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Simmondsia chinensis is a suitable plant candidate to be cultivated in soil of marginal fertility and under stress conditions

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Abstract: Jojoba tolerates drought, salinity and high temperatures and can be grown on marginal lands . Jojoba is the only plant known that synthesizes liquid wax, and stores in seeds up to 60% of their dry weight, and its wax has the wider applications in medicine. Jojoba shrubs do not seem to need fertilizers. To increase seed germination, seed sowing should be fulfilled during the warm months of the year (more than 21°C). since jojoba is a wind-pollinated and perennial dioecious shrub, the genetic heterogeneity of seed-obtained plants is relatively high and more than half of the seedlings are males. To avoid seed propagation problems, vegetative or in vitro propagation procedures can be used to avoid genetic variation and multiplication of elite shrubs. Vegetative propagation of jojoba shrubs can be established by stimulation of adventitious root formation on semi-hardwood cuttings, but the number of the obtained propagules is limited by plant size and period of the year. In vitro, shoot multiplication was registered on MS medium supplemented with a relatively high concentration of BAP (3.0 - 3.75 mg/ l) and a low concentration of NAA (0.1 – 1.3 mg/ l). The best data were recorded when jojoba explants were cultured on MS medium supplemented with 1 g/ l activated charcoal, 0.5 g/ l mannitol, 3 mg/ l BAP and 0.1mg/l NAA and incubated at 30 oC. In four weeks, 57 % of shoots were rooted on MS medium supplemented with 1.25 mg/ l BAP and 1.3 mg/ l NAA.

Keywords: Gene expression, Micro propagation, Cytokinin, Auxin.

Introduction

Simmondsia chinensis (Link) Schneider, is generally known as jojoba, it is a long-lived evergreen perennial woody desert shrub. Jojoba is the sole species in the family *Simmondsiaceae* (Mills *et al*., 1997) and possibly belongs to the subclass-*Hamamelididae* (Barabe *et al*., 1982). It is pronounced as ‘‘ho-ho-ba’’. Jojoba has many common names due to its wide distribution all over the world, e.g. bush nut, nut bush, black nut, coffee nut, coffee berry, coffee bush, deer nut, goat berry, goat nut, lemon leaf quinine nut, pig nut, sheep nut and wild hazel nut (Kuepper, 1981; Thomson, 1982; Alyousif *et al*., 2023). The chromosome number of jojoba is 2n = 52 (Weiss, 1983). Jojoba is native to the Sonoran desert of Arizona, South Western USA, and Northern Mexico (Hogan, 1979; Perry *et al*., 2012; Ahmed *et al*., 2023). Now, due to its economic importance, jojoba is grown commercially in several countries such as Australia, Argentina, Chile, Peru, Egypt, Israel, South Africa, and India.

Jojoba seedling has a tap root, which reaches a depth of 30 to 45 cm but mature jojoba shrub has several tap roots, which spread over 15 - 25 meters below the soil surface. Extensive parallel lateral and secondary roots are branched from the tap roots of each shrub giving an ability to draw moisture from a considerable volume of soil (Weiss, 1983). Consequently, jojoba plants can survive and grow where most of the other plants die (Begg, 1979).

Jojoba is a wind-pollinated and perennial dioecious shrub (Buchmann, 1987; Coates *et al*., 2005). Jojoba shrub height commonly ranges between 0.6 and 2 meters, sometimes it is over 3 meters (Kuepper, 1981). Jojoba has a natural long life span which appears to be between 100 and 200 years. Jojoba has xerophytic, simple, and opposite leaves. Leaves are pale green or yellowish and their petioles are less than 0.2 cm in length (Thomson, 1982). The leaves live through two or three successive seasons, but they depend on ecological conditions e.g. soil moisture content, temperature, and light intensity of the region

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(Thomson, 1982). The blades of the leaves are thick and leathery, oblong-ovate, rounded at both ends. Since jojoba is dioecious plant species and wind-pollinated, genetic variation is very high and it negatively affects strongly on vegetative growth and yields. Consequently, the dimensions of jojoba leaves varied in length (2.5 to 5.0 cm) and width (1.5 to 2.5 cm), variations were also detected in leaf size, shape, and color (Gentry, 1958). The leaves of one-year-old plants were pale green. In the open field, these characteristics provide the plant the ability to reflect light radiation, reduce the amount of intercepted light, reduce evapotranspiration, lower leaf temperature, and consequently improve water use efficiency (Doraiswamy & Rosenberg, 1974; Osman & Abohassan, 1998).

Flowers are unisexual, about 0.4 cm long, and yellow, they are petalous and formed on the axils of leaves (Yermanos, 1979). The male flowers are clustered (figure 2 (A)) but the female flowers are usually solitary, with one flower per two nodes. Furthermore, flowers in every node or cluster are not rare. Flower opening is influenced strongly by the water status of the soil; they are not open under the low water content of the soil (Eve & DeLange, 1966). Anthesis takes place in the spring, from March to June when the soil and air temperature is above 15°C. Ripen of jojoba fruits are from April to July.

After maturation, seeds fall to the ground in August and they are still dropping about three to six months (Eve & DeLange, 1966). Although jojoba tolerates low water content of the soil, irrigation in excessively dry years becomes an essential prerequisite, and it might ensure good flower production and good seed yield (Yermanos, 1979). The effect of genetic variation appears strongly on seed characteristics, where jojoba seeds showed great variation in size, color, and shape. The seeds are smooth - brown to curly- black (Thomson, 1982). Generally, they are smooth cylindrical capsules with about 2 cm long (figure 2 (D)). While they reach full size in about three months, they need about six months to mature. Shrub seed production is generally limited until the fourth year of growth (Orwa *et al*., 2009).

Under suitable conditions for seed germination, jojoba seeds can be germinated directly after harvesting because they do not go through a period of dormancy (Yermanos, 1982). Under field conditions, excessive cold may kill entire plantations, especially at the seedling stage. If the temperature dropped to -9 °C, the old plantations exhibited no serious frost damage. Frost damage in the early flowering stages is less destructive than that of later stages; where early frost gives enough time for new flowers to replace the damaged one. On the other hand, frost does not negatively affect strongly on the survival of the plantation but it may affect negatively the shrub yields.

Jojoba can grow in soil of marginal fertility, withstands several types of stress such as drought and salinity, and does not seem to need fertilizers. Under field conditions, jojoba does not need polluting chemical treatments because it is not affected yet by major diseases or insect pests. At the same time, jojoba shrubs can withstand many chemical sprays (Yermanos, 1979).

Chemical structure of jojoba oil

Jojoba seeds contain oil up to 60% of their dry weight, which can be obtained by cold pressing the seeds. The obtained jojoba oil is not oil, but it is a simple liquid wax. Jojoba oil is a clear, golden-colored (Alyousif *et al*., 2023), unsaturated liquid wax with no scent or greasy feel. Jojoba oil gives little or no calories when consumed as oil for cooking. Other than jojoba oil, plant oils, and solid fats are triglycerides with a glycerin backbone and branches of fatty acid. On the other hand, jojoba oil is a straight 42-carbon atom chain unsaturated ester of fatty acids and fatty alcohols (Perry *et al*., 2021), each of which is made up of 20-21 carbon atoms. Fatty acids in jojoba oil are not 16-18 carbon atoms as known in various oils and fats.

The economic value of the jojoba plant

Due to its economic value, jojoba is gaining rapid popularity and research work (Hassan, 2003; Saad, 2016; Hassanein *et al*., 2012; Alyousif *et al*., 2023), where many researchers as well as farmers all over the world are interested in jojoba propagation and plantation. Jojoba is planted commercially for its seed oil manufacturers as a replacement for sperm whale oil and is notably resistant to degradation by bacteria (Gad *et al*., 2021). Jojoba is the only plant known that synthesizes liquid wax and stores it in seeds up to 60% of their dry weight. Jojoba wax has the potential for wider applications in extenders (Mills *et al*., 1997; Benzioni *et al*., 1999). Jojoba is used as an efficient lubricant because of its superior lubricating ability and uniform viscosity over a wide range of temperatures (Yermanos, 1979; Low & Hackett, 1981). Therefore, it could be used as a lubricant for high-pressure machinery and other industrial purposes (Sardana & Batra, 1998). Jojoba oil is also used as antifoaming agents and resins, etc (Bashir *et al*., 2008). Jojoba oil contains specific lipids that can be used as anti-inflammatory, antimicrobial, antifungal, and anticancer and has been used in relieving headaches and throat inflammation and in treating wounds Therefore, it is widely used in cosmetics (El Gendy *et al*., 2023; Alyousif *et al*., 2023) and anticancer

compounds. In medicine, it was used as a medicine for stomach aches, cancer, easing childbirth, kidney disorders, and tending wounds (Weiss, 1983). Jojoba wax is also useful in the treatment of skin diseases such as eczema, acute acne, skin cancer, psoriases, and sores (Naqvi & Ting, 1990). Jojoba wax is also used in inks manufacture, varnishes, detergents, and plastics (Botti *et al*.,1998).

In addition to the utilization of jojoba wax for hair care and medicinal treatments, it is used by Indigenous Americans and Indians for cooking (Agrawal *et al*., 2002). It is edible and contains simmondisin, which depresses appetite. It has no cholesterol and therefore can be used as low-calorie edible oil (Undersander *et al*., 1990).

Because jojoba has a deep root system it can be used in highway and roadside plantings and hedges. It can also be used as a soil stabilizer in green belts around desert cities suffering from particulate air pollution.

Conventional jojoba propagation:

Conventionally, jojoba plantations are established by using seeds, seedlings, and rooted cuttings (Roussos *et al*., 1999; Singh *et al*., 2003; Roussos *et al*., 2006; Bashir *et al*., 2007a; Mohasseb *et al*., 2009). The main problem in jojoba plantations is the very slow growth either in the field (Begg, 1977) or under greenhouse conditions (Von & Farquhar,1981). When the plantation is established by seeds, the male plants outnumber the females (Harsh *et al*., 1987). In addition, jojoba shrubs obtained from seeds showed high variability in most traits including yield because jojoba is dioecious, and an obligate cross-pollinated species (Gentry, 1958). Previous reports indicated that less than 1% of the plant population originating from seeds of native plants has the potential to produce economically acceptable yields (Purcell & Purcell, 1988; Ramonet-Razon, 1988). Therefore, in a breeding program, comprehensive selection was fulfilled to obtain elite cultivars in many countries all over the world.

Female shrubs on average have large leaves and more open canopies than males but it is not sufficient as an effective indication to determine sex in jojoba (Harsh *et al*., 1987). It is extremely difficult to determine the sex of the shrub before flowering and it appears within 9 to 24 months (Dunstone & Begg, 1983). Jojoba shrub forms fruits within 3 years, but shrub maturity needs 10 to 12 years. The percentage of seed germination depends on several conditions, especially temperature. To increase seed germination and faster emergence of shoots, seed sowing should be fulfilled during the warm months of the year when the temperature of the soil becomes more than 21°C. Germination occurs within a week under a relatively high temperatures in summer but it needs more than one week during winter months. Generally, seed germination results in seedlings with tap roots but the emergence occurs within 20 days or more. The emergence is generally delayed when the seed sowing is fulfilled under low soil temperature; it may take place in 2 to 3 months. Within the first two months, irrigation should be carried out regularly, to ensure relatively high water content of the soil, and to ensure good germination and root development. On the other side, overwatering should be avoided during seed germination and seedling growth because it may cause disastrous seedling emergence and survival (Bashir, 2007_b).

Seedlings can be used to establish a plantation, where seeds can be germinated in large containers filled with vermiculite, sand, or similar material at about 27°C. Then, the germinated seeds are transplanted in small paper containers, which are open at both ends, containing potting mix. Under suitable conditions for seed germination, emergence occurs within three weeks. Seedlings are transplanted when they are 15 to 30 cm tall, usually in 8 to 10 weeks. To establish a jojoba farm, paper containers are naturally partly disintegrated and they may be left undisturbed or it may be peeled off before seedlings transplantation (Bashir *et al*., 2007_b).

Genetic heterogeneity resulting from seed propagation creates several difficulties, for example, more than half of the seedlings are males. However, under farm conditions, 8– 10% of males are necessary for pollination (Reddy & Chikara, 2010). To avoid this, setting up a plantation with asexual propagules may be more expensive than with seed but saves time in replanting plants as well as crops produced of known sex. Vegetative propagation from the selected jojoba shrub can be established by stimulation of adventitious root formation on semi-hardwood cuttings (Palzkill, 1988). Several authors described several asexual methods for jojoba propagation including air-layering, grafting, stem cuttings, and tissue culture (Reddy, 2003; Bashir *et al*., 2006, 2007_b; Kumar, 2012; Saad, 2016). Any of these asexual procedures have more advantages than seed propagation, where asexual propagation procedures result in the multiplication of a shrub of desirable characters (Bashir *et al*., 2006, 2007_b).

Vegetative propagation can be established using single-node, double-node, and three-node cuttings from selected shrubs of jojoba. Different plant hormones were used to increase the total number of propagules obtained from a stock plant (Cao & Gao, 2003). We can say that, to avoid the problems

resulting from seed propagation of jojoba, vegetative propagation could be achieved by rooting semi-hardwood cuttings, but the maximum number of the obtained propagules was limited by plant size and period of the year (Low & Hackett, 1981). Consequently, the application of micropropagation in jojoba becomes an essential prerequisite to overcome all the difficulties of traditional techniques.

Micropropagation

Micropropagation is an artificial technique better than seed propagation. In addition, it is an alternative method of vegetative propagation, which is well suited to the multiplication of elite clones. Micropropagation offers many advantages such as it is not limited by season, produces pathogen-free plants, and can provide commercial production within a limited time frame and space. The technique can also be used for the genetic improvement of certain plant species through the intended selection of elite strains and gene technology. Micropropagation can be used as a tool for commercial mass production in many plant species such as jojoba (Gaboor, 2014; Saad, 2016; Alyousif *et al*., 2023), banana, and date palm (Mills *et al*., 1997; Hassanein *et al*., 2023). Generally, *in vitro*, cultured plant materials give rise to multiple shoots that can be rooted and transferred to the soil after the acclimatization period. Consequently, a single explant (shoot tip, nodal segment, or any plant segment) could conceivably provide thousands of plantlets per year. Among the tissue culture techniques, micropropagation is an area of practical application for large-scale multiplication of elite-selected plant material.

Since several years ago, micropropagation reached the commercial level in many plant species (Chandra & Mishra, 2003; Hassanein *et al*., 2023). Multiplication or micropropagation is established by several means, i.e. multiplication of shoots obtained from already existing meristem such as shoot tips or auxiliary buds. On the other hand, multiplication can be initiated indirectly through the formation of adventitious buds or somatic embryos on any plant organ. Exploiting the totipotent nature of plant cells could be used for research and commercial applications.

Advantages of micropropagation in jojoba

In addition to the advantages of micropropagation which were previously stated, many others can be stated here. New harvest seeds give 80–90% germination, but it decreases with the seed age (Harsh *et al*., 2001) and it should be avoided when seeds are used to establish jojoba plantation. In addition, jojoba is a dioecious plant species, and true-to-type by sexual propagation is not guaranteed. In addition, propagation of jojoba can be established using stem cuttings. In conventional vegetative propagation, rooting of stem cuttings needs controlled conditions and the application of auxins, but the rate of propagation is very limited because the nodes are hard to root. Furthermore, clonal propagation of elite individuals of known sexuality is necessary to ensure that the plants in a commercial plot will be productive (Chaturvedi & Sharma, 1989). In jojoba and other plant species, the establishment of multiplication by rooting semi-hardwood cuttings of selected individuals was not sufficient when mass propagation was needed (Low & Hackett, 1981; Lee, 1988; Cao & Gao, 2003) because the number of possible obtained propagules was limited by plant size and time of year.

Due to the previous reasons, the application of micropropagation in jojoba is an essential prerequisite for commercial plantation on a large scale (Mohasseb *et al*., 2009), where tissue culture techniques offer opportunities for the production of thousands of plants from elite-selected individuals (Lee, 1988). Generally, tissue culture-obtained plants grow more vigorously than others obtained from seedlings and vegetative propagation where they are free from pathogens.

In vitro multiplication and growth in jojoba

In jojoba micropropagation , cytokinins such as BAP in culture media proved to stimulate cell division, induce shoot formation, and auxiliary shoot proliferation (Abobkar *et al*., 2012). Generally, shoot formation on MS medium supplemented with BAP and NAA was better than multiplication on MS medium supplemented with BAP alone (Bashir *et a*l., 2008; Gaboor, 2014; Saad,2016). To control organ differentiation of certain tissue relative concentrations of these two growth regulators must be applied. Generally, the mode of interaction between cytokinin and auxin often depends upon the plant species (Skoog & Miller, 1957; Moubayidin *et al*., 2009; Saad, 2016). The highest regeneration frequency was obtained when the MS medium was supplemented with 2 mg/l BAP and 0.1mg/l NAA. The auxins differ significantly in stability, effectiveness, and their influence on organogenesis (Rumary & Thorpe, 1984).

The auxin/ cytokinin ratio is important for inducing morphogenesis processes (Hassanein *et al*., 1999; Hassanein, 2004 a; Pati *et al*., 2006). It has been reported that low auxin and a high cytokinin concentration established the ratio that favored shoot initiation, while a reversed proportion of these two growth regulators promoted root formation (McCown & Amos, 1979). The effect of BAP with auxins was also found to be very effective for the multiplication of jojoba (Hassan, 2003; Kumar *et al*., 2009; Hassanein *et al*., 2015; Saad, 2016). According to Saad (2016), jojoba shoots were cultured on MS media

supplemented with several concentrations of BAP and NAA. In five weeks, the estimated parameters were significantly affected by the established ratios between the used BAP and auxins. In general, a reduction in shoot multiplication was detected when a relatively high concentration of NAA was used. Best shoot multiplication was registered on MS medium supplemented with a relatively high concentration of BAP (3.0 - 3.75 mg/l) and a low concentration of NAA (0.1 – 1.3 mg/l). The highest shoot multiplication was obtained on the full-strength MS medium containing 3.0 mg/l BAP + 0.1 mg/l NAA (Hassanein *et al*., 2015; Saad, 2016).

The number of shoots resulting from jojoba multiplication for three subcultures (four weeks each) under the influence of BAP concentrations was recorded by Saad (2016) and Hassanein *et al*. (2022). Data indicated that MS medium with 4 mg/l BAP is better than others containing 5 mg/l because it expressed the highest number of shoots after three subcultures. After 12 subcultures, the highest shoot number was detected when jojoba shoots were subcultured on MS medium supplemented with 3 mg/l BAP and 0.1 mg/l NAA. Comparison between data growth under the influence of BAP alone or in combination with NAA indicated that, after three subcultures, MS contained 3 mg/l BAP and 0.1 mg/l NAA is better than the other containing 4 mg/l BAP alone. In one year, one nodal segment resulted in 740 shoots after 12 subcultures; it indicated that tissue culture is a better alternative to the conventional method of vegetative propagation especially when propagation of the elite genotype is aimed. In addition, micropropagation does not need a large space or a long time (Nehra & Kartha, 1994; Rao *et al*., 1996, Hassanein *et al*., 2015) and it can be carried out at any time of the year. The obtained number of shoots per explant depends on the source of explants (Hassanein *et al*., 2015), the type of explants (Hassan, 2003), growth regulators and media composition (Gaboor, 2014), plant genotype (Tyagi & Prakash, 2004), and culture conditions (Benzioni *et al*., 2003; Mills *et al*., 2004). As was reported by Saad (2016) and Hassanein *et al*. (2022), MS medium was the best as was described by other authors (Mills & Benzioni, 1992; Mills *et al*., 1997; Agrawal *et al*., 2002; Bashir *et al*., 2008; Kumar *et al*., 2010 b; Hassanein *et al*., 2012 $_{b}$, 2015).

Saad (2016) and Hassanein *et al*. (2022) found that the number of formed shoots in jojoba tissue culture was significantly decreased when the concentrations of MS components were more than or less than full-strength MS components. The calculated growth parameters increased when the explants were cultured on an MS medium with chemical components higher than the normal basal content (full strength). Jojoba is a stress-tolerant plant (Al-Ani *et al*., 1972), and increased the osmotic stress of the medium by increasing the concentrations of medium components stimulated jojoba growth. In general, a decrease in the concentration of MS components to half strength resulted in a decrease in the values of the measured growth parameters (Saad, 2016 Hassanein *et al*., 2022). Mohasseb *et al*. (2009) found that half-strength MS medium containing growth regulators established multiple shoot formation. However, the used medium did not support shoot growth and the obtained shoots remained compact and stunted, and it needed a full-strength MS medium.

Although the most used medium by several investigators is Murashige & Skoog (1962) medium, many others were developed and used for several purposes. Schenk & Hildebrandt (1972) developed a medium for the callus culture of both monocot and dicot plants. Some of them are described as having high salt levels such as MS and SH, MS contains the highest concentrations of salts. Gamborg's B5 medium (Gamborg *et al*., 1968) was developed for soybean callus culture and contains a much greater proportion of nitrate compared to ammonium ions but its sum nitrogen was lower than MS. Nutritional requirements for optimal growth of the cultured explants varied with species (Bhojwani & Razdan, 1996) and it played a key role in morphogenesis and response of explants (Gautheret, 1955; Hassanein, 2004 $_{a}$; Salem & Hassanein, 2013). In four weeks, the highest jojoba shoot number was detected when shoots were cultured on a high salt medium (MS). Also, shoot growth was the best when explants were cultured on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA. The lowest growth values were obtained when B5 medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA was used. Comparison between data obtained in four and eight weeks indicated that shoot formation was delayed when B5 or SH was used as a multiplication medium, and SH was better than B5 (Saad, 2016; Hassanein *et al*., 2022). Increasing the osmotic potential of the medium improved *in vitro* multiplication of jojoba. Hassanein *et al.* (2015) reported that the addition of low concentrations of NaCl (2 g/l) improved jojoba micropropagation. Jojoba was previously propagated on different cultural media such as DKW (Driver & Kuniyuki, 1984), SH (Schenk & Hildebrandt, 1972), or MS (Murashige & Skoog, 1962) medium with varying levels of success. Generally, MS medium was the best (Kumar *et al*., 2012).

The success of *in vitro* regeneration depends on the control of morphogenesis, which is influenced by several factors, including culture environment, etc. (Rai *et al*., 2010). The effect of the physiological

condition of explant donors was examined. Three conditions were established: (1) Seedling grown in glass jars where high humidity, sterile-nutrient enriched conditions were established. (2) *In vitro* grown plant materials, where explants were cultured on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA. (3) Plants grew in soil under laboratory conditions. Explants obtained from the previous materials were cultured on MS medium supplemented with 3 mg/l BAP and 0.1 mg/l NAA for four weeks. The lowest survival frequency, shoot number/ explant, and other estimated parameters were recorded when explants from field-grown parameters were used (Saad, 2016). Improve the physiological condition improved the obtained data especially when the explants were derived from *in vitro* grown plant materials. The response of the cultured explants was primarily determined by the genotype and physiological state of the tissue (Murashige, 1974; Giri *et al*., 2004). In jojoba, organogenesis had been induced *in vitro* both from mature explants (Tyagi & Prakash, 2004; Kumar *et al*., 2009) and juvenile explants (Gao & Cao, 2001; Kumar *et al*., 2010$_b$; Hassanein *et al*., 2015).

Activated charcoal was often used in plant tissue culture to improve cell growth and differentiation (Thomas, 2008; Agrawal *et al*., 2002). In general, activated charcoal improved jojoba multiplication and *in vitro* growth. In six weeks, activated charcoal stimulated shoot multiplication and growth parameters. These values increased when the medium was supplemented with activated charcoal with BAP alone or in combination with a low concentration of NAA. The best data were recorded when jojoba explants were cultured on MS medium supplemented with 1 g/l activated charcoal in combination with 3 mg/l BAP and 0.1 mg/l NAA (Hassanein *et al*., 2015; Saad, 2016).

The influence of activated charcoal was studied by Prakash *et al*. (2003), they observed that cultured explants exhibited differential morphogenic behaviors under the influence of the applied adjuvant such as charcoal. Activated charcoal is characterized by a fine network of pores with large inner surface area where many substances can be adsorbed (Thomas, 2008) and it explained the positive effects of activated charcoal on morphogenesis. Consequently, stimulation of morphogenesis and *in vitro* plant growth under the influence of charcoal may be due to its ability to absorb of inhibitory compounds in the culture medium and decrease its toxic effect. In addition, activated charcoal is involved in several activities including the release of natural substances that promote growth, the darkening of culture media, and the adsorption of vitamins, metal ions, and plant growth regulators including abscisic acid and ethylene (Thomas, 2008).

While gibberellins stimulate cell elongation (Lyu, *et al*., 2021), gibberellins expressed a negative effect on jojoba shoot multiplication and shoot growth (Saad, 2016). The negative effect of GA_3 on shoot multiplication and shoot growth of *in vitro-grown* jojoba shoots could not be improved when GA_3 was used in combination with other growth regulators. Mohasseb *et al*. (2009) used MS medium supplemented with 1 mg/l BA and 0.5 mg/l GA_3 to enhance the frequency of bud break but only one shoot/ node was formed. Gibbrellic acid was also used by other authors (Llorente & Apóstolo, 2013).

Abiotic factors affect jojoba multiplication and *in vitro* growth

Salinity is considered one of the most detrimental environmental stresses that restrict horticultural production, especially in soils of the arid and semi-arid regions of the earth. Few economical plant species can be grown commercially in saline soil. Jojoba is known as a salt-tolerant plant species (Jensen & Salisbury, 1988; Benzioni *et al*., 1992). Plants grown *in vitro* respond to salinity in a similar way as the whole plant (Benzioni *et al*., 1992; Mills & Benzioni, 1992). Consequently, it is possible to examine the *in vitro* response and adaptation mechanisms of a certain plant species to salinity and other types of stresses and gather valuable information that can be used in open land cultivation. Jojoba is drought drought-resistant plant, but the application of *in vitro* culture techniques can be used to increase its stress tolerance (Predieri, 2001). In addition, the response of jojoba plants to salinity stress under *in vitro* conditions and that of the whole plant *in vivo* was similar, supporting the hypothesis that *in vitro* screening of organs on NaCl offered an efficient and inexpensive method for salt tolerance selection (Mills *et al*., 1992).

Water deficits such as drought are one of the major components of environmental stresses. Some plants such as jojoba can tolerate or adapt to grow economically under drought conditions. It is well known that the total area of arable land in the world is gradually decreasing due to the progressive salinization of the soil and soil pollution, and limiting the irrigation water resources. Jojoba can survive under these conditions.

The effects of environmental stresses show many physiological and molecular processes, e.g. increasing stomatal closure, decreasing water content, reducing the rate of photosynthesis, decreasing the rate of respiration, altering the gene expression, and/or increasing protein hydrolysis (Ahmed *et al*., 1987;

Hassanein & El-Khatib, 1998; Hassanein, 1999; 2004$_b$). This could lead to the accumulation of free amino acids, especially proline (Hassanein, 2004$_b$). In addition, environmental stress enhances the formation of oxygen free radicals, reducing DNA replication and cell division, and consequently reducing plant growth and yield (Hassanein, 1999; Mohamed *et al*., 2000; Demir & Kocacaliskan, 2001). The exact mechanism by which plant cells regulate the expression of macromolecules during their natural development or under stress conditions is not clear. Most probably they are either at the gene level or in the pathway between gene and functional enzymes (Scandalios, 1974). When plants experience water deficits, stomatal pores close (Saccardy *et al*., 1996; Tezara *et al*., 1999; Lawlor & Cornic, 2002) leads to decreases in photosynthetic, CO_2 assimilation due to restricted diffusion of CO_2 into the plant leaves (Pelleschi *et al*., 1997). Closure of stomata as a result of water deficit and consequent decrease in CO_2 concentration in the leaf mesophyll resulted in the accumulation of NADPH (nicotinamide adenine dinucleotide phosphate) in the chloroplasts. This situation resulted in a decrease in the cell content of NADP. Consequently, O_2 acts as an alternative electron acceptor (O_2 + e-⟶ O_2-) resulting in the formation of superoxide radicals (Gamble & Burke, 1984; Baisak *et al*., 1994; Sairam *et al*., 1998; Saad, 2016) leading to oxidative stress. Water stress due to drought can reduce photosynthesis (Tezara *et al*., 1999; Lawlor & Cornic, 2002), and changes in gene expression (Neill & Burnett, 1999; Pattanagul & Madore, 1999; Romo *et al*., 2001).

Tolerant clones accumulated less Na^+ and Cl^- ions and more total amino acids than sensitive clones. An increase in the amino acids in addition to ammonia could play a positive role in the salinity tolerance of jojoba clones (Fayek *et al*., 2010) and it explained the ability of the plant to multiply under salinity as was detected by Hassanein *et al*. 2012. Application of seawater for growing of jojoba plant was investigated where seawater in low levels (2000 ppm) did not inhibit the growth of jojoba shoots, while higher levels (8000 and 10000 ppm) decreased all parameters under investigation (Fayek *et al*., 2010). Low differences in morphological and anatomical parameters could be detected between jojoba plants grown under high or low concentrations of NaCl, but leaf and cuticle thickness showed a high tendency to increase under saline conditions (Botti *et al*., 1998).

Morphogenesis is a complicated process. As was reported by Saad (2016), shoots could be multiplied under the influence of relatively low concentrations of NaCl. *In vitro*, culture environments can be mutagenic and plants regenerated from organ cultures, calli, and protoplasts and via somatic embryogenesis sometimes exhibit phenotypic and/or genotypic variations (Orbović *et al*., 2008). Consequently, the stress tolerance expressed by the *in vitro-grown* plant materials may result from the mother plant or be created under the influence of *in vitro* culture conditions.

The ability of jojoba to multiply under salt stress as well as its ability to grow under salt stress *in vivo* confirms that jojoba is a salt-tolerant plant species (Botti *et al*., 1998; Fayek *et al*., 2010). The ability of jojoba to differentiate and grow *in vitro* under relatively low concentrations of NaCl indicated that jojoba can be recommended to understand the salt tolerant mechanisms in plants and to cultivate commercially in soils with relatively high concentrations of NaCl. The negative effect of relatively high concentrations of NaCl on the multiplication and growth of *in vitro* tissues may be due to the accumulation of reactive oxygen species (Pinho & Ladeiro, 2012). They lead to a series of biological processes in the stressed plants resulting in a reduction in plant growth and retardation of development. Shoot growth retardation due to the addition of 4 g/l NaCl in the growth medium may be due to disruption of the structure and function cell membrane and cellular homeostasis as was reported by other workers (Shinozaki & Yamaguchi-Shinozaki, 2000; Zhu, 2001).

Jojoba can also tolerate extreme temperatures ranging from -5 to 54ºC (Yermanos, 1979). Jojoba tolerates drought, salinity, and high temperature and hence can be grown on marginal lands where other plants cannot grow (Bhardwaj *et al*., 2010), it was recommended to be cultivated in great areas in the Arab world (Osman & Abo Hassan, 1998). Extreme temperatures as well as drought or salinity may induce similar cellular damage (Jewell *et al*., 2010), they induce the production of reactive oxygen species, and accumulation of hormones such as abscissic acid (Cheong *et al*., 2002). Ultimately, they induce the expression of specific genes leading to the creation of a defense system (Vinocur & Altman, 2005). As well as other plant species, the multiplication of *in vitro*-grown shoots of jojoba was influenced by the incubation temperature (Hassanein *et al*., 2012a). Plant responses to heat stress depend on the temperature value, its duration, and the plant type. In natural habitats, extensive agricultural losses are attributed to heat, but often heat stress combines with drought or other stresses (Mittler, 2006). The best temperature for jojoba multiplication on MS medium supplemented with 3 mg/ l BAP and 0.1 mg/ l NAA was 30ºC (Saad,

2016). Most plant species incubated at 25°C but jojoba multiplied efficiently when their explants were incubated at 30°C. Jojoba multiplication was reduced by decreasing or increasing the incubation temperature to less than or more than 30oC. The multiplication completely ceased when the jojoba explants were incubated at 40°C. Low or high temperature is responsible for the production of reactive oxygen species in plant cells (Shinozaki & Yamaguchi-Shinozaki, 2000) and it is responsible for the reduction of growth and retardation of shoot multiplication.

In vitro culture was used to investigate the ability of jojoba to resist water shortage. Consequently, the effect of several concentrations of mannitol on jojoba shoot multiplication and shoot growth was studied (Hassanein *et al*., 2012$_b$; Saad, 2016). For this purpose, jojoba micro shoots were subcultured on MS medium supplemented with 3 % sucrose, 3 mg/l BAP + 0.1 mg/l NAA, and several concentrations of mannitol (0, 0.5, 1, 2, 3, 4 g/l). Application of 0.5 g/l mannitol in shoot multiplication medium improved the shoot multiplication as well as shoot growth (leaves and nodes number and shoot length). Generally, the ability of the genotype to tolerate a particular stress can be determined by the ability of the regenerants to survive under the imposed pressure of the selection agent (Pérez-Clemente & Gómez-Cadenas, 2012). On the other hand, in three weeks, the determined growth parameters decreased when jojoba shoots were cultured on MS medium supplemented with relatively high mannitol concentrations (3 and 4 g/l) (Saad, 2016).

To overcome salt or drought stress, plants have developed protective mechanisms including an osmotic adjustment that is usually accomplished by accumulation of compatible solutes such as proline, glycine betaine, and polyols (Ghoulam *et al*., 2001). Under field conditions, the extensive root system of jojoba and its interaction with the shoot system established a biological-compact unit to avoid dehydration and establish resistance to drought (Maas & Nieman, 1977; Osman & Abohassan, 1997). Salinity and drought affect many physiological processes of stressed plants including reductions of growth parameters and yield. The incorporation of the plant antioxidant defense systems in these processes has positively improved the salt and drought tolerance in greenhouse, in the field, or *in vitro* grown plants (Hassanein & El-Khatib, 1998; El-Khatib *et al*., 1999; Hassanein *et al*., 1999; Hassanein, 2004 $_b$; Hassanein *et al*., 2012 $_b$).

Heavy metals are the most dangerous environmental pollutants, where industrialization and urbanization have increased the anthropogenic contribution of heavy metals in the biosphere. Lead is known to affect water imbalance, inhibits enzyme activities (Sinha *et al*., 1988a, $_b$), induces alterations in membrane permeability, and disturb mineral nutrition (Sharma & Dubey, 2005). High lead concentration also induces oxidative stress by increasing the production of reactive oxygen species in plants (Reddy *et al*., 2005). Under lead stress, plants possess several defense strategies to cope with lead toxicity. Such strategies include reduced uptake into the cell; sequestration of lead into vacuoles by the formation of complexes; binding of lead by phytochelatins, glutathione, and amino acids; and synthesis of osmolytes. In addition, the activation of various enzymes such as peroxidases to combat increased production of lead-induced reactive oxygen species constitutes a secondary defense system (Pourrut *et al*., 2011).

The plant tissue culture technique can be used as a tool to study the metal tolerance of a plant by subjecting a plant tissue to known quantities of the specific metal in culture media. This technique also offers the potential to study the effect of metal on tissue, organs, or whole plants (Saad, 2016). The growth of plants was negatively influenced by lead through reducing the uptake and transport of nutrients such as Ca, Fe, Mg, Mn, P, and Zn. In addition, lead interferes negatively with several physiological and biochemical processes (Gomes, 2011; Pinho, & Ladeiro, 2012). It is worth mentioning that seed germination was not influenced by lead acetate up to 0.02 g/ l (Saad, 2016).

Jojoba as a stress-tolerant species has different defense strategies to control the toxicity of heavy metals by avoiding the metal entry into the cell and/ or stimulating the formation of various antioxidants to combat the increased production of reactive oxygen species caused by metal toxicities (Rossato *et al*., 2012). Although, Pb is not essential for plants; it is absorbed and accumulated in different plant tissues (Kabata-Pendias & Pendias, 1999), especially in roots (Verma & Dubey, 2003). While the effects of lead acetate are more pronounced at higher concentrations, lower concentrations resulted in the stimulation of metabolic processes and the enzymes involved in them (Gomes, 2011).

Induction of callus formation

The application of callus culture techniques in physiological and molecular studies was recommended where the callus cells were directly contacted by the tested agent, and the physical environment and nutrient status parameters could be controlled (Saad, 2016).

To obtain jojoba callus, leaf explants of a one-year-old plant were cultured on MS medium containing 3 % sucrose and 0.56 mg/l BAP + 1 mg/l NAA + 0.11 mg/l 2, 4-D. The cultures were incubated in dark conditions

at 29 ± 1°C. The data indicated that the first leaf after the shoot tip of each shoot was the suitable material for callus formation and the resulting callus was used for further studies (Saad, 2016). Callus cultures were previously used to test plant responses to salt and osmotic stress in many plant species (Santos *et al*., 1996; Hassanein *et al*., 2022). Parallel response of callus and whole plant of different plant species under the influence of salt stress was detected (Smith & McComb, 1981). On the other hand, salt tolerance in intact plants is not necessarily matched by salt tolerance in callus (Smith & McComb, 1981). When calli were transferred on callus medium supplemented with several concentrations of NaCl (0.5, 1, 2, 3 and 4 g/l) or mannitol (0.5, 1, 2, 3 and 4g/l), the data indicated that jojoba callus can tolerate salinity and drought as was reported by several authors (Berrichi *et al*., 2010; Fayek *et al*., 2010; Hassanein *et al*., 2012 b, 2015; 2022). While the fresh weight of callus still without any negative effect under the influence of a relatively high concentration of NaCl (4 g/ l), it increased with the increase of mannitol concentrations in comparison to that of the control (Saad, 2016; Hassanein *et al*., 2022).

In three weeks, when jojoba calli were subcultured on callus medium supplemented with several concentrations of lead acetate (0, 0.004, 0.008, 0.012, 0.016, and 0.02 g/ l), the fresh weight of callus decreased with the increase of lead acetate concentrations in comparison to that of lead acetate free medium (Saad, 2016).

When jojoba calli were subcultured on callus medium and incubated at a relatively high temperature (30°C), the calli became compact in texture and resumed the best growth in comparison to those grown under low or extreme temperatures. Callus grew under extreme temperatures (40°C) and was friable in texture but it was better in texture than those grown under low temperatures. A relatively high temperature (30°C) was the best for seed germination, shoot multiplication, growth of shoots, and calli. While plant responses to high temperature vary across and within species, and developmental stages. Seed germination, shoot multiplication, shoot growth, and callus growth were the best when they were subjected to suitable culture conditions at 30°C (Saad, 2016). Under these conditions, jojoba accumulates different metabolites such as antioxidants, osmoprotectants, heat shock proteins, etc. (Hemantaranjan *et al*., 2014).

For induction of root formation, shoot cuttings were subcultured on half-strength MS medium supplemented with 1.5 % sucrose and 1.5 mg/l IBA for different periods (1, 2, 3, 4, 7, and 15 days) to induce root formation. Then, shoots were subcultured on MS medium without growth regulators and incubated in a tissue culture room (29 ± 1°C with 16-h photoperiod at 400 lux). In three months on MS basal medium, 1.5 mg/l IBA for 7 days was the most effective treatment for root induction (33%) and plant survival. MS medium supplemented with IBA was commonly used to induce root formation (Llorente & Apostolo, 1998; Shahzad & Siddiqui, 2001; Mohasseb *et al*., 2009). Generally, IBA alone was used to induce root formation in jojoba as well as other plant species (Hassanein, 2004$_a$; Salem & Hassanein, 2013; Hassanein *et al*., 2015). In jojoba, MS medium in full and half strengths with different concentrations of IBA did not stimulate root formations. It may need combinations of auxins and other growth regulators (Chaturvedi & Sharma, 1989; Bashir *et al*., 2008; Singh *et al*., 2008).

In the case of jojoba, auxins alone or in a combination with other hormones were used for induction of root formation (Chaturvedi & Sharma, 1989; Bashir *et al*., 2008; Singh *et al*., 2008). Therefore, in this work, auxin in a combination with BAP was used. Microshoots were transferred to a solidified half-strength MS medium supplemented with several concentrations of BAP, NAA, or IBA and they were incubated at 29 ± 1°C and under a 16/8 h day photoperiod. After four weeks, 57 % of shoots were rooted when MS was supplemented with 1.25 mg/l BAP and 1.3 mg/l NAA. In this work, half-strength MS medium expressed more root frequency than full strength. Several studies on jojoba indicated that a half-strength MS medium was better for root induction (Rost & Hinchee, 1980; Llorente & Apostolo, 1998; Kumar *et al*., 2009).

Rooting of micro shoots is an essential prerequisite to facilitate the transfer of the plantlets into the soil (Pati *et al*., 2006). After rooting, hardening of plantlets before transfer in the soil was necessary for successful plantlets transfer. Thea agro soil potting medium that was used in this work gave good results for acclimatization because its porosity permitted better aeration. The organic matter present in this potting mixture might also aid rapid shoot growth.

Detection of genetic variation

Thermal cycler (PCR) provides a simple, rapid, and powerful new tool for *in vitro* cloning of DNA sequences where a segment of DNA can selectively be amplified several million folds in a few hours. PCR techniques depend on the utilization of single random ten-base oligonucleotides for amplification of the given DNA sequence (Rasmussen & Rasmussen, 1995), as was described firstly by Williams *et al*., (1990). Since the amplification products frequently vary between genotypes, they can be used as genetic

markers and detection of genetic variation (Quiros *et al*., 1991; Kresovich *et al*., 1992; Stiles *et al*., 1993; Rasmussen & Rasmussen, 1995).

In the past, Restriction Fragment Length Polymorphism (RFLP) was used as a tool for genetic screening, but it needs a relatively large amount of extracted DNA, expensive enzymes, and radioactive labeling. On the other side, genetic screening using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) are simpler and faster than (RFLP) and they require very small amount of extracted DNA (Cipriani *et al*., 1996; Atienzar *et al*., 2000; Pradeep *et al*., 2002).

Consequently, RAPD and ISSR were successfully applied to detect the genetic variation in *vitro* and *in vivo* propagated plants (Carvalho *et al*., 2004; Martins *et al*., 2004; Ramage *et al*., 2004; Modgil *et al*., 2005; Lakshmanan *et al*., 2007). In addition, they are also used to assess intra-or inter-population genetic variability (Huff *et al*., 1993; Alberto *et al*., 1997). Molecular markers were used as efficient tools for genotype identification and estimation of relatedness through DNA fingerprinting. Seven random primers, as RAPD markers, were used to analyze the genomic DNA diversity among ten plants obtained from ten jojoba seeds and grown for one year under lab conditions (Saad 2016). The obtained polymorphism between the amplification products of the tested plants was investigated through the examination of agarose gel as was previously described by Williams *et al*. (1990).

The seven used primers showed a highly polymorphic nature of the studied plants. It is concluded that jojoba plants obtained from seeds may belong to different genotypes. Thus, the genetic variation among jojoba plants was correlated with the genotype of each sample (Gaber *et al*., 2006). Saad (2016) results are in agreement with Amarger & Mercier (1995) who reported significant differences among jojoba individuals grown from seeds of unknown origin. In addition, the RAPD technique was used to test the genetic integrity of jojoba plants obtained *in vitro* shoot multiplication (Kumar *et al*., 2011). They found that the amplified products were monomorphic across all the selected micropropagated plants and were similar to the mother plant. Consequently, *in vitro* multiplication of up to 12 subcultures was safe as was carried out in this work depending on the fact that axillary bud multiplication was safely used for the production of true-to-type plants (Kumar *et al*., 2011). Sex determination of jojoba using RAPD-PCR in distinguishing between jojoba sexes was used by several authors (Agrawal *et al*., 2007; Hosseini *et al*., 2011).

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