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Full Length Research Paper

Clonal propagation and medium-term conservation of *Capparis spinosa*: A medicinal plant

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Successful *in vitro* multiplication of *Capparis spinosa* was achieved on Murashige and Skoog (MS) medium supplemented with benzyl amino purine (BAP) at 0.8 mg/L. The highest shoot length (35.6 mm) was obtained with the use of 0.4 mg/L BAP and 0.2 mg/L 1-naphthaleneanacetic acid (NAA). Kinetin at 2.0 mg/L produced a multiplication rate of 9.7 microshoots per explants with an average shoot length of 21.3 mm. *In vitro* rooting was successfully achieved on MS media supplemented with different concentration of indole-3-butyric acid (IBA), indole acetic acid (IAA) or NAA at various concentrations. However, rooting did not occur in the absence of IBA, IAA or NAA. A total of 85% survival was achieved when rooted explants acclimatized *ex vitro* using a mixture of 1 perlite: 1 peat. In another experiment, *in vitro C. spinosa* were successfully stored without serious losses by using MS medium supplemented with an appropriate concentration of osmoticum (sucrose, sorbitol, mannitol or glucose) at various concentration (0, 3, 6, 9 or 12%). Two types of plant material were used (*in vitro* plantlets and *in vitro* plantlets without tips). The results obtained show that the two type of plant material could be successfully maintained *in vitro* and optimum treatments were identified for each plant material. Further studies are still needed on medium term conservation to enhance the survival percentages of different plant material type.

Key words: Clonal propagation, *Capparis spinosa*, medium term conservation, carbon source, roots formation.

INTRODUCTION

Caper (*Capparis spinosa* L.) is a summer perennial shrub species belonging to the Capparidaceae family. It usually thrives in rocky and anhydrous habitats fully exposed to the sunrays, and is able to withstand high temperatures above 40°C (Sozzi, 2001; Levizou et al., 2004). Caper has been used in traditional medicine against bacterial infection, which exploited its properties for several purposes (Sozzi, 2001). In addition, *C. spinosa* helps in soil and water conservation, desertification control and land reclamation. *C. spinosa* germination rates are very low and this is probably because the seed coat contains inhibitors. Indiscriminate cutting and collection of plant has threatened this species. On the other hand, *C. spinosa* has not been propagated through cuttings (Inocencio et al., 2002). Unfortunately, this plant has not been subjugated to its full potential either by the scientists or by the local farmers. Therefore, *C. spinosa* need to be protected from loss to ensure its availability for future plant improvement. Field preservation is not easy, risky, requires huge land areas and is very expensive. More importantly, the materials are threatened by attacks of pests, diseases, human activities, climatic disaster and other biotic and abiotic hazards (genetic erosion).

In vitro culture plays an important role in agricultural and horticultural research, since a high number of

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genetically identical plants can be produced from single mother stock (Shatnawi et al., 2004, 2007, 2011a; Makhadmeh and Shatnawi, 2008). This could help develop new type of plants that can be produced year round with a high quality price ratio and distributed far more widely than classically propagated methods. Thus, the use of tissue culture in the production of *C. spinosa* would alleviate these problems, aiding in the commercial success of the crop. Several reports on *in vitro* micropropagation appeared in literature using various explants such as axillary buds, and shoot tips (Rodriquez et al., 1990; Deora and Shekhawat, 1995; Tayagi and Kothari, 1997; Chalak et al., 2003; Caglar et al., 2005; Chalak and Elbitar, 2006; Carra et al., 2007; Musallam et al., 2011). These reports are based on one or two genotypes and mostly do not document the effects of genotype on micropropagation and germplasm conservation.

In vitro conservation is based on conditions which permit minimal rates of growth, generally reduces the culture temperature or uses growth retardant (Marin and Duran-Vila, 1991; Orlikowska, 1992; Shibli et al., 1999; Sheyab et al., 2010; Shatnawi et al., 2011b). Sucrose functions as both a carbon/energy source and as an osmotic agent (Brown et al., 1979; Bonnier and Van Tuyl, 1997). The storage of healthy germplasm enables extended subcultured intervals, thus reducing the time needed and cost of maintenances. No systematic work is reported so far about the conservation of wild species of *C. spinosa* using *in vitro* techniques. Therefore, the present study was under taken to develop an *in vitro* propagation and medium-term conservation protocols for *C. spinosa* grown wild in Jordan for future use and research programs.

MATERIALS AND METHODS

Plant materials and culture conditions

Shoots of *C. spinosa (local cultivar)* were obtained from the field of Al-Balqa' Applied University, Al-Salt-Jordan. Healthy shoot tips were selected and trimmed to approximately 15 mm in length. The leaflets were removed prior to sterilization. Shoot tips were sterilized in 70% alcohol for 30 s and placed in 1% NaOCl for 30 min. Two drops of Tween 20 were added to decrease surface tension. Disinfestations with NaOCl took place under vacuum to reduce trapped air around the explants, thus increasing surface contact with plant tissue. After sterilization, each explant was rinsed 3 times in sterile deionized water and cut to a single bud under sterile conditions in a laminar air flow cabinet.

Explants were initially cultured on Murashige and Skoog medium (1962) supplemented with 0.5 mM myoinositol, 0.34 mM thiamine HCl, 2.4 mM pyridoxine HCl, 4.1 mM nicotinic acid and 3% sucrose. The pH was adjusted to 5.8, and 7 g/L agar (Sharlau, Germany) was added prior to autoclaving. Furthermore, 60 ml of medium were dispensed into each of (250 Duran flasks). The breather hole of each flask was then plugged with cotton wool to facilitate gas exchange and the media were autoclaved for 20 min at 121°C. Five explants were incubated at 24 ± 2°C with a 16 h photoperiod and irradiance of (50 µmol $m^{-2}s^{-1}$) supplied by cool white florescent lamps. Shoots produced from these explants were subcultured every six weeks onto MS medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L indole acetic acid (IAA) to initiate enough plant material.

Shoot multiplication

Effect of *benzylaminopurine (BAP)* on shoot proliferation

Microshoots (15 mm in length) were subcultured on Murashige and Skoog (MS) medium supplemented with six concentration of BAP (0.0, 0.4, 0.8, 1.2, 1.6 or 2.0 mg/L) with or without the addition of 0.2 mg/L naphthalene acetic acid (NAA). For each replicate, 60 ml of the medium was dispensed into 250 ml culture vessels (Duran flask). Each treatment consisted of five replicates; each, with five microshoots. Culture conditions were as mentioned before. Data were collected after five weeks on shoot length and number of new shoot.

Effect of kinetin on shoot proliferation

Microshoots (15 mm in length) were subcultured on MS medium supplemented with kinetin 0.0, 0.4, 0.8, 1.2, 1.6 or 2.0 mg/L, with or without 0.2 mg/L NAA. For each replicate, 60 ml of the medium was dispensed into 250 ml (Duran flask). Each treatment consisted of five replicates, each with five microshoots. Culture conditions were as mentioned previously. Data were collected after five weeks on shoot length and number of new shoot.

In vitro root formation

Explants were grown on growth regulator free-medium before initiating experiments. Microshoots (15 mm in length) were then subcultured on MS medium containing six concentration (0.0, 0.5, 1.0, 1.5 or 2 mg/L) of indole-3-butyric acid (IBA), indole acetic acid (IAA) or naphthalene acetic acid (NAA). For each replicate, 60 ml of the medium was dispensed into 250 ml culture vessels (Duran flask). Each treatment consisted of 5 replicates, each with 5 microshoots. In another experiment, microshoot (15 mm in length) were subcultured on MS medium supplemented with five concentration (0.0, 0.3, 0.6, 0.9 or 1.2 mg/L) of IBA, IAA or NAA at with 0.5 g/L charcoal. Culture conditions were identical to those described previously. Six weeks later, data were collected for shoot length, number of roots/explants, root lengths and percentage of shoots developing roots.

Ex vitro acclimatization

In vitro rooted plantlets were extracted from the medium, and then agar was washed in water bath and transplanted to 5 × 5 cm plastic pots filled with sterile mixture of 1 peat: 1 perlite. Humidity was reduced gradually to ambient conditions over a period of 3 weeks, whilst the plants produced new leaves. Three weeks later, data were collected for the percentage of plants that survived the *ex vitro* acclimatized.

Medium-term conservation

Medium-term conservation of plantlets (with shoot tips)

Plantlets (with shoot tips) of 15 mm length were transferred to hormone-free MS medium supplemented with different concentrations of sucrose, sorbitol, mannitol or glucose at 0, 3, 6, 9

Table 1. Influence of different concentration of benzyl amino purine (BAP) on number of new shoots and maximum shoot length *of in vitro* grown *C. spinosa* on MS medium after five weeks growth periods.

Growth regulator (mg/L)		Average number of new shoots/explant	Maximum shoot length (mm)
NAA	BAP		
0	0	04.6^{d}	26.2^{b}
0	0.4	36.0^{b}	25.8^{b}
0	0.8	45.3^{a}	25.2^{bc}
0	1.2	34.5^{b}	28.2^{b}
0	1.6	35.3^{b}	21.5^{cd}
0	2	35.0^{b}	26.9^{b}
0.2	0	01.6^{d}	12.5^{e}
0.2	0.4	21.1^{c}	35.6^{a}
0.2	0.8	25.1^{c}	25.0^{bc}
0.2	1.2	25.1^{c}	18.7^{d}
0.2	1.6	36.2^{b}	24.6^{bc}
0.2	2	03.0^{d}	13.6^{e}
LSD		6.45	3.77

Values represent mean; n = 25. Means within a column with the same letter are not significantly different based on the least significant differences test (LSD) at P ≤ 0.05.

or 12% (w/v). Next, 30 ml of the medium was dispended into jar (5 x 5 cm) vessels. The stored explants were incubated in the growth room under 16 h light/8 h dark (50 µmol $m^{-2}s^{-1}$) or under dark conditions, at 24 ± 2°C. Each treatment consisted of 6 replicates, each with 5 microshoots. Survived data were collected after 17 weeks in storage; survival plantlets (with shoot tips) were subculture on MS medium hormone free. After four weeks, re-growth data were collected.

Medium-term conservation of plantlets (without shoot tips)

Plantlets (without shoot tips) of 15 mm length were transferred to hormone-free MS medium supplemented with different concentrations of sucrose, sorbitol, mannitol, or glucose at 0, 3, 6, 9 or 12% w/v, and then 30 ml of the medium was dispended into jar (5 × 5 cm) vessels. The stored explants were incubated in the growth room under 16 h light/8 h dark (50 µmol $m^{-2}s^{-1}$) or under dark conditions, at 24 ± 2°C. Each treatment consisted of 6 replicates, each with 5 microshoots. Survival data were collected after 17 weeks in storage; survived plantlets (without shoot tips) were subculture on MS medium hormone free. After four weeks, re-growth data were collected.

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA); differences between individual means were determined by least significant difference (LSD) test at 0.05 level of probability. The layout of the different experiments was a complete randomized design (CRD). Collected data were statically analyzed using SAS software version 9 (SAS, 2002).

RESULTS

In vitro propagation

In vitro growth and development of *C. spinosa* plants were highly influenced by the concentration of growth regulators that were supplemented to the culture medium (Tables 1 and 2). BAP application increased the number of new shoots and shoot lengths. The plants' multiplication rates in the control medium were minimal (4.6 microshoot per ex-plant). Maximum number of new shoot per explant (45.3) was obtained on medium supplemented with 0.8 mg/L BAP (Figure 1). *In vitro* shoot length responded positively to BAP, and the highest (35.6 mm) value was recorded with 0.4 mg/L BAP and 0.2 mg/L NAA (Table 1). In contrast, shoot length decreased with increasing BAP concentration to 2.0 mg/L. The lowest value of shoot length obtained was 12.5 mm at 0.2 mg/L NAA growth medium (Table 1). Increasing kinetin concentration (0.4 to 2.0 mg/L) increased number of new shoots from 4.8 to 9.7, respectively (Table 2). Shoot length was also increased with the increases in the kinetin concentration from 0.0 to 2.0 mg/L kinetin. In summary, kinetin significantly affected the number of shoots/explants, and shoot length (Table 2; Figure 2).

In vitro root formation

In vitro root formation of *C. spinosa* plants successfully occurred either with IBA, NAA or IAA (Table 3). Root initiation (Figure 3) was achieved from the bases of shoots, after incubation on media containing IBA, IAA, or NAA. There were differences in the percentage of shoots that were developed roots, among different IBA, IAA or NAA in the medium (Table 3). The use of IBA, IAA or NAA did not enhance shoot formation compared with the controls. The highest (41.9 mm) shoot length was occurred on medium supplemented with 2.0 mg/L IAA

Table 2. Influence of different concentration of kinetin on number of new shoots and maximum shoot length *of in vitro* grown *C. spinosa* on MS medium after five weeks growth periods.

Growth regulator (mg/L)		Average number of new shoots/explant	Maximum shoot length (mm)
NAA	Kin		
0	0	4.6^{de}	26.2^{a}
0	0.4	4.8^{cd}	23.4^{abc}
0	0.8	4.7^{cd}	19.1^{d}
0	1.2	5.3^{cd}	21.2^{bcd}
0	1.6	8.12^{ab}	20.5^{cd}
0	2	9.7^{a}	21.3^{bcd}
0.2	0	1.6^{f}	12.5^{e}
0.2	0.4	6.6^{bc}	26.7^{a}
0.2	0.8	4.8^{cd}	20.1^{cd}
0.2	1.2	9.1^{a}	20.1^{cd}
0.2	1.6	9.4^{a}	21.6^{cd}
0.2	2	8.9^{a}	24.9^{ab}
LSD		1.98	4.11

Values represent mean; n = 25. Means within a column with the same letter are not significantly different based on the least significant differences test (LSD) at P ≤ 0.05.

Figure 1. Formation of multiple shoots of *C. spinosa.* Extensive formations of shoots on (MS + 0.8 mg/L BAP) after five weeks. Blue bar represents 5 mm.

(Table 3). No callus formation appeared at the base of each cutting. Maximum root number (3.2 roots per explants) was obtained at 1.5 mg/L NAA. On the other hand, maximum root length (6.52 mm) was occurred on medium containing 2.0 mg/L IAA. Maximum root formation percentage (60%) was obtained on MS medium supplemented with 2.0 mg/L IAA (Table 3).

Furthermore, to improve root formation of *C. spinosa*, charcoal was added to the medium at 0.5 g/L. The mean number of root per shoot was affected with the supplement of IBA, IAA or NAA when charcoal was added to the medium (Table 4). Increased IBA concentrations decreased root percentage. The number of roots formed per microshoots was affected by the concentration of IBA, IAA or NAA (Table 4). The highest number (1.45) of roots per ex-plant was obtained on MS medium supplement with 0.6 mg/L IBA when charcoal was used. A maximum root formation (75%) was obtained at 0.6 mg/L IBA, when charcoal was add to the medium at 0.5 g/L. Root length was affected and value was ranged from 0.15 to 9.15 mm. Shoot length was influenced by different concentration of IBA, IAA or NAA.

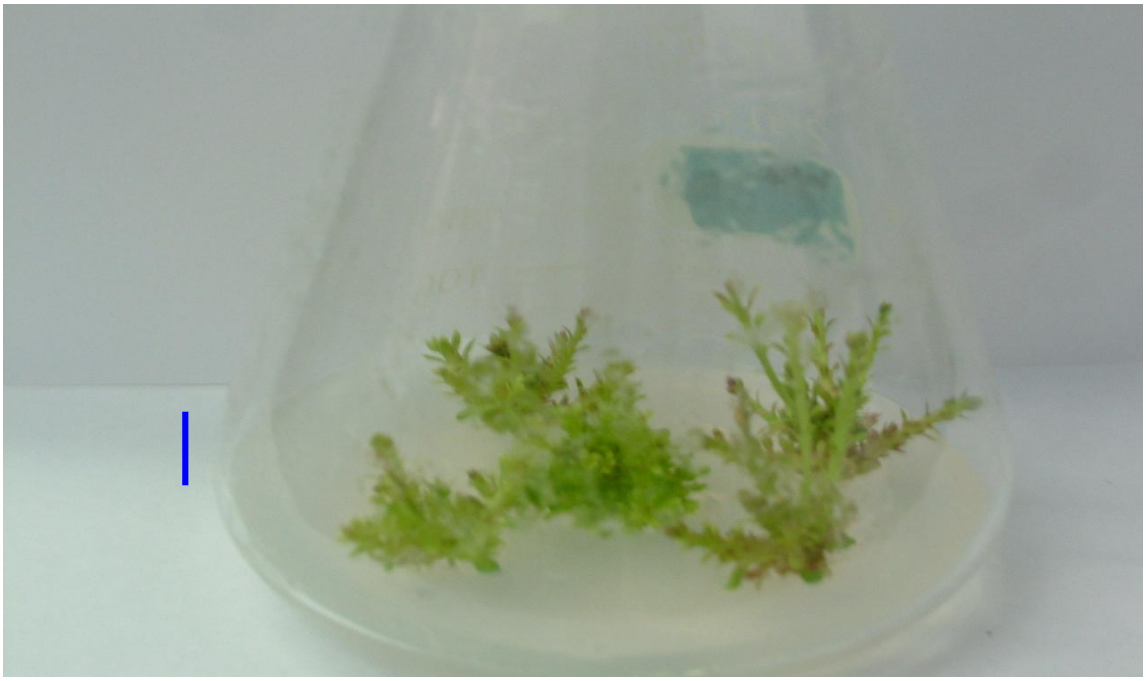

Figure 2. Formation of multiple shoots of *C. spinosa.* Extensive formations of shoots on (MS + 2.0 mg/L kinetin and 0.2 mg/L NAA) after five weeks. Blue bar represents 5 mm.

Table 3. Effect of indole-3- butyric acid (IBA), indole acetic acid (IAA) and naphthalene acetic acid (NAA) concentrations on number of new shoots, shoot length, number of roots, and root length of *in vitro* grown *C. spinosa* on MS media after six weeks growth periods.

Growth regulator (mg/L)	Average number of new shoots/explant	Shoot length (mm)	Average number of roots/explant	Root length (mm)	Rooting (%)
Control					
0	1.12^{bc}	26.44^{cd}	0.00^{e}	0.00^{f}	0
IBA					
0.5	1.16^{bc}	21.36^{d}	0.20^{e}	0.76^{def}	16
1	1.44^{bc}	22.88^{d}	0.60^{de}	2.60^{bcd}	32
1.5	1.20^{bc}	25.92^{cd}	1.56^{bcde}	2.16^{bcde}	56
2	1.04^{c}	24.48^{cd}	2.44^{abc}	2.24^{bcde}	48
IAA					
0.5	1.52^{abc}	40.48^{ab}	0.16^{e}	0.20^{ef}	4
1	1.60^{ab}	35.32^{b}	1.04^{cde}	2.16^{cdef}	28
1.5	2.00^{a}	40.72^{ab}	1.36^{cde}	3.40^{bc}	28
2	1.96^{a}	41.92^{a}	3.08^{ab}	6.52^{a}	60
NAA					
0.5	1.52^{abc}	29.76^{c}	1.12^{cde}	4.12^{b}	56
1	1.16^{bc}	24.16^{d}	0.60^{de}	1.24^{def}	28
1.5	1.52^{abc}	21.64^{d}	3.20^{a}	3.24^{bc}	48
2	1.96^{a}	21.24^{d}	2.08^{abcd}	3.00^{bc}	48
LSD	0.48	5.48	1.63	2.17	----

Values represent mean; n = 25. Means within a column with the same letter are not significantly different based on the least significant differences test (LSD) at P ≤ 0.05.

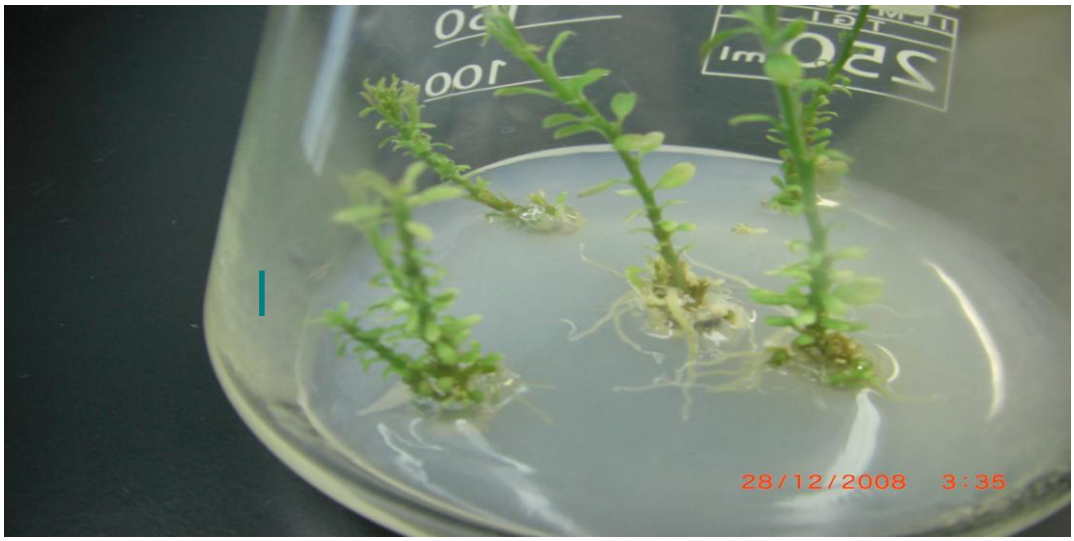

Figure 3. *In vitro* root formation of *C. spinosa* on MS + 2.0 mg/L IAA after six weeks growth periods. Blue bar represents 5 mm.

Table 4. Influence of IBA, IAA and NAA on number of new shoots, shoot length, number of roots, and root length of *in vitro* grown *C. spinosa* on MS media.

Growth regulator (mg/L)	Charcoal (mg/L)	Average number of new shoots/explant	Shoot length (mm)	Average number of root/explant	Root length (mm)	Rooting (%)
Control						
	0	1.15^{de}	27.05^{cde}	0.00^{e}	0.00^{f}	0
IBA						
0.3	500	1.70^{ab}	33.30^{abc}	1.25^{ab}	6.90^{ab}	65
0.6	500	1.75^{a}	38.15^{a}	1.45^{a}	9.15^{a}	75
0.9	500	1.25^{cde}	26.90^{de}	0.70^{cde}	3.05^{cde}	50
1.2	500	1.35^{abcd}	28.25^{cde}	0.90^{abc}	4.95^{bc}	50
IAA						
0.3	500	1.30^{bcde}	37.00^{ab}	1.35^{ab}	4.35^{bcd}	65
0.6	500	1.45^{abcd}	31.60^{bcd}	1.25^{ab}	5.60^{bc}	60
0.9	500	1.05^{de}	23.80^{e}	0.50^{cde}	1.55^{def}	50
1.2	500	1.65^{abc}	31.35^{bcd}	1.20^{ab}	3.40^{cd}	40
NAA						
0.3	500	1.30^{bcde}	26.55^{de}	0.10^{de}	0.15^{f}	6.6
0.6	500	1.25^{cde}	26.50^{de}	0.45^{cde}	1.80^{f}	25
0.9	500	1.00^{e}	27.10^{cde}	0.70^{bcd}	3.05^{cde}	35
1.2	500	1.05^{de}	25.55^{de}	0.50^{cde}	3.00^{cdef}	35
LSD		0.4	6.34	0.65	3	-

Values represent mean; n = 20. Means within a column with the same letter are not significantly different based on the least significant differences test (LSD) at P ≤ 0.05.

Acclimatization

Rooted plants, when moved to acclimatization conditions, showed 85% survival. Plantlets resumed normal growth in the greenhouse, developing new leaves within 15 days (Figure 4). The produced plants did not show abnormalities, which might indicate variation or mutation occurrence during the micropropagation procedures,

Figure 4. *C. spinosa* during acclimatization as transplanted plantlets were growing in the growth chamber. Bar represents 2 cm.

phenotypically plants appeared to be identical.

In vitro conservation of plantlets (with shoot tips)

After 17 weeks storage periods, the control treatment (no osmoticum) did not show any reduction in survival and regrowth under either light or dark conditions. Increasing sucrose to 12% decreased survival and regrowth rates under both light and dark conditions (Table 5). After 17 weeks storage periods, 40% of the plantlet that developed shoot tips was able to regrow on growth medium containing 6% sucrose. The plantlet that developed shoot tips did not regrow under light condition on medium containing 9% sorbitol, whereas 80% of plantlets that developed shoot tips were able to regrow in growth medium containing 3% sorbitol. Relative to the 6 and 9% mannitol, the survival and regrowth rates of the plantlet grown in growth medium contained 12% mannitol decreased dramatically under both light and dark conditions. The survival percentage was 53 to 20% under light conditions on media supplemented with 3 or 6% mannitol, respectively (Table 5), and it was lower than that of 12% mannitol. The survival percentage on media supplemented with glucose under light conditions was similar (Table 5). Storage under dark with glucose from 3 to 12% did not cause any regrowth after the storage periods (Table 5).

In addition, survival and re-growth of plantlets (without shoot tips) decreased as the concentration of sucrose increased in the medium. Plantlets (without shoot tips) were not able to re-grow when stored under dark condition on medium contained 3% sucrose (Table 6). After 17 weeks, plantlets (without shoot tips) were capable to resume growth and produce well developed shoots if stored on medium containing sorbitol under both light and dark condition (Table 6). Variations in survival and re-growth were obtained among the different concentrations of mannitol. Increasing concentrations of mannitol decreased both survival and re-growth. The survival rate of plantlets (without shoot tips) were stored under light condition re-growth on medium containing 6% was 33.3% and it was 40.0% in dark re-growth.

Moreover, the survival rate decreased when glucose was used, irrespective to its concentrations, while under light condition only 6.6% plantlets (without shoot tips) able to re-grow after the storage period (Table 6). A complete inhibition of re-growth was recorded when shoots were preserved on glucose supplemented medium when stored under dark condition (Table 6). Preservation on medium containing 3 - 12% glucose reduced (P=0.05) the growth of plantlets (without shoot tips). Thus, glucose could not be recommended for preservation of *C. spinosa* as compared to other osmoticums. In addition, plantlets (without shoot tips) did not increase in length and did not produce any new shoot or roots (Table 6).

DISCUSSION

In vitro shoot proliferation

High number of new shoots (45.3 shoot per explant) were obtained through the uses of 0.8 mg/L BAP (Table 1). Similar result was reported on *Coffea arabica* using

Table 5. Effect of sucrose, sorbitol, mannitol or glucose concentration on survival and re-growth percentage of *C. spinosa* plantlets with shoot tips stored under light or dark conditions at 24 ± 2°C.

Osmotic agent (w/v)	Survival % after 17 weak	Re-growth %	Survival % after 17 weeks	Re-growth %
	Light condition		Dark condition	
Sucrose				
0%	100^{a}	100^{a}	100^{a}	100^{a}
3%	66.7^{bc}	33.3^{bc}	40.0^{de}	33.3^{b}
6%	60.0^{bc}	40.0^{bc}	66.7^{bcd}	40.0^{b}
9%	60.00^{bc}	20.0^{cde}	40.0^{de}	26.6^{b}
12%	60.00^{bc}	26.6^{cd}	73.3^{abc}	20.0^{bc}
Sorbitol				
3%	100^{a}	80.0^{a}	60.0^{bcd}	0.0^{c}
6%	20^{egh}	20.0^{cde}	60.0^{bcd}	0.0^{c}
9%	33.3^{de}	0.0^{e}	60.0^{bcd}	0.0^{c}
12%	13.3^{egf}	0.0^{e}	53.3^{cd}	6.6^{bc}
Mannitol				
3%	73.3^{b}	53.3^{b}	73.3^{abc}	26.6^{b}
6%	46.6^{cd}	20.0^{cde}	86. 7^{ab}	26.6^{b}
9%	26.6^{def}	13.3^{de}	93.3^{a}	26.6^{b}
12%	20.00^{efg}	13.3^{de}	40.0^{de}	26.6^{b}
Glucose				
3%	20.00^{efg}	0.0^{e}	13.3^{ef}	0.0^{b}
6%	13.33^{fg}	13.3^{de}	0.0^{f}	0.0^{b}
9%	06.67^{fg}	0.0^{e}	6. 7^{f}	0.0^{b}
12%	00.00^{g}	0.0^{e}	13.3^{ef}	0.0^{b}
LSD	22.34	26.42	33	22.37

Values are the means of six replicates, each with 5 explants per replicate. Means within a column with the same letter are not significantly different based on the least significant differences test (LSD) at P ≤ 0.05.

8.0 mg/L BAP (Ebrahim et al., 2007). A study by Musallem et al. (2011) on *C. spinosa* showed that multiple shoot production was obtained when using woody plant medium (WPM) supplemented with 0.8 mg/L, 0.05 mg/L IBA and 1.0 mg/L GA_3 kinetin and NAA. Similarly, Chalak and Elbitar (2006) reported that multiple shoot would be obtained on MS medium supplemented with 1.0 mg/L zeatin. Shoot multiplication was optimized on the same media by subculturing shoot segment with 2 - 3 nodes every six weeks. In the current study, at 2.0 mg /L BAP + 0.2 mg/L NAA decreased shoot length as compared with the control significantly.

BAP promoted multiplication of *Vitis vinifera* and the rate of multiplication with BAP at 0.8 mg/L B (Shatnawi et al., 2011a). Similar results were obtained by Shatnawi et al. (2011b) on *Setivia rebuandia*. BAP had inhibitory effect on shoot elongation (Tables 1 and 2). Similar results have been obtained by Oztürk et al. (2004) in *Ludwigia repens*. In this study, significant differences were obtained between control, and BAP, or kinetin. *C. spinosa* plantlets developed symptoms of shoot tip necrosis with increased culture duration. This may be related to calcium deficiency in the plants; low evapotranspiration of plantlets in the culture vessels lead to insufficient mass flow of calcium to the growing tips (Mulwa and Bhalla, 2000).

In vitro root formation

The effects of IBA, IAA or NAA growth regulators on root induction were tested (Tables 3 and 4). Micropropagation protocols must produce plantlets with high quality shoots and roots, suitable for transfer to soil conditions. Percentage of root formation and number of roots per shoots were highly influenced by the concentration and the type of auxin. Auxin is necessary for the rooting process of *C. spinosa*. With the supplement of IBA, IAA, or NAA to MS media, mostly single shoots were produced. Low concentrations of NAA and IAA were effective in inducing rooting in *C. spinosa* and the development of roots was quite similar at the different

Table 6. Effect of sucrose, sorbitol, mannitol or glucose concentration on survival and re-growth percentages of *C. spinosa* plantlets without shoot tips stored under light/dark conditions at 24 ± 2°C.

Osmotic agent (w/v)	Survival % after 17 weak	Re-growth %	Survival % after 17 week	Re-growth %
	Light condition		Dark condition	
Sucrose				
0.0	100^{a}	73.3^{a}	100^{a}	0.00^{d}
3%	100^{a}	73.3^{a}	40.0^{d}	0.00^{d}
6%	100^{a}	80.0^{a}	100^{a}	80.0^{a}
9%	100^{a}	80.0^{a}	100^{a}	86.6^{a}
12%	93.0^{a}	60.0^{ab}	86.6^{ab}	40.0^{b}
Sorbitol				
3%	66.6^{b}	20.0^{de}	86.6^{ab}	46.6^{b}
6%	20.0^{cde}	20.0^{de}	60.0^{cd}	20.0^{c}
9%	26.6^{cd}	33.3^{cd}	53.3^{cd}	6.6^{cd}
12%	20.0^{cde}	6.6^{e}	60.0^{cd}	6.6^{cd}
Mannitol				
3%	66.6^{b}	46.6^{bc}	86.6^{ab}	20.0^{c}
6%	33.3^{c}	33.3^{cd}	86.6^{ab}	40.0^{b}
9%	26.6^{cd}	20.0^{de}	46.6^{cd}	20.0^{c}
12%	20.0^{cde}	6.6^{e}	66.6^{bc}	20.0^{c}
Glucose				
3%	26.6^{cde}	6.6^{e}	60.0^{cb}	0.0^{d}
6%	33.3^{c}	0.0^{e}	40.0^{d}	0.0^{d}
9%	6.6d^{e}	0.0^{e}	0.0^{e}	0.0^{d}
12%	0.0^{e}	0.0^{e}	0.0^{e}	0.0^{d}
LSD	22.72	21.84	22.3	19.89

Values are the means of six replicates, each with 5 explants per replicate. Means within a column with the same letter are not significantly different based on the least significant differences test (LSD) at P ≤ 0.05.

concentrations (Table 3). Musallam et al. (2011) reported that high frequency of rooting (80%) in *C. spinosa* rooting was achieved on half strength MS medium supplemented with 5.0 mg/L IBA. Chalak and Elbitar (2006) also reported the effect on the shoots of *C. spinosa* rooted on MS medium supplemented with 100 mg/L IAA. The highest number of roots of *C. spinosa* was obtained on medium supplemented with 2.0 mg/L IAA growth medium (Table 3). The growth medium containing 2.0 mg/L IAA produced higher number of roots, root length than that at 2.0 mg /L IBA (Table 3). It is clear that NAA was suitable to produce callus in comparison to other IBA, IAA or NAA. However, increasing NAA to 2.0 mg/L produced the highest root length (P=0.05). To improve root formation, charcoal at 0.5 g/L was added to the medium.

Activated charcoal is a black, shiny, odorless and tasteless substance made by burning certain types of wood under controlled conditions so that a very large adsorptive surface is produced. In this study, activated charcoal adsorbs toxic compounds released by the explants tissues. Using charcoal *in vitro* increased root formation up to 75% in medium supplemented with 0.6 mg/L IBA (Table 4), with a maximum root length of 9.15 mm. Activated charcoal adsorbs not only toxic compounds, but also excess growth regulators and other compounds that are added to the medium (Pierik, 1987).

Ex vitro acclimatization

Transfer of sterile rooted plantlets to greenhouse conditions is a critical step. When moved to acclimatization conditions, the rooted plants showed 85% survival. Plantlets resumed normal growth in the greenhouse and developing new leaves within 15 days (Shatnawi et al., 2011a). The produced plants did not show abnormalities and plants appeared to be identical phenotypically. In this study, rooted plantlets were easily acclimatized by transplanting to a potting mix. The procedure used in this study seems to be efficient method for acclimation; this method could be used for large scale production of *C. spinosa* and may be for other

important horticulture species without serious losses.

Medium term conservation

Sucrose (3 - 12%) was able to play an important role in the conservation of explants (Table 5). Bertrand-Desbrunais et al. (1992) found that elongation of *in vitro* stored shoot tips of C*offea* spp. was maximized at a sucrose concentration of 2%, and that shoot tip elongation decreased as the concentration was increased in the medium. High concentrations of osmoticum in the medium cause a negative water potential, and reduce the optimal turgor pressure needed for cell division and inhibit growth (Tahtamoni et al., 2001). *C. spinosa* plantlet could survive 17 weeks on the control treatment. This is because the lack of carbon source decreased cells and shoots proliferation as compared with the control. Survival of *C. spinosa* plantlets was 100% after 17 weeks with 3% sorbitol, if stored in the light conditions (Table 5). However, increased sorbitol up to 12% decreased survival and re-growth rates of plantlet, and plantlet without shoot tips under different treatments (Tables 5 and 6). Moges et al. (2003) reported that shoot height of African violet decreased when sorbitol was used as an osmotic agent. Tahtamouni et al. (2001) also reported that preserved microshoots of wild pear survived at the end of the preservation duration when sucrose and mannitol were used.

The supplement of 12% mannitol resulted in a significantly decreased in survival of plantlet and plants without shoot tips, compared with the 3% mannitol (Tables 5 and 6). With 6% mannitol, 86% of plant with shoot tips survived and 26% were able to re-grow after 17 weeks storage (Table 5). This is because physiological disorders increased with increased mannitol concentration in the medium; this is similar to previous finding in wild pear (Tahtamouni et al., 2001). High glucose concentration might result in a more negative water potential which would reduce the optimal turgor pressure needed for cell division and thus reduce growth. On the other hand, it may lead to some toxicity. However, no re-growth was obtained under dark condition if glucose was used (Tables 5 and 6). This may be because physiological disorders increased in the presences of glucose.

In the current study, no new shoot proliferation was obtained during storage at any osmoticum treatments. However, this could be due to the effects of osmoticum or due the absence of required hormones, as the medium used was hormone-free. In addition, the fact that no roots developed during the storage period could be due to the absence of the required hormones (mainly auxin) or to due the presence of osmoticum such sucrose, sorbitol, mannitol or glucose. Contamination did not cause serious problem and a very low levels being noticed. However, fungal contamination was observed in a few replicates, and this was no doubt due to the controlled room facilities in our laboratory. The leaf color of the explants stored in the dark faded from green to pale white green, with some brown and died leaves and shoots due to the degradation of chlorophyll pigments, prevention of pigment synthesis, and starch accumulation (Moges et al., 2003).

In conclusion, this study has shown that *C. spinosa* explant could be stored without serious losses on MS medium supplemented with different concentration of sucrose, sorbitol, mannitol or glucose at 24 $\pm$ 2°C, for up to 17 weeks. However, the results also demonstrated that the assessment of survival rates at the end of the storage period does not necessarily reflect the re-growth capacity of the material after conservation. In addition, conservation under light was found to be beneficial than dark. This appears to be the first report on *in vitro* conservation of *C. spinosa.* The procedures developed in this study can be used as the basis of Genebank conservation of this valuable species and can be repeated with some modification in other plant species.

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