



## Article

# Involvement of Nitric Oxide in Methyl Jasmonate-Mediated Regulation of Water Metabolism in Wheat Plants under Drought Stress

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**Abstract:** Drought is a serious challenge that causes significant crop loss worldwide. The developmental processes of plants are regulated by phytohormones and signaling molecules that crosstalk together in signaling cascades. We suppose that nitric oxide (NO) is a secondary messenger of the JAs signaling pathway, as  $10^{-7}$  M methyl jasmonate (MeJA) pretreatment regulates NO accumulation in wheat plants under drought stress, modulated by 12% polyethylene glycol (PEG), and in control plants. This study aimed to compare  $2 \times 10^{-4}$  M nitric oxide donor sodium nitroprusside (SNP) and MeJA pretreatments in regulating growth and water balance parameters at the vulnerable initial first-leaf stage of wheat growth. The application of 12% PEG decreased transpiration intensity twofold, relative water content (RWC) by 7–9%, and osmotic potential of cell sap by 33–40% compared with those of control plants. Under drought, MeJA- and SNP-pretreated plants decreased transpiration intensity by 20–25%, RWC by 3–4%, and osmotic potential of cell sap by 16–21% compared with those of control plants, and enhanced the proline content by 25–55% compared with MeJA- and SNP-untreated plants. Our results suggest that pretreatment with MeJA as well as SNP could mitigate drought stress in wheat plants. Similarities in MeJA- and SNP-induced shifts in plant water balance suggested that NO is a mediator of MeJA-induced regulation of wheat water content during water deficit.

**Keywords:** *Triticum aestivum* L.; water balance; drought stress; methyl jasmonate; nitric oxide; transpiration; proline; osmotic potential; polyethylene glycol 6000; sodium nitroprusside



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## 1. Introduction

The weather on Earth has become very changeable and unpredictable. More than 60% of the world's irrigated agricultural lands are exposed to prolonged or frequent drought stress [1]. Under the influence of this dangerous environmental stress, the relationship between water uptake and water loss in the plant changes significantly, limiting plant distribution and crop productivity worldwide [2,3]. Dehydration is a complex process that can occur under such restricting water supply abiotic stress factors as drought, salinity, high and low temperature, heavy metal exposure, and excess fertilizers [4,5]. In laboratory experiments, dehydration is achieved by the exposure of plants to water withdrawal or osmotic treatments. Osmotic stress factors affect plants on the morpho-physiological, biochemical and molecular levels of their organization, and decrease plant photosynthesis, respiration, biomass accumulation, and yield productivity [6,7]. A drought-induced decrease in water availability to a plant leads to the generation of excess reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, which results in damage to cell components. Plants survive due to two main strategies in response to water limitation: drought avoidance and drought tolerance [8]. Avoidance of drought involves the maintenance of water in plant tissue during water deficits, whereas tolerance implies the ability to withstand water deficits by regulating plant physiology, biochemistry, and gene expression. Changes in

tissue water status trigger some of these responses directly, while many others are caused by plant hormone-dependent signaling [9].

Plant tolerance of water limitation by maintaining transpiration under stress conditions is important for their adaptation. The stress factor causes a decrease in transpiration and CO<sub>2</sub> fixation by reducing conductivity inside the stomata and mesophyll cells, which also leads to reduced plant growth. Abscisic acid (ABA) regulates stomatal closure with the involvement of nitric oxide (NO) as a secondary messenger [8,10]. Along ABA, other phytohormones, such as salicylic acid, brassinosteroids, cytokinins, ethylene, auxines, cytokinines, ethylene, and jasmonates (JAs) regulate water loss by transpiration through stomatal apertures under both biotic and abiotic stresses [11]. Plant roots also integrate environmental influences with plant water status; the water absorptive capacity of plants is controlled not only by stomata openings in shoots, but by changes in the root hydraulic conductivity and length, and by the induction of lateral root growth [12]. It should be mentioned that methyl jasmonate (MeJA) treatment can regulate the hydraulic conductivity of tomato (*Solanum lycopersicum* L.) roots [13].

Jasmonic acid (JA) and over 30 different jasmonate compounds, which are derived in the metabolism of fatty acids, are found in many plant species [14]. Jasmonates (JAs) have diverse physiological roles closely related to plant defense and resistance to biotic and abiotic stresses. JA content transiently increases under water stress conditions [15]. The tolerant chickpea variety accumulated higher JAs levels than the sensitive variety, suggesting the role of this class of phytohormones in drought tolerance [16]. JAs applied externally in small amounts, JA enhanced plant tolerance of heavy metal toxicity [17], drought [18,19], and other osmotic stress factors, not only by stomata aperture regulation but also by reducing oxidative stress [9] and root xylem development [20,21]. In other studies, JAs has been reported as a negative agent that causes the induction of leaf senescence, a notable reduction in growth and yield, so the actual role of JA in drought stress remains controversial [9,22]. The effectiveness of plant treatment by JAs depends on their chemical formula, tested plant species, plant age, method of application, and JA concentration. In addition, it is known that the JA signaling system interacts with the signaling of other phytohormones [20,21,23,24], and thus acts as a hub modulating plant growth and development under optimal growth conditions, as well as under environmental stresses.

The most abundant RNS in plants is nitric oxide (NO), an important naturally occurring signaling molecule that regulates growth and developmental events throughout the life cycle of plants [25]. The increased production of NO in plants under water deficit suggests the universal requirement of NO during drought stress signaling [26]. NO is one of the first signaling molecules to be produced under ABA, and also during auxin, gibberellin, cytokinin, and JA application [9,27]. NO-induced stress tolerance might be ascribed to a combinatory effect of modulation of stomatal response and activation of the antioxidant enzymes [28]. During a water deficit, the exogenous NO treatment of plants ameliorates water loss by stomata closure regulation [29–32] and confers resistance to environmental stresses by the regulation of plant redox status [31–33], through transcriptional regulation and/or through the induction of post-translational modifications (PTMs) of target proteins, peptides, or fatty acids [34], which may be either positive or negative [35].

The effects of NO depend on the site of its application and concentration [36]. It should be mentioned that aside from NO, other secondary messengers, such as CO<sub>2</sub>, Ca<sup>2+</sup>, and H<sub>2</sub> are involved in JA-induced regulation of plant stress tolerance [37,38]. In turn, NO can modulate the activity of JAs biosynthesis enzymes allene oxide synthase and lipoxygenase (LOX2) in thale cress plants (*Arabidopsis thaliana* Heunh.) [39]. Nitric oxide may be associated with the downregulation of JA-biosynthetic genes encoding enzymes, such as LOX2 [27]. It is known that the interaction of NO with JA induces salinity tolerance in tomato plants [31].

Despite the growing knowledge regarding the many NO- and JA-mediated plant functions in different plants, the exact roles of these substances in a plant's response to water deficit are still mostly unclear. Due to similar NO and JA effects on stomata closure regulation [40], it could be supposed that these signaling molecules participate together in plant tolerance under water deficit. Similar changes in water balance parameters in response to sodium nitroprusside (SNP) or MeJA pretreatment during water deficit confirmed that NO is implicated as an important intermediate in MeJA-mediated signaling pathways of drought resistance in wheat plants. Owing to the lack of knowledge of the molecular mechanisms of NO generation in plants, the current research on NO effects in plant water relation relies on the exogenous application of NO donor SNP, and its comparison with the MeJA application. The focus of the present study was to identify the effect of a short water deficit, and to investigate how exogenous individual supplementation of SNP and MeJA influences the water balance regulation of wheat plants at the stage of the first leaf appearance.

## 2. Results

### 2.1. Growth Parameters

Exogenous application of  $10^{-7}$  M MeJA or  $2 \times 10^{-4}$  M active NO donor sodium nitroprusside (SNP/+NO) over 24 h increased the root length by 40% and 25%, respectively, increased shoot height by 7% and 5%, respectively, and increased leaf area by 13% and 11%, respectively, in comparison to the control (Table 1). Maximal enhancement in growth parameters was observed in MeJA-treated seedlings. Short drought stress for 24 h resulted in the decline of root length by 7%, shoot height by 8%, and leaf area by 7%. The application of MeJA or (SNP/+NO) followed by the treatment of polyethylene glycol 6000 (PEG) for 24 h increased root length by 16% and 12% and leaf area by 5% and 4%, respectively, as compared to the control wheat plants, but barely affected shoot height. Root length, shoot height, and leaf area in wheat seedlings pretreated with  $2 \times 10^{-4}$  M (SNP/−NO) sodium nitroprusside, which lacked NO activity after treatment of the active SNP by light and thus served as a control for the active NO donor, did not change in comparison to untreated and stressed plants.

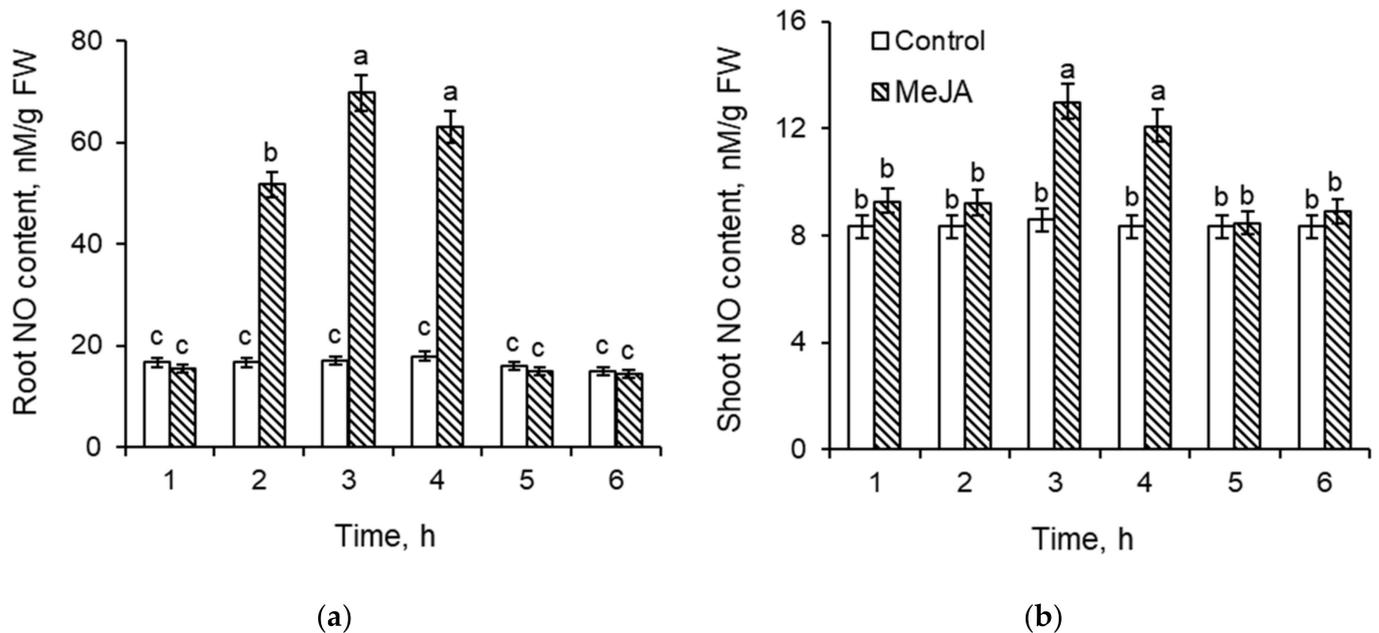
**Table 1.** Growth parameters of 7-day-old wheat plants pretreated with  $2 \times 10^{-4}$  M SNP or  $10^{-7}$  M MeJA over 24 h and subsequently applied by 12% PEG during 24 h.

Treatment	Root Length, cm	Shoot Height, cm	Leaf Area, cm <sup>2</sup>
Control	7.22 ± 0.80	15.75 ± 0.93	3.04 ± 0.55
(MeJA)	10.07 ± 1.32 (140) *	16.80 ± 1.51 (107)	3.43 ± 0.61 (113) ns
(SNP/+NO)	9.00 ± 1.29 (125) *	16.49 ± 1.45 (105)	3.36 ± 0.43 (111) ns
(SNP/−NO)	7.36 ± 0.74 (102) ns	15.77 ± 1.76 (100)	3.07 ± 0.43 (101) ns
12% PEG	6.71 ± 0.92 (93) ns	14.48 ± 1.84 (92)	2.83 ± 0.61 (93) ns
(MeJA)+PEG	8.34 ± 1.43 (116) ns	16.13 ± 2.08 (102)	3.19 ± 0.58 (105) ns
(SNP/+NO)+PEG	8.10 ± 1.23 (112) ns	16.05 ± 1.45 (102)	3.17 ± 0.38 (104) ns
(SNP/−NO)+PEG	7.19 ± 1.37 (100) ns	14.50 ± 1.38 (92)	2.88 ± 0.39 (95) ns

MeJA—methyl jasmonate; (SNP/+NO)—sodium nitroprusside, active nitric oxide donor; (SNP/−NO)—light-inactivated SNP; PEG—polyethylene glycol 6000. The data are mean ± SE (n = 30). \* indicate significant difference and ns indicate non-significant difference at  $p \leq 0.05$  between control and treatments. Values in brackets are the percentage of control.

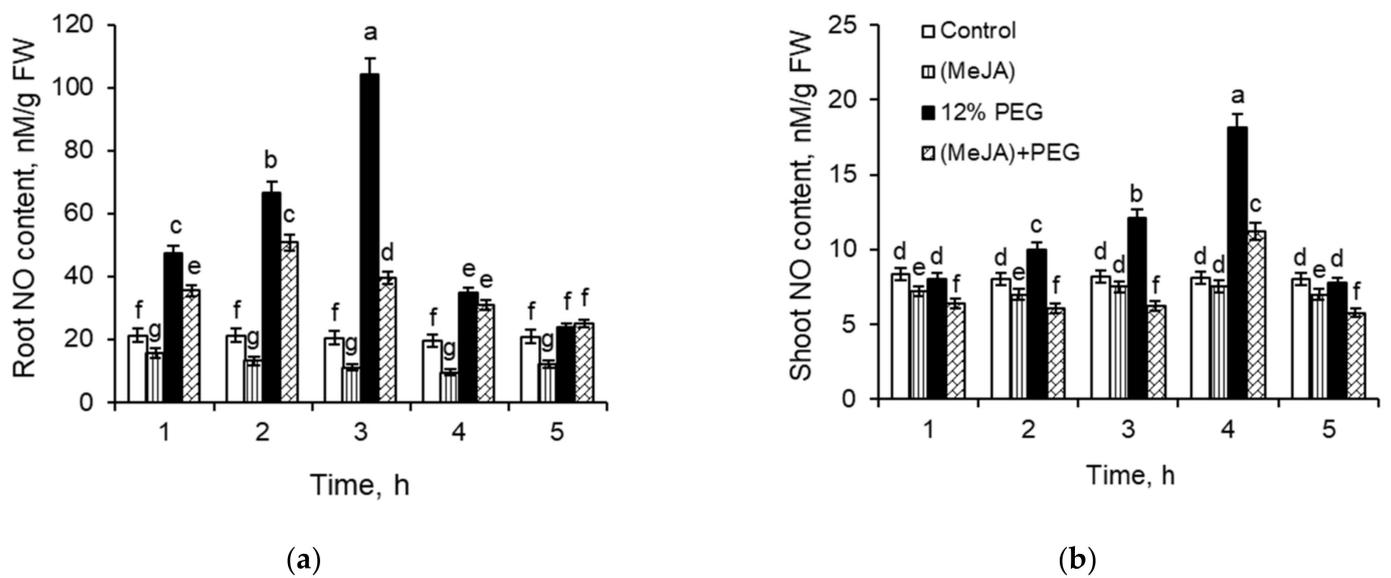
## 2.2. NO Content

Exogenous  $10^{-7}$  M MeJA application caused induction of NO production both in the roots and shoots of wheat plants. Under MeJA treatment, the endogenous NO content was increased three- and fourfold in wheat roots (Figure 1a), and by 45–50% in shoots (Figure 1b) compared with those of control plants. The maximum NO-accumulation occurred at 3–4 h, and dropped from the control level to 5 h of MeJA application.



**Figure 1.** Effect of  $10^{-7}$  M MeJA treatment during 6 h on nitric oxide (NO) generation in roots (a) and shoots (b) of 5-day-old wheat seedlings. MeJA—methyl jasmonate, FW—fresh weight, g. Data are the mean of three replicates; different letters show significant difference at  $p \leq 0.05$ , and bars indicate  $\pm$ SE (n = 30).

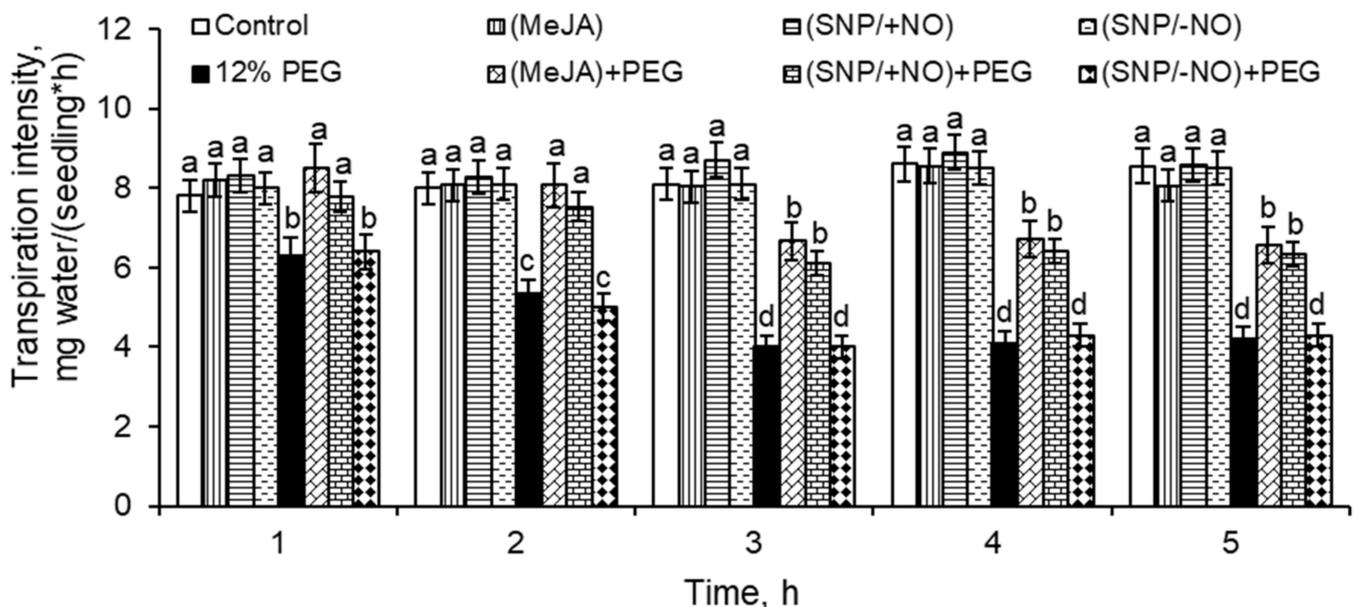
The NO content in MeJA-pretreated wheat seedlings under drought stress, modulated by 12% PEG, is depicted in Figure 2. MeJA-pretreated wheat roots showed NO levels reduced by 35–40% (Figure 2a), while the level of NO in shoots decreased by only 7–14% (Figure 2b) as compared to the control plants. Water deficit resulted in a rapid two- to fivefold increase in nitric oxide content in wheat roots in the first 3 h of stress influence, and a more than twofold increase in NO generation in shoots after 4 h of PEG application (Figure 1). MeJA pretreatment revealed a decrease in NO production of wheat seedlings under drought stress. Thus, NO production of these MeJA-pretreated plants during water deficit increased only twofold in roots and by 40% in shoots compared to control seedlings. The peak of NO production in shoots took place after 3 h of drought stress application in roots and after 4 h of PEG influence in shoots.



**Figure 2.** The level of NO in roots (a) and shoots (b) of 6-day-old wheat seedlings pretreated with  $10^{-7}$  M MeJA for 24 h and under subsequent 12% PEG treatment for 5 h. Data are the mean of three replicates; different letters show significant difference at  $p \leq 0.05$  and bars indicate  $\pm$ SE (n = 30).

### 2.3. Transpiration Intensity

The pre-stress treatment with only MeJA, (SNP/+NO), or (SNP/−NO) over 24 h did not change the transpiration intensity of the leaves of wheat seedlings (Figure 3). Water deficit over 2 h decreased the level of transpiration intensity in non-pretreated and (SNP/−NO)-pretreated shoots by 20–40%, while transpiration intensity of MeJA- and (SNP/+NO)-supplied seedlings stayed at the level of the control plants.

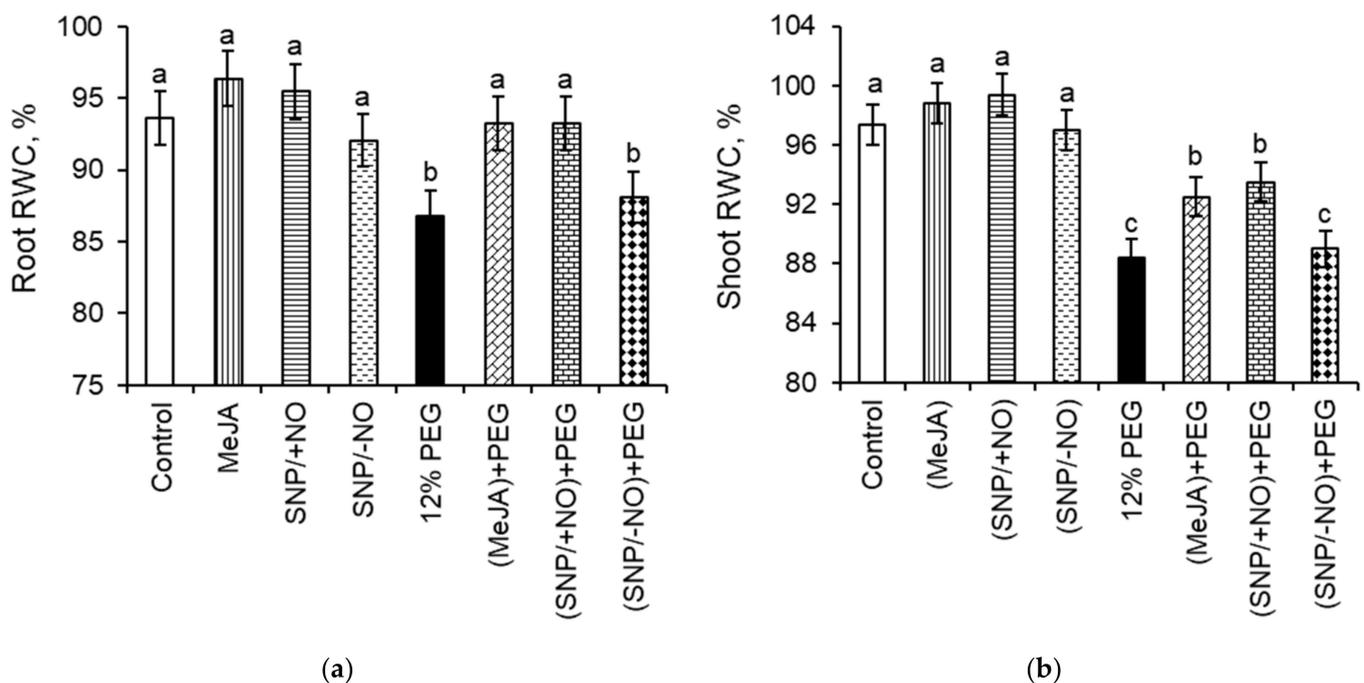


**Figure 3.** Effect of exogenous  $10^{-7}$  M MeJA and  $2 \times 10^{-4}$  M SNP pretreatments on transpiration intensity of wheat leaves under water deficit, modulated by 12% PEG for 5 h. Data are the mean of three replicates; different letters show significant difference at  $p \leq 0.05$  and bars indicate  $\pm$ SE (n = 20).

The next 3–5 h of drought stress influence caused a twofold decrease in transpiration intensity of non-pretreated and (SNP/–NO)-pretreated wheat seedlings, while (SNP/+NO) and MeJA pretreatment showed only 20–25% decreases in this parameter as compared to the control plants.

#### 2.4. Relative Water Content (RWC)

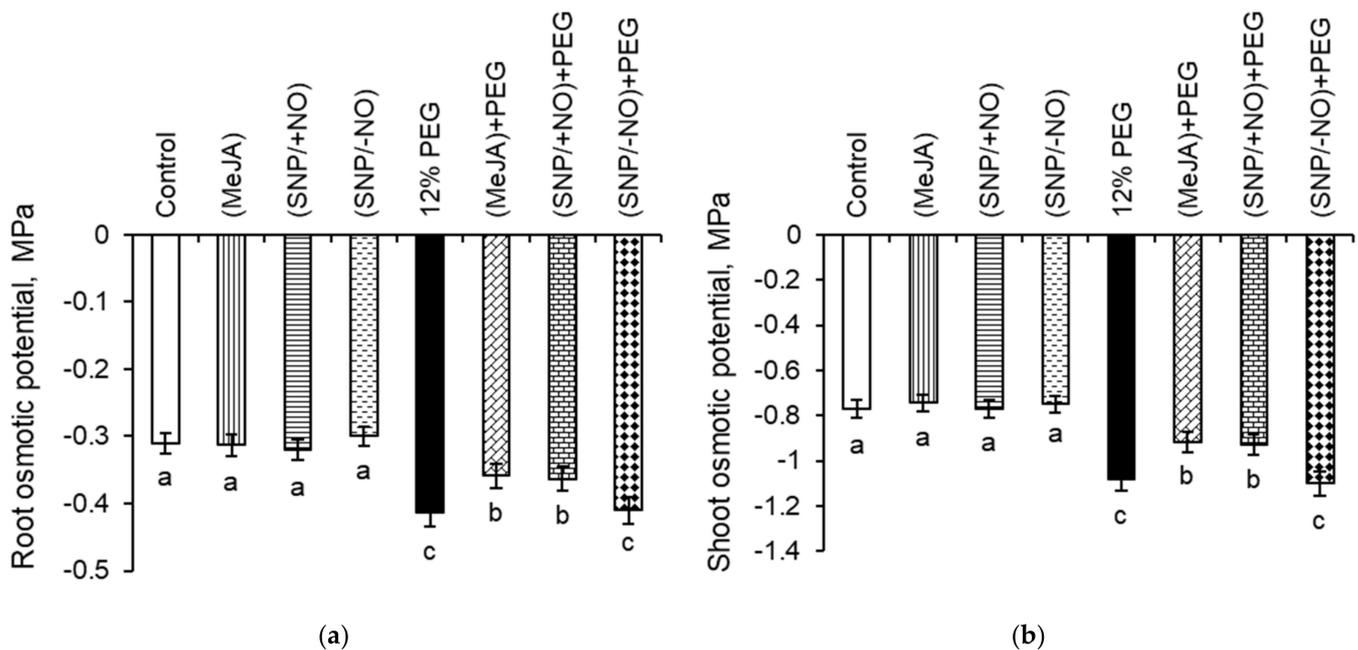
The shoots of MeJA-, (SNP/+NO)-, and (SNP/–NO)-pretreated and control wheat seedlings had a similar, near 97–99% plant hydration status, measured as relative water content (RWC) (Figure 4). The roots of the control, MeJA-, (SNP/+NO)- and (SNP/–NO)-pretreated seedlings had 93–95% RWC (Figure 4b). To compare the extent of dehydration, RWC was measured in shoots and roots of wheat plants after 5 h of water deficit, modulated by 12% PEG. The exposure of seedlings to drought stress caused a 9% water loss in shoots and a 7% water loss in roots during the analyzed dehydration period. However, MeJA or (SNP/+NO) pretreatment followed by short-time water deficit improved relative water content. The MeJA- and (SNP/+NO)-pretreated wheat plants had RWC in roots near the control level (Figure 4a). MeJA and (SNP/+NO) pretreatments under drought stress decreased RWC in shoots by 3–4% as compared with those of the control plants (Figure 4b). In order to verify whether exogenous (SNP/–NO) is capable of changing the RWC of wheat seedlings under water deficit, SNP without NO activity was applied. Under 12% PEG application, the root and shoot RWC levels of (SNP/–NO)-supplied seedlings were the same as those of (SNP/–NO)-untreated plants.



**Figure 4.** Effect of exogenous  $10^{-7}$  M MeJA and  $2 \times 10^{-4}$  M SNP pretreatments on relative water content (RWC) in roots (a) and shoots (b) of wheat plants exposed to 12% PEG for 5 h. Data are the mean of three replicates; different letters show significant difference at  $p \leq 0.05$  and bars indicate  $\pm$ SE ( $n = 15$ ).

### 2.5. Osmotic Potential Estimation

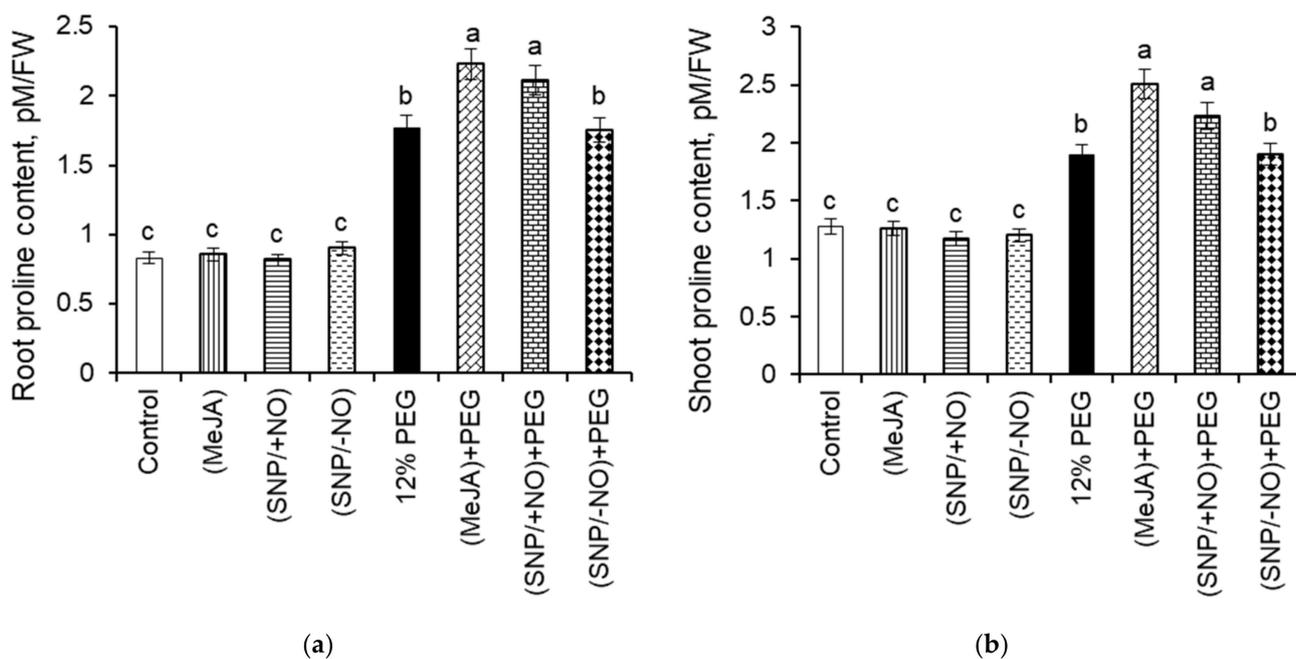
After MeJA, (SNP/+NO), or (SNP/−NO) pretreatment, the levels of osmotic potential of cell sap of wheat seedlings were the same as in control plants (Figure 5). The application of 12% PEG over 24 h caused a decrease in osmotic potential in roots by 33% and in shoots by 40% compared with the control plants. In MeJA- or (SNP/+NO)-pretreated plants under water deficit, the parameter of osmotic potential increased compared with that of stress alone. In these MeJA- or (SNP/+NO)-supplied seedlings under 12% PEG, the osmotic potential of wheat sap decreased by 16% and 17% in roots, and by 19% and 21% in shoots, respectively, compared with the control plants. Pretreatment of wheat seedlings with light-inactivated SNP under drought stress did not change the osmotic potential, and this parameter was the same as for the PEG-treated wheat plants.



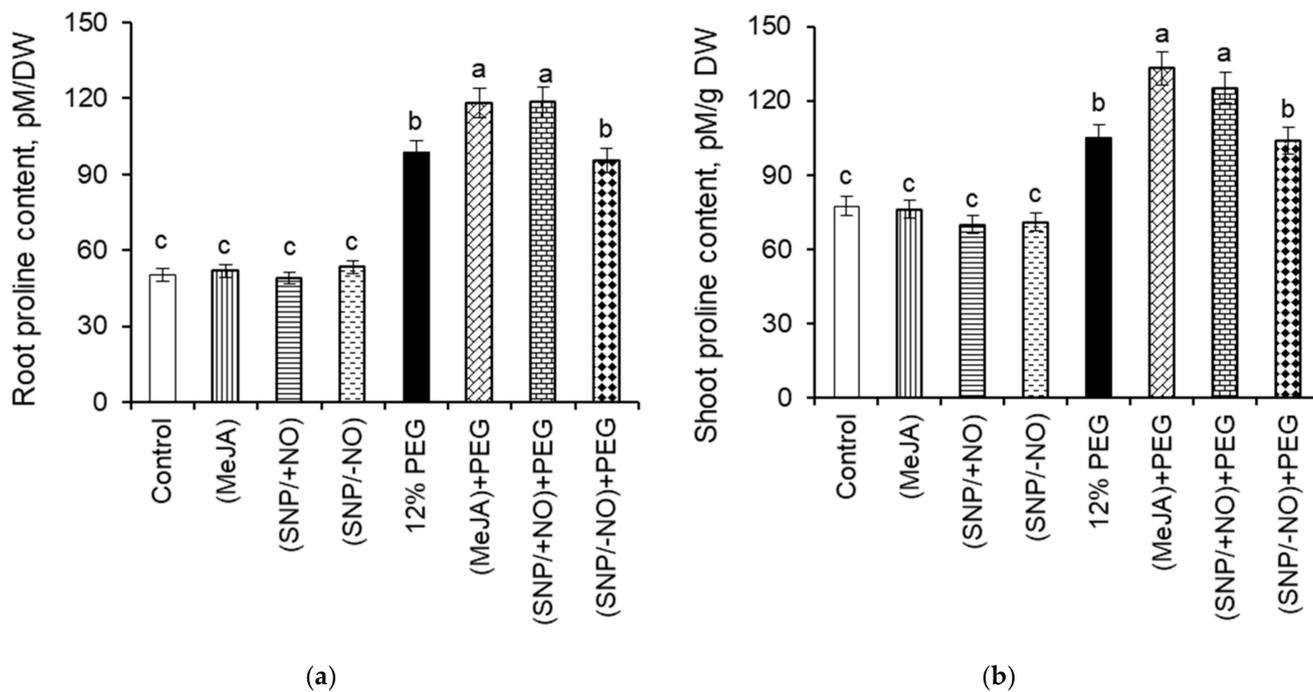
**Figure 5.** Effects of exogenous application of  $10^{-7}$  M MeJA and  $2 \times 10^{-4}$  M SNP on osmotic potential of (a) roots and shoots (b) of wheat plant sap under drought stress, modulated by 12% PEG. Data are the mean of three replicates; different letters show significant difference at  $p \leq 0.05$  and bars indicate  $\pm$ SE (n = 30).

### 2.6. Proline Content

It was observed that MeJA, (SNP/+NO), or (SNP/−NO) application for 24 h did not affect proline accumulation compared with the control plants (Figure 6). Relative to the control, proline accumulation was enhanced twofold in roots (Figure 6a) and by 50% in shoots (Figure 6b) in 12% PEG-treated wheat plants. Under water deficit, MeJA or (SNP/+NO) pretreatment further enhanced the proline content by 40% and 55% in roots, and by 25% and 50% in shoots, respectively, in comparison to PEG application alone. No significant difference in proline content was observed in control and (SNP/−NO)-pretreated wheat plants under water deficit induced by 12% PEG. As some treatments, especially drought stress, affected wheat seedlings' growth (Table 1) and water content (Figure 4), fresh weight parameters (FW) were also changed. The substitution of FW by dry weight during proline content calculation did not affect the accumulation trend of this amino acid, so under water deficit, MeJA or (SNP/+NO) pretreatment increased root and shoot proline accumulation compared with untreated wheat seedlings (Figure 7).



**Figure 6.** Proline content of roots (a) and shoots (b) of wheat plants pretreated by  $10^{-7}$  M MeJA or  $2 \times 10^{-4}$  M SNP under 12% PEG application during 24 h. Data are the mean of three replicates; different letters show significant difference at  $p \leq 0.05$  and bars indicate  $\pm$ SE (n = 15).



**Figure 7.** Proline content of roots (a) and shoots (b) of wheat plants pretreated by  $10^{-7}$  M MeJA or  $2 \times 10^{-4}$  M SNP under 12% PEG treatment for 24 h, calculated to DW—dry weight, g. Data are the mean of three replicates; different letters show significant difference at  $p \leq 0.05$  and bars indicate  $\pm$ SE (n = 15).

### 3. Discussion

Drought is a severe environmental stress. Plant tolerance under limited water availability is one of the most complex traits [41]. Nutrient and water supply through roots, air humidity and shoot transpiration intensity, water retention ability of plants, root morphology and length, and number of lateral roots all influence drought tolerance, so our work concerned the investigation and comparison of the parameters for both the roots and shoots. Drought significantly influences CO<sub>2</sub> assimilation and productivity by decreasing transpiration rate and stomatal conductance [42]. One plant strategy for withstanding water deficit relies on growth trait regulation to induce a water saving capacity in stressed plants. For example, the leaf shape impacts plant transpiration, regulating plant water content during water-limiting conditions [43]. Under drought stress, tolerant wheat varieties had less leaf area and maintained significantly higher leaf RWC. Plant organisms have evolved various defense mechanisms to optimize water uptake under a wide range of water-limiting conditions. The complex network of phytohormones, transcription factors (TFs) and signaling molecules regulate plant growth and development under non-challenging growth conditions and under environmental stresses [44,45]. Transcription factor (TF) SIWRKY81, for example, is a negative regulator of proline accumulation in tomato plants, decreasing drought tolerance by suppressing proline biosynthesis and/or accelerating proline catabolism, possibly by acting as a transcriptional repressor for SIP5C1 and a transcriptional activator for SIProDH [46].

NO and JAs are considered to be advanced plant growth regulators with both beneficial and deleterious effects on plant physiology, depending upon the concentration and exposure time. The similarities in the chemical characteristics of their molecules and properties in biological systems should be mentioned. NO is a well-known gaseous signal transmitter [30] and MeJA also has a strong volatility [47]. NO molecules can freely cross cell membranes or be transported by aquaporins into plant cells [30]; phytohormone MeJA has the ability to alter the cell membrane conductivity [48].

It is known that NO can crosstalk with auxins, cytokinins, gibberellins, ethylene, and ABA during early plant development [25]. NO has been proposed as an important player in JA-regulation without any stressful influence and under environmental stresses. JAs are known to induce NO accumulation [39], and in turn, NO also positively regulates JAs production in plants [49]. Despite the huge amount of the literature data, the functions of JAs and RNS under abiotic stresses are still only partially understood.

We investigated the growth and water relation parameters after  $10^{-7}$  M MeJA or  $2 \times 10^{-4}$  M active NO donor sodium nitroprusside (SNP/+NO) pretreatments followed by short-time drought stress at both roots and shoots of wheat seedlings at the vulnerable stage of growth of this crop variety. Generally, roots and shoots displayed similar changes in water status and growth parameters. The MeJA or (SNP/+NO) pretreatment stimulated the increase in root length, shoot height, and leaf area under optimal growth conditions and during water deficit (Table 1). MeJA was more effective as growth regulator than (SNP/+NO), probably due to the more stable MeJA molecule. Both MeJA and (SNP/+NO) pretreatments influenced the root length more than the shoot height, perhaps because the roots were the application site. Another reason for this could be irregular water distribution in soil under drought stress; plants, first of all, have to stimulate root growth to find water. According to literature data, NO is an important signaling molecule that has several effects on root architecture [25]. JAs can also regulate the growth of different plants [38]. We suppose that these signaling molecules boosted the growth as well as development of the wheat plants by producing complex responses under drought stress. This assumption is consistent with the JA promotion of plant senescence [39]. The differences in growth parameters in our experiments were small and probably due to short 2-day time period after pretreatment, which could limit the ability of plants to expand their growth significantly, or owing to regulation, result in more plant acclimation than growth under water deficit. The interaction of JA signaling with NO could generate specific useful outputs for plant tolerance and probably integration with other

phytohormones, including ABA [27,30]. We assumed that the MeJA-induced burst of NO could activate downstream signaling pathways of wheat plants cultivar Salavat Yulaev at the drought-sensitive one-leaf stage, and trigger efficient adaptive mechanisms that alleviate the negative effects of water deficit.

Plant tolerance under water deficit is one of the most complex traits [41]. Nutrient and water supply through roots, air humidity and shoot transpiration intensity, the water retention ability of plants, root morphology and length, and number of lateral roots influence it, so our work concerned the investigation and comparison of the parameters of both the roots and shoots. Plant organisms have evolved various defense mechanisms to optimize water uptake under a wide range of water-limiting conditions.

In our experiments, nitric oxide and MeJA had few and statistically insignificant effects on water relation parameters under well-watered conditions. The absence of changes in both plant water loss due to transpiration intensity (Figure 3) and in plant water-saving parameters, in RWC (Figure 4), in osmotic potential of wheat sap (Figure 5), and in proline content (Figures 6 and 7) in roots and shoots of wheat seedlings after MeJA or (SNP/+NO) pretreatment tends to indicate that they were not affected by stress. The application of MeJA or (SNP/+NO) on the initial stage of plant growth did not change the water relation of wheat seedlings without the influence of the stress factor. It appears from literature data that exogenous JA or NO application could regulate water relations under stresses and even enhance drought tolerance [15,19,40,50].

The ability of phytohormone MeJA to induce NO accumulation (Figure 1) suggests that this important signal molecule could be involved in the MeJA-mediated wheat plant response to water deficit. The MeJA- and 12% PEG-induced increases in NO generation in wheat seedlings at the same period (Figures 1 and 2) allow us to assume that MeJA and water deficit possibly trigger plant tolerance reactions close in time. In our experiments, the control level of NO in wheat roots was more than twofold than that in shoots, which is consistent with published data for rice [51]. The more conspicuous MeJA-induced NO accumulation in roots (Figure 1) is probably because roots were the site of action of this growth regulator, being the nitric oxide synthesis site.

Besides growth regulation under drought stress, alterations in MeJA and NO levels in wheat seedlings could also trigger plant tolerance. Various studies have reported NO production following water deficit in different species of the plant kingdom [35,36]. SNP treatment was able to induce plant tolerance to drought stress by retaining more water in plant organisms [40], reducing membrane damage and transpiration intensity due to stomatal closure [28,29,35]. For example, SNP can heal the water stress-induced damage in *Crambe* plants under water deficit conditions by improving the activity of antioxidant enzymes; increasing the osmotic potential of plant sap, proline content, chloroplast pigments, fluorescence parameters of chlorophylls; and stabilizing gas exchange [52]. As NO accumulation in plants was transient (Figure 1), nitric oxide should be considered a signaling molecule. This prediction is confirmed by the earlier and more significant nitric oxide accumulation in the site of its action (roots) than in shoots (Figures 1 and 2). In our experiments MeJA pretreatment for 24 h reduced NO production in unstressed wheat seedlings as compared to the control plants (Figure 2). This is probably due to the MeJA-induced stress tolerance of wheat seedlings, as it was observed that the increased drought tolerance in barley plants was associated with decreased NO generation under both well-watered and water-stress conditions [53]. The MeJA regulation of NO generation under drought stress allows us to suggest that NO is a key signaling intermediate in MeJA-mediated regulation of wheat plant acclimation under the initial stage of water deficit.

Water loss in plant tissues is the main cause of reduced growth in plants under water deficit. The growth inhibition (Table 1) and shifts in water balance parameters started during the initial stage of drought stress, and was modulated by 12% PEG. Under water deficit, the root and shoot RWC decreased (Figure 4). Plants have a variety of strategies to deal with water loss. Wheat plants tried to save water by decreasing transpiration intensity (Figure 3) through stomatal closure and accumulation of active osmotic substances (Figure 5), including proline (Figures 6 and 7). It is known that MeJA and NO take part in stomatal closure regulation [40,48] and generally in long-term alleviation of the adversities of water deficit [9]. Acclimation to water deficit is a great challenge for the non-tolerant wheat plant cultivar Salavat Yulaev. MeJA and (SNP/+NO) pretreatment appeared to have ameliorative benefits in warding off the effects on wheat water balance with the application of 12% PEG. Under drought stress, (SNP/+NO) and MeJA pretreatment enhanced transpiration intensity (Figure 3) and maintained higher RWC (Figure 4) and lower osmotic substance accumulation (Figures 6 and 7) as compared to untreated and stressed plants. (SNP/+NO) and MeJA application thereby increased the ability to support water balance and growth parameters at near normal values. At the organismal level, partial closures of stomata not only reduced water loss but also supported gas exchange for photosynthesis. Despite the defense effort of MeJA- and (SNP/+NO)-pretreatment, wheat plants were stressed because the parameters of transpiration intensity, RWC, and osmotic potential of wheat sap were not returned to the control level.

Osmoregulation is often achieved by accumulating osmotically active substances under environmental stresses to mitigate the detrimental effects of such stresses by reducing the water potential of plant cells, or by protecting various cellular structures, including proteins, during stress. Among these substances, the compatible osmolyte proline is important one. Accumulation of this free amino acid is considered to be an adaptive response of plants against abiotic stresses [54]. At the cellular level, the compatible osmolyte proline helps to reinstall turgidity under water stress [9], stabilize the sub-cellular structures, and scavenge ROS [54]. It also can modulate cellular functions and regulate gene expression. In this regard, it's no wonder that in MeJA- and (SNP/+NO)-pretreated wheat seedlings under 12% PEG application, the proportion of proline increased (Figures 6 and 7), consistent with published data [17,55].

The similarities in MeJA- and (SNP/+NO)-induced growth and water balance activation in wheat plants at the beginning of a drought stress influence lead us to suggest that these two growth regulators are closely related to one signaling pathway. There may be a link between MeJA and NO as a secondary messenger of the JA signaling pathway. It is known that most of the plant developmental processes are affected by various hormones and the whole cascade of signaling metabolites that act together to improve growth, reproduction, and stress tolerance [44,45]. Interestingly, (SNP/+NO) effects were reversed by changing the active NO donor to light-inactivated SNP. The growth and water relation parameters of (SNP/−NO)-pretreated plants were the same as control. Under water deficit, the growth and water relation parameters of (SNP/−NO)-supplied wheat seedlings were the same as (SNP/−NO)-untreated stressed plants.

## 4. Materials and Methods

### 4.1. Plant Material and Treatments

Wheat seeds (*Triticum aestivum* L.) of drought-sensitive cultivar Salavat Yulaev obtained from Chishminsky Crop Production Station, Russia. The seeds were surface sterilized with 96% ethanol, washed, and sown in Petri plates containing filter paper. Two-day-old seedlings were grown in glass jars for 3 days on 10% Hoagland-Arnon solution. Regular changing of the solution (every day) was sufficient. Wheat seedlings were grown in a climatic chamber under the illumination of  $200 \text{ mmol m}^{-2}\text{s}^{-1}$  at 22–24 °C and a 16-h photoperiod.

The degree of drought tolerance strongly depends on the developmental stage in most plant species, so we used the sensitive first leaf stage of soft spring wheat plants in our investigation. For carrying out the hydroponic experiments, we used phytohormone MeJA (MilliporeSigma, Germany) and the nitric oxide donor SNP (ChimReactivSnab, Ufa, Russia). MeJA and SNP were applied through the wheat roots as supplements in growth media. For the treatments, 5-day-old wheat seedlings were supplemented with the following final concentrations:  $2 \times 10^{-4}$  M SNP/+NO (active NO donor),  $2 \times 10^{-4}$  M SNP/−NO (exhausted NO donor), and  $10^{-7}$  M MeJA on 10% Hoagland-Arnon solution for 24 h. The dose of  $2 \times 10^{-4}$  M SNP/+NO was very beneficial in affecting plant growth [56]. Control plants were grown on 10% Hoagland-Arnon solution. The next day, some MeJA-, (SNP/+NO)- and (SNP/−NO)-pretreated 6-day-old wheat plants were exposed to the short-time drought stress, induced by 1–24 h of 12% PEG (PanReac AppliChem, Spain) exposure prepared in 10% Hoagland-Arnon solution. This osmotic stress reduces the ability of plants to take up water, thus modulating water deficit stress. The remaining wheat plants continued to grow on 10% Hoagland-Arnon solution. Further, six- or seven-day-old seedlings were analyzed for different parameters, including NO content, transpiration intensity, relative water content (RWC) measurements, and proline accumulation.

#### 4.2. NO Measurement

A measure of 0.5 g of plant tissues was ground into a powder with a mortar and pestle and homogenized in 2 mL ice-cold Na-acetate buffer (pH 3.6), followed by centrifugation at  $10,000 \times g$  for 10 min at 25 °C. The mixture of 1 mL of extract and 1 mL of the Greiss reagent was incubated at room temperature for 30 min. Nitrite and nitrate-derived NO content was measured at absorbance 540 nm using a Smart Spec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). NO content was calculated by comparison to a standard curve of NaNO<sub>2</sub>.

#### 4.3. Measurement of the Relative Water Content (RWC)

The fresh weight was determined immediately after separating the leaves and roots from the plant. To determine turgor weight, shoots were placed at room temperature for 24 h in the dark in closed vessels, loading the base into distilled water [57], while roots were completely immersed in water for the same period [15]. Then samples were dried in open containers, which provided free circulation air, in a hot air oven at 70 °C until constant dry mass was achieved. RWC was estimated by following the formula:

$$\text{RWC} = [(\text{Fresh weight} - \text{Dry weight}) / (\text{Turgid weight} - \text{Dry weight})] \times 100\%.$$

#### 4.4. Estimation of the Transpiration Intensity

Transpiration expressed per one seedling was measured gravimetrically in 10 plants per treatment. To prevent evaporation of 10% Hoagland-Arnon solution from the surface of the beakers, they were covered by aluminum foil. A small slit for the shoot was made on the top of the beaker to allow the plant to grow through. The initial and final (after each time point) beaker weight was taken, and transpiration was calculated by subtracting the final beaker weight from the initial weight divided by the number of seedlings [58].

#### 4.5. Osmotic Potential Estimation

Six-day-old wheat roots and shoots were fixed at −20 °C, then thawed and released sap centrifuged. The osmotic potential of cell sap was measured using a digital microsmometer (“Camlab Ltd.”, Cambridge, UK).

#### 4.6. Proline Accumulation

For proline determination, 0.5 g of fresh shoot and root material were soaked in 3 mL of hot distilled water and boiled in a water bath for 1 h at 100 °C [59]. The immediately cooled extract (1 mL) was then mixed with 1 mL of acid-ninhydrin reagent and 1 mL of glacial acetic acid in a test tube, and the mixture was placed in a water bath for 1 h at 100 °C. The absorbance of the cold reaction mixture was measured at 522 nm using a spectrophotometer Smart Spec Plus (Bio-Rad, Hercules, CA, USA). The proline concentration in each wheat tissue sample was calculated by comparison to a standard curve of proline.

#### 4.7. Estimation of Shoot Height, Root Length, and Leaf Area

Shoot and root lengths were measured using a manual scale. Leaf area was measured using a planar scanner and image software ImageJ.

#### 4.8. Statistical Analysis

All experiments were performed with three biological replications and three technical repetitions each. Data presented were mean values with standard errors ( $\pm$ SE). Statistical data processing was carried out using a statistical analysis package in Microsoft Office Excel 2010. Means were compared using analysis of variance (ANOVA) with  $p \leq 0.05$ .

### 5. Conclusions

Plants are sessile organisms, and severe harvest reduction occurs due to their exposure to various biotic and abiotic stress-factors. At present, understanding of the complex regulatory networks and metabolic processes of plants during adaptation to environmental stresses is still limited. Slight, but targeted shifts in the number and/or activity of signaling components might be explored to achieve tolerant crops [9]. Owing to the involvement of JAs and NO as second messengers in a large array of stress responses, the comparison of MeJA and SNP pretreatment proved that they acted similarly in growth and water balance regulation under drought stress. The available data show that NO is involved in MeJA-regulated water balance in response to the initial stage of water deficit. Taken together, these findings confirm that during water stress, the MeJA has ameliorative functions that involve NO as a key signaling intermediate and induce growth and water-balance regulation as well as additional proline accumulation. The investigation of the mechanisms of plant adaptation to water deficit could help create agricultural plants with the property of water-stress resistance. In conclusion, although our studies demonstrate that both MeJA and SNP regulate and enhance the adaptive responses against water deficit in wheat plants, there are numerous unanswered questions and important areas for further research.

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