In vitro Propagation of *Silybum marianum* L.

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ABSTRACT

Silybum marianum L. is a wild medicinal herbal plant found in Jordan. In vitro propagation of this medicinal crop was achieved by using explants from seedlings produced in vitro from seeds. Seeds were surface-sterilized and inoculated on the surface of hormone-free Murashige and Skoog, 1962 (MS) medium until full germination occurred. MS medium supplemented with 0.5 mg/l kinetin and 0.1 mg/l NAA was used for multiplication of mother stock obtained from developed seedlings. Proliferation was experimented with different levels (0.0, 0.4, 1.0, 1.6 or 2.0 mg/l) of kinetin, BA, or 2ip. Highest proliferation of *S. marianum* was obtained when BA and 2ip were used at (2.0 and 0.4 mg/l; respectively). Kinetin gave highest proliferation at 1.6 mg/l. Rooting was experimented at different levels (0.0, 0.4, 1.0, 1.6 or 2.0 mg/l) of IBA, IAA and NAA. Highest root number (4.0) and length (6.14 cm) was achieved at 1.0 mg/l NAA, and no roots were shown on MS media supplemented with IAA or IBA. Rooted transplants were acclimatized *ex vitro* successfully with 70% survival.

Keywords: *In vitro* propagation, Plant growth regulators (PGR), *Silybum marianum*.

Abbreviations: BA: 6-benzylaminopurine; kinetin: 6-Furfurylaminopurine; IBA: 3-Indolebutyric acid; IAA: Indole-3-acetic acid ; NAA: α-naphthalene acetic acid; 2iP: 6-(gamma,gamma-Dimethylallylamino)purine.

INTRODUCTION

Medicinal plants in Jordan are distributed all over the land of the Kingdom even over arid and semi-arid regions. Many examples of wild plants are used in Jordan as endemic species which are usually used for their sedative and curative properties. Considering that these plants have this importance, they are exposed to a great threat by collecting and overgrazing. Therefore, our medicinal wealth of these plants is decreasing continuously and facing extinction or severe genetic erosion in spite of the fact that some are endemic and very rare (Oran and Al-Eisawi, 1998). Some plant species that were reported as medicinal plants have the potential to be used in pharmaceutical industry. However, most of Jordanian medicinal plants have not been studied yet in terms of their economical value (Al-Eisawi et al., 1994).

Milk thistle *(Silybum marianum)* L. as shown in Figure 1, is one from Jordan's plant flora, that is widely used in folk medicine in Jordan and neighboring countries. Milk thistle is an edible plant which has an important medicinal value due to the presence of volatile oils and other secondary metabolites. Milk thistle has a number of useful secondary metabolites like silymarin, an isomeric mixture of flavonolignans (silybin, silychristin, and silydanin).

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Silymarin acts as a strong anti-hepatotoxic, which has been used for chronic inflammatory liver disease and liver cirrhosis (Corchete, 2008).

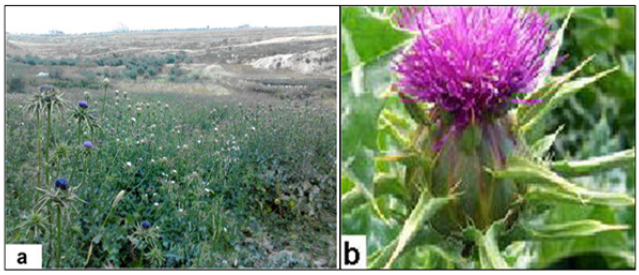

Figure 1. *Silybum marianum* in wild nature in Jerash, Jordan: a) whole plant , b) mature flower.

An efficient micropropagation system along with an *in vitro* production of secondary metabolites, would offer an alternative mean for propagation of plants and production of secondary metabolites. *In vitro* propagation would offer sufficient plant material year round and off season for the extraction of secondary metabolites. Plants are tremendous source for discovery of new products having a medicinal value for drug development and plant cell technologies are possible tools for producing plant secondary metabolites and having disease-free plant for further purposes (Vanisree et al., 2004). Single plant or few plants may produce thousands or even millions of plant depending on the capacity of the tissue culture system (Shibli et al., 1997). There are a huge number of publications which report the benefits associated with *in vitro* multiplication of medicinal plants (Elfahmi 2006; Gopi and Vatsala, 2006).

Milk thistle is considered an underutilized species in Jordan. Due to increase demand on milk thistle for medicinal purposes, there is a need to increase the mass production of this medicinal crop *in vitro*, because most of medicinal plants are subjected to loss.

To the best of our knowledge in Jordan, there are no studies relate to the micropropagation from seeds of this plant. Thus, the need of increasing the mass production of this medicinal crop, and conserve it to be used in other studies of in vitro secondary metabolites production; leads to start this study. Still, there is a need for further studies on in vitro conservation of this valuable genetic resource.

MATERIALS AND METHODS

Plant Material. Seeds of *Silybum marianum L* were thoroughly washed under running tap water for 20 minutes with few drops of mild detergent. Then antiseptic surface sterilized by solution of 4.0% sodium hypochlorite plus two drops of Tween–20 for 15 minutes (under the laminar – air flow cabinet), and were then rinsed with autoclaved distilled water for three times (5 min. each). Seeds were then transferred into 70% (V/V) ethanol solution for 30 seconds and rinsed with sterile distilled water 3 times (5 min. each). After that seeds were inoculated in test tubes containing hormone-free MS medium (Murshige and Skoog, 1962). Cultures were kept under dark until full germination. Germinated cultures were transferred to the growth room under daily light regime of 16-h (photosynthetic photon flux density (PPFD) = 40-45 µ mol. m^{-2} sec^{-1}) light, 8-h dark at 24 ± 1 °C. Light was supplemented using cool white fluorescent tubes.

Seeds Germination and Dormancy Breaking. Sterilized seeds were inoculated on the surface of half strength solid MS media supplemented with 2.0 mg/L GA_3 and 15 g/L sucrose. Seeds were kept under dark at 21±2 °C for 10 days until germination but there was full dormancy in the seeds; so, seeds were tested with tetrazolium test for viability then were treated with different treatments to break dormancy, 5 replicates (5 seeds/replicates) for each treatment: a) sterilized seeds were soaked in cold water for 72 h; b) seeds were soaked in 75% H_2SO_4 for 10 min; d) seeds were soaked in GA_3 (250 ppm, 500 ppm, 1000 ppm), c) seeds were soaked

in 7% potassium nitrate (KNO_3); for three days. Treated seeds were inoculated separately on the surface of MS medium for 14 days in the growth room under dark conditions. Data were reported for the date and percentage of germination of each treatment.

Initial culture medium was MS medium supplemented with 30 g/l sucrose, 8 g/l agar and medium was suplemented with plant growth regulators (PGR) according to experiment design. The value of pH was adjusted to (5.7-5.8). The medium was dispensed into 250 ml Erlenmeyer flasks (100 ml media \ vessel or flask), closed and then sterilized by autoclaving at 121°C and 15 kg\cm^2 pressure for 15 min.

In vitro multiplication of the mother stock cultures. For *in vitro* multiplication of mother stocks of milk thistle, MS media were prepared and used. It was supplemented with 6-furfurylaminopurine (kinetin) at 0.5 mg/l, and 1-Naphthaleneacetc acid (NAA) 0.1 mg/l. Subculture of microshoots (10-12 mm) to the prepared media under completely sterile conditions (Laminar air-flow cabinet). Cultures were transferred to growth room and maintained under daily light regime of 16-h (photosynthetic photon flux density (PPFD) = 40 µ mol. m^{-2} sec^{-1}) light, 8-h dark at 24 ± 1 °C. The mother stock cultures were incubated for 6 weeks in the growth room, then overall performance of the cultures and growth were monitored. The contaminated cultures were discarded.

In vitro Shoot Multiplication Nodal segments were subcultured in 250 ml glass bottles containing MS supplemented with BA, kinetin or 2-iP at different concentrations (0.0, 0.4, 1.0, 1.6 or 2.0 mg l^{-1}), and 0.1 mg l^{-1} NAA.

Data were reported after five weeks for the number of proliferated shoots, shoot height, number of leaves/explant. Plantlets performance was monitored. Treatments were arranged in a completely randomized design (CRD) with 5 replications (5 explants/ replicate).

Rooting. Microshoots were grown on hormone-free MS medium for one week to eliminate any carry over effect of any hormones that might affect rooting. To induce rooting, individual microshoot (5-7 mm long) were isolated and transferred to 25×150 mm culture tube containing 12 ml agar-gelled MS rooting medium supplemented with different concentrations (0.4, 1.0, 1.6, and 2.0 mg/l) of IBA, NAA or IAA. One set of cultures were inoculated on basal hormone-free MS medium without the addition of auxins and kept as control. Four weeks later, culture were evaluated for root formation (number and length of roots, number of leaves and shoots height) per explant. Five replicates were assigned (test-tube) per treatment and a single explant per replicate.

Ex vitro Acclimatization. Three days before acclimatization, the plugs were removed from culture tubes containing *in vitro* rooted microshoots. Plantlets with well-developed roots were then removed from test tubes and the agar was removed by gentle washing with tap water. Then plantlets were transferred to 1 peat: 1 perlite mixture in plastic pots and each was covered with a beaker; water was sprayed continuously to avoid wilting. Plantlets were acclimatized under 16 h of supplementary light of 40 µmol. /m^2/sec., 8-hours dark for three weeks at 24±2 °C. Beakers were removed after one week from transplanting of plantlets. Survival percentage was recorded at the end of three weeks. Acclimatized plants were transferred to green house (air temperature 25 ± 2 °C day/ 19 ± 2 °C night) and planted in a mixture of 1 soil (clay loam): 1 perlite in 17.5 cm × 17.5 cm plastic pots and overhead irrigated.

Statistical Analysis. Complete Randomized Design (CRD) was used to arrange all treatments involved in micropropagation. Each treatment was replicated five times. The obtained results were statistically analyzed by using SPSS analysis system. Analysis of variance

(ANOVA) was used and mean separation was done at probability level of 0.05 according to the Tukey's HSD.

RESULTS AND DISCUSSION

In vitro germination and establishment. Viability test showed that seeds were viable the viability percentage was 100 %, but seed germination failed or was very low with different treatments used. No germination for seeds which were treated with a) soaking in cold water for 72 h; b) soaking in 75% H_2SO_4 for 10 min. or c) soaking in GA_3 (250 ppm and 1000 ppm) for three days. Very low germination percentage was achieved with seed washed with 7% of potassium nitrate (KNO_3), the germination percentage was only 40 %. Germination percentage was only 10% when seeds were soaked in (500 ppm) GA_3 for three days. The germination failure in *Silybum marianum* L. seeds could be related to deep dormancy resulted from physiological (hormone types in seeds) (Al-Qudah et al., 2011) or mechanical case due to hard seed coat. The seed of *Silybum marianum* L is capable of remaining dormant in the soil for many years (Tamar Valley, 2011) .Figure 2 shows *in vitro* seed germination of milk thistle *Silybum marianum*.

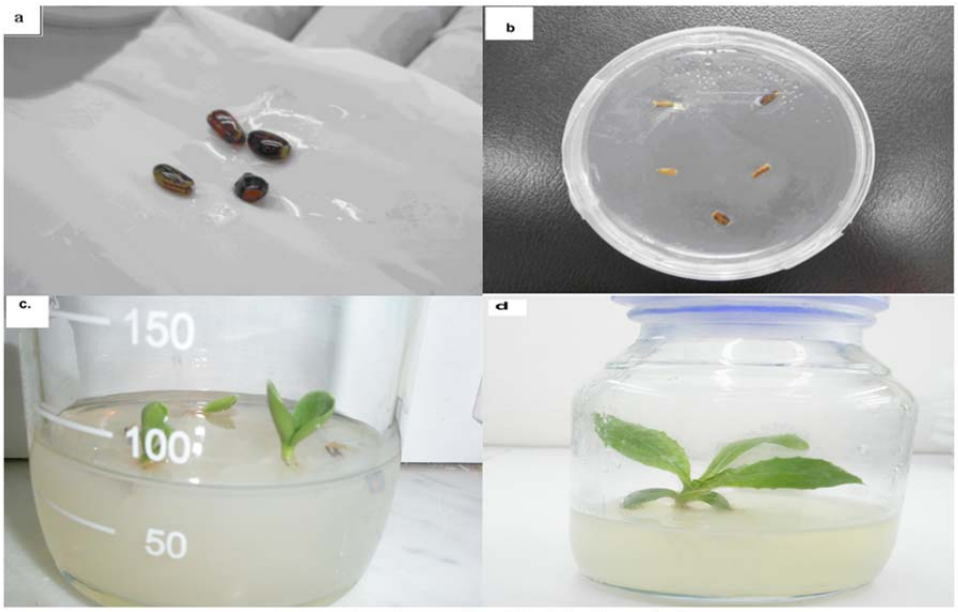

Figure 2. In vitro seed germination of milk thistle *Silybum marianum*; a. Seeds, b. Seeds on the surface of hormone-free MS media, c. germinated seedlings on the surface of hormone-free MS media, d. *In vitro* germinated plantlet on the surface of hormone-free MS.

In vitro Mother Stock Multiplication:

Milk thistle microshoots showed satisfactory plant size, growth and development (7-9 proliferated shoots, 3-3.5 cm shoot height and 5 leaves/microshoots) on MS medium supplemented with (0.5 mg/l) of kinetin and (0.1 mg/l) of NAA (Figure 3). Al-Qudah et al., (2011) indicated that MS mediumsupplemented with 0.5 mg/l of kinetin and 0.1 mg/l of NAA was suitable for mother stock propagation of *Teucrium polium*. Also, mother stocks of *Arbutus andrchne* were cultured on MS medium supplemented with 2.0 mg /L zeatin (Mostafa et al., 2009).

Figure 3. In vitro growth of *Silybum marianum* mother stock on MS media supplemented with 0.5 mg/l kinetin and 0.1 mg/l NAA.

In vitro Shoot Multiplication. In this study, no significant differences were noticed between control treatments C1 (without 0.1 mg l−1 NAA) and C2 (with 0.1 mg l−1 NAA) during the 4 wk of incubation. Table (1) shows the effect of different BA, kinetin, and 2iP concentrations on shoot multiplication of *Silybum marianum* L.

BA. It's clear that after four weeks of culture, multiplication parameters and growth performance of *Silybum marianum* L. responded to increased BA concentrations from 0.4 mg/l up to 2.0 mg/l. Shoot length and number of leaves produced from microshoots cultured on MS medium supplemented with (0.4, 1.0 and 1.6 mg/l) BA weren't different from each other as shown in Table 1. The best concentration of BA which enabled *Silybum marianum* L. to produce most shoot number (5.0 shoot per explants) was 2.0 mg/l BA and resulted in highest shoot length and highest number of leaves.

Shibli et al., (1998) found that increasing BA concentrations (1.5-2 mg/l) increased the number of shoots, but decreased the shoot length for *in vitro* propagated apple root stock (MM.106). As BA concentrations increased, shoot number increased to the maximum. The best concentration of BA which produced the highest shoots number of *Artemisia herba alba* was 1.0 mg/l BA (Sharaf, 2010). Almost similar results were found by Mneny and Mantell (2002) when they used BA in propagation of cashew *Anacardium occidentale L*. BA was also used to induce multiple shoots in *Artemisia pallens* (Sharief and Chandra, 1991), and *Artemisia vulgaris L*. (Sujatha and Kumari, 2007). Similarly, *Origanium vulgare* explants were reported to give the highest shoots number on MS medium supplemented with high levels (1.6 and 2.0 mg/l) of BA (Arafeh et al., 2006).

Kinetin. Shoot proliferation occurred on media that were supplemented with different kinetin concentrations. The highest proliferation rate (5.4 shoot per plant) was found on medium supplemented with 1.0 mg/l kinetin (Table 1). Otherwise maximum shoot height (6.72 cm) was obtained on medium supplemented with (0.4 mg/l). Maximum number of leaves (9.2 per shoot) was shown on medium supplemented with (1.0 mg/l).

Arikat et al., (2004) reported that the best shoot proliferation of *Salvia fructosia* was obtained at low levels (0.4 mg/l) of kinetin. Bouhouche and Ksiksi (2007) reported that, highest *in vitro* proliferation rate of *Teucrium stocksianum* Boiss was achieved on medium containing 3 mg/l kinetin and 0.5 mg/l IAA. Moreover, the highest shoot length in *Teucrium polium* L. was also obtained at low level (0.4 mg/l) of kinetin (Al-Qudah et al., 2011). However, the high concentration of kinetin oppositely affected proliferation of some plants.

2iP. The maximum number of shoots (5.2 shoots per plant) were obtained on medium supplemented with (1.0 mg/l), also for number of leaves (7.6 leaves) was obtained on the same concentration of 2iP. On the other hand the best result of shoot height (6.94 cm) was given on medium

supplemented with (1.6 mg/l) of 2iP (Table 1).

Sujatha and Kumari (2007) reported that increasing concentrations of cytokinin over 1.0 mg/l resulted in reducing the number of microshoots of *Artemisia vulgaris*. Catapan et al., (2000) reported that a high number of shoots were induced from each nodal segment of *Phyllanthus caroliniensis* using MS medium supplemented with (0.5 – 1.0) mg/l of 2iP. While, the lower concentration (0.25 mg/l) of 2iP failed to induce multiple shoots of *Psoralea corylifolia* L. on solid MS medium (Baskaran and Jayabalan, 2008). Also Sharaf (2010) reported that the best multiplication parameters and growth performance of *Artemisia herba-alba* were obtained at (1.0 mg/l) 2iP. At this concentration, the maximum number of microshoot per explants (22.9) and the longest shoots (2.61 cm) obtained.

When comparing growth regulators; kinetin gave the highest proliferation rate (5.4 shoot per plant) and the highest shoot height (6.72 cm) at 1.6 mg/l, than BA or 2ip. This means that kinetin is the best growth regulator used for micropropagation of *Silybum marianum* L.

Table 1. Effect of different BA, Kinetin, and 2iP concentrations on number of shoots, shoot length, and number of leaves of *in vitro* grown *Silybum marianum* L.

PGR concentration (mg/l)	Number of shoots	Shoot height (cm)	Leaves number
BA			
C1 [x]	1.4 bc [z]	2.68 c	9.2 b
C2 [x]	1.0 c	2.60 d	8.8 b
0.4	1.2 b	4.16 c	11.8 a
1.0	2.4 b	4.16 c	11.0 ab
1.6	3.8 ab	5.12 b	12.2 a
2.0	**5.00 a**	**5.65 a**	**13.2 a**
Kinetin			
C1 [x]	1.40 b[Z]	2.58 b	5.00 c
C2 [x]	1.80 b	2.54 b	4.60 c
0.4	4.20 a	3.96 ab	6.80 b
1.0	4.60 a	4.68 a	**9.20 a**
1.6	**5.40 a**	**6.72 a**	6.80 b
2.0	2.20 b	5.40 b	5.60 bc
2iP			
C1 [x]	1.6 cd[z]	2.60 c	4.6 c
C2 [x]	1.2 d	2.40 c	4.40 c
0.4	3.6 b	3.52 ab	5.0 bc
1.0	**5.2 a**	4.30 b	**7.6 a**
1.6	3.8 ab	**5.94 a**	5.3 bc
2.0	2.8 bc	4.22 c	6.0 b

[x] Control treatment (C1 and C2 represent control treatments (without and with 0.1 mg/l NAA) respectively)

[z] Means within columns having different letters are significantly different according to Tukey HSD at P≤0.05

Rooting. Different concentrations (0.0, 0.4, 1.0, 1.6 and 2.0 mg/l) of auxins (IAA, IBA and NAA) were tested for their effect on rooting. The maximum number of roots (3.6 roots per explant) and root length (2.04 cm) were resulted on media supplemented with 1.0 mg/l of NAA as shown in (Table 2). IBA and IAA did not give any root at all tested concentrations. Roots obtained only at levels of (0.4, 1.0, and 1.6 mg/l) of NAA with rooting percentages of 90%. The best rooting obtained at 1.0 mg/l NAA which gave high number of roots, root length and shoot height, but it gave large callus on the base of the plant (Fig. 4). These results revealed that *Silybum marianum* L. is sensitive to certain growth regulators. In *Salvia fruticosa* Mill, rooting was optimized at 0.6 mg/l IBA or 0.5 mg/l IAA (Arikat et al., 2004). Arafeh et al., (2006) showed that 1.6 mg/l IBA gave the highest number of roots for *in vitro* grown *Origanium vulgare*. Lopez et al., (2006) reported that rooting of *Helianthmum inaguaereported* was achieved on MS media supplemented with 1.0 mg/l IBA. Bouhouche and Ksiksi (2007) indicated that root induction in *Teucrium stocksianum* Boiss. was achieved on half-strength MS medium containing IBA. Also, in *Talinum portulacifolium* L., root development was induced by MS medium supplemented with both 0.2 mg/l NAA and 0.8 mg/l IBA (Thangavel et al., 2008). Sharaf, (2010) reported that IBA gave the best root formation (root number and length) of *in vitro* propagated *Artemisia herba-alba* plants. She found that maximum number of roots was obtained on medium supplemented with 1.0 mg/l of NAA. Highest rooting percentage of the in vitro rooted *Siybum marianum* (72%) was obtained on MS medium supplemented with 1.0 mg/l of NAA.

Figure 4. *In vitro* rooting of *Siybum marianum* on MS media supplemented with (1.0 mg/l) NAA.

Table 2. Effect of different NAA concentrations on number of roots, root length, shoot height, number of leaves/explants of *in vitro* grown *S.marianum* L.

NAA* conc. (mg/L)	Number of roots	Rooting percentage	Root length (cm)	Shoot height (cm)	Leaves number
C	0.00 c[z]	0%	0.00 d	2.34 b	**5.8 a**
0.4	2.0 bc	40%	1.02 b	**3.48 b**	3.4 ab
1.0	**3.6 a**	**72%**	**3.04 a**	2.04 b	3.8 ab
1.6	2.60 b	52%	2.62 c	1.84 b	3.8 ab
2.0	0.00 c	0%	0.00 d	1.20 b	3.2 b

*IAA & IBA at all tested concentrations (0.0, 0.4, 1.0, 1.6 & 2.0 mg/l) have no effects on rooting induction.

[z]Means within columns having different letters are significantly different according to Tukey HSD at P≤0.05

Ex vitro Acclimatization:

Rooted plantlets of *Silybum marianum* were successfully transferred to plastic cups (10-cm diameter) containing autoclaved growing mixture medium (1 peat: 1 perlite). *In vitro* rooted plants of *Silybum marianum* L and the survival percentage was 70%. Acclimatized plants appeared normal and did not exhibit any morphological abnormalities.

Arafeh et al., (2006) indicated that *in vitro* rooted plantlets showed 71% survival percentage when NAA was used in the acclimatization protocol for *Origanium vulgare* and *Origanum syriacum*. Also, Arikat et al., (2004) reported that *Salvia fruticosa* Mill resulted in good acclimatized plants. Pierik et al., (1997) mentioned that the *in vitro* grown roots are nonfunctional roots, thus plants *in vivo* tend to form new functional roots. In the current study, acclimatized plants were successfully grown in the green house due to sufficient root biomass and favorable conditions as shown in (Figure 5).

In *Origanium vulgare* the survival rate was 71% (Arafeh et al., 2006), also in *Capparis spinosa* it was 63%, (Musallam et al., 2011). *In vitro* rooted plants of *Artemisia herba-alba* showed survival percentage of 50%., the acclimatized plants appeared normal and did not exhibit any morphological abnormalities (Sharaf 2010).

Figure 5. Ex vitro acclimatized plantlets of *Silybum marianum* L.

CONCLUSION

The current study shows MS medium supplemented with plant growth regulators (0.5 mg/l Kinetin + 0.1 mg/l NAA) is considered suitable for mother stock propagation. High concentrations of kinetin (1.6 mg/l) or 2iP (1.0 mg/l) gave higher proliferation rate and BA gave good results at (2.0) mg/l; (5.4, 5.2, & 2.0 shoots/explants; respectively).

Among the different types of cytokinins (BA, kinetin, and 2iP) tested in this study, BA was the best. The explants cultured on BA medium performed healthy and strong plants. Rooting was achieved only with NAA, best rooting was achieved at 1.0 mg/l. Acclimatization of *Silybum marianum* L. resulted in (70%) of acclimatized plants that remain healthy and showed normal growth in the greenhouse.

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الإكثار الدقيق لنبات الخرفيش (*Silybum marianum* L.)

فيحاء الحوامدة[1]، رضا شبلي[1]، تمارا القضاه[2]

ملخص

تعد ظروف الإجهاد، مثل: نقص ماء التربة وقلة خصوبتها التربة من الأسباب الرئيسة لنقص الإنتاجية في المناطق الجافة. ونبات الخرفيش البري هو نبات طبي عشبي يعيش في الأردن. تم إكثار النبات في هذه الدراسة عن طريق الاكثار الدقيق، حيث تم تأسيس النبات داخل الأنابيب من البذور. وقد نجحت طريقة الاكثار الدقيق بعد التعقيم السطحي للبذور، وتمت زراعة البذور في بيئة موراشيغ و سكوج الخالية من منظمات النمو حتى تم الانبات. و قد تم تأسيس الأمهات اللازمة للتجارب على بيئة موراشيغ وسكوج المزودة ب (0.5 ملغ/لتر من منظم النمو كاينيتين) و(0.1 ملغ/لتر من منظم النمو نفثالين اسيتك اسيد) باستخدام نباتات المنماه داخل الانابيب. تمت دراسة تأثير السيتوكينينات (الكاينيتين و البنزيل- أدينين BA و تواي بي) عند مستويات (0.0، 0.4، 1.0، 1.6، و2.0، ملغم/لتر) على التفرع و النموات الخضرية و الطول وعدد الأوراق، حيث تم الحصول على أكبر عدد من التفرعات عند المستويات (2.0، 0.4 ملغم/ لتر على التوالي) من البنزيل ادينين و تو اي بي. وبالنسبة للكاينيتين؛ فقد اعطى اعلى تفرع عند (1.6 ملغم/لتر). أما بالنسبة للتجذير، فقد تم دراسة تأثير عدة مستويات (0.0، 0.4، 1.0، 1.6، و2.0) ملغم/لتر لثلاثة أنواع مختلفة من الأوكسينات (اندول بيوتييرك اسيد، اندول استك اسيد، و اندول نفثالين اسيتك اسيد) حيث استجاب النبات فقط لحمض النفثاليك اسيتك اسيد عند تركيز (1.0 ملغم/لتر) وبلغ عدد الجذور (4) و طول الجذر (6.14 سم)، وبلغت نسبة الجذير 70 % و لم يعطِ النبات اي جذور لكل من اندول بيوتييرك اسيد و اندول استك اسيد. وتم نقل النبيتات المجذرة داخل الأنابيب في مرحلة الأقلمة إلى البيت الزجاجي بنسبة بنجاح 70%.

الكلمات الدالة: الاكثار الدقيق، منظمات النمو النباتي، نبات الخرفيش.

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