EFFECTS OF CRYOPRESERVATION AND RELATIVE HUMIDITY ON VIABILITY AND NUTRITIONAL COMPOSITION OF SAINFOIN (ONOBRYCHIS VICIFOLIA SCOP.)

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ABSTRACT
Changes in seed viability due to environmental conditions are crucial factors to preserve seed germination ability in equatorial zones. Influence of relative humidity (RH) and frozen storage on the seed viability, nutritional compositions, trace metals and macro-nutrients of three Onobrychis viciifolia varieties (Golpaygan-181, Orumieh-1763 and Gorgan-1601) were investigated. Mean relative humidity of 84% hastened the loss of seed viability during 6 months, while frozen seeds at -20°C only exerted a minimal deteriorative effect on the germination of seed and vigor index. HPLC profiles differed in peak areas of the two important alkaloids, Berberine and Sanguinarine, in ambient humidity compared with control condition. Accumulation of crude protein (CP) and dry matter digestibility (DMD) were enhanced under ambient humidity and frozen storage in seed cells. Relative humidity indicated positive effects on seed quality by crude fibre (CF) reduction. The atomic spectroscopy analysis confirmed the increment of trace metals ratio (Fe, Mn and Cu) and reduction of macro-nutrients ratio (Ca, P and N) in frozen storage after 6 months. Although vigor index and germination speed were lower in frozen storage seeds compared with control seeds, the notable results can be optimized in future studies.

Keywords: viability, relative humidity, frozen storage, crude protein, alkaloid.

INTRODUCTION
Seed quality and viability determine which type of seeds can be maintained longer in the market. Seeds are kept as viable regenerative organisms until the place and time are suitable for the starting of a new generation. Nevertheless, they cannot maintain their viability forever and they ultimately deteriorate and perish (Copeland and McDonald, 1995). Furthermore, a crucial condition for effective protection of germplasm is maintenance of seed viability. Seed viability is the set of the qualities that influences the activity and performance of seed germination in different ecological circumstances (ISTA, 2009). The deterioration procedures consist of enhancing the free radical content, changing in protein structure, reducing of food reserves, developing of fat acidity, transforming in enzymatic activity, damaging membrane, chromosomal changes and increasing of respiration (Sastry et al., 2008).

Seed longevity is influenced by storage conditions, genetic and physiological factors. According to many previous researches, speed of deterioration is mainly determined by storage temperature, seed quality, relative humidity, seed moisture content and type of seeds (Yin et al., 2000). Most significant causes of seed longevity are temperature and relative humidity, even though the humidity percentage is generally more effective than temperature (Ellis et al., 1985). The decline of germinability is also related to the hygroscopic nature of seeds, especially under warm temperatures, which is associated with the relative humidity of the surrounding air (Khaldun and Haque, 2009).

Rao et al. (2006) stated that seed deterioration could be understood on the basis of relative humidity and storage temperature. Although they concluded that adoption of appropriate temperature storage and relative humidity technique would significantly affect the seed quality, Sisman and Delibas (2004) suggested that storage is used to keep the harvesting quality of seed not to improve it. However, it is necessary to improve the methods that increase the potential of seed viability at different environmental conditions (Coronado et al., 2007).

The present study was designed; to observe the effect of relative humidity and frozen storage on seed growth and quality, to investigate the feasibility of relative humidity and cryopreservation for improvement of alkaloid, macro-nutrients and trace metals found in the sainfoin seeds.

MATERIALS AND METHODS
Plant materials: Based on the previous study (Mohajer et al., 2011), seeds of three superior Onobrychis viciifolia varieties (Golpaygan-181, Orumieh-1763 and Gorgan-1601) were selected from the existing seeds at gene bank of natural resources in Iran. Some seeds were frozen at -
20 °C for 6 months, while some other seeds were kept at room temperature (Table 1) to assume the germination process at an interval of 2 months.

**In vitro culture:** Stock solution of MS media were prepared by dissolving the constituent’s amount of salt, iron and vitamin solution in 1 liter of distilled water and kept in dark-colored bottles in a refrigerator. The recommended amount of each stock solution was added to distilled water up to 85% of final volume required for the medium preparation. To prepare the Murashige and Skoog medium (MS), 3% (w/v) sucrose and 0.77% (w/v) agar were added to stock solution. The pH of the medium was adjusted to 5.6–5.8 using 1N NaOH or 1N HCl. Autoclaving was carried out at 120 °C and 20 psi for 20 min. The cultured seeds were transferred to growth room and maintained at 25±2 °C, under 16 h photo-period and 8 h dark-period.

Germination Speed (GS), judged by the appearance of the radicle, was counted daily up to 7 days (ISTA, 1985); 

\[ \text{GS} \% = \left( \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \right) \times 100 \]

The vigor index (VI) value was also computed as described by Abdul-Baki and Anderson (1970) by multiplying germination of seeds and total seedling length in millimeter following the formula:

\[ \text{Vigor index} = \left( \text{germination percentage} \times \text{mean (radicle length + plumule length)} \right) / 100 \]

**HPLC-UV analysis:** The standard solution of sanguinarine and berberine were purchased from Sigma (USA). The chemical structures of the alkaloids are shown in Figure 1. HPLC system (Knauer K-2600) coupled with UV detector was used for quantitative determination of the two alkaloids in both control and stored seeds. The UV detector was set at the wavelength of 280 nm. The area was used for quantification. Chromatographic separation was carried out on a Kromasil C18 analytical column (5 μm, 250 mm×4.6 mm) at 30 °C. A linear gradient evaluation of A (100% acetonitrile) and B (0.1% phosphoric acid aqueous solution) was used. The time program for the multi-step gradient was: initial 27% (A), 0–5 min keeping 27% (A), 5–17 min linear gradient of 54% (A), 17–20 min from 54% to 75% (A), 20–25 min from 75% to 80% (A), 25–35 min keeping 80% (A), 35–40 min linear gradient of 27% (A), keeping 27% (A) at 40–45 min. The flow rate was 0.8 ml/min, and the injection volume was 5 μl. Calibration curves were drawn based on the reference (standards) areas against their respective concentrations.

**Quality Traits Assessment:** Percentage of crude fibre (CF), crude protein (CP), dry matter digestibility (DM), water soluble carbohydrates (WSC), acid detergent fibre (ADF), neutral detergent fibre (NDF) and ash of both ambient humidity and frozen storage seeds were compared with control seeds using near infrared radiation (NIR) spectroscopy. After calibration, percentages of quality traits were calculated following the method by Jafari et al. (2003).

**Atomic Absorption Spectrometry:** Seed powder was also analysed for different elements of Mn, Cu, Ca, P and N (based on the ratio of stored samples with control) according to the methods described by AOAC (2003) using atomic absorption spectrometry (Young Lin AAS-8020).

**Statistical analysis:** Results were expressed as means ± standard error. The effects of treatments were tested by variance analysis and differences between samples were determined by Duncan’s multi-range test at P<0.05 using SAS 9.2 software.

## RESULTS

The control seeds showed better performance in germination and vigor characteristics. Germination speed in ambient humidity and frozen storage revealed that percentages of all three varieties decreased significantly at room temperature, although frozen storage had the notable results after 6 months (Table 2). Despite the fact that high vigor index was observed in the frozen storage, approximately the seeds lost 50% of their vigor after 2 months in the humid condition.

The content of two alkaloids (Berberine and Sanguinarine) in both control and humid conditions were also measured in this study. The chemical structures of both berberine and sanguinarine are shown in Figure 1. A standard stock solution containing two reference components (berberine and sanguinarine) was prepared by serial dilutions to appropriate concentrations, and utilized to construct the calibration curves. Calibration curves were drawn based on the areas of the standards against their respective concentrations. Good linearity (R²=0.995) was achieved in both calibration curves of the alkaloids. Sanguinarine content was increased when subjected to ambient humidity from 0.000121% to 0.002281%. However, berberine content declined when exposed to relative humidity from 0.000152% to 0.0000931% after 6 months (Figure 2).

Percentage of crude protein and digestibility of seeds were significantly increased in ambient humidity and frozen storage compared with control seeds. Highest percentage of crude protein (35.03%) and digestibility (94.12%) were observed once the seeds were exposed to frozen storage for 6 months (Figure 3). Percentage of ADF decreased with increasing time of storage in ambient humidity, although the percentage of NDF rose up from 9.99% to 11.03% in frozen storage seeds. In spite of the fact that there were no statistically significant difference among the water-soluble carbohydrates (WSC) treatments, the highest percentage was observed in frozen samples with 34.70% (Figure 4). Furthermore, percentage
of crude fiber (CF) was reduced when the seeds were exposed to both frozen storage and ambient humidity. Effect of relative humidity and cryopreservation for ash percentage at probability level of 5% were not significantly different, but the treated samples in humid condition had the highest percentage after 6 months with 6.21% (Figure 5).

The amounts of trace metals (Fe, Mn and Cu) and macro-nutrients (Ca, P and N) in storage and control seeds were determined by using atomic absorption spectrophotometry (AAS). The ratio of different elements in storage samples to the control seeds was depicted in Figure 6. It was observed that exposure to relative humidity had negative effects on trace metals and Ca. Unlike the samples in ambient humidity, effect of frozen treatments increased the Mn, Fe and Cu content. Despite the fact that relative humidity increased the content of P, negative influence on N was indicated. Ultimately, the amount of other elements such as N, P and Ca were found to decrease under frozen condition (Figure 6).

Table 1. Temperature and Relative Humidity range

<table>
<thead>
<tr>
<th></th>
<th>APR</th>
<th>MAY</th>
<th>JUN</th>
<th>JUL</th>
<th>AUG</th>
<th>SEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>T ºC 24 hour mean</td>
<td>26.8</td>
<td>27</td>
<td>26.7</td>
<td>26.4</td>
<td>26.4</td>
<td>26.2</td>
</tr>
<tr>
<td>T ºC Daily max. mean</td>
<td>32.8</td>
<td>32.7</td>
<td>32.3</td>
<td>31.9</td>
<td>32</td>
<td>31.8</td>
</tr>
<tr>
<td>RH% 24 hour mean</td>
<td>84.9</td>
<td>84.3</td>
<td>83.9</td>
<td>83.4</td>
<td>83.1</td>
<td>84.6</td>
</tr>
<tr>
<td>RH% Daily max. mean</td>
<td>98.4</td>
<td>98.2</td>
<td>98.1</td>
<td>97.8</td>
<td>97.9</td>
<td>98.3</td>
</tr>
</tbody>
</table>

Source: Director General Meteorological Service-Malaysia (2013)

Table 2. Effect of ambient humidity on vigor index and germination speed of *Onobrychis viciifolia*

<table>
<thead>
<tr>
<th>Humid condition</th>
<th>Varieties</th>
<th>Control</th>
<th>Two months</th>
<th>Four months</th>
<th>Six months</th>
<th>Frozen (6 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS (%)</td>
<td>Golpaygan-181</td>
<td>92.7±2.1</td>
<td>62.3±2.4</td>
<td>37.9±1.9</td>
<td>4.3±1.2</td>
<td>86.5±2.5</td>
</tr>
<tr>
<td></td>
<td>Orumieh-1763</td>
<td>94.4±2.2</td>
<td>60.3±2.6</td>
<td>32.6±2.1</td>
<td>2.1±1.1</td>
<td>82.3±2.4</td>
</tr>
<tr>
<td></td>
<td>Gorgan-1601</td>
<td>98.3±3.1</td>
<td>61.2±2.1</td>
<td>32.8±1.8</td>
<td>2.4±1.1</td>
<td>81.4±2.4</td>
</tr>
<tr>
<td>VI</td>
<td>Golpaygan-181</td>
<td>61.8±1.5</td>
<td>28.6±1.3</td>
<td>12.5±1.4</td>
<td>1.1±0.6</td>
<td>59.6±1.6</td>
</tr>
<tr>
<td></td>
<td>Orumieh-1763</td>
<td>66.8±1.5</td>
<td>28.4±1.5</td>
<td>11.2±1.6</td>
<td>0.8±0.4</td>
<td>60.5±1.4</td>
</tr>
<tr>
<td></td>
<td>Gorgan-1601</td>
<td>76.8±2.3</td>
<td>31.2±1.7</td>
<td>8.6±1.1</td>
<td>0.8±0.3</td>
<td>72.6±1.9</td>
</tr>
</tbody>
</table>

The means of the treatments with same connotations were not significantly different as per Duncan’s multi-range test at P<0.05.

VI: Vigor Index; GS: Germination Speed

Figure 1: Chemical structures of two alkaloids identified in *Onobrychis viciifolia*
Figure 2: HPLC chromatograms of control seed and ambient humidity according to standard mixture.
NB: Retention time has been adjusted based on the centesimal unit in this experiment.

Figure 3: Mean comparison of digestibility and crude protein after influence of humidity and freezing

The means of the treatments with same connotations were not significantly different as per Duncan's multi-range test at \( P<0.05 \).
Figure 4: Mean comparison of acid detergent fiber, water soluble carbohydrates and neutral detergent fiber after influence of humidity and freezing

The means of the treatments with same connotations were not significantly different as per Duncan’s multi-range test at P<0.05.

Figure 5: Mean comparison of ash and crude fiber after influence of humidity and freezing

The means of the treatments with same connotations were not significantly different as per Duncan’s multi-range test at P<0.05.
DISCUSSION

Several studies have documented the positive effects of relative humidity (RH) on growth of plants after cultivation, including wheat (*Triticum aestivum* L.), sugar beet (*Beta vulgaris* L.) and kale (*Brassica oleracea* L.) by Ford and Thorne (1973) and sweet-potato (*Ipomoea batatas* Lam.) by Mortley *et al.* (1994). In this regard, only a few researches have focused on the effect of relative humidity (RH) and frozen storage on seeds before implanting. In the current study, the germination percentages of all the three investigated varieties decreased from 98.3% to less than 5% under humid condition after 6 months. Although variety of Orumieh-1763 had more notable germination results compared with variety Golpoygan-181 in prior study (Mohajer *et al.*, 2013), it was less resistant to deterioration of humid condition after 4 and 6 months.

Ellis *et al.* (1982) reported a negative relationship between relative humidity and longevity. It has been found that the significant factors which influence the seeds longevity in storage are moisture content, temperature and oxygen pressure (Copeland and McDonald, 1995). Generally, longevity increases with decreasing temperature and relative humidity (Murdoch and Ellis, 1992; Copeland and McDonald, 1995). Deterioration effect by high humidity causes damage to cell membranes, prevents cellular building and destroy the enzymes activities (Hampton and Tekrony, 1995). Changes in protein and sugar were also observed during seed deterioration (Kapoor *et al.*, 2010), which was confirmed in this study. Increment of crude protein showed decomposing or composing of other factors to crude protein. The quality and nutritional value of seeds were directly correlated with crude protein (CP) and dry matter digestibility (DMD), and were inversely correlated with acid detergent fiber (ADF) and crude fiber (CF) (Mohajer *et al.*, 2011).

Seed moisture content and its interaction with relative humidity play an important role in seed longevity (Roberts, 1973). Also high relative humidity increases seed moisture content, which results in biochemical events such as enhanced respiration, enzyme activity and increment in free fatty acids. Based on De Villiers *et al.* (2000) study, once the relative humidity reached to 70%, the moisture content of seed increased to 13%, which enhanced probability of fungi infection. It has also been stated that longevity decreases to half by each one percent increasing of seed moisture content (Bewley and Black, 1994).

The average of relative humidity is about 85% during the year in Malaysia and equatorial zones. In reality, if the seeds are stored in an open ambient or outbuilding, the relative humidity damages the viability of seeds. However, high summer temperatures and high relative humidity increase seed deterioration and consequently, seed viability and longevity fall off.

Collected seeds of perennial species like *Onobrychis vicifolia* should not be stored for too long, since they are probably not as long-lived as seeds of the annual species. It was indicated that the longevity of seeds was not much negatively affected by subfreezing temperature. This has been established by a number of published reports by germination testing on a wide variety of vegetables, flowers and herb seeds at -7 °C for a long period of time (De Villiers *et al.*, 2000; Sisman and Delibas, 2004).

**Acknowledgements:** The authors would like to thank the
University of Malaya, Malaysia for the facilities and financial support provided (UMRG grant RP025-2012A).

REFERENCES


