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Master's thesis

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Jocelyn Cuevas

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :
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Biochar as a soil amendment, evaluating its potential on decreasing drought stress in *Arabidopsis thaliana* and *Medicago sativa* plants

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*Running title: *Biochar's potential as a soil amendment to reduce drought stress in plants*

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ABSTRACT

Drought is known to decrease plant productivity. In addition, population growth will increase the food demand, putting pressure on agricultural production. Hence, using biochar to mitigate the adverse effects of drought stress can improve plant growth and productivity. However, knowledge regarding the influence of biochar on the plant during drought stress is limiting. Therefore, this study aims to investigate the effect of biochar on growth and oxidative stress markers in *Arabidopsis thaliana* and *Medicago sativa* when exposed to drought.

Drought was simulated in the SAFETY^{96} liquid system using four polyethylene glycol (PEG) concentrations (0, 5, 10, and 20 m/m%). Subsequently, root length, fresh weight, lipid peroxidation, and antioxidative capacity were determined in *A. thaliana*. In addition to the SAFETY^{96} system pot experiments using soil and *M. sativa* were optimized.

In general, PEG-induced drought stress affects the growth of the *A. thaliana* seedlings (i.e., fresh weight & root length) by increasing lipid peroxidation. However, adding biochar in these conditions alleviates drought stress-induced lipid peroxidation. Nevertheless, the plant growth was not stimulated, so application of biochar in a PEG-induced drought condition, should be further investigated. Therefore, initial soil pot experiments were optimized. The reduced water-filled pore space water supply is the suggested method for future investigation in the pot experiment as it is easier to manipulate under controlled conditions, but further optimization is needed.

INTRODUCTION

Climate change is a well-known environmental threat that can be described as an increasingly unstable weather condition that alters temperature, humidity, precipitation, and other atmospheric conditions resulting in catastrophic weather events such as drought and heatwaves. According to IPCC (2021), a rise in global surface temperature by 0.99 °C was noted in the 21st century (from 2001 to 2020) compared to the years 1850 to 1900s (1). Subsequently, frequent exposures to extreme weather conditions like drought would eventually dry up the groundwater reserves, escalating the evapotranspiration rate that could reduce the plant growth resulting in decreased agricultural yield (2). Moreover, the world's population could exceed 9 billion people by 2050, which leads to higher food demand. Therefore, food production would need to increase by 70% to 85% putting additional strain on already deteriorating natural resources (3).

One of the major factors limiting crop production is drought stress. In plants, drought stress causes stomata regulation, osmotic adjustment, and antioxidative defense to alleviate the damage (4). In addition, almost all plant functions are affected, resulting in decreased growth and development of plants. Multiple studies have reported that drought causes a reduction in the height and stem length, biomass, and yield of different plants in response to drought stress (5, 6).

Plants, as sessile organisms, experience drought stress when there is a disturbance from the water supply to the roots or when the transpiration is exceptionally high (5). Subsequently, to prevent transpirational water loss, plants close their stomata

as their first response to acute water loss. This in its turn leads to a decrease in both photosynthetic rate and intracellular CO_2 concentration, which could induce the formation of oxygen-free radicals known as "reactive oxygen species (ROS)" such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radical (°OH), and singlet oxygen ($^1O_2$) leading to oxidative stress (7-9). During drought stress conditions, ROS production increases, damaging macromolecules such as proteins, DNA, and lipids (8). Damage to membrane fatty acids may form small hydrocarbon fragments such as malondialdehyde (MDA) (10). MDA is a by-product of lipid peroxidation in plant cell membranes and is an indicator of membrane system damage (11). A rise in MDA concentrations was found in *Brassica napus* cultivars under drought-induced stress (12). Furthermore, excessive ROS in thylakoids impairs chloroplast ultrastructure in several plant species, decreasing chlorophyll photosynthetic pigments such as chlorophyll *a*, *b*, and carotenoids (13-15). As a result, when plants are exposed to drought conditions, their photosynthetic pigments decrease.

Plant cells are protected from the harmful effects of ROS under optimal conditions by a complex antioxidant system that includes both non-enzymatic and enzymatic antioxidants (16). Antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, and antioxidants metabolites such as vitamins, glutathione and sugars help control the levels of ROS. Hence, they protect the plant's structural and molecular components from the effects of ROS accumulated in the cell. Besides the damaging effects of ROS, they may also be involved in signal transduction regulation, hence ROS may also play a critical role in modifying plant acclimation to drought stress (4)

Alfalfa (*Medicago sativa*) is a perennial forage legume crop widely known for its high agronomic interest, high yield, crude protein-richness with excellent digestibility, and low production cost. It is commonly grown in water-scarce regions. Compared to other crops, *M. sativa* has a better drought avoidance strategy due to its ability to utilize deep soil moisture via its deep root system (4). Although some varieties of *M. sativa* can adapt to drought conditions, drought stress still has a negative impact on the plant productivity of these plants. Therefore, increasing crop production on marginal soils is important to improve their physicochemical properties. This improvement can be done with *in situ* application of soil amendments, such as biochar.

Biochar is obtained through the conversion of e.g., biomass rest streams, i.e., flax, straw peat, etc., heated with temperatures ranging from 300-700°C under limited oxygen supply, a process called pyrolysis. Biochar addition to soil can improve soil physicochemical properties through its water holding capacity (WHC), surface area, and porosity (2, 4). The small particle size of biochar would facilitate for a higher surface area, allowing for increased WHC in amended soils (17).Therefore, this amendment has been proposed to enhance plant growth and crop productivity while mitigating the detrimental effects of drought. Biochar improves soil quality and water availability while increasing photosynthesis, transpiration, water potential, and stomatal conductance (18, 19).

Additionally, the water-retaining ability of biochar amended soils can vary depending on the soil type, the source and rate of biochar application, and the biochar preparation methods (20, 21). Furthermore, biochar properties vary greatly and are primarily determined by the feedstock from which it is produced (22). In addition, biochar also improves the physiological parameters, antioxidant enzyme activity, and crop yield while decreasing oxidative stress in wheat plants exposed to drought and cadmium stress (23).

The use of biochar to improve crop growth and yield has been reported under drought stress conditions. Its application mitigated the effect of drought on wheat by improving water use efficiency (24). In addition, biochar alleviates drought stress's negative impact by increasing the antioxidant activity, soil fertility, and rapeseed physiological processes (25). Furthermore, biochar addition to sandy soils increased tomato plants' resistance to wilting and drought stress (26). Moreover, applying biochar to drought-stressed soils increased soil WHC and plant performance (photosynthetic rate, plant biomass, chlorophyll content, and antioxidant activity) (27). Yet, contrasting results are associated with biochar application. Some studies show that biochar increased plant height and leaf area, biomass, and yield in drought-affected soils (28-30), but in some studies, biochar application reduced plant biomass (31).

Nonetheless, it is still unknown how biochar can reduce cellular drought stress responses in plants. Therefore, this study will examine whether biochar can stimulate growth and alleviate oxidative stress in *A. thaliana* under drought. Furthermore, to investigate the effect of biochar application in *M. sativa* plants under drought conditions by optimizing the water-filled pore space (WFPS) and water withholding method.

This study will be an effective way to get fundamental knowledge on the drought-induced oxidative stress responses in plants and how specific biochars can enhance plant growth under drought conditions by diminishing the stress responses. In conclusion, this will help to assess crop productivity improvements in drought-affected areas.

EXPERIMENTAL PROCEDURES

1.1 Experimental Design

The experiment consisted of an initial biochar screening, followed by pot experiments conducted at Hasselt University. Based on the Water holding capacity (WHC) and preliminany data, two biochars (straw peat and flax) were selected. These two biochars were first screened for their potential in decreasing drought stress using the SAFETY^{96} well system (patented). Polyethylene glycol (PEG) was used to simulate drought stress in this liquid-based system. The effect of drought stress and the application of biochar on it was assessed in *A. thaliana* seedlings via the determination of fresh weight and root length as well as malondialdehyde (MDA) concentrations as a measure for lipid peroxidation, and the ferric reducing antioxidant power (FRAP) assay for the total antioxidant potential.

Subsequently, pot experiments were conducted using Ravels soil (loamy sand) and *M. sativa* seedlings. This soil is classified as moderately poor to good according to Belgian soil classification. The sampling of the Ravels soil was based on the method of Xu et al., 2020 (32). Drought was simulated in two ways: A first experimental set-up was by giving the pot's water until 50% WFPS was reached, and plants were grown until the first true leaves emerged. Then, plants were divided into two groups: normal and drought conditions. The normal condition continued with 50% WFPS whereas plants in the drought condition were no longer given water. In a second experimental set-up, plants were exposed to drought from the beginning of the experiment, i.e., plants continuously grown on 50% WFPS soil (control) or 40% WFPS soil (drought).

1.2 Plant Material and Growth Conditions

1.2.1 Screening using the SAFETY^{96} well system

Wild type (WT) *A. thaliana* seeds were surface sterilized with 70% ethanol and stored in the dark for two nights at 4 °C before sowing. 1/4 Murashige and Skoog (MS) medium was prepared, and drought was simulated in the 96-well plate system via four different PEG concentrations (0, 5, 10 & 20 m/m% PEG). To investigate the potential mitigating effect of biochar, 0.5 m/m % biochar was added per condition. The seeds were sown in the 96-well plate system and allowed to grow for ten days. The growth conditions were set to a 12-hour photoperiod, 22/18 °C day/night temperatures, and relative humidity of 65%. A combination of blue, red, and far-red Philips Green-Power LED modules provided light, simulating sunlight's photosynthetic active radiation. The harvesting of samples was done at ten days after sowing. Additionally, the growth parameters such as root length and fresh weight were measured. The samples were snap-frozen in liquid nitrogen and stored at -80 °C until further analysis.

1.2.2 Biochar

The biochars used were conventionally pyrolyzed at a temperature of 450 °C and put in the oven for 24 hours at a temperature of 105 °C to remove the volatile polycyclic aromatic hydrocarbons. Then, the biochars were milled and sieved using a 63 mm sieve. This experiment used 2 types of biochar, flax, and straw peat (**Table 1**).

Table 1 Water holding capacity of biochars

Biochar Feedstocks	WHC Values (w/w%)
Flax	540
Straw peat	118

1.2.3 Pot Experiment with Medicago sativa

Medicago sativa seeds were surface sterilized with 70% ethanol and kept in the dark for 2 nights at 4°C before sowing. The seeds were sown into the pot filled with Ravels soil from now on called reference soil (RS) amended with or without 0.5 m/m % flax or straw peat biochar. These seeds were grown in the same controlled environment as the 96-well plate system. The plants were given water every day by weighing the trays and subtracting the measured weight by the reference weight of the trays. The plants were grown for at least 21 days. Drought stress was applied to the plants in two ways (cfr supra): (1) drought stress application after the first true leaves emerged and then without any water supply for nine days and (2) drought stress from the start of the experiment. In the second experimental set-up, plants of the control condition were given water up to 50% WFPS, whereas plants in the drought condition were given only 40% WFPS and grown for 21 days.

During the experiment, the following growth parameters were determined three times a week: the number of true leaves and stem length. Harvesting for lipid peroxidation and total antioxidant capacity was done after the drought exposure. The samples were harvested after nine days (first experimental set-up) and three weeks of exposure (second experimental set-up), respectively. For both experimental set-ups, leaf samples were snap frozen in liquid nitrogen and stored at -80 °C for MDA and FRAP analysis. Additionally, a part was harvested for chlorophyll determination for the second experimental set-up. The samples were immediately frozen in liquid nitrogen and stored at −80 °C. Additionally, the fresh weight was determined.

1.4 Lipid peroxidation

The malondialdehyde (MDA) concentration was used to determine the rates of lipid peroxidation in plant tissues (11). The thiobarbituric acid (TBA) reaction was used to determine the MDA concentration. A homogeneous powder was created by pulverizing the frozen sample using two stainless steel beads in the shredder (Retsch Mixer Mill MM 400). Subsequently, 750 µL of 80% ethanol was added to the frozen samples, and samples were vortexed. Afterwards, the homogenate was centrifuged for 30 minutes at 14000 rpm. Furthermore, 500 µL of 0.5% TBA (in 20% TCA) was added to 250 µL supernatant and incubated at 90°C for 1 hour. Subsequently, samples were incubated at 4°C for 5 minutes to stop the reaction. After centrifugation at 5000 rpm for 1 minute at 4°C. The absorbance was measured at 440, 532, and 600 nm in a 96-well plate using the FLUOstar Omega plate reader.

The amount of MDA (in nmol/mL) in the well was calculated using the following formula:

$$6.45*(A_{532}-A_{600}) - (0.56* A_{440}) *3$$

1.5 Antioxidative capacity

The total antioxidative capacity of *A. thaliana* and *M. sativa* plants was assessed using the FRAP assay. This method uses antioxidants in the sample to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) and produces a blue color when Fe^{3+} is reduced.

A homogeneous powder was created by pulverizing the frozen sample using two stainless steel beads in the shredder (Retsch Mixer Mill MM 400). Subsequently, 750 µL of 80% ethanol was added to the frozen samples, and samples were vortexed. Afterwards, the homogenate was centrifuged for 30 minutes at 14000 rpm. Then, from the homogenate, an aliquot of 200µL sample was made and stored at -80°C until actual analysis. 50 µL of each sample was pipetted in a 96-well plate. The reaction mix for each plate was made by combining 16.67 ml of 0.3 M acetate buffer,1670 µl of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), and 1670 µl of 20 mM $FeCl_3$. The mixture was kept at 4°C. Finally, 150 µL of the reaction mixture was added to 50 µl of sample in a 96-well plate and incubated for 20 minutes in the dark at 4°C. Afterwards, the absorbance of 600 nm was determined using the FLUOstar Omega plate reader. The concentrations of total antioxidative capacity were calculated via a trolox standard curve.

1.6 Chlorophyll Determination

A spatula tip of calcium carbonate was added to the frozen samples. A homogenous powder was created by pulverizing the frozen samples using two stainless steel beads in the shredder (Retsch Mixer Mill MM 400). Subsequently, 1 ml of 80% acetone

was added to the frozen samples and was kept in the dark. Afterwards, the samples were centrifuged for 5 minutes at 6000 rpm, 4°C. The absorbance was measured at 663 nm, 646 nm, 470 nm in the 96 well plate using the FLUOstar Omega plate reader.

The amount of chlorophyll *a* and *b* content is calculated using the formula:

Chlorophyll *a* (µg/ml) = 12,21 A_{663} – 2,81 A_{646}
Chlorophyll *b* (µg/ml) = 20,31 A_{646} – 5,03 A_{663}
Total carotenes (µg/ml) = (1000 A_{470} – 3,27 chl *a* – 104 chl *b*) / 229

Statistical Analysis

R software (R v64 4.0.5) was used for all statistical analyses. The Shapiro-Wilk and Bartlett's tests were used to confirm the data's normal distribution and homoscedasticity, respectively. The data were transformed (square root, inverse, exponent, logarithm) provided the assumptions were not met. Two-way ANOVA (p-value <0.05) was used to analyze the data, followed by a post-hoc Tukey-Kramer test to identify which group differed significantly. If the data were not normally distributed, the Wilcoxon and Kruskall-Wallis rank sum tests were used at a statistically significant level of P<0.05.

RESULTS

3.1 Effect of PEG-induced drought stress and biochar on morphological responses of A. thaliana seedlings in the SAFETY^{96} set-up

Based on preliminary screening of six biochars (See Appendix 1), two biochars (derived from Flax & Straw peat) were selected to investigate their potential and further decrease drought stress in plants. Results presented in fig. 1A show that the fresh weight of *A. thaliana* seedlings significantly decreased when exposed to 10% PEG compared to the control reference medium (RM; 0% PEG). Although biochar addition did not affect the fresh weight of *A. thaliana* seedlings in the control reference medium, straw peat biochar significantly reduced the plant fresh weight at 5% and 10% PEG concentrations, compared to the reference medium exposed to the same PEG concentration. No data is available for the fresh weight of the seedlings when exposed to 20% PEG, as seedlings germinated were too small to weigh when treated with flax or straw peat biochar.

As shown in fig. 1B, the root length of the seedlings of *A. thaliana* was increased at 5% PEG concentration compared to the control reference medium. In this condition, flax biochar significantly increased the root length of the plant when compared to the reference medium without biochar. However, this stimulating effect disappeared when plants were exposed to the different PEG concentrations.

In general, addition of biochar reduced the seedlings' root length as the concentration of the PEG increased compared to its own biochar-amended reference medium without PEG. Nevertheless, in comparison with the reference medium with similar PEG concentrations, the application of straw peat biochar, overall, resulted in shorter root lengths.

3.1.1 Effect of PEG-induced drought stress and biochar on lipid peroxidation and antioxidant capacity of A. thaliana seedlings

MDA concentrations of *A. thaliana* seedlings in the reference medium significantly increased at 10% PEG when compared to the control reference medium (fig. 2A). Adding biochar in this condition significantly decreased the MDA concentrations in the seedlings. Additionally, there is a trend towards a higher antioxidative capacity when the seedlings were exposed to 10% PEG compared to the control reference medium Although an overall lower antioxidant capacity was observed when biochar was added to the reference medium with similar PEG concentrations, this was only significant for straw peat biochar.

3.2 Optimization Of Drought Stress Using Pot Experiments

3.2.1 Water withholding-induced drought stress

In the water withholding-induced drought stress set-up, drought stress application was done by withholding water for nine days after the first true leaves emerged. The results show that a significant decrease was observed in the fresh weight of *M. sativa* in the reference soil when

drought was imposed (fig. 3) Although addition of flax biochar significantly improved the plant fresh weight when compared to the reference soil in the control condition, no significant differences were observed on fresh weight for either of both biochars compared to the reference soil under drought condition. In addition, MDA concentrations of the plants were significantly increased in the reference soil under drought condition when compared to the control condition (fig. 4A). Similarly, an increasing antioxidative capacity was also observed in the plants that were exposed to drought as compared to the control (fig. 4B).

Biochar addition under control condition had no significant effect on the MDA concentrations. However, flax biochar shows a decreasing trend in the MDA concentration level (fig. 4A) when compared to the reference soil under drought condition. A similar trend was also observed in the plant antioxidant capacity (fig. 4B) with flax biochar addition when drought was imposed as compared to the reference soil.

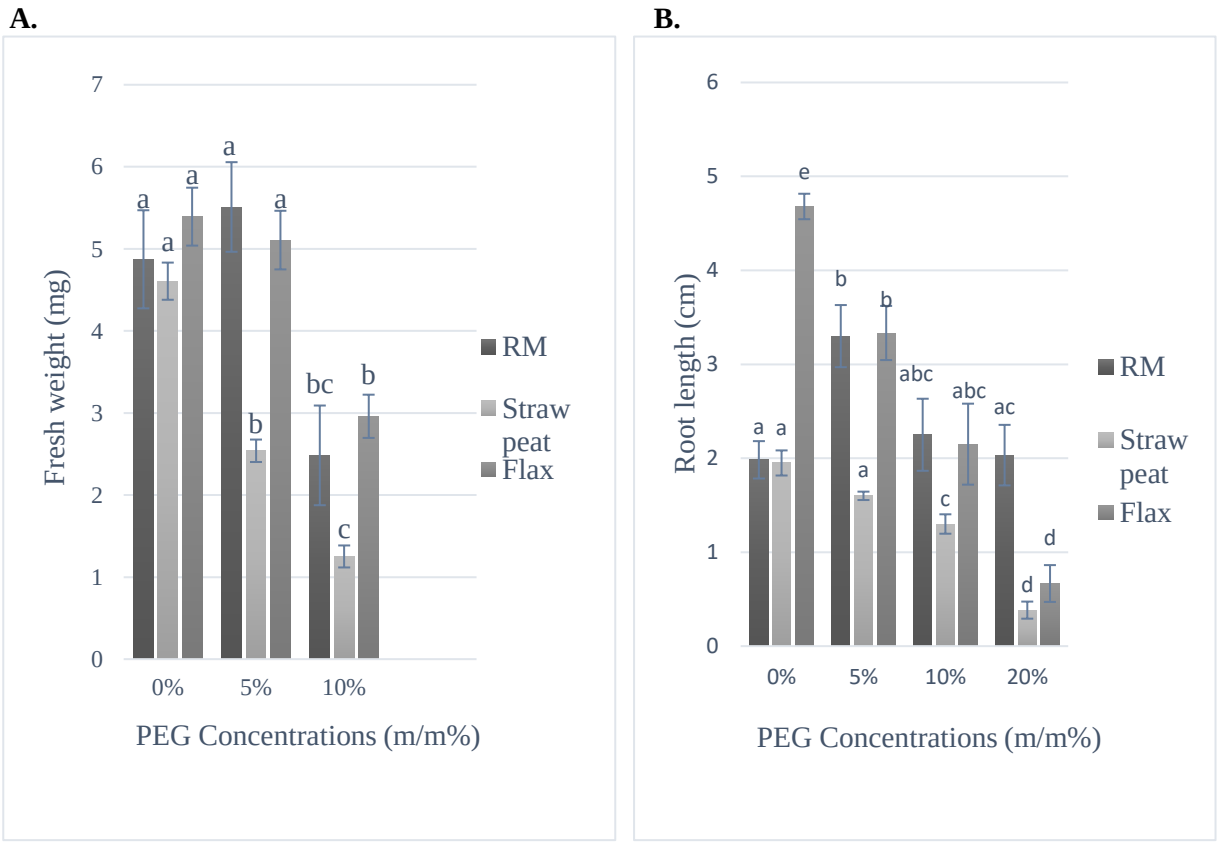


Fig. 1 Effect of PEG-induced drought stress on morphological responses such as A. Fresh weight and B. root length of 10 days old *A. thaliana* seedlings grown in a 96-well plate containing ¼ MS medium amended with 0.5 m/m% flax or straw peat biochar and exposed to different PEG concentrations (0%, 5%, 10%, & 20 m/m%). Values are the average ± SE of at least six biological replicates. Significant differences are indicated with different letters using non-parametric test (Wilcoxon & Kruskall-Wallis) with a p-value <0.05. RM: Reference medium.

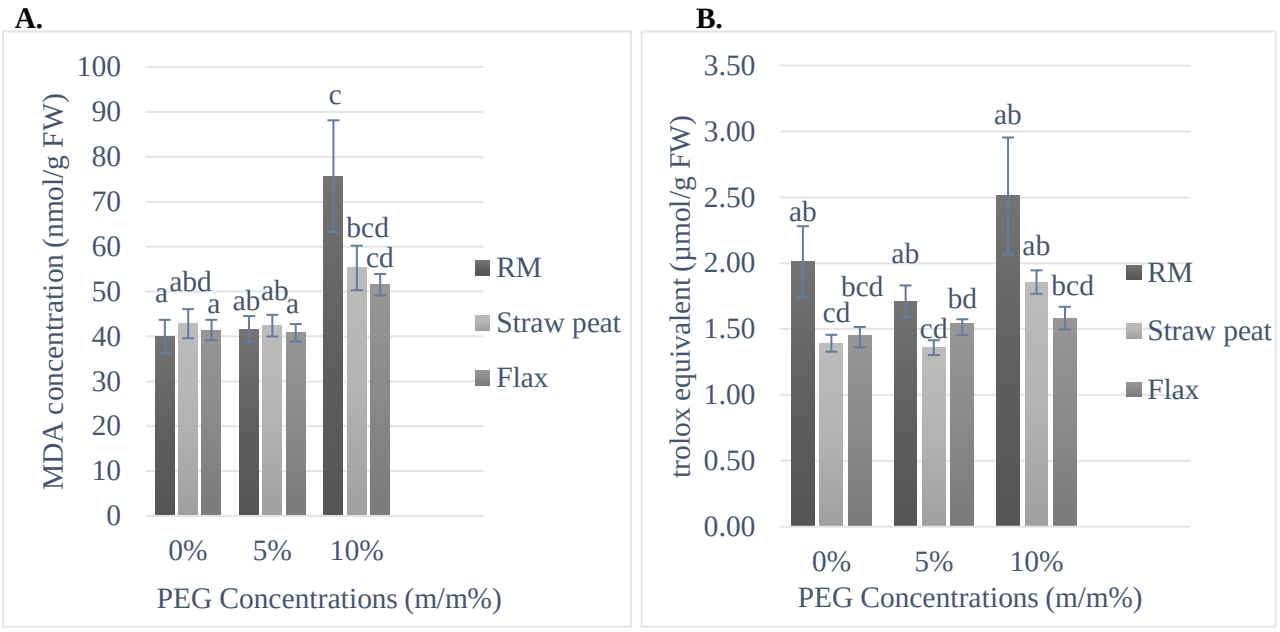


Fig 2. Lipid peroxidation (A.) and Antioxidative capacity (B.) of 10 days old *A. thaliana* seedlings grown in a 96-well plate containing ¼ MS medium amended with 0.5 m/m% flax or straw peat biochar and exposed to different PEG concentrations (0%, 5%, & 10% m/m%). Values are the average ± SE of at least six biological replicates. Significant differences are indicated with different letters using non-parametric test (Wilcoxon & Kruskall-Wallis) with a p-value <0.05. RM: Reference medium

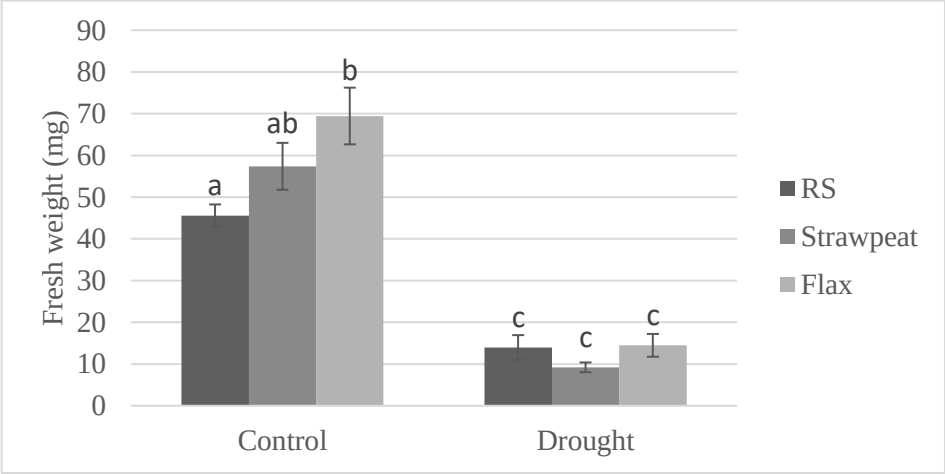


Fig. 3 Fresh weight of 21 days old *Medicago sativa* grown on Ravels soil amended with 0.5 m/m% flax or straw peat biochar exposed to drought stress (no watering) for nine days after the first true leaf emerged. Values are the average ± SE of eight biological replicates. Significant differences are indicated with different letters using Two-Way ANOVA with p-value < 0.05. RS: Reference soil

3.2.2 Reduced Water Filled Pore Space (WFPS) water supply to impose drought stress

In the WFPS-induced drought experimental set-up, drought stress was applied from the beginning of the experiment. The plants of the control condition were given water up to 50%WFPS, whereas in the drought condition plants were given only 40% WFPS and grown for 21 days.

The MDA concentrations as shown in fig. 7A, do not show any significant differences between drought and control condition in the reference soil. However, treatment with flax biochar shows a significant decrease in MDA concentration compared to the reference soil exposed to drought. Similar findings were noted in the antioxidant

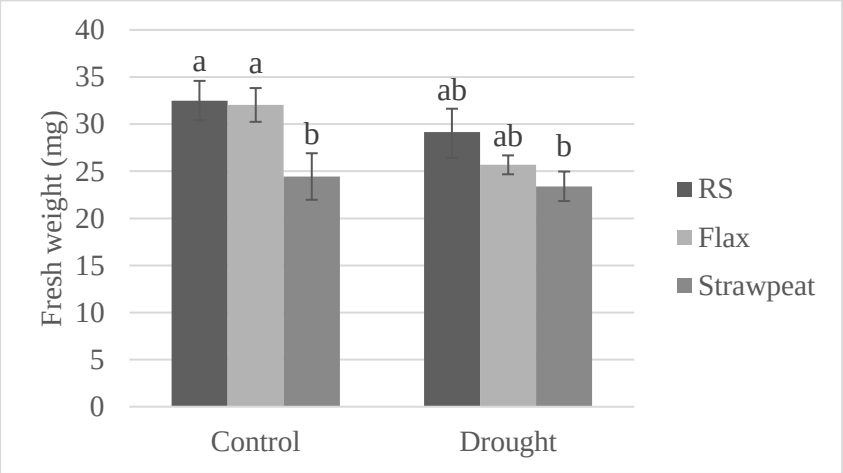


Fig. 5 Fresh weight of *Medicago sativa* exposed to drought (40% WFPS) and control (50% WFPS) conditions grown on Ravels soil amended with flax or straw peat biochar. The drought stress was applied from the beginning of the experiment and the plants under drought condition were subjected to drought stress for 21 days. Values are the average ± SE of at least eight biological replicates. Significant differences are indicated with different letters using Two-Way ANOVA with a p-value < 0.05. RS: Reference soil

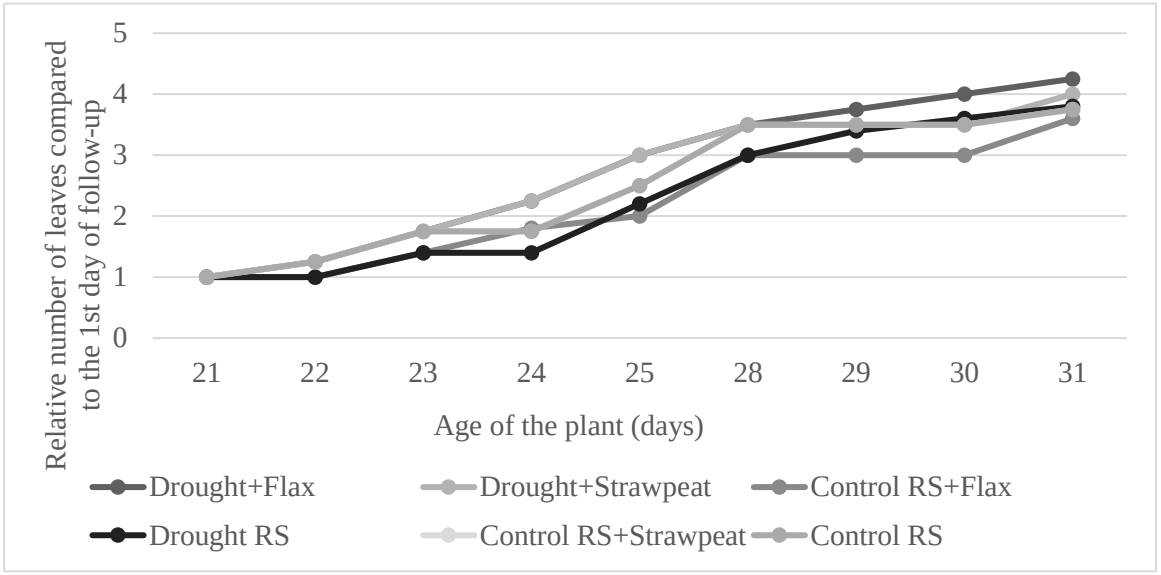
Fig. 5 shows that there are no significant differences in the plant fresh weight in the reference soil between drought and control condition. Yet, straw peat biochar negatively affects the fresh weight of the plant in the control condition as compared to the reference soil and a reducing trend is also seen under drought condition.

Growth parameters such as relative number of leaves, and relative stem length are presented in fig. 6A and 6B. Results indicate that plants treated with flax biochar and exposed to drought had the highest relative number of leaves, and the relative stem length was highest at drought RS compared to other treatments, respectively.

capacity of the plant in the reference soil between both conditions. Yet, flax biochar addition had a decreasing trend of significantly reducing the plant's antioxidative capacity compared to the reference soil (without biochar) under drought conditions.

There are no significant differences in the chlorophyll concentrations of the plants under drought and control conditions. Similar findings were also found for biochar addition (Table 2).

A.



B.

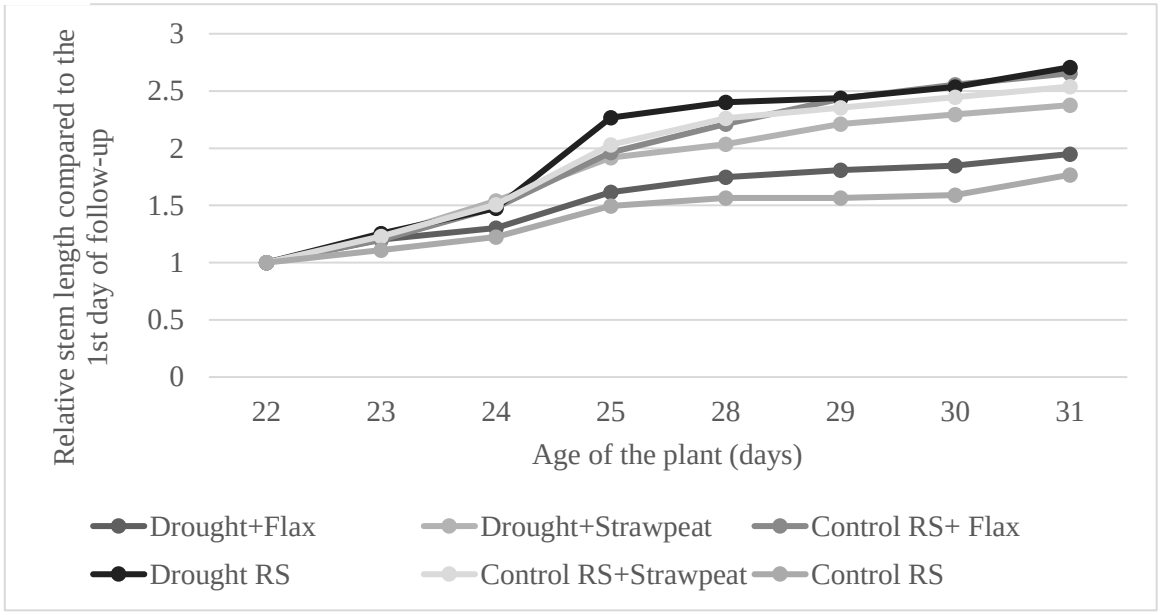


Fig. 6 Relative number of leaves (A.) and stem length (B) of *Medicago sativa* exposed to drought (40% WFPS) and control (50% WFPS) conditions grown in Ravels soil amended with flax and straw peat biochar. The drought stress was applied from the beginning of the experiment and the plants under drought condition were subjected to drought stress for 21 days. Values are the average ± SE of eight biological replicates. RS: Reference soil

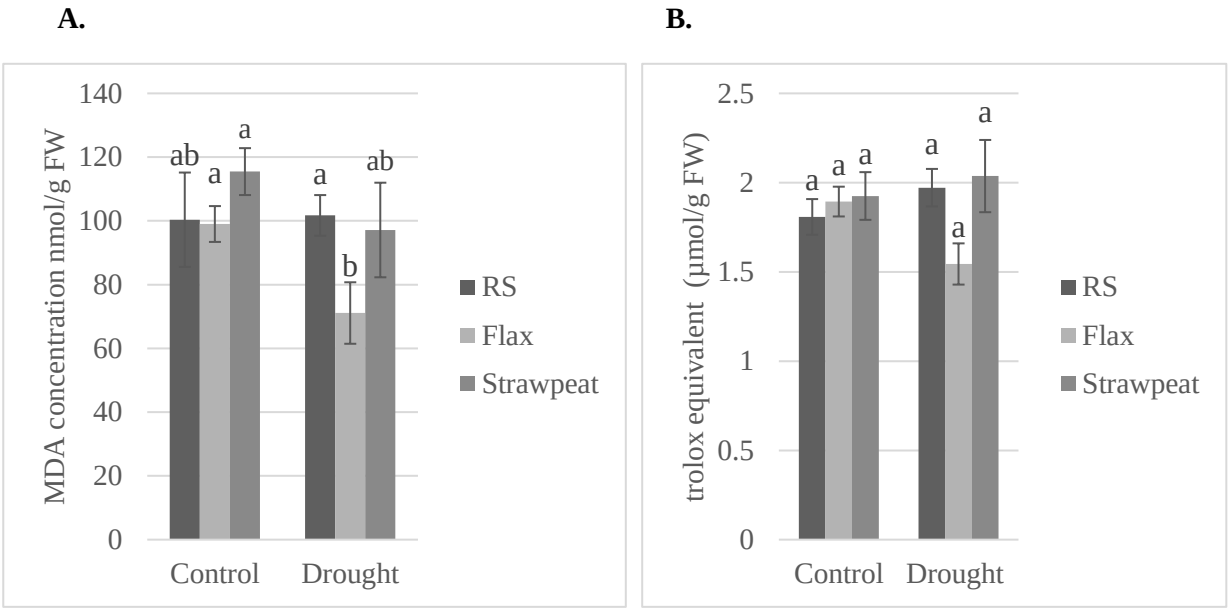


Fig. 7 Lipid peroxidation (A.) and Antioxidative capacity (B.) of *Medicago sativa* exposed to drought (40% WFPS) and normal (50% WFPS) conditions grown in ravels soil amended with flax and strawpeat biochar. The drought stress was applied from the beginning of the experiment and the plants under drought condition were subjected to drought stress for 21 days. Values are the average ± SE of eight biological replicates. Significant differences are indicated with different letters using non-parametric test (Wilcoxon test & Kruskall-Wallis test), and Two-Way ANOVA, respectively with p-value < 0.05. RS: Reference soil

Table 2. Concentrations of chlorophyll *a*, chlorophyll *b* and carotenes (µg/g FW) in leaves of *Medicago sativa* plants exposed to drought (40% WFPS) and control (50% WFPS) conditions grown in Ravels soil amended with flax and straw peat biochar. The drought stress was applied from the beginning of the experiment and the plants were grown for 21 days. Values are the average ± SE of eight biological replicates. No significant differences were found using Two- Way ANOVA with p-value <0.05. RS: Reference soil

Treatment	**Chlorophyll *a* (µg/g FW)**	**Chlorophyll *b* (µg/g FW)**	**Carotenes (µg/g FW)**
Control RS	656.56 +/- 67.08	342.85 +/- 94.04	101.35 +/- 19.51
Drought RS	681.91 +/- 27.73	281.51 +/- 47.46	47.46 +/- 15.44
Control RS+Flax	729.16 +/- 30.94	314.31 +/- 53.27	139.03 +/- 26.76
Drought RS+Flax	664.67 +/- 69.89	384.22 +/- 92.37	664.67 +/- 15.87
Control RS+Strawpeat	730.29 +/- 46.33	254.90 +/- 35.27	113.85 +/- 22.77
Drought RS+Strawpeat	795.31 +/- 75.74	219.66 +/- 25.10	155.18 +/- 9.60

DISCUSSION

Effect of PEG-induced drought stress and biochar on morphological & molecular responses of A. thaliana seedlings in the SAFETY96 set-up

To investigate drought stress and the potential mitigating effect of biochar in a fast-screening system (SAFETY96) different PEG concentrations and two biochars namely flax and straw peat were added to this liquid-based system.

In the current study, PEG-induced drought stress affected the growth of the *A. thaliana* seedlings. The fresh weight of the seedlings was significantly reduced under PEG-induced drought stress conditions. Reduction in plant fresh weight (both roots and shoots) has been reported by Hellal et al., 2018 and was said to be associated with an increasing PEG concentration (33). Moreover, Hamayun et al., 2010 found that soybean plants were susceptible to PEG-induced drought stress, as shown in the reduction of the fresh weight and dry weight at the pre-flowering stage compared to the later stage (34). In our study, the reduction in plant fresh weight could be because there might be an increase in ROS production leading to increased lipid peroxidation and antioxidant capacity of the seedlings or there is a suppression of the cell expansion and cell growth due to the low turgor pressure resulting in a decrease in the plant fresh weight (5). In addition, a significant root length increase was observed in seedlings grown in reference medium and exposed to 5% PEG. This result is in accordance with Jaleel et al., 2008 (8), who also reported that the root growth of *Catharanthus roseus* was increased initially. Still, the root length is reduced in the later stage, where drought stress becomes severe. MDA concentrations indicate membrane lipid peroxidation and may reflect the extent of damage under adverse conditions. In our study, the MDA concentrations significantly increased as the PEG concentration increased. Similarly, an increasing trend of antioxidant capacity in the plant was also observed as the concentration of PEG increased in the reference medium. When plants are subjected to environmental stresses such as drought, ROS production can exceed the antioxidant capacity, resulting in protein damage, DNA damage, and lipid peroxidation (35). Probably in our study, the plants invest in their defense to limit the damage but fail, leading to oxidative stress and limited plant growth. This is in accordance with the study of Mirzaee et al., 2013, which also showed that the level of MDA concentrations in two canola cultivars was also increased with an increasing PEG concentration (0-15%) (12). Another study revealed that there was an increase in MDA and H_2O_2 concentrations in *Brassica napus* under drought stress with 35- 40% withholding capacity (25). In our study, although biochar addition did not affect the fresh weight of *A. thaliana* seedlings in the control reference medium, straw peat biochar reduced the seedlings' fresh weight at 5% and 10% PEG concentrations when compared to the reference medium exposed to the same PEG concentration. A contradicting result was found by Yildirim et al., 2021. They found that 5% by weight biochar produced by the thermal conversion process increased the fresh weight of the cabbage seedlings at 50% irrigation (36). The effectivity of biochar to mitigate the effect of drought stress may depend on its properties such as increasing WHC, nutrient retention surface area, and pore volume. These are primarily determined by the feedstock from which it is produced (22).

Furthermore, biochar reduced the seedlings' root length as the concentration of the PEG increased. The result may suggest that a combined effect of biochar and PEG can have a toxic effect on the plant, decreasing plant growth and survival. The toxic effect could be caused by osmotic stress associated with an increased availability of nutrients as released by the biochar in the medium, which could become too much for the plants to handle. Additionally, there was no increase in the MDA concentration nor the antioxidative capacity of the plant. Therefore, it cannot be concluded that the decrease in the fresh weight under drought condition with biochar addition is caused by oxidative stress. Further investigation is still needed to have a better understanding. Additional measurements such as H_2O_2 concentrations, gene expression analysis, etc. would be needed.

Pot experiment optimization to induce drought stress

Drought stress was imposed in the pot experiment in two different ways: (1.) drought stress was applied by withholding water for 9 days after the first true leaves emerged, and (2) drought stress from the start of the experiment. Moreover, in the second experimental set-up, plants of the control condition were given water up to 50% WFPS. In contrast, plants in drought were given only 40% WFPS water supply, respectively, and grown for 21 days.

In the water withholding method, drought stress significantly affected the growth of *M. sativa* plants. Plants under drought started to wilt, have yellowish leaves and stunted growth (fig. 8).

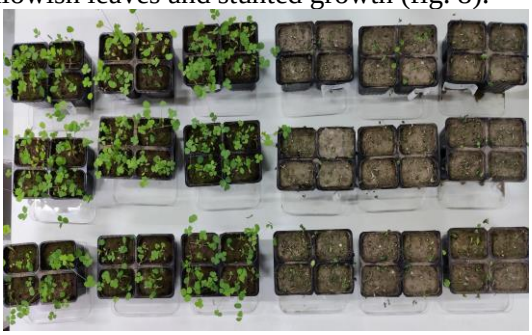


Fig. 8 Physical Growth of *Medicago sativa* plants under normal (left part of the image) and drought (right part of the image) conditions.

This observation is in line with Khan et al., 2021 (25) stating that the rapeseed cultivar Huayuoza 9 (HZ 9, Hybrid) has distinctive yellowing of the leaves when the plants were exposed to 35 to 40% water withholding capacity for at least one month. Furthermore, the MDA concentrations in plants were significantly higher in the reference soil under drought conditions when compared to the control condition (fig. 4A). Similarly, plants exposed to drought showed increased antioxidative capacity when compared to controls (fig. 4B). In this method the stress was too severe and therefore no biochar effect could be found. Weng et al., 2015 also found that oxidative damage was greater in the wheat seedlings cultivars under continuous drought stress (37). Li and Tan (2021) used rice straw-based biochar to identify mechanisms involved in drought stress alleviation in the soil-plant system. They reported that while biochar could improve soil water retention, drought stress cannot be alleviated without external water irrigation (17). On the other hand, the second method, the reduced WFPS water supply to impose drought stress, showed no significant differences between the control and drought conditions. This may suggest that no drought stress was induced or that both conditions were suffering stress.

The water withholding method provides a platform for plant survival during severe drought stress. Furthermore, information on stagewise water requirements or baseline data on daily evaporation rate could be helpful to control drought exposure for several days. In comparison, the reduced WFPS water supply method to induce drought stress appears to be easier to determine the volume and timing of the watering, and there will be enough plant material to harvest at the end of the experiment than the water withholding method. It is recommended to use the latter for future research. It is suggested that the difference in water percentages could be used (i.e., from 10% difference would be increased to 30-40%) to optimize the reduced WFPS water supply method., A range of the treatment e.g., 80%, 60%, 40%, and 20% WFPS could be used to allow the differences. Furthermore, it is advisable to have a dose range of biochars to compare which dose (m/m%) is effective in mitigating the effect of drought stress in plants.

CONCLUSION

In this study, drought stress substantially affected the growth of *A. thaliana* and *M. sativa* plants. Using the WFPS method, is suggested for future pot experiment to induce drought because it is easier to manipulate under controlled conditions. Soil amendments such as biochar can be a good organic alternative to enhance crop productivity under drought conditions. However, more research is still needed to determine the different effects of flax and straw peat biochar on plants.

In the 96 well set-up, PEG can be a good simulator of drought stress but not the best method in combination with biochar. If used in future experiments, one should be cautious of the combined effect of biochar and PEG because it may increase plant stress and exacerbate the situation. It may also be necessary to consider the osmotic potential of the biochar when added to the medium. Therefore, it is recommended for future research to have a constant osmotic potential in all conditions.

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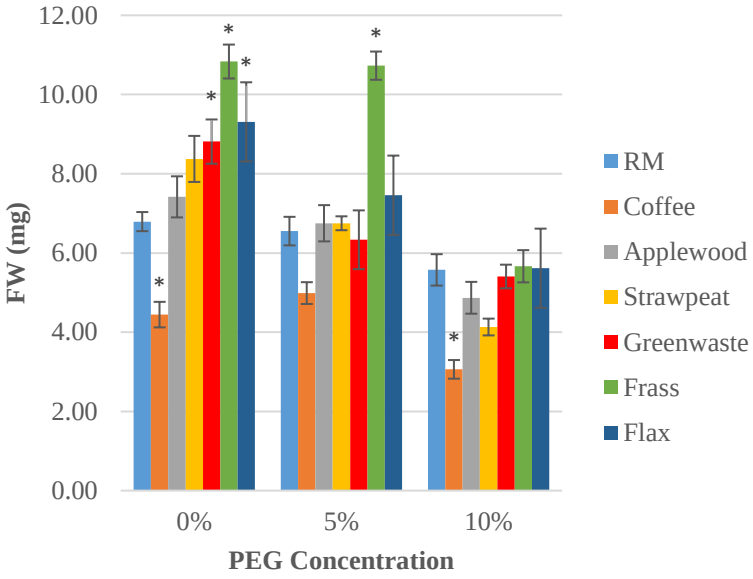
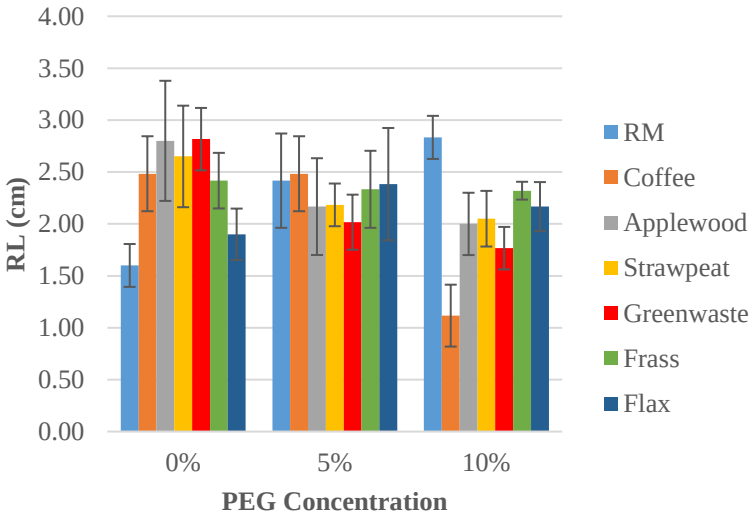
Author contributions – **Who did what**? JC and IP conceived and designed the research. JC and IP performed experiments and data analysis. All authors carefully edited the manuscript.

Appendix I

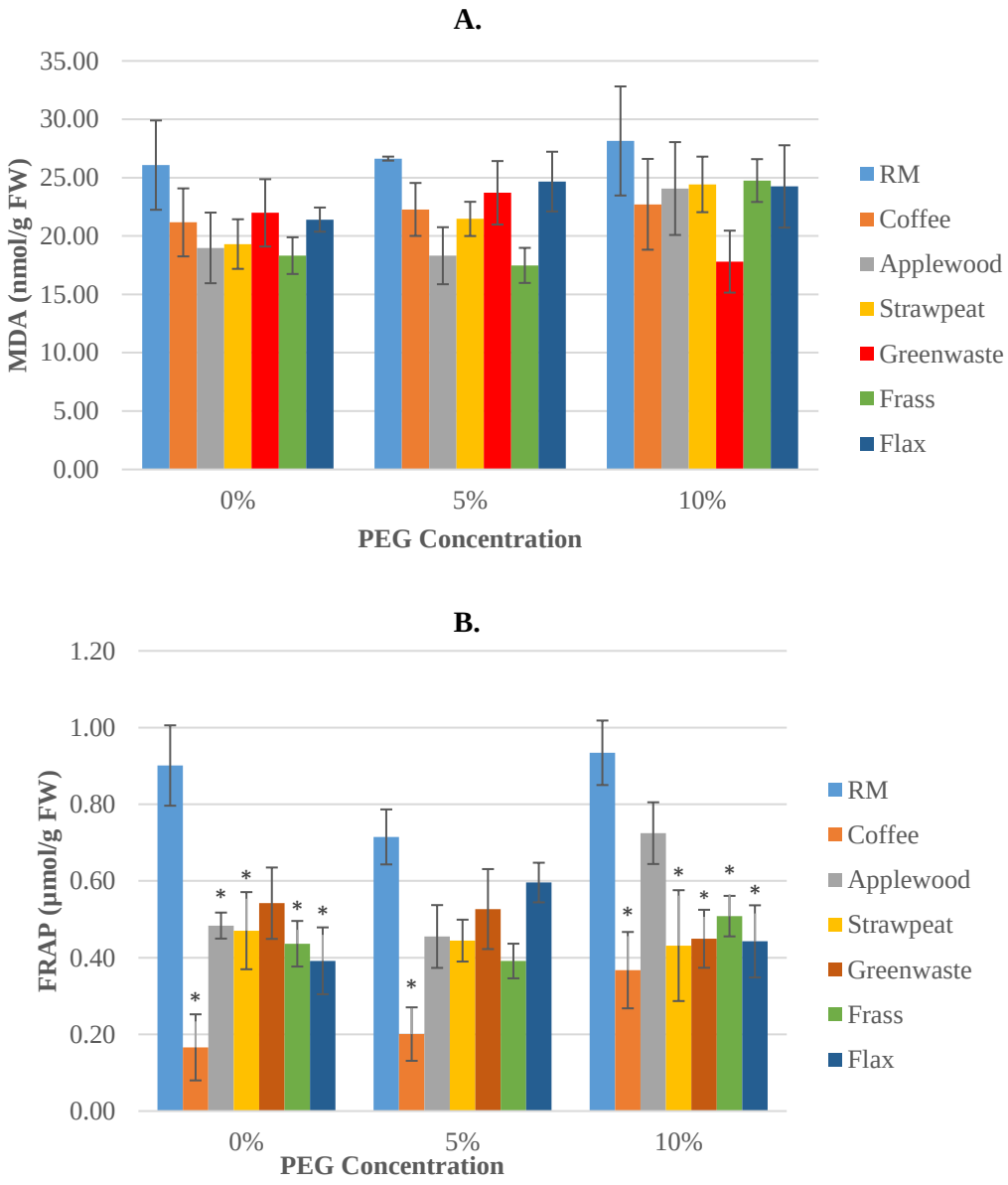
A.



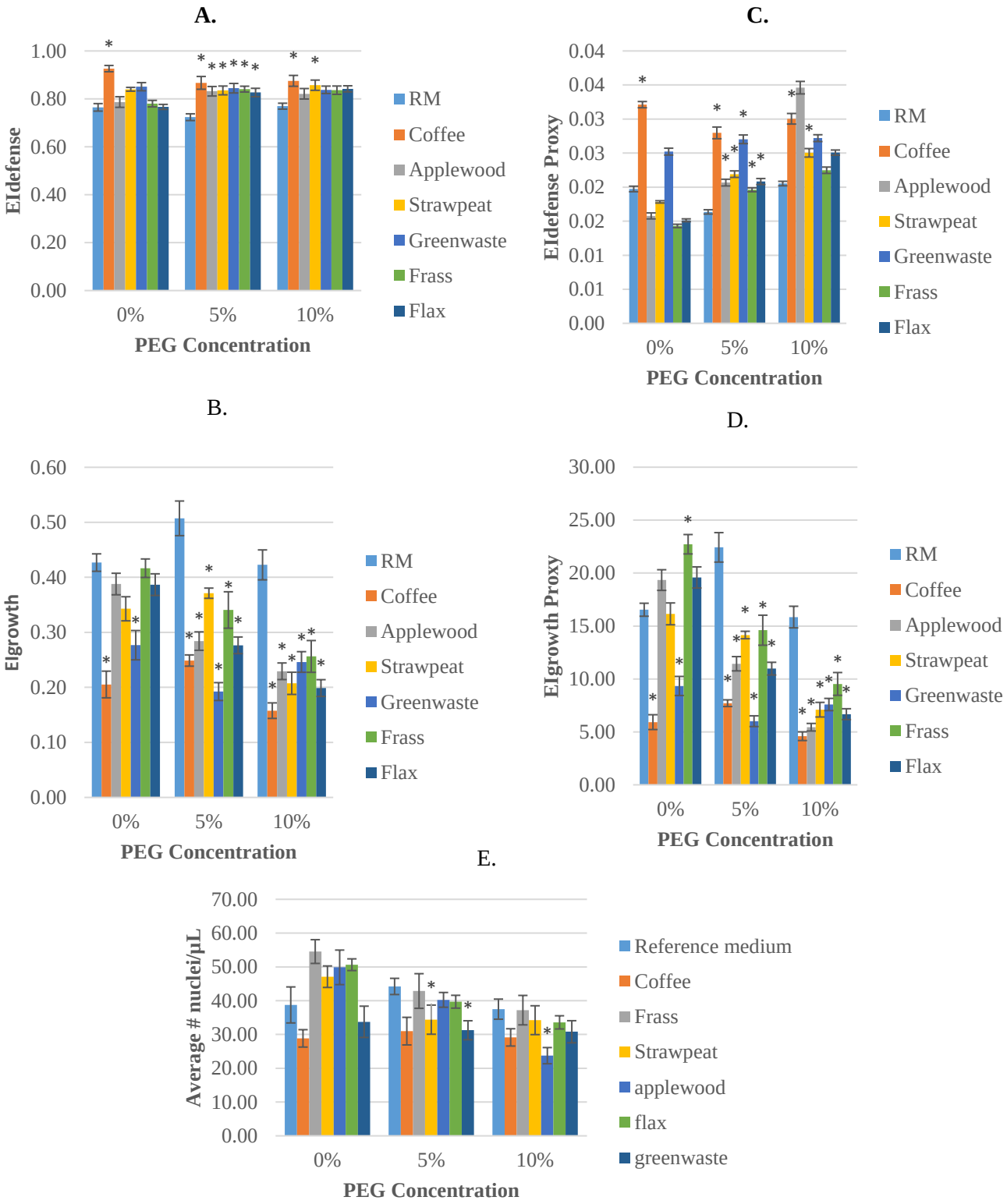
B.



Effect of the PEG induced drought stress on A. Fresh weight (Top graph) and B. Root length (Bottom graph) of *A. thaliana* seedlings harvested at 10 days after sowing and grown in ¼ MS medium amended with Coffee, Applewood, Greenwaste, Frass flax, and strawpeat biochar in the 96 well plate system exposed to three different PEG concentrations (0%, 5%, & 10%). RM: Reference medium. Values are the average ± SE of eight biological replicates. In fig.A & B, values obtained between treatment with the same PEG exposure are indicated using an asterisk using TWO-Way ANOVA with a p-value<0.05.



MDA content (A. top graph) and Antioxidative capacity (B. bottom graph) of *A. thaliana* seedlings exposed to three different PEG concentrations (0%, 5%, & 10%) amended with six biochars. Values are the average ± SE of eight biological replicates using non-parametric test (Wilcoxon & Kruskall- Wallis) with $P < 0.05$. The plants were sampled at 10 days after sowing. In fig B, values obtained between treatment with the same PEG exposure are indicated using an asterisk with a p-value<0.05.



Effect of the PEG induced drought stress on A. EIdefense and B. EIGrowth C. EIdefense Proxy D. EIgrowth Proxy and E. Proxy of *A. thaliana* seedlings harvested at 7 days after sowing and grown in ¼ MS medium amended with six different biochars in the 96 well plate system exposed to three different PEG concentrations (0%, 5%, & 10%). Values are the average ± SE of eight biological replicates. In fig.A & B, values obtained between treatment with the same PEG exposure are indicated using an asterisk using TWO-Way ANOVA with a p-value<0.05.