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Improving phenotyping in winter barley cultivars towards waterlogging tolerance by combining field trials under natural conditions with controlled growth condition experiments

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ABSTRACT

Additional rainfall in Northern Europe due to global climate change is increasing the incidences of field flooding. Flooding causes hypoxic stress that results in a reduced capacity for photosynthesis, reduction in nutrient availability and uptake, increased production of toxic metabolites by anaerobic bacteria in the soil, and ultimately yield losses and crop death. To overcome hypoxic environmental conditions, new cultivars need to be bred and tested for waterlogging tolerance. We scored 403 winter barley cultivars from the 'Association Genetics of UK Elite Barley' (AGOUEB) population, taking advantage of the phenotypic changes associated with hypoxic stress. This enabled us to identify an initial set of waterlogging sensitive and tolerant cultivars. Comparative analysis of a subset of 65 cultivars exposed to waterlogging stress under field and growth cabinet environments showed variability in scores due to varying sensitivity to waterlogging over multi-season field trials. In field trials, we observed waterlogging damage resulting in reductions in biomass, grain yield and crop height. However, the effects varied between seasons and the severity of waterlogging due to differences in the topography of the field and the amount of rainfall. To overcome the seasonal variations in environmental conditions in multi-season field trials, we developed in parallel, an enhanced phenotyping method by complementing field experiments with phenotyping under controlled growth conditions. The phenotyping scoring method allows for the grouping of cultivars by sensitivity and tolerance to waterlogging, with limited variance between cultivars scored in the field and controlled conditions. Together, these two complementary approaches maximise the data available to breeders, allowing for the reliable selection of more tolerant cultivars able to grow under flooding conditions.

1. Introduction

Global climate change is increasing the severity and frequency of extreme weather events which pose a threat to crop production. Extreme rainfall can be a major limiting factor in crop yield. A projected increase in rainfall of between 5% and 15% by 2071–2100 (Jacob et al., 2014) during winter months have been forecasted for Northern Europe. This is expected to cause additional crop losses, as the majority of Northern European cereal crops are planted in late autumn or early spring. Reported global crop losses due to flooding vary between studies due to numerous factors including no clear definition of flooding severity, variability in flood duration and weather patterns (Shaw and Meyer, 2015). While the percentage of land use and yield loss is the most effective reporting mechanism due to its accuracy and ease of understanding, the severity and duration of flooding are rarely reported when data is collected about national or global crop loss, leading to

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inconsistencies between reports (Shaw et al., 2013).

Flooding causes straw biomass, as well as grain and yield losses, and in the most severe instances, crop death. Globally 20–25% of the world's crops are affected by flooding (De San Celedonio et al., 2014). Flooding is classified into three categories: (1) waterlogging, (2) flooding with partial submergence, and (3) flooding with complete submergence (Voesenek and Bailey-Serres, 2013; Sasidharan et al., 2017). Waterlogging is the submergence of the root system in groundwater. It may not be visible, but the plant is affected and the root system becomes hypoxic shortly after the onset of waterlogging (Loreti et al., 2016). Groundwater flooding refers to when the water table has risen to or above the surface level of the soil. If the groundwater has covered the plant, it is referred to as submergence flooding, and the intermediate between these is partial submergence (Voesenek and Bailey-Serres, 2013). Flooding stress is caused by the low molecular diffusion rate of oxygen through water (Grable, 1966). This creates hypoxic (low oxygen) conditions for the plants and anoxic (lack of oxygen) conditions after prolonged periods of flooding (Zhou et al., 2013). The lack of oxygen reduces aerobic respiration, and the plant reverts to anaerobic respiration (Veen, 1981). In addition to the energy (ATP) reduction, increased glucose consumption can lead to carbohydrate depletion, which in turn affects recovery after flooding (Nishiuchi et al., 2012). This energy reduction is the primary cause of flooding damage, but there are many secondary issues involved in flooding and waterlogging stress.

Nutrient uptake and use are reliant on available oxygen and adequate ATP (Dracup et al., 1992; Guyomarc'h et al., 2016). Noticeably in cereal crops and grasslands, a reduction in manganese (Mn) and iron (Fe) due to prolonged flooding reduced growth (Zhou et al., 2013). Another detrimental secondary effect of flooding is the toxicity of flooded soil. Hypoxic soils have a higher concentration of toxic sulphide due to anaerobic microorganisms metabolising sulphates. Sulphide is phytotoxic and has an inhibitory effect on cytochrome oxidase, which is essential for respiration. Hydrogen sulphide (H_2S) is particularly phytotoxic and increasing concentrations negatively affect net photosynthesis and further reduces nutrient uptake (Shabala, 2011). Reduction of energy, limited nutrients and a toxic growing environment are the leading causes of flooding damages to crops. A plant's tolerance is usually mediated by adaptive mechanisms working together to increase oxygen uptake and decrease the effects of toxic metabolites and reactive oxygen species (ROS) (Fritschi et al., 2014; Mustroph, 2018; Voesenek and Bailey-Serres, 2013). Mechanisms of tolerance vary between species and include morphological, physiological and molecular changes. One such change is the formation of arenchyma in the roots (Videmšek et al., 2006). These gas pockets increase gas flow allowing plants to survive in hypoxia conditions. Metabolic changes to the plants such as the up-regulation of alcohol dehydrogenase (ADH), sucrose synthase (SUS) and pyruvate decarboxylase (PDC) are linked with tolerance to hypoxia. High levels of ADH may contribute to recovery due to the ethanol being converted to acetate and acetyl-CoA (Ismond, 2003). SUS contributes to hypoxia resistance through the breakdown of sucrose, providing more fructose to the glycolysis pathway resulting in more energy for the plant (Wang et al., 2014). Higher levels of PDC allow the plant to maintain ATP production through the ethanolic fermentation pathway (Xuan et al., 2016).

Phenotypic changes associated with flooding tolerance and sensitivity in *Hordeum vulgare* (barley) can be easily observed. One of the most common is yellowing starting at the tip of the leaves due to the reduction in chlorophyll (Zhou et al., 2013). After prolonged hypoxia, the plant will begin to wilt. Severe flooding will cause necrosis of the plant (Sundgren et al., 2018). The phenotype caused by flooding stress allows for the rapid ranking of flooding sensitivity in plants. Phenotypic scoring allows for the analysis of traits within a population and breeding desired traits into new cultivars. While phenotypic scoring methods are effective for grouping large populations, they are susceptible to errors due to trial design and conditions. For example, Mano and Takeda (2012) have highlighted the effect of soil differences when ranking cultivars for flooding sensitivity. The objectives of this study were (1) to phenotype a large set of winter barley cultivars under multi-season field trials and controlled growth conditions to develop a reliable phenotyping method for determining waterlogging tolerance, (2) to devise from these experiments an optimal method for breeders for selection of this trait, and (3) to characterize the cultivars for their waterlogging tolerance phenotypes with agronomic and physiological descriptors.

2. Materials and methods

2.1. Plant material

Hordeum vulgare (barley) seeds from 403 winter barley cultivars (Supplementary Table 1) of the AGOUEB population (Thomas et al., 2014) were coated with 2 μl g^{-1} Redigo Deter™ fungicide.

2.2. Development of a phenotypic scoring system

The phenotypic scoring system was primarily based on plant health (Miricescu et al., 2021). Signifying traits of decreased plant health due to hypoxic stress are chlorosis, necrosis and wilting (Sundgren et al., 2018). Chlorosis was scored using a gradient from the tip of the leaf to the base of the leaf. These traits were compiled into a scoring chart (Fig. 1) and cultivars were ranked from 1 to 6. Scoring was done once the plants had reached growth stages 30 and 31 (Zadoks et al., 1974). The scoring system was used in three situations. Firstly to select cultivars from the pilot study, secondly in two field seasons to evaluate the use of phenotypic scoring in waterlogging conditions, and lastly to evaluate cultivars response to waterlogging in controlled growth conditions. Plants were scored individually by eye in both the field and the growth cabinet. The exception to this was the pilot study in which the plants were scored from photographs.

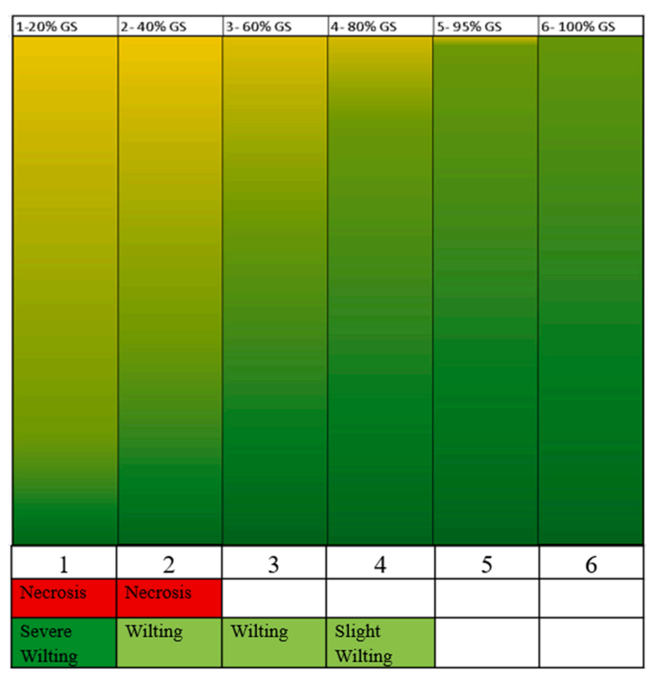

Fig. 1. Scoring chart used for phenotyping. A colour gradient represents the percentage leaf green space (GS) indicating chlorosis in sensitive cultivars (score 1) to tolerant cultivars (score 6). The presence of necrosis on the leaves signify sensitive cultivars. Wilting occurs on scores 1–4. Score 6 represents a tolerant cultivar with no phenotypic response to flooding. Score 6 is the common phenotype of control plants.

2.3. Population selection

An initial screening of the population consisting of 403 winter barley cultivars (Supplementary Table 1) was performed as a pilot study in 2016. The field site in Oak Park, Carlow, Ireland (52°51′57, 6°54′30) was selected due to its proximity to an artificial lake (Fig. 2). The level of this artificial lake can be controlled through pumps and adjustable barriers, essentially controlling the water table around the surrounding shores.

The field was ploughed and tilled before sowing. All 403 cultivars were planted in a small plot field design on 21st October 2016. The field design consisted of six blocks (Flooded blocks: W1, W2, W3; Control blocks: D1, D2, D3) (Fig. 2). Each block consisted of 70 plots and each plot was 1.1 m^2 and contained six rows (0.9 m in length) with six cultivars. Three grams of seeds per cultivar were planted in each row. A 0.5 m gap separated each plot. Plot design was randomised using an alpha design in the Agricolae package in R (Mendiburu, 2015). The plots were sown with a Haldrup SR-30 single row magazine seeder and artificially waterlogged on the 5th of December 2016 using a boom irrigator due to the dry conditions in this particular winter. Throughout three months, a total of 1620 mm of water was applied evenly over the waterlogged blocks to achieve artificial waterlogging. The cultivars were ranked using the phenotypic scoring system (Fig. 1). The plants were photographed on the 18th of January 2017 at GS31 and scored from the photos. The pictures were taken using a Nikon Dx AF-S Nikkor 18–55 mm 1:3.5–5.6G lens mounted on a camera stand. Twenty-eight of the most tolerant and 29 of the most sensitive cultivars were selected to undergo further trait analysis in field testing in two consecutive years, as well as in growth cabinets (Supplementary Table 2). An additional eight varieties were added from current recommended lists in Ireland: Infinity, Tower, KWS Cassia, Quadra, Carneval, Kosmos, Pixel and Belfry (Crops Evaluation Certification Division, 2018).

2.4. Field trait analysis

Sixty five cultivars (57 from the 2016 pilot screening trial and eight from the recommended list) were planted in field season 1 (2017) and field season 2 (2018). The field was tilled before sowing on the 10th of October 2017 for field season 1 and 1st October 2018 for field season 2. Sixty-five cultivars per block were planted, with a total of 6 blocks (three flooded - W1, W2, W3, and three control - D1, D2, D3) (Supplementary Figure 1). There were a total of 65 plots. Each 1.1 m^2 plot contained six rows of the same cultivar. Eighteen grams of seed were planted per plot. Plot design was randomised using an alpha design in the Agricolae package in R (Mendiburu, 2015). The flooding blocks were designed to allow for natural waterlogging. The blocks were levelled by the removal of 10–15 cm of topsoil to achieve even waterlogging in the blocks. In field season 1, 36 mm of additional water was added over a month to the blocks (Table 1). Phenotypic scoring was conducted on 20th November 2017 and 19th of December 2017 for field season 1, and 19th of December 2018 and 6th February 2019 for field season 2, when the plants were at growth stages 30 and 31. Plants reached growth stages 30 and 31 at different times in field seasons 1 and 2. The plots were manually harvested on the 9th of July 2018 for season 1 and 1st of August 2019 for season 2 and bundled. They were then weighed to obtain the total biomass, and three height readings were taken per

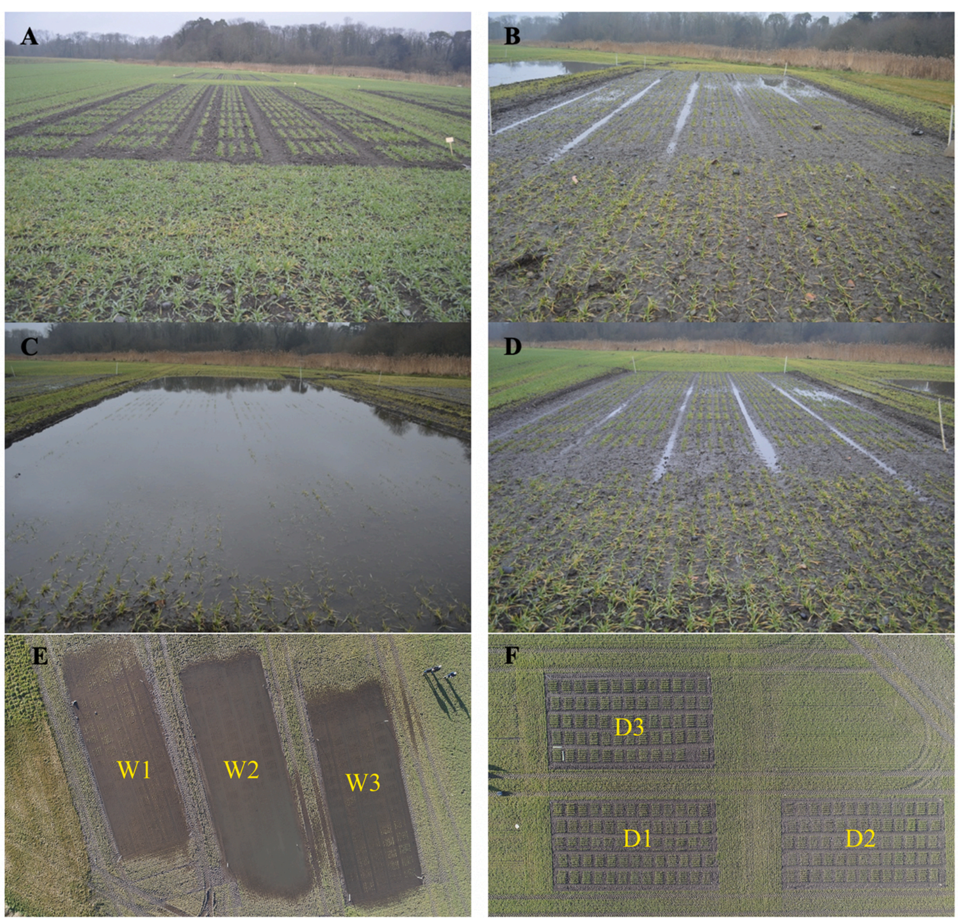

Fig. 2. Conditions of field trials. A) Field season 1, control block D1. B) Field season 1, block W1. C) Field season 1, block W2. D) Field season 1, block W3. E) Field season 2, flooded blocks. F) Field season 2, control blocks. (A-D) Taken on 19th December 2017 (field season 1) with a Nikon Dx AF-S camera. (E-F) Taken on 26th January 2019 (field season 2) using a DJI phantom 4 drone.

Table 1
Weather data for three seasons of flooding field trial. Data obtained from Met Eireann(Met Eireann., 2019) weather station at Oak Park, Carlow, Ireland. In addition to rainfall, water was added with a boom irrigator.

	Oct	Nov	Dec	Jan	Feb	Mar	Apr	Total
Pilot Study (2016)								
Rainfall (mm)	32.3	26.3	80.2	26.2	57.8	66.6	15.8	305.2
Evaporation (mm)	36.5	13.9	10.9	14.9	25.4	51.8	71.2	224.6
Day Flooded	3	3	15	9	13	8	0	51
Added artificial flooding (mm)	0	0	540	540	540	0	0	1620
Field Season 1 (2017)								
Rainfall (mm)	62.9	45.8	84.2	108.1	38.7	98.1	73	510.8
Evaporation (mm)	35.6	13	11.9	17.5	24	43.8	77.3	223.1
Day Flooded	4	12	19	17	10	15	8	85
Added artificial flooding (mm)	36	0	0	0	0	0	0	36
Field Season 2 (2018)								
Rainfall (mm)	58.3	160.5	119.3	30.9	36.8	122.9	72.5	601.2
Evaporation (mm)	37.7	17.9	14.7	15.5	17.9	34.9	47.7	186.3
Day Flooded	1	20	23	0	0	0	0	44
Added artificial flooding (mm)	0	0	0	0	0	0	0	0

bundle. The awns were separated from the plant and then threshed using a Haldrup LT-21 to separate the grain before cleaning and weighing.

Statistical analyses of height, biomass and grain weight were conducted using the ggplot2 and DYPLR packages in R to generate boxplots (Wickham, 2016, Wickham et al., 2018). Analysis of the flooded blocks compared to the control was performed using a two-way ANOVA with Tukey's post hoc analysis to compare treatments regardless of the cultivar. Biomass and grain yield data were scaled against the control blocks so that agronomic qualities could be used to rank the performance of the cultivars over three blocks. Block W2 in field season 2 was omitted due to extreme submergence conditions in this block leading to the death of all cultivars. Cultivars were categorised into tolerant performers, moderate performers and bad performers based on their ranking in their respective field season (Tables 4 and 5).

2.5. Controlled flooding conditions in growth cabinets

Climate controlled growth cabinets were used to simulate conditions that reflect the early growth period of winter barley in Ireland. Data obtained from Met Eireann (The Irish Meteorological Service) showed a mean October temperature of 10.1 °C for Oak Park, with a mean day time max temperature of 14 °C and a mean night-time minimum temperature of 7 °C (Met, 2019), and these temperatures were chosen as the day-time and night-time temperatures. Relative humidity was set at 80%, and plants were grown under a photoperiod of 12 h light and 12 h darkness. Growth Cabinet 1 (GC1) was a Snijders MicroClima High Specification Plant Growth Cabinet MC1750E and Growth Cabinet 2 (GC2) was a Snijders MicroClima MC1204. Easy Log USB data loggers were used to record the internal conditions of the growth cabinet (Supplementary Figures 1 and 2). A lux meter was used to determine that an average of ~140 μmol m^{-2} s^{-1} was received by the plants during the day (Environmental Growth Chambers, 2021). The soil used for plant growth was made up of 80% sterilised loam, 19.5% 3–6 mm lime-free grit, and 0.4% Osmocote mini. This soil mix was chosen as it closely resembles the soil in the field trials. For all trials, black 6 × 6×7 cm pots were used. 210 g of soil per pot were used and any

Table 2
Summary of pilot study scoring. Scoring was based on Fig. 1 scoring chart. A total of 403 cultivars were scored.

Score	Number of cultivars	Summary Statistics	
1	3	Mean	3.18610422
2	84	Median	3
3	165	Mode	3
4	139	Standard deviation	0.83314752
5	10	1st quartile	3
6	2	3rd quartile	4

Table 3
Summary statistics from scoring systems. Scores closer to 1 indicate crop sensitivity and scores closer to 6 indicate tolerance.

	Mean	Median	Standard Deviation	1st Quartile	3rd Quartile
Field Season 1 GS30	4.44	4.67	0.63	4	5
Field Season 1 GS31	3.12	3	0.79	2.67	3.67
Field Season 2 GS30	5.11	5	0.37	5	5.33
Field Season 2 GS31	4.08	4	0.60	3.67	4.67
Growth Cabinet Rep 1	3.55	4.00	1.2873	3.00	5.00
Growth Cabinet Rep 2	4.23	4.00	1.2963	3.00	5.00
Growth Cabinet Rep 3	4.11	4.00	1.0019	4.00	5.00

large stones or soil clumps were removed. The soil was soaked before potting to prevent soil loss from drainage holes. 15 pots were then placed in containers, each measuring 18 × 30 × 6 cm before planting. Plot design was randomised using the Agricolae package in R (Mendiburu, 2015).

To simulate waterlogging, a starch waterlogging solution was adapted from Mano et al. (2012). Three plants from each of the 65 cultivars were grown for 15 days and then waterlogged. The waterlogging treatment involved applying the 0.1% (w/v) soluble starch solution (Sigma Aldrich) to the tray until the water level reached 10 mm above the soil. The plants were waterlogged for 15 days before scoring. Controls followed a normal watering pattern. The experiments were repeated three times independently.

2.6. Calculation of agronomic performance at harvest

Plant height (cm), biomass (kg) and grain yield (kg) were scaled relative to the cultivars in the control blocks. Each parameter was scaled individually. Scaling was done using the scale function in R. All data were scaled from 0 to 10 with 0 being the worst performers. The individual parameters in the scaled data were averaged to give the performance of the cultivar in its given year (Tables 3 and 4).

2.7. Weather data

Weather data specific to Oak Park, Carlow, Ireland were collected by Met Eireann (The Irish Meteorological Service)(Met Eireann., 2019) from an on-site weather station. The key parameters of interest were

Table 4
List of cultivars and their performance score in field season 1. Performance was based on grain weight and biomass data scaled against control blocks. Cultivars are split into three categories, 1) Tolerant (Top 33.3% of range of performance scores), 2) Moderate (Middle 33.3% of range of performance scores) and 3) sensitive (Bottom 33.3% of range of performance scores).

Tolerant		Sensitive		Moderate	
Cultivar	Performance	Cultivar	Performance	Cultivar	Performance
Merode	3.50	Calliope	1.15	Plaisant	2.30
Alfeo	2.64	KWS B100	1.14	Arrow	2.21
Sunbeam	2.64	Monalisa	1.10	Maeva	2.19
Antonia	2.44	Chestnut	1.10	Amarena	2.13
Vesuvius	2.34	Breeze	1.07	Siberia	1.93
		Strider	1.03	Pixel	1.82
		Fakir	0.94	Tempo	1.74
		Infinity	0.93	Ceb02215–05	1.63
		Cavalier	0.89	Masquerade	1.58
		Dolphin	0.89	Kompolti	1.50
		Regina	0.88	Sumo	1.50
		Portrait	0.85	Louise	1.45
		Carneval	0.84	Pilastro	1.45
		Kold	0.83	Tamaris	1.43
		SWUB 01–41	0.81	Sonra	1.30
		Hasso	0.79	Mead	1.28
		Ager	0.79	Chintz	1.22
		Mortimer	0.75	Isa	1.20
		Arda	0.73	Arma	1.17
		Mahogany	0.71		
		5593 BH2	0.71		
		Retriever	0.68		
		Ravel	0.64		
		Quadra	0.64		
		Athene	0.63		
		Tsch Two Row	0.62		
		Talisman	0.59		
		CASSIA	0.57		
		Pioneer	0.55		
		Cathay	0.54		
		Kosmos	0.52		
		Madrigal	0.47		
		Dura	0.46		
		Liebniz	0.43		
		Grete	0.37		
		Mystique	0.36		
		Melanie	0.36		
		Tower	0.30		
		Belfry	0.20		
		SY Venture	0.16		
		Passport	0.00		

rainfall (mm h^{-1}), evaporation rate (mm h^{-1}) and soil moisture deficit (mm).

3. Results

3.1. Weather conditions during three consecutive years of field seasons

The on-site weather station allowed for the accurate collection of total rainfall, potential evaporation, and soil moisture deficit in the field trials. The drier growth conditions and the lack of excavated block during the pilot study (2016) required additional irrigation to achieve artificial waterlogging. Although the total rainfall was higher in the second field season (2018), the total days flooded was reduced (Table 1) compared to field season 1 (2017). In field season 1, the number of days waterlogged was spread out through the entire growth period compared to field season 2, where all of the waterlogging occurred in the first three months of growth. The irrigator was used early in field season 1 due to a dry period (Table 1) but was not needed afterwards. Variation in block waterlogging was also noticeable (Fig. 2) (Table 3). The central position of block W2 and narrow tramlines resulted in increased water accumulation in block two. Although this was observed in both years, it was most notable in field season 1 (Fig. 2). Future trial design should focus on maximizing the distance between waterlogging blocks to reduce overflow from blocks into the central blocks.

3.2. Summary of flooding phenotypes from pilot screening experiment 2016

The 2016 pilot study grouped cultivars into scoring from 1 to 6,) based on the phenotypic scoring system (Fig. 1)(Table 2). The mean score was 3.186, the first quartile was 3 and the third quartile was 4. The scores were normally distributed allowing for classifying the cultivars into sensitive and tolerant scores based on data distribution. On the basis of these group classifications, 57 cultivars from the pilot screening were chosen for additional field trials in 2017 and 2018. 28 of these cultivars were identified as tolerant and 29 were identified as sensitive.

3.3. Reduction of total biomass due to flooding

Waterlogging caused a significant reduction in harvested plot biomass in both field seasons (Fig. 3). Average plot biomass in control plots from field season 1 was 2.01 ± 0.75 kg per plot and 2.40 ± 0.47 kg per plot in field season 2. An average biomass reduction of 1.48 ± 0.5 kg per plot compared to the control plot was observed in field season 1 whereas a lower reduction of 0.34 ± 0.17 kg per plot was recorded in field season 2 (Fig. 3). It is clear from the biomass and weather data that the flooding was more severe in field season 1 than 2. The most extreme flooding was seen in block 2 (W2) in field season 1 that led to crop death (Fig. 3). The flooding in this block could be classified as total

Table 5
List of cultivars and their performance score in field season 2. Performance was based on grain weight and biomass data scaled against control blocks. Cultivars are split into three categories, 1) Tolerant (Top 33.3% of range of performance scores), 2) Moderate (Middle 33.3% of range of performance scores) and 3) sensitive (Bottom 33.3% of range of performance scores).

Tolerant		Sensitive		Moderate	
Cultivar	Performance	Cultivar	Performance	Cultivar	Performance
Fakir	7.29	Amarena	3.25	Isa	5.27
Tamaris	6.70	Grete	3.25	Kompolti	5.24
Infinity	6.59	Kold	2.81	Dura	5.21
Quadra	6.47	Hasso	1.88	Masquerade	5.17
Breeze	6.33	KWS B100	1.27	Ravel	5.16
Cavalier	6.33			Arma	5.09
Carneval	6.24			Madrigal	5.02
Louise	6.17			Retriever	5.01
Antonia	6.16			Alfeo	5.00
Passport	6.10			Plaisant	4.97
Maeva	5.98			Cathay	4.97
SWUB 01–41	5.87			Vesuvius	4.96
Strider	5.85			CASSIA	4.93
Liebniz	5.84			SY Venture	4.86
Monalisa	5.58			Mystique	4.86
Tower	5.56			Ager	4.81
Mead	5.50			Pilastro	4.79
Pixel	5.44			Tsch Two Row	4.78
Arrow	5.43			Arda	4.52
Chintz	5.42			Sumo	4.46
Mahogany	5.37			Merode	4.43
Tempo	5.34			Dolphin	4.41
Talisman	5.33			Sunbeam	4.39
Kosmos	5.31			Portrait	4.36
Athene	5.30			5593 BH2	4.30
Chestnut	5.28			Siberia	4.17
				Belfry	4.08
				Calliope	4.01
				Ceb02215–05	3.95
				Mortimer	3.82
				Melanie	3.82
				Sonra	3.64
				Pioneer	3.60
				Regina	3.52

submergence.

3.4. Reduction of grain biomass due to flooding

The average plot grain yield in control plots 2017 was 0.98 ± 0.3 kg per plot and in 2018 1.19 ± 0.35 kg per plot. Consistent with the severe reduction in average total biomass in field season 1 of waterlogging trials, we also observed a significant reduction in harvestable grain weight per plot (Fig. 4). This reduction averaged 0.85 ± 0.06 kg per plot compared to field season 2, where a non-significant average reduction of 0.06 ± 0.101 kg per plot compared to controls was observed.

3.5. Reduction of crop height due to flooding

The average plot plant height in control plots in field season 1 was 84.24 ± 13.61 cm per plot and 114.01 ± 11.32 cm per plot in field season 2. There was a significant height reduction in both field seasons (Fig. 5) from waterlogging. In field season 1, an average reduction in height of 45.51 ± 6.27 cm per plot was observed whereas a smaller average reduction of 7.43 ± 1.12 cm per plot was observed in field season 2. These results highlight again the severity of waterlogging in field season 1.

3.6. Cultivar ranking based on agronomic performance

The cultivars from field seasons 1 and 2 were ranked based on their performance in each season. The ranking combined biomass and grain weight data and scaled them against control blocks from that year. The scale was from 0 to 10, with 0 being the worst performer and 10 being the best performer. Comparing field season 1 (Table 4) and field season 2 (Table 5) reveals overall cultivars performed worse due to waterlogging, with a median performance score of 0.891 in field season 1 and 5.015 in field season 2. From field season 1 only five cultivars could be categorised as good performers. 19 cultivars were categorised as moderate performers and 41 of the cultivars were categorised as poor performers. This was in contrast with field season 2 which had 26 good performers, 34 moderate performers and seven poor performers. 49% of the cultivars having a change in performance score of ± 4 between field seasons 1 and 2 highlighting the severe weather in field season 1. Antonia was the only cultivar categorised as a good performer in both seasons and KWS B100, Kold, Hasso and Grete were categorised as poor performers in both seasons.

3.7. Phenotypic scoring of cultivar response to flooding in field conditions

The phenotypic scoring system was applied in field seasons 1 and 2 at GS30 and GS31. The severity of waterlogging in field season 1 was reflected in the variation in scores between the two seasons. The mean scores were 4.44 and 5.12 in field season 1 GS30 and field season 2 GS30, respectively (Table 3). The mean scores were 3.12 and 4.08 in field season 1 GS31 and field season 2 GS31, respectively (Table 3). The relative standard deviation from the mean scores were 24.9% in field season 1 (GS1 and GS2) and 10% in field season 2 (GS1 and GS2). The within-season variation can be at least partly explained by the variation in waterlogging duration with 85 days of flooding in field season 1 and 44 days of waterlogging in field season 2.

The scoring system used GS30 and GS31 scoring values to try and predict the performance of the cultivars. There was no strong correlation

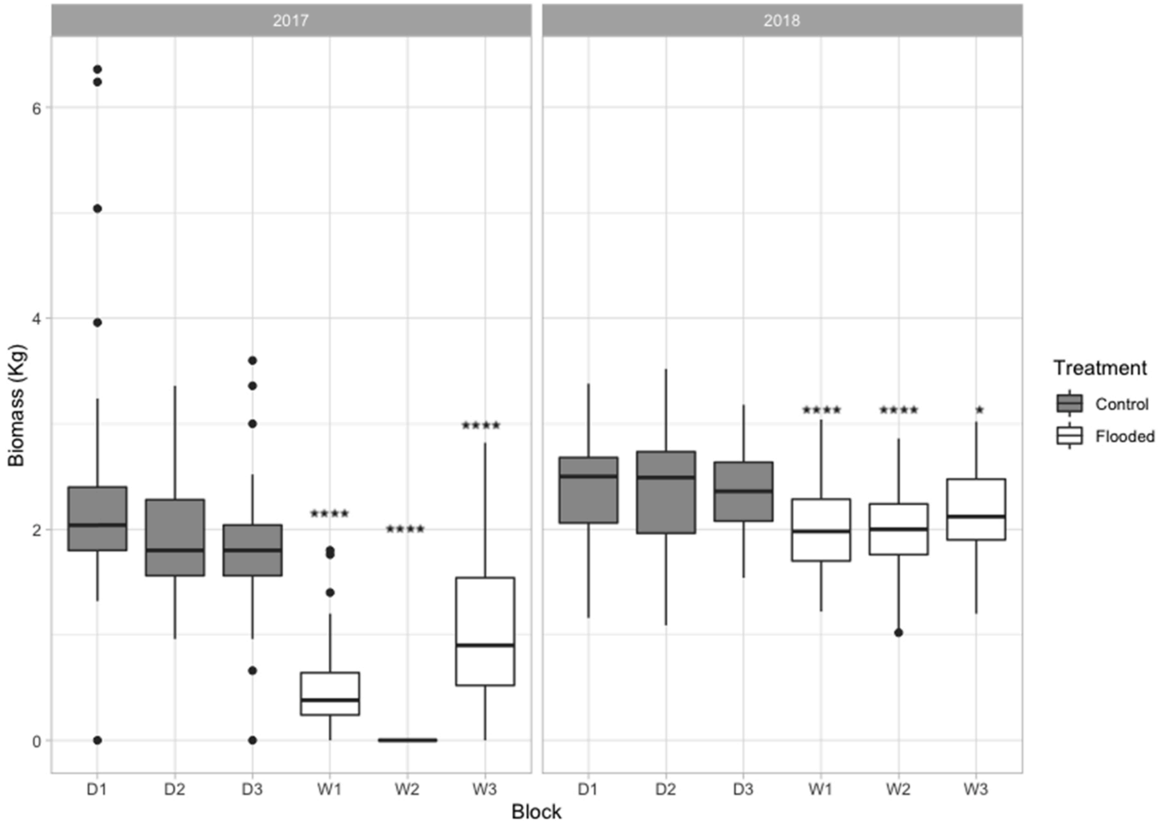

Fig. 3. Biomass reduction due to waterlogging. Total biomass (kg) per plot in field season 1 (2017) and field season 2 (2018). Plot size was 1.1 m^2. Whiskers indicate minimum and maximum values in the first and fourth quartile. The box boundaries indicate the upper (25th percentile) and the lower (75th percentile) quartiles. The bold line in the box represents the median. The significance of the difference from the control is represented by * $p > 0.05$, * * $p > 0.01$, * ** $p > 0.001$, * ** * $p > 0.0001$ (Tukey test).

between performance and score in field seasons 1 and 2 due to the variation in responses from the cultivars. Combining the scoring and cultivar data allows the extraction of four groups: 1) Tolerant, 2) Recovers, 3) Delayed sensitivity and 4) Sensitive (Table 6). By extracting these cultivar groups, differing responses have been identified (Table 7). There was no overlap between cultivars in these groups between the two field seasons.

3.8. Phenotypic scoring of cultivar response to flooding in controlled conditions

Due to the high level of variability in field trials arising from prevailing environmental conditions, the use of the growth cabinets provided the opportunity to rank and score plants in controlled and consistent conditions. Within the three replicates, the mean scores were 3.55 (growth cabinet rep 1), 4.23 (growth cabinet rep 2) and 4.11 (growth cabinet rep 3). The median score for all three reps was 4. The spread of the scores in the growth cabinet (relative standard deviation; growth cabinet replicate 1 = 36.3%, growth cabinet replicate 2 = 30.6%, growth cabinet replicate 3 = 24.4%) allows for clearer identification of cultivars which are tolerant or sensitive compared to the field trials (Table 2). The average relative variation between mean scores of each growth cabinet trial is 11.4% and the average relative variation between mean scores of each field season is 31.6% (Fig. 6).

The mean score of a cultivar from the three growth cabinet trials was compared to the mean score from the two field seasons. The scores were normally distributed and variance between the two scoring methods was calculated. 58.5% of the cultivars had a score variance of less than 1 between the two growth methods. 9.2% of cultivars had a score variance of greater than 2. The growth cabinet did not correlate with the cultivar performance in field seasons 1 and 2 due to the early stage the plants were scored at.

4. Discussion

4.1. Waterlogging causes a loss in agronomic outputs

Crops grown in field conditions are subject to both biotic and abiotic stresses. Waterlogging reduces the performance of the plant, its ability to grow and fill grains, and its yield (Araki et al., 2012). Waterlogging localized in areas of the field or near a water body can cause crop death and greatly reduce total yield (Shaw et al., 2013). The variability between field seasons 1 and 2 is indicative of the challenges faced in environmental variability in agricultural research and crop production. In field season 1, a total of 510.8 mm of rainfall and 85 days of flooding were recorded during the growing seasons, compared to 601.2 mm of rainfall and 44 days of flooding in field season 2. In field season 1, prolonged flooding resulted in a reduction in crop growth and complete crop death in block W2. The two other flooding blocks in field season 1 (W1 and W3) experienced a reduction in total biomass, grain weight and height. In field season 2, a significant reduction in biomass and height was observed, yet the reduction was not as extreme as in field season 1. The average biomass loss was 1.48 ± 0.5 (Standard deviation) kg per plot in field season 1 and 0.34 ± 0.17 kg per plot in field season 2. It is apparent that the frequency of flooding (85 days) throughout the growing season dramatically reduced yield compared to the 44 days of flooding in field season 2 which occurred mainly in the months of November and December. The block size of 1.1 m^2 allows the calculation of potential biomass loss per hectare. In field season 1 of flooding trials, there was a significant reduction in harvestable grain weight per

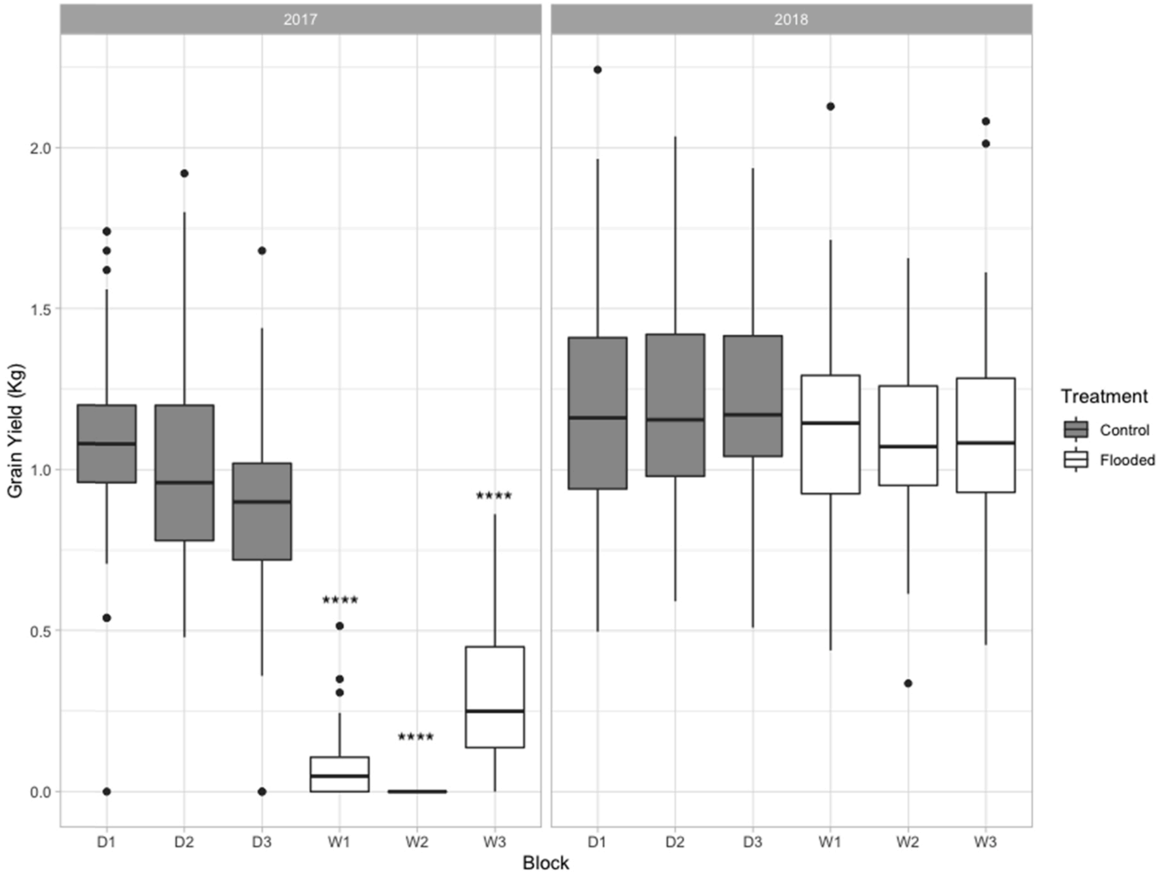

Fig. 4. Grain yield reduction due to waterlogging. Grain biomass (kg) per plot in field season 1 (2017) and field season 2 (2018). Plot size was 1.1 m^2. Whiskers indicate minimum and maximum values in the first and fourth quartile. The box boundaries indicate the upper (25th percentile) and the lower (75th percentile) quartiles. The bold line in the box represents the median. The significance of the difference from the control is represented by * $p > 0.05$, * * $p > 0.01$, * ** $p > 0.001$, * ** * $p > 0.0001$ (Tukey test).

plot with an average reduction of 0.77 ± 0.054 kg m^{-2} (7.7 t ha^{-1}). Irish winter barley yields per hectare ranged from highs of 10.2 t ha^{-1} (2015) to a low of 8.8 t ha^{-1} (2018) between the years 2012 and 2018 (Teagasc, 2020). This reporting primarily highlights the reductions in agronomic output but it also shows variability between field location and season.

By taking a large population of 403 cultivars and screening them, a subset could be tested for their performance in flooded conditions. The top performers in field season 1 and field season 2 differed due to the extreme waterlogging in field season 1. Antonia was a top performer in both field seasons 1 and 2. This does not, however, indicate it is the best performer out of the 403 cultivars. All 403 cultivars would need to be ranked based on agronomic qualities in order to find the top performers. The difference between field seasons 1 and 2 highlights the risk of using one year of field trials to assess the waterlogging tolerance. Here we look at the same site over two years and note substantial differences in the severity of waterlogging. These differences were largely due to weather conditions, notably longer periods of submergence in field season 1, however, some of these differences may be due to block depth as well. When the blocks were lowered by removing soil in field season 1, this created a topography which allowed waterlogging. When preparing the blocks for field season 1 the topography couldn't be matched exactly so there would have been slight differences in the site from year 1 and year 2. Care should be taken to avoid any major soil disruption over multi-year field trials, so ploughing should be avoided. While these differences may be negligible in other biotic or abiotic trials, waterlogging is difficult to control. We recommend using multiyear field trials on the same site to best capture the waterlogging response but using backups with more controlled conditions are recommended.

4.2. Comparative phenotypic scoring in field and controlled conditions

Modern crop breeding methods prioritize selection from large populations. This allows for greater diversity, trait variability and potential for increased genetic gain (Hernandez et al., 2020). The use of larger populations requires more resources and limits the time available to detect traits. This has led to the development of phenotypic scoring systems for specific traits (Fasoula et al., 2020). The effect of flooding on winter barley has a clear phenotype of yellowing wilting leaves with necrosis in severe cases (Fig. 1), allowing the severity of the waterlogging response to be estimated through monitoring of phenotypic changes (Sundgren et al., 2018). A phenotypic scoring system based on plant health allows for a higher throughput of cultivars in a shorter period.

The phenotypic scoring system responded to the varying severity of the waterlogging between field seasons 1 and 2. Field season 1 (2017) had severe and uneven waterlogging between the blocks. This resulted in crop death in one of the blocks, a common result of severe waterlogging. Total yield loss is highly disruptive to growers, resulting in loss of profits. Waterlogging in field season 2 (2018) was more evenly distributed across the field but the overall waterlogging stress was less severe. Yield data was obtained from all plots in field season 2 and the results showed that there was no significant effect of flooding on grain yield. The median score from field season 1 was 3. In comparison, the median score of the second field season was 4.33. The blocks W1 and W2 do not have much variation in their yield responses in field season 1 (2017). The plant response in the severely flooded block demonstrates environmental variation caused by topographical qualities of the field can become a limiting factor when conducting field experiments without

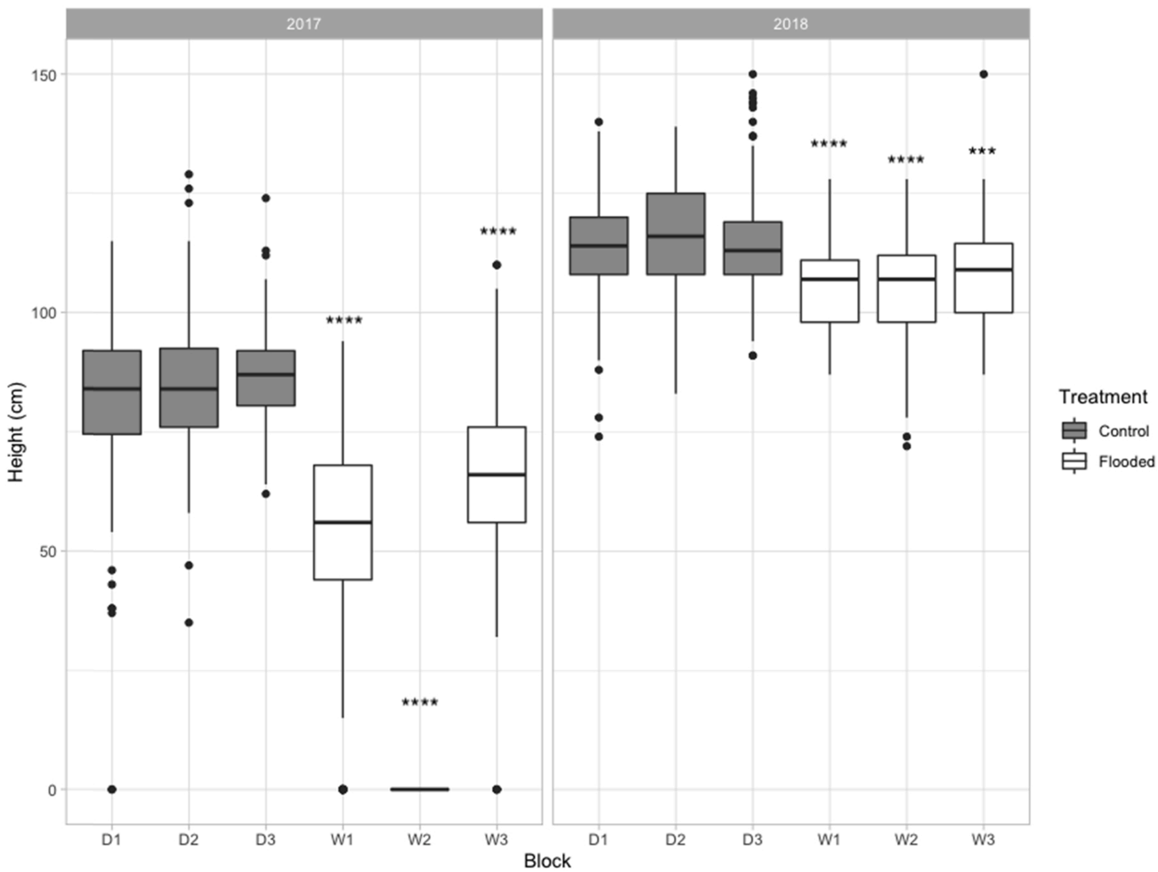

Fig. 5. Height reduction caused by waterlogging. Height (cm) per plot in field season 1 (2017) and field season 2 (2018). Plot size was 1.1 m^2. Whiskers indicate minimum and maximum values in the first and fourth quartile. The box boundaries indicate the upper (25th percentile) and the lower (75th percentile) quartiles. The bold line in the box represents the median. The significance of the difference from the control is represented by * $p > 0.05$, * * $p > 0.01$, * ** $p > 0.001$, * ** * $p > 0.0001$ (Tukey test).

Table 6
Categories of cultivars based on response to waterlogging. Scores above 4.5 are tolerant. Scores 4.5–2.5 are moderate and scores below 3 are sensitive. Rank at harvest is based on Tables 3 and 4.

	Tolerant	Recovers	Delayed sensitivity	Sensitive
Score at GS30	Tolerant	Moderate/ Sensitive	Tolerant/ Moderate	Moderate/ Sensitive
Score at GS31	Tolerant/ Moderate	Moderate/ Sensitive	Tolerant/ Moderate	Sensitive
Rank at Harvest	Tolerant	Tolerant	Sensitive	Sensitive

supplemental controlled conditions.

The use of growth cabinets to complement field trials allowed for more consistent environmental conditions. Controlled waterlogging using an artificial waterlogging method as described by Mano et al. (2013) gave a median score of 4. There was also a wider range of scores giving a clear spectrum of tolerant and sensitive cultivars (Fig. 6). The controlled growth method only takes 30 days to produce reproducible phenotypic data. The limitation of this method is the inability to collect yield data that is necessary for cultivar selection. Using the scoring system to compare cultivar responses in the growth cabinet complemented the field scoring with 58.5% of the cultivars having a score variance of less than one between methods. Variance in cultivar score arose due to waterlogging severity, with the growth cabinet allowing for more controlled conditions. However, the scores do not correlate well with the agronomic performance of the cultivars. This is likely due to the scoring at GS30 and GS31 being too early to predict the agronomic outcome. The cultivars respond differently to the waterlogging stress with some being greatly affected by the waterlogging early on and unable to recover, some recovering and some seeing lesser detrimental effects. There is, however, no consistency in the groupings between field seasons 1 and 2, highlighting once again the difficulty in using field trials for identifying waterlogging tolerance. The difficulty occurs due to the lack of control of water addition. It is clear that all cultivars are negatively affected by waterlogging, but there are two clear strategies employed by the best performers; a) tolerance to waterlogging b) recovery from waterlogging. Here we have outlined cultivars that have employed each strategy in each field season. Further investigation into the strategies would require taking these cultivars and investigating them in detail to monitor their recovery and tolerance. Tables 4 and 5 reveals the cultivar Antonia as a top performer in both seasons, despite waterlogging whereas the cultivars KWS B100, Kold, Hasso and Grete are consistently poor performers in both field seasons. These cultivars are clear candidates for further studies. The addition of scoring data to the performance data reveals how a cultivars classification of tolerance and sensitivity may change over the season. A top performer in both seasons is Antonia appears to recover from its waterlogging sensitivity as indicated by its low phenotypic scoring in field season 1. This is in contrast to field season 2 where Antonia did not score low, likely due to the reduced waterlogging severity in this field season.

With a reduced deviation of scores between the three growth cabinet trials, it is clear that this method is reproducible and not subject to variable climatic conditions. It is however important to note that field trials cannot be replaced by growth cabinet trials, as plants grow differently and yield differently in controlled conditions due to

Table 7
Grouping of cultivars based on response to waterlogging (Table 6) in field season 1 and 2.

Field Season 1				Field Season 2			
Tolerant	Recovers	Delayed sensitivity	Sensitive	Tolerant	Recovers	Delayed sensitivity	Sensitive
Alfeo	Antonia	5593 BH2	Ager	Antonia	Monalisa	Amarena	
Merode	Sunbeam	Athene	Dolphin	Arrow	SWUB 01–41	Grete	
Vesuvius		Belfry	Kold	Athene	Tamaris	Hasso	
		Breeze	Kosmos	Breeze		Kold	
		Calliope	Melanie	Carneval		KWS B100	
		Carneval	Monalisa	Cavalier			
		Cathay	Pioneer	Chestnut			
		Cavalier	Retriever	Chintz			
		Dura	Talisman	Fakir			
		Fakir	Tsch Two Row	Infinity			
		Grete		Kosmos			
		Infinity		Liebniz			
		KWS B100		Louise			
		Liebniz		Maeva			
		Madrigal		Mahogany			
		Mahogany		Mead			
		Mortimer		Passport			
		Mystique		Pixel			
		Passport		Quadra			
		Portrait		Strider			
		Quadra		Talisman			
		Ravel		Tempo			
		Strider		Tower			
		SY Venture					
		Tower					

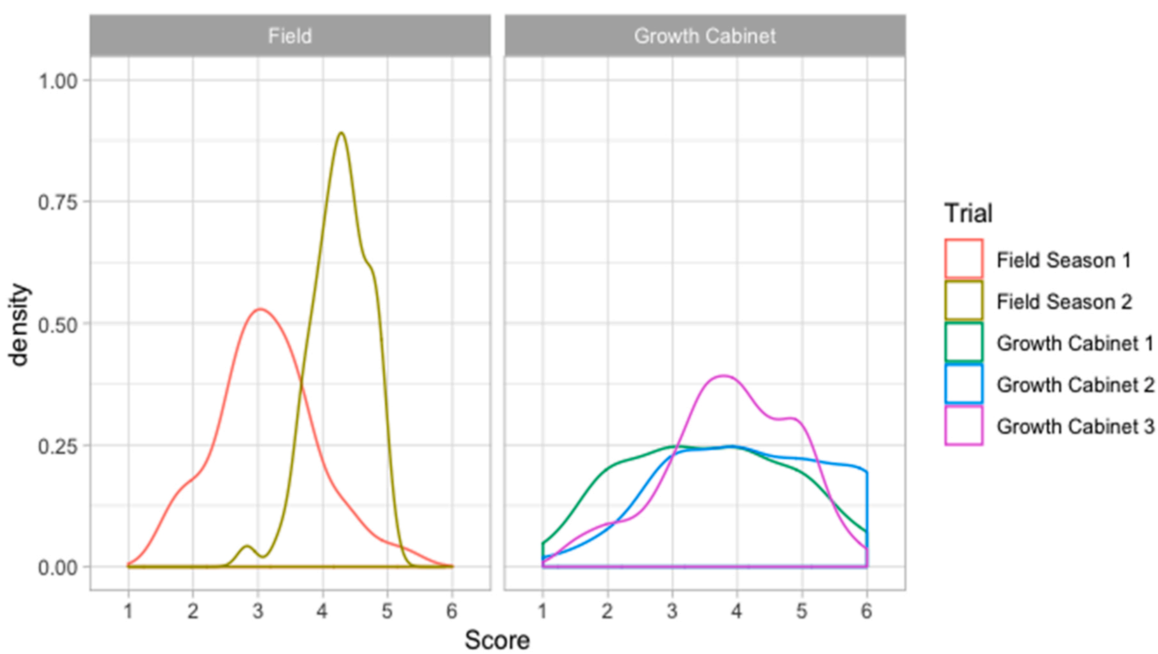

Fig. 6. Comparison of phenotypic scoring system applied to field and growth cabinet grown experiments: Phenotypic scores for waterlogging sensitivity are ranked from one (sensitive) to six (tolerant). The first panel compares the score distribution of field season 1 (2017) and field season 2 (2018). The second panel represents the distribution of scores from three separate waterlogging experiments performed in controlled growth conditions.

qualitative differences in growth conditions (Poorter et al., 2016). Growth cabinets allow for the control of light intensity and duration but often results in the loss of UV-B light unless specific bulbs are fitted (Caldwell and Flint, 1994), which were not present in this study. Growth cabinets also have a higher CO_2 concentration (500 μmol mol^{-1} in this study) compared to a field (~400 μmol mol^{-1}) (Poorter and Navas, 2003). While these variables can be controlled with investment into higher specification equipment, the controlled conditions of temperature, light quantity, humidity, water and soil allow for more accurate scoring (Poorter et al., 2016, 2012). Cultivar selection for breeding programs should proceed with regular protocols for field trials but the scoring should take place twice. Once at growth stage 30 and once before harvest. This will allow for the identification of tolerant cultivars but will also identify cultivars with the potential for recovery.

5. Conclusion

Crop breeding relies on high throughput systems to identify traits of interest in large populations with cultivar testing being performed using large scale field trials. This study compares the uses of field and controlled trials in the testing of cultivars for waterlogging tolerance. The variation in biomass loss between field season 1 (2017) and field season 2 (2018), highlights the variability in flooding severity while conducting multi-season field trials. The phenotype present in waterlogged winter barley allows for phenotypic scoring of tolerance. This method is quick and effective to use. The comparison of scores in field trials, growth cabinet trials and cultivar performance highlights the need for well-timed scoring to identify both tolerant cultivars and cultivars with the potential to recover from waterlogging damage. The need for new tolerant cultivars becomes apparent when looking at the loss in agronomic outputs reported here. The severity of flooding in the field greatly affects crop yield-reducing profits and food production. The addition of short controlled condition trials will allow for greater insights when selecting cultivars for recommended lists and breeding.

CRediT authorship contribution statement

TB, PKA, TM, LF, AM and SB conducted experiments. TB, JG, PKA, LF, AM, EG, JS, CKYN and SB designed experiments and analysed data. TB, CKYN and SB wrote the manuscript. WTBT provided plant material. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eja.2021.126432.

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