

Article

Single and combined abiotic stress in maize root morphology

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Abstract: Plants are continually exposed to multiple stresses, which co-occur in nature and the net effects are frequently more non-additive (i.e., synergistic or antagonistic) suggesting ‘unique’ responses respect to that of the individual stress. Further, plant stress responses are not uniform showing a high spatial and temporal variability among and along the different organs. In this respect, the present work investigated the morphological responses of different root types (seminal, seminal lateral, primary, primary lateral) of maize plants exposed to single (drought and heat) and combined stress (drought + heat). Data were evaluated by a specific root image analysis system (WinRHIZO) and analyzed by uni- and multi-variate statistical analysis. The results indicated that primary root and their laterals were the types more sensitive to the single and combined stresses while the seminal laterals specifically responded to the combined only. Further, antagonistic and synergistic effects were observed for the specific traits in the primary and their laterals and in the seminal lateral roots in response to the combined stress. These results suggested that maize root system modified specific root types and traits to face with different stressful environmental conditions highlighting that the adaptation strategy to the combined stress may be different from that of the individual ones. The knowledge of “unique or shared” responses of plant to multiple stress can be utilized to develop varieties with broad spectrum stress tolerance.

Keywords: combined stresses; drought stress; heat stress; maize; root morphology; root types.

1. Introduction

The European climate change scenarios will be characterized by extreme temperature, heat waves and warmer days along with dry days and droughts, especially in Southern Europe [1]. Emerging evidences indicate that these climate change-related events will negatively impact on the plant/crop/forest productivity in both natural and agro-ecosystems [2-7]. To date, the effects and the plant responses to each stressor have been extensively studied at both morpho-physiological and molecular level. For example, both drought and heat stress reduced the photosynthetic activity, modified the oxidative metabolism inducing membrane instability [8], changed the phosphoproteome [9] and, consequently affected grain yield and quality in maize [10]. However, under field conditions, these abiotic stresses co-occur concurrently during the plant life cycle stimulating fine-tuned and early-prompted plant responses to allocate resources efficiently for the adaptation to the coexistent threats. Recent studies uncover that plants evoke a “unique response” to the drought and heat combined stresses [11-13], suggesting that their effects are mostly non-additive (i.e., synergistic and antagonistic) and, therefore, cannot be predicted through single-stressor results. Although few studies have been performed so far, the impacts of the combined drought and heat stress on maize growth, development and yield production and quality have been pointed out [9, 14-17]. In particular, the maize responses to the combined abiotic stress were mainly focused on the aerial part of plant and its reproductive organs, probably for breeding aims. To the best of our knowledge, no information on the maize root system responses to the combined drought and heat stress are available. This knowledge is even more interesting considering that single maize root types, such as

the embryonic roots (primary and seminal) and the post-embryonic roots (nodal and lateral) [18], differently responded to environmental cues and could be a “source” of stress adaptation. For example, different responses among root types to drought stress [19,20], allelochemicals [21,22], P deficiency [23], and to combined N deficiency/drought stress [24] were already reported. In this respect, a microcosm experiment was setup for studying several growth and morphological parameters of different root types in response to drought (30% of field capacity) and heat stress (32° air temperature) and their combination in maize plants. Further, the additive, synergic and antagonistic effects in combined drought and heat stress were also evaluated.

Considering the cooperation or the antagonism mechanisms of diverse root traits or phenes for the root phenotypes adaptations in diverse environments [25-27], the use of the multivariate approach for identifying the root architecture strategy in terms of functional traits and mechanisms, which operate independently or jointly, was raised [28]. In this respect, the maize root architecture responses to the single and combined stress were evaluated with the sparse Partial Least Squares-Discriminant Analysis (sPLS-DA), a novel multivariate approach, which differently to the unsupervised methods (i.e. principal component analysis, the PCA) pointed out very satisfying predictive performances being able to select informative variables [29].

Maize (*Zea mays* L.) is a major cereal crop, and food for both humans and animals, widely used as resource for industrial use and for bio-energy production worldwide.. It is highly productive under water suitable by irrigation, but the water scarcity, high temperature, and their combination as observed in the semi-arid environments caused a reduction on its yield and quality. Hence, the maize tolerance improvement to drought, heat, and their combination has become a challenge for the breeding programs [30].

2. Results

2.1. Univariate analysis of the root morphological data

One-way ANOVA revealed that each stress (H and D), alone or in combination, significantly affected plant growth in terms of both fresh and dry weight. In particular, the combined stress reduced the fresh weight more than heat ones respect to the control but similarly to the drought stress. By contrast, all the stresses diminished the plant dry weight at similar extent (Fig. 1).

Figure 2 showed the single root types of maize seedlings were also modified by single and combined abiotic stress.

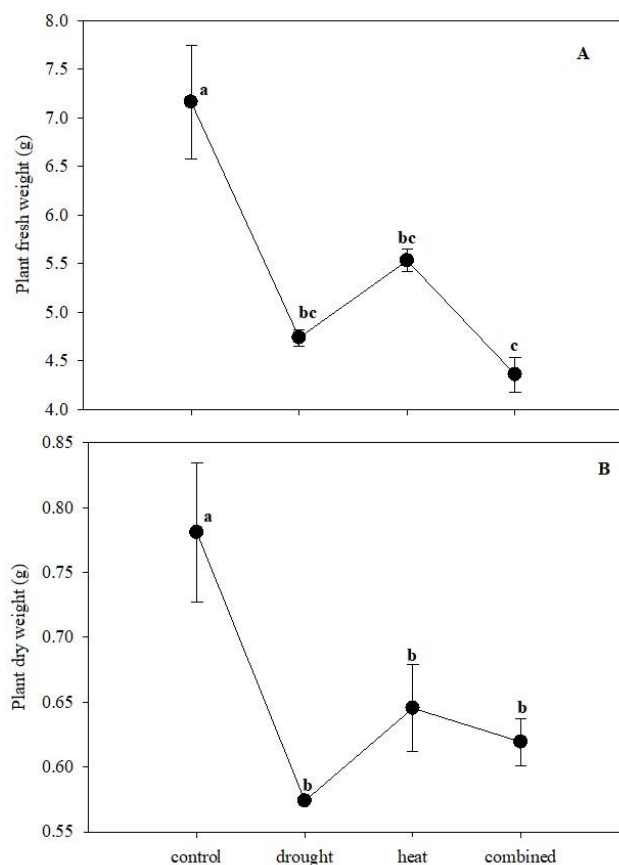


Figure 1 – The maize growth in terms of fresh (A) and dry weight (B) in presence of single (drought and heat stress) and combined stress. The bars represented the error standard (N=4).

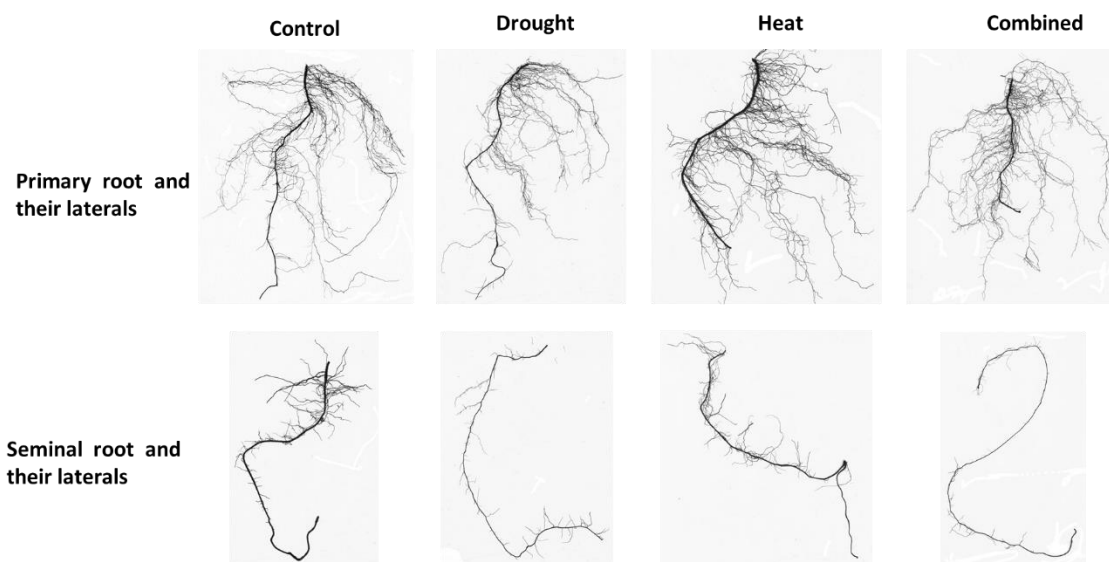


Figure 2 – Primary and seminal roots and their laterals of maize seedlings exposed to drought, heat and their combination (Combined).

Out of the eleven traits of the primary roots, eight were significantly modified by stresses in comparison to the control (Table 1). The drought stress significantly increased the RLR, RMR, fineness, tissue density, branching zone reducing the branching density; conversely, the RLR and branching zone were not affected by heat stress, which, in turn, weakly increased and reduced the

fineness and branching density, respectively (Table 1). The combined stress pattern was similar to the drought stress but with a sharply increase of the dry weight and, consequently, of both the RMR and tissue density (Table 1).

Table 1 – Morphology of primary root of maize plants exposed to single (drought and heat) and combined stress (drought + heat).

Category	Parameters	Treatments			
		<i>Control</i>	<i>Drought</i>	<i>Heat</i>	<i>Combined</i>
<i>biometric</i>	Fresh weight (g)	0.128 (0.008)a	0.131 (0.001)a	0.147 (0.001)a	0.162 (0.021)a
	Dry weight (g)	0.0111(0.0009)b	0.0116 (0.0006)b	0.0131(0.0003)b	0.0168 (0.0017)a
<i>geometric</i>	Length (cm)	49 (1)a	71 (6)a	57 (4)a	66 (12)a
	Surface area (cm²)	9.460 (0.009)a	10.669 (0.896)a	9.723 (0.587)a	10.415 (1.766)a
<i>Length components</i>	RLR (cm g⁻¹)	63 (4)b	123 (12)a	83 (2)b	117 (15)a
	RMR (g g⁻¹)	0.01381(0.00016) c	0.01840(0.00002)b	0.02004(0.00051)b	0.02702(0.00205)a
	Fineness (cm cm⁻³)	336 (11)a	531 (11)b	396 (8)c	500 (6)d
	Tissue density (g cm⁻³)	0.075 (0.007)c	0.097 (0.006)b	0.097 (0.003)b	0.117 (0.007)a
<i>branching</i>	Root zone formation (cm)	9 (1)ab	3 (1)b	9 (3)a	7 (1)ab
	Branching zone formation (cm)	42 (2)b	65 (5)a	48 (1)ab	64 (11)a
	Branching density (n cm⁻¹)	4.961 (0.101)a	3.389 (0.051)c	4.276 (0.027)b	4.152 (0.284)b

Different letters along the rows indicated significant differences among the means at $p < 0.05$ (Test of Tukey). The values within the brackets are the error standards (n=4).

The morphology of the seminal roots was lesser affected and not by all the stresses (Table 2). In particular, the drought stress reduced the fresh weight increasing the fineness only; the heat stress increased the fineness but, differently from the drought stress, raised the branching density (Table 2). Conversely, the combined stress did not modify the seminal root morphology respect to the control (Table 2).

Table 2 – Morphology of seminal root of maize plants exposed to single (drought and heat) and combined stress (drought + heat).

Category	Parameters	Treatments			
		Control	Drought	Heat	Combined
<i>biometric</i>	Fresh weight (g)	0.285 (0.031)a	0.172 (0.029)b	0.205 (0.043)ab	0.176 (0.018)b
	Dry weight (g)	0.021 (0.004)a	0.015 (0.003)a	0.014 (0.004)a	0.018 (0.002)a
<i>geometric</i>	Length (cm)	64 (7)a	64 (7)a	63 (8)a	51 (2) a
	Surface area(cm²)	11 (1)a	10 (1)a	12 (2)a	10 (1)a
<i>Length components</i>	RLR(cm g⁻¹)	82 (13)a	103 (14)a	97 (14)a	83 (1)a
	RMR (g g⁻¹)	0.027 (0.002)a	0.023 (0.002)a	0.027 (0.007)a	0.031 (0.003)a
	Fineness (cm cm⁻³)	294 (47)b	463 (21)a	435 (55)a	362 (24)ab
	Tissue density (g cm⁻³)	0.094 (0.007)ab	0.064 (0.023)b	0.091 (0.003)ab	0.124 (0.001)a
<i>branching</i>	Root zone formation (cm)	7 (1)ab	8 (2)ab	12 (3)a	6 (1)b
	Branching zone formation (cm)	54 (5)a	55 (6)a	57 (14)a	44 (3)a
	Branching density (n cm⁻¹)	2.8 (0.1)b	2.9 (0.1)b	4.5 (0.4)a	3.0 (0.2)b

Different letters along the rows indicated significative differences among the means at $p < 0.05$ (Test of Tukey). The values within the brackets are the error standards ($n=4$).

Seven traits of the primary lateral roots were differentially modified by the stress (Table 3). The RLR and fineness were increased by drought stress in comparison with the control; while the heat stress increased the fresh weight, length, surface area and RLR but not the fineness. The combined stress pointed out a significant and marked modification of the primary lateral root morphology, increasing by 84%, 56%, 124%, 43% and 57% the length, surface area, RLR, fineness and average length, respectively (Table 3).

Table 3 – Morphology of primary lateral root of maize plants exposed to single (drought and heat) and combined stress (drought + heat).

Category	Parameters	Treatments			
		Control	Drought	Heat	Combined
<i>biometric</i>	Fresh weight (g)	0.212 (0.012)b	0.280 (0.047)ab	0.315 (0.040)a	0.225 (0.001)ab
	Dry weight (g)	0.022 (0.004)a	0.016 (0.001)a	0.021 (0.003)a	0.020 (0.001)a
<i>geometric</i>	Length (cm)	557 (8)c	691 (35)bc	757(82)b	1028 (33)a
	Surface area (cm²)	34.6 (0.7)b	37.2 (2.7)b	45.8 (4.8)a	53.3 (0.1)a
	number (n)	208 (16)a	225 (14)a	200 (2)a	247 (27)a
	Average length (cm)	2.860 (0.136)b	3.076 (0.039)b	3.640 (0.384)ab	4.486 (0.578)a
<i>Length components</i>	RLR (cm g⁻¹)	736 (41)c	1195 (63)b	1125 (75)b	1650 (90)a
	RMR (g g⁻¹)	0.0324 (0.0079)a	0.0220 (0.0003)a	0.0308 (0.0031)a	0.0330 (0.0016)a
	Fineness (cm cm⁻³)	3257 (44)c	4018 (56)b	3512 (76)c	4670 (288)a
	Tissue density (g cm⁻³)	0.134 (0.027)a	0.093 (0.005)a	0.097 (0.005)a	0.093 (0.006)a

Different letters along the rows indicated significant differences among the means at $p < 0.05$ (Test of Tukey). The values within the brackets are the error standards (n=4).

The seminal lateral roots were affected by the stress in eight out of ten traits (Table 4). However, the single stress lesser influenced these root types which reduced the RMR (drought stress) and the dry weight and surface area (heat stress) in comparison to the control (Table 4). Conversely, the combined stress strongly affected the seminal lateral roots, reducing the dry weight, length, surface area and RMR and increasing the fineness (Table 4).

Table 4 – Morphology of seminal lateral roots of maize plants exposed to single (drought and heat) and combined stress (drought + heat).

Category	Parameters	Treatments			
		Control	Drought	Heat	Combined
<i>biometric</i>	Fresh weight (g)	0.0600(0.0114) ab	0.0355 (0.0048)b	0.1323 (0.0459)a	0.0149 (0.0002)b
	Dry weight (g)	0.0051 (0.0010)a	0.0035(0.0006)a b	0.0025(0.0008)b	0.0013 (0.0002)b
<i>geometric</i>	Length (cm)	113 (7)a	104 (1)ab	105 (13)ab	79 (14)b
	Surface area (cm²)	8.5 (1.3)a	7.5 (0.1)a	3.7 (0.6)b	4.5 (0.9)b
	number (n)	149 (21)ab	169 (18)ab	202 (23)a	134 (5)b
	Average length (cm)	0.872 (0.151)a	0.707 (0.081)a	0.563 (0.136)a	0.530 (0.094)a
<i>Length components</i>	RLR (cm g⁻¹)	147 (1)ab	178 (2)a	151 (12)ab	128 (19)b
	RMR (g g⁻¹)	0.0066 (0.0010)a	0.0032 (0.0001)b	0.0041(0.0015)a b	0.0021(0.0003) b
	Fineness (cm cm⁻³)	2728 (48)b	2416 (0)b	3630 (376)b	6283 (1176)a
	Tissue density (g cm⁻³)	0.095 (0.004)a	0.085 (0.015)a	0.101 (0.036)a	0.102 (0.022)a

Different letters along the rows indicated significant differences among the means at $p < 0.05$ (Test of Tukey). The values within the brackets are the error standards ($n=4$).

2.2. Additive, synergistic and antagonistic effect of combined stress

In order to evaluate the non-additive effects on the root traits significantly modified by the combined stress, we used the multiple risk model [31] that eluded the over-inflated response estimated by a simple additive model.

The response to the combined stress in maize root types were the result of the additive, synergistic and antagonistic effects of the single stress depending on the root types and traits (Figure 3). Indeed, a significant increase in the fineness and a decrease in the branching density in the primary roots under the combined stress (Table 1) was the result of an antagonistic effect of the single stress; whereas an additive effect was evoked for the enhance of dry weight, RLR, RMR, tissue density and branching zone (Fig 3A). The increase of the fresh weight in the seminal root, the only trait significantly modified by the combined stress (table 2), was the result of the additive effect (Fig. 3B). A synergistic effect could justify the increase of the length, surface area in the primary lateral roots exposed to the combined stress while an additive effect for the RLR, fineness and average length (Figure 3C). Finally, the only increase in the length of the seminal lateral roots in response to the combined stress was due to a synergistic effect, whereas an additive effect was responsible for the modifications of the dry weight, surface area, RMR and fineness (Fig. 3D).

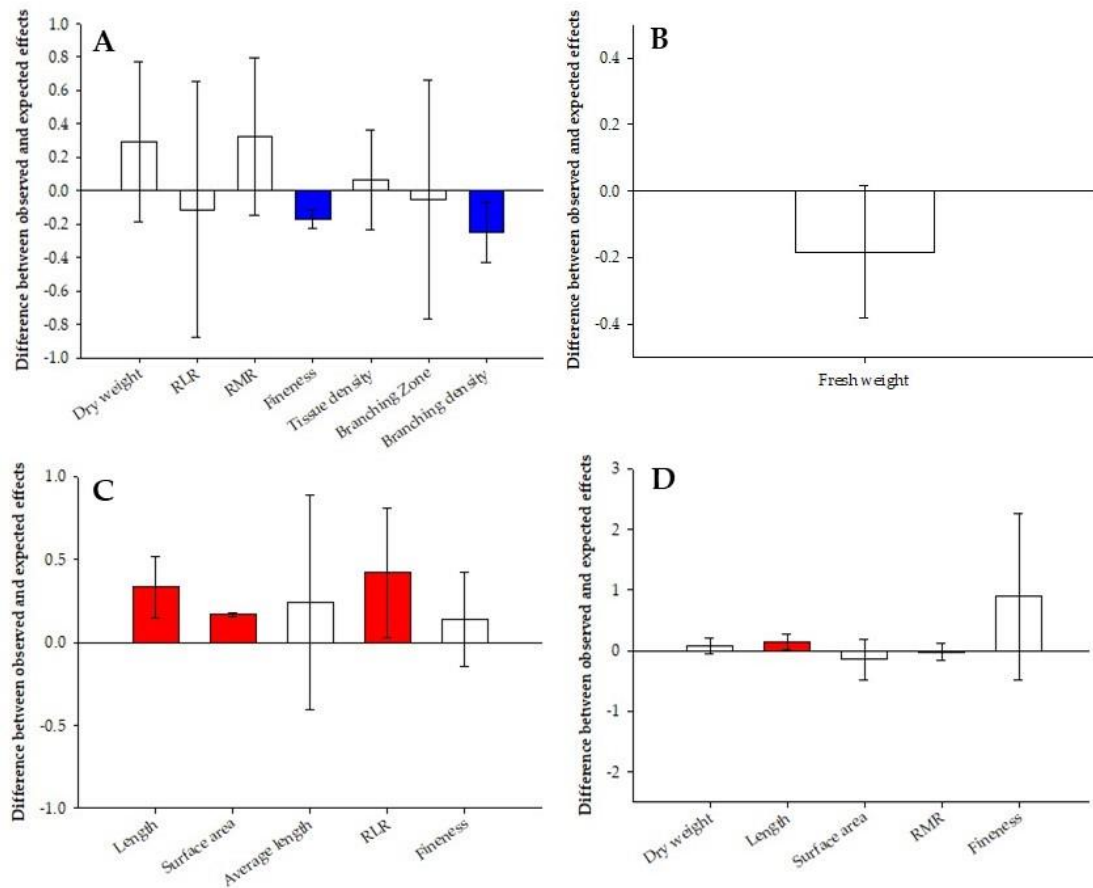


Figure 3– The combined impacts from drought and heat stress on selected traits of root primary (A), seminal (B), primary lateral (C) and seminal lateral (D) of maize plants. The combined impact of single stressors was estimated as synergist (red colour), additive (white colour) or antagonistic (blue colour) (greater than, equal to or less than expected effects, respectively, based on single stressor effect sizes). The vertical and error bars represent, respectively, the mean and the 95% confidence interval of the overall effect size difference between the observed and expected additive effects from combined drought and heat on root traits of maize plants. The zero line represents the expected additive effects from combined stressors. When the means (and their 95% confidence limits) were higher than or less than the zero line, they were considered synergistic or antagonistic, respectively.

2.3. Root responses to the single and combined stress: a supervised analysis with PLS-DA.

A PERMANOVA analysis revealed that all the treatments determined a significant difference ($p=0.001$) in the root morphology as reported in Table 5. The Pairwise PERMANOVA comparisons suggested that the combined and drought stress but not the heat ones triggered a significant difference respect to the control. Furthermore, the drought stress caused a different root morphology in comparison to both the heat and combined stress (Table 5).

Table 5 - Results of the PERMANOVA analysis testing the root morphology of maize plants against stress treatment.

Factor	df	R ²	F	P
Stress treatment	3	0.6693	8.0955	<0.001
Residual	12	0.3307		
Total	15	1.0000		
Pairwise contrasts				
	F model	R2	p	
<i>C vs D</i>	9.262202	0.6068719	0.031	
<i>C vs H</i>	3.877963	0.3925873	0.053	
<i>C vs HD</i>	15.925573	0.7263469	0.027	
<i>D vs H</i>	7.056074	0.5404438	0.034	
<i>D vs HD</i>	13.677155	0.6950779	0.023	
<i>H vs HD</i>	7.751958	0.5636985	0.054	

In order to select informative and relevant root traits, we used the sparse Partial Least Squares-Discriminant Analysis (sPLSDA), a multivariate method characterized by a very satisfying predictive performance for the multiclass classification in plant biological studies [29, 32].

The performance step for the selection of the number of components suggested that 3 were enough to sharply reduce the balanced error rate (Figure 4A). Further, the final model obtained by tuning process pointed out that each component was constituted by one root morphological trait with BER around 0.18 (Fig. 4B). The sample plots on the three components permitted to visualize a sharply discrimination among the treatments with 52% of total explained variability split up by 27%, 14% and 11% for the first, second and third components, respectively (Fig. 5A and B). In particular, plotting the first two components, the combined and, at lesser extent, the drought stress were sharply separated from the control by the first component, whereas the second component discriminated the drought and, at lesser degree, the heat plants respect to the control (Fig. 5A). The addition of the third component permitted to separate the heat plants from the control ones (Fig. 5B).

Figure 5C, D and E showed the selected root traits and relative loading weights for each component and the colour indicated the treatments for which the selected root traits has a maximal mean loading weight value. In particular, the root traits identified as performants for the sPLS-DA model were the primary lateral RLR in the combined stress, primary root branching density in the control and seminal root branching density in the drought stress for the first, second and third component, respectively (Fig. 5C, D and E).

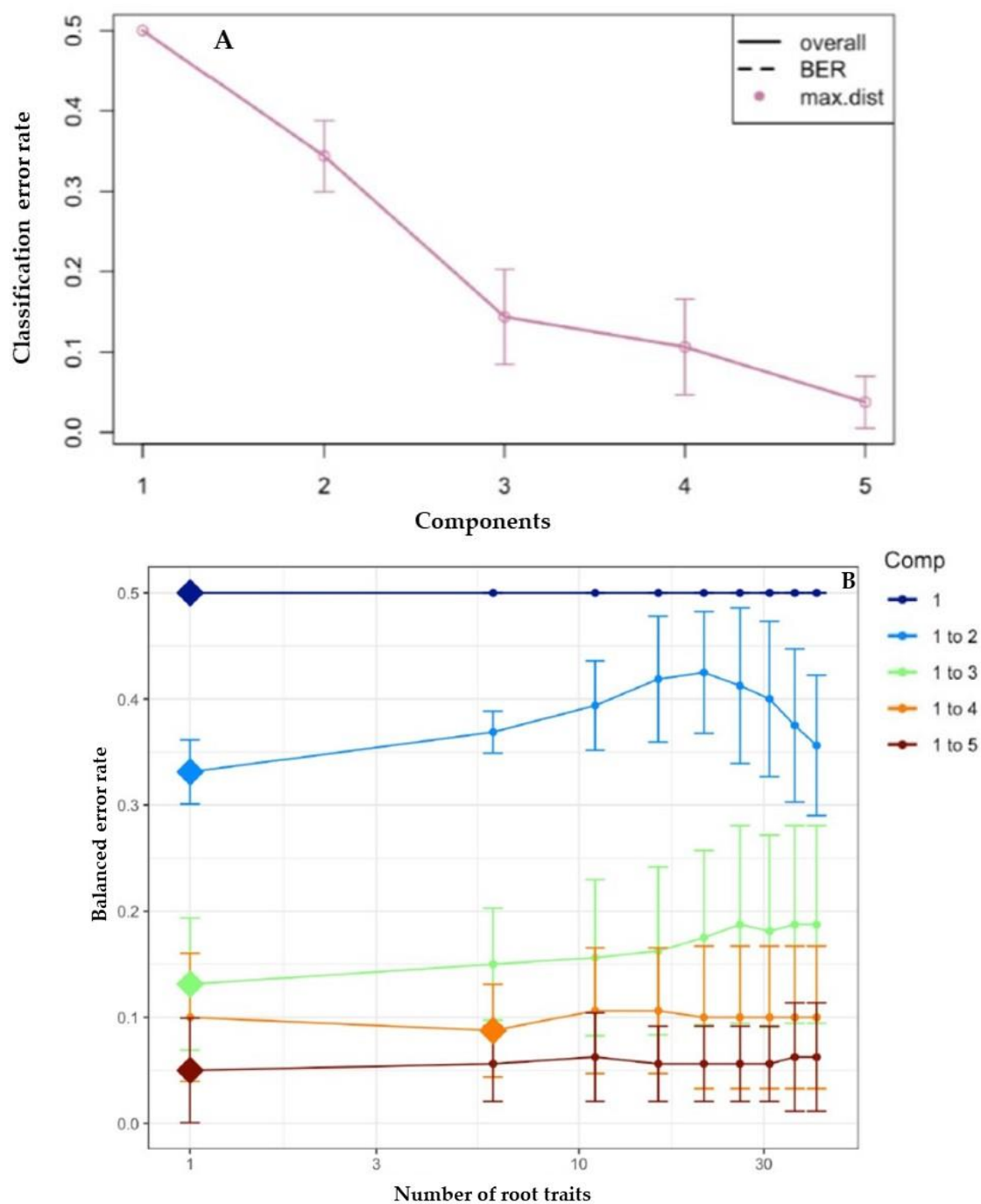


Figure 4 – Choosing the number of components in sPLS-DA by performance test (A). Mean classification by overall and balanced error rate (5 cross-validation averaged 50 times) for each sPLS-DA component. **Choosing the number of root traits for each sPLS-DA components by tuning test (B).** Estimated classification balanced error rates for root morphology data set (5 cross-validation averaged 50 times) with respect to the number of selected root traits for the sparse exploratory approaches

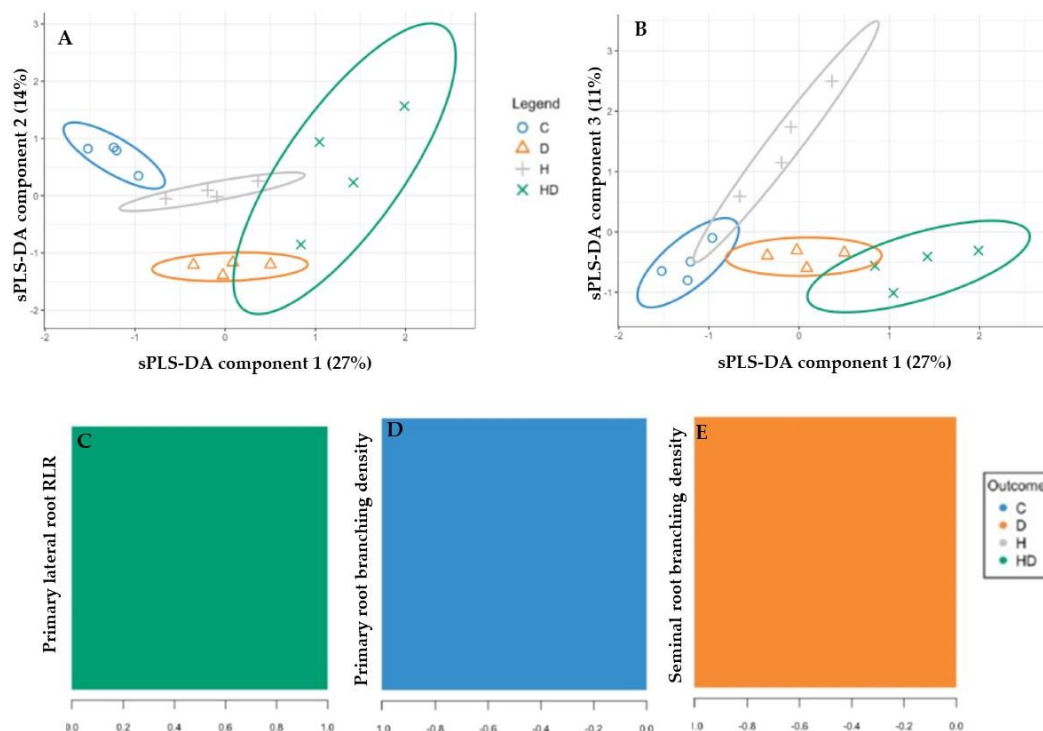


Figure 5 - sPLS-DA sample plot for the different components using 95% confidence ellipses. (A) Component 1 vs. Component 2, (B) Component 1 vs. Component 2.

Contribution plots by loading weights of the root traits selected for each sPLS-DA component. (C) Component 1, (D) Component 2, (E) Component 3.

3. Discussion

3.1. Single stress determined different root type-related morphological responses.

Drought as well as heat stress affected the primary root more than the other types. Indeed, differently from the seminal root, the primary improved its length both in term of absolute (LR) and relative value (RLR) but with different intensities: higher in drought than heat plants. The primary root is the very early dominant type in maize seedlings determining the early vigor and, by deepening in the subsoil strata, the survival under water deficit and higher air-temperature [20, 33]. Further, the rooting depth is a phenon very interesting as it confers drought tolerance/resistance in several plant species such as rice [34] and wheat [35] improving the subsoil water capture. In order to evaluate the “morphological pattern” determining the increase in the RLR, trait that better than absolute length has been related to the plant’s potential for water and nutrient acquisition under stress conditions [36], we estimated its “morphological components”, that is RMR, fineness and tissue density. In according to Ryser and Lambers (1995) [37], the higher RLR could be due to an increase of the RMR and/or fineness and/or decrease of the tissue density as explained by the equation 1. The results indicated that the increase of RLR in the primary root, under both single stress conditions, was due to a concomitant enhancement of the biomass allocation (RMR) and fineness, accompanied by a higher RTD trait positively correlated with the degree of lignification and cell wall thickness [38-40]. Hence, the maize seedlings under both the drought and heat stress, increased the length of the primary root, which appeared more fineness and, at the same time, thickness, useful traits to penetrate the hard soil layers under water stress [41]. To note that, the RLR of the primary root under the drought stress was also higher than to heat ones and this difference was due to a higher fineness rather than biomass allocation variation (Table 1). Extensive transcriptomic and proteomic studies

revealed specific transcripts and proteins related with cell wall extension properties in the primary root of maize seedlings exposed to the water stress (see reference in [42,43]). Therefore, according to our results, the drought stress could induced a different molecular mechanism respect to the heat one, which differently regulated the elongation of the primary roots as observed in this study.

The seminal root axes were the lesser modified by the single stresses, which determined an increase in their fineness only (Tab. 2). The maize seminal roots responded to the drought and heat stress by reducing their emergence angle and length that resulted in the soil deepening [20, 24]. These results were not observed in this study, probably, for the pot volume that limited the soil exploration.. Besides the primary and seminal root axes, the laterals, as post-embryonic roots arising from these axes, played an important role for the water acquisition from soil allowing an improvement of the soil exploration due to their higher surface to volume ratio [44]. Differently to the seminal lateral roots, the primary laterals were more modified by both single stresses: the drought-stressed plant pointed out similar lateral length to the heat plants but with a higher fineness (Table 3 and 4). Again, the trait 'fineness' was differently regulated by drought respect to the heat stress allowing a higher surface soil contact of the laterals, fundamental for water uptake. To note that, although the length of the primary lateral roots was increased, the branching density was decreased in both stressed plants. This reduction associated with an increase of length the primary lateral roots constituted an important root "phenotypic pattern", which improved drought resistance in maize plants by reducing both the intra-plant competition for the photosynthates and the capture of mobile soil resources such as water [19].

3.2. Combined stress caused different root type-related morphological response respect to the single stress with non-additive effects.

The recent studies on combined stress, such as drought and heat, were focused on the morpho-physiological and molecular responses of plant aerial traits such as yield and quality [45, 46], plant growth [47], foliar chemistry [48] and leaf physiology [14, 49]. Conversely, very few studies have focused on the root system responses to combined stress and no information are available for the different root types. Here, for the first time, the responses of the single root types of maize seedlings to the combined stress were reported. It induced similar morphological pattern to the single stresses resembling those of the drought and heat stress for the primary and seminal root axes and those of the heat for the primary lateral roots only. Conversely, the morphology of the seminal lateral roots of the combined stress was completely different from that of the single stresses: length and biomass were sharply inhibited. Probably, the water scarcity exacerbated by the heat rendered not useful the exploration and resource exploitation of the topsoil strata; hence, the maize plants engaged their internal resource towards to the root classes, such as the primary and its laterals, mainly localized in the subsoil to reach the water reserve .

Analyzing the pattern of the combined stress on the root traits is interesting to understand their additive (equal to the sum of the single-stress effects), synergistic (higher than expected) or antagonistic (lower than expected) effects. Besides a useful information on morphological pattern, these could provide a hypothesis on signaling pathways and molecular mechanisms underlying the plant strategy in presence of simultaneous stress. For example, the synergistic effect of NaCl and ABA in *Arabidopsis thaliana* was induced by the expression of *Responsive-to-Dehydration 29A (RD29A)* that cannot be explained by the sum of responses to the single stresses [50]. Furthermore, the antagonistic effect of drought and insect herbivory could be explained by synergistic interactions between JA and ABA signaling [51]. In our study, the combined stress produced mostly additive effects, but synergistic and antagonistic effects for specific trait and root types were also observed (Fig. 3) suggesting, for these latter, an unique molecular and signaling interaction mechanisms. These results were below discussed for their ecological role and, plant fitness in environment characterized by co-occurring drought and heat stress. Differently to the seminal and their lateral roots, primary lateral root traits were mostly synergized whereas those related to the primary root axes were antagonized (Fig. 3). Why non-additive effects (synergistic and antagonistic ones) were observed in the primary and their lateral respect to the seminal roots? Their different location within the soil environment that

caused diverse efficiency for the resource acquisition could be hypothesized. Indeed, the primary and their lateral roots are placed in the water-rich subsoil strata making these root types more important under water scarcity due to lesser rainfall/irrigation, further aggravated by a simultaneous heat stress. The root deepening during drought stress was revealed in maize plants [20,24,35]. However, our results pointed out that combined stress antagonized the fineness and the branching density of the primary roots (Fig. 3A). Probably, the thicker primary roots could be more useful for the deeper penetration in soil compacted caused by both drought and heat stress [52,53]. At the same time, the reduction of the branching density of the primary root axes along with the root deepening is a useful strategy for the root adaptation in water scarcity environments as observed by [54]. Next to the antagonistic effects in the primary roots, the synergistic effects were observed on the length, surface area and RLR traits of the primary lateral roots (Fig. 3C). Sustained by the soil penetration of the thicker primary roots, the longer laterals with higher surface contact with soil could increase the exploration of subsoil strata characterized by higher soil moisture under combined drought and heat stress.

3.3. Primary lateral RLR, primary and seminal root branching density discriminated the root phenotypes in drought and heat stress and their combination.

Currently, for understanding the root strategy for the plant adaptation to the abiotic stress, the multivariate analysis were applied. This suitable method allowed to identify the efficient and meaningful “multi-trait classifiers” of the root systems [28] and the detection of the functional root traits and mechanisms, which operate independently or jointly. For example, the principal component analysis (PCA) permitted to identify the root ideotypes in drought stress for the peanut landraces [55], sugar beet genotypes [56], bean landraces [57] and soybean genotypes [58]. Conversely, the sparse PLS-DA, a supervised technique, made efficient the trait selection and dimension reduction simultaneously by imposing sparsity to the solution [59], making it a novel approach to investigate high dimensional and redundant root data.

The sPLS-DA clearly separated the root phenotypes of the combined and, at lesser degree, the drought stress from that of the control by the component 1 (Fig. 5A). This component showed high positive loading for the RLR of the primary lateral roots and the combined stress was the group for which the selected variable had a maximal mean value. Hence, the root phenotypes of the combined stress were different from that of the control plants as firstly suggested by Permanova analysis and the RLR of the primary lateral roots was the triggering root trait as pointed out from sPLS-DA. As already described, this trait, which expresses the relative investment of the plant in root length, was strictly related to the soil resource availability and hence for their capture [37,60], depended on the nutrient availability [61, 62] and drought stress [56]. The component 2 and 3 separated the root phenotypes of the drought stress plants from that of the control and heat ones, respectively (Fig. 5A and B). The primary and seminal root branching density were the root trait with a maximal negative mean value for the control and drought group, respectively confirming that the inter-branch length could play a fundamental role in the drought stress condition [19, 54].

4. Materials and Methods

4.1. Plant material and growth condition and treatment

The experiments were conducted at the University “Mediterranea” of Reggio Calabria, Italy. Maize seeds (*Zea mays* L.) (genotype KXB7554, provided by KWS Italia) were surface sterilized with 20% NaClO for 20 min, rinsed and then soaked in aerated deionized water at room temperature for 36h. Afterwards, five seeds were sown in each of sixteen sterilized pots (16 cm diameter x 12 cm height), which were filled with sand:soil mixture (70:30 v/v). The soil physico-chemical values were reported in Gelsomino et al. [63]. Then, the pots were randomly placed in the growth chamber at 25°C, 70 % relative humidity and 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic photons flux density at plants' height (LI-190SA quantum sensor, Li-Cor, Lincoln, NE) with a 14 h photoperiod. The planted pots received, for two weeks, 200 mL of tap water every four days, necessary to compensate the water

losses by evapotranspiration, as suggested by preliminary trials. After twelve days from seeding, five seedlings were thinned to one for each pot.

4.2. Treatments.

From the third week, planting pots were subjected to stresses. In particular, eight pots were transferred to a second growth chamber with the same environmental condition except for the temperature setted to 32°C (Heat condition, H), whereas the remaining eight were left in the previous growth chamber at 25°C. In four pots of both growth chambers were imposed the drought stress (D) by the water withholding until to reach the theoretical fraction of 30% of field capacity (FC) measured by gravimetric method. To the remaining pots, conversely, were ensured the well-watered conditions in order to maintain the theoretical fraction of 80% FC. The desired percentage of FC was maintained by daily surface addition of water. Overall, the following treatments were imposed: drought (D) (25° and water at 30% FC), heat (H) (32°C and water at 80% FC), combined (Comb) (32°C and water at 30% FC) and control (Con) (25° and water at 80% FC).

4.3. Morphological root analysis

After 7 days of treatments, the seedlings were harvested and separated in shoot and root. The shoot fresh (ShFW, g) was measured and then the shoot dry weight (ShDW, g) were determined after oven-drying at 70 °C for 48 h.

After gently removing the adhering substrate, the root system was washed by tap water, paper-blotted and then stained with 0.1 % toluidine blue solution for 5 min. Afterwards, it was divided into primary and seminal roots with their laterals and scanned at a resolution of 300 dpi (WinRhizo STD 1600, Instruments Régent Inc., Canada). with the WinRHIZO image analysis (WinRhizo STD 1600, Regent Instruments Inc., Canada). Then, the length (L, cm), surface area (SA, cm²) and volume (V, cm³) of the seminal (S) and primary axes (P) and seminal (SL) and primary lateral roots (PL) were measured by WinRhizo Pro v. 4.0 software package (Instruments Régent Inc., Canada). The number of both primary and seminal lateral roots was manually counted from the scanned image. The root and branching zone formation (RZF and BZF, respectively; cm) and the branching density (BD, n cm⁻¹) were also measured and calculated, respectively, as reported on Drubvosky et al. [64].

The fresh and dry weights of the primary (PrFW, g; PrDW, g), seminal (SFW, g; SDW, g), primary lateral (PrLFW, g; PrLDW, g) and seminal lateral roots (SLFW, g; SLDW, g) were measured as reported above. The fresh (RFW, g) and dry weight (RDW, g) of root system were calculated as the sum of each root type and the plant fresh (PFW, g) and dry weight (PDW, g) were calculated by the sum of the ShFW and RFW and the ShDW and RDW, respectively.

Finally, based on the above measurements, the root length ratio (RLR, root length/whole plant dry weight, cm g⁻¹), root mass ratio (RMR, root dry weight/whole plant dry weight, g g⁻¹), root fineness (RF, root length/root volume, cm cm⁻³) and root tissue density (RTD, root dry weight/root volume, g cm⁻³) were calculated for each root type. The functional significance of these root parameters are reported on Ryser [65]. Further, according to Ryser and Lambers [37], the RLR and its 'morphological components' (RMR, RF, RTD) are related as follows:

$$RLR = RMR \times \frac{RF}{RTD} \quad (1)$$

4.4. Statistical analysis

All the experiment was arranged in a randomized complete design with four replicates per treatment.

All the root morphological parameters were firstly analyzed by one-way ANOVA followed by the Tukey's test to compare the mean values among the treatments (Control, D, H and Comb) at p<0.05.

To determine if the combination of the H and D stress exerted additive, synergistic or antagonistic impacts on root traits, we used the Bansal et al. method [66] and, specifically, we

compared the observed effects (Ob) to expected additive effects (Ex) for the plants exposed to the Comb treatment. The Ob effect sizes were calculated as the absolute value of:

$$Ob = \frac{(ob - \bar{x}Con)}{\bar{x}Con} \quad (2)$$

where ob is the measured trait value for each plant and treatment and $\bar{x}Con$ is the mean trait value for the control plants. The Ex additive effect sizes for the Comb treatment were defined in two steps by first determining and then summing the independent effects (Ind) of each treatment. The Ind effect sizes were calculated as the absolute value of:

$$Ind = \frac{(\bar{x}stress - \bar{x}Con)}{\bar{x}Con} \quad (3)$$

where $\bar{x}stress$ is the mean trait values from each stress (H and D), and $\bar{x}Con$ is the mean trait value for the control plants. Then, the Ex additive effect size for the Comb treatment was calculated using a multiplicative risk model as suggested by Darling et al. [31], i.e. the sum of two Ind effects minus their product. Finally, the Ex additive values for Comb treatment were compared to the actual Ob additive effects. In particular, we calculated a mean difference (\pm 95% confidence interval) between the effect sizes of Ob and Ex for each seedling of the Comb treatment. When $Ob - Ex > 0$ and the lower 95% confidence limit was greater than zero, the impact from the combination of both stressor was classified as synergistic. Conversely, the effects were antagonistic when the $Ob - Ex < 0$ and the upper 95% confidence limit was less than zero, and additive when the 95% confidence interval crossed the zero line.

Furthermore, we analyzed the effects of the single and combined stress on the entire dataset of the root morphological parameters using a multivariate approach with R statistical software 3.5 (R Core Team 2013). First, the differences among treatments were inferred through PERMANOVA multivariate analysis (999 permutations) using the package *vegan*. Pairwise comparisons among the groups were calculated using a custom script and correcting P values using the False Discovery Rate (FDR) method. In order to identify the root morphological key predictors that could constitute a root strategy among the treatments, we used a preliminary unsupervised (Principal Component Analysis, PCA) followed by the supervised analysis (Sparse Projection to Latent Structure-Discriminant Analysis, sPLS-DA) using the package *mixOmics* [67]. The *perf.plsda()* and *tune.plsda()* functions were used to predict the number of latent components (associated loading vectors) and the number of discriminants root traits for sPLS-DA, , respectively.

In particular, the optimal number of components were chosen by the averaged overall and balanced classification error rate with centroids distance over 50 repeats of 5-fold cross-validation (*perf.plsda()*). The optimal number of root traits for each component was then selected by the lowest average balanced classification error rate with centroids after tuning of the sPLS-DA model (*tune.plsda()*) using the selected number of components and 5-fold cross-validation with 50 repeats. Single samples were showed on a score plot and differentiated by treatments with color and 95% confidence ellipses. Furthermore, discriminant root traits were plotted according to their contribution weight to the component 1, 2 and 3 of sPLS-DA and discriminated by treatments with color.

Finally, the Pearson product-moment correlations between the plant fresh and dry weight with the scores of the latent components determined by sPLS-DA, were run for verifying the root strategy for plant adaptation to the abiotic stress.

The statistical software was the SPSS Inc., V. 10.0, 2002 (SPSS Inc., Evanston, IL, USA).

5. Conclusions

The present study pointed out, for the first time, the responses of different root types to the combined abiotic stress in maize seedlings.

The single and combined stress caused fine variation in growth and morphology of the single root types in the maize root phenotype. The seminals were the least modified root types, whereas the primary and their lateral roots were stimulated with an increase of the length together with higher biomass allocation and fineness by the single stress conditions. The combined stress determined similar effects but associated with a specific inhibition of growth and morphology of the seminal lateral roots. Non-additive effects (synergistic and antagonistic) were only observed in the primary and their lateral roots under the combined stress suggesting that single molecular mechanisms could

underlying their growth and morphological responses. Further, the results of the sPLS-DA supported the idea that the primary and their lateral roots could be the “root type” with an important role for the adaptation to the combined abiotic stress.

List of abbreviations: Partial Least Squares-Discriminant Analysis (sPLS-DA); Principal Component Analysis (PCA); Drought stress (D); Heat stress (H); Combined stress (Comb); Field Capacity (FC); Control (Con); Shoot Fresh Weight (ShFW); Shoot Dry Weight (ShDW); Length (L); Surface Area (SA); Volume (V); Seminal root (S); Primary root (Pr); Seminal lateral root (SL); Primary Lateral roots (PL); Root Zone Formation (RZF); Branching Zone Formation (BZF); Branching Density (BD); Primary Fresh Weight (PrFW); Primary Dry Weight (PrDW); Seminal Fresh weight (SFW); Seminal Dry Weight (SDW); Primary Lateral Fresh Weight (PrLFW); Primary Lateral Dry Weight (PrLDW); Seminal Lateral Fresh Weight (SLFW); Seminal Lateral Dry Weight (SLDW); Root Fresh Weight (RFW); Root Dry Weight (RDW); Plant Fresh Weight (PFW); Plant Dry Weight (PDW); Root Length Ratio (RLR); Root Mass Ratio (RMR); Root Fineness (RF); Root Tissue Density (RTD); False Discovery Rate (FDR).

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