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ORIGINAL ARTICLE

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Rapid phenotyping of adult plant resistance in barley (*Hordeum vulgare*) to leaf rust under controlled conditions

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Abstract

Breeding for adult plant resistance (APR) is currently impeded by the low frequency of annual field-based testing and variable environmental conditions. We developed and implemented a greenhouse-based methodology for the rapid phenotyping of APR to leaf rust in barley to improve the efficacy of gene discovery and cloning. We assessed the effects of temperature (18 and 23°C) and growth stage (1–5 weeks) on the expression of APR in the greenhouse using 28 barley genotypes with both known and uncharacterized APR. All lines were susceptible in week 1, while lines carrying *Rph20* and several with uncharacterized resistance expressed resistance as early as week 2. In contrast, lines lacking *Rph20* and carrying either *Rph23* and/or *Rph24* expressed resistance from week 4. Resistant phenotypes were clearest at 18°C. A subset of 16 of the 28 lines were assessed for leaf rust across multiple national and international field sites. The greenhouse screening data reported in this study were highly correlated to most of the field sites, indicating that they provide comparable data on APR phenotypes for screening purposes.

KEYWORDS
adult plant resistance, barley, leaf rust, phenotyping, *Puccinia hordei*, resistance screening

1 | INTRODUCTION

Barley is the fourth most important cereal crop worldwide by area harvested and yield (FAOSTAT, 2015). Leaf rust of barley (caused by *Puccinia hordei* Otth.) is an economically significant disease in the major cereal production regions worldwide (Clifford, 1985). In Australian barley crops, leaf rust is the most common and damaging of the rust diseases (Park et al., 2015). It has been estimated to cost Australian barley growers $21 million per annum, with yield losses of up to 62% in untreated susceptible varieties (Cotterill, Rees, Platz, & Dillmacky, 1992; Murray & Brennan, 2009). Economic and ecologically sustainable control of leaf rust can be achieved through the use of resistance genes (Golegaonkar, Park, & Singh, 2009). To date, 26 leaf rust resistance (*Rph*) genes have been designated. Two classes of *Rph* genes are recognized: all stage resistance (ASR) genes (*Rph1-19*, *21*, *22*, *25* and 26) and adult plant resistance (APR) genes (*Rph20, 23* and *24)* (Kavanagh, Singh, Bansal, & Park, 2017; Park et al., 2015; Ziems et al., 2017). Lines that carry APR genes are seedling susceptible, but as adult plants show a medium to strong reduction in the number and size of pustules (Smit & Parlevliet, 1990). Many APR genes have demonstrated their ability to provide durable resistance to cereal rusts, for example, gene *Lr34/Yr18/Sr57/Pm18* in wheat has provided pleiotropic resistance against multiple pathogens for over 50 years (Dyck, 1987; Dyck & Samborski, 1982; Krattinger et al., 2009).

Of the currently designated *Rph* genes, only three confer APR. The main limitations that prevent rapid characterization and use of barley APR genes include reliance on seasonal field trials and

phenotypic variation due to fluctuations in temperature and disease pressure. The plant response to disease under field conditions is dependent on variable environmental conditions, decreasing the reliability of year to year comparisons (Riaz, Periyannan, Aitken, & Hickey, 2016). At present, most phenotyping for APR is conducted in the field annually over successive seasons (Singh, Dracatos, Derevnina, Zhou, & Park, 2015). This is a very slow process in comparison with the greenhouse seedling trials used for ASR gene discovery and characterization. A typical germplasm survey for APR sources requires repeated measurements across different locations and field seasons to elucidate useful APR sources. This process is currently limited by the annual turnover of standard in-field testing (Dracatos, Singh, Bansal, & Park, 2015),

In order to overcome the limitations associated with screening germplasm for the presence of APR in the field, greenhouse-based screening of adult plants can be used. The efficacy and speed of this approach has been demonstrated in several studies in diverse pathosystems. Pretorius, Park, and Wellings (2000) utilized accelerated growth conditions to produce flag leaves 28 days after sowing in wheat for testing APR against leaf rust (caused by *P. triticina*) and stripe rust (caused by *P. striiformis* f. sp. *tritici*) in the greenhouse. In a similar study, Riaz et al. (2016) utilized greenhouse accelerated growth conditions to screen for novel APR phenotypes in wheat to leaf rust (*P. triticina*). Similar methodology was used by Wallwork, Butt, and Capio (2016) to screen a significant number of barley accessions in the greenhouse under accelerated growth conditions to assess APR responses to *Pyrenophora teres* f. sp. *teres*, the causal agent of net form net blotch.

While there has been some attention paid to greenhouse APR screening for rust diseases in wheat, by comparison barley rust diseases have received little attention. Several studies have focussed singly on the APR gene *Rph20*, utilizing greenhouse screening in parallel to field measurements. To map *Rph20*, a combination of field and greenhouse screening was used. The greenhouse screening was shown to accurately reflect field screening, with similar QTL results (Hickey et al., 2011). A separate study was conducted to test the expression of *Rph20* in the greenhouse at early growth stages in a range of barley genotypes. Separate cohorts were rust tested at 1-week intervals for 5 weeks under standard greenhouse conditions. The *Rph20* phenotype was reliably observed in the 5-week cohort, while some backgrounds clearly expressed the *Rph20* phenotype at 3–4 weeks (Singh, Macaigne, & Park, 2013). While there has been some effort made to study *Rph20*, it is unknown whether *Rph23*, *Rph24* or other uncharacterized sources of APR can be screened reliably in the greenhouse at early growth stages. In this study, we aimed to ascertain whether this greenhouse methodology could be applied to known and uncharacterized APR genes. If such resistances can be phenotyped in a greenhouse setting, this methodology will enable more rapid screening of germplasm for the presence of APR and hence gene discovery and cloning in barley.

2 | MATERIALS AND METHODS

2.1 | Plant and pathogen material

A panel comprising 28 barley lines was assembled to characterize APR to leaf rust under field and greenhouse conditions. These lines were selected from Australian and international breeding lines and cultivars, and included selected reference genotypes carrying known APR genes (Table 1).

The pathogen material used for Australian field and greenhouse testing was pathotype 5457 P+ (virulent on *Rph1-4*, *6*, *9*, *10*, *12*, *19* and *25*). A standard isolate of this pathotype (culture number = 612) is maintained at PBI, Cobbitty, New South Wales (NSW). The pathotype used in Uruguay for field screening was UPh3 (virulent on *Rph1-5*, *Rph9-12*), maintained at the Instituto Nacional de Investigación Agropecuaria (INIA), La Estanzuela, Colonia, Uruguay.

2.2 | Seedling APR testing

Two seeds of each line were sown in individual cells of an 8 × 5 cell seedling trays in a mixture of bark fines and coarse sand and fertilized at sowing using Aquasol® (Hortico Pty Ltd, Revesby, NSW, Australia) (100 g/10 L H_2O). Twenty seedling trays were sown simultaneously and maintained at 18°C in a rust free greenhouse growth room. Four seedling trays were taken at weekly intervals for inoculation, two trays for each of the temperature treatments. Inoculation was conducted in a closed inoculation chamber. Urediniospores (10 mg/10 ml) were suspended in light mineral oil (Isopar L, Univar, Ingleburn, NSW, Australia) and sprayed above seedlings using an atomizer with a hydrocarbon propellant pressure pack. After 5 min, seedlings were moved from the inoculation room to a dark incubation chamber maintained at 100% humidity by an ultrasonic humidifier for 12–18 hr. Plants were maintained at either 18 or 23°C in separate greenhouse microclimate chambers. Disease response was measured 9 days post-inoculation using a modified Stakman "0–4" scale (Park et al., 2015). Both plants in each well were scored separately as were the two trays in each temperature treatment. Greenhouse scores were converted to a "0–9" scale for analysis (Ziems et al., 2014) (Figure S1). Variations in infection type (IT) were indicated using "+" (higher response than average for that class), "–" (lesser response than average for that class), "c" (chlorosis present) and "n" (necrosis present).

2.3 | Field APR testing

From the panel, 16 lines were selected for field testing (Table 1) across five locations in 2016: La Estanzuela, (Uruguay), Cobbitty (NSW; 2 sites), Gatton (QLD) and Toowoomba (QLD). Each line was sown as a block as part of large scale field trials. The lines were scored again in 2017 at one site in Cobbitty NSW. Field scoring in NSW and Uruguay was conducted using the modified Cobb scale (Peterson, Campbell, & Hannah, 1948), which was converted to a Coefficient of Infection score (CI) by multiplying the modified Cobb

TABLE 1 Summary of barley leaf rust APR and ASR gene information and pedigree for germplasm panel

Cultivar[a]	APR gene(s)[b]	ASR gene(s)[b]	Identifier[c]	Pedigree
Baronesse[+]	*Rph20+Rph24* (1)	–	PI 568246	Mentor/Minerva//Vada mutant/4/Carlsberg/Union//Opavsky/Salle/3/Ricardo/5/Oriol/6153P40
Barque[+]	*Rph20* (2)	*Rph2+Rph12*	–	Triumph/Galleon
Beecher	*Rph23* (2)	–	–	Atlas/Vaughn
ISR950.11	*Rph23+Rph24* (3)	–	–	Canadian breeding line
Clho9776	Uncharacterized APR (4)	–	AUS490069	Moroccan landrace
CPI 36396[+]	*Rph24* (3)	–	–	Not known
Flagship[+]	*Rph20* (2)	–	–	Chieftain/Barque//Manley/VB9104
Franklin[+]	Uncharacterized APR (2)	*Rph12*	–	Shannon/Triumph
Fumai 8[+]	Uncharacterized APR (5)	–	–	76-22///Zaoshu 3//Humai 1/8-2
Gairdner[+]	*Rph23* (2)	–	–	Onslow//Shannon/Triumph
Henley	*Rph20+Rph24* (2,3)	*Rph3*	–	Not known
Klimek[+]	*Rph20+*? (2)	*Rph9.am*	–	–
Lenka	*Rph20+Rph23+Rph24* (1)	*Rph3*	–	HVS-5013-74/Q-496-72
Morex J	Uncharacterized APR (3)	–	–	Cree/Bonanza
Nagrad[+]	*Rph20+*? (5,3)	–	–	RPB393173/Georgie
Tallon[+]	Uncharacterized APR (3)	–	AUS 406324	Triumph/Grimmet
Volla[+]	*Rph20+Rph23* (6)	*Rph3*	PI 280423	Breuns Wisa/Heines Haisa 1
YAN90260[+]	Uncharacterized APR (5)	–	–	Chinese breeding line
YAN90260XBaronesse F34632	*Rph20* + ? (3)	-	-	YAN90260/Baronesse F3 Line
YAN90260XBaronesse F34741	*Rph20+*? (3)	–	–	YAN90260/Baronesse F3 line
Yerong[+]	*Rph23* (3)	*Rph2*	–	M22/Malebo
YF11	–	–	–	Yerong/Franklin DH line
YF229	–	–	–	Yerong/Franklin DH line
YF291	–	–	–	Yerong/Franklin DH line
YF70	–	–	–	Yerong/Franklin DH line
Zhoungdamei[+]	Uncharacterized APR (5)	–	–	Chinese landrace
Zug 161[+]	Uncharacterized APR (5)	–	–	Breeding lines from Zhejiang University, China
Gus[+]	–	–	PI494521	–

Note. APR: adult plant resistance; ASR: all stage resistance.

[a]Lines selected for field testing denoted by + superscript.

[b]Cultivars with unknown or nil resistance genes are represented by a dash, gene source reference in brackets.

[c]Genotype identifier prefix; AUS—Australian Winter Cereals Collection number. PI—US National Small Grains Collection number.

References: 1: Drijepondt et al. (1991), 2: Kavanagh et al. (2017), 3: Singh & Park unpublished, 4: Smit and Parlevliet (1990), 5: Singh et al. (2015), 6: Hickey et al. (2011).

score severity value (0–100) by the severity of the infection (0.10, 0.25, 0.50, 0.75 or 1.00 for host response ratings of R, MR, MR–MS or S, respectively) (Pathan & Park, 2006). Field scoring in Queensland was conducted using the Resistance Index (RI) 0–9 scale where 0 = immune and 9 = totally susceptible (Akhtar et al., 2002). RI scores were converted to the CI scale through multiplication by a factor of 10 (Figure S2).

2.4 | Chitin assay and histology

The fungal biomass was determined in third leaves of the barley genotypes "Zhoungdamei", "Gus", "Zug161", "Volla", "Tallon", "Flagship", "ND24260", "Gairdner", "Baronesse" and "RAH1995" with the method described in Ayliffe et al. (2013). Infected parts were cut from three different plants (replicates) of each barley genotype maintained at 18°C 10 days after inoculation and placed individually in 15 ml Falcon tubes. The leaf material was weighed and 1 M KOH added to fully cover the leaves. Samples were left for clearing at room temperature for 48 hr with a change of KOH after the first 24 hr. The leaves were then washed twice in 50 mM Tris-HCl (pH = 7) buffer for neutralization. Fresh Tris-HCl was added to a concentration of 50 mg leaf per ml. The leaf material was homogenized by sonication. Leaf homogenate (200 µl per sample) was added to PCR tubes with three technical replicates per biological replicate. 10 µl of WGA-FITC (1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) was added to each tube. Samples were spun at 184 *g* for 2 min and the

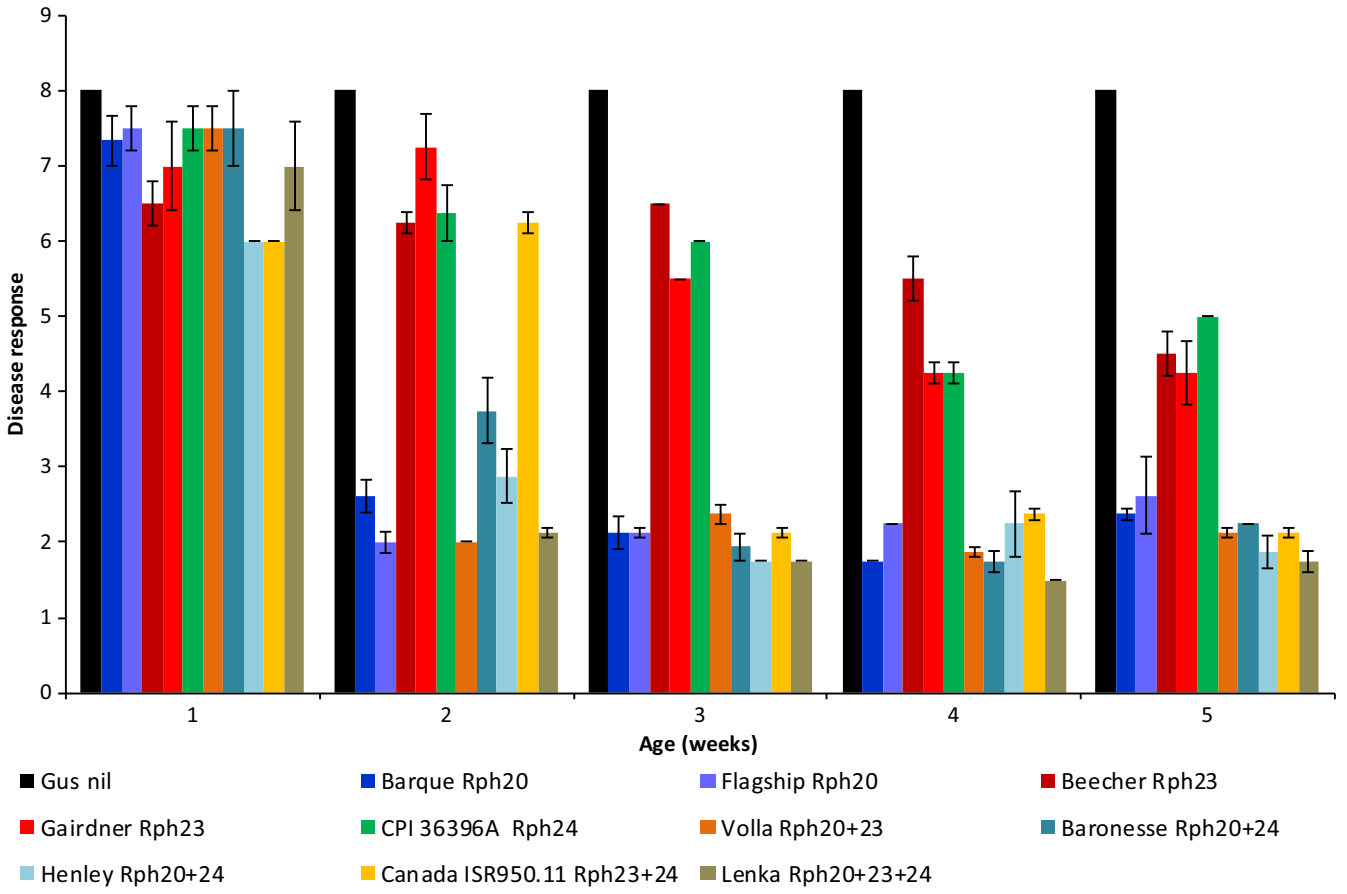

FIGURE 1 Mean disease response of characterized adult plant resistance lines to leaf rust under controlled greenhouse (18°C treatment). Gus is included as a susceptible control. Error bars are the standard error of the mean [Colour figure can be viewed at wileyonlinelibrary.com]

supernatant removed. Samples were resuspended in 200 µl 50 mM buffer, spun and again the supernatant was removed. This washing step was repeated three times. Samples were then transferred to black 96-well plates suitable for fluorometry. After the first run samples were diluted 4x within linear range of the standard curve as described in Ayliffe et al. (2013), fluorescence was measured with a Wallac Victor 1420 multilabel counter (Perkin-Elmer Life Science, MA, USA) using 485 nm adsorption and 535 emission wavelengths and a 1.0 s measurement time. Leaf samples for histological observations were collected and cleared in the same way as described for the biomass assay. The staining method followed that described in Ayliffe et al. (2011). In brief, the KOH was poured off after clearing and samples were neutralized in 50 mM Tris-HCl (pH = 7) buffer. New buffer was added to fully cover the leaves. WGA-FITC (1 mg/ml) (Sigma-Aldrich) was added to a final concentration of 20 µg/ml Tris-buffer in order to stain the fungal structures. Samples were then rinsed in buffer and mounted for microscopy. The specimens were examined under blue light with a Zeiss Axio Imager 2 microscope.

2.5 | Statistical methods

Statistical analyses were conducted using Genstat 18th Edition (VSN International Ltd). A linear mixed model (REML) was used to investigate interactions between experimental effects in the greenhouse. Converted CI scores from the greenhouse and field trials were evaluated using a fitted multiple linear regression model to assess the relationship between field and greenhouse response. A correlation contingency table was generated using the greenhouse and field scores. A principal component analysis (PCA) was performed and visualized using a biplot. Analysis of the data from the chitin assay was performed using R (R Development Core Team, 2017). A one-way ANOVA model was utilized for analysing the general effect of the explanatory factor "variety" on the dependent variable "fluorescent units." Model assumptions were evaluated graphically and data were log-transformed to meet the assumptions of equal variance and normality. Multiple comparisons were performed using a Tukey honestly significant differences test.

3 | RESULTS

3.1 | Greenhouse screening

The timing and expression of APR under greenhouse conditions in the barley accessions used in this study was dependent mainly on the specific genotype and gene combination present. A REML analysis found a significant interaction between plant growth stage and gene combination ($p < 0.001$), reflecting the clear groupings of genotypes by their gene combination and variable effects of each gene

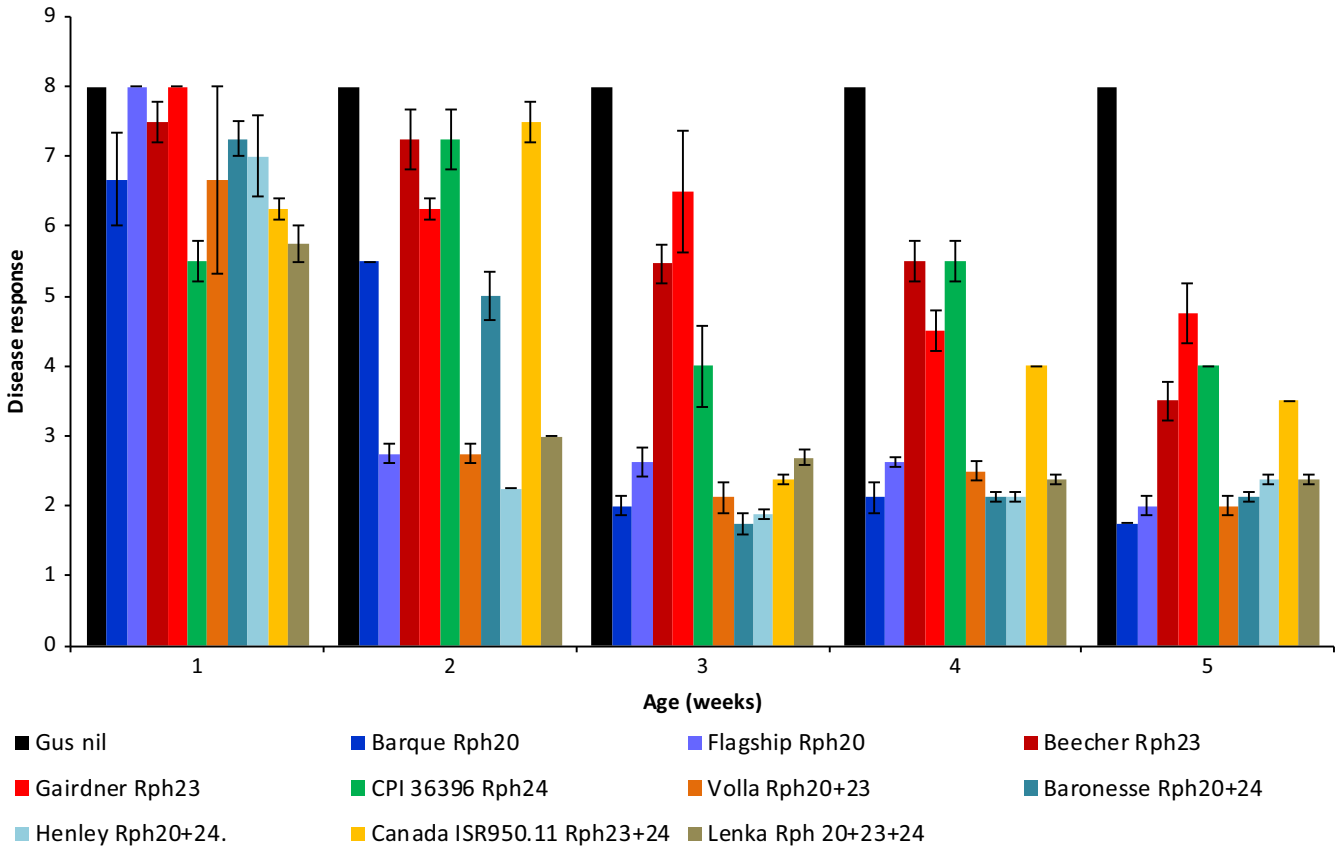

FIGURE 2 Mean disease response of characterized adult plant resistance lines to leaf rust under controlled greenhouse (23°C treatment). Gus is included as a susceptible control. Error bars are the standard error of the mean [Colour figure can be viewed at wileyonlinelibrary.com]

across the growth stage of the plants tested. Out of the 18°C treatment group, eight of the 10 characterized APR lines showed susceptibility (IT score 7–9) at the seedling stage (Figures 1 and 2). Both Beecher and Henley produced IT score 6, outside the susceptible range, indicating that there may be minor effect partial resistance genes present in addition to the known APR genes or epistasis between ASR and APR genes. The susceptible standard Gus was susceptible at all growth stages tested. Lines with *Rph20* were seen to express resistance at earlier growth stages than lines without *Rph20*. All lines carrying *Rph20* were classed as resistant in the second week with IT scores in the range of 2–4. Lines lacking *Rph20* (Beecher, Gairdner, CPI 36396A, Canada ISR950.11) had a higher disease response (IT scores of 6–7) in the second week. The leaf rust response of lines carrying *Rph20* was consistent (IT score of 2–3) across weeks 2–5. The one exception was the cultivar Baronesse, which had an IT score of 4 in the second week. Non-*Rph20* lines produced high IT scores (6–7) in the second week. Only one line included in this study (Canada ISR950.11) carried both *Rph23* and *Rph24* in combination. Our data suggested that when both genes are present singly that resistance was expressed in later weeks and was more pronounced at 23°C, however Canada ISR950.11 was more resistance suggesting a possibly additive effect of *Rph23* and *Rph24*. In the 23°C treatment, the IT scores were higher for all gene combinations while still exhibiting the same trends (Figures 3 and 4).

The lines with uncharacterized APR components exhibited a wide range of leaf rust responses. Lines carrying *Rph20* in addition to an uncharacterized gene exhibited high levels of resistance, similar to the resistance observed in lines carrying only *Rph20*. Only one line carrying *Rph23* plus additional uncharacterized APR was assessed, which exhibited moderate (IT score 5 in week 5) resistance. The remaining lines with uncharacterized APR showed a wide range of leaf rust responses, from moderately susceptible to highly resistant.

3.2 | Correlation of field and greenhouse disease scores

The correlation between field scores and greenhouse screening was calculated using a fitted multiple linear regression models (Table 2). The majority of the sites had high correlation values (R^2 = 0.65–0.77), with the two outliers being Gatton (R^2 = 0.51) and Toowoomba (R^2 = 0.36). Field sites within NSW and Uruguay were better correlated to the greenhouse scores than those in Queensland (Gatton and Toowoomba). A correlation contingency table was generated (Table 3) to assess how the greenhouse scores correlated to each field site and to compare how well this fitted with the correlation between different field sites. The correlation values between both temperature treatments and the field site scores reflected the variation in correlation seen among the different field treatments.

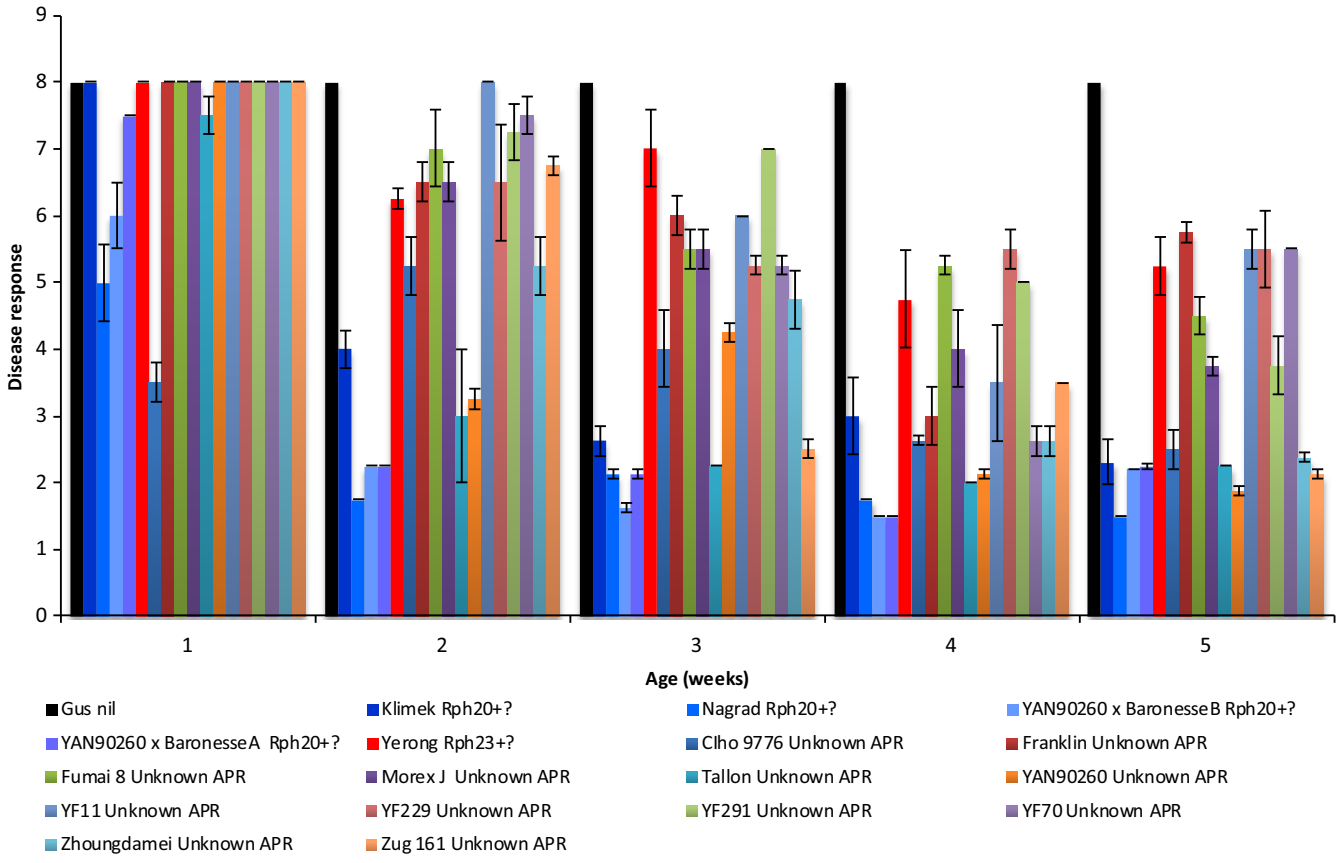

FIGURE 3 Mean disease response of uncharacterized adult plant resistance (APR) lines to leaf rust under controlled greenhouse (18°C treatment). Gus is included as a susceptible control. Lines with a question mark (?) are hypothesized in prior studies (see Table 1) to carry additional uncharacterized APR. Error bars are the standard error of the mean [Colour figure can be viewed at wileyonlinelibrary.com]

The field sites with the lowest correlation values were again Toowoomba and Gatton, in addition to Cobbitty B (2016) (Table 3).

A PCA biplot was generated to assess the correlation between different field sites and greenhouse treatments (Figure 5). The horizontal axis (PC-1 73.5%) accounted for the majority of the variation between the individuals. The susceptible control genotype Gus is at the right extreme of the horizontal axis, while the most resistant lines, primarily comprised of lines carrying *Rph20*, are located on the opposite extreme as expected. The field sites are displayed as the biplot axes, with the most closely correlated axis being those with the smallest angle between them. The two greenhouse temperature treatments (GH_18 and GH_23) are closely aligned to each other. The most highly correlated field site to the greenhouse treatments is Cobbitty_A_2017. As with the previous regression analysis, the least correlated sites are Gatton and Toowoomba (Figure 5).

3.3 | Fungal biomass and histology

The assessment of APR based on the chitin assays confirmed the strong effect of the resistance gene *Rph20* observed in the greenhouse and field trials (Figure 6). All varieties with *Rph20* either singly or in combinations had a significantly lower level of chitin compared to varieties with *Rph23* and *Rph24*. The variety Flagship with only *Rph20* was as resistant as the varieties with other resistance genes in addition to *Rph20*. The varieties with only unidentified resistance genes showed variable responses as for the IT assessment. The resistance in Tallon equalled that in varieties with *Rph20* whereas Zhoungdamei was level with the most susceptible varieties. The resistance in Zug161 was in between that of Tallon and Zhoungdamei. The varieties with *Rph23* and *Rph24* could not be differentiated from the susceptible control (Gus) based on the assay. Histological observations indicated a correlation between the chitin level and the relative number of colonies with pustule formation at the time of sampling (Figure S3).

4 | DISCUSSION

In order for controlled environment greenhouse screening to capture adult plant response accurately, several criteria must be met. The effect of any ASR needs to be eliminated so as to screen purely for APR genes. It is clear that none of the lines carry ASR effective against the pathotype used for this test given their susceptible scores in the seedling stage (first week) for both temperature treatments. As the effect of ASR was overcome by the pathotype used, the greenhouse methodology used clearly permitted the detection of APR within 5 weeks.

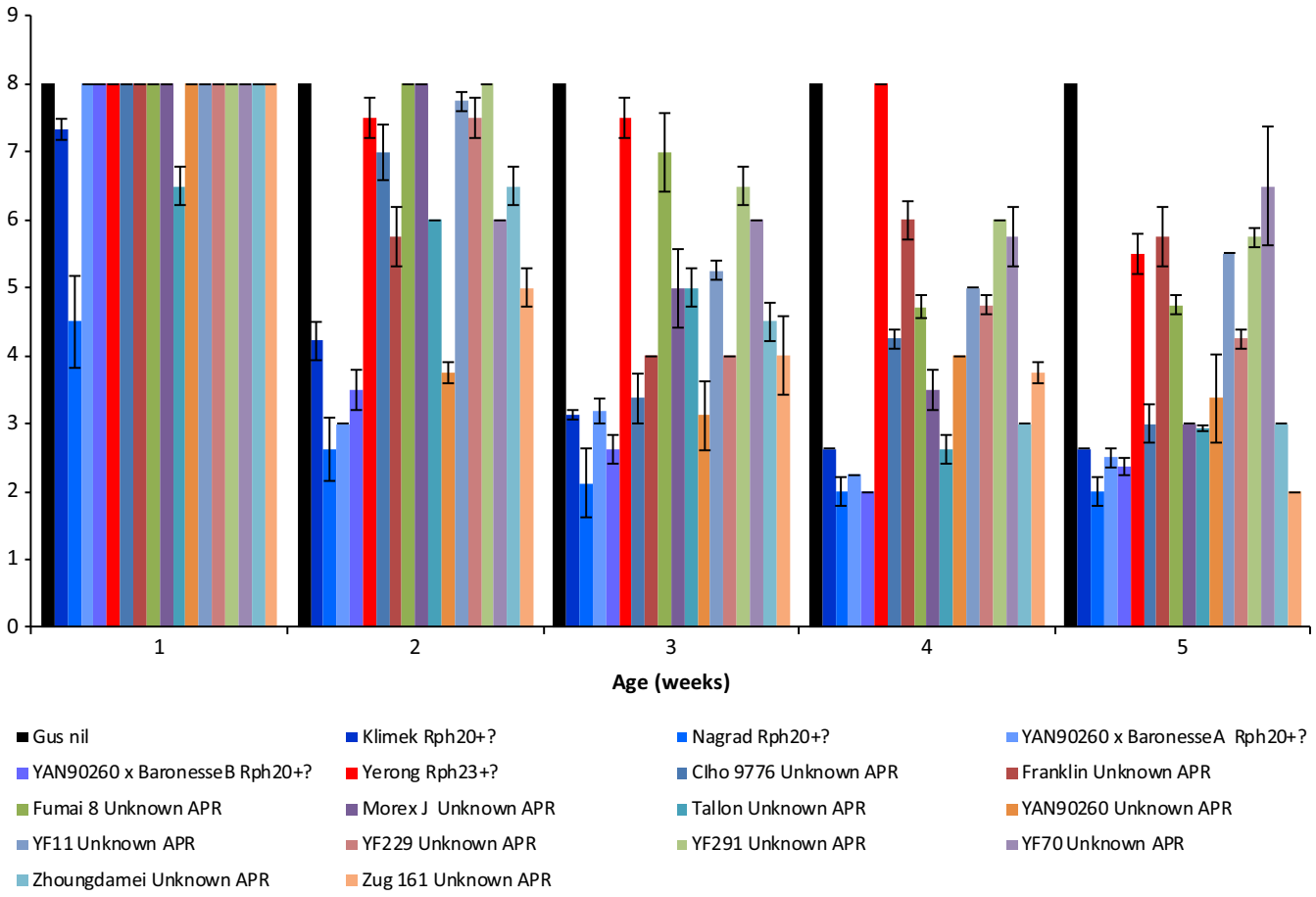

FIGURE 4 Mean disease response of uncharacterized adult plant resistance (APR) lines to leaf rust under controlled greenhouse (23°C treatment).Gus is included as a susceptible control. Lines with a question mark (?) are hypothesized in prior studies (see Table 1) to carry additional uncharacterized APR. Error bars are the standard error of the mean [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Greenhouse adult plant resistance scoring to field scoring regression models correlation values

Field location	Fitted regression model correlation to greenhouse scores (R^2)
Uruguay (La Estanzuela, Uruguay)	0.71
Cobbitty_A (2017) (NSW, Australia)	0.71
Cobbitty_A (2016) (NSW, Australia)	0.65
Cobbitty_B (2016) (NSW, Australia)	0.76
Gatton (QLD, Australia)	0.51
Toowoomba (QLD, Australia)	0.36

In order to classify lines according to the possible gene combination they carry based on disease response, clear variation in phenotype among different genes is required. The differences between the three characterized APR genes were apparent by the second week, particularly in the 18°C treatment. Lines carrying *Rph20* were more resistant at earlier growth stages than those lacking *Rph20* (Figure 1). The additive nature of the characterized APR genes (*Rph20*, *Rph23*, *Rph24*) was also observed in this study, with lines such as Henley (*Rph20+Rph24*) and Lenka (*Rph20+Rph23+Rph24*) showing a stronger resistant response than lines carrying *Rph20* only, such as Flagship. The additive relationship between *Rph20* and a number of characterized and uncharacterized APR genes was described previously by Derevnina, Singh, and Park (2013), while Ziems et al. (2017) demonstrated additivity between *Rph20* and *Rph24*. Our results were in agreement with Ziems et al. (2017); *Rph24* provided a very low level of resistance on its own, however when combined with *Rph20* additivity was observed in all cases. Similarly, additive effects were also observed in lines with gene combinations *Rph20+Rph23* and to a lesser extent *Rph23+Rph24*. An additive effect between *Rph23* and *Rph24* was observed in the line carrying this combination (Canada ISR950.11). This line was more resistant from the third week onward that lines carrying *Rph23* or *Rph24* singly in the 18°C treatment group. This effect was not observed in the 23°C treatment group, indicating that low temperature may increase the additive effect. The combination of all three known APR genes did not provide a higher level of resistance than that conferred by *Rph20* and *Rph24* (Ziems et al., 2017), indicating that the interaction between *Rph23* and *Rph24* is likely masked by presence of *Rph20*. Caution however must be taken interpreting variations in additivity between gene combination lines in this study as they are not near-isogenic and therefore may carry additional minor effect alleles that modify the resistance phenotype. The strong effect of *Rph20* was

TABLE 3 Correlation contingency table for field sites and greenhouse scores

Cob_A_2016_F	1	–						
Cob_B_2016_F	2	0.3807	–					
Cob_A_2017_F	3	0.4261	0.5111	–				
Uruguay_F	4	0.4947	0.4704	0.6802	–			
Gatton_F	5	0.8051	0.7236	0.6269	0.6201	–		
Toowoomba_F	6	0.6670	0.5447	0.5950	0.6424	0.7576	–	
Wk5_18degC_G	7	0.5507	0.2294	0.7026	0.2550	0.5151	0.3354	–
Wk5_23degC_G	8	0.5121	0.3916	0.8517	0.5154	0.6120	0.4165	0.8787
		1	2	3	4	5	6	7

Note. Field site (_F) or greenhouse (_G) data are indicated. Cob_A and Cob_B are two field sites located at Cobbitty, NSW.

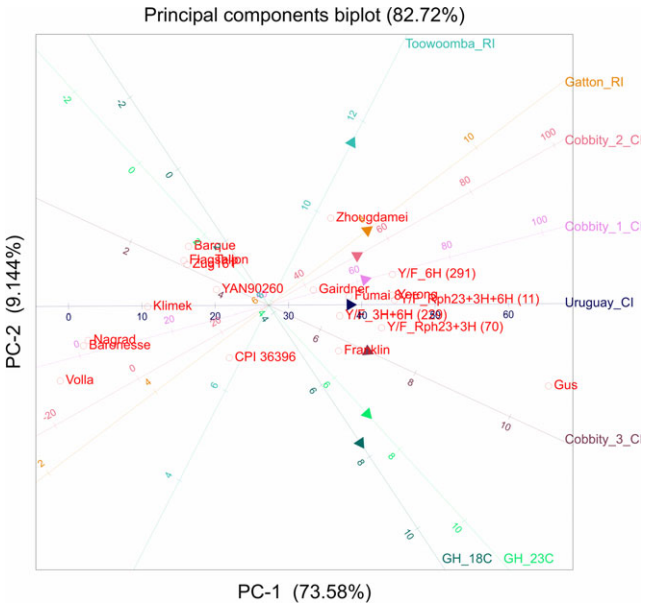

FIGURE 5 Principal component analysis biplot generated from a combined field and greenhouse screening dataset. The individual cultivars are denoted by the labelled red circles arrayed along the horizontal (PC-1, accounting for 73.58% of observed variation) and vertical (PC-2, accounting for 9.14% of observed variation) axes. The field trial sites and greenhouse treatments for all cultivars are represented by the biplot axes intersecting at the centre of the figure. Axes with the smallest angle at the intersection point are the most correlated to each other [Colour figure can be viewed at wileyonlinelibrary.com]

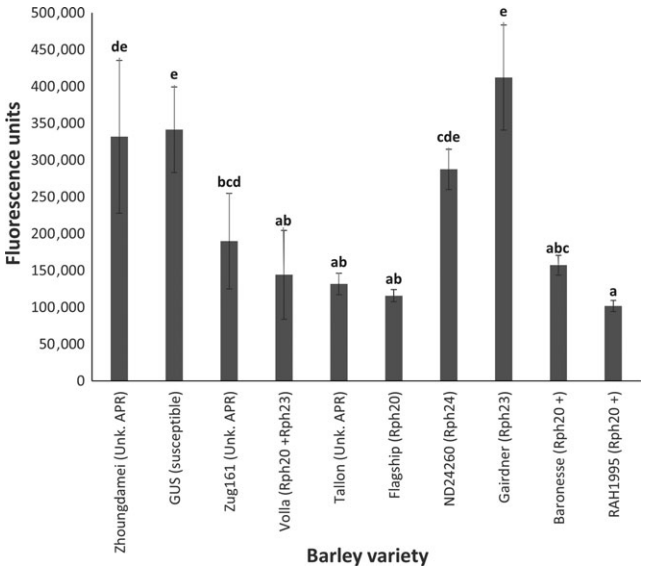

FIGURE 6 The relative level of fungal biomass in 10 barley varieties with either no (susceptible) or different genes for adult plant resistance (*Rph20*, *Rph23*, *Rph24* and unknown APR = unidentified gene[s]) assessed as fluorescent units after chitin staining. Bars with different letters are significantly different at $\alpha = 0.05$. "+" = denotes additional but unknown resistance gene(s) [Colour figure can be viewed at wileyonlinelibrary.com]

also observed when a chitin assay was used for resistance assessment. The additive effect of different genes could not be detected with this method which indicates that chitin assays require support by IT assessment for a more accurate APR evaluations.

Screening lines with all three known APR genes at 18°C provided a stronger, more stable disease response than 23°C. A stronger resistance response at lower temperature has been shown previously for *Rph20* (Singh et al., 2013); however, this effect has not been demonstrated for *Rph23* and *Rph24*. Research on genes conferring APR to rust in wheat also found a relationship between temperature and expression of APR. The expression of the pleiotropic APR locus *Lr34/Yr18/Sr57/Pm18* in wheat was tested at both the seedling and adult stage in the Thatcher background across a range of temperatures in the greenhouse by Singh and Gupta (1992). They found that *Lr34/Yr18/Sr57/Pm18* was most strongly expressed in seedlings at 7°C. Adult plants were most resistant at 15°C in studies by Drijepondt, Pretorius, and Rijkenberg (1991). A similar study using Mexican-derived pathotypes of *P. triticina* saw the strongest expression of resistance in seedlings at post-inoculation temperatures of 14–17°C (Singh & Gupta, 1992). Our study suggests the expression of *Rph20* in barley and other uncharacterized APR genes such as *RphTallon* and *RphZug161* is enhanced under cooler temperatures either when deployed on their own or in combination with other minor APR loci. However, the cooler temperature treatment (18°C) used in our study had no effect on the expression of *Rph23* or *Rph24* either singly or in combination.

A range of disease responses was expressed by the genotypes carrying uncharacterized APR. Several lines exhibited high levels of

resistance, providing similar levels of resistance to lines with *Rph20*. Derevnina et al. (2013) reported that three of the lines used in the present study (Zug161, Zhoungdamei and Yan 90260) carried moderate levels of APR under field conditions. A previous survey of African germplasm found the line CIho9776 exhibited high levels of APR under field conditions, consistent with results obtained in the greenhouse in the present study (Elmansour, Singh, Dracatos, & Park, 2017). A number of other lines carried weaker APR components. The line Fumai8 was among lines with the lowest level of APR, mirroring the field response seen in a previous germplasm survey (Derevnina et al., 2013). The range of APR responses seen in this study are indicative of the spectrum of potential APR sources in international germplasm that could be identified and exploited through utilizing greenhouse APR screening.

The greenhouse response of the lines tested also needs to correlate with their field response in order for this methodology to be truly representative of resistance under field conditions. Multiple linear regression analysis demonstrated a high degree of correlation between field and greenhouse response for the lines tested. The R^2 values for the most highly correlated field sites from this analysis are similar to those found in a similar study conducted on wheat leaf rust (R^2 = 0.77) (Riaz et al., 2016). The measurements in Riaz et al. (2016) were taken at the flag-2 leaf stage under accelerated growth conditions, indicating that scores taken at 5 weeks of age can provide a similar picture to those taken at a much later growth stage in the greenhouse. The correlation analysis also illustrated the accurate reflection of field trial scores by greenhouse APR screening. There were similar levels of correlation between different field sites when the two temperature treatments were compared to each other, indicating that greenhouse screening data provide an accurate approximation of field response, just as one field site will give an approximation of field response at another site. Greenhouse APR screening can thus provide rapid, accurate estimation of APR response under controlled environmental conditions.

The correlation between field and greenhouse scores from the fitted regression model was strongest for the Uruguay and NSW field sites. The two Queensland field sites, Toowoomba (R^2 = 0.36) and Gatton (R^2 = 0.51), were the least correlated. The remaining field sites all had high regression scores, indicating that the greenhouse screening was representative across the range of field sites tested. The Gatton field site had the highest mean maximum daily temperature and lowest rainfall of any field site during the scoring window, which may have had a negative impact on APR gene expression (Table 4). Toowoomba in contrast had similar average temperature and rainfall readings to the southern Australia sites. The only clear difference to the southern field sites is the northern latitude and altitude at Toowoomba, which may have some effect on the APR expression. The trends seen in the fitted regression model were mirrored in the PCA. The field sites that are most closely correlated with the greenhouse testing are La Estanzuela-Uruguay and Cobbitty, particularly Cobbitty_A_2017. The two 2016 Cobbity field sites, especially Cobbity_B, were less well correlated. This is potentially due to the fact that 2016 was a drier field season than 2017 (Table 4). While Cobbity_A is located next to a small river, Cobbity_B is located several hundred metres from water, potentially leading to differing exposure to overnight moisture that could affect disease progress. The two Queensland sites in contrast were again not highly correlated with the greenhouse data.

TABLE 4 Climatic summary for field sites for the month when field scoring took place (October)

Field site	Mean maximum daily temperature (Celsius)	Mean minimum daily temperature (Celsius)	Cumulative rainfall (mm)	Elevation above sea level (m)
Cobbitty 2016	24.4	9.2	18.4	61
Cobbitty 2017	26.1	12	51.2	61
Gatton	28.7	10.6	16	89
Toowoomba	23.7	11.2	29	641
LE, Uruguay	21.2	11.3	111	72

The two greenhouse temperature treatments in the PCA biplot (GH_18°C and GH_23°C) and the correlation contingency table (18°C_Wk5, 23°C_Wk5) are clearly associated to each other, indicating that they are fairly equivalent measures of field response. Therefore, the methodology developed in this study can be seen to capture the effect of novel APR genes both alone and in combination with known APR genes. Given the similar levels of correlation to field response the cooler temperature treatment provides a better platform for APR screening due to the clearer APR phenotype seen at lower temperatures.

The presented APR phenotyping methodology allows for rapid greenhouse-based screening of APR candidate lines under controlled conditions without the temporal and environmental constrains of traditional field screening. Screening APR candidate lines in the greenhouse at 5 weeks post sowing captured APR expression. Maintaining lines post-inoculation at 18°C provided the clearest phenotype. Greenhouse scores were shown to correlate with field measurements from a number of sites. Greenhouse-based screening was thus validated as a representative methodology for APR phenotyping. Taken together the effect of temperature on resistance gene expression has important implications for the deployment of cultivars across different growing environments. The data from this study can be used to propose varietal deployment across diverse barley growing environments as well as greatly improving the rate of APR phenotyping and hence gene discovery.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

AUTHORS' CONTRIBUTIONS

CR contributed to the experimental design and conducted the greenhouse testing, statistical analysis and manuscript composition. DS carried out field testing at Cobbity. FvO was a major contributor to the statistical analysis. RF carried out field testing at Gatton and Toowoomba. CS carried out the histology. SG carried out field testing in Uruguay. RP contributed to the study design and manuscript drafting. PD designed the study and contributed to the manuscript drafting.

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SUPPORTING INFORMATION

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