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Water deficit overrides the effects of sulfur deficiency on pea growth, N_2 fixation, root architecture, and nutrient uptake under combined stress during vegetative development

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Abstract

Aims: Grain legumes such as pea are key components of low-input agroecological systems because they rely on symbiotic N_2 fixation to meet their nitrogen (N) demand. However, water deficit (WD) and sulfur (S) deficiency increasingly constrain pea production. We investigated the individual and combined effects of these stresses on growth, nodulation, root system architecture, and nutrient acquisition.

Methods: Two contrasting pea genotypes, the drought-sensitive Caméor and the drought-resilient Kayanne, were grown exclusively on symbiotic N_2 fixation. Plants were cultivated on a non-destructive phenotyping platform under control, WD, S deficiency, or combined stress conditions, and a structural–functional analysis of pea responses was performed.

Results: WD reduced biomass by 30%, decreased leaf N and S concentrations, increased carbon concentration and the root-to-shoot ratio, and impaired nutrient uptake more strongly than S deficiency. By contrast, S deficiency reduced biomass only in Kayanne but modified root architecture in both genotypes, increasing shallow-root density and stimulating root elongation in Caméor. Root growth was maintained under all stress conditions, with greater allocation to nodulated roots. Nodule size and biomass declined, reducing plant N content, although N_2-fixation efficiency was unaffected. Kayanne responded to stress by initiating more nodules, whereas Caméor showed increased nodule senescence. Under combined stress, WD largely overrode the effects of S deficiency, including on molybdenum uptake.

Conclusions: Overall, WD was the dominant constraint during vegetative growth, while the contrasting adaptive strategies of Caméor (root architectural plasticity) and Kayanne (enhanced nodulation) identify complementary traits for breeding pea varieties resilient to multiple abiotic stresses.

Introduction

Climate change has increased the frequency and intensity of extreme events like drought since the 1950s, impacting crop performance, their yield and the nutritional quality of harvested products (Dai, 2013; IPCC, 2023). In legume species like pea, soil water deficit is a major limiting factor, with the extent of yield reduction depending on the intensity, duration and timing of the water deficit (Daryanto et al. 2015; Lecomte et al. 2023). Early water deficit during vegetative growth was most detrimental to seed quality, particularly nitrogen (N) and associated protein content, because it impaired plant N uptake through N_2 fixation (Guilioni et al. 2003; Prudent et al. 2016). This stress is of particular concern in the context of the agroecological transition, where N-fixing-legume crops play key roles in lowering nutrient inputs in agroecosystems and act as a source of plant-based protein for healthy diets (Burstin et al. 2011; Voisin et al. 2014; Willett et al. 2019; Zhao et al. 2022). Indeed, symbiotic soil bacteria Rhizobia perform N fixation in root nodules, allowing legumes to meet their N requirements for growth and seed protein accumulation without N fertilization while legume residues can provide N for the following crop, contributing to a beneficial pre-crop effect that enhances yield by 20 % on average (Zhao et al. 2022).

At the plant level, water deficit decreases growth, causes stomatal closure, decreases C fixation and induces changes in C allocation (Brodribb and McAdams, 2013; Durand et al. 2016; Muller et al. 2011). The increase of C allocation towards the root system in response to water deficit is linked to changes in root system architecture (RSA) such as the development of a sinking architecture with steeper growth towards deeper horizon or a greater number of lateral roots to improve moisture foraging (Brodribb and McAdams, 2013; Klein et al. 2020; Lynch 2022; Muller et al. 2011; Tixier et al. 2025). In legumes, water deficit also impairs N fixation through reduced nodule initiation and growth, the inhibition of N fixation activity, and induces nodule senescence, which are linked to preferential C allocation towards root (and water acquisition) at the expense of nodule (and N fixation) (Dhanushkodi et al. 2018; Prudent et al. 2016). The subsequent N uptake decline compromises plant overall productivity since this element is a major constituent of proteins and nucleic acids, playing key roles in photosynthesis and reproduction (Kuehn and McFadden 1969; Marschner 2012). It is worth noting that water deficit can also lead to nutrient deficiencies by reducing the diffusion of soluble nutrients from the soil matrix to the roots and their transport within plants through decreased transpiration, transporter activity and cell membrane permeability (D'Oria et al. 2022; Hu et al. 2007; Jacques et al. 2023). Further, macronutrients (N, S, phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg)) or micronutrients (iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni), molybdenum (Mo) boron (B) and chlorine (Cl) sometimes exhibits compensatory mechanisms when deficient, notably because they may share non-specific root transporters (de Bang et al. 2021; Jacques et al. 2021; Nath and Tuteja, 2015) and these transporters can share common regulators at the transcriptional or post-translational level (Dong et al. 2021; Saito and Uozumi 2020).

Among macronutrients, sulfur (S) deserves particular attention because its availability has markedly declined in many agricultural soils following the reduction in atmospheric S dioxide emissions and sulfur deposition over recent decades (Feinberg et al. 2021). S deficiency affects both yield and seed protein composition in legumes (Henriet et al. 2019; Scherer and Lange 1996). It impairs directly growth and photosynthesis through its pivotal role in proteins with disulfide bond and Fe-S clusters but also indirectly through its impact on N acquisition via nitrate uptake or N_2 fixation (Almagro-Lopez et al. 2025; Varin et al. 2010; Zuber et al. 2013). In legumes, S deficiency impairs nodulation, inhibits N_2 fixation activity and alters N allocation, conspicuously through limitations of C allocation towards nodules and because S allows for the synthesis of key S-dependent components of the N_2 fixation machinery, including nitrogenase and leghemoglobin (Avenhaus et al. 2016; Becana et al. 2018; Brosnan and Brosnan, 2006; Varin et al. 2010; Zuber et al., 2013). Additionally, S deficiency has been reported to increase allocation of C and N towards roots when stress was applied during the vegetative phase as well as to alter some root architecture traits (Gao et al. 2016; Joshi et al. 2019; Yang et al. 2025; Zuber et al. 2013). For example, increased root length on seedlings of *Arabidospis thaliana* and increased lateral root

initiation in the root apical region of *Medicago trunculata* have been observed. These adaptations are likely established to enhance mineral foraging in deeper soil horizons (López-Bucio 2003). As a constituent of reactive oxygen species (ROS)-scavenging glutathione, S deficiency can hinder plant ability to counteract ROS production, especially in response to environmental stress such as water deficit (Brodribb and McAdam 2013; de Bang et al. 2021; Johansson et al. 2025; Wang et al. 2018). S deficiency also affects the uptake and homeostasis of other nutrients such as Mo and Fe (Coppa et al. 2026; Maillard et al. 2016a, 2016b).

The increased incidence and co-occurrence of water deficit (WD) and S deficiency requires to evaluate their impact when alone or in combination to assess if they behave as additive, synergistic or antagonistic stresses for crops, especially for legumes which are crucial for sustainable cropping systems. Synergistic and antagonistic interactions occur when the combined effect of the stresses exceeds or is lower than the sum of the effects of each individual stress, while additive interaction occurs when the effect of combined stress is equal to the sum of individual effects. It has already been shown in the literature that it is difficult to predict combined stress behavior based on individual stresses. This depends on the process being studied, on whether the processes involved in the stress response are common to the combined stresses (Sato et al. 2024), and on the intensity of each stress (Zandalinas and Mittler, 2022). Henriet et al. (2019) examined the combined effects of WD and S deficiency on yield components in pea during the reproductive growth, and in absence of N-fixing symbiosis, but the impact of these combined stresses during vegetative growth and under N-fixing conditions remains largely unexplored. While this developmental period is key for plant N acquisition and therefore for seed N content and yield quality, no data are available on the effect of combined WD and S deficiency on N fixation, C and N allocation. Further, a comprehensive analyses of RSA responses to these stresses, as well as the functional responses of hydromineral acquisition would be valuable to identify traits that improve water and nutrient uptake under WD and S deficiency. Indeed, developing low- input cropping systems adapted to climate change requires optimizing crop root systems for water and mineral acquisition, which relies on identifying and prioritizing key root architecture traits and their associated C and N costs to maintain yield stability.

In this context, this study aims to understand how pea plants and their nodulated root system respond to WD and S deficiency during the vegetative stage. We hypothesized that (1) the combination of WD and S deficiency has a higher impact on plant growth, N-fixation, C and N allocation, RSA and nutrient uptake than when applied individually; (2) plants allocate more biomass to the roots under both treatments, at the expense of shoot, nodules and associated N fixation to maintain root growth and hydromineral acquisition with the root system extending towards deeper soil horizons; (3) nutrient uptake is affected by both treatment through several processes ranging from impaired uptake of some nutrients to compensatory uptake of other nutrients. In the present study, WD and S deficiency were applied to pea plants either individually or in combination and we evaluated the growth, C and N allocation, nutrient uptake and N fixation using a structure-function analysis. Non-destructive observation of the RSA throughout the experiment made it possible to access the temporal and spatial dynamics of its responses to these treatments. Two genotypes contrasted for their WD responses were used: previous studies have found Kayanne to be more resilient to water deficit than Caméor (Couchoud et al. 2020; Henriet et al. 2019). This choice enabled us to evaluate whether the genotype's sensitivity to water deficit could exacerbate its response to combined stress.

Materials and Methods

Plant growth conditions and sample collection

Two contrasted genotypes for their response to WD of *Pisum sativum* L. were used: Kayanne, a semi-leafless spring pea cultivar (obtained from KWS Momont, Mons-en-Pévèle, France) and Caméor, a leaflet garden pea cultivar. After disinfection, seeds were germinated in the dark at 21-22 °C for 3-4 days. The seedlings were then transferred to RhizoTubes® which are cylindrical rhizotrons that allow the non-destructive-2D observation of the complete root system throughout the experimentation (Jeudy et al. 2016). Two seedlings per RhizoTube® were placed between an outer transparent layer and an inner permeable membrane that allows the passage of water and nutrients from subtrate but not roots. The seedlings were immediately inoculated with Rhizobium leguminosarum bv. viciae, strain P221 (MIAE01212, 10^8 bacteria. mL^{-1}). A total of 104 plants, with 50 for Kayanne genotype and 54 for Caméor genotype were grown under controlled conditions in the 4PMI (Plant Phenotyping Platform for Plant and Microorganism Interactions, Dijon, France). A perlite:sand (3:1, v:v) mix was used and a N-free nutrient solution was applied following methods in Couchoud et al. (2020). The plants were grown under 16 H photoperiod and mean day/night temperatures of 20/16 °C. Artificial light (PAR of 280 µmol m^{-2} s^{-1}) was supplied by 600 W sodium lamps to complement natural light.

During the first two weeks, the plants were irrigated with the N-free solution to maintain the substrate at 100 % of their water holding capacity, which was gravimetrically measured every day before each watering. Following this, the plants of each genotype were exposed for 2 weeks to either control condition (sufficient water and sulfur; **WW S+**), water deficit (complete cessation of irrigation ; **WD S+**), sulfur deficiency (leaching of S from substrate with a S-free and N-free solution and following irrigation at 100 % of water holding capacity with this solution ; **WW S-**) or combined stress (leaching of S from substrate using the S-free and N-free solution followed by complete cessation of irrigation; **WD S-**). For each treatment of each genotype, six or seven RhizoTubes® were used, corresponding to 12-14 plants. Images of the RSA were taken automatically every 3 to 4 days for a total of 10 times throughout the experiment.

After two weeks of treatment, stomatal conductance was measured on the last fully expanded stipule using a porometer (AP4 porometer; Delta T device, Cambridge, United Kingdom), and leaf water potential was measured with a pressure chamber (PMS, Corvallis, Oregon, USA). The next day, six to seven plants per condition and genotype were harvested for sample analysis. Shoot height was measured and the plant development was measured by counting the number of leaves. Leaf and stem area were measured using a planimeter (LI 3100c area meter; LI-COR, Inc., Lincoln, NE, USA). Nodules were separated from roots and counted by dividing them into three groups according to their color (white/pink/green-brown). All tissue samples (leaf, stem, root, nodules) were placed in a drying oven during 72h at 60°C for further analysis.

Samples analysis

After drying, the dry biomass (DW) of the tissues was measured with shoot DW (g) as the sum of leaves and stem biomass. Nodulated root to shoot biomass ratio was calculated as:

$$Nodulated\ root{:}\ shoot = \frac{DW_{root} + DW_{nodule}}{DW_{leaf} + DW_{stem}} \quad (1)$$

The ratio of nodule and nodulated root was calculated as:

$$Nodule{:}\ nodulated\ root = \frac{DW_{nodule}}{DW_{root} + DW_{nodule}} \quad (2)$$

The dried plant tissues were ground into a fine powder using a ball mill (Retsch MM 400) and encapsulated for elemental analyses. Carbon (C) and nitrogen (N) concentrations for each plant organ were determined with the Dumas method using a C-N analyser (Thermo electron NC2500, Courtaboeuf, France).

The concentration of macronutrients (P, K, S, Ca, Mg) and micronutrients (Fe, Mn, B, Ni, Cu, Mo, Zn), were determined at the PLATIN' Platform (Plateau d'Isotopie de Normandie, France) using a 40 mg aliquot of powder, and high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS, Element 2, Thermo Scientific, Bremen, Germany) following methods described in Maslard et al. (2026). Briefly, a microwave acid (HNO_3 with H_2O_2) sample digestion (Multiwave ECO, Anton Paar, Les Ulis, France) was followed by spiking the samples with gallium and rhodium internal standards to final concentrations of 5 and 1 mg L^{-1} respectively. Further dilution to 50 mL with Milli-Q water yielded solutions containing 2.0 % (v/v) nitric acid, which were filtered through a 0.45 µm Teflon filtration system (Digifiltre, SCP Science, Villebon-sur-Yvette, France). Nodule samples often yielded insufficient powder for ionomic analysis; therefore, biological replicates were occasionally pooled to obtain enough material for measurement. The concentration of each element ([E], in mg g^{-1}) was used to calculate total amount of element in each organ following the formula:

$$Organ\ E\ amount\ \ (mg)\ \ = [E]_{organ} * DW_{organ} \quad (3)$$

The total amount of an element (Plant E amount) was calculated as the total amount of E in the plant, summing leaf, stem, root and nodule amount. The calculation for elemental uptake efficiency (EUpE, mg g^{-1} day^{-1}) was adapted from Jacques et al. (2021):

$$EUpE\ \ = \frac{Plant\ E\ amount_{t_h} - Plant\ E\ amount\ _{t_0}}{\int_{t0}^{th} DW_{root}\ .dt} \quad (4)$$

where t_h (day 28) refers to the time of harvest and t_0 (day 0) refers to the start of the RhizoTube® experiment. Plant E amount at t_0 was considered as the total amount of E in the seed.

Since N fixation occurs in nodules, the N fixation efficiency was calculated per nodule biomass instead of root biomass following the equation:

$$N2\ fixation\ efficiency = \frac{Plant\ N\ amount_{t_h} - Plant\ N\ amount\ _{t_n}}{\int_{tn}^{th} DWnodule\ .dt} \quad (5)$$

Here, t_h refers to the time of harvest and t_n refers to the first day pink nodules were observed (day 11).

Root system architecture (RSA) analysis

The root images obtained from the RhizoTubes® were analysed by the 4PMI Platform through methods described in Jeudy et al. 2016. Root depth (cm), root length (cm), root width (cm), root diameter (cm), root area (cm^2), root depth: width, root prospection area (cm^2), root growth rate (mm^2 h^{-1}) and root density were measured using image analysis as described by Maslard et al. (2021) and Tixier et al. (2025).

Furthermore, these RSA traits were also measured for 10-cm-deep-sections of the RhizoTube ® (0 - 10 cm, 10 - 20 cm, 20 - 30 cm, 30 - 40 cm and 40 - 48 cm). Root density was calculated as the ratio of root area and root bounding box area (root height x root width). For each RhizoTube® sections, the bounding box area was calculated by multiplying the width of the entire RS by the thickness of the section. Nodules were manually counted based on their color which corresponds to their phenological stages: newly formed inactive nodules were white, mature active nodules were pink while senescent inactive nodules were green/brown (Sprent, 2009). Nodule size was quantified by tracing their outline and measuring their area.

The kinetic of root depth, root length, total number of nodules and nodule size was tracked throughout the experiment. Nodule number and size kinetics were fitted to a sigmoidal curve following the simple logistic equation:

$$y = \frac{y_{max}}{1 + e^{-k(t - t_{50})}} \quad (6)$$

where t is time (days), y_{max} = maximum trait value, k= maximum rate or steepness of the curve, t_{50} = time of maximum rate where 50 % of y_{max} is reached. Because y approaches asymptotically y_{max}, growth duration was the time required to reach y_{max} (t_{max}) which was estimated as the time needed to reach 99 % of y_{max} using the formula:

$$t_{max} = t_{50} + \frac{1}{k} \text{ x } \log(99) \quad (7)$$

Statistical analysis

An unpaired two-sample Student's t-test was used to compare traits between the 2 genotypes under control conditions. A two-factor analysis of variance (ANOVA) for each genotype was performed on all measured traits to evaluate the effects of water deficit and S deficiency as well as their interaction, followed by a post hoc Duncan multiple range test (DMRT). For traits that did not meet normality criteria, non-parametric Kruskal-Wallis rank sum test followed by Kruskal-Wallis multiple comparison test was performed. All statistical analyses were performed in R software (R Core Team, 2024).

Results

Water deficit and S deficiency increased biomass allocation towards roots in both genotypes

Under control condition (WW S+), plants from Caméor genotype had 43.18 % higher biomass than plants from Kayanne genotype at harvest, with corresponding greater root and nodule biomass (Fig. 1, Table S1).

WD reduced total plant biomass by 27.27 % and 30.16 % in Kayanne and Caméor genotypes, respectively, and this decrease was associated with a decrease in shoot area and an increased biomass allocation towards the nodulated root system in both genotypes (Fig. 1 A-D). Indeed, shoot and nodule biomass decreased in response to WD while root biomass remained unaffected, resulting in increased nodulated-root:shoot ratio. WD decreased stomatal conductance from 144.50 ± 31.42 to 54.12 ± 11.42 mmol m^{-2} s^{-1} in Caméor genotype while a non-significant decrease was observed in Kayanne (Fig. 1 E, F).

S deficiency reduced plant biomass, shoot biomass and shoot area only in Kayanne, but it increased biomass allocation towards roots for both genotypes with increased nodulated-root:shoot ratio and reduced nodule biomass by 41.8 % in Kayanne and 27.8 % in Caméor (Fig. 1 A-D, Table S1). The increase of the nodulated-root:shoot ratio in Caméor was attained through increased root biomass while maintaining shoot and plant biomass.

The combination of WD and S deficiency (WD S-) induced similar responses in terms of biomass, biomass allocation and stomatal conductance as under WD S+ for both genotypes with similar values observed.

S deficiency induced more RSA changes than water deficit, especially in Caméor genotype

Caméor exhibited higher root length, area and prospection area, than Kayanne genotype under control condition (Fig. 2), which is in line with the higher root biomass observed in this genotype (Fig. 1, Table S1). Both genotypes had most of their root distributed in the upper part of the root system with 73 - 78 % and 67 - 75 % of the total root area distributed in the top 20 cm of the root system for Kayanne and Caméor genotype, respectively (Table S2).

At the whole root system level, WD alone (WD S+) had no effect on studied traits of RSA in Kayanne but it significantly increased root width and decreased depth:width ratio in Caméor (Fig. 2, Table S2). This increase in width of 21.23 % was mainly observed in the top 20 cm of the RS (Table S2), and was associated with lower root density in the lower 30-40 cm of the RS (Fig. 3).

Conversely, S deficiency alone (WW S-) decreased root depth in Kayanne genotype while it increased root width, root length, growth rate and root area in Caméor genotype (Fig. 2, Fig. 3). These increases in width, length and area were mainly located in the top 20 cm of the root system although increased root density was also observed

in the lower 40 - 48 cm section (Fig. 3, Table S2). The greater root length in Caméor under WW S- was associated with a higher root growth rate from 11 days after S deficiency initiation until harvest (Fig. 2J).

Combined WD and S deficiency (WD S-) had no effect on studied traits of RSA in Kayanne showing a response similar to WD alone. In Caméor, WD S- significantly increased root system width and decreased its depth:width ratio while decreasing root density in the lower 30 - 40 cm section, to similar extent as under WD S+ (Fig. 2, 3, Table S2). Lateral expansion in the top 20 cm with increased root length and area was also observed in a similar manner to the changes observed under S- alone but these local changes under combined stress (WD S-) led to smaller increase of root area and root length at the whole root system level. Finally, a specific and significant 4.62 % increase in root depth was observed under WD S- treatment in Caméor (Fig. 2B).

WD and S deficiency decreased nodule size for both genotypes, but only Kayanne showed an increase in nodule number in response to S deficiency

Nodule initiation was identified by the first appearance of immature white nodules on roots after growing 8-11 days in RhizoTubes® for both genotypes under all treatments. Functional pink nodules were the most abundant at harvest under control conditions (WW S+) representing 84 % and 77 % of the total 57.9 ± 21.3 nodules in Kayanne and 85.2 ± 22.2 nodules in Caméor; respectively; while only 1.2 % and 1.5 % were senescent with a brown/green color (Table 1). WD alone (WD S+) and S deficiency alone (WW S-) significantly increased nodule initiation occurrence in Kayanne genotype with increased number and proportion of white nodules (Table 1, Table S1) which ultimately led to the increase of total number of nodules which was only significant for WW S- (Fig. 4 A-D). In Caméor, WD induced nodule senescence with increased number and proportion of brown/green nodules (Table 1). S deficiency alone (WW S-) also increased nodule senescence in Caméor but with no significant changes. The combination of WD and S deficiency (WD S-) in Kayanne cancelled the independent effect of both stress on nodule initiation and no difference in terms of nodule number and proportion were observed when compared to control conditions (WW S+). In contrast, the effects of WD S- on nodule senescence for Caméor were similar to those recorded under WD S+ conditions (Table 1)

Average nodule size increased throughout the experiment following a sigmoidal pattern, reaching maximum values at harvest of 2.3 ± 0.4 mm^2 and 2.5 ± 0.3 mm^2 for Kayanne and Caméor, respectively, under control conditions (Fig. 4 E-H). While nodule initiation coincided among treatments, the mean nodule size and pink nodule size were consistently lower under WD S+, WW S- and WD S- than under control conditions (WW S+) after the onset of treatments in both genotypes which ultimately resulted in significant differences of nodule size at harvest (Fig. 4. E-H, Fig. S1). This reduction of nodule size by WD and S deficiency when alone and combined was associated with a shorter nodule growth duration (t_{max} in days, time to reach maximum nodule size), reduced pink nodules growth rate and size at harvest, as well as a decrease in their contribution to the total nodule number (Fig. 4I-L, Table 1, Tables S1, S2).

WD and S deficiency decreased plant S and N concentration while only WD combined or not with S deficiency, increased plant C concentration

WD increased plant C concentration and decreased plant N and S concentration in both genotypes, under WD S+ and WD S- treatments, regardless of S supply (Fig. 5 A-F). The increase in C concentration in response to WD was observed in all organs for both genotypes except for nodules in Kayanne (Table S3). However, the reduction of plant N concentration in response to WD was mostly linked to changes observed in shoots and nodules, whereas S concentration declined mainly in shoots and roots of plants grown under WD S+ and WD S- for both genotypes (Table S3).

S deficiency decreased N and S concentration in both genotypes under WW S- and WD S- but it only decreased plant C concentration in Caméor genotype under WW S-. The decrease of S concentration in response to S

deficiency was observed in all organs for both genotypes while N concentration decline was mostly observed in shoots and nodules (Table S3).

While plants grown under WD S+, WW S- and WD S- showed similar levels of plant N concentration within each genotype, the decline of plant S concentration caused by S deficiency was significantly stronger than the one observed under WD (Fig. 4 C-F; Table S3). Finally, when WD and S- were combined under WD S- treatment, plant C concentration increased, plant N and S concentration decreased, reaching altogether similar plant C, N, S concentrations as the ones observed under WD S+.

WD impacted more elements uptake and their concentration than S deficiency, both treatment having negative effects on N, S, P and K while they showed antagonist effects on Mo uptake and B concentration in both genotypes

WD did not impair the N_2 fixation and the uptake efficiency of Mn, Fe, Ni and Cu for both genotypes but it impaired the uptake efficiency of P, K, S, Mg, Ca, Mo and B, regardless of S supply (Fig. 6, Table S4). Only Kayanne exhibited a decline of Zn uptake efficiency under WD S+ and WD S-. S deficiency decreased S, P, K, Mg uptake efficiency in both genotypes (Fig. 6, Table S4). When applied alone (WW S-), it increased Mo uptake efficiency in both genotypes but Mo uptake efficiency declined under WD S- in a similar fashion as in WD S+ treatment (Fig. 6). Similarly, under WD S-, P and K uptake efficiency values were similar to the ones observed in WD S+ and significantly lower than the ones observed in WW S- (Fig. 6, Table S4). Further, if B uptake efficiency was decreased by WD regardless of S supply, plants grown under combined stresses (WD S-) showed higher B uptake efficiency than the ones subjected only to a WD alone (WD S+) (as a tendency in Kayanne, or significant effect in Caméor; Fig. 6A, 6B, Table S4). Finally, the combination of WD and S- under WD S- treatment lead to a significant increase of Fe uptake efficiency in Caméor (Table S4).

WD increased C concentration and decreased N, P, K, S, Mo and B plant concentration under WD S+ and WD S- treatments in both genotypes (Fig. 7, Table S3). Under WD S-, plant B concentration was significantly higher than under WD S+. Further, K, B and Mo concentrations declined in all organs of both genotypes under WD S- and WD S+. However, if the decline in P concentration in response to WD was observed in all organs of Caméor plants, this P concentration decline was mainly observed in the roots of Kayanne plants (Table S3). In Caméor, a decrease in Mg and an increase in Fe plant concentration was also observed under WD S+ and WD S- treatments (Fig. 7H, 7J, Table S3).

S deficiency decreased plant N, K, S, and Mg plant concentration in both genotypes under WW S- and WD S- except for Mg which remained stable in Kayanne genotype under WD S- treatment when S deficiency was combined with WD (Fig. 7A, B, G). When applied alone under WW S- treatment, S deficiency nearly doubled Mo concentration in both genotypes (Fig. 7K, L). Plant P concentration decreased in response to S deficiency only in Caméor, although this reduction was smaller than under WD (Fig. 7E, F). This decrease was primarily associated with a reduction in shoot P concentration (Table S3).

Discussion

WD and S deficiency impairs pea growth, N fixation and nutrient uptake during vegetative growth

Water deficit, regardless of S availability, decreased total plant biomass and total plant N in both genotypes due to decline in plant growth, the amount of C fixed and N fixation (Fig. 1, Table S5), which is consistent with previous findings (Jacques et al. 2023; Prudent et al. 2016). The increase in C concentration is in line with the common report that C demand for growth declines earlier than gas exchange and photosynthesis (Körner 2015; Muller et al. 2011; Tixier et al. 2025). Similarly, the changes of biomass allocation and CN allocation towards root at the expense of shoot and nodules (Fig. 1, Table S6) fits the general hypothesis that plants prioritize root growth under WD to improve access to water and nutrients (Farooq et al. 2009; Prudent et al. 2016). However

few RSA adaptations were observed in response to WD (Fig. 2, 3). While Caméor increased the root system width and its deeper root density for hydromineral foraging, Kayanne showed no morphological changes under WD S+ and WD S- in contrast with previous findings which can be accounted for the milder stress level and shorter duration performed in this study (Couchoud et al. 2020; Klein et al. 2020; Lynch 2022; Tixier et al. 2025). Indeed, in RhizoTube® systems (Jeudy et al. 2016) —unlike in conventional pots—withholding water for two weeks from small plants makes it possible to induce moderate water stress (minimum leaf water potential at -0.37 MPa, Table S1). Both genotypes exhibited impaired growth of nodules which ultimately led to reduced nodule size -especially the functional ones- and the decrease of plant N levels (Fig. 4,5) which is consistent with previous findings in common bean (Fenta et al. 2020). While our study shows that this reduction is the consequence of reduced growth rate and shorter growth duration (Fig. 4, Table S2), it highlights that N fixation is maintained, suggesting that nodule activity only declines under more severe water stresses (Prudent et al. 2016). Reduced nodule growth was associated with increased senescence in Caméor and increased nodule initiation in Kayanne (Table 1). The acceleration of nodule senescence under WD in Caméor is likely due to oxidative damage of protein and lipids as demonstrated under drought (Dhanushkodi et al. 2018). Kayanne increased nodule initiation under both WD S+ and WD S- conditions as evidenced by the higher number and proportion of white nodules (Table 1, Table S1), suggesting a rapid adaptation of N fixation rates to meet the N demand associated with potential post-stress growth (Couchoud et al. 2020). Finally, WD constrained uptake efficiency of K, P, S, Mg, Ca, Mo and B in both Kayanne and Caméor genotypes regardless of S availability and it resulted in decreased concentrations of K, P, S, Mo and B at the whole plant level in both genotypes, while plant Mg concentration only declined in Caméor genotype (Fig. 6, 7). Similar decline of these elements in response to WD were observed in rapeseed and wheat (D'Oria et al. 2022). These constraints in nutrient uptake can be attributed to reduced transpiration and concomitant mass flow from the soil matrix to the roots although decreased transporter activity and cell membrane permeability under WD cannot be ruled out (Hu et al. 2007; Jacques et al. 2023). The decline in element uptake and tissue concentration can have detrimental effect on the plant ability to adapt to the water stress. For example, Mo has key roles in water utilization, anti-oxidative defense and osmotic adjustment (Wu et al. 2014).

S deficiency increased biomass allocation towards roots, probably to improve nutrient uptake in both genotypes, but it declined growth only in Kayanne genotype, while Caméor exhibited several RSA adaptations such as higher root growth and RSA width as well as increased root area in the upper part of the root system and increased root density in deeper parts (Fig. 1, 2, 3). These adaptations seem to be tailed for nutrient foraging, although mainly increased branching in deeper horizon have been reported (López-Bucio et al. 2003). This could be due, in particular, to the modulation of the expression of genes involved in root branching in response to S deficiency (Kutz et al. 2022). Furthermore, S deficiency impaired N fixation primarily by affecting nodule development, reducing both rate and duration of nodule growth and ultimately decreasing nodule size, whereas N_2 fixation efficiency remained unchanged (Fig. 4, Table 1). The lack of an effect of S deficiency on nodule function and activity contrasts with previous reports but may be explained on the one hand by the relatively moderate severity of the S deficiency imposed in this study, as reductions in nodule activity are generally observed only under severe S deficiency (Scherer et al. 2006, 2008; Zhao et al. 1999), and on the other hand by the fact that the plant could remobilize sulfur, which has been stored during the two weeks of growth preceding the deficiency, to support essential functions, such as N_2 fixation. Nevertheless, impaired nodule development reduced total nodule biomass and plant N status. In Kayanne, this decline was accompanied by a compensatory increase in nodule initiation, similar to that observed under WD, resulting in a higher number and proportion of immature nodules. In both genotypes, S deficiency decreased the uptake efficiency of macronutrients P, K, S and Mg, resulting in decreased plant concentrations of K, S and Mg while total P concentration decreased only in Caméor (Table S2), and Fe uptake efficiency only decreased in Kayanne (Table S3). These changes in several nutrient uptake may

be linked to the coordinated regulation of various transporters, as shown though transcriptomic analysis in various species, such as rice (Dai et al. 2023), rapeseed and wheat (Courbet et al. 2021; D'Oria et al. 2022). The concomitant decrease of plant K and Mg concentration under S deficiency may indicate the role of these elements as counter ion to sulfate to maintain electroneutrality (Bäucker et al. 2003; Reich et al. 2016). Also, the higher plant Mo concentration under S deficiency in both genotypes support the hypothesis of potential interactions between S and Mo uptake, possibly involving shared transport mechanisms (Maillard et al. 2016a, Reich et al. 2016).

Under combined stresses, WD predominates over S deficiency in shaping growth, RSA, N fixation and nutrient uptake

In this study, WD and S deficiency were imposed individually at comparable severity, eliciting similar reductions in growth, RSA, N fixation and nutrient uptake, which enabled to assess cumulative, dominant and compensatory responses under combined stress (Table 2). Indeed, comparable responses to WD and S deficiency applied individually in terms of growth, biomass allocation, RS width, nodule size and nodule biomass were observed. Further, under combined stress no cumulative effect of WD and S- were observed as the responses of above-mentioned traits were similar to the ones under stress applied individually. Yet few traits showed cumulative effect of treatment such as stomatal conductance in Kayanne and RS depth in Caméor. However, WD appeared as the prevailing limiting factor for most traits, overshadowing the effect of S deficiency on growth, RSA traits and nutrient uptake such as plant N, S, K, P, Mg, Mo and B plant concentrations (Fig. 7, Table 2). In fact, the effect of S deficiency on root growth, deeper root density, nodule number, C, N, S, Mg, Mo and B concentrations were compensated under combined WD and S deficiency. These results contrast with previous report of synergistic effect of both stresses on traits measured at maturity (Henriet et al. 2019) or improved drought resistance after the application of S (Lehr et al. 2025). However, in these studies, the individual effect of one stressor was greater than that of the other, and nitrogen acquisition did not rely on N2 fixation. Indeed, our study indicates WD is the prevailing limiting factor during the vegetative growth, but the long-term effects of combined WD and S deficiency can ultimately lead to yield reduction and lower seed quality at the end of the growth cycle (Henriet et al. 2019). While water uptake seems of paramount importance during the vegetative growth, the influence of S uptake during that time of the growth cycle on yield and seed quality through remobilization process should be studied in the near future to rule out the benefits of S fertilization during vegetative growth.

Genotypic comparison highlights potential traits for improving stress resilience

The two studied genotypes differed in their susceptibility to treatments. They exhibited different adaptation prioritizations, with Kayanne showing more nodule development adaptations while Caméor exhibited more root system architecture adaptations. On one hand, Caméor exhibited changes in stomatal conductance, RSA, Mg and Fe uptake under WD that were not observed in Kayanne (Fig. 1-7, table 2), consistent with previous studies identifying Caméor as more susceptible to water deficit than Kayanne (Couchoud et al. 2020; Henriet et al. 2019). Specifically, the rapid initiation of nodule formation in Kayanne suggests the prioritization of C allocation towards root growth for improved water uptake must be balanced with investment in N fixation in legumes to ensure resilient growth and development under drought conditions (Fig. 4). Further, it suggests maintenance of nodule growth and rapid nodule initiation might be interesting targets for the breeding of resilient ideotype in the near future. On the other hand, Kayanne exhibited changes in growth, root system depth and deeper root density, nodule number and B under S deficiency that were not observed in Caméor suggesting that Kayanne genotype has a higher sensitivity to S deficiency, potentially through the combination of higher prioritization of N fixation in this genotype and detrimental effect of S deficiency on this physiological process.

Furthermore, having confirmed that it was not possible to predict a pea genotype's behavior under combined stress based on its response to individual stresses, and that genetic variability exists in the response to such

combined stress, it would be worthwhile to extend the study to a broader panel of genotypes. Because this study identified key traits to be considered when investigating the response to combined stresses, including nodulation and N_2 fixation, C allocation, RSA, a broader panel of genotypes could be established based on contrasting RSA characteristics. Such an approach would allow further investigation of the hypothesis that enhanced root branching capacity, improving nutrient foraging under nutrient deficiency, represents a valuable trait for developing resilient low-input cropping systems (Lynch 2022).

Conclusions

This study provides the first characterization of the effects of water deficit and S deficiency, applied individually or in combination during vegetative growth in a legume, on plant growth, C and N allocation, root system architecture, nutrient uptake and N fixation. Both treatments increased allocation towards roots, impaired structural components of N_2 fixation and the uptake and concentration of key elements like K and S in both pea genotypes. Temporal dynamics of the RSA revealed that WD and S deficiency impaired nodule growth and development in both genotypes. WD impaired the growth of both genotypes while S deficiency only impaired plant biomass of Kayanne, the maintenance of plant biomass in Caméor being associated with RSA adaptations. Conversely, Kayanne showed compensated impaired nodule growth and associated N_2 fixation by increasing nodule initiation. Little additive effect of WD and S- on plant growth, RSA and nutrient uptake were revealed but instead WD appeared as the prevailing limiting factor, overshadowing the effect of S deficiency on RSA and plant C, N, S, Mg, and Mo plant concentrations. Overall, these results demonstrate that the early vegetative stage of pea is highly sensitive to water deficit and that sulfur supply is not sufficient to alleviate the effects of water stress or improve plant tolerance at this developmental stage.

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Tables

Table 1: Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S) on the proportion of functional (pink), initiated immature (white) and senescent (green/brown) nodules in Kayanne and Caméor genotypes. The proportion of the number of nodules in each of the three groups was calculated at harvest on 6-8 pea plants per treatment and genotype.
The average nodule size was calculated for each nodule group, by combining the four treatments together. Mean values are presented with standard deviation. Different letters for nodule proportion indicate significant difference between treatments while different letters for nodule size indicate significant difference between nodule developmental stages (DMRT, $p < 0.05$). Control: WW S+.

Genotypes	Treatments	Immature (white)	Functional (pink)	Senescent (green-brown)
Proportion of nodules (%)				
Kayanne	WW S+	14.400 ± 8.427 *b*	83.555 ± 9.488 *a*	2.045 ± 2.972 *ab*
	WD S+	29.639 ± 4.946 *a*	64.864 ± 2.062 *b*	5.497 ± 4.022 *a*
	WW S-	30.978 ± 10.827 *a*	67.637 ± 10.369 *b*	1.385 ± 1.547 *b*
	WD S-	15.415 ± 4.664 *b*	81.923 ± 5.326 *a*	2.662 ± 1.715 *ab*
Caméor	WW S+	20.973 ± 12.146 *a*	77.536 ± 10.534 *a*	1.491 ± 1.877 *b*
	WD S+	18.017 ± 9.635 *a*	64.465 ± 10.748 *ab*	17.519 ± 14.418 *a*
	WW S-	21.020 ± 10.664 *a*	70.170 ± 9.639 *ab*	8.809 ± 11.383 *ab*
	WD S-	26.989 ± 10.246 *a*	60.559 ± 9.411 *b*	12.452 ± 8.784 *a*
Nodule size (mm^2)				
Kayanne		0.260 ± 0.083 *b*	2.271 ± 0.802 a	2.045 ± 2.972 *b*
Caméor		0.335 ± 0.085 *c*	2.307 ± 0.556 *a*	1.031 ± 0.729 *b*

Table 2: Effect and interaction of Water deficit (WD), sulfur deficiency (S-) and when they are combined (WD S-) on studied traits in Kayanne and Caméor génotypes. Upward blue arrows indicate a decreased trait value whereas downward red arrows indicate an increased trait value and horizontal black arrows indicate no changes when compared to control (WW S+). Under WD S -, responses were categorized as non-additive when WD S+, WW S- and WD S- produced similar effects, WD prevailing when WD S- exhibited a response similar to WD alone under WD S+ treatment and antagonistic, when the effect of individual stress was reduced or offset under the combination WD S-. Arrows are in bold when genotypes exhibited different responses.

Trait	Genotype	WD	S-	WD S-	Interaction
Plant biomass	Kayanne	⇩	**⇩**	⇩	Non-additive
	Caméor	⇩	**⇔**	⇩	**WD prevails**
Shoot area	Kayanne	⇩	**⇩**	⇩	Non-additive
	Caméor	⇩	**⇔**	⇩	**WD prevails**
Stomatal conductance	Kayanne	**⇔**	⇔	⇩	**Additive**
	Caméor	**⇩**	⇔	⇩	**WD prevails**
Nodulated root:shoot	Kayanne	⇧	⇧	⇧	Non-additive
	Caméor	⇧	⇧	⇧	Non-additive
Root growth	Kayanne	⇔	**⇔**	⇔	Non-additive
	Caméor	⇔	**⇧**	⇔	**Antagonistic / WD prevails**
RSA width	Kayanne	**⇔**	**⇔**	**⇔**	Non-additive
	Caméor	**⇧**	**⇧**	**⇧**	Non-additive
RSA depth	Kayanne	⇔	**⇩**	**⇔**	**Antagonistic / WD prevails**
	Caméor	⇔	**⇔**	**⇧**	**Additive**
Deeper root density	Kayanne	**⇔**	**⇩**	**⇔**	**Antagonistic / WD prevails**
	Caméor	**⇩**	**⇧**	**⇩**	**Antagonistic / WD prevails**
Nodule biomass	Kayanne	⇩	⇩	⇩	Non-additive
	Caméor	⇩	⇩	⇩	Non-additive
Nodule size	Kayanne	⇩	⇩	⇩	Non-additive
	Caméor	⇩	⇩	⇩	Non-additive
Nodule number	Kayanne	⇔	**⇧**	⇔	**Antagonistic / WD prevails**
	Caméor	⇔	**⇔**	⇔	Non-additive
N_2 fixation efficiency	Kayanne	⇔	⇔	⇔	Non-additive
	Caméor	⇔	⇔	⇔	Non-additive
[C]	Kayanne	⇧	**⇔**	⇧	**WD prevails**
	Caméor	⇧	**⇩**	⇧	**Antagonistic / WD prevails**
[N]	Kayanne	⇩	**⇩**	⇩	Non-additive
	Caméor	⇩	**⇩**	⇩	**Antagonistic / WD prevails**
[S]	Kayanne	⇩	⇩	**⇩**	**Antagonistic / WD prevails**
	Caméor	⇩	⇩	**⇩**	**Antagonistic**
[K]	Kayanne	⇩	⇩	⇩	**WD prevails**
	Caméor	⇩	⇩	⇩	**WD prevails**
[P]	Kayanne	⇩	**⇔**	⇩	**WD prevails**
	Caméor	⇩	**⇩**	⇩	**WD prevails**
[Mg]	Kayanne	**⇔**	**⇩**	**⇔**	**Antagonistic / WD prevails**
	Caméor	**⇩**	**⇩**	**⇩**	**Antagonistic / WD prevails**
[Fe]	Kayanne	**⇔**	⇔	**⇔**	Non-additive
	Caméor	**⇧**	⇔	**⇧**	**WD prevails**
[Mo]	Kayanne	⇩	⇧	⇩	**Antagonistic / WD prevails**
	Caméor	⇩	⇧	⇩	**Antagonistic / WD prevails**
[B]	Kayanne	⇩	**⇧**	⇩	**Antagonistic / WD prevails**
	Caméor	⇩	**⇔**	⇩	**Antagonistic**

Figures

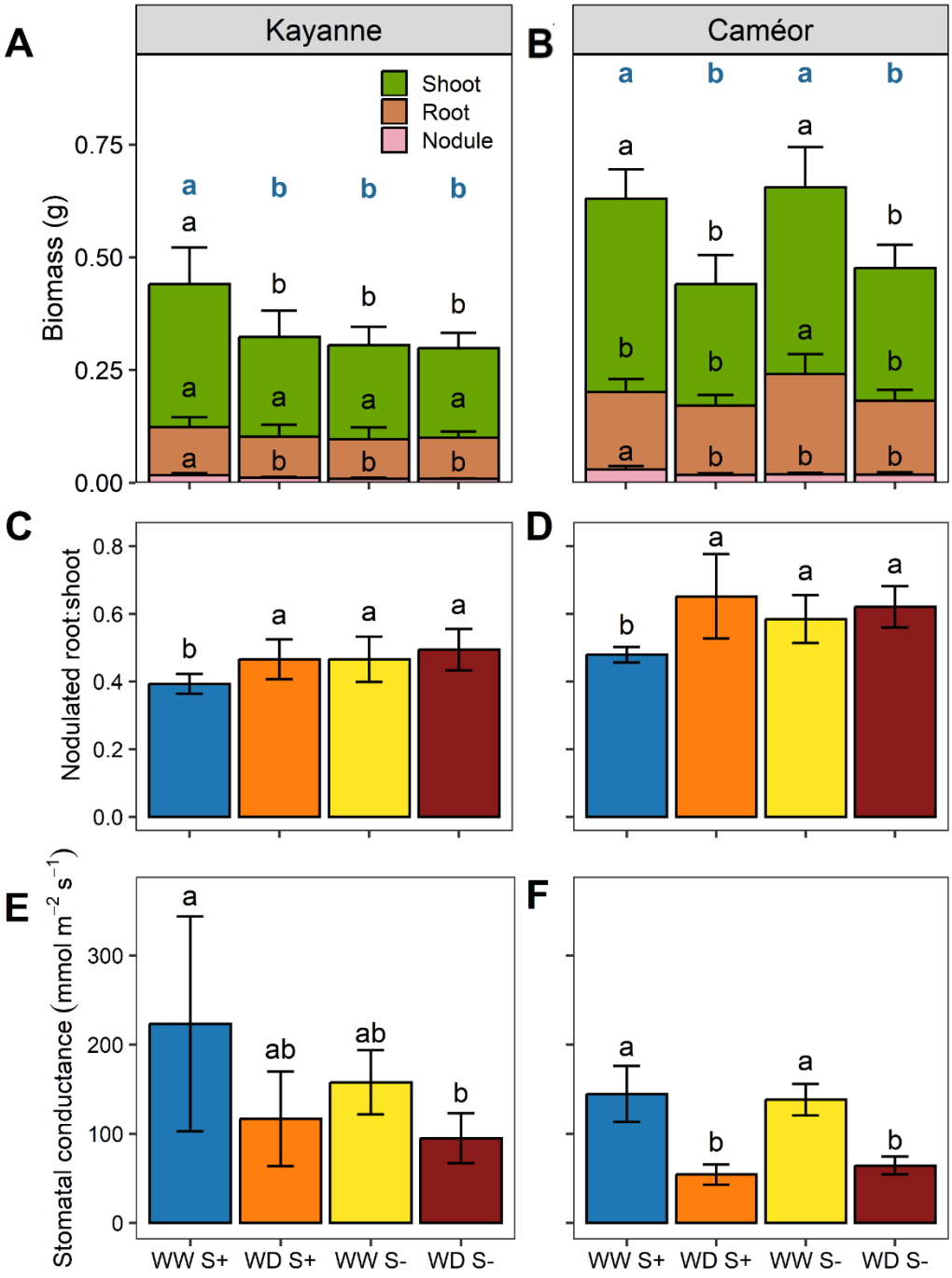


Fig. 1 Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on plant and organ biomass (A-B), nodulated-root:shoot-ratio (C, D) and stomatal conductance (E, F) in Kayanne (A, C, E) and Caméor genotype (B, D, F). Plant biomass and its allocation in shoot, root and nodules were measured on 4-7 pea plants per treatment and genotype and on 4 or 5 plants for stomatal conductance after 4 weeks of growth in RhizoTubes®. Error bars represent standard deviation (SD) and different letters on top of the error bars indicate significant differences between treatments (DMRT, $p < 0.05$). A-B, letters in blue indicate significant differences for total plant biomass and letters in black indicate significant differences for organs. Control: WW S+.

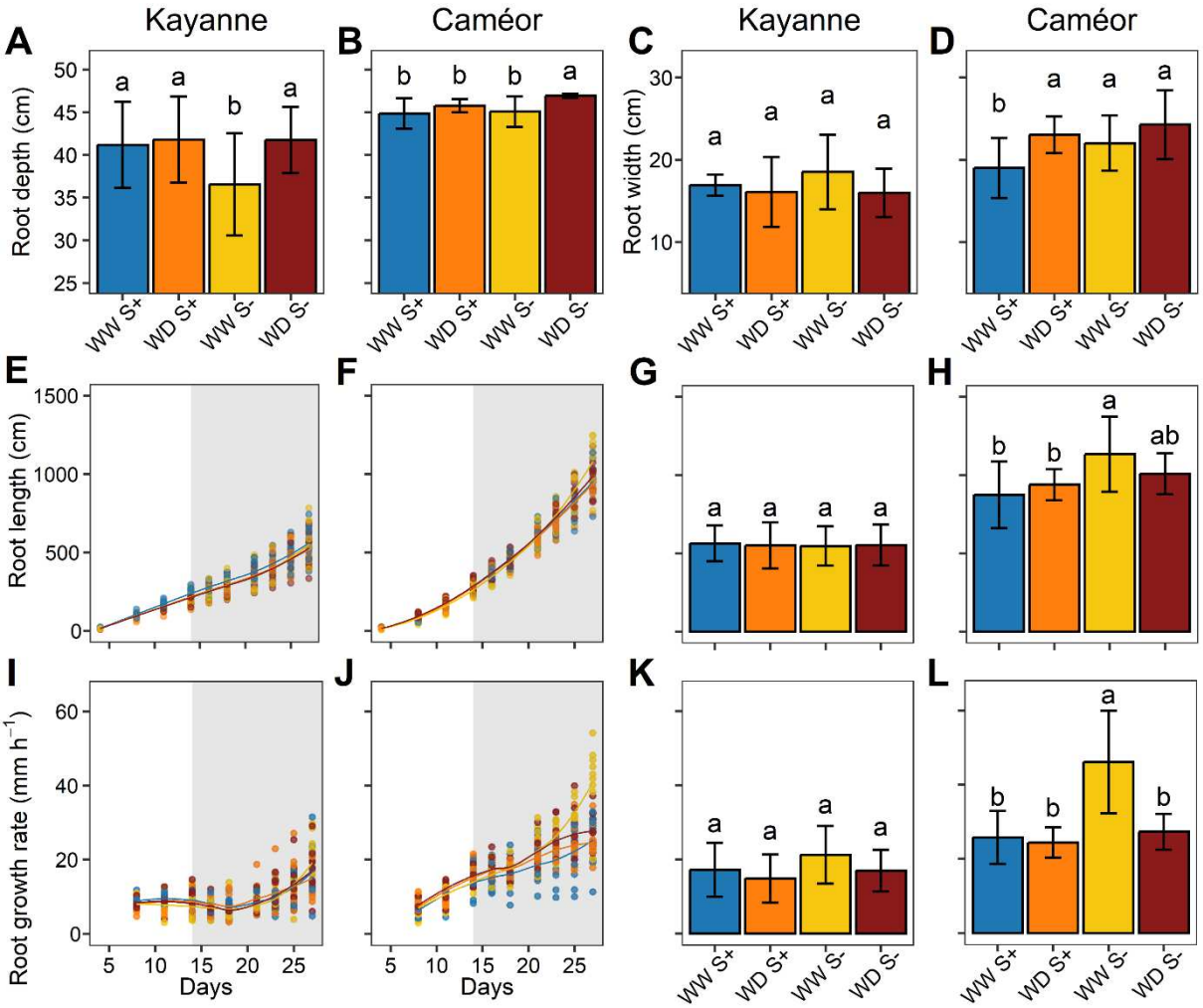


Fig. 2 Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on root depth (A, B) and root width (C, D), total root length (E-H) and root growth rate (I-L) in Kayanne (A, C, E, G, I, K) and Caméor genotype (B, D, F, H, J, L). Images were taken every 3-4 days (n= 9-13- throughout the experiment (E, F, I, J) and traits were compared at harvest after 4 weeks of growth in RhizoTubes® where mean values are presented with standard deviation as error bars (A-D, G, H, K, L). Root growth rate was calculated as the difference of root length between two time points and temporal trends of root growth and root length were smoothed using locally estimated scatterplot smoothing (LOESS). Grey area represents the 2 weeks of treatment application. Different letters indicate significant difference between treatment conditions (DMRT, $p < 0.05$). Control: WW S+.

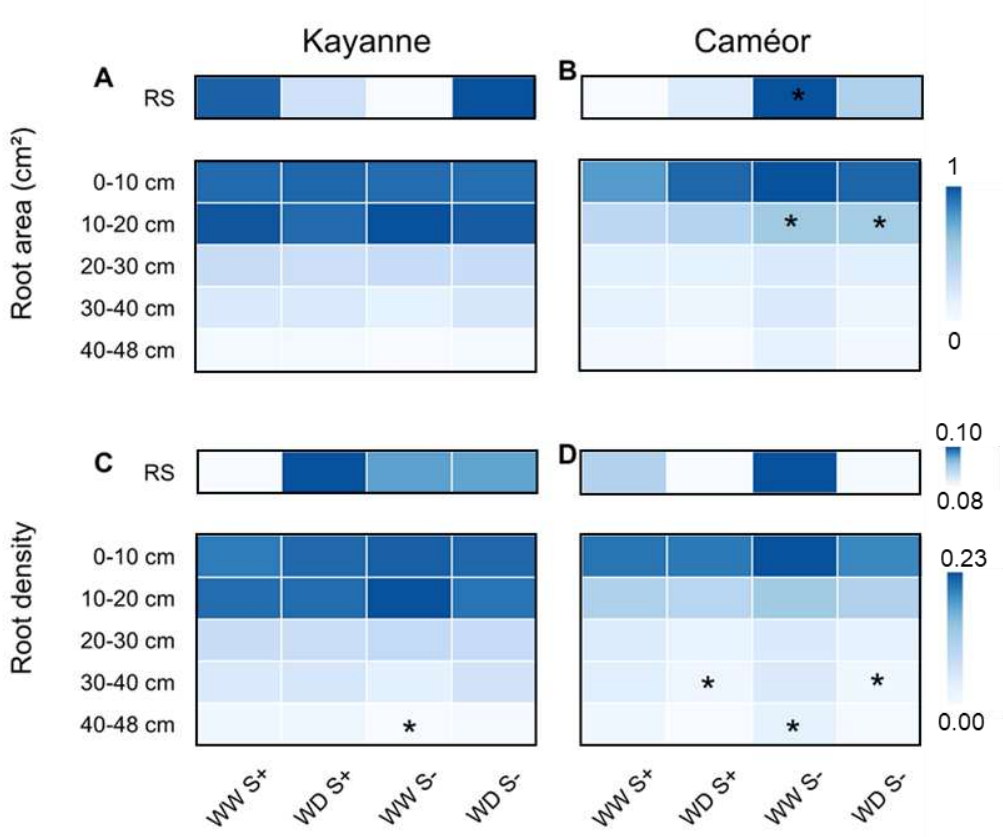


Fig. 3 Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on root area (A, B) and root density (C, D) at the whole root system level (RS) and on 10-cm-deep-sections of the RS in Kayanne (A, C) and Caméor genotype (B, D). Heatmaps show mean values of root traits measured on 9-13 plants per treatment and genotype. Values of traits were normalized for each trait and genotype so intensity of color reflects trait value with blue representing high values and white representing low values with different scales for whole RS analysis and the 10-cm-deep-sections analysis. Asterisks indicate significant difference between treatments and control (WW S+, DMRT, $p < 0.05$).

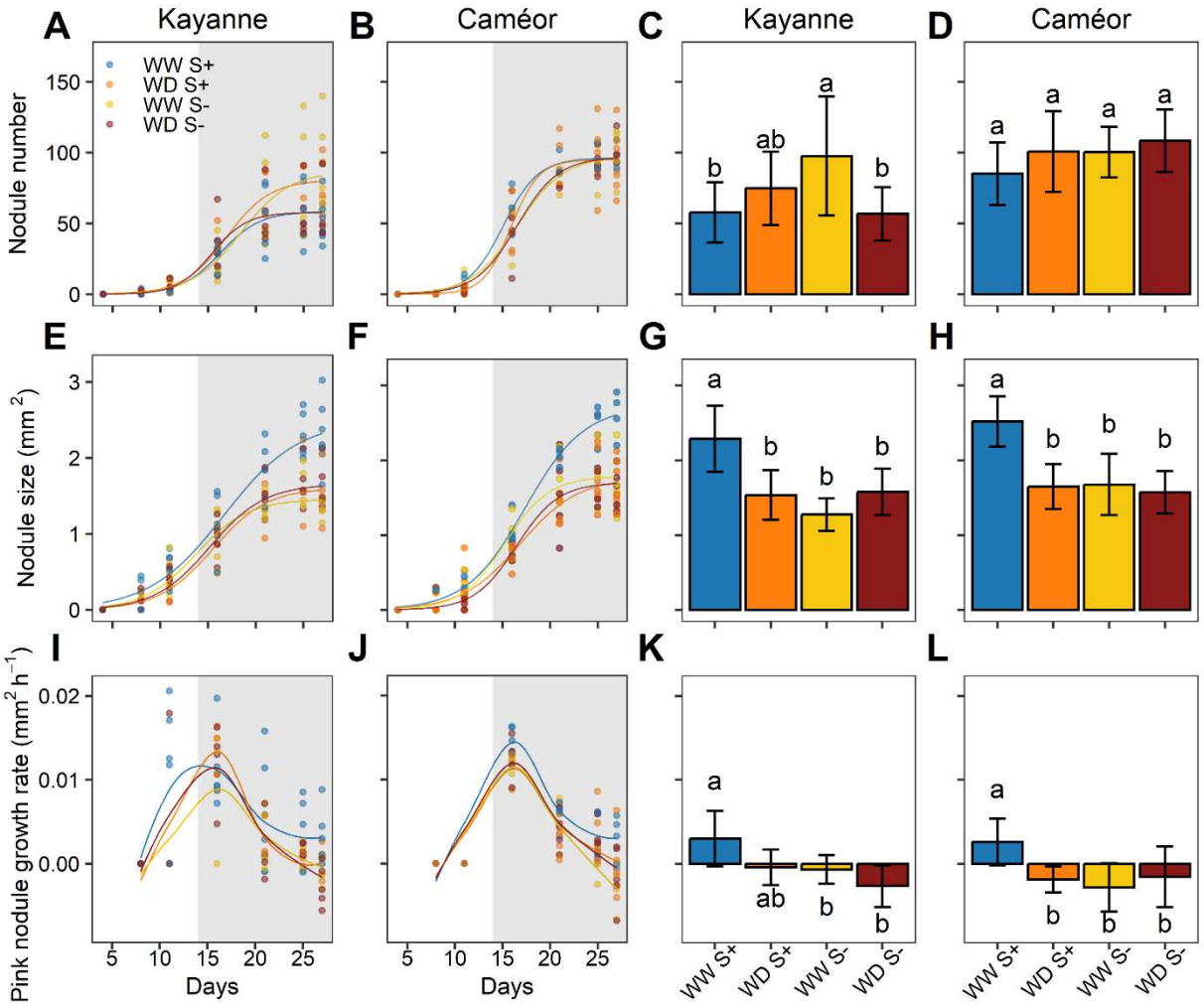


Fig. 4 Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on nodule number (A-D), nodule size (E-H) and pink nodule growth rate (I-L) in Kayanne (A, C, E, G, I, K) and Caméor genotypes (B, D, F, H, J, L). Images were taken every 3-4 days on 6-8 plants per treatment and genotype, while nodule phenological stage, number and size were measured throughout experiment (A, B, E, F, I, J) and at harvest where mean values are presented with standard deviation as error bars (C, D, G, H, K, L). Nodule size and number data were fitted to sigmoidal curves. Nodule growth rate was calculated as the difference of root length between two time points and temporal trends were smoothed using locally estimated scatterplot smoothing (LOESS). Grey area represents the 2 weeks of treatment application. Different letters indicate significant difference between treatment conditions (DMRT, $p < 0.05$). Control: WW S+.

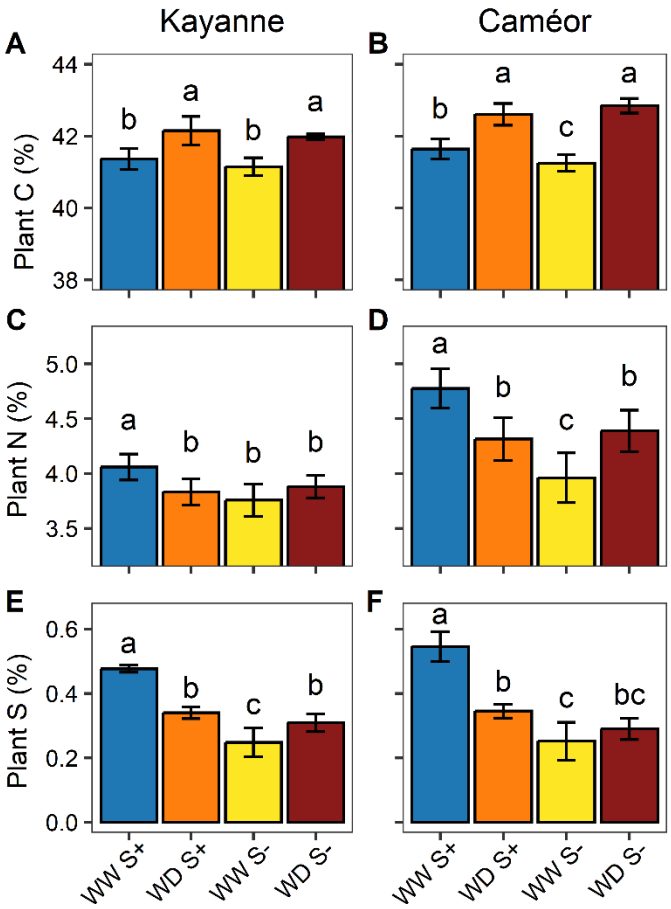


Fig. 5 Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on total plant concentration of carbon (A, B), nitrogen (C, D) and sulfur (E, F) in Kayanne (A, C, E) and Caméor genotype (B, D, F). Concentration of elements in the whole plant was measured on 4 to 6 pea plants per treatment and genotype. Mean values are presented with standard deviation as error bars. Different letters indicate significant difference between treatment (DMRT, $p < 0.05$). Control: WW S+.

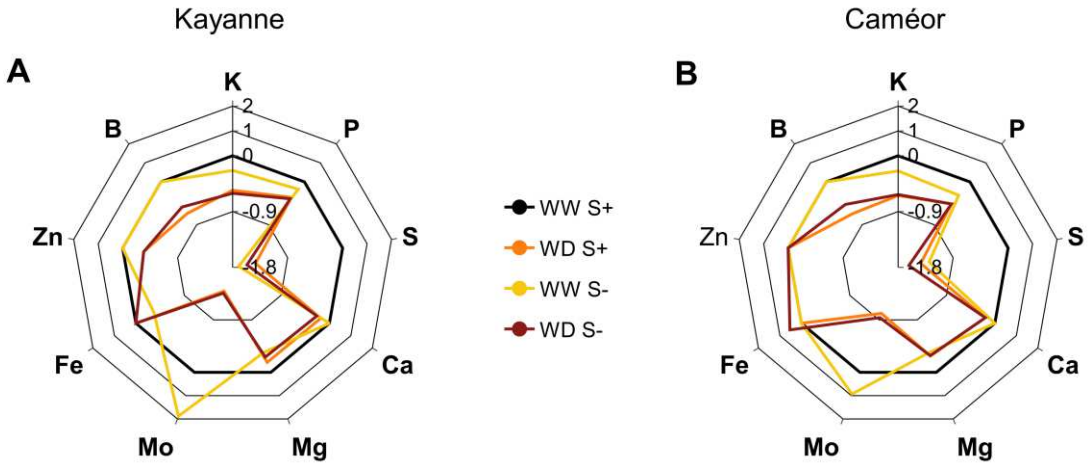


Fig. 6 Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on the uptake efficiency of essential elements in Kayanne (A) and Caméor genotype (B). Uptake efficiency was measured on 4-6 pea plants per treatment and genotype. (A, B) Radar diagrams summarising scaled changes in uptake efficiency relative to control conditions. The negative axis is scaled 2.5 times the original size. Element in bold letters exhibit significant concentration changes in response to treatment for a given genotype (DMRT, $p < 0.05$). Control: WW S+.

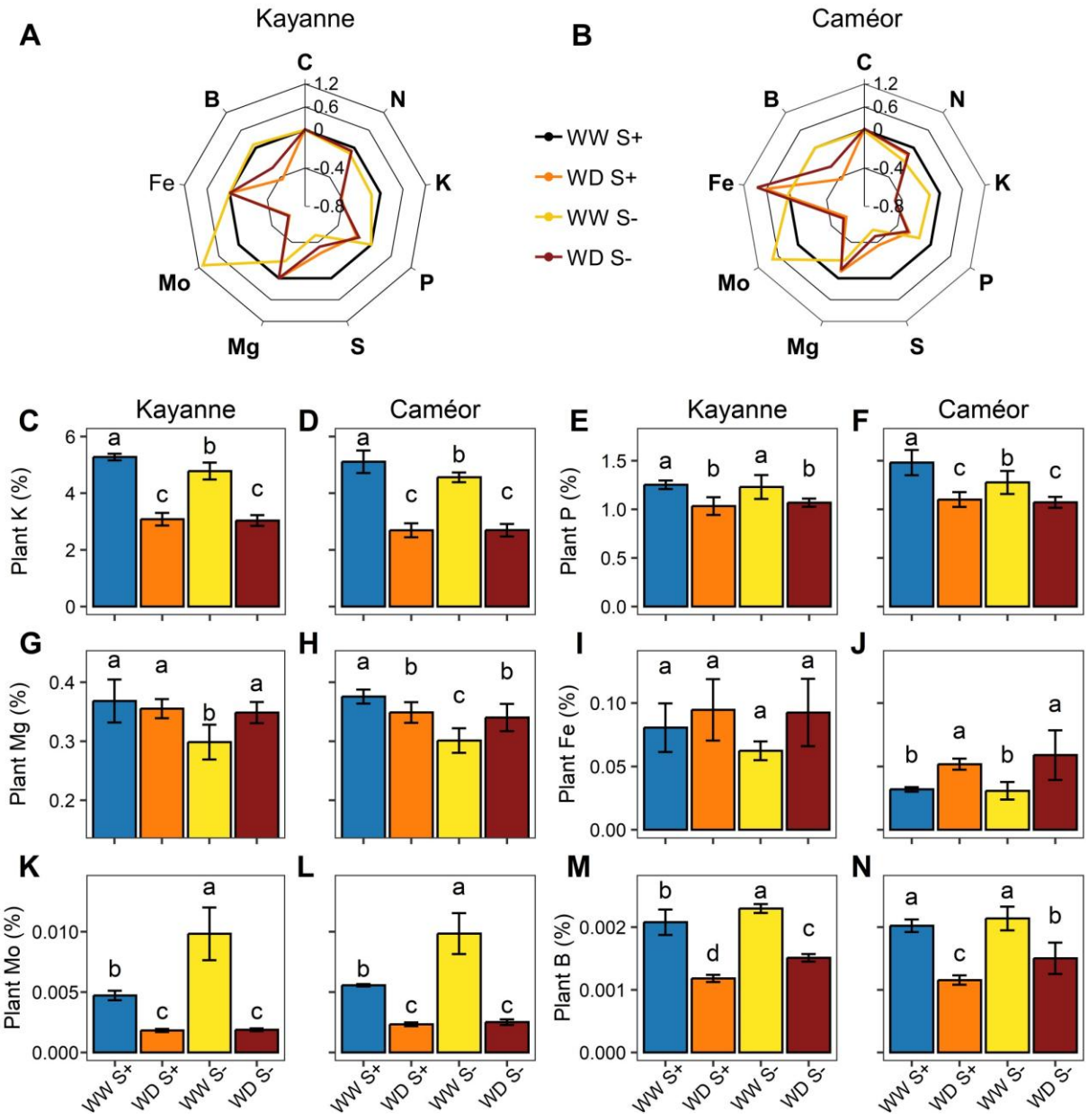


Fig. 7 Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on total plant concentration of essential elements in Kayanne (A, C, E, G, I, K, M) and Caméor genotype (B, D, F, H, J, L, N). Plant elemental concentration was measured on 4-6 pea plants per treatment and genotype. Radar diagrams summarize significant changes in plant elemental concentration relative to control conditions (A, B). The negative axis is scaled 2.5 times the original size. Element in bold letters exhibit significant concentration changes in response to treatment for a given genotype (DMRT, $p < 0.05$). Different letters indicate significant difference between treatment (DMRT, $p < 0.05$). Control: WW S+.

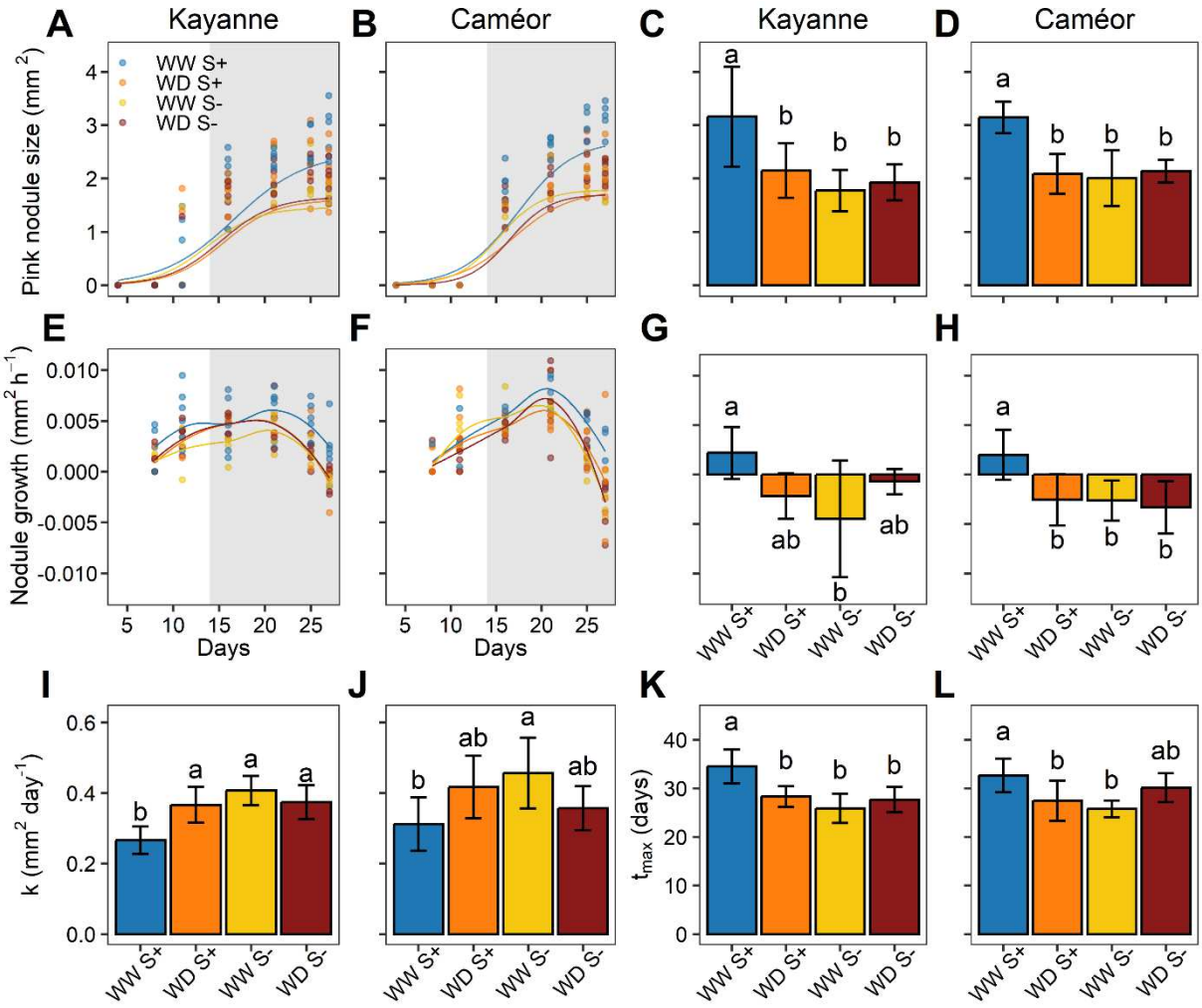


Figure S1: Influence of water deficit (WD S+), sulfur deficiency (WW S-) and their combination (WD S-) on pink nodule size (A-D) and nodule growth (E-H) temporal dynamics during experiment (A, B, E, F) and at harvest (C, D, G, H), nodule growth rate (k; I, J) and nodule growth duration (t_{max}; K, L) in Kayanne (A, C, E, G, I, K) and Caméor genotype (B, D, F, H, J, L). The nodule size growth rate (k) and nodule growth duration (i.e., number of days to reach maximum nodule size) were obtained from nodule size temporal dynamics. The growth rate for nodule size was calculated between each of the two imaging time points to study the evolution of nodule growth. Images were taken every 3-4 days on 6-8 plants per treatment and genotype, nodule size were measured and data were fitted to sigmoidal curve. Each point represents a plant and mean values are presented with standard deviation as error bars. Different letters indicate significant difference between treatment conditions (DMRT, $p < 0.05$). Control: WW S+.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTablesJoshietal.xlsx