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Article

Proline Enhances Resistance and Recovery of Oilseed Rape after a Simulated Prolonged Drought

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Abstract: This study was carried out to evaluate the effect of exogenous proline on growth, biochemical responses and plant recovery of drought-stressed oilseed rape plants after renewed irrigation. The experiment was conducted under controlled laboratory conditions. After 21 days of cultivation, 3–4 leaf stage seedlings were sprayed with proline (1 mM), then subjected to prolonged drought stress for 8 days to achieve a severe water deficit, then irrigation was resumed and recovery was assessed after 4 days. The results show that exogenous application of proline reduced drought-induced growth inhibition of seedlings while maintaining relative water content (RWC) and growth parameters closer to those of irrigated plants. Proline had a positive effect on chlorophyll accumulation and membrane permeability while decreasing ethylene, H₂O₂ and MDA levels. Moreover, after 4 days of recovery, the H₂O₂ content of the proline-treated plants was significantly lower (2-fold) and the MDA content was close to that of continuously irrigated plants. Thus, all these biochemical reactions influenced plant survival: after drought + proline treatment, the number of surviving plants was 2 times higher than that of drought-treated plants. The findings show that exogenous proline has antioxidant, osmotic and growth-promoting properties that improve the drought tolerance of winter oilseed rape plants and is, therefore, beneficial for drought adaptation in oilseed rape.

Keywords: *Brassica napus*; exogenous proline; recovered growth; simulated drought

1. Introduction

Drought stress has detrimental effects on plant growth and development and poses a major threat to sustainable crop production in a rapidly changing environment worldwide. Europe's climate has already become more extreme than previously predicted and rainfall deficits can occur at any time of year [1–3]. Plants achieve different physiological and molecular defences to be tolerant to drought stress. It has been estimated that about 80–95% of the fresh biomass of the plant body is water, which plays a vital role in various physiological processes, including many aspects of plant development, and metabolism [4]. One of the adaptive metabolic responses to drought is the accumulation of proline [5]. Proline accumulation is a common physiological response in many plants to a variety of biotic and abiotic stresses [6,7]. Proline accumulates in the cytosol without damaging cellular structures, and it is an essential part of the physiological adaptations to stress in many plant species [8,9]. Farooq et al. [10] reported that the use of proline as an osmotic protection against water deficit in wheat plants resulted in the accumulation of high levels of chlorophyll, proline, glycine betaine, and all soluble phenols. In addition, the use of osmoprotectants has attracted a lot of attention due to their high efficiency, ease of use, low cost, and no need for advanced equipment. Osmoprotectants or compatible solutions are small, highly soluble organic molecules at physiological

pH with a neutral charge and low toxicity [11]. It has been shown that proline can protect cells by increasing water uptake potential and facilitating enzyme activation [12]. The relationship between proline content and abiotic stress tolerance in plants is still not clearly understood. However, there is a consensus among plant researchers that the accumulation of proline is beneficial for plants, especially during recovery from stress. In recent years, various studies have suggested that the exogenous application of proline by foliar spraying may play an important role in enhancing abiotic stress tolerance in plants [13–17]. Furthermore, many reports reveal that different plant species have different responses to drought stress, which are generally dependent on the intensity of the stress and the species of plant [18–20]. *Brassica napus* L. is an important oilseed plant in the world and the oil extracted from it is used for human and industrial applications due to its fatty acid composition [21,22].

Drought stress adversely affects germination, seedling establishment, photosynthetic efficiency, mineral uptake, shoot elongation, yield, and quality in rapeseed [23]. It is a problem that needs to be addressed by knowing the targeted pathways and processes. For this reason, the application of exogenous proline in sustainable agriculture practices has emerged as innovative and environment-friendly technology for improving rapeseed productivity. Understanding the role of proline in rapeseed growth and development under drought stress can help the selection of technologies that may be beneficial to regional climate conditions. For example, in Europe, higher temperatures will accelerate the development of winter rapeseed in autumn, making the crop more susceptible to low spring temperatures, but higher temperatures in summer can also cause drought stress and affect plant growth and development [24]. Although the application of exogenous proline effectively reduces the adverse effects of stress, the effect of proline on rapeseed plants depends on the growth stage, the timing and method of application, and the proline concentration. That is why the advanced study on proline metabolism in stress response should be attentive. Thus, the exogenous application of proline may be an effective approach to reducing the adverse effects of water deficit stress, however, the potential role of proline in improving resistance to prolonged drought in rapeseed has not been investigated.

We hypothesize that exogenous proline application will improve drought tolerance and the growth of rapeseed under simulated drought conditions. The objectives of this study were to (1) explore does proline improves shoot growth under water deficit conditions, (2) investigate rape seedling biochemical responses to proline exposure during prolonged drought stress and (3) determine the effect of proline on plant recovery and survival after watering.

2. Results

2.1. Options of Different Concentrations of Proline to Improve RWC and Growth Recovery of Drought-Stressed Oilseed Rape

Differences in RWC of oilseed rape leaves became more pronounced on the 8th day of drought: plants treated with 1 mM proline at 3 and 12.5 ml per pot had RWC of 71% and 73% (mild stress), respectively, whereas drought-only treated plants had RWC of 63% and 52% (high stress). It should be noted that low doses of proline also slightly increased the RWC of leaves of continuously watered plants, i.e. from 83% to 86%. Thus, at 1 mM proline at 12.5 mL per pot, plants were less wilted compared to the drought control (Table 1).

Table 1. Effect of Proline Treatment on RWC of Leaves with a Continuous Increase in Water Deficit (Simulating Drought).

Treatment (3 ml)	RWC, %	
	4 days	8 days
Control, H ₂ O	82.3 a	82.5a
Proline 0.1 mM	81.5 a	81.7a
Proline 1 mM	82.3 a	80.7a
Proline 10 mM	84.0 a	85.6 a
Drought	70.1 c	63.2 d
Proline 0.1 mM + Drought	74.7 b	58.8 e
Proline 1 mM + Drought	73.6 b	70.7 c
Proline 10 mM + Drought	70.9 c	59.8 f
Treatment (12.5 ml)		
Control, H ₂ O	84.2 a	83.5 a
Proline 0.1 mM	85.2 a	87.2 a
Proline 1 mM	80.7 a	87.3 a
Proline 10 mM	80.1 a	86.8 a
Drought	63.1 d	51.9 e
Proline 0.1 mM + Drought	73.7 b	60.1 d
Proline 1 mM + Drought	74.6 b	72.6 bc
Proline 10 mM + Drought	70.4 c	58.7 d

* Mean (\pm SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Duncan's Multiple Range Test (DMRT).

The choice of proline concentration and dose to improve the growth of oilseed rape under simulated prolonged drought conditions showed that the most suitable concentration was 1 mM at 12.5 ml per pot (Table 2). It was observed that this dose of proline enhanced the final average weight of recovered seedlings after 4 days of irrigation.

Table 2. Effect of Proline on the Weight of Oilseed Rape Recovering from Prolonged Drought after 4 Days of Irrigation.

Treatment (3 ml)	Average Weight (g)	
	Fresh	Dry
Control, H ₂ O	0.91 ab	0.051 a
Proline 0.1 mM	0.88 b	0.052 a
Proline 1 mM	1.01 a	0.055 a
Proline 10 mM	1.24 a	0.052 a
Drought	0.84 c	0.041 c
Proline 0.1 mM + Drought	0.75 cd	0.045 ab
Proline 1 mM + Drought	0.78 c	0.049 ab
Proline 10 mM + Drought	0.97 ab	0.044 b
Treatment (12.5 ml)		
Control, H ₂ O	1.13 a	0.057 a
Proline 0.1 mM	1.26 a	0.056 a
Proline 1 mM	1.47 a	0.059 a
Proline 10 mM	1.35 a	0.057 a
Drought	1.02 b	0.043 c
Proline 0.1 mM + Drought	1.07 ab	0.043 c
Proline 1 mM + Drought	1.18 a	0.055 ab
Proline 10 mM + Drought	1.11 a	0.051 b

* Mean (\pm SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying DMRT.

Thus, treatment with 1 mM proline 12.5 ml per pot, was selected for further experiments because it promotes plant RWC and drought-stressed plants growth recovery (Figure 1).

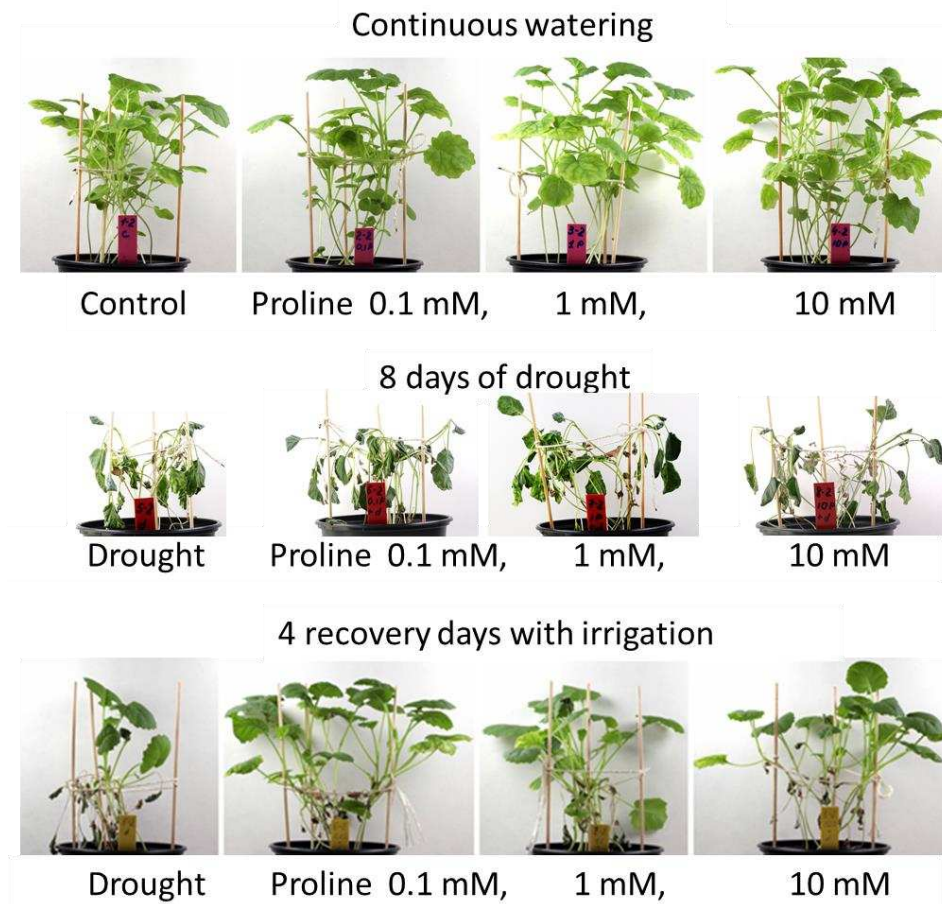


Figure 1. Photograph of *Brassica napus* seedlings exposed to proline (12.5 ml) after 8 days of drought compared to continuously watered plants.

2.2. Impact of Proline Application on Morphometric Parameters of Oilseed Rape Seedlings Exposed to Drought

Drought stress caused a decrease in fresh and dry weight and length of rapeseed seedlings. Proline treatment had no reliable effect on seedling length both in continuous watering and in drought-stressed. Exogenous application of proline improved the weight of drought-stressed oilseed rape: fresh weight was increased by 31% and 9%; dry weight was increased by 20% and 15% on the 4th and 8th day of drought, respectively (Table 3). The best result was that after 8 days of prolonged drought and recovery after 4 days of irrigation, the length and final average fresh weight of the seedlings treated with 1 mM proline were the highest and close to the continuously watered control.

Table 3. Effect of Proline (1 mM) on Morphometric Parameters of Oilseed Rape Seedlings (per Plant) under Simulated Drought Conditions.

Treatment	Average Length (cm)			Average Weight (g)					
				Fresh			Dry		
	4 days	8 days	4 days recovery	4 days	8 days	4 days recovery	4 days	8 days	4 days recovery
Control H ₂ O	15.26 a	17.27 a	18.00 b	0.68 b	0.74 b	0.77 b	0.045 a	0.045 a	0.046 a
Proline	15.76 a	17.93 a	19.03 a	0.74 a	0.87 a	0.89 a	0.048 a	0.048 a	0.049 a
Drought	14.64 b	14.74 c	15.71 c	0.51 c	0.45 d	0.46 c	0.030 c	0.041 b	0.038 b
Proline + Drought	14.89 ab	15.29 b	17.75 b	0.67 b	0.49 c	0.70 b	0.036 b	0.047 a	0.039 b

* Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying DMRT.

2.3. Impact of Proline Application on RWC of Prolonged Drought-Stressed Oilseed Rape leaves

After the first 4 days of drought, the plants were highly stressed according to the Hsiao [X] standard, as shown by the reduction of the RWC of oilseed rape leaves by up to 61% (Figure 2). Proline treatment had a positive effect on the RWC of drought-stressed oilseed rape leaves, increasing it to 79% (moderate stress). Even more, after 8 days of prolonged drought, the RWC of the proline-treated seedlings was much closer to the irrigated control than that of the drought-stressed plants, which had only 40% RWC. After 4 days of seedling recovery by irrigation, the RWC of the proline-treated plants was close to that of the continuously irrigated control.

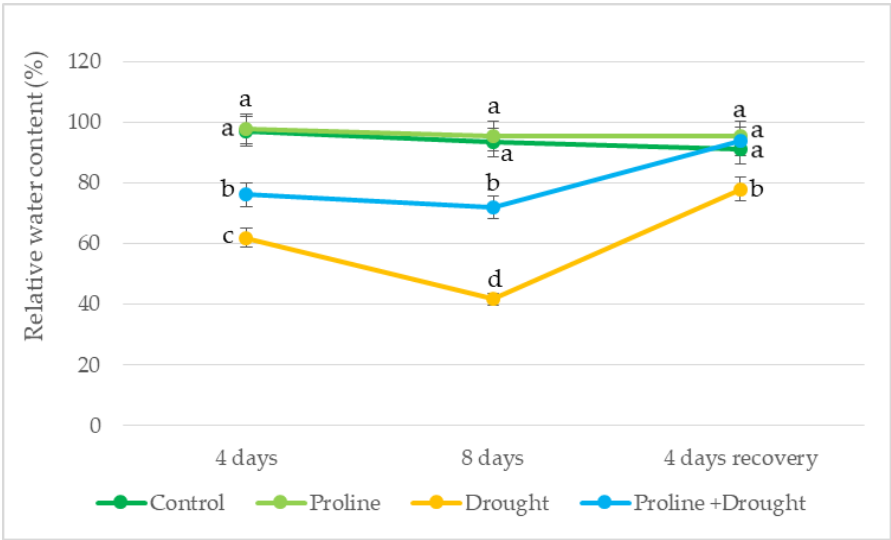


Figure 2. Impact of proline application on RWC of oilseed rape leaves after simulated prolonged drought and recovery by watering. The horizontal axis indicates the duration of prolonged drought and recovery by irrigation. Vertical error bars represent the standard deviation of the mean of three replications (n = 3). Different lowercase letters indicate statistically significant differences ($p < 0.05$).

2.4. Impact of Proline Application on Chlorophyll Content of Drought-Stressed Oilseed Rape Leaves

Analysis of chlorophyll a and b content in oilseed rape leaves showed that after 8 days of drought, the concentration of chlorophyll a decreased to $0.57 \text{ mg g}^{-1} \text{ FW}$, while the chlorophyll b to $0.12 \text{ mg g}^{-1} \text{ FW}$, as compared with watered control 0.99 mg g^{-1} and $0.23 \text{ mg g}^{-1} \text{ FW}$, respectively. Proline significantly increased chlorophyll accumulation after 4 days of drought vs untreated drought control, whereas after 8 days of drought, there was no significant difference between chlorophyll accumulations in leaves of these variants. The highest content of total chlorophylls was

found in plants exposed to 8 days of prolonged drought and recovered after 4 days of irrigation (Table 4).

Table 4. Effect of Proline Application on Chlorophyll Content of Oilseed Rape Seedlings under Drought Stress.

Treatment	Chlorophyll contents (mg g ⁻¹ FW)								
	Chlorophyll a			Chlorophyll b			Chlorophyll a+b		
	4 days	8 days	4 days recovery	4 days	8 days	4 days recovery	4 days	8 days	4 days recovery
Control, H ₂ O	0.98 a	0.99 a	0.85 a	0.24 a	0.23 a	0.24 a	1.22 a	1.22 a	1.09 a
Proline	0.97 a	1.02 a	0.85 a	0.25 a	0.24 a	0.25 a	1.22 a	1.23 a	1.11 a
Drought	0.78 c	0.57 c	0.67 b	0.21 a	0.12 b	0.18 b	0.98 c	0.69 c	0.85 b
Proline + Drought	0.93 b	0.62 b	0.89 a	0.21 a	0.13 b	0.22 a	1.13 b	0.75 b	1.10 a

* Mean (±SE) was calculated from three replicates for each treatment. Different letters in columns designate statistically significant difference at p ≤ 0.05 applying DMRT.

2.5. Impact of Proline Application on Ethylene Emission of Drought-Stressed Oilseed Rape Leaves

In plants treated with proline, ethylene emission was significantly lower by 30% and 19% than only drought treated on the 4th and 8th day, respectively. After 8 days of drought and 4 days of recovery by watering, ethylene levels in proline-treated plants were reduced to those of irrigated control (Figure 3).

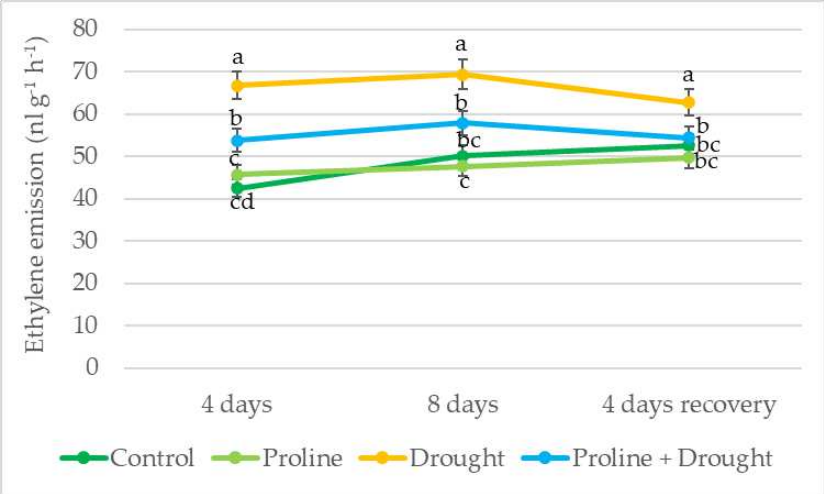


Figure 3. Impact of proline application on ethylene emission of oilseed rape leaves after simulated prolonged drought and recovery by watering. The horizontal axis indicates the duration of prolonged drought and recovery by irrigation. Vertical error bars represent the standard deviation of the mean of three replications (n = 3). Different lowercase letters indicate statistically significant differences (p < 0.05).

2.6. Impact of Proline Application on H₂O₂ Levels of Drought-Stressed Oilseed Rape Leaves

Spraying with exogenous proline significantly reduced H₂O₂ content in watered oilseed rape leaf tissues. Impact of exogenous proline manifested on 4-day and 8-day of drought: H₂O₂ content decreased to 33 % compared to the respective drought control. In addition, after 8 days of drought and 4 days of recovery by watering, H₂O₂ levels in proline-treated plants were lower by 2-fold (Figure 4).

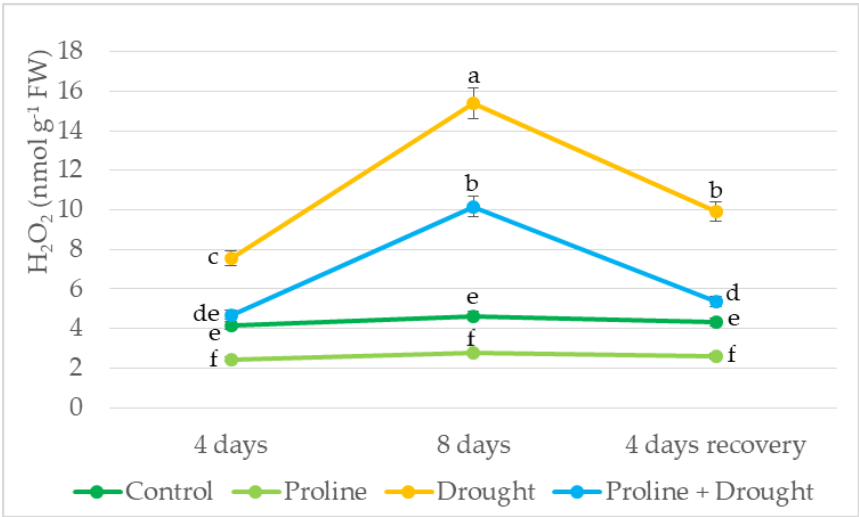


Figure 4. Impact of proline application on H_2O_2 level of winter oilseed rape leaves after simulated prolonged drought and recovery by watering. The horizontal axis indicates the duration of prolonged drought and recovery by irrigation. Vertical error bars represent the standard deviation of the mean of three replications ($n = 3$). Different lowercase letters indicate statistically significant differences ($p < 0.05$).

2.7. Impact of Proline Application on MDA Content of Drought-Stressed Oilseed Rape Leaves

MDA level showed that drought stress significantly increased lipid peroxidation in oilseed rape leaves: after 4 days of drought stress, the amount of MDA in the plants increased by 2 fold, and after 8 days even up to 2.5 fold. In plants treated with proline, the MDA content on the 4th and 8 the day of the drought was significantly lower by 56% and 75% than only drought treated, respectively. In addition, after 8 days of drought and 4 days of water recovery, both proline + drought-treated plants and only drought-treated ones had lower MDA content than continuously irrigated plants (Figure 5).

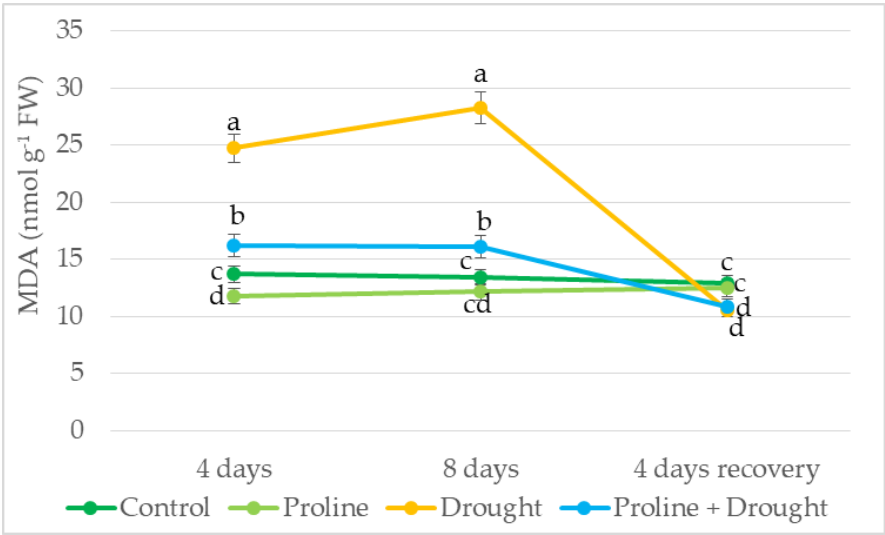


Figure 5. Impact of proline application on MDA content of winter oilseed rape leaves after simulated prolonged drought and recovery by watering. The horizontal axis indicates the duration of prolonged drought and recovery by irrigation. Vertical error bars represent the standard deviation of the mean of three replications ($n = 3$). Different lowercase letters indicate statistically significant differences ($p < 0.05$).

2.8. Effects of Exogenous Proline on PM ATPase activity of Rapeseed Seedlings Exposed to Drought

After 4 days of drought, PM ATPase activity decreased by 4-fold in oilseed rape leaf cells. Application with exogenous proline increased the H⁺-ATPase activity of drought-exposed plants by 62% vs only drought-treated plants. After 8 days of drought PM ATPase activity of proline + drought-treated plants increased by 5 fold vs drought test variant, and approached that of the control after recovery of 4 days of irrigation (Figure 6).

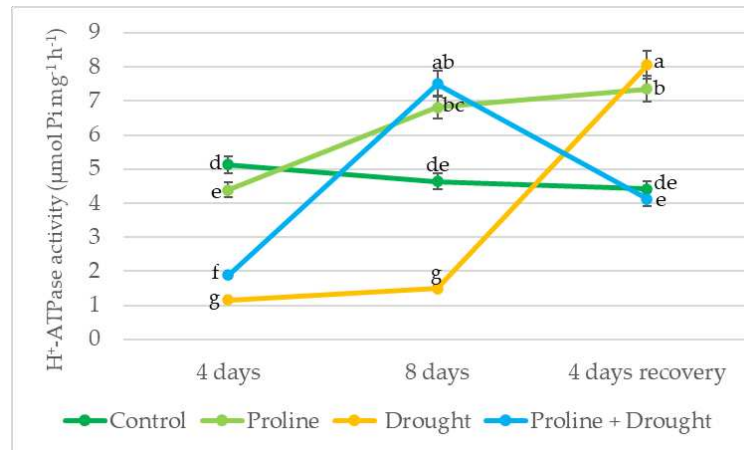


Figure 6. Impact of proline application on PM ATPase activity of winter oilseed rape leaves after simulated prolonged drought and recovery by watering. The horizontal axis indicates the duration of prolonged drought and recovery by irrigation. Vertical error bars represent the standard deviation of the mean of three replications (n = 3). Different lowercase letters indicate statistically significant differences (p < 0.05).

2.9. Effect of Exogenous Proline on Endogenous Proline Content of Rapeseed Seedlings Exposed to Drought

The continuously irrigated oilseed rape plants sprayed with proline had an average of 32 % more endogenous free proline than the control plants throughout the study period. Drought stress significantly increased proline levels: during the first 4 days of drought, proline levels increased by a 2 fold, and as the drought progressed by up to 4 fold. Rapeseed plants in the proline-drought variant had 20-23% more free endogenous proline than plants subjected to 8 days of drought alone. In plants treated with proline and exposed to drought for 8 days and recovering after 4 days of irrigation, the endogenous proline content has come closer to that of irrigated plants, still, in the drought test variant, the level of endogenous proline decreased to a lesser extent (Figure 7).

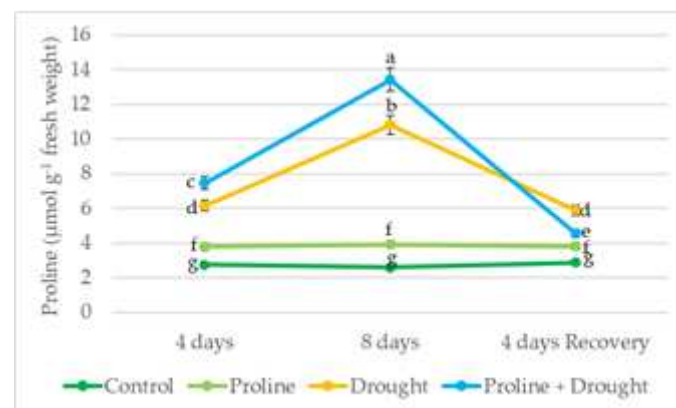


Figure 7. The effect of exogenous proline application and drought stress, on winter rapeseed proline content. Vertical error bars represent the standard deviation of the mean of three replications (n = 3). Different lowercase letters indicate statistically significant differences (p < 0.05).

2.10. Effect of Exogenous Proline on Survival of Plants

Survival evaluation of winter oilseed rape seedlings after 8 days of simulated drought stress and 12 days of recovery by watering showed that the number of surviving plants was significantly higher (more than 2 fold) after proline treatment compared to drought test variant plants (Table 5).

Table 5. Effect of proline on *Brassica napus* plant survival after 8 days of prolonged drought recovered after 12 days of irrigation.

Treatment	Number of survived plants (%)
Control H ₂ O	100.00 a
Proline	100.00 a
Drought	19.79±2.21 b
Proline + Drought	44.10 ±3.15 c

Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying DMRT.

3. Discussion

Different crop species respond differently to drought on the intensity of stress and type of crops [26]. Also drought is one of the many factors with a negative effect morphology, physiology and development of the economically important rapeseed plants [27,28]. Rapeseed is highly sensitive to drought stress, and global climate change leading to severe and prolonged drought in some parts of the world is expected to reduce productivity of rapeseed. There are few studies that water deficit induce metabolic changes in plants which led to the accumulation of specific metabolites such as proline [29,30]. Therefore, proline may help plants to regulate the osmotic potential of cells and to improve water absorbance and translocation under drought conditions [31]. Moreover, data from the literature indicate that exogenous application of osmoprotectants has shown positive effects on plants growing under drought stress, promoting growth and antioxidant activity [32,17]. In addition, proline plays an essential role in protecting plants from various stresses and helping them recover faster from stress [8]. Previous studies have shown that the relative water content (RWC) of leaves is one of the main indicators of water status and the ability of plants to grow and develop under drought stress [33–35]. So, the RWC informs of the relative water content in leaves and is directly influenced by the soil water content. On the other side, Abdelaal et al. [36] showed that the leaf RWC of barley plants during drought was restored by exogenous proline. We found that exogenous proline affected relative water content (RWC) in rapeseed leaves under continuously increasing water deficit (simulated drought). Our data showed that drought-treated plants exposed to 1 mM proline had the best leaf water retention. It should be noted that low doses of proline also slightly increased the RWC of leaves of continuously watered plants compared to drought control.

Many authors have provided evidence that drought stress negatively affected the morphological parameters, including the fresh and dry weight of shoots, which decreased under drought stress. Thus, plant biomass formation can be considered an important indicator for drought stress assessment [27,32,35,37]. This coincides with the data of our study which revealed that drought stress significantly reduced rapeseed plant growth in terms of fresh and dry biomass. According to the literature, exogenous proline increased the growth of maize [38] and wheat [39] plants and the dry weight of barley plants [36] under drought conditions. Our research has shown that application of exogenous proline (1 mM) was effective in increasing fresh and dry mass of shoots, both under normal water supply and under drought stress. The best result was obtained after irrigation recovery of drought-stressed seedlings sprayed with 1 mM proline: the average fresh weight was the highest and close to the continuously irrigated control.

There is no doubt that one of the most important changes in metabolic functions during drought is the loss of photosynthetic pigments or a decrease in their synthesis. Numerous studies have reported that drought-induced oxidative stress significantly reduces chlorophyll content in crop plants [28,34,35,40]. This was also confirmed by the data of our study showing that moderate and

severe drought stress significantly reduced the content of chlorophyll a, b, and compared to control plants. It is important to note that exposure of plants to exogenous proline before drought significantly improved photosynthetic pigment levels not only throughout the drought but also during plant recovery after the resumption of watering. Other researchers also noted that exposure to exogenous proline improved chlorophyll content in wheat [10], barley [36], and rice [41] plant leaves under drought conditions.

Literature data suggested that ethylene emission in plants increases during drought stress [42]. In our study, ethylene content significantly increased during drought stress. In addition, application with exogenous proline decreased ethylene accumulation vs only drought-treated plants, indicating reduced stress levels. After growth recovery by watering, phytohormone ethylene emission of proline-treated plants remained close to irrigated control. Our data contribute to the suggestion that ethylene content may help to remove the inhibitory effect of drought stress on plant growth [43].

Drought may also induce stress responses which result in the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide H_2O_2 [8,44]. Our study showed that H_2O_2 concentration during 8 days of drought increased 3.3-fold compared to plants grown under irrigation conditions. Similar drought-induced oxidative stress was recorded in oilseed rape plants in studies by other authors [28,34,36]. Excessive concentrations of free radicals, including H_2O_2 , can cause damage to cell membranes, ion leakage, and osmotic imbalance, so maintaining their level is very important [34,40]. Plant spraying with exogenous proline significantly reduced H_2O_2 content in prolonged drought-stressed rapeseed leaf tissues. In addition, after 4 days of recovery by watering, the H_2O_2 level in proline + drought-treated plants decreased even by 2-fold. These data agreed with the results obtained by Abdelaal [36] where exposure to exogenous proline significantly reduced H_2O_2 content in leaves of barley plants under drought conditions. There are reports in the literature [45,46] characterizing the antioxidant properties of proline in detoxifying ROS. In addition, Rejeb and colleagues [47] in their study concluded that the role of proline as a free radical scavenger is more important than its role as a simple osmolyte.

The stability of cell membranes in drought-stressed plants is adversely affected by ROS, which is evident from the increase in MDA [28,36]. In our study, lipid peroxidation results showed significant membrane damage due to drought. MDA content was elevated in drought-stressed plants and increased with increasing drought time from 1.8 to 2.1 fold that of irrigated plants at 4 and 8 days of drought, respectively. Furthermore, after 8 days of drought and 4 days of recovery by watering, MDA content in proline-treated plants was lower than in continuously irrigated plants. Similar results of increased MDA in rapeseed under drought were also obtained by other researchers [28,33,36]. Meanwhile, a significantly lower amount of MDA - 1.5–1.7 fold - was detected in the cells of plants exposed to exogenous proline before experiencing drought stress, which indicates better stability of the cell membranes of these plants. Similar results where exposure to exogenous proline reduced malondialdehyde levels under drought were obtained in wheat [10], barley [36], and rice [41] plants. This reduction in MDA contents could be attributed to the putative role of osmolytes in alleviating the deleterious effects of stress on the structure of cell membranes and activities of PM enzymes as well as reducing the generation of highly destructive free radicals [38,41,48].

The properties, integrity and composition of the cell membrane can have a major impact on the activity of the PM ATPase, which displaces protons from the plant cell, creates an electrochemical gradient across the plasma membrane and plays an important role in various aspects of plant physiology. PM ATPase activity has been reported to be altered by drought in many plant species [49,50]. In our study, PM ATPase activity is significantly reduced by drought stress, a reduction that has been previously reported by other investigators [51,52]. These findings are also in agreement with the results of Mi et al [53], who showed that Na^+,K^+ -ATPase and Ca^{2+},Mg^{2+} -ATPase activities of winter rapeseed leave cells gradually decreased with increasing drought severity. In our study: the exogenous application of proline significantly increased PM ATPase activity (from 62 to 400%) compared to drought-exposed, proline-untreated plants. These findings are in line with other researchers who have shown that proline not only protects cells by increasing water uptake potential but also facilitates enzyme activation [12]. In the present study, we showed that the PM ATPase

activity of proline-treated oilseed rape recovering from drought stress approached that of control continuously watered plants.

The role of the accumulation of the osmoprotectant proline under drought stress is being actively investigated in order to clarify plant resistance to dehydration. Several reports indicate that under drought stress, plants accumulate higher levels of osmolytes such as proline, which prevent cellular water loss and play an important role in maintaining tissue turgor [54,55]. Our study found that prolonged drought stress significantly increased proline levels, with a 2-fold increase in proline levels during the first 4 days of drought, and as much as a 4-fold increase as the drought progressed. Literature data demonstrate that the exogenous application of proline can increase its endogenous levels in plant tissues exposed to drought stress contributing to the maintenance of drought adaptation in plant tissues [17]. In our research, we confirmed that exogenous proline treatment increased the concentration of endogenous proline in rapeseed leaves under prolonged drought. In addition, it is suggested that the higher levels of proline accumulated in plants exposed to severe and moderate stress conditions may be essential in plant recovery from stress [56]. Our research showed that after watering was renewed, endogenous proline levels of proline + drought-treated plants approached those of irrigated plants.

It was also evident that exogenous proline (1 mM) improved the survival of winter canola seedlings upon resumption of irrigation after 8 days of drought stress. We estimated that the number of surviving plants after 12 days of recovery was more than 2 fold higher proline + drought test variant compared to plants exposed only to drought. Thus, the response of plants to drought stress is complex and involves many physiological, biochemical, cellular, and molecular changes to ensure plant survival.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Oilseed rape seeds (*Brassica napus* L. cv. 'Visby') were sown in plastic cubic pots with a peat moss substrate (pH 5.5-6.5). Plants were germinated and grown under controlled conditions of a constant temperature of 23 ± 1 °C, a photoperiod of 16/8 h and a fluorescent light photon flux of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ at soil level, and at 65% humidity in a Climacell plant growing chamber (Medcenter Einrichtungen GmbH). Soil moisture was maintained at ~70%.

4.2. Treatments

4.2.1. L-proline aqueous solutions (Roth) was used for seedling spraying at the 3–4 leaf stage (BBCH-scale 13-14 [57]).

4.2.2. Drought treatment-for the drought stress control studies. During the simulated drought, irrigation was stopped to allow gradual drying of the soil. Soil moisture was estimated using a soil moisture meter (Biogrod, China).

4.3. Determination of the Active Proline Concentration

To determine the active proline concentration, seeds of oilseed rape were sown in 16x3 pots with peat substrate. Each experimental unit consisted of 14 seeds. Pots without proline served as control. Three treatments of the study of proline impact were used: 1) control, H₂O 3 ml per pot; 2) proline 0.1 mmol 3 ml per pot; 3) proline 1 mmol 3 ml per pot; 4) proline 10 mmol 3 ml per pot; 5) control, H₂O 12.5 ml per pot; 6) proline 0.1 mmol 12.5 ml per pot; 7) proline 1 mmol 12.5 ml per pot; 8) proline 10 mmol 12.5 ml per pot, other eight treatments were the same plus drought treatment. Seedlings at the BBCH 13–14 third-fourth leaves stage [57] were foliar sprayed with water solutions of proline, control was sprayed with water.

4.4. Experimental Design of Drought Stress Control Studies

Plants were sown in plastic cubic pots (15x35 cm) (12 pots in total), 60 seeds per pot, in a peat moss substrate. Four pots were used for the experiment: two for rational watering and two for drought simulation, repeated three times with four pots each. Aqueous solution of L-proline 1 mmol 12.5 ml per pot was sprayed according to the following scheme: 1) control watering, 2) proline and watering, 3) drought, 4) proline and drought (Figure 8.).

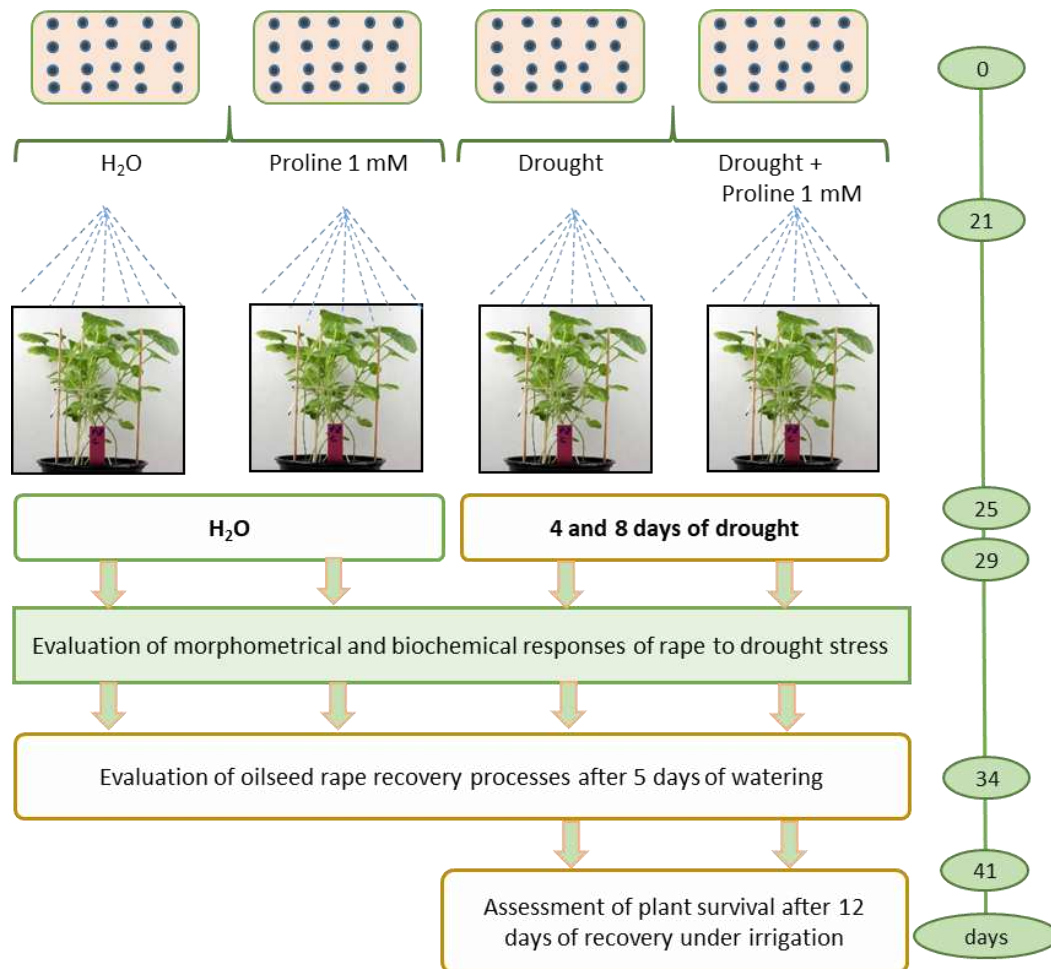


Figure 8. Experimental design of simulated drought stress control studies.

4.5. Sampling

Plant samples were taken for analysis on three occasions: on the 4th day of the drought (soil moisture 40%), on the 8th day of the drought (soil moisture 20%), and on the 4th day of plant recovery after watering when soil moisture was 70%. The watered plants, used as controls, were sampled at the same time (soil moisture 70%). Shoots of thirty wheat seedlings were sampled for morphometrical measurements. For biochemical analysis, three independent replicates were carried out using the third leaves of rapeseed plants. Freshly harvested samples were used for ethylene emission analysis and pigment measurement. For H⁺-ATPase activity, MDA, H₂O₂ and proline assays the samples were immediately frozen in liquid nitrogen and stored in a low-temperature freezer (Skadi Green line, EU) at -80 °C until the analysis. Survived seedlings were counted after 41 days of cultivation right away.

4.6. Morphometrical Measurements

Shoot length, fresh and dry mass were taken after 4, 8, and 12 days of growth after treatments using a ruler and balances (Kern EWJ, Germany and Sartorius BP 110S, Korea).

4.7. Recovery Evaluation

The same morphometric and biochemical parameters were measured as after 4 and 8 days of drought.

4.8. Survival Evaluation

The plant survival test was carried out by starting to water the plants after 8 days of prolonged drought stress. The test needs to wait for 5-12 days to confirm that winter oilseed rape seedlings are recovering by generating green shoots at the apical meristem growth point in the middle leaf rosette [58]. Thus, plants in each pot were scored as dead or alive after a 12-day recovery period. Plant survival was presented as a percentage of recovered plants.

4.9. Relative Water Content (RWC)

To determine RWC rapeseed leaves were collected and weighted as fresh weight (FW). Then leaves were allowed to fully hydrate on the surface of pure water for 1 d at 4°C in the dark and their turgid weight (TW) was recorded. The leaves were drying for 2 d at 80°C in a drying chamber and weighted to determine the dry weight (DW). RWC have been calculated according to the formula: $RWC = 100 \times (FW - DW)/(TW - DW)$ [59].

4.10. Assessment of Biochemical Parameters

4.10.1. Photosynthetic Pigments.

The photosynthetic pigments were extracted from fresh leaves with N, N'-dimethyl-formamide (DMF) (Sigma-Aldrich). Light absorption was measured at 480, 664, 647 nm. Chlorophyll a/b ratio and chlorophyll a and b contents were calculated according to Wellburn [60].

4.10.2. Ethylene.

Method of Child et al. [61] was used to evaluate ethylene emission from freshly harvested leaves. Samples with known mass were placed in 40 mL clear glass vials (Agilent technologies) sealed with PTFE/Si septa caps and incubated for 24 h at 21 °C in darkness. Following incubation, 1 mL of gas sample from each vial was sampled using a gas-tight syringe (Agilent technologies) and injected into a gas chromatograph equipped with a stainless-steel column (Propac R) and hydrogen flame ionization detector. The temperature of the in-jector, column and detector was 110.90 and 150 °C, respectively. Helium (AGA) was used as the carrier gas. Calibrations were made with ethylene standard (Messer). Results were expressed as $\text{nl g}^{-1} \text{FW h}^{-1}$.

4.10.3. Hydrogen Peroxide (H₂O₂).

Rapeseed leaves (0.5 g) were homogenized using 5% trichloroacetic acid (TCA) (Sigma-Aldrich). H₂O₂ content in leaves was determined according to [62]. The supernatant was mixed with 10 mM, pH 7.0 potassium phosphate buffer (Alfa Aesar) and 1 M potassium iodide (Alfa Aesar) in a ratio of 1:1:2. The reaction solution was incubated for 30 minutes at 25 °C in the dark. The absorbance of the supernatant was measured at 390 nm. The amount of H₂O₂ was calculated using a standard curve. The results expressed in $\mu\text{mol g}^{-1} \text{FW}$.

4.10.4. MDA content.

For analysis of lipid peroxidation, MDA leaf material (0.5 g) was homogenized using 5% trichloroacetic acid (TCA) (Sigma-Aldrich). The method of Hodges et al. with slight modifications, have been used to estimate MDA. The homogenates were centrifuged at 13 g for 17 min (centrifuge MPW-351 R), and supernatant was added to 20% TCA containing 0.5% thiobarbituric acid (TBA) (Alfa Aesar). The homogenate was incubated in a heater at 95 °C for 30 min (Blockthermostat BT 200, Kleinfeld, Labortechnik) and then subsequently cooled on the ice. The optical density was measured

at 532 and 660 nm by spectrophotometer (Analytik Jena Specord 210 Plus). The results were expressed in $\mu\text{mol g}^{-1}\text{ FW}$ [63].

4.10.5. H^+ -ATPase activity assay.

Membrane-enriched microsomal fraction was extracted from plant samples. Protein content was measured using the Bradford dye-binding procedure [64] at 595 nm. The H^+ -ATPase activity of microsomal fraction was evaluated according to re-leaked inorganic phosphate (P_i) that accumulates as a result of ATP hydrolysis [65]. The colour reaction for P_i measurement was performed with ammonium molybdate and stannous chloride at 750 nm. The activity of H^+ -ATPase was expressed as $\mu\text{mol P}_i \text{ mg}^{-1}$ of protein h^{-1} .

4.10.6. Proline.

1. A color reaction of acidified ninhydrin was used to determine the content of proline [66]. The equal volume of supernatant of a ground plant material (0.5 g), acetic acid, and acidified ninhydrin was mixed and heated for 1 h 15 min at 108.5°C in a heater. The formed chromophore was extracted with toluene. The absorbance was read spectrophotometrically at 520 nm using a multi-sample quartz cuvette and Rainbow microplate reader. The corresponding content of proline was determined using the standard curve. Calculations were provided using the SLT program (SLT Labinstruments). Results were expressed as μmol of proline $\mu\text{mol g}^{-1}\text{ FW}$.

4.11. Statistical analysis

2. The results are presented as mean \pm standard deviation (SD) of three independent experiments with at least three replicates. The data were analysed using analysis of variance (ANOVA). Tukey's test was performed to test the statistical significance of differences ($p < 0.05$) between means. Post hoc test Duncan's Multiple Range Test (DMRT) was used for comparing the means of different groups after performing an analysis of variance (ANOVA).

5. Conclusions

In the current study, we determined that exogenous proline improved the tolerance of rapeseed growth to prolonged drought by maintaining water status, the accumulation of photosynthetic pigments, osmoprotective substances, and the stability of the plasma membrane in leaf tissues and thus affected the recovery of irrigated rapeseed seedlings after drought stress. Exogenous proline initiated changes in endogenous proline levels of leave tissues. The accumulation of proline is beneficial for plants, especially during recovery from stress: the number of survived plants was significantly higher (2-fold) after proline treatment than the drought variant. the analysis of the stress-mitigating role of exogenous proline leads to conclude that it could be proposed for improving plant drought-stress resistance and increasing the amount of oilseed rape products.

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