, along with heterogenous genetic variance for each TraitTimeExp [57]. Factor analytic models of increasing order were iteratively fitted to the genotype by TraitTimeExp interaction effects, with an appropriate order of FA model ("final model") determined as that which minimized the AIC [58]. In the case of the durum wheat crop type, a diagonal genetic variance model was fitted to capture heterogeneous genetic variance across TraitTimeExps. However, due to the limited number of durum genotypes in common between experiments, genetic correlations were unable to be estimated for this crop type, and results for durum will not be presented.

Predictions of genotype performance for each TraitTimeExp were generated from the final model as empirical best linear unbiased predictions (eBLUPs). The analysis was performed using the asreml-R package [52], which estimates variance parameters using residual maximum likelihood (REML) estimation [51], in the R statistical computing environment [53]. Mean predictions from the 16 experiments for NDVI1, NDVI2, TR1, TR2, and grain yield were determined from these eBLUPS.

Correlation coefficients between assessment methods were determined from the genotypes' predicted values (eBLUPs) using Genstat for Windows 22nd Edition [54]. These correlation analyses were conducted with genotypes grouped by intended region for production and breeding status, as specified in Section 2.6.3 above. The number of genotypes per group ranged from 13 to 101 for ASlow and AABL, respectively.

2.8. Generation of Tolerance Groupings by Grain Yield, Normalized Difference Vegetation Index (NDVI), and Visual Tolerance Rating (TR) for the Queensland Commercial Genotypes

Tolerance categories for NDVI1, NDVI2, TR1, TR2, and grain yield for the Queensland commercial genotypes (QComm) were constructed as previously described for grain yield [12]. The range of genotype eBLUPs between the minimum (most intolerant genotype) and the maximum (most tolerant genotype) for each method was subdivided into nine arithmetically equal sub-ranges. These sub-ranges were given nine alpha descriptors ranging from very intolerant to tolerant, and each genotype was assigned to one of these categories based on its eBLUP value for the respective method used for measuring tolerance. Thus, each genotype had for each assessment method both a quantitative measure of

tolerance and an ordinal alpha descriptor of tolerance used for communication of tolerance levels in the grains industry.

3. Results

3.1. Genetic Correlations from the Three Wheat Experiments with Low and High Population Densities of Pratylenchus thornei

From the three experiments that had low and high initial population densities of *P. thornei*, the genetic correlations between visual assessment tolerance ratings, NDVI, and grain yield of the 36 wheat genotypes are given in Table 4. The genetic correlation coefficients with grain yield range from 0.1 to 0.96 for NDVI, 0.21 to 0.99 for TR by the ETA, and −0.06 to 0.99 for the TR by the ITA. The mean correlation coefficients with yield across all experiments and all initial population densities were 0.73, 0.64, and 0.50 for ETA, NDVI, and ITA, respectively. For all three experiments, the genetic correlations between NDVI and TR as well as the correlations between both TR and NDVI with grain yield were greater on high population densities compared to low population densities.

Table 4. Genetic correlations derived from best linear unbiased predictions (BLUPs) among grain yield, normalized difference vegetation index (NDVI), and tolerance rating (TR) made by either the experienced (ETA) or inexperienced (ITA) assessors for wheat genotypes growing on low and high initial population densities (*P. thornei*/kg soil) for each experiment.

Experiment	Nematode Population Density [a]	*P. thornei*/kg Soil [b]			CTT [e]	Genetic Correlation				
		Log_e (x + 1)	SED [c]	BTM [d]		NDVI [f] and ETA [g]	NDVI and ITA [h]	NDVI and Yield [i]	ETA and Yield	ITA and Yield
1	Low	7.85 a	0.06	2570	1021	0.83	NR [j]	0.56	0.65	NR
					1265	0.99	NR	0.70	0.69	NR
	High	9.12 b		9091	1021	0.95	NR	0.89	0.93	NR
					1265	0.99	NR	0.96	0.95	NR
2	Low	6.88 a	0.11	975	985	0.89	0.81	0.79	0.85	0.77
					1320	0.79	NR	0.66	0.83	NR
	High	8.01 b		3018	985	0.95	0.93	0.93	0.99	0.99
					1320	0.88	NR	0.84	0.98	NR
3	Low	6.36 a	0.10	578	985	0.95	0.99	0.32	0.35	0.27
					1320	0.80	0.95	0.10	0.21	−0.06
	High	7.13 b		1245	985	0.92	0.93	0.69	0.74	0.72
					1320	0.79	0.98	0.28	0.60	0.30

[a] Low population density (after resistant wheat), high population density (after susceptible wheat); [b] Initial *Pratylenchus thornei* population densities log_e(x + 1) 0–90 cm with different letters after mean values denoting significant differences between initial *P. thornei* population densities within each experiment; [c] SED: standard error of difference, [d] BTM: back-transformed mean; [e] CTT: cumulative thermal time (°Cd); [f] NDVI: normalized difference vegetation index; [g] ETA: experienced tolerance assessor; [h] ITA: inexperienced tolerance assessor; [j] NR: not recorded; [b–i] from Robinson et al. 2019 [14].

The influence of the initial population density of *P. thornei* on the genetic correlation of grain yield with (a) NDVI and (b) trial ratings is shown in Figures 1a and 1b, respectively. There were significant asymptotic relationships between the yield/NDVI genetic correlation and *P. thornei* population density (R^2 = 0.55, p = 0.011, n = 12) (Figure 1a) and between the yield/TR genetic correlation and *P. thornei* population density (R^2 = 0.66, p = 0.003, n = 12) (Figure 1b).

3.2. The 16 Field Experiments Grown on High Population Densities of Pratylenchus thornei

3.2.1. Initial Nematode Population Densities of *Pratylenchus thornei* and Plant Available Water (PAW)

For all 16 experiments grown on high population densities of *P. thornei* over the period from 2011 to 2019, the initial *P. thornei* population densities and the initial PAWs are given in Table 5. The initial *P. thornei* population densities of the 16 experiments ranged from 2580 in 2019 to 7777 *P. thornei*/kg soil (0–90 cm) in 2013. The initial PAW in the 0–90 cm soil profile ranged from 96 mm in 2012 to 192 mm of water in 2015.

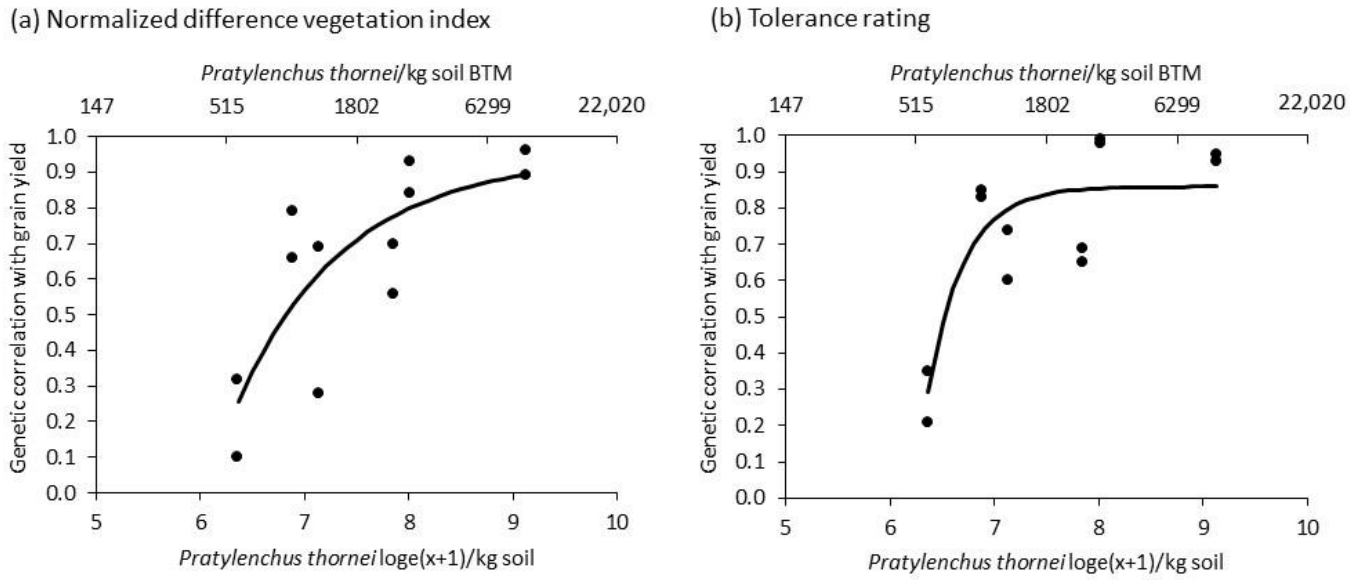

Figure 1. The relationship between initial density of *Pratylenchus thornei* and the genetic correlation coefficients between grain yield with either (**a**) normalized difference vegetation index, y = 0.943 − 287 × (0.387x), R^2 = 0.55, *p* = 0.011, n = 12, or (**b**) the tolerance rating (TR) by the experienced trial assessor (ETA), y = 0.8591–61743124 × (0.0545x), R^2 = 0.66, *p* = 0.003, n = 12.

Table 5. Average plant available water (PAW, mm) and average initial *Pratylenchus thornei* population densities (*Pratylenchus thornei*/kg soil) in the soil profile to 90 cm for each year when the 16 experiments were grown on high population densities only. Different letters after means indicate statistically different values by the Bonferroni multiple range test.

Year	Plant Available Water (PAW)		*Pratylenchus thornei*/kg Soil 0–90 cm		
	Mean (mm)	s.e.m. [a]	loge(x+1)	s.e.m	BTM [b]
2011	167 ab	5.89	8.55 a	0.10	5186
2012	96 d	5.89	8.72 a	0.10	6129
2013	164 abc	11.77	8.96 a	0.21	7777
2014	140 bc	7.45	8.21 ab	0.13	3691
2015	192 a	8.33	7.89 b	0.15	2659
2016	139 bc	8.33	8.64 a	0.15	5624
2017	135 bc	8.33	8.65 a	0.15	5692
2019	121 cd	7.85	7.86 b	0.14	2580

[a] s.e.m.: standard error of mean. [b] BTM: back-transformed means.

3.2.2. Genetic Correlation Analysis for Each of the 16 Experiments When Grown on High Population Densities of *Pratylenchus thornei*

The genetic correlations between pairs of the measured traits for each of the 16 experiments are displayed as a heat map in Figure S1. The genetic correlations were derived from the MET analysis of the trial ratings and NDVI readings for up to two times of assessment per experiment and the MET analysis of grain yield for each experiment. Generally, the grain yield for each experiment was highly correlated with the grain yield of the other experiments. Exceptions to this were 16TOS1, 16TOS2, and 19TOS1. In these three experiments, the genetic correlations between NDVI and TR were also generally less than in other experiments.

The genetic correlation for NDVI1, NDVI2, TR1, and TR2 with grain yield ranged from 0.12 to 0.88, 0.18 to 0.88, 0.13 to 0.94, and 0.30 to 0.88, respectively (Table 6). The grain yield ranged from 946 kg/ha (19TOS2) to 5862 kg/ha (16TOS1). The mean grain yield of all the first sown (TOS1) experiments (n = 8) was 3327 kg/ha and the mean grain yield of all second sown (TOS2) experiments (n = 8) was 2768 kg/ha.

Table 6. The mean grain yield (kg/ha) and the genetic correlations between grain yield and normalized difference vegetation index (NDVI1 and NDVI2) or visual tolerance ratings (TR1 and TR2) by the experienced tolerance assessor (ETA) at two times for each of the 16 experiments grown from 2011 to 2019.

Experiment [a]	Mean Yield (kg/ha)	Genetic Correlation with Grain Yield			
		NDVI1 [b]	NDVI2	TR1	TR2
11TOS1	4827	0.43	0.35	NR	0.52
11TOS2	4168	NR	0.71	0.73	0.74
12TOS1	3308	0.59	NR	0.83	NR
12TOS2	2412	0.36	0.19	0.84	0.74
13TOS1	2738	NR	0.62	0.67	0.67
13TOS2	2390	0.88	0.84	0.86	0.88
14TOS1	2880	0.12	NR	0.60	NR
14TOS2	1718	0.82	NR	0.94	NR
15TOS1	3668	0.50	0.47	0.50	0.59
15TOS2	3869	0.50	0.53	0.57	0.61
16TOS1	5862	0.35	NR	0.40	NR
16TOS2	5542	0.34	0.46	0.36	NR
17TOS1	1887	0.85	NR	0.77	NR
17TOS2	1096	NR	NR	0.94	NR
19TOS1	1443	0.23	0.18	0.13	0.30
19TOS2	946	NR	NR	0.64	0.68

NR: not recorded. [a] Year: yy; TOS1: time of sowing 1: TOS2: time of sowing 2; the average initial *Pratylenchus thornei* population density for each trial year is in Table 5. [b] The suffix 1 or 2 after NDVI and TR is either the first or second measurement.

Tolerance ratings by the ETA had higher genetic correlation with grain yield when assessed in the TOS2 experiments compared to the TOS1 experiments each year (Figure 2). The genetic correlation coefficients with yield were similar for both the first (TR1) or second (TR2) assessment time in both experiments. The genetic correlations between NDVI and yield were similar for the TOS1 or TOS2 experiments and for the timing of the NDVI reading (NDVI1 and NDVI2). The mean CTT for NDVI1 and NDVI2 was 1073 °Cd (SE ± 90) and 1350 °Cd (±92), respectively, and the mean CTT for TR1 and TR2 was 1126 °Cd (±84) and 1379 °Cd (±89), respectively.

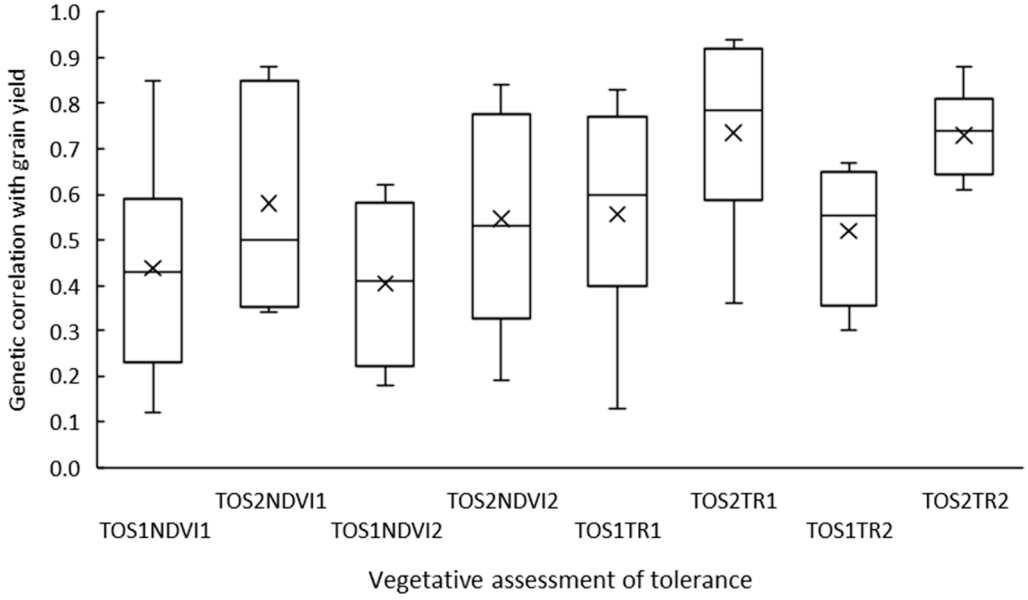

Figure 2. Box and whiskers plot of the genetic correlation of grain yield with either NDVI or TR at two times of tolerance assessments in trials with two times of sowing. The upper and lower extremities of

the box denote the upper and lower quartile values, respectively. The upper and lower extremities of the whiskers denote the maximum and minimum values. The median is shown as a horizontal line, while the mean is shown as a cross. TOS1 and TOS2 represent the first and second sown experiments each year. NDVI1 and NDVI2 represent normalized difference vegetation readings, and TR1 and TR2, respectively, represent first and second tolerance ratings during the growing season.

3.2.3. Classification of the Wheat Genotypes into Their Recommended Regions for Production or Breeding Background

The yield of the wheat commercial genotypes recommended for Queensland (QComm) had the highest correlation (r) with both NDVI values and both TR values compared to the commercial genotypes that are recommended for other regions in Australia (AComm) (Table 7). The r-values with yield were 0.83 and 0.90 for NDVI and TR respectively, for QComm genotypes. For the AComm genotypes, the correlations with yield were less than for the QComm but still highly significant ($p < 0.001$), with r ranging from 0.73 to 0.77 for NDVI, and ranging from 0.75 to 0.78 for TR. When genotypes were winter types or very slow to mature (ASlow), the relationships between yield and NDVI and yield and TR were not statistically significant for both times of assessment. The correlation between yield with either NDVI or TR for the advanced breeding lines of wheat were all significant ($p < 0.001$), but r-values were greater for the QABL compared to AABL. There was no correlation between the grain yield with either NDVI measurement for QPBL. There was a correlation between grain yield with both TR1 and TR2 for the QPBL. For the first assessment time, the correlation between NDVI1 and TR1 ranged from r = 0.87 to 0.96 for QPBL and QABL, respectively. For the second assessment time, the correlation between NDVI2 and TR2, ranged from r = 0.85 to 0.96 for the QPBL and QABL, respectively.

Table 7. The correlation coefficients (r) and the respective *p*-value for the relationships between predictions of mean grain yield, normalized difference vegetation index (NDVI1 and NDVI2), and tolerance ratings by an experienced tolerance assessor (TR1 and TR2) from the 16 field experiments for the wheat genotypes in relation to the intended area of production and their adaptation to Queensland, Australia.

Group	n	Grain Yield								NDVI1		NDVI2	
		NDVI1		NDVI2		TR1		TR2		TR1		TR2	
		r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
QComm	60	0.83	<0.001	0.83	<0.001	0.90	<0.001	0.90	<0.001	0.94	<0.001	0.94	<0.001
AComm	86	0.77	<0.001	0.73	<0.001	0.78	<0.001	0.75	<0.001	0.94	<0.001	0.94	<0.001
ASlow	13	−0.02	ns	0.05	ns	0.23	ns	0.33	ns	0.89	<0.001	0.90	<0.001
QABL	50	0.77	<0.001	0.76	<0.001	0.76	<0.001	0.77	<0.001	0.96	<0.001	0.96	<0.001
AABL	101	0.72	<0.001	0.66	<0.001	0.80	<0.001	0.78	<0.001	0.94	<0.001	0.93	<0.001
QPBL	79	0.16	ns	0.16	ns	0.40	<0.001	0.46	<0.001	0.87	<0.001	0.85	<0.001

QComm: commercial genotypes recommended for Queensland; AComm: commercial genotypes not recommended for Queensland; AABL: advanced breeding line; QABL: breeding line developed with adapted parents to Queensland; QPBL: pre-breeding line developed with a synthetic hexaploid parent; ASlow: unsuitable to be grown in Queensland due to winter wheat type or very slow maturity; n = number of genotypes; seven extremely slow genotypes were omitted from this analysis. ns: non-significant.

For QComm, the distribution of the genotypes (n = 60) into the nine tolerance categories is shown in Figure 3. For NDVI and TR, more genotypes were ranked in category 8 than any of the other categories for each assessment. For grain yield, more genotypes were ranked in category 7 than any of the other categories. The greatest number of genotypes (n = 18) that were ranked into any one category (category 8) was by NDVI1.

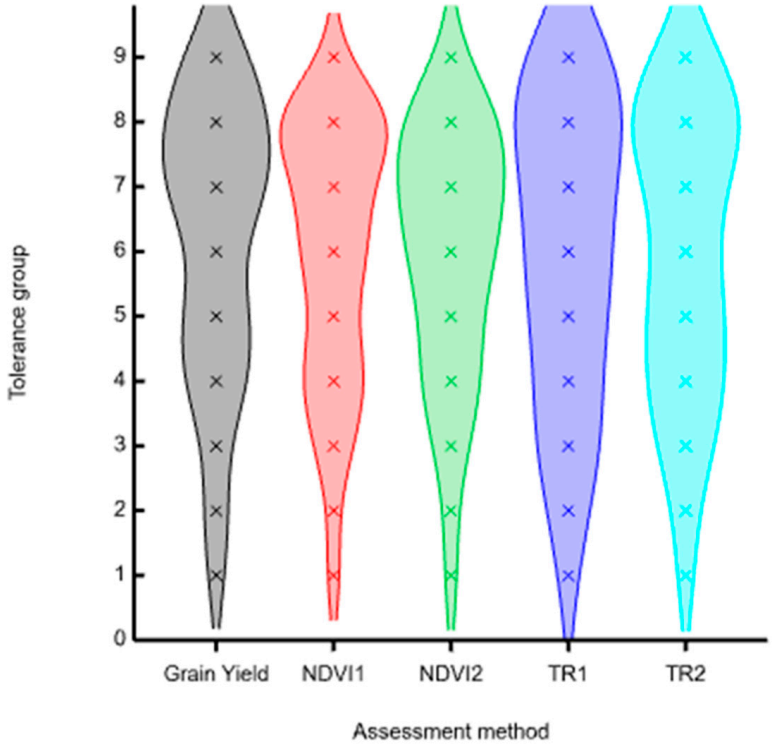

Figure 3. The distribution of the Queensland commercial (QComm) genotypes (n = 60) into the respective tolerance categories (where 1 = very intolerant and 9 = tolerant) derived either from normalized difference vegetation index at two times (NDVI 1 and NDVI2) or tolerance rating at two times (TR1 and TR2) or grain yield. *Y*-axis: 9 = tolerant, 8 = tolerant to moderately tolerant, 7 = moderately tolerant, 6 = moderately tolerant to moderately intolerant, 5 = moderately intolerant, 4 = moderately intolerant to intolerant, 3 = intolerant, 2 = intolerant to very intolerant, 1 = very intolerant.

There was an overall yield loss of 54% between the mid-predicted yield of the tolerant category compared to the mid-predicted yield of the very intolerant category for the QComm genotypes. This represents a yield change of 6.7% between successive tolerance categories. For NDVI1 and NDVI2, there was a 3.2 and 4.2% change in NDVI values between the successive categories, respectively. The overall reduction in NDVI1 and NDVI2 between the mid-predicted tolerant compared to the mid-predicted very intolerant categories was 26 and 34%, respectively. For TR1 and TR2, there was a 7.8% and 8.3% change in the ratings between the successive categories, respectively. The overall reduction in TR1 and TR2 between the mid-predicted tolerant compared to the mid-predicted very intolerant categories was 62 and 66%, respectively.

3.2.4. Identification of a Single Genotype for Each of the Tolerance Groups

For six of the nine tolerance categories, a single QComm genotype was identified that was representative of its corresponding tolerance group by yield, NDVI, or TR (Table 8). An exception to this is the wheat genotype SEA Condamine that was identified as the tolerant (T) reference genotype based on yield and TR but was categorized as tolerant–moderately tolerant (T-MT) by NDVI1 and NDVI2. There were no genotypes identified that were deemed to be tolerant by all of the five assessment methods. The wheat genotype Kennedy was rated as MI by all assessment methods except for TR2 (MI-I). Strzelecki was categorized as MI-I for both NDVI assessment times, I for yield and TR2, and I-VI for TR1.

Table 8. The nine wheat genotypes identified from each tolerance group that were categorized consistently for tolerance by grain yield, two normalized difference vegetation index assessments (NDVI1, NDVI2), and two tolerance ratings by an experienced tolerance assessor (TR1, TR2).

Tolerance Group [a]	Genotype	Tolerance Group by Assessment Method				
		Yield	NDVI1	NDVI2	TR1	TR2
T	SEA Condamine	T	*T-MT*	*T-MT*	T	T
T-MT	EGA Wylie	T-MT	T-MT	T-MT	T-MT	T-MT
MT	Sunzell	MT	MT	MT	MT	MT
MT-MI	Merinda	MT-MI	MT-MI	MT-MI	MT-MI	MT-MI
MI	Kennedy	MI	MI	MI	MI	*MI-I*
MI-I	Elmore CL Plus	MI-I	MI-I	MI-I	MI-I	MI-I
I	Strzelecki	I	*MI-I*	*MI-I*	I-VI	I
I-VI	EGA Stampede	I-VI	I-VI	I-VI	I-VI	I-VI
VI	Lincoln	VI	VI	VI	VI	VI

Categories that are italicized are different to the tolerance category derived by yield. [a] T = tolerant; T-MT = tolerant–moderately tolerant; MT = moderately tolerant; MT-MI = moderately tolerant–moderately intolerant; MI = moderately intolerant; MI-I = moderately intolerant–intolerant; I = intolerant; I-VI = intolerant–very intolerant; VI = very intolerant.

4. Discussion

This study found both NDVI and TR to be robust methods suitable for use by researchers and plant breeders to phenotype genetically diverse germplasm in the vegetative stage for tolerance on high population densities of *P. thornei* only, and without the need to harvest grain yield. Thus, the use of these in-season assessment methods provides an efficient alternative that requires less resources than using grain yield for assessment of tolerance to *P. thornei*. Also, routine use of these vegetative assessment methods for tolerance of wheat genotypes is a valuable safeguard should grain yield be lost through adverse weather conditions. The genetic correlations of TR and NDVI with grain yield can be increased by sowing later in the recommended sowing window for the region.

The first experimental approach that used low and high initial *P. thornei* population densities showed significant and positive asymptotic exponential relationships of genetic correlations between grain yield and NDVI or TR with population densities. Both NDVI and TR assessment methods were more predictive of grain yield at higher initial *P. thornei* population densities. The symptoms of intolerance are more severe when populations are higher, thus having a greater range of NDVI values between intolerant and tolerant genotypes. Therefore, managed sites with high initial population densities of *P. thornei* provide reliably superior assessments of genotype tolerance using NDVI and/or TR. Our study also showed that TR was more predictive of tolerance than NDVI when experiments had lower initial population densities of *P. thornei.*

The initial population density of *P. thornei* was not the only factor that influenced the accuracy of the tolerance ratings. The genetic correlations between TR and NDVI improved when the assessor was experienced (ETA) (>15 years) compared to the inexperienced assessor (ITA). Both assessors performed best on experiments where the initial population densities of *P. thornei* were the greatest. When compared to NDVI, TR by the experienced assessor gave higher genetic correlation with grain yield, while TR by the inexperienced assessor gave lower genetic correlation than the NDVI with grain yield. Shi et al. [59] reported that although training will improve the accuracy of visual assessors, inconsistences will remain in their subjectiveness.

The second approach used in this study was to evaluate greater numbers of genotypes and greater genetic diversity on only high population densities of *P. thornei*. This approach was advocated as the most practical method to assess genotypes for tolerance in plant breeding based on grain yield [12]. Furthermore, tolerance assessed in this way encompasses the interactions between the plant genotype, the nematode species, and the environment [60] and directly relates to the relative yield growers can expect from sowing various wheat genotypes in *P. thornei*-infested fields [12]. Although there is a greater range

in the yields between intolerant and tolerant genotypes when grown in heavily infested fields, yield loss still occurs when intolerant genotypes are grown in fields that are less infested [12]. Furthermore, if a genotype is tolerant to *P. thornei*, this does not imply that this genotype is tolerant to *P. neglectus* [12].

A priority for evaluating genotypes on high populations alone is to have a field site that is managed to have damaging population densities of *P. thornei* without other disease constraints. Previously, it was found from MET analysis of 29 field experiments that greater discrimination of wheat genotypes for tolerance based on grain yield was obtained for greater initial population densities of *P. thornei* (range 1775–9402 *P. thornei*/kg soil at 0–90 cm) and greater pre-sowing PAWs (range 61–208 mm at 0–120 cm) [12]. Similarly, it was concluded that initial population densities of >2500 *P. thornei*/kg soil at 0–90 cm were required to be a robust discrimination of tolerance of wheat genotypes based on the NDVI [14]. However, if populations were low, the area under the disease progress curve could be used if more frequent NDVI assessments were made [14]. All our experiments for the second approach of testing on high nematode population densities exceeded thresholds for damage, with the population densities of *P. thornei* ranging from 2580 to 7777/kg soil in the profile to 90 cm depth. However, the 2019 site had the lowest population density of 2580 *P.* thornei/kg soil at 0–90 cm, the second lowest PAW of 121 mm at 0–90 cm, and the lowest in-crop rainfall of only 14 mm. As a result, the genetic correlations of TR and NDVI with respect to grain yield in 2019 were generally the lowest in our study. Caution is required when using NDVI in extremely water-deficient experiments or seasons [61]. Chenu et al. [62] refer to this as Environment Type 3 (ET3), where water is limited through the vegetative stages, and continues to be extremely limited during grain fill. The 2019 experiments experienced ET-3 conditions, and NDVI and TR were valuable methods when grain yield is extremely limited by water deficiency. Improvement in the genetic correlation between grain yield with TR1 or TR2 was observed for the second sown experiment (19TOS2) compared to the first sown experiment (19TOS1). If the criteria outlined above are met, our study shows that wheat genotypes can be non-destructively screened for tolerance to *P. thornei* by TR and NDVI, thereby providing an efficient phenotyping platform of genotypes for plant breeding and characterization of genotypes for growers' sowing guides.

It is interesting to note that the second sown experiments had the best discrimination (genetic correlation with grain yield) for tolerance using either NDVI or TR, despite these experiments yielding ~17% less on average than the first sown experiments. It should be noted that tolerant genotypes out-yielded intolerant genotypes in both TOS1 and TOS2 experiments and that genotype rankings were consistent regardless of sowing time. Therefore, if the objective is to effectively discriminate wheat genotypes for *P. thornei* tolerance in the vegetative stages, experiments should be delayed and sown later in the normal planting window, when the expression of symptoms are more severe. The recommended sowing window for the region is between late May and late June, comparable to the mean TOS2 date of June 30 (excluding 14TOS2—sown 25 August 2014) in the present study. By sowing in the recommended window, the soils are cooler and outside the optimum temperature range that is conducive to *P. thornei* reproduction [63,64]. These cooler soil conditions are desirable for growers but not for researchers. For research purposes, by sowing in the later part of the window, the higher reproductive rates and the increased activity of *P. thornei* mean plants are under more disease pressure. This would contribute to both NDVI and TR having higher genetic correlation with grain yield than the earlier sown experiments (mean sowing date was June 10). However, the later sown crops are more likely to experience heat and water stress during flowering and damaging weather events that impact grain yield. If grain yield is impacted by stress other than *P. thornei*, in-season assessments by TR and NDVI can be better estimates of tolerance than grain yield. On the other hand, if the objective is to demonstrate the potential of *P. thornei*-tolerant genotypes to produce maximum grain yields for growers, then earlier sowing times are preferred.

Pratylenchus thornei reduces the biomass, yield components, and yield of intolerant wheat genotypes [8]. When an intolerant genotype was grown on high compared to low

populations of *P. thornei*, the NDVI of the plant canopy was reduced [14]. The reduction in NDVI commenced at 50 days after sowing [14], and plant growth declined between 50 and 70 days after sowing [9]. However, the predictiveness of NDVI can be limited when the canopy cover exceeds 80%, as there is then no relationship between biomass and NDVI [65]. In these circumstances, it would be worth investigating normalized difference red edge (NDRE) or other vegetation indices (VIs) as substitutes. For instance, NDRE outperformed NDVI at the flowering stage of wheat using an unmanned aerial vehicle (UAV), and it is reported that using a combination of different vegetation indices, as opposed to relying on just one index, enhances the predictability for yield [66].

Despite TR by the ETA being more predictive than NDVI of grain yield, and hence genotype tolerance, the subjectiveness of TR means there will always be more potential for error surrounding this assessment type [28,67]. To improve the accuracy of visual assessments, illustrations of disease severity called standard area diagrams (SADs) can be used as aids to assist with disease severity assessments [68]. For *P. thornei* tolerance, there is no SAD, but Table 1 details each of the tolerance categories with respect to the symptoms that are likely to be visible in the field. Inclusion of a check (control) genotype for each of the nine *P. thornei* tolerance categories in every experiment provides an in situ SAD for *P. thornei* tolerance assessors to train or 'fine-tune' ratings based on actual crop appearance at the assessment time. This meets some of the best-operating procedures [29]. In our study and that of Bock et al. [29], there was consistently a reduction in the genetic correlation (or accuracy) with grain yield when the experiment was assessed by an ITA compared with an ETA. More training would likely improve the accuracy of the inexperienced assessor [29,58,69], but subjective errors are not eliminated. In addition, the cost and time required to train the assessors need to be considered when implementing visual tolerance ratings. In comparison, the handheld Greenseeker™ is a device requiring very limited experience on the part of the operator, while the instrument costs approximately AUD 1000. Our NDVI assessments were achieved at a similar or faster speed than trial ratings by an ETA.

The normalized difference vegetation index provides an objective assessment of the canopy, without discriminating whether the changes in the canopy were caused by abiotic or biotic stresses affecting the physiology of the plant. Our study showed that NDVI1 had greater genetic correlation with grain yield than NDVI2 had for both the TOS1 and TOS2 experiments. On the other hand, for both TOS1 and TOS2 experiments, the genetic correlations between TR1 or TR2 and grain yield were similar and thus less reliant on assessment timing than NDVI. From two experiments, Robinson et al. [14] found that when NDVI was assessed at CTT in the range from 695 °Cd to 1538 °Cd, the relationships between grain yield and NDVI were strong ($R^2 > 0.8$), and that a single NDVI reading at ~1000 °Cd would be predictive of tolerance based on grain yield, provided the experiments were grown on population densities greater than 2500 *P. thornei*/kg soil. Furthermore, the R^2 values were the greatest at a CTT of 1159 °Cd and 695 °Cd for Experiments 1 and 2, respectively [14]. In the present study, for both sowing times, the average CTT for NDVI1 and NDVI2 was 1073 °Cd and 1350 °Cd, respectively, and thus at the later part of the effective sensing window [14]. This reinforces that NDVI is sensitive to not only the damage caused by *P. thornei* but also confounding influences like plant growth stages, maturity or stay-green in wheat [30], and other diseases that reduce canopy greenness [38]. The advantage of visual tolerance ratings is that allowances can be made by the assessor to reduce any other influences on canopy growth, like maturity or stay-green or plant architecture, that NDVI is not able to do. But, NDVI has the advantage of providing tolerance estimates earlier in the growing season, a factor likely to be desirable in a commercial wheat breeding program. To further improve decision capabilities, more research is required to investigate vegetation indices other than NDVI and determine whether UAV could be used to improve data capturing efficiency for experiments with thousands of plots.

Our study demonstrated that TR and NDVI are robust in-crop methods that predict the grain yield of wheat genotypes belonging to the QComm, AComm, QABL, and AABL

groups ($p < 0.001$ for TR and NDVI for these groups). The correlations between TR or NDVI with grain yield were the greatest for the commercial genotypes recommended for production in Queensland (QComm) compared to the other groups of genotypes. Our methods (TR and NDVI) also reliably assessed genotypes ($p < 0.001$) that are not recommended for production in Queensland (AComm) and the advanced breeding lines (AABLs) where the anticipated production region is not confirmed. However, this was not the case for the genotypes belonging to the ASlow group (these genotypes take a very long time to mature and thus are not suitable for production in Queensland). Our methods reported here offer all Australian wheat breeders an opportunity to screen their spring wheat germplasm for tolerance to *P. thornei*.

This study found that TR1 and NDVI1, and TR2 and NDVI2 were significantly correlated with each other, but each of these was poorly correlated to grain yield for the genotypes that derived from a synthetic parent, CPI133872 (QPBL). CPI133872 is more resistant to *P. thornei* than other sources derived from common wheats [70]. The introgression of a synthetic parent into common wheats may result in considerable genetic drag of undesirable traits that limit their yield potential [71]. In our study, the least desirable trait is the reduced yield potential compared to the current commercial and advanced breeding lines that were also in this study. Despite this, the high visual tolerance ratings and high NDVI scores indicate these genotypes were tolerant and could be effectively selected using either TR or NDVI. Not only do these synthetic derived genotypes offer novel sources of resistance but they also potentially offer new sources of genetic tolerance to *P. thornei*. Further targeted backcrossing of these genotypes with commercial genotypes is required to improve their yield performance in this region.

Another objective of this study was to determine a robust method that researchers and breeders could use to screen hundreds or thousands of genotypes without measuring grain yield. To facilitate this, nine alpha categories, as reported by Thompson et al. [12], were determined for the QComm genotypes by grain yield, NDVI, or TR in this study. There was a mean yield loss of 6.5% between the successive tolerance categories and a total yield loss of 52% between the mean of the tolerant and the mean of the very intolerant groups. To compare this with a similar study that included a wider range of genotypes, the reduction in grain yield between successive tolerance groups was 7.5%, with a total yield loss of 60% between the tolerant and the very intolerant categories [12]. For the other methods in our study, the total reductions in NDVI1, NDVI2, TR1, and TR2 values were 28, 31, 60, and 63%, respectively. Although there is almost a two-fold difference in the total percentage reduction between NDVI and TR, the correlations with grain yield were similar at 0.83 and 0.90 for NDVI and TR, respectively.

Tolerance should always be measured using grain yield when disseminating information to growers to select the best genotypes to grow in their fields [12]. However, our results demonstrate that NDVI and TR are suitable in-crop methods that wheat breeders could use to select genotypes that are tolerant to *P. thornei* without needing to harvest grain. To further improve both methods, one genotype from the QComm group for each of the nine tolerance categories was identified that ranked similarly no matter whether tolerance was assessed by grain yield, NDVI, or TR. These nine genotypes are suitable reference or check genotypes that should be included in every trial to measure tolerance fully and reliably, independent of what assessment method is being used. These genotypes will add value to each experiment by representing the full range of tolerance ratings from very intolerant to tolerant for each assessment method and providing the assessor with an in situ SAD to refine their tolerance ratings at each time of observation. The results of this study showing that the vegetative assessment of tolerance of these check genotypes by either NDVI or TR is predictive of tolerance assessed by grain yield will increase the confidence of researchers in their use in screening experiments.

5. Conclusions

This study demonstrated that normalized difference vegetation index (NDVI) and visual tolerance ratings (TRs) are effective in-crop methods for wheat breeders to identify genotypes that are tolerant to high population densities of *P. thornei* without the need to harvest plants to determine tolerance by grain yield or to grow experiments on low and high *P. thornei* population densities. The advantages of this approach to tolerance testing are (i) the reduced costs, (ii) the reduced land area required, (iii) the use of in-season data to aid selection of tolerant genotypes, and (iii) the fact that the experiments can be sown later when the soil temperature is more conducive to rapid nematode reproduction and higher disease pressure. Our research showed both vegetative methods to be robust and well suited to test diverse genotypes that are commercially available, are breeding germplasm or pre-breeding lines, and that have an adapted or unadapted parent. Nine genotypes were identified that represent the complete range of tolerance levels and will score comparably irrespective of whether tolerance is determined by grain yield, NDVI, or TR.

These methods offer wheat breeders a robust phenotyping platform that has a high throughput and incurs less cost. For NDVI and TR to be effective methods, genotypes need to be grown on high population densities (>2000 *P. thornei*/kg soil), to be sown towards the later part of the normal planting window within a region, and to include check genotypes that represent each of the nine tolerance categories from very intolerant to tolerant. An additional requirement of NDVI is that assessments are conducted prior to leaf senescence as the crop matures. Our study successfully screened a diverse range of commercially released genotypes and advanced breeding lines suited to Queensland and other Australian cropping areas, and genotypes that were hybridized with a *P. thornei* resistant synthetic hexaploid parent. Genotypes that are tolerant are highly desired by growers to minimize the yield losses caused by *P. thornei*, and our study identified NDVI and TR as two robust methods that plant breeders could use to select tolerant genotypes in their programs.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14123043/s1, Figure S1: A genetic correlations heat map for the first (TOS1) and second (TOS2) times of sowing (TOSs) conducted from 2011 to 2019. The genetic correlations are derived pairwise among grain yield, normalized difference vegetation index (NDVI), and visual ratings (TRs). For each NDVI and TR, the number suffix 1 or 2 indicates the first or second reading taken in each experiment. Table S1: Daily maximum and minimum air temperature (°C) for each of the experimental years. Data were acquired from the Queensland Government weather station located at Dalby (Dalby Post Office, weather station ID: 41023) [49].

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Abbreviations

AABL	Australian advanced breeding line
AComm	Australian commercial
AIC	Akaike information criterion
ANOVA	Analysis of variance
ASlow	Australian slow
BD	Bulk density
CLL	Crop lower limit
CR	Cumulative rainfall
CTT	Cumulative thermal time
DAS	Days after sowing
eBLUP	Empirical best linear unbiased prediction
ETA	Experienced tolerance assessor
FA	Factor analysis
GRDC	Grains Research and Development Corporation
GWC	Gravimetric water content
I	Intolerant
ITA	Inexperienced tolerance assessor
MET	Multi-environment trial
MI	Moderately intolerant
MT	Moderately tolerant
NDVI	Normalized difference vegetation index
PAW	Plant available water
QABL	Queensland advanced breeding line
QComm	Queensland commercial
REML	Residual maximum likelihood
T	Tolerant
TOS	Time of sowing
TR	Tolerance rating

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