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Effects of Salinity and Laser Radiation on Proline Accumulation in Seeds of Spring Wheat

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Abstract
Laser is one of the sources that induce a biological effect in higher plants. The positive effect of laser on some crops has been proved. This research was carried out to study the effect of laser radiation on the accumulation of proline in spring wheat (*Triticum aestivum* cv. Kavir) under different NaCl treatment levels. Semi-coherent red beam, infrared beam and one-second harmonics of Neodymium-Yttrium-Alminum Garnet (Nd:YAG) laser were chosen as illumination sources. Irradiation on seeds was conducted for 12 minutes once or twice and non-irradiated seeds were used as the control. Experiment was conducted as factorial based on completely randomized design with three replications. The results revealed proline accumulation in leaves after laser treatment and subsequently salt stress tolerance enhanced significantly. Each dosage of radiation imposed a different effect on proline content. The highest proline accumulation was observed in one-time exposure of Nd:YAG laser radiation. This is the first investigation report on using different types of lasers and their effect on proline content in the spring wheat. Results show the powerful effect of laser treatment on the improvement of wheat tolerance to salinity stress.

Keywords: Diode laser, Proline, Salinity tolerance, *Triticum aestivum*

Introduction
Commonly, environmental stresses negatively influence the plant growth, developmental stage and crop yield. Salinity and drought are the most important and widely spread abiotic stresses in the world. The climate changes are expected to intensify the damage caused by these kinds of stresses (Viana and Cruz 2002). High salinity disrupts homeostasis in water potential and ion distribution in plants; so, during evolution, different plant species have developed diverse mechanisms to cope with these effects (Dash and Panda 2001; Munns 2002).

Osmotic adjustment or reduction of cellular pressure potential by solute contents such as amino acids, proline, etc. is an important procedure to increase salt tolerance in plants (Hasegawa *et al*. 2000). Therefore, determining the basic mechanisms involved in plant salinity tolerance is imperative to develop resistant crop plants in the saline conditions. At first, proline accumulation in the dehydrated plant tissues was shown in 1954 (Kemble and Macpherson 1954). Proline counteracts the effects of osmotic stress in response to salinity, drought, heat, chilling and heavy metal exposure. Moreover, proline accumulates in higher plants in reaction to salinity stress as a conventional metabolic characteristic (Stewart and Larher 1980; Rhodes 1987; Annicchiarico 1992; Samaras *et al.* 1995; Rhodes *et al.* 1999; Ashraf and Har0ris 204). Some researchers have shown that proline as a non-toxic and protective osmolyte compound significantly increases in the high salinity condition that may cause osmotic adjustment, protection of membrane stability and neutralize the effect of salinity accumulation in the vacuole (Gasim 1998; Maggio *et al.* 2000; Khatkar and Kuhad 2000; Muthukumarasamy *et al*. 2000; Jain *et al.* 2001).

Appropriate preparation of cereal seeds is one of the ways to increase crop yields. Chemical and physical factors are used in the pre-sowing step. Laser treatment is a physical phenomenon in which light energy is absorbed by grains. It increases the energy potential of seeds, which in turn impacts the physiological processes in germinating seeds, thus accelerating maturity, increasing resistance to disease as well as raising the biological and processing quality of the yield (Drozd and Szajsner 1999). In recent years, many investigators have used laser beam to solve various scientific problems in medicine and biology. Laser can be successfully used for other purposes in plant genetics and breeding. A laser micro beam combines various tools for cell and molecular biologists in one instrument (Rybiñski *et al.* 1993). In comparison with conventional light sources, laser is distinguished by characteristics such as mono-chromatism, polarization, coherence and high density. Changes that occur in the physiological state of an organism depend closely on the laser radiation type, its wavelength, laser power, exposure time, intensity, species and cultivar of the plants (Rybiñski *et al.* 1993; Drozd and Szajsner 1999). The effect of laser radiation on plant and seed may be a result of its light and electromagnetism effects (Xiang 1995). Although the results of the investigations are not constant but the majority of researchers declare that laser pretreatment brings positive results in accelerating the plant growth and metabolism and suitable doses of laser irradiation improves germination capacity of plant seeds, enzymatic activities and adaption in poor environmental conditions and increases yield quantity and quality (Drozd and Szajsner 1999; Chen *et al.* 2005). The best effects of laser treatment were achieved in vegetable plants (cucumbers, tomatoes, lettuce, etc.), and in some degree in cereals (wheat, rye, broad bean, maize, etc.) and root crops (sugar beet, etc.) (Wilde *et al.* 1969; Koper 1994; Wójcik and Bojarska 1998; Podleœny 2002). Abundant data concerning the influence of lasers on wheat have been obtained. These studies have illustrated that laser treatment could protect cells of wheat from enhanced UV-B radiation damage (Qiu *et al.* 2007). Laser's radiation can have a beneficial effect in wheat growing and increase the crop yield in some variants that leads to economical profits (Dinoev *et al.* 2004). It has been shown that laser pretreatment could protect the cells of a plant from drought stress damage with enhancing the activity of many enzymes. Moreover, proper laser pretreatment can accelerate physiological and biochemical metabolism and accordingly enhance the seedling growth (Qiu *et al*. 2007). Consequently, this method can be used for quick determination of the stress responses in plants (Drozd and Szajsner 1999).

This research was conducted to evaluate the effect of spring wheat seed pretreatment by laser irradiation on proline content under different salinity treatments to improve salinity tolerance.

Materials and Methods

Plant materials: Seeds of spring wheat (*Triticum aestivum* L. cv. Kavir) were obtained from Seed and Plant Improvement Institute in Karaj. The seeds with similar size were selected and after sterilization for 10 minutes in 0.1% $HgCl_2$, were washed in distilled water for 50 minutes.

Laser treatment: Two diodes lasers, emitting 660 nm semi-coherent red beam (power intensity: 110 mW mm^{-2}; beam diameter: 10 mm) and 980 nm infrared beam (power intensity: 250 mW mm^{-2}; beam diameter: 10 mm), and one-second harmonics of Neodymium-Yttrium-Alminum Garnet (Nd:YAG) laser emitting 532 nm (power intensity: 75 mW mm^{-2}; beam diameter: 10 mm) were chosen as illumination sources. The seeds were irradiated for 12 minutes in separate experiments including one time and two time irradiation. The non-irradiated seeds were used as the control or reference seeds.

Salinity levels: The experiment was conducted during 2009 at the greenhouse and central laboratory of College of Abouraihan, University of Tehran, Iran. After irradiation of seeds, they were immediately sown in the pots and irrigated with four salinity solutions (0, 100, 200 and 300 mM NaCl).

Proline determination: Free proline content at tillering stage was determined according to the method described by Bates *et al*. (1973). Leaf samples (0.5 g) of fresh plant material were homogenized in 10 mL of 3% sulpho-salycylic acid and filtered through Whatman's filter paper. Afterwards, 2 mL of filtrate was mixed with 2 mL acid ninhydrin and 2 mL of glacial acetic acid in a test tube and heated at 100°C for an hour in water bath. Reaction was then stopped by using ice bath. The reaction mixture was extracted with toluene and the absorbance of fraction with toluene aspired from liquid phase was recorded at 520 nm. Appropriate standard proline was included obtaining calibration curve and data expressed as $\mu mol.g^{-1}$ of fresh weight (F.W.).

Statistical analyses and methods: A factorial experiment was carried out based on completely randomized design with three replications. Factors included four salinity levels (0, 100, 200 and 300 mM NaCl), three types of laser (Red, Infra-red and Nd:YAG laser) and two exposures of irradiation. Non-irradiated seeds were used as the control. All data were subjected to analysis of variance, using the general linear model of SAS (version 9.1). Treatment means were tested by Duncan's multiple range test.

Results and Discussion

Effect of different types of laser pretreatment and salinity solutions on proline accumulation are shown in Table 1. Salinity, laser and radiation dosage had significant effect on proline accumulation. In addition, there were significant interactions among evaluated factors (Table 1). The results suggest that the choice of factor levels was appropriate and choosing the best combination of factors could be fruitful in improving genotypes under saline condition. The results support the idea that proline accumulation occurs in higher monocotyledon plants under salt stress (Ashraf and Harris 2004; Poustini *et al*. 2007). The effect of salinity on proline content of Kavir wheat cultivar was reported previously by Goudarzi and Pakniyat (2009). Proline accumulation in response to salt stress has been extensively reported in many plants (Gasim 1998). In wheat, there is a positive relationship between proline levels and osmotic potential, and it is inferred that proline is an osmo-regulatory component (Bradford 1976; Naidu *et al.* 1990).

Table 1. Analysis of variance for proline content of wheat (*Triticum aestivum* L.) irradiated with three types of lasers at two exposures in four NaCl levels

Sources of variation	df	MS
Laser	3	223.82***
Exposure	1	19.64 *
Salinity	3	2789.29***
Laser × Exposure	3	10.33*
Laser × Salinity	9	50.26***
Exposure × Salinity	3	83.54***
Laser × Exposure × Salinity	9	8.19*
Error	64	2.90

ns, not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Mean comparison among laser types when irradiated once reveals that Nd:YAG had the highest effect on proline accumulation. However, when irradiation was replicated (two-times exposure) Nd:YAG and Infra-red laser had the same effect on proline increment. Results indicate that using the laser irrespective of the type of laser increases proline content (Table 2). There are some procedures to increase the endogenous proline concentration for salt defense mechanism (Ashraf and Foolad 2007). Present research showed that laser irradiation successfully enhances proline content of the evaluated wheat. Researchers have previously shown that certain types of laser irradiation accelerate growth, improve seed germination and enhance the content of useful compounds in plants and alleviate stress damages such as UV-B radiation that causes plants to generate reactive oxygen compounds, which interfere with the normal metabolism (Qiu *et al.* 2007; Ashrafijou *et al.* 2010). The laser beam, as a specific light, can be absorbed effectively through the macromolecules and cause some photochemical impacts (Xiang 1995). Seeds pretreated with suitable laser irradiation have to absorb further energy from the surrounding than that of the control samples during their development because the laser break the kinetic equilibrium of germinating seeds and increase the internal energy of seeds (Chen *et al.* 2005). Furthermore, the results in this experiment demonstrated clearly that prior laser irradiation on seeds, irrespective of the laser type, has significant effect ($p<0.0001$) on proline content under the same salinity concentration. These results are in accordance with the recent report of Yi-Ping Chen (2009) that showed the concentration of the proline increase progressively with UV-B irradiation, laser irradiation and He-Ne laser irradiation plus UV-B irradiation compared with the control. One possible reason is the stimulation effects of laser irradiation that can raise the enzymes' activities and increase plant growth exposed to UV-B radiation stress. In addition, laser radiation may stimulate the signal system transaction functions (Chen 2009). It is known that the effects of laser irradiation on an organism chiefly depend on light, electromagnetism, temperature and pressure effects (Xiang 1995).

Table 2. Mean comparisons of proline content in wheat exposed once (a) and twice (b) to laser treatment

Laser	Proline content (a)	Duncan's grouping (a)	Proline content (b)	Duncan's grouping (b)
Nd:YAG	19.06±4.38	A	18.74±4.32	A
Infrared laser	16.63±3.82	B	17.78±2.50	A
Red laser	14.42±3.09	C	16.72±2.69	B
No-laser	9.99±1.68	D	9.99±1.68	C

Means followed by the same letters are significantly different at 0.05 probability level according to Duncan's test

The effect of laser irradiation time (once or twice) and salinity concentrations on proline content are presented in Table 3. Regardless of the laser type, the two-times exposure had higher effect on proline accumulation than the single exposure. Except Red laser there was no difference in the effect of each laser type on proline content when was applied once or twice. It seems that pretreatment with laser for a longer time (two-times illumination) may have cumulative effect in conveying resistance to salt solution in wheat. Chen *et al.* (2005) reported that laser pretreatments have evidently induced the enzymatic activities, change thermodynamic parameters, accelerate physiological and biochemistry metabolism, and accordingly enhance the growth of seedlings. They expressed that the influence of the laser beam exerts on an organism is a long term effect. Also, Qiu *et al.* (2007) have reported on the connection between drought stress and laser treatment. Their results showed that laser treatment increases the drought resistance of the wheat plant, enhancing the activity of many similar enzymes. Their experiments to optimize the exposure time to the laser showed that 3 minutes (among laser radiation for 0, 1, 3 and 5 minutes) was optimal. In a word, laser light is one of the sources for inducing a bio stimulation effect and genetic changes in many plants. It is known that lower doses of laser radiation may increase the bio-energetic potential of the cells and inhance the activation of their biochemical and physiological processes. However, higher doses influence the heredity material leading to genetic changes of plant traits (Rybinski 2004).

Table 3 also shows that higher salt concentration greatly increase proline value in wheat as 300 mM NaCl induced the highest amount of proline while control treatment showed the lowest content (Table 3). Proline accumulation in plant cells under salt stress is a universal phenomenon that can serve as an osmotic regulator (El-Sayed *et al.* 2007) and is widely documented in the cell pressure adjustment, detoxification of injurious ions and membrane stabilization in plants under salinity conditions (Kishor *et al*. 2005; Ashraf and Foolad 2007).

Table 3. Mean comparisons of different exposure radiation (a) and different salinity concentrations (b) on proline accumulation in wheat

Laser dosage (a)	Proline content	Duncan’s grouping
Twice	17.75±1.59	A
Once	16.70±2.15	B
Salinity level (b)		
300 mM	32.43±1.17	A
200 mM	21.99±0.82	B
100 mM	9.11±0.43	C
Control	5.37±0.57	D

Means followed by the same letters are significantly different at 0.05 probability level according to Duncan's test

Mean comparison of all two order combinations are shown in Table 4. Means of exposure × salinity combination, revealed that laser dosage for single exposure × 300mM cause the highest amount of proline accumulation (35.07 $\mu mol.g^{-1}$ F.W.). Nd:YAG laser had also the highest increasing effect on proline when it was combined with different salinity levels and exposure time. However, there was no difference between one-time or two-times exposure of Nd:YAG. The lowest influence on proline accumulation was related to Red laser when it was used once. Moreover, the lowest amount of proline was observed in the salt free solution (Table 4). Figure 1 presents that the Nd:YAG laser results in the highest proline accumulation at 300 mM NaCl when there was a single exposure. The same result was obtained when there was double dosage (Figure 2). However, in the single exposure the weakest effect belonged to the Infra-red laser in the control solution (Figure 1) while in the double dosage the lowest influence was related to the use of no laser in the control solution (Figure 2).

Table 4. Mean comparison of different factor levels in three order interactions on proline accumulation of spring wheat

Laser×Exposure×Salinity	Duncan Grouping	Proline (μmol g^{-1} F.W)
Nd:YAG×300 mM× Dosage1	A	40.46
Infrared laesr ×300 mM× Dosage1	B	34.33
Nd:YAG×300 mM× Dosage2	BC	32.23
Red laesr ×300 mM× Dosage1	DC	30.41
Red laesr ×300 mM× Dosage2	DC	29.56
Infrared laesr ×300 mM× Dosage2	DE	27.59
Nd:YAG×200 mM× Dosage2	EF	26.13
Infrared laesr ×200 mM× Dosage2	FG	23.91
Nd:YAG×200 mM× Dosage1	FG	23.66
Infrared laesr ×200 mM× Dosage1	GH	21.84
Red laesr ×200 mM× Dosage2	HI	20.37
No laesr ×300 mM	IJ	17.88
Red laesr ×200 mM× Dosage1	J	16.02
No laesr ×200 mM	K	12.40
Infrared laesr ×100 mM× Dosage2	K	12.28
Red laesr ×100 mM× Dosage2	KL	9.55
Nd:YAG×100 mM× Dosage1	L	9.05
Nd:YAG×100 mM× Dosage2	L	8.72
Infrared laesr ×100 mM× Dosage1	LM	8.38
Nd:YAG×0 mM× Dosage2	LM	7.89
Red laesr ×0 mM× Dosage2	LMN	7.40
Infrared laesr ×0 mM× Dosage2	LMN	7.33
Red laesr ×100 mM× Dosage1	LMN	6.69
No laesr ×100 mM	MNO	5.29
Red laesr ×0 mM× Dosage1	NOP	4.54
No laesr ×0 mM	NOP	4.39
Nd:YAG×0 mM× Dosage1	OP	3.06
Infrared laesr ×0 mM× Dosage1	P	1.99

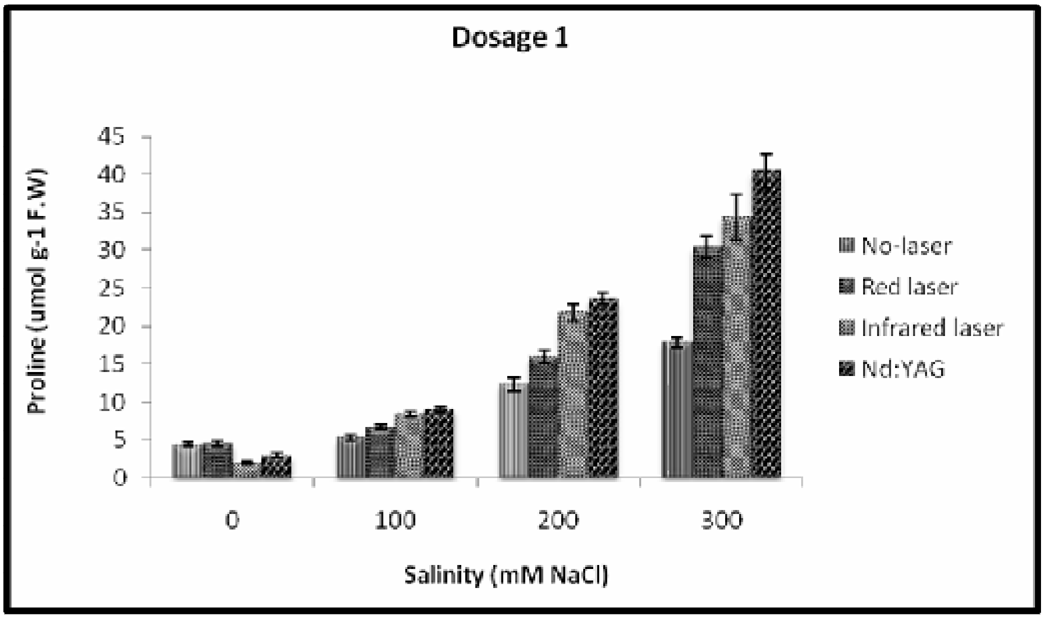

Figure 1. Effects of different lasers types at single exposure period on proline content of wheat

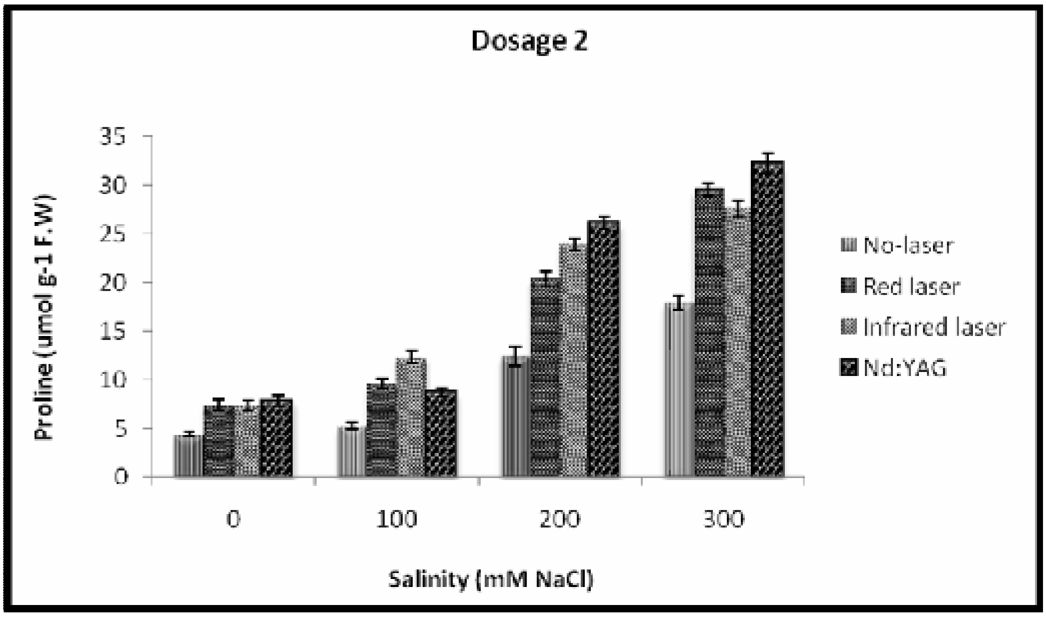

Figure 2. Effects of different lasers types at double exposure period on proline content of wheat

Conclusion

These results enable us to formulate the following conclusions: (1) increasing of the salinity, enhance proline content and 300 mM NaCl induced the highest amount, (2) all different types of evaluated laser radiations (Red, Infra-red and Nd:YAG lasers) increased proline content significantly in comparison to the control samples, however, Nd:YAG laser was the most efficient beam, (3) comparison of proline content of the control with irradiated plants at the same salinity concentration revealed that laser types had considerable effect on the proline content, (4) single exposure by laser was more efficient than the double exposure and (5) the best combination was Nd:YAG × single dosage × 300 mM causing the highest amount of proline. As a conclusion, stimulating effect of laser light can be used in wheat breeding and investigating the use of laser beam on other plants is recommended.

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