Original Article

The influence of ultra-high diluted compounds on the growth and the metabolites of *Oryza sativa* L.

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Abstract

Background: The ultra-high dilutions (UHDs) have been widely used in the field of human, animal and plant treatment. In the present research, the effects of the potentized ultra-high dilutions and potentized (UHDs) on physiological and biochemical variations in *Oryza sativa* L. (rice) were investigated. **Methods**: To study the effect of UHDs (*Calendula officinalis* Calen. and *Arnica montana* Arn.) on the physiological and biochemical variations of the *Oryza sativa L.* (rice), 28 experiments were designed and statistically analyzed using the Design Expert 7.0.1 software over the general factorial design methodology. Three qualitative factors were studied including the UHDs/placebo usage, sterile/non-sterile experimental condition and the type and timing of the UHDs usage. The validated analysis was subjected to more extended studies on the variations in physiological growth, carbohydrate, protein content, pigment production, and amino acid patterns. To evaluate the effects of UHDs on rice, a desirable response percentage was formed from a number of healthy seedling productions, and the height percentage of the aerial parts and main roots were studied. **Results**: The statistical analysis resulted in a prediction model which was more than 97% correlates with experimental results. The results showed that the UHDs increased the pH variations, carbohydrate, protein and pigment levels each by ~2.5, ~1.5, ~1.4, and ~1.4 folds, respectively. Also compared to placebo, the amount and proportion of amino acids has significantly varied, showing a statistical effect on the germination and seedling growth of the rice, as well as the stress conditions caused by the sterilization process, seedlings entrance into the light and their transition into hydroponic culture medium. **Conclusion**: The use of UHDs leads to an increase in the production of chlorophyll, as well as carbohydrate and protein content. Moreover, it causes significant variations in the amino acid profile and the production of amino acids along with the photosynthesis, germination, and metabolism processes.

Keywords: Amino acid, GC/MS, Rice, Physiological parameters, Ultra-High Dilution and Potentized compounds

Introduction

The challenges of ultra-high diluted (UHD) materials are increased through the limits of Avogadro's molar constant (1, 2). Therefore, their effects are concealed due to the lack of materiality by current laboratory methods (3). Despite the serious criticisms voiced by scientists and rationalists, UHDs have been used in the field of humans, animals, and plants treatments (4-11). The usage of UHDs in the field of plants is associated with their prevention and empowerment against stress conditions such as radiations, drought/dehydration, salinity or exposure to pathogens and pests (11, 12). The use of common or routine pesticide/herbicide are known to cause the resistance of pathogens and bring about the need for more powerful types of chemicals. It resulted in the depreciation of soil, loss of its organic materials and its hunching, minimize the nutritional value, and contaminate the product and food sources and underground resources (13-17). Reportedly, UHDs usage on the other hand is expected to not only solve these problems, but also to improve the quality and fertility of soil and reduce water consumption. Moreover, UHDs are harmless to humans, livestock and different ecosystems as they cause no pathogen resilience, and in long-term, they increase the product efficiency (18-25). They may also cause a remarkable increase in stem growth and stability, seed germination rate, survival of buds, defense of enzyme expressions, and resistance to a variety of mutagenic agents (25-30).

Given these brilliant effects, UHDs can be highly practical in agriculture and plant science. The most important criticism in the field is, however, that these UHDs are at a diluted stage, which may make them nothing more than a "placebo" (31, 32). Despite the ambiguity of the mechanism, it must change the research paradigm related to the lack of adequate information, the essence of scientific discovery, and UHDs effects. There is not enough information on plant molecular and chemical variations treated with the UHDs; in most cases, it reduces the acceptance of their effect (11).

The current study presents some molecular and chemical results of the UHDs effect on the *Oryza sativa* L. (Rice; a scientific model plant). Rice is one of the most important grains and food items worldwide (33). It is a short-term growth herbaceous plant that is suitable for laboratory studies. Given that, its genome and proteome have been thoroughly investigated, rice can be easily used in the development and continuation of the experiments (34, 35). Here, the potentised ultra-high dilutions of *Arnica montana* and *Calendula officinalis* were used. Previous studies showed that *Arnica montana* and *Calendula officinalis* UHDs were used for the plant aerial parts and root damages, respectively (11). To produce knowledge and more information on the effects of UHDs on plants, the physiological growth features, total carbohydrates, proteins, photosynthetic pigments productions, and the amino acid profile variations of rice under the mentioned UHDs treatment are considered herein.

Materials and Methods

Materials and growth conditions

Seeds (3 months old harvested) of *O. sativa* L. cv. IR651 were obtained from the Genetic and Agricultural Biotechnology Institute of Tabarestan, Sari University of Agricultural Sciences and Natural Resources, Mazandaran, Iran. The UHDs of *Calendula officinalis* (Calen. C30) and *Arnica montana* (Arn. C200) were purchased from DHU Co., Germany. Both UHDs were in solid granules in the size of 1 mm. Placebo (as a control material), was obtained in the same size of granules, from DHU Co., Germany. Ultrapure ICP-MS grade water, Sartorius Co., Germany was used throughout the

experiment. The solutions of granules and placebo were used in the experiments and treatments at a ratio of 1 globule per 10 mL ultrapure water. The UHDs were dissolved in water for 20 min, and freshly used 30 min after preparation. A conventional laboratory culture was performed in a sterile condition, while normal culture is in non-sterile conditions. So, two study groups were noted: non-sterile and sterile conditions. In the sterilization process, the autoclave was applied in 121°C at a pressure of 1.1 bar for 20 min. The method of using UHD is briefly referred to the general principles of the previous publication (11). Seedlings (7 seeds) were placed on Petri dishes (ID: 7 cm) with 7 ml of water based on the experiments described in Table 1 (35, 36). In the first stage of seed germination, in each petri dish (containing 7 seeds), 7 mL of Calen. solution was used. Petri dishes were kept at 27 ± 0.5°C in a dark place for four days. On the fifth day and for the growth of the seedlings and production of chlorophyll, the dishes were transferred into light and kept for at least three days (second stage of growth). The 1 mL of Arn. solution UHDs was applied before the transferring the Petri dishes into the light. At the third stage of growth and for continuing the study on the growth of the seedlings (5 per pot), they were transferred into a pot (1L volume) containing rice specific hydroponic growth cultivation media (50 mL of Yoshida, Sigma Aldrich Co., Germany). The transfer process may damage the root, and, in this step, 2 mL of Calen. solution was used. Each experiment was conducted in a separated pot. The contents of the pots were not intertwined, and the pots were placed 25 cm apart. Seedlings were grown in a constant and controlled media, using phytotrons, maintained at a thermoperiod of 27 ± 0.5°C, photoperiod of 16 h, with a relative humidity of 70%, and a photon flux density of 220 mM/m²/S. Every 24 h, the pH was adjusted into 5.25 ± 0.5. The cultivation continued for 15 days (35, 36).

Statistical design of the treatments data analysis

To investigate the effects of UHD treatments and the sterilization conditions on the rice seedlings, the Design Expert 7.0.1 software and the general factorial design methodology was used. Three influential qualitative factors (A-C) were studied on UHDs treatments and growing conditions (Table 1). To this end, 28 experiments (in three replications) were designed (Table 1). The conditions of the experiments were designed based on the general factorial design methodology algorithm to evaluate three qualitative factors including the UHDs/placebo usage (Factor A), sterile/non-sterile experimental condition (Factor B), and the type and timing of the UHDs usage (Factor C). The factors A and C were selected to study the effects of UHDs on rice seed germination and growth. It is obvious that the sterilization conditions (applying the high temperature and pressure or sterilizing solutions) lead to changes in the seeds nature or the properties of the UHDs. So, the factor B is chosen to study the effect of sterilization conditions on germination and growth. The UHDs treatments (Factor C) encompassed three stages. The Calen. treatment was used at the first stage of seedling, when the seeds were put in water and dark conditions. It was also used at the third stage of the planting, during the seedlings transfer into the hydroponic medium. The Arn dilution was used in the second stage before transforming the seedlings into the light. Placebo was used instead of UHDs in the corresponding conditions in a similar procedure. Tools, seeds, and water were sterilized in all tests designed in the sterile conditions. A total of 28 experiments were designed. Each of which was conducted in three randomly replications based on the standard randomization order (Table 1). A desirable function (Eq. 1) was used to study the influence of the mentioned factors. To find the effective factors on rice seedlings, the desirability function was used as a response, obtained from Eq. (1):

$$\textit{Desirable response \%} = \frac{\sum 50\,\Delta\left(\frac{m_i}{M}\right) + 20\,\Delta(n_i/N) + 30\Delta\,(p_i/P)}{100} \qquad \text{Eq. (1)}$$

Table1: General factorial design methodology experimental design for the study of Rice seedlings.

Std	Run	UHDs/Placebo treatment	General condition	Stages of treatments	healthy seedlings %	aerial parts height %	Main root height %	Desirable response %
26	1	UHDs	Sterile	1st, 2ed and 3ed stage	97.18	67.09	69.72	70.89
15	2	Placebo	non-Sterile	1st and 2ed stage	43.66	30.79	52.78	38.67
10	3	UHDs	Sterile	3ed stage	69.01	68.35	58.47	65.45
8	4	UHDs	non-Sterile	2ed stage	87.32	89.21	59.72	80.17
12	5	UHDs	non-Sterile	3ed stage	59.15	69.24	70.83	68.71
2	6	UHDs	Sterile	1st stage	59.86	40.72	63.89	49.58
4	7	UHDs	non-Sterile	1st stage	84.23	55.76	76.39	64.79
3	8	Placebo	non-Sterile	1st stage	51.69	32.19	41.94	37.07
21	9	Placebo	Sterile	2ed and 3ed stage	45.35	29.60	28.06	30.71
19	10	Placebo	non-Sterile	1st and 3ed stage	53.52	29.32	44.44	36.28
6	11	UHDs	Sterile	2ed stage	74.08	96.40	50.00	80.25
16	12	UHDs	non-Sterile	1st and 2ed stage	97.18	54.86	69.58	63.51
25	13	Placebo	Sterile	1st, 2ed and 3ed stage	43.66	32.84	36.11	34.90
14	14	UHDs	Sterile	1st and 2ed stage	69.15	57.59	58.33	58.97
5	***15***	***Placebo***	***Sterile***	***2ed stage***	***45.07***	***28.60***	***27.92***	***30.04 ****
11	16	Placebo	non-Sterile	3ed stage	46.48	32.91	42.50	37.15
22	17	UHDs	Sterile	2ed and 3ed stage	78.59	78.24	58.33	72.30
17	18	Placebo	Sterile	1st and 3ed stage	42.39	27.19	31.94	30.14
18	19	UHDs	Sterile	1st and 3ed stage	59.44	57.55	56.94	57.56
20	20	UHDs	non-Sterile	1st and 3ed stage	92.96	64.75	91.67	75.64
24	21	UHDs	non-Sterile	2ed and 3ed stage	87.32	90.83	72.22	84.90
28	***22***	***UHDs***	***non-Sterile***	***1st, 2ed and 3ed stage***	***100.00***	***100.00***	***100.00***	***100.00 *****
9	23	Placebo	Sterile	3ed stage	43.66	32.55	30.56	33.07
1	24	Placebo	Sterile	1st stage	42.39	28.99	29.17	30.39
7	25	Placebo	non-Sterile	2ed stage	48.59	29.50	43.06	35.47
23	26	Placebo	non-Sterile	2ed and 3ed stage	43.94	29.60	41.81	34.70
13	27	Placebo	Sterile	1st and 2ed stage	43.94	29.21	31.11	31.25
27	28	Placebo	non-Sterile	1st, 2ed and 3ed stage	45.07	32.81	41.94	36.77

1st stage: The use of Calen., at the start of seedling when the seeds were put in water and dark conditions.
2ed stage: The use of Arn., before transforming the seedlings into the light.
3ed stage: The use of Calen., during the seedlings transfer into the Yoshida medium.
Std: Standard randomization order of experiments
* and **: lowest and highest desirable function

We investigated the height percentage of the aerial parts (n) and main root (p) to obtain the desirable response percentage, the number of healthy seedlings production (m). The tallest shoot leaves and the main root length were measured with a ruler (with the precision ± 0.01 cm) as soon as they were collected. This response emerges from the desirability of each mentioned response that was 50% of

m (healthy seedling production percentage), 20% of *n* (the aerial parts height percentage) and 30% of *p* (the root height percentage). In this equation, *i* is the experiment's run order and the *M*, *N* and *O* are the highest response percentage corresponding to run No. 22 in Table 1.

The analysis of variance (ANOVA) report is available either with or without annotation and includes a full analysis of variance, prediction equations, and case statistics. The implication of the F-value depends on the degree of freedom of the model. Data were analyzed with an accuracy of 99%≤. The F-probability ≤0.0001 were considered to be statistically significant. The values of R-Squared and adjusted R-Squared were evaluated as the accuracy extent of the experimental results and the statistical model, respectively. The standard error of the estimation was evaluated for the standard deviation of the residuals. After statistical analysis and determination of the most effective laboratory conditions, chemical and physiological studies were performed. Accuracy and validity of the results and graphs were determined using MSTAT-C software. Different mean values were mentioned with letters (a, b, c and ab) at P-value <0.05.

Physiological analysis and growth quality assay

To conduct a physiological analysis and the growth quality assay, the shoots and roots were dried individually by freeze dryer (333Bi5, DorsaTech Co., Iran) at -80°C, for 24 h. For the total carbohydrate content, 100 mg of dried root/shoot powder was extracted using ethanol 80% for 60 min. Five mL of HCl 1.1% was added into the supernatant, and the mixtures were heated in a water bath (97°C) for 30 min. The samples (1 mL) were mixed with 5 mL of ice-cold anthrone reagent (72% sulfuric acid containing 0.2% anthrone) and reheated (11 min). The solutions absorbance was measured at 630 nm (36). The total protein content of 0.1 g root/shoot tissues were extracted over TRIzol reagent protocol (Molecular Research Center, Inc., Cincinnati, OH, USA). Thereafter, the protein concentration was determined according to the Bradford assay kit (BioRad Co., Hercules, CA, USA) in the comparison with BSA as the standard (35). For all photosynthetic pigments, 20 mg of dry shoot powder (70 ° C for 60 min) was extracted using dimethylsulfoxide (DMSO). We measured the absorbance of extract at 470, 646 and 663 nm. The chlorophyll "a" (Chl a) and "b" (Chl b) contents, their ratio, and the carotenoids content were determined (36).

Amino acid extraction and identifications

To study the shoot and root amino acid pattern, 0.5 mg of dried powder samples were made homogeneous using 200 µL extraction solution (α-1-aminobutyric acid solution; 0.1 nM, D-hystidine; 500 ppm as internal standards, and 1.8 mL triflurouric acid (TFA); 10% v/v) at 4 ºC. The supernatant (15 min, 4°C, and 12,000 rpm) was collected; the process was repeated twice, and dried. 10 ml of chloroform was added into 0.3 mg of dried extract and refluxed (under nitrogen atmosphere, 24 h, at 250 ºC). Then, the solution was cooled, completely dried (45ºC), and dissolved in water (20 mL, pH 6.6-8.6). The filtered (0.22 µm) sample was dried (under vacuum, at 40ºC) and dissolved in dry methanol (3 mL). For derivatization, 0.03 mg of the sample was vigorously combined in the derivatization reagent (32 mg/mL hydroxylamine hydrochloride, 40 mg/mL of 4-(dimethylamino) pyridine in pyridine 4:1 methanol) and then heated (30 min, 75°C). Thereafter, it was cooled, receiving an added acetic anhydride (1 mL) and reheated for 20 minutes. The cooled sample was then mixed with dichloromethane (2 mL) and hydrochloric acid (1 mL, 1M). The solution was finally stirred vigorously for 30 sec and dried. Eventually, it was dissolved in 300 µL of ethyl acetate 1:1 n-hexane and was used for gas chromatography mass spectrometry (GC-MS) analysis (37-43). For this,

the CP-3800 gas chromatography (GC) equipped with EZ faast™ Physiological Amino Acid column ID: 30 m × 0.25 mm × 0.25 µm (Phnomenex Co., USA) in 1:30 injection ratio to study the amino acid derivatives is used. The temperature program began at 90°C (kept for 5 min), increasing up to 250°C with a ramp of 5°C/min and was kept for 1 min. Afterwards, it reached 270°C at a ramp of 10°C/min and was kept for 5 min. The GC was connected to a 4000 MS-ionTrap (40-2000 m/z) ionizer with temperatures of 120°C, 100°C, 170°C and 220°C for trap, manifold, transfer line, and detector, respectively (41, 44-46).

Results

In order to answer whether or not the ultra-high dilutions (UHDs) have an effect on rice seed germination and the growth of seedlings, 28 experiments (in three replications) were designed (Table 1). The previous studies have shown that Calen. was useful in treating plant's root damages and it began to treat the root at the start of seeds germinations (stage 1) and the seedlings transformation into hydroponic media (stage 3). Moreover, Arn. was useful in treating aerial parts damages. It was used at the time of transmitting the seedlings into light (stage 2). In each experiment, the physiological examinations were performed (details can be seen in supplementary file).

Table 2: ANOVA for the study of Rice seedling growth under the variety of treatments.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.000207	21	9.86E-06	52.71876	< 0.0001 **	**significant**
A-UHDs	0.000186	1	0.000186	997.0628	< 0.0001 **	
B-Sterilization	1.04E-05	1	1.04E-05	55.73225	0.0003 *	
C-Treatment stage	3.65E-06	6	6.09E-07	3.255437	0.0883	
AB	1.72E-06	1	1.72E-06	9.211211	0.0229 *	
AC	3.59E-06	6	5.99E-07	3.201224	0.0913	
BC	1.19E-06	6	1.98E-07	1.057959	0.4736	
Residual	1.12E-06	6	1.87E-07			
Lack of Fit	4.351E-03	10	4.351E-04	0.005258	0.9198	**not significant**
Pure Error	0.029501	5	0.0059		**Std. Dev. :**	4.324E-004
Adeq Precision:	19.446		**R-Squared :**	0.9946	**Adj R-Squared :**	0.9757

** Highly significant parameters
* Significant parameters

Data were analyzed with an accuracy of 99%≤ and the F-probability of ≤0.0001 was considered statistically significant (Table 2). The Model (F-value of 52.72) and the factors A, B, C, AB, AC, BC were significant. The values of R2 and adjusted R2 were 99.46% and 97.57%, respectively. The standard error of the estimation showed a ~0.0004 value for the standard deviation of the residuals. "Adeq Precision" measures the signal to noise ratio, and a ratio greater than 4 is desirable. Adeq precision of 19.446, as obtained in this study indicates that this model is robust enough. Figure 1 shows the response interaction pattern by the generated model considering the variations of the effective parameters. Chart (A) shows the desirable response of each experiment and their relations; chart (B) shows the variations of response levels in the sterile condition compared to the non-sterile condition. As it can be seen in Fig. 1A, the best growth was achieved in the UHD treatment and in non-sterile conditions. Also, the best growth conditions were for the seeds that receive UHDs in all three stages of growth. This growth condition was compared to similar experiments in the sterile conditions for further evaluation. On the other hand, Fig. 1B shows that the UHDs treatment growth response

efficiency increased for approximately four times in comparison with non-UHDs treatments (placebo usage at sterile conditions), which is discussed more later on.

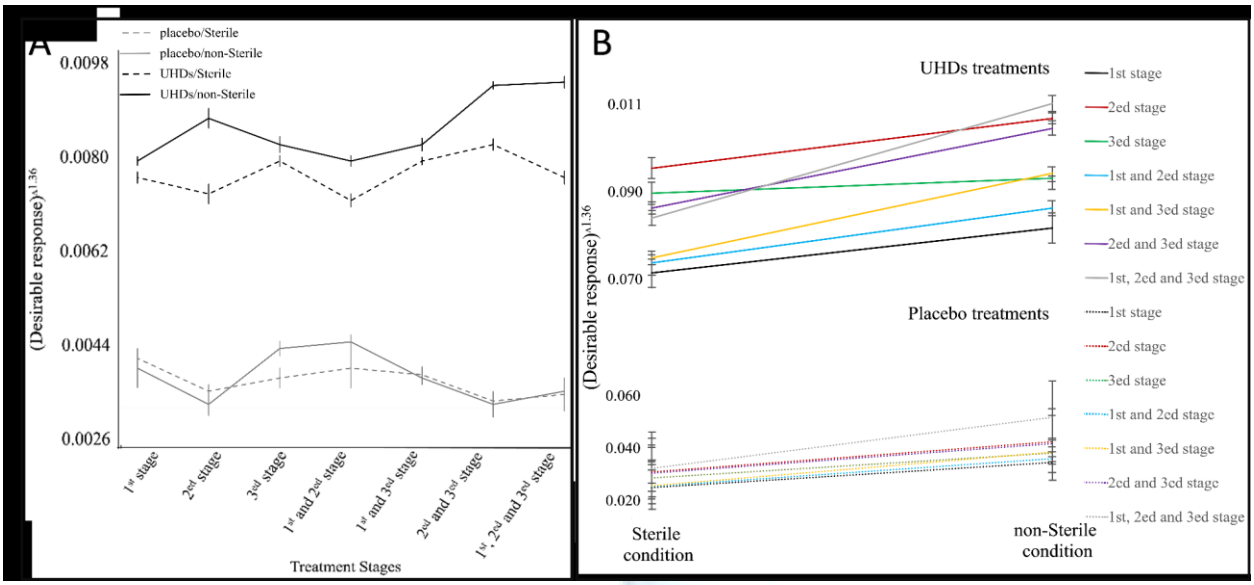

Figure 1: Statistical Investigation of variations of Rice plant in germination and growth. 1st stage: The use of Calen., at the start of seedling, when the seeds were put in water and dark conditions. **2ed stage**: The use of Arn., before transforming the seedlings into the light. And the **3ed stage**: The use of Calen., during the seedlings transfer into the Yoshida medium. **Chart A** shows the desirable response under the different treatment conditions. Lines (—) are for non-sterile cultivation conditions and dotes (···) are for sterile cultivation. The black color is UHD treatment and gray color is the placebo treatment. **Chart B** shows the desirable response variations of each treatment under sterile and non-sterile culture conditions. Lines (—) are for UHDs treatment and dotes (···) are for placebo. The statistical study generates by the Design Expert 7.0.1 software and the general factorial design methodology based on the desirable response calculated over Eq1.

According to the results of the statistical analysis, two experimental conditions were selected for the studies of daily pH variations, total carbohydrate, chlorophyll and protein content, and amino acid pattern investigation, (Fig. 2 and 3). The two treatment conditions (in three replications) were "UHDs treatment under non-sterile conditions" and "placebo treatment under sterile conditions". Figure 2A shows that the rate and magnitude of daily pH variations were much greater in the UHDs treatment. Also, compared to sterile-placebo treatments, the total amount of chlorophyll (Fig. 2B), protein (Fig. 2C), and carbohydrate production (Fig. 2D) in these conditions have increased significantly. As the last part of this research investigation, the amino acid pattern of hydroponic rice cultivation under UHDs/non-sterile and placebo/sterile treatments were studied via GC-MS variations (Fig. 3). This figure shows that the amino acid production pattern in UHDs conditions is

Table 3: Amino acid percentage in UHDs and non-UHDs conditions.

Amino acids	Concentration percentage	
	UHDs	non- UHDs
Alanine	5.67	≥ 1%
Tryptophan	11.03	7.74
Glutamic acid	9.54	≥ 1%
Aspartic acid	8.66	≥ 1%
Tyrosine	≥ 1%	19.71
Threonine	≥ 1%	16.42
Glycine	7.33	≥ 1%
Valine	≥ 1%	13.67
Leucine	7.54	6.8
Isoleucine	9.64	≥ 1%
Histidine	6.21	10.38
Cysteine	≥ 1%	3.99
Lysine	11.21	≥ 1%

totally varied in comparison with non-UHDs ones. The details of amino acid production variations are mentioned in Table 3.

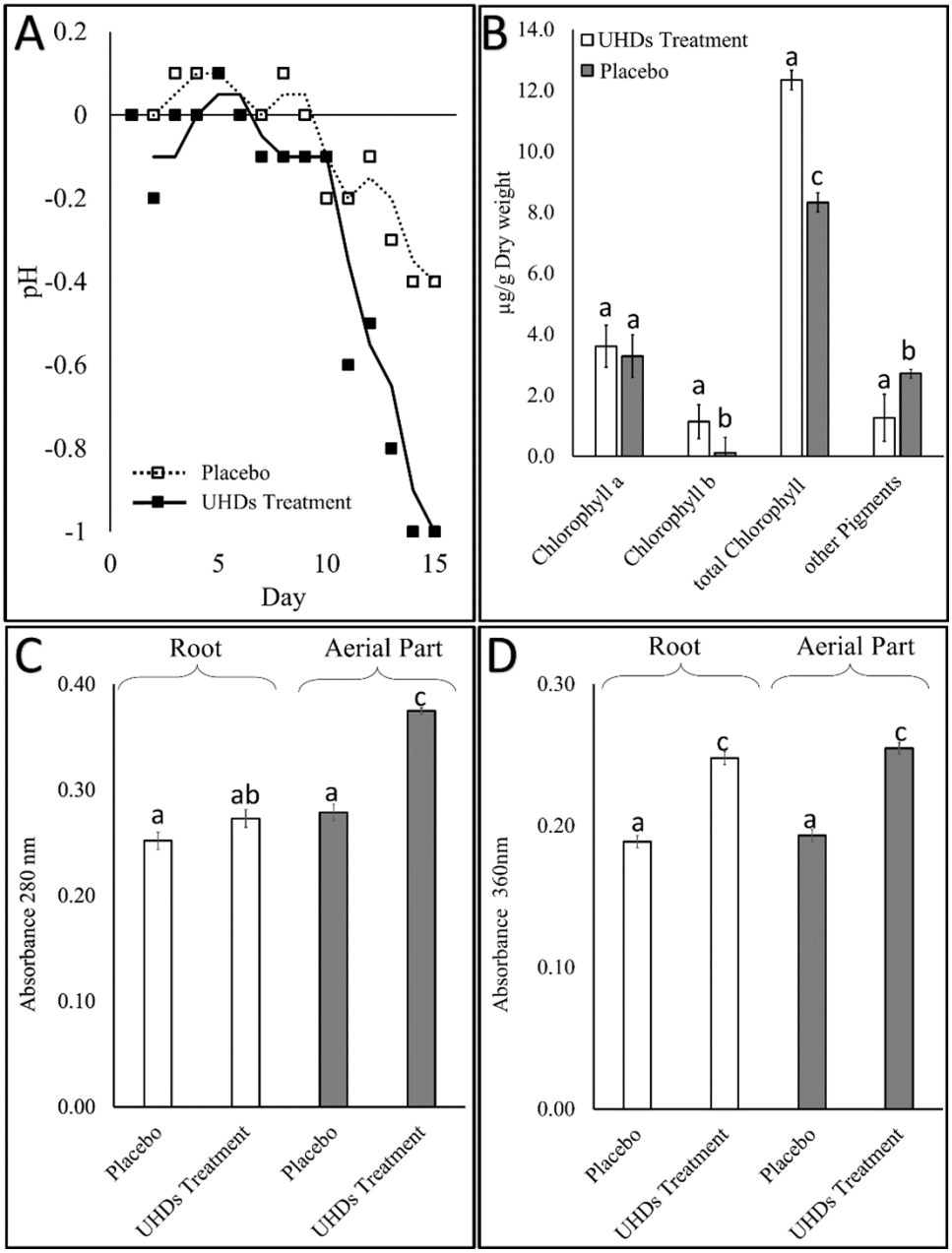

Figure 2: variations of daily pH of hydroponic media (A), the total content of photosynthetic and non-photosynthetic pigments (B), total protein (C) and carbohydrate content (D) of aerial parts and roots of plant cultivated under UHDs treatments and placebo conditions. Accuracy and validity were determined using MSTAT-C software. Different mean values were mentioned with letters (a, c and ab) at P-value <0.05. Letters a/c means high validated differences in results.

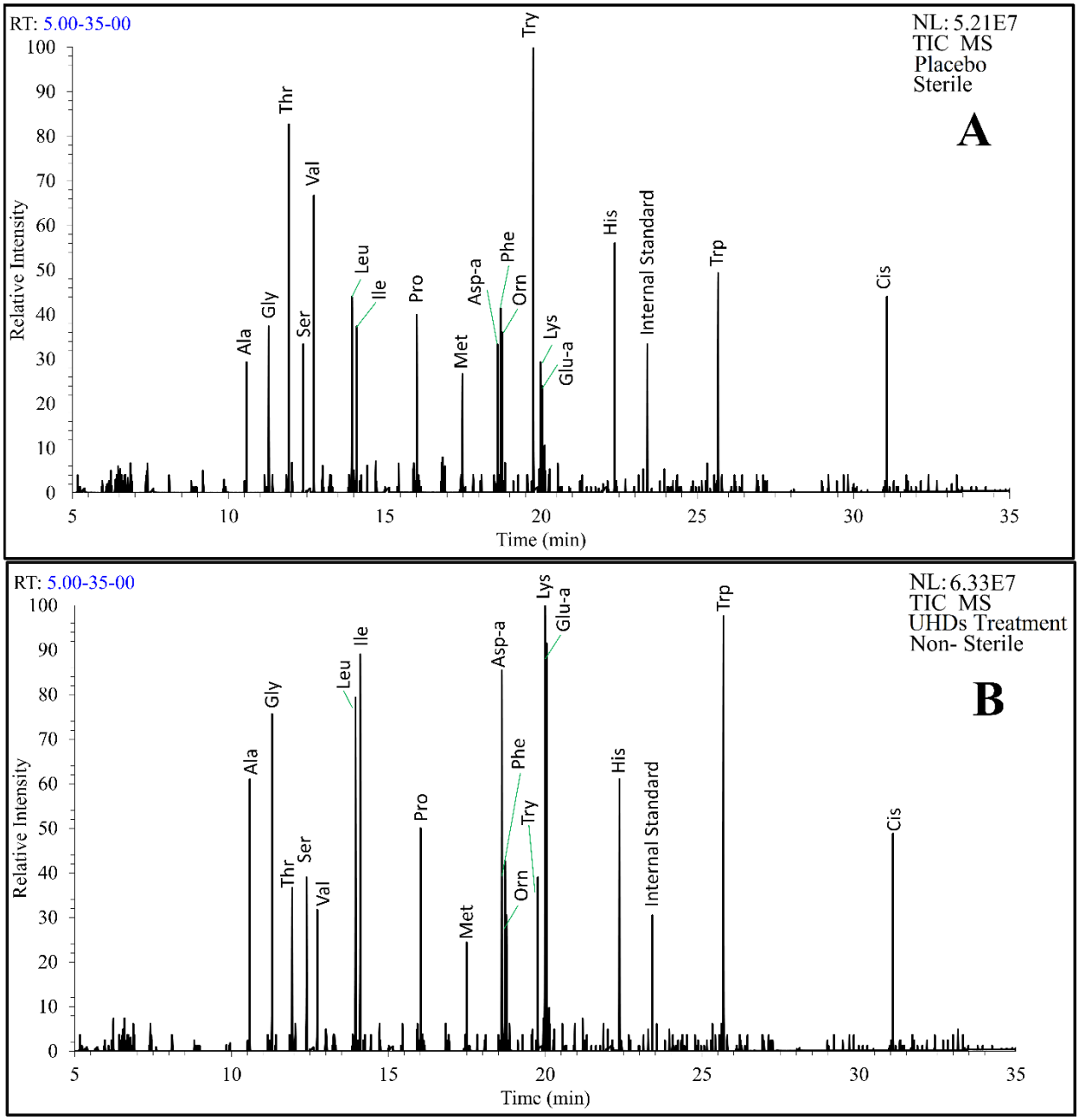

Figure 3: Amino acids GC-MS spectrum pattern of non-UHDs (Placebo)/sterile conditions (A) and UHDs/sterile conditions (B). Instrumental condition: CP-3800 gas chromatography (GC) equipped with EZ faast™ Physiological Amino Acid column ID: 30 m × 0.25 mm × 0.25 µm (Phnomenex Co., USA) in 1:30 injection ratio, the temperature program: 90°C (5 min), increasing to 250°C with a ramp of 5°C/min, increasing to 270°C at a ramp of 10°C/min and was kept for 5 min. The 4000 MS-ionTrap (40-2000 m/z) ionizer with temperatures of 120°C, 100°C, 170°C and 220°C for trap, manifold, transfer line, and detector, respectively.

Discussion

To study the UHDs impact on the germination and growth of rice, 28 experiments were designed and analyzed statistically. As mentioned in the previous section, the study was statistically significant and presenting validated results (Table 2). In addition to UHD vs. placebo treatment, this project studied the timing and stages of treatment as well as influence of sterilization on the growth conditions. Since the sterilization process (the usual lab cultivation method) is not in accordance with the nature of the seeds and it may also damage the UHDs, it is necessary to study the experiments under different sterile conditions simultaneously. The results of the statistical analysis (Table 2) showed that the studied factors were statistically valuable and capable of mathematical evaluations and further analysis. The correlations of the theoretical and experimental results have more than 97% of agreement with a standard deviation of about 0.0004.

Clearly, the most influential factors were the use of the UHDs and the sterilization conditions. The simultaneous impact of these two factors was also significant (Table 2). Figure 1 shows the detailed results of the experiments and the interaction of the factors. The most significant point in Fig. 1A is that in the responses of both, experiments performed via the UHDs, were better than those of placebo. Although the sterilization conditions slightly reduce the effect of the UHDs, the decrease is not significant and can be ignored. Also, the overall response in placebo usage does not change considerably. In this regard, the interference and standard errors of the experimental points under placebo conditions were not valuable (Fig. 1A, gray lines). Both parts of the Figure 1 indicate that the response of the experiments (desirable function calculated from Eq. 1) under the UHDs treatments, in all three growth stages, was more significant than that of placebo. The maximum effect of the UHDs was related to UHDs treatment in all the three growth stages. Noting the amount of response in single- and two-step treatments helps to evaluate the most effective phases of the treatment. The two-step usage of UHDs-in the 2nd (Arn., treatment in the entrance of light) and 3rd (Calen., treatment in the transition into culture medium) stages has the next highest responses (Fig 1A). Moreover, the single-stage treatment-in the 2ed stage (Arn., treatment in the entrance of light), had the third highest desirable response (Fig. 1A). The impact of the UHDs is reduced by sterilization. Also, the response under placebo usage (in both sterile and non-sterile conditions) not only had the least amount, but also it shows no validated differences. For ease of comparison, the physiological results of the experiments can be seen separately in the supplementary file.

These results bring about two hypotheses. First, the 2nd and 3rd treatment stages cause stress conditions for plant germination and growth. Second, the sterilization reduces the effect of UHDs. The following experiments were considered for closer examination of these two hypotheses. According to these two evaluation situations, the difference between UHD and placebo can be shown as: "UHDs treatment under non-sterile conditions" and "placebo treatment under sterile conditions". The samples were experimented in the two conditions, to analyze the pH variations of the media in 24h, as well as total carbohydrate/protein/ pigments/amino acid pattern (Fig. 2). Figure 2A shows that the pH decreased in the UHDs treated sample a week after the beginning of cultivation in a hydroponic environment. The pH was set to 5.2 every 24 h (35, 36). The pH decrease started from the 7th or the 8th day by only 0.2 degrees and from the 14th or the 15th by ~1 degree daily. A decrease of pH over 24 h in the last two days, compared to a decrease of 0.2 units at the end of the first week of cultivation, shows increased metabolism and mineral uptake by the roots. The rate of pH reduction in non-UHDs (placebo) samples was lower than in UHDs conditions (Fig. 2A). Also, the first decrease in pH on the non-UHDs samples was ~3 times larger than non-UHDs ones, which happened from the 9th to the 10th day, by ~0.2 units. The reduction of pH in the last two days reached a maximum of

about 0.4 units within 24 h. In addition to enhancing root growth and facilitating metabolism, the acidic constituents were transferred to the medium through the roots so that environmental elements could be absorbed (36). The pH of the environment becomes more acidic every 24 h during growth, indicating the intensity of the process of absorption and metabolism of minerals in the roots.

The overall content variations of the pigments were examined for a closer examination of the photosynthetic metabolism. Unlike the non-UHDs samples, a general review on UHDs photosynthetic pigmentation showed that the relevant levels were increased. The pattern of changes in chlorophylls "a" and "b" showed that the growth of samples in UHDs conditions were associated with higher amounts of chlorophyll "a" and "b". As it can be seen in Figure 2B, the variation of chlorophyll "a" variations under UHDs treatments were not as significant, as chlorophyll "b". In addition, the total amount of chlorophyll pigments increased significantly in the samples treated with UHDs. In non-UHDs samples, the relative amount of chlorophyll "b"/"a" was higher than those treated with UHDs. On the other hand, non-chlorophyll pigments had higher concentrations in non-UHDs samples. The pure chlorophyll "a" is a blue/green compound and chlorophyll "b" is a green/yellow one. Thus, not only the absorption of chlorophyll "a" spectrum differs from that of "b", but also the type of protein is different. Chlorophyll "b", having less energy (465 and 665 nm), absorbs longer wavelengths, and chlorophyll "a", with a higher level of energy (430 and 660 nm), absorbs shorter wavelengths (47, 48). Therefore, chlorophyll "a"-based photosynthesis is mainly activated in higher energies in comparison with chlorophyll "b". By increasing the amount of chlorophyll "b", plant photosynthesis in an expanded wavelength spectrum does not require intense light and proceeds on lower energy levels. Regarding the increase in chlorophyll "b" content in UHDs conditions, the process of photosynthesis is facilitated and accelerated, and there is no need to use high-energy wavelengths. Increasing the plant's chlorophyll "a" content causes photosynthesis on low- and high-energy wavelength increments, which leads to an increase in the possibility of the production of reactive oxygen species (ROS). Following this phenomenon, other photosynthetic pigments (carotenoids and anthocyanins) can be increased to reduce ROS concentrations.

Total protein and carbohydrate production are two important biochemical factors that indicate the plant metabolism and growth (36, 37, 44, 48). Total protein and carbohydrate levels were assessed for metabolism. To this end, the grown plants in hydroponic media (15 days of cultivation) were collected and examined through the previously mentioned methods. These compound productions and their distribution in different tissues of the plant express the status and quality of the growth. Figure 2C and 2D show the variations in total protein and total carbohydrate produced by the aerial parts and roots of the two groups of UHDs and non-UHDs treated plants. As can be seen in this figure, total protein production of aerial parts under the UHDs treatment increased meaningfully. Moreover, the carbohydrate content, of root and aerial part under the UHDs treatment increased significantly. Accordingly, the amounts of protein and carbohydrate produced in the body and root of plants treated with UHDs are more than those in non-UHDs treated samples. This difference is not significant in the case of root carbohydrates and can be statistically neglected. However, in cases of aerial part carbohydrate and aerial part/root proteins, they gain significance. The production of these two biomolecules increasing results in an active and healthy metabolism state in UHDs specimens.

Along with germination and growth, metabolites production and concentration, including amino acids, will vary depending on the environmental conditions and potential stresses. Other changes in amino acids include other plant compounds and their properties, such as carbohydrates, lipids, and the cell wall. Amino acids can be soluble or bonded to other compounds and have different rules. Some amino acids enter the chlorophyll synthesis, regulating the opening of plant apertures, pollination, germination of seeds and resistance to environmental stress conditions (39, 40, 42, 49,

50). This study experimented the variations in the pattern of amino acids after hydroponic growth in UHDs and non-UHDs conditions. UHD and non-UHD-treated hydroponic samples were prepared in the laboratory and the amino acid pattern was evaluated using GC-MS.

Figure 3 shows that, the pattern of amino acid production under UHDs was quite different from that of non-UHDs. Moreover, relative percentages of amino acids in non-UHDs samples indicate that the highest percentages of chemical products were as follows: Amino acids were Tyrosine (19.71%), Threonine (16.42%), Valine (13.67%), Histidine (10.38%), Tryptophan (7.74%), Leucine (6.8%) and Cysteine (3.99%), (Table 1). Also, amino acids with the highest ratio in samples prepared under UHDs conditions were Lysine (11.21%), Tryptophan (11.03%), Isoleucine (9.64%), Glutamic acid (9.54%), Aspartic acid (8.66%), Leucine (7.54%), Glycine (7.33%), Histidine (6.21%) and Alanine (5.67%) (Table 3).

Studies on amino acid activity showed that alanine and tryptophan were involved in the germination and chlorophyll synthesis. Glutamic acid was active in seed germination as well as being a precursor in the synthesis of chlorophylls and other amino acids. Aspartic acid was effective in seed germination and amino acid metabolism. Tyrosine and threonine contributed to the pollination and resistance in accordance with the environmental condition. Glycine played an important role as a precursor in the formation of phytol groups and chlorophyll synthesis. Valine was responsible for pollination and resistance to environmental conditions and seed germination. Leucine and isoleucine were effective in the resistance to salinity and the germination of pollen. Histidine was involved in the regulation of leaf apertures opening. Cysteines, as a part of the nitrogenase enzyme structure, played a significant role in the biological nitrogen fixation. Lysine, as a part of the chlorophyll synthesis process, was effective in regulating the leaf apertures opening and germination of pollen (15, 38, 39, 42, 49). According to Figure 3 and the description of the activity of amino acids, their production in UHDs samples were mainly involved the germination process, chlorophyll synthesis, metabolism, and aerobic respiration control. However, the amino acids produced in non-UHDs samples were associated with environmental resistance, germination and chlorophyll synthesis.

Conclusion

The main results obtained in this research can be summarized as follows:

a) Inspection of the results showed that the UHDs usage, in comparison with placebo, causes a significant and statistically validated increase in the rice germination and seedlings growth (Table 2). This effect is slightly reduced under sterilization process.
b) The entrance of seedlings into light and their transition into hydroponic culture medium causes stress, and negatively influences the plant germination and growth. The administration of UHDs can compensate the negative effects of the culturing process.
c) The use of UHDs leads to increase in the production of chlorophyll, carbohydrate and protein.

Biochemical studies show that the use of the UHDs lead to a spike in the formation of active amino acids in chlorophyll production along with an increase in the quality and quantity of photosynthesis, germination, and metabolism processes.

Acknowledgments

The results presented in this article relate to the joint research project of the Atlas Peydayesh Institute and Shahid Beheshti University with the number of SAD/600/35.

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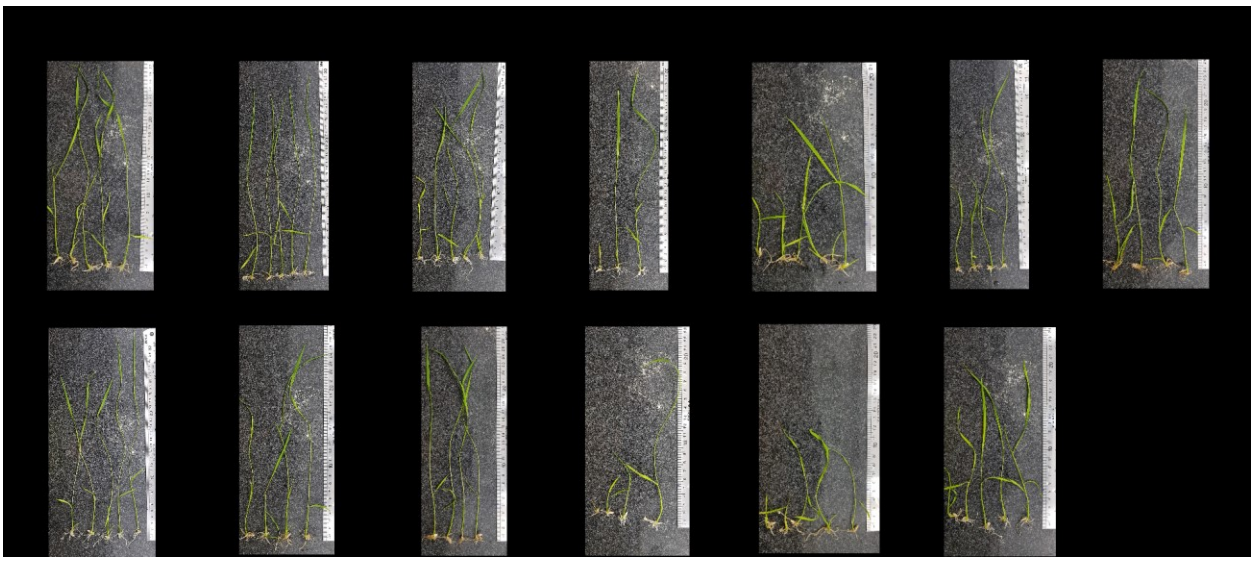
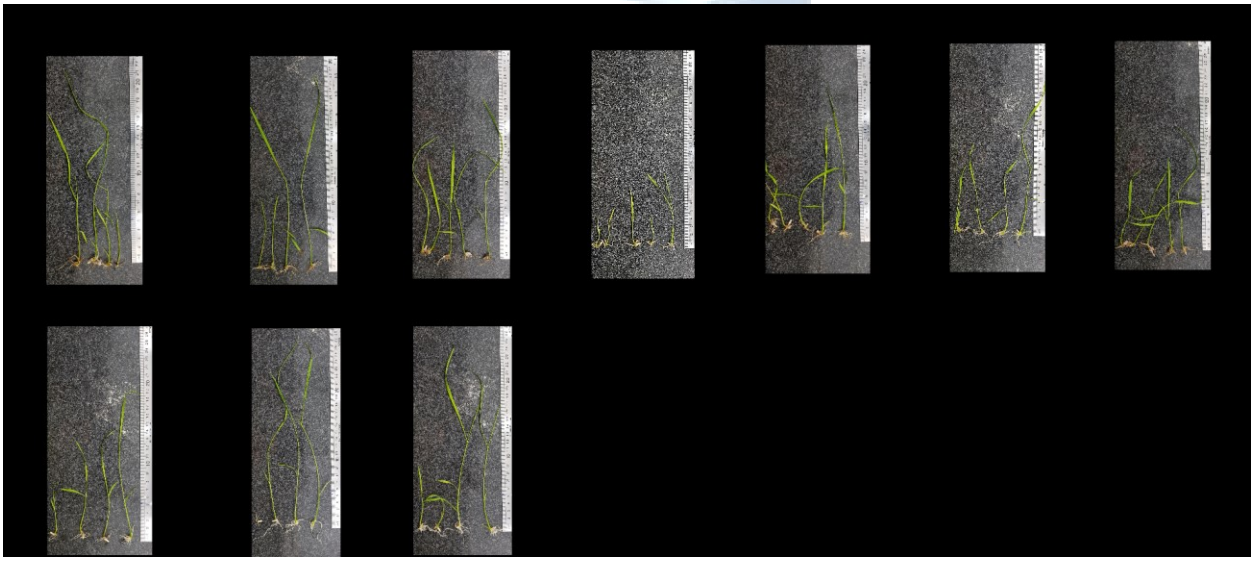
Supplementary file



Results of Rice seedling growth under the different sterilization conditions and variety of UHDs treatments. Samples infected with bacteria or fungi were not imaged.



Results of Rice seedling growth under the different sterilization conditions and variety of placebo treatments. Samples infected with bacteria or fungi were not imaged.

Received: Jan 18,2020. Accepted: Apr 14 ,2020.