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DROUGHT STRESS

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Drought tolerance in perennial ryegrass (*Lolium perenne* L.) as assessed by two contrasting phenotyping systems

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Abstract

The objectives of this research were to establish a practicable phenotyping platform for assessing the drought stress response of perennial ryegrass (*Lolium perenne* L.; *Lp*), and to use this platform for evaluating the variation for drought tolerance among a panel of 39 diverse *Lp* populations. A moderate-to-strong correlation was assessed between the performance of plants grown in a hydroponics system, where the stress was generated by the addition of polyethylene glycol (PEG), and those grown in the field in a rainout shelter. Following the application of drought stress, tetraploid *Lp* populations, along with a small number of reference *Festulolium* and *Festuca* sp. accessions, were able to develop more shoot and root dry matter than diploid *Lp* populations. The onset of drought symptoms was also delayed within these accessions and the plants recovered better once drought had been relieved. Although most of the diploid *Lp* populations were drought susceptible, there was a considerable accession-to-accession variation for performance under drought stress conditions. Measuring biomass production and post-drought recovery in rainout shelter experiments in combination with the assessment of root biomass accumulation in PEG-supplemented hydroponics represented a viable means of screening *Lp* germplasm for drought tolerance.

KEYWORDS
after-drought recovery, hydroponics culture system, rainout shelter

1 | INTRODUCTION

The impact of incipient changes to the world's climate presents a major challenge for crop research. The predicted rise in atmospheric temperature can be expected to intensify both the frequency and severity of heat waves, drought and flooding (Jacob et al., 2013; Trnka, Hlavinka, & Semenov, 2015). The future productivity of perennial ryegrass (*Lolium perenne* L.; *Lp*), presently among the most widely grown grass species in temperate zones (Sampoux et al., 2011, 2013), will be compromised by drought, as—unlike many grass species—it forms a rather shallow rooting habit, limiting its inherent level of drought tolerance (Sheffer, Dunn, & Minner, 1987). As irrigation is generally not an economically viable option for pasture land, there is a need to breed more drought tolerant varieties (Lobell & Gourdji, 2012), unless the choice is to abandon the species for other, more drought tolerant grasses.

One of the most critical choices to be made in breeding for drought tolerance is that of the selection environment. Intensity, timing and duration of the stress cannot be easily controlled in a field experiment. Hatier et al. (2014) chose a rainout shelter facility to screen above-ground traits of a segregating *Lp* population under drought conditions, but could only speculate about the influence of rooting on drought stress response. Methods for field-based phenotyping of roots are mostly destructive and laborious (Böhm, 1979). Hydroponics allows a higher throughput and direct monitoring of root development but depend on artificial liquid media (Mathieu, Lobet,

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Tocquin, & Perilleux, 2015) with great influence on root growth behaviour. Because of this, results are often not transferable to field conditions (Araus & Cairns, 2014). Rainout shelters exclude untimely rain events from undisturbed field plots (Kant et al., 2017) and are thus a compromise between controlled laboratory and greenhouse conditions and a completely uncontrolled field environment.

Genetic variation for drought tolerance in *Lp* was described in several studies. Cui, Wang, Wang, and Jiang (2015) have compared the performance of six diploid genebank accessions in a greenhouse trial by measuring leaf water content, leaf wilting, plant height and chlorophyll fluorescence, but field performance of these accessions remained unclear. Genetic variation in *Lp* for the ability to recover after drought has been described by both Cheplick, Perera, and Koulouris (2000) and Turner, Holloway-Phillips, Rawnsley, Donaghy, and Pembleton (2012). The accumulation of specific carbohydrates to form an energy reserve may be important in this context (Volaire, 1995). Typically, drought halts leaf growth, which only restarts when sufficient soil water becomes available (Volaire & Norton, 2006). Developing a strong root system clearly promotes a plant's access to soil water. However, due to the challenging characterization of root systems, little emphasis has been given so far to selection on the basis of root traits. A distinction needs to be made between drought tolerance with the maintenance of biomass production under moderate water limitation and drought survival with no or minor productivity during severe drought but a good recovery after rehydration. Most studies investigated a limited amount of genotypes and addressed many different drought-related traits. This makes studies of drought tolerance in *Lp* hard to compare.

The aims of this study were (i) to identify suitable selection traits for assessing drought tolerance in *Lp* that can be handled in a larger number of genotypes, (ii) to investigate genetic variation of drought tolerance within a broad range of *Lp* populations and (iii) to compare the results between a rainout shelter experiment and greenhouse-based results of a hydroponics experiment. The comparison was possible due to the use of genetically identical plant material within the two selection environments.

2 | MATERIALS AND METHODS

2.1 | Plant material

The investigated germplasm panel comprised 39 diverse *Lp* populations (Table 1), including current or candidate varieties as well as genebank accessions (ecotypes and historic varieties). The basis for the selection of these 39 populations was their performance under drought stress during the first year in a multilocation field trial with a total of 200 accessions. These 200 accessions were ranked according to the multilocation mean of visual scoring of drought symptoms, which permitted to cover a broad range of drought response types within the selected accessions. A set of eight references with good drought tolerance under field conditions were included as controls, namely meadow fescue (*Festuca pratensis* Huds.; *Fp*) (two populations), tall fescue (*Festuca arundinacea* Schreb.; *Fa*) (two populations) and *Festulolium* (*Festuca* × *Lolium*) (four populations; Table 1). Each of the 47 accessions was represented by a random sample of 40 genotypes of the population, which were clonally propagated for the different experiments. The plant material was obtained from the Bavarian State Research Center for Agriculture (LfL), the breeding companies Deutsche Saatveredelung AG (DSV), Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ) and Saatzucht Steinach (SZS) as well as the genebank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK).

2.2 | Simulated drought stress in hydroponic experiments

For the hydroponics experiment, three tillers per genotype were split off from plants cultivated under greenhouse conditions (temperature set at 18/10°C and regular ventilation from a critical 20/12°C; natural daylight was supplemented between 6 a.m. and 8 p.m to ensure a minimum light intensity of 6,000 Lux). Tillers were placed in plastic mesh pots (Ø 5.5 cm) and fixed with expanded clay pellets in the wells (230 cm^3) of a 24-cavity plug tray, which was mounted over a tank (60 × 40 × 24 cm) containing 20 L of water. After 1 week, the water was replaced by a half-strength Hoagland and Arnon's (1950) nutrient solution, which was continuously aerated. To expose the plants to osmotic stress, either 10% w/v PEG 6000 (PEG10) or 20% w/v PEG 6000 (PEG20) was added to the solution; a control treatment lacking any PEG (PEG0) was included. The set of experiments was carried out in eight batches over a 2-year period. Every randomized batch consisted of five tanks and three treatments, whereby each tank was equipped with six populations and four genotypes per population with identical genotypes in each treatment. In sum, 47 accessions with 20 genotypes each were investigated. After 4 weeks of PEG exposure, the chlorophyll content of the youngest fully developed leaf was measured using a SPAD-502 chlorophyll meter (Konica Minolta Inc., Tokyo, Japan). The root and shoot fresh weight was determined. Finally, the material was dried at 105°C until constant weight was reached.

2.3 | Simulated drought stress in the rainout shelter experiments

The rainout shelter experiments were conducted at two locations, one at IPK-Malchow/Poel in Northern Germany (53°59′40″ N, 11°28′26″ E; soil type loamy sand) and the other at LfL Pulling/Freising in Southern Germany (48°21′53″N, 11°42′49″ E; soil type sandy loam). The experiments were designed as randomized complete blocks with two replications, each replication containing 20 genotypes of every population. Each plot (1.25 m^2) comprised two rows of ten plants with a 0.5 m spacing between both rows and plants. The material was planted at Malchow/Poel on 2012-10-29, and at Pulling/Freising on 2012-10-11. The plots were maintained by regular cutting and fertilizer application according to standard agricultural practice. Nitrogen was applied as calcium ammonium nitrate at Malchow/Poel and as a liquid fertilizer (Wuxal P 5-20-5; Wilhelm Haug GmbH & Co., KG, Düsseldorf, Germany) at Pulling/Freising. At both locations, 80 kg/ha N fertilizer was

TABLE 1 Plant material examined in greenhouse and rainout shelter experiments

No.	Variety	Origin	Species	Ploidy	Ranking[a]	Comment
01	Lipalma	b	Fa	6×	5	
02	Belfine	b	Fa	6×	13	
03	Cosmolit	b	Fp	2×	4	
04	Preval	b	Fp	2×	11	
05	Hykor	b	FEL	6×	2	FEL similar to *Fa*
06	Lesana	b	FEL	6×	3	FEL similar to *Fa*
07	Lofa	b	FEL	4×	15	FEL similar to *Lm* (*Lm* × *Fa*)
08	Prior	b	FEL	4×	43	FEL similar to *Lp* (*Lp* × *Fp*)
09	Arvicola	b	Lp	4×	195	
10	Activa	b	Lp	4×	188	
11	410*	b	Lp	2×	14	
12	411*	b	Lp	2×	71	
13	412*	b	Lp	2×	37	
14	413*	b	Lp	2×	141	
15	414*	b	Lp	2×	31	
16	415*	b	Lp	2×	38	
17	416*	b	Lp	4×	200	
18	417*	b	Lp	2×	60	
19	418*	b	Lp	2×	44	
20	419*	b	Lp	2×	198	
21	420*	b	Lp	2×	128	
22	GR 3107	e	Lp	2×	53	
23	GR 3109	e	Lp	2×	74	
24	GR 3352	e	Lp	2×	75	
25	GR 3467	e	Lp	2×	19	
26	GR 3511	e	Lp	2×	9	
27	GR 5112	e	Lp	2×	61	
28	GR 5559	e	Lp	2×	45	
29	GR 6594	e	Lp	2×	23	
30	GR 6598	e	Lp	2×	17	
31	GR 7457	e	Lp	2×	12	
32	GR 7607	e	Lp	2×	196	
33	GR 7965	e	Lp	2×	16	
34	GR 8422	e	Lp	2×	6	
35	GR 8611	e	Lp	2×	10	
36	GR 12735	e	Lp	2×	21	
37	Brio	b	Lp	2×	20	
38	Limonica	b	Lp	2×	7	
39	442*	b	Lp	4×	24	
40	Forestro	b	Lp	2×	197	
41	444*	b	Lp	4×	70	
42	446*	b	Lp	4×	54	
43	447*	b	Lp	4×	26	
44	449*	b	Lp	4×	107	
45	450*	b	Lp	4×	118	
46	Respect	b	Lp	2×	73	
47	Astonenergy	b	Lp	4×	145	

*, candidate variety; b, breeders material; e, ecotype; *Lp*, *Lolium perenne* L.; *Fa*, *Festuca arundinacea* Schreb.; *Fp*, *Festuca pratensis* Huds.; FEL, *Festulolium*; *Lm*, *Lolium multiflorum* Lam.

[a]Drought symptoms ranking according to a multilocation pre-experiment under natural drought conditions (data not shown).

applied at the beginning of the vegetation period and a further 60 kg/ha N after each biomass cut. Two drought treatments were applied to simulate pre-summer and midsummer stress (Figure 1). The volumetric water content of soils ranged between 34.3% and 52.6% at Pulling/Freising under optimal irrigation and dropped to 7.6% to 22.0% during drought stress. At Malchow/Poel, the volumetric water content ranged between 17.0% and 32.9% under optimal irrigation and dropped to values between 4.5% and 17.0% during drought stress. Information on ambient temperature and solar radiation at both locations is given in Figure 1. Dry matter (DM) yield was determined three to 6 weeks after re-watering of plants (Cut3 and Cut5 in 2013, both locations; Cut3 in 2014 both locations and additionally Cut5 at Malchow in 2014). To obtain the moisture content of the fresh biomass, a 500 g of sample was dried at 60°C for 72 hr and then re-weighed. A set of morphological parameters was scored on a 1–9 scale for each plot during and after drought stress, namely above-ground plant biomass prior to harvest (1 = poor, 9 = productive), above-ground plant biomass ten days after harvest (1 = poor, 9 = productive), drought symptoms (1: no symptoms, 9: wilted leaves) and post-drought recovery (1: no green plants, 9: 100% recovery). Recovery was scored 3 days after re-watering.

2.4 | Statistical analysis

Statistical analyses for the hydroponics-derived data were performed using the procedures PROC MEANS and PROC GLM implemented in SAS v9.4 software (SAS Institute Inc., Cary, NC, USA). Tukey's test was used to carry out *post hoc* comparisons of differences among means, applying a significance threshold of $p < .05$. The Pearson's correlation coefficient was used to reveal intertrait correlations. A stress tolerance index (STI) was determined from adjusted population means according to the Fernandez (1992) formula.

$$STI_i = \frac{(Yp_i)(Ys_i)}{\overline{Yp}^2}$$

where Yp is the performance of the *i*-th population under controlled conditions, Ys is the performance of the *i*-th population under stress conditions, $\overline{Yp}$ is the mean performance of all populations under controlled conditions. Populations recording an STI value close to or above one were considered as drought tolerant. Based on the STI

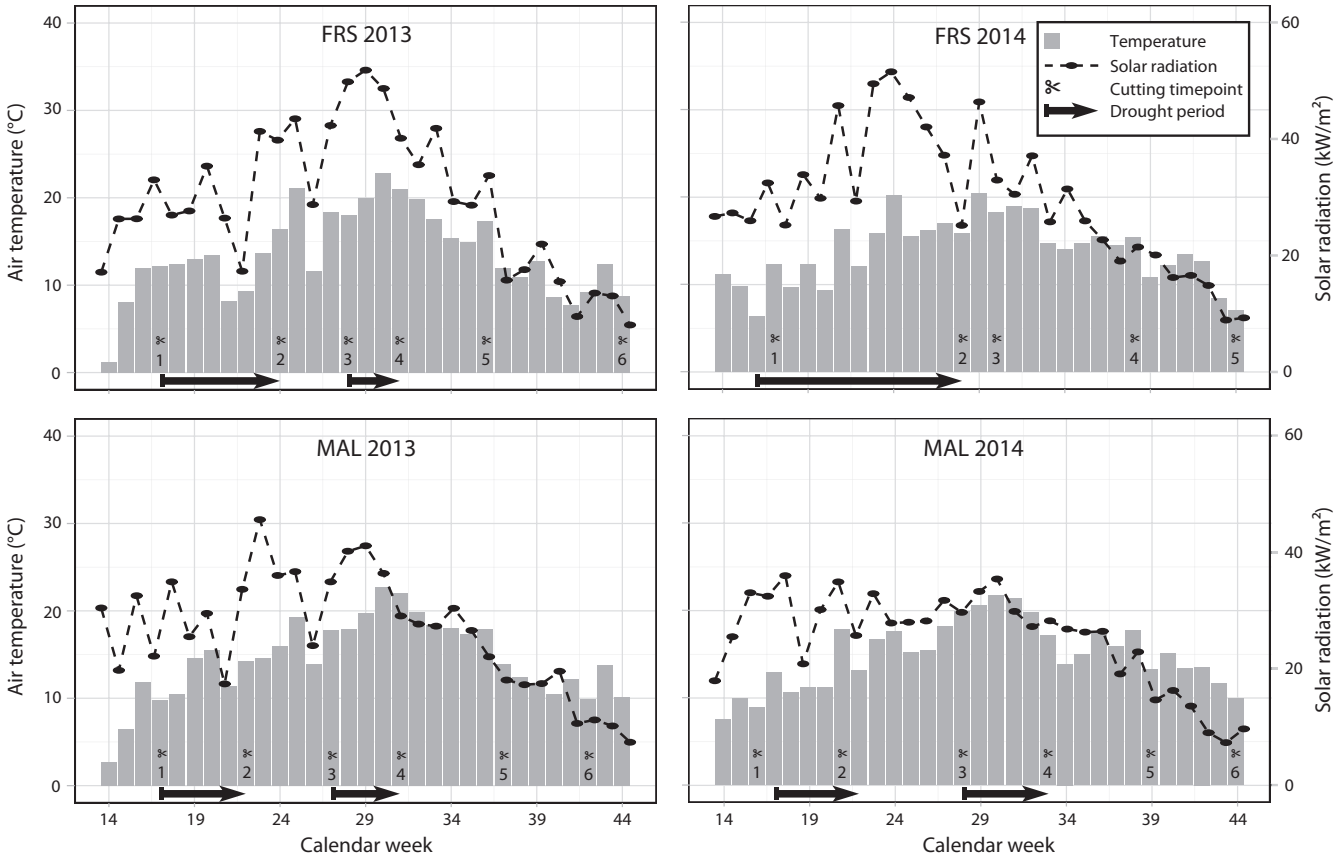

FIGURE 1 Sum of solar radiation per week, mean air temperature and cutting timepoints at the experimental sites Malchow and Pulling during the growing seasons 2013 and 2014

values for whole plant DM, shoot DM, root DM and chlorophyll content, a membership function value of drought stress tolerance (MFVD) was calculated, following Chen, Min, Yasir, and Hu (2012) to evaluate the overall drought tolerance of the grass populations.

$$U_{ij} = \frac{\mathrm{STI}_{ij} - \mathrm{STI}_{j\min}}{\mathrm{STI}_{j\max} - \mathrm{STI}_{j\min}} \qquad U_{\mathrm{i}} = \frac{1}{n}\sum_{j=1}^{n} U_{ij}$$

where U_{ij} is the membership function of the trait (j) for the population (i) for stress tolerance index; $\mathrm{STI}_{j\max}$ is the maximum value of the stress tolerance coefficient for the trait (j); $\mathrm{STI}_{j\min}$ is the minimum value of the stress tolerance coefficient for the trait (j); U_i is the average value of the membership function of the traits whole plant DM, shoot DM, root DM and chlorophyll content for the population (i).

Statistical analyses for the rainout shelter-derived data were performed using SAS Studio 3.4 web application (SAS Institute Inc.). Values were based on plot means. For analysis of variance, population and location (location-year combination) were considered as fixed effects. The MIXED procedure involving a type III test of fixed effects was applied, and the significance threshold was set as 0.05. A principal component analysis was calculated to integrate the various drought tolerance associated traits and to estimate the relevance of the assessed parameters. For this analysis (based on the PROC PRINCOMP procedure in SAS), the data were normalized around a mean of 0 and a variance of 1.

Spearman rank correlation coefficients between the various parameters evaluated in both phenotyping platforms were determined from mean values of each accession, using the PROC CORR procedure in SAS.

3 | RESULTS

3.1 | Variation for drought-related traits in perennial ryegrass under hydroponics conditions

The hydroponics system allowed the monitoring of both shoot and root growth as affected by simulated drought stress (Figure 2). The accumulation of shoot and root DM was influenced both by the intensity of the stress and by populations. There was also a significant population x stress level interaction (Table 2). Under PEG0, the mean shoot DM of *Lp* ranged from 0.2 to 4.9 g/plant (mean 1.31 g), while under PEG10 and PEG20, the shoot DM was reduced on average to, respectively, 53.8% and 44.9% of the PEG0 level. Similarly, root growth of *Lp* was inhibited by the stress, falling to 80.3% of the PEG0 level under PEG10 and to 67.1% under PEG20. Root growth was less affected by the stress than shoot growth or even stimulated

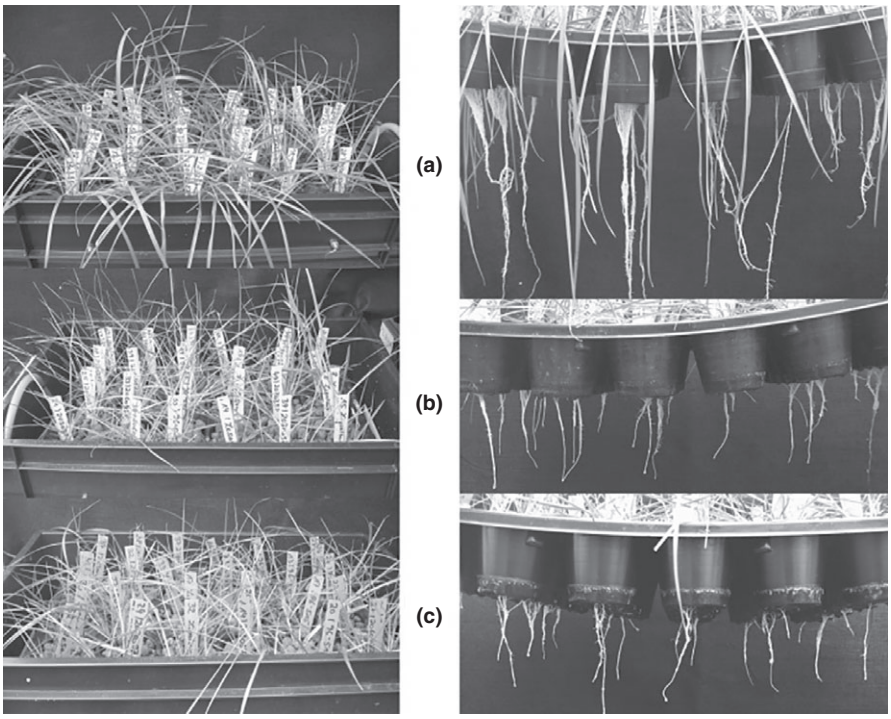

FIGURE 2 Plant development in hydroponics culture after two weeks in nutrient solutions with different concentrations of PEG 6000 for simulation of drought stress; a = control; b = moderate stress (10% PEG); c = severe stress (20% PEG)

by limited water availability. Correspondingly, the root/shoot ratio tended to increase in the stress treatments: it averaged 0.36 (range 0.1–1.8) at PEG0, 0.56 (0.1–3.1) at PEG10 and 0.59 (0.1–3.1) at PEG20. When shoot DM in the two levels of stress was expressed in relation to the performance under PEG0, the response varied markedly between populations, but there was no population x stress level interaction. However, a significant interaction was noted when considering the relative root DM. The mean chlorophyll content of the youngest fully developed leaf fell from 39.1 at PEG0 to 31.1 under PEG10, but only to 38.8 under PEG20. Although the stress even promoted leaf chlorophyll accumulation in some populations, in others its effect was very negative.

Variation in the population performance was noted in respect to both shoot (Figure 3b) and root (Figure 4b) DM accumulation. The diploid *Lp* populations, whether breeding material or ecotypes, were more sensitive to drought stress than the tetraploids. The breeding material was more productive than the genebank accessions at both PEG0 and at each of the two levels of PEG-induced stress. The response of the reference *Festuca* sp. and *Festulolium* populations was comparable to that of the tetraploid *Lp* populations. The STI values related to both shoot and root DM are shown in Figures 3a and 4a, respectively, grouped according to the different status of the plant material. The range for shoot DM STI at PEG10 was 0.0 to 2.4 (mean 0.64), and 0.0 to 1.7 (mean 0.47) at PEG20. None of the diploid breeding material recorded an STI > 1, but one diploid ecotype (GR 3467) did so at PEG10, as well as five of the tetraploid *Lp* populations at PEG10 (446, 449, Astonenergy, 450 and 444) and four populations under PEG20 (449, Astonenergy, 450 and 444). The STI values for root DM ranged from 0.1 to 2.3 (mean 0.93) at PEG10 conditions and from 0.1 to 1.7 (mean 0.74) at PEG20 conditions. Four of the diploid breeding populations (412, 417, 414 and 410) and eight representatives of the tetraploid group (449, 416, Astonenergy, 446, 447, 442, 444 and 450) recorded an STI > 1 at PEG10. The STI values related to chlorophyll content had substantially smaller ranges than the STI values for shoot and root DM, ranging from 0.5 to 1.4 (mean 0.80) at PEG10 and from 0.6 to 1.3 (mean 1.01) at PEG20 conditions. A correlation analysis revealed that shoot DM was positively correlated with root DM at both PEG10 (r_g = 0.67) and PEG20 (r_g = 0.38) (Table 3), but there was no correlation between chlorophyll content and root DM or shoot DM. The strength of the correlations was higher in plants exposed to PEG10 than to PEG20.

3.2 | Variation for drought-related traits in perennial ryegrass under rainout shelter conditions

The rainout shelter experiment allowed simultaneous investigation of a large number of genotypes under controlled drought conditions similar to a field environment. Analysis of variance showed that a highly significant genotypic variance was found for all recorded traits, whereas there was no effect of the trial site on the visual scorings but on the DM yield measurement (Table 4). The population × location interaction was significant for both post-drought recovery and DM yield. A summary of the performance data is shown in Table 5. The *Lp* DM yield in 2013 decreased from Cut3 (1.34–53.67 dt/ha) to Cut5 (0.00–43.99 dt/ha) and was higher in the first year than in the second year (0.00–36.87 dt/ha and 0.00–24.40 dt/ha, respectively). Across years and cutting times, the references yielded more consistently than the tested *Lp* germplasm. The DM yield of the *Lp* populations in 2013 declined by about 7.9 dt/ha from Cut3 to Cut5. The visual scores for biomass assigned before and after the imposition of drought stress were strongly correlated

TABLE 2 Plant performance in hydroponics culture at different levels of osmotic stress

	PEG0			PEG10			PEG20			F-statistics		
Trait	Mean	Max/min	Variance	Mean	Max/min	Variance	Mean	Max/min	Variance	PEG	Population	PEG*access
39 *Lp* populations												
DM shoots [g]	1.31	4.92/0.21	0.61	0.66	1.92/0.05	0.14	0.53	1.45/0.16	0.06	551.96***	43.18***	6.70***
Rel DM shoots [%]				57.17	149.19/12.87	466.41	49.62	135.46/16.74	442.49	19.05***	6.67***	0.87$^{n.s.}$
DM roots [g]	0.38	1.08/0.05	0.03	0.03	0.70/0.01	0.03	0.25	0.58/0.03	0.02	130.97***	36.75***	2.62***
Rel DM roots [%]				82.36	175.03/8.00	1052.31	70.81	143.61/13.92	837.90	21.78***	5.61***	2.13***
R/S ratio DM	0.36	1.75/0.11	0.05	0.56	3.07/0.07	0.17	0.59	3.08/0.12	0.21	45.56***	18.96***	1.27$^{n.s.}$
SPAD	39.13	60.40/23.00	44.92	31.05	49.20/18.90	19.63	38.84	56.70/19.10	48.96	255.11***	12.45***	5.68***
8 reference populations												
DM shoots [g]	1.67	4.41/0.60	0.70	0.92	1.71/0.23	0.14	0.73	1.17/0.31	0.07	82.68***	19.99***	4.65***
Rel DM shoots [%]				64.22	139.57/25.46	612.97	51.48	96.79/26.82	317.51	10.02**	5.56***	1.29$^{n.s.}$
DM roots [g]	0.48	1.19/0.19	0.04	0.43	0.78/0.18	0.02	0.34	0.60/0.13	0.01	11.56***	6.30***	2.87***
Rel DM roots [%]				103.09	187.03/45.86	1213.91	84.79	190.21/29.71	1406.21	10.31**	12.31***	1.02$^{n.s.}$
R/S ratio DM	0.31	0.54/0.14	0.01	0.60	1.97/0.22	0.13	0.60	1.78/0.21	0.13	32.99***	22.14***	4.64***
SPAD	36.29	47.10/20.30	49.13	30.24	41.40/22.80	24.14	41.23	58.00/28.30	65.17	80.42***	9.51***	13.94***

PEG0, control; PEG10, moderate osmotic stress; PEG20, severe osmotic stress; DM, dry matter (g/plant); rel, relative biomass calculated in per cent of control; data determined after 5 weeks of culture, thereof 4 weeks under stress conditions; R/S ratio, root-shoot ratio; SPAD, chlorophyll content.

Significance level *$p < .05$; **$p < .01$; ***$p < .001$; $^{n.s.}$not significant.

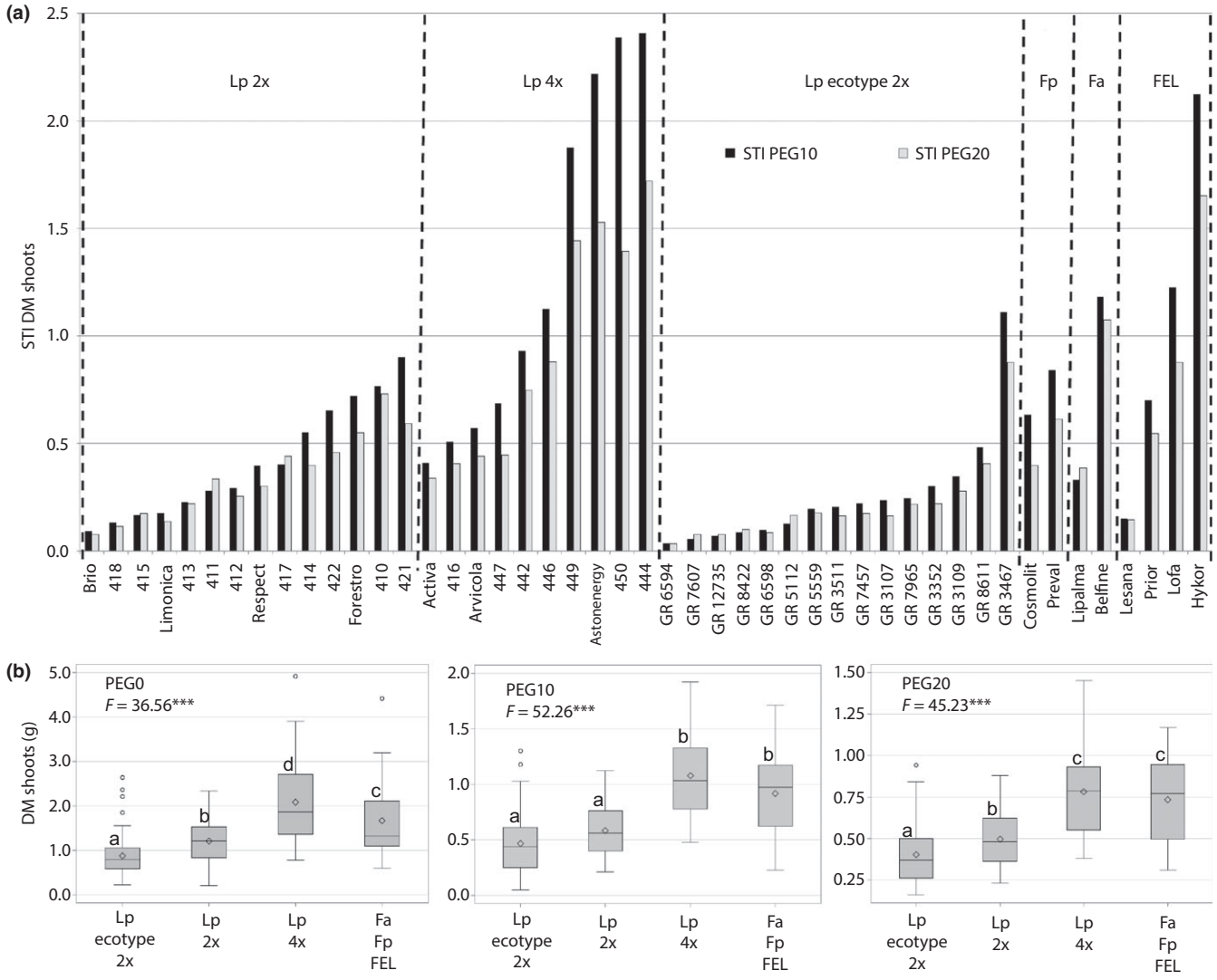

FIGURE 3 (a) Stress tolerance indices (STI) for dry matter of shoots produced after 4 weeks of culture under moderate (PEG10) and severe (PEG20) stress conditions; (b) median and mean dry matter of shoots produced by different plant material groups after 4 weeks of culture under control (PEG 0) and stress conditions (PEG10, PEG20). Lp ecotype 2× = *Lolium perenne* L. genebank accessions diploid; Lp 2× = *Lolium perenne* L., diploid; Lp 4× = *Lolium perenne* L. tetraploid; Fa, Fp, FEL = reference populations *Festuca* sp. and *Festulolium*; different letters mark significant differences between groups at the respective PEG level (Tukey, $p < .05$)

with measured yields. As for DM yield, the variability of visual scores was considerably higher in the second year than in the first year. The rating for drought symptoms in *Lp* ranged between 0.85 and 7.30 in 2013 and the estimates for post-drought recovery between 1.15 and 6.70. In 2014, the range of drought symptoms rating was smaller (0.10 to 4.50) and recovery ranged between 0.10 and 6.60.

A principal component (PC) analysis was performed to determine the shares of single phenotypic traits regarding the variance seen for drought tolerance. The analysis comprised 16 traits evaluated for all 47 populations within 2 years and across two locations. The first three principal components (PC1–PC3) explained 89.5% of the total variance of drought tolerance (Table 6). PC1 accounted for 72.8% of total variation and was equated with post-drought recovery after Cut3 and Cut5 according to the corresponding eigenvector value; PC2 accounted for 10.6% of total variance and corresponded to the rating of the drought symptoms. Four populations (Belfine, Hykor, Lesana and Lipalma) all of which belonged to the references group achieved a positive value for PC1 (high DM yield) and a negative one for PC2 (low drought symptoms rating). Most of the *Lp* ecotype accessions formed a distinct cluster in PC, separated from both the tetraploid *Lp* populations and the *Festuca* sp. indicating drought susceptibility of this group of *Lp*.

3.3 | Comparison of the phenotyping platforms hydroponics and rainout shelter

The interplatform correlations were highly significant for the hydroponics PEG20 treatment and the 2014 rainout shelter experiment

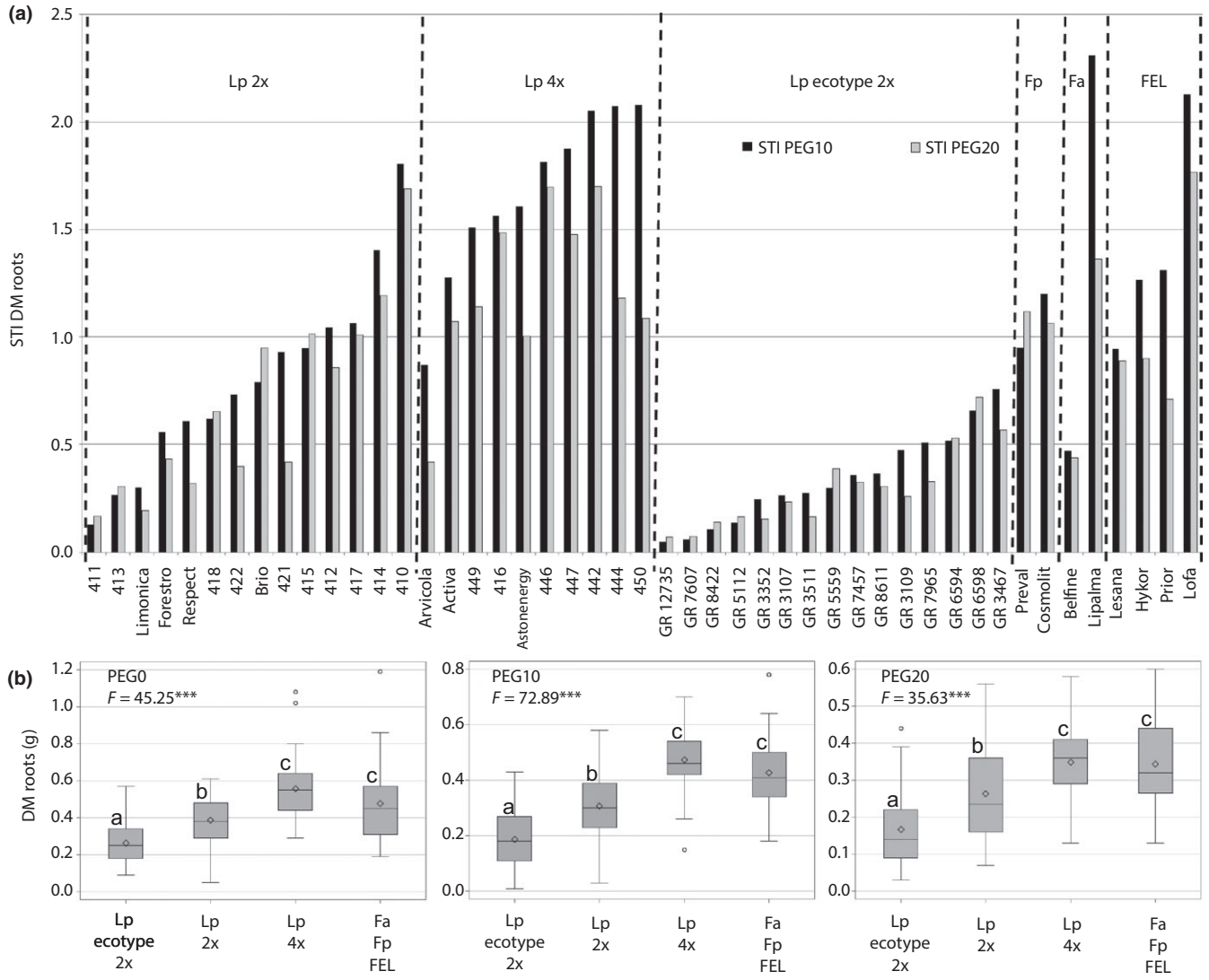

FIGURE 4 (a) Stress tolerance indices (STI) for dry matter of roots produced after 4 weeks of culture under moderate (PEG10) and severe (PEG20) stress conditions; (b) median and mean dry matter of roots produced by different plant material groups after 4 weeks of culture under control (PEG 0) and stress conditions (PEG 10, PEG20). Lp ecotype 2× = *Lolium perenne* L. genebank accessions diploid; Lp 2× = *Lolium perenne* L., diploid; Lp 4× = *Lolium perenne* L. tetraploid; Fa, Fp, FEL = reference populations *Festuca* sp. and *Festulolium;* different letters mark significant differences between groups at the respective PEG level (Tukey, $P < .05$)

(Table 7). Shoot DM of the PEG20 treatment was highly and positively correlated with plant biomass scorings in the 2014 rainout shelter experiment prior to both Cut3 (r = .69***) and Cut5 (r = .64***) as well as with visual scores of plant biomass after Cut3 (r = .67***). Correlations of 0.50–0.75 were obtained between root DM in PEG10 treatment and both estimates of plant biomass in the 2014 rainout shelter experiment as well as DM yield at Cut5 in the 2013 rainout shelter experiment. In 2014, post-drought recovery was moderately to strongly correlated with both root and plant DM measured in hydroponics at PEG10 (Figure 5) and PEG20, as well as with the STI values for plant DM and root DM. No significant correlations were found between chlorophyll content or its STI and any of the rainout shelter experiment parameters. The MFVD values calculated from the data obtained on both phenotyping platforms were moderately to highly correlated with each other. For the PEG10 treatment, the correlation coefficients ranged from 0.30 to 0.55, increasing to 0.67 for the PEG20 treatment. An above average post-drought recovery (STI > 1) was observed both for the tetraploid *Lp* and the reference populations; the diploid *Lp* breeding materials recorded an intermediate performance, while the genebank accessions—with the exception of population GR 3467—all performed poorly with respect to shoot DM under the PEG10 treatment, and also exhibited a poor post-drought recovery in the rainout shelter. Overall, the entries best able to produce DM under PEG-mediated stress were those which were most capable of recovery after drought stress.

TABLE 3 Pearson correlation coefficients (r_g) among examined traits of 47 divergent populations evaluated in hydroponics culture system (PEG10 and PEG20)

	DM shoots	DM roots	SPAD
DM shoots			
PEG10	–	0.67***	0.26
PEG20	–	0.38***	0.30*
DM roots			
PEG10		–	0.04
PEG20		–	0.18
SPAD			
PEG10			–
PEG20			–

DM, dry matter; PEG10, moderate osmotic stress; PEG20, severe osmotic stress; SPAD, chlorophyll content.
Significance level *$p < .05$; ***$P < .001$.

4 | DISCUSSION

4.1 | Assessing variation for drought tolerance of perennial ryegrass grown in hydroponics

The imposition of osmotic stress obtained by introducing PEG to the cultivation medium of in vitro or hydroponics grown plants has been promoted as a convenient means of screening germplasm for drought tolerance (Anithakumari, Dolstra, Vosman, Visser, & van der Linden, 2011). The approach has been implemented in various di- and monocotyledonous plant species, including *Lp* (Guthridge et al., 2001; McFarlane, Guthridge, Smith, Jones, & Forster, 2001). Variation in the vegetative development and metabolic profiles of two different *Lp* genotypes grown in hydroponics has been demonstrated by Foito, Byrne, Shepherd, Stewart, and Barth (2009). In our experiments, the 39 *Lp* populations comprising germplasm panel displayed considerable genetic variation for all of the targeted traits, when plants were exposed to either moderate (PEG10) or severe (PEG20) simulated drought stress. The PEG treatments reduced both absolute root and shoot DM, while chlorophyll content was only affected by the PEG10 treatment (Table 2). Overall, shoot growth was reduced to a larger extent than root growth under osmotic stress conditions. Furthermore, some populations even enhanced root growth under moderate water-stress conditions. This result is in accordance with results of Jupp and Newman (1987), who also found enhanced growth rates of lateral roots in *Lp* in a pot experiment and results from another hydroponics experiment (Van Loo, 1992). Similar effects were described for *Fa* under field conditions (Ebrahimiyan, Majidi, & Mirlohi, 2013). In our study, the ability to maintain a high root DM under conditions of osmotic stress was positively correlated with the accumulation of shoot DM (Table 3). In contrast, testing of the *Lp* cultivar S24 in nutrient solutions revealed a reduction in both shoot and root DM induced by severe stress, however without an effect on the root/shoot ratio (Gales, 1979). This result may indicate that S24 is a particularly drought-sensitive cultivar. The differential effect of stress on root and shoot growth, which we observed in our experiments, resulted in an increase in the root/shoot ratio and may be interpreted as ability of specific populations to cope with drought stress (Blum, 1996). The mean root/shoot DM ratio increased from 0.36 in the PEG0 to 0.56 in the PEG10 and 0.59 in the PEG20 treatment, consistent with the suggestion of Blum (1996). As this redirection of metabolism is incompatible with the need to sustain above-ground biomass yield, Lynch (2015) proposed that a favourable ideotype would be represented by a plant able to channel a high proportion of its root system into root types metabolically not that demanding and/or having a reduced respiratory requirement.

Barnes, Kopecký, Lukaszewski, and Baird (2014) have shown that the superior drought tolerance of *Festulolium* (compared to *Fa*) can be explained by a large root/shoot ratio. *Fa* populations selected on the basis of their ability to produce a high root/shoot ratio also showed an improvement in respect to both their drought tolerance and their post-drought recovery (Karcher, Richardson, Hignight, & Rush, 2008). Screening for root characteristics under

TABLE 4 Analysis of variance—Type III test of fixed effects on parameters investigated in rainout shelter experiments conducted at Malchow and Pulling in the years 2013 and 2014

		Scoring plant biomass before		Scoring plant biomass after				DM yield	
Source of variation	*df*	Cut3	Cut5	Cut3	Cut5	Drought symptoms rating	Recovery rating after stress	Cut3	Cut5
Population (P)	46	***	***	***	***	***	***	***	***
Location (L)	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	**
P × L	46	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
Year (Y)	1	n.s.	n.s.	n.s.	n.s.	**	n.s.	***	***
P × Y	46	***	***	***	***	***	***	***	***
L × Y	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
P × L × Y	46	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	***

df, degrees of freedom; Cut3, cutting timepoint 3; Cut5, cutting timepoint 5; DM, dry matter.
Significance level *$p < .05$; **$p < .01$; ***$p < .001$, [n.s]not significant.

TABLE 5 Mean, maximum, minimum value and variance of 16 scored parameters of 47 populations evaluated across two experimental sites (Malchow and Pulling) in 2013 and 2014

		39 *Lp* populations			8 reference populations*		
Year	Trait	Mean	Max/min	Variance	Mean	Max/min	Variance
2013	Plant biomass rating before Cut3	3.10	4.40/0.85	0.58	3.22	4.90/1.70	0.90
	Plant biomass rating after Cut3	2.02	3.35/0.50	0.29	2.47	4.00/1.00	0.79
	Plant biomass rating before Cut5	3.08	4.75/0.60	0.89	3.44	4.90/1.70	1.03
	Plant biomass rating after Cut5	3.07	4.55/0.65	0.80	3.47	4.75/1.70	0.98
	Drought symptoms rating	3.90	7.30/0.85	1.75	3.54	6.25/1.55	1.15
	Recovery rating after drought	4.49	6.70/1.15	1.45	4.89	8.00/1.55	3.23
	DM yield Cut3 [dt/ha]	24.39	53.67/1.34	121.00	24.87	46.03/10.52	84.77
	DM yield Cut5 [dt/ha]	16.45	43.99/0.00	77.51	24.52	42.85/2.30	95.36
2014	Plant biomass rating before Cut3	2.66	4.40/0.30	1.07	3.38	5.55/1.60	0.98
	Plant biomass rating after Cut3	1.93	3.45/0.20	0.62	2.78	4.75/1.15	0.75
	Plant biomass rating before Cut5	2.75	5.20/0.10	1.66	3.83	5.95/0.80	1.86
	Plant biomass rating after Cut5	2.90	4.90/0.05	1.43	3.67	5.15/0.85	1.23
	Drought symptoms rating	2.12	4.50/0.10	0.93	1.89	3.80/0.95	0.77
	Recovery rating after drought	4.36	6.60/0.10	2.21	5.62	7.20/1.60	1.91
	DM yield Cut3 [dt/ha]	8.81	36.87/0.00	94.51	17.65	61.06/0.00	250.11
	DM yield Cut 5 [dt/ha]	6.29	24.40/0.00	34.39	13.25	39.55/0.00	154.79

Cut3, cutting timepoint 3; Cut5, cutting timepoint 5; DM, dry matter; *including *Festuca* sp. and *Festulolium*.

controlled drought conditions can thus be efficient for enhancing drought tolerance (Blum, 1996; Crush, Nichols, Easton, Ouyang, & Hume, 2010; Crush, Nichols, & Ouyang, 2010; Lelièvre, Seddaiu, Ledda, Porqueddu, & Volaire, 2011; Lilley & Fukai, 1994; Songsri et al., 2008). Similarly, Bonos, Rush, Hignight, and Meyer (2004) suggested that recurrent selection for longer roots could be an effective strategy for improving tolerance in both *Fa* and *Lp*. The STI concept has been applied to characterize plant performance in a range of crop species, including *Fa* (Ebrahimiyan, Majidi, Mirlohi, & Gheysari, 2012; Pirnajmedin, Majidi, & Gheysari, 2015). In the experiments carried out by Ebrahimiyan et al. (2012), STI values ranged from nearly 0 to about 4. In our set of *Lp* populations, the highest recorded STI value was about 3 (involving root DM), a value which should be a sufficient basis for effective selection of drought tolerant genotypes.

It has been proposed repeatedly that stress sensitivity can be correlated to the extent of chlorophyll degradation induced by moisture deficit (Li, Guo, Baum, Grando, & Ceccarelli, 2006 and references therein). However, there was no evidence for such relationship in our experiments, which is in accordance with results of Yu et al., 2013; who also found no effect of drought stress on chlorophyll fluorescence in *Lp*. For most of the populations, the chlorophyll content of the youngest fully developed leaf was lower at PEG10 than in the PEG20 treatment. This stress intensity-dependent response may help to explain apparently contradictory observations involving Kentucky bluegrass (*Poa pratensis* L.), *Fa* (Ebrahimiyan, Majidi, Mirlohi, & Noroozi, 2013; Fu & Huang, 2001; Pirnajmedin et al., 2015) and blue grama (*Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Griffiths; Garcia-Valenzuela, Garcia-Moya, Rascón-Cruz, Herrera-Estrella, & Aguado-Santacruz, 2005). An increase in chlorophyll content induced by drought stress in various pot-grown grass species has been described by Kocon and Staniak (2014). Here, the boost of chlorophyll content induced by the PEG20 treatment may reflect two distinct and opposing processes. The first involves the disruption of chlorophyll metabolism and the second the development of smaller leaves, which as a consequence contain a higher concentration of chlorophyll. The balancing of these processes implies that chlorophyll content is unlikely to be a suitable parameter for assessing drought tolerance.

4.2 | Assessing variation for drought tolerance of perennial ryegrass in rainout shelters

For post-drought recovery of *Lp*, a considerable variation was found within the investigated germplasm set (Table 4) explaining 72.8% of the total variance of drought tolerance. Genetic variation in *Lp* for drought recovery has been described by Cheplick et al. (2000), Turner et al. (2012) and Barnes et al. (2014). Within a segregating *Lp* population, Hatier et al. (2014) found that the trait "leaf lamina regrowth after defoliation" explained up to 57% of the variation in DM production after rehydration of plants. This confirms the observation of our study with post-drought recovery as the most important trait for describing drought tolerance in the field. Genetic variation for drought survival and post-drought recovery has been also documented in other grass species like *Fa* (Ebrahimiyan, Majidi, & Mirlohi, 2013) and Kentucky blue grass (Chai, Jin, Merewitz, &

TABLE 6 Eigenvectors and eigenvalues of the first three principal components (PC) of 47 divergent populations evaluated in rainout shelter experiments across two locations (Malchow and Pulling)

	Eigenvectors		
Traits	**PC 1**	**PC 2**	**PC 3**
2013			
Plant biomass rating before Cut3	0.25	0.10	−0.46
Plant biomass rating after Cut3	0.27	−0.01	−0.32
Plant biomass rating before Cut5	0.28	0.13	−0.13
Plant biomass rating after Cut5	0.28	0.10	−0.10
Drought symptoms rating	0.16	0.56	−0.20
Recovery rating after drought	0.24	0.03	−0.25
DM yield Cut3	0.23	0.18	−0.15
DM yield Cut5	0.27	−0.07	0.10
2014			
Plant biomass rating before Cut3	0.28	−0.02	0.22
Plant biomass rating after Cut3	0.27	−0.06	0.27
Plant biomass rating before Cut5	0.28	−0.09	0.22
Plant biomass rating after Cut5	0.27	−0.07	0.25
Drought symptoms rating	0.12	0.56	0.46
Recovery rating after drought	0.27	−0.09	0.29
DM yield Cut3	0.24	−0.36	−0.07
DM yield Cut5	0.24	−0.38	−0.06
Eigenvalues	11.65	1.70	0.97
% of total variance explained	72.78	10.63	6.04
% of cumulative variance explained	72.78	83.42	89.46

Cut3, cutting timepoint 3; Cut5, cutting timepoint 5; PC 1, principal component 1; PC 2, principal component 2; PC 3, principal component 3; DM dry matter.

Huang, 2010). The latter authors included two *Lp* cultivars in their study and concluded that neither was able to express complete post-drought recovery, corresponding well to our findings for most of the *Lp* populations analysed here. Possible adaptations supporting post-drought survival include formation of a deeper root system, maintenance of a low water and osmotic potential in the leaf, accumulation of plentiful supply of soluble carbohydrate reserves in the tiller base, minimization of the accumulation of proline in stubble and rapid uptake of nitrogen upon re-watering (Volaire, Thomas, & Lelièvre, 1998). Data by Chai et al. (2010) showed that drought tolerance in *Lp* is correlated with osmotic adjustment, high cell wall elasticity and cell membrane stability. Furthermore, drought recovery is likely associated with both the accumulation of carbohydrates in leaves and rhizomes during the stress episode and with a rapid burst of root development after re-watering. The observed variation displayed by the present set of germplasm may be based on such underlying physiological mechanisms.

Leaf wilting was suggested as a suitable selection trait for drought tolerance in *Lp* by Yu et al. (2013). Results of this study show that visual scoring of drought symptoms (e.g. wilting) in *Lp* can complement selection for drought tolerance. However, as it here only contributes 10.6% to the total variance of drought tolerance, this trait is of minor importance.

4.3 | Comparison of hydroponics and rainout shelter phenotyping platforms

Assessment of plant response to naturally occurring drought stress is complex, largely because the stress is experienced unpredictably both in time and space. Experiments in which the duration and/or intensity of the stress is controlled can at best identify just a few of the components required for tolerance. Here, a hydroponics-based test, in which stress intensity can be readily controlled and where the entire root system experiences the stress, was compared with a rainout shelter experiment, more closely reflecting the natural situation in which the stress level is not constant over time or space. The correlations obtained between shoot DM in hydroponics grown plants and both visually scored and measured DM yield in the rainout shelter were medium to high (Table 7), which indicates that yield potential in drought-prone environments can be predicted reasonably well from hydroponics experiments. This is irrespective of the fact that the plants investigated had a different physiological age, as plants in the rainout shelter were monitored over a period of 2 years, while the hydroponics plants were continuously propagated clonally. The higher correlations obtained from the PEG20 rather than from the PEG10 treatment might imply that this higher stress level was more comparable to the stress experienced in the rainout shelter experiments. However, the correlations obtained between root DM and both DM yield and post-drought recovery were higher for the PEG10 treatment.

Performance varied significantly not only among populations, but also between the various groups of material (diploid genebank accessions, diploid and tetraploid breeding material, and references). Both the tetraploid populations (all of which were selected by breeders) and the diploid breeding materials were more productive in terms of DM yield and biomass development in the rainout shelter, as well as in the ability to develop shoot and root DM in the hydroponics experiment compared to unselected ecotypes and historical varieties. Their general vigour reflects their selection history, as has been documented the same way in other crops. Notably in maize, Tollenaar and Lee (2002) have shown that modern cultivars are not only more productive, but also more stress tolerant than older ones. Although most of the diploid *Lp* ecotypes were rather drought sensitive, there was one exception (accession GR 3467). Polyploids are in general more vigorous than their diploid counterparts (Otto & Whitton, 2000), although the mechanistic basis for their advantage remains unclear. The *Festulolium* and *Festuca* sp. reference populations responded diversely to drought in both experimental platforms (Table 5, Figures 3 and 4). Their generally higher productivity in the rainout shelter experiments can be attributed to a superior ability to form an extensive root system (Ebrahimiyan, Majidi, & Mirlohi, 2013; Huang & Gao, 2000; Karcher et al., 2008; Turner et al., 2012). Nevertheless, their shoot DM measured in the hydroponics experiment did not differ significantly from that attained by the tetraploid *Lp*

TABLE 7 Spearman's rank correlation coefficients (r_g) among examined traits of 47 divergent populations evaluated under drought stress in hydroponics culture system and rainout shelter experiments based on adjusted means of two locations

	Scoring plant biomass before				Scoring plant biomass after				Drought symptoms rating		Recovery rating after drought		DM yield			
	Cut3 2013	Cut5 2013	Cut3 2014	Cut5 2014	Cut3 2013	Cut5 2013	Cut3 2014	Cut5 2014	2013	2014	2013	2014	Cut3 2013	Cut5 2013	Cut3 2014	Cut5 2014
DM shoots																
PEG10	0.47***	0.55***	0.62***	0.57***	0.50***	0.51***	0.64***	0.51***	0.54***	0.38*	0.34*	0.57***	0.49***	0.51***	0.52***	0.48***
PEG20	0.59***	0.64***	**0.69***	0.64***	0.62***	**0.67***	**0.67***	0.59***	0.61***	0.28	0.45**	0.62***	0.54***	0.59***	0.60***	0.54***
DM roots																
PEG10	0.34*	0.55***	**0.67***	**0.69***	0.45**	0.63***	**0.72***	0.63***	0.33*	0.24	0.36*	**0.65***	0.33*	**0.65***	0.60***	0.57***
PEG20	0.21	0.36*	0.50***	0.55***	0.26	0.39**	0.52***	0.52***	0.11	0.09	0.21	0.51***	0.25	0.52***	0.49***	0.45**
SPAD																
PEG10	0.17	0.21	0.16	0.10	0.18	0.16	0.06	0.16	0.20	0.08	0.26	0.02	0.24	0.14	0.09	0.17
PEG20	0.18	0.35*	0.32*	0.28	0.29	0.36*	0.22	0.28	0.19	0.00	0.35*	0.18	0.13	0.27	0.18	0.27
STI DM shoots																
PEG10	0.52***	0.58***	**0.65***	0.59***	0.54***	0.53***	**0.66***	0.53***	0.58***	0.38**	0.37*	0.58***	0.51***	0.53**	0.57***	0.50***
PEG20	0.57***	0.61***	**0.65***	0.59***	0.58***	0.64***	**0.65***	0.53***	0.63***	0.37*	0.40**	0.59***	0.54***	0.55***	0.58***	0.50***
STI DM roots																
PEG10	0.33*	0.53***	0.64***	**0.66***	0.42**	0.59***	**0.71***	0.59***	0.38**	0.31*	0.32*	0.62***	0.36*	0.61***	0.56***	0.54***
PEG20	0.30*	0.48***	0.61***	0.64**	0.36*	0.51***	**0.65***	0.60***	0.27	0.22	0.29	0.61***	0.33*	0.60***	0.57***	0.52***
STI SPAD																
PEG10	0.01	−0.07	−0.10	−0.12	−0.07	−0.07	−0.13	−0.07	0.02	0.00	−0.06	−0.14	0.08	−0.11	−0.09	−0.05
PEG20	0.04	0.05	0.02	−0.04	0.02	0.09	−0.06	0.00	0.10	−0.04	0.05	−0.11	0.04	−0.05	−0.03	0.01
MFVD																
PEG10	0.38**	0.47***	0.55***	0.52***	0.40**	0.48***	0.58***	0.48***	0.44**	0.32*	0.30*	0.50***	0.41**	0.48***	0.47***	0.43**
PEG20	0.41**	0.52***	0.61***	0.59***	0.44***	**0.67***	0.63***	0.54***	0.44**	0.26	0.31*	0.57***	0.41**	0.54***	0.54***	0.48***

DM shoots, dry matter shoots; DM roots, dry matter roots; SPAD, chlorophyll content; STI stress tolerance index; MFVD, membership function value of drought stress tolerance; PEG10, moderate osmotic stress; PEG20, severe osmotic stress, correlations above .65 are given in bold.

Significance level *$p < .05$, **$p < .01$, ***$p < .001$.

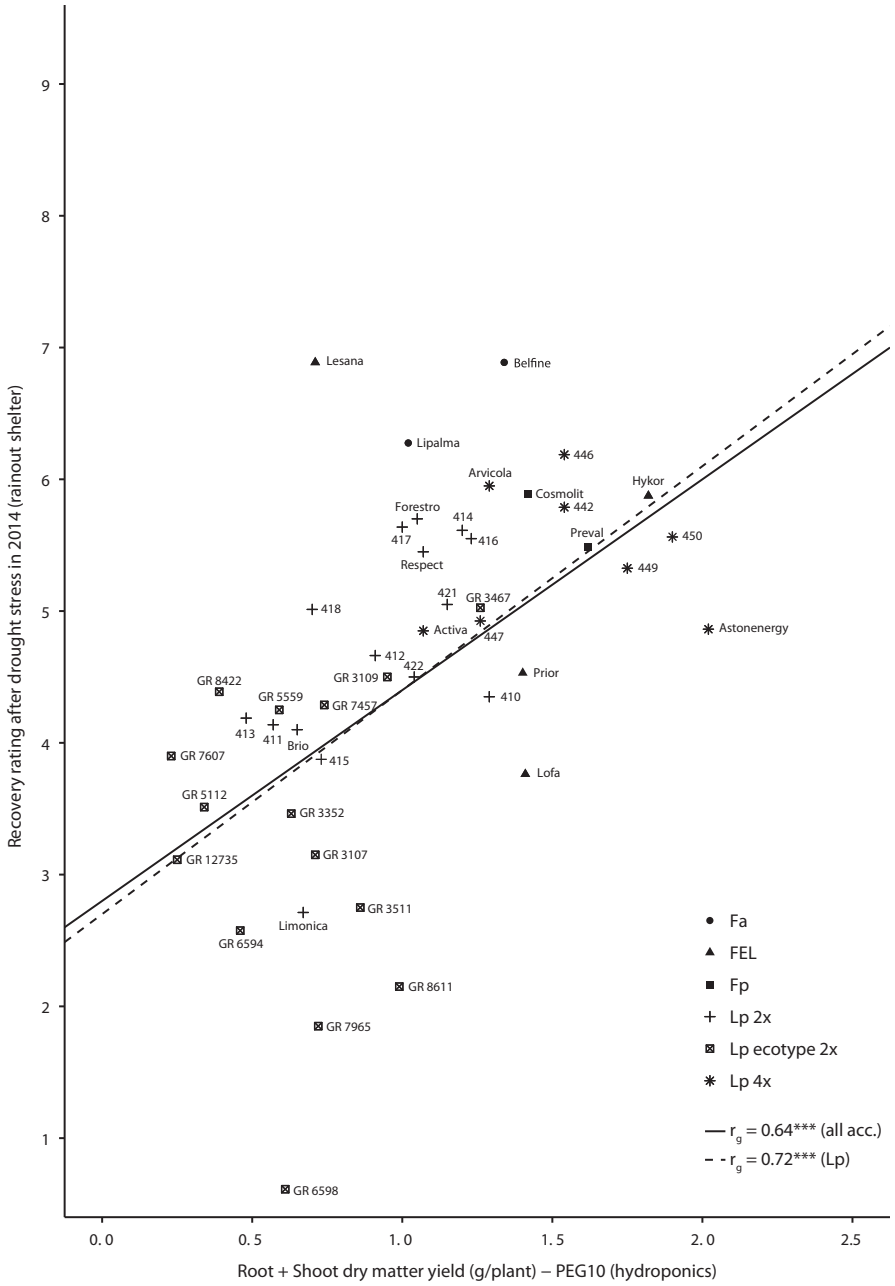

FIGURE 5 Correlation between whole plant dry matter yield of 47 divergent populations assessed under moderate stress conditions (PEG 10) in hydroponics culture system and scores for the recovery after drought stress recorded in rainout shelters in 2014 (adjusted means for two locations). Lp ecotype 2× = *Lolium perenne* L. genebank accessions diploid; Lp 2× = *Lolium perenne* L., diploid; Lp 4× = *Lolium perenne* L. tetraploid; Fa, Fp, FEL = reference populations *Festuca sp.* and *Festulolium*; *** significance level $p < .001$

populations (Figure 3b), nor did they generate a higher root DM. A possible explanation for this unexpected result is that in the 2013 growing season, the pre-summer drought period was applied before the reference accessions could fully develop their root system. By the time of the later drought periods (Cut5 in 2013, Cut3 and Cut5 in 2014), the *Festulolium* and *Festuca* sp. plants were able to access water from deeper soil layers compared to the *Lp* plants. Regarding the hydroponics experiments, it has to be noted that the length of the growing period (5 weeks) was probably too short to allow the reference populations to fully develop their roots. In addition, the complete root system is exposed to stress under hydroponics, whereas in field-grown plants, only the more shallow roots are under stress. Thus, while the hydroponics system may be suitable for comparing performance within a species, interspecific comparisons need to be treated with caution.

Although it is difficult to successfully simulate the environment of a plant growing under field conditions (Araus & Cairns, 2014), the presented experiments demonstrate the feasibility of selection for drought tolerance within *Lp* germplasm using PEG-supplemented hydroponics. They allow the determination of genotypic differences in root biomass and root/shoot ratios induced by drought stress in a high number of individuals at the reasonable input of labour and time requirement, offering the potential for especially within-species assessment of drought tolerance. In comparison, rainout shelter experiments can provide new insights into the physiology of plants raised under moisture-limited conditions. The two systems complement each other usefully in investigating drought tolerance in perennial ryegrass and other forage grass species, and their combination constitutes an effective selection strategy for breeding *Lp* with enhanced drought tolerance. Nevertheless, as both phenotyping

platforms represent artificial growing environments, the performance of preselected promising genotypes needs to be validated under field conditions.

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