Extended drought periods in grasslands: impacts on the number of photosynthetically active leaves and on leaf senescence in grass and clover species

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Abstract—Water availability in soil is a key aspect in the context of global change. More frequent and more severe extreme events including droughts, flooding and heat waves must be expected for the next decades in some regions. Since a high percentage of the area used for agriculture is covered by grasslands, the response of grass and legume species to severe drought is important for the evaluation of drought impacts. Differences between the species considered (*Lolium perenne* L., *Dactylis glomerata* L., *Phleum pratense* L., *Trifolium repens* L., *Trifolium pratense* L.) in their response to a low water potential in the root medium were evident. In general the number of photosynthetically active leaves per plant decreased under drought. This decrease was partially due to a smaller number of new leaves produced and partially to senescence of the oldest leaves. The strongest reduction in the number of active leaves was observed in *Trifolium repens*, while *Trifolium pratense* was less susceptible. The grasses were even less affected than the clover species. Temperature of sun-exposed leaves was increased in drought-stressed plants as a consequence of decreased transpiration. The elevated temperature may cause additional effects on leaf metabolism (e.g. inactivation of Rubisco activase). Modifications in leaf senescence and leaf emergence under drought stress are species-specific and influence plant performance during the stress and the subsequent recovery phase.

Keywords—Climate change, drought stress, grassland plants, recovery

I. INTRODUCTION

A shorter version of this paper was presented orally at the 9th International Conference on Energy & Environment (EE '14) in Geneva, Switzerland (December 29-31, 2014) and was included in the Conference Proceedings [1]. Water availability in agriculturally used soil is besides an increasing CO_2 level in the atmosphere and an increase in the average ambient temperature an important aspect in the context of global change caused by the combustion of fossil energy sources [2, 3]. More frequent and/or more severe drought periods must be expected during the next decades in some regions as a consequence of climatic changes, while other regions may be characterized by increased precipitation with favorable (increased water availability and plant productivity) or unfavorable (waterlogging) effects on crop plants [2-5]. Even in cases when the yearly precipitation will remain quite constant, a shift from the main growth period to the cold season may cause summer droughts and negatively influence plant productivity. Such changes are of ecological and economical relevance. Economic consequences of global change on agriculture were recently discussed by Lanfranchi et al. [6] and possible impacts on sustainable development and tourism were listed by Mazilu [7].

Various species present in grasslands may be influenced differently by such extreme events [8-10]. A series of reports refer to the overall productivity of grasslands [9, 11], to the species composition [11-14], to the expansion of weed populations [15] and to gas exchange with the atmosphere [16]. The weed *Rumex obtusifolius* may be less affected by drought than the desired grassland species and cause serious problems for farmers [17]. On the level of individual plants, stomatal regulation [18 and references therein], photosynthetic activities [19-21], summer dormancy [22, 23] and symbiotic nitrogen fixation in legumes [24, 25] were key aspects addressed during the past decades. Stomatal and non-stomatal limitations may negatively influence CO_2 assimilation [26]. Water availability in the root medium influences stomatal opening, transpiration and as a consequence leaf temperature [18]. Physiological processes on the whole plant level are less well investigated, but leaf production/expansion and senescence were identified as crucial points in this context [27, 28].

The experiments reported here are focused on the number of active leaves per plant and its dependence on the formation of new leaves and senescence of older leaves. A direct comparison of selected grass and clover species was envisaged by growing them on the same pots either in soil culture with natural illumination in a green house or in hydroponic culture

in a growth cabinet with a controlled light and temperature regime.

II. MATERIALS AND METHODS

A. Plant Species

Three grass species (*Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*) and two clover species (*Trifolium pratense*, *Trifolium repens*) were germinated on wet tissue paper in darkness. Afterwards the young plants were transferred to a light/dark cycle as reported previously [29]. Three experiments were performed. In one experiment the plants were transferred to soil, while the other experiments were performed with hydroponic cultures (one with two clover and a grass species focused on an extended drought period and one with three grass species including a recovery phase after the drought stress).

B. Pot Experiment in a Greenhouse

Young plants (3 weeks old) were transferred to pots with nutrient-rich soil. Each pot contained 4 *Dactylis glomerata* and 4 *Trifolium repens* plants. The pots were incubated for additional 2 weeks in a growth cabinet and afterwards put outside. When the plants were 8 weeks old, the pots were transferred to a large green house and the drought experiment was started. Control pots were watered regularly, while water was withheld from pots subjected to drought. Green and yellow/senesced leaves were counted throughout the drought period. Additionally air temperature (with a common greenhouse thermometer) and leaf temperature (with an infrared thermometer from below the leaf to avoid shadowing with the equipment) were recorded. Relative chlorophyll contents per leaf area were determined with a SPAD meter (Konica Minolta Inc, Osaka, Japan).

C. Artificial Drought in Hydroponic Culture

Young plants of *Lolium perenne* (7 d old), *Trifolium pratense* (17 d old) and *Trifolium repens* (17 d old) were transferred to pots with 150 mL standard medium [29] for hydroponic culture. The experiment with artificial drought was started 13 d later by replacing the nutrient medium with 165 mL fresh nutrient solution (control) or with a mixture of 165 mL of the same medium and 16.5 g polyethylene glycol 6000 (PEG treatment) in a growth chamber with a light/dark cycle as reported previously [29]. Polyethylene glycol is suitable to decrease the water potential in the root medium [30,31]. Initially the drought stress was moderate. It became more severe afterwards, since water was taken up by plants (but not polyethylene glycol) and the water potential in the medium decreased further and reached very low values [30,31]. The actual water potential in the medium was calculated according to Michel and Kaufmann [31]. To avoid nutrient depletion during the experiment the following solutions were added to all pots (controls and artificial drought) at day 9 and day 15: 2 mL 100 mM $Ca(NO_3)_2$, 2 mL 200 mM KNO_3 and 0.1 mL micronutrient solution (containing

1.96 mM $MnCl_2$, 9.86 mM H_3BO_3, 0.34 mM $ZnSO_4$, 0.4 mM Na_2MoO_4, 0.1 mM $Ni(NO_3)_2$ and 0.22 mM $CuSO_4$) [32]. To control pots (but not to artificial drought pots) deionized water was added at day 9 and day 15 up to the original level of the nutrient medium (165 mL). Transpiration was determined gravimetrically. F_v/F_m (a good indicator for the intactness of the photosynthetic electron transport) was determined in dark-adapted plants according to Maxwell and Johnson [33]. Means and standard deviations of 5 independent replicates are shown. Missing leaves were entered as 0.0. The number of photosynthetically active leaves was calculated by determining F_v/F_m in the various leaves of each plant and dividing the sum of these measurements by 0.8 (average value for healthy leaves).

In a separate series three grass species (*Phleum pratense*, *Dactylis glomerata*, *Lolium perenne*) were compared with essentially the same protocol as mentioned above. The plants were subjected to a stress phase (addition of polyethylene glycol 6000 to the nutrient medium) for 27 days. At day 27 all nutrient media were replaced by fresh standard nutrient solution for the recovery phase.

D. Statistical analyses

Data represent means and standard deviations of 4 or 5 replicates. Significant differences between control and drought-stressed plants were identified with Student's t-test.

III. RESULTS

The number of green leaves per pot containing 4 *Dactylis glomerata* (grass) and 4 *Trifolium repens* (clover) plants are documented in Figure 1a. For clover this number was for drought-stressed plants considerably lower than for controls and even decreased in the stressed plants at the end of the drought period reaching values of nearly 50% of the controls. In contrast to clover, the number of green leaves in *Dactylis glomerata* was less affected by drought, increased throughout the stress period and reached finally values of nearly 70% of the controls. Since the number of green leaves depends on two processes (emergence of new leaves and senescence of older leaves) a further differentiation was necessary.

Leaf senescence caused an increase in the number of yellow and brown leaves. Senesced leaves accumulated more rapidly in drought-stressed than in control plants (Figure 1b). However, the difference in the number of senesced leaves cannot explain the smaller number of green leaves in drought-stressed plants. Therefore anticipated senescence and a smaller number of newly expanded leaves contributed to the difference. Leaf senescence also started in well watered plants and was not restricted to plants under abiotic stress, but the number of senescing and senesced leaves was higher under drought.

Relative chlorophyll levels per leaf area were higher in photosynthetically active leaves of drought-stressed plants than in controls (Figure 2a). This can be explained by a smaller leaf area as it become obvious from macroscopic inspection. The smaller leaf area of young leaves (emerged during the stress

phase) was primarily caused by a negative effect on leaf expansion. In older leaves (fully expanded before the onset of drought stress) shrinkage as a consequence of deceased turgor caused the higher chlorophyll levels per leaf area.
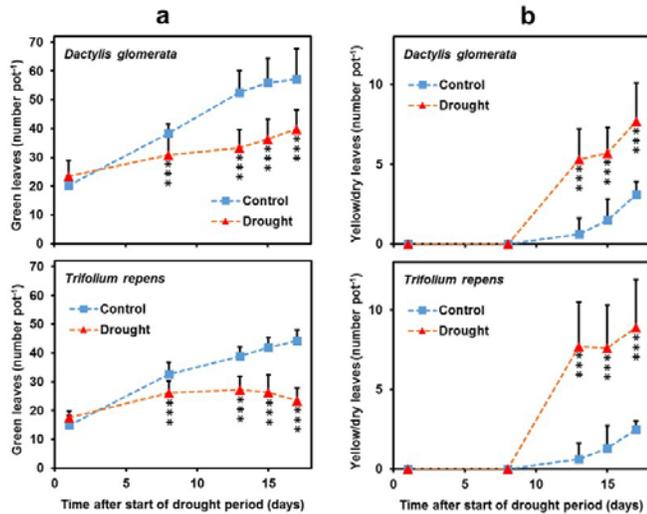
Fig. 1 number of (a) green and of (b) senescing/senesced leaves on well watered pots (Control) and pots with limited water supply (Drought). *Dactylis glomerata* and *Trifolium repens* plants were grown together on the same pots. Means of 4 independent replicates and standard deviations (on one side only for clarity) are shown. Significant differences between control and drought-stressed plants at the $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) level are indicated.

Air and leaf temperatures were analyzed throughout the experiment (Figure 2b). Air temperature strongly depended on the actual weather. It was highest during warm and sunny days, intermediate during cloudy days and low during cold and rainy days. For both plant species leaf temperature was in general higher in drought-stressed plants than in leaves of well watered controls. This difference was most pronounced at the end of the experiment when drought stress was most severe (3 to 5°C difference). No major differences between drought-stressed and control plants were observed on cloudy/rainy days when the air temperature was low.

In the second experiment two clover (*Trifolium pratense* and *Trifolium repens*) and a grass species (*Lolium perenne*) were grown hydroponically on the same pot to ensure identical conditions. Artificial drought was caused by the addition of polyethylene glycol 6000 to the nutrient solution. The calculated water potentials in the nutrient medium and the transpiration rates are shown in Figure 3a.

The water potential in pots with standard nutrient medium was always close to 0, while it decreased in the polyethylene glycol (PEG) treatments to very low values. This decline was caused by uptake of water (transpiration) but not of PEG leading to increasing PEG concentration and as a consequence decreasing water potentials (Figure 3b).

The transpiration rates per pot were until day 10 very similar for controls and PEG treatments, then they increased more slowly in PEG treatments than in controls until day 13

and decreased finally in the PEG treatments, while they further increased in the controls.
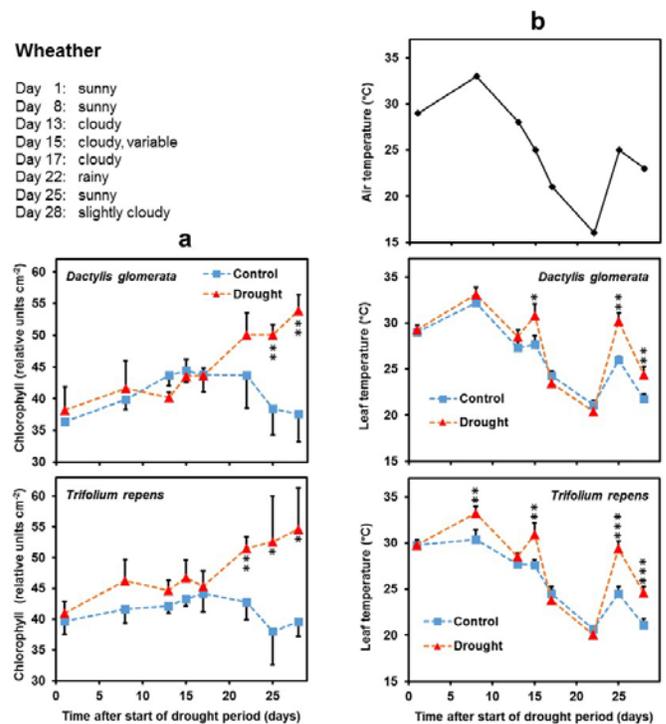
Fig. 2 (a) relative chlorophyll contents per leaf area; (b) air temperature and temperatures of green leaves on well watered pots (Control) and pots with limited water supply (Drought). *Dactylis glomerata* and *Trifolium repens* plants were grown together on the same pots. Means of 4 independent replicates and standard deviations when exceeding the size of the symbol (on one side only for clarity) are shown for leaf temperatures. Significant differences between control and drought-stressed plants at the $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) level are indicated.
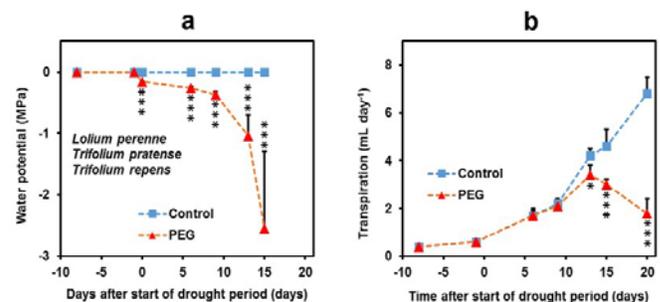
Fig. 3 (a) water potential and (b) transpiration rates in hydroponic cultures without (Control) and with polyethylene glycol 6000 (PEG). *Lolium perenne, Trifolium pratense* and *Trifolium repens* plants were grown together on the same pots. Means of 5 independent replicates and standard deviations when exceeding the size of the symbol (on one side only for clarity) are shown. Significant differences between control and drought-stressed plants at the $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) level are indicated.
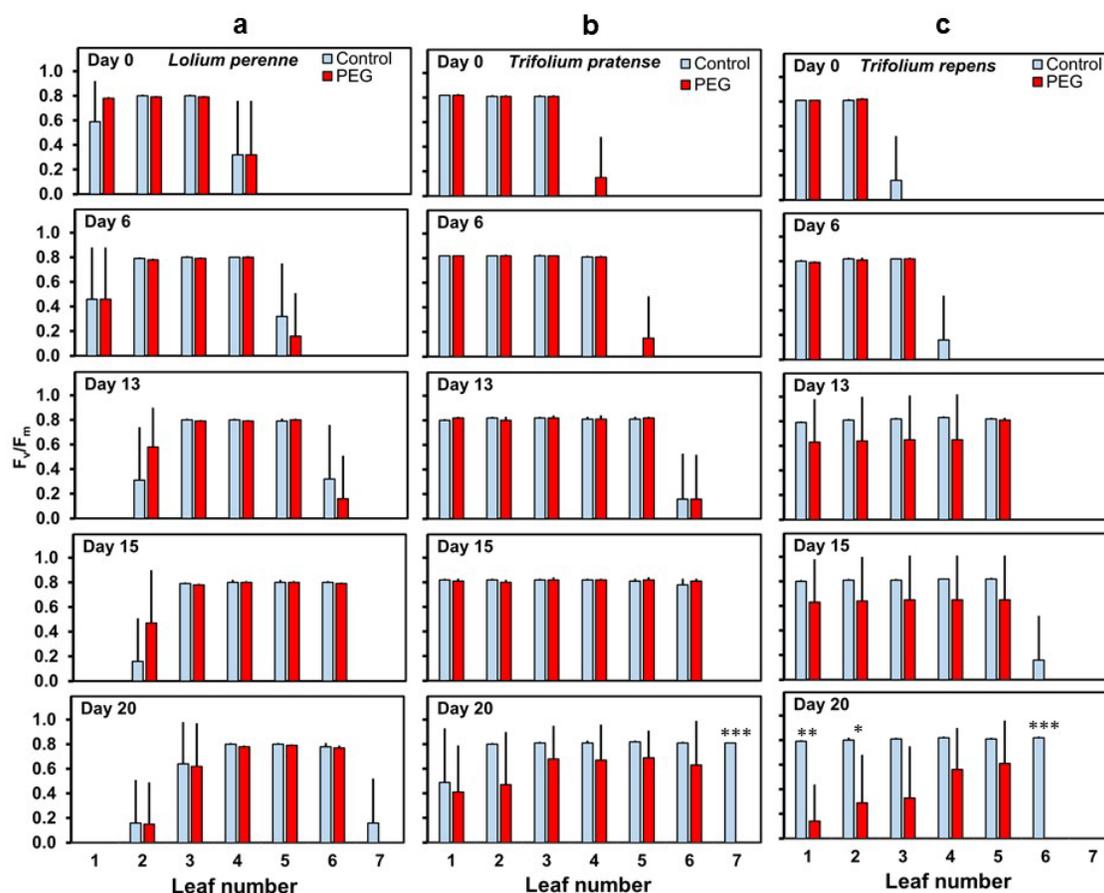
Fig. 4 intactness of photosystem II (F_v/F_m) in (a) *Lolium perenne*, (b) *Trifolium pratense* and (c) *Trifolium repens* leaves from hydroponic cultures without (Control) and with polyethylene glycol 6000 (PEG). The three species were grown together on the same pots. After the stress phase (days 0 to 27) the plants were transferred to fresh standard nutrient medium for the recovery phase (days 27 to 36). Leaves are numbered from the oldest (1) to the youngest (7). A value of 0.0 was entered for missing leaves. Means of 5 independent replicates and standard deviations (on one side only for clarity) are shown. Significant differences between control and drought-stressed plants at the P<0.05 (*), P<0.01 (**) and P<0.001 (***) level are indicated.

F_v/F_m was measured in dark-adapted plants (in the morning before the lights were switched on) and used as an indicator for the intactness of the photosynthetic electron transport. The sequentially emerging leaves were analyzed separately throughout the stress period lasting for 20 days. In healthy leaves a value around 0.8 can be expected.

The measurements for *Lolium perenne* are documented in Figure 4a. Initially (day 0) 4 to 5 leaves were present. In mature leaves the standard deviations were extremely small, while they were large in emerging and senescing leaves. These large standard deviations can be explained by the fact that the timing for leaf emergence and senescence differed to some extent between the pots and a given leaf was active in some plants and not yet (later emergence) or no longer (earlier senescence) in other plants. The number of active leaves was for *Lolium perenne* similar for controls and stressed plants. A slightly better performance of control plants was observed for the youngest leaves.

The F_v/F_m measurements for *Trifolium pratense* are shown in Figure 4b. Until day 15, the values were very similar for control and stressed plants. At the end of the experiment (day 20) differences became obvious. The youngest leaf (leaf 7) was expanded in controls, but not in PEG-treated plants. Furthermore expanded (especially older) leaves in stressed plants were partially damaged.

Compared to the other two species, the differences between controls and stressed plants were most pronounced in *Trifolium repens* (Figure 4c). A delay in the emergence of new leaves under drought stress was one important factor. Additionally, the decline in F_v/F_m was anticipated or accelerated in older leaves of drought-stressed plants. The youngest expanded leaves remained active longest in stressed plants. The number of active leaves throughout the experiment is summarized in Figure 5. No major effects for this parameter were observed for *Lolium perenne* (Figure 5a). However, it must be borne in mind that this figure refers only to the number of active leaves and that the size of the leaves
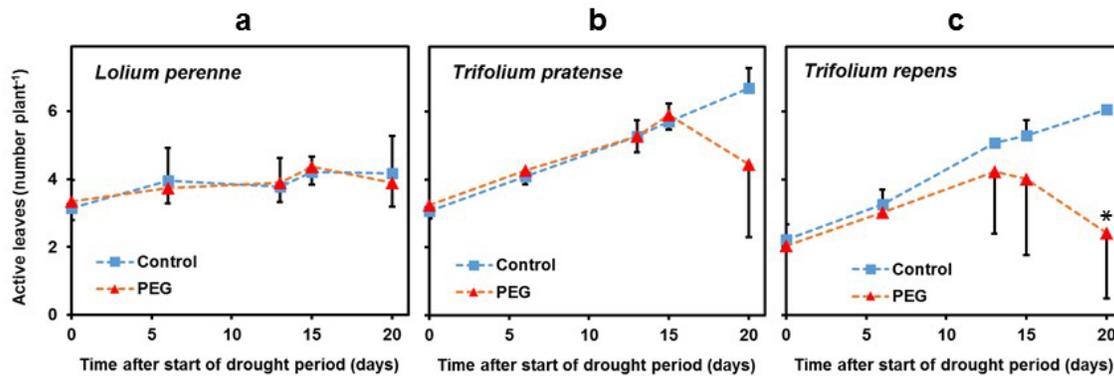
Fig. 5 number of photosynthetically active leaves in (a) *Lolium perenne*, (b) *Trifolium pratense* and (c) *Trifolium repens* grown hydroponically without (Control) and with polyethylene glycol 6000 (PEG). The three species were grown together on the same pots. Means of 5 independent replicates and standard deviations (on one side only for clarity) are shown. Significant differences between control and drought-stressed plants at the $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) level are indicated.
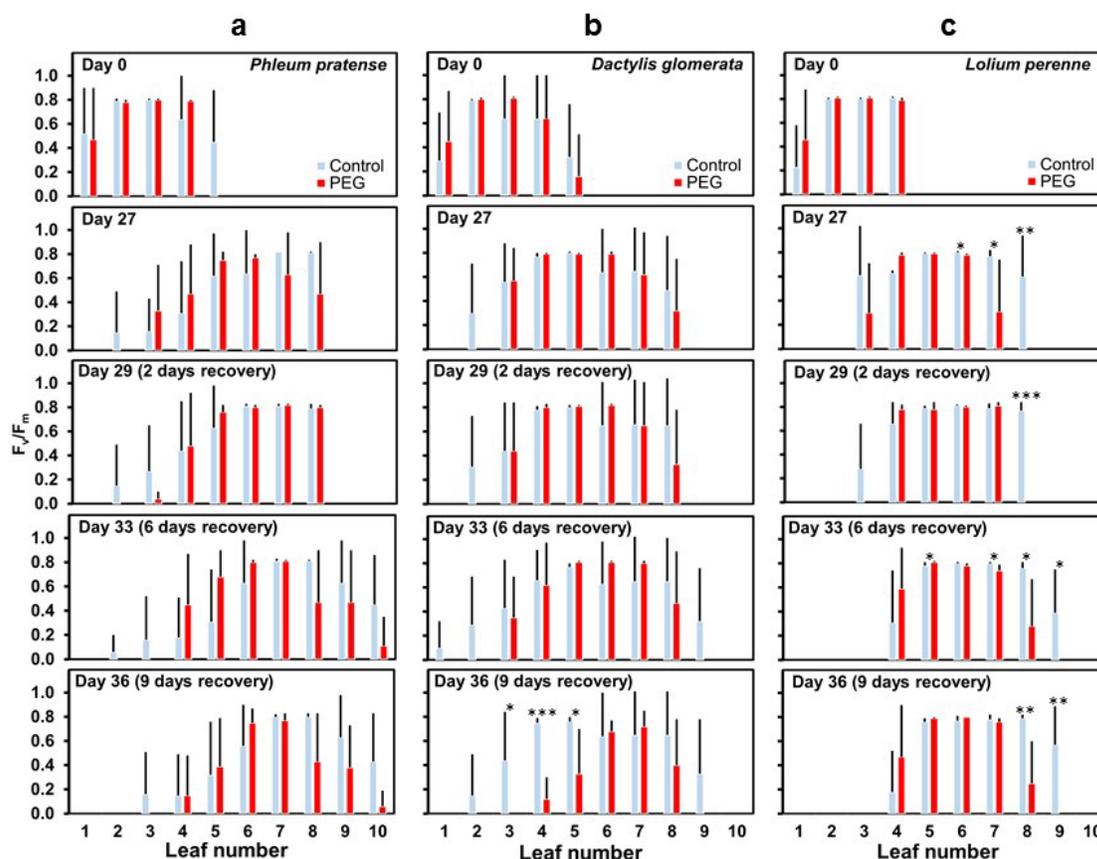
Fig. 6 intactness of photosystem II (F_v/F_m) in (a) *Phleum pratense*, (b) *Dactylis glomerata* and (c) *Lolium perenne* leaves from hydroponic cultures without (Control) and with polyethylene glycol 6000 (PEG). The three species were grown together on the same pots. After the stress phase (days 0 to 27) the plants were transferred to fresh standard nutrient medium for the recovery phase (days 27 to 36). Leaves are numbered from the oldest (1) to the youngest (10). A value of 0.0 was entered for missing leaves. Means of 5 independent replicates and standard deviations (on one side only for clarity) are shown. Significant differences between control and drought-stressed plants at the $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) level are indicated.

(shrinkage of previously older leaves and reduced expansion of the youngest leaves) and the actual photosynthetic activity may be considerably affected by drought. The number of active leaves was at the end of the experiment slightly decreased in *Trifolium pratense* (Figure 5b). The strongest response was observed in *Trifolium repens* (Figure 5c). In this species the number of active leaves was affected earlier and more severely than in the two other species grown simultaneously under identical conditions in the same containers. From these data it became obvious that the

response of various grassland species to severe drought may differ considerably. Leaf expansion and leaf senescence contribute to this effect in a species-specific manner. The duration of a drought period is important for the relative impact on various grassland species.

Three grass species (*Phleum pratense*, *Dactylis glomerata*, *Lolium perenne*) were compared in an additional experiment

grown on the same nutrient medium (Figure 7). It became obvious that the recovery phase must be considered for an overall evaluation of the performance. Only minor effects of the lowered water potential were detected in *Phleum pratense* and *Dactylis glomerata,* but a marked decline was observed during the subsequent recovery phase (Figure 7a,b). Stress-induced senescence of the older leaves continued during the
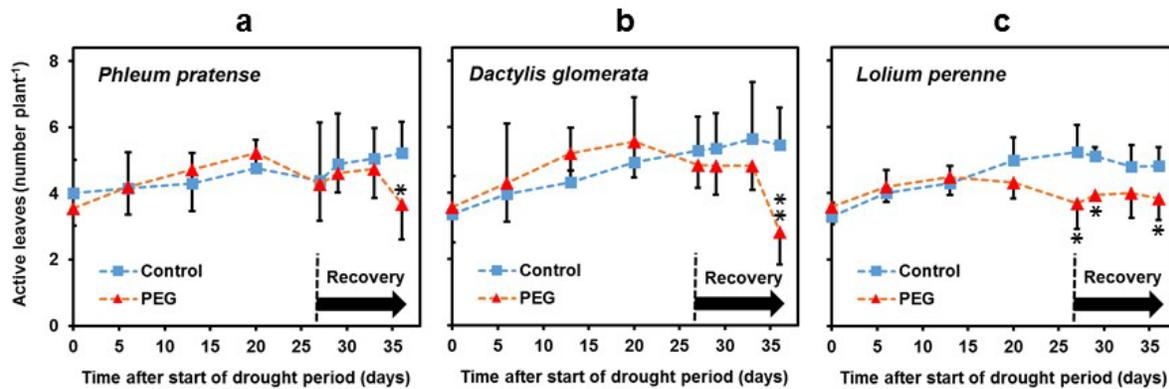
Fig. 7 number of photosynthetically active leaves in (a) *Phleum pratense*, (b) *Dactylis glomerata* and (c) *Lolium perenne* grown hydroponically without (Control) and with polyethylene glycol 6000 (PEG). The three species were grown together on the same pots. Means of 5 independent replicates and standard deviations (on one side only for clarity) are shown. Significant differences between control and drought-stressed plants at the $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) level are indicated.

including the recovery phase after the drought period (Figure 6). From the control plants of all three species it became evident that senescence started in the oldest leaves during the experiment without drought stress. However, the lowered water potential in the medium influenced senescence of the oldest leaves as well as the expansion of new leaves. Characteristic differences between the three species were observed. The oldest leaves as well as the youngest leaves were affected in *Phleum pratense*, while leaves 6 and 7 remained active throughout the stress and the recovery phase (Figure 6a). A recovery phase of 9 days was not sufficient to increase the number of active leaves again indicating that the differences between control and drought-stressed plants remained relevant after the stress phase for several weeks and were not easily compensated by the formation of new leaves. A stronger drought effect was detected in *Dactylis glomerata* (Figure 6b). The early senescence of the oldest leaves under drought was obvious, while the youngest leaves were far less affected. Leaves 6 and 7 remained again active throughout the experiment. Effects of the previous drought treatment remained relevant during the recovery phase. In contrast to the two other species, the production of new leaves was in *Lolium perenne* far more influenced by drought than the emergence of new leaves (Figure 6c). Leaves 5 to 7 remained in this species most active during the drought stress. Senescence progressed in control and drought-stressed plants similarly, but a smaller number of younger active leaves was detected in the drought-stressed plants.

The time courses for the number of active leaves in control and drought-stressed plants differed for the three grasses

recovery phase, while this loss of active leaves was not yet compensated by the expansion of new leaves (Figure 6a,b). The different time course in drought-stressed *Lolium perenne* (Figure 7c) can be explained by the fact that in this species senescence of older leaves was not or only marginally affected by drought (Figure 6c).

IV. DISCUSSION

The cultivation of various species on the same pot allowed a direct comparison. In hydroponic culture the roots of all species were exposed to the same water potential, while in soil culture there might still be water potential gradients which may be used unequally by the various species. From the results it became evident that the active leaf biomass in grassland plants was strongly influenced by water limitation. Both, leaf expansion and leaf senescence contributed to the decrease in the number of active leaves under drought. Furthermore the relative importance of leaf emergence and leaf senescence was species-specific. Leaf expansion was affected in all species. Effects on leaf senescence were most pronounced in *Dactylis glomerata*, *Phleum pratense* and *Trifolium repens*. Modifications in plant morphology were considered as important responses to drought [34, 35]. Bevan et al. [34] reported that tall vegetation may be an advantage for grasslands under drought. Solute allocation to roots, root growth and metabolism in various parts of the root system are in soil culture not easily accessible, but were considered as relevant for the drought response [28 and references therein]. Modifications in the geometry of the root system may allow the access to so far unusable soil water.

Fv/Fm values for *Lolium perenne* under drought were reported previously [28] from a different experiment with grasses only in the same pots (*Lolium perenne*, *Poa pratense*, *Festuca rubra*). This previous experiment [28] and the investigations reported here (Figures 4a, 5a, 6a, 7a) were performed in the same growth chamber under the same conditions, but not simultaneously. Direct comparisons should therefore be handled with caution. The drought effects in *Lolium perenne* were very similar in all experiments. It can be concluded that the other species present in the same pots caused no major differences in the drought response of individual *Lolium perenne* leaves.

Reactive ogygen species (ROS) play an important role in abiotic stress responses [36-39]. ROS are involved in intracellular signaling [36, 37]. Under various abiotic stresses ROS may accumulate and finally damage cell constituents [36-38]. Protective solutes and activities of enzymes involved in ROS detoxification must be considered as important players in the protection of cells under drought and other abiotic stresses [28, 38, 39].

Effects of the stress period are often very obvious, but the response of plants to a subsequent recovery phase are equally important for an overall evaluation of drought impacts [13, 28, 35]. A down-regulation of physiological activities may allow a species to survive and to recover more efficiently than a species which is more active at the beginning of the stress phase but with a limited potential to recover after an extended drought period.

Leaf temperature can be indirectly influenced by water availability in the soil [18]. Abscisic acid transported via the transpiration stream to the leaves or produced in the leaves may cause stomatal closure and as a consequence reduced transpiration. Especially under full sunlight and reduced cooling by transpiration leaf temperature may reach high values and negatively affect physiological processes in the leaves [18, 28]. Strong effects of drought stress on leaf temperature were detected in oak [41, 42] and beech [43] leaves. Higher leaf temperatures under drought were also found in herbaceous species [18, 28], but it must be borne in mind that many other factors influence these values (air temperature, air convection, irradiation, nutritional status of the plants). It must be considered that combined effects of heat and drought stress may be quite complex and not simply additive [40]. Heat may reversibly and later also irreversibly damage important cellular constituents in fully expanded leaves [44, 45]. Rubisco activase was identified as a highly heat-sensitive enzyme [45]. This enzyme might be primarily the cause for non-stomatal limitations at elevated temperature, since deactivation of Rubisco negatively influences photosynthesis and plant productivity [26, 45].

The spatial resolution for climate change modelling was considerably improved during the past decade [2, 46]. This high resolution in space and time is necessary for agriculture, since impacts of extreme events can be very local. Therefore caution is recommended when generalizing drought effects.

Irrigation of large areas will for most locations not be possible, since during a drought period the quantity of water available for irrigation will be also limited. Improvements in the use of the limited water for plant cultivation may be possible by the installation of more complex irrigation systems [47]. For grasslands however, breeding and selection of suitable grass and legume genotypes, use of optimized species mixtures and agronomic practices (e.g. fertilization, time of mowing or grazing) are highly important for minimizing negative effects of extreme climatic events such as extended drought periods or heat waves.

V. CONCLUSIONS

Species-specific and even variety-specific responses must be considered and are important for genotype selection and breeding. As documented in this paper, even closely related plant species may be affected differently by drought (e.g. formation/expansion of new leaves versus senescence of the oldest leaves). Besides the performance of a species or of a variety during the stress period, the potential to recover after the stress period is highly important for the overall response. This investigation was focused on the response of individual plants to severe drought, while other aspects (e.g. root activities or species competition) were not or only marginally addressed. Especially the following points remain to be elucidated in more detail for grasslands exposed to severe drought:

- Root development and root physiology: Root growth allows the exploration of new soil regions. Water availability may differ vertically between soil layers [20], but horizontal gradients caused by inhomogeneous soil or interactions with other plants must also be considered. Therefore the response of roots or of parts of the root system remain to be further investigated [28, 32].
- Competition between plants in mixtures of species and/or varieties: The competition between species/varieties can be influenced by drought [15]. Since the weather during the main growth period is not known when grasslands are established, changing frequencies of droughts must be included in planning and risk evaluation.
- Conditioning of plants exposed to moderate drought for a severe drought stress: A mild drought followed by a recovery phase may condition plants for a more severe drought period [48].
- Effects of combined environmental stresses: Several environmental factors (e.g. drought, temperature and elevated CO_2) may be influenced by climate change and affect grassland performance [49]. Our knowledge concerning plant responses to multiple abiotic stresses is still quite limited.
- Comprehensive evaluation considering time courses (e.g. changes throughout the day) [50] besides the spatial situation (various leaves, roots, reproductive organs) [51]: Daily time courses for stomatal opening and

photosynthesis are important for optimal water use efficiency under drought. Furthermore, senescence of some leaves accompanied by a reduced water loss of the plant may contribute to plant survival and performance after the drought period [51]. A positive effect of abscisic acid-induced stomatal closure during a drought period was reported to be advantageous for gradual repair of xylem embolism in grapevine after rewatering [52].

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