ORIGINAL ARTICLE

Liquid in vitro culture system allows gradual intensification of osmotic stress in *Solanum tuberosum* through sorbitol

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Abstract
Because of their shallow root system, drought stress is a major problem in potato cultivation. Due to climate change more severe drought periods are expected to occur in the vegetative growth phase of potato growth. Therefore, there is a great need for drought tolerant potato genotypes. Potato responds to drought stress in the field in various ways, including osmoregulation. Osmotic stress can be induced in vitro by adding an osmotic agent and thus lowering the osmotic potential of the medium. In this study, a new, cost-effective in vitro test system is presented, in which the osmotic agent can be gradually added after root formation to prevent an osmotic shock. This is achieved by using sieves as plant holders and liquid medium, which, allows an improved simulation of gradually drying soil. Responses to osmotic stress in four potato genotypes were analysed and an increase in proline under osmotic stress was detected. Moreover, genes of interest that were postulated to be linked to drought stress were shown by quantitative qRT-PCR to be regulated under osmotic stress. Furthermore, we showed that the content of sorbitol, which was used as osmotic agent, was 700- fold higher for 'Eurostarch' after seven days under osmotic stress and 1093- fold higher after 14 days, respectively, compared to control plants without sorbitol addition. Therefore, further investigations must show, whether it was taken up through the roots, is metabolised, stored or de novo synthesised by the potato plants.
Keypoints The established novel in vitro test system for potato allows gradually increasing stress exposition of rooted plants. Sorbitol seems not an ideal osmotic agent as it is likely taken up.

Keywords Osmotic stress · Gene expression · In vitro test system · Sorbitol uptake

Abbreviations

13-LOX	Lipoxygenase
APRT	Adeninphosphoribosyltransferase
Cyclo	Cyclophilin
DM	Dry mass
Ef1α	Elongation factor α
FM	Fresh mass
Glyx	Lactoylglutathione lyase/glyoxalase I
GOI(s)	Gene(s) of interest
INH1	Cell wall / vacuolar inhibitor of fructosidase
LC-MS	Liquid chromatography – mass spectrometry
MS medium	Medium according to Murashige and Skoog (1962)
PEG	Polyethylene glycol
PES	Polyester
POD	Peroxidase 51-like
PP	Polypropylene
RPT5a	Regulatory particle triple-A ATPase 5A
SBT1.7	Subtilase family protein
SHMT	Serine transhydroxymethyltransferase
ZBD	Zinc-binding dehydrogenase family protein

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Introduction

Drought stress is a major limiting abiotic factor for the yield quality and quantity of many crops including potato. In the temperate regions of the world, climate change will

lead to more drought periods in spring and early summer, when potato plants are in their vegetative growth phase (Haverkort und Verhagen 2008). Potato is a rather drought sensitive crop due to its shallow root system (Iwama und Yamaguchi 2006). Therefore, it is of utmost importance to select potential new genotypes that display increased drought tolerance and to better understand mechanisms that allow plants to withstand drought stress. Osmotic stress is a term used for a situation, in which plant growth and development is limited by insufficient water availability due to changes in the solute concentration around the cell. In contrast to drought stress, osmotic stress can be induced under in vitro culture conditions, and is considered one part of drought stress (Zhu et al. 1997; Chen und Jiang 2010). Plants are able to alleviate drought stress by osmoregulation, i.e., by synthesis of compounds like glycine betaine, proline or polyols (Mullet und Whitsitt 1996). As osmotic stress is part of drought stress, responses to osmotic stress are therefore indicators for drought stress responses.

In vitro tests systems are characterised by highly controlled environments and are less time intensive than field studies. However, the plant responses under the artificial mixotrophic in vitro conditions may not fully reflect the responses of plants grown ex vitro. Nevertheless, since potato breeders establish in vitro cultures of their important germplasm for reasons of sanitation during breeding, the potentially new genotypes are also available as in vitro shoot cultures. Therefore, an in vitro pre-test to determine osmotic stress tolerant genotypes would be helpful for breeders to narrow down the number of genotypes which have to be tested for drought tolerance in the field and thereby cutting breeding costs until market introduction.

In previous in vitro studies, potato plants were mostly grown on solid medium to which an osmotic agent was added. Dobránszki et al. (2003) used four concentrations of mannitol to induce osmotic stress in vitro. They managed to group five potato genotypes into three osmotic tolerance groups. However, a severe disadvantage of using mannitol as an osmotic agent was reported by Lipavsk und Vreugdenhil (1996). The authors showed in an in vitro study with wheat, rape, and potato, that mannitol was taken up by the plants, transported to the shoots and accounted for up to 20% of shoot dry mass. Another osmotic agent commonly used for inducing osmotic stress in vitro is polyethylene glycol (PEG). Stefan et al. (2020) tested several potato breeding lines with different concentrations of PEG 6000 in solid MS medium for their osmotic stress tolerance. However, Gopal und Iwama (2007) stated that PEG might limit O_2 diffusion due to its high viscosity. The most widely used osmotic agent in potato to date is sorbitol (Gopal und Iwama 2007; Bündig et al. 2016a; Mawia et al. 2020; Hanász et al. 2022). Sorbitol is nontoxic to plants

and not as viscous as PEG. However, Bündig et al. (2016b) reported a possible uptake of sorbitol through the freshly cut surface of the shoots after their cultivation on solid sorbitol-containing medium.

In this study, we tested four starch potato genotypes for their responses to osmotic stress in a liquid MS medium (Murashige und Skoog 1962). This allowed the shoots to form roots prior to being exposed to the osmotic agent. In addition, the stress could be intensified gradually through the stepwise addition of sorbitol. The intact roots were expected to prevent the uptake of sorbitol and transfer to the shoots because of the barrier of the Casparian strip (Łotocka et al. 2016). We investigated fresh and dry mass of shoots and roots, proline content in shoots, and normalised gene expression of candidate genes, which had been selected based upon a proteomic study of drought stressed potatoes (Wellpott et al. 2021). Also, the sorbitol content in the shoots was measured by LC–MS to determine whether sorbitol might be taken up through the intact roots in vitro.

Material and methods

Plant material

Four starch potato genotypes with contrasting responses to osmotic stress based on trials in a solid medium test system (Bündig et al 2016a), were used in this study. 'Eurobravo', 'Eurostarch', and 'Tomba' originated from EUROPLANT Pflanzenzucht GmbH, Lüneburg, Germany. 'Maxi' was bred by Bayerische Pflanzenzuchtgesellschaft eG &Co KG, Hamburg, Germany. In vitro material was kindly provided by the Julius Kühn-Insitute (JKI), Federal Research Centre for Cultivated Plants – Institute for Resistance Research and Stress Tolerance, Groß Lüsewitz, and cultivated in 500 ml polypropylene (PP) vessels (Plastikbecher.de GmbH, Giengen, DE) on 80 ml solid MS (Murashige and Skoog 1962) medium (3% (w/v) sucrose, 7.5 g/l Plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands), pH 5.8) at 18 ± 2 °C in a long day photoperiod (16 h light/8 h dark) with a photon flux density of $\sim 35 \pm 8$ μmol m^{-2} s^{-1}. Subcultures were done using nodal cuttings every four to five weeks. Ten single node cuttings were placed in each vessel for propagation purpose.

Further information on starch content and yield of the genotypes can be found in Meise et al. (2019).

Osmotic stress experiment

Plants were grown for three weeks before five shoots were placed in each experimental vessel, which contained 45 ml liquid MS-medium each (Murashige und Skoog 1962) with

3% (w/v) sucrose. Only shoot tips were used for the experiments. The plant holders were made in-house in order to establish the liquid culture test system. Through a screen mesh, which was melted onto a polypropylene tube section, the shoots were fixed ensuring that the stem base accessed the medium (Fig. 1). The holder was made of a 1.5 cm high pipe ring with a 75 mm diameter (Ostendorf Kunststoffe GmbH, Vechta, Germany) and an attached PES (polyester) screen mesh (mesh size 1600–1800 μm, Franz Eckert, Waldkirch, Germany). The holder was then placed into a 500 ml vessel (Plastkbecher.de GmbH, Giengen, Germany).

In their study, Bündig et al. (2016a) indicated several drawbacks of the solid medium approach, as plants were wounded through cutting of the explants, had to undergo root formation and cope simultaneously with the osmotic stress applied by adding sorbitol directly to the solid medium at one single time point. Based on the in vitro test system using solidified medium by Bündig et al. (2016a) the aim was to establish a protocol for osmotic stress tests in vitro in a liquid culture system where stress could be applied to rooted plants and gradually increased over time. The stress response of the plants was measured through growth parameters, as well as by proline content, and candidate gene expression as described in Wellpott et al. (2023). During a series of experiments, the following parameters were altered in order to optimise the system (compare Table S1 and S2):

– Rooting time was varied between seven and eleven days. Nine days of rooting provided the plants with enough initial roots to continue root growth and secure stability.

– The final concentration of the osmotic agent (here: sorbitol) tested in the medium ranged from 0.3 M to 0.6 M. The concentration in the medium of 0.3 M displayed first differences between control and stressed plants without causing excessive damage to the plants.

– Stress was applied exponentially over 4 application time points (0.1 M, 0.13 M, 0.28 M and 0.6 M) or linearly over three time points (0.1 M, 0.2 M, 0.3 M) to determine optimal application intervals. Both application schemes resulted in differences of growth (FM and DM of shoots and roots) between the treatments, however, linear application was chosen for simplicity.

Based on these findings, the following optimised osmotic stress test system was applied in the experiments reported here: After nine days of root formation in 45 ml of MS-medium, autoclaved sorbitol solution was added as osmoticum in three steps each with an increase of sorbitol concentration of 0.1 M (0.76 ml on day 0, 0.79 ml on day 2, and 0.82 ml on day 5 of a 6 M solution of sorbitol) until an end concentration of 0.3 M sorbitol in the medium was reached (Fig. 2). Addition of the same amount (0.76/0.79/0.82 ml, respectively) of autoclaved deionised water served as a control (2.37 ml in total) as sorbitol was dissolved in water. The experiment was conducted six times with five to ten replicates (one replicate corresponds to one vessel with 5 shoots each) per genotype. Samples were collected from three experimental replications (hereafter termed experiment 1, experiment 2, and experiment 3) for growth and proline and two additional experiments (4 and 5) for gene expression analysis. A sixth experiment was conducted to measure sorbitol content in the shoots. Shoot length, as well as shoot and root fresh mass were measured from plants
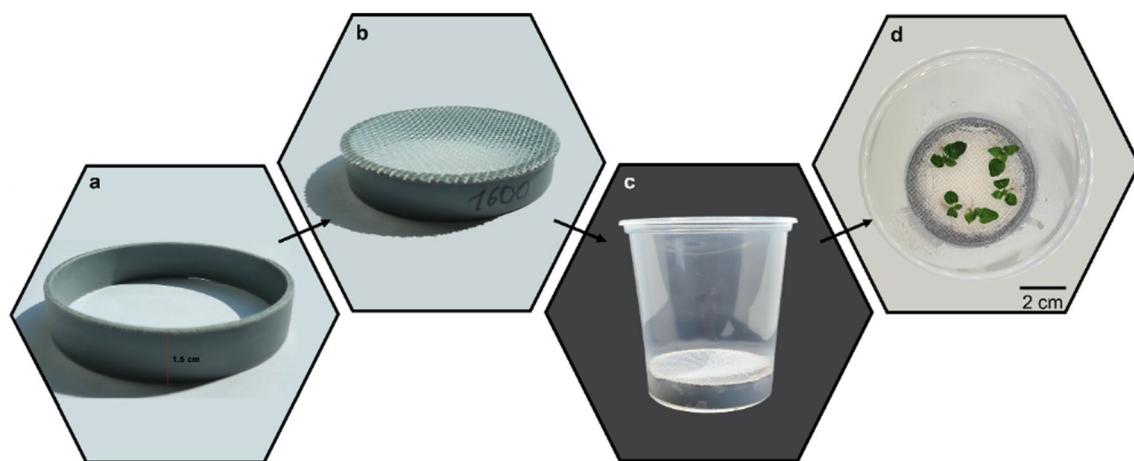

Fig. 1 **Overview of the established in vitro liquid medium stress system. a**: A polypropylene (PP) pipe was cut into rings of 1.5 cm height and deburred with sandpaper. **b**: Sieve mesh (1600 μm diameter, polyester) was melted onto the pipe ring (with a 75 mm diameter). **c**: The sieve fits into 500 ml PP vessels. d: 45 ml medium was filled into the vessel up to the height of the sieve and five shoots were placed into the holder, top view after seven days of osmotic stress
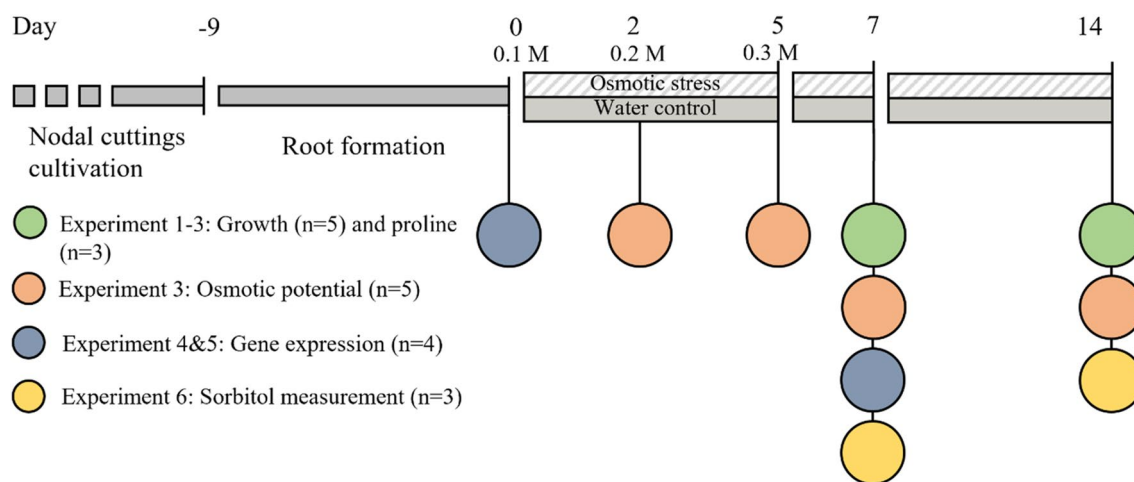
Fig. 2 **Timeline of osmotic stress experiments and samplings.** Four starch potato genotypes were propagated in vitro through nodal cuttings for three weeks. Osmotic stress treatment received sorbitol as osmotic agent in three steps until an end concentration of 0.3 M was reached. Control treatment received the same volumes of deionised water instead. Samples were taken after nine days of root formation (day 0), on day 2, day 5, day 7, and day 14 from different experiments for different analyses (see colours). Green: Experiment 1–3 for growth data (n=5). Orange: Experiment 3 for osmotic potential (n=5). Blue: Experiment 4&5 for gene expression analysis (n=4). Yellow: Experiment 6 for sorbitol measurement (n=3)

of five vessels per treatment and genotype after seven and 14 days, respectively. After 48 h at 70 °C in an oven, shoot and root dry mass were recorded. Samples were kept at RT in tubes over silica beads until further use. For gene expression analysis, about 100 mg of fresh shoot material from four vessels (=replicates) per treatment and genotype was collected, blotted dry with sterile paper and immediately frozen in liquid nitrogen. For sorbitol measurement, shoots were washed in deionised water to remove sorbitol, which might be adhering in condensation water on the shoot surface. 100 mg were collected, blotted dry, frozen in liquid nitrogen and stored at -80 °C until further use.

Gene expression analyses

Gene expression was performed as described in Wellpott et al. (2023) with minor changes:

RNA isolation and cDNA synthesis

Frozen shoot material from four vessels (corresponds to four biological replicates) per treatment (control, C; osmotic stress, S) and genotype ('Eurobravo', 'Eurostarch', 'Maxi', and 'Tomba') was ground in a mixer mill at 25 Hz for 2 min (MM400, Retsch, Haan, Germany). RNA was extracted by following the manufacturer's instructions for the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany) using the DCT lysis buffer. Genomic DNA was removed with DNase I according to the manufacturer´s instructions (Thermo Scientific, Waltham, MA, USA). The integrity of RNA was examined in a 1% (w/v) agarose gel before cDNA

was synthesised by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with the oligo-dT primer and 1 µg RNA as a template. The cDNA was diluted 1:10 (v/v) and stored at -20 °C.

Primer selection

Eight candidate genes were selected based on proteins found differentially abundant in different genotypes under drought stress based on a previous rain-out shelter experiment (Wellpott et al. 2021). Primers were designed upon the following criteria: 18–24 bp length, GC content 40–60%, 80–250 bp amplification product, melting temperature 60 °C (for sequence information see Table S3). The primers were tested for specificity with BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov) aligning it to the *Solanum tuberosum* subsp. *tuberosum* genome (NCBI: txid4113). Sequence information was obtained from Spud DB using *Solanum tuberosum* group Phureja Dm1-3 v6.1. Primers were tested in a standard PCR with cDNA of 'Eurostarch', with $T_A = 60$ °C on a 1.5% (w/v) agarose gel. Amplification products were sequenced by Sanger sequencing (Sanger et al. 1977).

RT-qPCR

RT-qPCR was performed by Applied Biosystems QuantStudio 6 Flex System (Thermo Fisher Scientific, Waltham, MA, USA). All primers were tested with a pool of all cDNAs for efficiency. Primer efficiencies were calculated with the software QuantStudio™ Real-Time PCR Software v1.3.

EF1a (elongation factor α), *APRT* (adeninphosphoribosyltranferase), and *Cyclo* (cyclophilin) served as reference genes (Nicot et al. 2005). After a test for stability in RStudio (2022.07.1 Build 554) based on R version 4.1.3 using the NormFinder algorithm (Andersen et al. 2004) *EF1a* was excluded from calculations of the normalised gene expression because of a stability value > 0.25. Four biological and three technical replicates were measured for experiments 4 and 5 on day 0 and day 7, respectively. Diluted cDNA of 96 samples was mixed with Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) diluted 1:4 (v/v) for analysis with every primer pair (final concentration in reaction: 0.2 µM). Following PCR conditions were used: one cycle at 95 °C for 60 s, 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Hereafter, melting curve analysis (60 °C to 95 °C with an increment of 0.5 °C/15 s) was conducted to determine specificity of amplification. Data were further processed with QuantStudio™ Real-Time PCR Software v1.3. Data are shown as normalised gene expression (Pfaffl 2001).

Sorbitol measurement

The extraction of sorbitol from plant material was performed acccording to Salem et al. (2016) with minor modifications. In detail, approximately 100 mg plant material was weighed into a 2 ml safe-lock centrifuge-vial and frozen in liquid nitrogen together with five 5 mm steel beads. The exact sample weight was noted (Table S4) and used for calculating analyte concentrations. The tissue was disrupted using a MM 400 beadmill (Retsch, Haan, Germany) at 30 Hz for 3 min. A mixture of precooled methyl tert-butyl ether (MTBE) and methanol (3:1, v:v; 1 ml per sample) was added and the disruption step was repeated. Samples were incubated on a tube rotator (20 rpm) for 15 min at 4 °C and subsequently sonicated in an ice-cooled sonication bath for 15 min. The samples were centrifuged for 10 min at 4 °C and 10,000 × g. Subsequently, 800 µl of the supernatant was transferred to a new reaction tube. A mixture of water and methanol (3:1, v:v; 800 µl per sample) was added and mixed by vortexing. The samples were centrifuged for 10 min at 4 °C and 10,000 × g and the lower phase was collected in a new reaction tube. Samples were dried in a vacuum concentrator until no liquid was left and reconstituted in mobile phase A (0.1% formic acid in water) prior to LC–MS analysis.

The method for the chromatographic separation of sorbitol was inspired by a protocol from Antonio et al. (2007) also using a porous graphitic carbon column for the analysis of sugars. An Agilent 1290 Infinity II LC System coupled with an Agilent 6460 triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) was used. Chromatographic separations employed a 50 × 4.6 mm Hypercarb column with 5 µm particle size (Thermo scientific, Waltham, MA, USA).

The column was operated at a flowrate of 0.2 ml min^{-1} and a temperature of 30 °C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient given in Table 1 was employed.

The injection volume was 5 µl and analysis was carried out in negative mode employing the multiple-reaction-monitoring (MRM) mode. Transitions (precursor ions and product ions) as well as collision energies, fragmentor energies and retention time were as presented in Table 2.

The in-source parameters were: gas temperature 150 °C, gas flow 11 l min^{-1}, nebulizer pressure 40 psi, sheath gas temperature 300 °C, sheath gas flow 11 l min^{-1}, capillary voltage 2,000 V, and nozzle voltage 2,000 V. The analyte was eluted in a single peak with a full width at half maximum (FWHM) between 0.2 and 0.4 and a signal to noise ratio (SNR) over 500. Pure D-sorbitol (Sigma Aldrich, St. Louis, MO, USA) as a standard eluted with the same retention time as the analyte from plants. A similar ratio of product ion abundances was observed for the pure standard and the analyte in matrix. Different concentrations of the standard dissolved in water were used for external calibration (tentative absolute quantification). The signal obtained for the quantifier product ion was converted to a concentration with the help of a standard calibration curve. The concentration was normalised to the measured weight of the respective sample. Measured values are shown in Table S4.

Osmotic potential

The measurement of the liquid medium's osmotic potential took place after root formation (day 0), after the first sorbitol addition (day 2), after the second sorbitol addition (day 4), and on both days on which the evaluation took place (day 7 and day 14) for samples from both, control and stress treatments. Medium was analysed by vapor pressure osmometry (VAPRO 5600; Wescor, Logan, UT). Distilled water and medium without plants were measured in addition. Measurements were carried out against three osmolality standards (Opti-Mole 100 mmol kg^{-1}, 290 mmol kg^{-1}, and 1000 mmol kg^{-1}). Three

Table 1 Gradient for chromatographic separation of sorbitol

Time (min)	Mobile phase A (%)	Mobile phase B (%)
5.00	92	8
7.00	75	25
10.00	75	25
12.00	50	50
16.00	50	50
18.00	92	8
28.00	92	8

Table 2 Transitions (precursor ion and product ion), fragmentor, collision energy, and retention time

Analyte		precursor ion [M-H]- (m/z)	production	fragmentor	collision energy (V)	retention time (min)
Sorbitol	Quantifier	181.1	71	127	21	3.96
	Qualifier	181.1	89	127	5	3.96

biological replicates and two to three technical replicates were measured. Osmolality was transformed into osmotic potential (Bündig et al. 2016a).

Proline analysis

Proline analysis was performed according to (Bates et al. 1973). To 25 mg of dried and ground shoot material, a total of 1.8 ml of sulphosalicylic acid (3%) was added in two steps (2 × 900 µl). The samples were incubated on ice for 30 min, mixed, and centrifuged at 16,162 xg for 15 min. The supernatant (150 µl) was transferred into new tubes per sample and 90 µl glacial acid and 90 µl ninhydrin reagent were added and mixed. The samples were placed in boiling water for 45 min before they were cooled down on ice. After the addition of 1.5 ml toluene, three technical replicates containing 200 µl of the toluene phase were put on a microtiter plate. Absorption was measured at 520 nm. Toluene served as a blank. For each sample, five biological replicates were measured.

Statistical analysis

Illustration of data and statistical analysis were performed in R version 4.1.3 (R Core Team 2022) using RStudio v. 2022.07.1 Build 554 (RStudio Team 2022). Packages used for figures included 'ggplot2' (Wickham 2016), 'ggpubr' (Kassambara 2020), 'ggsci' (Xiao 2018) 'cowplot' (Wilke 2020), and 'Rcolorbrewer' (Neuwirth 2014). An analysis of variance (ANOVA) was calculated to assess treatment and genotype effects and interactions. Means were compared pairwise by Tukey's test ($p < 0.05$). When normal distribution was not given, data were log transformed or analysed by a Kruskal–Wallis-Test with Bonferroni adjustment. Packages used for statistics were 'agricolae' (Mendiburu 2021), 'emmeans' (Lenth 2022), and 'multcomp' (Hothorn et al. 2008).

Results

Osmotic potential of the liquid medium

The measured osmotic potential of the liquid medium was -0.5 MPa in the liquid MS medium without plants and did not differ significantly when plants had been cultured in it for two, four, seven, and 14 days as shown for 'Eurobravo' and 'Eurostarch' (control treatment). In the stress treatment of all genotypes, the osmotic potential decreased gradually to -1.5 MPa in MS medium with 0.3 M sorbitol. At day 14, there was a significantly lower osmotic potential than on day seven observed for 'Eurobravo' and 'Tomba' (Fig. 3).

Shoot dry mass decreased in all genotypes after 14 days of osmotic stress

After seven days of osmotic stress in liquid medium, the plants showed decreased shoot length in all genotypes and overall more roots in the three genotypes 'Eurobravo', 'Eurostarch', and 'Maxi'. After 14 days of stress, these changes became more pronounced (Fig. 4 and 5).

After seven days of osmotic stress, shoot dry mass of the genotype 'Tomba' was significantly lower in experiment 2 when compared to the other three genotypes, while the other three genotypes performed similar under stress (Fig. 6a-c). The same was observed for the root mass of 'Tomba' in experiment 2 and 3. However, it is noteworthy, that for the genotype 'Tomba' the root development was also reduced under control conditions (Fig. 4, 5, 6 and 7). Root dry masses were significantly higher after osmotic stress than in the control in experiment 1 and 3 for the genotypes 'Eurostarch' (experiment 1: 53%, experiment 3: 28%), and 'Maxi' (212% and 33.6%, respectively).

After 14 days, the plants displayed significantly lower shoot dry mass under osmotic stress only in genotype 'Eurobravo' in experiment 1 and in 'Eurostarch' in experiment 3. In contrast, in experiment 2, all genotypes showed lower shoot mass under stress compared to the control conditions. 'Eurobravo' showed the greatest decrease among all genotypes (56.9%), followed by 'Tomba' (54.0%) and 'Eurostarch' (41.5%), whereas 'Maxi' showed the smallest decrease in shoot mass (34.7%). The root mass difference between control and stressed treatment was only significant for 'Eurobravo' (44.6%) in experiment 2 (Fig. 7), whereas in all other comparisons no pronounced differences in root dry mass were observed.
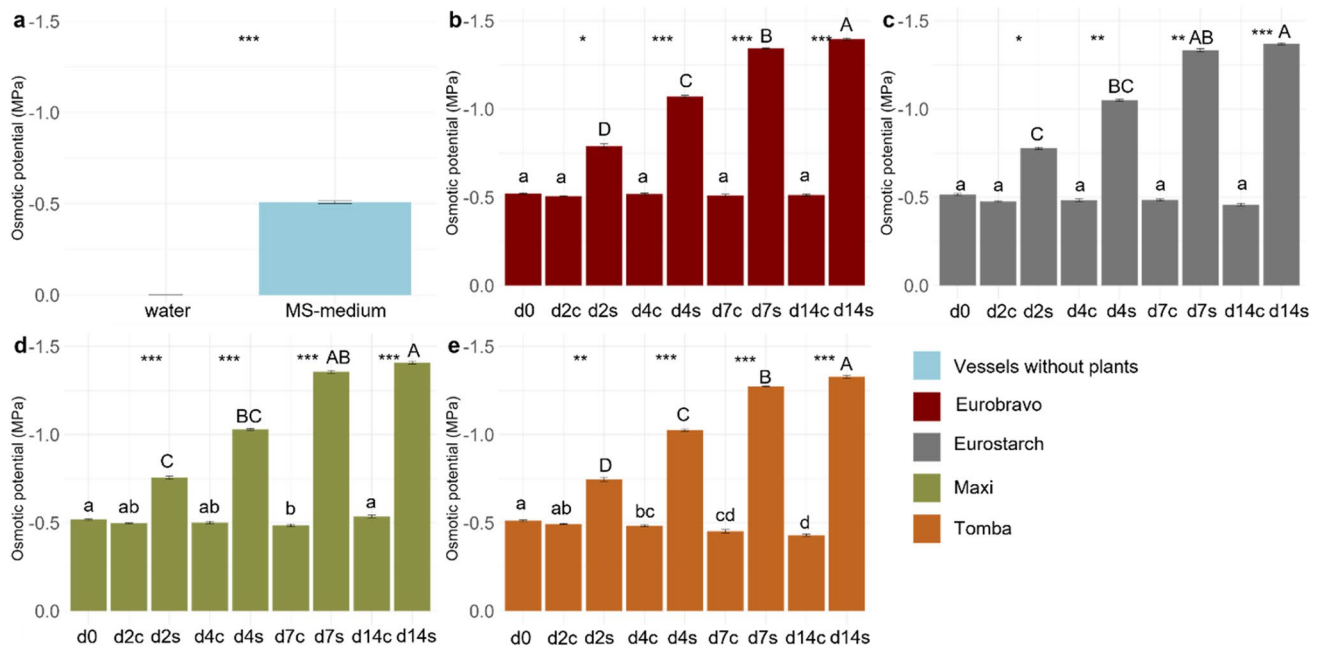
Fig. 3 Osmotic potential in MPa of liquid MS medium with or without sorbitol on day 0, 2, 4, 7, and 14. The osmotic vapor pressure was converted to osmotic potential (MPa). Sorbitol content increased from day 2 (0.1 M) over day 4 (0.2 M) to day 7 and 14 (0.3 M). a = H_2O: water. MS medium: liquid MS medium without plants. b = 'Eurobravo' on day 0 (d0), day 2 control (d2c) and under stress (d2s), day 4 control (d4c) and stress (d4s), day 7 control (d7c) and stress (d7s), and day 14 control (d14c) and stress (d14s). c = 'Eurostarch'. d = 'Maxi'. e = 'Tomba'. Lower case letters compare control treatments between the days, whereas upper case letters compare stress treatments between days using Kruskal–Wallis Test with Bonferroni correction. Asterisks compare control and stress treatment within one day. Significance codes after Kruskal–Wallis test: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Given are means and standard deviations of n = 3 replicates

The root/shoot ratio based on the dry mass (DM) was similar under control and stress conditions in all experiments after seven days (Fig. S1). After 14 days the ratio under osmotic stress was higher compared to the ratio under control conditions in 'Eurobravo' in experiment 1 (0.20 ± 0.01/0.27 ± 0.03), 'Maxi' in experiment 2 (0.19 ± 0.04/0.30 ± 0.03), and 'Eurostarch' (0.23 ± 0.0/0.32 ± 0.05) in experiment 3 (data shown at https://doi.org/10.25835/u5gj5bdx).

Gene expression

Two independent experiments (experiment 4 and 5), in which plants were treated with osmotic stress for seven days, were conducted to show early responses of the plants to osmotic stress by selected novel candidates for drought stress indicator genes from Wellpott et al. (2021). Results for *RPT5a*, *POD*, and *SBT1.7* are shown separately for each experiment (Table 3), whereas results of the statistical analyses allowed the presentation of combined data from both experiments for *Glyx*, *ZBD*, *INH1*, *SMHT*, and *13-LOX* (Table 4 a, b).

Expression of *RPT5a* was not regulated significantly after seven days of stress. *Glyx* showed downregulation in all genotypes. The expression of this gene showed high variations between vessels for the genotypes 'Eurobravo' and 'Maxi', nevertheless, leading to a significant alteration in 'Eurostarch' with a fold change (FC stress/control) of 0.39 and 'Tomba' with a fold change of 0.42.

Expression of *SBT1.7* was lower in genotype 'Tomba' than in the other genotypes on day 0 (Table 3 a). After seven days of osmotic stress, *SBT1.7* was downregulated in all genotypes in experiment 4 (fold changes 'Eurobravo' 0.22, 'Eurostarch' 0.27, 'Maxi' 0.32, 'Tomba' 0.33), as well as in 'Eurostarch' (0.03) and 'Tomba' (0.09) in experiment 5 (Table 3 b).

POD expression was similar in all genotypes on day 0 (Table 3 a). The gene was significantly lower expressed after seven days of osmotic stress in 'Eurobravo' (FC experiment 4: 0.08 and experiment 5: 0.03), 'Eurostarch' (FC 0.09 and 0.03), and 'Tomba' (FC 0.16 and 0.01). Gene expression was also reduced in 'Maxi', however, this was not statistically significant (Table 3 b).

Fig. 4 Morphology of potato plants after 7 days of culture under control or osmotic stress conditions. Control = medium with addition of water, Stress = medium with addition of sorbitol up to an end concentration of 0.3 M sorbitol. Day 7 = seven days after first addition of sorbitol, Side view of all plants of a representative culture vessel from experiment 3

The expression of *SHMT* differed between the genotypes on day 0 (Table 4 a). 'Tomba' (3.490 ± 0.902) showed a significantly higher expression than 'Eurobravo' (1.871 ± 0.419), 'Eurostarch' (2.163 ± 0.570), and 'Maxi' (2.460 ± 0.453) (Table 4 b). All genotypes showed downregulation of *SHMT* after seven days of osmotic stress. 'Tomba' (FC 0.51) displayed the highest fold change, followed by 'Maxi' (FC 0.38), 'Eurobravo' (FC 0.34) and 'Eurostarch' (FC 0.34) (Table 4 b). 'Tomba' (fold change 0.61) and 'Maxi' (0.42) also displayed a downregulation of *13-LOX*. Downregulation was significant in all genotypes for *ZBD* with fold changes of 0.56 ('Eurobravo'), 0.60 ('Eurostarch'), 0.66 ('Maxi'), and 0.54 ('Tomba').

INH1 was the only analysed gene to show upregulation after osmotic stress (Table 4 a). The gene was expressed similarly in all genotypes on day 0 and displayed a significant upregulation in 'Eurostarch' (3.44) and 'Tomba' (2.42) after seven days of stress (Table 4 b).

Proline accumulated in stressed shoots

Proline was analysed after seven and 14 days under osmotic stress in 'Eurobravo', 'Eurostarch', 'Maxi', and 'Tomba' in the shoot dry mass. The results showed an increase of proline in all genotypes under osmotic stress after seven and 14 days, except for 'Eurostarch' and 'Maxi' in experiment 3 after seven days and 'Tomba' in experiment 1 after 14 days. After seven days, the lowest fold change (stress/control) was diplayed by 'Tomba' in experiment 3 (FC 0.58) and the highest increase by 'Eurostarch' in experiment 2 (FC 7.76). After 14 days, 'Tomba' (FC 1.34) showed the lowest increase in experiment 3 and 'Eurostarch' (FC 6.67) the highest in experiment 1 (Fig. 8).

Sorbitol was detected in shoots of plants rooted prior to stress application

Sorbitol was measured in shoot samples of stressed and control plants of genotypes 'Eurostach' and 'Maxi' to

Fig. 5 **Morphology of potato plants after 14 days of culture under control or osmotic stress conditions.** Control = medium with addition of water, Stress = medium with addition of sorbitol up to an end concentration of 0.3 M sorbitol. Day 14 = 14 days after first addition of sorbitol. Side view of all plants of a representative culture vessel from experiment 3

determine whether the osmotic agent was taken up by plants after rooting (Table S2). 'Eurostarch' showed a sorbitol content in shoots of control plants of 3.8 µg/g fresh mass (FM). After 14 days sorbitol content dropped to 0.9 µg/g shoot FM. After seven days of stress treatment with sorbitol, the shoot content rose to 2696.5 µg/g shoot FM and to 939.3 µg/g shoot FM after 14 days. This resulted in fold changes (FC stress/control) of 702 and 1093 for seven and 14 days, respectively. 'Maxi' showed a sorbitol content in shoots of control plants of 1.9 µg/g FM after 7 days and 2.2 µg/g FM after 14 days. They increased to 1211.5 µg/g and 769.3 µg/g in plants treated with sorbitol, resulting in fold changes of 630 and 349 after 7 and 14 days, respectively (Table S2).

Discussion

General response to osmotic stress in vitro

With climate change and severe drought periods in temperate regions, there is growing need for drought tolerant potato genotypes (Haverkort und Verhagen 2008). Osmotic stress arises in vivo as part of e.g. drought and salt stress and can be achieved in vitro by adding an osmotic agent to the culture medium. Literature shows that in vitro screenings are time- and cost-efficient systems for the detection of tolerance in newly bred genotypes (Gopal und Iwama 2007).

Growth reduction is one of the first responses to osmotic stress (Dobránszki et al. 2003). Likewise, in our study, seven
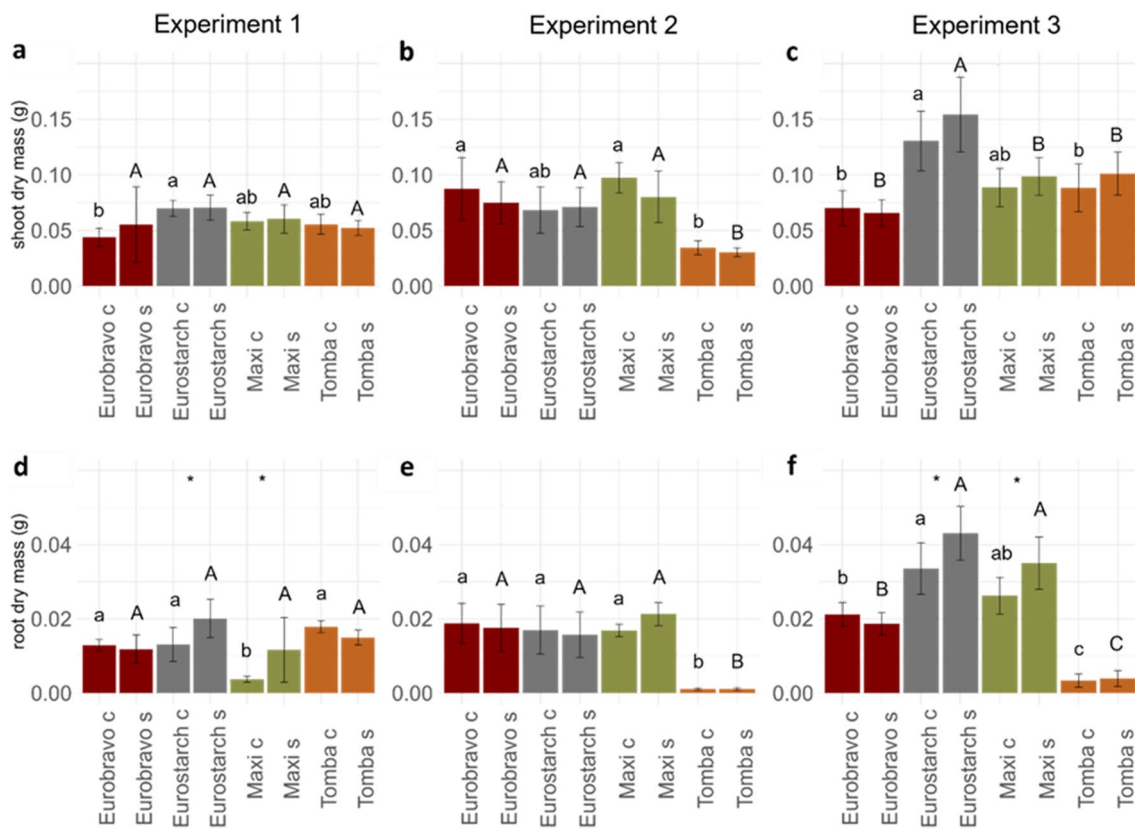
Fig. 6 **Shoot and root dry mass after 7 days of osmotic stress.** a-c: shoot dry mass from experiment 1, 2, 3, d-f: root dry mass from experiment 1, 2, and 3. c: control, s: stress. Lower case letters compare control treatments between the genotypes, whereas upper case letters compare stress treatments between genotypes using Tukey's test (Kruskal–Wallis Test with Bonferroni correction for root DM of experiment 2). Asterisks compare control and stress treatments within one genotype. Significance codes after Tukey's test or Kruskal–Wallis test: *** = p < 0.001; ** = p < 0.01; * = p < 0.05. Given are means and standard deviations of n = 5 replicates

days after the first sorbitol application, the plants showed a visible reduced shoot growth (Fig. 4), which was also detectable in shoot fresh mass in experiment 2 and 3 (Table. S5). Since the difference between control and stressed plants was no longer reflected in the dry mass of the shoots (Fig. 6), it can be assumed that the plants without osmotic stress primarily contained more water. Water loss was higher for all stressed genotypes compared to their control. The decrease of water content in the shoots from control to stressed shoots ranges from 14% ('Maxi') to 36.8% ('Eurostarch') in experiment 1, from 50.8% ('Tomba') to 64.6% ('Maxi') in experiment 2 and from 27.1% ('Tomba') to 54.7% ('Maxi') in experiment 3 (Table S6). At day 14, all genotypes expressed a shoot growth reduction also in their dry mass in experiment 2 (Fig. 7 b). 'Maxi' displayed the highest DM after osmotic stress in our test set. This is in agreement with previous results showing that 'Maxi' better coped with osmotic stress in vitro than the genotype 'Eurobravo' using solidified media (Bündig et al. 2016a). Interestingly, 'Maxi' was also rated rather tolerant to drought stress compared to a test set under greenhouse and rain-out shelter conditions in which

'Eurobravo' was also represented and presented as rather sensitive genotype (Sprenger et al. 2015; Meise et al. 2019). However, decreased shoot mass under osmotic stress after 14 days was only shown for 'Eurobravo' in experiment 1 and 'Eurostarch' in experiment 3. For that reason the osmotic stress intensity should be considered to be further increased in future studies.

However, it is noteworthy that there was a genotype-dependant significant increase from 7 to 14 days of stress in osmotic potential measured in medium from plants of genotype 'Tomba' and 'Eurobravo' despite the fact that no additional sorbitol was added (Fig. 3). This might be explained by exudates, which these genotypes might release into their surrounding medium or a higher transpiration. Further analyses are needed to explain this observation. Overall, root growth was not as severely affected as shoot growth. This reaction of potato to osmotic stress in vitro was also postulated by Dobránszki et al. (2003). Similarity in root mass between stress and control could be due to the previous rooting phase. All plants were able to form roots prior to the stress treatment, which were initially sufficient
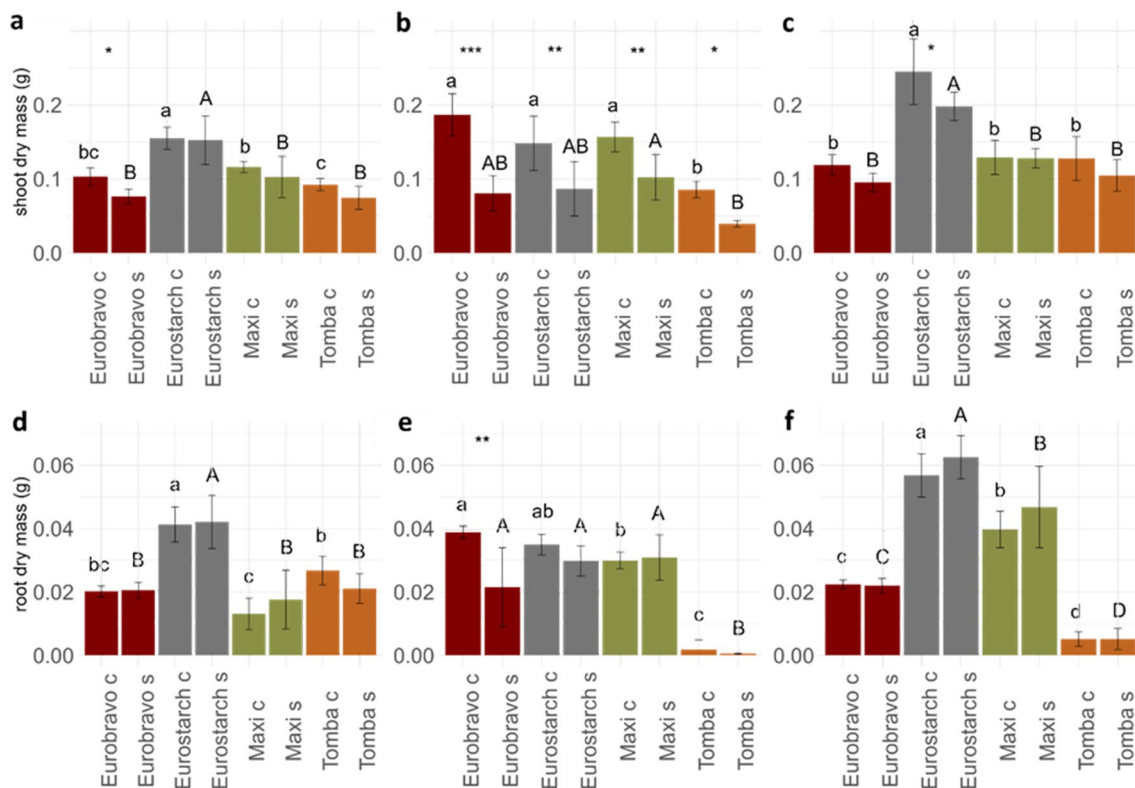
Fig. 7 Shoot and root dry mass after 14 days of osmotic stress. a-c: shoot dry mass from experiment 1, 2, 3, d-f: root dry mass from experiment 1, 2, and 3. c: control, s: stress. Statistical analysis: ANOVA and Tukey's test (Kruskal–Wallis Test with Bonferroni correction for root DM of experiment 2). Lower case letters compare control values between the genotypes. Upper case letters compare stress values between genotypes. Asterisks compare control and stress treatment within one genotype. Significance codes after Tukey's test or Kruskal–Wallis test: ***=p<0.001; **=p<0.01; *=p<0.05. Given are means and SD of n=5 replicates

for them to continue growing. The root/shoot ratio was significantly shifted towards the roots for 'Eurobravo' in experiment 1, in 'Maxi' in experiment 2, and in 'Eurostarch' in experiment 3 (Fig. S1). A shift towards root growth may be a sign for stress tolerance (Bündig et al. 2016a). Adaptation of individual genotypes to osmotic stress by shifting their root to shoot ratio towards the roots more consistently may be recorded at a later stage or with a higher end concentration of osmotic pressure and needs further investigation. The same holds true for the up to now unexplained variability of results when comparing the different experiments. Although conducted under highly controlled in vitro culture conditions and using explants of the same age and size, the genotypes reacted slightly or even strongly ('Tomba') different in the different experiments. One possible source of variation could be endophytic bacteria, which were observed from time to time, especially in 'Tomba'. This might also explain the poor root growth in the genotype 'Tomba' as these were also present in the control variant. Which kind of endophytes might play a role would have to be tested further, for instance through amplicon sequencing. However, the number of subcultures of the explants prior to the installation of the experiments, the inhomogeneity of light on the shelves in the growth chamber and the handling of the explants, e.g. small differences in cutting of the explants, might also lead to these differences.

Abiotic stress like drought stress and osmotic stress lead to an accumulation of proline in the plants by both, activation of proline biosynthesis and inhibition of degradation (Hayat et al. 2012). The amino acid acts as an osmoprotectant, as well as prevents damage caused by reactive oxygen species (ROS), and stabilises DNA, membranes and proteins (Ben Rejeb et al. 2014). In several studies on osmotic stress, proline showed to be higher abundant in the stress treated plant material (Bündig et al. 2016a; Mawia et al. 2020). This correlates with the results presented. However, Schafleitner et al (2007) already indicated, that proline accumulation in potato should not be considered as a tool for the prediction of stress tolerance, but rather as a sign for plant stress. Therefore, with accumulated proline in stressed potato shoots, we can prove the successful application of osmotic stress in vitro for experiment 1 and 2, as well as for 'Eurobravo' and 'Tomba' in experiment 3.

Table 3 Mean values under control conditions (C) or osmotic stress (S) and fold changes (S/C) of normalised expression of *RPT5a*, *POD*, *SBT1.7* in four potato genotypes at the start of the experiment (day 0) and after seven days (day 7)

a

Day	Variant	Genotype	Experiment	RPT5a	POD	SBT1.7
0	Start	Eurobravo	4	0.352 ± 0.049 a	0.034 ± 0.022 a	0.079 ± 0.016 a
			5	0.413 ± 0.029 A	0.023 ± 0.002 A	0.039 ± 0.003 AB
		Eurostarch	4	0.334 ± 0.014 a	0.022 ± 0.011 a	0.080 ± 0.027 a
			5	0.381 ± 0.023 AB	0.023 ± 0.004 A	0.056 ± 0013 A
		Maxi	4	0.369 ± 0.060 a	0.014 ± 0.005 a	0.052 ± 0.007 ab
			5	0.329 ± 0.021 B	0.051 ± 0.005 A	0.057 ± 0.013 A
		Tomba	4	0.423 ± 0.107 a	0.039 ± 0.020 a	0.042 ± 0.005 b
			5	0.376 ± 0.026 AB	0.079 ± 0.047 A	0.030 ± 0.006 B
7	Control	Eurobravo	4	0.308 ± 0.017 a	0.026 ± 0.007 a	0.073 ± 0.018 a
			5	0.299 ± 0.041 A	0.050 ± 0.025 A	0.031 ± 0.015 A
		Eurostarch	4	0.340 ± 0.050 a	0.020 ± 0.017 a	0.067 ± 0.026 a
			5	0.273 ± 0.088 A	0.033 ± 0.017 A	0.039 ± 0.006 A
		Maxi	4	0.379 ± 0.033 a	0.020 ± 0.008 a	0.054 ± 0.015 a
			5	0.222 ± 0.022 A	0.029 ± 0.013 A	0.027 ± 0.006 A
		Tomba	4	0.359 ± 0.045 a	0.037 ± 0.015 a	0.036 ± 0.007 a
			5	0.405 ± 0.035 A	0.038 ± 0.016 A	0.034 ± 0.014 A
	Stress	Eurobravo	4	0.356 ± 0.039 a	0.002 ± 0.001 a	0.016 ± 0.004 a
			5	0.254 ± 0.085 A	0.001 ± 0.001 AB	0.013 ± 0.008 A
		Eurostarch	4	0.376 ± 0.075 a	0.002 ± 0.000 a	0.018 ± 0.004 a
			5	0.311 ± 0.066 A	0.001 ± 0.000 AB	0.008 ± 0.002 A
		Maxi	4	0.406 ± 0.041 a	0.006 ± 0.004 a	0.017 ± 0.004 a
			5	0.316 ± 0.053 A	0.005 ± 0.003 A	0.010 ± 0.003 A
		Tomba	4	0.361 ± 0.020 a	0.006 ± 0.009 a	0.012 ± 0.007 a
			5	0.293 ± 0.045 A	0.001 ± 0.000 B	0.003 ± 0.002 A

b

Genotype	Eurobravo		Eurostarch		Maxi		Tomba	
Experiment	4	5	4	5	4	5	4	5
RPT5a	1.15	0.85	1.11	1.14	1.07	1.42	1.01	0.72
POD	0.08 **	0.03 ***	0.09 *	0.03 **	0.30	0.18	0.16 ***	0.01 ***
SBT1.7	0.22 ***	0.42	0.27 ***	0.21 ***	0.32 **	0.38	0.33 *	0.09 ***

Asterisks display significant differences in mean normalised expression between control and stress treatments, significance codes: *** = p < 0.001; ** = p < 0.01; * = p < 0.05.)

a: mean values. Data are means of 4 biological replicates ± SD and are displayed for experiment 4 & 5 separately. Lower and upper case letters compare values of one treatment between the genotypes within one gene of interest in experiment 4 and 5, respectively. Heat map colours reach from white (lowest value) to grey (highest value) and were calculated for each column, separately. b: Fold changes (stress/control). Significant downregulation is marked by light grey cells (b). Statistical analysis: Kruskal–Wallis test with Bonferroni correction (for all genes day 7 of experiment 5 and *POD* day 7 of experiment 4) or Tukey's test (for remaining comparisons), p < 0.05, n = 4. *RPT5a*: regulatory particle triple-A ATPase 5A, *POD*: Protein peroxidase 51-like, *SBT1.7*: Subtilase family protein

Normalised gene expression indicated osmotic stress responses for all genotypes

Normalised gene expression was analysed seven days after the first sorbitol addition. This time point was chosen based on the visible alteration in growth (Fig. 4 and 6) in order to examine rather early responses to osmotic stress. Early molecular responses can occur even minutes, hours, or days after onset of stress (Kollist et al. 2019). Response to osmotic stress was therefore visible for most GOIs despite no significant alteration in shoot dry mass after seven days.

Plants can induce osmoregulation in vitro (Dobránszki et al. 2003). This was demonstrated by expression analyses of genes linked to osmotic adjustment, like *lipoxygenase* and *subtilisin* (Ueda et al. 2004). Upregulation of *lipoxygenase* was assigned to osmotic stress. Daneshmand et al. (2010)

Table 4 Mean values under control conditions or osmotic stress and fold changes (S/C) of normalised expression of *Glyx*, *ZBD*, *INH1*, *SHMT*, and *13-LOX* of four potato genotypes at the start of the experiment (day 0) and after seven days (day 7)

a

Experiment	Day	Genotype	Variant	*Glyx*	*ZBD*	*INH1*	*SHMT*	*13-LOX*
		Eurobravo		0.365 ± 0.111 a	0.104 ± 0.013 c	0.365 ± 0.093 a	1.871 ± 0.419 b	0.100 ± 0.052 a
	0	Eurostarch	Start	0.349 ± 0.091 a	0.115 ± 0.027 bc	0.422 ± 0.187 a	2.163 ± 0.570 b	0.084 ± 0.055 a
		Maxi		0.426 ± 0.147 a	0.156 ± 0.043 b	0.492 ± 0.419 a	2.460 ± 0.453 b	0.102 ± 0.056 a
		Tomba		0.382 ± 0.090 a	0.234 ± 0.033 a	0.744 ± 0.526 a	3.490 ± 0.902 a	0.104 ± 0.043 a
4&5		Eurobravo		0.282 ± 0.069 b	0.122 ± 0.015 b	0.344 ± 0.182 ab	2.353 ± 0.618 a	0.0510 ± 0.027 b
		Eurostarch	Control	0.389 ± 0.121 ab	0.127 ± 0.050 b	0.190 ± 0.067 b	2.294 ± 0.829 a	0.031 ± 0.017 b
		Maxi		0.453 ± 0.151 a	0.160 ± 0.054 ab	0.343 ± 0.171 ab	3.406 ± 1.071 a	0.112 ± 0.044 a
	7	Tomba		0.310 ± 0.070 ab	0.224 ± 0.059 a	0.442 ± 0.192 a	2.329 ± 0.651 a	0.146 ± 0.053 a
		Eurobravo		0.193 ± 0.092 ab	0.068 ± 0.012 b	0.484 ± 0.101 b	0.795 ± 0.222 a	0.055 ± 0.028 ab
		Eurostarch	Stress	0.152 ± 0.060 b	0.076 ± 0.013 b	0.654 ± 0.135 b	0.786 ± 0.248 a	0.034 ± 0.006 b
		Maxi		0.351 ± 0.197 a	0.105 ± 0.035 ab	0.385 ± 0.158 b	1.282 ± 0.560 a	0.048 ± 0.041 ab
		Tomba		0.132 ± 0.045 b	0.120 ± 0.044 a	1.068 ± 0.391 a	1.186 ± 0.393 a	0.090 ± 0.040 a

b

Genotype	Eurobravo		Eurostarch		Maxi		Tomba	
Experiment				4&5				
Glyx	0.68		0.39	***	0.78		0.42	**
ZBD	0.56	*	0.60	*	0.66	*	0.54	***
INH1	1.41		3.44	***	1.12		2.42	***
SHMT	0.34	***	0.34	***	0.38	***	0.51	**
13-LOX	1.08		1.11		0.42	***	0.61	**

Asterisks display significant differences in mean normalised expression between control and stress treatments (Tukey's test, n = 8, significance codes: *** = p < 0.001; ** = p < 0.01; * = p < 0.05.)

a: mean values. Data are displayed for experiment 4&5 combined,. Letters a-c display significant differences in between a box of four genotypes in one treatment and one gene of interest. Statistical analysis: Tukey's test ($p < 0.05$; n = 8). Heat map colours reach from white (lowest value) to grey (highest value) and were calculated for every column separately. b: Fold changes (stress/control). Significant upregulation is marked by orange, significant downregulation is marked by light grey cells (b). *Glyx*: Lactoylglutathione lyase / glyoxalase I, *ZBD*: Zinc-binding dehydrogenase family protein, *13-LOX*: lipoxygenase, *SHMT*: serine transhydroxymethyltransferase, *INH1*: cell wall / vacuolar inhibitor of fructosidase

showed that NaCl, as well as PEG 6000 provoked osmotic stress leading to increased activity of a lipoxygenase in *Solanum stoloniferum* in vitro. The lipoxygenase cascade in plants is linked to oxylipin biosynthesis, which includes jasmonates that are involved in plant defence mechanisms (Royo et al. 1996; García-Marcos et al. 2013). In our study, *13-LOX* was downregulated in 'Maxi' and 'Tomba', but not in 'Eurobravo' and 'Eurostarch'. There seem to be genotypic differences in early regulation of *13-LOX*. Expression should be investigated by studies including earlier and later sampling time points under increased osmotic stress.

Expression of *ZBD* was downregulated in all genotypes under osmotic stress. The most likely protein for *ZBD* found in Wellpott et al. (2021) was an allyl alcohol dehydrogenase (Soltu.DM.03G015960), which is part of a family that can be linked to plant growth, development, and to adaptation (Jörnvall et al. 2010; Strommer 2011). Allyl alcohol dehydrogenases are known to be NADP$^+$ dependent (Ying et al. 2014), which is a cofactor for e.g. photosynthesis and the Calvin cycle. Downregulation of *ZBD* can therefore point to

reduced photosynthesis rate, which is a common response to abiotic stress (Sharma et al. 2020). It is important to consider that in vitro plants grow mixotrophicly. Mixotrophy describes the ability to use different carbon sources for growth and is characteristic for in vitro growth as light intensity is usually low, sugar is added as a C source in the culture medium, high relative air humidity and a decreased gas exchange through vessel lids are predominant. All this leads to a less active photosystem of in vitro cultured plants (Kozai und Kubota 2001).

SBT1.7, POD, and *SHMT* showed consistent downregulation, whereas *INH1* displayed upregulation in all genotypes after osmotic stress

*SBT1.*7, a subtilase family gene, was downregulated under osmotic stress in vitro. Subtilases are linked to cell growth and development (Schaller et al. 2018), leading to the conclusion, that reduced expression under osmotic stress can

be assigned to reduced cell growth and thus, smaller shoot and root systems.

Another group of proteins, which are linked to osmotic stress, are peroxidases (Csiszár et al. 2012). Peroxidases are involved in detoxification of hydrogen peroxide (H_2O_2), which can be related to oxidative stress (Boguszewska et al. 2010). A gene of the peroxidase family was strongly downregulated in our study in all genotypes. The same gene was strongly downregulated in a study in an open greenhouse (Wellpott et al. 2023). Sprenger et al. (2016) also showed a gene of the peroxidase family (Peroxidase 21, PGSC0003DMG400001774) to be downregulated in potato after drought stress, linking the response of drought and osmotic stress. However, this was not the same peroxidase as found in this study (Peroxidase 51, PGSC0003DMT400038514), pointing to the fact, that not all peroxidases were addressed under osmotic or drought stress. A consequence of oxidative stress is ROS production and thereafter damage of the plant cells. A strategy to overcome this damage and protect the plants is the detoxification of ROS. *SHMT* is involved in this response (Hourton-Cabassa et al. 1998; Ambard-Bretteville et al. 2003). Gene expression of *SHMT* was significantly reduced in all genotypes under osmotic stress. This, paired with the higher abundant associated enzyme observed under drought stress in Wellpott et al. (2021), may point to a more rapid response of *SHMT*. This should be investigated further by analysing earlier time points after stress onset and by linking this to ROS production.

Genotype-specific responses were recorded for *Glyx,* a protein of the glyoxalase system (Kaur et al. 2014; Hoque et al. 2016) which was downregulated and *INH1*, an invertase inhibitor, which was upregulated in 'Eurostarch' and 'Tomba'. *Glyx* is known to detoxify methylglyoxal, which is potentially cytotoxic (Upadhyaya et al. 2011). A downregulation could have happened after a quick upregulation early after stress onset. This was identified for e.g. salt stress application to *Arabidopsis thaliana* and *Marchantia polymorpha* on transcriptomic level where expression clusters were identified (Wu et al. 2021). This has to be clarified in future studies with further sampling dates. High variation was identified in gene expression between vessels of the genotypes 'Eurobravo' and 'Maxi'. Biological replicates in the in vitro culture, i.e. explants and vessels, display the still unexplained physiological variation of individual plants or variation in physical conditions of vessels, even under the relatively well-controlled culture conditions. Meanwhile, *INH1* was also found to be regulated after drought stress treatment in potato in a field study in Chile (Aliche et al. 2022). In contrast to our study, *INH1* was downregulated there, and linked to reduced growth. *INH1* plays a role in drought stress-mediated stomatal closure in vivo (Kulik et al. 2011; Matsuoka et al. 2021) and the primary metabolism by

hydrolysis of sucrose into glucose and fructose (Ruan et al. 2010). In vitro, the significance of stomatal closure is not as pronounced as in vivo, because of the mixotrophic growth. A test set including diverse genotypes regarding drought and osmotic stress tolerance and knock-down mutants of these genes would be helpful to understand the role of these genes in osmotic stress response.

Through the demonstrated regulation of genes, that are postulated to be linked to drought stress tolerance (Wellpott et al. 2021) as well as a general stress response of potato to abiotic stress (*13-LOX*, *SBT1.7*, *POD*, *ZBD*, and *SHMT*), we were able to show that the potato plants of this study indicated osmotic stress before it was measurable in the shoot dry mass. *Glyx* and *INH1*, reacted genotype-specific in our test setup, which therefore might be interesting GOIs for the identification of biomarkers for osmotic stress tolerance. Further studies under osmotic, drought and other abiotic stressors should be conducted to show if this is a general or osmotic and drought stress-specific response.

Sorbitol concentration drastically increased in rooted shoots under osmotic stress

Osmotic agents for an in vitro stress test should fulfil several criteria, such as reducing the osmotic potential in the medium, being inert, nontoxic to the plant and plants should not take up the osmotic agent. Several studies reported experiments using mannitol or PEG for osmotic stress in vitro (Gopal and Iwama 2007; Sahoo et al. 2020; Jiroutova et al. 2021; Hanász et al. 2022; Lipavsk und Vreugdenhil 1996). However, in literature it was already shown, that mannitol was taken up and influenced growth (Lipavsk und Vreugdenhil 1996). Also Gopal und Iwama (2007) proposed PEG to limit O_2 movement resulting in O_2 deficiency in roots, and therefore postulated that PEG might not be an ideal osmotic agent.

Being less viscous, sorbitol is an often used osmotic agent to induce osmotic stress in potato in vitro (Gopal und Iwama 2007; Bündig et al. 2016a; Mawia et al. 2020; Sajid und Aftab 2022). Bündig et al. (2016b) stated that nodal cuttings presumably take up sorbitol through cut surfaces of shoots. In our study, plants in the osmotic stress experiments were rooted prior to the stress treatment to avoid an uptake of sorbitol through the wound. Measurement of sorbitol in the shoots of 'Eurostarch' and 'Maxi' displayed much higher concentration of sorbitol in shoots after sorbitol treatment than in control shoots after treatment with water. However, this does not necessarily mean, that sorbitol is taken up from the medium. The related tomato (*Solanum lycopersicum*) is classified as a non-usual sorbitol producer (Pleyerová et al 2022). Schauer et al. (2005) found up to 0.05 μmol g^{-1} FW (corresponds to 9108.5 ng g^{-1} FW) in leaves of tomato and consistently we only
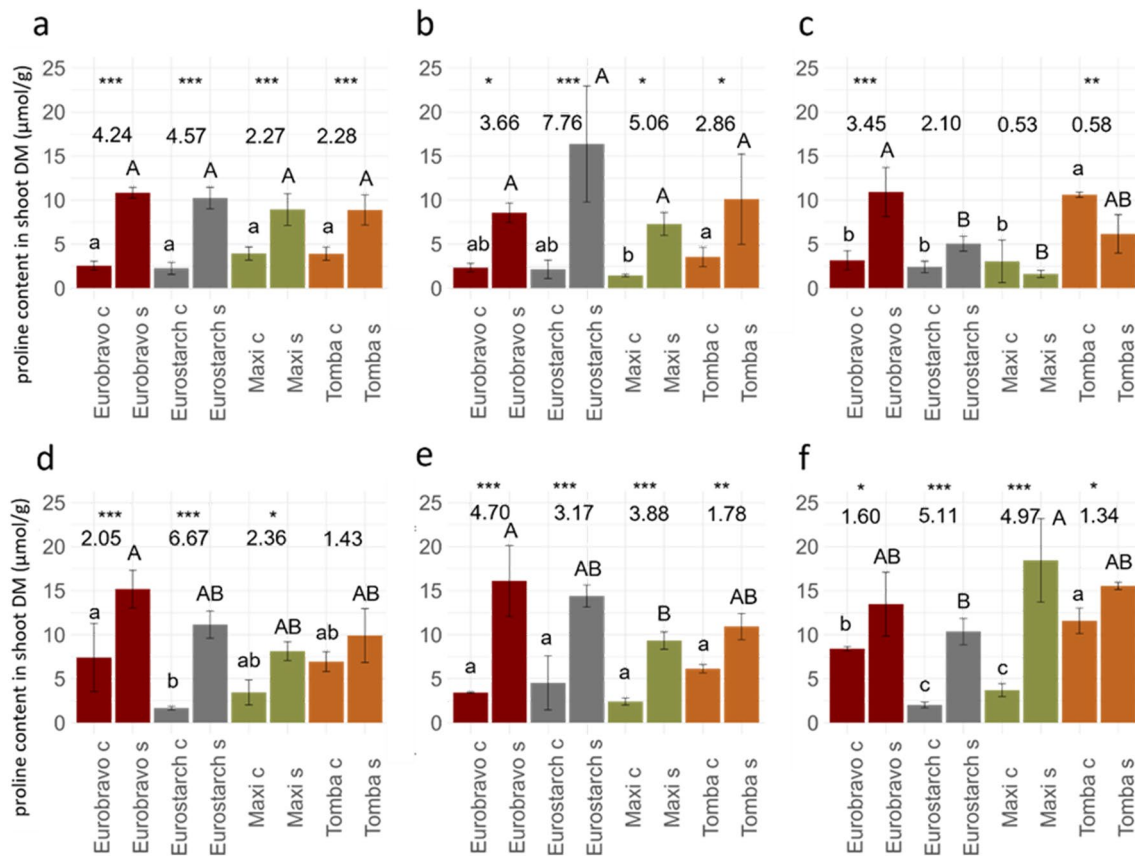
Fig. 8 Proline content of in vitro shoots after seven and 14 days under osmotic stress in vitro. a-c: proline content in shoot dry mass from experiment 1, 2, 3 after seven days, d-f: proline content in shoot dry mass from experiment 1, 2, and 3 after 14 days. Values above bars represent the fold change (stress/control). c: control, s: stress. Statistical analysis: ANOVA and Tukey's test. Lower case let- ters compare control values between the genotypes. Upper case letters compare stress values between genotypes. Asterisks compare control and stress treatment within one genotype. Significance codes after Tukey's test or Kruskal–Wallis test: *** = p < 0.001; ** = p < 0.01; * = p < 0.05. Given are means and SD of n = 5 replicates

detected small amounts of sorbitol in the related species *Solanum tuberosum* in the absence of a sorbitol treatment (up to 3843.5 ng g^{-1} FW). Furthermore, it was shown that drought stress in tomato results in the increased production of sorbitol (Almaghamsi et al. 2021). Since we cannot distinguish the sorbitol supplied by the stress treatment from the endogenously formed sorbitol through this experiment, we cannot exclude that the increase of sorbitol in the shoots upon stress treatment is not the result of its formation in the plant upon sensing drought-like conditions. Nevertheless, the most likely conclusion up to date would be that it was indeed taken up as sorbitol was added to the medium and might easily been taken up as it is water-soluble. However, for the conveyance, sorbitol transporters would be needed as were recently described in e.g. *Malus domestica* (15 SOTs) as a usual sorbitol producer or *Solanum lycopersicum* as a non-usual sorbitol producer (1 SOT) (Li et al. 2022). Yet, these have not been documented for potato. The treatment with isotopically labelled sorbitol would enable us to determine the uptake of sorbitol from the medium

and give a conclusive answer to the possibility of sorbitol uptake by rooted potato plants in vitro.

Conclusion

In this study, we introduce a novel and low-cost test system with liquid medium, in which the plants were allowed to root prior to the stress treatment with sorbitol. In addition to osmotic stress, it is possible to add substances to trigger other abiotic stresses like salt stress or add e.g. inhibitors, at a desired time point. Unlike in solid medium, it is also possible to transfer the plants with roots in the plastic sieves to new medium and continue the test system while the conditions in the medium may change.

Furthermore, finding a suitable osmotic agent is crucial for the induction of osmotic stress with the purpose of classification of potato genotypes according to their tolerance level. In this study, we propose that sorbitol is

probably taken up by plants into the shoot. Whether sorbitol is taken up through the roots, metabolised or stored in the plants remains unclear, and should be further investigated. If however, the osmotic agent is indeed taken up through the roots and can be measured in the shoots, this would indicate that sorbitol has to be replaced in future osmotic stress studies.

In summary, we could show that in vitro plants show morphological responses to osmotic stress in our new liquid test system. In this test system, we were able to apply the osmotic agent gradually, therefore allowing us to mimic the development of drought stress in the field more closely than was described before with solid medium. In addition, the novel described genes for drought stress tolerance displayed alterations in gene expression for the majority of the analysed GOIs. *INH1* and *GLYX* might represent possible candidates for abiotic stress tolerance, namely to osmotic stress.

Author contribution Material preparation and data collection were performed by KW and CB. MH performed and supervised part of the data collection. KW and CB contributed to data analysis. CB and TW conceived and coordinated the project. The first draft of the manuscript was written by KW. The manuscript was revised by MH, CB and TW. All authors have read and approved the final document.

Data availability The data that support the findings of this study are openly available in Research Data Repository of the Leibniz University Hannover at https://doi.org/10.25835/u5gj5bdx.

Declarations

Competing interests All the authors declare that there is no conflict of interest. Traud Winkelmann is one of the Associate Editors of the journal. As such she was fully excluded during all the evaluation period of this work, had no access to its handling during peer-refereeing, and her status had no bearing on the editorial consideration of the manuscript.

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