Effect of *Nicotiana benthamiana* exposed to long- or short-chain perfluoroalkyl substances: Levels, transfer and potential risk analysis

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Abstract

Background: Perfluoroalkyl substances (PFAS) can endanger human health through the food chain. However, the physiological mechanisms of crops exposed to PFAS are still unclear.

Objectives: The physiology, phytotoxicity and accumulation of Solanaceae model crops *Nicotiana benthamiana* exposed to perfluorooctanoic acid (PFOA), hexafluoropropylene oxide-dimer acid (HFPO-DA) and their mixed contaminants have been studied.

Results: (i) Biomass, relative electrical conductivity and catalase all decrease; (ii) Chlorophyll, peroxidase, hydrogen peroxide and malondialdehyde all increase; (iii) Superoxide dismutase and soluble sugar both increase and then decrease; (□) The absorption and transport of K, Ca, Mg and Na are affected, particularly shoots; (V) PFOA has the highest toxicity and bioaccumulation.

Conclusion: PFAS will damages the economic benefits of crops and people's health. So, its production and uses should be curtailed except for essential uses.

Future direction: Substitute substances may be more harmful, so grouping strategies and evaluation framework should be established as soon as possible.

Highlights

• No competition between PFOA and HFPO-DA, but HFPO-DA is the easiest to transport.

• The toxicity and accumulation of PFASs lead to abnormal plant physiology and affect crop yield.

• Human exposure of PFASs via the food chain of contaminated crops represents a health risk.

• The structural diversity of PFAS poses a challenge to grouping and assessment.

Introduction

Non-stick cookware was once a miniature of 'better living through chemistry', owing to their excellent surface activity and hydrophobic-oleophobic property given by perfluoroalkyl substances (PFASs).(Gluge et al. 2020; Lindstrom et al. 2011) However, the disadvantages of PFASs were obvious, including persistence,(Cousins et al. 2020b) bioaccumulation(W Zhang et al. 2019) and potential biological toxicity. (Holmquist et al. 2020) Nowadays, PFASs have been detected in the soil in Himalaya Mountains(Wang et al. 2019a) and the *Ursus maritimus* in polar regions.(Wang et al. 2019b) Researchers gradually realized that these emerging substances were also harmful to human health(Cardenas et al. 2017; Liu et al. 2021) and the ecological environment.(H Chen et al. 2020) Perfluorooctanoic acid (PFOA) was the most widely used and researched PFASs. It was estimated that PFOA had a half-life of 3.8 years in the human body(Olsen et al. 2007) and was listed as a new persistent organic pollutants (POPs) in 2019 (“Stockholm Convention”).(Hogue 2019) Furthermore, PFOA had been phased out in European Union,

North America, China and other regions or countries. The main elimination policy at this stage was to completely replaced long-chain PFASs (number of carbon atoms ≥ 7) with short-chain PFASs (C < 7). For instance, a quintessential case of PFOA (C = 8) substitute substance was hexafluoropropylene oxide-dimer acid [HFPO-DA (C = 6), also known as GenX]. Both PFOA and HFPO-DA had low volatility and high solubility, and could be retained in various media for a long time once entering into environment.(Tian et al. 2018; Xiao 2017) Due to the long-term retention of PFOA and HFPO-DA in the environment, this might increase their accumulation and transfer from the medium to the organism.(Houtz et al. 2013)

To our knowledge, a multitude of studies had focused on the accumulation and toxicity of PFOA and HFPO-DA in persons(Gebbink and Van Leeuwen 2020; Wen et al. 2020) and animals.(Blake et al. 2020; Coperchini et al. 2020; Gaballah et al. 2020; Hassell et al. 2020) Some investigations had also been conducted that other PFASs could be easily absorbed by crops.(Gredelj et al. 2020b; Liu et al. 2019; W Wang et al. 2020; Xiang et al. 2018) Crops belong to the starting point of the food chain and constitute the first trophic level. According to the principle of food webs and energy flow, once PFOA and HFPO-DA were absorbed by crops, they could enter into various nutrition levels through bioaccumulation and biomagnification, especially for humans. However, the precise environmental health risks and economic losses caused by PFOA and HFPO-DA in crops production were still unknown. The occurrence of these hazards was closely related to the physiological responses of crops. In order to solved this knowledge gap, this experiment mainly designed the model plant *Nicotiana benthamiana*. Many pioneering researches had used it as standard materials (e.g., photoperiod, nutrient absorption and genetic modification, etc.).(Goodin et al. 2008; J Li et al. 2019; Shan et al. 2013) *N. benthamiana* was also closely associated with the research of important agronomic crops, such as *Capsicum annuum* L., *Solanum lycopersicum*, *Solanum melongena* L. and *Solanum tuberosum*. This significant helped us to use these characteristics to explored the physiological and biochemical regulations of how crops tolerated, accumulated and responded under PFASs stress.

This study proposes to deeply understand the influence and damage mechanism of PFOA, HFPO-DA and their mixture pollutants on *N. benthamiana*; Predict the effects and harm of PFASs in crops production. And conducted the following work: (i) Investigating different concentrations stress of PFOA, HFPO-DA and their mixed contaminants (v/v, 1:1) on the germination. (ii) Finding morphological effects, phytotoxicity, uptake rate and translocation efficiency of each group in seedlings. (iii) Revealing physiological and biochemical regulations of seedlings under stress. Including biomass; Relative electrical conductivity; Chlorophyll content; Catalase (CAT), Superoxide dismutase (SOD) and Peroxidase (POD) activity; Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA) and Soluble sugar content; And potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) translocation were also measured.

Materials And Methods

2.1. Main chemicals

PFOA (96% pure, CAS no. 335-67-1) and HFPO-DA (97% pure, CAS no. 13252-13-6) were purchased from official website of MACKLIN reagent, Shanghai, China (http://www.macklin.cn/).

2.2. Growth conditions

Placed the vernalized seeds in a petri dish containing half-strength Murashige and Skoog (½ MS) agar medium,(CH Chen et al. 2020) pH = 5.8 and different concentrations of PFOA, HFPO-DA or (PFOA + HFPO-DA) (0, 5, 15, 50, 150 and 350 mg/L). Finally, placed vertically on racks and incubated with a 16/8 hours light/dark period with a light intensity of 120 μmol/(m^2·s) at 22 ± 2 ℃ for one, two and three weeks.

2.3. Calculation of seed germination index

Did three parallel experiments, and each group would germinate 100 seeds. Daily germination: count the number of germinated seeds in the three groups of experiments every day until no seeds germinated.

 Germinating percentage (GP):

$$\mathrm{GP} = \frac{\mathrm{Numberofgerminatedseeds}}{\mathrm{Totalnumberofseeds}} * 100\%$$

 Relative Germinating percentage (RGP):

$$\mathrm{RGP} = \frac{\mathrm{StressedGP}}{\mathrm{NostressedGP}} * 100\%$$

 Germinating energy (GE):

$$\mathrm{GE} = \frac{\sum(\mathrm{Gtmax})}{\mathrm{Totalnumberofseeds}} * 100\%$$ (Gt is the number of germinating seeds per day.)

 Relative Germinating energy (RGE):

$$\mathrm{RGE} = \frac{\mathrm{StressedGE}}{\mathrm{NostressedGE}} * 100\%$$

 Germinative index (GI):

$$\mathrm{GI} = \sum \frac{\mathrm{Gt}}{\mathrm{Dt}} * 100\%$$ (Dt is the number of days of seeds.)

 Relative Germinative index (RGI):

$$\mathrm{RGI} = \frac{\mathrm{StressedGI}}{\mathrm{NostressedGI}} * 100\%$$

 Vitality index (VI):

$$\mathrm{VI} = \mathrm{GI}*\mathrm{S} * 100\%$$ (S is the average length grown to the 7th day.)

 Relative Vitality index (RVI):

$$\mathrm{RVI} = \frac{\mathrm{StressedVI}}{\mathrm{NostressedVI}} * 100\%$$

 Inhibition rate (plants length)

$$\mathrm{Inhibitionrate} = \frac{\mathrm{Nostressed} - \mathrm{Stressed}}{\mathrm{Nostressed}} * 100\%$$

2.4. Extraction and analysis of target pollutants

A method for the determination of PFOA, HFPO-DA and (PFOA + HFPO-DA) in shoot and root samples was developed.(CH Chen et al. 2020; Fan et al. 2020) Additional details was given in the Supplementary Information **(S1)**.

Since the *N. benthamiana* had visible roots and shoots, the translocation factors (TFs) for PFOA, HFPO-DA and (PFOA + HFPO-DA) were designed by using the following formula:(Zhou et al. 2017)

$$\mathrm{TFs} = \frac{\mathrm{Concentraioninshoots\,(mg/kg)}}{\mathrm{Concentraioninroots\,(mg/kg)}}$$

Bioconcentration factors (BCFs). BCFs had been calculated to characterize plant uptake and translocation efficiency of PFASs.

$$\mathrm{BCFs} = \frac{\mathrm{Concentraioninrootsorshoots\,(mg/kg)}}{\mathrm{Concentraionin\,MSmedium\,(mg/L)}}$$

2.5. Agronomic traits in seedling stage

The experiment was also repeated three times.

a. Counted the root and shoot lengths of 10 plants and took the average.

b. Number of lateral roots was the sum of 10 plants.
c. After 3 weeks of growth of the *N. benthamiana*, samples were collected. Freeze and vacuum dry at -30°C.

2.6. Determination of physiological indicators

 $\mathrm{Watercontent} = \frac{(\mathrm{FW} - \mathrm{DW})}{\mathrm{FW}} * 100\%$

 $\mathrm{Relativeelectricalconductivity} = \frac{\mathrm{R1}}{\mathrm{R2}} * 100\%$ (Additional details was gave in the **S1**)

Chlorophyll content: determined with chlorophyll meter (SPAD-502Plus, Konica Minolta, Japan).

CAT, SOD and POD activity; H_2O_2, MDA and soluble sugar content: the above indicators were determined using the kit **(Table 1)**.

K, Ca, Mg and Na were measured, additional details were giving in the **S1**.

2.7. Statistical analysis

SPSS 17.0 software was used to perform analysis of variance (One-way ANOVA) on the measured data. The least significant difference method (LSD method) was used to make multiple comparisons of the significance of the difference between different treatments for each indicator. The mapping analysis software was Origin Pro 2020b.

Result And Discussion

3.1 Effects of PFASs on germination

Under the stress of PFASs, the germination time of a part of seeds was prolonged, and some seeds could not germinate. Although the seeds in different concentrations (0, 5, 15, 50, 150 and 350 mg/L) of PFOA, HFPO-DA and (PFOA + HFPO-DA) stress were reached the peak of germination on the 3rd day, (85.0, 83.0, 81.7, 74.3, 74.3, 0%), (85.0, 84.0, 82.0, 82.0, 73.7, 59.0%) and (85.0, 80.0, 83.7, 71.7, 70.0, 16.7%), respectively **(Fig. 1, a to c)**, the germination time of them differed greatly. For example, when the concentrations of PFOA and (PFOA + HFPO-DA) overtopped 50 mg/L, the germination of seeds was hindered, and the germination time might be extended by up to 5 days **(Fig. 1, a/c)**. Then, the results were indicative of low concentrations of PFASs (5, 15 mg/L) did not affect the GP and GE, but the germination would be significantly inhibited when the concentration of PFASs increased, GP: (PFOA: 98.3, 98.0, 97.7, 95.3, 91.3, 28.7%), (HFPO-DA: 98.3, 98.0, 97.3, 97.3, 95.3, 92.3%) and [(PFOA + HFPO-DA): 98.3, 96.0, 96.7, 95.0, 95.7, 75.3%], respectively **(Fig. 1, d);** GE: (PFOA: 85.0, 83.0, 81.7, 74.3, 74.3, 14.0%), (HFPO-DA: 85.0, 84.0, 82.0, 82.0, 73.7, 59.0%) and [(PFOA + HFPO-DA): 85.0, 80.0, 83.7, 71.7, 70.0, 37.0%], respectively **(Fig. 1, e)**. And the effect of PFASs on the GI and VI were proportional to the concentration, GI: (PFOA: 31.7, 31.4, 31.2, 30.0, 28.6, 5.1%), (HFPO-DA: 31.7, 31.5, 31.2, 31.2, 30.0, 27.5%) and [(PFOA + HFPO-DA): 31.7, 30.7, 31.1, 29.4, 28.8, 17.0%], respectively **(Fig. 1, f);** VI: (PFOA: 64.3, 57.4, 55.0, 35.7, 15.5, 0%), (HFPO-DA: 64.3, 60.1, 59.4, 53.0, 36.2, 5.1%) and [(PFOA + HFPO-DA): 64.3, 56.6, 48.5, 39.2, 30.2, 2.0%)], respectively **(Fig. 1, g)**. Finally, the low concentrations of PFASs (5, 15 mg/L) had no effect on the RGP, RGE and RGI **(S-Fig. 1, a to c)**, but had a significant effect on RVI **(S-Fig. 1, d)**. And the high concentrations of PFASs (≥ 50 mg/L) had a significant impact on the RGP, RGE, RGI and RVI **(S-Fig. 1)**.

The results suggested that the toxic effects of three PFASs on germination were PFOA > (PFOA + HFPO-DA) > HFPO-DA. Low concentrations of the PFASs (5, 15 mg/L) have weaker toxicity to seeds.(Zhang et al. 2020) The feasible reason was that the effect of inhibiting the production of reactive oxygen species (ROS) and reactive nitrogen species (such as NO and others) in the seeds was not obvious.(Xie et al. 2020) This leads to a slight decrease in the level of oxidative stress, which slows down the rate of nutrient decomposition, thereby reducing nutrient supply and slowing down the breathing rate.(Liu et al. 2020) High concentrations of PFASs (> 50 mg/L) have strong inhibitory effects on germination.(Gredelj et al. 2020a) It might be that the stress had a greater impact on cell osmotic regulation, the composition of membrane lipids and fatty acids, and the activities of physiological metabolic enzymes.(P Li et al. 2019)

3.2 Effects of PFASs on the seeding growth

Researched on seeding found that the concentration increased, the growth rate of shoots (Final inhibition rate: 18.2, 25.6, 61.8, 91.5%, D, respectively) and roots (Final inhibition rate: 7.7, 17.0, 57.8, 84.9%, D, respectively) were decreased under the PFOA stress **(Fig. 2, a)**. If the HFPO-DA exposure levels were the same or greater than 15 mg/L, it would significantly inhibit the shoots (Final inhibition rate: 18.4, 21.0, 32.4, 62.0, 88.1%, respectively) and roots (Final inhibition rate: 1.4, 0.3, 32.6, 58.0, 95.2%, respectively) growth **(Fig. 2, b)**. Under the stress of (PFOA + HFPO-DA), the shoots (Final inhibition rate: 13.3, 20.4, 50.3, 72.7, 99.0%, respectively) and roots (Final inhibition rate: 5.7, 12.3, 45.6, 79.9, 98.2%, respectively) growth were all inhibited **(Fig. 2, c)**.

Next, the work indicated that with the increase of PFOA concentration, the germination time of lateral roots was prolonged and the number of lateral roots was decreased (Average total number: 38, 27, 19, 11, N, D, respectively), the result showed significant differences **(Fig. 2, d)**. Under the HFPO-DA stress, low concentration (5 mg/L) had little effect on the germination and growth of lateral roots. When the concentration increased, the germination and growth of lateral roots (Average total number: 38, 36, 29, 15, 10, 5, respectively) were both inhibited **(Fig. 2, e)**. Under the stress of (PFOA + HFPO-DA), the growth of lateral roots (Average total number: 38, 34, 25, 13, 8, N, respectively) were inhibited obviously, too. When the concentration was equal or greater than 15 mg/L, there was a quite difference. Once the concentration was equal or more than 350 mg/L, the lateral roots could not germinate **(Fig. 2, f)**. Besides, the growth morphology of *N. benthamiana* after exposed 7 days, 14 days and 21 days shown in **S-Fig. 2**.

Above results illustrated that the toxicity of PFASs to seedlings was: PFOA > (PFOA + HFPO-DA) > HFPO-DA. Tolerance had been widely used to evaluated the ability of crops under biological and abiotic stress. (CH Chen et al. 2020; Li et al. 2020) The difference between stress group and control group was more significant, which was manifested as weaker tolerance and greater damage to crops. In general, PFASs exposure levels were equal or greater than 15 mg/L, the tolerance of *N. benthamiana* decreased rapidly. It was well known that the growth and antioxidant defense responses of different species might be different. Differences in defense capabilities might result in different tolerance and phytotoxicity. However, the *N. benthamiana* used in this experiment and *C. annuum* L., *S. lycopersicum*, *S. melongena* L., *S. tuberosum* and other crops were belonging to the Solanaceae family, and their morphology, life form, reproduction mode, internal structure and genes were the same or similar. Therefore, above results have a high reference value.

3.3 Bioaccumulation and translocation of PFASs

It was found that the accumulation of PFOA, HFPO-DA and (PFOA + HFPO-DA) was directly proportional to the stress concentration (Shoots: 0, 41.3, 155.7, 457.3, N, D mg/kg; Roots: 0, 164.7, 465.7, 1324.0, N, D mg/kg) **(Fig. 3, a)**, (Shoots: 0, 37.0, 97.0, 318.1, 1022.7, N mg/kg; Roots: 0, 47.7, 140.3, 418.0, 1182.7, N mg/kg) **(Fig. 3, b)** and [Shoots: PFOA (0, 22.3, 73.0, 231.0, 716.7, N mg/kg); HFPO-DA (0, 18.3, 50.3, 168.0, 522.7, N mg/kg)]; [Roots: PFOA (0, 72.3, 247.3, 654.3, 1316.7, N mg/kg); HFPO-DA (0, 24.3, 70.3, 218.0, 604.7, N mg/kg)] **(Fig. 3, c)**, respectively. Under the same concentration, the HFPO-DA had the lowest

accumulation and the PFOA had the highest accumulation. Meanwhile, the PFASs were more likely accumulated in roots.

And TFs was an overall upward trend (PFOA: 0.25, 0.33, 0.35, N, D) **(Fig. 3, d)**, (HFPO-DA: 0.78, 0.69, 0.76, 0.86, N) **(Fig. 3, e)** and [(PFOA + HFPO-DA): PFOA (0.31, 0.30, 0.35, 0.54, N); HFPO-DA (0.75, 0.71, 0.77, 0.86, N)] **(Fig. 3, f)**, respectively, but the transport ability was weak (TFs < 1). At the same concentration, the TFs of the HFPO-DA exposed could be higher than those of the PFOA-stressed. Besides, BCFs of PFOA, HFPO-DA and (PFOA + HFPO-DA) was comparable (Shoots: 32.9, 31.0, 26.5, N, D; Roots: 8.3, 10.4, 9.1, N, D), (Shoots: 9.5, 9.4, 8.4, 7.9, N; Roots: 7.4, 6.5, 6.4, 6.8, N) and (Shoots: 19.3, 21.2, 17.4, 12.8, N; Roots: 8.1, 8.2, 8.0, 8.3, N) **(S-Table 2)**, respectively, with the highest BCFs under PFOA exposure group.

The bioaccumulation characteristics of PFASs in *N. benthamiana* and organs demonstrated that there were three main parts related to root absorption: apoplasts (space outside the cell membrane), symplasts (space inside the cell membrane) and plant vacuoles (storage organelles). In order to be transported through the xylem sap to the upper part of the crop, PFASs must passed through the cell membrane into the symplast, and entered the xylem through the casparian zone.(Muller et al. 2016) The paper also showed that PFASs were more easily enriched in roots.(TT Wang et al. 2020b; W Zhang et al. 2019) With high bioaccumulation of the PFOA might be interpreted by their better hydrophobicity and stays in the crops body longer.(Zhu and Kannan 2019) In addition, the observed root concentrations for PFOA and HFPO-DA was consequently mainly flow from adsorption, but the effective sucked in the roots was a portion of the observed.

Due to the biological amplification effect of PFASs in the food chain,(Sznajder-Katarzynska et al. 2018) the higher the nutrient level is, the higher the PFASs content should be, in especial in mammals. And the effect of bioaccumulation, the human beings were exposed to higher concentration of food chain and accumulated for a long time.(Semerad et al. 2020; TT Wang et al. 2020a) Now, there a multitude of investigations on high-trophic species, and few studies on low-trophic species.(Wen et al. 2013) This research completes the gap in the field of the first trophic level research. However, the strength of the interaction between single and compound PFASs stresses was not concluded. The different chain lengths of PFASs, both the molecular size and polarity of the molecules vary. PFOA was increasingly lipophilic and might be adsorbed onto crop roots surface quickly and strongly, while HFPO-DA might diffused through the plasma membranes of roots cell at higher concentration as a result of their smaller molecular size.(Pi et al. 2017) Moreover, crops might be under PFOA stress with higher BCFs, but under HFPO-DA stress the transport capacity was higher, this mean that the potential harm of HFPO-DA would be greater. (Gomis et al. 2018) In general, PFOA and HFPO-DA manifested a preference for bioaccumulation in different tissues, which should be further demonstrated.

3.4 Physiological response to PFASs stress

3.4.1 Regulations of the biomass

The biomass and water content decreased. PFOA, HFPO-DA and (PFOA + HFPO-DA) still significantly impacted the weights, especially PFOA **(Fig. 4, a)**. As the exposure levels increased, the water content

decreased, (PFOA: 95.4, 95.3, 94.5, 93.8, 89.6%, D), (HFPO-DA: 95.4, 95.4, 95.5, 95.0, 94.4, 90.8%) and [(PFOA + HFPO-DA): 95.4, 95.5, 95.0, 94.0, 92.0, 90.3%], respectively **(Fig. 4, b)**.

In the above study, low concentration (5 or 15 mg/L) HFPO-DA group stress could promote the growth of seedlings.(Gredelj et al. 2020a) The biomass and water content under different levels and each group types of PFASs also demonstrated differences. This was probably because PFASs could destroyed respiration and photosynthesis, affected nutrient and water uptake and transport, disrupted gene and protein expression, and enhanced ROS accumulation and lipid peroxidation. These would leaded to physiological metabolism disorder, affected the growth and development of crops.

3.4.2 Regulations of conductivity and chlorophyll in leaves

The relative electrical conductivity of leaves increased significantly. The PFOA, HFPO-DA and (PFOA + HFPO-DA) exposure levels increased (30.1, 37.7, 39.0, 44.2, 48.8%, D), (30.1, 36.8, 37.1, 43.1, 47.3, 59.0%) and (30.1, 37.6, 38.6, 42.7, 49.0%, N), respectively **(Fig. 5, a)**. When the stress time prolonged, the relative conductivity of leaves also increased (PFOA: 28.4, 39.7, 57.6, 59.9%, N, D), (HFPO-DA: 28.4, 36.4, 49.6, 54.0, 59.7%, N) and [(PFOA + HFPO-DA): 28.4, 37.6, 51.8, 53.7, 60.4%, N], respectively **(Fig. 5, b)**. **And the chlorophyll content illustrated a total downward trend.** 14 days after exposure: (PFOA: 17.5, 14.2, 13.1, 13.0, 2.6, D SPAD), (HFPO-DA: 17.5, 13.1, 16.8, 19.1, 12.3, 7.6 SPAD) and [(PFOA + HFPO-DA): 17.5, 12.9, 13.9, 12.9, 5.5, N SPAD], respectively **(Fig. 5, c)**. 21 days after exposure: (PFOA: 16.0, 15.0, 15.4, 10.8, 0.7, D SPAD), (HFPO-DA: 16.0, 15.1, 15.5, 13.6, 11.9, 1.4 SPAD) and [(PFOA + HFPO-DA): 16.0, 14.7, 14.4, 13.0, 10.3, N SPAD], respectively **(Fig. 5, d)**. Compared with each group, the chlorophyll content demonstrated a trend of first increased and then decreased in a short time under the HFPO-DA stress **(Fig. 5, c)**. At last, while on the same concentration (≥ 15 mg/L), the chlorophyll content of leaves was the highest under the HFPO-DA stress.

The relative conductivity of normal leaves was significantly different from the stressed. The results showed that when crops tissue was injured by stress, the cell membrane was damaged by mechanical damage and membrane lipid peroxidation, resulting in the destruction of membrane structure, the increase of membrane permeability and the exosmosis of intracellular substances (especially electrolytes), resulting in the increased of relative conductivity of tissue soaking solution.(Chow et al. 2018) Beside chlorophyll content decreased with PFASs increased, might signify PFASs involvement (phytotoxic) in modulating about physiological responses.(Moulick et al. 2017) This was because the increased of chlorophyll degradation (etiolation) or the inhibition of chlorophyll synthesis.

3.4.3 Regulations of CAT, SOD, POD, H_2O_2, MDA and soluble sugar

The CAT showed an overall downward trended under the stress of PFOA, HFPO-DA and (PFOA + HFPO-DA) (31.3, 27.4, 24.8, 23.7, N, D U/g·min), (31.3, 30.0, 28.7, 26.7, 15.7, N U/g·min) and (31.3, 28.9, 27.1, 25.6, 12.7, N U/g·min), respectively **(Fig. 6, a)**. The SOD and soluble sugar illustrated a trend of first increased and then decreased (PFOA: 64.0, 82.5, 88.7, 88.6, N, D U/g·min; 7.2, 7.8, 9.2, 9.7, N, D mg/g), (HFPO-DA: 64.0, 67.2, 78.0, 81.1, 70.6, N U/g·min; 7.2, 7.4, 8.0, 8.9, 8.1, N mg/g) and [(PFOA + HFPO-DA):

64.0, 68.6, 80.0, 83.1, 75.2, N U/g·min; 7.2, 7.7, 8.6, 9.5, 9.1, N mg/g], respectively **(Fig. 6, b/f)**. The POD, H_2O_2 and MDA demonstrated an overall upward trend (PFOA: 51.1, 63.8, 79.9, 107.7, N, D U/g·min; 1.6, 2.2, 3.1, 4.4, N, D umol/g; 8.1, 9.9, 10.8, 12.5, N, D umol/g), (HFPO-DA: 51.1, 57.4, 73.3, 95.3, 142.9, N U/g·min; 1.6, 1.8, 2.6, 3.2, 4.4, N umol/g; 8.1, 8.5, 9.7, 11.1, 13.2, N umol/g) and [(PFOA + HFPO-DA): 51.1, 61.2, 75.6, 97.8, 150.1, N U/g·min; 1.6, 2.0, 2.8, 3.5, 4.8, N umol/g; 8.1, 9.4, 10.2, 11.4, 13.5, N umol/g], respectively **(Fig. 6, c/d/e)**. All results indicated that the physiological indexes under the HFPO-DA stress were the lowest, then under the PFOA stress were the highest.

CAT manifested a downward trend, SOD increased first and then decreased, POD illustrated an upward trend. CAT, SOD and POD enzymes form an antioxidant system during the crops metabolism, and the three enzymes can effectively remove active oxygen free radicals.(Zhao et al. 2017) The increase and decrease of antioxidant enzyme activity were related to the resistance of crops. With stronger resistance could maintain the enzyme activity at a higher level under favorable conditions for themselves.(W Zhang et al. 2019) The CAT enzyme activity declines faster under high concentration (> 50 mg/L) stress. It showed that as the stress concentration increases, the harmful free radicals produced exceed the normal disproportionation ability, the antioxidant enzyme system was destroyed, and the enzyme activity decreases. SOD enzyme activity gradually increased under low concentration (< 50 mg/L) stress, and rapidly decreased under high concentration (> 50 mg/L) stress.(Moradi et al. 2019) It showed that under the treatment of low concentration of the PFASs, the defense function in the plant was stimulated, and the enzyme activity rises rapidly to deal with the harm caused by the increase of active oxygen triggered by PFASs. However, as the stress concentration increases, PFASs accumulate in the plant, causing the crop's physiological metabolism to be disordered, and the enzyme activity was inhibited and begins to decline. POD enzyme activity has been increasing, indicating that under the current concentration of the PFASs treatment, the increase in enzyme activity catalyzes the oxidation of phenols and amines by H_2O_2, eliminating the toxic effects of H_2O_2, phenols and amines. So, SOD and POD enzymes play an active role in this process.

H_2O_2 demonstrated an overall upward trend. The increased of H_2O_2 oxidizes biological macromolecules, such as nucleic acid and protein in the cell, and damages the cell membrane, thereby accelerating the aging and disintegration of cells. It illustrated that as the concentration increases, the toxic effect of PFASs on crops was stronger.(CH Chen et al. 2020)

The MDA illustrated an overall upward trend. While the crops was stressed by PFASs, with the increase of the concentration, the higher the degree of peroxidation of cell membrane plasm, the greater the degree of cell membrane damage.(Zhang and Kirkham 1993)

Soluble sugar manifested a trend of first increased and then decreased. When the crops were stressed by low concentrations of the PFASs, they could promote the synthesis of soluble sugars in seedlings by adjusting the balance of carbon and nitrogen metabolites, which enhances resistance.(Gill et al. 2003) As the concentration increases, the cell structure of crops would be destroyed, which leads to the imbalance of osmotic regulation and hinders the synthesis of soluble sugar.

3.4.4 Regulations of mineral elements absorption and transport

K, Ca and Mg content generally illustrated a trend of first increased and then decreased. **The Na content had a rose trend in the shoots, but did not change much in the roots**. To sum it up, the stress of PFOA, HFPO-DA and (PFOA + HFPO-DA) significantly effects on shoots. For K: (25.5, 28.5, 21.9, 16.7, N, D mg/g), (25.5, 29.4, 24.0, 19.2, 10.8, N mg/g) and (25.5, 27.7, 22.6, 17.5, 9.1, N mg/g), respectively **(Fig. 7, a)**; For Ca: (8.1, 9.6, 8.1, 6.1, N, D mg/g), (8.1, 9.8, 9.0, 7.4, 5.3, N mg/g) and (8.1, 9.4, 8.5, 6.7, 4.9, N mg/g), respectively **(Fig. 7, b)**; For Mg: (2.6, 2.5, 2.1, 1.4, N, D mg/g), (2.6, 2.8, 2.3, 1.9, 1.3, N mg/g) and (2.6, 2.6, 2.3, 1.5, 1.3, N mg/g), respectively **(Fig. 7, c)**; For Na: (0.2, 0.2, 0.4, 0.5, N, D mg/g), (0.2, 0.2, 0.3, 0.4, 0.6, N mg/g) and (0.2, 0.2, 0.4, 0.5, 0.8, N mg/g), respectively **(Fig. 7, a)**. When compared with the control group, TF for K, Ca and Mg have little change, whereas Na was significantly affected (PFOA: 1.42, 1.95, 3.45, 3.95, N, D), (HFPO-DA: 1.42, 1.30, 2.67, 2.67, 6.34, N) and [(PFOA + HFPO-DA): 1.42, 1.60, 2.10, 3.95, 7.13, N], respectively **(S-Table 3)**. At the same time, there were also differences under different levels of stress.

K was an essential massive nutrient element for crops, and it plays a key role in the composition and metabolism of substances in the body. Under the low concentration of the PFASs (5 mg/L), the content of K in crops decreased sharply. It was resulted in the absorption and transport capacity of nutrients in crops to decline, resulting in metabolic disorders, and the external morphology of crops present corresponding symptoms. In addition, it was also led to the membrane lipid peroxidation of plant roots, which leads to the increase of membrane permeability and the leakage of small molecules.

Ca and Mg were playing an indispensable role in the regulation of osmotic pressure, the maintenance of metabolic balance, and the synthesis of substances in plants.(Knight et al. 2020) At the low concentration of the PFASs (5 mg/L), the content of Ca and Mg in the plant was relatively reduced. The reduction of Ca and Mg elements was not conducive to alleviating the stress and toxicity of PFASs, and might lead to increased absorption and accumulation of PFASs. Furthermore, the reduction of Ca and Mg ions was harmful to the maintenance of the normal osmotic system of root cells, and might lead to enhanced stress effects of PFASs.

Na was important to the transportation and metabolism of important substances in crops. With the increase of the PFASs stress concentration, the Na content increased. It was changing the structure and function of the cell membrane, leading to increased toxicity. Moreover, excessive sodium element had produced sodium salt stress and affect the photosynthesis of crops.

Conclusion And Future Direction

The area of PFASs to agricultural soil is more and more large, some studies have detected PFASs in cabbages, soybeans, wheat, etc.(Liu et al. 2017; Liu et al. 2019; M Zhang et al. 2019) Based on this reality, this work systematically evaluated the effects of PFASs exposure on the physiological regulations of *N. benthamiana* germination and seeding growth. The results showed that PFASs exposure will reduce the economic benefits of crops, and individuals will pose health risks by eating contaminated crops.

Future direction 1: Since the inherent characteristic of PFASs are not degradable, we completely and totally agree that speed up the process of phasing out and replacing 'non-essential' uses of PFASs with green chemicals.(Cousins et al. 2019; Gluge et al. 2020; Zimmerman et al. 2020) Future direction 2: PFOA is manifest higher bioaccumulation capacities, but HFPO-DA have greater mobility. Which means the short-chain compounds are commonly used to replace long-chain may be more harmful that need to be building up a specifically ecotoxicity life cycle impact assessment framework (Holmquist et al. 2020) and new strategies for grouping PFASs.(Cousins et al. 2020a; Cousins et al. 2020b).

Declarations

• Ethical Approval

Not applicable.

• Consent to Participate

Not applicable.

• Consent to Publish

Approved by all authors.

• Authors Contributions

Xin Li and Wenli Hu designed research; Huinian Liu, Sihui Lu, Fangwen Hu, Zhuang Zhang, Yanni Xi and Zhu Su performed research; Huinian Liu, Yanfen Liu, Tanghuan Xie, and Chang Zhang analyzed data; and Huinian Liu, Wenli Hu, and Xin Li wrote the paper.

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• Competing Interests

No conflict of interest.

• Availability of data and materials

All data are fully available without restriction.

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Tables

Table 1
The kit information

Brand	NO.	Assay Kit	Link
Solarbio	BC0205	CAT	http://www.solarbio.com/goods-12544.html
Solarbio	BC0175	SOD	http://www.solarbio.com/goods-9124.html
Solarbio	BC0095	POD	http://www.solarbio.com/goods-9116.html
Solarbio	BC3595	H_2O_2	http://www.solarbio.com/goods-9127.html
Solarbio	BC0025	MDA	http://www.solarbio.com/goods-9102.html
Solarbio	BC0035	Soluble sugar	http://www.solarbio.com/goods-9104.html

Figures

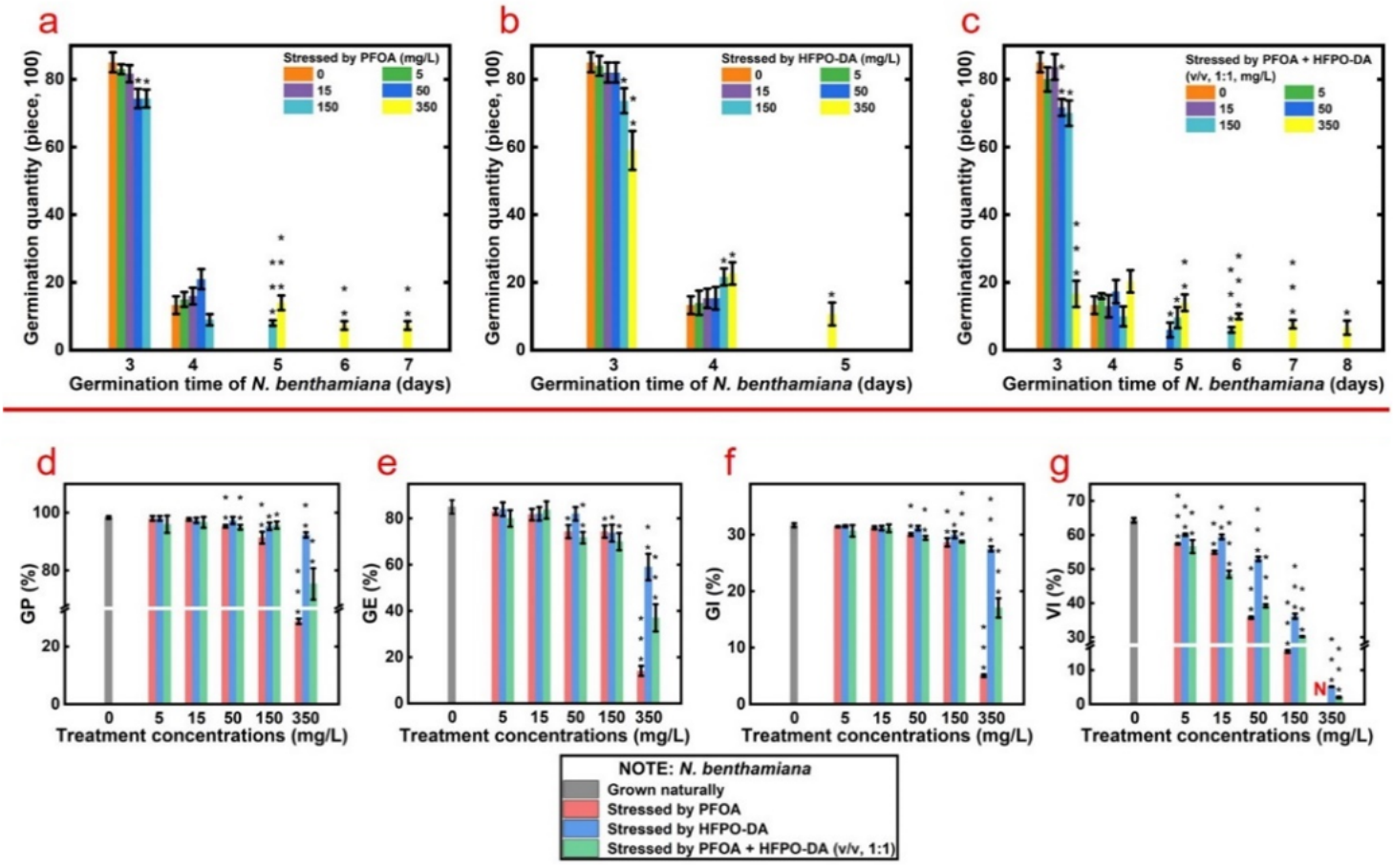

Figure 1

Effects of different concentrations of PFASs exposure on germination. a-c: germination time and quantity of seeds stressed by PFOA, HFPO-DA and (PFOA + HFPO-DA). d-f: GP, GE, GI, VI. NOTE | N: plants not obtain biomass. Each group use 100 seeds. Data were means ± SE of three independent groups. Asterisks indicate significant differences between the treatments and the control, where $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

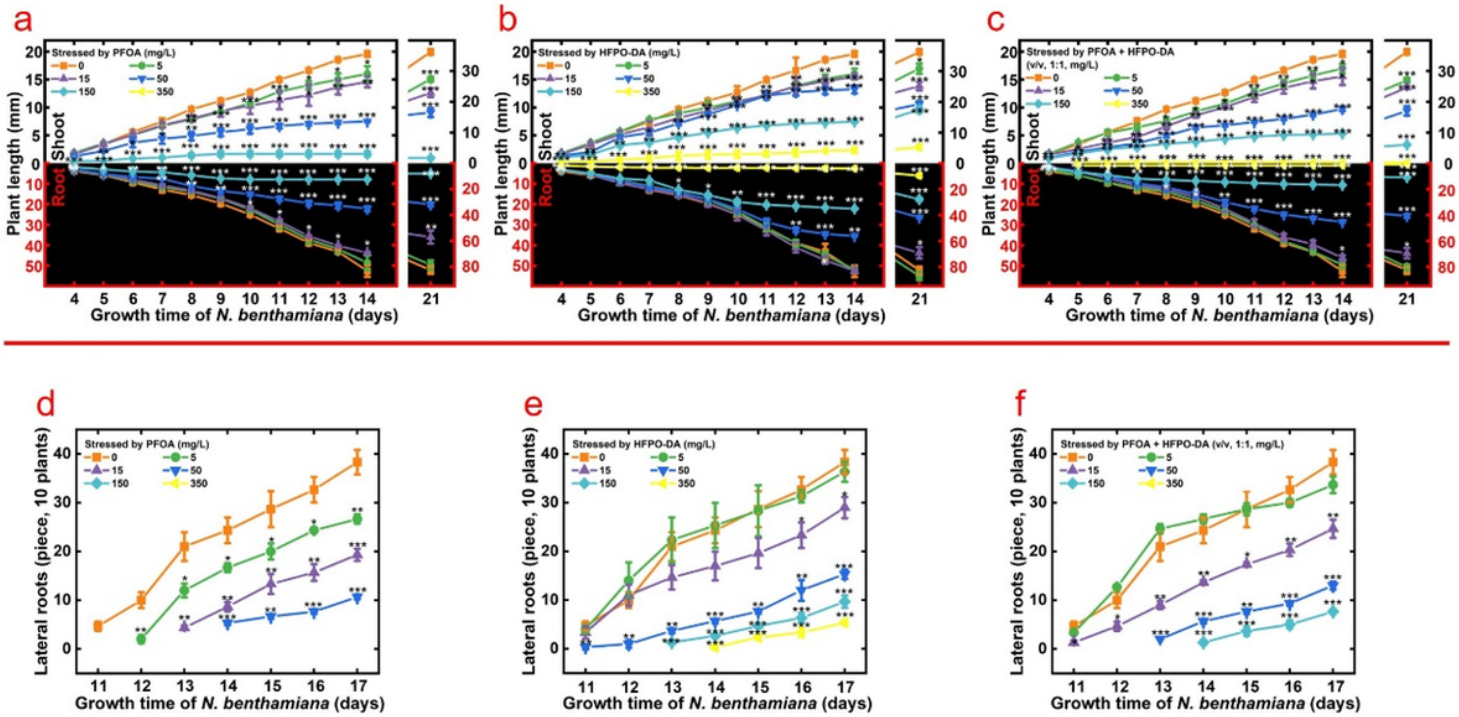

Figure 2

Effects of different levels of PFASs exposure on agronomic traits. The length of roots and shoots changes with the growth time under stressed by PFOA (a), HFPO-DA (b) and (PFOA + HFPO-DA) (c). The number of lateral roots sprouted with the stress time under stressed by PFOA (d), HFPO-DA (e) and (PFOA + HFPO-DA) (f) (The total number of 10 plants was counted).

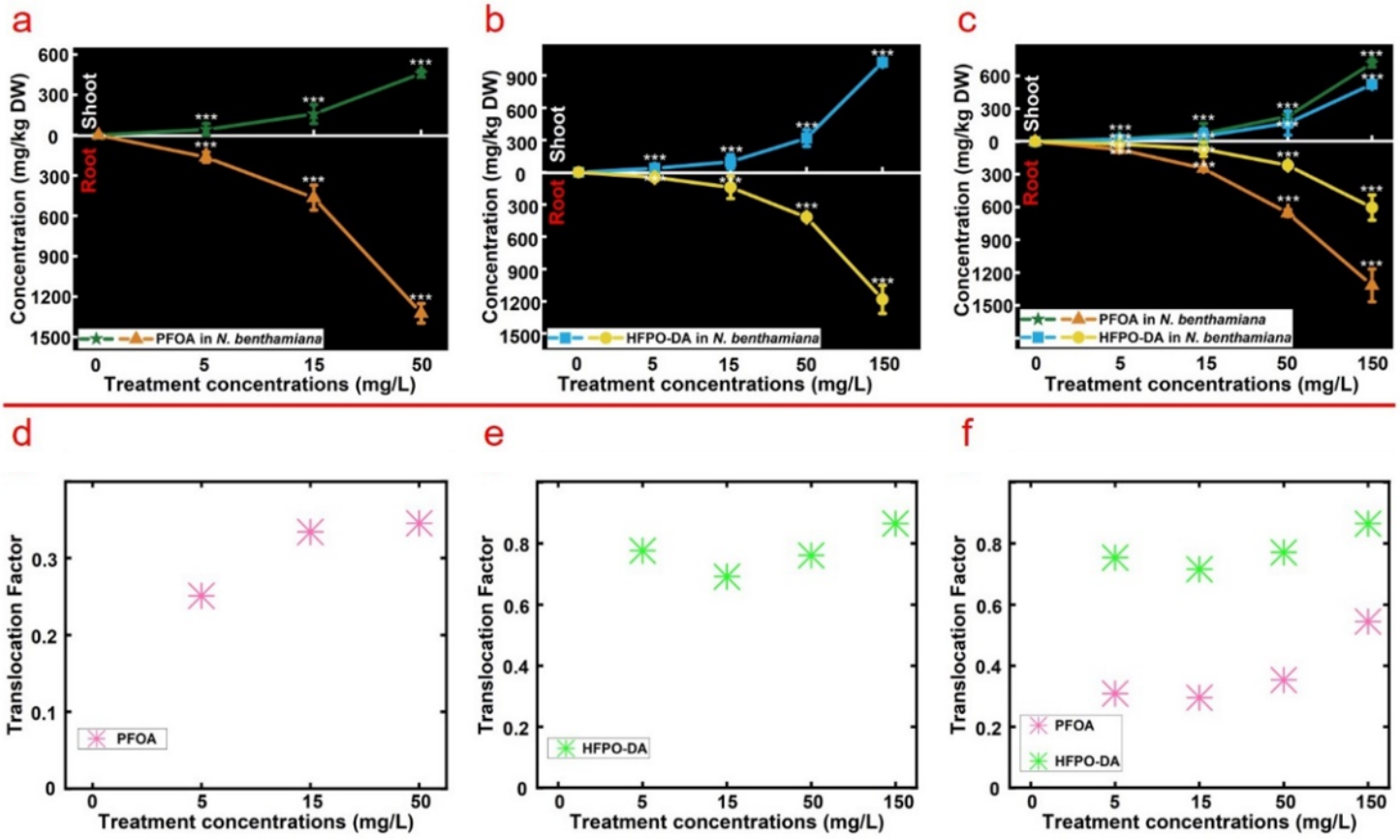

Figure 3

Emerging contaminants content after exposed 21 days. (a, d) Stressed by PFOA. (b, e) Stressed by HFPO-DA. (c, f) Stressed by (PFOA + HFPO-DA), v/v, 1:1. The test biomass under high concentration stressed was not obtained.

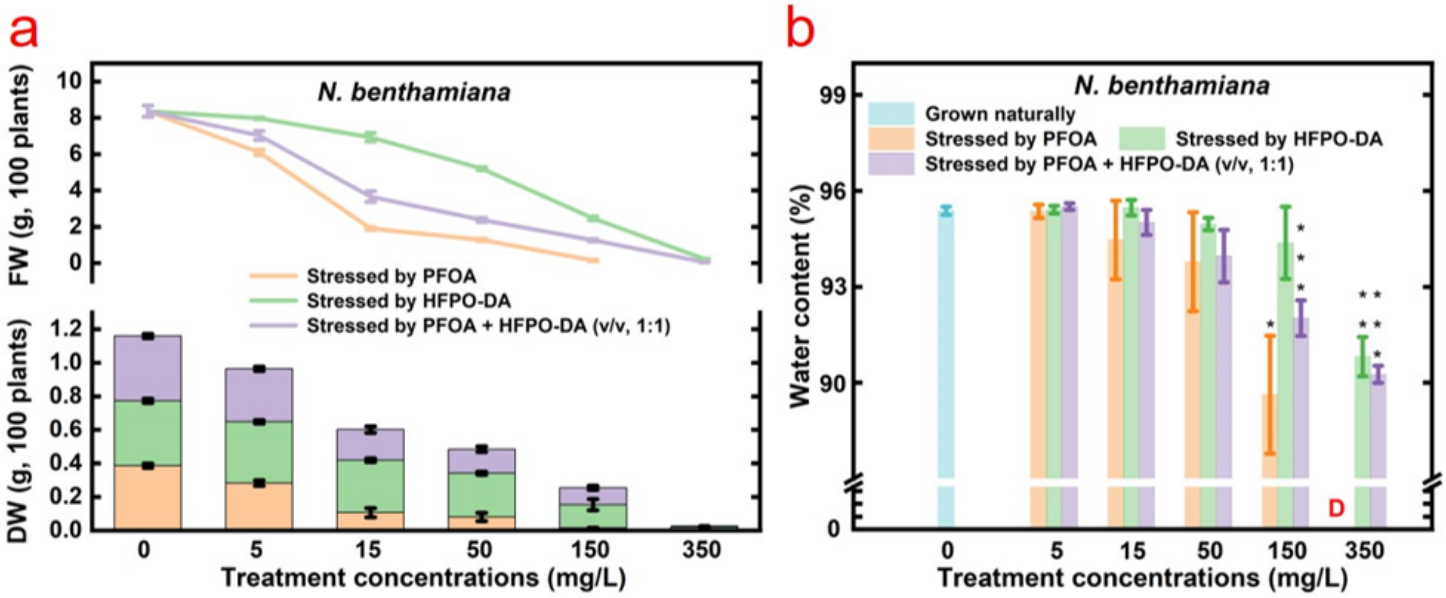

Figure 4

Agronomic traits after stressed 21 days. (a) The fresh weight (FW) and dry weight (DW) under stressed by PFASs, the total number of 100 plants was counted. (b) The water content under stressed by PFASs. D: Plants with no signs of life. Each group use 100 plants.

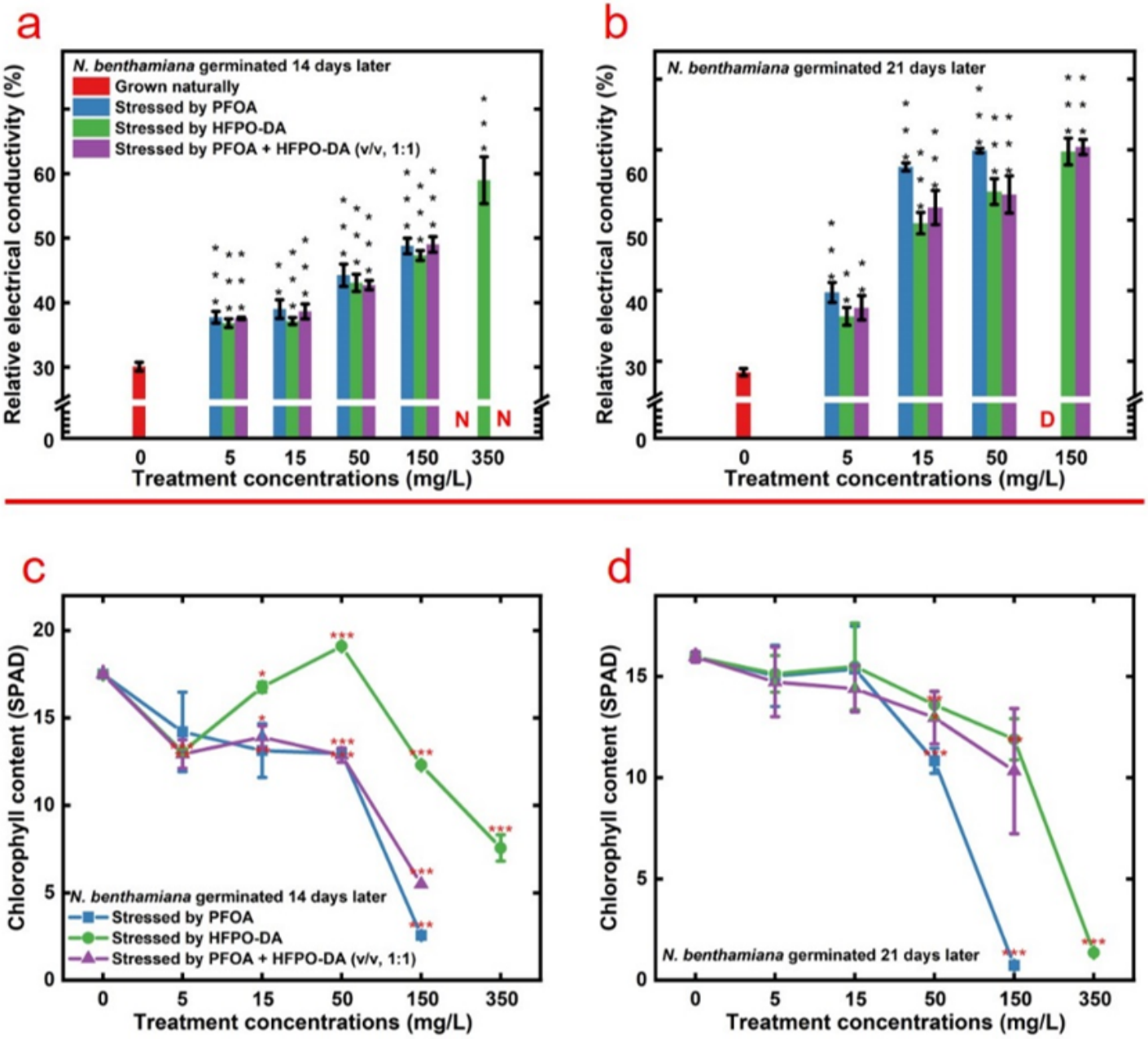

Figure 5

Physiological and biochemical indicators after stressed. The relative electrical conductivity after 14 days (a) and 21days (b) on stressed. The chlorophyll content after 14 days (c) and 21days (d) on stressed. D: Plants with no signs of life. N: Plants not obtain biomass.

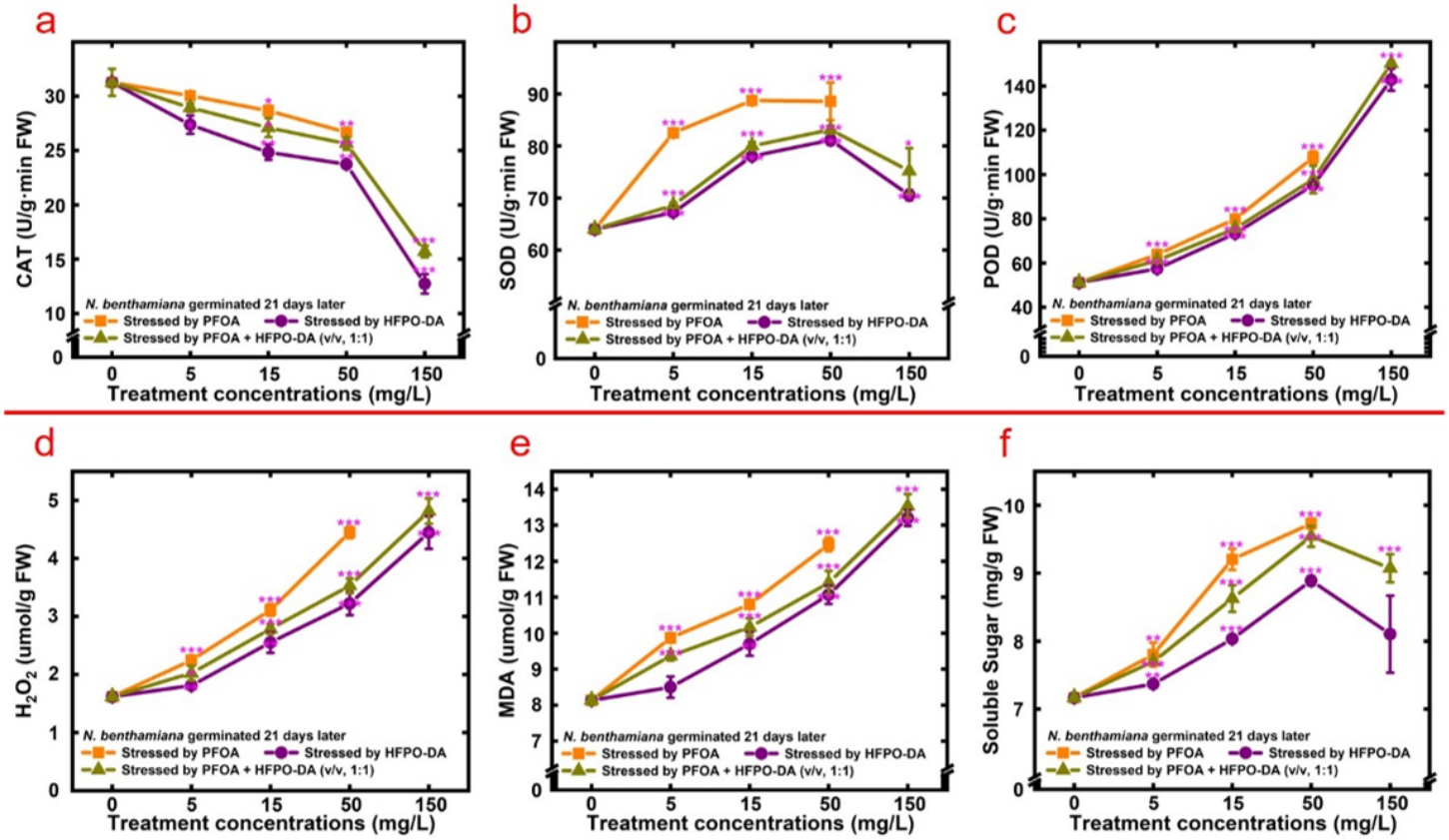

Figure 6

Physiological and biochemical indicators after stressed 21 days. (a) CAT activity. (b) SOD activity. (c) POD activity. (d) H2O2 content. (e) MDA content. (f) Soluble sugar content. The test biomass under high concentration (150 mg/L) PFOA or (PFOA + HFPO-DA) stressed was not obtained.

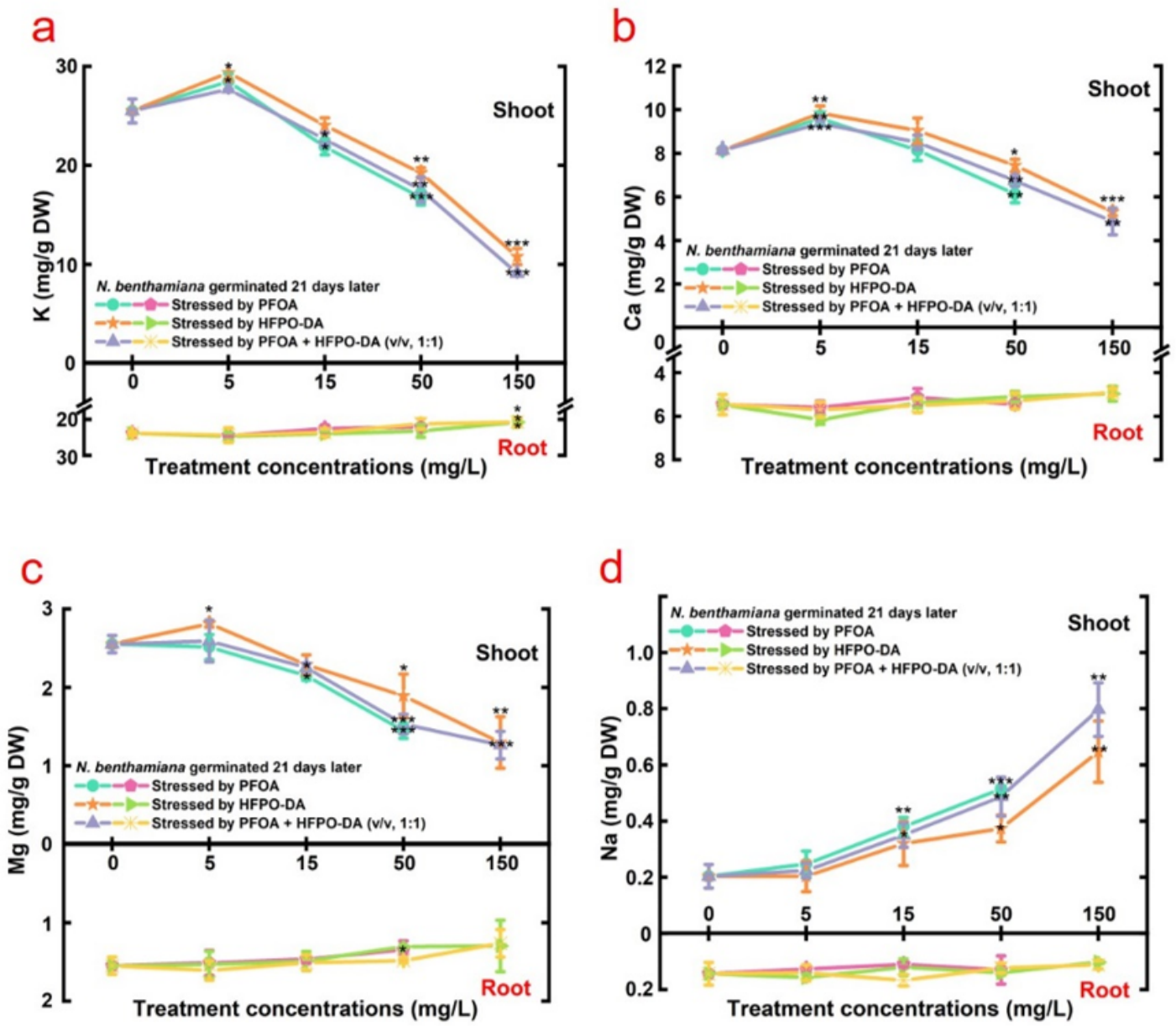

Figure 7

Mineral element content in roots and shoots after stressed 21 days. (a) K content. (b) Ca content. (c) Mg content. (d) Na content. The test biomass under high concentration (150 mg/L) PFOA or (PFOA + HFPO-DA) stressed was not obtained.

Supplementary Files

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- SupplementalMaterial.docx