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Full Length Research Paper

Propagation physiology of *Juniperus phoenicea* L. from Jordan using seeds and *in vitro* culture techniques: Baseline information for a conservation perspective

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Seeds of *Juniperus phoenicea* L. collected from Shouback city, south of Jordan, were cold-moist stratified at 5°C for 1 and 3 months. Non-stratified seeds were used as the control. The recalcitrant nature of *J. phoenicea* was clear as shown by low germinability of seeds. The highest germination percentage was recorded for seeds stratified for three months. Germination bioassay of wheat grains indicated that stratification is a prerequisite for the germination of *J. phoenicea* seeds and that reduction in inhibitors found in mature seeds occurred with longer stratification periods. In a separate set of experiments, *in vitro* culture of *J. phoenicea*, using shoot tips with axillary buds from young seedlings and mature seeds from adult trees as explants was attempted. Explants were established on Murashige and Skoog (MS), and Rugni Olive (OM) media. The effects of kinetin (kin), 6-benzyl amino purine (BAP) and thidiazuron (TDZ) on the differentiation of axillary buds were tested at 0.5 mg/L and compared with that using plane media without hormones. Mature seeds were completely contaminated one month after culture establishment. The grooves along the surface of the seeds may have increased the chance for latent microbes to grow with prolonged culture time. In contrast, shoot tips with axillary buds responded better than seeds *in vitro*.

Key words: Germination, *in vitro* culture, recalcitrance, seed morphology, thidiazuron (TDZ).

INTRODUCTION

The genus *Juniperus* consists of 67 species and 37 varieties. All the taxa grow in the northern hemispheres, except *J. procera*, which grows along the Rift Mountains in east Africa into the southern hemisphere (Adams, 2004). *J. phoenicea* is an evergreen monoecious or dioecious tree native to some regions of the Mediterranean basin from Portugal to Lebanon, Jordan and Western Saudi Arabia, and North Africa in Algiers, Morocco and Egypt, as well as the Canary Islands (Adams et al., 2002; Adams, 2004; El-Bana et al., 2010). *J. phoenicea* is a small tree, normally 6 to 8 m tall, or sometimes only a shrub with scaly leaves and brown to reddish 8 to 14 mm cones that usually contain on average five seeds (Adams, 2004; Mazur et al., 2003; Piotto et al., 2003). These plants grow in areas characterized by persistent drought and arid climate with high temperature ranges (El-Bana et al., 2010). A reproductive cycle of about 20 months, from cone differentiation to seed dispersal has been reported in *J. phoenicea* subsp. *phoenicea,* while in *J. phoenicea* subsp. *turbinata* it is two years (Arista et al., 1997). *J. phoenicea* occurs in the southern part of Jordan, usually at high altitudes, over 1000 m, on sandy rocks (Syouf and Duwayri, 1996; Al-Qura'n, 2005). *Juniperus* species are used for many purposes including landscaping, wood and medicinal purposes (Dale and Greenway, 1961; Lind and Morrison,

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Abbreviations: MS, Murashige and Skoog; **OM,** Rugni Olive media; **Kin,** kinetin; **BAP,** 6-benzyl amino purine; **TDZ,** thidiazuron.

1974; Loureiro et al., 2007). *J. phoenicea* is used largely in traditional medicine to treat diarrhea (Qnais et al., 2005). Leaves and berries of this plant are used to prepare mixtures that act as an oral hypoglycaemic agent (Amer et al., 1994). Oils isolated from leaves showed also inhibition effects against bacteria and fungi (Mazari et al., 2010).

Lack of pollination, low pollen viability and / or embryo degeneration are important causes for reduced seed production in *Juniperus* (Ortiz et al., 1998). Furthermore, it is reported for the genus *Juniperus* like *J. communis* and *J. sabina* L. that seed quality tends to decrease in drier and or colder regions (Garcia et al., 2000; Wesche et al., 2005) and only very small percentage of the collected cones contain viable seeds (Wesche et al., 2005). *J. phoenicea*, similar to other *Juniperus* species, does not have a high rate of plant production through seed germination (Ortiz et al., 1998). Moreover, it is not efficiently propagated by traditional methods and results are extremely inconsistent and not reproducible (Brito, 2000). Piotto et al. (2003) reported that no completely effective pre-treatments are known that will improve germination for *J. phoenicea* from Mediterranean countries. However, they suggested cold treatments of naked seeds at 3 or 4°C for 30 to 90 days. Moreover, some nurseries in Sardinia sow seeds of *J. phoenicea* in October and transplanting of the seedlings usually occurs the following March (Piotto et al., 2003). Stratification was also recommended at 3 to 5°C for 6 to 8 weeks to get good germination in seeds of *J. procera* depending on the collection site and seed lot (Tigabu et al., 2007).

Tissue culture had emerged as a tool to improve the propagation of many plants. Gomez and Segura (1995) reported the proliferation of *J. oxycedrus* by axillary shoots and later found some morphogenic capacity in calluses derived from single cell culture of the same species (Gomez and Segura, 1996). Harry et al. (1995), reported plantlet regeneration using embryogenic explants of *J. cedrus*. The induction of adventitious buds was achieved on excised cotyledon segments and embryo explants of *J. excelsa* (Negussie, 1997). Explants with axillary buds prepared from microcuttings of mature plants were used for the micropropagation of Portuguese *J. phoenicea* and *J. narvicularis* (Loureiro et al., 2007; Castro et al., 2011). The shoot branching process generally involves the formation of axillary meristems in leaf axils and the growth of axillary buds (Shimizo -Sato and Mori, 2001). The mechanisms of axillary bud growth depend on the ratio of the plant hormones auxin and cytokinin. Regulation of initiation and outgrowth of axillary meristems is important for controlling the overall plant growth and architecture (Shimizo-Sato and Mori, 2001).

Due to the prevailing drought conditions in the southern part of Jordan and the difficulty of *J. phoenicea* plant regeneration through seeds, this plant is considered as an endangered plant species. This is true as Jordanian *J. phoenicea* trees were noticed to dry in the last few years in its natural habitats. Furthermore, the over-exploitation of forest trees timber as heat-generating source in winter, in light of the increasing prices of oil, has contributed to the gradual decline of this plant species. To the best of our knowledge, *Juniperus* species have not been investigated in relation to their level of physiological dormancy. Furthermore, only one study on the *in vitro* regeneration of *J. phoenicea* using explants with axillary buds from mature trees has been reported (Loureiro et al., 2007). In Jordan, no reports were published on the propagation of *J. phoenicea*. This research, therefore, was undertaken to understand the propagation physiology of *J. phoenicea* in order to develop methods for mass production through seeds and vegetative means. For this purpose the following objectives were investigated: the effects of stratification on the germination of seeds collected from Shouback south of Jordan, physiology of seed germination and dormancy in *J. phoenicea* utilizing wheat grains germination bioassay, and the effects of hormones and media on the *in vitro* regeneration of *J. phoenicea* using mature seeds and axillary buds of young seedlings.

MATERIALS AND METHODS

Study area and cones collection

Mature reddish Cones of *J. phoenicea* were collected from its habitat (Al-Jhayer Mountains), approximately 1700 m above sea level, latitude 30° 31′ N, longitude 32° 35′ E, in Shouback region in the Southern part of Jordan. The soil of the seed collection site is silt loam with pH of 7.7 and EC of 0.47 (dS/m). The mean annual temperature, precipitation and relative humidity of shouback were 13.3°C, 176.5 mm and 59.9%, respectively (the data was obtained from the National Meteorological Department of Jordan). Cones were harvested from the periphery of infections- and insects- free trees based on visual inspection. Usually, cones were collected from the upper part of small to medium-sized trees. The height and diameter measured at breast height of *J. phoenicea* trees were variable and ranged from (2 to 10 m) and (30 to 80 cm), respectively. The cone and seed characteristics are given in Table 1. The cones served as the starting material for seed germination tests.

Seed extraction

Cones of *J. phoenicea* were soaked in distilled water for 24 h, which allowed for the softening of the hard pericarp of the fruits. The seeds were then manually extracted from cones and kept at room temperature for later experiments.

Morphological features of the seeds

The morphological features of seeds obtained from mature cones were observed and photographs were taken to identify seed coat morphology using Labomed 7GA9 microscope (USA, SN: 09010289) (Program Software::Progres Capture Pro 2.7.7).

Cold-moist stratification and nursery experiments

Seeds collected in 2008 were soaked in distilled water for 24 h.

Then un-imbibed or damaged seeds were excluded. Seeds were placed in perforated plastic bags filled with moistened perlite and were placed in a refrigerator at 5°C for 1 and 3 months. Non-stratified seeds served as the control. Bags containing seeds were checked periodically for moisture availability and were moistened with the fungicide Seed Guard at 1g/L. The seeds stratified for 1 and 3 months as well as non-stratified seeds (control) were allowed to germinate in plastic trays filled with peat and perlite at (2:1) ratio, respectively. The seeds were placed in the greenhouse in National Center for Agricultural Research and Extension (NCARE) in Baqa´a city North of Jordan. The trays were irrigated when needed with tap water and every two weeks with 1g/L benomyl fungicide. The germination process was monitored every two days for 100 days and considered as the emergence of new seedlings.

The following parameters were determined: germination capacity (GC) and mean germination time (MGT). GC is the proportion of total number of germinated seeds to that of sown seeds expressed in percentage. MGT was calculated as follows:

$$MGT\ (days) = \sum(tini)/\sum ni$$

Where, *ti* is the number of days starting from the date of sowing and *ni* is the number of seeds germinated at each day (Bewley and Black, 1994). The total number of seeds per treatment was 216 seeds in four replicates (54 seeds per replicate) and the experiment had a completely randomized design.

Biological determination of inhibitors to *J. phoenicea* seeds germination (wheat germination bioassay)

To determine the promotion or inhibition in germination of wheat grains by extracts obtained from seeds of *J. phoenicea* stratified for 1 and 3 months as well as extracts of non-stratified seeds. The promotion in grains germination acted as an indicator for the change in seed inhibitors of *J. phoenicea* in response to increased stratification period. This, in turn, could explain the germination behavior of seeds of *J. phoenicea* stratified for the assigned durations. This procedure was based on several reports utilizing wheat coleoptiles bioassay, barley endosperm bioassay and bioassay utilizing *Brassica camprestris* L. seeds (Al-Ramamneh, 1998; Boucaud and Ungar, 1973; Zhou and Bau, 2011)

Sensitivity of wheat grains to filtrates of *J. phoenicea* seeds

This aimed at determining extract concentration of *J. phoenicea* seeds that can induce minimum germination of wheat grains. Extracts of *J. phoenicea* from seeds collected in 2010 were prepared using a modified procedure of Saxena et al. (1996). Seeds were ground in a blender to give a fine powder. The dry tissue was extracted with distilled water to prepare concentrations of 100, 33 and 20 g/L. The seed powder solutions were homo-genized by stirring gently for 10 min. The extract was then filtered through Whatman no. 1 filter paper and the filtrate was collected. Wheat grains cv. Hourani were germinated on filter paper on 9-cm Petri dishes. Then 5 ml of each prepared extract (100, 33 and 20 g/L filtrates) was added to each Petri dish containing wheat grains. Distilled water was used as a positive control for the germination of wheat grains. The Petri dishes were kept at 23 to 25°C. Each treatment consisted of three replicates of 10 grains each in a completely randomized design (a total of 30 grains). At the end of a 5-day germination period, the following parameters were recorded for wheat grains: GC, coleoptiles length, number and length of seminal roots.

In vitro culture

Microcuttings from young seedlings of *J. phoenicea* (3-year old) obtained from the Forestry Department in Shouback and mature seeds from adult plants provided explants for tissue culture techniques.

Preparation and sterilization of the plant material

Cuttings from terminal branches of the young seedlings were first washed in tap water, and then treated with household detergent for five minutes. This was followed by washing cuttings with tap water to remove all the traces of the detergent. Cuttings were immersed in 2.5% active chlorine from sodium hypochlorite for 15 min and then washed three times with sterilized distilled water. Cuttings were then divided to provide explants that consisted of shoot tips 1.5 cm in length (containing three axillary buds). The mature seeds were sterilized using the same procedure employed for the cuttings.

Influence of medium composition and growth regulators

The resulting explants were cultured in tubes (25 mm × 150 mm) on approximately 20 ml of either MS (Murashige and Skoog, 1962) or OM (Rugini, 1984) media supplemented with kinetin, thidiazuron (TDZ) and 6-benzyl amino purine (BAP) at 0.0 or 0.5 mg/L. All cultures were incubated in growth chamber at 25±2°C, with a 16-h photoperiod and illuminated by 40 W (watts) white fluorescent lights. The experiments were arranged in a completely randomized design with ten replicates. Each replicate was the average readings for three explants (total 30 explants for each treatment).

Shoot survival and morphological characters as number of shoots per explant, shoot length, number of branches per explant and branch length were evaluated three weeks after culture establishment. *In vitro* cultures lasted for two months to collect more observations. The formation of callus on explants was also evaluated.

Statistical analysis

All the described experiments were repeated twice. The data were analyzed with a general linear model using the Statistical Analysis System (SAS, 2001) and least significant difference (LSD) test at 5% probability level was applied to compare the treatment means.

RESULTS

Morphological features of the seeds

J. phoenicea trees produce small cones (mean cone weight equals 0.25 g), each containing 5 small seeds (Table 1). The cones are green-colored and become reddish when mature. The shape of seeds in *J. phoenicea* is ovoid. However, seeds tend sometimes to be tapered at both ends. They are wrinkled and charac-terized by the presence of protrusions and furrow-like grooves along their surface (Figure 1A to C).

Seed germination in response to cold-moist stratification

Stratification had a significant effect ($P<0.01$) on GC and MGT for seeds collected from Shouback region (Table 2). Seeds stratified for three months had significantly higher

Table 1. Morphological characteristics of cones and seeds of *J. phoenicea* collected from Al-Jhayer in Shouback south of Jordan.

Cone and seed characters	Overall mean
No. of seeds / cone	4.65 ± 0.24[a]
Cone weight (g)	0.25 ± 0.01[b]
Seed size (mm × mm × mm)	(5.03 ± 0.17) × (3.18 ± 0.13) × (2.58 ± 0.08)[c]
1000 seed weight (g)	16.27 ± 0.25[d]

The values are the mean ± standard error of [a](n:20), [b](n:30), [c](n:20) and [d](n:3).

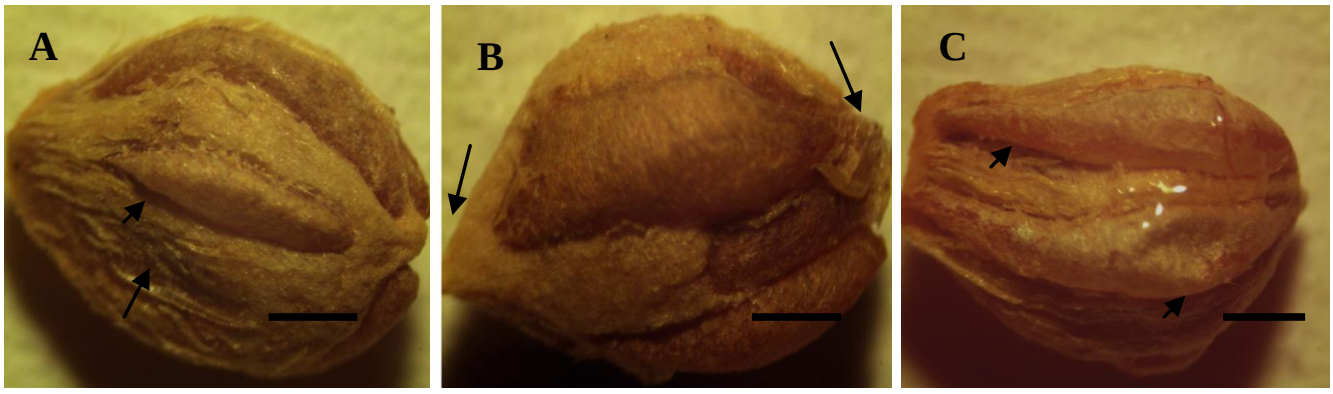

Figure 1. Morphology of *J. phoenicea* seeds (A) Ovoid seed shape, wrinkled surface of the seed (arrow) and furrow-like grooves (arrowhead). (B) Tapered ends of the seed (arrows). (C) furrow-like grooves (arrowheads). Scale bars: 1 mm.

Table 2. Germination capacity (GC) and mean germination time (MGT) of *J. phoenicea* seeds from Shouback stratified at 5°C for 0, 1 and 3 months.

Parameter	GC	MGT
Control	0.0±0.0[c]	0.0±0.0[b]
5°C × 1 month	3.2±0.8[b]	74.9±2.1[a]
5°C × 3 month	9.3±1.3[a]	73.4±3.6[a]
LSD (*P*=0.05)	2.9	7.7

Control: Non-stratified seeds of *J. phoenicea*. Means in each column followed by the same letter are not significantly different at *P<0.05.*

GC (9.3%) than seeds stratified for one month (3.2%). However, MGT was on the same level of significance for seeds stratified for one and three months (74.9 and 73.4, respectively). Meanwhile, the non-stratified seeds failed to germinate.

Sensitivity of wheat grains to filtrates of *J. phoenicea* seeds

The germination of wheat grains was highly sensitive to increase in filtrate concentration of *J. phoenicea* seeds (*P<0.01*) (Table 3). The filtrate concentration of 100 g/L resulted significantly in the least germination response of wheat grains (GC=23%). This was also coupled with the least average length (0.5 cm) of the seminal roots of the germinated grains. Wheat grains germinated in distilled water free of *J .phoenicea* filtrate recorded significantly the highest GC (77%), coleoptiles length (2.1 cm), mean number and length of seminal roots (4.1 seminal roots and 2.7 cm, respectively). However, data from further experiments indicated that 110 g/L filtrate of *J. phoenicea* seeds resulted in 10 % GC of wheat grains (Table 4) and therefore, was used as the concentration of *J. phoenicea* filtrate required to induce the minimum accepted germination of wheat seeds.

Biological determination of inhibitors to germination of *J. phoenicea* seeds

It is shown in Table 4 that germination of wheat grains was significantly stimulated when moistened with seed-filtrates of *J. phoenicea* that received longer stratification periods. In this sense, germination percentages of 10, 53 and 77 were obtained when wheat grains were moistened with filtrates of *J. phoenicea* seeds that were non-stratified, stratified for 1 month and those stratified for 3 months in that order, respectively. Moreover, germination efficiency of wheat seeds as indicated by coleoptile length, number and length of seminal roots was also enhanced for wheat grains that were treated with seed-

Table 3. Effects of seed filtrate of *J. phoenicea* on the germination of wheat grains cv. Hourani.

Filtrate concentration (g/L)	GC (%)	Coleoptile length (cm)	Number of seminal root	Length of seminal root (cm)
0	77[a]	2.1[a]	4.1[a]	2.7[a]
20	55[b]	0.8[b]	3.2[ab]	1.8[b]
33	57[b]	0.4[b]	2.5[bc]	1.5[b]
100	23[c]	0.8[b]	1.7[c]	0.5[c]
LSD (*P*=0.05)	14.1	0.6	1.4	0.7

0: Distilled water used as positive control. Means in each column followed by the same letter are not significantly different at *P<0.05.*

Table 4. Effects of seed filtrates (110 g/L) of *J. phoenicea* stratified for 0, 1 and 3 months on the germination of wheat grains cv. Hourani.

Stratification period	GC	Coleoptile length (cm)	Number of seminal roots	Length of seminal roots (cm)
Control	10.0[c]	0.9[b]	0.6[b]	0.1[b]
5°C × 1 month	53.3[b]	1.1[b]	2.3[ab]	0.5[ab]
5°C × 3 month	76.7[a]	2.6[a]	4.0[a]	0.7[a]
LSD (*P*=0.05)	14.8	1.3	2.2	0.5

Control: Non-stratified seeds of *J. phoenicea*. Means in each column followed by the same letter are not significantly different at *P<0.05*.

Table 5. Effect of basal media and growth regulators on the growth parameters of *J. phoenicea* three weeks after culture establishment.

Medium	Growth regulator (mg/L)	Number of shoots per explant	Shoot length (cm)	Number of branches per explant	Branch length (cm)	Callus [a]
MS	0.0	0[e]	0[d]	0[d]	0[c]	-
	0.5 KIN	2.6[cd]	1.3[c]	3.6[b]	1.8[b]	+
	0.5 BAP	0[e]	0[d]	0[d]	0[c]	-
	0.5 TDZ	2.9[c]	1.5[c]	4.4[b]	2.2[b]	+
OM	0.0	4.6[b]	2.1[b]	2.4[bc]	1.4[b]	-
	0.5 KIN	5.3[b]	2.6[ab]	7.0[a]	3.3[a]	+
	0.5 BAP	1.6[d]	0.9[c]	0.7[cd]	0.3[c]	-
	0.5 TDZ	6.6[a]	3[a]	8.3[a]	3.6[a]	+
LSD (*P*=0.05)		1.2	0.6	2.1	1.0	

Explants consisted of shoot tips 1.5±0.2 cm with three axillary buds. [a]Qualitative characterization of callus production: -, absence; +, presence. Means in each column followed by the same letter are not significantly

filtrates of *J. phoenicea* that were stratified for up to three months (Table 4).

In vitro culture - Mature seed explants

Mature seed explants showed microbial contamination few days after culture irrespective of media and hormones used. The contamination developed gradually to cover all cultures one month after culture establishment. Contamination varied from creamy to green areas

In vitro culture - Microcutting explants

Microcuttings grown on MS medium without hormones or with 0.5 mg/L BAP failed to show any morphogenic response. These explants showed progressively necrotic areas and were browned at the end of the culture. Explants growth on OM medium without hormones were significantly better than explants grown on the same medium containing 0.5 mg/L BAP (Table 5). The best response was for explants that were grown on OM medium supplemented with 0.5 mg/L TDZ (Table 5). This medium-hormone combination stimulated axillary bud differentiation and thus significantly produced the highest number of shoots per explant (6.6). However, shoot length, number of secondary branches per explant and secondary branch length were on the same level of significance for explants grown in the presence of 0.5 mg/L TDZ or 0.5 mg/L kinetin in both media with

significantly higher response in OM compared to MS medium. Shoots and secondary branches developed in the presence of 0.5 mg/L TDZ were thick, swelled and dark green compared to shoots formed in media containing 0.5 mg/L Kinetin.

However, irrespective of the media and hormones used, it was necessary to perform sub-culturing after three to four weeks from start of the experiment. After this, shoots began browning starting from the base of the explants which gradually spread to the whole shoots and secondary branches. Prolong exposure of shoots to TDZ (2 months) caused callusing of shoots (data not shown). Callus production in the present study formed in OM and MS media when containing 0.5 mg/L Kin or TDZ.

DISCUSSION

Seed germination in response to cold-moist stratification

Stratification of seeds in a cold-moist medium at 5°C is recommended to overcome dormancy in many plant species, especially those from temperate regions (Baskin and Baskin, 2004). In the present study, stratification for one and three months significantly raised the GC of *J. phoenicea* seeds collected from Shouback region compared to non-treated seeds. However, the GC was poor and did not exceed 9.3% for seeds that was stratified for three months. In agreement with the results of the present study, seeds of *J. procera* collected from five provenances in Ethiopia have shown significant differences in seed germinability in response to stratification at 5°C (Tigabu et al., 2007). Furthermore, seeds of *J. procera* collected from Gra-Kassu, Wolf-Washa and Arib-Gebeya provenances in Ethiopia germinated poorly (< 8%) (Tigabu et al., 2007) which is similar to the germination response of *J. phoenicea* seeds collected from shouback region and cold-stratified for up to three months. However, Tigabu et al. (2007) recorded a GC between 60 and 80% for seeds stratified for 6 weeks and collected from Hirna and Yabelo provenances characterized by high temperature. High temperature that prevails during seed maturation can induce an after-ripening effect, which in turn enhances the growth of the embryo necessary for seed germination (Tigabu et al., 2007). The collection site (Al-Jhayer) for *J. phoenicea* seeds in this study is characterized by the decrease in temperature during seed maturation (from October to December), which induces dormancy and poor germination response in seeds when they mature.

Physiology of *J. phoenicea* seed germination

Grains of wheat germinated poorly when moistened with filtrate of non-stratified *J. phoenicea* seeds. However, germination of wheat grains was enhanced when grains were moistened with filtrate obtained from *J. phoenicea* seeds stratified for one month and was significantly the highest (GC: 77%) for grains that were treated with filtrate of *J. phoenicea* seeds stratified for up to three months. These results indicate clearly that mature seeds of *J. phoenicea* contained high amounts of inhibitors and that stratification was associated with a reduction in the inhibitors found in the mature seeds. Consistence with this, dormancy release of seeds in many plants involves the decrease in sensitivity of seeds to inhibitors involved in the signal transduction pathway controlling germination (Chien et al., 1998; Goggin et al., 2009; Koornneef et al., 2002; Nonogaki et al., 2010). These results highlighted the fact that stratification is a prerequisite for the successful germination of *J. phoenicea* seeds. This is understood by the gradual significant increase in the germination of *J. phoenicea* seeds that received stratification for one and three months in that order.

The length of the chilling moist treatment necessary to break seed dormancy varies among different plant species. The present study indicated that mature seeds of *J. phoenicea* possess deep physiological dormancy as indicated by slow germination (MGT; 73.4) and poor GC (9.3%) of *J. phoenicea* seeds that were stratified for up to three months. This, when compared to the enhanced germination of wheat grains that were moistened with filtrate of *J. phoenicea* seeds stratified for up to three month, indicated that the effect of changes in inhibitors in repressing/stimulating germination of *J. phoenicea* seeds is probably modulated by the inhibitor/promoter ratio alongside other minor factors, in response to stratification (Piotto et al., 2003). The role of promoters, in particular gibberellins (GA), in breaking seed dormancy is well documented in many plant species (Dissanayake et al., 2010; Fang et al., 2006; Güleryüz et al., 2011; Nadjafi et al., 2006; Penfield et al., 2005; Goggin et al., 2011).

This principle of inhibitors/promoters ratio was supported in earlier studies by barley endosperm bioassay and wheat coleoptile bioassay that supported the presence of an inhibitor activity and the absence of gibberellin-like activity in dormant non-treated varieties of the genus *Suaeda* (Boucaud and Ungar, 1973). Al-Ramamneh (1998) reported an increase in the germination of *Tilia argentea* seeds with cold stratification for up to four months, and found by utilizing wheat germination bioassay, a significant decrease in free ABA with stratification of *T. argentea* seeds for up to four months. Moreover, Piotto et al. (2003), pointed out that after breaking dormancy of *J. phoenicea* seeds, germination may be encouraged by the temperature of about +15 °C, while higher temperatures (+20/25 °C) do not seem optimal. In this respect, the germination temperature that prevailed throughout the present study (22±2) may not be optimal to induce the required increase in seed germination of *J. phoenicea*. The recalcitrant nature of seeds of *J. phoenicea* as indicated in this study could

result from the adaptation to the particular environment where these trees grow in Al-Jhayer Mountains (approximately 1700 m above Sea level) with both cold winters and summers.

In vitro culture

The contamination noticed in mature seed cultures involved, although not identified, bacterial and fungal contaminant. The failure to establish seed cultures *in vitro* in the present study indicates that sterilization techniques employed needs fine tuning. The morphological features of seeds in *J. phoenicea* seem to play a role in the contamination of the established cultures. The grooves along the surface of seeds reduced the effectiveness of surface sterilization and thus latent microbes can have the chance to develop with prolonged culture time. This is further understood in light of the fact that *Juniperus* species can be attacked by pest insects (García, 1998; Tigabu et al., 2007) and pest-damaged seeds, in turn, may harbor infective pathogens (El Atta, 1993). The results of the present study showed that MS medium without hormones was not suitable to establish axillary bud multiplication *in vitro* using shoot tips as explants from young seedling of *J. phoenicea*. For the two media tested in this investigation, growing explants on OM medium resulted in significantly highest response in terms of shoot number per explants, shoot length, number of secondary branches and average length of secondary branches. Loureiro et al. (2007) showed the favorable response of OM medium compared to MS medium on explants from microcuttings collected from old trees of *J. phoenicea* in Portugal.

The browning of explants that extends to shoots and secondary branches made it necessary to do subculturing after three to four weeks from the start of the experiment in the present study. Adverse conditions were also reported for the *in vitro* cultures of *J. excelsa* (Negussie, 1997). Original explants, undifferentiated adventitious domes and well differentiated buds / shoots of *J. excelsa* showed gradual yellowing and subsequent death. This condition might be due to physiological and / or physical causes (Negussie, 1997). Axillary bud differentiation on explants in the present study was favored in the presence of TDZ and kinetin in the media. When BAP was included in OM media, it was noticed that number of shoots, number of secondary branches and shoot elongation were significantly less than in the presence of either TDZ or kinetin. This led us to conclude that the concentration of BAP (0.5 mg/L) used in the present study was too high and toxic to the explants especially when using MS medium. BAP at 0.09 mg/L produced a better response for the *in vitro* culture of *J. phoenicea* and *J. navicularis* on explants from adult trees (Castro et al., 2011; Loureiro et al., 2007).

TDZ effects appeared to be promising on the stimulation of axillary bud differentiation using microcuttings from young seedling of *J. phoenicea*. Although how TDZ functions as a cytokinin is not known, improved regeneration using TDZ rather than cytokinins like BA is well documented in many plants (Canli and Tian, 2008; Huetteman and Preece, 1993; Wojtania et al., 2004). Similar to the results of the present study, TDZ was also reported to induce thickened and swelled tissues in many plants (Mundhara and Rashid, 2006; Singh et al., 2003).

Conclusion

The results of the present study provide evidence that seeds of *J. phoenicea* have deep physiological dormancy. In this sense, cold-moist stratification of mature seeds for up to three months was not sufficient to produce efficient germination response. Therefore, further studies should be carried out to investigate the effects of longer stratification periods on germination of *J. phoenicea* seeds. These studies should also investigate the effects of using various temperature regimes throughout the germination process on subsequent GC.

However, studies concerning treatment of seeds with plant growth promoters or other stimulants of seed germination should be of high priority to replace longer stratification periods. *In vitro* culture techniques provide a great potential for the conservation of *J. phoenicea*. The use of OM medium combined with TDZ was promising for the multiplication and elongation of shoots using shoot tips containing few axillary buds from young seedlings as explants. And in conclusion, the surface-sterilization of mature seeds of *J. phoenicea* should be further optimized to reduce contamination.

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