RESEARCH ARTICLE:

Short title: Membrane failure and fluorescence predict mortality

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Dead or alive? Using membrane failure and chlorophyll a fluorescence to predict plant mortality from drought

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One Sentence Summary:

Chlorophyll fluorescence and cellular leakage from disrupted membranes are reliable indicators of the precise timing of plant mortality from drought and beetle kill across species.

List of Authors Contributions:
CRG, BEE conceived the research
CRG supervised the experiments and analyzed the data
CRG, HNS, TLA, BJH, SBD, JTL, RNS performed the experiments
CRG, BEE, CW wrote the paper

Funding Information:
This research was funded by the Wyoming Water Development Commission, United States Geological Survey and National Science Foundation grants IOS-1025965, EAR-0910831 and EPS-1208909.

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Abstract:

Climate models predict widespread increases in both drought intensity and duration in the next decades. Although water deficiency is a significant determinant of plant survival, limited understanding of plant responses to extreme drought impedes forecasts of both forest and crop productivity under increasing aridity. Drought induces a suite of physiological responses; however, we lack an accurate mechanistic description of plant response to lethal drought that would improve predictive understanding of mortality under altered climate conditions. Here, proxies for leaf cellular damage, chlorophyll \(a\) fluorescence and electrolyte leakage, were directly associated with failure to recover from drought upon re-watering in \textit{Brassica rapa} (genotype R500) and thus define the exact timing of drought-induced death. We validated our results using a second genotype (imb211) that differs substantially in life history traits. Our study demonstrates that while changes in carbon dynamics and water transport are critical indicators of drought stress, they can be unrelated to visible metrics of mortality, i.e. lack of meristematic activity and re-growth. In contrast, membrane failure at the cellular scale is the most proximate cause of death. This hypothesis was corroborated in two gymnosperms (\textit{Picea engelmannii} and \textit{Pinus contorta}) that experienced lethal water stress in the field and lab. We suggest that measurement of chlorophyll \(a\) fluorescence can be used to operationally define plant death arising from drought, and improved plant characterization can be used to test and enhance surface model predictions of drought mortality and its consequences to ecosystem services at a global scale.

Keywords: plant mortality - plant hydraulics - carbon starvation - chlorophyll \(a\) fluorescence - electrolyte leakage - drought
Introduction:

During the last few decades, climate change has caused rising temperatures, heat waves, more severe and frequent droughts and insect outbreaks, all of which may increase plant mortality (O’Grady and Mitchell, 2015; Allen et al., 2010; Bentz et al., 2010; Elderd et al., 2010). Existing climate models predict a further increase in periodic water stress, with severe consequences on plant growth and distribution (Engelbrecht, 2012; IPCC, 2014; Van Mantgem et al., 2009; McDowell et al., 2015; Anderegg et al., 2012a). Climate and land surface models currently lack a realistic mechanism of plant death; consequently, drought-induced tree and forest die-off have been extensively documented but only poorly predicted (O’Grady and Mitchell, 2015; Allen et al., 2010; Adams et al., 2010; McDowell et al., 2015; Steinkamp and Hickler, 2015; Anderegg et al., 2012a; Choat, 2013; Anderegg et al., 2015b; McDowell, 2011; Sala et al., 2010; Tyree and Sperry, 1988).

In parallel to forest research, crop studies have focused on predicting responses to non-lethal levels of water stress in order to inform breeding programs and/or water management (Tardieu, 2012; Blum, 2005; Jones and Corlett, 1992), even though severe dehydration can lead to total crop loss especially in developing countries (http://www.africanfarming.net, 2015; http://www.ipsnews.net, 2013; Xoconostle-Cásarez et al., 2011). Death has been defined as the point of equilibrium between living organisms and their environment (McDowell, 2011; Bernat et al., 1981), or as the complete interruption of organic interactions (Anderegg et al., 2012a; McDowell, 2011). Operationally, plants are often considered dead when stress is alleviated yet they fail to recover (Anderegg et al., 2012a). None of these definitions provide testable mechanisms
that can precisely explain the physiology of plant stress responses that lead to mortality. Also, no existing process model can accurately predict when a plant will die from severe water stress (Zeppel et al., 2011; Anderegg et al., 2012; Brodribb and Cochard, 2009; McDowell et al., 2013; Adams et al., 2013; Xu et al., 2013; Meir et al., 2015; Mencuccini et al., 2015). A quantifiable definition of drought mortality is needed that resolves death on the daily time scale so that we can predict if the timing of drought alleviation (through rain or irrigation) will allow plant recovery.

Currently, there are two preeminent hypotheses regarding mechanisms of plant mortality in response to drought, hydraulic failure and carbon starvation. In both hypotheses, drought causes hydraulic limitation, with the subsequent development of high tension in the xylem water column and the closure of stomata (Tyree and Sperry, 1988). Under the first hypothesis, if water tension in the xylem does not recover upon re-watering, then progressive cavitation will lead to hydraulic failure and death of the plant (Sperry, 2000; Sperry et al., 1988; Urli et al., 2013; McDowell et al., 2008). The carbon starvation hypothesis proposes that the reduction in carbon assimilation due to stomatal closure forces plants into a sustained negative carbon balance and ultimately leads to death of the plant. Although various studies have found a decline in carbohydrates in drought conditions (Dietze et al., 2014; McDowell, 2008), this decrease seems to reduce plant survival only in droughted trees that also experienced shade or high temperature stress (Sevanto et al., 2014a; Adams et al., 2009). A more recent hypothesis recognizes that these two mechanisms are fundamentally inextricable; indeed, they may be simultaneously triggered, leading to a failure of sugar transport in the phloem (Anderegg et al., 2015a; Sala et al., 2010; McDowell et al., 2013; Sevanto, 2014b). Plant researchers
have used a variety of methods to test these hypotheses (Sperry, 2000; Sperry et al., 1988; Urli et al., 2013; McDowell et al., 2008; Adams et al., 2009; Sevanto et al., 2014a). Some approaches are extremely laborious; others present practical limitations (Brodribb and Cochard, 2009). Regardless of the method chosen, all current models fail to realistically predict the time of death, namely when droughted plants will not recover upon renewed irrigation or a precipitation event (Anderegg et al., 2012a; Brodribb and Cochard, 2009; McDowell et al., 2013; Adams et al., 2013; Xu et al., 2013; Meir et al., 2015; Mencuccini et al., 2015; Mackay et al., 2015).

Both hydraulic limitation and changes in carbon metabolism dynamics are intrinsically related to cell membrane failure, such that mechanical destabilization of cell membranes can be critical to predict the timing of plant mortality. When drought causes changes in the homeostasis of electrolytes, such as $K^+$ and $Na^+$ (Wang et al., 2013), cell membrane mechanical destabilization results in mortality. The apoplastic water potential depends on the concentration of sugars within the cell, and membranes cease to function upon extreme fluctuations in sugar content (Bajji et al., 2001; Sutinen et al., 1992).

Moreover, in cases of prolonged desiccation, the accumulation of reactive oxygen species (ROS) may lead to biochemical disruption of membranes and result in mortality (Petrov et al., 2015; Suzuki et al., 2012). A fatal failure at the cellular scale can happen in various organs at different times, which permits differentiation of remaining parenchymatic cells and re-sprouting at the whole-plant scale if stress is alleviated (Thomas, 2013; Klimešová et al., 2015). Here we propose to connect the cell theory of life (i.e. all life is made of cells, which are capable of maintaining internal homeostasis using cell membranes) (Karling, 1939) to an empirical measurement and predictive framework of plant
mortality.
We experimentally tested the hypothesis that drought mortality occurs on the day that cell membrane failure of whole organs occurs by measuring responses to drought and subsequent re-watering in *Brassica rapa* (R500, *Sarson seed oil*). This is a globally cultivated annual crop plant with a short life cycle, enabling experimental assessment of mortality. We grew plants in well-watered conditions for 28 Days After Sowing (DAS) and then started a water-withholding treatment (Fig. S1A). At different times after the start of the drought treatment, subsets of plants were re-watered until no plant in the re-watered cohort showed any sign of visual recovery (assessed as new visible growth).

Measurements at the whole-plant and organ levels included leaf gas exchange, hydraulics from soil to leaf, and sugar content of leaves and roots (Anderegg et al., 2012b; McDowell, 2011; McDowell et al., 2008). We also monitored physiological traits that are considered direct proxies for cellular damage within a tissue, such as chlorophyll *a* fluorescence in leaves and electrolyte leakage in leaves and roots (Bajji et al., 2001; Sutinen et al., 1992; Baker and Rosenqvist, 2004; Woo et al., 2008). In R500 (*B. rapa*) the measurement of cellular damage was straightforward and proved to be a flawless metric of the time of death. We confirmed the accuracy of the recovery approach in a second genotype of *Brassica rapa*, imb211, derived from the Wisconsin Fast Plant, which expresses a different life history than R500. Then, to assess the generality of the mechanism observed in the herbaceous, angiosperm *B. rapa*, we applied the same measurement protocols to two conifer species, Engelmann spruce (*Picea engelmannii*) and lodgepole pine (*Pinus contorta*) that experienced lethal water stress in the field or during a controlled bench-drying test. For the field samples, twigs were harvested from
several individuals, including a variety of ages, dominance, and health status due to attack by bark beetles, species *Dendroctonus ponderosae* and *Dendroctonus rufipennis*, which causes mortality by hydraulic failure from blue-stain fungal occlusion of xylem (Frank *et al.*, 2014). We observed that our proxies for cellular damage, chlorophyll *a* fluorescence and electrolyte leakage, were strongly associated with mortality in both phenotypically differentiated genotypes (R500 and imb211) of a herbaceous species as well as in tree species.

We conclude that changes in carbon dynamics and water transport are important signs of drought stress; however, these mechanisms do not adequately define plant mortality (McDowell, 2011; McDowell *et al.*, 2008; Sevanto *et al.*, 2014a; Skelton *et al.*, 2015). Our results precisely identify the time of death from drought for individual plants and membrane failure at the cellular scale resulted in the most proximate cause of death. This mechanism should be accounted for in future models of plant response to extreme environmental stresses. We show chlorophyll *a* fluorescence to be a quantitative and rapid screen for membrane failure, which can be applied at large scales and will improve predictive capacity of global models forecasting climate impacts on both forests and crops.

**Results and discussion:**

Four weeks after sowing (28 DAS) as plants reached maturity, cohorts of *B. rapa* plants (R500 genotype) were assigned to one of three watering treatments for the duration of the experiment (51 DAS): well-watered plants were watered every day for the entire duration of the experiment, droughted plants were never re-watered after 28 DAS, and re-watered plants experienced multi-day drought (of variable duration) followed by re-
watering for 7 days (Fig. S1A). At 32 DAS, droughted plants were harvested every other day along with well-watered controls. At the end of each harvest day, a group of plants was re-watered and harvested after 7 days of full re-watering (Fig. S1A). At each
harvesting time, we measured/assayed at least 6 plants per treatment (well-watered, droughted, and re-watered). The soil water potential ($\psi_s$) decreased from -0.2 ± 0.1 MPa at the beginning of the drought treatment to a -5 ± 0.1 MPa at the end (Fig. S1B). Low water potential in the dry soil limited evaporation and caused $\psi_s$ to stay constant for plants droughted for longer than 14 days. Hereafter, recovery will be defined as the visual evidence of new above ground growth, according to previous work (Brodribb and Cochard, 2009; Petrov et al., 2015), which described the process of recovery as evidence of reactivated physiological processes and meristematic activity. Throughout the experiment, we measured leaf-level gas exchange of both water vapor and carbon dioxide before dawn (4:00 a.m. – 5:00 a.m.) and during the day (9:00 a.m. – 12:00 p.m.). After 4 days of drought, stomatal conductance ($g_s$) of re-watered plants showed a dramatic drop ($p < 0.01$) with respect to the well-watered controls, and approached near-zero values at day 12 after drought, until the end of the experiment (Fig. 1AB). Only after 6 days of drought did the leaf net assimilation significantly ($p < 0.01$) decrease from 16 ± 0.8 μmol CO$_2$ m$^{-2}$ s$^{-1}$ to 10 ± 0.9 μmol CO$_2$ m$^{-2}$ s$^{-1}$. Re-watered plants, that experienced 12 or more days of drought, recorded negative average values of net assimilation (Fig. 1B). This result reflects elevated leaf respiration, which occurred despite restoring midday soil moisture (%) to pre-drought levels (Fig. S2). The average value for the re-watered cohort was closer to zero at day 14 of drought (Fig. 1A). We ascribe the largest drop in net assimilation (between 8 and 12 days of drought treatment) to the simultaneous decline in leaf water potential ($\psi_l$) (Fig. 1A). Overall, the water potential of the aboveground tissue decreased from -0.2 ± 0.1 MPa to -4.0 ± 0.7 MPa. At night, after an initial significant decline ($p < 0.01$), respiration ($R$) in re-watered plants stayed constant, except at 16 days
of drought when it was not significantly different from zero (Fig. 1A). The difference between night \( R \) and the recorded day \( R \) is likely explained by the significant difference in temperature (32 ± 2°C and 25 ± 4°C) between the day and night conditions in the greenhouse and the metabolic pathways activated by light during day time (Hew et al., 1969). In the droughted cohort some plants showed near-zero \( A \) after 6-8 days of drought when their soil moisture was still close to 20% (Fig. S2). Taking into account the same period of drought, re-watered plants showed restored photosynthetic activity up to ~62% (on average) of that observed in the well-watered controls (Fig. 1A).

In trees, more than one study has shown that prolonged near-zero gas exchange may translate into a higher risk of mortality (Poyatos et al., 2013; Mitchell et al., 2014). It is clear that gas exchange, water transport, and growth decrease during drought, but the temporal sequencing of these events in relation to mortality is still unclear in both herbaceous and woody plants (Mitchell et al., 2013). Considering the high level of segmentation in plants (Zimmermann, 1983; Tyree and Ewers, 1991), the onset of near-zero gas exchange in the droughted plants may be partly the result of sacrificed mature organs of the plant (i.e. leaves), a process that preserves meristematic tissues that are crucial to re-sprouting following stress (Sutinen et al., 1992; Petrov et al., 2015). Our results confirm that near-zero gas exchange is one component of drought stress response. However, here we show that very low values of gas-exchange can appear relatively early in droughted plants, when re-watered plants are (on average) able to recover to levels prior to drought (Fig. 1A). Deleted

In several studies of gymnosperm tree death, visual mortality has been defined as reddening or browning of needles (Anderegg et al., 2012b; Zimmermann, 1983); here we
applied the same approach to \textit{B. rapa} re-watered for 7 days. Since prolonged lack of
meristematic activity (assessed in terms of new visible growth) eventually leads to death
(Sutinen et al., 1992), we scored a plant as dead when the last meristematic tissues
remained brown and brittle in the re-watered plants (Fig. S3A). Also, visual mortality can
be defined as the percentage of replicates plants that fail to recover (100\% being equal to
the entire cohort of re-watered plants) when the stress is relieved (Fig. S3B). For
herbaceous plants, the first visual sign of water stress is wilting of the aboveground
biomass when compared to well-watered controls (Fig. S4AB). In our droughted plants
this loss of turgor pressure was only partly evident on the sixth day of drought treatment
(Fig. S4C), and none of the plants showed visual mortality after 7 days of re-watering
over the same period of drought (Fig. S4E). After 8-10 days of drought, all droughted
plants displayed multiple leaves visibly bleached of pigments (Fig. S4D), corresponding
to the reddening of needles in conifers. However, after 8-10 days of drought, re-watered
plants were able to largely recover gas exchange levels (Fig. 1AB). After 11 days of
drought, several leaves began to crumble when clamped for gas exchange measurements
(Fig. S4F). This symptom was variable among plants and among leaves of the same
plant; nevertheless, recovery of gas exchange after re-watering was still possible among a
small number of plants at this time (Fig. 1A, S2). Interestingly, some re-watered
individuals were able to produce new growth during the recovery time. However,
regardless of the number of days of treatment, the final response to drought stress was the
loss of the active meristematic tissue located at the innermost part of the stem (Fig.
S4GH). Elsewhere, (Sala et al., 2010) it has been suggested that the lack of a vital
meristem could be utilized in mortality assessment. Here, the assessment of visual
mortality confirmed that near-zero gas exchange values in droughted plants do not always indicate a lack of live meristems or an inability to regrow after re-watering. Since individual re-watered plants experienced death at different times during the course of the experiment, we next explored what processes could consistently predict the time of mortality between day 8 (25% of plants visually dead) to day 14 (100% of plants visually dead) of the drought treatment (Fig. S3B). We specifically tested for traits that failed under drought and that could not be recovered after re-watering. Such collapse in one of the measured physiological traits would allow for a reliable definition of mortality and alleviate the need for continued visual assessment of mortality after the stress is relieved.

We tested carbon dynamics by separately examining water-soluble nonstructural carbohydrates (NSC) and starch, in above- and below-ground tissue that was harvested on the same days that gas exchange was measured. Prior studies have shown that the dynamics of carbohydrates in drought varies considerably among species and with the duration and intensity of the stress (Adams et al., 2009; Beck et al., 2011; McDowell, 2011). When separated into above and below-ground components, the dynamics of NSC differed significantly at some time points \( p<0.01 \) but not others, due to variation among replicates and microenvironments, and no significant differences were noted among treatments (Fig. S5A). When compared to the initial content (28 DAS), the NSC for droughted plants (subjected to drought treatment for longer than 8 days) increased more than 100%. Similarly, starch content increased with a maximum gain of 48% in plants droughted for 12 days (Fig. S5B). The trend at the whole-plant level (i.e. the sum of leaf, stem and root contents) clearly shows that the sum of NSC plus starch was not significantly different between well-watered vs. both droughted and re-watered plants.
when compared at either 12 or 14 days of drought treatment (Fig. 2A).

Our results are consistent with previous experiments in which herbaceous plants, short-lived with respect to woody plants, show constant or increased carbon storage
during drought, very likely because of reduced translocation (Müller et al., 2011). An increase in the carbon storage pool (Fig. S5B) can also reflect the role that soluble sugars play in osmotic adjustment and ROS regulation, both of which lead to alkalization of the cytosol that in turn disrupts membrane function (Sundaresan et al., 2015). None of the droughted or re-watered plants experienced carbon starvation going from mild to lethal drought stress in our experiment (Fig. 2A; S5AB). During our experiment stomata closed only two days before all plants were dead (14 days into drought), but near-zero or negative values for daily carbon gas exchange were experienced starting from day 6 in the droughted cohort and day 10 in the re-watered one (Figure S2). Although photosynthesis was low for longer than 5-6 days, several plants recovered, demonstrating that factors in addition to the negative carbon balance determine plant carbohydrate metabolism. Carbon starvation may be a macroscopic symptom of drought at the whole plant scale (McDowell et al., 2008; Adams et al., 2009; Sevanto et al., 2014a), and our results confirm that this mechanism is neither universal nor adequate to describe the time of plant death from drought.

In order to test for hydraulic limitations at decreasing soil water potential values, we calculated the leaf specific hydraulic conductance ($K_L$), using transpiration measurements and pre-dawn and midday leaf water potentials. After 6-8 days of drought treatment, all droughted plants had lower values of $K_L$ that were not significantly different from zero (Fig. 2B). Once re-watered, most replicates (100% after 6 days, 75% after 8 days of drought) recovered gas exchange activity (Fig. 1A). It is noteworthy to mention that stomatal closure is controlled by both hormones and water status of the leaf, with the relative contribution of each likely related to the degree of isohydry (Fuchs and
Livingston 1996; Daszkowska-Golec and Szarejko, 2013; Brodribb and MacAdam, 2013). Deleted Plant water transport influences carbon metabolism in both drought and recovery, as assessed elsewhere in plants with differing drought sensitivity (Tyree and Sperry, 1988; Brodribb and Cochard, 2009; Rolland et al., 2015). However, $K_L$ as measured here is a problematic proxy for embolism and our results cannot completely rule out the significance of hydraulic failure in determining the time of death. Here, the presence of embolism was indirectly assessed and our $K_L$ measurements may be partly influenced by $g_s$ reductions. Although $g_s$ was independent of $K_L$ at the lowest $\Psi_L$ values (data not shown), this may be due to the small difference between pre-dawn and midday water potential when $\Psi_s$ is really low. There is still an open debate on which method is the most accurate measurement of xylem vulnerability (Tixier et al., 2013; Cochard et al., 2013), especially in herbaceous plants but new visual methods such as X-ray tomography and the optical vulnerability method, are showing some promise (Skelton et al., 2017). There is no consensus on how to define hydraulic failure (Anderegg et al., 2012a; Meir et al., 2015; Mencuccini et al., 2015; McDowell et al., 2008; Sevanto et al., 2014a), although it is at times described as the absence of hydraulic conductance after re-watering (Brodribb and Cochard, 2009; Sperry et al., 1988; Mackay et al., 2015). The recovery ability of xylem itself makes hydraulic failure extremely difficult to quantify and with high chance for artifacts (Sperry, 2013). While clear thresholds defining hydraulic failure have been shown (Brodribb and Cochard, 2009), the importance for the amount of time spent by an organ at low leaf water potentials is still debated (Anderegg et al., 2012a; Urli et al., 2013; Sevanto et al., 2014a). Although we confirm hydraulic dysfunction is a clear symptom of drought stress in herbaceous plants, we show that plant hydraulic behavior in
drought is often transient and does not fully determine the potential for recovery upon re-watering. In sum, measures of hydraulic limitations alone are not a reliable metric of plant death from drought.

We tested whether the electrolyte leakage from the combined thylakoid and cellular membranes (Bajji et al., 2001), assessed as the percent increase in electroconductivity, could be used to define membrane failure and thereby provide a reliable indicator of death from drought. In order to do so, we measured the electroconductivity of the incubating solutions from leaf discs, and values were expressed as the percentage of maximum values obtained by subsequently boiling the samples. This percentage increased over time in droughted plant tissue, with 25% of the plants reaching values higher than 80% after 8 days of drought treatment (Fig. 3A). The increase in electroconductivity (%) at death correlated consistently with assessed visual mortality after re-watering (Fig. S3B). None of the plants that were re-watered after 14 days of drought showed any visible signs of recovery (new growth), and all droughted plants showed an electroconductivity increase higher than 80% after 14 days of drought treatment (Fig. S3AB; 3A). Consequently, we advocate the use of electroconductivity increase (%) as a reliable metric for the time of death from drought, alleviating the need for visual assessment of mortality after re-watering.

In a follow-up experiment, utilizing the same experimental design, B. rapa (R500) plants were analyzed for electrolyte leakage at both the leaf and root level. Interestingly, root electrolyte leakage increased at the same rate as leakage in the leaves (Fig. S6), suggesting that, in herbaceous plants, cellular failure may be triggered simultaneously in different organs as supported by recent work in Solanum lycopersicum (Skelton et al.,...
However, in trees and perennial plants, a higher level of hydraulic segmentation may cause differences in the time to death among organs (Tyree and Ewers, 1991; Muller et al., 2011). Electrolyte leakage derives from cellular membrane failure, which has been...
widely studied due to its central role in programmed cell death (PCD) and regulation of
growth, development and differentiation in both plants and animals (Foyer and Noctor,
2003; Pennell and Lamb, 1997). Prior studies have shown that plant cell death can also
result from unbalanced redox homeostasis, and generally an electrolyte leakage threshold
of 50% has been utilized to assess stress damage across species (Bartels, 2001; Smirnoff,
1998). Under optimal conditions, ROS are produced within photosystems II and I, but
antioxidant activity maintains homeostasis. In cases of severe or prolonged
environmental stresses, such as drought, antioxidant capacity can be depleted causing
cellular necrosis through membrane failure.

Our study agrees with previous work on desiccation tolerance mechanisms in
resurrection plants, such as Selaginella or Ramonda spp., where membrane integrity is
crucial for plants, both to withstand drought and to resume metabolic activity in recovery
(Griffiths et al., 2014; Mitra et al., 2014; Gashi et al., 2013; Farrant et al., 2012). In
resurrection plants, mechanisms of cell wall folding and pigment-protein complex
formation cause an increase in $F_{v}'/F_{m}'$ (maximum efficiency of photosystem II in light
conditions) and serve as protective strategies against photo-inhibition and ROS
accumulation. However, these defensive processes, which allow quick recovery after
rehydration in resurrection plants, can fail, allowing for fatal dehydration and death.
Because variable fluorescence signals specifically require the presence of intact
photosystems to arise (Franck et al., 2002), we speculated that in case of drought stress,
membrane damage could be the primary cause for the impairment in photosystem
activity. Then, we tested if variable fluorescence correlates with electrolyte leakage
among plants in well-watered to extremely droughted conditions. Using a whole-plant
imaging fluorometer, we found a substantial decrease in variable chlorophyll \( a \) fluorescence (\( F_{v} \)) with increasing drought (Fig. 3B). At each harvesting time, droughted plants with an electroconductivity increase greater than 80% also showed no sign of variable chlorophyll \( a \) fluorescence (Fig. 3AB). The visible decrease of \( F_{v} \) during the drought treatment resulted in a steady and significant decline in the average \( F_{v}/F_{m} \) with respect to well-watered plants (maximum efficiency of photosystem II under dark acclimated conditions) for both droughted and re-watered plants (Fig. S7).

Both the percent electroconductivity increase and the image of \( F_{v} \) in droughted plants (Fig. 3AB) correlated with the visual assessment of percent mortality of re-watered plants (Fig. S3AB). Visible mortality reached 100% for re-watered plants experiencing 14 days of drought (Fig. S3). Correspondingly, 100% of droughted plants showed an electroconductivity increase higher than 80% and no sign of variable chlorophyll \( a \) fluorescence after 14 days of drought treatment (Fig. 3AB). Both electroconductivity increase and fluorescence have been identified previously as direct, mechanistic indicators of abiotic and biotic stress at the leaf level, and they are systematically interconnected (Griffiths et al., 2014; Babu et al., 2004). However, these two traits have never been considered in the debate on the causes of drought-induced plant mortality, and none of the current models accounts for them (Steinkamp and Hickler, 2015; Anderegg et al., 2012a; Choat, 2013; Meir et al., 2015; Mencuccini et al., 2015).

Plant survival during our experiment was variable; single plants experienced mortality between day 8 and 14 of drought (Fig. S3AB). Figure S8 shows the single values of \( F_{v}'/F_{m}' \) (maximum efficiency of PSII in light-acclimated samples), here recorded with the pulse amplitude method (PAM), plotted against \( \Psi_{L} \). At the onset of the
stress, PAM measurements indicate there was high variation in photosynthetic activity, consistent with high variability in the gas exchange values at that stage (Fig. 1; Fig. S2); for both traits, this variation decreased when the stress became more severe. This physiological variability might be related to the use of a semi-controlled environment such as a greenhouse, where each plant can experience changes in temperature, light, and ventilation that may differentially amplify or mitigate the severity of drought among replicates. This physiological variability affected the survival of each plant, regardless of drought and re-watering. Within the droughted treatment, fresh aboveground biomass values at harvesting correlated with survival, but this trait correlated only weakly ($R^2 < 0.2$) with survival in re-watered plants (Fig. S9). Fresh biomass at harvest might be a good indicator of survival in the cohort of droughted plants but not for re-watered plants.

Utilizing the same experimental design, a second genotype of *B. rapa*, imb211, showed identical trends to R500 in all measured physiological traits. Relative to R500, imb211 is differentiated at 331K SNPs (Devisetty et al., 2014), flowers 51% earlier, and accumulates less biomass. As expected, the changes in gas exchange observed in R500 also preceded mortality in imb211, but the timing was slower, with negative net assimilation occurring after 14 days of drought, near-zero gas exchange values occurring at 18 days, and 100% visual mortality occurring in re-watered plants only after 22 days of drought treatment. In imb211, hydraulic limitation started later, and this genotype died after a longer period of drought, most likely due to its smaller size with respect to R500 and its correspondingly slower use of soil water (Fig. S10). Despite the substantial genetic and phenotypic differentiation between these genotypes, variable chlorophyll $a$ fluorescence and percent increase in electroconductivity remained the best indicators of
death (data not shown).

Analysis of conifer needles reinforces our hypothesis that fatal membrane failure is the proximal cause of mortality at a cellular scale and can be used as a direct, mechanistic metric of mortality in extremely different plant types. Branches from Engelmann spruce and lodgepole pine were collected from the Medicine Bow Mountains, Wyoming, USA (see Materials and Methods). Sampled trees included a variety of ages, dominance, and health status due to attack by bark beetles, which results in death by desiccation due to blue-stain fungi occlusion of xylem. Branches were used in a bench-drying assay to define the time of death under drought; undamaged green branches were included, to control for the additional stress imposed by beetle attack. In 2013, branches were collected in the fall, and death occurred in the needles over the course of the following 38 days in the lab. In 2014 branches were collected in early summer, and died over the course of 10 days; more rapid death in 2014 is likely attributable to the lower initial water content. In contrast to the experiments with B. rapa, it was not possible to apply a re-watering treatment to the branches collected from the field, and death was scored as the time when all needles were completely brown (Mitchell et al., 2013; Anderegg et al., 2012b; Zimmermann, 1983).

For both species, in both field and lab conditions, the time-course of measured sugars (Fig. S11AB) over time was indicative of needle death. Also, a plot of electroconductivity increase (\% versus $\Psi_L$) (Fig. S11C) shows how leaf water potential, was not significantly correlated to death assessed as complete browning of the needles. For spruce only undamaged green needles from the field were analyzed because this species turns yellow and loses its deleted needles upon beetle attack (Wiley, 2015).
Interestingly, the percent electrolyte leakage results pooled from all field samples showed two peaks in the lower and upper end of the distribution (Fig. S12B). In both years, the bench-drying experiment provided better temporal resolution of membrane failure following drought (Fig. S12A). However, in both conifer species, $Fv'/Fm'$ and electrolyte leakage were good indicators of viability and eventual death, as was the case in imb211 and R500 (Fig. 4).

Overall, our results show that both carbon starvation, if present, and hydraulic limitations are macroscopic signals of mortality, which take place at whole-plant scale.
Theoretical mechanism of plant mortality

PLANT SCALE

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<th>Prolonged stress events</th>
<th>Hydraulic Failure</th>
<th>Carbon Starvation</th>
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<td>Relative short &amp; strong stress events</td>
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CELL SCALE

<table>
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<th>Membrane Failure</th>
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<td>Membrane Disruption</td>
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OUTCOME

| Plant Death |

(A) (B) (C) (D)

Figure 5: Theoretical framework of plant mortality. (A) In nature there are several biotic or abiotic stressors, which can have different duration and/or intensity. (B) At the onset of the stress, diverse symptoms appear at the whole-plant scale, e.g. in case of a drought event, hydraulic failure and carbon starvation are two symptoms of the stress. Although one or another symptom can be prominent or multiple symptoms can occur at the same time, (C) they all eventually lead to membrane disruption. Complete membrane failure can be considered the most proximal cause of plant mortality. (D) Death is the final outcome when the plant reaches the species-specific threshold of membrane disruption.

and they can be recovered to certain extent (Fig. 2AB). Drought events may vary in intensity and duration causing either of these two macroscopic symptoms to prevail over the other (Fig. 5AB). Simultaneously, either one or both these distal symptoms may trigger membrane disruption at a cellular scale (Fig. 5C). When the stress is severe, plants can cross a threshold to complete membrane failure, which results in plant death (Fig. 5D). Dehydration may lead to fatal membrane failure through either physical or biochemical effects in cells. In the first case, the lack of water will alter the osmotic potential of cells, leading the cell membrane structures to collapse. Alternatively, ROS production along with pigment depletion causes the deactivation of fatty acids and
proteins, again causing failure of the membrane structure. Membrane failure may take
place in cases of several environmental stresses (i.e., insect outbreak, temperature stress,
light stress), suggesting that a wide range of applications exist for the indicators of
mortality that we defined (Smirnoff, 1998; Spichal, 2015).

Based on the results of our drought and re-watering experiment, we functionally
define plant mortality as a threshold in membrane disruption from which plants cannot
recover. Our proxies for point of death, e.g. fluorescence and electrolyte leakage, are very
likely correlated to hydraulic failure, even though further and more direct analyses of
embolism are needed to confirm this hypothesis.

We confirmed fluorescence to be a non-destructive and rapid screening method to
assess the degree of drought stress from mild to lethal and we suggest the loss of variable
fluorescence as an operational definition of plant mortality. Passive solar-induced
fluorescence is currently a fast and reliable tool to evaluate canopy-scale fitness through
satellite imaging of large vegetated areas (Guanter et al., 2014; Joiner et al., 2011;
Schlau-Cohen and Berry, 2015). As indicated in Porcar-Castell et al. (2014), future work
will combine spectrally resolved fluorescence of large vegetative areas and PAM (Pulse
Amplitude Method) measurements at the leaf scale, incorporating a more mechanistic
understanding of fluorescence signals to eco-physiological implications.

**Conclusions:**

During the last decade, the scientific community has focused on determining
hydraulic thresholds and sugar content levels that possibly lead to plant death. However,
modeling these traits has not successfully predicted plant mortality (Anderegg et al.,
2012a; Anderegg et al., 2012b; McDowell, 2011; Brodribb and Cochard, 2009; Skelton et
No current model is capable of establishing the exact time of death, namely when a plant does not recover upon re-watering. Defining the exact time of death, rapidly and at large spatial scales, will enable ecosystem and land surface models to more accurately predict climate change-induced drought mortality, including whether precipitation events are likely to occur in time to prevent cell membrane failure. Future work is necessary to clarify the heterogeneity of chlorophyll \( a \) fluorescence in leaves of different species (Bresson et al., 2015) and to define mortality thresholds for deciduous and evergreen plants. For these species the electroconductivity measurements should still be reliable on twigs, even if some methodological adjustment is necessary. In regards to re-sprouting species, possibly circumventing permanent cellular damage at the leaf scale, we can be quite confident in proposing the utilization of both fluorescence and electroconductivity to assess mortality. In fact, \textit{Brassica rapa} (R500) was flushing out new growth in some re-watered individuals, but this did not change our conclusions since re-sprouting is only possible with an active meristematic tissue.

Our results represent a crucial path to accurate mechanistic modelling of plant death and hence of shifts in ecosystem services that will occur as a consequence of plant mortality under climate change. The next challenge for plant researchers will be to estimate the structural and/or metabolic causes of cellular breakdown and to define species- and genotype-specific thresholds for electrolyte leakage. For instance, we advocate the analysis of ROS pools (Foyer and Noctor, 2003; Bartels, 2001) to complement current models for plant mortality. Because an accurate quantification of these highly reactive molecules is challenging (Noctor et al., 2016), lipid and protein peroxidation or glutathione accumulation are among candidate proxies for estimating...
ROS-dependent changes in tissue redox state (Noctor and Foyer, 2016; Kranner et al., 2006). Our new insights into the mechanisms of plant mortality will assist crop breeding for drought resistance, by enabling rapid phenotypic screens of death and survival. Similar to predicting the leaf water potential that leads to stomatal closure and transport failure (Breshears et al., 2009), mortality mechanisms can be implemented in process models to predict the timing of the electrolyte leakage, and these mechanisms can allow for more accurate predictions of plant mortality at the ecosystem level and beyond.

Materials and Methods:

Seeds of *Brassica rapa*, var. *Yellow Sarson* (R500) were planted in pots (500 ml) filled with a soil mixture (Miracle-Gro Moisture control Potting Mix (20% v/v), Marysville, OH, USA and Profile Porous Ceramic (PPC) Greens Grade (80% v/v), Buffalo Grove, IL, USA) with the addition of 2 ml of Osmocote 18-6-12 fertilizer (Scotts, Marysville, OH, USA) to each pot. Plants were grown in a greenhouse (1,800 μmol of photons m⁻² s⁻¹ maximum PPFD, 16-h photoperiod, 25-30°C /18-22°C day/night). For 4 weeks after sowing, all plants were regularly watered to maintain volumetric soil water content at 38 ± 5 % (ECH₂O probe, Decagon, Pullman, WA, USA). On day 29 after sowing (DAS), watering was withheld, starting an incremental decrease in the volumetric soil water content for the droughted plants (Fig. S1). At this point, every other day subsets of at least 6 plants were measured and harvested from the droughted plants along with 8 well-watered controls (Fig. S1A). At the end of each harvesting day, subsets of 6 droughted plants were re-watered for the subsequent 7 days.
Starting on 39 DAS, every other day subsets of at least 6 re-watered plants were measured and harvested along with 6 well-watered controls of the same age (Fig. S1A). The controls did not show any significant variation in their gas exchange, which might be anticipated if plants were senescing, over the course of the experiment. Each day, droughted and re-watered plants were visually examined and photographed for mortality assessment (Fig. S3). Plants were considered living if they maintained any meristematic growth and/or leaf production. For the determination of leaf water potential ($\Psi_L$), plants were cut at the base of the stem at ~ 5:00 a.m. and at 1:00 p.m. and the main stalk was immediately inserted into a Scholander pressure chamber (Model 600 Pressure Chamber Instrument, PMS Instrument Company, Albany, Oregon, USA) (Koide et al., 2000). To determine the response to the drought/recovery treatment, gas exchange (Long and Bernacchi, 2003) was measured on the youngest fully expanded leaves every other day. At every time point, photosynthetic capacity ($A_{max}$) and stomatal conductance ($g_s$) were measured, using two portable gas exchange systems (LI-COR-6400XT; LI-COR Biosciences Inc., Lincoln, NE, USA). All spot measurements were taken from 9:00 a.m. to 12:00 p.m. in the greenhouse where plants were growing, and environmental conditions in the cuvette matched ambient conditions in the greenhouse. Specifically, the cuvette settings were: flow rate, 300μmol s$^{-1}$; CO$_2$, 400μmol mol$^{-1}$; VPD, 1.1 – 3.2 kPa and the cuvette fan was set to the fast mode, with temperature at 26°C. For each replicate ($n$ = 6 to 8), gas exchange values were recorded after stabilization of the readings (3-6 min). Night respiration measurements were taken with the same cuvette settings except in the dark and temperature set at 22°C; measurements were taken predawn (4:00 a.m. to 5:00 a.m.), using a dim green light for visibility. Along with daytime gas exchange,
chlorophyll *a* fluorescence measurements were taken using two leaf chamber fluorometers (Li-COR 6400-40, LI-COR Biosciences Inc., Lincoln, NE, USA). After gas exchange was recorded, with an actinic light of 800 μmol photons m\(^{-2}\) s\(^{-1}\) (λ = 470 nm, 10% blue), a saturating pulse (0.800 sec; ~3,000 μmol photons m\(^{-2}\) s\(^{-1}\)) was applied. Values of steady-state fluorescence (*F*\(_{s}\)), minimum fluorescence after light induction (*F*\(_{o}'\)), maximum fluorescence in light conditions (*F*\(_{m}'\)), the maximum efficiency of photosystem II in light conditions (\((Fm'-Fo')/Fm'=Fv'/Fm'\)), and the efficiency of photosystem II photochemistry (\((Fm'-F_s)/Fm'=ΔF/Fm'=Φ_{PSII}\)) were calculated. A short (3 sec) Far-Red pulse was used to record the *F*\(_{o}'\) value at the end of the induction (Baker, 2008). Each day, chlorophyll *a* fluorescence was also monitored at predawn (\(*F_v/Fm*, maximum efficiency of photosystem II in dark adapted conditions) and midday conditions (\(*F_v'/Fm'*, maximum efficiency of photosystem II in light conditions) with a hand-held fluorimeter (Fluorpen, PSI, Brno, Czech Republic) (Humplík et al., 2015).

Measurements of *F*\(_v/Fm* at predawn were taken according to Murchie and Lawson, 2013. For every measurement, only leaf material naturally dark-adapted overnight in the greenhouse was utilized. Using large aluminum foil sheets each plant was kept darkened continuously until the measurements of *F*\(_v/Fm* were complete. Initially, for a subset of plants, the FluorPen’s detector was applied onto the leaf with the measuring beam off, ensuring a reading of zero for *F*\(_o*). After switching the measuring beam on, we quantified *F*\(_o* three times at one minute intervals to ensure that neither quenching nor fluorescence variations introduced error. The measuring light of the FluorPen was set at ~1,500 μmol photons m\(^{-2}\) s\(^{-1}\) throughout the experiment. Then, we applied a saturation pulse at ~2,200 photons μmol m\(^{-2}\) s\(^{-1}\) to obtain *F*\(_v/Fm* At midday, before each set of measurements,
ambient light conditions were determined with a portable light meter (Li-COR 250, LI-COR Biosciences Inc., Lincoln, NE, USA) and the internal light source of the FluorPen matched to the average recorded value. Throughout the experiment, the actinic source was maintained between 700 and 1,000 μmol photons m\(^{-2}\) s\(^{-1}\). After placing the cuvette on each leaf and allowing for a three-minute acclimation period, we measured \(F_s\) three times at one minute intervals to make sure it was the true \(F_s\) and that no quenching or fluorescence variation occurred between measurements. After three minutes, \(F_{v'}/F_{m'}\) was measured using a saturation pulse (0.800 sec; ~2,200 photons μmol m\(^{-2}\) s\(^{-1}\)).

Calculations of \(F_o'\) used the following equation from Oxborough and Baker (1997) where \(F_o'=F_o/(F_vF_m+F_o/F_{m'})\). Drought affected both \(F_{v'}/F_{m'}\), measured with the FluorPen, and \(\Phi_{II}\), measured with the Li-COR 6400-40, in the same fashion, and no significant difference was found at any time between the methods (data not shown). To avoid redundancy, we present only data collected with the FluorPen, because this instrument is both accurate and allows for higher phenotyping throughput than the Li-COR 6400-40.

A kinetic fluorescence imaging system (Closed FluorCam, PSI, CZ) was used to measure chlorophyll fluorescence kinetics parameters on intact leaves according to Schreiber et al. (1986). At predawn, dark-adapted plants were exposed to a short (0.800 s) pulse of saturating blue light (6000 μ mol photons m\(^{-2}\) s\(^{-1}\); \(\lambda = 470\) nm) provided by the camera photodiode (Woo et al., 2008). At midday, samples were dark adapted for 20 min prior to all measurements in light in order to allow for the closure of the reaction centers. Midday fluorescence measurements (~800 μ mol photons m\(^{-2}\) s\(^{-1}\); \(\lambda = 470\) nm) were recorded at light levels that matched the average light conditions in the greenhouse with actinic light applied for 15 minutes.
Water-soluble sugars in the collected extracts were determined using the anthrone method (Seifter et al., 1950). At dusk every other day plant material was collected, flash-frozen and pulverized. After air-drying, 0.1 g of the resulting sample was put into a 15-ml centrifuge tube and 10 ml of 80% ethanol were added. The mixture was incubated at 80°C in a water bath for 30 min, and then centrifuged at 4000 RPM for 5 min. The pellets were extracted two more times with 80% ethanol. An aliquot of the extract was hydrolyzed in 5 ml anthrone solution (4 g anthrone in 1000 ml 95% H2SO4; Sciencelab.com, Houston, TX) in a boiling water bath for 15 min. After cooling, the sugar concentration was determined spectrophotometrically (UV/Vis BioSpec Mini Shimadzu) at 620 nm. Glucose (Sigma Aldrich, Inc., St. Louis, MO) was used as a standard. The sugar concentration was calculated on a dry matter basis (% DW). The ethanol-insoluble pellet was used for starch extraction. Ethanol was removed by evaporation. Starch in the residue was released in 2 ml distilled water for 15 min in a boiling water bath. After cooling to room temperature, 2 ml of 9.2 M HClO4 were added. Starch was hydrolyzed for 15 min. Distilled water (4 ml) was added to the samples. The samples were then centrifuged at 4000 RPM for 10 min. The pellets were extracted one more time with 2 ml 4.6 M HClO4. Supernatants were retained, and the starch concentration was measured spectrophotometrically at 620 nm using an anthrone reagent. Glucose was used as a standard. The starch concentration was described on a dry matter basis (% DW).

In order to determine the time course of electrolyte leakage during the drought and recovery, two leaf discs (1 cm²) from the uppermost fully expanded leaf blade of each plant were collected and immediately weighed (Bajji et al., 2001; Babu et al., 2004).
Discs from the same leaf were put into 20 ml of deionized water in a test tube and an initial electrical conductivity measure ($EC_i$) was taken (BlueLab ECmeter, NZ). Then, the tubes containing the discs were left in the dark at room temperature, and a second measurement ($EC_f$) of the same sample was recorded after 24 hrs. Following these readings, samples were boiled in a waterbath, cooled at 25°C, and the total electrical conductivity ($EC_t$) was measured. Electrolyte leakage was expressed as: \[ \frac{(EC_f - EC_i)}{(EC_t - EC_i)} \times 100 \]. This same procedure was later utilized in a follow-up experiment where B. rapa (R500) plants were also analyzed for electrolyte leakage from roots. In that occasion, ~0.01 g of root material were also collected to determine the time course of electrolyte leakage during the drought and recovery time. The same experimental protocol and growth environment were utilized to obtain cohorts (well-watered, droughted and re-watered) of imb211 plants. In this case, the recovery was performed until 22 days of drought treatment with harvesting and measurement days prolonged to day 56 after sowing for droughted plants and 63 for the re-watered ones. All techniques described for R500 were also applied to imb211 genotype.

Similar techniques were used on conifer needles collected from the field. Lodgepole pine (Pinus contorta) and Engelmann spruce (Picea engelmannii) branch samples were harvested from the field during the summers of 2013 and 2014. Samples were collected from a variety of field sites located in Wyoming’s Medicine Bow Mountain, 55 km west of Laramie, WY (41°20'39"N, 106°12'47"W) with altitude ranging from 2700 to 3200 m. Sampled trees included a variety of ages, dominance, and health status due to attack by bark beetles, species Dendroctonus ponderosae and Dendroctonus rufipennis, which causes mortality by hydraulic failure from blue-stain fungal occlusion.
of xylem (Wiley et al., 2015; Hubbard et al., 2013; Frank et al., 2014). Immediately after branch collection, needles were removed to test chlorophyll $a$ fluorescence, water potential, carbohydrates, and electrolyte leakage. After these initial tests, a subset of the branches was left out to bench dry in uncontrolled lab conditions and was systematically resampled until needle death (electrolyte leakage >80%). The bench drying was required to populate the center of the distribution for trees because biweekly measurements in the field were not sufficient to capture this dynamic. For all measured traits, we used analysis of variance (ANOVA) to test for significant differences between treatments (R 2.15.1 GUI 1.52, Development Core Team, 2012).

Acknowledgements

We thank Kaleb Kenneaster, William Mandeville and Sam Kacmarsky for assistance running experiments. We thank Urszula Norton for the use of the spectrophotometer, and Jon Pleban, Scott Mackay and Steven Jansen for comments on an earlier version of the manuscript.
**Figure legends:**

**Figure 1:** Water vapor (A), leaf carbon dioxide exchange and leaf water potential ($\Psi_L$) (B) for re-watered R500 *Brassica rapa* plants. Specifically: leaf net assimilation (green), stomatal conductance, $g_s$ (sky blue), and night respiration, $R$ (navy) measured on leaves of plants that experienced different lengths of drought treatment (from 0 to 16 days) and were all re-watered for 7 days. $\Psi_L$ (violet red) progression over the days was measured on the same plants after the gas exchange assessments. Error bars are standard errors ($n = \text{at least 6}$).

**Figure 2:** (A) Sugar pools (sum of nonstructural carbohydrates and starch) at the whole-plant level (sum of roots, stem, and leaves) in droughted and re-watered R500 *Brassica rapa* plants and in well-watered controls. The values refer to day 12 and day 14 into the drought treatment. Contents are referred to dry weight and error bars are standard errors ($n = 8$). (B) Leaf specific hydraulic conductance ($K_L$) relationship to soil water potential ($\Psi_S$) changes in well-watered (blue rectangle), droughted (red rectangle) and re-watered (grey rectangle) R500 *Brassica rapa* plants. Each circle and triangle indicates a single plant measurement. For droughted plants (red rectangle), to clarify the time sequence of the drought treatment, each small rectangle (yellow, gold, orange, light red, firebrick red) groups together the measurements taken on the same day and the labels indicate the number of days into drought treatment. Correspondently, for re-watered plants (big gray rectangle) the length of drought experienced prior to re-watering is indicated by different colors (yellow, gold, orange, red, firebrick red).

**Figure 3:** (A) Electrolyte leakage (solid dots), measured as percent electroconductivity increase (%) in droughted R500 *Brassica rapa* plants ($n = 8$) across Days of Drought
Treatment. The correspondent midday soil water content (%) for each plant is represented by open triangles. Each dot and triangle indicates a single plant measurement, green and red representing live and dead plants. (B) Representative fluor-CAM pictures of chlorophyll a fluorescence (actinic light: 800 μmol photons m⁻² s⁻¹; pulse: 0.800s, 6000 μmol photons m⁻² s⁻¹; λ = 470 nm) as variable fluorescence (Fv) for droughted R500 Brassica rapa plants at increasing drought stress. At each time point, the top row represents plants with the highest values of Fv (values averaged across the same plant), while the bottom row the lowest values (values averaged across the same leaf).

**Figure 4:** Electrolyte leakage, measured as electroconductivity increase (%), and the corresponding Fv’/Fm’ (maximum efficiency of photosystem II in light conditions, measured with Fluor-CAM; actinic light: 800 μmol photons m⁻² s⁻¹; pulse: 0.800s, 6000 μmol photons m⁻² s⁻¹; λ = 470 nm), across species and drought levels. Both droughted and re-watered plants of R500 and imb211 genotypes of Brassica rapa, which all experienced 8, 10, 12 or 14 days of drought, are presented. Field droughted branches of Picea engelmannii and Pinus contorta measured right after harvesting and bench-droughted branches measured at four different time points after the harvesting day. For all species and treatments error bars are standard errors (n= at least 8) and R = 0.85.

**Figure 5:** Theoretical framework of plant mortality. (A) In nature there are several biotic or abiotic stressors, which can have different duration and/or intensity. (B) At the onset of the stress, diverse symptoms appear at the whole-plant scale, e.g. in case of a drought event, hydraulic failure and carbon starvation are two symptoms of the stress. Although one or another symptom can be prominent or multiple symptoms can occur at the same time, (C) they all eventually lead to membrane disruption. Complete membrane failure
can be considered the most proximal cause of plant mortality. (D) Death is the final outcome when the plant reaches the species-specific threshold of membrane disruption.

**Supplemental figure legends:**

**Figure S1:** (A) Experimental design with days of harvesting/measurement for well-watered (blue), droughted (brown) and re-watered (yellow) *Brassica rapa* R500 plants.

**Figure S2:** Leaf net carbon assimilation of well-watered controls (green), droughted (red) and re-watered (orange) R500 *Brassica rapa* plants (*n* = at least 6) over the days of drought treatment.

**Figure S3:** (A) Schematic of re-watered plant cohorts over the days of drought.

**Figure S4:** Pictures of well-watered R500 *Brassica rapa* plants utilized as controls at (A) day 34 and (B) 42 after sowing (DAS).

**Figure S5:** (A) Nonstructural carbohydrates (NSC) progression in well-watered (blue), droughted (red) and re-watered (yellow) R500 *Brassica rapa* plants after 10 days from the start of the drought treatment.

**Figure S6:** Electrolyte leakage, measured as percentage increase of electroconductivity in re-watered R500 *Brassica rapa* plants from a follow-up experiment, utilizing the same experimental design but harvesting from both leaves and roots.

**Figure S7:** *Fv*/*Fm* (maximum efficiency of photosystem II in dark acclimated conditions) normalized to the well-watered controls (100) for droughted (orange) and re-watered (green) plants from 4 to 14 days of drought.

**Figure S8:** Spot measurements of *Fv’*/*Fm’*, as maximum efficiency of photosystem II in light conditions (actinic light: 800 μmol photons m⁻² s⁻¹; pulse: 0.800s, 6000 μmol photons m⁻² s⁻¹; λ = 470 nm), measured with the Fluorpen for well-watered (green),
droughted (red) and re-watered (yellow) R500 Brassica rapa plants.

**Figure S9:** Fresh aboveground biomass in droughted (solid triangles) and re-watered (solid squares) R500 Brassica rapa plants \((n = 8)\) over the days after sowing (DAS).

**Figure S10:** Soil water potential values \((\Psi_S)\) for the two Brassica rapa genotypes, R500 (blue) and imb211 (pink) during the drought period.

**Figure S11:** Figure S11:(A) Non-structural carbohydrates (NSC) and (B) starch content in dry weight for needles of Pinus contorta (red triangles) and Picea engelmannii (brown dots).

**Figure S12:** (A) Electrolyte leakage, measured as percentage increase of electroconductivity for both healthy, un-attacked needles from Pinus contorta and Picea engelmannii air-dried in the lab during the Summer of 2013 (blue) and the Fall of 2014 (black).

**Figure S1:** (A) Experimental design with days of harvesting/measurement for well-watered (blue), droughted (brown) and re-watered (yellow) Brassica rapa R500 plants. The x-axis represents the time of the experiment in days after sowing (DAS), broken axis represents the initial four weeks of well-watering regime for all cohorts of plants. The dotted red line represents the start of the drought after four weeks from sowing for droughted and re-watered plants. (B) Soil water potential progression for well-watered (blue), droughted (brown) and re-watered (yellow) Brassica rapa R500 plants after the start of the drought treatment.

**Figure S2:** Leaf net carbon assimilation of well-watered controls (green), droughted (red)
and re-watered (orange) R500 *Brassica rapa* plants (*n* = at least 6) over the days of drought treatment. Numbers represent the days into the drought treatment. At every time point the well-watered controls and droughted plants were the same age.

**Figure S3:** (A) Schematic of re-watered plant cohorts over the days of drought. Visual mortality (red) was assessed as cessation of new meristematic tissue after 7 days of re-watering and expressed as a percentage of dead plants in the re-watered cohort. Green represents the plants considered still alive. (B) The percentage of visual mortality (red) is inversely proportional to the recovery (%) of physiological traits (green) over the days of drought treatment. The time of death for all plants in the re-watered cohort: none of the re-watered plants (*n* = 8) subjected to a previous drought treatment of 14 days showed any new meristematic growth (equal to 100% visual mortality) at recovery assessment.

**Figure S4:** Pictures of well-watered R500 *Brassica rapa* plants utilized as controls at (A) day 34 and (B) 42 after sowing (DAS). Droughted *Brassica rapa* plants pictures at (C) 34 or (D) 42 DAS correspondent to 6 and 14 days of drought treatment. Re-watered plants at (E) 41 or (F) 47/49 DAS. (G-H) Close-up pictures of an active meristematic tissue and lack of active meristematic tissues from a dead plant. Both are pictures of re-watered plants subjected to a drought treatment of 10 or 14 days, respectively.

**Figure S5:** (A) Nonstructural carbohydrates (NSC) progression in well-watered (blue), droughted (red) and re-watered (yellow) R500 *Brassica rapa* plants after 10 days from the start of the drought treatment. For each treatment above-ground (stems and leaves) (Ag) and below-ground (roots) (Bg) contents are shown. Concentrations are referred to dry weight and error bars are standard errors (*n* = 8). Letters represent the significance of *P* < 0.01 for droughted plants between time points. (B) Percentage change in
concentration of nonstructural carbohydrates (NSC) (purple) and starch (cornsilk) for
droughted (D) and well-watered (W) plants after 8, 10 and 12 days of drought treatment.
Days of drought correspond to 36, 38 and 40 DAS, respectively. Percentage change was
calculated with respect to the time zero of drought (28 DAS). Contents are referred to dry
weight and error bars are standard errors (n = 8).

**Figure S6:** Electrolyte leakage, measured as percentage increase of electroconductivity
in re-watered R500 *Brassica rapa* plants from a follow-up experiment, utilizing the same
experimental design but harvesting from both leaves and roots. At increasing drought
stress (at 1, 7 and 10 days of drought), the leakage was measured on both leaves (green)
and roots (dark red). Error bars are standard errors (n = 8). Letters represent the
significance of $P < 0.01$ between treatments and time points.

**Figure S7:** $F_v/F_m$ (maximum efficiency of photosystem II in dark acclimated conditions)
normalized to the well-watered controls (100) for droughted (orange) and re-watered
(green) plants from 4 to 14 days of drought. Error bars are standard errors (n = 8). Stars
represent the significance of $P < 0.01$ (*) or $P < 0.001$ (**).

**Figure S8:** Spot measurements of $F_v'/F_m'$, as maximum efficiency of photosystem II in
light conditions (actinic light: 800 μmol photons m$^{-2}$ s$^{-1}$; pulse: 0.800s, 6000 μmol
photons m$^{-2}$ s$^{-1}$; $\lambda = 470$ nm), measured with the Fluorpen for well-watered (green),
droughted (red) and re-watered (yellow) R500 *Brassica rapa* plants. The values are
plotted against the leaf water potential ($\Psi_L$). Numbers represent the days into the drought
treatment. At every time point the well-watered controls were as old as the droughted
plants.

**Figure S9:** Fresh aboveground biomass in droughted (solid triangles) and re-watered
(solid squares) R500 Brassica rapa plants \((n = 8)\) over the days after sowing (DAS).

Each triangle and square indicates a single plant measurement, green and red representing live and dead plants.

**Figure S10:** Soil water potential values \(\left(\Psi \right)\) for the two Brassica rapa genotypes, R500 (blue) and imb211 (pink) during the drought period. Days of drought from 0 to 23 correspond to 28 DAS to 51 DAS. Error bars are standard errors \((n = 8)\). Pictures (right top corner) compare the size of the two genotypes at 32 DAS in well-watered conditions.

**Figure S11:** Figure S11: (A) Non-structural carbohydrates (NSC) and (B) starch content in dry weight for needles of Pinus contorta (red triangles) and Picea engelmannii (brown dots). Values in the green and red shaded areas correspond to field data. Values in the central white area show the behavior of healthy needles dried in the lab over 30 days. Error bars are standard errors \((n = \text{at least } 6)\). For the same samples (C) the leaf water potential \(\left(\Psi \right)\) is reported versus the electroconductivity increase (%), here used as a proxy of needle death.

**Figure S12:** (A) Electrolyte leakage, measured as percentage increase of electroconductivity for both healthy, un-attacked needles from Pinus contorta and Picea engelmannii air-dried in the lab during the Summer of 2013 (blue) and the Fall of 2014 (black). (B) Histogram of the distribution of field observations for electrolyte leakage. Un-attacked needles are represented in green while Beetle-attacked needles are shown in red.

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