# Improved Seed Handling Techniques for *Juniperus polycarpos*

## Implications for Active Restoration of Degraded Juniper Forests in Iran

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Cover: A typical *Juniperus polycarpos* natural stand in Chaharbagh, NE Iran (Photo: Abolfazl Daneshvar)

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### Abstract

*Juniperus polycarpos* (K. Koch) is one of six native juniper forest tree species in Iran whose population size is declining continuously. The ultimate aim of the research presented in this thesis was to increase the regeneration potential of *J. polycarpos* by identifying factors influencing seed quality and develop methods that increase germination capacity and vigor. The research focused on impact of infection by dwarf mistletoe on reproductive output, methods for sorting unproductive seeds from seed lots, improving germination capacity and vigor through sorting techniques and by breaking seed dormancy. The results show that moderate level of infection significantly ($p < 0.05$) reduced mean number of cones per unit area of the host crown, increasing the number of damaged seeds, reducing seed size and germination capacity. For sorting viable seeds from non-viable seeds, three methods were applied: Incubation, Drying and Separation (IDS), modified specific gravity (SG) separation and near infrared spectroscopy (NIRS). For the IDS experiments, seven days incubation followed by nine hours of drying and sedimentation in pure water or 200 g sucrose /l water solution resulted in 75% and 82% sunken-viable seeds, respectively. For seeds soaked for 48 hours, SG separation in 600 g/l sucrose solution also resulted in 77% sunken-viable seeds. For both sorting techniques, only 4% of viable seeds were lost into the discarded floating fraction. NIR spectroscopy of individual seeds discriminated between viable and non-viable seeds of *J. polycarpos* with 98% and 100% accuracy, respectively based on spectral differences attributed to seed coat chemical composition and storage reserves. For finding optimal dormancy breaking treatment and subsequent stimulation of germination capacity and vigor of *J. polycarpos* seeds, different pretreatments were tested. The results showed warm stratification for sixteen weeks followed by twelve weeks of cold stratification resulted in 72% germination after 12 days compared with 16-week cold-stratification alone (42%) and the control (8%). Exogenous application of phytohormones alone or in combination with cold stratification resulted in less than 50% germination, though significantly ($p < 0.01$) higher than the control (8%). As a whole, the results have strong implication for active restoration by increasing seedling production potential in nurseries.



*Keywords: dormancy, IDS, NIRS, seed sorting, stratification, seed quality, seed vigor*



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# Dedication

To my parents
To my wife, Marziye
To my daughters: Monireh and Maedeh
&
To my new-born son: Hossein

*Scholars are the torches of the earth, the prophet's representatives, and heirs to me and the other prophets.*

Prophet Mohammad Peace be Upon Him

# Contents

# List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Daneshvar, A., Tigabu, M., Karimidoost, A., Farhadi, M., Oden, P.C. (2014). Growth characteristics and reproductive output of dwarf mistletoe-infected *J. polycarpos* in Iran. *Journal of Forestry Research* 25(4), 827-834.

II Daneshvar, A., Tigabu, M., Karimidoost, A., Oden, P.C. Improved sorting system for upgrading the quality of *J. polycarpos* seed lots. (Submitted manuscript).

III Daneshvar, A., Tigabu, M., Karimidoost, A., Oden, P.C. Single seed NIR spectroscopy discriminates viable and non-viable seeds of *J. polycarpos*. *Silva Fennica.* 49 no. 5, article id 1334. 14 p.

IV Daneshvar, A., Tigabu, M., Karimidoost, A., Oden, P.C. Stimulation of germination in dormant seeds of *J. polycarpos* by stratification and hormone treatments (Submitted manuscript).

Papers I and III is reproduced with the permission of the publishers.

The contribution of Abolfazl Daneshvar to the papers included in this thesis was as follows:

I I developed the research idea with Mulualem Tigabu. I collected data with the help of Assadollah Karimidoost. I did the statistical analysis and was the main responsible for writing the paper which was critically revised by co-authors. Overall input of my contribution during the complete study was ca. 80%

II Mulualem Tigabu and I planned the experiment and I collected the data. I did the statistical analysis and was the main responsible for writing the paper which was critically revised by co-authors. Overall input of my contribution during the complete study was ca. 80%

III Mulualem Tigabu and I planned the research idea. I collected data and analyzed it with the help of Mulualem Tigabu. I was main responsible for writing the paper which was critically revised by co-authors. Overall input of my contribution during the complete study was ca. 70%

IV I developed the research idea and planned the experimental design together with Mulualem Tigabu. I did the lab works. I carried out the calculations and statistical analysis. I was the main responsible for writing the paper which was critically revised by co-authors. Overall input of my contribution during the complete study was ca. 80%

# Abbreviations

| | |
|---|---|
| BAP | Benzyl Amino Purine |
| DMR | Dwarf Mistletoe Rating |
| GA | Gibberellic Acid |
| GC | Germination Capacity |
| IDS | Incubation – Drying – separation |
| ISTA | International Seed Testing Association |
| MGT | Mean Germination Time |
| NIRS | Near Infra-Red Spectroscopy |
| OPLS-DA | Orthogonal Projection to Latent Structures-Discriminant Analysis |
| PD | Physiological Dormancy |
| PLS-DA | Partial Least Squares – Discriminant Analysis |
| SG | Specific Gravity |
| TTC | Triphenyl-Tetrazolium Chloride |

# 1 Introduction

## 1.1 Overview of Juniper forest

The *Juniperus* genus (Cupressaceae family) comprises valuable evergreen species highly tolerant to harsh climatic- and environmental conditions in arid and semi-arid forests (Ciesla, 2002; Olano *et al*., 2008; Ahani *et al*., 2013). The genus has worldwide geographical distribution among forest tree species (Tylkowski, 2009), and can be found throughout the Northern hemisphere, except for *J. procera* native to Africa and the Southern hemisphere (Farjon, 1998, 2005; Gonny *et al*., 2006; Adams, 2014).

Members of this genus range from low prostrate shrubs to well-recognized trees including several subspecies (Farjon, 2005; Mao *et al*., 2010; Yashwant and Colin, 2012). Regarding the number of species in this genus, so far different numbers have been claimed, from 54 (Ciesla, 2002), 67 (Farjon, 2005), 68 (Adams, 2004; Gonny *et al*., 2006), to 75 (Adams, 2014). The cause of this discrepancy is mainly due to difference in classification as a main species or subspecies.

The wood of Juniper species is suitable for making a variety of products. It is aromatic and highly decay resistant and can be used as construction timber and for furniture, paneling and fence posts (Wilhite, 1990). In addition, the juniper species are sources of various non-wood products. Notably juniper berries are sources of essential oils that are used as flavor and spice (Bajaj, 1986) and in pharmaceutical preparations as microbe antagonist in the treatment of infectious-, fungal- and contagious diseases such as colds and bronchitis (Unlu *et al*., 2008). It has been also used as a natural remedy for haemorrhoids, gynaecological diseases, wounds, tumors and for stabilizing blood glucose (Akkol *et al*., 2009, Orhan *et al*., 2011).

Furthermore, Podder and Lederer (1982) reported that *J. occidentalis* berries contain 46% carbohydrates, 16% lipids, and 4% proteins, enough to feed domestic birds and mammals during winter. Juniper species are highly appreciated as windbreaks and shelter. They play a crucial role to control soil erosion, especially in the highlands and slopes of their natural habitats (Lawson, 1990; Ciesla *et al.*, 1998; Korouri *et al.*, 2012).

At present, due to human interference resulting in degradation of their natural sites, in combination with slow growth habit and poor natural regeneration, most juniper forest around the world are heavily degraded (Ciesla, 2002; Korouri *et al*., 2012; Ahani *et al*., 2013). In addition, junipers generally have long reproductive cycles, produces seeds of low quality with deep dormancy adding to the concern for their future restoration (Garcia *et al*., 1999; Korouri *et al*., 2012). Therefore, juniper forests are in the need of well concerted management programs to facilitate regeneration and restoration of pure and mixed stands.

## 1.2 Juniper forests in Iran

Iran is located in South Western Asia in the dry part of the Northern hemisphere and is characterized as a low forest area (Marvie Mohajer, 2006). The presence of high mountain chains in Alborz (800 km length) in the North and Zagros (1500 km length) in the Western part of the country creates diverse climatic conditions for a special vegetation composition in the region (Sagheb Talebi *et al.*, 2014). Iran has ca. 13 million ha of forests as compared to Turkey with ca. 21 million ha forest (Kleine *et al.*, 2009). Forest management programs in Iran were initiated in 1963 when the forests were nationalized. Prior to that, all forests were under extreme pressure and seriously degraded by human and other interference (Soltani, 2003).

Despite the diverse flora of deciduous tree species in Iran, only a few conifers are naturally present (Sagheb Talebi *et al.*, 2014). The Juniper genus is represented by six native species including *Juniperus excelsa* M. Bieb., *J. foetidissima* Willd., *J. communis* L., *J. oxycedrus* L., *J. sabina* L. and *J. polycarpos* K. Koch. Among these juniper species, *J. polycarpos* and *J. excelsa* have the highest frequency and the largest geographical distribution (Figure 1) in the highlands of the country (Djavanshir, 1974; Assadi, 1997; Kharazipour *et al.*, 2008; Korouri *et al.*, 2012).

Throughout its natural range, the juniper trees have remained an integral part of the culture landscape of Iranian natives (Pirani *et al.*, 2011). Juniper forests are considered important for maintaining soil fertility and preventing erosion. Various parts of the juniper trees are used for medicine, food, incense, constructing timber, fencing posts, firewood, household articles and decoration. Some ethnic tribes respect juniper as a sacred tree. Juniper forests and their degradation are consequently of great social concern. Their outstanding resistance to both drought and frost, their high potential for protection against soil erosion in the mountains, their use as food and habitat for mammals and birds are main issues that trigger interest in restoration of *J. polycarpos* among managers and researchers in Iran (Djavanshir, 1974; Kroroui *et al.*, 2012; Ahani *et al.*, 2013).

*J. polycarpos*, locally known under a range of names including many-fruited-, Eastern-, mountain- and water-reaching juniper, is one of the most important conifers in the Alborz mountains across Northern Iran (Djavanshir 1974; Korouri *et al.*, 2012; Sagheb Talebi *et al.*, 2014) and in mountain stretches in Western and South-Western Iran (Korouri *et al.*, 2012; Adams, 2014). Its natural distribution range around the world also includes adjacent parts of Turkmenistan in the East, essentially all of Azerbaijan and adjacent parts of Turkey, Armenia, Georgia and Russia. In some areas, it occurs sympatric with *J. excelsa*, *J. communis*, *J. foetidissima*, *J. seravschanica* and/or *J. sabina*.

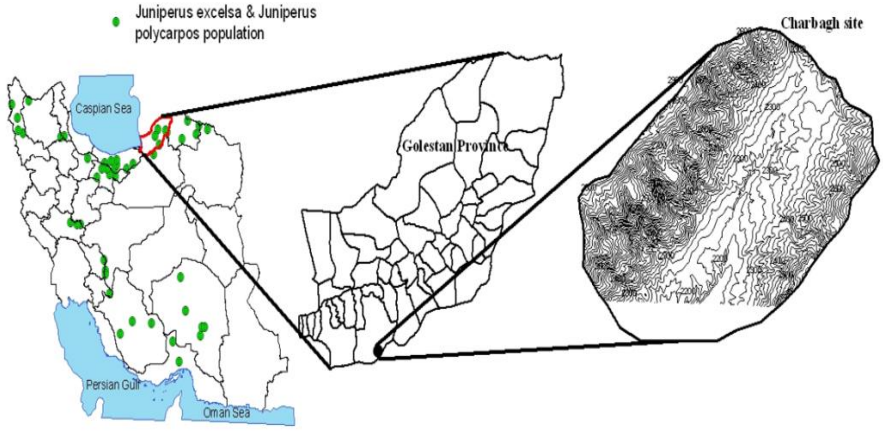


*Figure 1.* Geographic distribution of *J. polycarpos* and *J. excelsa* and location of the study area, Charbagh, Golestan province, Iran.

Until recently *J. polycarpos* was classified as a sub-species of *J. excelsa*, which is closely related, but confirmed to be genetically and morphologically distinct (Franco, 1964; Farjon, 2005; Adams, 2014). However, Djavanshir (1981) and Korouri *et al.*, (2012) claimed that *J. polycarpos* is dioecious ('male' and 'female' reproductive structures borne on separate plants) and *J. excelsa* is monoecious (reproductive structures of both sexes borne on a single plant) that can easily be recognized in the field. Korouri *et al.*, (2012) further emphasized that the two mentioned species are seen side by side in Iran, with varying proportions in different stands. In the Chaharbagh stand (our study place), only 1.58% of the trees are *J. excelsa*, which is the lowest proportion compared to other natural geographical distribution range of the species in the country.

In fact, *J. polycarpos* and *J. excelsa* are the two most important species in the highland natural forest ecosystems after pistachio (*Pistacia vera* L.) with wide geographical range and altitudinal distribution among the Iranian native tree species (Korouri *et al*., 2012). They occur in the ecoregion with semi-arid climate with cold winters and low annually precipitation (Kharazipour *et al.*, 2008) where other species cannot compete with them (Ahani *et al.*, 2013).

Despite the wide range of ecosystem services provided by juniper forests in Iran, natural forests of *J. polycarpos* (the most abundant juniper species in Iran) are under heavy human pressure and at present mainly characterized as open woodlands of scattered trees and sometimes mixed with other junipers (Adams, 2014; Djavanshir, 1974; Korouri *et al.*, 2012; Sagheb-Talebi *et al.*, 2014).

*Figure 2.* Instance of illegal cutting of *J. polycarpos* trees by local people for their consumptions.

*Figure 3.* A typical big-size *J. polycarpos* tree that was newly scorched by local people.

The open degraded forests result mainly from the combination of slow growth, a serious lack of regeneration due to poor seed quality and an excessive use by man that include irrational harvesting (Figure 2), anthropogenic wildfires (Figure 3) and grazing/browsing (Figure 4). These factors are also responsible for a substantial reduction in the area stocked with *J. polycarpos*, from around 1.2 million ha in the early 1970s (Djavanshir, 1974) to approximately 0.5 million ha by the early 2000s (Kharazipour *et al.*, 2008).

*Figure 4.* Evidence of grazing and browsing disturbance on the quality of *J. polycarpos* stand in Chaharbagh.

Although commercial harvesting of *J. polycarpos* in Iran has been prohibited since 1989, the population size of juniper forests continues to decline. At present, there is a growing interest in conserving and restoring degraded juniper forests for economic and ecological purposes (Okasaka *et al*., 2006; Akkol *et al*., 2009; Korouri *et al*., 2012). The current conservation and restoration practice in Iran is prohibition of commercial harvesting and establishment of protected areas to reduce anthropogenic disturbances and encourage natural regeneration. However, passive restoration, which relies on natural regeneration alone has been insufficient due to the long reproductive cycle, irregular flowering (seed mast year every 4-8 years) and poor seed quality of *J. polycarpos* (Ahani *et al*., 2013).

## 1.3 Regeneration barriers of *J. polycarpos* in Iran

Many factors have been presented by researchers as regeneration barriers for *J. polycarpos* (Djavanshir, 1974; Korouri *et al*., 2012; Ahani *et al*., 2013). This thesis focus on issues related to seed production and seed quality.

### 1.3.1 Dwarf mistletoe

Dwarf mistletoe is a parasitic flowering plant that infects conifers, producing characteristic yellow to orange or green to brown leafless aerial shoots on the host plant. Recently, forest managers are arguing that infections of juniper forests by dwarf mistletoe (Figure 5) may influence tree vigor and reproductive output, thereby contributing to insufficient natural regeneration of juniper forests in Iran (Korouri *et al*., 2012).

The dwarf mistletoe associated with juniper (hereafter referred to as juniper dwarf mistletoe) is *Arceuthobium oxycedri* (DC.) M. Bieb, (Viscaceae), which has a wider geographic distribution ranging from North Africa, Southern Europe to Eastern and Central and Western Asia as far as near East and Western China (Hawksworth and Weins, 1996; Sarangzai *et al*., 2010).

*Figure 5*. Dwarf mistletoe infection on *Juniperus polycarpos* trees in Chaharbagh stand.

Dwarf mistletoe depends on their host tree for supply of nutrients and water. Infections cause branch swelling, reduced growth, dieback and abnormal proliferation of host branches known as "witches' brooms" (Figure 6), which varies with infection severity and vigor of the host. Generally, severe infection is associated with growth loss and mortality (Geils and Hawksworth, 2002; Stanton, 2006; Scott and Mathiasen, 2012), alteration of physiological processes of the hosts (Glatzel and Geils, 2009) and predisposition of host trees to other pests (Kenaley *et al*., 2006) and climate factors (Stanton, 2007).

*Figure 6*. Witches brooms on the *Juniperus polycarpos* trees in Chaharbagh stand.

Infection by dwarf mistletoe may also influence host reproduction through cone production, seed quantity and quality, and seedling survival (Geils and

Hawksworth, 2002). Previous studies, although few, showed that some conifer species infected by dwarf mistletoe produce fewer cones, fewer seeds, lighter seeds and lower germination capacity (Singh, 1981; Singh and Carew, 1989; Schaffer *et al*., 1983; Sproule, 1996). However, Lamien *et al*. (2006) could not see an effect on flowering and fruiting behavior of mistletoe-infected Shea trees (*Vitellaria paradoxa* CF Gaertn.). Despite extensive studies on dwarf mistletoe and host interactions elsewhere, so far no detail studies have been carried out regarding the extent of damage incurred by dwarf mistletoe on *J. polycarpos*. Such information is useful for forest managers relying on natural regeneration to mitigate damages associated with infection of dwarf mistletoe in juniper forest. In addition, as Djavanshir (1974) emphasized, mature seeds of *J. polycarpos* can be infested by *Megastigmus*-insects thereby seriously decreasing seed quality. Kapuscinski (1946) and Bobinski (1976) reported the same problem for *J. communis*.

### 1.3.2 Seed quality (viability)

Seed viability is the capacity of seeds to germinate and produce a normal seedling under suitable conditions (Copeland & McDonald, 2001). Seed viability is an important variable for determining the number of seeds needed to create the desired seedling density (Tauer, 2010). Seed lot quality is a measure of characters that will determine the performance of the seeds when sown or stored (Hampton, 2002), which often encompasses technical, physiological and genetic qualities that affect seed viability and vigor (Basu, 1995). The main causes of poor seed lot quality are pollination failure and post-zygotic degeneration, insect infestation, infection by seed borne pathogens and parasitic plants (Djavanshir, 1974; Slobodník and Guttenberger, 2000; Sivakumar *et al*., 2007; Ahani *et al*., 2013; Daneshvar *et al*., 2014).

The germination performance of *J. polycarpos* seed lots is generally reported to be very low; ca. 12% if seeds are collected at the right time (Khoshnevis *et al*., 2008). The reasons for poor germination are believed to be poor seed lot quality and dormancy (Ahani *et al*., 2013). Seed lots of *J. polycarpos* are often composed of large quantity of empty seeds due to unproductive (sterile) pollen, unproductive (sterile) ovule, asynchronous male and female maturation and undesirable climate condition for fertilization (Djavanshir, 1974). Under natural condition, male and female strobili mature at different time of the year; i.e., male strobili matures too late to fertilize the female strobili which is receptive for only 10 to 12 days before the cone scales start to close (Djavanshir, 1974). Cones of *J. polycarpos* take 16 to 18 months

to mature while other Iranian juniper species need shorter time (Korouri *et al*., 2012).

The low proportion of viable seeds in *J. polycarpos* cones is a major problem for natural regeneration as well as for the production of seedlings in the nursery. Previously it was suggested to remediate the problem through artificial pollination followed by planting of seedlings raised in nurseries (Djavanshir, 1974). However, this was never implemented to an extent of any practical significance. Vegetative propagation is another alternative for the production of transplants. Methods have been successfully developed (summarized by Ahani *et al*., 2013), but these are being used only on an experimental basis. The rooted cuttings, however, are difficult to establish. Based on these premises development of operational methods for separating viable from empty, dead-filled and damaged seeds is a major step that may help initiate large-scale seedling production in nurseries.

### 1.3.3 Seed dormancy

Seed dormancy is a phenomenon in which a seed fails to germinate and produce a normal seedling under favorable germination conditions, i.e. moist substrate, sufficient oxygen supply and optimal temperature. It has been evolved as an adaptation for persistence in unpredictable environments, to escape competition and for optimizing reproductive success in randomly varying environmental conditions by dispersing germination in space and time (Baskin and Baskin, 2001). The cause of dormancy can be due to impermeable seed coat, restricting imbibition (physical dormancy); underdeveloped embryo (morphological dormancy) or chemical and physiological inhibitory mechanisms (physiological dormancy) and combinations of the above factors (Baskin and Baskin, 2004).

In other terminology, the term seed dormancy comprises of three groups of endogenous dormancy (physiological, morphological, and morpho-physiological), exogenous dormancy (physical, mechanical and chemical), and combination of these. Specific knowledge of each of them needs to be at hand when efforts to release them are made (Baskin and Baskin, 1998). Exposure of seeds to warm-cold-moist conditions are the main procedures to overcome the seeds dormancy in many plants seeds exhibiting physiological and/or morpho-physiological dormancy (Bewley and Black, 1994). Some species have relatively non-deep physiological dormancy for which periods of cold stratification ranging from 5 days to 60-90 days can easily release their dormancy. By reducing the seed dormancy barriers, seedling production cost

can decrease and seed efficiency will increase considerably in the nurseries (Cooke *et al*., 2002).

In most juniper species, dormancy is characterized as an physiological dormancy. In *Juniperus procera*, cold-moist stratification for 6 weeks can alleviate seed dormancy (Tigabu *et al*., 2007). Circumstantial evidence suggests that *J. polycarpos* seeds possess physiological or morpho-physiological dormancy for which efficient dormancy releasing method is not yet available (Korouri *et al*., 2012; Ahani *et al*., 2013).

## 1.4 Upgrading seed lot quality

### 1.4.1 Seed lot quality and improvement techniques

As previously mentioned, seed quality varies considerable due to pollination failure, post-zygotic degeneration, insect infestation, infection by seed borne pathogens and parasitic plants (Djavanshir, 1974; Slobodník and Guttenberger, 2000; Sivakumar *et al*., 2007; Ahani *et al*., 2013; Daneshvar *et al*., 2014). As a consequence, it is common to encounter a large proportion of non-viable seeds (empty, shriveled, anatomically underdeveloped and insect-infested seeds) in seed lots during collection. Thus, these unproductive seeds must be eliminated from the seed bulk to upgrade seed lot performance when sown in the nursery to produce the desired quantity of seedlings for active restoration program.

The quality of a given seed lot can be upgraded using a variety of seed sorting techniques, notably, the Pressure-Vacuum (PREVAC) to separate mechanically and insect-damaged seeds from seed lot (Lestander and Bergsten, 1985; Bergsten and Wiklund, 1987), Incubation-Drying-Separation (IDS) that has proven successful for upgrading seed lot quality of conifers (Simak, 1981, 1983, 1984; Downie and Bergsten, 1991; Downie and Wang, 1992; Singh and Vozzo, 1994; Poulsen, 1995; Demelash *et al*., 2002) and broad-leaved species (Falleri and Pacella, 1997; Demelash *et al*., 2003). In addition, recently near infrared (NIR) spectroscopy, has proven its potential as an extremely rapid and non-destructive technique for sorting non-viable seeds from viable ones (Lestander and Odén, 2002; Tigabu and Odén, 2002, 2003a, 2003b, 2004; Soltani, 2003; Tigabu *et al*., 2004, 2007; Agelet and Hurburgh, 2014) for improving the seed lot quality of many species.

### 1.4.2 *J. polycarpos* seed quality improvement

Specific gravity (SG) separation in water is the common nursery practice in Iran to remove non-viable seeds of *J. polycarpos*. For seed lots used in this

study with 13% initial moisture content, this technique resulted in complete separation of empty and insect-damaged seeds as floaters. However, assessment of the viability of sunken seeds by tetrazolium and cutting tests revealed that only 48% of the sunken fraction is viable; i.e. 52% of the sunken seeds are still non-viable that should be further sorted using other flotation medium than water or other sorting techniques. Thus, developing appropriate sorting system that enables the removal of unproductive seeds from the seed lot of *J. polycarpos* is highly needed.

## 1.5 Breaking the seed dormancy of *J. polycarpos*

Seed dormancy is one of the major hurdles for raising the desired quantity of *J. polycarpos* seedlings in the nursery in Iran (Ahani et al., 2013). Germination capacity of freshly collected seeds of *J. polycarpos* is very low and erratic, ca. 10%. Hitherto, there is no well-defined treatment regime for breaking dormancy in *J. polycarpos* seeds. Thus, developing appropriate dormancy breaking treatment that enables complete and synchronous germination of *J. polycarpos* is highly needed to produce the desired quantity of seedlings in the nursery for restoration planting plans.

# 2 Aim and objectives

The overall objective of the research presented in this thesis was to enhance seed quality and overcome dormancy of *J. polycarpos* seeds in order to increase the potential of seedling production in nurseries for active afforestation and restoration programs in Iran.

The overall hypothesis of the study is that the low success of seedling production of *J. polycarpos* in the nurseries of Iran firstly depends on the health status of seed production trees. Trees infected by parasite plants and pests produce seeds of lower quality due to competition of nutrients. In addition, a high proportion of remaining non-viable seeds in seed lots after specific gravity separation in water reduce the success of seedling production of *J. polycarpos*. Applying IDS and SG separation techniques or NIRS technique increases seed quality by efficiently sorting non-viable seeds, thereby increasing the success of seedling production. Also, after seed sorting, cold- and warm stratification and growth promoters, solely or in combination, will break seed dormancy and stimulate germination.

Thus, to address the first objectives, the influence of dwarf mistletoe on cone and seed quality and quantity of *J. polycarpos* was investigated (Paper I). The potential of IDS and SG was then tested for separation of dead-filled seeds from viable-filled seeds of *J. polycarpos* (Paper II). To further improve sorting efficiency, the potential of NIRS in combination with multivariate analysis was assessed to discriminate between filled viable and non-viable seeds (Paper III). Finally, after sorting out viable-filled seeds from the seed lot, stratification for different periods and exogenous application of phytohormones, solely or in combination, were tested to develop appropriate treatment regime for breaking dormancy (paper IV).

Therefore, the specific objectives of this thesis were to:

- Investigate the impact of dwarf mistletoe infection on *J. polycarpos* growth characteristics, cone production and seed quality and quantity (Study I).

- Define appropriate conditions for the application of IDS and SG techniques for separation of viable and non-viable seeds of *J. polycarpos* (Study II).

- evaluate the potential of Near Infrared Spectroscopy for separation of viable and non-viable seeds of *J. polycarpos* (Study III).

- Develop appropriate treatment regime for dormancy release and stimulation of germination of *J. polycarpos* seed (Study IV).

# 3 Material and methods

## 3.1 The study area

The study was conducted in *J. polycarpos* stand at Chaharbagh in Hyrcanian region Northeastern Iran, at 36° 36´ to 36° 41´ N and 54° 28´ to 54° 35´ E and an altitude of 2150-3150 meter above sea level. The climate is characterized as semi-dry with an average annual precipitation of 305 mm, which appears mostly in the form of snow. The study area has cold winters and moderately warm summers with the absolute minimum temperature of -14 °C that occurs in December and the maximum of 31 °C in July (Korouri et al., 2012). The *J. polycarpos* stand in the study site is nearly pure stand (Figure 7) and unique in the country.

*Figure 7*. An overview of *J. polycarpos* stand from Chaharbagh in NE Iran.

## 3.2 Methodological aspects

### 3.2.1 Dwarf mistletoe infection and impact on reproductive outputs

In study I, the extent of dwarf mistletoe infection and its impact on reproductive outputs were assessed using a total of 40 sample trees (n = 20 for infected and uninfected trees each). The severity of dwarf mistletoe-infested tree was assessed using Hawksworth's 6-class dwarf mistletoe rating (DMR) system (Hawksworth 1977). The number of witches' brooms and dwarf mistletoe colonies were also counted. The number of dwarf mistletoe colonies was counted in the middle part of the crown along the four sides of the crown (north, south, east and west) using 1 × 1 m wooden-frame mounted on a long pole. Figure 8 illustrates the assessment procedure in the field.

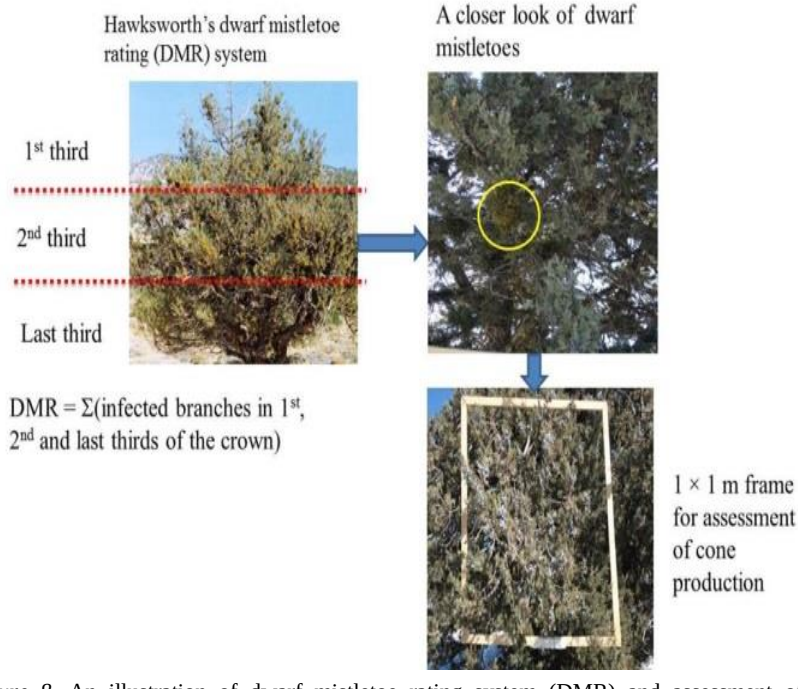


*Figure 8.* An illustration of dwarf mistletoe rating system (DMR) and assessment of cone production using the frame method.

To examine the impact of dwarf mistletoe on reproductive outputs, mature cones were collected from 40 sample trees with comparable morphological features (n = 20 for infected and uninfected trees each),

occurring at the same altitudinal range. Prior to collection of cones, the number of cones produced by infected and uninfected trees was counted to the main stem using 1 × 1 m wooden-frame, which was also used to assess mistletoe colonies (Fig. 8). Thereafter, one kg of mature cones with dark blue or dark blue with a white and waxy coating was picked from each infected and uninfected sample trees. The following cone characters were determined: number of cones per unit area, number of cone per kg of cones, cone diameter and mean number of seeds per cone. For seed traits, seed length, seed width, 1000-seed weight, number of filled-, and damaged-seeds as well as seed viability were assessed. Seeds with visible embryonic axis and mega-gametophyte were recognized as filled seeds while damaged seeds were composed of empty, insect-damaged and shriveled seeds. Seeds were considered as empty if the mega-gametophyte and embryo was absent, as insect-damaged if visible exit holes made by the emerging insect was observed and as shriveled seeds if black wrinkled internal tissues were observed.

### 3.2.2 Seed sorting systems

To upgrade the quality of *J. polycarpos* seed lots, two sorting systems were evaluated: flotation technique (study II) and NIR spectroscopy (Study III). The flotation technique involved Incubation, Drying and Separation (IDS) and specific gravity (SG) separation in water or different concentrations of sucrose solution. For the IDS experiments, seeds were pre-imbibed to 30% moisture content, incubated in plastic tubes at 15 ºC and ca. 85% relative humidity for seven days in a climate chamber (Inventum DK 11). Each incubation tube was capped with a one-way membrane (polytetrafluoroethylene, Trade mark GORE-TEX) that allows gas exchange but not water. After seven days of incubation, seeds were evenly distributed on a piece of netted cloth and dried in a fan-ventilated drying cabinet set at 20 ± 2 ºC and ca. 40% relative humidity for 3, 6, 9, 12 and 15 hours. Immediately after drying, seeds were placed in a bowl containing Millipore-filtered water, stirred to facilitate sedimentation of the seeds, and the floating and sunken seeds were collected separately after five minutes. The IDS experiment was repeated using different concentrations of sucrose solutions (0, 100, 150, 200, and 250 g/l water) as flotation media.

To examine whether SG separation in sucrose solution could be a simple alternative to IDS technique, a modified SG separation was tested. Unlike the conventional SG separation, seeds were left to imbibe for 24, 48 and 72 hours at a constant room temperature (20 ºC) in plastic boxes filled with 400 ml

water. Then seeds soaked for different lengths of time were immediately placed in bowls containing 0, 100, 200, 400, 600, 800 and 1000 g/l sucrose solutions; intermittently stirred to facilitate sedimentation of the seeds. The floating and sunken seeds were collected separately after five minutes, and the number of seeds in each fraction was counted. For each treatment, four replications of 100 resin-cleaned seeds were used.

The viability of floating and sunken seeds was determined by topographical tetrazolium (TTC) test, which is well-standardized and internationally accepted biochemical test for seed viability (ISTA, 2010). Note that juniper seeds, including *J. polycarpos*, are dormant (Baskin and Baskin, 1998) and dormancy-breaking method is not yet available for this species. For juniper species, the recommended TTC test involves pre-moistening the seeds at 20 ºC for 18 hours, cutting transversely 1/3 from the distal end to open embryo cavity, immersing the seeds in 1% staining solution for 18 hours in darkness, and finally cutting longitudinally through the endosperm to expose the embryo for examining the staining pattern. In this study, dry seeds were soaked in water for 24 hours to make sure that the seeds were sufficiently softened; thereby punctured more readily and stained evenly. Based on the red staining, the number of viable seeds in floating and sunken fractions was recorded.

For NIR spectroscopy application, seeds of *J. polycarpos* were sorted into viable, empty, insect-attacked and shriveled seeds by taking a series of X-ray images. A total of 400 seeds (100 seeds per each seed lot fraction) were correctly identified by X-ray analysis and TTC test and used for NIR spectroscopic analysis. In addition, 160 seeds (40 seeds per seed lot fractions) were scanned separately to serve as test set. NIR reflectance spectra from single seed, expressed in the form of log (1/R), were collected with XDS Rapid Content Analyzer (FOSS NIRSystems, Inc.) from 400 to 2498 nm with a spectral resolution of 0.5 nm. For every seed, 32 monochromatic scans were made at stationary position that allowed collection of radiation reflected from the entire surface of the seed, and then the average value of 32 scans was recorded for each individual seed.

### 3.2.3 Pre-treatments to break dormancy

In study IV, a series of experiments were conducted to identify the best pre-treatment for breaking dormancy in *J. polycarpos* seeds. The first experiment involved cold stratification at controlled moisture content (30%) in a

refrigerator at 1 ºC for four, eight, twelve, and sixteen weeks. The second experiment involved warm-cold stratification where seeds with 30% moisture content were first warm-stratified at 20 ºC for four, eight, twelve, and sixteen weeks and then cold-stratified for 12 weeks in a refrigerator at 1ºC.

The effects of gibberellic acid (GA3) and 6-Benzylamino purine (BAP) on the release of dormancy in *J. polycarpos* seeds were also tested. For this purpose, seeds were soaked in three concentrations of GA3 and BAP (250, 500, and 1000 ppm) solutions for 72 hours at 20 ºC to reach 30% of moisture content. Afterwards, seeds were washed with tap water for 5 minutes before sowing for germination tests. Based on the findings of the above experiments, a combination of 500 ppm GA3 and BAP solutions and cold stratification were tested to examine their effect on breaking dormancy. For this purpose, seeds were soaked in 500 ppm GA3 and BAP solutions for 72 hours at 20 ºC to reach 30% moisture content. Thereafter, seeds were washed for 5 minutes under tap water and stratified in polyethylene tubes in the refrigerator adjusted to 1 ºC for 12 weeks. The polyethylene tubes were covered with one-way GOURTEX cloth and placed in desiccators filled with moist sand to maintain high relative humidity around the tubes.

For the germination test, four replicates of 50 seeds each for each treatment were sown on standard moistened filter paper in petri dishes (9.5 mm) and placed on Jacobsen's apparatus that was set at 20 ± 1 ºC constant temperature during day and night with a continuous illumination of ca. 20 µE m-2s-1 (Floresent lamp F 40 W / 33 RS cool white light) for 30 days. To serve as control, untreated seeds, four replicates of 50 seeds, were also sown. The germination process was monitored every day and germinated seeds were counted when the radicle reached 2 mm long and had a normal appearance.

## 3.3 Data Analysis

In study I, two-sample T-test was performed to compare differences in mean number of dwarf mistletoe colonies per unit area and witches' brooms per trees between infected trees. One-way ANOVA was also performed to compare differences in growth characteristics (DBH, total height, crown surface area and crown volume), cone and seed characters across infection classes. The crown surface area and volume were calculated from crown width and height assuming a cone shape, which is a typical crown shape of *J. polycarpos*. Prior to ANOVA, germination capacity was arcsine-transformed to meet the

normality assumption for the analysis of variance. A Chi-square analysis of 2 × 6 contingency table was also performed to test the null hypothesis that the proportion of cones with varying number of seeds per cone is independent of the health status of trees (infected versus uninfected trees).

In study II, the proportion of viable seeds (PVS) in floating and sunken fractions was calculated separately using the following formula:

$$PVS(\%) = \frac{No.\,viable\ seeds\ in\ ith\ fraction}{Total\ no.of\ seeds\ in\ ith\ fraction} \times 100$$

Sorting efficiency, defined as the overall gain in viable seeds after sorting, was computed as the difference in proportion of viable seeds in the sunken fraction to that of the discarded floating fraction. Prior to ANOVA, the proportion of viable seeds was arcsine transformed to meet the assumptions of homoscedasticity and normal distribution (Zar, 1996). For the IDS experiment, One-way ANOVA was performed to examine the effect of different drying times, and concentrations of sucrose solution on the proportion of viable seeds in the floating and sunken fractions as well as sorting efficiency. For the SG experiment, Two-way ANOVA was performed to examine the effects of soaking time and different concentrations of sucrose solution on the proportion of viable seeds in each seed lot fraction and overall sorting efficiency. Means that exhibited significant differences were compared using Tukey's test at 5% level of significance.

In study III, multivariate classification models were derived by Partial Least Squares-Discriminant Analysis (PLS-DA) and Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA). The calibration set was composed of 400 NIR spectra (100 spectra per seed lot fraction) and the test set was composed of 160 NIR spectra (40 spectra per seed lot fraction). Initially four-class PLS-DA and OPLS-DA models were developed using the absorbance values in the full NIR spectral region (780-2500 nm) as regressor and a matrix of dummy variables as regressand (1 for member of a given class, 0.0 otherwise). The discriminant models were also fitted using the shorter (780-1100 nm) and longer (1100-2500 nm) NIR spectral regions. To further improve the model performance, two-class models were developed by merging empty, insect-attacked and shriveled seeds into a non-viable class while keeping the filled-viable seeds as a viable class. All calibrations were developed on mean-centered data sets and the number of significant model

components was determined by a seven-segment cross validation. Finally, the predictive ability of the fitted models was evaluated using independent test set that was drawn from seed lots not included in the calibration. Seeds were considered as member of a given class if predicted values were greater than an acceptance threshold (≥ 0.5), and the classification accuracy was computed as the proportion of number of samples in a given class predicted correctly to the total number of samples in that class. The spectral region accounted for the discrimination of filled-viable and non-viable seeds was determined using a parameter called Variable Influence on Projection (VIP). All calculations were performed using Simca-P + software (Version 13.0.0.0, Umetrics AB, Sweden).

In study IV, germination capacity (GC) and mean germination time (MGT) were calculated for each treatment and replicate as follows:

$$GC\ (\%) = \left(\frac{\sum n_i}{N}\right) \times 100$$

$$MGT\ (days) = \frac{\sum (t_i \times n_i)}{\sum n_i}$$

Where $\sum n_i$ is the number of germinated seed after 30 days, and N is the total numbers of seed sown; $t_i$ is the number of days starting from the date of sowing and $n_i$ is the number of seeds germinated at each day (Bewley and Black, 1994). All percentage data sets were arcsine-transformed prior to statistical analysis to approximate the normality assumption for analysis of variance (Zar, 1996). Two-way analysis of variance (ANOVA) was performed to examine significant differences in GC and MGT between stratification treatments (cold versus warm-cold) and length of stratification period as well as between hormones ($GA_3$ versus BAP) and their concentrations. One-way ANOVA was also performed to determine significant differences in germination response among the combined treatments of hormones and 12 weeks of cold-stratification. Means that showed significant differences were compared by Tukey's honestly test at 5% level of significance. All statistical analyses in study I, II, and IV, were performed with Minitab 17.0 statistical software (Minitab Inc., State College, PA, USA).

# 4 Results and Discussion

## 4.1 Growth and reproductive outputs of dwarf mistletoe-infected *J. polycarpos* trees

The results show that the severity of infection by juniper dwarf mistletoe on its host was low to moderate. According to DMR system, 40% of the sample trees were lightly infected while the remaining 60% were moderately infected. There were no significant differences in mean number of witches' brooms ($p = 0.078$) and mean number of dwarf mistletoe colonies per unit area ($p = 0.398$) between light and moderate infection severity classes. Similarly, growth characteristics (diameter, total height, crown volume, and crown area) did not vary significantly ($p > 0.05$) among uninfected, lightly and moderately infected trees.

The severity of infection generally depends on host quality and dispersal agents. According to differential parasitism theory, mistletoes selectively parasitize or survive better on hosts with high nutrient (Panivi and Eickmeier, 1993; Dean *et al*., 1994) and water status (Gregg and Eleringer, 1990). The poor growth conditions in the natural habitat of *J. polycarpos* (semi-dry climate and poor soil quality) may predispose the trees to high water stress and nutrient deficiency, and hence affecting the fitness (growth and flower production) of juniper dwarf mistletoe. In addition, the negative impact of infection by mistletoe on growth of its host is usually associated with high infection severity and time since infection. Compared to low and moderate infection, significant growth losses and less annual variation in growth was observed for severely infected bristlecone pines (Scott and Mathiasen, 2012), western hemlock (Shaw *et al*., 2008) and ponderosa pine (Stanton, 2006). Moreover, Stanton (2006) observed that broom abundance does not uniquely impact recent radial growth. We also found weak insignificant correlations

between growth characteristics and number of witches' brooms and number of dwarf mistletoe colonies per unit area (data not shown). The severity of infection and the associated impact on growth of the host progresses with time since infection. At the beginning, infection occurs at a slow rate with little effect, but it increases progressively over time and culminates at large damaging effect (Geils and Hawksworth, 2002). Although there is no record of time since infection of *J. polycarpos* by dwarf mistletoe in Iran, it seems that the infection is of recent phenomenon as can be inferred from the low to moderate level of infection observed in the present study.

Regarding cone characters, significant differences in mean number of cones per unit area ($p = 0.001$) and number of cones/kg ($p = 0.024$) among the infected and uninfected trees were detected, but not for mean cone diameter ($p = 0.095$). Uninfected trees produced nearly twice more cones per unit area than infected trees, but both lightly and moderately infected trees produced similar number of cones per unit area (Table 1). On the contrary, moderately infected trees produced 1.1 times more cones/kg compared to uninfected trees. The mean number of seeds per cone was similar ($p = 0.503$) across infection classes (Table 1), but the proportion of cones with varying number of seeds per cone was dependent on the health status of trees ($\chi 2 = 12.859$, d.f. = 5, $p = 0.025$). Uninfected trees produced slightly more cones with one, two and five seeds per cone than infected trees, whereas infected trees produced slightly more cones with three and four seeds per cone than their uninfected counterparts.

*Table 1.* Cone characters of *J. polycarpos* trees across three levels of infection (mean ± SE). For each cone character, means followed by different letter across the row are significantly different.

| Cone characters | Infection level | | |
|---|---|---|---|
| | Low | Moderate | Uninfected |
| Mean No. cone $m^{-2}$ | 342 ± 37b | 332 ± 70b | 637 ± 58a |
| No. cone $kg^{-1}$ | 2815 ± 152ab | 3063 ± 78a | 2754 ± 56b |
| Mean no. seed per cone | 3 ±0.2a | 3 ± 0.1a | 3 ± 0.1a |
| Mean cone diameter (mm) | 8.10 ± 0.32a | 8.02 ± 0.2a | 8.65 ± 0.2a |

For seed characters, significant differences in 1000-seed weight ($p = 0.001$), seed width ($p < 0.001$), seed length ($p < 0.001$), number of damaged seeds/kg ($p = 0.008$) and total number of seeds/kg ($p = 0.021$) were detected among

uninfected, lightly and moderately infected trees, but not for number of filled seeds ($p = 0.640$). Seeds from uninfected trees were heavier than seeds from moderately infected trees; whereas seeds from uninfected trees were longer and wider than seeds from both lightly and moderately infected trees. The number of filled seeds was generally low for both uninfected and infected trees compared to damaged seeds, which were more for moderately infected trees than uninfected trees (Table 2). As a whole, infected trees produced more seeds than uninfected trees.

*Table 2.* Comparison of seed quantity across three levels of infection (mean ± SE). For each variable, means followed by different letter across the row are significantly different.

| Seed quality | Infection level | | |
|---|---|---|---|
| | Low | Moderate | Uninfected |
| No. filled seed / kg | 844 ± 192a | 744 ± 137a | 940 ± 140a |
| No. damaged seed / kg | 8164 ± 296ab | 8656 ± 139a | 7370 ± 307b |
| Total no. seed / kg | 9009 ± 324ab | 9400 ± 173a | 8310 ± 286b |

It thus appears that infection by juniper dwarf mistletoe has a significant effect on the reproductive output of its host by significantly reducing the mean number of cone per unit area of the host crown, and increasing the number of damaged seeds. Generally, reproductive output is governed by changes in resource allocation patterns (Bazzaz *et al*., 2000). Mistletoes drain nutrient (particularly nitrogen) from its host by both active and passive uptake mechanisms (Okubamichael *et al*., 2011), which offer the mistletoes competitive advantage over the neighboring uninfected branches, thereby perturbing resource allocation for reproduction at individual tree level. In addition, a plant may allocate the available resource to the production of many smaller seeds under limited resources availability. This is evident in the present study where infected trees (subjected to resource competition with the dwarf mistletoe) produced 1.1 times more cones/kg (Table 1) but smaller seeds than uninfected trees.

It is interesting to note that the proportion of damaged seeds was slightly higher for infected than uninfected trees, and most of the damaged seeds were empty seeds in both infected and uninfected trees. Poor seed production is a typical problem of many conifers due to lack of pollination and fertilization, pollination by non-viable pollen grain, perturbations in the pollination

mechanism, degeneration of ovule or early embryo (e.g. Slobotník and Gutternberger, 2000). However, infection by juniper dwarf mistletoe appears to aggravate this problem. We also observed significant difference in the number of seeds per cone between infected and uninfected trees. Uninfected trees produce slightly more cones with one and two seeds per cone than uninfected trees indicating a resource trade-off that can be interpreted as evidence of ovule competition (Wulff, 1995).

With regard to germination, infection by juniper dwarf mistletoe resulted in reduced overall and speed of germination compared to seeds from uninfected trees. The observed difference in the speed of germination might be related to amount of reserve compounds (seeds from infected trees were significantly lighter than those from uninfected trees), and their subsequent conversion into energy to trigger radicle protrusion. Vigorous seeds exhibit increased fumarase activity, a key respiratory enzyme in the tricarboxylic acid cycle (Shen and Oden 2000) that fuels rapid germination. As a whole, our results are consistent with previous studies that have reported fewer cones, fewer seeds, lighter seeds and lower germination for dwarf mistletoe infected than uninfected conifer species (Singh, 1981; Singh and Carew, 1989; Schaffer *et al*., 1983; Sproule, 1996).

## 4.2 Improved sorting system for upgrading seed lot quality

### 4.2.1 Sorting viable from non-viable seeds by flotation technique

The results show that the number of floating seeds increased while that of sunken seeds deceased linearly with increasing drying time when water was used as a flotation medium (Figure 9A). No viable seed was found after three hours of drying in the floating fraction, but the share of viable seeds in the floating fraction was significantly ($p < 0.01$) lower after 6 and 9 hours of drying than after 12 hours, which in turn had significantly higher viable seeds than 15 hours of drying (Figure 9B). For the sunken fraction, the proportion of viable seeds was significantly ($p = 0.01$) higher after 9 and 12 hours of drying than the rest of the drying times tested. As a whole, nine-hour drying appeared to be efficient in terms of the net gain of viable seeds after sorting while 15 hours of drying was the least efficient (Figure 9C).

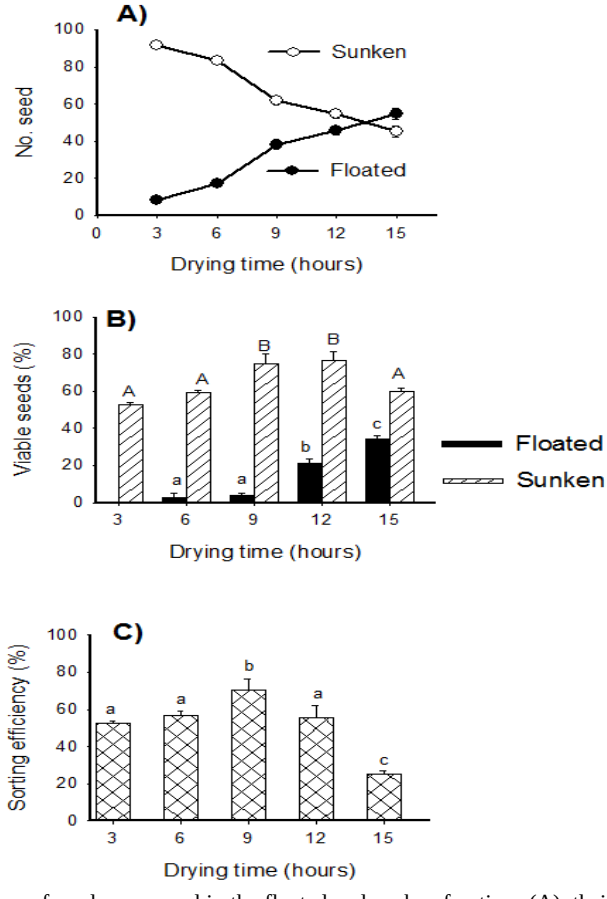


*Figure 9.* Number of seeds recovered in the floated and sunken fractions (A), their corresponding proportion of viable seeds (B) and overall sorting efficiency (C) of IDS in water and different drying times (mean ± SE). For each seed lot fraction, bars with different letters are significantly different ($p < 0.05$).

Sorting viable seed and non-viable seeds was also tested using different concentrations of sucrose solution as separation medium. For seven-day incubated seeds followed by 9 hours drying, significant differences in number of floating and sunken seeds and the proportion of viable seeds in each seed lot fraction were detected among different concentrations of sucrose solution ($p < 0.01$). The number of floating seeds ranged from 38% in pure water (0 g/l) to

55% in 250 g/l sucrose solution; whereas the proportion of sunken seeds ranged from 45% in 250 g/l sucrose solution to 62% in pure water (Figure 10A). The proportion of viable seeds in the floating fraction was less than 5% for pure water and all other concentrations of sucrose solutions tested except 250 g/l that had a larger share of viable seeds in the floating fractions (Figure 10B). For the sunken fraction, the proportion of viable seeds was the highest (82%) in 200 g/l sucrose solution compared to pure water and low concentrations of sucrose solution (Figure 10B). The net gain in viable seeds after sorting (overall sorting efficiency) did not vary significantly ($p = 0.173$) among concentrations of the sorting media; albeit a tendency of higher gains in 200 g/l (Figure 10C).

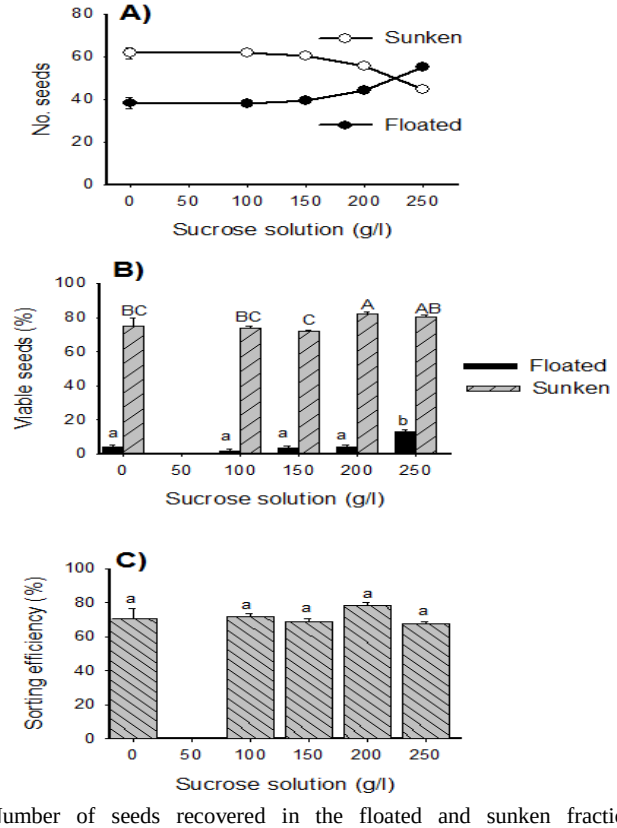


*Figure 10.* Number of seeds recovered in the floated and sunken fractions (A), their corresponding proportion of viable seeds (B) and overall sorting efficiency (C) of IDS in different concentrations of sucrose solution as sorting media (mean ± SE). For each seed lot fraction, bars with different letters are significantly different ($p < 0.05$).

As a whole, the IDS conditions tested in the present study substantially upgraded the quality of *J. polycarpos* seed lot from 48% to more than 75%. Among the IDS conditions tested in the present study, seven days incubation followed by nine hours of drying and sorting in either water (Figure 9) or 200 g/l sucrose solution (Figure 10) were effective in terms of yielding high proportion of viable seeds in the sunken fraction and low in the discarded floating fraction (4%). Compared to three days incubation originally used by Simak (1981), seven days incubation allows viable seeds to metabolically bind the absorbed water, thereby losing the moisture content slowly during the drying process. The fact that the proportion of viable seeds in the floating fraction increased with drying time is attributed to loss of moisture during extended drying (e.g. 15 and 12 hours) by viable but low vigor seeds, as also observed in other species (e.g. Simak, 1984).

In the present study, IDS in 200 g/l sucrose solution resulted in recovery of more viable seeds in the sunken fraction than IDS in water (Figure 10). This indicates that the sorting efficiency depends not only on the specific density of the sorting medium but also on its viscosity (a measure of the extent to which a fluid opposes the movement of an object through it). At a given temperature, the higher the concentration of the solution is, the higher the viscosity will be and the lower the movement of an object through the solution. At 20 °C, the specific density and viscosity of 200 g/l sucrose solution are 1.081 g/l and 1.941 mPa.s, whereas pure water has a specific density of 1 g/l and a viscosity of 1.002 mPa.s. Thus, the rate of sedimentation of seeds decreases with increasing viscosity of the sucrose solution, as evidenced from the low number of sunken seeds with increasing concentration of the sucrose solution for the IDS experiment (Figure 10).

Specific Gravity (SG) using sucrose solutions was another sorting method that was tested in this study for separation of viable from non-viable seeds of *J. polycarpos*. SG separation showed significant variations among pre-sorting soaking times, concentrations of the sorting medium and their interaction ($p < 0.01$ in all cases). As the concentration of sucrose solution increased from 0 g/l (pure water) to 1000 g/l, the proportion of floating seeds increased while the proportion of sunken seeds decreased continuously irrespective of the initial soaking time, although the decrease/increase was sharp for seeds soaked for 24 hours (Figure 11). At lower concentrations of sucrose solution (0 g/l and 200 g/l), all seeds sunk irrespective of the soaking times tested.

For 24-hour soaked seeds, SG separation in higher concentration of sucrose solution (≥ 600 g/l) returned more viable seeds in the sunken fraction than lower concentrations (200-400 g/l) and pure water; whereas the proportion of viable seeds in the floating fraction was larger when very high concentrations of sucrose solution were used as sorting medium (800-1000 g/l) than moderate (600 g/l) and low concentrations; particularly no viable seed was recorded for pure water and 200 g/l sucrose solution (Figure 11). While SG separation in 600 g/l and 800 g/l sucrose solutions resulted in higher viable seeds in the sunken fraction for 48-hour soaked seeds than lower concentrations, the proportion of viable seeds recovered in the sunken fraction was higher when the concentration of the sorting medium was very high (800-1000 g/l) for 72-hour soaked seeds (Figure 12). As a whole, the efficiency of SG separation was better when seeds were soaked for 48 hours and then sorted using 600 g/l sucrose solutions, where a net gain of viable seeds in the sunken fraction amounted to 72% (Figure 12).

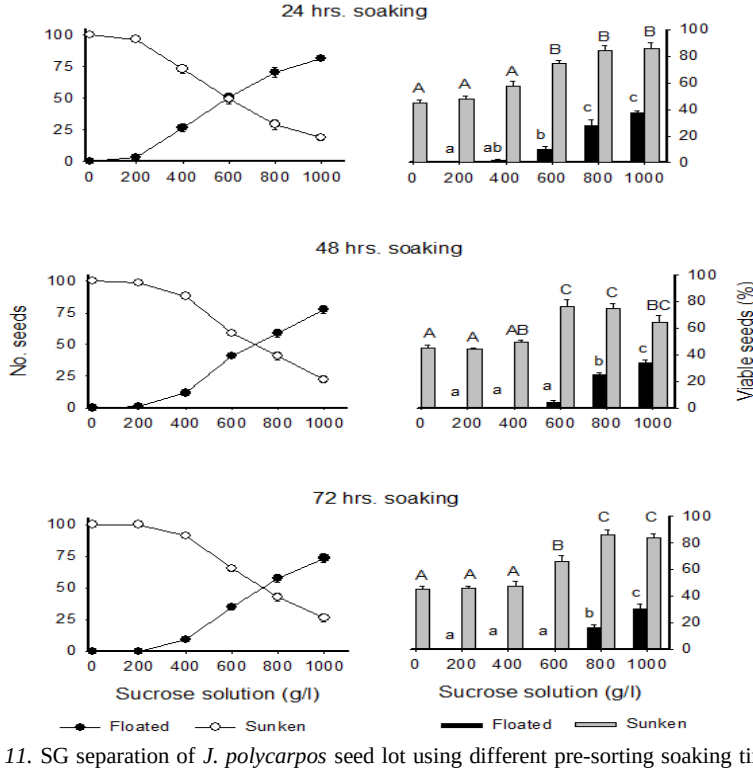


*Figure 11.* SG separation of *J. polycarpos* seed lot using different pre-sorting soaking times and different concentrations of sucrose solutions as sorting medium (mean ± SE). For each soaking time and seed lot fraction, bars with different letters are significantly different ($p < 0.05$).

Our results confirmed that 48 hours soaking prior to sorting and sorting using 600 g/l sucrose solution results in comparably the same sorting efficiency as IDS technique. This result underscores the feasibility of SG separation in sucrose solution as a simple alternative to IDS technique, particularly for local nurseries where modern facilities for performing IDS (such as controlled climate chamber, appropriate drier etc.) are not easily available.

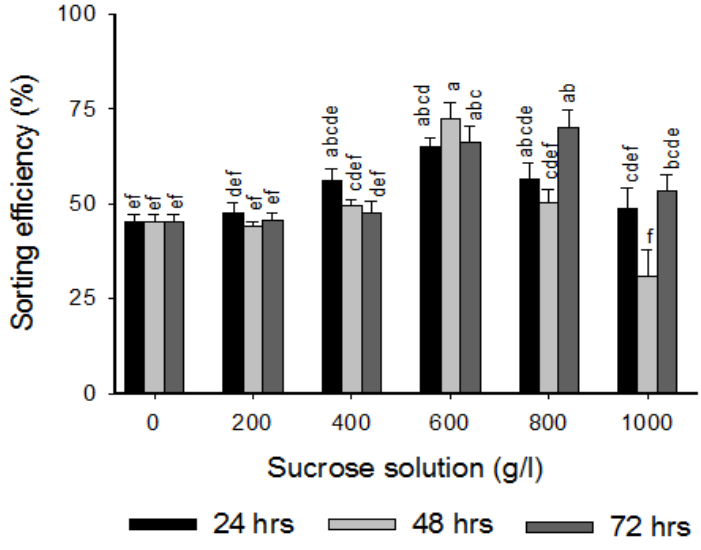


*Figure 12.* Overall sorting efficiency of SG in different concentrations of sucrose solution and soaking times (mean ± SE). Bars with different letters are significantly different ($p < 0.05$).

For the best IDS and SG sorting system found in this study, the proportion of viable seeds discarded as floaters is remarkably low (4%). This is an additional advantage of the present sorting system because high loss of viable seeds into the discarded floating fraction may not be economically feasible from seed supplier's point of view and may have an impact on maintaining the genetic diversity of seed lots.

### 4.2.2 Discrimination of viable and non-viable seeds by NIR spectroscopy

The success of simultaneous discrimination of empty, insect-attacked, shriveled and filled-viable seeds by NIR spectroscopy was limited. The

computed multivariate classification models described less 75% of the class variation ($R^2Y$) with 66% prediction accuracy according to cross validation and 64-71% accuracy according to independent test set.

When two-class models were fitted to the spectral data to discriminate between viable and non-viable seeds, the modeled ($R^2Y$) and predicted ($Q^2_{cv}$) class membership of the calibration set were improved substantially (more than 90%) for both PLS-DA and OPLS-DA models compared to the four-class models (Table 3). The prediction accuracy for the test set was also improved to 99% compared to the four-class models. However, the OPLS-DA models required only one significant factor for modelling 89-93% of the class variation ($R^2Y$) using small fraction of the total spectral variation ($R^2X_p$ = 1.2%-6.8%) while large proportion of the spectra was uncorrelated to the class variation ($R^2X_{o=}$93.2%-98.8%), depending on the NIR region analyzed (Table 3).

*Table 3.* Statistical summary of two-class models developed using the entire (780-2500 nm) shorter (780-1100 nm) and longer (1100-2500 nm) NIR regions for discriminating viable and non-viable seed of *J. polycarpos.*

**A) PLS-DA**

| Spectra (nm) | A | $R^2X$ | $R^2Y$ | $Q^2_{CV}$ | $Pred._{test}$ (%) |
|---|---|---|---|---|---|
| 780-2500 | 11 | 0.999 | 0.913 | 0.871 | 99 |
| 780-1100 | 6 | 0.999 | 0.927 | 0.915 | 99 |
| 1100-2500 | 7 | 0.999 | 0.914 | 0.888 | 99 |

**B) OPLS-DA**

| Spectra (nm) | A | $R^2X_P$ | $R^2X_0$ | $R^2Y$ | $Q^2_{CV}$ | $Pred._{test}$ (%) |
|---|---|---|---|---|---|---|
| 780-2500 | 1+10 | 0.0312 | 0.969 | 0.916 | 0.905 | 99 |
| 780-1100 | 1+9 | 0.0683 | 0.932 | 0.927 | 0.920 | 99 |
| 1100-2500 | 1+8 | 0.0121 | 0.988 | 0.892 | 0.878 | 99 |

A = number of significant components to build the model (for OPLS-DA models , the first value is for predictive component and the second value is for the orthogonal component), $R^2X$ = the explained spectral variation (1 - SS(E)/SS(X)), $R^2Y$ = the variation between seed classes explained by the model (1 – SS(F)/SS(Y)), $R^2X_p$ = the predictive spectral variation; $R^2X_o$ = Y-orthogonal variation (spectral variation uncorrelated to class discrimination), $Q^2_{cv}$ = the predictive power of a model according to cross validation, and $Pred_{test}$ = the overall prediction accuracy of the models for the test set.

The two-class PLS-DA models assigned samples in the test set correctly to viable and non-viable classes, except one viable seed that was misclassified as non-viable seed. The overall classification accuracy was 98% for filled-viable seeds and 100% for non-viable seeds (empty, insect-attacked and shriveled seeds). The OPLS-DA models also resulted in similarly higher classification accuracy for the test set (Figure 13).

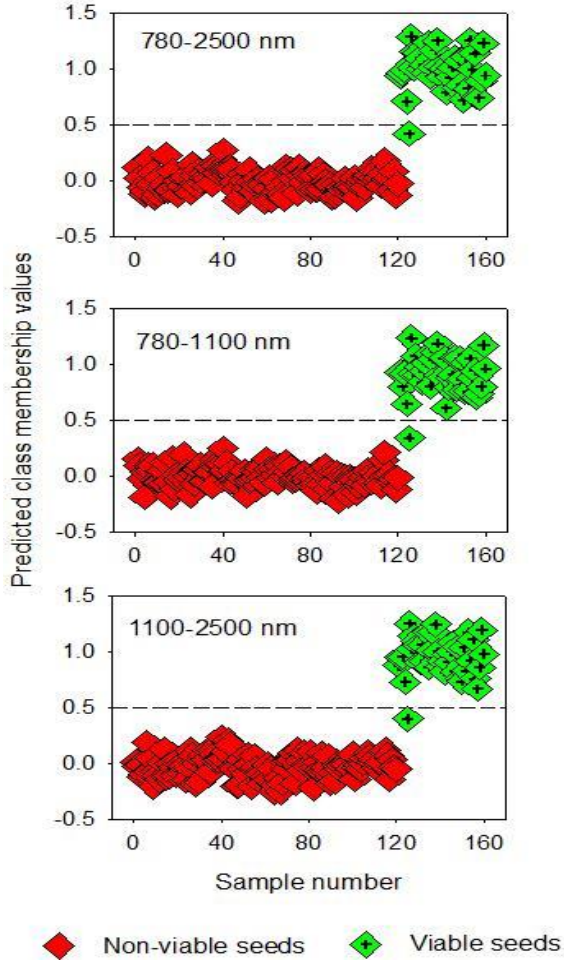


*Figure 13.* Discrimination of non-viable (empty, insect-attacked and shriveled) and viable seeds in the test set by two-class OPLS-DA modelling of different NIR spectral region. The horizontal dotted line is the class limit ($Y_{predicted} > 0.5$) for assigning the test sets into viable seed class.

The results demonstrate that NIR spectroscopy can discriminate viable and non-viable seeds of *J. polycarpos* successfully, although the success of discriminating among the non-viable seed lots fractions (empty, insect-attacked and shriveled seeds) was limited. Cutting and examining the internal content of the non-viable seed lot fractions confirmed that some of the insect-attacked seeds were totally devoid of its contents while some were partially consumed; whereas the shriveled seeds differed in the content of the black undifferentiated mass of tissues from a quarter to half the size of the seed. This divergence in the internal content of insect-attacked and shriveled seeds might not be large enough compared with the empty seeds that are totally devoid of stored reserves, and hence limited the success of discriminating fully among the non-viable seed lot fractions.

Both OPLS-DA and PLS-DA modelling approaches result in identical predictive capacity of class membership of the test set samples, however the OPLS-DA modelling results in dimensionally less complexity and parsimonious models (A = 1 for OPLS-DA versus A = 10 for PLS-DA). Dimensional complexity is an important factor in the interpretation of multivariate analysis and parsimonious models with few dimensions (components) are often highly preferred (Trygg and Wold, 2002).

Our attempt to discriminate viable and non-viable seeds of *J. polycarpos* using the shorter (780-1100 nm) and longer (1100-2500 nm) NIR regions has resulted in similar prediction accuracy of class membership as the full NIR region. This suggests that discrimination of *J. polycarpos* seeds according to their viability using NIR spectroscopy is not sensitivity to the change in detection system from Silicon-detector in the shorter to InGaAs-detector in the longer NIR regions. This finding shed light on the prospect of developing cost-effective automated sorting system for large-scale seed handling operations.

Absorption bands that were highly relevant for discriminating viable and non-viable seeds appeared in 780-1200 nm and 1210-1389 nm regions (Figure 14). The absorption bands in 780-1100 nm and 1200-1389 nm are characterized by third and second overtone of C - H stretch, respectively due to absorption by methyl and methylene (the common molecular moieties in fats and oils), and benzene (Osborne *et al*., 1993; Workman and Weyer, 2012). In addition, the 780-1100 nm is a region characterized by the O - H stretching second overtone where absorption spectra of aliphatic and aromatic hydroxyl groups as well as starch and water overlap. Previous studies have shown that

juniper berries are rich in essential oils with 15-74 different components; the dominant being monoterpenes (α-pinene, myrcene, sabinene, limonene and β-pinene), followed by small amounts of sesquiterpenes and non-terpene components such as undecanone-2 and tricyclene (Okasaka et al. 2006; Rezvani et al. 2009; Sela et al. 2011; Höferl et al. 2014). As the non-viable seeds are totally devoid of storage reserves due to consumption by insect larvae (insect-attacked seeds) and developmental arrest of the storage organs and seed filling (empty and shriveled seeds), the terpenoids in the seed coats possibly dominates the spectral signature. The viable seeds showed unique absorption in the longer wavelength regions (1850-2000) with small but notable peaks at 1890 nm and 1996 nm. While the absorption band around 1900 nm is characterized by O–H stretch and combination of C–O stretch due to absorption by molecular moieties of protein and starch, the absorption band around 2000 nm is typical of N–H, O–H and C–H bond vibrations due to absorption by protein, lipid and carbohydrate moieties (Shenk et al. 2001). Thus, the discriminant models utilized spectral difference attributed to seed coat chemical compositions coupled with storage reserves as a basis to discriminate viable and non-viable seeds of *J. polycarpos*. Tigabu et al. (2007) have also found these regions as useful for discriminating sound and insect-damaged seeds of *J. procera*.

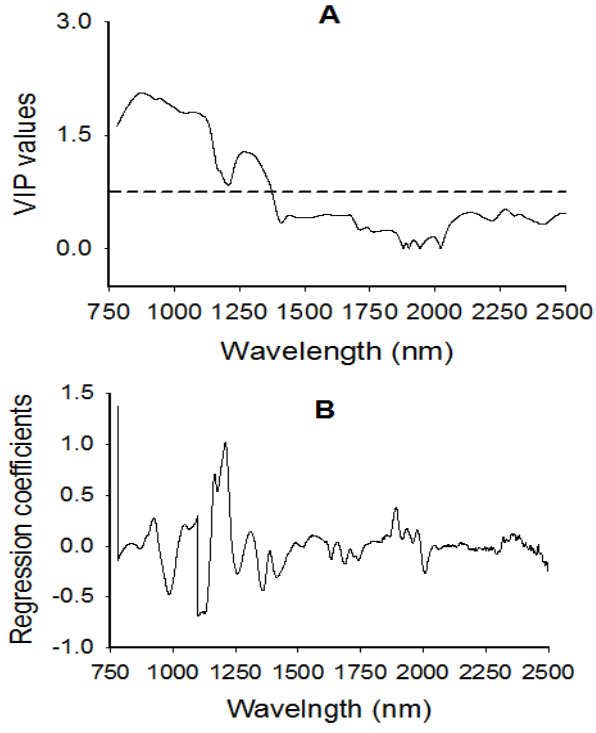


*Figure 14.* Plots of Variable Influence on Projection, VIP, (A) and regression coefficients (B) showing spectral regions that influenced the discrimination of viable from non-viable (empty, insect-attacked and shriveled) *J. polycarpos* seeds. The horizontal dotted line in the VIP plot is the cut-off limit (0.75) for discriminating relevant and irrelevant predictors. The regression coefficient plot is for the viable seeds class.

## 4.3 Alleviation of physiological dormancy in *J. polycarpos* seeds

### 4.3.1 Germination responses to stratification

Significant difference in germination capacity was detected between cold and warm-cold stratification treatments ($p < 0.01$), among durations of stratification treatments ($p < 0.01$) and for the interaction effect ($p < 0.01$). Warm-cold stratification resulted in 40%-72% germination capacity compared to 12%-42% germination for cold stratification, depending on the duration of treatments (Figure 15).

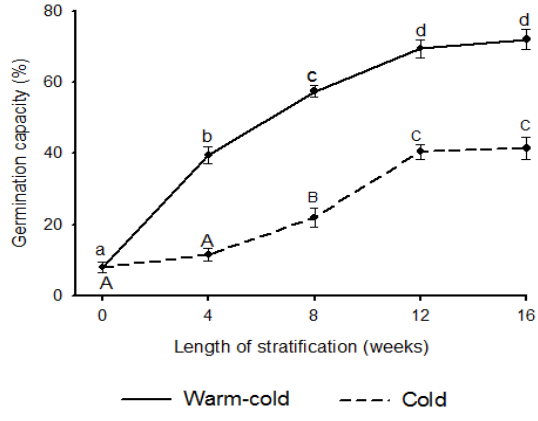


*Figure 15.* Germination capacity (%) of *J. polycarpos* seeds in response to different lengths of cold and warm-cold stratification period (mean ± SE). For each stratification treatment, means followed by different letter are significantly different according to Tukey's test ($\alpha = 0.05$).

Among all durations of stratification treatments tested in the present study, germination capacity was higher after 12 and 16 weeks than eight and four weeks of stratification, which in turn resulted in significantly higher germination capacity than the control, particularly for warm-cold stratified seeds. For cold-stratified seeds, four weeks appeared to be insufficient to release dormancy and stimulate germination compared to the control.

The limited germination response to 16 weeks of cold stratification (42%) suggests that there are two groups of seeds within the seed lot – one with non-deep dormancy and the other with deep dormancy for which cold stratification alone might not be sufficient. The length of the stratification period required for dormancy release largely depends on the level of dormancy (Baskin and Baskin, 2001) and varies between and within populations (Tigabu *et al*., 2007). In addition, within seed lot variability in dormancy level and germination can occur due to genetic and environmental effects during seed development (Mamo *et al*., 2006).

Germination was, however, substantially improved (72%) by warm stratification for 16 weeks followed by 12 weeks of cold stratification compared with cold-stratification alone (42%) and the control (8%). This suggests that the embryos were not fully developed at the time of maturation

and dispersal to a critical size and prolonged warm stratification promotes sufficient embryo growth. Santaigo *et al*. (2015) have reported an increase in embryo length from 1.3 mm in fresh *Viburnum lantana* L. seeds to 3.0 mm after 20 weeks of warm stratification while the embryo hardly grew after 24 weeks of cold stratification. Similarly, Chen *et al*. (2014) have reported that *Sambucus chinensis* Lindl. seeds require warm stratification for dormancy break and germination. Our result gives indication that dormancy in *J. polycarpos* seeds could be morpho-physiological; presumably intermediate simple morpho-physiological dormancy (*sensu* Baskin and Baskin, 2004). Seeds exhibiting this type of dormancy often need up to four months of stratification at 18-20°C followed by four months of cold-stratification at 0-3°C for maximum germination (Baskin and Baskin, 2001; Baskin *et al*., 2002).

### 4.3.2 Germination responses to exogenous application hormones

Germination capacity did not vary significantly with respect to the type of hormones applied ($p = 0.772$), but significant differences were detected among concentrations of hormones ($p < 0.001$) and for the interaction effect ($p < 0.01$). While high concentration of GA3 totally inhibited germination (Figure 16), application of 500 ppm GA3 stimulated germination (25%) better than 250 ppm GA3 (11%) and the control (6%). Application of 500 ppm BAP also trigger as high as 16% germination compared with 6% for the control; however, germination capacity remained the same across concentration of BAP tested (Figure 16).

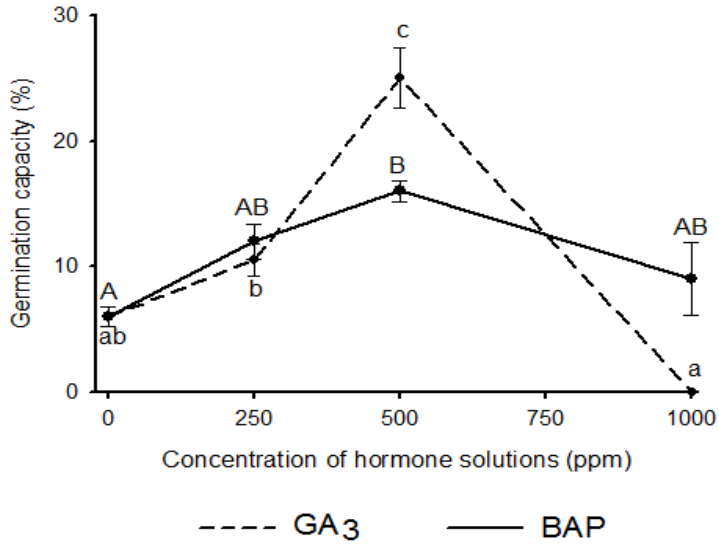


*Figure 16.* Germination capacity (%) of *J. polycarpos* seeds in response to exogenous application of $GA_3$ and BAP (mean ± SE). For each hormone treatment, means followed by different letter are significantly different according to Tukey's test ($\alpha = 0.05$).

For the combined treatment of hormones and cold stratification, significant differences in germination capacity ($p < 0.01$) and mean germination time ($p < 0.01$) were detected compared to the control. Exogenous application of $GA_3$ followed by 12 weeks of cold stratification stimulated more germination than 500 ppm BAP, which in turn resulted in higher germination than the control (Table 4). The mean germination time was shorter for seeds subjected to combined treatment of hormones and cold stratification than the control; but it remained the same between $GA_3$ and BAP treated seeds subjected to 12 weeks of cold stratification afterwards (Table 4).

*Table 4.* Germination capacity and mean germination time of *J. polycarpos* seeds in response a combined treatment of hormones (500 ppm of BAP and $GA_3$) and cold stratification for 12 weeks (mean ± SE). Means followed by the same letter across the column for each stratification treatment are not significantly different using Tukey's test ($\alpha = 0.05$).

| Treatment | GC% | MGT (days) |
|---|---|---|
| Control | 6 ± 1a | 23.4 ± 0.6A |
| BAP + cold stratification | 31 ± 2b | 15.5 ± 0.3B |
| $GA_3$ + cold stratification | 48 ± 2c | 14.9 ± 0.2B |

Exogenous application of phytohormones, such as GAs and BAP, alone or in combination with cold stratification induces dormancy release and germination in some species, depending on the concentration and length of incubation (Hidayati *et al*., 2000; Baskin and Baskin, 2001; Sivakumar *et al*., 2006; Ahmadloo *et al*., 2015). The mechanism of dormancy release and germination stimulation by $GA_3$ treatments is often attributed to the mobilization of stored reserves (Bewley and Black, 1994) and weakening of the mechanical resistance of the endosperm cells around the radicle tip due to an increased activities of cell wall degrading enzymes (Downie *et al*., 1997). Cytokinins are also opined to contribute to the promotion of dormancy release and germination by enhancing ethylene biosynthesis, which is implicated in the promotion of germination in some species (Matilla, 2000; Kucera *et al*., 2005). In the present study, germination decreased with increasing concentration of the hormones; while the germination of $GA_3$- and BAP-treated seeds that were subsequently cold stratified for 12 weeks was 31% and 48%, respectively. Apparently, the hormone treatment alone or in combination with cold stratification is not effective in breaking dormancy and stimulating germination of *J. polycarpos* seeds.

# 5 Conclusions and implications for active restoration

Poor seed lot quality is a major barrier for the regeneration of *J. polycarpos* in Iran. The findings presented in this thesis demonstrate that infection by juniper dwarf mistletoe and production of large quantities of non-viable seeds due to pollination failures and post-zygotic degeneration coupled with some degree of insect attack are the major factors influencing seed quality.

The study provides evidence that moderate infection by juniper dwarf mistletoe has a negative effect on the reproductive output of its host by significantly reducing the mean number of cone per unit area of the host crown, increasing the number of damaged seeds, reducing seed size and seed germination. The low germination performance of *J. polycarpos* seed lot after cleaning using the conventional nursery practice (flotation on water) is due to the occurrence of a large quantity of shriveled seeds in the sunken fraction. Such unproductive seeds can be effectively removed using the IDS or SG separation techniques.

The best IDS conditions are seven days incubation at 15 °C, nine hours of drying and sorting in water or 200 g/l sucrose solution. For SG separation, soaking seed for 48 hours followed by sorting in 600 g/l is the best condition to get high recovery of viable seeds in the sunken fraction. NIR spectroscopy has successfully discriminated viable from non-viable seeds; thus it would be an efficient and rapid technique once on-line sorting system is available. For now, NIR spectroscopy can serve as a rapid technique to estimate seed crop and guide decisions during seed collection, as seed yield in *J. polycarpos* varies from year to year.

Another factor that hinders the production of *J. polycarpos* seedlings in the nurseries is seed dormancy. Dormancy in juniper species is variously contemplated as physiological or morpho-physiological; but the findings in this thesis suggest that morpho-physiological dormancy is the most likely dormancy type in *J. polycarpos* seeds, and tentatively the level of dormancy is opined as intermediate simple morpho-physiological dormancy. This class of dormancy could be an adaptive mechanism for relatively warmer autumn temperature during seed maturation and the subsequent cold winter temperature under natural conditions. Warm stratification for 16 weeks at 20°C followed by 12 weeks of cold stratification at 1°C induces 72% of the seeds to germinate in 12 days.

Juniper forests once occupied large mountainous terrain in the north, north-west, central and southeast highlands of Iran but today their populations are highly fragmented due to anthropogenic disturbance. Owing to their immense economic and ecological importance, interests in conserving and restoring of degraded juniper forests have been continuously growing. Its ability to grow on poor soil conditions where no other species can thrive coupled with its tolerance to harsh climatic conditions make *J. polycarpos* a candidate species for reforestation/restoration program. Previous efforts to restore juniper forests in Iran using area closure method; i.e., passive restoration, has been insufficient due to long seed production cycle (seed mast year every 4-8 years) and poor seed lot quality. Thus, forest managers relying on natural regeneration (passive restoration) should consider enrichment planting as the number of filled seeds produced is low irrespective of the health status of the tree.

For active restoration, involving seedling planting, it is advisable to collect seeds from uninfected trees to get fairly good germination. In addition, nurseries in Iran could further increase the potential seedling production by adopting IDS technique compared to the current nursery practice, where flotation technique is used to sort out empty and insect-damaged seeds only. As sucrose is affordable and readily available, local nurseries can applying SG separation in 600 g/l sucrose solution as a simple alternative to IDS technique, which needs modern facility. Once the quality of seed lots is upgraded, warm stratification for 16 weeks at 20°C followed by 12 weeks of cold stratification at 1°C is recommended to break dormancy in *J. polycarpos* seeds.

# 6 Further research

As the studies presented in this thesis are the first attempt to systematically assess interaction between juniper dwarf mistletoe and its host in the Central and South Western Asian (c.f. Sarangzai et al. 2010), we recommend stand level dwarf mistletoe inventory to confirm whether the observed severity of infection in the present study is a stand level phenomenon. Further study is also needed to understand the mechanisms of selection of hosts and the distribution of infection within individual trees and across the landscape.

To further improve the sorting efficiency of the IDS technique, it could be beneficial to test drying times between 9 and 12 hours and concentrations of sucrose solutions between 200 and 250 g/l. For SG separation, manipulating the concentration of sucrose solution in the range between 600-800 g/l is recommended. Although the findings suggest that *J. polycarpos* seeds possess morpho-physiological dormancy, further study is recommended to determine the critical embryo length, examine whether GA substitutes for cold stratification and whether alternating the temperature regime shortens the length of warm-cold stratification, as these treatments have been opined to alleviate intermediate morpho-physiological dormancy (Baskin and Baskin, 2001). Further study should also be made to optimize the warm-cold stratification treatments for seeds from different elevation of each region and from different provenances among the natural distribution of species around the country (mid-north, northwest, central, and southern highlands).

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