Investigating *in vitro* mating preference between or within the two forms of *Pyrenophora teres* and its hybrids

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Keywords: Back crosses, Hybridisation, Introgression, Recombination, Reproduction vigour, Sexual reproduction.

ABSTRACT

Net blotch diseases result in significant yield losses to barley industries worldwide. They occur as net-form and spot-form net blotch caused by *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), respectively. Hybridisation between the forms was proposed to be rare, but recent identifications of field hybrids has renewed interest in the frequency and mechanisms underlying hybridisation. This study investigates the mating preference of *Ptt*, *Ptm* and laboratory-produced hybrids *in vitro*, using 24 different isolates and four different experimental setups. Two crosses in our study produced ascospores during two intervals separated by a 32-35 day period of no ascospore production. For these crosses *Ptt* isolates mated with isolates of the same form during the early ascospore production interval and produced hybrids during the later interval. *Ptm* isolates did not mate with isolates of the same form, instead hybridised with *Ptt* isolates. Analyses based on DArTseq™ markers confirmed that laboratory-produced hybrids, when given the choice to mate with both *Ptt* and *Ptm*, mated with *Ptt* isolates. These results unravel a novel concept that *Ptt* seems to have a greater reproduction vigour than *Ptm*, which could lead to increased prevalence of hybrid incidences *in vivo.*

Keywords: Back crosses, Hybridisation, Introgression, Recombination, Reproduction vigour, Sexual reproduction.

Barley net blotches caused by the fungal pathogen *Pyrenophora teres* (syn. *Drechslera teres*) are important foliar diseases resulting in yield losses and reduced grain quality of barley (Smedegård-Petersen 1974). The pathogen exists as two forms: *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), causing net form net blotch (NFNB) and spot form net blotch (SFNB) of barley, respectively. Lesions caused by *Ptt* are characterized by dark-brown narrow, net-like transverse and longitudinal necrotic striations while *Ptm* is distinguished by dark-brown circular to elliptic lesions on the infected leaf sheaths (Smedegård-Petersen 1971). Both forms can co-exist in the same field and there is no clear evidence of morphological or life cycle differences except that *Ptt* can be transmitted through infected seeds while *Ptm* is not known to be seed-borne (Liu et al. 2011; McLean et al. 2009).

Pyrenophora teres reproduces both sexually and asexually. Asexual reproduction of *P. teres* occurs via the production of genetically identical conidia. As *P. teres* is a heterothallic Ascomycetous fungus, two opposite mating types are needed for sexual reproduction (McDonald 1963). Sexual reproduction in *P. teres* is controlled by a single mating type locus (*MAT1*), which exists as two alternative forms or idiomorphs, i.e., *MAT1-1* and *MAT1-2* (McDonald 1963). During successful mating between *MAT1-1* and *MAT1-2* genotypes, fertile ascomata are formed. Ascomata contain asci and each ascus contains four pairs of ascospores with each pair being genetically identical (Finchman 1971). *In vitro* progeny isolates resulting from sexual recombination (sexual reproduction within form) were reported to have a great genetic diversity (Liu et al. 2011; McDonald 1963; McLean et al. 2009) and exhibited different levels of virulence to those of the parental isolates (Afanasenko et al. 2007).

Hybridisation, i.e. successful genetic crossing between non-conspecific individuals, plays an important role in the exchange of genetic material between species. Hybridisation is proposed as one of the major factors shaping the evolution of fungal plant pathogens, which has resulted in the emergence and adaptation of novel crop pathogens (Brasier 2001). In fungi,

hybridisation may occur between species due to both sexual recombination and asexual fusion of hyphae (Kohn 2005). However, hybrids are found to be rare in nature as a result of reduced fitness compared to parental isolates (Stukenbrock 2016) and genetic incompatibilities like Dobzhansky-Muller interaction (negative epistatic interactions) (Kohn 2005; Kondrashov et al. 2002). Occurrence of hybrids, including ascomycetous species in natural conditions indicates that both genetic incompatibilities and reduced fitness could be overcome under certain environmental conditions, potentially enhancing adaptive diversity and accelerating adaptive evolution of crop pathogens (Stukenbrock 2016).

Many population genetic studies have identified *Ptt* and *Ptm* as genetically distinct groups, and sexual reproduction between the two *P. teres* forms, also known as hybridisation, has been reported to be rare (Bakonyi and Justesen 2007; Campbell et al. 2002; Lehmensiek et al. 2010; Leišova et al. 2005b; Leišova et al. 2005a; Rau et al. 2003; Serenius et al. 2005). Seven hybrid isolates have thus far been reported in barley fields, including one each from South Africa (Campbell et al. 2002), Japan (Dahanayaka et al. 2021) and Hungary (Dahanayaka et al. 2021), and two from the Czech Republic (Leišova et al. 2005b) and Australia (McLean et al. 2014; Turo et al. 2021). A hybrid isolate collected from barley fields in Western Australia showed increased resistance to some group 3 fungicides (azole or demethylase inhibitor) and was also found to be rapidly propagated by asexual reproduction suggesting that field hybrid isolates could also be genetically stable, fertile, and potentially possess increased virulence similar to the *in vitro* hybrid isolates (Turo et al. 2021). The recent increase in the identification of hybrids in nature indicates the necessity to broaden the knowledge of the sexual reproduction pattern of this pathogen. Acquiring virulence from both *Ptt* and *Ptm* could lead to the development of complex host and fungicide resistant pathotypes through evolutionary changes, as was recently seen in Western Australia (Turo et al, 2021).

A number of studies have produced hybrid isolates following mating between *Ptt* and *Ptm in vitro* (Campbell et al. 1999; Crous et al. 1995; Jalli 2011; Louw et al. 1995; Smedegård-Petersen 1971). The progeny resulting from these hybrid crosses have produced net-like, spot-like or intermediate disease symptoms described as jagged-type spots on the host (Campbell et al. 1999; Smedegård-Petersen 1971). Laboratory-hybrids retained their fertility and virulence, and were genetically stable under laboratory conditions over the course of years (Campbell and Crous 2003). Some laboratory-hybrids were less sensitive to triazole fungicides compared to parental isolates while others were reported to show virulence patterns different to both the parental isolates, with some hybrid isolates being virulent on barley cultivars on which both parents were avirulent (Jalli 2011). Exchange of genetic material between *Ptt* and *Ptm* due to hybridisation can, therefore, lead to enhanced virulence and novel pathotypes that may overcome available host resistances. This could directly challenge existing disease management strategies which may become ineffective. Thus, having a better understanding of mating preference of *P. teres* would help in predicting the virulence profile of the pathogen and the development of novel disease resistant varieties.

Hybridisation of *Pyrenophora* forms within barley fields may be more prevalent than previously assumed as hybrid isolates may not have been identified in the past due to a lack of an appropriate molecular marker system. For example, isolate CBS 282.31 (collected in 1931 in Japan), previously described as *P. japonica* and recently synonymised with *P. teres* based on multi-locus phylogenetic analyses (Crous et al. 1995; Marin-Felix et al. 2019), was revealed to be a hybrid between *Ptt* and *Ptm* based on genome wide DArTseq™ markers ((Dahanayaka et al. 2021). The availability of molecular markers specific to *P. teres* hybrids (Poudel et al. 2017) provides the opportunity to rapidly and more reliably screen *P. teres* populations for occurrence of hybrids in the field. Interestingly, previously identified field hybrids, including CBS 282.31, H-919 (Dahanayaka et al. 2021) and WAC10721 (McLean et al. 2014) seemed

to be genetically more closely related to *Ptm* than *Ptt*, having a larger percentage of *Ptm* alleles. This would suggest that hybrid isolates themselves more frequently undergo sexual reproduction with *Ptm* rather than *Ptt* in subsequent matings.

In order to assess the prevalence of natural hybridisation between the two forms of *P. teres* in the field, a field study was designed where a barley variety susceptible to both forms was inoculated with *Ptt* and *Ptm* isolates of opposite mating types (Poudel et al. 2018). Results indicated that *Ptt* and *Ptm* isolates preferred to undergo sexual recombination within their respective forms as no hybrids were collected from the field during three years of trials. A number of reasons were given for the lack of sexual reproduction between the two forms, including pre-mating barriers like sexual selection and temporal difference, and post-mating barriers like gametic compatibility (Giraud et al. 2008; Kohn 2005). It was suggested that competition mating assays involving individuals of both *Ptt* and *Ptm* of opposite mating types need to be conducted under laboratory conditions to observe competition in mating within and between forms.

Hence, this study was designed to use molecular assays and genotyping-by-sequencing approaches to i) test the hypothesis that there is a preference of *Ptt* and *Ptm* isolates to undergo sexual recombination within forms rather than hybridising between forms *in vitro*; and ii) investigate whether hybrid isolates have a greater preference for *Ptm* isolates than *Ptt* isolates for mating *in vitro*.

MATERIALS AND METHODS

Fungal material. For this study**,** 10 *Ptt* (NB50, NB29, NB81, NB63, NB85 HRS09127, NB90, HRS11093, NB73 and 97NB1) (Lehmensiek et al. 2010; Martin et al. 2020), eight *Ptm* (HRS07033, 07-047, 16FRG073, SNB320, SNB113, SG1, SNB 171 and U7) (Ellwood et al. 2019; Lehmensiek et al. 2010; McLean et al. 2014) and six laboratory-hybrids (unpublish data)

were used. For conidia production, infected barley leaf samples of *Ptt* and *Ptm* isolates were incubated in Petri plates with sterile moist filter paper at 15 ± 1°C under 12 hours of white fluorescent light and 12 h of dark for two days in the incubator (Thermoline, New South Wales, Australia). Single conidium from each isolate was transferred aseptically using a sterile glass needle onto Petri plates containing potato dextrose agar (PDA) medium (20 g/litre; Biolab Merck Darmstadt, Germany). Petri plates were incubated at 25 ± 1°C for ten days to produce mycelium. The six laboratory-hybrids were retrieved from 15% glycerol tubes stored at -80°C and were cultured on PDA.

Establishment of crosses. To investigate the preference of *Ptt* and *Ptm* for mating**,** three experiments each with two *Ptt* and two *Ptm* isolates belonging to alternate mating types were conducted (Experiments 1 to 3; Table 1). An additional experiment (experiment 4) was conducted using six laboratory-hybrids, crossed with seven *Ptt* and six *Ptm* isolates (Table 2) to investigate the preference of hybrids mating with either *P. teres* form.

Each of the experiments 1 to 3 consisted of two *Ptt* (selected from NB50, NB29 and NB81: *MAT1-1*; and NB63, NB85 and HRS09127:*MAT1-2*) and two *Ptm* (selected from HRS07033, 07-047 and 16FRG073: *MAT1-1*; and SNB320, SNB113 and SG1:*MAT1-2*) isolates and comprised of 15 different crosses, including five competition (Cm) crosses, four positive control crosses (Co), and six negative control crosses. Competition crosses (Cm) were established to assess the mating preference of *Ptt* or *Ptm* isolates, where opportunity for both within-form and between-form mating was provided by crossing three or four isolates of different forms and mating types. Positive control crosses (Co) were used to i) confirm the reproductive viability of isolates used in competition crosses under given laboratory conditions by crossing alternate mating types of the same form and ii) establish the ability of isolates to produce hybrids (Table 1). Negative control crosses were established in two settings (data not shown): i) the same isolate was used as both the maternal and paternal isolate and ii) two

isolates possessing the same *MAT1* idiomorph from different forms were placed on to the same crossing plate (*Ptt MAT1-1* and *Ptm MAT1-1, Ptt MAT1-2* and *Ptm MAT1-2*). The crosses of experiment 1 and 2 were established on the 28th of April 2019 and experiment 3 on the 23rd of May 2019.

Experiment 4 was established according to Table 2 on the 12th of September 2019, with 12 competition hybrid crosses (H) and six control crosses (HCo). For hybrid crosses, laboratory- hybrid isolates, previously confirmed though real time PCR form-specific markers (Dahanayaka et al. 2021) and mating type markers (Lu et al. 2010), were placed on the crossing plate along with a *Ptt* and *Ptm* isolate of opposite mating type to the hybrid. Positive control contained hybrids with one of the parents used in the original cross.

Crosses were established according to the method described by Martin et al. (2020). Five 50 mm long autoclaved pieces of wheat straw were placed onto Sach's agar (Hebert 1971) plates before the agar had set. An approximately 25 mm^2 mycelial plug was taken from isolates grown on PDA plates and placed adjacent to the barley straw. Mycelial plugs with different isolates were placed at equal distance from each other mycelial plug. The plates were then kept in transparent plastic bags to prevent desiccation and placed inside an incubator at 15°C with a 12 h light/12 h dark photoperiod.

Ascospore collection. Once ascomata had matured (when ascomata formed a short cylindrical beak or neck), lids of Petri plates were replaced with 2% water agar (Sigma LifeScinece, María de Molina, Spain) plates for ascospore collection. After establishing crosses, crosses of experiment 1 to 3 were monitored daily for one year while those for experiment 4 were monitored for 8 months. The number of days taken from producing the crosses to the production of the first ascospore and the ascospore production time period were recorded for each cross. Single ascospores were collected under a dissecting microscope (Nikon SMZ 745, New York, USA) and transferred to PDA plates using a sterile glass needle.

Water agar plates were replaced with new plates each day after collecting ascospores. All ascospores produced from the competition crosses were collected. A maximum of 12-13 ascospores from control crosses were collected. Plates inoculated with ascospores were placed in an incubator at 20°C to facilitate mycelial growth for fungal DNA extraction.

DNA extraction. Aerial mycelium of each isolate was scraped aseptically from two-week-old PDA isolates and used to extract DNA using a Wizard Genomic DNA Extraction Kit (Promega Corporation, New South Wales, Australia) according to manufacturer's instructions.

PCR amplification for hybrid identification. DNA of all the ascospores obtained from experiment 1, 2 and 3 plus positive controls (*Ptt* isolates NB29 and NB50 (Lehmensiek et al. 2010; Martin et al. 2020) and *Ptm* isolates HRS06033 and SNB113 (Lehmensiek et al. 2010; McLean et al. 2014) and two laboratory-produced hybrids Pop37.1 and Pop37.8 (unpublished data) were amplified using six *Ptt* and six *Ptm* specific PCR markers (Poudel et al. 2017). Amplification was conducted as described in Dahanayaka et al. (2021)

DArTseq genotyping and data analyses. DNA samples extracted from progeny and parents in experiment 4 were confirmed for integrity as described by Dahanayaka et al. (2021) and submitted to Diversity Arrays Technology Pty. Ltd., Canberra, Australian Capital Territory, Australia, for DArTseq™ genotyping. DArTseq™ data (SNP and SilicoDArTs) were filtered following the method described by Dahanayaka et al. (2021) using 10% as the cut off value for the maximum number of missing data points for loci and isolates. SilicoDArTs and SNPs were combined for further analyses. Clonal isolates of each cross were identified by the function *clonecorrect* in *poppr* package (Kamvar et al. 2014) in RStudio version 4.0.1 and were removed.

Phylogenetic relationship among isolates. A neighbor-net network was used in this study in order to depict the relationship among isolates, which provides information about the exchange of genetic material among isolates (Bryant and Moulton 2004). From this

information, the respective parental isolates for each progeny were identified. Based on clone-corrected DArTseq™ data, a neighbor-net network was constructed for isolates obtained from competition crosses in experiment 4 using SplitsTree version 4.16.2 (Huson 1998). Networks were produced for each of the hybrid competition crosses based on neighbor-joining (NJ) algorithm described by Saitou and Nei (Saitou and Nei 1987) following the method depicted by Bryant and Moulton (2004). Bootstrap analysis with 1000 replicates was used to test the support of branches on the network.

Multivariate cluster analyses. Discriminant analysis of principle components (DAPC) is a multivariate cluster analysis which has been developed to detect clusters of genetically related individuals (Jombart 2008). Hence, in this experiment, DAPC was used to identify the structure and the clustering of the progeny isolates and parental isolates to reveal their genetic relatedness. DAPC was calculated from the clone-corrected DArTseq™ data of the isolates using the *dapc* function in the R package *adegenet* version 2.1.2 (Jombart 2008) in the RStudio. The optimum number of clusters in each cross was obtained using the Bayesian information criterion function *find.clusters* and the optimum number of principal component axes to include in the DAPC analysis were calculated via the *xvalDapc* function in *adegenet.*

RESULTS

Ascospore collection. Ascomata emerged four to six weeks after establishing the crosses. In experiment 1, four out of five competition crosses (Cm18-1, Cm18-2, Cm18-4 and Cm18-5) produced 2-63 ascospores between 65-190 days after crossing (Table 1). The period of ascospore production of these crosses varied from 41 to 152 days. Out of the four control crosses, two (Co18-1 and Co18-5) produced ascospores 56-83 days after the crosses were established (Table 1).

In experiment 2, three competition crosses (Cm18-6, Cm18-7 and Cm18-9) produced 8-63 ascospores between 65-186 days after crossing. The ascospore production period ranged from 11 to 225 days. Three control crosses (Co18-7, Co18-10 and Co18-11) produced ascospores between 76-108 days after establishing crosses (Table 1).

In experiment 3, all competition crosses (Cm19-1, Cm19-2, Cm19-3, Cm19-4 and Cm19-5) produced 12-100 ascospores between 50-163 days after crosses were established. The ascospore production period varied from 132 to 73 days. Except for the crosses Cm19-1 and Cm19-2, all other crosses produced ascospores continuously throughout their respective ascospore production period. Crosses Cm19-1 and Cm19-2 had a period of 32 and 35 days, respectively, after the collection of the first ascospores where no ascospores were produced (Table 1). After this period, 12 and 39 further ascospores were collected from crosses Cm19-1 and Cm19-2, respectively. Three control crosses (Co19-6, Co19-7 and Co19-10) produced ascospores 56-162 days after establishing the crosses (Table 1).

In experiment 4, three crosses (H9, H10 and H12) and two control crosses (HCO-14 and HCo-17) produced 3-16 ascospores. The first ascospore of each cross was observed 141-172 days after establishing the crosses (Table 2).

PCR amplification of *Ptt* and *Ptm* specific markers. PCR amplification of DNA samples obtained from experiment 1 with *Ptt* and *Ptm* specific markers indicated that all ascospores collected from competition crosses Cm18-4 ($n = 2$) and Cm18-5 ($n = 37$) amplified both *Ptt* and *Ptm* markers. On the other hand, DNA samples obtained from competition crosses Cm18-1 ($n = 63$) and Cm18-2 ($n = 18$) resulted in amplification of only *Ptt* specific markers (Table 1). DNA samples from isolates of control cross Co18-1 amplified only *Ptt* specific markers while Co18-5 amplified both form-specific markers.

In experiment 2, DNA of all isolates from crosses Cm18-6 and Cm18-7 showed amplification only for *Ptt* specific markers. Seven out of eight isolates from Cm18-9 showed

amplification for both *Ptt* and *Ptm* markers while the remaining isolate showed amplification only for *Ptm* markers. Isolates of control crosses Co18-7 and Co18-8 of experiment 2 amplified *Ptt* specific markers while Co18-11 amplified both *Ptt* and *Ptm* specific markers.

Many isolates from competition crosses in experiment 3 [Cm19-1 (n = 5), Cm19-2 (n = 39), Cm19-3 (n = 8), Cm19-4 (n = 24) and Cm19-5 (n = 10)] showed amplification for both *Ptt* and *Ptm* markers, while 64 isolates from Cm19-1, 61 isolates from Cm19-2 and two isolates from Cm19-5 amplified only *Ptt* markers. Additionally, one isolate from Cm19-3 and 26 isolates from Cm19-4 amplified only *Ptm* specific markers. Isolates from control crosses Co19-6 and Co19-7 amplified only *Ptt* markers while isolates from Co19-10 amplified both *Ptt* and *Ptm* specific markers.

Overall, more crosses were observed involving a *Ptt* isolate than a *Ptm* isolate and the crosses involving *Ptt* isolates yielded 63, 18, 63, 39, 64 and 61 ascospores, whereas those involving *Ptm* isolates yielded 1, 1 and 26 ascospores. We therefore, consider *Ptt* isolates to have a higher reproduction vigour than *Ptm* isolates.

Clone correction and data filtering. After quality filtering of DArTseq™ data obtained for progeny isolates of experiment 4, 1,444 markers (273 SNPs and 1,171 SilicoDArTs) were retained. Clone-correction results showed that two pairs of isolates from cross H9 and one pair from H12 were clonal, hence, one clonal isolate from each pair was removed for subsequent analyses.

Phylogenetic relationship among isolates. The neighbor-net network results for *P. teres* and parental hybrid isolates from experiment 4 divided *Ptt* and *Ptm* isolates into two distinct groups. Hybrid isolates used as the parental isolates were positioned in between *Ptt* and *Ptm* isolates (Fig. 1). The neighbor-net network of the progeny from experiment 4 showed two distinct subdivisions each for the isolates from hybrid crosses H9 (n = 13), H10 (n = 10) and

H12 (*n* = 15) (Fig. 2A, B and C) and were highly reticulated. All progeny from the hybrid crosses grouped close to the parental *Ptt* and hybrid isolates.

Multivariate cluster analyses. The optimum number of clusters in the DAPC analysis was found to be four for the progeny isolates of H9 cross (Fig. 3A). One cluster (red: cluster 3) out of four consisted of the *Ptm* isolate SG1 used in the H9 cross and cluster 1 (blue: *n* = 7) and cluster 2 (green: *n* = 6) consisted of progeny isolates along with the *Ptt* isolate 97NBi of the H9 cross and the parental hybrid isolate 37_416, respectively. Cluster 4 contained two progeny isolates: H9_3 and H9_12. Cross H10 showed three distinct clusters in DAPC analysis. Six progeny isolates clustered with crossed hybrid isolate 37_416 in cluster 2 (green: *n* = 7) and four progeny isolates clustered with the crossed *Ptt* isolate HSR09127 in cluster 3 (blue: *n* = 5) (Fig. 3B). Cluster 1 (red: *n* = 1) contained the *Ptm* isolate SNB320 of the cross. The progeny isolates of cross H12 and its crossed isolates NB81, U7 and 37_407 were best fit into four clusters according to the DAPC analysis. Cluster 1 (blue: *n* = 6) and cluster 2 (green: *n* = 4) consisted of progeny isolates with the crossed *Ptt* and hybrid isolates, respectively. Cluster 4 (cyan: *n* = 7) consisted of only progeny isolates while the crossed *Ptm* isolate formed a separate cluster, cluster 3 (Fig. 3C).

DISCUSSION

In this study for the first time, we investigated the sexual reproduction patterns and preference of *P. teres* and its hybrids for mating *in vitro*. A previous study aimed at inducing natural hybridisation between two *P. teres* forms under field conditions reported that *Ptt* and *Ptm* isolates preferred to mate within their respective forms (Poudel et al. 2018). Therefore, the current study was established to investigate whether there is a preference of *P. teres* isolates for mating *in vitro*.

Some of the crosses in experiments 1, 2, and 3, established to identify the preference of mating within or across *Ptt*, showed that *Ptt* isolates preferred to undergo recombination within the same form *in vitro* rather than hybridizing with *Ptm* at early stages of ascospore production. A similar observation was made in a field trial, where originally *Ptt* and *Ptm* isolates were co-inoculated in a barley field to facilitate hybridisation, but all the progenies were detected as *Ptt* or *Ptm* (Poudel et al. 2018). It was suggested that this was due to *Ptt* and *Ptm* being reproductively isolated, which prevented the exchange of genetic material between the two forms (Giraud et al. 2008). It was proposed that the genetic isolation could have resulted from pre-mating barriers like sexual selection (Fernández-Meirama et al. 2017), temporal difference, and post-mating barriers like gametic compatibility, which lead to unfit or nonviable hybrids (Giraud et al. 2008; Kohn 2005) or ineffectual meiosis occurring in the crosses between *Ptt* and *Ptm* (Serenius et al. 2005). Furthermore, sexual incompatibility between the two forms might have arisen due to the environmental conditions, such as temperature and rain fall which favoured *Ptt* to mature while hindering *Ptm* maturity (Giraud et al. 2008).

The crosses in this study showed that mating preference for some *Ptt* isolates changed over time. Some crosses (Cm19-1 and Cm19-2) in experiment 3 were established to assess whether there is a preference of *Ptt* to recombine with the same form rather than hybridizing with *Ptm*. Even though the majority of progeny were found to be *Ptt* isolates, many progeny isolates produced in the second round of ascospore production were hybrids, confirming that the preference of *Ptt* under *in vitro* conditions could change with time. In the current study, even though *Ptt* and *Ptm* were crossed at the same time, *Ptm* mycelia involved in sexual recombination might have matured later than *Ptt* mycelia. Late maturity of *Ptm* mycelia for mating in the first round of ascospore production may have led to the generation of only *Ptt* offspring. Hybrid offspring found in Cm19-1 and Cm19-2 crosses in the second round of ascospore production could have resulted from the availability of mature *Ptm* mycelium for

329 sexual reproduction., This suggests that pre-mating genetic isolation barriers/genetic
330 incompatibility could be overcome over time (Stukenbrock 2016).

331 Progenies of some crosses revealed that *P. teres* isolates do not possess any preference
332 to mate within the same form when both forms and mating types were present. Progeny isolates
333 from cross Cm18-5 and more than 80% of isolates from cross Cm19-5 were found to be
334 hybrids, indicating that in these crosses *Ptt* and *Ptm* did not have any preference for the same
335 form but sexual reproduction occurred randomly under *in vitro* conditions. Furthermore, these
336 crosses needed longer to produce the first ascospores compared to other crosses, suggesting
337 that maturity of mycelia for sexual reproduction for some of the parents in these crosses could
338 have been delayed.

339 Our results showed that *Ptm* isolates preferred *Ptt* isolates for mating when given the
340 choice between *Ptm* and *Ptt* isolates of opposite mating type. Of the crosses established to
341 identify the mating preference of *Ptm in vitro*, only three (Cm18-9, Cm19-3 and Cm19-4)
342 produced ascospores. Majority of progeny isolates collected from all three crosses produced
343 progenies of hybrid isolates, suggesting that crossed *Ptm* isolates do not have a preference for
344 the respective *Ptm* isolate to recombine *in vitro* conditions. In comparison to most of the *Ptt*
345 crosses (Cm18-1, Cm18-6, Cm18-7, Cm19-1 and Cm19-2), *Ptm* crosses (Cm18-4, Cm18-9
346 and Cm19-4) required a longer period to produce the first ascospore. The laboratory conditions
347 set in this study could have delayed the maturity of the *Ptm* mycelium. Also, delaying of the
348 mycelium for sexual reproduction could be the reason behind the absence of ascospores from
349 the *Ptm* cross Cm18-8. Hybrid formation in these *Ptm* crosses could also be due to *Ptt* having
350 greater vigour to recombine/hybridise with a suitable mating type regardless of the form of the
351 isolate. A similar observation was demonstrated for strains of *Microbotryum violaceum*, an
352 anther smut fungus, taken from two *formae speciales* of *Silene latifolia* and *S. dioica* (Van
353 Putten et al. 2003). Of the strains from the two *formae speciales*, strains from *S. latifolia*

outcompeted and had higher frequency of conjugation than strains from *S. dioica* in both male hosts of *S. latifolia* and *S. dioica*, which was similar to how *Ptt* outcompeted *Ptm* in the presence of both *Ptt* and *Ptm*. Further studies are warranted with different laboratory conditions, *e.g.*, different temperature or light intensities to determine whether *Ptm* isolates prefer to mate with *Ptm* in the presence of *Ptt* under different laboratory conditions or whether these hybrids were the result of high *Ptt* reproduction vigour.

Results of the current study showed that the preference of laboratory-produced hybrid isolates was to mate with *Ptt*. Previous field collected hybrids from Leišova et al. (2005a) (PTM-15 and PTM-16), McLean et al. (2014) (WAC17021), Dahanayaka et al. (2021) (H-919 and CBS 281.31), and Turo et al. (2021) were genetically closer to *Ptm* than *Ptt*. Hence, experiment 4 was established to get an insight into the nature of progeny arising from crosses between hybrids in the presence of both *Ptt* and *Ptm*. Clustering of all progeny isolates of H9, H10 and H12 with the respective *Ptt* parent of the cross indicated the preference of hybrid isolates to mate with *Ptt* isolates rather than *Ptm* isolates. These results also suggest that *Ptt* isolates have a higher ability or higher reproduction vigour compared to *Ptm* under the given laboratory conditions. Environmental conditions used in the experiment could have favoured the crossing between hybrid and *Ptt* isolates. Successful production of ascospores of these crosses confirms the fertility of hybrid isolates and their ability to integrate with the *P. teres* population.

Progeny isolates from crosses H9, H10 and H12 showed evidence for introgressive hybridization of laboratory-hybrids to parental forms of *P. teres*, namely *Ptt*. Repeated introgression/backcrossing of hybrids in oomycetes proposed to "dilute" or reduce the genetic material of hybrids and change the hybrid genome towards the parental species while retaining adaptive traits from both species (Baack and Rieseberg 2007). The neighbor-net networks and DAPC analyses of this study showed that genomic characters of these laboratory-hybrid

379 progenies shifted toward the *Ptt* genome and reduced the hybrid genetic characters. Repeated
380 introgression of *P. teres* hybrids with their parental forms in fields may have left these
381 unrecognised in nature. Introgression/backcrossing may accelerate the adaptive evolution
382 through descending heritable/adaptive genetic characters between species (Arnold 2004) and
383 result in novel pathogenic fungi (Menardo et al. 2016). Progeny of natural hybrids, along with
384 introgressive hybrids, occurred between *Melampsora medusae* and *M. occidentalis*, two rust
385 pathogens of *Populus deltoides* and *P. trichocarpa*, respectively (Newcombe et al. 2000).
386 These progeny isolates were found to be virulent on a hybrid population of *Populus deltoides*
387 and *P. trichocarpa*, which was originally developed against *M. occidentalis* (Newcombe et al.
388 2000). Hybrids and introgressive hybrids of *P. teres* could also have devastating effects on
389 barley varieties which have been developed against either *Ptt* or *Ptm*. Identifying heritable
390 genes of *P. teres* through developing backcrosses would allow us to recognize inheritable
391 genes/genomic regions and expand the knowledge of this challenging pathogen.

392 The identification of field hybrids in recent studies has led to the understanding that field
393 hybrids may not have been detected in previous studies due to the absence of an appropriate
394 marker system and not due to the absence of field hybrids (Dahanayaka et al. 2021). The
395 possibility of retaining the fertility, virulence and genetic stability of laboratory-produced
396 hybrids (Campbell and Crous 2003) and decreased fungicide sensitivity and rapid asexual
397 reproduction of field hybrids (Turo et al. 2020) suggest potential for integration with the local
398 *P. teres* population. Thus, regular monitoring of *P. teres* isolates in barley fields is vital. Also,
399 further studies should be conducted under different laboratory conditions including
400 temperature, light intensities, culture medium and field conditions, including glasshouse
401 experiments, to gain comprehensive knowledge on the sexual reproduction patterns and
402 reproduction vigour of *P. teres* and its hybrids.

In conclusion, the *P. teres* crosses that were established to identify the mating preference of *Ptt* isolates in this study revealed that *Ptt* isolates preferred to undergo recombination with the respective *Ptt* isolates at the early stages of their maturity but over time *Ptt* preferred to undergo hybridisation with *Ptm* isolates. In contrast to *Ptt*, *Ptm* isolates did not have preference to undergo recombination with *Ptm* and instead showed preference towards hybridisation with *Ptt*. The laboratory-hybrids preferred to undergo sexual reproduction with *Ptt* rather than *Ptm* isolates. These results suggest that *Ptt* isolates have a greater reproduction vigour than *Ptm* hence, *Ptm* and hybrid isolates were forced to undergo sexual reproduction with *Ptt*. These findings indicate the high potential for production of hybrids *in vitro* and would support the development of a reproductive model and a better understanding of speciation/form differentiation and evolution of *P. teres.* The potential for more frequent occurrences of field hybrids under suitable environmental conditions, could lead to novel, more complex and highly virulent pathotypes with both *Ptt* and *Ptm* characteristics. Thus the development of novel barley lines which can withstand both *Ptt* and *Ptm* infections is vital.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Francisco Lopez-Ruiz, Centre for Crop and Disease Management, Curtin University, Australia for providing some of the isolates used in this study. We are also grateful to Dr Michael D. Thompson (The University of Queensland, Australia) for the technical support.

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542 Table 1. Meta data of *Pyrenophora teres* crosses of experiment 1 to 3.The number of ascospores produced per cross, results of PCR

543 amplification with the form-specific markers and ascospore production time are given

Cross ID[a]	*Ptt 1*[b]	*Ptt 2*[c]	*Ptm 1*[d]	*Ptm 2*[e]	No. of ascospores[f]	*Ptt* specific markers[g]	*Ptm* specific markers[h]	Both types of markers[i]	Days to produce the first ascospore[j]	Production period (Days)[k]
Experiment 1										
Cm18-1	NB50	NB63	HRS07033	-	63	63	0	0	65	125
Cm18-2	NB50	NB63	-	SNB320	18	18	0	0	190	44
Cm18-3	NB50	-	HRS07033	SNB320	0	0	0	0	NA	0
Cm18-4	-	NB63	HRS07033	SNB320	2	0	0	2	190	41
Cm18-5	NB50	NB63	HRS07033	SNB320	37	0	0	37	185	152
Co18-1	NB50	NB63	-	-	11	11	0	0	56	NA
Co18-2	-	-	HRS07033	SNB320	0	0	0	0	NA	NA
Co18-4	NB50	-	-	SNB320	0	0	0	0	NA	NA
Co18-5	-	NB63	HRS07033	-	3	0	0	3	83	NA
Experiment 2										

Cm18-6	NB29	NB85	07-047	-	63	63	0	0	65	225
Cm18-7	NB29	NB85	-	SNB113	39	39	0	0	65	31
Cm18-8	NB29	-	07-047	SNB113	0	0	0	0	NA	NA
Cm18-9	-	NB85	07-047	SNB113	8	0	1	7	186	11
Cm18-10	NB29	NB85	07-047	SNB113	0	0	0	0	NA	NA
Co18-7	NB29	NB85	-	-	12	12	0	0	76	NA
Co18-8	-	-	07-047	SNB113	12	0	12	0	107	NA
Co18-10	NB29	-	-	SNB113	0	0	0	0	NA	NA
Co18-11	-	NB85	07-047	-	2	0	0	2	108	NA
Experiment 3										
Cm19-1	NB81	HRS09127	16FRG073	-	69	64 (57+7[l])	0	5[l]	50	131 (1-76 and 109-131)[m]
Cm19-2	NB81	HRS09127	-	SG1	100	61	0	39[n]	55	132 (1-71 and 107-132)[o]
Cm19-3	NB81	-	16FRG073	SG1	9	0	1	8	82	80
Cm19-4	-	HRS09127	16FRG073	SG1	50	0	26	24	101	119

Cm19-5	NB81	HRS09127	16FRG073	SG1	12	2	0	10	163	73
Co19-6	NB81	HRS09127	-	-	13	13	0	0	56	NA
Co19-7	-	-	16FRG073	SG1	11	0	11	0	91	NA
Co19-9	NB81	-	-	SG1	0	0	0	0	NA	NA
Co19-10	-	HRS09127	16FRG073	-	12	0	0	12	162	NA

544

545 [a] Identity of cross

546 [b] Mating type 1 *Ptt* isolates used in the experiment

547 [c] Mating type 2 *Ptt* isolates used in the experiment

548 [d] Mating type 1 *Ptm* isolates used in the experiment

549 [e] Mating type 2 *Ptm* isolates used in the experiment

550 [f] Number of ascospores produced by the respective cross

551 [g] Number of isolates which only amplified with *Ptt* specific markers

552 [h] Number of isolates only amplified with *Ptm* specific markers

553 [i] Number of isolates amplified with both *Ptt* and *Ptm* specific markers

554 [j] Number of days to produce the first ascospore

555 [k] Number of days ascospores were produced and collected

556 [l] Ascospores were produced in the second round (n = 12)

557 [m] Ascospores were produced in two time periods, day 1 to day 75 first period, day 76 to day 108 no ascospore production and day 109 to day 131
558 second period

559 [n] Ascospores were produced in the second round (n = 39)

560 [o] Ascospores were produced in two time periods, day 1 to day 71 first period, day 72 to day 106 no ascospore production and day 107 to day 132
561 second period

562 NA Not available

563

564 Table 2. Meta data for *Pyrenophora teres* isolates used in experiment 4 with the number of ascospores produced by each cross and ascospore

565 production time given

Cross ID[a]	Hybrid[b]	Mat[c]	Hybrid cross[d]	*Ptt*[e]	Mat[f]	*Ptm*[g]	Mat[h]	No. of ascospores[i]	Days to produce the first ascospore[j]	Production period (Days)[k]
H1	30_1	*Ptm*1	NB73 × SNB171	97NB1	2	SG1	2	0	NA	NA
H2	30_1	*Ptm*1	NB73 × SNB171	HRS09127	2	SNB320	2	0	NA	NA
H3	30_3	*Ptt*2	NB73 × SNB171	HRS11093	1	16FRG073	1	0	NA	NA
H4	30_3	*Ptt*2	NB73 × SNB171	NB81	1	U7	1	0	NA	NA
H5	34_8	*Ptm*1	NB90 × HRS07033	97NB1	2	SG1	2	0	NA	NA
H6	34_8	*Ptm*1	NB90 × HRS07033	HRS09127	2	SNB320	2	0	NA	NA
H7	34_18	*Ptt*2	NB90 × HRS07033	HRS11093	1	16FRG073	1	0	NA	NA

H8	34_18	*Ptt*2	NB90 × HRS07033	NB81	1	U7	1	0	NA	NA
H9	37_416	*Ptm*1	NB63 × HRS07033	97NB1	2	SG1	2	15[l]	141	48
H10	37_416	*Ptm*1	NB63 × HRS07033	HRS09127	2	SNB320	2	10	155	52
H11	37_407	*Ptt*2	NB63 × HRS07033	HRS11093	1	16FRG073	1	0	NA	NA
H12	37_407	*Ptt*2	NB63 × HRS07033	NB81	1	U7	1	16[m]	146	49
HCo-13	30_1	*Ptm*1	NB73 × SNB171	NB73	2	-	-	0	NA	NA
HCo-14	30_3	*Ptt*2	NB73 × SNB171	-	-	SNB171i	1	3	172	NA
HCo-15	34_8	*Ptm*1	NB90 × HRS07033	NB90	2	-	-	0	NA	NA
HCo-16	34_18	*Ptt*2	NB90 × HRS07033	-	-	HSR07033	1	0	NA	NA

HCo-17	37_416	*Ptm*1	NB63 × HRS07033	NB63i	2	-	-	8	170	NA
HCo-18	37_407	*Ptt*2	NB63 × HRS07033	-	-	HSR07033	1	0	NA	NA

[a] Identity of the crosses

[b] Hybrid isolates used in the crosses

[c] Mating type of the hybrid isolates used

[d] Parental genotypes of the hybrid isolates

[e] *Ptt* isolates used in experiment 4

[f] Mating type of the *Ptt* isolates used

[g] *Ptm* isolates used in experiment 4

[h] Mating type of the *Ptm* isolates used

[i] Number of ascospores produced by the respective cross

576 [j] Number of days to produce the first ascospore

577 [k] Number of days ascospores were produced by the respective cross

578 [l] Out of 15, 13 isolates were retain as two pairs of isolates were clones based on DArTseq™

579 [m] Out of 16, 15 isolates were retain as one pair of isolates were clones based on DArTseq™

580 - Not applicable

Figure captions

Fig. 1. Neighbor-net network constructed for *Pyrenophora teres Ptt* (blue), *Ptm* (red) and the parental hybrid isolates (green) used for hybrid competition crosses H9, H10 and H12, with 1000 bootstrap replicates, based on DArTseq™ data.

Fig. 2. Neighbor-net networks constructed using neighbour-net distance matrix with 1000 bootstraps for progeny isolates of *Pyrenophora teres* hybrid competition crosses (A) H9 (B) H10 and (C) H12 based on DArTseq™ data. Different colours depict progeny (black), parental *Ptt* (blue), parental *Ptm* (red), and parental hybrid (green) isolates used to establish the cross.

Fig. 3. Discriminant analysis of principal components for progeny isolates of *Pyrenophora teres* hybrid competition crosses (A) H9 (B) H10 and (C) H12 with their respective *Ptt*, *Ptm* and parental hybrid isolates. Blue and green circles include progeny isolates as well as with the *Ptt* and parental hybrid isolates for each cross. Cyan circles represent only progeny isolates, and red circles *Ptm* isolates of the respective cross. The distribution of the eigenvalues of principal component analysis (PCA) and discriminant analysis (DA) indicate that the first two principal components explain approximately 50% of the total genetic variation.

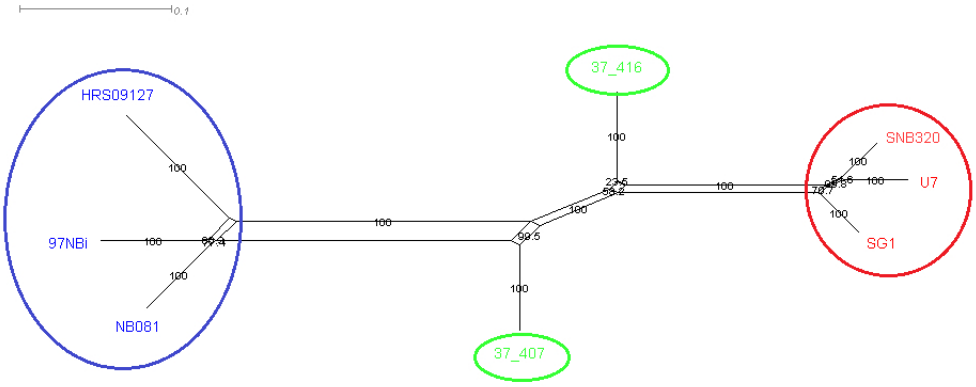

Fig. 1. Neighbor-net network constructed for *Pyrenophora teres*, *Ptt* (blue), *Ptm* (red) and the parental hybrid isolates (green) used for hybrid competition crosses H9, H10 and H12, with 1000 bootstrap replicates, based on DArTseq™ data.

247x109mm (96 x 96 DPI)

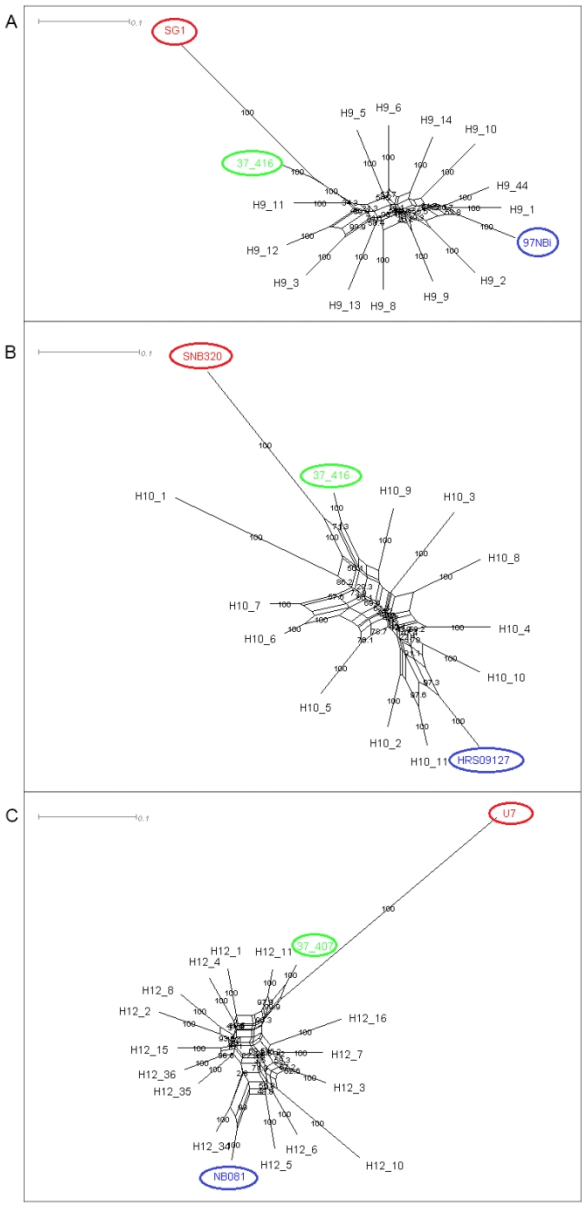

Fig. 2. Neighbor-net networks constructed using neighbour-net distance matrix with 1000 bootstraps for progeny isolates of *Pyrenophora teres* hybrid competition crosses (A) H9 (B) H10 and (C) H12 based on DArTseq™ data. Different colours depict progeny (black), parental *Ptt* (blue), parental *Ptm* (red), and parental hybrid (green) isolates used to establish the cross.

202x411mm (96 x 96 DPI)

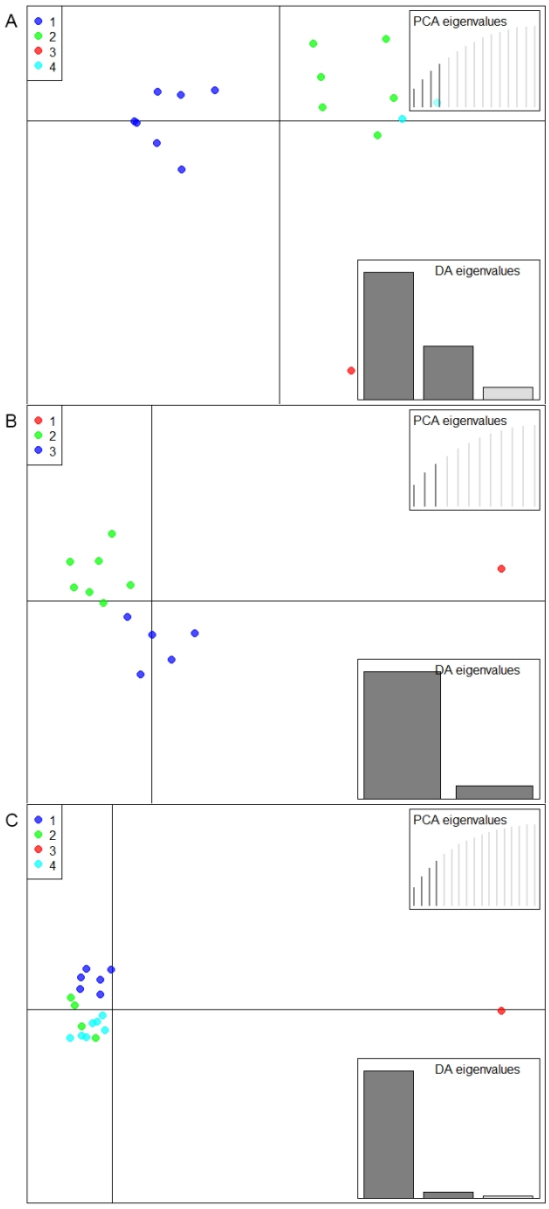

Fig. 3. Discriminant analysis of principal components for progeny isolates of *Pyrenophora teres* hybrid competition crosses (A) H9 (B) H10 and (C) H12 with their respective *Ptt*, *Ptm* and parental hybrid isolates. Blue and green circles include progeny isolates as well as with the *Ptt* and parental hybrid isolates for each cross. Cyan circles represent only progeny isolates, and red circles *Ptm* isolates of the respective cross. The distribution of the eigenvalues of principal component analysis (PCA) and discriminant analysis (DA) indicate that the first two principal components explain approximately 50% of the total genetic variation.

189x409mm (96 x 96 DPI)