

Introduction:

Drought is a major constraint limiting plant growth and threatening global food security. Epicuticular waxes play a key role in reducing non-stomatal water loss and improving drought tolerance. In barley, variation in wax load, structure, and composition affects cuticle permeability, reflectivity, and water retention. Another adaptive strategy is earliness, enabling a drought-escape mechanism in varieties from arid environments (Ogradowicz et al. 2023). However, the combined effects of wax traits and heading date on drought resilience across diverse barley genotypes remain poorly understood.

This study examines drought-induced responses in barley lines differing in wax phenotypes and earliness. Our goal is to elucidate how wax characteristics and developmental timing jointly shape drought tolerance, offering insights for breeding more resilient cereal crops. In this study, we applied a Random Forest (RF) machine learning framework to predict treatment identity based on a comprehensive phenomic dataset including morphological and physiological traits. This approach enabled the identification of the most informative traits differentiating the studied genotypes, independent of environmental treatment, and revealed key phenotypic markers capturing genotype-specific variation.

Plant material, experimental setup and methods

Five spring barley DH lines with contrasting wax phenotypes and heading times were examined (Table 1). The study included cross combinations between BW1 (Bowman cer mutant, glossy, early-heading genotype), two reference lines with strong and very strong wax coverage (Wa1, Wa2), an old Polish cultivar Lubuski, and a Syrian breeding line CamB. These genotypes represent diverse combinations of wax load and developmental timing, enabling assessment of their responses to drought stress (Fig. 1).

Table 1. Description of the plant materials

Genotype	Leaf surface type	Description
CamBW1	Non-waxy (glossy)	a glossy line derived from a cross between CamB × BW408
CamWa1	Waxy (glaucous)	a glaucous line derived from a cross between CamB × Wa1
CamWa2	Waxy (glaucous)	a glaucous line derived from a cross between CamB × Wa2
LubBW1	Non-waxy (glossy)	a glossy line derived from a cross between Lubuski × BW408
LubWa1	Waxy (glaucous)	a glaucous line derived from a cross between Lubuski × Wa1

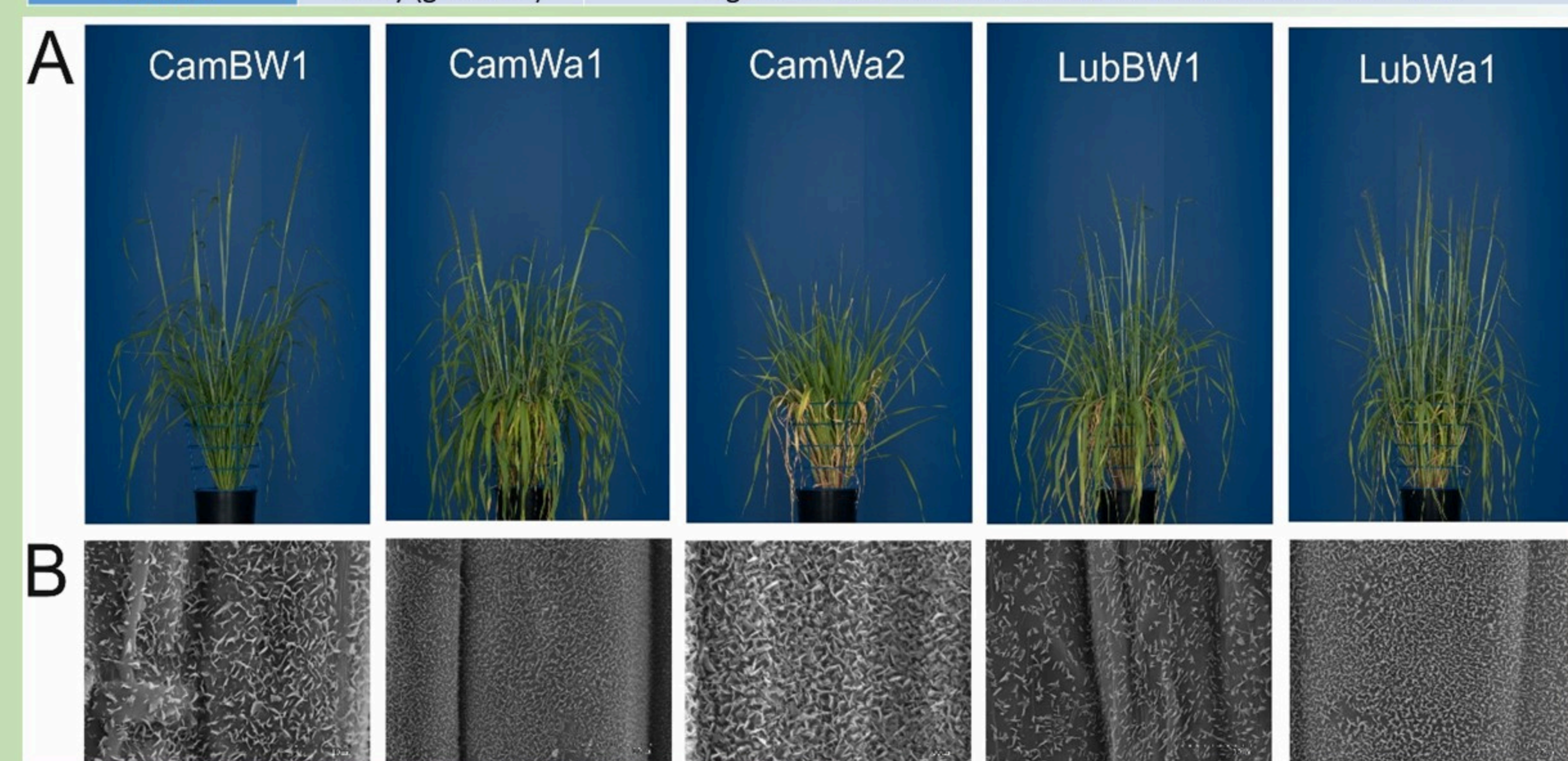


Figure 1. Morphological variation of the studied plant material grown under control condition – visible differences in plant habit and earliness (A). Variation in the distribution of wax crystals on the leaf surface – in glaucous line, a noticeable densification of crystals is observed, as shown in SEM images (B).

Phenotyping workflow under drought stress: The experiment involving drought application at the flag leaf stage, which was conducted using the PlantScreen™ Modular phenotyping platform (PSI, Czech Republic). Multiple imaging sensors were used, including RGB, chlorophyll fluorescence, and thermal IR imaging, to monitor stress response over time.

SEM observation procedure: Leaf samples were observed using a Hitachi S-3000N scanning electron microscope. **Data-driven approach to trait selection and sample classification:** Machine learning algorithms, specifically Random Forest, were applied to identify key phenotypic traits that contribute to treatment classification, rank trait importance and discriminating between experimental conditions associated with plant stress responses (Fig. 2). A set of selected traits related to plant morphology, and parameters associated with whole plant canopy temperature were analyzed, including, among others, Plant Volume (digital biomass derived from RGB top and side views) and deltaT (difference between leaf and air temperature known as canopy temperature depression).

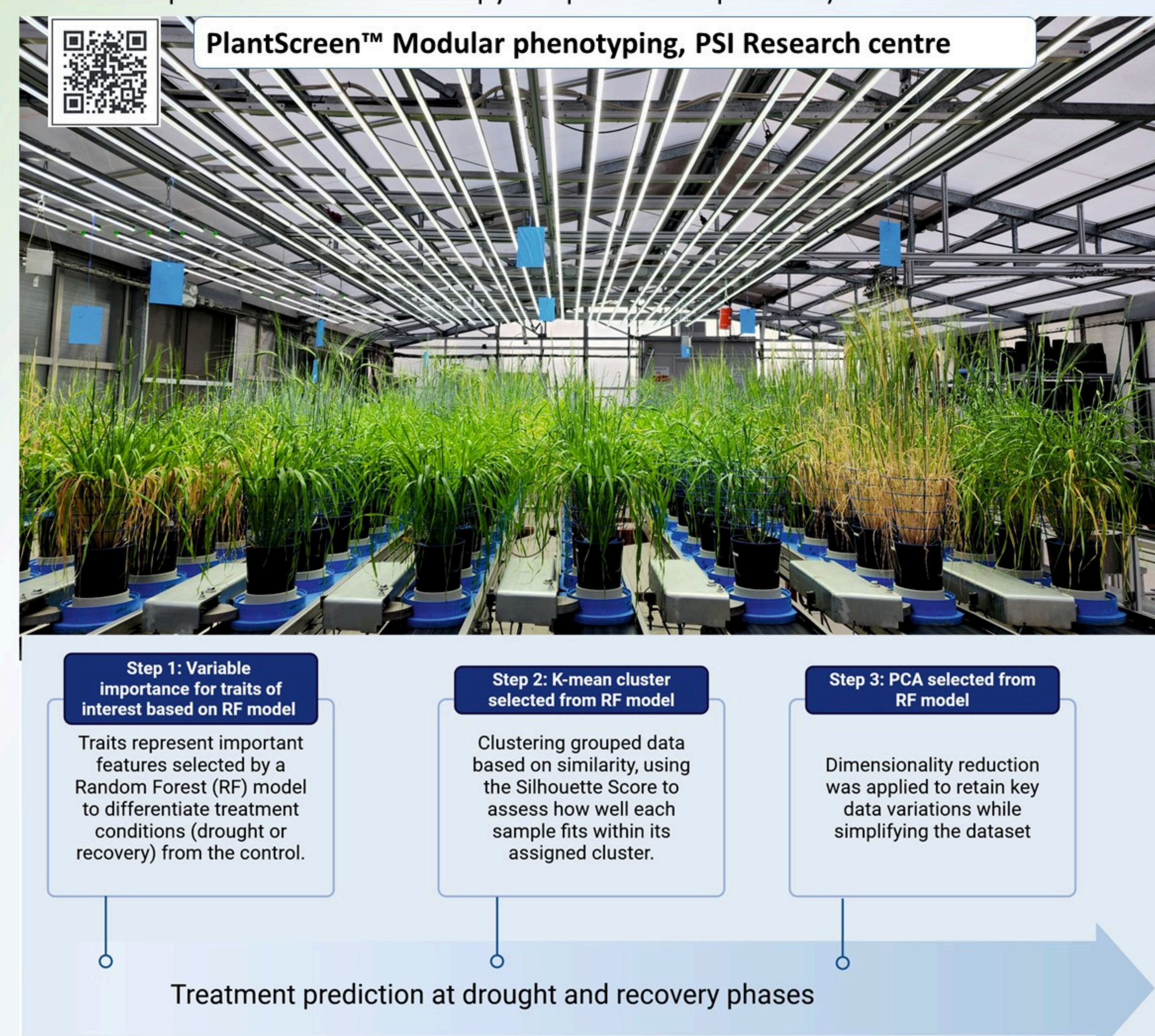


Figure 2. Workflow for treatment prediction under drought and recovery phases using Random Forest (RF) model for better data integration and trait identification

Results and Discussion:

Drought stress significantly reduced plant volume across all genotypes, confirming its role as a sensitive indicator of water-deficit responses. However, the magnitude of reduction varied, reflecting genotype-specific adaptive strategies (Fig. 3). Both CamBW1 and LubWa1 showed an increasing trajectory of the curves during the recovery period in drought-treated plants, indicating enhanced recovery capacity. LubWa1 maintained higher tolerance, supported by better recovery dynamics and a return to almost control-like values. CamWa1, CamWa2, and LubBW1 displayed intermediate responses, with CamWa1 showing poor recovery and CamWa2 and LubBW1 maintaining relatively stable but reduced volumes. These findings demonstrate that plant volume is a robust indicator for assessing drought-induced growth responses and differentiating genotypic strategies of tolerance and recovery.

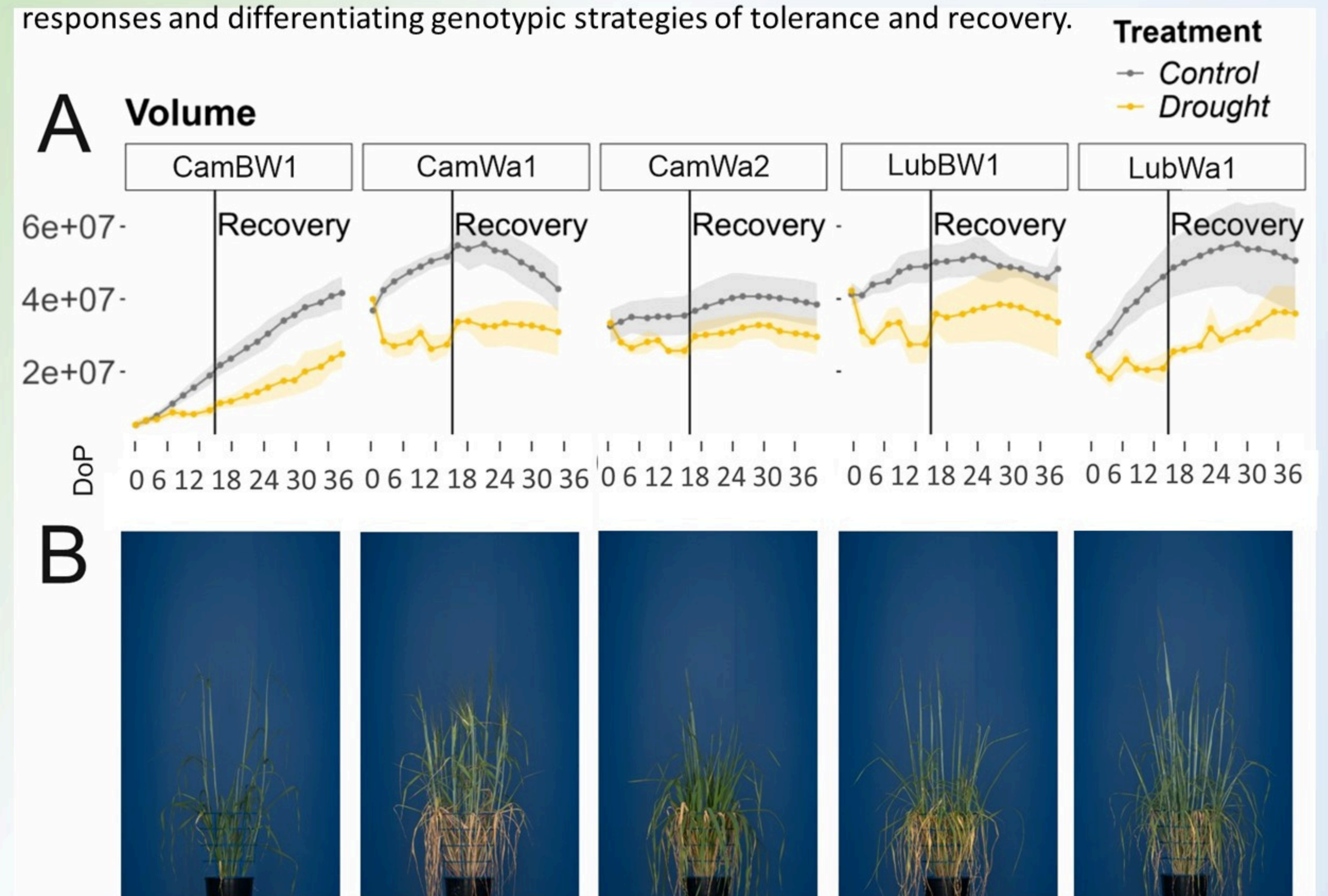


Figure 3. The distribution of Plant Volume values of the studied plants during the drought and recovery periods plotted for the two experimental conditions. DoP = day of phenotyping. (A). Images of plants subjected to drought stress – visible differences related to yellowing and loss of turgor (B)

For better data visualization, the top 20 variables contributing to treatment prediction were identified under both drought and recovery. DeltaT emerged as the most important common parameter to discriminate between stressed plants (Fig. 4).

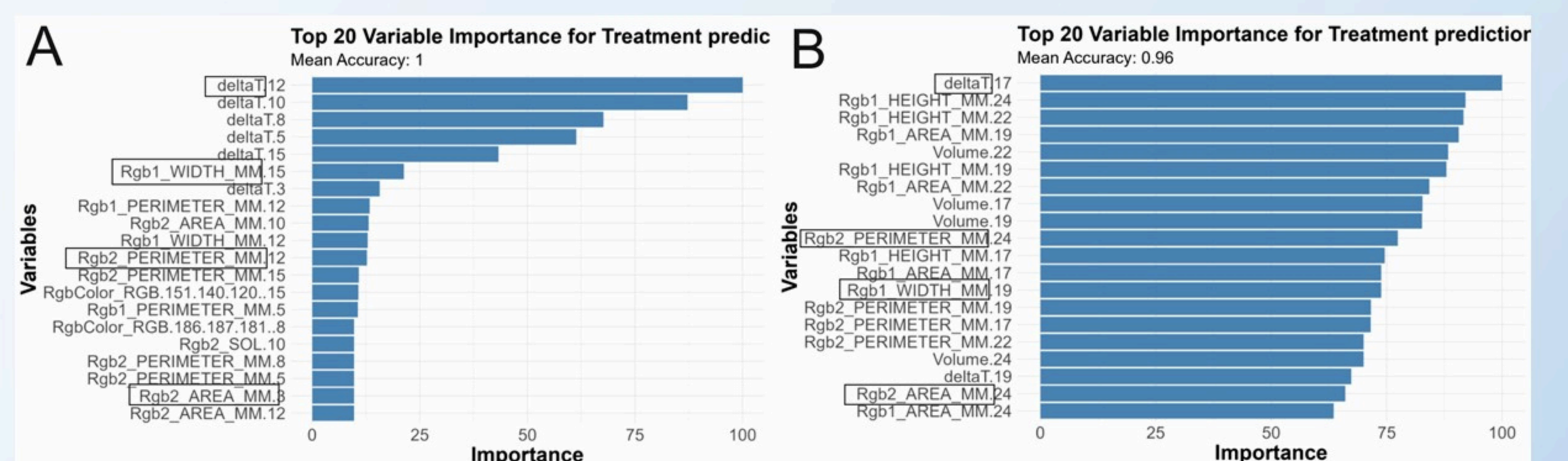


Figure 4. Top 20 most important traits identified by the Random Forest model for treatment prediction under drought condition (A) and recovery period (B).

The distribution of the deltaT trait revealed clear genotypic differences in drought responses. In CamBW1, significant changes appeared only on day 5, whereas in other genotypes they were already detected by day 3 (Fig. 5). This delayed thermal response suggests more efficient regulation of transpiration and leaf temperature in CamBW1 under early drought. This genotype showed a rapid initial increase in deltaT, followed by a gradual decline after 12 DoP, with recovery dynamics closely resembling control conditions, indicating effective post-stress restoration. A similar but less pronounced pattern was observed in CamWa2 and LubWa1, while other genotypes maintained higher and more sustained deltaT values in drought condition, which may reflect reduced cooling capacity and delayed stomatal regulation (Pflüger et al. 2024).

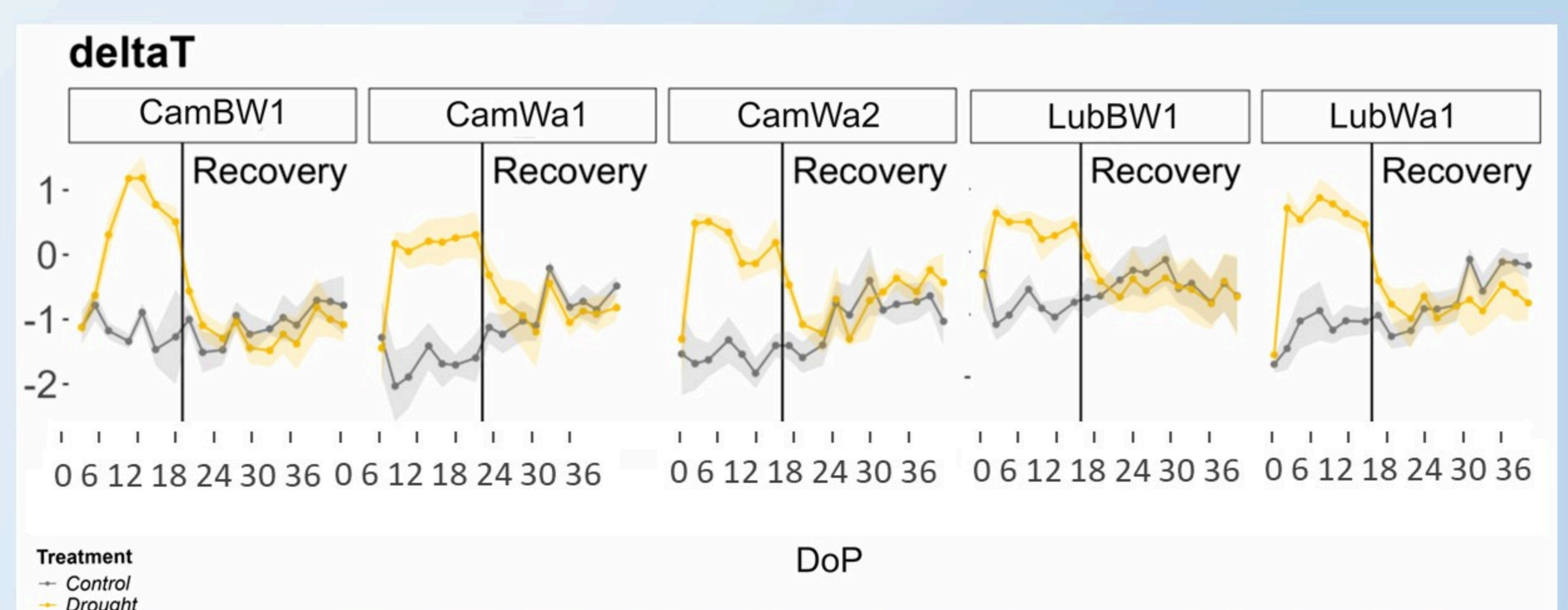


Figure 5. Time-course line plots with shaded confidence intervals depict the deltaT trajectories for each genotype. DoP = day of phenotyping

Key Findings:

- Heading date strongly affects genotype-specific drought responses.
- Combining phenological, morphological, and physiological traits improves prediction of adaptive strategies.
- Epicuticular wax quantity and composition boost thermal regulation.
- Drought tolerance involves complex, multi-trait interactions.

References:

- Ogradowicz, P., Wojciechowski, M.K., Kuczyńska, A., Krajewski, P., Kempa, M. (2023). The Effects of Growth Modification on Pollen Development in Spring Barley (*Hordeum vulgare* L.) Genotypes with Contrasting Drought Tolerance. *Cells*, 12, 1656.
- Pflüger T., Signe M., Jensen S.M., Fulai Liu F., Eva Rosenqvist E. (2024). Leaf gas exchange responses to combined heat and drought stress in wheat genotypes with varied stomatal density. *Environmental and Experimental Botany*, Volume 228, Part A, 105984.