agronomy

MDPI

Article

Quantitative Determination of the Effects of He–Ne Laser Irradiation on Seed Thermodynamics, Germination Attributes and Metabolites of Safflower (*Carthamus tinctorius* L.) in Relation with the Activities of Germination Enzymes

Rashida Perveen [1], Xiukang Wang [2,*], Yasir Jamil [1], Qasim Ali [3,*], Shafaqat Ali [4,5,*], Muhammad Qaisar Zakaria [6], Muhammad Afzaal [7], Rehan Ahmed Kasana [3], Muhammad Hamzah Saleem [8] and Sajid Fiaz [9]

1 Department of Physics, University of Agriculture, Faisalabad 38040, Pakistan; rashida_phy@yahoo.com (R.P.); yasirjamil@yahoo.com (Y.J.)
2 College of Life Sciences, Yan'an University, Yan'an 716000, China
3 Department of Botany, Government College University, Faisalabad 38000, Pakistan; rehangujar786@gmail.com
4 Department of Environmental Sciences and Engineering, Government College University, Faisalabad 38000, Pakistan
5 Department of Biological Sciences and Technology, China Medical University, Taichung 40402, Taiwan
6 Department of Physics, The University of Lahore (Sargodha Sub-Campus), Sargodha 40100, Pakistan; muhammad.zakaria@phys.uol.edu.pk
7 Department of Botany, University of Salford, 43 Crescent, Salford M5 4WT, UK; afzaal1@edu.salford.ac.uk
8 College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China; saleemhamza312@webmail.hzau.edu.cn
9 Department of Plant Breeding and Genetics, The University of Haripur, Haripur 22620, Pakistan; sfiaz@uoh.edu.pk
* Correspondence: wangxiukang@yau.edu.cn (X.W.); qasimbot_uaf@yahoo.com (Q.A.); shafaqataligill@yahoo.com (S.A.)

check for updates

Citation: Perveen, R.; Wang, X.; Jamil, Y.; Ali, Q.; Ali, S.; Zakaria, M.Q.; Afzaal, M.; Kasana, R.A.; Saleem, M.H.; Fiaz, S. Quantitative Determination of the Effects of He–Ne Laser Irradiation on Seed Thermodynamics, Germination Attributes and Metabolites of Safflower (*Carthamus tinctorius* L.) in Relation with the Activities of Germination Enzymes. *Agronomy* **2021**, *11*, 1411. https://doi.org/10.3390/agronomy11071411

Academic Editors: Helen D. Skaltsa, Antonios Chrysargyris and Maria Konstantopoulou

Received: 6 June 2021
Accepted: 9 July 2021
Published: 14 July 2021

Abstract: The present investigation was undertaken to assess the effects of different doses (100, 300, and 500 mJ) of low power He–Ne laser (632.8 nm) irradiation on seed germination and thermodynamics attributes and activities of potential germinating enzymes in relation with changes in seed metabolites. He–Ne laser seed irradiation increased the amylase (Amy), protease (Pro) and glucosidase (Gluco) activities, with a significant improvement in seed thermodynamics and seed germination attributes. A fast increase was found in free fatty acids (FFA), free amino acids (FAA), chlorophyll (Chl), carotenoids (Car), total soluble sugars (TSS) and reducing sugars (RS) in laser treated seeds in parallel with fast decline in seed oil contents and total soluble proteins (TSP). Significant positive correlations were recorded in laser-induced enhanced seed energy levels, germination, activities of germination enzymes with levels of FAA, FFA, Chl, TSS and RS, but a negative correlation with the levels of TSP and oil. In conclusion, the seed treatment with 100 and 300 mJ He–Ne laser was more effective to improve the seed germination potential associated with an improvement in seed energy levels due to increased activities of germination enzymes due to the speedy breakdown of seed reserves to simple metabolites as building blocks.

Keywords: thermodynamics; metabolites; germination vigor; amylase; protease; glucosidase

1. Introduction

Among different factors responsible for better crop production, a good and uniform crop stand establishment is of prime importance [1]. It depends on fast and speedy seed germination and seedling emergence [2]. The better, fast and uniform seed germination as well as seedling emergence is the function of the activities of seed metabolic enzymes [1].

Various methods and techniques are being currently used to increase/speed up the seed germination for uniform crop stand leading to better final crop yield [2]. These methods are categorized as biological, chemical and physical ones [3]. Among these, the interest in physical methods are increasing. These include irradiation with low-power non-destructive laser, microwaves, ultrasound, as well as magnetic fields. These are considered as most suitable due to their low cost, without any side effects to other organisms, and their nature-friendly emissions [4]. For example, it was found that germination of wet lettuce seeds increased when treated with low doses of red light [5], and it was depicted that it is due to the action of red light on membrane photoreceptors known as phytochromes also present in seeds that are sensitive to light, which have a potential role in seed germination [6–9]. In some earlier studies, it has been found that some metabolic processes during seed germination and activity of enzymes involved in seeding growth significantly improved under infrared and visible light of various wavelengths [7,10,11].

Furthermore, it was found that laser irradiation of low doses are also effective to prolong the plant vegetative period and to delay the reproductive stage [12,13]. In earlier studies it was depicted that in corn seed irradiation with diode laser, CO_2 laser, argon laser and He–Ne laser were found effective in boosting seed germination, plant growth, and enhanced tolerance against pathogenic diseases [13–15]. Seed irradiation with lasers also provided protection to cells against damages by UV-B radiations [15–17]. Similarly, pre-treatment of seeds with low doses of lasers have been reported to influence the thermodynamic parameters of seeds [8,13]. Furthermore, irradiation of seeds with lasers has found useful for tomato [18,19], Chinese medicinal herb *Isatis indogotica* [13], maize [12], spring barley [20], green gram [21], broad bean [16], sunflower [8], Chinese pine seeds [22] as well as wheat [23]. The studies reveal that the main effect of the applied low power laser was the increase in the seed internal energy due to exogenous applied laser. Furthermore, it has been found that the seeds with high stored energy showed speedy germination [23]. However, the suitable applied laser energy for better germination depends upon the type of seed and type of the applied laser. After laser seed treatment, the given laser energy is used to activate the germination related process at high-energy doses that results in speedy metabolic activities with the involvement of activities of germination enzymes that generates energy, improves seed thermodynamics and increases the simple metabolites that later on has use in seedling building blocks. In view of the information available, it can be explained that laser-applied influences on seed germination and seedling emergence is a complex mechanism that is the function of various metabolic activities. It still has various missing gaps that need to be fully explored. Moreover, as the literature depicts, both the positive and negative influences of different types of lasers, including the He–Ne lasers, on seed treatment for seed germination and seedling emergence are due to laser type, dose of applied laser and specific plant species. However, these laser-energy induced processes are laser-energy dose dependent [23,24].

Safflower (*Carthamus tinctorius* L.) is an annual plant belonging to family Asteraceae. Its flower is a source of natural dyes to dye the clothes. Its seed is a source of edible oil, which reduces risk of heart attack due to the high percentage (more than 70%) of linoleic acid that is effective to reduce the hardening of blood vessels. It is grown as a winter crop in Pakistan in the months of October–November. In these days, there are seed germination and seedling emergence problems, leading to uniform crop stands that are necessary for better crop production [25]. The seed germination of safflower normally takes 10–15 days and in months of seed sowing the seed containing soil surface dries between watering, creating a problem in proper seed germination [25]. So, it was hypothesized that He–Ne laser seed treatment might be effective to speed up the seed germination and emergence.

The present experiment was planned with the aim to assess the most effective laser dose for better seed germination and seedling vigor of safflower, in relation with changes in activities of germination enzymes, seed thermodynamics and the changing trends in seed metabolite levels. Until now very little or no work has been reported in relation to studying the enhancement of seed germination of safflower (*Carthamus tinctorius* L.)

through He–Ne laser irradiation, considering seed thermodynamics and germination in relation with activities of germination enzymes and the seed metabolite levels.

2. Materials and Methods

Safflower (*Carthamus tinctorius* L.) seeds were purchased from Ayub Agricultural Research Institute, Faisalabad, Pakistan. Before laser irradiation the seeds were soaked in distilled water for three hours, then the seeds were air-dried after absorbing the excess water using a blotting paper. Seeds were irradiated with continuous wave portable He–Ne laser. The laser (Model No. 1508P-1256, JDS Uniphase USA, wavelength 632.8 nm and beam diameter 1.5 mm) was used for seed irradiation. The laser output power was measured using power/energy meter (Quantel, France). The seeds were irradiated one by one with He–Ne laser beam with energies of 100, 300 and 500 mJ by following the procedure reported by Chen et al. [13]. For irradiation purposes, more uniform and healthy seeds were selected.

2.1. Measurement of Seed Thermodynamic Parameters

Seed thermodynamic parameters were measured using PARR Oxygen Bomb Calorimeter, 6000 Series, Moline Illinoise, USA. From each treated seed lot, ten seeds (ca. 0.5 g) were taken in calorimeter at constant temperature (25 °C). The thermograms of germinating seeds were recorded continuously up to 72 h after taken in calorimeter. Then different thermodynamic attributes of seeds were using different formulas. The change in enthalpy (ΔH) was calculated from thermogram area of germinating seeds i.e., (ΔH = R *ptdt*), entropy generation $(\Delta S)_c = \Delta H$ divided by absolute temperature (T), entropy flux $(\Delta S)_e = -(\Delta S)_c$, entropy generation ratio $(\Delta S)_c/\Delta t$ is $(\Delta S)_c$ divided by change in time (Δt) and entropy flux $(\Delta S)_e/\Delta t$ is $-(\Delta S)_e$ divided by Δt.

2.2. Estimation of Seed Germination Attributes

For the estimation of different germination attributes, the experimental setup was arranged in the research area of the Department of Botany, University of Agriculture Faisalabad, under natural environmental conditions. The design of the experiment was CRD with three replications of each treatment. The laser irradiated seeds were sown in plastic pots (25 cm diameter × 30 cm height) filled with properly washed river sand and supplied with full strength Hoagland's nutrient solution. Ten seeds were sown in each plastic pot. Before sowing the seeds were treated with 0.05% $HgCl_2$ solution for five minutes and washed with distilled water for ten minutes. Then the pots were kept in a glass house at 25.8 °C. After germination of seeds, light of approximately 900 mol m^{-2} s^{-1} μ "photosynthetic photon flux" was supplied to the seedlings for 8 h day^{-1} as daily photoperiod. The germinated seeds were counted on daily basis until the constant count was reached. The instructions given in Handbook of the Association of Official Seed Analysis [26] were followed. Then different attributes regarding seed germination and vigor were estimated from recorded data.

2.2.1. Germination Percentage

Germination percentage of seeds was recorded on alternate days for 5 days and calculated by using the formula:

$$\text{Germination \% age} = (\text{Number of germinated seeds}/\text{total number of seeds}) \times 100$$

2.2.2. Days to 50% Germination

Days to 50% seedling emergence (E_{50}) was worked out following the formula of Coolbear et al. [27]:

$$E_{50} = t_i + [(N/2 - n_i)\,(t_j - t_i)/n_j - n_i]$$

where N represents the final number of seeds emerged, and n_i and n_j represents the cumulative number of seeds emerged by adjacent counts at times t_i and t_j, respectively, when $n_i < N/2 < n_j$.

2.2.3. Mean Emergence Time

Mean emergence time (MET) was calculated following Ellis and Roberts [28]. The equation used for estimation of MET is as under:

$$\text{MET} = (\Sigma\, Dn/\Sigma\, n)$$

where n represents the number of seeds, emerged on day D, and D represents the number of days from the initiation of seed germination.

2.2.4. Coefficient of Uniformity of Emergence (CUE)

CUE was worked out following Bewley and Black [29] using the following equation:

$$\text{CUE} = \Sigma\, n/\Sigma\, [(t^{/} - t)^2 \times n]$$

In the equation above t represents the time in days, starting from the day of sowing, and n is the number of seeds that have completed emergence on day t, and $t^{/}$ represents the mean emergence time (MET).

2.2.5. Emergence Index

Emergence index (EI) was calculated following the method described in the Association of Official Seed Analysis [30] using the following formula:

$$\text{EI} = (\text{No. of germinated seeds/days of first count}) + \ldots\ \ldots$$

$$+ (\text{No. of germinated seeds/ days of final count})$$

2.2.6. Germination Energy

Energy of germination (GE) was calculated on the 4th day after sowing of seeds following Ruan et al. [31]. The percentage of germinating seeds 4 d after sowing is relative to the total number of seeds tested.

2.3. Activities of Seed Germination Enzymes

He–Ne laser pretreated seeds of safflower were surface sterilized for 5 min with 0.1% solution of $HgCl_2$. Then the seeds were washed properly in distilled water. After washing the sterilized seeds was placed in 14 cm glass Petri-dishes containing double-lined filter paper. Ten mL of Hoagland's nutrient solution were used for each Petri-dish and four replications of each treatment (radiated and un-radiated) were placed in a completely randomized design in the growth room at 26 ± 2 °C, then the seeds allowed to germinate in dark. After 24 h of sowing of seeds, fresh samples of each treatment were collected with 24 h interval up to 72 h for the assay of the activities of α-amylase and protease.

2.3.1. Estimation of Amylase Activity

Ten seedlings from each replicate were ground properly in a pestle and mortar. Extraction was carried out with cold 1% NaC1 solution prepared in 0.2 mM phosphate buffer (pH 5.5). Then the homogenate was centrifuged for 10 min at 10,000 rpm and the supernatant used for the assay of enzymes following Chrispeel and Varner [32]. The activity of enzymes was expressed as mg of starch hydrolyzed g^{-1} fresh weight h^{-1}.

2.3.2. Estimation of Protease Activity

Protease activity was appraised following Ainouz et al. [33]. From each replicate five seedlings were ground in a pestle and mortar. The extraction was carried out with cold

1% NaCI solution prepared in 0.2 m*M* phosphate buffer (pH 7.0). Then the homogenate was centrifuged for 30 min at 12,000 rpm. One ml of the supernatant in 5 mL of 1% casein solution in 0.2 M phosphate buffer (pH 6.0) was incubated at 50 °C. After one hour the reaction was terminated with 1 mL of 40% TCA (trichloroacetic acid) solution. The proteolytic activity in TCA soluble fraction after reaction with Folin phenol reagent was measured at 570 nm following Lowry et al. [34].

2.3.3. Estimation of Glucosidase Activity

The incubation mixture contained 0.1 mL of maltose of desired concentration, prepared in McIlvaine buffer [35], pH 5, 0.3 mL of the same buffer, and 0.1 mL of glucosidase preparation. The reaction was started by the addition of enzyme. The assay mixture was incubated for 30 min at 37 °C. The glucosidase activity was determined from the glucose liberated from maltose by the glucose oxidase method [36]. After glucose reagent addition the reaction mixture was incubated for 50 min at 37 °C, and the reaction was terminated by adding 2.5 mL of 5 N HCl with a vigorous mixing. The absorbance at 525 nm was measured.

2.4. Estimation of the Levels of Different Seed Metabolites during Germination at Different Time Intervals

2.4.1. Estimation of Chl Content of Germinating Seeds

The estimation of total chlorophyll content in the germinating seeds and seedlings was performed following Arnon [37]. Briefly, fresh germinating seeds (0.25 g) were homogenized in 80% acetone and centrifuged at 10,000× *g* the absorbance of the supernatant was read using a spectrophotometer (Hitachi U-2001, Tokyo, Japan) at 645, 663 and 480 nm. The Chl content was measured following the equation:

$$\text{Total chl } (\text{mgg}^{-1}) = [(0.0202 \times A_{645}) + (0.00802 \times A_{663}) \times 10]/\text{sample weight}$$

Here A is the absorbance at respective wavelength.

2.4.2. Estimation of Carotenoid Content of Germinating Seeds

For the estimation of total carotenoid content of germinating seeds, acetone-hexane (4:6) solvent (10 mL) was added on 0.25 g sample and homogenized well. Then the homogenate was settled down of two prepared phases, the absorbance of the upper phase was read using a spectrophotometer Hitachi U-2001, Tokyo, Japan) at 480, 645 and 663 nm wavelengths. Total carotenoid content was calculated according to Kirk and Allen [38] using the equation:

$$\text{Total carotenoids } (\text{mgg}^{-1}) = A_{480} + (0.114 \times A_{663} - 0.638 \times A_{645})$$

2.4.3. Estimation of TSS and RS Contents of Germinating Seeds

TSS and RS from the samples were extracted following Tonguç et al. [39]. The content of TSS was estimated following Dubois et al. [40] using phenol sulfuric acid assay and RS content was estimated following the Somogyi [41]. The quantification of TSS and RS was performed using a standard curve prepared from pure glucose.

2.4.4. Estimation of TSP and FAA Contents of Germinating Seeds

The method described by Larson and Beevers [42] was used for the extraction of protein from the germinating seeds and fresh leaf (0.5 g) was homogenized in 5 mL phosphate buffer (pH 7) in a pre-chilled pestle and mortar according to Bradford [43]. After centrifugation, 100 µL of the supernatant was mixed with 5 mL of the Bradford reagent. The absorbance of the mixture was noted at 595 nm using a spectrophotometer. The free amino acids from the germinating seeds were extracted using the method as described by Noctor et al. [44] and content was estimated using ninhydrin method as ascribed by Lee and Takahashi [45]. Standard curve was prepared using L-valine as standard.

2.4.5. Estimation of Oil and FFA Contents of Germinating Seeds

Nuclear magnetic resonance (NMR, Brükermqone) was employed for the estimation of oil content from the germinating seeds and the method described by Lowry and Tinsley [46] was employed for the estimation of the free fatty acid content of lipids. Samples (0.5 g) were extracted with hexane and the extracted lipids (4 µL) were dissolved in 5 mL benzene. Cupric acetate-pyridine reagent (1 mL) was added, and the samples were vortexed for 90 s. After centrifugation, the 3 mL of the supernatant were used to determine the free fatty acid contents of the samples. The total free fatty acid content of samples (%) was determined as oleic acid equivalents.

2.5. Statistical Analysis

To find out the significant variation among treatments of studied attributes CoStat Computer Program (window version 6.303, PMB 320, Monterey, CA, 93940 USA) was used. The Least Significant Difference Test (LSD) at 5% level of probability was used to find out the significant differences among means of studied attributes. XLSTAT Computer Program was used for PCA analysis and to find out correlations among studied attributes.

3. Results

3.1. Effect of He–Ne Laser Energy on Seed Internal Energy during Germination

Seed internal energy increased significantly of safflower seeds irradiated with different doses of He–Ne laser (Figure 1). This increase in seed energy was recorded in both laser treated, and non-treated seeds after starting of seed germination and the maximum increase was recorded after 48 h of seed germination. However, this increase in seed internal energy was comparatively more in seeds irradiated with He–Ne laser in comparison with non-treated ones. Comparatively, the maximum increase in seed internal energy was in seeds treated with 100 mJ followed by 300 mJ.

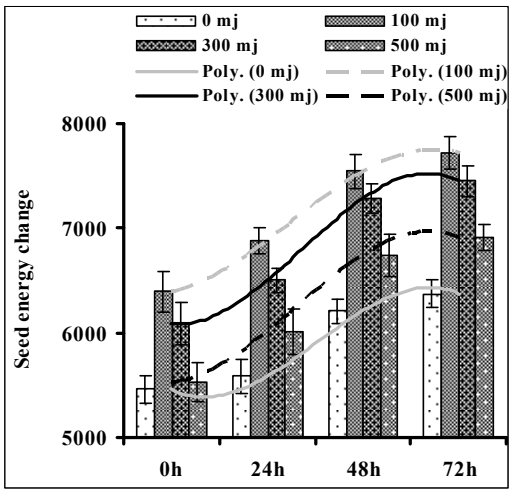

Figure 1. Seed energy during three days of germination of safflower seeds irradiated with He–Ne laser of different energies (mean ± SE; n = 4); (here poly. (0 mJ), poly. (100 mJ) . . . corresponds to polynomial regression to find out the trend).

3.2. Effect of He–Ne Laser Energy on Seed Thermodynamics during Germination

Data presented in Table 1 for different thermodynamic attributes of germinating seeds shows that seed irradiation with different He–Ne laser doses significantly improved them. Regarding seed enthalpy (ΔH), the maximum increase was found in seeds treated with a 500 mJ energy dose of He–Ne laser that was not significantly different with other energy doses. Like enthalpy, other thermodynamic attributes were also increased due to the He–Ne laser seed treatment. Increase in other thermodynamic parameters was also observed in laser pretreated seeds during germination. For example, entropy generation

$[(\Delta S)_c]$ and entropy generation ratio $[(\Delta S)_c/\Delta t]$ improved by 53% while entropy flux $[(\Delta S)_e]$ and entropy flux ratios $[(\Delta S)_e/\Delta t]$ increase by 51% as compared with non-treated ones. Comparatively the seeds treated with 300 and 500 mJ of energies doses showed more increase and this increase in these attributes was more after 24–48 h of seed sowing.

Table 1. Different seed thermodynamic attributes of germinating safflower seeds irradiated with He–Ne laser of different energies (mean ± *S.E*).

	He–Ne Laser Energy Doses (mJ)				
	0	**100**	**300**	**500**	**LSD 5%**
ΔH (J)	108.57 ± 3.43 [a]	158.15 ± 2.98 [b]	162.83 ± 3.82 [b]	164.58 ± 4.60 [b]	10.01
(ΔS)c (J/K)	0.36 ± 0.011 [a]	0.530 ± 0.12 [b]	0.54 ± 0.013 [b]	0.55 ± 0.015 [b]	0.03
(ΔS)e (J/K)	−0.36 ± 0.011 [a]	−0.531 ± 0.012 [b]	−0.546 ± 0.013 [b]	−0.552 ± 0.015 [b]	0.03
(ΔS)c/Δt (μW/K)	1.41 ± 0.045 [a]	2.048 ± 0.040 [b]	2.108 ± 0050 [b]	2.13 ± 0.061 [b]	0.13
(ΔS)e/Δt (μW/K)	−1.41 ± 0.045 [a]	−2.05 ± 0.038 [b]	−2.108 ± 0.050 [b]	−2.14 ± 0.052 [b]	0.12

Mean values with different letters in superscript in a row differ significantly at $p \leq 0.05$.

3.3. Effect of He–Ne Laser Energy on Seed Germination Related Attributes

Seed irradiation with different energies of He–Ne laser significantly affected the different studied germination attributes (Figure 2). Significant reduction in mean emergence time ($p \leq 0.001$) and time to 50% germination (E_{50}) ($p \leq 0.01$) was recorded and the maximum reduction was found in E_{50} and mean emergence time in seeds irradiated with 300 mJ followed by 100 mJ dose of energy, respectively. However, there was a significant ($p \leq 0.001$) increase in CUE and GI due to seed irradiation with the He–Ne laser and the maximum was found in the seeds irradiated with the 100 mJ energy dose, followed by the 300 mJ energy dose. Similarly, seed G% and GE was maximum at the 100 mJ energy dose of He–Ne laser energy dose.

3.4. Effect of He–Ne Laser Energy on Activities of Seed Germination Enzymes

Seed treatment of different energies with the He–Ne laser significantly influenced the activities of the studied seed germination enzymes. The seed Amy activity increased in the laser treated germinating seeds and the maximum increase was found in the seeds treated with 300 mJ of laser energy dose. The maximum increase in seed Amy activity was found after 48 h of seed sowing in all treatments, but this increase was maximum in germinating seeds treated with 300 mJ energy dose. However, a little decrease in activity after 72 h of seed sowing was recorded in laser treated as well as non-treated seeds, but the activity was more in laser treated seeds as compared to non-treated ones (Figure 3).

A significant increase in seed Pro activity was also found due to seed treatment with He–Ne laser. Among different applied laser doses, the 300 mJ energy dose was found most effective. The maximum increase in seed Pro activity was found during 48–72 h after seed sowing in all treatments and then a decreasing trend was found. However, this decrease in seed Pro activity was more in non-irradiated seeds as compared with irradiated seeds. Furthermore, increased activities of Amy and Pro at 300 mJ level of laser energy were positively related with the seed G% (Figure 3).

Seed Gluco activity increased significantly due to the He–Ne laser seed treatment; comparatively, the 300 mJ energy dose was found effective and maximum activity of Gluco was recorded after 48 h of seed sowing and a decreasing trend in the activity was found after 72 h of seed sowing. However, this decrease in seed Gluco activity was more in non-treated seeds as compared with laser treated seeds. Furthermore, increased activities of Amy and Pro at 300 mJ level of laser energy were positively related with the seed germination percentage (Figure 4).

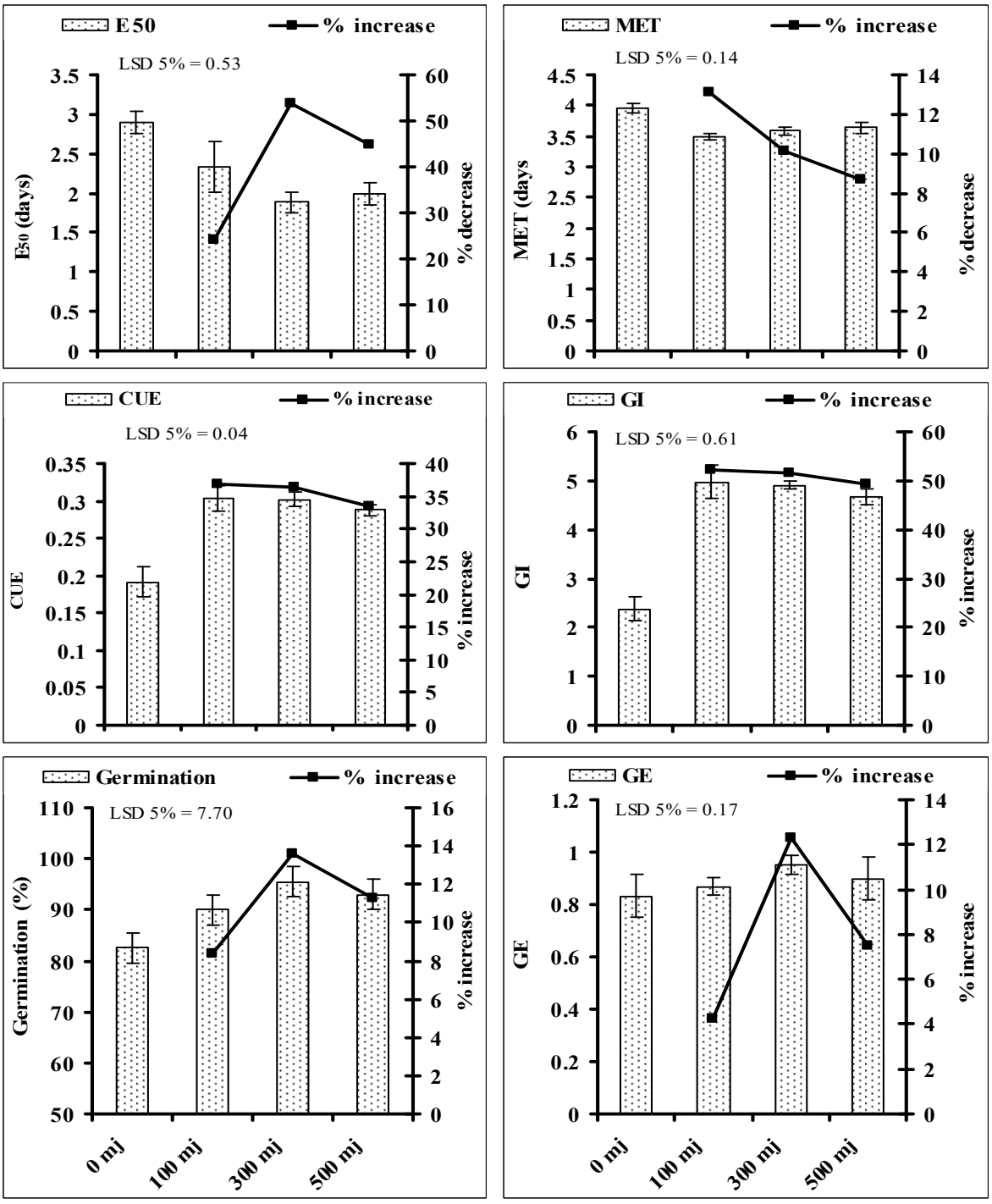

Figure 2. Different germination attributes of safflower seeds irradiated with laser of different energies (mean ± SE; n = 4). GE = germination energy; GI = germination index; CUE = coefficient of uniformity of emergence; MET = mean emergence time; E_{50} = time to 50% emergence; % increase or decrease = increase or decrease in laser treated seeds relative to non-treated ones (0 mJ).

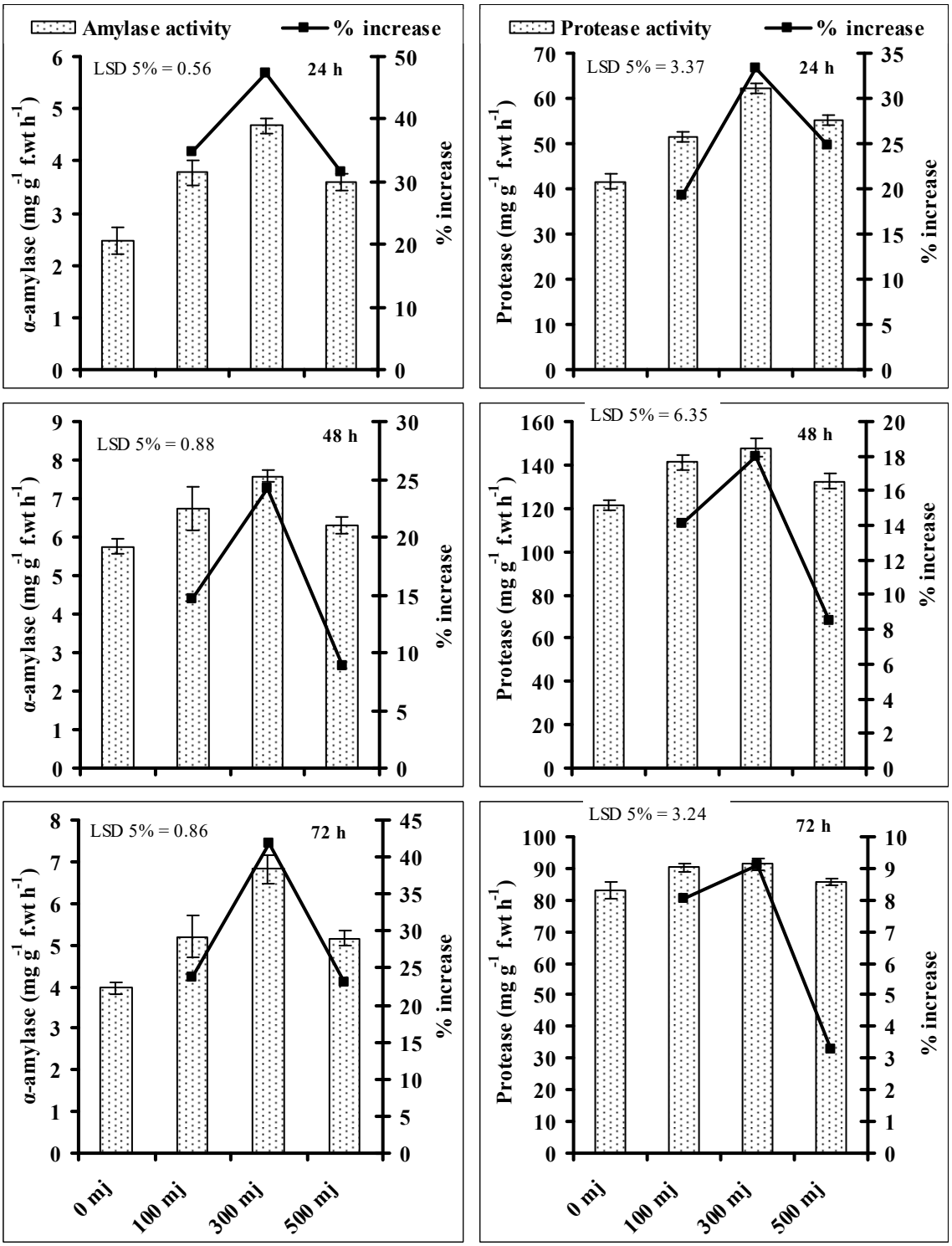

Figure 3. α-Amylase and protease activities during germination of safflower seeds irradiated with He–Ne laser of different energies (mean ± SE; $n = 4$).

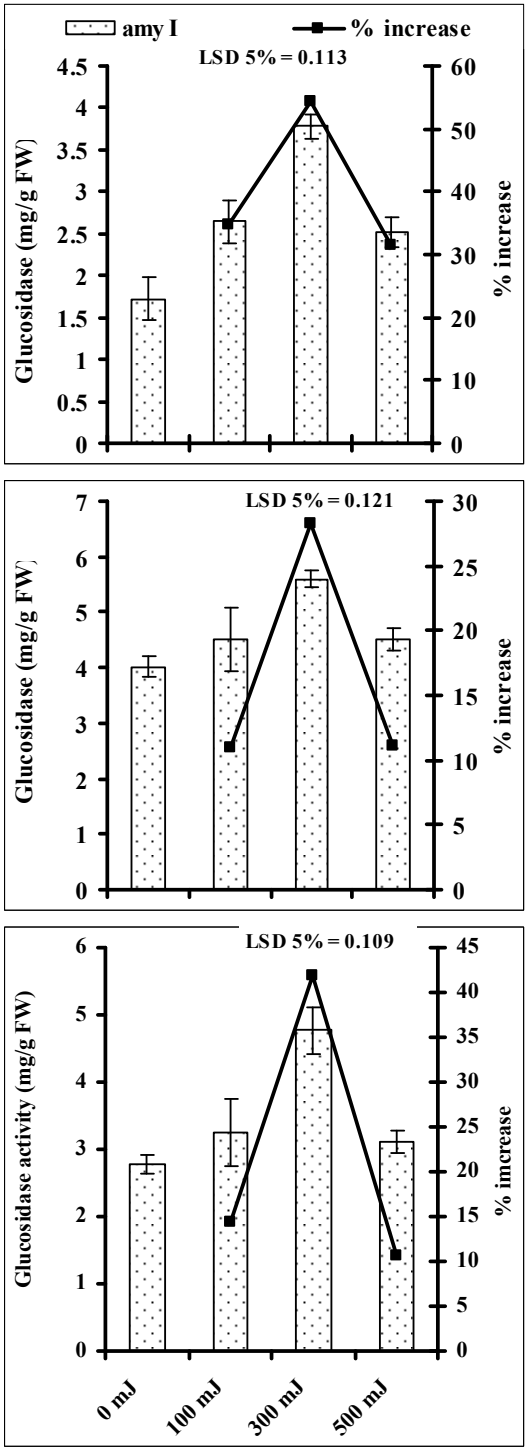

Figure 4. Activity of glucosidase during germination of safflower (*Carthamus tinctorious* L.) seeds irradiated with He–Ne laser of different energies (mean ± SE; $n = 4$).

3.5. Effect of He–Ne Laser Energy on Seed Metabolites during Seed Germination

Data presented in Table 2 for the Chl and carotenoids biosynthesis shows that He–Ne laser seed treatment significantly increased their biosynthesis, but the extent of increase was laser dose specific. This increased biosynthesis of Chl and carotenoids in seeds and was found maximum at the 300 mJ laser energy dose, followed by 100 mJ compared to non-treated ones, and the minimum increase was recorded at the 500 mJ laser energy dose (Table 2).

Table 2. Seed/seedlings chlorophyll, carotenoids, total soluble sugars and reducing sugar contents at different time intervals during germination when grown after irradiation with different doses of He–Ne laser (mean ± SE; n = 4).

Chlorophyll (mg/g of Germinating Seeds) (LSD 5% = 0.25)					Carotenoids (µg/g of Germinating Seeds) (LSD 5% = 0.065)			
Time Interval (h)	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**
0	^{w} 0 ^{c}	^{w} 0 ^{b}	^{w} 0 ^{c}	^{w} 0 ^{d}	^{w} 0 ^{c}	^{w} 0 ^{c}	^{w} 0 ^{c}	^{w} 0 ^{c}
24	^{w} 0.09 ± 0.002 ^{c}	^{w} 0.16 ± 0.070 ^{b}	^{w} 0.122 ± 0.005 ^{c}	^{w} 0.114 ± 0.005 ^{d}	^{w} 0.014 ± 0.006 ^{c}	^{w} 0.042 ± 0.003 ^{b}	^{w} 0.034 ± 0.001 ^{c}	^{w} 0.028 ± 0.002 ^{c}
48	^{w} 0.19 ± 0.010 ^{c}	^{w} 0.42 ± 0.037 ^{b}	^{x} 0.710 ± 0.029 ^{b}	^{w} 0.410 ± 0.031 ^{c}	^{x} 0.054 ± 0.003 ^{c}	^{x} 0.110 ± 0.006 ^{b}	^{w} 0.142 ± 0.005 ^{b}	^{x} 0.113 ± 0.006 ^{b}
72	^{w} 0.61 ± 0.028 ^{b}	^{x} 1.09 ± 0.073 ^{a}	^{x} 1.221 ± 0.065 ^{a}	^{x} 1.082 ± 0.060 ^{b}	^{z} 0.150 ± 0.021 ^{b}	^{y} 0.260 ± 0.015 ^{a}	^{w} 0.349 ± 0.016 ^{a}	^{x} 0.280 ± 0.014 ^{a}
96	^{w,x} 1.08 ± 0.055 ^{a}	^{w,x} 1.28 ± 0.076 ^{a}	^{w} 1.429 ± 0.069 ^{a}	^{w} 1.371 ± 0.069 ^{a}	^{y} 0.280 ± 0.013 ^{a}	^{x} 0.320 ± 0.016 ^{a}	^{w} 0.407 ± 0.017 ^{a}	^{x} 0.329 ± 0.018 ^{a}
Total Soluble Sugars (mg/g of Germinating Seeds) (LSD 5% = 6.56)					**Reducing Sugars (mg/g of Germinating Seeds) (LSD 5% = 4.10)**			
Time Interval (h)	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**
0	^{w} 16.25 ± 0.92 ^{e}	^{w} 18.24 ± 0.88 ^{e}	^{w} 20.35 ± 0.75 ^{e}	^{w} 16.45 ± 0.88 ^{e}	^{w} 5.90 ± 0.25 ^{d}	^{w} 5.99 ± 0.31 ^{d}	^{w} 6.15 ± 0.33 ^{d}	^{w} 6.05 ± 0.33 ^{d}
24	^{y} 17.31 ± 0.88 ^{d}	^{x} 24.33 ± 0.99 ^{d}	^{w} 32.25 ± 1.33 ^{d}	^{x,y} 21.12 ± 0.55 ^{d}	^{w} 7.25 ± 0.28 ^{c,d}	^{w} 7.12 ± 0.33 ^{d}	^{w} 8.11 ± 0.35 ^{d}	^{w} 7.43 ± 0.25 ^{d}
48	^{x} 34.33 ± 1.55 ^{c}	^{x} 39.55 ± 1.95 ^{c}	^{w} 52.21 ± 2.15 ^{c}	^{x} 38.23 ± 1.55 ^{c}	^{y} 11.35 ± 0.58 ^{c}	^{yx} 14.65 ± 0.61 ^{c}	^{w} 20.23 ± 0.61 ^{c}	^{x} 15.65 ± 0.55 ^{c}
72	^{x} 47.56 ± 1.99 ^{b}	^{x} 51.65 ± 2.25 ^{b}	^{w} 67.65 ± 1.99 ^{b}	^{x} 49.33 ± 1.55 ^{b}	^{z} 16.35 ± 0.91 ^{b}	^{x,y} 34.15 ± 1.82 ^{b}	^{w} 42.11 ± 1.55 ^{b}	^{y} 31.12 ± 0.99 ^{b}
96	^{x} 58.65 ± 1.97 ^{a}	^{x} 64.33 ± 3.01 ^{a}	^{w} 72.15 ± 3.15 ^{a}	^{x} 61.23 ± 1.85 ^{a}	^{y} 32.11 ± 0.99 ^{a}	^{x} 43.00 ± 1.98 ^{a}	^{w} 53.23 ± 1.99 ^{a}	^{x} 41.25 ± 2.01 ^{a}

Mean values with different letters in a column (with letters a, b, c . . .) and in a row (with letters w, x, y . . .) in superscript differ significantly to each other.

Seed TSS and RS during germination increased significantly in safflower seeds due to the He–Ne laser seed treatment, but this increase was laser dose specific. The more increase in TSS and RS was recorded due to seed treatment with 300 mJ He–Ne laser dose followed by 100 and 500 mJ laser doses and the minimum increase in TSS and RS was recorded due to seed treatment with the 500 mJ dose of laser energy comparative to the non-treated ones (Table 2).

Data presented in Table 3 shows that seed TSP and oil contents decrease significantly during seed germination. Seed laser treatment with different levels of He–Ne laser significantly speed up the decrease in TSP and oil contents, but the extent of decrease was laser dose specific. The speedier decrease in these metabolites during seed germination was recorded in seeds irradiated with the 300 mJ laser energy dose as compared with other doses, and the minimum decrease was at 500 mJ laser energy dose.

A significant increase in seed contents of FAA and FFA increased significantly during seed germination, and this accumulation in seed FAA and FFA contents further increased with time. Seed treatment with He–Ne laser further sped up the increment in seed FAA and FFA contents during seed germinations up to 72 h, but a decrease in FAA and FFA was recorded after 72 h of seed sowing. The more accumulation in these metabolites was found due to seed treatment with the laser dose of 300 mJ energy as compared with other laser doses and non-treated ones (Table 3).

Table 3. Seed/seedlings total proteins, total free amino acid, seed oil and free fatty acid contents at different time intervals during germination when grown after irradiation with different doses of He–Ne laser (mean ± SE; n = 4).

Total Protein (mg/kg of Germinating Seeds) (LSD 5% = 62.40)					Total Free Amino Acids (mg/kg of Germinating Seeds) (LSD 5% = 51.70)			
Time Interval (h)	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**
0	^{w} 688 ± 8.9 ^{a}	$_{w}$ 699 ± 5.56 ^{a}	^{w} 711 ± 4.45 ^{a}	^{w} 710 ± 5.6 ^{a}	^{w} 55 ± 1.99 ^{d}	^{w} 67 ± 2.33 ^{e}	^{w} 63 ± 2.34 ^{e}	^{w} 67 ± 2.34 ^{d}
24	^{w} 600 ± 5.7 ^{b}	^{w} 570 ± 4.99 ^{b}	^{w} 540 ± 3.65 ^{b}	^{w} 592 ± 4.3 ^{b}	^{x} 75 ± 1.65 ^{d}	^{w,x} 121 ± 3.21 ^{d}	^{w} 135 ± 3.15 ^{d}	^{w,x} 99 ± 3.25 ^{d}
48	^{w} 460 ± 4.5 ^{c}	^{x,y} 340 ± 7.11 ^{c}	^{y} 305 ± 4.35 ^{c}	^{x} 401 ± 6.3 ^{c}	^{x} 175 ± 2.24 ^{c}	^{w,x} 225 ± 3.22 ^{c}	^{w} 242 ± 2.99 ^{c}	^{w,x} 201 ± 3.43 ^{c}
72	$_{w}$ 290 ± 6.3 ^{d}	^{w,x} 240 ± 5.11 ^{d}	^{x} 215 ± 7.12 ^{d}	^{w,x} 262 ± 8.0 ^{d}	^{x} 345 ± 2.65 ^{b}	^{w} 405 ± 3.21 ^{b}	^{w} 435 ± 3.45 ^{b}	^{w} 399 ± 3.45 ^{b}
96	$_{w}$ 230 ± 5.2 ^{d}	^{w,x} 190 ± 4.10 ^{e}	^{x,y} 165 ± 4.98 ^{d}	^{y} 215 ± 8.1 ^{e}	^{y} 499 ± 3.11 ^{a}	^{w} 601 ± 2.44 ^{a}	^{w} 605 ± 3.65 ^{a}	^{x} 561 ± 3.55 ^{a}
Oil Content (LSD 5% = 3.36)					**Free Fatty Acids (LSD 5% = 7.61)**			
Time Interval (h)	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**	**0 mJ**	**100 mJ**	**300 mJ**	**500 mJ**
0	^{w} 33.05 ± 2.16 ^{a}	^{w} 33.1 ± 0.13 ^{a}	^{w} 33.05 ± 0.12 ^{a}	^{w} 32.95 ± 0.14 ^{a}	^{w} 45 ± 2.33 ^{d}	^{w} 38 ± 2.44 ^{d}	^{w,x} 41 ± 2.45 ^{e}	^{x} 36 ± 2.78 ^{d}
24	^{w} 32.9 ± 3.25 ^{a,b}	^{x} 28.7 ± 0.08 ^{b}	^{y} 24.65 ± 0.32 ^{b}	^{w,x} 30.85 ± 0.22 ^{a}	^{w} 65 ± 1.55 ^{c}	^{w} 67 ± 2.34 ^{c}	^{w} 65 ± 2.44 ^{c}	^{w} 70 ± 1.56 ^{c}
48	^{w} 26.1 ± 2.14 ^{b}	^{x} 20.5 ± 0.11 ^{c}	^{y} 15.90 ± 0.22 ^{c}	^{w} 24.90 ± 0.11 ^{b}	^{x} 105 ± 2.34 ^{b}	^{w} 120 ± 2.54 ^{b}	^{w} 115 ± 2.45 ^{b}	^{w} 115 ± 1.67 ^{b}
72	^{w} 18.9 ± 0.15 ^{c}	^{x} 14.6 ± 0.21 ^{d}	^{y} 8.80 ± 0.23 ^{d}	^{w,x} 15.85 ± 0.10 ^{c}	^{x,y} 125 ± 1.65 ^{a}	^{x} 131 ± 2.56 ^{a}	^{w} 201 ± 2.36 ^{a}	^{y} 121 ± 1.22 ^{a}
96	^{w} 12.5 ± 0.08 ^{d}	^{w} 10.2 ± 0.11 ^{e}	^{x} 5.45 ± 0.12 ^{e}	^{w} 9.40 ± 0.04 ^{d}	^{x} 120 ± 1.45 ^{a}	^{w} 128 ± 2.45 ^{a}	^{x} 120 ± 2.34 ^{b}	^{wx} 123 ± 2.33 ^{a}

Mean values with different letters in a column (with letters a, b, c . . .) and in a row (with letters w, x, y . . .) in superscript differ significantly to each other.

3.6. Correlations of Studied Attributes

Correlation data regarding the activities of germination enzymes (Amy, Pro, Gluco) at different time intervals, seed germination attributes and different thermodynamic attributes are presented in Table 4 and Figure 5. The activities of different germination enzymes are strongly correlated with each other at all time intervals during germination and positively correlated with GI, CUE, ΔH, (ΔS)c, GE, G% and (ΔS)c/Δt. However, these attributes are negatively correlated with MET, (ΔS)e, (ΔS)e/Δt, and E_{50} at all intervals. Data presented in Figure 5 further shows that the maximum contribution was of the components, F1 and F2 to find out the variation among all studied attributes. Among the components the major contribution was of F1 (85.56%) followed by F2 (11.39%) with accumulative contribution of 96.95%. The F1 component divided the studied attributes in three groups as encircled in Figure 5. The first group includes the activities of seed germination enzymes at different intervals, group II includes the GI, CUE, ΔH, (ΔS)c, GE, G% and (ΔS)c/Δt and the 3rd group include the MET, (ΔS)e, (ΔS)e/Δt, and E_{50}. The parameters given in group III are strongly negatively correlated with the parameters in group I and group II.

Table 4. Spearman's correlation coefficients for different seed germination attributes of safflower treated with different laser energies.

Variables	Amy (24)	Amy (48)	Amy (72)	Pro (24)	Pro (48)	Pro (72)	Gluco (24)	Gluco (48)	Gluco (72)
Amy (24)	1	0.972 ***	0.982 ***	0.967 ***	0.972 ***	0.916 ***	0.982 ***	0.942 ***	0.890 ***
Amy (48)	0.972 ***	1	0.977 ***	0.897 ***	0.972 ***	0.939 ***	0.985 ***	0.962 ***	0.945 ***
Amy (72)	0.982 ***	0.977 ***	1	0.961 ***	0.932 ***	0.863 ***	0.999 ***	0.988 ***	0.955 ***

Table 4. *Cont.*

Variables	Amy (24)	Amy (48)	Amy (72)	Pro (24)	Pro (48)	Pro (72)	Gluco (24)	Gluco (48)	Gluco (72)
Pro (24)	0.967 ***	0.897 ***	0.961 ***	1	0.882 ***	0.787 ***	0.950 ***	0.924 ***	0.852 ***
Pro (48)	0.972 ***	0.972 ***	0.932 ***	0.882 ***	1	0.985 ***	0.941 ***	0.882 ***	0.841 ***
Pro (72)	0.916 ***	0.939 ***	0.863 ***	0.787 ***	0.985 ***	1	0.880 ***	0.809 ***	0.780 ***
Gluco (24)	0.982 ***	0.985 ***	0.999 ***	0.950 ***	0.941 ***	0.880 ***	1	0.988 ***	0.960 ***
Gluco (48)	0.942 ***	0.962 ***	0.988 ***	0.924 ***	0.882 ***	0.809 ***	0.988 ***	1	0.988 ***
Gluco (72)	0.890 ***	0.945 ***	0.955 ***	0.852 ***	0.841 ***	0.780 ***	0.960 ***	0.988 ***	1
E 50	−0.902 ***	−0.781 ***	−0.871 ***	−0.972 ***	−0.801 ***	−0.694 **	−0.852 ***	−0.810 ***	−0.708 ***
MET	−0.811 ***	−0.727 **	−0.687 **	−0.738 **	−0.866 ***	−0.870 ***	−0.691 **	−0.571 *	−0.473 *
CUE	0.885 ***	0.784 ***	0.784 ***	0.854 ***	0.892 ***	0.860 ***	0.781 ***	0.679 **	0.575 *
G index	0.874 ***	0.767 ***	0.770 ***	0.849 ***	0.878 ***	0.844 ***	0.766 ***	0.663 **	0.555 *
G %	0.854 ***	0.728 **	0.752 ***	0.855 ***	0.839 ***	0.791 ***	0.743 **	0.643 **	0.525 *
G.E	0.855 ***	0.718 **	0.763 ***	0.880 ***	0.816 ***	0.751 ***	0.751 ***	0.660 **	0.539 *
ΔH	0.855 ***	0.718 **	0.763 ***	0.880 ***	0.816 ***	0.751 ***	0.751 ***	0.660 **	0.539 *
(ΔS)c	0.855 ***	0.718 **	0.763 ***	0.880 ***	0.816 ***	0.751 ***	0.751 ***	0.660 **	0.539 *
(ΔS)e	−0.855 ***	−0.718 **	−0.763 ***	−0.880 ***	−0.816 ***	−0.751 ***	−0.751 ***	−0.660 **	−0.539 *
(ΔS)c/Δt	0.855 ***	0.718 **	0.763 ***	0.880 ***	0.816 ***	0.752 ***	0.751 ***	0.660 **	0.539 *
(ΔS)e/Δt	−0.850 ***	−0.711 **	−0.757 ***	−0.877 ***	−0.809 ***	−0.744 **	−0.744 **	−0.653 **	−0.530 *

*, ** and *** = significant at 0.05, 0.01 and 0.001 levels respectively.

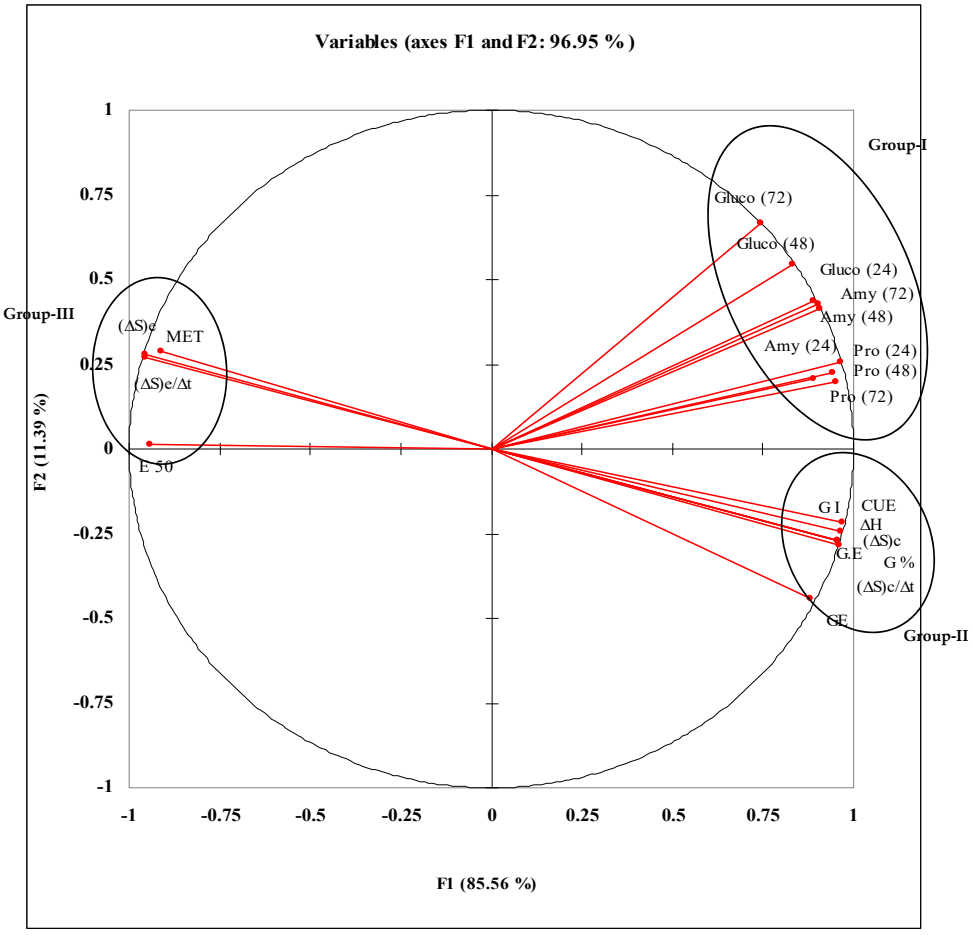

Figure 5. PCA of seed germination attributes of germinating enzymes of laser treated safflower seeds. List of abbreviations Δ*H* = enthalpy change; (Δ*S*)*c* = entropy generation; (Δ*S*)*c*/Δt = entropy generation ratio; (Δ*S*)*e* = entropy flux; (Δ*S*)*e*/Δt = entropy flux ratio; GE = germination energy; G % = germination percentage; G I = germination index; MET = mean emergence time; E_{50} = time to 50% seed germination; Amy (24) = activity of α-amylase after 24 h of seed sowing; Amyl (48) = activity of α-amylase after 48 h of seed sowing; Amy (72) = activity of α-amylase after 72 h of seed sowing; Pro (24) = activity of protease after 24 h of seed sowing; Pro (48) = activity of protease after 48 h of seed sowing; Pro (72) = activity of protease after 72 h of seed sowing; Gluco (24) = activity of glucosidase after 24 h of seed sowing; Gluco (48) = activity of glucosidase after 48 h of seed sowing; Gluco (72) = activity of glucosidase after 72 h of seed sowing.

4. Discussion

The responses of seeds to light irradiation are dependent on the light transmittance of seed coat, intensity of light and duration of exposure, which is the function of wavelength and energy dose [8,23,47]. Generally, all the biological, chemical and physical phenomena lead to thermal changes in system, that are of theoretical and practical importance. In living organisms, each metabolic process is associated with heat change. During seed germination and seeding growth the heat generation is a well-known example of thermogenesis that has the involvement of conversion of matter to energy [48]. However, in case of laser application to plant cells, the applied laser energy is stored by living tissues by radiation energy. Similarly, regarding the seed light treatment, where applied light energy is absorbed by seed living tissue that later on is being used in different growth processes in the form of chemical energy [8,18,49,50].

In present investigation, seed treatment with low-energy continuous wave He–Ne laser doses were investigated on seed activities of germination enzymes, germination parameters, different thermodynamic parameters, and the levels of metabolites of safflower. It was found that a rapid heat was evolved during germination after 48 h of sowing, rising to a maximum, and then with a decreasing trend after 72 h of seed sowing in all treatments. Moreover, the change in ΔH was recorded as maximum in seeds not pre-treated with 500 mJ laser energy dose, but regarding the physio-chemical attributes 100 and 300 mJ energy doses were most suitable. The same was in the case of grand energy change that was recorded at the maximum at 100 as well as at 300 mJ energy. ΔH which is related to internal energy is also known as 'growth heat effect'. In the present study, seed enthalpy change due to laser seed treatment is positively related to change in seed internal energy that takes place during seed germination. This ΔH is an indicator of internal molecular force during germination [51]. Moreover, it has also been found that ΔH is the function of entropy flux and free energy change, and the former one is considered as the of molecular randomness, positional uncertainty, arbitrariness and 'chaos' [52]. In the present study, the 500 mJ laser energy dose as compared with the 100 and 300 mJ laser doses was not suitable, because the extra energy in the form of 500 mJ given to seeds resulted in an adverse impact on biochemical reactions. In the present study, the thermograms of germinating seeds were obtained at constant temperature (25 °C). So, it can be concluded that He–Ne laser treated seeds were at higher temperatures during germination as compared with non-treated ones (with an increased ΔS of laser treated germinating seeds leading to increased ΔH) with a decrease in activities of biomolecules at the specific laser dose.

In general, it is found that seed irradiation with visible and infra-red light of various wavelength results in modulations in seed germination process [53]. In earlier studies, it was found that the germination of photosensitive buried seeds under field condition can be saturated with a milli second exposure with sunlight and a few seconds exposure with moonlight [10,47]. Furthermore, it was also found that in wet seeds of lettuce, irradiation with monochromatic red light (0.1–10 nmol m^{-2}) is sufficient to break seed dormancy [5] because red light directly effects the photochromes (pr and pfr), that have a direct role in seed germination [6,54,55]. Furthermore, it was reported that irradiation of dry dormant seeds with weak He–Ne laser (λ = 632.8 nm) is helpful in boosting up many germinations related metabolic reactions [8,23,56]. The literature has many studies regarding laser-induced modulations in optical, biochemical and electrochemical properties of seeds and most of them describe the laser stimulation on growth and seed germination related processes [8,13,17,56–62]. Irrespective to the positive influences of He–Ne laser seed treatment on seed germination and seedling emergence, it was found that other types of laser were also found effective to influence the seed germination potential. For example, Rasam et al. [63] reported an increase in germination percentage of hard wheat seeds irradiated with Nd:YAG pulsed laser at 532 nm, but the effects were laser dose specific, where the higher doses showed the negative effects on the studied attributes. Pulse laser was also found to be involved in improving seed germination in soybean when applied at 532 nm [64]. Germination in many plants also improved by irradiating the seeds with

diode laser, such as hard wheat seeds when applied at 650 nm and salt stressed wheat seeds when applied at 980 nm [65].

In the present study, seed G%, GI, CUE and GE increased and E_{50} and MET decreased due to laser seed treatment. Maximum positive response due to laser seed treatment in the studied germination attributes was recorded due to laser seed treatment with 100 and 300 mJ energies. The maximum increase in seed G%, GI, CUE and GE was 13.60, 52.26, 36.75 and 12.33%, respectively, in comparison with non-treated ones. A similar finding has also been found in sunflower [8], where 300 mJ energy level of He–Ne laser was found most effective. In some earlier studies, Vasilevski [66], Jamil et al. [23], Perveen et al. [8], and Muszyñski and Gladyszewska [7] reported an average increase of 20% to 35% in seed germination due to laser irradiation in wheat, sunflower and radish respectively. Furthermore, in some earlier studies it was reported that laser treated promotion in seed germination was due to the fact of enhanced levels of seed thermodynamics due to laser treatment in various plant species [8,13,14,67,68]. In their findings, they narrated that laser stimulated seed germination might be a fact of phytochrome-mediated light energy transformation due to phytochromes—part of photoreceptor system that is part of chloroplast membranes, mitochondrial and plasmalemma, as well as part of the cell nuclei. It is known that these phytochromes regulate the energy storage and transferring processes such as photosynthetic and oxidative phosphorylation [69]. Moreover, several studies reveal that endoplasmic reticulum, tonoplast, and plasmalemma have energy-transducing functions and have the ability to equalize the changes in membrane potential due to transforming light energy into electrochemical potential ($\Delta\mu H^+$) across the membranes [69,70]. Simply, we can say that in a living plant cell a well-organized membrane system is working that has the ability to transform and utilize the additional supplied energy up to an extent [66]. Studies reveal not only the positive influences of laser irradiation on seed germination, but also the negative impacts that have been found when the laser is applied in high doses, as despite the positive effects of laser there are reports that unsuitable doses of He–Ne laser may inhibit the germination and the emergence of seedlings [8,23,56,71]. The decreasing effects might be due to damaging effects of the high energy dose on the cellular membrane and activities of biomolecules [72].

In an earlier study, while working on maize and wheat Dinoeve et al. [12] reported positive influences of laser seed treatment on seed germination attributes including the seed germination energy and seed physiological state leading to both positive and negative influences on growth and development due to the function of the laser wavelength, intensity and its type. They explained that this might be due to the fact that laser treatment enhanced the activities of seed enzymes that speed up break down of macromolecules, resulting in generation of more heat energy and then the biological system works at a high-energy level due to the enhanced entropy level of the system. Comprehensively, it can be explained that seed treatment with laser led the system from higher order to disorder [73,74].

In the present study, laser seed treatment positively influenced the different studied thermodynamic parameters, germination percentage, GI, CUE and GE, MET and E_{50}. During the germination process the newly developed seedling uses the stored reserves in seeds for the purpose of energy and building blocks. These include sugars and amino acids that fulfill the cellular energy demand, building blocks for structural purposes as well as the biosynthesis of secondary metabolites [23,24]. These all processes are dependent on the activities of specific enzymes. In the present study, seed treatment with He–Ne laser of different energies promoted the biosynthesis of Chl, carotenoid, reducing and non-reducing sugars along with increased contents of free amino acids and free fatty acids, but decreased total soluble protein and oil content and the maximum improvement in breakdown or in biosynthesis of these metabolites was due to treatment with the 300 mJ laser dose and was maximum was after 48 h and 72 h of seed sowing. Moreover, the content of FAA, FFA, and sugars increased with a decrease in TSP and oil that is the function of the increased activities of amylolytic enzymes such as amylase, protease, glucosidases

that improved the breakdown of macromolecules to simple molecules with an improved energy generation. The best of these processes was found in seeds treated with 300 mJ laser dose, where maximum activities of amylolytic enzymes were recorded. The increase in chlorophyll and carotenoids might be best attributed to their better biosynthesis, with the availability of basic simple metabolites as building blocks. As a result, there was speedy germination and seedling emergence with better thermodynamics.

External environmental factors influence significantly physiological processes of growth and development that are limited with cellular enzyme activities and suitable laser doses have positive influences on activities of enzymes [8,15]. The present findings revealed that seed laser treatment with different energies enhanced the activities of amylases, proteases and glucosidases and the 300 mJ treatment was found most effective. These findings can be correlated with findings of previous studies on faba bean seeds of the variety Nadwilanski, as it was found that seed treatment with laser increased activity of the seed amylolytic enzymes during the early period of germination [75], and all applied laser doses were found equally effective. However, in some other studies it was found that lower doses of laser as seed treatment were more effective in boosting up the activities of germination enzymes as well as the biosynthesis of de-novo proteins [8,14,76]. Similarly, like the findings of present study, it was reported by Hong and Lin. [77] that laser treatment of seeds increased the activity of amylase in Bailan melon. In the present findings, the increase in activities of seed germination enzymes has a positive correlation with seed thermodynamic attributes such as ΔH, $(\Delta S)c$, $(\Delta S)e$, $(\Delta S)c/\Delta t$ and $(\Delta S)e/\Delta t$ that increased significantly with seed laser treatment, as similar to the findings of Chen et al. [13] on *Isatis indogotica*. The present findings reveal a positive correlation of different seed germination attributes (germination percentage, GI, CUE and GE) with thermodynamic attributes (ΔH, $(\Delta S)c$ and $(\Delta S)c/\Delta$t), but a negative correlation with MET and E_{50} was found.

5. Conclusions

In conclusion, laser seed irradiation increased the activities of seed germination enzymes, which was the result of improvements in different seed thermodynamic attributes that boosted the physiological and biochemical metabolisms, leading to speed up the seed germination related phenomena. Laser-applied improvements in cellular metabolism were associated with the increased activities of germination enzymes at higher-energy levels, which resulted in the speedy breakdown of larger metabolites such as proteins, starch and lipids. It resulted in more energy generation and provided the building blocks to structural molecules for the newly developing seedlings that sped up the germination and emergence processes of safflower seeds. In the future, further studies are needed to find out the impacts of seed laser treatment on physiological and biochemical mechanism of safflower plants at later growth stages as well as on final seed yield and its quality.

Author Contributions: Conceptualization, R.P., X.W., Y.J., Q.A., S.A. and M.H.S.; Data Curation, R.P., M.Q.Z., M.A. and R.A.K.; Formal Analysis, R.P., M.Q.Z. and R.A.K.; Funding Acquisition, X.W.; Investigation, M.Q.Z.; Methodology, R.P. and R.A.K.; Project Administration, Y.J.; Resources, X.W. and M.H.S.; Software, R.P., S.A., S.F. and M.A.; Supervision, Y.J. and Q.A.; Validation, S.A. and M.Q.Z.; Writing—Original Draft, R.P., S.F. and Q.A.; Writing—Review and Editing, X.W., S.F., S.A., M.A. and M.H.S. All authors have read and agreed to the published version of the manuscript.

Funding: The publication of the present work is supported by the Natural Science Basic Research Program of Shaanxi Province (grant no. 2018JQ5218) and the National Natural Science Foundation of China (51809224), Top Young Talents of Shaanxi Special Support Program.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors are highly thankful to the Higher Education Commission (HEC), Pakistan to support financially this research work.

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

References

1. Ali, Q.; Javed, M.T.; Noman, A.; Haider, M.Z.; Waseem, M.; Iqbal, N.; Waseem, M.; Shah, M.S.; Shahzad, F.; Perveen, R. Assessment of drought tolerance in mung bean cultivars/lines as depicted by the activities of germination enzymes, seedling's antioxidative potential and nutrient acquisition. *Arch. Agron. Soil. Sci.* **2018**, *64*, 84–102. [CrossRef]
2. Noman, A.; Ali, Q.; Maqsood, J.; Iqbal, N.; Javed, M.T.; Rasool, N.; Naseem, J. Deciphering physio-biochemical, yield, and nutritional quality attributes of waterstressed radish (*Raphanus sativus* L.) plants grown from Zn-Lys primed seeds. *Chemosphere* **2018**, *195*, 175–189. [CrossRef] [PubMed]
3. Ali, Q.; Ali, S.; Iqbal, N.; Javed, M.T.; Rizwan, M.; Khaliq, R.; Shahid, S.; Perveen, R.; Alamri, S.A.; Alyemeni, M.N.; et al. Alpha-tocopherol fertigation confers growth physio-biochemical and qualitative yield enhancement in field grown water deficit wheat (*Triticum aestivum* L.). *Sci. Rep.* **2019**, *9*, 12924. [CrossRef] [PubMed]
4. Aladjadjiyan, A. The use of physical methods for plant growing stimulation in Bulgaria. *J. Cent. Eur. Agric.* **2007**, *8*, 369–380.
5. Van Der Woud, W.J. A dimeric mechanism for the action of phytochrome: Evidence from photothermal interactions in lettuce seed germination. *Photochem. Photobiol.* **1985**, *42*, 655–661. [CrossRef]
6. Shinomura, T. Phytochrome regulation of seed germination. *J. Plant Res.* **1997**, *110*, 151–161. [CrossRef]
7. Muszyñski, S.; Gladyszewska, B. Representation of He–Ne laser irradiation effect on radish seeds with selected germination indices. *Int. Agrophys.* **2008**, *22*, 151–157.
8. Perveen, R.; Ali, Q.; Ashraf, M.; Al-Qurainy, F.; Jamil, Y.; Ahmad, M.R. Effects of different doses of low power continuous wave He–Ne laser radiation on some seed thermodynamic and germination parameters and potential enzymes involved in seed germination of sunflower. *Photochem. Photobiol.* **2010**, *86*, 1050–1055. [CrossRef]
9. Dirk, L.M.A.; Kumar, S.; Majee, M.; Downie, A.B. PHYTOCHROME INTERACTING FACTOR1 interactions leading to the completion or prolongation of seed germination. *Plant Signal. Behav.* **2018**, *13*. [CrossRef]
10. Hartmann, K.M.; Grundy, A.C.; Market, R. Phytochrome- mediated long-term memory of seeds. *Protoplasma* **2005**, *227*, 47–52. [CrossRef]
11. Azad, M.O.K.; Kim, W.W.; Park, C.H.; Cho, D.H. Effect of artificial LED light and far infrared irradiation on phenolic compound, isoflavones and antioxidant capacity in soybean (*Glycine max* L.) prout. *Foods* **2018**, *7*, 174. [CrossRef] [PubMed]
12. Dinoeve, S.; Antonov, M.; Stoyanov, T.; Georgieva, C. Spectral impact of low-power laser radiation on wheat and maize parameters. *Prob. Engr. Cybern. Robot.* **2004**, *54*, 74–85.
13. Chen, Y.P.; Yuea, M.; Wang, X.L. Influence of He–Ne laser irradiation on seeds thermodynamic parameters and seedlings growth of *Isatis indogotica*. *Plant Sci.* **2005**, *168*, 601–606. [CrossRef]
14. Cai, S.W.; Qi, Z.; Ma, X.L. The effect of He–Ne laser irradiation on soluble protein synthesis of corn seedling. *Chin. J. Lasers* **2000**, *27*, 284–288.
15. Qi, Z.; Yue, M.; Wang, X.L. Laser pretreatment protects cells of broad bean from UV-B radiation damage. *J. Photochem. Photobiol.* **2000**, *59*, 33–37. [CrossRef]
16. Qi, Z.; Yue, M.; Han, R.; Wang, X.L. The damage repair role of He–Ne laser on plants exposed to different intensities of UV-B irradiation. *Photochem. Photobiol.* **2002**, *75*, 680–686. [CrossRef]
17. Han, R.; Wang, X.L.; Yue, M. Influence of He–Ne laser irradiation on the excision repair of cyclobutyl pyrimidine dimers in the wheat DNA. *Chinese Sci. Bull.* **2002**, *47*, 818–821. [CrossRef]
18. Gładyszewska, B.; Koper, R.; Kornarzyski, K. Application and results of pre-sowing laser bio-stimulation of tomato seeds. *Inz. Rol.* **1998**, *2*, 37–44.
19. Koper, R.; Rybak, P. Effect of pre-sowing laser biostimulation of glasshouse tomato seeds on physicochemical properties of the fruits. *Inz. Rol.* **2000**, *5*, 105–114.
20. Koper, R.; £aziak, S.; WoŸniak, Z. A device for pre-sowing laser stimulation of seeds of chosen cultivated plants and the results of its application. *Agric. Engin.* **1997**, *1*, 63–69. (In Polish)
21. Govil, S.R.; Agrawal, D.C.; Rai, K.P.; Thakur, S.N. Growth responses of *Vigna radiata* seeds to laser irradiation in the UV-A region. *Physiol. Plant.* **2006**, *63*, 133–134. [CrossRef]
22. Wu, J.; Gao, X.; Zhang, S. Effect of laser pretreatment on germination and membrane lipid peroxidation of Chinese pine seeds under drought stress. *Front. Biol. China* **2007**, *2*, 314–317. [CrossRef]
23. Jamil, Y.; Perveen, R.; Ashraf, M.; Ali, Q.; Iqbal, M.; Ahmad, M.R. He–Ne laser-induced changes in germination, thermodynamic parameters, internal energy, enzyme activities and physiological attributes of wheat during germination and early growth. *Laser Phys. Lett.* **2013**, *10*, 1–13. [CrossRef]
24. Abu-Elsaoud, A.M.; Shahda, R.H. Role of He–Ne laser pre-treatment in protecting *Zea mays* against the deleterious effects of ultraviolet radiations. *Egypt. J. Exp. Biol.* **2017**, *13*, 403–422. [CrossRef]
25. Tabatabaei, S.A.; Ansari, O. Predicting seed germination of safflower (*carthamus tinctorius)* cultivars using hydrotime model. *Cercet. Agron. Mold.* **2017**, *1*, 79–87. [CrossRef]
26. Association of Official seed Analysis (AOSA), Rules for testing seeds. *J. Seed Technol.* **1990**, *12*, 1–112.
27. Coolbear, P.; Francis, A.; Grierson, D. The effect of low temperature pre-sowing treatment under germination performance and membrane integrity of artificially aged tomato seeds. *J. Exp. Bot.* **1984**, *35*, 1609–1617. [CrossRef]

28. Ellis, R.A.; Roberts, E.H. The qualification of aging and survival in orthodox seeds. *Seed Sci. Technol.* **1981**, *9*, 373–409.
29. Bewley, J.D.; Black, M. *Seed Physiology of Development and Germination*; Plenum Press: New York, NY, USA, 1985.
30. Association of Official Seed Analysis (AOSA). *Seed Vigor Testing Handbook, 1st ed*; Contribution No. 32 to the handbook on seed testing; Association of Official Seed Analysis; AOSA: East Lasing, MI, USA, 1983.
31. Ruan, S.; Xue, Q.; Tylhowska, K. The influence of priming on germination of rice (*Oryza sativa* L.) seeds and seedlings emergence and performance in flooded soils. *Seed Sci. Technol.* **2002**, *30*, 61–67.
32. Chrispeel, M.J.; Vaner, K.E. Gibberelic acid enhanced synthesis and release of alpha-amylase and ribonuclease by isolated barley aleurone layers. *Plant Physiol.* **1967**, *42*, 398–406. [CrossRef]
33. Ainouz, I.I.; Filho, J.X.; Filho, E.G. Atividade proteolitica msementes de *Vigna sinensis* cv. serida Cienc. *Cult. Sao Panlo Suppl.* **1972**, *24*, 104.
34. Lowry, O.H.; Roebrough, N.J.; Randall, R.J.; Farr, A.L. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]
35. Dawson, R.C.M.; Elliot, D.C.; Elliot, W.H.; Jones, K.M. *Data for Biochemical Research*; Oxford at the Claren don Press: London, UK, 1969; p. 484.
36. Lloyd, J.B.; Whelan, W.J. An improved method for enzymatic determination of glucose in the presence of maltose. *Anal. Biochem.* **1969**, *30*, 467–469. [CrossRef]
37. Arnon, D.I. Copper enzyme in isolated chloroplast Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* **1949**, *24*, 1–15. [CrossRef] [PubMed]
38. Kirk, J.T.O.; Allen, R.L. Dependence of chloroplast pigment synthesis on protein synthesis: Effect of actidione. *Biochem. Biophys. Res. Commun.* **1965**, *21*, 523–530. [CrossRef]
39. Tonguç, M.; Elkoyunu, R.; Erbaş, S.; Karakurt, Y. Changes in seed reserve composition during germination and initial seedling development of safflower (*Carthamus tinctorius* L.). *Turk. J. Biol.* **2012**, *36*, 107–112.
40. DuBois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
41. Somogyi, M. Notes on sugar determination. *J. Biol. Chem.* **1952**, *195*, 19–23. [CrossRef]
42. Larson, L.A.; Beevers, H. Amino acid metabolism in young pea seedlings. *Plant Physiol.* **1963**, *40*, 424–432. [CrossRef]
43. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
44. Noctor, G.; Bergot, G.L.; Mauve, C.; Thominet, D.; Lelarge-Trouverie, C.; Prioul, J.L. A comparative study of amino acid measurement in leaf extracts by gas chromatography-time of flight-mass spectrometry and high-performance liquid chromatography with fluorescence detection. *Metabolomics* **2007**, *3*, 161–174. [CrossRef]
45. Lee, P.Y.; Takahashi, T. An improved colorimetric determination of amino acids with the use of ninhydrin. *Anal. Biochem.* **1966**, *14*, 71–77. [CrossRef]
46. Lowry, R.R.; Tinsley, I.J. Rapid colorimetric determination of free fatty acids. *J. Am. Oil Chem. Soc.* **1976**, *53*, 470–472. [CrossRef] [PubMed]
47. Hartmann, K.M.; Mollwo, A. The action spectrum for maximal photosensitivity of germination. *Sci. Nat.* **2000**, *87*, 398–403. [CrossRef]
48. Criddle, R.S.; Breidenbach, R.W.; Hansen, L.D. Plant calorimetry: How to quantitatively compare apples and oranges. *Thermochim. Acta* **1991**, *193*, 67–90. [CrossRef]
49. Dziwulska, A.; Koper, R. Effects of pre-sowing laser bio-stimulation on germination of Lucerne seeds. *Acta Agrophysica* **2003**, *82*, 33–39. (In Polish)
50. Saleem, M.H.; Rehman, M.; Zahid, M.; Imran, M.; Xiang, W.; Liu, L. Morphological changes and antioxidative capacity of jute (*Corchorus capsularis*, Malvaceae) under different color light-emitting diodes. *Braz. J. Bot.* **2019**, *42*, 581–590. [CrossRef]
51. Raffa, R.B. Thermodynamics of the drug receptor interaction. *Life Sci.* **1999**, *59*, 967–980. [CrossRef]
52. Yan, L.F.; Zhan, Y.L. *Molecular Biology*; Agriculture University of China Press: Beijing, China, 1997. (In Chinese)
53. Alam, H.; Khattak, J.Z.K.; Ksiksi, T.S.; Saleem, M.H.; Fahad, S.; Sohail, H.; Ali, Q.; Zamin, M.; El-Esawi, M.A.; Saud, S. Negative impact of long-term exposure of salinity and drought stress on native *Tetraena mandavillei* L. *Physiol. Plant.* **2020**, 1–16. [CrossRef]
54. Shinomura, T.; Nagatani, A.; Chory, J.; Furuya, M. The induction of seed germination in *Arabidopsis thaliana* is regulated principally by phytochrome B and secondarily by phytochrome A. *Plant Physl.* **1994**, *104*, 363–371. [CrossRef]
55. Shichijo, C.; Katada, K.; Tanaka, O.; Hashimoto, T. Phytochrome A-mediated inhibition of seed germination in tomato. *Planta* **2001**, *213*, 764–769. [CrossRef]
56. Perveen, R.; Jamil, Y.; Ashraf, M.; Ali, Q.; Iqbal, M.; Ahmad, M.R. He–Ne laser-induced improvement in biochemical, physiological, growth and yield characteristics in sunflower (*Helianthus annuus* L.). *Photochem. Photobiol.* **2011**, *87*, 1453–1463. [CrossRef] [PubMed]
57. Gladyszewska, B.; Koper, R. Determination of free radical concentration in tomato seeds biostimulated by laser irradiation (in Polish). *Inynieria Rol.* **2000**, *4*, 35–42.
58. Podleoeny, J.; Misiak, L.; Koper, R. Concentration of free radicals in faba bean seeds after the pre-sowing treatment of the seeds with laser light. *Int. Agrophys.* **2001**, *15*, 185–189.

59. Salyaev, R.K.; Dudareva, L.V.; Lankevich, S.V.; Ekimova, E.G.; Sumtsova, V.M. Effect of low-intensity laser radiation on the lipid peroxidation in wheat callus culture. *Russ. J. Plant Physl.* **2003**, *50*, 498–500. [CrossRef]
60. Rubinov, A.N. Physical grounds for biological effect of laser radiation. *J. Phys. D Appl. Phys.* **2003**, *6*, 2317–2330. [CrossRef]
61. Ali, Q.; Ahmar, S.; Sohail, M.A.; Kamran, M.; Ali, M.; Saleem, M.H.; Rizwan, M.; Ahmed, A.M.; Mora-Poblete, F.; do Amaral Júnior, A.T. Research advances and applications of biosensing technology for the diagnosis of pathogens in sustainable agriculture. *Environ. Sci. Poll. Res.* **2021**, 1–18.
62. Samuilov, F.D.; Garifullina, R.L. Effect of laser irradiation on microviscosity of aqueous medium in imbibing maize seeds as studied with a spin probe method. *Russ. J. Plant Physl.* **2007**, *54*, 128–131. [CrossRef]
63. Rassam, Y.Z.; Mashhadani, F.A.A.; Boya, A.F. Laser treatment may enhance growth and resistance to fungal infection of hard wheat Seeds. *J. Agric. Vet. Sci.* **2012**, *2*, 47–51.
64. Khalifa, N.S.; El-Ghandoor, H. Investigate the effect of Nd-Yag laser beam on soybean (*Glycin max*) leaves at the protein level. *Int. J. Biol.* **2011**, *3*, 135. [CrossRef]
65. Hameed, A.; Akram, N.A.; Saleem, M.H.; Ashraf, M.; Ahmed, S.; Ali, S.; Abdullah Alsahli, A.; Alyemeni, M.N. Seed Treatment with α-Tocopherol Regulates Growth and Key Physio-Biochemical Attributes in Carrot (*Daucus carota* L.) Plants under Water Limited Regimes. *Agronomy* **2021**, *11*, 469. [CrossRef]
66. Vasilevski, G. Perspectives of the application of biophysical methods in sustainable agriculture. *Bulg. J. Plant Physliol.* **2003**, *29*, 179–186.
67. Li, Y.W.; Feng, W.X. The effects of He–Ne laser treatment on seeds germination and growth of *atractylodes macrocephala*. *Chin. J. Appl. Laser* **1996**, *16*, 37–41.
68. Qiu, Z.B.; Zhu, X.J.; Li, F.M.; Liu, X.; Yue, M. The optical effect of a semiconductor laser on protecting wheat from UV-B radiation damage. *Photochem. Photobiol. Sci.* **2007**, *6*, 788–793. [CrossRef]
69. Kuznetsov, V.l.V.; Dmitrieva, G.A. *Fiziologiya Rastenii (Plant Physiology)*; Vysshaya Shkola: Moscow, Russia, 2005.
70. Shakhov, A.A. *Fotoenergetika Rastenii i Urozhai (Photoenergetics of Plants and the Yield)*; Nauka: Moscow, Russia, 1993.
71. Koper, R.; Dziwulska, A. Effects of laser stimulation of white lupine seeds. *Acta Agrophysica* **2003**, *82*, 99–106. (In Polish)
72. Zhang, H.; Gao, Z.; Li, Z.; Du, H.; Lin, B.; Cui, M.; Yin, Y.; Lei, F.; Yu, C.; Meng, C. Laser radiation induces growth and lipid accumulation in the seawater microalga *Chlorella pacifica*. *Energies* **2017**, *10*, 1671. [CrossRef]
73. Liburdy, R.P. Microwave bioeffects in erythrocytes. *Bioelectromagnetics* **1985**, *6*, 283–289.
74. Danie, R.M. The upper limits of enzyme thermal stability. *Enzyme Microb. Technol.* **1996**, *19*, 74–79. [CrossRef]
75. Podlesny, J. Effect of laser irradiation on the biochemical changes in seeds and the accumulation of dry matter in the faba bean. *Int. Agrophys.* **2002**, *16*, 209–213.
76. Xu, M.F. Biological effects of lasers in wheat. *Appl. Laser* **1995**, *153*, 131. (In Chinese)
77. Hong, F.; Lin, L. Effects of laser irradiation on amylase in the seeds of Bailan melon during germination. *J. Southw. Uni. Natl.* **2005**, *2*.