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Research Article

Application of Brown Algae (*Sargassum angustifolium*) Extract for Improvement of Drought Tolerance in Canola (*Brassica napus* L.)

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Background: Abiotic environmental stresses, especially drought stress, is one of the most important problems in arid and semi-arid regions. Like other major crops, Brassica napus is vulnerable to drought stress.
Objective: The present study was conducted to evaluate efficacy of *Sargassum angustifolium* extract on mitigating adverse effects of drought stress on *B. napus* seedlings during vegetative growth under greenhouse conditions.
Materials and Methods: Seedlings were periodically sprayed with the seaweed extract until they reached 7-leaf stage. Then water deficit stress was imposed and measurements were performed at morphological, biochemical and molecular levels on three phases: 80% field capacity for 20 days (Phase I), 60% field capacity for 20 days (Phase II) and 40% field capacity for 20 days (Phase III). Real-Time PCR assay was carried out to monitor the changes in expression of the genes involved in proline biosynthesis.
Results: Morphological measurements revealed that seaweed treatment improved shoot height and dry weight compared to control ($p<0.05$). Biochemical analyses indicated that foliar application of seaweed extract significantly enhanced the photosynthetic pigments' content, free radical scavenging and superoxide dismutase activity ($p<0.05$). Moreover, proline content was significantly increased in plant tissues treated with seaweed extract ($p<0.05$). The results of Real-Time PCR assay showed that the increase in proline content is due to enhanced expression of P5CS which is involved in biosynthesis of proline, and to decreased expression of *PRODH* which catalyzes proline degradation.
Conclusions: Overall, the results obtained in this research suggest that application of *S. angustifolium* extract as a biostimulant is able to protect canola seedlings against deteriorating effects of drought stress.

Keywords: Drought Stress, Proline, Sargassum Angustifolium, Seaweed Extract

1. Background

Existence of abiotic environmental stresses, especially drought stress, is one of the most important problems in arid and semi-arid regions of the world (1). Stress is the presence of an organism under the influence of the severity of an environmental factor that causes loss of appearance, efficiency or value (2). Drought stress occurs when plant receives less water than its losses (3). Drought is one of the most important environmental stresses that negatively affects the crops and hence has major adverse effects on agricultural production (4, 5). The drought stress is regarded as the most important and common stress in the environment, causing huge damage to crop products in the world (6). In most parts of the world, the drought stress that results from the shortage of rainfall, especially in those stages where the water requirement of plants and its evapotranspiration potential is increased, negatively affects sensitive phases of crop growth, even in irrigated areas (7). When growth is restricted, it's difficult to achieve high performance. According to the estimates, about 40% of the earth's land is located in semi-arid regions which shows the importance of drought and calls for remedies to protect the crop against this stress (8).

Canola (Brassica napus L.) is a major crop widely cultivated as a source for production of edible oil. According to FAO reports, canola cultivation area had increased to 68.9 million metric tons, and the harvested area had expanded to 33.7 million hectares (9). Like other major crops, B. napus is vulnerable to environmental stress combinations, such as heat and drought. Plants are equipped with a polygenic defense mechanism against drought and other stresses composing of interrelated dynamic processes (10). Plant adaptation to drought stress is accompanied by expression of stress-specific genes, accumulation of metabolites, extension of root network and reduced leaf area (11-14). Application of plant biostimulants has been proved to be an efficient and cost-effective approach for improvement of drought tolerance among crops. This approach has gained much attention in both scientific societies and marketplace (15, 16).

Seaweed extracts represent a major category of products accepted as a biostimulants. Extracts of brown seaweeds are increasingly used in agri-horticultural crop productions (14, 17, 18). Soil or foliar application of seaweed extract has been shown to enhance chlorophyll content (19), water retention capacity and generally ameliorating biotic and abiotic stresses (20, 21). Positive effects of the extract of Sargassum species on plant tolerance against stresses has been reported by a number of researchers (22-24). Although positive and considerable effect of seaweed extracts in improving crops' tolerance to both biotic and abiotic stresses has been reported in many studies (25-27), the mode of action of seaweed extract in improving stress tolerance is not fully understood and various genes and metabolite pathways have been proposed as possible mechanisms justifying this effect (15, 28). To this end, the present study was conducted to evaluate the impact of S. angustifolium extract on drought tolerance of canola (*B. napus*) and to unravel the physiological, biochemical and molecular mechanisms involved in this process. The novelty of this research lies in the fact that together with investigating morphological and physiological changes caused by seaweed extract treatment, molecular basis of these changes, at least for proline which is a prominent agent in endowing drought tolerance, is also studied.

2. Objective

The present study was conducted to evaluate efficacy of *Sargassum angustifolium* extract on mitigating adverse effects of drought stress on *Brassica napus* seedlings during vegetative growth under greenhouse conditions.

3. Materials and Methods

3.1. Algae Source

The brown algae S. angustifolium was collected from Chabahar shores and extraction procedure was followed by the method developed before (29).

3.2. Plant Cultivation and Induction of Drought Stress

Canola seeds were sown under controlled conditions. When canola seedlings reached 3-leaf stage, control group was sprayed with distilled deionized water while the experimental groups were sprayed with 7.0 mL of 1:1000 (w/v) concentration of the algae extract. When the seedlings reached 7-leaf stage (70 days after spraying), water stress was started. Measurements were made in three phases: 80% field capacity for 20 days (Phase I), 60% field capacity for 20 days (Phase II) and 40% field capacity for 20 days (Phase III).

3.3. Plant Height and Dry Weight

Plant height was measured in cm in all phases of the experiment. The canola seedlings were collected and transferred to the laboratory. Their fresh weight was carefully weighed. For measurement of dry weight, above-ground parts of the canola seedlings were dried at 80 °C until constant dry weight was obtained.

3.4. Photosynthetic Pigments

The amount of photosynthetic pigments of leaves including chlorophyll a, b, total chlorophyll and carotenoids was measured using Lichthen Thaler 1987 method (30).

3.5. Antioxidative Capacity

Free radical scavenging activity was measured according to the method proposed by Shukla *et al*. (28).

3.6. Proline Measurement

The proline concentration in plant tissues was determined according to the method of Bates *et al*. (31).

3.7. Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was assayed according to Mansori *et al*. (32).

Table 1. Primer details of proline biosynthesis and degradation genes (P5CS and PRODH, respectively) and a housekeeping gene (Elongation factor 1-alpha) used in this study.

Gene	Primer*	T_m (°C)
Elongation factor 1-alpha	F: 5′ ACAAAATCCCATTCGTCCCCATC 3′	55.2
	R: 5′ ACTGGCACCGTTCCAATACCAC 3′	57.2
P5CS	F: 5′ GCTACAGCACAAGAAGCTGGAC 3′	56
	R: 5′ TCCAAAACAAGACCATCTGCCAC 3′	55.6
PRODH	F: 5′ CTGAAGACACAATCCTCCAACCC 3′	55.2
	R: 5′ CACCTCTCACCAACTTAAACCCC 3′	55.1

*F: forward, R: reverse.

Table 2. Effect of algae extract (AE) treatment on morphological traits (plant height and dry weight) of canola seedling: 80% field capacity for 20 days (Phase I), 60% field capacity for 20 days (Phase II) and 40% field capacity for 20 days (Phase III).

Traits	Plant height			Dry weight		
	Phase I	Phase II	Phase III	Phase I	Phase II	Phase III
Control	49.01±1.32^{c}	47.56±2.22^{d}	44.33±1.07^{e}	12.98±1.76^{c}	12.45±0.34^{cd}	11.21±1.16^{d}
AE	61.64±1.19^{a}	59.48±1.13^{a}	56.22±2.18^{b}	20.27±1.81^{a}	19.06±1.45^{a}	16.13±1.28^{b}

3.8. Real- Time PCR analysis

Expression of two genes involved in proline metabolism viz. P5CS (delta-1-pyrroline-5-carboxylate synthetase A), PRODH (proline dehydrogenase) was evaluated in transcription level by Real-Time PCR assay and Elongation factor 1-alpha was applied as housekeeping gene (**Table 1**). Quantitative Real-Time PCR experiments were performed in duplicate for each sample the relative-fold expression was determined using 2^{-DDCT} method as described by Livak and Schmittgen (33). Elongation factor 1-alpha gene was used as a housekeeping gene.

3.9. Statistical Analysis

The research was conducted as a factorial experiment in the form of completely randomized design (three levels of drought and two levels of seaweed extract with three replications). Duncan test was used for mean comparison. Statistical analyses were performed at 95% confidence interval ($p<0.05$). All of the statistical analyses were performed with SPSS software version 16.0. REST 2009 V2.0.13 software was used for Real-Time PCR analysis.

4. Results

4.1. Morphological Traits

The results of measurement of plant height and dry weight are presented in **Table 2**. According to the results, application of algae extract (AE) mitigated the negative impact of drought stress on plant height. The same results were observed for dry weight in all three phases which was greater in AE-treated plants compared to control ($p<0.05$).

4.2. Photosynthetic Pigments

Figure 1 represents the impact of AE on photosynthetic pigment contents of canola seedlings. According to the results, the highest content of chlorophyll a was observed in AE-treated plants and control group during Phase I. Chlorophyll a content during drought stress (in both Phase II and III) was significantly higher in AE-treated plants compared to control group ($p<0.05$).

4.3. Free Radical Scavenging

Free radical scavenging activity of AE-treated and control seedlings was investigated by common DPPH assay and the results are presented in **Figure 2**. Accordingly, AE treatment significantly improved reactive oxygen species (ROS) scavenging capacity of the canola plants compared to control group ($p<0.05$). An ascending trend was found in ROS scavenging activity of the plants through different phases of the experiment.

4.4. SOD Activity

Activity of SOD as an antioxidant mechanism during occurrence of environmental stress was measured and the results are presented in **Figure 3**. Increased SOD activity was observed in both control and AE treatment groups, however, the increase in SOD activity was significantly higher in AE-treated group compared to control ($p<0.05$).

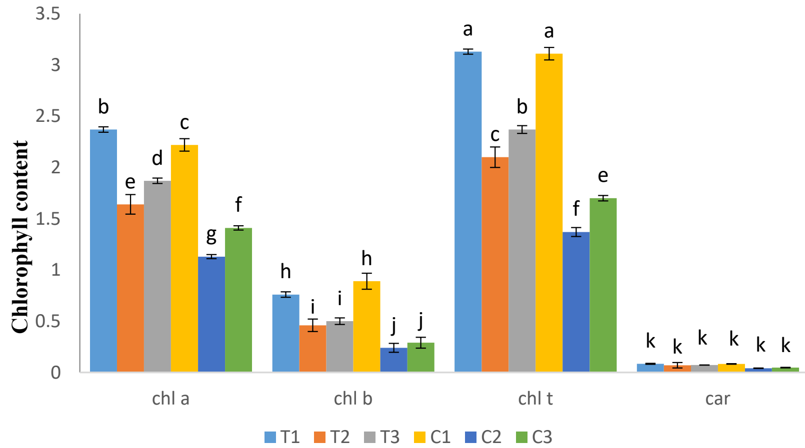

Figure 1. Increase in photosynthetic pigments by AE treatment during three phases of drought stress. (T1, T2 and T3: AE treatment; C1, C2 and C3 : control condition during Phase I, II, and III; respectively. chl a: chlorophyll a; chl b: chlorophyll b: chl t: total chlorophyll, car: carotenoid).

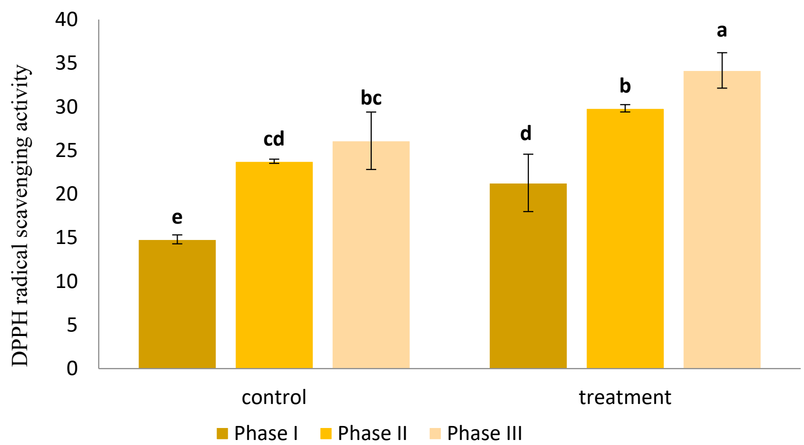

Figure 2. Impact of AE on ROS scavenging activity of canola seedlings during three phases of drought tolerance. Free radical scavenging activity followed an ascending trend in both control and treatment groups. The values represented in the graph are the mean of three replications.

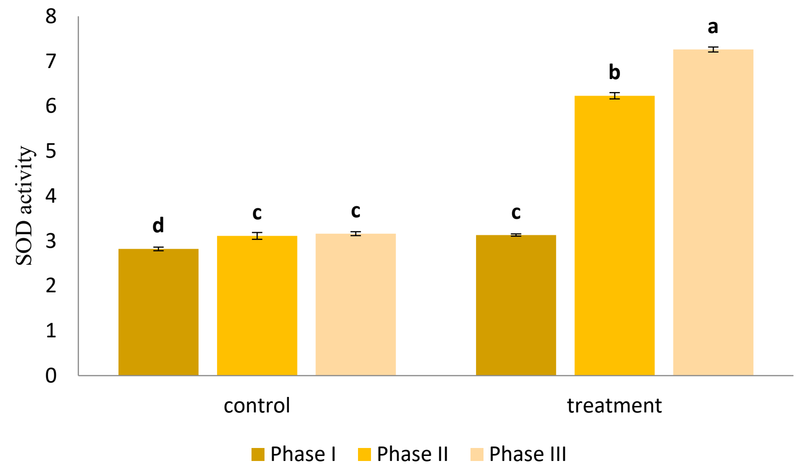

Figure 3. Variation in SOD activity under AE treatment during three phases of drought stress. The values represented in the graph are the mean of three replications.

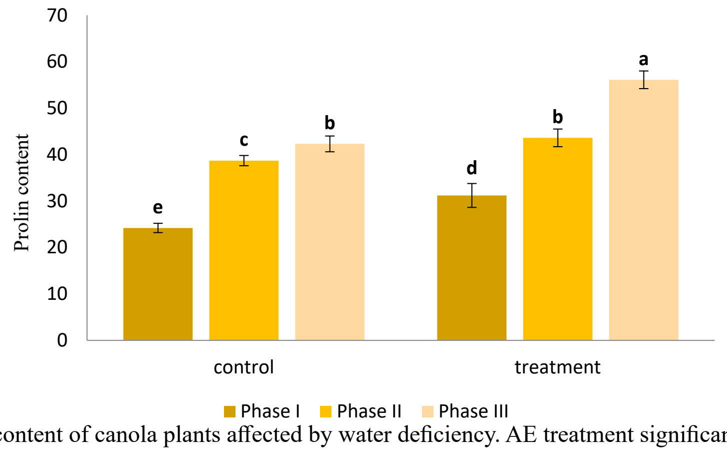

Figure 4. Variation in proline content of canola plants affected by water deficiency. AE treatment significantly improved proline content of the plants compared to control ($p<0.05$).

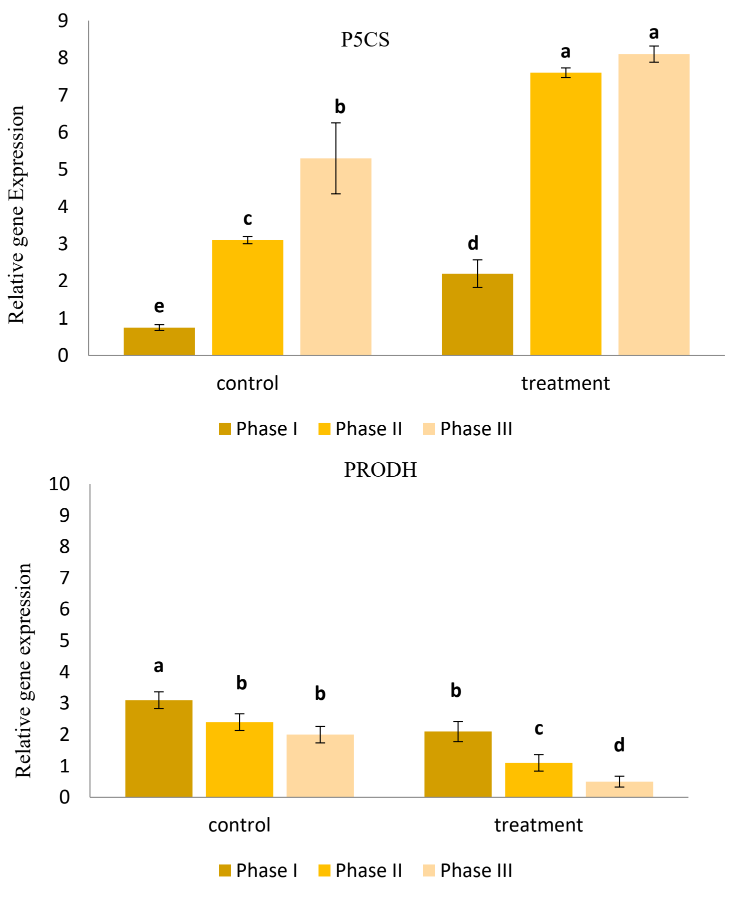

Figure 5. The results of Real-Time PCR assay on expression of genes including P5CS (top) and PRODH (bottom) involved in biosynthesis of proline.

4.5. Proline Measurement

Proline content was measured during Phases I, II and III and the results are presented in **Figure 4**. Comparison of AE treatment and control groups revealed that seaweed treatment significantly increased proline content in canola seedlings ($p<0.05$) as proline content of AE-treated plants even during Phase II was higher than that observed during Phase III of control group (**Fig. 4**).

4.6. Proline Biosynthesis-related Gene Expression

Figure 5 shows the results of Real-Time PCR assay as fold change in gene expression. According to the result, expression of P5CS gene was enhanced by induction of drought stress in both AE and control groups. In contrast, PD expression was decreased through stress period. The main finding of Real-Time PCR assay was that P5CS expression was significantly higher in AE-treated seedlings than in control plants ($p<0.05$), whereas PD expression was significantly lower in AE plants than in control plants ($p<0.05$).

5. Discussion

The results obtained from this experiment revealed that foliar spray of AE significantly improved both shoot dry weight and seedling height compared to control group. This positive impact is consistent with those reported for application of seaweed on other crops (16, 31, 34, 35). The promoting impact of seaweed on growth parameters might be attributed to the presence of auxin (32, 36), cytokinin (10) and other growth promoting agents (28) as well as both micro- and macronutrients (37) in algae extract. Moreover, bioactive compounds contained in seaweed extract may exhibit synergic effect on plant growth (15). The results obtained in this research also revealed positive effect of AE on photosynthetic pigments. Promoting photosynthesis machinery may justify enhanced shoot dry weight and plant growth as seen in this research. The positive effect of AE on photosynthetic pigments has been reported by other authors as well (19, 38). It has been speculated that promoting effect of seaweed extract on photosynthetic pigments is primarily due to reduction of chlorophyll degradation during water shortage (39). Betaines is a notable biological agent in seaweed extract that enhances chlorophyll content of plants (40), thus improved chlorophyll content of canola plants as treated with AE may be due to betaines content of the seaweed extract.

Due to their ability for ROS scavenging, seaweed extracts have gained much attention as novel source of natural antioxidants (41). The results obtained in this study indicated that application of AE promoted ROS scavenging capacity (1.31 fold compared to contorl) of canola seedling so that the highest rate of ROS scavenging was obtained on Phase III of the experiment in the seedlings treated with AE. This positive effect of AE on ROS scavenging capacity has been reported in other studies (42, 43). It was shown here that SOD activity was enhanced by foliar application of *S. angustifolium* on canola plants. SOD catalyzes the dismutation of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide. Uncontrolled formation of superoxide is responsible for many types of cell damages during abiotic stresses (2), therefore, activity of SOD is a major mechanism to attenuate adverse effects of drought stress on crops' growth and yield (44). Increased SOD activity of AE-treated plants has been reported by many studies (26, 32, 45). Aaccording to the results, it can be concluded that increased activity of SOD (82 % compared to control) and promoted ROS scavenging capacity in canola plants promotes plant vigor during drought stress which results in better growth of the seedlings compared to control.

A notable finding of the present study was increased proline content of canola seedling as a result of AE application. Proline is major component of plant defense mechanism during water shortage and drought stress and is known as the most accumulated osmolyte under various stressful conditions. Many researchers have reported positive effect of proline on plant tolerance to abiotic stresses (1, 9, 46, 47). Accumulation of proline in control group shows that increase of this amino acid during drought stress is a natural mechanism of plants to cope with water shortage. However, proline content was significantly higher in AE-treated seedlings ($p<0.05$) which shows another positive effect of seaweed extract on plant tolerance to water deficit. This is consistent with previous reports on the impact of seaweed extract on proline content of crops (48, 49).

Real-Time PCR assay revealed that enhanced accumulation of proline in foliar tissues of canola was the result of a combination of up-regulation of proline biosynthesis gene (P5CS) and down-regulation of proline degradation gene (PRODH). P5CS is a key gene through biosynthesis pathway of proline which is overexpressed under stressful conditions. The enzyme encoded by P5CS is composed of two domains acting as kinase and dehydrogenase and is accumulated in cytosol and plastids (50). This finding is of great importance because it implies a sophisticated mechanism for accumulation of proline during water stress. This finding accords with previous results suggesting that proline accumulation in plants is the result of both increased proline biosynthesis and decreased proline degradation (51). As a whole, these findings support early evidences that seaweed extract act as an elicitor the boost growth and yield of crops under normal and stress conditions (12, 15, 27, 28, 38, 49).

6. Conclusion

The results obtained in this study reconfirmed early evidence suggesting high potential of algae as efficient bioresources for enhancing crop tolerance to biotic and abiotic stresses. Considering the magnitude of drought stress in many areas across the world, together with the growing demand for natural substance as alternatives to chemicals for promotion of crop yield, our results may have practical implications for farmland managers and agriculture policymakers. Particularly, regarding economic importance of canola, the results of this research indicate that application of the brown algae S. angustifolium may be an eco-friendly, available and cost-effective approach for mitigating adverse effect of drought stress on canola plant. Further research in the field of algae-based fertilizers and biostimulants production will pave the road for extension of practical application of marine algae.

Conflict of interest

There is no conflict of interest.

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