# Alfalfa and Its Symbiosis Responses to Osmotic Stress

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Additional information is available at the end of the chapter

### Abstract

Alfalfa (*Medicago sativa* L.) is one of the most cultivated forage legumes in Morocco thanks to its great adaptation to the climatic conditions of this country, its high protein content and its ability to fix atmospheric nitrogen in symbiosis with rhizobia. Environmental stresses such as drought and salinity constitute a major factor limiting the symbiotic nitrogen fixation and legume productivity. In the last decades, this process has interested scholars in understanding the implication of these strains in legume stress tolerance in order to make these symbioses more efficient under difficult conditions. Seed osmopriming is a great technique in the amelioration of seed germination and seedlings growth in responses to several abiotic stress conditions. In this chapter, the effects of water deficit on the Moroccan alfalfa populations and their symbiotic association with rhizobia were discussed. Besides, osmopriming could make these symbioses more efficient especially under stress conditions.

**Keywords:** alfalfa, drought, salinity, $N_2$-fixing, osmopriming, photosynthesis

## 1. Introduction

Alfalfa (*Medicago sativa* L.) is a frequently cultivated legume forage in the Mediterranean region thanks to its high leaf protein content, effects on soil fertility and deep root system [1]. In Morocco, this crop occupies 455,000 ha, which represents about 25% of the area devoted to forage crops where 40% is in irrigated systems. In the southeast oases of Morocco, alfalfa cultivation contributes to socioeconomic life as the main forage crop. Local alfalfa populations have many characteristics of agronomic interest such as tolerance to grazing (capacity for rooting and regrowth and diseases) [2], and are considered moderately tolerant to abiotic

stresses, including drought and salinity in these areas, but they show significant variation within many of them depending on their habitats of origin.

Water deficit in these regions is one of the major factors influencing the productivity and persistence of many crops. This stress aggravates the impact of other abiotic or biotic stresses to which plants are exposed. In addition, the increase in water demand by other sectors of the national economy (industry and tourism) and the high incidence of drought due to climate change have led to low water availability for agriculture. In addition, climate change is expected to increase the extent of drought and temporal variation in our Mediterranean region [3]. Moreover, this constraint limits the forage production of alfalfa in several regions of the world via its negative effects on germination, the rate of photosynthesis, the metabolism of the plant and its ability to establish and function in the $N_2$ fixing symbiosis. A severe and prolonged water deficit decreases the number of rhizobia and affects its genetic diversity in the soil as well as in the rhizosphere [4], by inhibiting the process of root infection and directly influencing the functioning of the nodules and the survival of rhizobia in the soil. As a result, large quantities of chemical fertilizers have to be brought to the soil, some of which (about 1%) are used by plants, while the rest are rapidly converted into insoluble complexes. These lead to the need for frequent application of fertilizers; however, its regular use has become costly and ecologically undesirable, and it raises soil salinity in the long term, hence the need to develop economic and environmentally friendly technologies. The selection, the characterization of drought-tolerant alfalfa populations, their symbiosis and the understanding their responses to theses abiotic stresses are of great importance by taking advantage of the genetic biodiversity of both local populations and rhizobia strains in the soil. Seed osmopriming could be also an effective technique for improving germination and vigour of young seedlings of many species. It is a useful tool to overcome the problems of drought and salinity, ensuring the rapid and successful establishment of seeded seeds and the induction of tolerance mechanisms in young seedlings in post-germination, especially under conditions of stress [5].

In this context, the major objective of this review is dedicated to the presentation of the recent knowledge on the effects of water stress and salinity on the growth and development of alfalfa (*M. sativa* L.), first in the germination stage, during their stage of development and in association with rhizobia isolated from different Moroccan soils. The study was focused on physiological, hydric, growth, biochemical and photosynthesis parameters related to water deficit and salinity tolerance, as well as the presentation of recent knowledge about the impact of seed priming on the tolerance of alfalfa to drought conditions.

## 2. Alfalfa origins and domestications

It is difficult to recognize the origins of the first domestications of alfalfa (*M. sativa* L.). It was cultivated according to different authors 9000 years ago, in its centre of origin. In Ref. [6], the centre of origin of *M. sativa* is the Near East, Asia Minor, Transcaucasia, Iran and the high areas of Turkmenistan. The most common geographic centre is Iran (**Figure 1**). These regions are characterized by cold winters and dry, warm summers and well-drained and neutral pH soils [7]. In Ref. [8], a second centre of origin, Central Asia, characterized by a dry climate and mild winters was added.
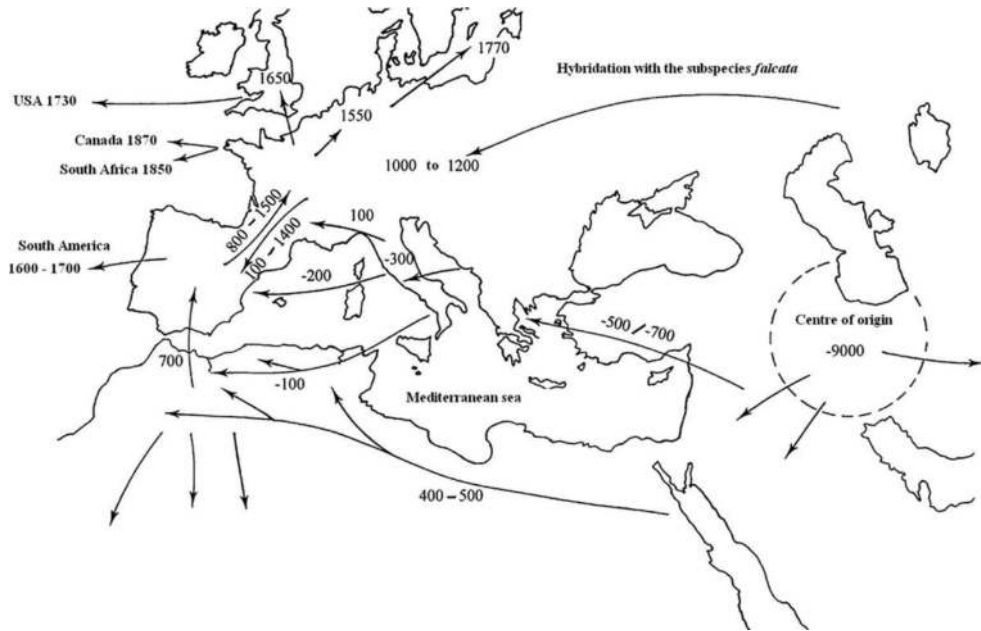
**Figure 1.** The different routes and approximate dates of the spread of alfalfa cultivated from its centre of origin. (-) Correspond to the dates before JC [11].

Alfalfa cultivation was introduced to North Africa 400–500 years BC through Egypt and 100 years BC by the Roman Empire [9]. These introductions concerned the Atlas Mountains. Other more recent introductions were made in the eighth century by Arab Muslims and concerned the pre-Saharan oases [9], and thanks to Arab Muslims during the seventh and eighth centuries, it was reintroduced into Europe via Africa, and the name "alfalfa" in English is a word from Arabic meaning "al alfa" [10]. العلفة.

## 3. The responses of alfalfa to osmotic stress

### 3.1. Alfalfa responses of plants to water deficit

The response of alfalfa to water deficit mainly depends on the severity of the stress and growth stage and its physiological state. It results in a 49% decrease in biomass and an 18% increase in leaf-to-stem ratio [12]. The three main mechanisms that reduce the yield of alfalfa due to water deficiency are (i) reduction of the absorption of photosynthetic radiation by the canopy, (ii) decrease of radiation efficiency and (iii) reduction of the harvest index [13].

### 3.2. Effect on seed germination

Seed germination and seedling growth are the most vulnerable stages to drought. Thus, water stress is one of the main fatalities to seed germination in alfalfa. Germination is a metabolic

process requiring three main factors—water, oxygen and temperature—in addition to light as another factor in some species. At seeding, water stress inadequacy delays the germination process and reduces germination percentage and growth rate. It induces irregular germination and non-synchronized emergence of alfalfa seedlings, resulting in low stand populations and reduced yields [14]. In Refs. [1, 15], the exposure of alfalfa seeds to high concentrations of polyethylene glycol (PEG) significantly decreased their germination rate, radicle length and velocity index, and thus the seed germination is inhibited beyond an osmotic pressure of −0.9 MPa. However, under moderate stress, the root length remains intact and even increases in some cases to resist water shortage, and this is probably due to the essential role of roots in the life and function of the plant [1].

### 3.3. Effect on growth and water uptake

A very moderate water deficit, which does not cause flagrant symptoms, results in a significant change in the morphology and physiology of the plant in many species [16]. It acts negatively on cell division, enlargement and differentiation due to loss of turgor and induces decreased energy supply and synthesis of impaired enzymes. This stress causes, in alfalfa, as in other legumes, reduction of the leaf area, the number of leaves, the closure of the stomata limiting the assimilation of $CO_2$, photosynthetic activity and growth [17].

The most severe effects of water deficiency occur at the root level and directly affect the water absorption process. The ability of alfalfa root to absorb water is the result of the intrinsic hydraulic properties of the root [18]. Generally, this constraint causes an increase in root/stem ratio due to reduced aerial growth and low root change. This mechanism allows the plant to explore more soil volume to absorb water from deeper layers that are not affected by less developed roots. However, no relationship has been demonstrated between root/stem ratio and water deficit tolerance [19]. In addition, length or root density may have a direct relationship to this tolerance [20].

### 3.4. Effect on nutrient uptake

Water deficiency negatively affects the nutritional balance of legumes through its adverse effects on assimilation, transport and nutrient distribution. Depending on severity and duration, it reduces the bacterial mineralization of organic matter and negatively affects the ability of the roots to absorb nutrients despite the availability of these nutrients in the soil through its direct effect on root hydraulic conductance and transpiration [21] (**Figure 2**).

### 3.5. Effect on photosynthesis

The rate of photosynthesis is negatively affected by water deficit due to stomatal and non-stomatal changes [22]. The reduction of stomatal conductance ($g_s$) and stomatal density is among the remarkable responses to water deficit in alfalfa plants [23, 24]. As a consequence of the stomata closure, the diffusion of $CO_2$ from the atmosphere to the carboxylation site is reduced, which influences the activity of RuBisCO as well as other enzymes such as sucrose phosphate synthase (SPS) and nitrate reductase (NRA), whose inhibition is often considered to be the main cause of the decrease of water-deficient photosynthesis [25]. It has been shown that decreasing water
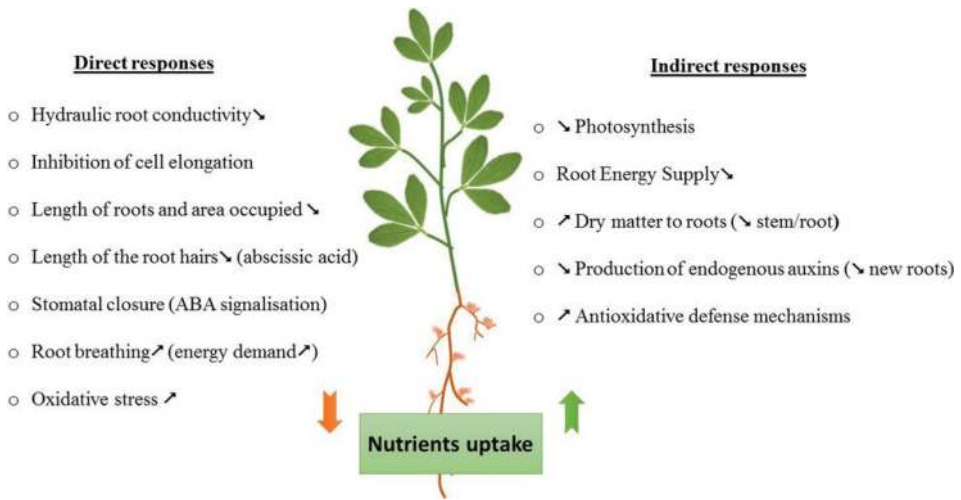
**Figure 2.** The direct and indirect effects of water deficit on the assimilation of nutrients by roots [21].

content and ion concentration in water-deficient leaves are more limiting for photosynthesis than closure of stomata [26]. Water stress also causes considerable disturbance of photosynthetic pigments in the photosystem (PS) II, leading to degradation of thylakoid membranes [27] and reduction of chlorophyll content, affecting their components [28]. Chlorophyll b (Chl b) is more affected than chlorophyll a (Chl a) [29]. However, fluorescence produced by Chl after excitation by light is a non-destructive and rapid biomarker for the estimation of microbiological and environmental stress responses at the PS II level and its structure and function [30].

## 4. Alfalfa responses to salinity

Through the enhancement of osmotic pressure, salinity leads to the reduction of water uptake which subsequently affects several other metabolic and physiological processes leading to a prolongation of the germination period [31].

### 4.1. Effect on growth and nutrient uptake

Salinity affects all physiological processes of the plant. Its effects are mainly reflected by a growth reduction. In Ref. [32], it has shown that salt stress significantly inhibits alfalfa growth. The roots are often more affected than the aerial parts [33].

Salt stress causes an imbalance of the plant mineral nutrition that results from a disruption in the absorption and transport of essential items. In general, the presence of NaCl inhibits of $K^+$, $Ca^{2+}$, $P_i$, $NO_3^-$ and $NH_4^+$ uptake and reinforces $Na^+$ and $Cl^-$ salt ions, which accumulate to become toxic for the plant [32]. Thus, in Ref. [34], salt stress resulted in significant $K^+$ reductions and $Na^+$ accumulations in young seedlings of *M. sativa* L.

### 4.2. Effect on photosynthesis

In response to salt stress, a substantial decrease in a plant's stomatal opening can be observed, but the rates of photosynthesis per unit leaf area sometimes remain unchanged [35]. Following stomatal closure, the internal reduction of $CO_2$ decreases the activity of several enzymes including RuBisCo [36]. In Ref. [37], it has been found that there is a large reduction in stomatal conductance ($g_s$) at two genotypes of durum wheat. Thus, limiting carboxylation and reducing the net photosynthetic rate, the effects of salinity on photosynthesis can be caused by alterations in the photosynthetic metabolism or else by secondary effects caused by oxidative stress [36].

### 4.3. Effect on the cell membrane

The plasma membrane is the main site of interaction between salt and alfalfa plant. Salt stress induces a perturbation of the lipid and protein composition in the cell membrane leading to an uncontrolled electrolyte leakage, thus affecting its permeability and stability [33, 38]. In sugar beet, it has been reported that saline treatment caused a significant leaf electrolyte loss reflecting the disruption and destabilization of cell membranes [38].

### 4.4. Effect on metabolic activity

Some enzymes with carboxylase activity are influenced by salinity. Indeed, the activity of phosphoenolpyruvate carboxylase (PEPC) and ribulose bisphosphate carboxylase oxygenase (RubisCO), enzymes involved in the fixation of atmospheric carbon dioxide, has been negatively affected by salt stress [39]. This modulation is variable according to the species considered and the stage of development of the plant.

The activities of some enzymes involved in nitrogen nutrition of plants are not immune to turn the effect of salinity. In fact, it turned out that the salt stress has a negative influence on the activities of nitrate reductase and glutamine synthetase [38]. This effect varies according to the species, the variety and its nutrition in nitrogen ions.

## 5. Alfalfa rhizobia symbiosis under osmotic stress

Alfalfa is one of the legumes capable of fixing large amounts of $N_2$ (200–400 kg ha$^{-1}$ yr$^{-1}$) in symbiosis with *Ensifer* (*Sinorhizobium*), and drought can indirectly reduce its productivity through impaired performance of the nodules. This stress affects not only the biomass of the nodules but also their functioning. In addition, several studies have reported that biological $N_2$ fixing (BNF) is conditioned by the active interaction of nodules with the leaves and roots [40–42].

The effect of salinity on symbiosis manifests at different levels. Indirectly, salt stress can affect this symbiosis by reducing the growth of the host plant and affecting some of its physiological processes or directly by inhibiting the infection process and nodule development [43]. Under saline conditions, many studies have reported that the activity of the key enzyme in the $N_2$ reduction process, nitrogenase, is greatly reduced [44].

## 6. Effect on multiplication and survival of rhizobia

Drought is one of the important factors influencing the proliferation of soil microflora. It reduces the availability of water around the soil particles and increases the salt concentration in the soil solution, which subsequently leads to adverse effects on growth, rhizobia persistence, movement and ultimately and their diversity in the soil [45]. Water deficiency has been shown to reduce the survival of almost all species of rhizobia, whether or not capable of nodulating legumes and often influencing the map of genetic diversity of different species in the soil. Rapidly growing rhizobia is the most affected in comparison with slow-growing rhizobia [46]. Variability of the genetic tolerance potential has been observed in several rhizobia species such as *Sinorhizobium* [47]. The survival of these water-deficient strains mainly depends on their ability to enter into symbiosis even if this symbiosis is not very effective [48].

## 7. Effect on alfalfa plant in symbiosis

The detrimental effect of drought on BNF is manifested at several levels of symbiotic interaction, namely, the early stages of infection during development and the functioning of the nodules. The formation of new root hairs and the lengthening of previously dissociated hairs are reduced in response to water deficiency which results in a strong reduction in the plant-bacterial interaction as well as the formation of the infection cord [49]. The aerial and nodular biomass also shows a considerable reduction under these conditions, followed by a reduction in the efficiency of the nitrogenase complex for BNF [50]. This reduction cannot be mainly explained by the decrease in the rate of photosynthesis, whereas other alternative causes can regulate the BNF under water deficit. In Refs. [51, 52], $O_2$ limitation, C shortage and N-feedback are the three main factors that could be involved in this inhibition. Indeed, severe water deficiency disrupts the nitrogenase activity of the nodules by causing a rapid decrease in the supply of oxygen to the nodules and consequently an interruption of the adenosine triphosphate (ATP) supply to the nitrogenase [53]. It can also slowdown the transport of photosynthate towards the nodules, which limits the contribution of the energy substrates [54].

In Ref. [55], it has been reported that, in general, rhizobia strains appear to be more tolerant to water deficiency than host plants, but these strains exhibit variation in their growth and survival under this stress. The reduction of the nodular biomass under water deficit could be explained by the reduction in the number and diameter of the root hairs or the inhibition of the emergence and the elongation of these organs [56] and, on the other hand, Limited rhizobia growth, which reduces the initiation and development of nodules [57]. Studies reported that $g_s$ was reduced in all of the studied symbiotic combinations subject to water deficiency. This confirms the results found in Refs. [42, 58]. The fact that these combinations exhibited lower $g_s$ combined with high forage yield even under limiting water conditions supports the hypothesis that tolerant cultivars should develop mechanisms for better use of fixed $CO_2$ and that these mechanisms are related to day/night differential control [59].

In Ref. [24], it has been reported that no significant correlation was observed between leaf area and relative water content (RWC) and between $g_s$ and RWC, while leaf area was found to be highly correlated ($r^2 = 0.674$) at $g_s$. Thus, we suggest that this could have positive indirect effects on RWC by controlling stomata behaviour, maintaining the leaf area and facilitating water uptake in the event of water deficit [60]. The rate of electrolyte loss is considered to be a good physiological index which reflects the degree of alteration of the plants in the plasma membranes of the cells under stressful conditions [38]. An increase in electrolyte loss indicates that the integrity of the membrane is affected. The results of our study showed a significant increase of this parameter under water deficit in all the studied symbiotic combinations. However, this increase was not significant for the more tolerant combination *Ad-RcRh09* which proves the importance of this parameter in the osmotic stress tolerance [24]. The same results have been reported in Refs. [61, 62].

## 8. Drought tolerance mechanisms in alfalfa

### 8.1. Osmotic adjustment and water potential

Water potential ($\Psi$) and osmotic adjustment (AO) can be used as selection criteria to improve drought tolerance in many legume species grown in arid and semiarid regions. Maintaining a low $\Psi$ is considered one of dehydration avoidance mechanisms developed by plants in order to survive in extreme drought conditions. It depends on access and absorption of soil water by roots, sweating, canopy size, leaf area, leaf rolling and internal water transport [63]. On the other hand, substances that can contribute to AO include organic acids, cations (such as $K^+$) and inorganic anions, carbohydrates and amino acids. These substances are often associated with easy protective functions such as hydroxyl (OH), cyclitol, proline and glycine betaine [64]. Active AO is the net increase in osmotically active solutes leading to the decrease of the osmotic potential ($\Psi_s$) in the cell and consequently the total $\Psi$ decrease. This is seen as a drought adaptation and not a mere response. On the other hand, a second form of AO has been proposed according to the Ref. [65], called "passive AO", considered as a response to water deficit and associated with loss of water and therefore a reduction of the cell volume.

### 8.2. Antioxidant defence

Water deficiency induces the appearance of oxidative stress, that is, the accumulation of reactive oxygen species (ROS) causing damage to cellular structures [66]. These are molecules showing redox states between oxygen ($O_2$) and water ($H_2O$) including superoxide anion $O_2^-$, hydrogen peroxide ($H_2O_2$), hydroxide ($OH^-$) and oxygen singlet ($^1O_2$) which are extremely reactive and tend to reduce the water molecule rapidly (ms to ps). As a result, legumes have developed a powerful antioxidant system that is finely regulated in time and space to maintain adequate levels of ROS. This system could detoxify the ROS and radicals of lipid peroxides and maintain an adequate redox balance in the cell [40]. An antioxidant is defined as a molecule capable of releasing $H^+$ electrons or protons with a low reduction potential in order to have a radical either harmless or effectively quenched by other electron donors and have

properties that correlate with oxidative stress [67]. Numerous studies have shown the induction of nonenzymatic and enzymatic antioxidants in legumes subjected to water deficiency. Antioxidant enzymes are proteins that catalyse the detoxification of free radicals by using protons or electrons released by nonenzymatic antioxidants [68]. In the case of water deficit and salinity, several workers have reported the increase of these activities in the nodules under stress [41]. It has been shown in Refs. [40, 69] that tolerant alfalfa-rhizobia symbiotic combinations synthesize more peroxidase, catalase and superoxide dismutase in their leaves and nodules than the sensitive ones.

## 9. Deficit irrigation and water productivity (WP)

Irrigation schemes can be classified as full and deficit irrigation regimes, depending on the crop species, the physiological conditions of the plant, the soil and the climate of the region in question. With full field capacity irrigation, high growth and stable yield could be achieved but require high amounts of water and high cost of accompanying farming practices. The water requirements of each crop vary at different stages of plant development and genotype, sensitivity of its physiological state, soil structure and property, climatic conditions and agricultural practices [28]. To avoid water stress damage during the growth phase, deficit irrigation could be an additional method to improve water productivity (WP) in alfalfa and other crops especially in areas where water resources are limited or production costs are high [70], as in the case of the arid regions of Morocco where the cultivation of lucerne is conducted only by irrigation. It has been reported in Refs. [71, 72] that deficit irrigation could improve the WP and hence the growth and yield of plants relative to full irrigation. However, in Ref. [73] summer deficit irrigation reduces the yield of alfalfa without impeding its growth.

## 10. Seed osmopriming and drought tolerance in alfalfa

Seed priming with chemical agents such as sodium nitroxide, hydrogen peroxide, sodium hydrosulphide, melatonin, polyamines and polyethylene glycol (PEG) or biological agents such as bacterial suspensions improves plants tolerance to different abiotic stresses by improving cellular homeostasis and plant growth [74]. The most commonly used priming agents share the same modes of action, especially under stressful conditions. Moreover, when used against different abiotic constraints, their modes of action exhibit similarities, but also distinct specificities and their performance mainly depend on concentration of the priming agent, priming period and temperature [74].

The purpose of seed osmopriming is to reduce germination time and to improve germination percentage especially under adverse environmental conditions. Treated seeds have been shown to have the potential to rapidly restart germinative metabolism, thereby improving germination rates [75]. The impact of abiotic stress on the physiology and growth of alfalfa, maize and soybean plants from treated seeds has been shown to be remarkably reduced compared to plants from untreated seeds [15, 33] (**Figure 3**). Moreover, those plants whose seeds
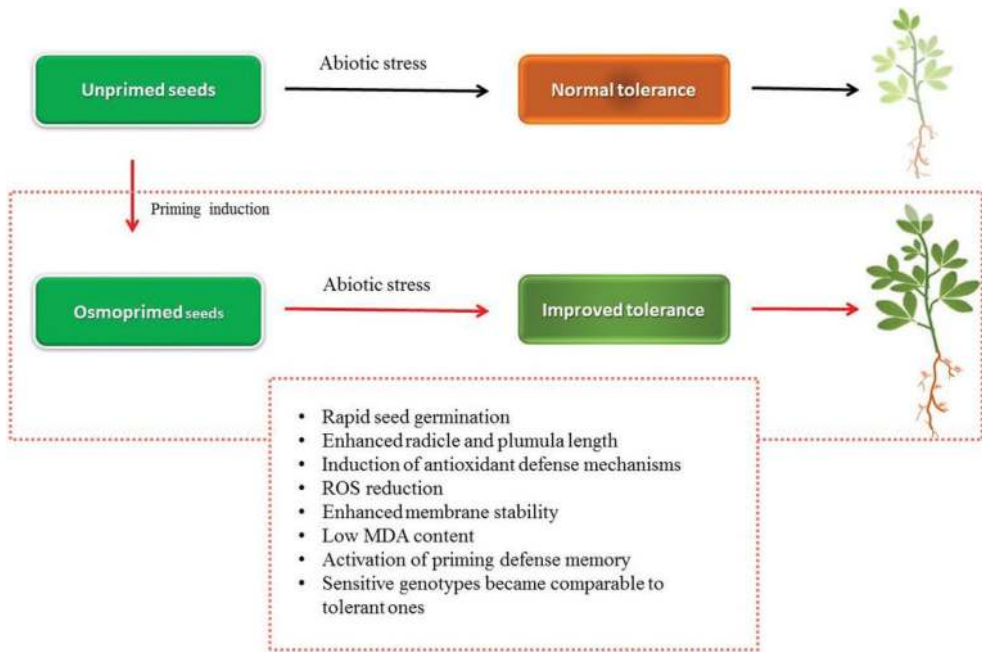
**Figure 3.** Seed osmopriming as a way to improve drought tolerance in alfalfa.

have been previously exposed to a pretreatment agent such as a natural or synthetic chemical compound present opportunities for better use in the study and management of the physiology of biotic and abiotic stresses in plants.

### 10.1. Osmopriming and pregerminative metabolism

In the osmotic priming technique, seeds are soaked in PEG or other osmotic solution during the period of time estimated to complete the first two phases of the germination process. Several osmotic agents can be used, mainly $KNO_3$, $KH_2PO_4$, $K_2HPO_4$, $CaCl_2$, $ZnSO_4$, $MgCl_2$, $MnSO_4$, NaCl, $NaSO_4$ and organic compounds, namely, fumaric, succinic, malic and citric acids; purines; and pyrimidines. PEG is most commonly used as a $\Psi$ reducing agent due to its non-toxic nature and large molecular size, without penetrating the seeds during soaking [76]. This technique has beneficial effects on plant germination and tolerance, especially under osmotic stress conditions. During the treatment, the amount of absorbed water is controlled in such a way to induce the pregerminative metabolic activities necessary for germination but prevents the actual emergence of the radicle [77]. Different physiological and biochemical activities occur in the seed at different moisture levels. Generally, this treatment improves seed rate, uniformity and germination time [78]. This effect can be attributed to the activation of seed repair mechanisms after exposure to adverse conditions, accumulation of germination-promoting substances, nutrient accumulation and osmotic adjustment (AO) [78, 79]. This technique has been shown to be strongly associated with increased antioxidant defence in

germinated seeds, which allows better tolerance to oxidative stress, reduction of lipid peroxidation and increase in membrane stability under water deficit conditions [15, 80].

## 10.2. Other techniques of seed priming

There are several techniques for seed priming, which differ according to the used agent. Among the most frequently used are hydropriming and biopriming. The first technique consists in exposing the seeds to a limited quantity of water in a continuous or successive manner at a suitable temperature. This is an inexpensive technique often used for field-grown cereals and legumes to accelerate germination [81]. The second technique consists in treating the seeds with microbiological agents such as rhizobia, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Trichoderma*, *Gliocladium* and other species and aims to improve the vigour and viability of the seeds. This technique uses a combination of hydration and seed inoculation with beneficial organisms in order to improve their germination, especially to protect them under stressful environmental conditions [77].

## 10.3. Osmopriming drought tolerance

The beneficial effects of seed priming to improve the germination rate under abiotic stress have been reported in some alfalfa genotypes [15, 33] and other legumes such as faba bean [82] and soybeans [83] and other crops such as cumin and rice [84]. At the molecular level, seed priming may be strongly linked to tolerance to water deficit. Indeed, it has been proposed in Refs. [85] that priming involves the accumulation of inactive cell kinase cascades and the modification of the chromatin structure and thus allows the amplification and activation of stress defence genes. In Ref. [86], two strategies have been proposed in which osmopriming probably improves abiotic stress tolerance including water deficit and salinity. In the first place, the treated seeds mobilize activities related to germination, for example, respiration, weakening of the endosperm, transcription and translation of genes, etc., thus facilitating the transition of dry seeds from the state of resting towards germination and leading to the improvement of their germination potential. Secondly, osmopriming initially imposes a certain level of abiotic stress on the seeds that suppresses the emergence of the radicle but stimulates stress-related reactions such as the accumulation of abundant proteins of late embryogenesis (LEA). These two strategies constitute a sort of priming memory, which could participate in the mechanisms of tolerance of germinated seeds during subsequent stress exposure [87]. In Ref. [15], priming of alfalfa seeds at −0.6 MPa of PEG6000 for 24 h at 25°C improved the water deficit tolerance in germinated seeds. Similar results have been reported in Refs. [84, 88] for rice and cumin, respectively.

In general, tolerant genotypes have the ability to protect themselves by stimulating the synthesis of enzymes and antioxidant molecules [89]. These compounds can thus neutralize the toxic capacity of the peroxide, superoxide and the hydroxyl radicals present in the tissues [90]. In our studies, we observed a significant increase in peroxidase (PO) and catalase (CAT) activities under water deficiency in young seedlings of all tested alfalfa genotypes [15]. These increases showed relative variations between genotypes. In addition, CAT activity was positively correlated ($r = 0.161*$) to germination performance under water deficiency, which could be explained by the induction of CAT enzyme synthesis, which plays a key role in the

protection and repair systems under water deficiency, especially during PEG priming [91]. The results showed that priming improves membrane protection in most alfalfa seedlings under severe water stress (−0.75 MPa PEG), especially in seedlings treated with −0.6 MPa PEG6000. Similar results have been reported in Refs. [92] after 48 h of osmopriming for spinach seeds and after 12 h of osmopriming in [33] for alfalfa under saline stress. Under similar conditions, the *Adis-Tata* population showed the greatest stability of the membrane compared to the other genotypes studied. It presented low levels of malondialdehyde (MDA) and low electrolyte loss values in comparison to other tested populations, and these results could be explained by the high levels of antioxidant enzyme activity such as PO and CAT [90]. The low accumulation of MDA in tolerant cultivars could be explained by the decomposition of ROS via increased CAT and PO activities and is consistent with improved protection of some *Medicago* cultivars against oxidative damage [41].

## 10.4. Osmopriming and $N_2$-fixing symbiosis

Seed osmopriming is a good technique to improve the germination rate and vigour of young seedlings as well as the plant growth in many species. It has been shown that it can improve nodulation, $N_2$ capacity in legumes and nutrient acquisition, especially in less fertile soils [81]. Studies have reported that plants from treated seeds have shown high capacity to form nodules and accumulate large amounts of N, $K^+$ and P, especially under stress conditions [93, 94]. In addition, rapid germination of seedlings could emerge and produce deep roots before the upper soil layers are dried and crusty, which may result in better legume placement and improve their ability to form a large number of nodules [93]. This technique has been reported to be effective in improving growth and yield of legumes [95]. It has been showed in Ref. [96] that the application of *Rhizobium* with seed priming significantly increased photosynthesis and nodulation and consequently nitrogenase activity in *Cicer arietinum*. It has been suggested that the combination of a tolerant rhizobia with some stress-tolerant alfalfa cultivars could improve the ability of plants to grow and survive under water and salinity deficiency conditions [15, 93].

In Ref. [93], osmopriming increased significantly (p<0.001) the chlorophyll-fluorescence (Fv/Fm) ratio, time to maximal fluorescence (TFm) and electron transport rate (ETR) in almost all symbiotic associations subjected to water deficit. These results indicated that this treatment may reduce the adverse effects of water deficit and salinity in alfalfa plants [97]. Several traits in these studies such as high chlorophyll contents, ETR and leaf area (canopy), could be behind the improvement of photosynthesis efficiency in combination with osmoprimed seeds in comparison to those from unprimed ones. In addition, we suggest that osmoprimed seeds and improved symbiotic $N_2$ fixing, high leaf relative water content (RWC) and photosystem (PS) II efficiency in the tolerant symbiotic combinations could avoid leaf senescence under water stress.

Under water deficiency, it has been reported that nutrient level was reduced in alfalfa plants from unprimed seeds in comparison to those from osmoprimed seeds [93]. However, the $K^+$ content was significantly (p < 0.001) increased in tolerant combinations from the treated seeds compared to those of untreated seeds. Seed priming has been shown to intensify seed supply

consumption, depletion rates, and dry seedling biomass [93, 98]. In addition, a high absorption of nutrients depends on good seed germination, vigorous establishment, root growth and activation of tolerance mechanisms such as osmoregulators and ROS detoxification enzymes [15, 40].

## 11. Conclusion

The negative effects of osmotic stress on legumes could strongly determine the interaction between rhizobia and alfalfa symbioses. These conditions have negative effects on plant metabolism and photosynthesis. However, several symbiotic interactions between alfalfa and tolerant rhizobia have shown high tolerance to drought and salinity with significant variation in their behaviour. Osmopriming treatment improves water deficit tolerance in young seedlings of alfalfa as well as the $N_2$ fixing capacity in growth stage. This enhancement is strongly related to the induction of antioxidant enzymes and due to also to the presence of tolerant rhizobia. This technique is very effective for the less tolerant genotypes and could make them comparable to the tolerant ones under water deficit.

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