Agar-based polyethylene glycol (PEG) infusion model for pea (*Pisum sativum* L.) — perspectives of translation to legume crop plants

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Abstract

Due to the oncoming climate changes water deficit represents one of the most important abiotic stressors which dramatically affects crop productivity worldwide. Because of their importance as the principal source of food protein, legumes attract a special interest of plant scientists. Moreover, legumes are involved in symbiotic association with rhizobial bacteria, which is morphologically localized to root nodules. These structures are critical for fixation of atmospheric nitrogen and highly sensitive to drought. Therefore, new drought-tolerant legume cultivars need to be developed to meet the growing food demand. However, this requires a comprehensive knowledge of the molecular mechanisms behind the plant stress response. To access these mechanisms, adequate and reliable drought stress models need to be established. The agar-based polyethylene glycol (PEG) infusion model allows a physiologically relevant reduction of soil water potential (Ψ_w), although it is restricted to seedlings and does not give access to proteomics and metabolomics studies. Earlier, we successfully overcame this limitation and optimized this model for mature Arabidopsis plants. Here we make the next step forward and address its application to one of the major crop legumes — pea. Using a broad panel of physiological and biochemical markers, we comprehensively prove the applicability of this setup to legumes. The patterns of drought-related physiological changes are well-interpretable and generally resemble the stress response of plants grown in soil-based stop-watering models. Thus, the proposed model can be efficiently used in the study of stress-related metabolic adjustment in green parts, roots and root nodules of juvenile and flowering plants.

Keywords: agar-based PEG infusion model, biochemical stress markers, drought, osmotic stress, oxidative stress, physiological stress markers, polyethylene glycol (PEG)

Introduction

The ongoing climate changes and rapid growth of human population are the key factors negatively affecting the global food security (Lesk, Rowhani and Ramankutty, 2016). In this context, as water deficit represents one of the most important abiotic stressors limiting crop productivity (Osmolovskaya et al., 2018), it attracts a special attention of plant scientists and breeders. In general, drought can be defined as a period of below-average precipitation (Verslues et al., 2006), when the amounts of available water in the plant rhizosphere drop below the limits required for efficient

growth and biomass production (Deikman, Petracek and Heard, 2012). The decrease in crop yields, accompanying drought onset, is mediated by multiple deleterious effects of water deficit on different aspects of plant physiology — growth, reproduction, photosynthesis, respiration and uptake of mineral nutrients (Wang et al., 2016; Fahad et al., 2017). Therefore, new cultivars of economically important crops featured with improved drought tolerance are required to meet the growing food demand. However, the development of drought-tolerant crops, among other aspects, requires a comprehensive knowledge of the molecular mechanisms behind the plant stress response and genetic control of the contributing traits at different stages of plant development (Farooq et al., 2009).

As plant response to dehydration is accompanied with the accumulation of osmoprotective metabolites and proteins (Wang et al., 2016), an analysis of alterations in plant metabolome and proteome is absolutely mandatory for deep insight in the underlying mechanisms (Shumilina et al., 2021). Moreover, comprehensive metabolomics and proteomics studies might deliver promising molecular markers, useful in breeding new cultivars with improved drought tolerance and in designing new stress-protective synthetic phytoeffectors (Lamaoui, Jemo, Datla and Bekkaoui, 2018). However, both proteomics and metabolomics experiments require larger sample amounts (Leonova et al., 2020), which needs to be considered when selecting an experimental model.

To date, multiple drought stress models, relying on the reduction of water potential of the substrate (Ψ_w), were proposed (Osmolovskaya et al., 2018). However, reliable establishment and maintenance of well-defined values of the substrate Ψ_w can be quite difficult to achieve. Indeed, in soil-based models, the substrate Ψ_w changes continuously along with water evaporation from its surface and absorption by the plant (Bodner, Nakhforoosh and Kaul, 2015), affecting reproducibility of such setups. In contrast, models based on hydroponic designs allow precise estimation of the substrate Ψ_w. From the point of physical chemistry, this approach is based on the simulation of drought by the establishment of osmotic stress, i. e. increasing osmotic pressure of the medium by supplementation of high-molecular-weight osmolyte polyethylene glycol (PEG) with an average molecular weight of 6000 Da or more (Hohl and Schopfer, 1991; Ji et al., 2014; Sunaina et al., 2016). Nevertheless, PEG-containing nutrient solutions are characterized by high viscosity and can interfere with root morphology and function (Verslues et al., 2006; Chen and Fluhr, 2018). The accompanying partial root dysfunction might impact leaf dehydration and plant stress response. It is especially important for legume crops, the yield of which directly depends on the legume-rhizobial symbiosis localized in the root system. Thereby, changes in the architecture of the root system and the efficiency

of nodule formation represent an important marker of drought response (Khatun et al., 2021).

The agar-based PEG infusion model represents an elegant way to overcome this limitation, being to date the method of choice for osmotic stress experiments with *Arabidopsis thaliana* seedlings (van der Weele et al., 2000). In comparison to other setups, this model has two fundamental advantages. Firstly, it provides a stable and reproducible reduction of the substrate Ψ_w. Secondly, it engages a solid substrate mimicking the conditions of a real solid substrate and minimizing hypoxia. Thus, the agar-based model currently is an ideal choice to address the mechanisms of drought tolerance (Verslues et al., 2006). Previously, we successfully modified this model for adult plants of *Arabidopsis thaliana,* which gave us access to comprehensive metabolomics and proteomics studies (Paudel et al., 2016; Frolov et al., 2017). Here, we address its applicability to crop legume (pea, *Pisum sativum* L.) plants, which represent one of the major sources of protein-rich foods.

Materials and methods

Reagents, plant material and rhizobial culture

Unless stated otherwise, the following materials were obtained from Carl Roth GmbH and Co (Karlsruhe, Germany): agar-agar Kobe I, 2-(N-morpholino) ethanesulfonic acid, MES (p. a.), polyethylene glycol (PEG) 8000 (p. a.). All other chemicals were purchased from Merck KGaA (Darmstadt, Germany). Water was purified in house on a water conditioning and purification system Millipore Milli-Q Gradient A10 system (resistance 18 mΩ/cm, Merck Millipore, Darmstadt, Germany).

Pea seeds of the cultivar SGE and rhizobial culture (*Rizobium leguminosarum* bv. *viciae* CIAM 1026) were provided by Dr Vladimir Zhukov (the All-Russia Research Institute for Agricultural Microbiology, St Petersburg, Russia).

Plant experiment

Pea seeds were sterilized for 7 minutes in 33 % (*v/v*) ethanol, stratified in the dark at 4 °C for two days and germinated in the dark at 22 °C for two days. Afterwards, all seeds used in this work were transferred to 1 L pots (two plants per pot) filled with vermiculite and inoculated with rhizobial culture according to a previously published protocol (Leonova et al., 2020). The plants were grown for the following two weeks in a phytotron MLR-351H (SANYO Electric Co., Ltd, Moriguchi, Japan) in a closed plastic box at 16 h light/8 h dark regimen (199 µmol photons m^{-2} s^{-1}) at 22 °C under 75 % relative humidity. Afterwards, following the rinsing of roots with distilled sterilized water, the plants were transferred to the agar medium (0.8 % *w/v* in 200 mL of 6 mmol/L MES
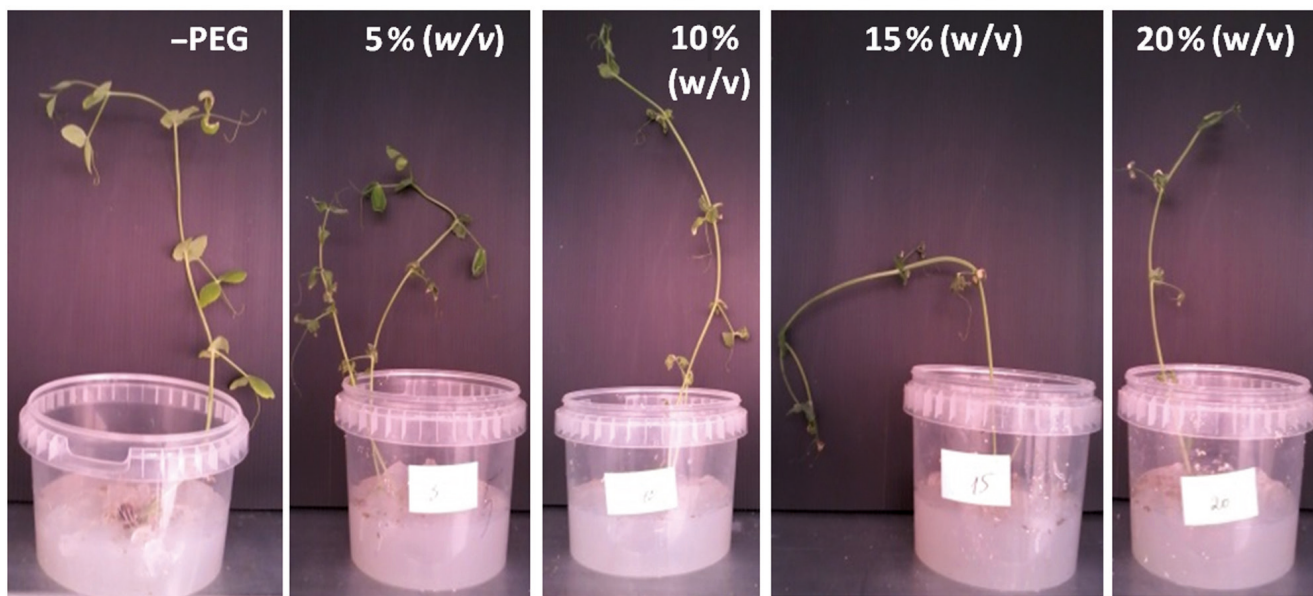
PEG content in the agar medium, % (*w/v*)

Fig. 1. The 19-day-old pea (*P. sativum*) plants grown during three days on 0.8 % (*w/v*) agar medium containing 200 mL of 6 mmol/L MES buffer (pH 5.7) saturated with 5, 10, 15 and 20 % (*w/v*) PEG 8000 solutions in half-strength Murashige and Skoog medium containing 6 mmol/L MES buffer, pH 5.7 (300 mL per pot) in parallel to control plants grown on PEG-free agar medium (–PEG). Prior to the treatment, the plants were germinated in the dark for two days and grown on vermiculite during the following two weeks in a closed plastic box at 16 h light / 8 h dark regimen (199 μmol photons m^{-2} s^{-1}) at 22 °C under 75 % relative humidity.

buffer, pH 5.7) filled in polypropylene pots (Combiness Europe, Nazareth, Belgium) and saturated for three days with 5, 10, 15, 20 % (*w/v*) PEG 8000 solutions (n = 6 per treatment group, one plant per pot) in half-strength Murashige and Skoog medium prepared in 6 mmol/L MES buffer, pH 5.7 (300 mL per pot) (Frolov et al., 2017). Thereby, the final agar concentration did not change. When the saturation was completed, the overlay solution was discarded, and the plants were transferred on agar, in approximately 1-cm-deep wells made by scalpel. Thus, the roots were placed in the body of the agar layer, but opened, i. e., in well-aerated conditions. The control plants were transferred to the pots filled with the agar saturated with a PEG-free medium and grown for the same time as the corresponding experimental groups. On the third day after stress application physiological parameters were assessed, and the plants were harvested for the analysis of biochemical stress markers (see below). For this, the shoots were frozen in liquid nitrogen and ground in a Mixer Mill MM 400 ball mill with a 20 mm stainless steel ball (Retsch, Haan, Germany) at a vibration frequency of 30 Hz for 1 min. The ground material was stored at –80 °C until further biochemical assays.

Physiological assays

Stomatal conductivity was assessed with a portable porometer (AP4, Delta-T Devices Ltd, Cottbus, Germany) as described by Leonova et al., 2020. Photosys-

tem II (PS II) efficiency (variable fluorescence (Fv) / maximal fluorescence (Fm)) was determined by means of a MINI-PAM-IIB fluorometer (Heinz Walz GmbH, Effeltrich, Germany) as described by Paudel et al., 2016. The measurements of relative chlorophyll contents relied on a diffusion chlorophyll meter SPAD-502 (Konica Minolta, Langenhagen, Germany), whereas the leaf relative water contents (LRWC) were calculated based on the difference of fresh and dry weight (3 d at 80 °C) using the equation: LRWC (%)=(fresh weight–dry weight) x100 %/fresh weight (Paudel et al., 2016).

Biochemical assays

Lipid peroxidation products were determined as malondialdehyde (MDA) equivalents by the thiobarbituric acid (TBA) method (Velikova, Yordanov and Edreva, 2000), whereas total ascorbate, ascorbic acid and dehydroascorbate contents were assessed by the ascorbate oxidase method, as described in detail by Paudel et al., 2016 (Paudel et al., 2016). The determination of leaf abscisic acid (ABA), jasmonic acid (JA) and its derivatives relied on the procedure of Balcke et al. The sample preparation relied on solid phase extraction (SPE) on strong cation exchange polystyrene/divinylbenzene (PS/DVB) Cromabond® HR-XC cartridges (Macherey-Nagel, Düren, Germany). The following analysis was performed with ACQUITY H-Class UPLC ultrahigh performance liquid chromatography system (Waters GmbH, Eschborn, Germany) coupled online to a QTRAP 6500 (AB

Sciex, Darmstadt, Germany) triple quadrupole-linear ion trap instrument operating in negative multiple reaction monitoring (MRM) mode (Balcke et al., 2012).

Statistical analysis

Due to the small number of technical replicates of each sample, we used the nonparametric Mann — Whitney U-test to identify statistically significant differences between the two groups (control and experimental plants).

Results and discussion

Due to its convenience, high throughput, reliability, physiological relevance and robustness, the agar-based PEG infusion model became a versatile tool in plant drought stress research (van der Weele et al., 2000). Originally, this model relied exclusively on seedlings as an object and on stress-related alterations in growth of their roots as the principal output (Verslues et al., 2006). The small size of seedlings did not allow this setup to yield sufficient amount of plant material for a comprehensive study of metabolic shifts behind the observed physiological effects. In the best case scenario, only targeted methods of biochemical analysis, i. e. determination of principal oxidative stress markers (tissue contents of pro- and antioxidants, as well as the activities of key antioxidant enzymes) or basic metabolomics analyses could be accomplished upon pooling multiple seedlings (Wang et al., 2009). However, comprehensive metabolite profiling requires multiple orthogonal and/or complementary separation techniques, and proteomics analyses (especially those employing gel-based methods) require much higher sample amounts of up to 1 g (Min et al., 2015; Leonova et al., 2020). Obviously, pooling the appropriate number of seedlings would make biological variability of stress response and statistical confidence of the observed metabolic shifts difficult to address.

Therefore, we have recently proposed an extension of the agar-based PEG infusion model, employing mature Arabidopsis plants, and demonstrated its applicability for a post-genomic analysis of plant metabolism (Frolov et al., 2017). Here, we are making the next step forward and propose a translation of this approach to crop plants. Thereby, we are focusing on legumes — economically important crops which represent the main source of cheap food protein available all over the world. It is important to note that high protein contents of legume seeds are to a large extent attributable to their ability to form symbiotic association with nitrogen fixing rhizobial bacteria (Maphosa and Jideani, 2017; Dellagi, Quillere and Hirel, 2020). This symbiosis is sensitive to water stress, and its efficiency is dramatically reduced under drought conditions (Kibido et al., 2020). As the agar-based PEG infusion model allows precise defining of the substrate Ψ_w, it repre-

sents a perfect tool for dissecting the metabolic alterations in root nodules associated with osmotic stress, especially in combination with the determination of leaf (Paudel et al., 2016) and nodule (Larrainzar et al., 2009) water potential. Therefore, in this study, we have chosen pea as a representative of this group, as the experience with modeling drought for this plant is already available in our lab (Leonova et al., 2020; Shumilina et al., 2021), and the results of this work can be directly used in legume-rhizobial symbiosis research. Based on the available literature data (Ceylan, Türkan and Sekmen, 2013; Jiang et al., 2013) we employed 5 % (w/v) PEG 8000 in the overlay solution as the lowest stress dose and 20 % (w/v) as the highest tolerated amount of PEG in growth medium.

Plant growing and model establishment

The seeds of the cultivar SGE demonstrated quantitative and concerted germination which indicated their good quality (Smolikova, 2014). Such concerted character of plant development could be followed in further steps of ontogenesis and was seen at the moment of transfer to the agar medium. This observation was in agreement with our previous experience with this cultivar (Leonova et al., 2020; Shumilina et al., 2021), and confirmed the SGE line to be the perfect choice for the study of drought response in pea. Indeed, the size and general vigor of individual plants were similar at the step of stress application. This might positively affect the reproducibility of the setup and reduce intra-group dispersion. In this first proof-of-concept study with a broad range of applied PEG concentrations in agar overlay solutions we decided for a relatively short treatment time period (three days) to ensure survival of the plants in presence of higher PEG dosages. This was the case in our previous study employing liquid PEG-containing media (Shumilina et al., 2021), and could not be excluded for the agar-based approach as well.

As can be seen from Fig. 1, the effect of dehydration was clearly different in the treatment groups. Thus, similarly to the observations done with our aqueous PEG-based model (Shumilina et al., 2021), supplementation of 5 % (w/v) resulted in symptoms of low-severity stress (minimal loss of turgor), whereas 10 % (w/v) PEG triggered moderate drought, characterized with pronounced turgor loss and chlorosis. Application of higher dosages of the stressor (15 and 20 % PEG (w/v)) resulted in pronounced dehydration of plant tissues and the development of severe stress symptoms.

The most critical step in the establishment of an agar-based PEG infusion model is transferring juvenile plants from solid substrate (or liquid medium) to the PEG-saturated agar. On the one hand, overlying of the agar gel with PEG might result in its detachment from the bottom and walls of the pot (Frolov et al., 2017). On the other hand, this manipulation often results in con-
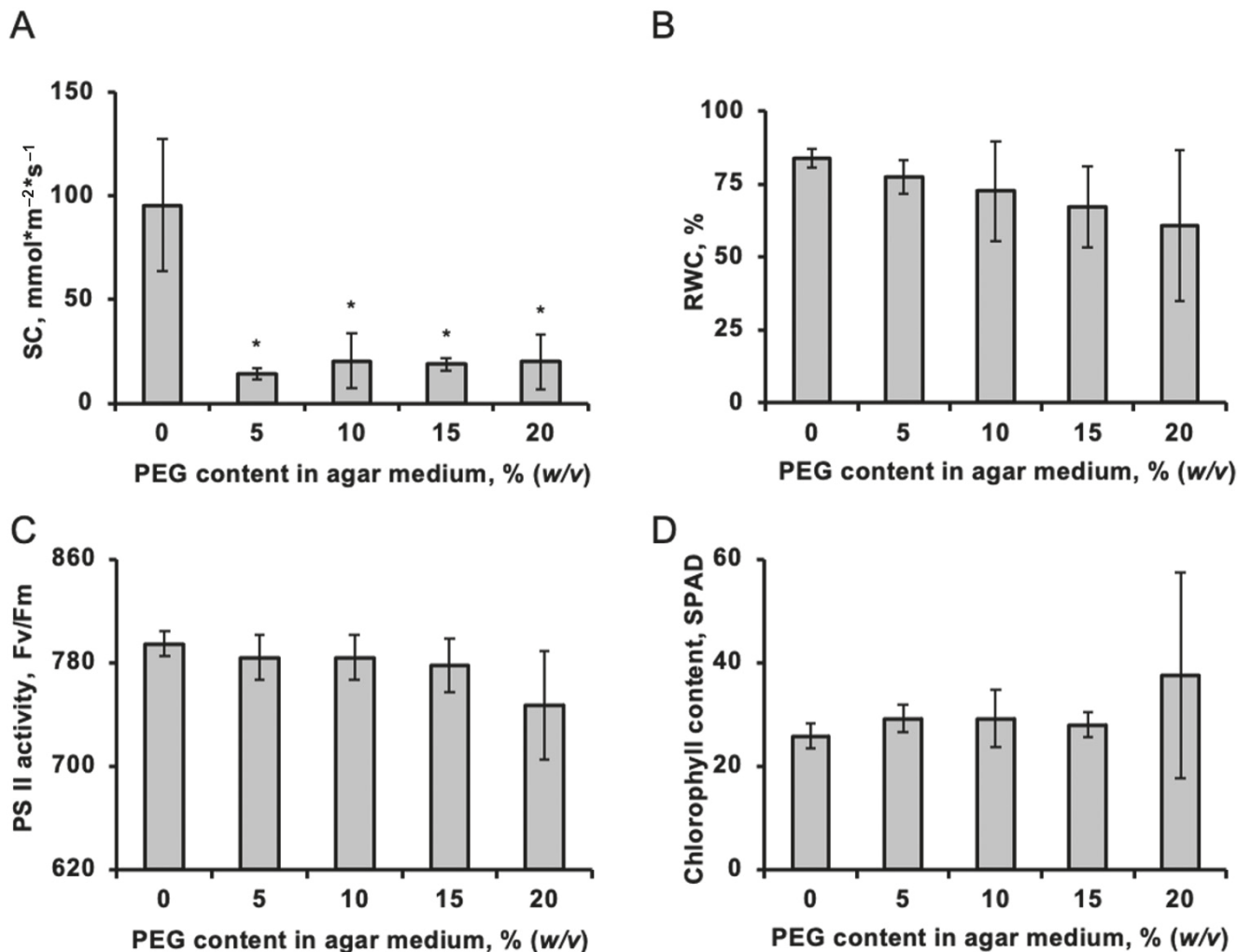
Fig. 2. Characterization of plant stress after a three-day exposure of pea (*Pisum sativum* L.) plants to the 0.8 % (*w/v*) agar medium saturated with 0, 5, 10, 15 and 20 % (*w/v*) PEG 8000 solutions by stomatal conductivity (SC, A), leaf relative water content (RWC, B), photosystem II (PSII) efficiency (C), chlorophyll content (D). * Denotes statistical significance (Mann — Whitney U-test) at the confidence level $p \le 0.05$.

tamination of the agar medium with fungi. When working in the scale of 300-mL tightly closed individual pots with Arabidopsis plants, we succeeded to establish a reliable procedure to avoid contamination. In this study, open pots were placed in one tightly closed sterile plastic box within the phytotron. As this setup seems to be more prone to fungi infection, we carefully observed each pot (both the plant itself and the agar surface) prior to harvesting in order to exclude contaminated plants from the analysis. However, no growth of mycelia was observed after three days of treatment.

Physiological markers of stress response

To probe the severity of osmotic stress, generated by introduction of the osmotically active compound in the agar medium, we assessed the alterations in transpiration, water homeostasis, integrity, and functional activity of photosynthetic apparatus.

Analysis of stomatal conductance revealed an approximately 5-fold drop of this parameter in response to the minimal dosage of the stressor (5 % *w/v* PEG) introduced in the overlay solution (Fig. 2A). Thus, even the lowest stress dosage resulted in a dramatic and significant drop of stomatal conductivity. This is an early reaction, which is triggered even with minimal dehydration (Wan et al., 2009; Yang, Isabel Ordiz, Jaworski and Beachy, 2011). Further increase of the PEG concentrations in the overlay solutions used for saturation of agar did not yield any further significant alteration of stomatal conductance. Similarly, although the average values of the leaf relative water content gradually decreased with the increase of PEG in the overlay solutions, the decrease was not significant (Fig. 2B). The obtained data generally corresponded to the observation done with soil-based setups in legumes (Juzoń et al., 2019), whereas aqueous PEG-based systems yielded a rapid and concerted drop of this parameter to significantly lower values in comparison to the untreated

controls (Leonova et al., 2020). This clearly indicates higher physiological relevance of agar as a substrate in comparison to the aqueous PEG-containing growth media.

The response of the leaf photosynthetic machinery to the PEG-induced osmotic stress was much less pronounced. Thus, the activity of the photosystem II (PSII) did not show any significant reduction on the third day upon stress application, although this tendency could be observed at the highest stressor dosage (Fig. 2C). This fact was in agreement with the absence of alterations in chlorophyll contents (Fig. 2D) which might be explained by the absence of severe tissue dehydration at this time point, and a low degree of oxidative stress triggered by the shifts of osmotic homeostasis accompanying it.

Biochemical markers of stress response

To address this aspect in more detail, we assessed the principal markers of reactive oxygen species (ROS) production and antioxidant defense along with alterations in the tissue levels of abscisic acid and jasmonates. Thus, approximately 30 % up-regulation in the contents of thiobarbituric acid (TBA)-reactive substances (TBARS, expressed as malondialdehyde, MDA, equivalents) was observed after the application of even a minimal dosage of osmotic stress with a clear tendency of a further dose-dependent increase (Fig. 3A). This response at the level of lipid peroxidation indicated stress-dependent up-regulation of ROS metabolism and the development of oxidative stress. As up-regulation of lipid peroxidation and MDA production are the markers of a well-pronounced drought response, our results are in agreement with the published data generated with both PEG- and soil-based models (Khaleghi et al., 2019).

As metabolism of ascorbic acid (Asc) is critically dependent on multiple enzymatic systems involved in cellular ROS detoxification machinery, the plant antioxidant status can be adequately characterized by the balance of Asc and the product of its oxidation — dehydroascorbic acid (DHA) (Osmolovskaya et al., 2018). In our study, the contents of Asc were not affected by the PEG concentrations of up to 15 % (w/v), whereas the highest stressor dosage resulted in the significant drop of Asc contents (Fig. 3B). In contrast, the levels of DHA were low, but significantly increased in the presence of even the minimal stressor concentration (Fig. 3C). Accumulation of DHA was dose-dependent with approximately 35 % up-regulation in presence of the maximal PEG dosage. The data on DHA tissue concentration matched ideally to the stress-related increase of MDA abundance in the leaf (Fig. 3A), indicating continuous detoxification of the overproduced ROS by Asc. The capacity of the Asc pool proved to be sufficient to sustain PEG doses up to 15 % (w/v), i. e., only the most severe osmotic stress resulted in ROS production sufficient for exceeding an-

tioxidant capacity of Asc (Fig. 3B). This high capacity of the Asc pool might be caused by a minimal but continuous increase of its synthesis, as it can be seen from dose-dependently increasing total ascorbate contents (not shown). The observed ROS detoxification processes can be seen even in a more contrasting form by studying the Asc/DHA ratio, which decreased by at least 40 % in the presence of the lowest PEG doses (Fig. 3D).

Finally, the levels of the principal drought-related phytohormones were assessed. Although the contents of ABA increased in all treatment groups, these alterations were mostly non-significant (Fig. 3E). Indeed, the observed PEG-induced alterations of ABA contents were rather small in comparison to a 10-20-fold increase commonly accompanying drought responses. This effect might be related to the relatively low water potential of agar medium (in comparison to the aqueous one) even in the absence of PEG, as was observed earlier for Arabidopsis plants (Frolov et al., 2017). As up-regulation of ABA contents and stomata closure belong to early stress responses (Osmolovskaya et al., 2018), the observed data might correspond to the post-maximal decrease of this phytohormone. This assumption is indirectly confirmed by the stomata conductance data: it reduced 5-fold already on the 3rd day of the experiment and did not change afterwards, i. e., the kinetics of the response, most likely, developed before this time point.

The analysis of jasmonic acid (JA), its precursor 12-oxo-phytodienoic acid (OPDA) and its active form — isoleucyl conjugate (JA-Ile) revealed no effect on the first two, whereas formation of Ja-Ile was significantly up-regulated upon application of even minimal stress dosage (Fig. 3F). It is known, that jasmonates contribute to the plant resistance to drought stress, as their accumulation (including JA-Ile) accompanies the onset of drought tolerance (De Domenico et al., 2012). Thus, as the phytohormone determination was done after three days of osmotic stress application, the increase of the JA-Ile contents can be explained by the development of drought tolerance, which typically is accompanied with multi-level metabolic adjustment and requires several days to be established (Frolov et al., 2017). Remarkably, the dose-dependent signature of JA-Ile was in a good agreement with the data on ABA contents (Fig. 3E) and stomatal conductance (Fig. 2A), which confirms the existence of a cross-talk between ABA and JA signaling and the involvement of JA-Ile in the regulation of stomata closure (Riemann et al., 2015).

It is interesting to note that application of 10 % (w/v) PEG in the overlay solution resulted in the recovery of biochemical markers of stress response to the levels comparable with control (Fig. 3). We hypothesize that this relatively low stress dosage might trigger metabolic rearrangement in stress plants which allows a complete recovery of stress-related alterations. Thus, this relative-
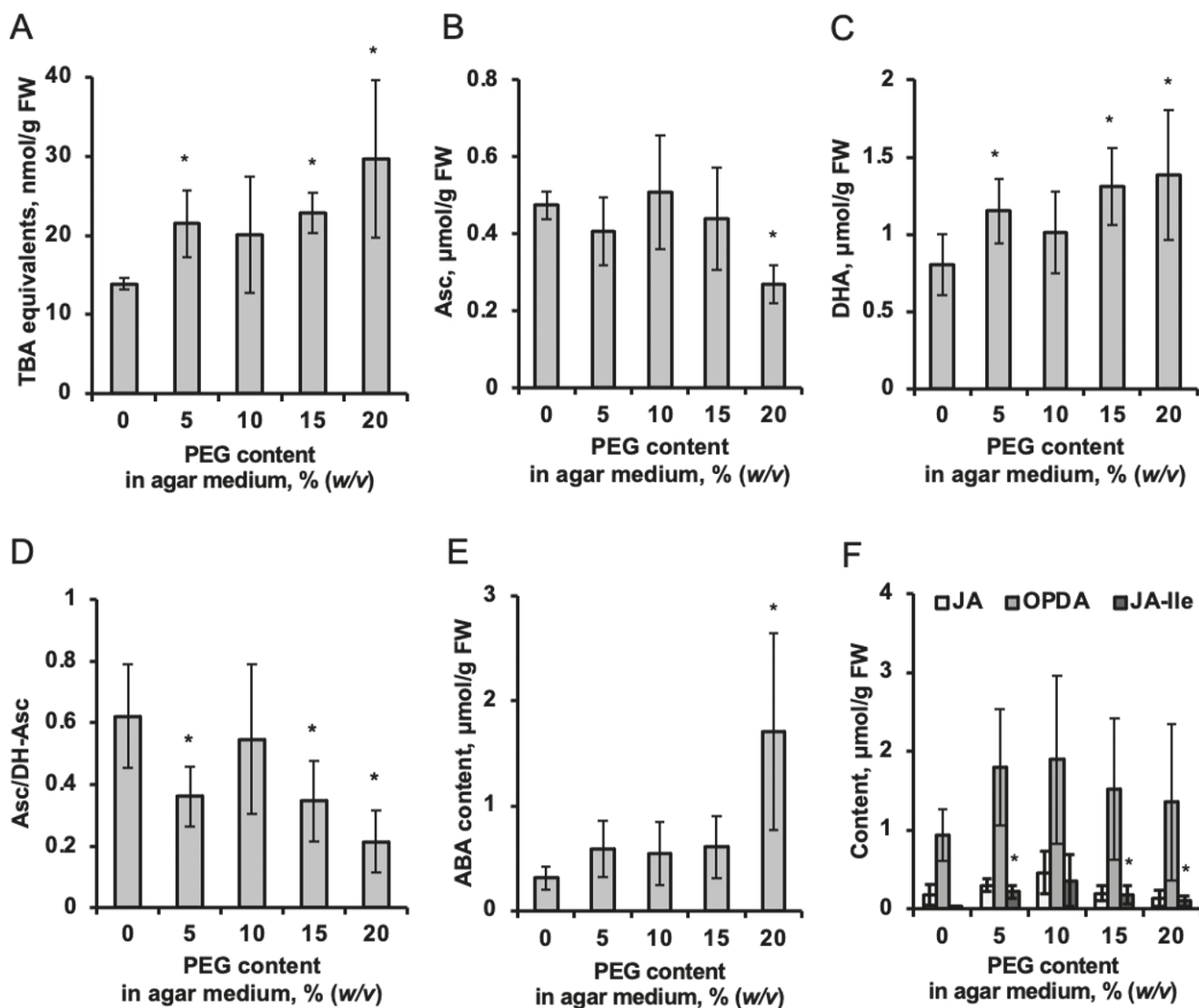
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Fig. 3. Characterization of plant stress after a three-day exposure of pea (*Pisum sativum* L.) plants to the 0.8 % (*w/v*) agar medium saturated with 0, 5, 10, 15 and 20 % (*w/v*) PEG 8000 solutions by thiobarbituric acid-reactive substances (expressed as malondialdehyde equivalents, A), content of ascorbate (Asc, B), content of dehydroascorbate (DHA, C), ascorbate/dehydroascorbate ratio (D), content of abscisic acid (ABA, E), content of jasmonic acid (JA) and its derivatives (OPDA, JA-Ile, F). * denotes statistical significance (Mann — Whitney U-test) at the confidence level $p \leq 0.05$.

ly low level of dehydration is sufficient to activate plant adaptation mechanisms which enhance plant tolerance against severe stress.

Conclusion

The agar-based PEG infusion model represents a powerful tool for the study of drought stress response in plants. Unfortunately, in its original design, this model employs early-stage seedlings, which, due to their small weight, do not allow comprehensive characterization of plant metabolic response to drought. In this context, the extension of this model onto mature Arabidopsis plants was an essential step forward, which was assumed to close this gap in application of the agar-based setup and to give access to proteomics and metabolomics stud-

ies. The next logical step was to switch to agriculturally valuable crops, giving the basis for the translational proteomics and metabolomics approach, which is currently underrepresented in the application of the agar-based PEG infusion model. In this study we have accomplished this step and demonstrated good applicability of this approach to mature legume plants. Thereby, the signatures of individual physiological and biochemical stress markers are in good agreement with each other and generally resemble the stress response of plants grown in soil-based stop-watering models. We are convinced that our setup can be efficiently used in the study of stress-related metabolic adjustment in green parts, roots and root nodules of juvenile and flowering plants. However, the effect of drought on seed development would be difficult to address in this model. As the next

closest step, stress kinetics and possible experiment duration need to be characterized for minimal and moderate stress setups. This knowledge will make further proteomics and metabolomics experiments with this stress model possible.

Acknowledgements

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