

Article

# Root and Agro-Morphological Traits Performance in Cowpea under Drought Stress

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**Abstract:** Drought is responsible for major yield losses in many worldwide crops and is expected to occur more frequently due to climate change. Cowpea, one of the most drought tolerant legumes, stands as a promising crop in the future climatic context. The screening for genotypes well adapted to this constraint is an essential step to improve cowpea production. A collection of 29 cowpea genotypes (*Vigna unguiculata* L. Walp.) from the Iberian Peninsula and 11 other countries from worldwide regions was grown and submitted to drought stress using pipes with 30 cm (control) and 90 cm (stress) of height in which water was supplied through the bottom. A set of root and agro-morphological parameters were evaluated, including shoot and root dry weight, root:shoot ratio and stem greenness. Overall, results show that under drought stress, plants seem to invest in root development and reduce shoot biomass. Higher root dry weight under drought conditions could be related to a higher drought tolerance in cowpea. Based on the evaluated traits, it was possible to identify genotypes, particularly C47 (Iran), C56 and C11 (Portugal), which might represent promising cowpea genetic resources for improved drought tolerance breeding.

**Keywords:** *Vigna unguiculata* L. Walp; drought stress tolerance; legumes diversity; genetic resources; landraces; phenotypic descriptors; water scarcity; Southern European agriculture

## 1. Introduction

Climate change predictions point towards an increase in extreme events in the coming years, namely long periods of drought [1]. Drought causes yield losses in major agricultural crops [2]. Since the global population is expected to reach over 9 billion people by 2050, agricultural systems are required to be more productive and to assure sustainable food production under the expected climate challenges [3].

Cowpea is an important crop in semi-arid tropics, particularly in Africa, the continent that is responsible for 96.4% of worldwide production [4]. Nowadays, Europe is facing a deficit of about 70% of grain legumes (including cowpea), leading a negative trade scenario. For this reason, the increasing of cowpea production in Europe is fundamental [5]. The dry and immature seeds, immature pods and leaves of cowpea plants can be consumed, providing a balanced source of nutrients with numerous health benefits [6]. The dry grain has high protein levels (20.0–39.4%) and low fat content

(3.1–30.4%) [6,7]. As with other legumes, cowpea establishes effective symbiosis with mycorrhizae, allowing the fixation of atmospheric nitrogen. Hence, it is presented as a key rotating crop in farming systems with infertile soils [8]. Furthermore, cowpea is one of the most drought and high temperature tolerant legume crops [9,10] producing high yields under terminal drought stress conditions and requiring less water than other cultivated legume species [11]. All these characteristics emphasize cowpea as a good opportunity to increase the sustainability of agricultural systems and counteract climate changes. Screening genotypes that are more tolerant to drought is an important step to improve the production of this crop under the expected future climatic constraints. The ways by which plants cope with drought stress are displayed as a complex expression of mechanisms and factors, thereby the screening for drought tolerant genotypes is still a difficult task [12]. A set of different drought tolerance parameters has been used to perform screenings in cowpea genotypes [13–16]. Shoot biomass variations under different drought conditions show correlation with the plants' water status, in which small shoot biomass leads to less water consumption, conferring higher drought tolerance [15].

Root traits of different crops have been suggested as an important feature related with drought tolerance [17]. Deep root systems are important for the uptake of water from deeper soil layers in drought conditions [16]. Plants may increase root length through different processes such as increased biomass allocation and/or promoting primary root development while suppressing lateral root growth [17]. Some reports suggested that an increase in root mass may indicate a higher plant capability to maintain their water status [17,18]. However, these mechanisms can reduce the plants' yield potential [12], with it being important to also include production related traits when screening for tolerant genotypes. The evaluation of drought tolerance mechanisms using root traits is hampered by the complexity of the methodology involved [15].

In the present study, we evaluated the performance of 29 cowpea genotypes under drought conditions where different shoot and root related agronomic parameters were evaluated. The objectives of this work were the selection of drought tolerant genotypes that might be particularly important in the climate change scenario for Southern Europe and to further understand the relevance of root related parameters in drought tolerance screenings. These results will hopefully contribute to obtaining cowpea genotypes with greater drought tolerance, allowing increased cowpea production in Europe.

## 2. Materials and Methods

### 2.1. Plant Material and Experimental Design

A total of 29 cowpea genotypes were selected based on previous research [13,19], including one drought susceptible and one drought tolerant genotype, Bambey21 and IT93K-503-1, respectively. The genotypes were originated from worldwide countries with a particular focus on Iberian Peninsula genotypes (8 from Portugal and 8 from Spain) (Table 1). All the genotypes were previously tested in field experiments, revealing that they have an erect growth habit [19–21].

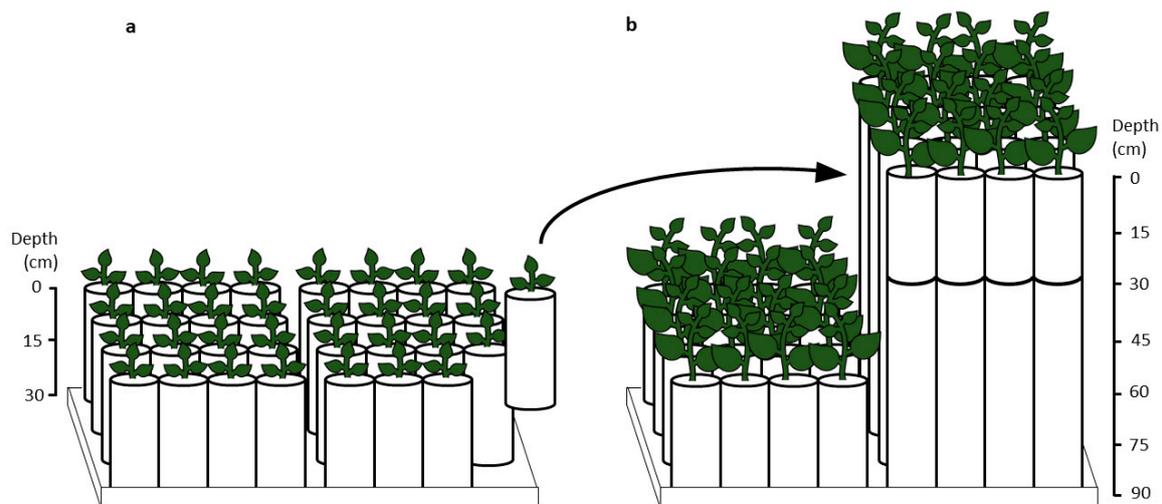
This study followed the experimental design proposed by Iseki et al. [15] with some modifications. The experiment took place under open field conditions at the Universidade de Trás-os-Montes e Alto Douro (41°17'10" N 7°44'8" E) and was divided into two stages: (a) all plants were grown in 30 cm pipes; (b) the pipe height of half of the replicates was increased to 90 cm, to simulate drought conditions (Figure 1). Pipes were protected by a small wire structure that allowed the sliding of a retractable cover that was only used when it was raining, being removed as soon as it stopped.

**Table 1.** List of the cowpea genotypes used in this study including their origin and type.

Code	Bank Code	Origin	Donor Institution #	Type
C3	Cp 4924	Évora, Portugal	INIAV	Landrace
C5	Cp 5648	Abrantes, Portugal	INIAV	Landrace
C8	Vg 50	Pinhel, Portugal	UTAD	Landrace
C11	Vg 56	Macedo de Cavaleiros, Portugal	UTAD	Landrace
C15	Fradel	Portugal	INIAV	Variety
C56	Vg 59	Fundão, Portugal	UTAD	Landrace
C57	Vg 62	Covilhã, Portugal	UTAD	Landrace
C58	Vg 72	Mogadouro, Portugal	UTAD	Landrace
C18	BGE038478	Málaga, Spain	CRF-INIA	Landrace
C59	IT97K-499-35	Nigeria	UCR	Breeding line
C41	VIG 58	Angola	IPK	Landrace
C43	VIG 90	Egypt	IPK	Landrace
C45	VIG 49	Senegal	IPK	Landrace
C47	VIG 1649	Iran	IPK	Landrace
C49	VIG 206	Cuba	IPK	Landrace
C51	VIG 10	China	IPK	Landrace
C52	NI 778	India	BGM	Landrace
C53	Baio Coaofom	Brazil	EMBRAPA	Variety
C19	BGE002195	Orense, Spain	CRF-INIA	Landrace
C20	BGE019751	Girona, Spain	CRF-INIA	Landrace
C21	BGE024703	Baleares, Spain	CRF-INIA	Landrace
C22	BGE025213	Caceres, Spain	CRF-INIA	Landrace
C23	BGE047731	Pontevedra, Spain	CRF-INIA	Landrace
C24	BGE036461	Huelva, Spain	CRF-INIA	Landrace
C29	BGE039237	Cordoba, Spain	CRF-INIA	Landrace
C31	MG 113779	Puglia, Italy	CRF-INIA	Landrace
C39	AUA 1	Crete, Greece	AUA	Landrace
<b>References</b>				
IT93K-503-1	IT93K-503-1	Nigeria	UCR	Breeding line
Bambey21	Bambey21	Senegal	UCR	Cultivar

# AUA—Agricultural University of Athens, Greece; BGM—Botanic Garden Meise, Belgium; CRF-INIA—National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research, Spain; INIAV—Instituto Nacional de Investigação Agrária e Veterinária, Portugal; IPK—Leibniz Institute of Plant Genetics and Crop Plant Research, Germany; UCR—University of California Riverside, USA; UTAD—Universidade de Trás-os-Montes e Alto Douro, Portugal.

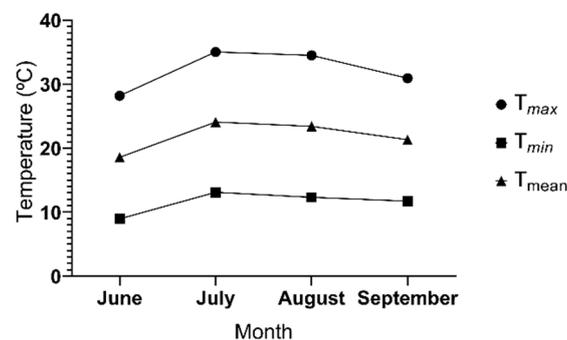
In the first stage (Figure 1a), 6 seeds of each cowpea genotype were sowed individually in the polyvinyl chloride (PVC) pipes, completely randomized on the 28th of June 2019. These pipes were 30 cm in height and 12.5 cm in diameter, were filled with a mixture of vermiculite, soil and peat (1.5:3:6) and were watered to field capacity. After 30 days of plant development, the water stress was imposed on half of the replicates by placing the 30 cm pipes on top of 60 cm pipes, previously filled with the same soil mixture of the 30 cm pipes and watered to ensure water conductivity between both pipes (Figure 1b). This was the timepoint for the onset of drought stress. Plants in the 30 cm pipes and 90 cm pipes are hereafter designated as control and stress, respectively. The trial was conducted during the next 42 days, which amounted to a final duration of 72 days since sowing took place. The pipes were placed on top of a wood tray that was leveled and impermeabilized with a plastic sheet. This made it possible to irrigate the base of the pipes with a thin layer of water during the first 35 days upon drought imposition. The bottom of the pipes had 4 holes of 0.5 cm diameter to ensure water absorption. This prevented complete dehydration of the pipes and ensured that water would be available in the deepest soil layers. No water was supplied in the last 7 days of the trial.



**Figure 1.** Graphical representation of the experimental trial. Sowing of 6 replicates per genotype occurred in pipes with 30 cm in height (a). At the end of 30 days, half of the replicates in the 30 cm pipes were put on top of 60 cm pipes to obtain a final height of 90 cm (b). The pipes were submerged in a thin layer of water to ensure that deeper layers remained humid.

## 2.2. Climate Data and Soil Water Content

Air temperatures during the trial were recorded by a weather station positioned at the experimental location. Daily mean ( $T_{\text{mean}}$ ), maximum ( $T_{\text{max}}$ ) and minimum ( $T_{\text{min}}$ ) air temperature were measured and averaged for each month (Figure 2).



**Figure 2.** Minimum, maximum and mean air temperatures (°C) registered for the months throughout the experiment.  $T_{\text{max}}$ —maximum air temperature;  $T_{\text{min}}$ —minimum air temperature;  $T_{\text{mean}}$ —mean temperature.

For the soil water content (SWC) measurements, six additional 30 cm pipes were sowed with a cowpea genotype and submitted to control and drought stress following the steps described in “2.1. Plant material and experimental design”. The pipes’ SWC was monitored 14, 28 and 42 days after drought stress imposition. For each measurement, a pipe of 30 and 90 cm was cut in two and six sections of 15 cm, respectively, and the soil was weighed before and after drying at 100 °C for 72 h. The SWC, on a dry mass basis, was calculated according to Muñoz-Perea et al. [22] as follows:

$$SWC (\%) = \frac{M_w - M_s}{M_s} \times 100$$

where  $M_w$  and  $M_s$  correspond to the mass of soil (g) before and after drying, respectively.

### 2.3. Data Collection

The days to germination (DG) and to first flower (DFF) were recorded for each plant. Seeds were considered germinated when hypocotyl was visible. By the end of the experiment, all plants were carefully removed from the pipes and the soil attached to the roots was rinsed with running water. Root, shoot and pods were separated and dried at 80 °C for 72 h to obtain root dry weight (RDW), shoot dry weight (SDW) and pod dry weight (PDW). Root:shoot ratio (RSR) was calculated as the quotient between RDW and SDW. The total number pods per plant (NPP) was counted and classified as “no grain” (pod without grains), “immature” (pods with not fully developed grains) and “mature” (pods with fully developed grains). The grains from immature and mature pods were counted to obtain the total number grains per plant (NGP). As all the genotypes shared the same erect growth habit, plant height (PH) was defined as the length of the main stem in cm from ground level to the tip of the plant [23]. The stem greenness (SG) was scored on a scale of 0 to 5, with 0 being “completely yellow” and 5 “completely green” following Muchero et al. [14].

### 2.4. Data Analysis

Means and standard deviations (SD) for each of the traits were calculated based on the replicates for each genotype and treatment ( $n = 3$ ). A non-parametric two-way analysis of variance (Aligned Ranks Transformation ANOVA) was performed to assess the effect of genotype, water stress treatment and interaction between them. For each genotype, differences between both water treatments were assessed using the Mann-Whitney test. All statistical analyses were performed using R (version 3.6.3) and a  $p$ -value of  $<0.05$  was considered significant. The principal component analysis (PCA) was performed using Past (version 4.0) [24]. For the PCA, the ratio between the mean of the three replicates for each genotype in control and drought treatments was calculated and then transformed to Z scores by subtracting the mean value measured in each parameter ( $obs - \mu$ ) and dividing the result by the respective standard deviation ( $\sigma$ ) as follows:

$$Z \text{ score} = \frac{obs - \mu}{\sigma}$$

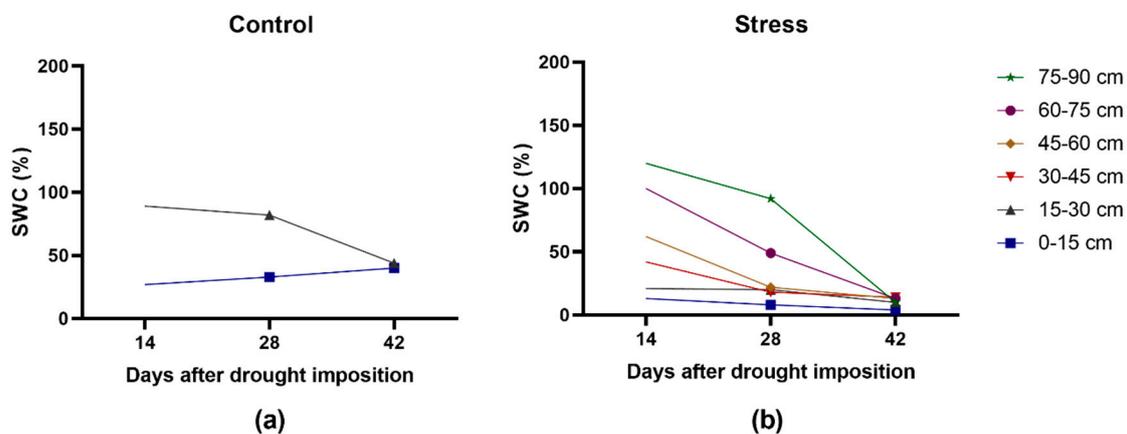
where *obs* represents the observed measurement. The same software was used to elaborate a hierarchical clustering based on the Manhattan distances between the ratios of the measured SDW under drought and control treatments using the UPGMA (unweighted pair group method with arithmetic mean) method. For the heat map, the measurements were first normalized to percentage accordingly to the minimum and maximum values obtained in each parameter. All graphical representations were created using *GraphPad Prism* (version 8.0.0).

## 3. Results and Discussion

### 3.1. Soil Water Conditions

Several parameters have been described as indicators of water deficit in crop plants, including plant-based parameters, such as stomatal conductance and biochemical markers (e.g., proline and anthocyanins contents), and soil-based parameters such the SWC, which seem to be indicative of water stress condition [15,25–27]. The differences in the SWC during the trial period are summarized in Figure 3. Soil humidity gradually decreased in all pipes and this decrease was more evident in the 90 cm pipes (drought stress). Nonetheless, the bottom layers were always more humid than the upper layers, indicating that a successful water content deficit between both ends of the pipes was obtained. In the upper soil layers of the drought stress pipes, a more accentuated decrease in the water content could be observed comparatively to the control pipes. The same result was also achieved by Iseki et al. [15]. During the first 28 days of drought treatment, humidity levels in control pipes were maintained between 27 and 89%, in the 0–15 cm and 15–30 cm soil layers, respectively, while humidity levels of these layers in drought stress pipes varied from 8 to 21%. The soil water content levels across

different depths in the 90 cm pipes show an uniform increase in humidity towards the bottom of the pipes, which is similar to what occurs in field and was observed in a previous study of Gebre and Earl in soybean [26]. In these measurements, it was possible to observe roots in the bottom layers of the pipes, suggesting that plants under drought stress treatment had to develop deeper roots in order to be able to reach more humid soil layers. A significant decrease in water content of the deepest layers was observed at the 42nd day, as a result of ending the water supply through the bottom of the pipes. At the end of the experiment, the SWC in the drought stress pipes in all the depths was very low (<15%), while in the control pipes the SWC was approximately 50%. These results indicate that the conditions used in this experiment were adequate, confirming that genotypes in the pipes of 90 cm were effectively under drought stress conditions. Moreover, as mentioned by Gebre and Earl [26], experiments that explore plant-water relationships using rooting cylinder, as opposed to small pots, allow a more realistic simulation of drought in field environments and allow further information on root development, namely rooting depth [26]. Kashiwagi et al. [27] also revealed that a cylinder protocol in chickpea could be a good choice for screening root traits.



**Figure 3.** Soil water content (SWC) in different depths (from pipes' top to base) of control (a, from 0 to 30 cm) and stress pipes (b, from 0 to 90 cm) measured in 3 different days after imposition of drought.

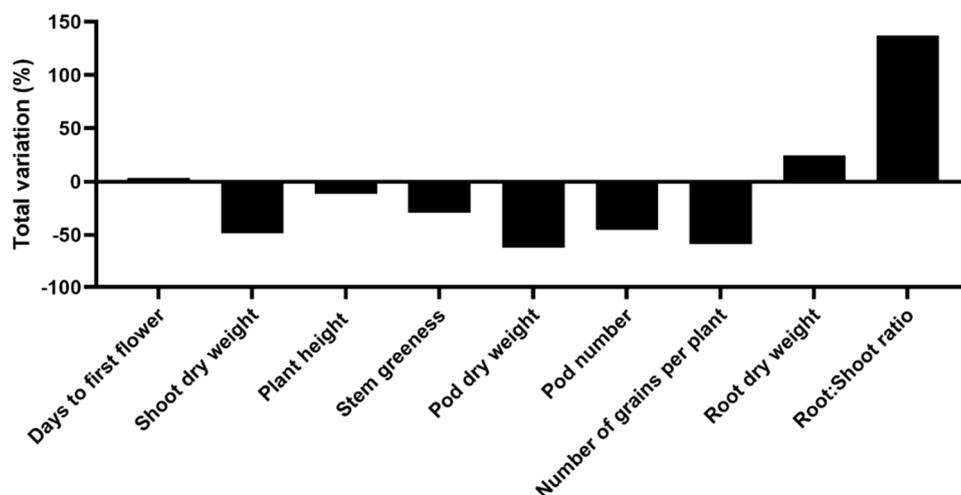
### 3.2. Evaluation of Agro-Morphological Parameters

For all evaluated parameters, the effect of treatment and genotype was significant ( $p < 0.05$ ), except for the treatment on DFF (Table 2). The genotype  $\times$  treatment interaction was significant ( $p < 0.05$ ) for the majority of the evaluated parameters except PH, RDW and DFF, indicating that the effect of the drought treatment differed between genotypes. Apart from DFF, results showed an overall reduction in the above-ground parameters' measurements (SDW, PH, SG, NPP, PDW and NGP) in the drought stress treatment compared to the control. Contrarily, root-related parameters were increased under drought stress conditions (Figure 4) suggesting that cowpea drought tolerance may be related to the development of the root system.

**Table 2.** Aligned Rank Transformation ANOVA analysis of the different measured parameters in the 29 studied cowpea genotypes under control (30 cm pipes) and drought stress (90 cm pipes) at 42 days of water stress.

Parameter #	Source	DF *	Residual DF	F	Prob < F
SDW	Treatment	1	116	293.1	<0.001
	Genotype	28	116	3.1	<0.001
	Treatment: Genotype	28	116	2.7	<0.001
PH	Treatment	1	116	13.493	<0.001
	Genotype	28	116	6.6	<0.001
	Treatment: Genotype	28	116	1.2	0.234
PDW	Treatment	1	112	253.5	<0.001
	Genotype	27	112	4.6	<0.001
	Treatment: Genotype	27	112	2.6	<0.001
SG	Treatment	1	116	130.5	<0.001
	Genotype	28	116	6.9	<0.001
	Treatment: Genotype	28	116	2.3	0.001
NPP	Treatment	1	112	151.5	<0.001
	Genotype	27	112	5.3	<0.001
	Treatment: Genotype	27	112	2.1	0.004
NGP	Treatment	1	112	186.8	<0.001
	Genotype	27	112	5.2	<0.001
	Treatment: Genotype	27	112	4.4	<0.001
RDW	Treatment	1	116	15.5	<0.001
	Genotype	28	116	3.4	<0.001
	Treatment: Genotype	28	116	1.1	0.352
RSR	Treatment	1	116	294.6	<0.001
	Genotype	28	116	3.3	<0.001
	Treatment: Genotype	28	116	2.7	<0.001
DFF	Treatment	1	116	0.0	0.906
	Genotype	28	116	5.1	<0.001
	Treatment: Genotype	28	116	1.3	0.178

# SDW—shoot dry weight; PH—plant height; PDW—pod dry weight; SG—stem greenness; NPP—number of pods per plant; NGP—number of grains per plant; RDW—root dry weight; RSR—root to shoot ratio; DFF—days to first flower. \* DF—degrees of freedom.

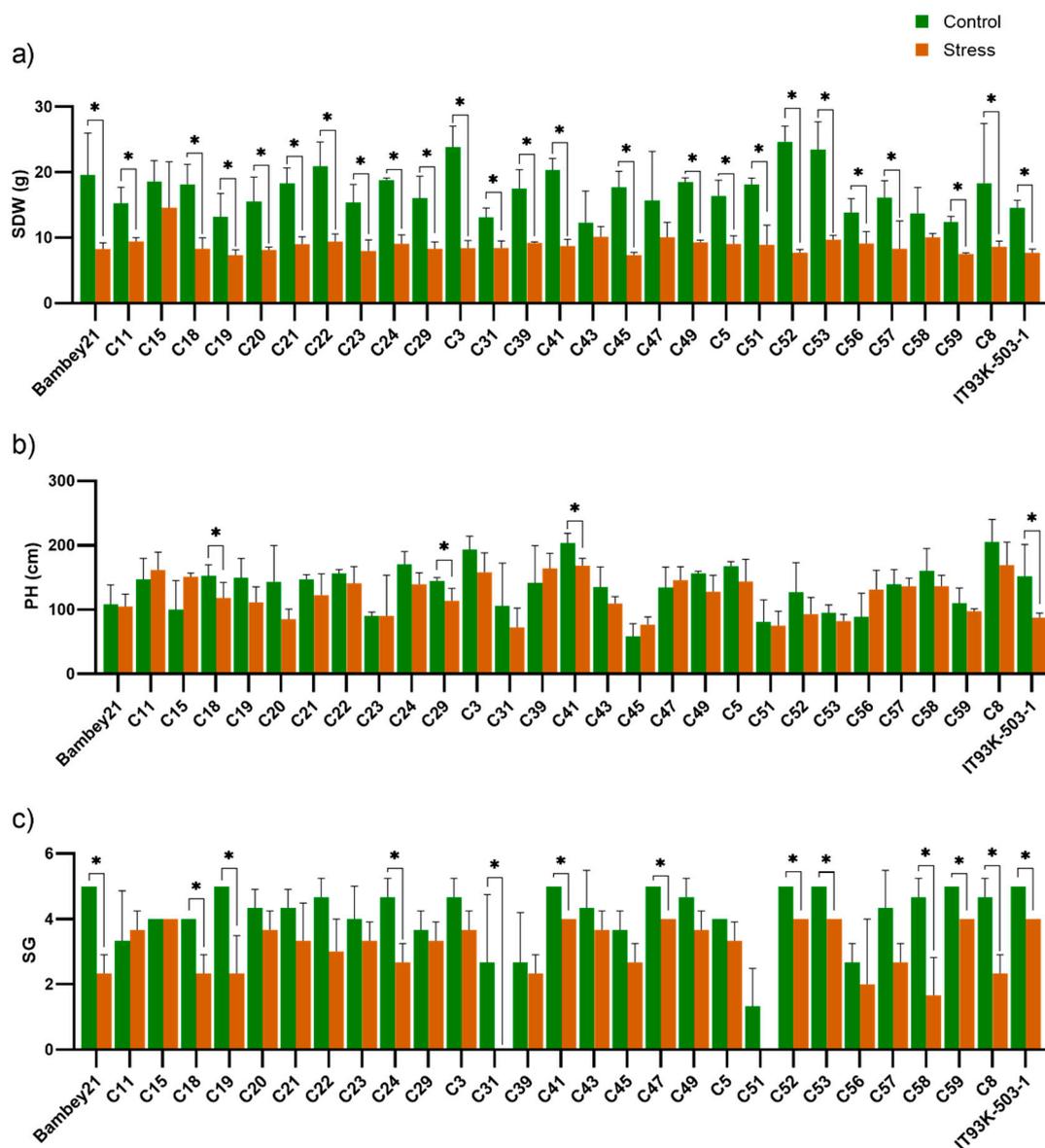


**Figure 4.** Variation percentage observed in the average of all cowpea genotypes in the drought stress condition compared to the plants under control condition, after 42 days of drought stress treatments.

### 3.2.1. Shoot-Related Parameters

SDW, SG and PH suffered a reduction of 48.46, 28.95 and 11.40%, respectively (Figure 4). Differences in the average of PH were relatively small between both treatments (136.68 and 121.10 cm in control and drought treatments, respectively), with only C18, C29, C41 and IT93K-503-1 genotypes presenting significant reductions ( $p < 0.05$ ) (Figure 5b). Contrarily, differences in mean SDW were significant ( $p < 0.05$ ) (except for C15, C43, C47 and C58) with average mean values in control plants being twice (17.24 g) the values recorded in plants under drought treatment (8.89 g) (Figure 5a). These results indicate that the plants were able to maintain similar heights in both treatments, but their shoot biomass was largely reduced. The reduction in SDW in cowpea under drought stress conditions was also verified in other studies [28–30], including other *Vigna* species [15], with this being considered a common effect of drought stress in legumes [31]. Plants with lower shoot biomass are likely to have lower water consumption, which allows them to maintain an adequate water status under drought conditions [15]. SG is an important indicator of drought tolerance in cowpea genotypes at a seedling stage [14], but it is not commonly evaluated in cowpea screenings at the vegetative and reproductive stage. In other crops, such as sorghum, the ability to maintain stem greenness under drought conditions at a post-flowering stage indicates higher drought tolerance [32]. SG was also clearly reduced under drought stress, in which the average of SG across genotypes was 2.97, while, in control pipes, it was 4.18. Significant differences ( $p < 0.05$ ) in SG between treatments were found in the genotypes Bambey21, C18, C19, C23, C31, C41, C47, C52, C53 C58, C59, C8 and IT93K-503-1 (Figure 5c).

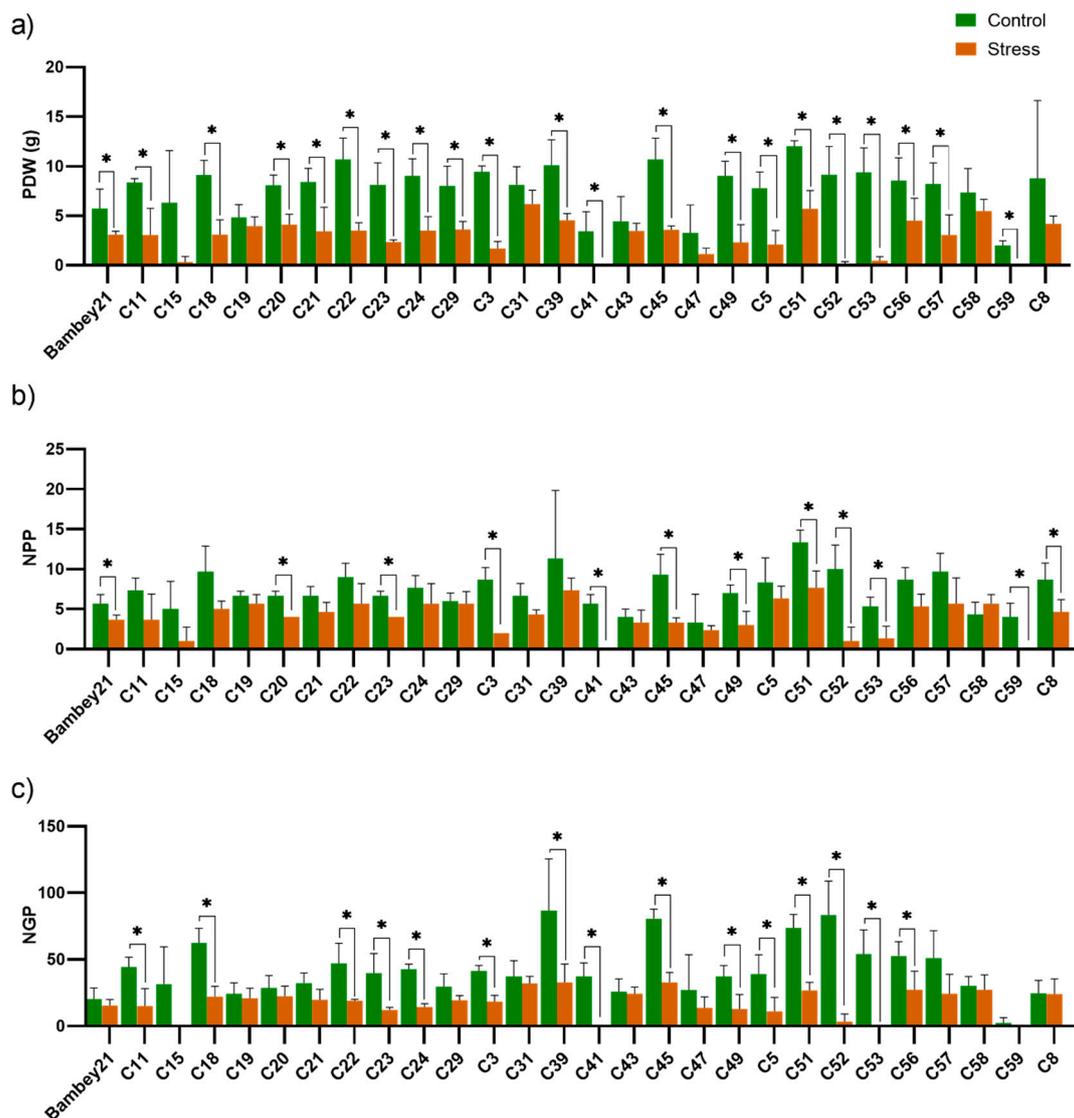
Despite the treatment effect being non-significant ( $p > 0.05$ ) for DFF (Table 2), it was observed that genotype C59 only reached flowering stage in the control treatment but not under drought stress, indicating that water availability may have affected its flowering time (Table S1). Similarly, not all replicates of the genotypes C11, C15, C41 and C52 reached flowering in drought treatment. However, it was also observed that some replicates of the genotypes C41 and C59 also did not flower under control conditions. All genotypes, with the exception of IT93K-503-1, presented similar DFF, suggesting identical photoperiod-sensitivity. Since IT93K-503-1 did not reach flowering, it was not included in the analysis of yield-related parameters (PDW, NGP and NPP).



**Figure 5.** Differences in the shoot dry weight (SDW) (a), plant height (PH) (b) and stem greenness (SG) (c) of the evaluated genotypes after 42 days in control and drought stress conditions. The data presented corresponds to the means  $\pm$  SD (error bars). \* represents significant differences between treatments according to Mann-Whitney U Test at  $p < 0.05$  level.

For the yield-related parameters, namely PDW, NGP and NPP, considerable reductions were also observed under drought stress (62.19, 58.66 and 45.48%, respectively) (Figure 4). The mean NPP and NGP found in these drought stress treated plants (3.86 and 16.91g, respectively) was significantly lower than the means observed in the control plants (7.08 and 40.93 g, respectively). Due to this decrease, total PDW in the drought stress treatment was 2.84 g, contrasting with 7.52 g in the control. Significant differences ( $p < 0.05$ ) between genotypes C23, C3, C41, C45, C49, C51, C52 and C53 were found in all yield related parameters (NPP, NGP and PDW), demonstrating a clear effect of the drought stress in these genotypes (Figure 6). This significant decrease in the NPP, NGP and PDW under drought stress for the genotypes C41 and C52 is in accordance with the absence of flowering previously mentioned. Overall, PDW significantly decreased under drought conditions, while the decreases in NPP and NGP were not so marked, wherein statistically significant differences were only found in some genotypes. Genotypes C15, C19, C31, C43 and C58 did not show significant differences ( $p > 0.05$ ) in any of the

yield-related parameters ( $p < 0.05$ ), suggesting a lower effect of drought treatment. In the study developed by Mendes et al. [30], cowpea accessions, when subjected to drought stress, were less affected in their source capacity (leaf related parameters) but presented considerable reductions in their sink size, namely in the number of pods and grains per plant. Some studies mention that the number of pods per plant in cowpea is much affected by drought stress, thus being an important trait in studies of drought tolerance [30,33,34]. The number of pods might be the major contributor to the decreased cowpea grain yield under drought stress [33]. Leite et al. [35], performed a study where a specific cowpea genotype (cv. EMAPA-821) was subjected to drought stress in vegetative, pre-flowering and pod filling phases, showing that the effect of drought was most severe during pod filling. Based on these results, the authors suggested that the development of less pods could be a drought tolerance mechanism used by this crop to seek for better conditions.



**Figure 6.** Differences in the pod dry weight (PDW) (a), number of pods per plant (NPP) (b) and number of grains per plant (NGP) (c) of the evaluated genotypes after 42 days under control and drought stress conditions. The data presented corresponds to the means  $\pm$  SD (error bars). \* represents significant differences between treatments according to Mann-Whitney U Test at  $p < 0.05$  level.

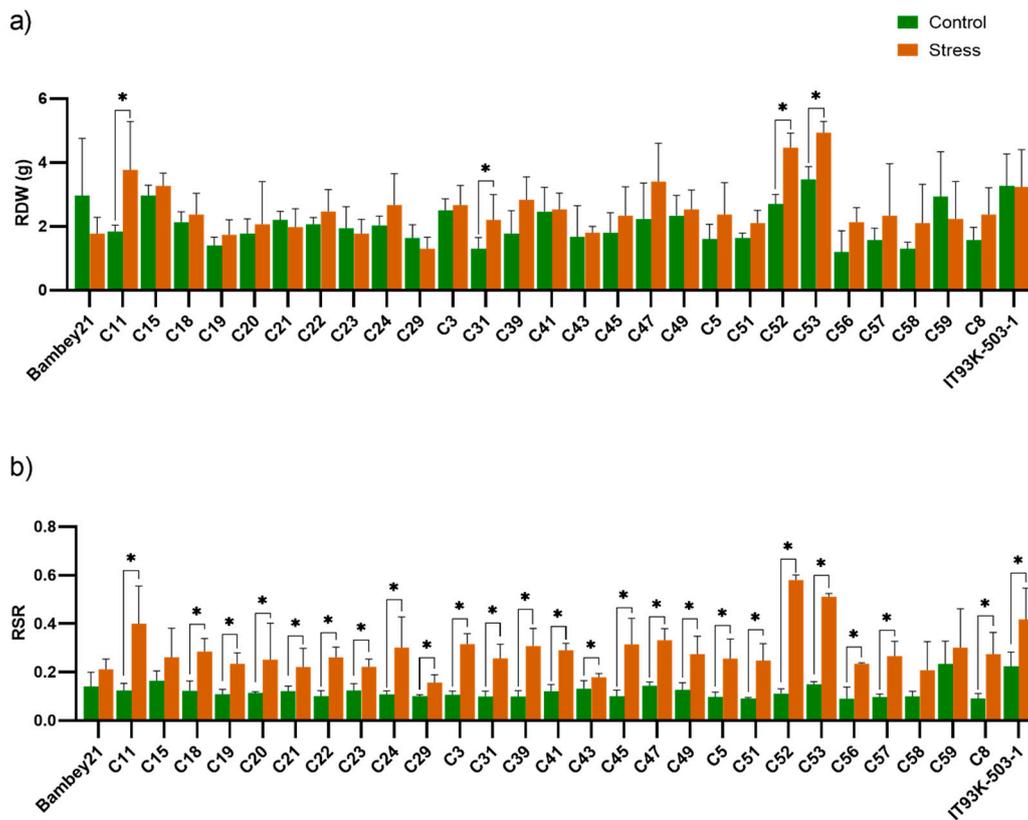
### 3.2.2. Root-Related Parameters

The root system seems to be strongly related with drought tolerance in legumes [31] and has been highlighted for its potential applicability in drought tolerance screenings in soybean (*Glycine max* L.) [36,37], chickpea (*Cicer arietinum* L.) [18,27,38], common bean (*Phaseolus vulgaris* L.) [39,40], faba bean (*Vicia faba* L.) [41] and lentil (*Lens culinaris* ssp. *culinaris* Medikus) [42]. In cowpea, root traits were suggested to have an important role in drought avoidance [12] but their use in drought tolerance screenings is still not commonly applied, as they might involve complicated procedures using specific chambers that often require access to recently developed imaging techniques and high-throughput phenotyping software that allow the visualization of the three dimensional distribution of the root in the soil [16,43]. Different legume crops present distinct responses to drought regarding root development: common bean [39], faba bean genotypes [41] and soybean [37], show a reduced root development, while, in chickpea genotypes [18,27,38] and in some species of the genus *Vigna* [15], root growth seems to be stimulated. Moreover, Iseki et al. [15] verified that the genotypes' tolerance levels were different between terminal and non-terminal drought treatments. Hence, the effect of drought in the root development varies among species and the type of drought stress imposed.

To avoid interpretation errors that could be induced by the differential size of the control and stress tubes, the root length data is not shown. Our results show that root-related parameters varied differently from the shoot-related parameters. Contrarily to shoot-related parameters, the RDW and RSR were 24.57 and 136.98% higher, respectively, under drought stress conditions comparatively to control (Figure 4), suggesting that droughted plants, overall, prioritized root development over shoot. Accordingly, mean RDW was also higher in drought stress conditions (2.59 g) than in control (2.08 g), although this increment was less pronounced (24.57%) and significant differences ( $p < 0.05$ ) were only observed in genotypes C11, C31, C52 and C53 (Figure 7a). The difference between mean RSR in the two treatments was larger, varying from 0.13 to 0.31 in control and droughted plants, respectively. All genotypes, except Bambey21, C15, C58 and C59, showed significant differences ( $p < 0.05$ ) in the RSR between the two treatments (Figure 7b). Similar results were obtained by Gebre and Earl [26] in soybean, where RSR increased by 18% under drought conditions. In other crops, an increased RSR in response to drought stress was associated with more sucrose being transported from leaves to roots [44]. In *Vigna* species, Iseki et al.'s [15] study showed that deeper roots alone do not guarantee enough water absorption to sustain shoot growth. Rather, a greater root biomass, which suggests a higher number of roots, appears to be a key factor to overcome poor water absorption. More tolerant genotypes seem to increase RDW when subjected to drought stress, while susceptible genotypes decreased RDW. The increased root biomass under drought conditions might be related to the accumulation of abscisic acid (ABA) in the roots [45]. Moreover, roots with higher biomass values are associated with an increased hydraulic conductivity that allows better tolerance to drought conditions [46].

### 3.3. Principal Component Analysis

In this study, we performed a PCA in order to determine the most contributive agro-morphological traits for the distribution of this set of genotypes in the two treatments (Table 3; Figure 8) and, consequently, the traits that could be contributing the most to drought tolerance. PCA distribution based on parameters of interest has been considered a useful complementary tool in drought tolerance screenings and, consequently, in the selection of the most tolerant genotypes, namely in drought tolerance screenings of cowpea [47] and other crops, such as common bean [48], chickpea [49] and bread wheat [50]. Results showed that the first three principal components presented eigenvalues superior to 1, and together explained 81.72% of the total variation (PC1: 40.57%, PC2: 21.73% and PC3: 19.42%) (Table 3). The first principal component was primarily associated with yield components (NPP, PDW and NGP), while the second principal component was more correlated with the evaluated root characteristics (RDW and RSR) and the third principal component with SDW and PH.

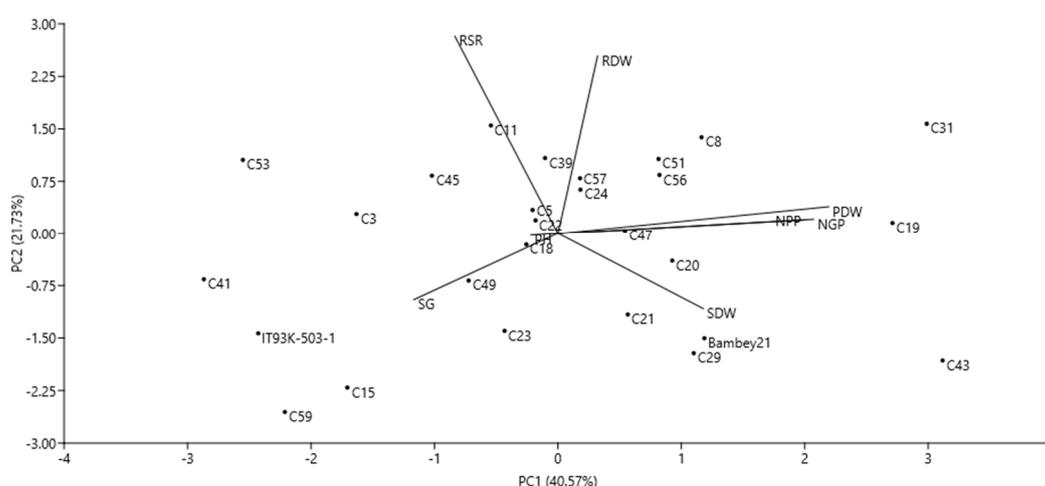


**Figure 7.** Differences in the root dry weight (RDW) (a), and root:shoot ratio (RSR) (b) of the evaluated genotypes after 42 days under control and drought stress conditions. The data presented corresponds to the means ± standard deviations (error bars). \* represents significant differences between treatments according to Mann-Whitney U Test at  $p < 0.05$  level.

**Table 3.** Principal component analysis (PCA) of the effect of drought treatment on morphological parameters from 29 cowpea genotypes. Measured values under drought treatment were divided by the respective control to obtain a ratio between both treatments.

Parameters #	PC 1	PC 2	PC3
SDW	0.29	−0.26	0.51
PH	−0.05	−0.01	0.67
SG	−0.29	−0.23	0.37
NPP	0.49	0.05	0.06
PDW	0.54	0.10	−0.01
RDW	0.08	0.62	0.38
NGP	0.51	0.05	−0.12
RSR	−0.20	0.69	0.01
Eigenvalue	3.36	1.80	1.61
% variance	40.57	21.73	19.42

# SDW—shoot dry weight; PH—plant height; PDW—pod dry weight; SG—stem greenness; NPP—number of pods per plant; NGP—number of grains per plant; RDW—root dry weight; RSR—root to shoot ratio.



**Figure 8.** Principal component analysis (PCA) of the effect of drought treatment in 29 genotypes. Measured values under drought treatment were divided by the respective control to obtain a ratio between both treatments (control—white squares; drought—black dots). The lines indicate eigenvectors representing the strength (given by the length of the vector) and the direction of the parameter correlation relative to the first two principal components (PC1 and PC2). SDW—shoot dry weight; SG—stem greenness; PH—plant height; NPP—number of pods per plant; NGP—number of grains per plant; PDW—pods dry weight; RDW—root dry weight; RSR—root:shoot ratio.

Our results show that NPP, PDW and NGP were the most contributive factors for the dispersion of the genotypes along the PC1 axis. At the seedling stage, Nkomo et al. [47] also denoted a heavy contribution of pod and grain related components, namely PDW, for the screening of drought tolerant cowpea genotypes. Alidu [51] observed that the reduction in shoot biomass as the result of drought imposition at the vegetative phase is one of the main reasons for a decreased yield. Our results seem to corroborate this observation, since genotypes with higher SDW also present a higher number of pods and grains per plant. In contrast, a negative correlation between average grain yield and shoot length, root length and fresh and dry weight of the plants was found in chickpea genotypes under drought stress [49]. Furthermore, Alidu [51] denoted a linear correlation between NPP and grain yield, thus suggesting that the pod number can act as an indicator of potential yield.

RDW and RSR were the main contributing factors to the variation along the PC2 axis, establishing a positive association. In bread wheat (*Triticum aestivum* L.) genotypes, RSR also was among the main contributing characteristics to the variation observed under drought conditions [50]. RDW, among other traits, was identified by Abdou Razakou et al. [52] as a reliable parameter for the screening of drought tolerant cowpea seedlings. According to Wasaya et al. [17], root phenotyping is as important as shoot phenotyping, since roots are the main responsible organ for the plant's performance in relation to water and nutrient absorption. The negative association of SDW, SG and, to some extent, PH in PC2 indicates that drought stressed genotypes are either increasing shoot biomass at the expense of root biomass or the opposite. This result has also been observed in other studies [15,51].

The variation observed in PC3 was predominantly and positively associated with SDW and PH. Higher shoot height and biomass likely translates to a larger photosynthetic apparatus able to sustain higher grain yield [51]. However, in the study of Ayala et al. [53], an inversed correlation between shoot development (SDW and PH) and water use efficiency was observed, suggesting that greater shoot development leads to a higher loss of water being prejudicial under drought conditions. Likewise, Iseki et al. [15] also found shoot biomass to be related with plant water status, where lower shoot biomass could confer better drought tolerance.

The distribution of the genotypes based on the effects of drought stress relative to control for all parameters suggests a wide variability of the studied genotypes. This variability was expected as the cowpea genotypes had origins in different worldwide countries. However, the dispersion of the

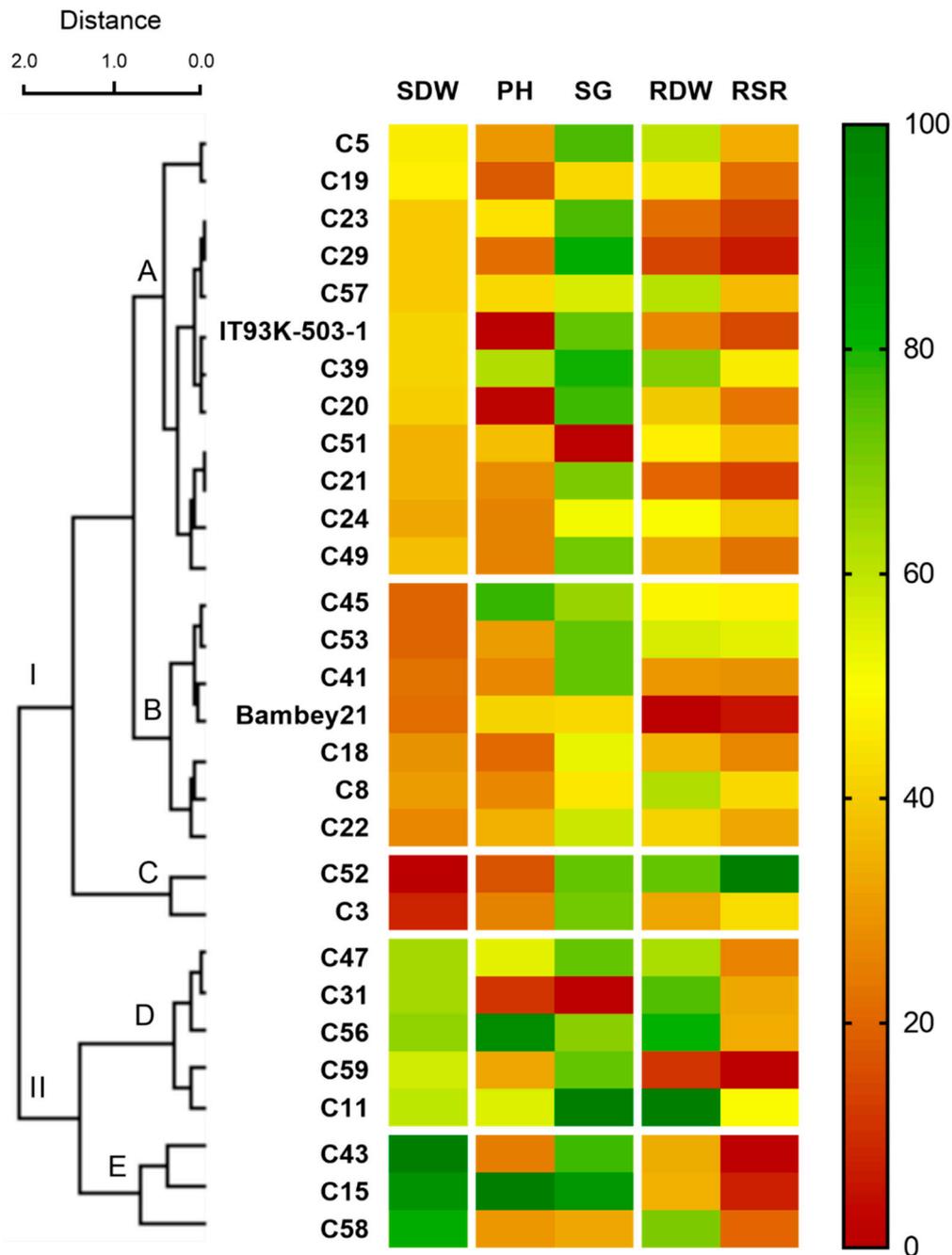
genotypes in the PCA did not reflect any obvious groupings based on similar origins. This result is in accordance with a previous study [19]. All evaluated parameters revealed considerable contributions to the variation observed between genotypes under drought treatment. This phenotypic variability under drought stress might represent an important resource for plant breeders.

#### 3.4. Screening for Drought Tolerant Genotypes

A dendrogram was elaborated based on the ratio of SDW between drought treatment and control for each genotype (Figure 9). Relative SDW translates into how much vegetative growth (expressed as shoot biomass) was affected by drought, and hence was used as a drought tolerance indicator. As suggested by the PCA, drought tolerance in cowpea seems to result from a complex of traits that can act simultaneously or singly and vary between different genotypes. The complexity and variability of the traits related to drought tolerance has been observed in previous studies [12,54]. Hence, the use of different traits, such as the ones here presented, might provide a more complete resource of phenotypic diversity to drought tolerance breeding. In order to assess the resulting clustering in a more integrated and holistic manner, a heat map with the ratios of SDW, PH, SG, RDW and RSR between drought and control treatment was included (Figure 9). This made it possible to make inferences about how the different traits combine with vegetative growth and which genotypes present the most interesting set of characteristics that might confer drought tolerance. Since drought was imposed at an early vegetative phase, not all plants were able to produce fully matured grains (hence the overall low number of mature pods seen in Table S2). Therefore, all parameters related to the reproductive phase (PDW, NGP and NPP) were not used for the screening of the most drought tolerant genotypes.

The clustering divided this set of genotypes into two main groups (I and II) that were further subdivided into A, B, C and D and E subgroups, respectively. Group I comprised most of the evaluated genotypes. Drought stimulated a lower reduction in shoot biomass in the genotypes from subgroup A (48%) than those observed in genotypes from subgroups B (56%) and C (67%). This subgroup A includes IT93K-503-1, the drought tolerant reference. Despite all genotypes in subgroup A having similar relative SDW, the same homogeneity was not observed in the other parameters. Regarding root parameters (RDW and RSR), C5, C24, C39, C51 and C57 genotypes presented the highest increases under drought treatment, whilst the opposite was found in C21, C23, C29 and IT93K-503-1 genotypes. SG was maintained in genotypes C5, C20, C21, C23, C29, C39, C49 and IT93K-503-1. When subjected to drought at the seedling stage, IT93K-503-1 also maintained SG, and this phenotype was related to higher drought tolerance [14]. Similarly, maintenance of SG under drought conditions is also an indicator of delayed senescence, and thus, drought tolerance [54]. Despite being considered drought tolerant, the IT93K-503-1 genotype still suffered a reduction in shoot biomass. This could be explained as a response to drought, where shoot metabolism is deactivated to lower the uptake of nutrients and water [55]. Genotypes in subgroup B (composed by Bambey21, the drought susceptible reference genotype and C8, C18, C22, C41, C45 and C53 genotypes) and subgroup C (C3 and C52 genotypes) presented the highest reductions in SDW under drought treatment, thus being considered the most susceptible to drought. Despite being the most affected by drought treatment in terms of shoot biomass, genotypes C52 and C3 were able to maintain SG, suggesting that shoot biomass reduction might have allowed maintenance of a more adequate plant water status. Overall, genotypes from subgroup B and C were characterized by low scores in all evaluated parameters, with some exceptions. C45, C52 and C53 presented superior values of relative RDW and RSR and were able to maintain their stem greenness under drought conditions. Contrarily, Bambey21 clearly differentiates from the rest of genotypes in subgroups B and C by the affected root development in terms of RDW and RSR. The Bambey21 genotype has been demonstrated to be susceptible to drought stress in previous works [14,25,56,57]. Contrarily to most of the remaining genotypes, in which drought promoted the allocation of biomass to root, the RDW of Bambey21 was slightly decreased. Nonetheless, this decrease was non-significant ( $p > 0.05$ ). RSR was slightly increased under drought stress, probably due to a more intense reduction in shoot biomass. The role of root development in drought tolerance, among other factors, might

explain the susceptibility of this genotype. Similar to our results, in the study of Ogonnaya et al. [57], Bambey21 presented lower root biomass and RSR than the other tested genotypes. In light of these results, we hypothesized that low RDW and RSR could be related to the increased susceptibility of cowpea. Finally, subgroup C consists of the two genotypes (C3 and C52) with the lowest relative SDW, indicating that vegetative growth was severely affected by drought treatment.



**Figure 9.** Analysis of morphological characteristics on 29 cowpea genotypes under drought stress (90 cm pipes) relative to control (30 cm pipes). The dendrogram shows the Manhattan distances between genotypes based on the ratio of SDW between drought treatment and control using UPGMA method. The heat map shows the ratios of SDW, PH, SG, RDW and RSR between drought and control treatment obtained for each genotype, normalized to percentage, where 0 and 100% represents the minimum and maximum values registered in each parameter. SDW—shoot dry weight; SG—stem greenness; RDW—root dry weight; RSR—root: shoot ratio.

Group II comprises the genotypes with the highest relative SDW, suggesting that these were the least affected by drought in the vegetative growth. Interestingly, under control conditions, the genotypes in subgroup D (C11, C31, C47, C56, and C59) and E (C15, C43 and C58) presented the lowest SDW among all genotypes. These genotypes were also the ones that suffered the least reduction under drought treatment (SDW reduction percentages in subgroups D and E were 37 and 22%, respectively). Previous studies have showed that genotypes with smaller shoot biomass achieve better water status and thus are more able to tolerate drought [15,52]. Even though a higher shoot biomass could lead to detrimental water losses through transpiration under drought conditions [15], an increase in net photosynthesis, as a consequence of a higher number of leaves, might support a higher accumulation of dry matter in pods, thus promoting a better yield [31,51]. Although genotypes in subgroup E maintained a higher shoot biomass compared to subgroup D, the genotypes in the last-mentioned subgroup presented a better performance in the rest of the evaluated parameters, particularly regarding RDW. The importance of root traits to drought tolerance in cowpea has been suggested in previous works [12], wherein root biomass seems to play an important role in a greater water absorption capacity [15]. One of the mechanisms of drought tolerance in common bean seems to involve a higher secondary lateral root development [39], which possibly translates into higher root biomass. Our results show that genotypes that were able to develop higher root biomass under drought treatment (particularly genotypes in subgroup D) were able to sustain shoot development with smaller reductions in SDW, PH and SG relatively to control conditions. In light of these observations, increasing root biomass might confer higher drought tolerance. Therefore, subgroup D seems to group the genotypes better adapted to drought regarding both root and shoot development, with the exception of C31 and C59, which presented considerable reductions in shoot (PH and SG) and root traits (RDW and RSR), respectively. Our results made it possible to corroborate a previous study where C11 and C47 genotypes were classified as drought tolerant at the germination level [13]. The indication that some of these genotypes are also drought tolerant at the germination level further reveals their importance for future breeding programs. As a final remark, reductions in NPP, NGP and PDW varied greatly among the evaluated genotypes. In group II, the subgroup E, particularly C43 and C58, was less affected than genotypes in subgroup D in the NPP, NGP and PDW. The genotypes C31, C43, C56 and C58 suffered reductions inferior to 50% in NPP, NGP and PDW, indicating that these genotypes might be able to provide better yield under drought conditions than the remaining genotypes in group II. Contrarily, genotypes C15 and C59 were severely affected, with little to no production of pods under drought treatment. However, as previously mentioned, further studies that focus on yield are required and the data here presented should be only interpreted as indicators of potential yield under drought stress.

#### 4. Conclusions

Cowpea is known for its drought tolerance and thus is considered an important legume crop to face the future constrains due to climate change. Our results show that when subjected to an intense drought stress, cowpea seems to prioritize root development over shoot, which reflected the overall significant reduction in SDW, SG, PDW, NPP and NGP, whereas RDW and RSR significantly increased. PCA depicted that all evaluated parameters had considerable contributions for the variation observed between both water treatments among genotypes, indicating that besides above-ground characteristics, root-related parameters are also important factors for drought tolerance screenings in cowpea. The hierarchical clustering of the genotypes under drought stress based on vegetative growth retardation (differences in shoot biomass between control and drought conditions) revealed that the majority of the genotypes with the least reductions in shoot biomass under drought conditions also presented the highest increases in root biomass, suggesting the importance of this trait to drought tolerance. In light of these results, a set of genotypes with the lowest vegetative growth retardation was identified, namely C11, C15, C31, C43, C47, C56, C58 and C59. From this group, the most drought tolerant genotypes were C11, C47 and C56 since these showed generally higher drought/control ratios in all measured parameters. It is important to mention that, being the result of several mechanisms,

drought tolerance variations cannot be explained only by a simple tolerance factor. In further cowpea experiments using pipes methodologies exploring root morphology and its role in drought tolerance, it would be advantageous the use of pipes of equal height and to complement plants' agro-morphological performance with physiological, biochemical and other developmental responses.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/10/1604/s1>, Table S1: Days to germination and flowering expressed as means  $\pm$  standard deviations of the replicates ( $n = 3$ ) for each genotype and Table S2: Percentage of immature (I), mature (M) and no grain (N) pods for each genotype under control and drought conditions.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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