CHAPTER 9

Effect of Storage Conditions and Storage Periods on Seed Germination

9.1 Introduction

Storage of seeds as *ex situ* germplasm is an essential step for the long term conservation of plant genetic resources. Maintaining seed viability for longer period is very essential to preserve the genetic integrity in stored samples. Since very early days, simple techniques have been adopted to maintain the seed viability in both domesticated and wild sources (Ellis *et al.*, 1991b, 1992; Onyekwelua and Fayose, 2007; Roberts, 1973, 1975; Vertucci and Roos, 1990, 1991). Inappropriate storage medium (Hezewijk *et al.*, 1993; Muller *et al.*, 2011) such as room temperature storage often results in low seed germination, seed deterioration and loss of viability, which is a natural phenomenon during storage (Nasreen *et al.*, 2000; Schmidt, 2002). Several factors, viz., temperature, nature of the seeds, seed moisture content, relative humidity, etc., influences seed longevity during storage (Roberts, 1972; Bonner, 1990; Gordon, 1992; Copeland and McDonald, 1994; Bradbeer, 1998; Takos, 1999; Butola and Badola, 2004ab; Yang *et al.*, 2005; Onyekwelua and Fayose, 2007). There is a close relationship between the loss of seed viability during storage and the accumulation of genetic damage in the surviving seeds, (Ellis and Roberts, 1981; Roos, 1982; Rao *et al.*, 1987). Seed moisture content, temperature and storage periods are among the main factors affecting above relationship (Roberts, 1988). Slight increase in temperature and moisture may promote fungal growth (Roberts, 1972) and insect development in seeds (Christensen, 1971). According to Harrington (1973), 1% rise in moisture content and 5^0C in temperature reduces the half storage life of seeds. Depending on the duration and method adopted, drying and long term storage may lead to considerable reduction in germination or to eventual death of the seeds. Before storage, if the seeds are not properly dried, the high moisture content may reduce the seed viability by promoting fungal growth. Such deterioration could further results in decline of seed germination capacity (Romanas, 1991). Proper storage conditions however, may effectively retain substantial viability in seeds over a considerable

storage period (Butola and Badola, 2004ab; Chen *et al.*, 2007). Such approaches are especially crucial in case of endangered species, where judicial use of seeds as valuable genetic material through standardizing proper storage mechanism is a precondition to strengthen species conservation programme.

Swertia chirayita (Roxb. ex Fleming) H. Karst (Gentianaceae) is one of the highly marketed (national and international) endangered medicinal herbs of Himalaya and prioritized at the top for the conservation through *ex-situ* cultivation by an international experts' exercise (Badola and Pal, 2002). Critically endangered standing of *S. chirayita* (Ved *et al.*, 2003b) further necessitates its propagation and mass multiplication, for which a protocol targeting an appropriate and relatively longer period of storage of seeds would be vital and urgent need of the time. This will strengthen both *in-situ* conservation as well as *ex-situ* cultivation of the species. We assessed the seeds of different populations of *S. chirayita* under long term storage, as an initial effort, but the germination testing was limited to only 4^0C storage (refer chapter 7). As an erect, about 3-5 ft, biannual or triennial herb S. *chirayita* is locally known as Chirowto or Pothi Chirowto or Kalo Chirowto, and distributed in temperate Himalaya from Kashmir, Nepal, Bhutan along 1200-3000m asl (Kirtikar and Basu, 1984). The stems are robust, branching; the leaves are broadly lanceolate; the flowers occur in large panicles and are lurid greenish yellow tinged with purple; the capsules are egg-shaped, many-sided, and sharp pointed; the seeds are smooth and many-angled. The breeding in *S. chirayita* is through self pollination (Chakroborty *et al.*, 2009); however, cross pollination in the species has also been reported (Khoshoo and Tendon, 1963). S. *chirayita* bears huge pharmacological importance (see chapter 1); whole plant is used in traditional medicine; however the root is mentioned to be the most effective and bitter part. Plant has anti-inflammatory (Chowdhary *et al.*, 1995; Banerjee *et al.*, 2000), hypoglycemic (Bajpei *et al.*, 1991; Banerjee *et al.*, 1994; Chandrasekhar *et al.*, 1990), anti-pyretic (Bhargava *et al.*, 2009), anti-fungal, anti-bacterial, anti-malarial (Bhat and Surolia, 2001) and anti-oxidant (Scartzzini and Speroni, 2000) properties, and considered to be a bitter tonic, febrifuge and laxative and is used in fever, burning of body, intestinal worms and skin diseases etc. *S. chirayita* is used in all kinds of fever in a variety of forms and in combination with

other medicines (Dutt and King, 1877). Entire plant contains gentiamine alkaloids, and xanthones is found in aerial part (Sharma, 1982). Community people in Sikkim use the juice, obtained through boiling the entire plant, to cure fever, cold, cough, diarrhea, and stomach-ache (Pradhan and Badola, 2008). Also, plant is used in mental disorders (Chowdhary *et al.*, 1995), effective in curing gastric ulcers (Rafatullah *et al.*, 1993), liver diseases (Krishnaraju *et al.*, 2005), and possess anti-carcinogenic properties (Lin *et al.*, 1996; Das *et al.*, 2004). Further, the species is practiced in the preparation of herbal drug, Diabecon (Kohli *et al.*, 2004), D-400 (Sundaram *et al.*, 1996), and Himoliv (Bhattacharya *et al.*, 2003).

The present study was undertaken to test the hypotheses: (1) seed germination varies amongst the population, (2) storage conditions and storage period affects seed germination in *S. chirayita.* The study was conducted on eleven populations from different parts of Sikkim, India. Till present reporting, there is no publication available on the effect of long term storage on seed germination of *S. chirayita*, and first time covering different storage conditions.

9.2 Materials and Methods

9.2.1 Experimental design

During November-December, freshly ripened seeds of *Swertia chirayita* were collected from 11 populations located along 1600m asl to 2700m asl in different parts of Sikkim Himalaya, India ($27^0$04' – $28^0$07' North and $88^0$00' – $88^0$55' East). Seeds from four of the above populations (Sc1 to Sc4), earlier tested from 2005 collection for storage at 4^0C (refer chapter 7), were recollected after one year time in 2006. Seeds from all populations were tested for three storage conditions over time. During seed collection, 15 – 20 plants/site were selected randomly from the middle of each population, to avoid edge effect if any and not to disturb natural spread of the population, practicing sustainable harvesting. The seeds procured were pooled separately for each population and brought to the laboratory, cleaned thoroughly for impurities, and dried in room temperature for 15 days. Seed moisture content was determined by oven drying (60^0C; 48 hrs) 50 seeds in 3 replicates from each

population. For each population 10 healthy fruits were considered for seed counting. Seed size was measured using 30 seeds per population under microscope (10 seeds each in 3 replicates). Seed viability test using 2,3,5, triphenyle tetrazolium chloride solution could not be conducted because of the minute seed size and the difficulty in finding the detached embryo. For each population, immediately after room drying, seeds were tested for their initial germination potential. The remaining seed lots were stored in three different experimental conditions, viz., room temperature (25 $\pm$ 5^{0}C), and in refrigerator at 4^{0}C and at -15^{0}C, in properly sealed specimen tubes. The seeds were periodically tested for their germination viability at six months interval for next 24 months.

For each germination test, seeds were at first disinfected with Sodium hypochlorite solution (4% w/v available Chlorine) for 5 sec. to reduce the incidence of fungal attack. Disinfected seeds were washed thoroughly with double distilled water (DDW) and soaked in DDW for 24 hours. The soaked seeds were placed in Petri-plates (90mm dia.) lined with single layer filter paper (Whatman No. 1) saturated with DDW. For each population, three replicates of 30 seeds each for each of three storage condition were used; and Petri-plates then placed in a seed germination chamber at 25^{0}C $\pm$ 2^{0}C; with alternating light (14/10 hrs. photoperiod). The experiment was performed in a randomized design. The seeds were kept moist (DDW) and checked every day. Visible protrusion of the radical was the criterion to score seed germination. The germinated seeds were counted and removed. Seeds were observed daily until constant reading obtained. The germination experiment was observed up to 45 days in each of the testing at six months interval.

9.2.2 Statistical analysis

MANOVA (multivariate analysis of variance) was performed using General Linear Model in SPSS 10.0 for windows (SPSS Inc. 1989) to determine the effect of populations, storage conditions and storage period and their interaction on seed germination percent and mean germination time (MGT). MGT was calculated using equation, $MGT = \sum(nd)/\sum N$; where n = number of newly germinated seeds after each incubation period in days d, and N = total number of seeds germinated at the end

of the test (Hartmann and Kester, 1989). Bonferroni test ($p<0.0001$) was employed to determine the variation in means of seed germination and MGT. The present experiment was designed to determine aging rates of seeds of *S. chirayita* stored in different storage conditions. Percent germination value obtained for the different populations prior to storage served as control value and a way to evaluate the variability in percent germination for periodical storage period. By recording changes in percentage germination and the time required for seeds to germinate, deterioration was evaluated.

9.3 Results

Populations of *Swertia chirayita* differed significantly for various seeds characteristics (Table 20). MANOVA revealed significant variations in seed germination percent and mean germination time amongst populations, storage conditions and storage period and their interaction (Table 21). At the initial test, seed germination percent ranged from 87.78% (Sc5) to 100% (Sc2) and showed negative correlation with altitude of the collection sites (r = -0.087). Minimum 12 to 16 days (average: 13 days) was required for onset and 20 to 40 days (average: 31 days) for the completion of seed germination in most of the populations for all the three storage conditions and storage period (Figure 26). Collectively, significant reduction in germination percent was observed with the elapse of storage period compared to initial test ($p<0.0001$) in populations of *S. chirayita*. Cumulatively for all the populations, seeds stored at 4^0C showed significantly ($p<0.0001$) higher germination percent than -15^0C and room temperature stored seeds. Similarly, the seed germination was significantly higher in -15^0C stored seeds over room temperature stored seeds ($p<0.0001$). For the seeds stored at 4^0C, 73% (8 populations) of the total populations recorded over 90% seed germination after 6 months; 64% (7 populations) of the total populations recorded over 80% germination after 12 months; 91% (10 populations) of the total populations recorded over 60% germination after 18 months and 70% (8 populations) of the total populations recorded over 50% seed germination after 24 months of storage (Figure 27A). For the seeds stored at -15^0C, Sc3 recorded highest and Sc10 recorded the lowest germination after 6 months, Sc1 and Sc10 recorded the highest and the lowest seed germination after 12 and 18 months of

storage, respectively; however, after 24 months of storage, Sc2 recorded the highest and Sc6 recorded the lowest seed germination (Figure 27B). Room temperature storage resulted in above 80% seed germination in 82% (9 populations) of the total populations after 6 months. Here, the percent seed germination ranged between 48.89% (Sc9) to 73.33% (Sc3) after 12 months; 45% of the total populations recorded above 50% germination after 18 months, and the seed germination recorded below 33% in 82% of the total populations after 24 months of storage (Figure 27C).

Significant variation ($p<0.0001$) in mean germination time (MGT) was observed amongst the storage conditions compared to the initial test. For example, seeds stored for 12 months at 4^0C recorded lowest MGT in all the populations (Figure 28A); similarly, 64% of the total populations recorded lowest MGT after 6 months of storage at -15^0C (Figure 28B) and 12 months of storage in room temperature (Figure 28C). Significant fall ($p<0.01$) in seed germination was observed in all the three storage conditions with the increase in storage time (Figure 29). The rate of fall in germination percent per 6 month was higher in room temperature (15.86%) followed by deep freeze (13.26%) and 4^0C (10.89%) stored seeds. At this rate of fall, seed looses its viability completely by approximately 36 months in 4^0C stored seeds which is slower compared to room temperature and deep freeze (Figure 29).

9.4 Discussion

To retain good seed longevity, 10% to 40% seed moisture content is desirable (Hampton and Hill, 2002). Improper drying of seeds results in rapid loses of viability during storage owing to high moisture content (Siddique and Wright, 2003). According to McDonald (2004), seed loses viability and vigor during processing and storage mainly because of high seed moisture content, i.e. greater than 18%. In our present study, however, seeds of *S. chirayita* from all 11 populations maintaining with 19% to 44% moisture content after drying resulted in high percent germination for the freshly collected seeds, which helped retaining substantial viability under storage for longer period. We recorded 100% germination in freshly collected seeds of *S. chirayita* for majority of populations with 16 to 42% moisture content (refer Chapter 7). However, Bhatt *et al.*, (2005b) recorded low seed germination with the moisture

content of 22% to 29% in freshly collected seeds of *Swertia angustifolia*. In our study, even after maintaining the moisture content of 15% to 21% in the domesticated seeds of *S. chirayita*, low germination at the initial testing was observed (refer chapter 11). This indicates species specific requirement of seeds for desirable moisture content for long term viability.

The length of time for which the seeds remain viable varies greatly by species and storage conditions (Siddique and Wright, 2003). Our present investigation, testing seeds for long term storage (at 4^0C, -15^0C and room temperature) confirm that the storage temperature significantly affects the seed germination capacity as stated by (Bradbeer, 1988). Our study indicates that the seed deterioration rates may vary depending on the storage conditions, however, the germination percent and/or seed viability gradually declines with increase in storage period (Yilmaz and Aksoy, 2007) irrespective of different storing conditions. The reported variability in percent seed germination for the seeds collected from different populations at same time or from same population at different times even if they are provided with the same treatments or test conditions (Perez-Garcia *et al.*, 1995; Baskin and Baskin, 2001) is not applicable to *S. chirayita* in Sikkim Himalaya. Our present study with *S. chirayita* revealed that seeds collected from the same location for two different years in case of four populations, i.e. Sc1, Sc2, Sc3 and Sc4 after a year gap resulted in 98% to 100% germination (roughly the similar trend) in comparison to the earlier result of 100% germination for the same populations (refer chapter 7). This is an indication that the initial germination potential may remain similar over different times if right collection strategy is maintained, especially the time of collection and selection of same spots.

In our present study, six month storage did not show much variation in percent germination among the storage conditions suggesting the suitability of all three conditions for short-term storage, which is often practiced in several Himalayan herbs (Butola and Badola, 2004ab, 2006a). For 4^0C storage, all the population showed higher percent germination after 12 months, comparing to -15^oC storage. In general, above 60%, 70% and 80% germination after 12 month storage in room temperature, at -15^0C, and at 4^0C, respectively, suggest that up to 12 months, seed storage in room

temperature can also be opted provided due care be taken in respect of moisture content level prior to storage. After 18 months, abrupt fall in percent seed germination below 48% in 55% populations indicates the room temperature as non appropriate condition of storing seeds for longer period in *S. chirayita*. Seed storage at -15^{0}C maintained > 60% germination with majority of the populations (7 populations) after 18 months of storage. Continued storage for 24 months at -15^{0}C resulted in decrease in seed germination (below 50%) for nine populations, which suggests this as unsuitable condition for long term seed storage.

Comparatively, seeds stored at 4^{0}C resulted in higher percent germination than the seeds stored at room temperature and at -15^{0}C, on subsequent testing. For all three storage conditions, progressive and significant reductions in percent germination were observed with increasing duration of storage. On an average basis, loss of seed viability was much faster at room temperature followed by -15^{0}C stored seeds but gradual at 4^{0}C suggesting it as most appropriate condition for the long term storage of *S. chirayita* seeds. Chauhan and Nautiyal (2007) reported much faster loss of seed viability at room temperature (10-35^{0}C) and retaining of seed viability for more than two years (storage at 0-4^{0}C in refrigerator) in *Nardostachys jatamansi*. Onyekwelua and Fayose (2007) stated that the seeds cannot be stored at sub-zero temperature probably due to freezing injury resulting from ice formation, which can alternatively be controlled by placing them in air tight containers. Our present study suggests that storing seeds of *S. chirayita* at -15^{0}C can be a second option after 4^{0}C; however, here the seeds loose their viability early compared to 4^{0}C even if they are placed in air tight containers. Many studies reported that the seed storage at 4^{0}C was effective for germination after 6 months (Butola and Badola 2004b) to 12 months (Chen *et al.*, 2007; refer chapter 7). In several other species, loss of seed viability is observed within a few months of storage at room temperature (Douglas, 1995; Verma *et al.*, 1996). Seed moisture content is adjusted as per the relative humidity of the surrounding air which changes with the air temperature and seeds differ in the way they adjust their moisture content to humidity. In refrigerator (4^{0}C), both temperature and the relative humidity are properly maintained thereby retaining the seed viability for longer period, relatively.

For *Swertia chirayita*, in our study (see chapter 7), an increasing trend in MGT was recorded after six months of storage at 4^0C, on subsequent testing for 18 months. Whereas, in our current study with four re-tested populations (Sc1 to Sc4) in second year and the remaining sets of populations, the case little differed as MGT declined till 12 months in majority of the populations, and then MGT enhanced subsequently when provided with similar storage condition. Similar trend in MGT reduction was observed for the seeds stored in room temperature. Seed moisture content plays a critical role in the seed germination process. The increase or decrease in the moisture content during the storage period of seed may be responsible for such variation in the MGT value which requires further detailed investigation for *S. chirayita*. For the seeds of *Kochia prostrate* stored in laboratory and under shed, mean germination time decreased as storage time increased, and varied unpredictably for cold room-stored seed, and remained unchanged for freezer-stored seed (Kitchen and Monsen, 2001), is not applicable to *S. chirayita*. Non-uniformity in MGT observed in the seeds on subsequent tests is rather confusing in the case of -15^0C stored seeds in *S. chirayita*. However for the seeds stored in room temperature and at 4^0C, the MGT first decreased up to 12 months of storage compared to initial test, which increased with the prolonged storage period.

9.5 Conclusion

Our present study confirms that various populations of *Swertia chirayita* in Sikkim Himalaya possess high germination potential. It also showed that the seed germination (1) varies amongst the populations; (2) is highly affected by the storage condition and the storage period. It further confirms that the 4^0C is the best/effective storage condition for the seeds of *S. chirayita* that retains seed viability for the longer period than other storage conditions. Also, for the shorter period up to one year, the other storage conditions may be opted, provided due care taken during storage. Equally, important is the recommendations on sustainable harvesting taking care of precise time and exact spot in the site, where earlier collection were made in case of using potential populations fro conservation programmes in case of endangered species.

Table 20. Seed characteristics of different plant population of *Swertia chirayita* collected in Sikkim

Name of Populations	Altitude (m asl)	No. Seeds/fruit	Seed weight (mg/50 seeds)	Seed length (µm)	Seed width (µm)	Moisture content (%)
Sc1 - Luing (ES)	2126	209	1.5	486	355	25.8
Sc2 - Railgaon (ES)	1948	297	1.6	532	383	22.4
Sc3 - U Pangthang (ES)	2176	309	1.6	502	380	29.1
Sc4 - Jaunbari (SS)	1651	222	1.2	454	374	39.1
Sc5 - Tiffin dara 2 (SS)	1744	269	1.4	406	362	26.7
Sc6 - Dhupi Dara (ES)	2124	258	1.8	418	378	18.8
Sc7 - Deewani Taar (WS)	2055	294	1.2	358	325	36.5
Sc8 - Gumpa Dara (WS)	1987	260	1.2	372	351	29.7
Sc9 - Hilley (WS)	2697	260	1.4	389	369	44.3
Sc10 - Tendong (SS)	2099	230	1.6	390	372	27.0
Sc11 - Raavngla (SS)	2160	256	1.3	320	297	25.6
(P<0.05)		38.49	0.14	21.53	21.14	10.54
F value		5.23	15.95	76.93	12.94	4.23

ES: East Sikkim; WS: West Sikkim; NS: North Sikkim; SS: South Sikkim

Table 21. Result of Three way ANOVA showing the effect of populations, storage conditions and storage periods and their interaction on seed germination and mean germination time in *Swertia chirayita*

Source	F value	Sig.	F value	Sig.
	Germination (%)		Mean Germination Time	
Population (P)	14.443	.000	42.392	.000
Storage Condition (SC)	279.387	.000	716.784	.000
Storage Period (SP)	1038.901	.000	519.862	.000
P x SC	8.914	.000	9.144	.000
P x SP	3.993	.000	5.728	.000
SC x SP	6.789	.000	147.827	.000
P x SC x SP	1.810	.001	4.878	.000

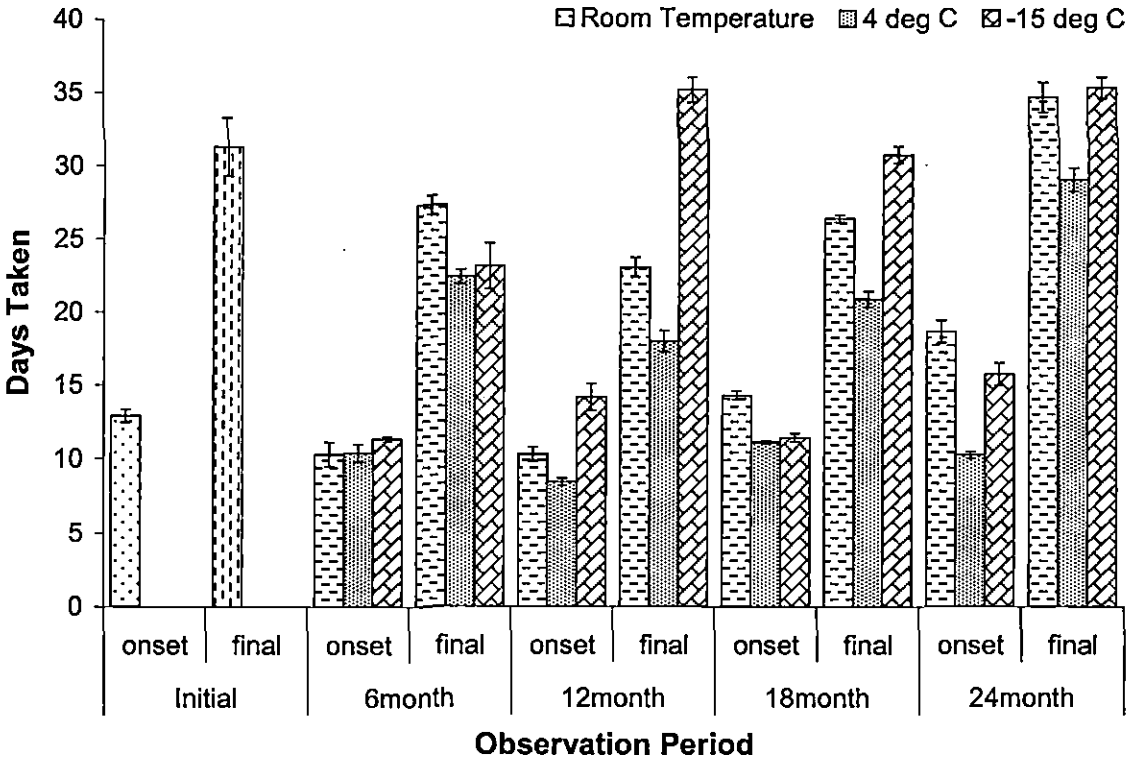

Figure 26. Storage effect on onset and final seed germination in *Swertia chirayita*

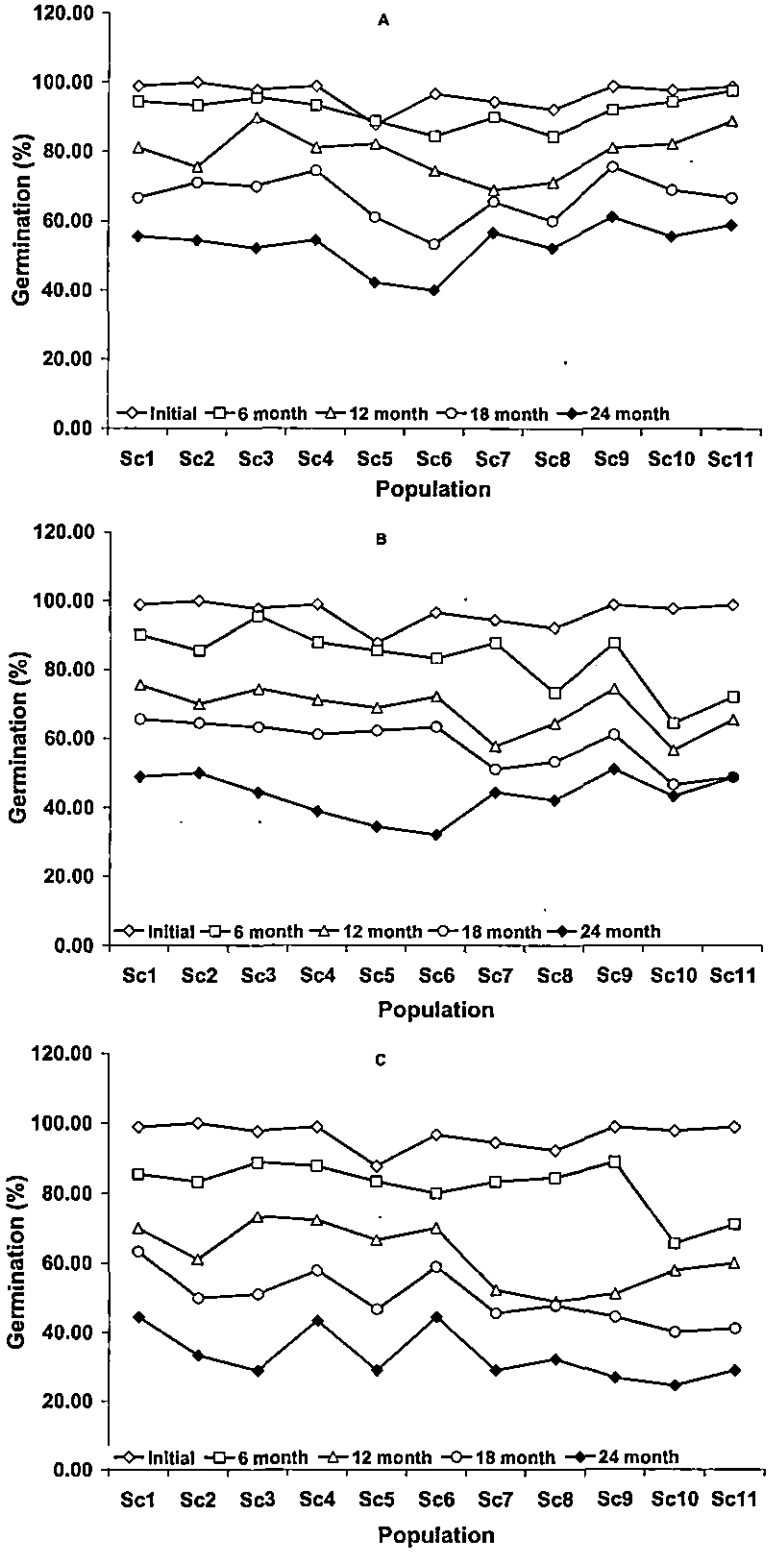

Figure 37. Effect of storage conditions and storage period on seed germination in populations of *Swertia chirayita*; A: 4^0C; B: -15^0C; C: Room temperature

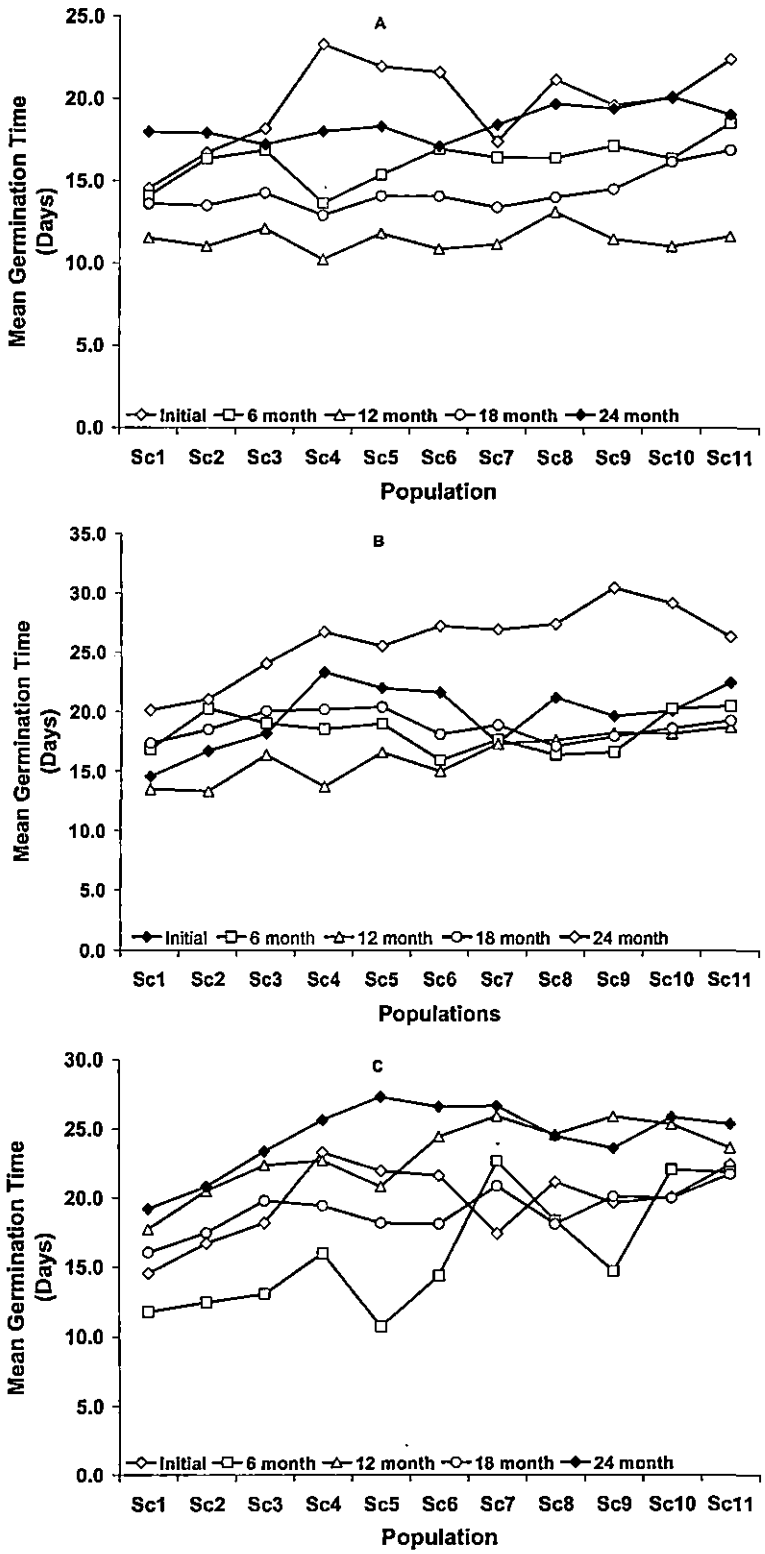

Figure 28. Effect of storage conditions and storage period on mean germination time in populations of *Swertia chirayita*; A: 4^0C; B: -15^0C; C: Room temperature

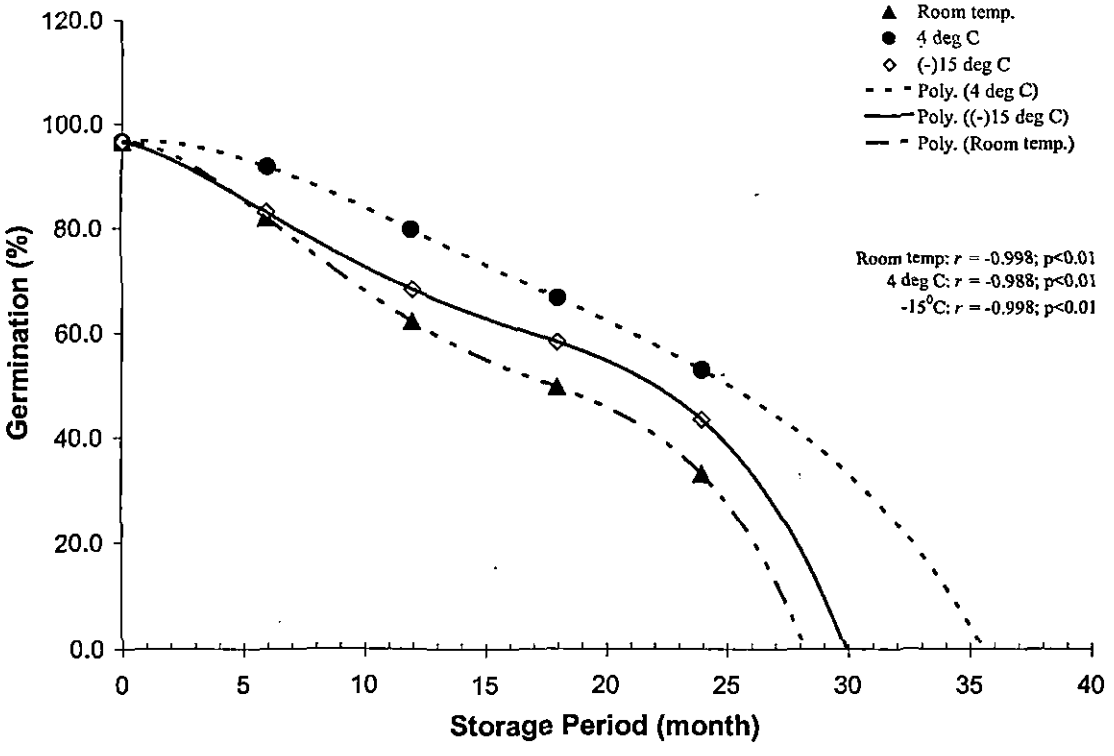

Figure 29. Seed viability as affected by storage period and storage condition in *Swertia chirayita*