IMPACT OF EXOGENOUS PROLINE ON VEGETATIVE GROWTH OF JOJOBA GROWN *IN VITRO* UNDER SALT AND DROUGHT STRESSES

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ABSTRACT

The present paper investigates the ability of exogenous proline to counteract salt and drought inhibitory effects in jojoba grown *in vitro*. Shootlets cultures were obtained *in vitro* by explanting nodal segments on MS medium supplemented with 1.5 mg/l BA + 1 mg/l IAA. Four concentrations (1,2,3 and 4 g/l) of proline were added to the culture medium to investigate the ability of proline to counteract the harmful effect of salt (induced by 3000 ppm of salt mixture) and drought (induced by 60 g/l mannitol) stresses. Generally, the addition of proline to saline or osmoticum medium improved the growth parameters i.e., shootlet number, shootlets height, leaf number, and fresh mass of shootlets even in the low proline application compared to control (proline-free medium). Results reported that 3 g/l of proline would be the most effective concentration for ameliorating the adverse effects of both salinity and drought. Under salt stress, the measured growth parameters were increased as proline increased till 3 g/l, and then decreased except shootlets height which took the opposite trend. On the other hand, all vegetative parameters and growth dynamics of shootlets grown under drought stress were increased with increasing the proline in the culture medium till 3 g/l. Salt or drought combating ratio took the same trend of growth dynamics. According to the obtained results, exogenous application of proline provided osmoprotection and enhanced the growth of shootlets of jojoba grown under salt and drought stresses. This can open the possibility of using exogenous applications of proline to alleviate stresses in the agronomic situation.
Keywords: jojoba, salinity, mannitol, proline, *in vitro* culture

1. INTRODUCTION

Jojoba (*Simmondsia chinensis*), a promising dioecious shrub of arid and marginal zones, is commercially precious for its seeds. Jojoba oil has various commercial uses which include cosmetics, lubricants, and adhesives, medicines, pharmaceuticals, supply of acids and alcohols, electric-powered insulators, foam control agents, and plasticizers (Jacoboni and Standarti, 1987). Besides its advanced lubricating properties, jojoba has attracted interest as a panorama and soil conservation plant due to the fact it may develop in regions of high atmospheric temperatures, low humidity, and low fertilizer requirements (Yermanos, 1982). Furthermore, jojoba plants had been discovered to be physiologically active during the whole year, indicating their capability in keeping advantageous carbon stability even under extreme drought at very low (-36 bar) water potential (Rashmi *et al*., 2016). Although jojoba is adapted to the aridity situations, salt accumulation in irrigated soils derived from both irrigation and groundwater sources can increase salinity to such levels which can reduce growth and yield. Also, drought is one of the maximum vast abiotic stress that affects jojoba growth and development. Salinity and drought are taken into consideration to be the most serious growth-limiting elements for crop plants. Salinity is a significant abiotic stress factor that threatens agriculture in both arid and semiarid environments, affecting over 20% of the world's irrigated land (Wu *et al*., 2017). The effects of salinity are typically summarized as water stress, salt stress, and stress because of ionic imbalance (Greenway and Munns, 1980). The primary purpose of salinity risks is enhanced ion toxicity causing impaired sequestering of sodium ions into the vacuoles. Likewise, the effects of drought or osmotic stress on plants are the reduction in vegetative growth via way of means of influencing various morphological and physiological processes. In this regard, mannitol is used as an osmotic agent in plant tissue subculture research (Bekheet *et al*., 2016). The addition of mannitol to the plant tissue culture medium decreases the water potential of the media inducing water stress that adversely affected the growth. The components of drought and salt stress cross-talk with every different as each of those stresses, in the long run, bring about dehydration of the cell and osmotic imbalance. Therefore, there's an absolute need for salt-tolerant cultivars or species to carry the poorly utilized saline lands into the right cultivation. Plants have evolved diverse approaches to reduce the negative effects due to abiotic stressors, several of that have been associated with the metabolism of amino acids (Batista Silva et al., 2019). The exogenous application of proline guarantees an additional way for enhancing growth under environmental stresses (Heuer, 2003). High levels of proline enable plants to maintain low water potentials, permitting additional water to be taken up from the environment. In this respect, some research has confirmed that exogenous proline can mitigate the damaging effects of salt and drought stresses (Heuer 2003; Hoque *et al*., 2007; Islam *et al*., 2009). Despite data displaying that jojoba tolerates fairly high levels of salinity (Benzioni *et al*., 1996) and water stress (Foster and Wright, 1980), the selections to this point have now no longer been meant for use in areas with extremely excessive levels of salinity and water stress (Botti *et al*., 1998). Consequently, *in vitro* culture constitutes a valid system for the study of the reaction of the plant to situations of drought and salinity and the isolation and selection of tolerant lines. Otherwise, tissue culture permits the management of stress homogeneity and the characterization of cell behavior under strain situations, unbiased of the regulatory systems present at the whole plant level (Lutts *et al*., 2004). At this point, many reviews have defined the use of nodal segment as explants as the *in vitro* propagation of jojoba (Agrawal *et al*., 2002; Tyagi and Prakash, 2004; Singh *et al*., 2008; Llorente and Apóstolo, 2013; Bekheet *et al*., 2015). Moreover, the effect of various salt concentrations on jojoba seedlings has been studied, and the substantial improve of proline and total sugar contents as salt concentrations increased has been discovered. Given the promising results on different crops, the the impact of the exogenous application of proline on the vegetative growth of jojoba tissue cultures grown under salt and drought stresses would be of great importance for the present investigation.

2. MATERIALS AND METHODS

2.1. Establishment of *in vitro* cultures

Nodal segments with axillary buds taken from women flowers have been used as explants for the *in vitro* regeneration of jojoba. Nodal explants have been washed with walking faucet water for 30 minutes. Once washed, the nodal explants were dipped in ethanol 70% for 30 seconds. The explants have been then sterilized with Clorox (50 %) for 10 minutes. Once sterilized, the explants wereA after which rinsed 3 instances in sterilized distilled water to take away all strains of the disinfectants. All steps of sterilization have been completed under aseptic situations with the use of a laminar air-float cabinet. The explants have been trimmed to 0.5 cm long then and cultured into 350 ml glass jars containing (MS) medium Murashige and Skoog (1962) supplemented with 1 mg/l benzyl adenine (BA) for 5 weeks. The proliferated shootlets have been sub-cultured instances (5 weeks interval) on MS medium contained 1.5 mg/l BA + 1 mg/l IAA for shootlets multiplication (Bekheet *et al*., 2018). When the explants began out to multiply, properly grown micro shoots have been separated with the assist of a sterile scalpel under the hood and placed in the same media for multiplication. Contaminated and vitrified cultures have been discarded and the healthful shoot buds have been used for the subsequent experiments. Small clusters of shoot buds (approximately 0.5 g weight) taken from the third subculture have been used in the following experiments.

2.2. Effect of proline on vegetative growth of shoot buds grown under salt stress

Various concentrations (1, 2, 3 and 4 g/l) of proline were used to investigate the ability of proline to mitigate the detrimental effect of salt stress. Uniform shoot buds (about 0.5g weight and 1 cm length) were cultured on multiplication medium (MS medium contained 1.5 mg/l BA + 1 mg/l IAA). To induce salt stress, the medium was supplemented with 3000 ppm of salt mixture [3 NaCl : 1 (3 $MgCl_2$:1 $CaCl_2$)] as described in (Ibrahim and El-Kobbia 1986).

2.3. Effect of proline on vegetative growth of shootlets grown under drought stress

To assess the influence of proline on the proliferation and growth of jojoba shootlets grown under drought stress, proline was added to the multiplication medium at a concentration of 1, 2, 3 and 4 g/l. Drought or osmotic stress was induced by supplementation of culture medium with 60 g/l mannitol. Shoot buds (0.5 g weight and 1 cm length) were cultured on the medium with different treatments for 8 weeks.

2.4. Recorded data

- Shootlets number: the number of proliferated shootlets recorded after 8 weeks of culturing.
- Shootlet height: the length (cm) obtained by the main shootlet that arose from the explant was recorded after 8 weeks of culturing.
- Leaf number: the number of leaves that arose from all the shootlets was recorded after 8 weeks of culturing.
- Fresh mass: fresh weight (g) at the end of the experiment.

$$Growth\ value = \frac{Final\ fresh\ mass - Initial\ fresh\ mass}{Initial\ fresh\ mass}$$

$$Salt\ (drought) combating\ ratio = \frac{Fresh\ mass\ on\ proline\ containing\ medium}{Fresh\ mass\ on\ proline - free\ medium}$$

2.5. Culture conditions and statistical analysis

Culture media were solidified with 0.7 % agar, amended with 30 g/l sucrose, and adjusted to pH 5.8 before autoclaving at 126 ºC and 1.5 Ib/M2 for 20 min. Cultures were incubated in a growth chamber at 25± 2ºC under 16 h light (2000 Lux) and 8 h dark. Experiments were designed in completely randomized design and data were statistically analyzed from 20 replicates using standard error (SE) (Snedecor and Cochran, 1967).

3. RESULTS AND DISCUSSION

3.1. Effect of proline on the vegetative increase of shootlets grown under salt pressure

3.1.1. Effect of proline on growth characteristics

Salt tolerance in plants is a complicated phenomenon that involves morphological and developmental modifications in addition to physiological and biochemical processes. The exogenous application of proline was found to be powerful in alleviating the negative results of salt stress. This experiment was performed to examine the impact of proline on the growth characteristics of jojoba shootlets grown *in vitro*. Proline in concentrations of 1, 2, 3 and 4 g/l was added to the grown medium which was salinized with 3000 ppm of a salt mixture. The shootlets characters determined in this experiment were the number of proliferated shootlets, the height of shootlets, and the number of leaves. Table 1 shows that in salt stress (proline-free medium) the shootlets and leaves numbers were reduced. Proline was found to improve the negative results of salt stress on *in vitro* multiplication of jojoba. The increase in proline level applied to culture medium triggered a significant increase in the shootlets and leaves' number. However, shootlets height values have been reduced in high levels of proline added to the saline culture medium. The maximum value (3.00 cm) of shootlets height was observed in the proline-free medium (Table 1 and Fig. 1-A). Shootlets had chlorosis. As a result, these explants appeared light green. While the highest numbers of shootlets (6.40) and leaves (12.00) have been obtained on the medium contained 3 g/l of proline (Table 1 and Fig. 1-B). The proliferated propagules in this treatment were healthy and had dark green color. It is also apparent from the coefficient of correlation obtained that the number of leaves was correlated with the proliferated shootlets. According to the results, it appeared clearly that the growth characteristics of the jojoba shootlets have been very affected by the proline added to the saline culture medium. These findings can offer perception for growing salt-tolerant lines of jojoba via *in vitro* studies.

3.1.2. Effect of proline on growth dynamic

The impact of diverse concentrations (1, 2, 3, and 4 g/l) of proline on the growth dynamic of shootlets of jojoba grown on medium salinized with 3000 ppm of the salt combination was determined. Table 2 reports the impact of the addition of proline on fresh mass, growth value, and salt combating ratio. It was found that the impact of proline on the growth of the shootlets system was highly perceptible. Compared to the control, the proline increased the 3 growth parameters of all concentrations of proline tested. The results confirmed that the growth parameters increased as the proline increased in the saline medium. However, the addition of proline at concentrations above until 3 g/l reduced the shootlets growth dynamic, thereby signifying 3 g/l of proline to be the top-rated concentration for alleviating the damaging consequences of salt stress. At this proline level, the highest fresh mass (3.50 g), and growth values (6.00) had been registered (Table 2). Also, the salt combating ratio calculated on fresh mass values took a comparable trend since the maximum value (1.75) was found in the 3g/l proline-containing medium. Consequently, these effects indicated that exogenous proline at an optimal concentration may be utilized for mitigating the suppressive consequences of imposed salt stress and thereby enhance the mechanisms of salt tolerance in jojoba tissue cultures Exogenous application of proline provided osmoprotection and additionally more advantages on the growth of plants grown under salt stress. Under salinity, proline has been suggested to enhance growth, physiological, biochemical, and anatomical characteristics, and also to improve the antioxidant system defense of plants (Abdelhamid *et al*., 2013; Dawood *et al*., 2014). Despite the useful consequences of exogenous proline application, it imparts poisonous effects if is over-accumulated or implemented at immoderate concentrations. The consequences of the prevailing research verified that the damaging consequences of salinity on jojoba grown *in vitro* might be partly offset with the aid of using the exogenous application of proline. Application of 3 g/l is the most effective concentration for combating the salt stress effects in the jojoba plants grown *in vitro*. At this concentration, the best results of growth characteristics (proliferated shootlets and leaves) and growth dynamics (fresh mass, growth value, and salt combating ratio) had been obtained. These results are in line with (Lone *et al*. 1987) who cited that the addition of exogenous proline to cultured barley (Hordeum vulgare L. cv. Maris Mink) embryos increased shoot proliferation under saline conditions. In addition, Athar and Ashraf (2009) confirmed that exogenously implemented proline on the germination and seedling stages alleviated the damaging consequences of salt stress on canola cultivars. In alfalfa callus (Medicago sativa) cultures, 1g/l of exogenous proline was very effective at lowering the consequences of salt stress and promoting traditional growth, and increased the dry weight (Ehsanpour and Fatahian, 2003). Similarly, (Khalid *et al*., 2010) suggested that reduction in germination, growth and chlorophyll contents caused by salt stress changed into improved by exogenous software of proline in sorghum. In a similar study, the exogenous proline at concentrations starting from 200-3000 mg/L, was found to be efficient for ameliorating the damaging consequences of salt stress (100 mM sodium chloride) on *in vitro* regeneration of Sorghum bicolor (L. Moench) (Amali *et* *al*., 2013). Likewise, Okuma *et al*., (2000) found that exogenous proline improved the growth of salt-stressed tobacco cell cultures and the development changed into attributed to the function of proline as an osmoprotectant for enzymes and membranes against salt inhibition. Regardless of the role of proline, jojoba tissue cultures tolerate salinity up to a level of sodium chloride concentration (113 mM), without showing any stress symptoms. Above this level, the salinity pressure effect was found as succulence and chlorosis of leaves and shoots (Roussos *et al*., 2007). Furthermore, Taha (2014) cited that growing seawater levels in tissue culture medium as much as 2000 ppm increased callus fresh weight in addition to shoots multiplication of jojoba. In this regard, Benichi *et al*., (2010) suggested that 3 g/l of NaCI marked the beginning of negative affect at the growth of jojoba seedlings, while 5g/l of NaCI inhibited completely the emergence of plumules.

Table1. Effect of exogenous proline on growth characteristics of jojoba shootlets grown on medium contained 3000 ppm of the salt mixture

Proline concentration (g/l)	Shootlets number ± SE	Shootlets height (cm) ± SE	Leaf number ± SE
0.0	2.50 ± 0.02	3.00 ±0.11	6.00 ±0.11
1.0	3.50 ± 0.10	2.70 ±0.16	8.20 ±0.15
2.0	5.30 ± 0.12	2.50 ±0.22	9.00 ±0.17
3.0	6.40 ± 0.20	2.20 ±0.20	12.00 ±0.10
4.0	5.00 ±0.23	2.00 ±0.31	10.00 ±0.09

Table 2. Effect exogenous proline on growth dynamic of jojoba shootlets grown on medium contained 3000 ppm of the salt mixture

Proline concentration (g/l)	Fresh mass (g) ± SE	Growth value	Salt combating ratio
0.0	2.00 ±0.13	3.00	-
1.0	2.40 ±0.20	3.80	1.20
2.0	3.00 ±0.10	5.00	1.50
3.0	3.50 ±0.15	6.00	1.75
4.0	2.70 ±0.09	4.40	1.35

3.2. Effect of proline on vegetative growth of shootlets grown under drought stress

3.2.1. Effect of proline on growth characteristics

The exogenous application of amino acid proline has been employed as an osmoprotectant substance that improves the performance of plants in drought conditions. Although the mechanism of drought tolerance is poorly understood, osmotic adjustment is considered to be associated with dehydration tolerance by allowing plants to maintain cell turgidity and physiological processes. In this experiment, the effect of proline as an osmoregulator and its role in jojoba combating drought stress was studied by the addition of proline (1, 2, 3 and 4 g/l) in the tissue culture medium that contained 60g/l mannitol. Under these conditions, the *in vitro* response of jojoba growth characteristics in terms of the number of proliferated shootlets, the height of shootlets, and the number of leaves were recorded. Data obtained revealed that all measured growth characters improved with proline treatments and improvements were recorded even in the low proline application. Obviously, the growth parameters gradually increased to 3 g/l of proline application and then decreased. The highest values of shootlet number (7.20), the height of shootlets (4.10 cm), and the number of leaves (13) were registered with 3g/l proline containing medium. However, higher proline concentration (4 g/l) doesn't prove to be of benefit. In contrast, the lowest values of the three growth parameters were observed with the control (proline-free medium) treatment. On the treatment, shootlets were vitrified and the leaves were relatively narrow and had pale green color (Table 3 and Fig. 1-C). In contrast, shootlets proliferated on medium contained 60 g/l mannitol and amended with 3 g/ l proline were healthy and had dark green color leaves (Fig 1-D). The obtained results proved that the addition of proline to the multiplication medium efficiently improved the growth characteristics of jojoba shootlets grown in the medium contained a high level of mannitol. The results could provide a platform for improving the sustainability and agricultural productivity of jojoba grown under drought conditions.

Table 3. Effect exogenous proline on growth characteristics of jojoba shootlets grown under drought stress induced by 60 g/l mannitol

Proline concentration (g/l)	Shootlets number ± SE	Shootlet height (cm) ± SE	Leaf number ± SE
0.0	3.00 ± 0.12	3.30 ±0.21	9.10 ±0.31
1.0	4.10 ± 0.15	3.70 ±0.26	10.20 ±0.19
2.0	6.20 ± 0.14	3.50 ±0.20	12.00 ±0.15
3.0	7.20 ± 0.22	4.10 ±0.10	13.00 ±0.10
4.0	5.30 ±0.19	4.00 ±0.18	11.50 ±0.12

3.2.2. Effect of proline on growth dynamic

Growth dynamics of jojoba shootlets grown on medium contained 60 g/l mannitol (drought inducer) and various concentrations (1, 2, 3 and 4 g/l) of proline as osmoprotectant were evaluated. Generally, all growth parameters are affected positively with proline treatments under drought conditions. Results reported that proline applications provided an increase in the growth parameters presented as fresh mass, growth value, and salt combating ratio compared to the control (proline-free medium). Although proline induced improvement in growth dynamics of jojoba shootlets at all applied levels, application of 3 g//l proved to be more effective in inducing drought stress tolerance as compared to the other level. At this treatment, the best results of fresh mass (3.90), growth value (6.80), and salt combating ratio (1.56) were obtained. A remarkable reduction of the three growth parameters was obtained with the 4 g/l proline containing medium (Table 4). These results show that exogenous applications of proline could be used to reduce the harmful effect of drought on the growth parameters of jojoba. It is important here to notice that both the number of proliferated shootlets and fresh mass values on proline-free medium (control) were higher in the mannitol-containing medium compared to the salt-containing medium. This may be explaining the differences of growth parameters reflected counteracting salt and drought stresses by application of proline.

Table 4. Effect of proline concentration on growth dynamics of jojoba shootlets grown under drought stress induced by 60 g/l mannitol

Proline concentration (g/l)	Fresh mass (g) ± SE	Growth value	Drought combating ratio
0.0	2.50	4.00	-
1.0	2.80	4.60	1.12
2.0	3.20	5.40	1.28
3.0	3.90	6.80	1.56
4.0	3.00	5.00	1.20

Drought stress is an important restricting factor in plant increase. Of diverse abiotic stresses recognized in nature, drought stress poses the first-rate hazard to crop production because water is essential at each stage of plant growth from seed germination to plant maturation (Chaves *et al*., 2003; Athar and Ashraf, 2005), so any degree of water imbalance may also produce deleterious results on crop growth. In this respect, numerous techniques had been proposed to reduce the harm due to drought stress and to increase the tolerance of plants towards this stress. As one of the compatible osmolytes, proline considers as a key signaling molecule capable of triggering a couple of responses and represents part of the adaptation process. At this point, preceding studies have proven that exogenous proline application effectively regulates osmotic ability and performs an important role in sustaining plant increase under osmotic stress (Ali *et al*., 2007; Ashraf and Foolad, 2007; Hoque *et al*., 2007). The current research found that the addition of proline accelerated both growth characteristics and growth dynamics of jojoba shootlets grown under drought stress caused by mannitol in comparison to untreated shootlets. Application of 3 g//l of proline proved to be more effective in inducing drought stress tolerance compared to the control or other levels. Our findings reveal that exogenous applications of proline might be used to alleviate the unfavorable results of and drought in jojoba. These effects are following the ones of Ali et al. (2007) who suggested that exogenous application of proline at specific growth stages improved the shoot fresh and dry weights of maize and application of 30 mM proline triggered the most increase in shoot fresh and dry weights. Meanwhile, the connection among increased proline content and better biomass were observed under osmotic stress in Arabidopsis (Roosens *et al*., 2002) and rice (Wu *et al*., 2003). Because of some earlier reviews, it's far suggested that exogenously applied would possibly have triggered enhanced endogenous proline accumulation under water stress situations which not only protects enzymes, 3-D structures of proteins and organelle membranes, but, it additionally supplies energy for growth and survival thereby assisting the plant to tolerate stress (Chandrashekar and Sandhyarani, 1996; Hoque *et al*., 2007; Ashraf and Foolad, 2007). Although obviously it's far from specific reviews that exogenous application of proline induces abiotic stress tolerance in plants, there are a few reviews that reveal that excessive concentrations of proline can be dangerous to plants, including inhibitory effects on growth or deleterious effects on cell metabolisms (Nanjo *et al*., 2003).

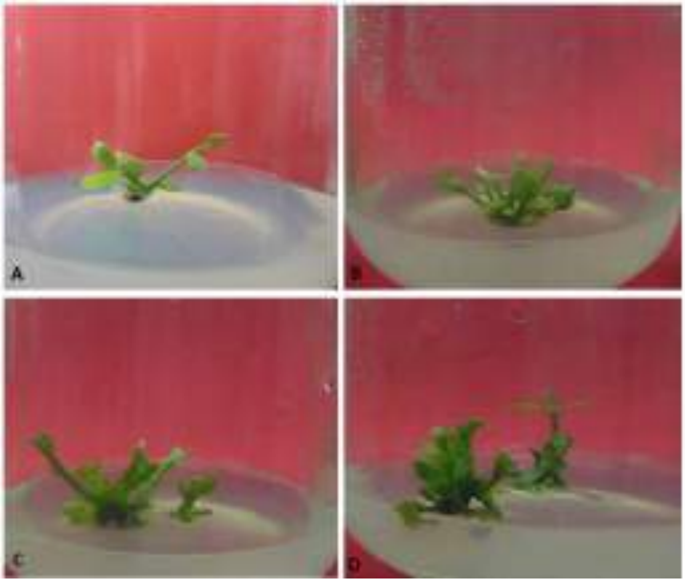

Fig. 1: a) Jojoba shootlets proliferated on: a) salt with proline-free medium, b) salt medium supplemented with 3 g/l of proline, c) drought with proline-free medium and d) medium contained 60g/l mannitol and 3 g/l proline.

4. CONCLUSION

Salinity and drought are consideration the most critical growth-restricting factors for crop plants. Although jojoba is adapted to aridity conditions, excessive salinity levels and excessive drought conditions are the most significant abiotic stresses that affect jojoba growth and production. The exogenous application of amino acid proline is thought to be effective in alleviating the damaging results of salt and drought stresses. In the present study, our findings indicated that the addition of proline to saline or osmotic medium improved the vegetative characteristics and growth dynamics of *in vitro* grown shootlets. Among numerous levels, three g/l of proline was found to be the most effective level on counteracting salt and drought inhibitory results. The acquired effects simply show that exogenous applications of proline could be used to reduce the dangerous effects of salt and drought stresses in jojoba. Also, the effects could offer a platform for enhancing the sustainability and agricultural productiveness of jojoba grown under salinity and drought conditions.

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