Isolation, protocol optimization and screening of genotypes against net blotch and scald of barley in controlled condition

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Swedish University of Agricultural Sciences, SLU
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Isolering, protokolloptimering och screening av genotyper för resistens mot bladfläcksjuka och sköldfläcksjuka i vårkorn i en kontrollerad miljö

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Abstract

Net blotch and scald are two economically important diseases of barley, causing significant losses in yield in Nordic and Baltic states (NBS) countries and in Sweden. Identification and deployment of resistant cultivars is the most effective method for controlling both the diseases. However, response of cultivars towards the pathogen is a complex phenomenon. It depends on the genetics of plants, environmental factors, isolates of pathogen, developmental stages of plants, and the resistance source for the plant. Due to this complexity, assessment of the cultivars reaction in the field requires consideration of multiple factors and along with that, has to face climate variability every year. Therefore, in this work, we isolated both the disease-causing pathogen from Swedish fields. A new optimized protocol was developed for culturing both pathogens in laboratory conditions. Then, using one isolate each of the two pathogens, Swedish official trial cultivars and breeding lines from NBS countries were tested for seedling-stage resistance against the two diseases using the developed optimized protocol under controlled conditions. Afterwards, Best Linear Unbiased Estimates (BLUEs) were used to categorize/rank the genotypes after scoring. Also, a rank correlation analysis was used to compare the net blotch resistance of official trial cultivars under controlled conditions and in a field experiment. 25 cultivars out of 37 showed similar resistance type in both field and controlled condition. The results of this study provide data regarding the source of resistance available in tested material and will help decision makers to recommend cultivars repleted with resistance towards both the disease.

Keywords: Barley, net blotch, scald, isolation, seedling resistance

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Abbreviations

AUDPC	Area under disease progressive curve
CZV8MC	Czapek-Dox broth V8 Malt Calcium carbonate
LBA	Lima bean agar
NBS	Nordic and Baltic states
NB	Net Blotch
NFNB	Net form of net blotch
PDA	Potato dextrose agar
POA	Peanut oatmeal
QTL	Quantitative trait loci
UV	Ultraviolet
WA	Water agar
WGA	Wheat germ agar

1. Introduction

1.1 The host- Barley

Acreage and growing conditions

Barley (*Hordeum Vulgare* L.) is one of the oldest and most important cereal crops globally (Taner et al., 2004; Newton et al., 2011; Dawson et al., 2015). It is grown widely worldwide and is ranked as the 4th most-grown cereal crop after rice, wheat, and maize. Europe has the maximum production, followed by Asia, America, Africa, Oceania. Russia, Germany, and France are the top three Barley producing countries in Europe (FAOSTAT, 2020). In Nordic and Baltic states countries (NBS countries[1]), Denmark has the highest production and harvested area over the period (1970-2020) (Figure 1). Sweden produced 1.5 million tonnes of barley in 0.29 million hectares of land in 2019. It has been one of the most dominant cereal crops in Sweden over the period (1970-2020) after wheat (FAOSTAT, 2020).

Generally, long and cool growing seasons are best for barley production (Robertson & Stark, 2003; Pettersson, 2006). The growth period changes significantly between countries in the NBS Countries (Brantestam, 2005). Although the vegetation period is short in the Nordic countries (Denmark, finland, Norway and Sweden), the more extended photoperiod in the spring and summer complements the crop growth rate in a limited time (Brantestam, 2005). Meanwhile, approximately 130-135 days are required until the crop harvest in the Baltic states countries (Estonia, Latvia, Lithuania) (Mukula & Rantanen, 1987; Wiberg, 1993).

Utilization

Barley is a food crop. However, over the years, barley consumption as food source has declined (Newton et al., 2011). It is predominately consumed as a food in the areas where other cereals crops do not perform well due to extreme climate and poor soil conditions. Apart from that, due to its high protein content compared to

[1] NBS Countries: Denmark, Estonia, Finland, Latvia, Lithuania, Norway, Sweden

maize, barley is preferred when used as animal feed. On the other hand, barley with low protein content is suited for malting (Boyles et al., 2001). Presently, 65% of global barley is used as animal feed, 30% for malting and brewing and only 2-3 % as food for humans (Aldughpassi et al., 2016).

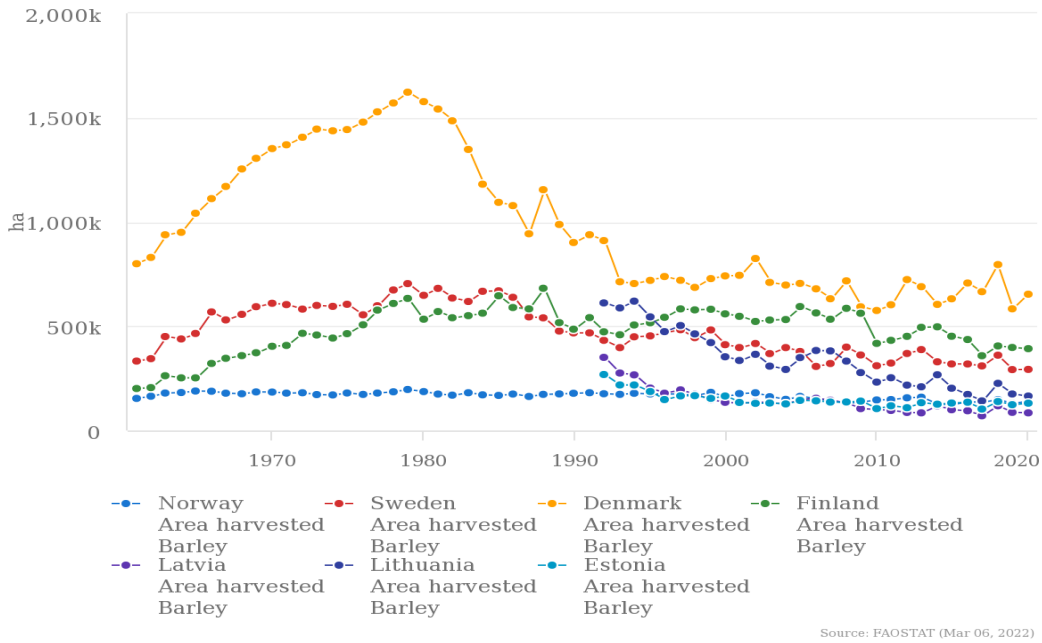


***Figure 1**:Figure and legend taken from (FAOSTAT, 2020) with permission. Graphical representation of the harvested area of barley in Nordic and Baltic states (NBS) countries.*

Over time, use of barley as a food source in the NBS counties has also changed (Fishbeck, 2002). Nowadays, barley in these regions is mainly used as malt for brewing industry and as feed for animals (Brantestam, 2005). Because of higher malt extract potential than six-rowed barley, two-rowed barley is primarily grown in southern Scandinavian areas, favouring malting industries (Ortiz et al., 2002).

Domestication and plant breeding

Barley has a long history of domestication for over the last eight to ten thousand years in different parts of the world (Badr et al., 2000). The cultivated barley is supposedly domesticated from *H. Vulgare ssp. Spontaneum* (Von Bothmer & Komatsuda, 2011) in the Fertile Crescent, Zagros mountains, and the Horn of Africa, becoming major sources of gene pool for the areas (Von Bothmer, Sato, Komatsuda, et al., 2003; Wang et al., 2015).

From the middle of the 19th century, crop improvement and breeding programme started using Mendelian principles in barley. Before that, selection based on

superior ones according to the trait of interest was common (Fischbeck, 1992). The successful crossing of different genetic backgrounds and geographical material followed by selection resulted in today's varieties and progenitors of barley (Hintum, 1994). In the mid-twentieth century, changing climate, population growth, consumer's needs, and economic stability urges for continued development of new cultivars fulfilling the world demand of feed, malt and future food security. Presently, introgression of traits related to biotic stress, abiotic stress and better end-user quality are the main breeding targets in barley (Hernandez et al., 2020).

Breaking of host resistance, fungicides insensitivity towards pathogen, climate variability and increase in importance of minor pathogens necessitates the development of efficient cultivars for disease control in barley. Diseases such as rust, powdery mildew, smuts, fusarium head blight, net blotch, spot blotch, scald, and ramularia are the targets of resistance breeding (Walters et al., 2012). Barley wild relatives are major contributors of resistance genes utilized in breeding programs for increased disease resistance in modern cultivars. *H. Vulgare ssp. spontaneum and H. bulbosum* are some of the wild barley progenitors carrying resistance genes against many foliar, viral, and rust diseases. However, introgression of resistance genes from wild relatives into a genotype would also come with trade-offs, i.e., lower quality and agronomic performance (Jahoor & Fischbeck, 1993; Garvin et al., 1997; Fetch Jr et al., 2003; Giura, 2002). Thus, a holistic approach in breeding is needed to get the required outcomes.

Among the barley diseases, the two foliar blotch diseases, net blotch and scald are significantly impacting barley production and quality when conducive environmental conditions are available (Clare et al., 2020; Zhang et al., 2020). The fungal pathogen *Pyrenophora teres f. teres* is the causal organism for net form of net blotch, and *Rhynchosporium commune* causes scald of barley. Both net blotch (bladfläcksjuka in Swedish) and scald (sköldfläcksjuka in Swedish) are two economically important diseases of barley in the NBS countries. Annually, on an average, both the diseases including ramularia cause yield losses of approximately 1114 kg/ha in these regions (Jalli et al., 2020).

1.2 Pathogens and diseases

1.2.1 *Pyrenophora teres f. teres* and net blotch

Pyrenophora (Syn. Helminthosporium) is an ascomycetes fungus belonging to class *dothideomycetes*; order *pleosporales*, and family *pleosporaceae*. Initially, the genus *Helmisporium* described the pathogen (Link, 1809). Later, the generic name changed to *Helminthosporium* illicitly (Persoon, 1822). Saccardo classified the

species *teres* in 1882, and Diedicke, 1902 described the teleomorphic stage, *i.e.*, *Pyrenophora*. Until early 1900, there were numerous species under the genus *Helminthosporium*, and most of them attacked graminaceous plants in colder regions. In response to that, Drechsler classified in 1923 *Helminthosporium* pathogen common in the Gramineae family. Subsequently, the "Drechsler" name described the genus's anamorphic stage (Nisikado, 1929; ITO, 1930). Additionally, based on symptoms in the host organism, Smedegård-Petersen, 1971 classified two forms of pathogen: the net form of net blotch (*P. teres f. teres*.) and spot form of the net form (*P. teres f. maculata*).

The teleomorphic stage of the *P. teres f. teres* produces septate dark spherical pseudothecia measuring 1-2mm in diameter, primarily found in the final period of the growing season on crop residue (Mathre, 1997). The fertile pseudothecia give rise to club-shaped asci, and the light brown ellipsoidal ascospore arises from asci. Ascospores are then dispersed through wind or rain splash and serve as a primary source of inoculum (Jordan, 1981; Jordan & Allen, 1984).

Conidia forms in the anamorph stage of the pathogen. After the colonization of the pathogen, these conidia become a secondary source of inoculum. Conidia are multi-segmented structures borne on top of the conidiophore. The infection starts when the conidia start germinating on the leaf surface (Backes et al., 2021).

The pathogen penetrates the outer epidermal cells and develops in primary vesicles (Liu et al., 2011). *P. teres f. teres* generally penetrates the epidermal layer within 48 hours of infection (Lyngs Jørgensen et al., 1998). Later, it forms secondary hyphae inside the sub-stomatal chamber (Walters et al., 2012). Therefore, it is a necrotrophic pathogen, grows intercellular during the infection period (Backes et al., 2021).

Environmental conditions play an essential role in developing the infection in the host plant (Keane & Kerr, 1997)**.** Conidiophores sporulate around 90-100% humidity after the first infection, thus maintenance of high humidity is crucial for disease development. Along with that, the pathogen grows well at a temperature between 15-25°C (Shaw, 2007).

Leaf infection and degraded seeds/harvested grain quality are the main sources for economic losses (Shipton, 1966). However, leaf infection is most common and defines the disease of the host plant. Net-like symptoms could be seen when the plants are attacked by *P. teres f. teres* (Smedegård-Petersen, 1976). Minor dot-like necrotic symptoms could be seen in resistant genotypes, while both necrotic and chlorotic symptoms that eventually lead to the death of the whole leaf is a typical symptom for susceptible genotypes (Tekauz, 1985; Backes et al., 2021).

In laboratory conditions, whole-plant leaf inoculation and detached leaf assays are common methods for disease assessment of net blotch (Backes et al., 2021). For whole-plant assays, the leaves of healthy grown plants are inoculated at a specific growth stage. On the other hand, detaching leaves from various growth stages of plants followed by inoculation of the pathogen is another method (El-Mor et al., 2018). Host, environment and pathogen interaction consideration and optimization plays a crucial role for success of the assessment.

Disease management and resistance of net blotch

Different management practices, such as integrated pest management (IPM) tools, are used and suggested to minimize losses due to net blotch. Foliar application of fungicides and seed treatment are the most common methods used (Shipton, 1966). Fungicides applied at the grain filling stage of crops are more effective than in the early stages (Liu et al., 2011). In addition to this, various cultural practices like field management and crop rotation are used to reduce the primary inoculum (McLean et al., 2009). Besides, biocontrol agents like *Trichoderma* species are also being suggested for controlling the disease loss. However, for its field level applicability, future research will need in the future. Using a resistant cultivar coupled with other tools of IPM has been the most effective method for net blotch control (Adhikari et al., 2020).

The genetic basis for net blotch resistance in barley has been demonstrated both quantitatively and qualitatively. The first monofactorial dominant resistance gene was described from Tifang (CI 4407-1), designated as *Pt1* (Schaller, 1955). A similar gene, *Pt2* (linked to *Pt1*), was identified from Harbin (CI 4929), Manchurian (CI 2335) and Ming (CI 4797) and two other unlinked genes were identified in two accessions, CI 4922 and CI 2750 (Mode & Schaller, 1958). Later, with the discovery of QTL mapping, various loci were mapped for resistance in different populations (Backes et al., 2021). Based on different QTL studies, the 6H chromosome, especially the Rpt5 locus, is essential for resistance (J. Richards et al., 2016). Although the resistance genes/locus involved in the resistance are identified within various populations, the resistance nature is still dependent on the source of resistance, isolate used, and developmental stage of the plant (Adhikari et al., 2020a). Considering the boom-and-bust cycle of plant and pathogen, introgression of a single gene into barley germplasm has confined applicability. On the other hand, few breeding programs have succeeded to introgress the mapped genes/QTL (Ma et al., 2004; O'Boyle et al., 2011).

Culture of pathogen P. teres f. teres

Regarding *P. teres f. teres.* isolation and culture, different media and methods have been used for its isolation, spore multiplication and storage. *Table 1* shows some

common media and methods used for *P. teres f. teres* culture. In summary, water agar plates are used for isolation of pathogen from diseased leaves sample. These plates are then placed in alternating white light/dark conditions to ignite the pathogen to produce mycelium. Later, the mycelia are transferred to other nutrient media to induce sporulation.

Media			**Plates growth condition**	**References**
Isolation	**Sporulation**	**Storage**		
WA plates	WA plates	NA	Incubation at complete dark condition during mycelial growth and kept under florescent/near-ultraviolet (UV) light for 24 hrs. at 20°C to induce sporulation	(Scott, 1992)
V8 Agar plates (15%)	V8 Agar plates (15%)	Infected leaves	Incubation of plates in alternating Light and dark conditions for 5-7 days at 20-22°C.	(Gilchrist-Saavedra et al., 1997)
WA plates	V5 Agar (25%)	Glycerol stock store at -80°C	WA: incubation at 12 hours of Light at 20 °C V5 Agar: NA	(Jonsson et al., 1997)
Petri plates with water-absorbent pad	V8 Agar plates (15%) & POA (peanut oatmeal agar) plates	NA	Plates with water-absorbent pad: incubation at 19 °C in 12 hrs. of diurnal fluorescent white and near UV light. V8 Agar: incubate in the dark for 5–6 days at 25 °C (±1 °C) POA: incubate at 19 °C (±1 °C) under diurnal Light for 9– 10 days	(R. A. Fowler et al., 2017)

Table 1 *Common media, methods used for Pyrenophora teres f. teres culture in laboratory*

1.2.2 *Rhynchosporium commune* and scald

The genus *Rhynochosporium* is classified under the class Leotiomycetes of Ascomycota (Goodwin, 2002; Penselin et al., 2016). The pathogen was first reported in 1897 in the Netherlands on rye and named *Marsonia secalis* Oud. (Oudemans, 1897). In the same year, the pathogen was reported in many parts of Germany and seen in rye and barley (Frank, 1897). Observation of the beaked conidiophore structure, obliged to give the genus name *Rhynchosporium* (derived

from the Greek word *rhynchos,* meaning beak) (Heinsen, 1901). In 1937, later the name Scald or leaf blotch disease was coined (Caldwell, 1937).

Like *P. teres f. teres*, *R. commune* spores remains in crop debris, forms conidia, and acts as the primary source of inoculum (Ayesu-Offei & Clare, 1970). The conidia can spread within a few distances due to their minimal size either through wind or rain splash and acts as a secondary source of inoculum. It can go to new leaves of the plants, starts new infection, and remains polycyclic in fields (Knogge, 2018a). Infected seeds could also be a source of inoculum (Brunner et al., 2007). The sexual form of *R. commune* is not known yet (Zhang et al., 2020a).

Scald symptoms develop in multiple phases. Upon contact with the surface of the leaves, conidia start to germinate 12 hours after inoculation. Thereafter, germinated conidia forms hyphae which penetrates the cuticle of leaves (just above the epidermal cells) and succeeding hyphal growth occurs longitudinally confined to the subcuticular region of the epidermis (Jones & Ayres, 1974; Carisse et al., 2000). During this fungal infection period, the pathogen grows around the pectin rich cell wall layer or stomata guard cell but not inside the stomata (Thirugnanasambandam et al., 2011; Zhang et al., 2020a). Dense stroma in the subcuticular region becomes the region for sporulation, and conidia formed from conidiophores erupt outside of the leaf cuticle into healthy leaf tissue (Avrova & Knogge, 2012). This leads to elongated pale blotches with brown margin symptoms on leaves (Ayesu-Offei & Carter, 1971; Zhan et al., 2008). In susceptible cultivars, the growth of the pathogen expands from epidermal cell to mesophyll cell and eventually leads to total collapse of leaves (Lehnackers & Knogge, 1990; Thirugnanasambandam et al., 2011). Until the mesophyll tissue is collapsed, the typical symptoms of scald cannot be seen. Before it was classified as necrotrophy pathogen, however, re-classification it as a hemi-biotroph has been proposed(Zhan et al., 2008). Wet, humid and cool conditions are favourable for pathogen infection and enhance disease severity.

As net blotch, disease assessment in barley for scald can be done by both methods: whole plant inoculation and detached leaf assay (Coulter et al., 2019).

Disease management and resistance to leaf scald

Numerous management practices are used to control leaf scald. Agronomic practices like crop rotation, destroying infected stubble are implemented to reduce the inoculum concentration in field (Elen,2002; Mayfield & Clare,1984). Similarly, different kinds of fungicides are also used to keep the disease below the threshold level (Knogge, 2018). However, use of resistant variety is found to be economical and sustainable method for disease control (Avrova & Knogge,2012).

Similar to net blotch, both qualitative major resistance genes and quantitative minor resistance genes, providing full or partial resistance against scald, have been

described in host barley (Hugh Wallwork & Grcic, 2011; Zhan et al., 2008). Multiple dominant or recessive genes have been expressed from various barley accessions localized in different chromosomes (Knogge, 2018b; Zhang et al., 2020b). Notably, a complex locus in the 3H chromosome was found to be a source for many resistance genes (*Rrs1–Rrs*$_{Rh4/Rh10}$). Other than 3H, genes have also been identified in chromosome 1HS (1), 2 HS (2), 4H (4), 6HS (6), 7 HS (1), except for 5H. Similarly, various QTLs have been mapped in similar chromosomes where significant R genes have been described. Pyramiding the detected genes/QTLs into a cultivar would be a sustainable and feasible method of disease control and will reduce the dependency on chemical control of the disease.

Culture of pathogen R. commune

For *R. commune* isolation and culture, WGA (wheat germ agar), LBA (Lima bean agar), CZV8MC, and PDA (potato dextrose agar) are commonly used media. It is necessary to sub-culture the pathogen in new media plates within 10-12 days for maintaining the viability of the pathogen (Gilchrist-Saavedra, 1997). However, storing pathogens in silica and beads is also common for long-term use. Some of the common media and methods used for *R. commune* isolation, sporulation and storage are mentioned in *Table 2*.

Table 2 *Common media, methods used for R. commune culture in laboratory*

Media			**Petri Plates' growth condition**	**References**
Isolation	**Sporulation**	**Storage**		
PDA (Potato dextrose agar) plates (3.9%)	LBA (Lima bean agar) plates	Cryotubes containing spore solution and sterilized silica -80°C	PDA plates: Incubate at 16°C for 12-h photoperiod for 2-10 days LBA plates: Incubate at 16°C for 12-h photoperiod, later transfer and spread the colonies to new LBA plates and keep it for 7 days at the same	(H. Wallwork et al., 2014)

			temperature and photoperiod.	
LBA plates	LBA plates	Dried infected leaves at 4°C	LBA plates: Incubate at 18-20°C for 10-14 days	(Gilchrist-Saavedra et al., 1997)
WGA (Wheat germ agar) plates	WGA plates	Stored in porcelain insulating beads kept in Duran bottle under -25 °C	WGA plates (for isolation): Incubation at 18°C in dark conditions for 7-14 days	(Salamati & Tronsmo, 1997)

1.3 Disease and field trials in Sweden

Net blotch has more impact on yield than scald in the NBS countries (Jalli et al., 2020). In Finland and Norway, net blotch is the most impacting disease of barley for yield. In Sweden, there is a long trend for incidence of net blotch and scald of barley, reaching a peak in specific years due to favourable climatic conditions in the particular year (Figure 2 and Table 3). This trend shows the potential of the pathogen to develop as an epidemic under optimal conditions in these regions. Therefore, monitoring of disease in field is necessary. It maintains the disease incidence below the threshold level and precautionary method can be implemented before it rises. Swedish board of agriculture (Jordbruksverket) is responsible authority in Sweden which conduct such monitoring activities.

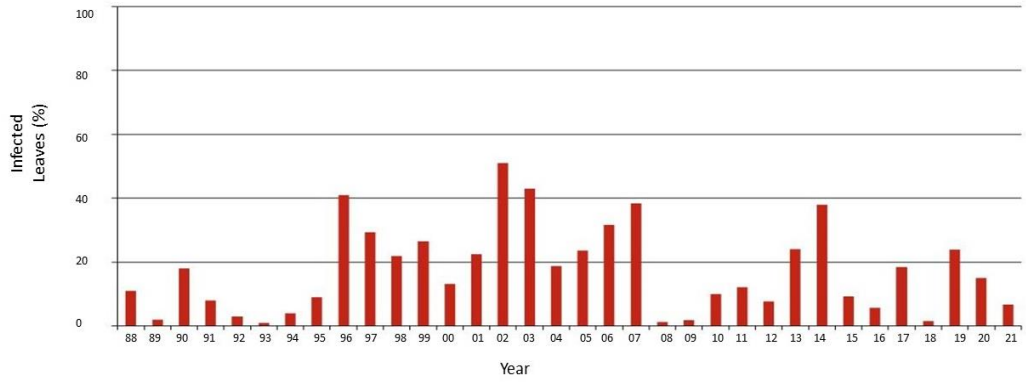


Figure 2. *Final attack (DC 75–83) of barley net blotch disease in spring barley from 1988–2021. Averages for Halland, Skåne and Blekinge regions of Sweden (Alden, L., Berg, G., Christerson, T., Gerdtsson, A., Ostlund, R. (2021).*

Table 3 *Mean severity (%) and standard deviation (SD) of net blotch and scald of barley in Sweden at DC 73-77 from 2013 to 2017. N= Number of trials (Jalli et al., 2020)*

Year	Net blotch			Scald		
	N	Mean	SD	N	Mean	SD
2013	12	15.7	15.4	11	0.8	1.5
2014	11	8.4	14.2	10	0	0.1
2015	10	8.6	10.4	10	9	5.9
2016	3	6.2	10.8	3	0	0
2017	13	15	23.5	5	0.2	0.3

Besides this, every year, many trials are conducted to assess the performance of cultivars grown in Sweden for various traits including diseases. Precautionary measures can be implemented based on the disease spread in the field and its progress over time.

Since the use of resistant cultivars is the most suited method for disease control, development of ideal cultivar with resistance to almost all diseases along with good yield and quality is the objective of breeders nowadays. Therefore, as the part of cultivar release process (Figure 3), various cultivars down the line are tested for various diseases in the field, along with other traits including productivity and quality every year before reaching the market. It is one of the precautionary methods for the future for various traits. Most of the testing is done in the multi-location of the country. It allows for assessing the performance of cultivar in specific environments and helps concerned agencies to recommended varieties for a particular location to farmers.

However, due to complexity of disease symptoms, resistance and uncertainty in climate, field assessment of disease in these trials' cultivar becomes difficult, varies within years and location, and could not reveal the true genetics of the cultivars. To categorize these trials cultivar (described in notes) against different disease, study of interaction is needed in a controlled environment. It will help to answer the true disease symptoms and genetics of the cultivars and pathogen interaction. Therefore, study of responses of cultivars against locally collected isolates in controlled environment was aimed in this work. Additionally, to see the resistance level in old cultivars and breeding lines of NBS countries, it was thought to include those genotypes for screening experiment.

However, since disease occurrence depends on the isolate of the pathogen, environment and host plant interactions, optimization of factors governing the disease symptoms as well as procedure for disease assessment is very important. This study also aimed at such optimization i.e., pathogen isolation, culture and symptoms assessment method.

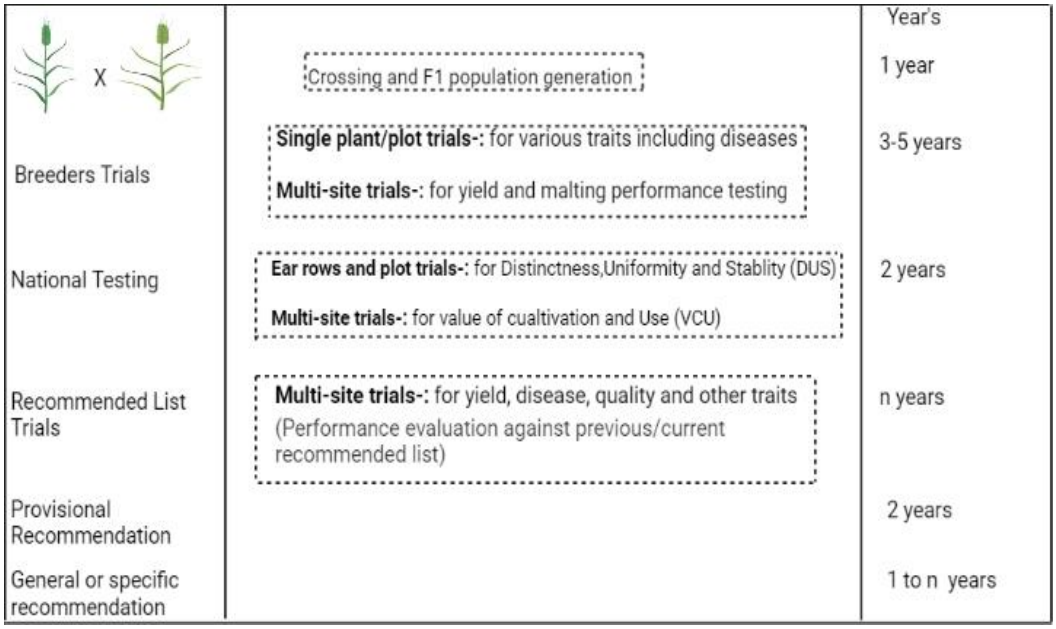


Figure 3*. Schematic representation of cultivar development from crossing to commercialization (Adopted from Acquaah,2009). Figure created in Biorender (https://biorender.com/)*

Project objectives

1. Isolation of pathogen *Pyrenophora teres f. teres* and *Rhynchosporium commune* from various fields situated in the South of Sweden.
2. Development/optimization of protocol for culture of both pathogens.
3. Evaluate seedling stage resistance of genotypes for net blotch and scald disease under controlled climatic conditions.
4. Comparison of official trials cultivars field experiment results with controlled climate condition results for net blotch.

2. Material and methods:

2.1 Collection of pathogens

For NFNB, 3 locations (within 50 km radius) were the source of leaf samples. One of the locations was a field near to Swedish University of Agricultural Sciences, Alnarp campus. The second and third locations were from a field near Ystad situated in the Skåne region of Sweden for net blotch leaves sample. For scald, most of the samples were collected from fields of barley in Ystad.

2.2 Isolation, storage, and inoculum preparation for net blotch (NB) experiment

Collected leaves were kept in a paper envelope, dried, and stored at room temperature. They were cut into small pieces (as per disease infection level), surface-sterilized in alcohol (70%) for 30 s, followed by rinsing in sodium hypochlorite (5%) for 2 minutes and finally washed with sterile water for 3 times. The sterile cut leaves were then placed on filter paper (sterile) for allowing it to dry in 90 mm Petri plates. Later, the leaves were placed in water agar (WA) plates containing 100 µg/L of rifampicin. The WA plates were then incubated under white light/dark conditions for 12/12 hours at 19 ºC for 4-5 days until mycelial growth was seen protruding outside of cut leaves under a stereomicroscope. Subsequently, mycelium was collected from the cut leaves using a sterile scalpel and placed in 20% V8 media plates. These plates were then incubated under white light for 3 days under 12/12 h light and dark conditions at 19-20 °C. Before preparation of inoculum for the screening experiment, 20% V8 media plates were kept in 8 hours of UV light. Spores counting was done using haemocytometer to confirm the effect of spores count by UV light. Later, with the confirmation of increase in spores count by placing the plates in more duration of UV light. Plates were transferred into the UV chamber for 12-14 days under 12 hours of UV and kept again in white light under similar conditions mentioned above. This duration of UV light was eventually used for preparation of inoculum for NB experiment.

Condia/conidiophores were observed under the microscope after following the protocol mentioned above. Later, the plates were flooded with tap water (approximately 10 ml) and scrapped gently with a sterile rubber spatula. All spore containing liquid was collected in a beaker and the concentration of inoculum was adjusted to 10000 conidia per ml using a haemocytometer. Vortexing of inoculum was done for 30s to help mycelium fragmentation and conidia dispersion. Tween® 20 with a concentration of 0.02% (V/V) was added to the prepared inoculum to facilitate adhesion to the leaf surface.

2.3 Isolation, storage, and inoculum preparation for scald experiment

Leaves infected with scald in the field were kept in a paper envelope, dried, and stored at room temperature. Leaves with a higher infection rate were cut into small pieces, rinsed in 5% sodium hypochlorite for 2 minutes, and eventually washed up to 3 times in sterile water. Then, the leaves were allowed to dry in filter paper placed in a sterile 90 mm petri dish.

Later, the disinfected leaves were placed in WA plates with 100 µg/L of rifampicin and kept at room temperature for approximately two weeks. Afterwards, the plates were positioned under the stereomicroscope and grown mycelium (occasionally spores) were transferred into modified CZV8MC (containing Czapek-Dox broth, 20% V8, Malt extract and calcium carbonate) media using a sterile scalpel. The CZV8MC plates were incubated in dark condition at 17–18ºC until white mycelium (1-3cm diameter) were seen in the plates.

2.3.1 Method 1: Inoculum preparation from mycelium

Mycelium and spores growing in an old CZV8MC media plate was transferred into new CZV8MC plates, 400-500 µL of sterile water was added and spread all over the plate using a sterile L-shaped spreader. The plates were then incubated in dark conditions at 17-degree Celsius for 1 week.

Alternatively, mycelium and spores grown in CZV8MC media was also transferred into the WGA (Wheat germ agar) plate, added sterile water, spread all around the plates, and incubated in the dark condition for 2 weeks 17 degrees Celsius.

Both steps and media (CZV8MC and WGA) mentioned above required sub-culture of pathogen within 10 to 12 days for preparing the inoculum. Therefore, spore stock suspension of the mycelium and spores found in both new CZV8MC, and WGA

plates were prepared in Eppendorf tubes to facilitate the storage of pathogen and save time.

2.3.2 Method 2: Inoculum preparation from frozen spore stock suspensions

Spore found in both the media (CZV8MC and WGA) were collected in the Eppendorf tube at 50/50 V/V of spores and glycerol. It was stored at -80 degrees Celsius and was used to prepare the inoculum for scald-controlled condition experiment. 20 µL of stock spore was taken, placed at the WGA plate and 450 µL of sterile water was added to the plate. Finally, the spore/glycerol/water solution was spread on the plate evenly using a sterile L shaped spreader. The plates were placed in dark condition at 16ºC temperature in the upside-down position. After 24 hours, the plates were flipped and were allowed to grow in similar conditions for the next two weeks.

Tap water was added to the plates (around 20-30 ml) and scrapped forcefully with the paintbrush to release spores. Finally, after collecting spores from each plate, inoculum concentration was adjusted to 5×10^5 spores/ml before inoculation. Also, 0.02% of the tween 20 (V/V) was added to the final inoculum to aid adhesion to the leaf surface.

2.4 Plant material

37 cultivars that are part of the national trials in Sweden in 2021-22 were assessed for resistance against NB and Scald disease under controlled conditions (Appendix table 1). Along with these cultivars, 42 other genotypes representing cultivars and breeding lines from Nordic and Baltic regions were included (Appendix Table 2). For which, seven to eight cultivars/breeding lines were selected randomly from Estonia, Denmark Latvia, Lithuania, Sweden to study the resistance of the cultivars and breeding lines.

2.5 Experimental design

In both experiments (net blotch and scald experiment), three replicates of the genotypes were arranged in random augmented block design making a total of four blocks per replicate. The three replicates were designed using agricolae package (design.dau function) in R (De Mendiburu, 2014)**.** Four genotypes RGT planet, Dragoon, Laureate and Flair were assigned as control checks in the NB experiment,

while Ingrid, Nordal, Freja and RGT planet were check genotypes for the scald experiment.

2.6 Plant growth conditions

Up to six seeds of each genotype were sown in pots of 9×9×8 cm size filled with peat soil from Emmaljunga Torvmull AB, Sweden. After successful germination, the plants were thinned and only two seedlings per pot were left to grow. The experiment was performed in the climate-controlled chamber in the Biotron, a facility situated in the Swedish University of Agricultural Sciences, Alnarp.

Prior to inoculation, growth conditions of barley plants (2-3 leaf stage) were set to 22ºC with 12 hours of light (250 µmol/m^2s^{-1}) and 12 hours of light/dark cycle and humidity maintained at 65% for the net blotch experiment. Similarly, seedlings (2-3 leaf stage) of barley were grown at 18°C with 14 hours of light and 8 hours of dark condition and 65 % humidity condition for the scald experiment. 1 ml of high soluble nitrogen SW-BOUYANT 7-1-5 and 0.5 g of KH_2PO4 per block were applied two times, before and after inoculation for both the experiment.

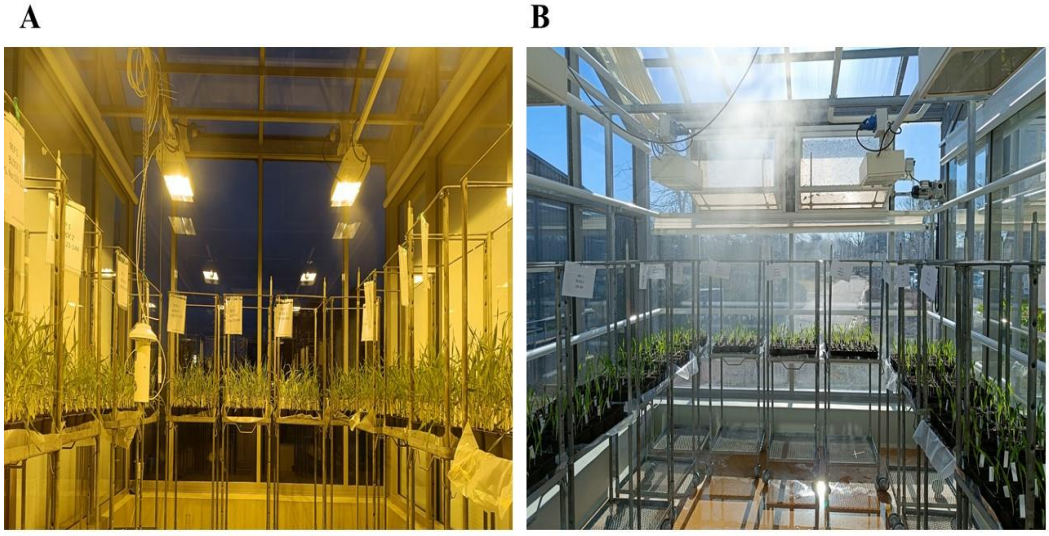


Figure 4. *Barley plants and pre-inoculation chambers for net blotch and scald experiment.* ***(A)****Barley plants (at Zadoks stage 14) and pre-inocualtion chamber for net blotch.* ***(B)*** *Barley plants (at Zadoks stage 12) and pre-inoculation chamber for scald.*

2.7 NFNB inoculation condition

Plants were spray inoculated at 3 leaf stage until leaves were entirely wetted with inoculum using a hand-held sprayer. Afterwards, the climate inside the controlled chamber (Biotron) was adjusted to the temperature of 19 ºC, 90% humidity and complete dark condition created with black polythene plastic for 24 hours (Figure 5 (A)). Subsequently, humidity was lowered to 70%, light condition to 300 µmol/m²s and the temperature of 19 ºC was maintained in the following days after inoculation.

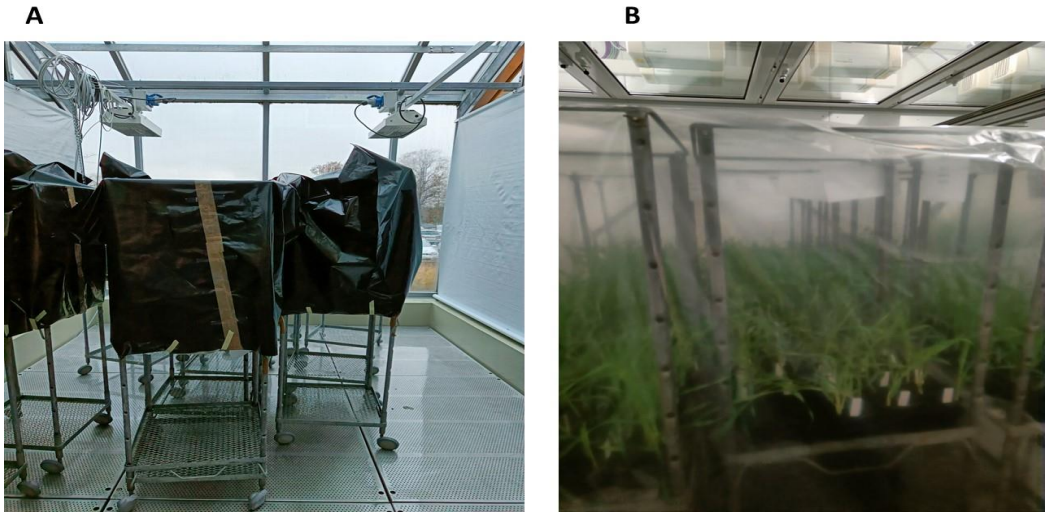


Figure 4. *Post-inoculation chambers for net blotch and scald experiment.* ***(A)*** *Trolley covered with black plastics to create dark condition for net blotch experiment.* ***(B)*** *Trolley covered with transparent plastics to create higher humidity condition for scald experiment*

2.8 Scald inoculation condition

As net blotch disease screening experiment, the resistance of genotypes against *R. commune* was also tested in the seedling stage i.e., 2-3 leaf stage of barley. After spraying the inoculum, blocks containing the pots were covered with transparent plastics to create a closed humidity condition and the climate in the chamber was adjusted to around 90% humidity for 72 hours at a temperature of 16 degrees Celsius (Figure 5(B)). After 72 hours of dark conditions, the plants were allowed to grow in 16 hours of light (250 µmol$m^{-2}s^{-1}$) at 17 ºC maintaining the humidity to around 75-80% until symptoms developed.

2.9 Disease assessment for net blotch experiment

The second and third leaves of respective genotypes were marked with a thin metal or thread ring. From 5 days post inoculation (dpi) to 17 dpi, leaves were assessed for the infection of NFNB response following a 1-10 scale as per Tekauz, 1985 and Jalli, 2010(Figure 6).

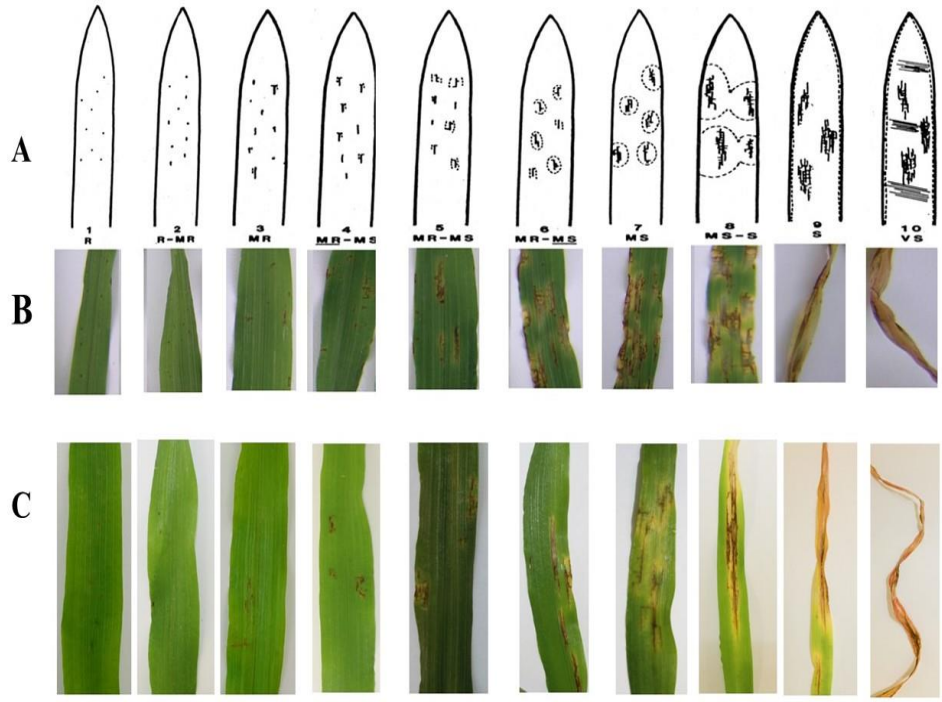


Figure 5. *Scale for net form of net blotch (NFNB) scoring on leaves.* ***(A)****Scale proposed by (Tekauz, 1985).* ***(B)*** *The scale used by (Jalli,2010).* ***(C)*** *The scale used in this study. Scoring was based on a scale of 1-10 where 1 represents the most resistant type and 10 represent the most susceptible type of genotype.*

2.10 Disease assessment for scald experiment

The second and third leaves of respective genotypes were marked with the help of a thin metal ring. Symptoms in the marked leaves were assessed from 7 dpi to 16 dpi and on a scale of 1 to 10 (Figure 7).

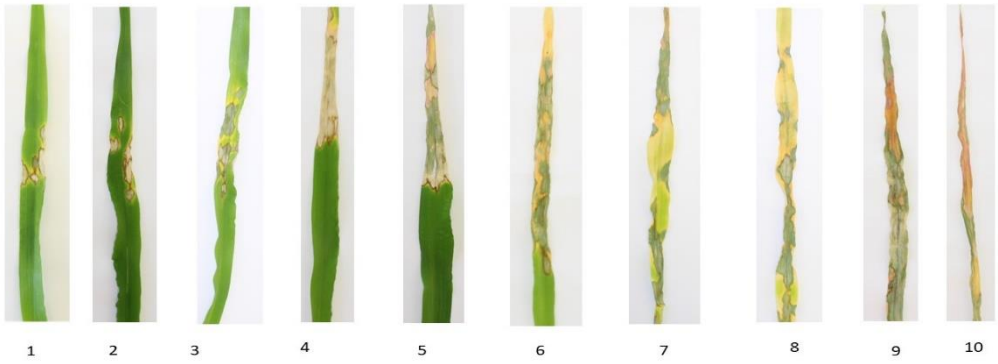


Figure 6. *Scoring scale for scald disease severity on leaves of barley. Scale from 1 to 10, 1 representing the less disease spread in whole leaf and 10 represents whole leaf collapsed due to disease.*

2.11 Phenotypic analyses

Net blotch experiment

An unadjusted mean score was calculated by averaging the scores from four leaves per pot. Then, the checks in each block in each augmented design were used to adjust the mean for all the genotypes using dau.test function in the agricolae package in R (De Mendiburu, Felipe & Yaseen, 2020) according to the following model:

$$Y\_il=\mu+ G\_il+ B\_l + \varepsilon\_il$$

Where Y_il is the adjusted mean of ith genotypes in the lth block, μ is the general mean value, G_il is the effect of ith genotype in the lth block, B_l is the block effect and ε_il is the residual.

Similarly, the adjusted mean was calculated for each time point of scoring (in total five time scoring points) and the audpc function in agricolae package in R was used to calculate Area under disease progressive curve (AUDPC) (De Mendiburu, Felipe & Yaseen, 2020). AUDPC calculation was based on following equation:

$$AUDPC=\sum_i^{n-1}\left(\frac{y_i+y_{i+1}}{2}\right)(t_{i+1}-t_i)$$

Where y_i is the disease score in the i^{th} date, t_i is the i^{th} day, n is the number of dates on which disease was recorded.

Finally, for calculating BLUEs (Best Linear Unbiased Estimates), the adjusted mean data from each augmented design was analysed in Meta R 6.04 (Alvarado et al., 2015) under the RCBD option using the following model:

$$Y\_im = \mu + G\_im + R\_m + \varepsilon\_im$$

Where Y_im is the BLUE of ith genotype in mth replicate, μ is the general mean value, G_im is the ith genotype effect in mth replicate, R_m is the effect of mth replicate and ε_im is the residual effect.

Scald experiment

Unadjusted mean scores were calculated from four diseased leaves samples from all the blocks and replication. Checks in each block were used to adjust the mean using the same package and model used in the net blotch experiment. Afterwards, adjusted mean scores from each time point were used to calculate AUDPC using the same procedure as outlined above for the net blotch experiment. Eventually, AUDPC values from each replication were used to calculate BLUEs using the same software and model mentioned in the net blotch experiment.

2.12 Field experiment design

The field trails in Sweden were arranged in alpha design with two replications. The plot represent minimum of $15m^2$ for each cultivar. The locations for field trials were in following places of Sweden: Vintrosa, Bålsta, Smedby, Ljungsbro, and Västerås (Figure 8).

2.13 Rank correlation between field data and controlled condition data for net blotch.

Percentage mean for individual cultivars was calculated from individual field scores for the cultivars (percentage infected). Later, a grand mean was calculated from all the mean scores of individual cultivars. Genotypes were categorized in two classes: resistance and susceptible, based on deviation of percentage mean value of individual cultivar from grand mean. Finally, a rank correlation table was prepared for the controlled condition experiment of net blotch with field trials results of net blotch infection in Sweden 2021.

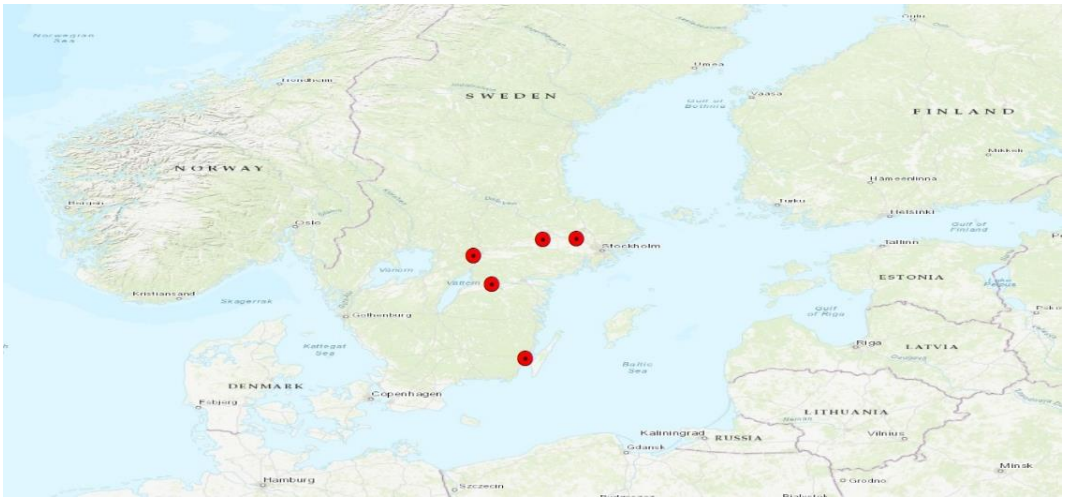


Figure 7. *Map of Sweden and NBS countries. Red dots showing location of fields for net blotch data from field trials of Sweden 2021. (Map extracted from ArcMap)*

3. Results

3.1 Isolation of pathogens

To inoculate the plant material and study the level of resistance in trials cultivar and breeding lines, isolation of both the pathogens *P. teres f. teres* and *R. commune* were done. Sampling was carried out from various fields situated in the southern region of Sweden and showed prevalence of pathogens in the barley fields in Skåne region of Sweden.

3.2 Protocol for *P. teres f. teres* isolation and culture

To isolate and culture the pathogen *P. teres f. teres* an optimized protocol was developed. The protocol consisted of four major steps: (a) leaf sample collection and disinfection;(b) developing mycelium by placing infected leaf disks on WA media;(c), followed by incubating plugs of mycelium-containing plugs of WA on V8 white light/dark conditions; (d) then, stress treatment on V8 media using near ultraviolet UVA radiation; and finally(e) V8 media plates incubation in light/dark condition. Figure 9 shows a summary chart of the protocol used from isolation of the pathogen till spore induction after stress treatment. In order to maintain a viable source of the pathogen that can be readily available for inoculum preparation, spore stocks were generated and stored at -80ºC. Glycerol was added to spore suspension with a ratio of 50/50 V/V as a cryopreserving agent to limit the damaging effect of lower deep freezing. These stocks were used directly for growing the pathogen, skipping steps a and b, respectively in net blotch experiment.

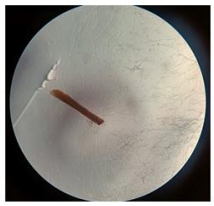
A) Disinfection	B) Water agar incubation	C) V8 media incubation in light/dark condition	D) V8 media plates incubation in UV	E) V8 media incubation in light/dark condition
Surface sterilize the cut diseased leaf sample.	Place the disinfected pieces of sample in water agar plates and under white light/dark condition for 12/12 hr at around 19-21 °C for 3-4 days. Collect the sporulating asexual conidiophore or mycelium using sterile scalpel by observing the WA plates under light microscope and place them in V8 media plates.	Keep the plates containing conidia or mycelium under white light/dark condition 12/12 hr at 19 °C for 3-4 days.	Then, incubate the plates for 12-14 days under UV light and dark condition for 12/12 hr at 19 °C.	Finally, keep the plates in same condition mentioned in steps **(C).**
				

Figure 8. *Protocol for P. teres f. teres culture under laboratory condition.*

Among the samples collected, most of them gave the expected fungi structure. However, one of the isolates collected from Ystad did not sporulate the same conidia as *P. teres f. teres* when kept in UV. Although, it showed typical mycelial structure for the pathogen when kept in white light/dark conditions.

While optimizing the protocol, it was also found that the spore count of *P. teres f. teres* was low when kept under white light/dark condition plus 8 hours of UV light. confirmed by haemocytometer count (around 4000-5000 spores/ml). But when the duration of UV treatment was increased to 12 hrs, the spore count increased by 2 times i.e., 10000-15000 spores/ml.

3.3 Protocol for *R. commune* isolation and culture

A revised protocol was developed for isolation and culture for *R. commune* under laboratory conditions. The protocol mainly comprised of five vital steps: (a)leaf disinfection; (b)water agar plates incubation; (c) modified CZV8MC media incubation; and (d)wheat germ agar (WGA) plate incubation (Figure 10).

It was found that CZV8MC media works well for developing mycelium, while WGA media plates are suited for the growth of mycelial structures and sporulation of the pathogen respectively.

Sub-culture of *R. commune* from frozen spore stock suspension extracted good number of spores count per plate as confirmed by counting using haemocytometer.

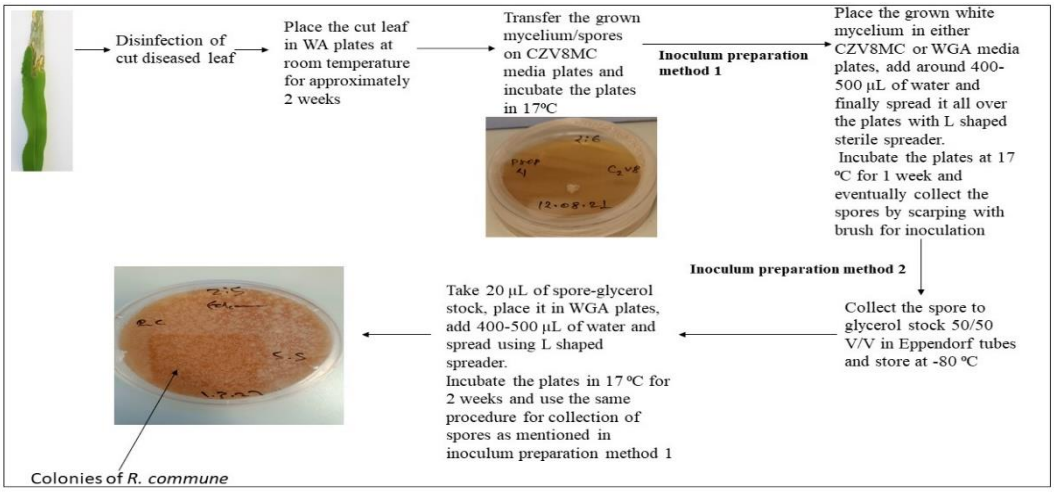


Figure 9. *Protocol for R. commune culture in laboratory condition.*

3.4 Screening for resistance in barley genotypes against net blotch

Differential responses were seen from both trials' cultivars and genotypes\breeding lines against NFNB disease. Tagged leaves were assessed for multiple time points from five days after inoculation (DPI) to seventeen DPI. Accordingly, the area under the disease progression curve (AUDPC) was calculated (Figure 11).

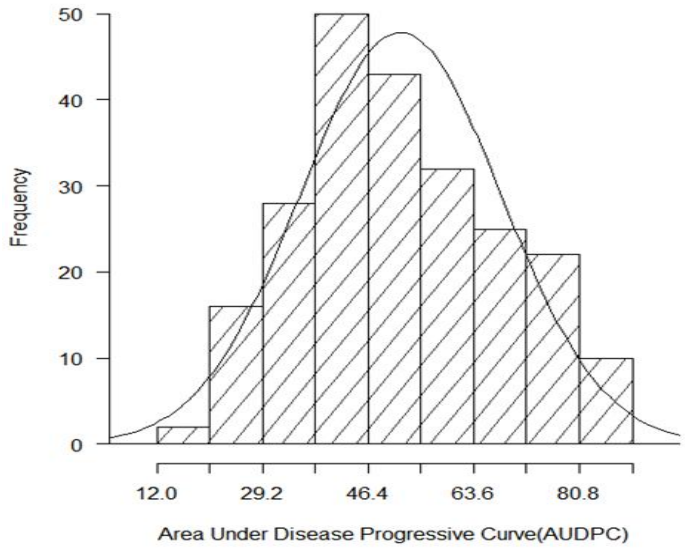


Figure 10. *Area under disease progressive curve for net blotch experiment.*

Analysis of variance showed significant differences between barley genotypes against net blotch resistance (Appendix table 3 and table 5). The adjusted mean disease scores ranged from 1.90 to 10.50 in all the replications, with a mean of 6.11(Figure 12) and broad sense heritability for the trait was found 0.62 (Table 5).

Based on BLUEs (described in notes) scores for individual genotypes, genotypes were categorized into various resistance types: Medium Resistant (MR), Medium Susceptible (MS), Susceptible/very susceptible (S/VS)) against NFNB. BLUEs (described in notes) scores were categorized accordingly (Table 4).

Regarding official trials cultivars, Firefoxx, SY Splendor, SY tungsten and SY Lowry were found to be MR, while Yoda, stairway and Flair were categorized as S/VS (Table 6). Similarly, breeding line 4953.6.5.3.2 originally developed in Estonia was found MR against NFNB (Table 6).

Out of 37 official trials cultivar studied in the NFNB experiment, 12 cultivars did not correlate with field data (Table 7).

Table 4 *BLUEs score, colour indication and type of resistance for NB experiment.*

BLUE SCORE	**Colour**	**Abbreviation**	**Type of resistance**
3		MR	Moderately Resistant
4		**MR**-MS	**Moderately Resistant**/Moderately susceptible
5		MR-MS	Moderately Resistant/Moderately susceptible
6		MR-**MS**	Moderately Resistant/**Moderately susceptible**
7		MS	Moderately susceptible
8		S	Susceptible
9		VS	Very susceptible

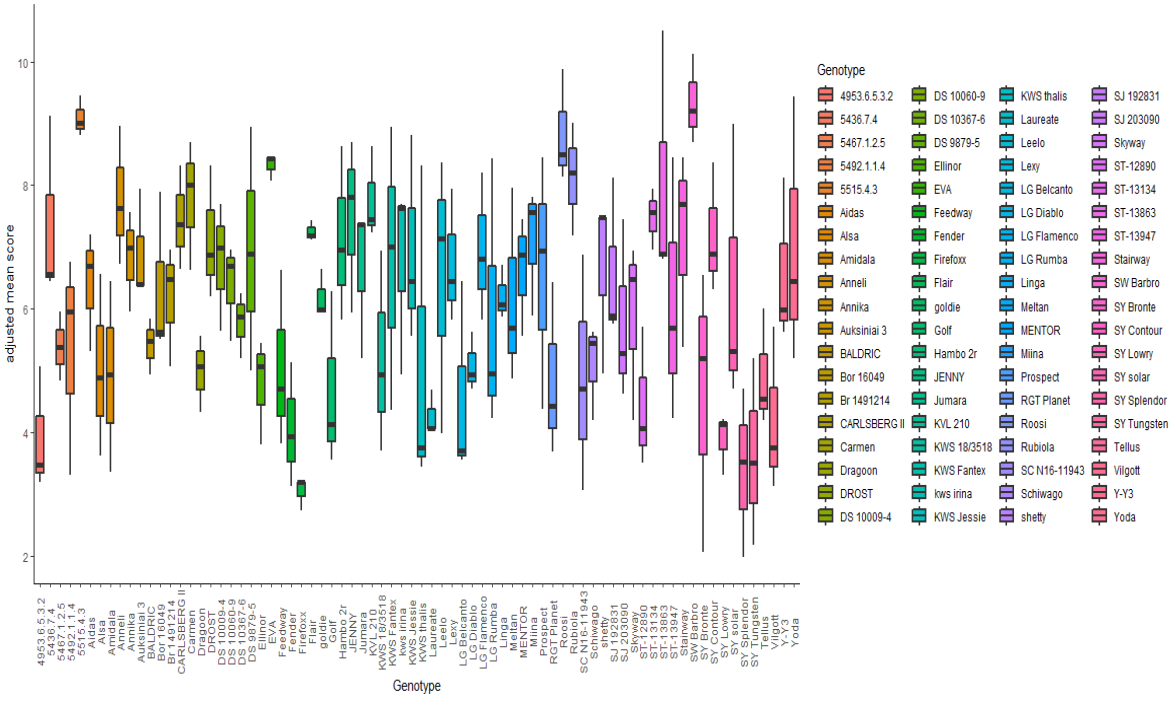


Figure 11. *Adjusted mean scores of genotypes across all the replications for the NB experiment.*

Table 5 *Broad sense heritability (H^2), genetic variance, coefficient of variation, and grand mean for NB experiment.*

Statistic	BLUP_Score	BLUE_Score
Heritability	0.62	NA
Genotype Variance	1.14	NA
Residual Variance	2.04	2.04
Grand Mean	6.11	6.11
LSD	1.82	2.30
CV	23.39	23.39
n Replicates	3	3
Genotype significance	3.13E-07	1.48E-07
	***	***

***Significant at P<0.0001

Table 6 *Category of gneotypes into different resistance types based on BLUEs score for NB experiment under controlled condition*

Trials cultivar	BLUE SCORE	Resistance type	Breeding lines/cultivar	BLUE SCORE	Resistance type
Firefoxx	3.04	MR	4953.6.5.3.2	3.91	MR
SY Splendor	3.40	MR	Vilgott	4.36	**MR**-MS
SY Tungsten	3.71	MR	ST-12890	4.42	**MR**-MS
SY Lowry	3.88	MR	Golf	4.65	**MR**-MS
Fender	4.06	**MR**-MS	Alsa	5.02	MR-MS
Laureate	4.27	**MR**-MS	LG Diablo	5.09	MR-MS
LG Belcanto	4.56	**MR**-MS	5492.1.1.4	5.34	MR-MS
SY Bronte	4.60	**MR**-MS	5467.1.2.5	5.38	MR-MS
Ellinor	4.77	**MR**-MS	BALDRIC	5.41	MR-MS
RGT Planet	4.85	**MR**-MS	DS 10367-6	5.77	MR-MS
SC N16-11943	4.88	**MR**-MS	ST-13947	6.12	MR-**MS**
Amidala	4.91	**MR**-MS	Leelo	6.16	MR-**MS**
Tellus	4.91	**MR**-MS	Meltan	6.17	MR-**MS**
Dragoon	4.98	**MR**-MS	goldie	6.18	MR-**MS**
Feedway	5.04	MR-MS	Linga	6.21	MR-**MS**
Schiwago	5.09	MR-MS	DS 10060-9	6.37	MR-**MS**
KWS thalis	5.17	MR-MS	Aidas	6.40	MR-**MS**
KWS 18/3518	5.19	MR-MS	DS 9879-5	6.44	MR-**MS**
LG Rumba	5.45	MR-MS	MENTOR	6.63	MR-**MS**
SJ 203090	5.78	MR-MS	Jumara	6.65	MR-**MS**
Skyway	5.87	MR-MS	DS 10009-4	6.77	MR-**MS**
SY solar	6.08	MR-**MS**	KWS Fantex	6.77	MR-**MS**
Y-Y3	6.10	MR-**MS**	Auksiniai 3	6.90	MR-**MS**
Br 1491214	6.16	MR-**MS**	Miina	7.09	MS
shetty	6.30	MR-**MS**	DROST	7.13	MS
Bor 16049	6.34	MR-**MS**	SY Contour	7.19	MS
Prospect	6.59	MR-**MS**	5436.7.4	7.38	MS
SJ 192831	6.59	MR-**MS**	CARLSBERG II	7.44	MS
Hambo 2r	6.71	MR-**MS**	JENNY	7.48	MS
Lexy	6.73	MR-**MS**	ST-13134	7.48	MS
kws irina	6.75	MR-**MS**	Anneli	7.77	MS
Annika	6.83	MR-**MS**	KVL 210	7.77	MS
KWS Jessie	6.94	MR-**MS**	Carmen	7.77	MS
LG Flamenco	6.94	MR-**MS**	ST-13863	8.06	S
Yoda	7.02	MS	Rubiola	8.13	S
Stairway	7.17	MS	EVA	8.31	S
Flair	7.25	MS	Roosi	8.84	S
			5515.4.3	9.09	S/VS
			SW Barbro	9.34	S/VS

Table 7 *Field data for net blotch infection from Swedish field trial 2021 and comparison with controlled condition results for NB experiment. Trials field located in: Vintrosa, Balsta, Smedy, Ljungsbro, and Vasteras municipality of Sweden. Mean field data=Mean taken from each location individual data (percentage of field coverage). Grand mean= sum of all mean field data (%). Resistant(R)≤Grand Mean; Susceptible(S)≥Grand mean; NA-Not available.*

Official Trials Cultivars	**Resistance type in controlled condition**	**Resistance type in the field**	**mean field data (%)**
Firefoxx	MR	R	6.9
SY Splendor	MR	S	14.4
SY Tungsten	MR	R	9.8
SY Lowry	MR	R	8.8
Fender	**MR**-MS	R	3
Laureate	**MR**-MS	R	6.6
LG Belcanto	**MR**-MS	R	10
SY Bronte	**MR**-MS	R	9
Ellinor	**MR**-MS	R	10.4
RGT Planet	**MR**-MS	S	16
SC N16-11943	**MR**-MS	S	15
Amidala	**MR**-MS	R	10.4
Tellus	**MR**-MS	R	6.2
Dragoon	**MR**-MS	R	5.2
Feedway	MR-MS	S	11.8
Schiwago	MR-MS	S	11.2
KWS thalis	MR-MS	S	14.2
KWS 18/3518	MR-MS	R	9.6
LG Rumba	MR-MS	S	13.4
SJ 203090	MR-MS	R	9.6
Skyway	MR-MS	R	10.6
SY solar	MR-**MS**	S	14.4
Y-Y3	MR-**MS**	S	12.2
Br 1491214	MR-**MS**	NA	NA
shetty	MR-**MS**	R	9.6
Bor 16049	MR-**MS**	NA	NA
Prospect	MR-**MS**	S	11.8
SJ 192831	MR-**MS**	NA	NA
Hambo 2r	MR-**MS**	S	13.2
Lexy	MR-**MS**	R	10.4
kws irina	MR-**MS**	R	10.4
Annika	MR-**MS**	S	15.6
KWS Jessie	MR-**MS**	R	10.8
LG Flamenco	MR-**MS**	S	16.2
Yoda	MS/S	R	5.8
Stairway	MS/S	R	7.6
Flair	MS/S	S	11.2
Grand mean			**11.21470588**

3.5 Screening for resistance in barley genotypes against scald

Phenotypic diversities were observed in both trials' cultivar and breeding lines against scald, and analysis of variance confirmed the significant differences between them. Leaf samples were assessed at different time points, i.e., from 7 DAI to 16 DAI, and AUDPC was calculated from each replication (Figure 13). Later genotypes were ranked based on BLUE (described in notes) AUDPC value. Carmen showed the most resistance towards scald and differ larger with other genotypes in BLUEs (described in notes) AUDPC value. In official trials cultivar, Y-Y3 and SY lowry were found to be most resistant towards scald (Table 9).

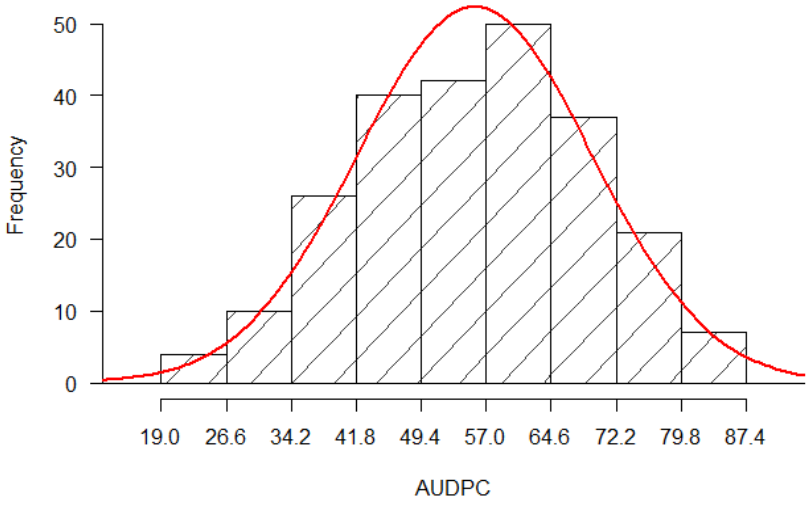


***Figure 12**. Area under disease progressive curve (AUDPC) for scald experiment.*

Table 8 *Broad sense heritability (H^2), genetic variance, coefficient of variation and grand mean for scald experiment.*

Statistics	**BLUP_AUDPC**	**BLUE_AUDPC**
Heritability	0.46	NA
Genotype Variance	28.11	NA
Residual Variance	96.90	96.90
Grand Mean	55.53	55.53
LSD	10.83	15.87
CV	17.72	17.72
n Replicates	3	3
Genotype significance	0.001	0.0004
	**	***

***Significant at P<0.0001; ** Significant at P<0.001

Table 9 Ranking of genotypes against scald of barley in controlled condition.

Rank	**Official Trials Cultivars**	**BLUE_ AUDPC**	**Rank**	**Cultivar/ breeding lines**	**BLUE_ AUDPC**
1	Y-Y3	37.57	1	Carmen	33.1
2	SY Lowry	38.85	2	DS 10060-9	37.7
3	Amidala	44.35	3	LG Diablo	44.1
4	LG Flamenco	45.1	4	Aidas	48.04
5	SJ 203090	45.17	5	Leelo	50.23
6	Shetty	45.48	6	Anneli	51.39
7	Fender	46.35	7	5515.4.3	51.51
8	Schiwago	47.39	8	KWS Fantex	51.76
9	SY Splendor	47.54	9	ST-13134	52.32
10	Dragoon	47.95	10	5492.1.1.4	53.1
11	Laureate	48.42	11	Auksiniai 3	53.92
12	RGT Planet	49.9	12	DS 10009-4	53.98
13	KWS 18/3518	50.95	13	5436.7.4	54.23
14	Bor 16049	51.82	14	BALDRIC	55.01
15	LG Rumba	52.54	15	DS 9879-5	56.48
16	SJ 192831	52.82	16	DROST	56.54
17	Hambo 2r	53.14	17	EVA	56.6
18	LG Belcanto	53.17	18	SY Contour	56.7
19	SY Tungsten	53.2	19	ST-13947	57.01
20	Firefoxx	54.79	20	JENNY	57.42
21	SC N16-11943	55.14	21	Rubiola	57.64
22	KWS Jessie	55.29	22	Miina	58.14
23	Annika	55.57	23	Meltan	58.29

24	Feedway	56.26	24	Freja	58.81
25	SY Bronte	56.51	25	Nordal	59.03
26	Prospect	56.89	26	DS 10367-6	59.23
27	Br 1491214	56.95	27	Jumara	59.51
28	Skyway	57.23	28	ST-13863	59.73
29	Tellus	57.29	29	Golf	59.85
30	SY solar	58.14	30	5467.1.2.5	60.73
31	Lexy	59.51	31	Vilgott	61.07
32	Flair	59.79	32	Roosi	61.14
33	KWS thalis	61.48	33	Linga	61.7
34	kws irina	65.26	34	Ingrid	61.81
35	Yoda	65.82	35	ST-12890	62.73
36	Stairway	72.6	36	Goldie	62.85
			37	CARLSBERG II	64.26
			38	SW Barbro	64.45
			39	Ellinor	66.1
			40	4953.6.5.3.2	69.23
			41	MENTOR	69.76
			42	KVL 210	70.85
			43	Alsa	72.23

4. Discussion

Net blotch and scald are two important diseases of barley having a potential to cause huge losses in yield and quality under favourable environmental condition (S Adawy et al., 2013; El-Mor et al., 2018). Chemical control as well as various agronomic practices are the implemented tools to minimize the losses caused by the diseases (Bartlett et al., 2002; Jordan & Allen, 1984;Avrova & Knogge, 2012). However, the use of resistant cultivars is found to be a more reliable, economical, and sustainable method. The responses of any genotypes/cultivars towards the pathogens are complex phenomenon (Prell & Day, 2001). For this, genetics of plants and environmental condition are key governing factors. But it also depends on severity of isolates, developmental stages of plants (Keane & Kerr, 1997). To reveal the true genetics architecture of the genotypes/cultivars, optimization on these factors is needed. In this work, we optimized the protocol for pathogen culture, procedure for inoculum preparation and environmental condition to study the various cultivars against both the diseases.

Since the cultivar response towards the pathogen is specific to the isolate of the pathogen, studying the performance of genotypes/cultivar against locally collected isolates becomes important for correct recommendation of cultivars in specific areas to farmers. Because of uncertainty and variation in climatic conditions in field, it becomes challenging to conduct these disease screening experiments in the field. Moreover, in the limited growth period of barley, such as for example in Sweden, it requires consideration of the many governing factors such as climate variability, seedling/adult resistance, isolate of pathogen to screen the genotypes in field. Therefore, testing of responses of cultivars for any disease resistance traits in a controlled environment condition becomes easy to handle as well as saves time, providing the true genetic behaviour of the trait (Jalli, 2010). However, validation of resistance types of cultivars/breeding lines in field is needed as enivrinoment condition in the fields and controlled condtions varies significantly.

Isolation of pathogen

To check the performances of different official trials cultivars grown in Sweden and breeding lines of NBS countries, both *P. teres f. teres* and *R. commune*

pathogens were isolated from barley fields in the south of Sweden. A previous study involving isolation of *P. teres f. teres.* from Swedish fields was done by Jonsson et al., 1997 and found significant differences between barley lines against each isolate. This showed the importance of isolate specific studies to characterize the performance of cultivars towards specific isolates.

In terms of Scald, there was a lack of studies related to pathogen isolation, its culture from the earliest in Sweden. This study was aimed at filling the gap by sampling the pathogen from the field and its isolation. This will allow for further study related to pathogen severity as well as screening study with plethora of germplasm.

Protocol for culture of pathogens

Previous protocols for culture of *P. teres f. teres* in laboratory conditions have mainly proposed water agar, V8/V5 and peanut oatmeal agar media's (Scott, 1992;Gilchrist-Saavedra, 1997; Jonsson et al., 1997;Ryan A Fowler et al., 2017). However, different growth conditions are used for incubation of the plates. In this work, WA and V8 media were used for the pathogen culture. Growth conditions such as light (UV-A and white fluorescent light) and temperature was used for stimulating sporulation of pathogen *P. teres f. teres*. It was found that UV-A light is important factor for *P. teres f. teres* sporulation (result 3.2). Further work of single spore culture of pathogens is required to get more spores per plates and to use it in bigger studies.

Regarding *R. commune* culture protocol, PDA, LBA, WGA and WA are the most used media. For growth conditions of plates, we found the temperature range of 15ºC to 18ºC along with dark condition is suitable. It was found that the sub-culture of pathogen in every 10-12 days is required to maintain the viability of pathogen (Gilchrist-Saavedra, 1997). In this study, we used an equal volume of glycerol and spore inoculum kept in Eppendorf tube and stored in -80ºC and again sub-cultured from the freezer stock in WGA plates. The viability of the pathogen remained the same and good number of spores could be extracted from this method. This could be one of the methods for storage of pathogen. Although, multiple tests are needed to verify the current result regarding the storage of pathogen in the future.

Net blotch disease

Net blotch is a necrotic disease, differentiating totally in between resistant and susceptible cultivar. Minor dot like symptoms appears in resistant cultivar, restricting their development of size after a period. Susceptible cultivard show typically net like symptoms along with chlorosis which eventually cause coalesce

of entire leaf (Tekauz, 1985). In this work, we tracked marked leaves for progression of disease over the period. Disease reactions followed the normal distribution(figure 11), however, most of the genotypes fall under MR-MS categories. This also explains the quantitaive nature of the traits. In total of 37 trials cultivar, 4 cultivar (Firefoxx, SY splendor, SY tungsten, and SY lowry) were found resistance(Table 6).

A rank correlation analysis was performed using data collected in the trial cultivars, to study the correlation between net blotch resistance shown in controlled conditions and in the field. The analysis showed that 12 out of 36 cultivars were not correlating with each other (Table 7). A comparison between current experiment result and companies recommendation across the globe was also tried to made, but consistency could not be seen between current results and companies finding based on trials results(Comaprison on shown). Various factors such as different environment, isolates host specific interaction, developmental stages of plants could be the reason for such variation.

Broad sense heritability of 0.62 was found for net blotch resistance in this study. Previous published work on net blotch has also found the heritability ranging from 0.62-0.99 (Grewal et al., 2012; König et al., 2013; J. K. Richards et al., 2017; Novakazi et al., 2019). Along with genetics and environment, type of population, stages for screening also governs the broad sense heritability. With wider range of susceptible to resistant reactions in a population, genotypic variance is higher and ultimately the heritability.

Scald disease

Scald symptoms in leaves of barley protrudes over time and eventually leads to total collapse of leaves in both resistant and susceptible cultivars. Temporal difference in cultivars for the pathogen development in marked leaf can be a method for understanding the virulence and resistance level present in the cultivars towards the disease. In current scald screening experiment, marked leaves were scored for multiple time points and AUDPC difference between genotypes were used for the ranking the genotypes. Carmen, Y-Y3, SY lowry and DS10060-9 were the genotypes showing resistant reaction (Table 9). The broad sense heritability of 0.46 was found for scald experiment (Table 8) and before, 0.20 to 0.97 range of heritability has been estimated (Spaner et al., 1998; Aoki et al., 2011; Xi et al., 2019).

5. Conclusion

The current work isolated the pathogens that cause two important diseases in barley (net blotch and scald), optimized their culture methods and screening methods in controlled conditions. In changing climatic condition and pathogen co-evolution, the importance for both the diseases will increase in the future. Incorporation of these disease as breeding target needs to be in breeding programmes. Therefore, collected pathogen, optimized protocol and screening methods could be used in breeding programmes, research studies to test more genotypes and find source of resistance in the future. Along with that, resistant/susceptible cultivars in Swedish official trials cultivars and NBS countries cultivars/breeding were found, reducing the uncertainty, time span for assessment compared to field. This could help national field trials agencies, breeding companies, and farmers for best cultivar selection.

Visual assessment of responses of cultivars/breeding lines against the pathogens is the most realistic method when a large set of genotypes is tested. However, with the advancement of image analysis and machine learning methods, precision for such evaluation could be gained in the future.

For both the disease, assessment of genotypes in seedling stage and in controlled condition could be the good alternative method for finding the resistance. However, multi-environment testing/trials are still needed to know part environmental effect on the genotypes and pathogen reactions in the environment. In summary, both greenhouse test along with field trials is necessary to select the best cultivars.

In molecular point of view, already identified QTLs/candidate gene from different population could be validated in currently used population by converting identified SNP-based markers into breeders friendly KASP markers. This will allow for confirmation for genes controlling the resistance for particular disease and also markers assisted selection for breeders. The goal is to develop effective, economical, and reliable cultivars which will resist almost all diseases along with good productivity and quality.

6. Notes

i. *BLUE and BLUP:* Linear mixed model allows to estimate both the fixed effect and random effects. A fixed factor is estimated with Best linear unbiased estimates (BLUE) and random effects with Best linear unbiased prediction (BLUP) (Piepho et al., 2008). In BLUE, the term "best" is given as it minimises the variances. "Linear" referring to linear functions of observed value. "Unbiased" referring to expected values of the estimates are equal to their true values. While in BLUP, the term "best" is given as it minimises the variances as in BLUEs. "Linear" indicating the predictions are linear functions of observed values. "Unbiased" as predictions are equal to their true values. Generally, fixed effect explained by BLUEs is considered when we are interested in particular value and random effect explained by BLUPs is more suited when we are more interested in prediction instead of estimation(McCulloch & Searle, 2004). In BLUE i.e., fixed effect, mean of the individual is the estimation parameter. On the other hand, in BLUP i.e., random effect, dispersion or variance of data is the estimate parameters. Therefore, it represents whole population(Buntaran, 2019).

ii. *Official Trials cultivars:* Every year, various cultivars developed in different countries must go through multi-environment trials as a precondition of certified seed in Sweden and in EU. After that, if the variety makes is in Swedish list of varieties or is in catalogue of agricultural variety in Sweden, then only it can enter market as cultivar (Jordbruksverket,2021). Besides, it is also checked for breeder right, which entails a new variety going to be cultivated as cultivar should have distinctness, uniformity, and stability (DUS) characteristics. The term official trials cultivars used in this text refers to variety/cultivars which were part of assessment mentioned above.

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Appendices

Appendices 1. *Official Trials cultivars used in both net blotch and scald experiment*

Trials cultivar	**Breeding company**	**Parents**
Firefoxx		Chanson/Acorn
SY Splendor	Syngenta[a]	Octavia/Dioptric
SY Tungsten	Syngenta[a]	RGT Planet/Ovation
SY Lowry	Syngenta[a]	
Fender		
Laureate	Syngenta[a]	Sanette/Concerto
LG Belcanto	Limagrain[f]	
SY Bronte	Syngenta[a]	Laureate/F1 or selection
Ellinor	BR SEEDS[g]	
RGT Planet	RAGT[e]	
SC N16-11943		
Amidala		
Tellus	Lantmannen[h]	Streng Franken III /3/binder/Opal/2/Balder
Dragoon	Syngenta	Shuffle x F1 selection
Feedway	Nordic seeds[c]	
Schiwago		
KWS thalis	KWS[b]	
KWS 18/3518	KWS[b]	
LG Rumba	Limagrain[f]	
SJ 203090		
Skyway	Nordic seeds[c]	RGT planet/Nordic seeds 2105-11
SY solar	Syngenta[a]	
Y-Y3		
Br 1491214		
shetty		
Bor 16049		
Prospect	Sejet plant breeding[d]	Overture /KWS irina
SJ 192831		
Hambo 2r		
Lexy		
kws irina	KWS[b]	Quench/Conchita

Annika	Sejet plant breeding[d]	
KWS Jessie	KWS[b]	
LG Flamenco	Limagrain[f]	
Yoda	BR SEEDS[g]	
Stairway	Nordic seeds[c]	
Flair	Sejet plant breeding[d]	

a Syngenta (https://www.syngenta.com)

b KWS (https://www.kws.com)

c Nordic Seed, Denmark (https://nordicseed.com)

d Sejet, Denmark (https://sejet.com)

e RAGT Seeds (https://ragt.com)

f Limagrain (https://www.limagrain.com)

g BR seeds (http://www.brseeds.com)

h Lantmannen Sweden (https://www.lantmannen)

Appendices 2 *Cultivars and breeding lines used in both net blotch and scald experiment*

Breeding lines/Cultivar	**Breeder/Origin**	**Parents**
4953.6.5.3.2	Estonian breeding lines	
Vilgott	Lantmannen(Sweden)	
ST-12890	Latvia	
Golf	United Kingdom	Armelle/Lud/2/ Luke
Alsa	Lithuania	Mirena/mutant derived from Gintariniai/2/Abava x Emir
LG Diablo	Limagrain	Overture/Sanette
5492.1.1.4	Estonian breeding line	
5467.1.2.5	Estonian breeding line	
BALDRIC	United Kingdom	
DS 10367-6	Lithuania	
ST-13947	Latvia	
Leelo	Estonian	Ansgar/Sv 2552/2/Elo
Meltan	WW	

goldie	North Dakota origin (US)	
Linga	Latvia	
DS 10060-9	Lithuania breeding line	
Aidas	Lithuania	KM 1192/ofir/2/Effendi
DS 9879-5	Lithuania breeding line	
MENTOR	CENEX, Oregon	Balder/Weihenstephan II
Jumara	Latvia	
DS 10009-4	Lithuania	
KWS Fantex	KWS	
Auksiniai 3	Lithuania	Carina/Tarra 26
Miina	Estonia	
DROST	Denmark	
SY Contour	Syngenta	
5436.7.4	Estonian breeding line	
CARLSBERG II	Denmark	Developed from carlsberg I which was cross between Prentice/Maja
JENNY	Sweden	Kristina /3/Pallas 5/Rupee/2/Hellas 2
ST-13134	Latvia	
Anneli	Lantmannen(Sweden)	
KVL 210	Czech reuplic	
Carmen	Lantmannen(Sweden)	
ST-13863	Latvia	
Rubiola	Latvia	
EVA	Common variety (Sweden)	Birgitta/Mari
Roosi	Estonia (Jõgeva Pl.Br.Inst.)	Nadja/Piggi/2/Abava
5515.4.3	Estonian breeding line	
SW Barbro	Lantmannen (Sweden)	
Frejas	Sweden	
Ingrid	Sweden	
Nordal	Denmark	

Information for origin and parentage extracted from:

[a]Seedstor(https://www.seedstor.ac.uk)

[b] Nordic Baltic genebank information system (https://nordic-baltic-genebanks.org)

Appendices 3 *ANNOVA table for augmented replication 1,2 and 3 in net blotch experiment*

ANOVA REP 1

Treatment adjusted

```
Response: SCORING
                          Df  Sum Sq Mean Sq F value   Pr(>F)
block.unadj                3  52.099 17.3665
trt.adj                   75 199.317  2.6576  3.9225 0.015681 *
Control                    3  18.012  6.0039  8.8616 0.004739 **
Control + control.VS.aug. 72 181.306  2.5181  3.7167 0.019002 *
Residuals                  9   6.098  0.6775
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Block adjusted

```
Response: SCORING
                     Df  Sum Sq Mean Sq F value   Pr(>F)
trt.unadj            75 246.311  3.2842
block.adj             3   5.105  1.7018  2.5119 0.124418
Control               3  18.012  6.0039  8.8616 0.004739 **
Augmented            71 218.684  3.0801  4.5461 0.009306 **
Control vs augmented  1   9.616  9.6160 14.1929 0.004435 **
Residuals             9   6.098  0.6775
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

coefficient of variation: 13.7 %
Means: 6.029356
```

ANOVA REP 2

Treatment adjusted

```
Response: SCORING
                          Df  Sum Sq Mean Sq F value    Pr(>F)
block.unadj                3  33.251 11.0836
trt.adj                   75 276.090  3.6812  13.089 0.0001403 ***
Control                    3  24.562  8.1875  29.111 5.789e-05 ***
Control + control.VS.aug. 72 251.528  3.4934  12.421 0.0001759 ***
Residuals                  9   2.531  0.2813
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Block adjusted

```
Response: SCORING
                     Df  Sum Sq Mean Sq F value    Pr(>F)
trt.unadj            75 300.435  4.0058
block.adj             3   8.906  2.9687  10.556 0.0026414 **
Control               3  24.562  8.1875  29.111 5.789e-05 ***
Augmented            71 271.576  3.8250  13.600 0.0001206 ***
Control vs augmented  1   4.297  4.2969  15.278 0.0035719 **
Residuals             9   2.531  0.2813
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

coefficient of variation: 8.4 %
Means: 6.34375
```

ANOVA REP 3

Treatment adjusted

```
Response: SCORING
                              Df  Sum Sq Mean Sq F value   Pr(>F)
block.unadj                    3   4.830  1.6099
trt.adj                       75 268.232  3.5764  3.9496 0.015306 *
Control                        3  30.793 10.2643 11.3352 0.002066 **
Control + control.VS.aug.     72 237.439  3.2978  3.6418 0.020384 *
Residuals                      9   8.150  0.9055
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Block adjusted

Response: SCORING					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt.unadj	75	271.279	3.6171		
block.adj	3	1.783	0.5942	0.6562	0.599137
Control	3	30.793	10.2643	11.3352	0.002066 **
Augmented	71	224.003	3.1550	3.4841	0.023747 *
Control vs augmented	1	16.483	16.4831	18.2027	0.002091 **
Residuals	9	8.150	0.9055		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					
coefficient of variation: 16.6 %					
Means: 5.735795					

Appendices 4 *ANNOVA table for augmented replication 1,2 and 3 in scald experiment.*

ANOVA REP 1

Treatment adjusted

Response: SCORING					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
block.unadj	3	89.503	29.8343		
trt.adj	78	136.689	1.7524	2.5554	0.06412 .
Control	3	7.141	2.3802	3.4709	0.06401 .
Control + control.VS.aug.	75	129.549	1.7273	2.5188	0.06715 .
Residuals	9	6.172	0.6858		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Block adjusted

Response: SCORING					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt.unadj	78	220.020	2.8208		
block.adj	3	6.172	2.0573	3.0000	0.08771 .
Control	3	7.141	2.3802	3.4709	0.06401 .
Augmented	74	206.287	2.7877	4.0650	0.01384 *
Control vs augmented	1	6.593	6.5930	9.6141	0.01271 *
Residuals	9	6.172	0.6858		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					
coefficient of variation: 9.5 %					
SCORING Means: 8.760989					

ANOVA REP 2

Treatment adjusted

Response: SCORING					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
block.unadj	3	186.241	62.080		
trt.adj	78	169.297	2.170	1.5392	0.2488
Control	3	10.512	3.504	2.4848	0.1269
Control + control.VS.aug.	75	158.785	2.117	1.5013	0.2632
Residuals	9	12.691	1.410		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

Block adjusted

Response: SCORING					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt.unadj	78	331.31	4.2475		
block.adj	3	24.23	8.0768	5.7276	0.01794 *
Control	3	10.51	3.5039	2.4848	0.12695
Augmented	74	316.87	4.2820	3.0366	0.03737 *
Control vs augmented	1	3.93	3.9258	2.7839	0.12956
Residuals	9	12.69	1.4102		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					
coefficient of variation: 15.2 %					
SCORING Means: 7.815934					

ANOVA REP 3

Treatment adjusted

```
Response: SCORING
                            Df  Sum Sq Mean Sq F value  Pr(>F)
block.unadj                  3  55.262 18.4208
trt.adj                     78 112.502  1.4423  3.0074 0.03840 *
Control                      3   6.293  2.0977  4.3738 0.03689 *
Control + control.VS.aug.   75 106.209  1.4161  2.9527 0.04087 *
Residuals                    9   4.316  0.4796
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Block adjusted

```
Response: SCORING
                      Df  Sum Sq Mean Sq F value  Pr(>F)
trt.unadj             78 161.815 2.07456
block.adj              3   5.949 1.98307  4.1348 0.04242 *
Control                3   6.293 2.09766  4.3738 0.03689 *
Augmented             74 154.647 2.08982  4.3574 0.01080 *
Control vs augmented   1   0.876 0.87578  1.8261 0.20958
Residuals              9   4.316 0.47960
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

coefficient of variation: 7.7 %
SCORING Means: 9.021978
```

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