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Germination and short-term storage of *Hippophae rhamnoides* L. seeds and its *ex-situ* reintroduction potential assessment under North East Indian conditions

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Abstract: Germination efficiency of freshly harvested *Hippophae rhamnoides* seeds collected from cold desert of Ladakh outside its native place under *in vivo* glass house condition recorded a highest of 73% germination in soilrite at $22 \pm 2^\circ\text{C}$ and 60–70% RH within three weeks at Guwahati, North East India. Germination of seeds was significantly enhanced to a maximum of 90% under *in vitro* condition in 1/4th Murashige & Skoog (MS) medium supplemented with 3% sucrose and 2% activated charcoal within two weeks. Glass house acclimatized healthy seedlings after been introduced to natural climatic conditions of Shillong and Guwahati of North East India showed inadequate survivability. Unlike storage at room temperature which is detrimental for seed viability, low temperature storage under refrigeration and natural climatic condition of Ladakh retains viability for prolonged period.

Additional key words: *in vitro*, *in vivo*, polyphenols, viability, introduction, substratum

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Introduction

Hippophae rhamnoides L. commonly known as Seabuckthorn is a medicinal shrub predominantly found in temperate Himalayan regions in the cold desert of Ladakh and can withstand temperature from -43 to 40°C (Lu 1992; Yao 1994). There are four *Hippophae* species namely, *H. rhamnoides*, *H. salicifolia*, *H. tibetana*, and *H. gyantsensis* that represent the genus in India (Naithani 2004). Ladakh hosts *H. rhamnoides* and *H. salicifolia* (Ali and Kaul 2011) whereas *H. salicifolia* has been reported in Sikkim and Arunachal Pradesh of North East India (Naithani 2004; Raina et al. 2012). Plant of *Hippophae* species are dioecious

with separate male and female characteristics. The fruit of *H. rhamnoides* commercially known as novel ‘super-fruit’ are rich source of complex vitamins (A, B, C, D, E, F, K and P); mineral (Na, K, Ca); carbohydrates, proteins, amino acids, tannins; triterpenoids, phospholipids, caumarin, catechins, leucoanthocyanins, flavonols, alkaloids, serotonin as well as omega 3 and one 7 unsaturated fatty acids (Kalia et al. 2011; Kanayama et al. 2012). Concentration of vitamins B₂, B₃, B₅, B₆, B₁₂, C and E is much higher than other fruits such as apricot, banana, mango, orange and peach (Stobdan et al. 2010). Seabuckthorn pulp, seeds, leaves and stem bark contain high levels of phenolic content and antioxidants (Korekar et al. 2011). The

plant is commonly used as a remedial of gastric, thrombosis, hepatic and ligament injuries, reducing cholesterol level and cancer (Stobdan et al. 2008). Besides medicinal uses the plant has been regularly used for soil and water conservation, desertification control, land reclamation in fragile cold desert ecosystem due to their natural potentialities to symbiotically fix atmospheric nitrogen with actinomycetes mainly *Frankia* species to promote effective growth in marginal soil (Akkermans et al. 1983; Zhang 2000; Tylkowski 2010). In nature, plant generally propagates through seeds and is the only means to inhabit new area (Lisenkov et al. 1969; Leme 1976). Seeds of *H. rhamnoides* form the store house of several crucial uncharacterized functional genes that encodes for its medicinal and cold tolerant properties. Germination of seed in plants is an essential phenomenon as the germination frequency varies from species to species and depends upon several biotic (genotype, metabolic activities, moisture content, plant animal interaction etc) and abiotic factors (light intensity, temperature, humidity, growth regulators, soil conditions etc). Seed germination in *Hippophae* sp. is also inhibited by several biotic factors such as hard coat and embryo dormancy (Landis et al. 1996; Sankhyan et al. 2005; Airi et al. 2009; Frochot et al. 2009).

To achieve higher productivity and select suitable genotypes for future breeding programmes, seed testing is very important (Mamo et al. 2006). Seeds also form the source and end material for genetic manipulation of plants. Efficiency of seed germination of *H. salicifolia* has been reported currently (Sankhyan et al. 2005; Airi et al. 2009). However, the literature cited in recent years does not deal with any comprehensive report on *in vitro* seed germination and storage of *H. rhamnoides*. Since North East India is one of the mega hot-spot regions of the world with diverse climatic conditions, introduction of *H. rhamnoides* that is reported to grow at different climatic condition would be very significant. Moreover, in view of the economic importance of the species assessment for possibilities of *ex situ* introduction at diverse geographic locations of North East India would be a great boon in future. The objective of the present study is to analyse the short-term storage and germination potentialities of *H. rhamnoides* seeds under *in vivo* and *in vitro* conditions and assess the acclimatization response of the species in North East India.

Materials and methods

Collection of seeds

The berries of *H. rhamnoides* enclosing seeds were collected from Shey area of Ladakh (31°44'57" – 32°59'57" N 76°46'29" – 78°41' E; 3524 m amsl), India during late September of 2010. Pulp from the berries

were removed and seeds were sun dried (28–30°C) for 2–3 days to prevent fungal infection. The seeds were stored in refrigerator (4°C) and closed container at room temperature (28–35°C) of Indian Institute of Technology, Amingaon, Guwahati, Assam, (26°11'5" N; 91°40'9" E; 31m amsl) and (–30–30°C) of Defence Institute of High Altitude Research, Leh-Ladakh (31°44'57" – 32°59'57" N; 76°46'29" – 78°41' E; 3524 m amsl) until the germination experiments were initiated. All the experiments were conducted at Indian Institute of Technology, Amingaon, Guwahati, Assam.

In vitro seed germination

Freshly harvested seeds (2–3 weeks, 3–5 mm in size) were surface disinfected by treating with Tween-20 (HiMedia Laboratories Pvt. Ltd, India) for 20 min followed by incubation in 0.5% fungicide (Bevastin, India) for 20 min and finally washed with sterile distilled water for 4–5 times. Finally, the seeds were surface sterilized in 1.2% sodium hypochloride and 0.2% mercuric chloride for 20 min and 5 min respectively followed by 4–5 washes with sterile distilled water. The aseptic seeds were dehydrated with 70% ethanol for 3 min as final treatment. The seeds (10 nos.) were placed in 150 ml glass bottle containing 50 ml of sterilized MS (Murashige and Skoog 1962) nutrient medium of different salt strength (1/4, 1/2, and full) supplemented with 3% sucrose and 2% activated charcoal and incubated in dark for 3 days followed by transferring to light condition. Seeds were also inoculated in non-nutrient medium supplemented with 3% sucrose and 2% activated charcoal in distilled water. The medium was solidified with 0.8% extra pure agar (HiMedia Laboratories Pvt. Ltd, India) and pH was adjusted to 5.8, prior to autoclaving for 15 min at 1.06 kg cm⁻² (121°C). Seeds inoculated in media bottles were incubated at 25±2°C under 12 h photoperiod with a PPFD of 40.5 μmoles m⁻²s⁻¹ provided by cool white fluorescent lamps. The experiments were repeated thrice with fifty replicates per experiment. Statistical analysis was done by Analysis of variance (ANOVA) and means compared using Tukey's test (p=0.05) (Origin 7.0 NORTHAMPTON, MA, USA). Observation was made on the percentage of seed germination after two weeks of culture.

Determination of seed moisture content, viability and storability

The dried pulps were completely removed and moisture content of fresh intact seeds was determined by drying seeds in the oven at 102°C for 48 h (Fabre and Dereuddre 1990). The percentage of moisture content was expressed on fresh weight (FW) basis using the formula

$$\text{Moisture content (\%)} = (\text{Fresh weight} - \text{Dry weight}) / \text{Fresh weight} \times 100$$

Moisture content was also determined for 4°C and RT stored seeds after 6 month using above method. Similarly, moisture content of 1 year old seeds kept under the climatic condition of Laddakh was determined at Indian Institute of Technology, Guwahati. The experiment was repeated thrice with 50 replicates per experiment.

Seed viability was tested using 1% solution of TTC (2, 3, 5 triphenyl tetrazolium chloride, Sigma) with pH adjusted at 6.5. Around 25 dry seeds (6 month at 4°C and RT stored at Guwahati, Assam, India and 1 year old seeds at Ladakh, J&K, India) were soaked in 1% aqueous solution of TTC for 24 h and kept in dark at room temperature. Seeds were bisected longitudinally and examined visually to determine viability percentage. Seeds with a red-stained embryo were considered to be viable. Storability of seeds was determined by analysing the percentage of seed germination for 6 month and 1 year old stored seeds in their pre-optimized (competent *in vitro* medium showing highest seed germination) medium. The experiments were repeated thrice with fifty replicates (50 seeds) per experiment. Statistical analysis was done by Analysis of variance (ANOVA) and means compared using Tukey's test ($p=0.05$) (Origin 7.0 NORTHAMPTON, MA, USA). Observation was made on the percentage of seed germination after two weeks of culture.

In vivo seed germination in glass house

To study *in vivo* seed germination, sand and soil was ground to fine particles and mixed with vermicompost (Krishi Jigyas, Guwahati) at different ratios. Similarly, soilrite (a mixture of perlite, Irish peat moss and vermiculite) alone, purchased from Keltech Energies Pvt. Ltd, Bangalore was used according to manufacture instructions. Around 50 (3–5 mm) seeds were kept for germination in four different pots (20 × 20 cm) comprising only soilrite and different mixtures of sand, soil and vermicompost at 1:1:2, 1:1:1 and 2:1:1 ratios respectively. The pH of these mixtures was determined using pH meter (Sartorius India Pvt. Ltd). The seeded pots were regularly sprinkled with water every alternate day for maintaining optimum humidity (70–80% RH) under 12 h photoperiod with a photosynthetic photon flux density (PPFD) of $40.5 \mu\text{moles m}^{-2}\text{s}^{-1}$ and constant temperature of $22 \pm 2^\circ\text{C}$ inside glass house. Experiments were conducted with 50 replicates and repeated thrice. Statistical analysis was done by Analysis of variance (ANOVA) and means compared using Tukey's test ($p=0.05$) (Origin 7.0 NORTHAMPTON, MA, USA). Observation was made on the percentage of seed germination after 50 days.

Acclimatization and seedling establishment

About 2 months-old *in vitro* raised seedlings (5–8 cm) were removed from culture vessels and washed with water to remove the adhering agar. These were then potted in cups containing the optimized seedling establishing substratum. The cups were covered with perforated polybags and sprinkled with water alternately at two days interval for the initial two weeks, and acclimatized at $22 \pm 2^\circ\text{C}$ and 50–60% RH under glass-house conditions. Seedlings (3–5 cm) were transferred to bigger pots (30 × 30 cm) containing various mixtures of surface black soil and bottom porous red soil collected from hill areas of the institute. Soil mixtures were prepared with these two types of soil alone as well as with vermicompost at 5:1 ratios respectively. In addition, a mixture of red soil, black soil and vermicompost at 1:1:1 ratios were also prepared. Substratum containing soilrite alone was considered as the control. The pH of these mixtures was determined using pH meter (Sartorius India Pvt. Ltd). The seedlings were regularly sprinkled with water every fourth day for maintaining optimum humidity (50–60% RH) and grown under 12 h photoperiod with a Photosynthetic photon flux density (PPFD) of $40.5 \mu\text{moles m}^{-2}\text{s}^{-1}$ at $22 \pm 2^\circ\text{C}$ inside glass house. Observation was made on the percentage of seedling establishment, growth and mortality after 50 days. Statistical analysis was done by Analysis of variance (ANOVA) and means compared using Tukey's test ($p=0.05$) (Origin 7.0 NORTHAMPTON, MA, USA).

Ex situ reintroduction in North East India

One month old healthy seedling (15–20 cm) established under glass house conditions were transferred to the natural hilly areas of Laban, Shillong, Meghalaya ($25^\circ34'32''$ N $91^\circ52'23''$ E, 1600 m amsl) and Amingaon, Guwahati, Assam ($26^\circ11'5''$ N $91^\circ40'9''$ E, 31 m amsl) during the month of July 2011. Irrigation was performed initially for two weeks manually followed by natural rain water irrigation of monsoons.

Results

In vitro seed germination

For rapid mass multiplication of *H. rhamnoides in vitro* seeds germination was found to be very suitable. Under *in vitro* condition excessive secretion of polyphenols from the seeds were recorded. This permitted only 15% seed germination on non-nutrient medium lacking activated charcoal that could be further enhanced to a maximum of 36% on activated charcoal containing non nutrient medium. On addition of full MS nutrients along with activated charcoal germina-

tion got further enhanced to an extent of 50%. However, of the different strength of MS tested, a highest of 90% seed germination was recorded in 1/4th MS strength supplemented with 2% activated charcoal within 10–12 days (Table 1; Fig. 1A, B). It was noted that addition of activated charcoal in the medium had a profound effect in enhancing seed germination. As compared to 1/4th MS medium containing activated charcoal wherein a highest of 90% germination was recorded, germination got reduced to 40% in the same MS strength lacking activated charcoal.

Table 1. Mean seed germination percentage of *H. rhamnoides* under *in vitro* condition ($25\pm 2^\circ\text{C}$) on different MS strength supplemented with activated charcoal

MS medium strength	Activated charcoal (2%)	Germination (%)
–	+	32.0 \pm 4.6 ^c
1/4	+	85.33 \pm 5.8 ^a
1/2	+	59.33 \pm 4.8 ^b
Full	+	42.66 \pm 5.9 ^b
–	–	13.33 \pm 1.7 ^e
1/4	–	36.0 \pm 4.1 ^c
1/2	–	27.33 \pm 4.6 ^{cd}
Full	–	23.33 \pm 4.0 ^d

Values are mean \pm SEM of three experiments with fifty replicates/experiment.

Values followed by same letter are not significantly different according to ANOVA.

(–) represents non MS medium containing only water solidified with 0.8% agar.

($P = 0.05$) and Tukey's test.

Determination of seed moisture content, viability and storability

In the present study, moisture and polyphenol content of seeds were found to be key factors for regulating higher seed germination competency and viability of *H. rhamnoides*. Seeds collected immediately from ripened fruits (7th days) had an initial moisture content of 28.79%. About 91% of the seeds were found to be significantly viable at this moisture level as revealed by TTC test. These viable seeds could also germinate significantly to a highest of 90% when kept under the pre-optimized *in vitro* medium. However, there was sharp decline in moisture content of seeds after prolonged storage (6 months) inside container at room temperature ($27\pm 7^\circ\text{C}$) and refrigerator at 4°C . The difference in storage condition affected the shelf life of seeds as revealed from their viability test which gradually reduced under prolonged storage. Seeds stored at 4°C refrigeration recorded a minimum of 79% viability with decline in moisture level to 8.33%. Out of the 79% viable seeds, only 63% could germinate successfully in their optimized germination medium (Table 2). On the contrary, 6 month old seeds stored in Assam condition at RT inside container became entirely non-viable with a sharp fall in moisture level to a minimum of 3.72%. Probably such low moisture content in seeds resulted into their mortality (Table 2). Morphological observation of the water imbibed de-coated 6 month old seeds also showed noticeable changes that occurred during different storage conditions. The cotyledons of seeds stored at room temperature ($27\pm 7^\circ\text{C}$) turned brownish after 6 months whereas 4°C seeds remained whit-

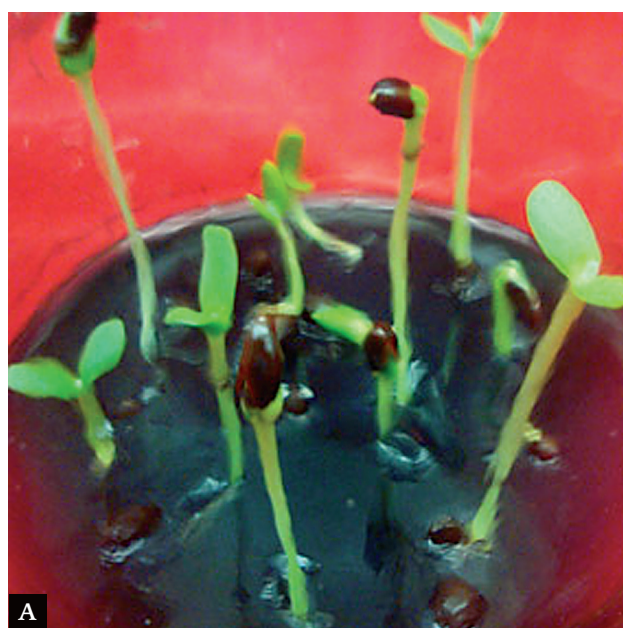


Fig. 1 A. *In vitro* seed germination of *H. rhamnoides* in 1/4th MS medium supplemented with and B. without activated charcoal within 15 days

ish-creamy in appearance even after its storage for 6 months. During storage at room temperature there was excessive secretion of polyphenols in the seeds leading to browning and non-viability of seeds (Fig. 2A, B). However, the phenolic secretion was comparatively lesser at 4°C leading to its higher viability even after 6 month of storage at Guwahati, Assam. Due to low temperature of Ladakh through-out the year, chances of polyphenol secretion in the seeds was comparatively lesser that permitted storage even for a year with around 85% seed viability and 77% germinability (Table 2; Fig. 2C, D). However, as the seeds were exposed to warmer temperature of Guwahati, Assam two phenomenal changes occurred within the seeds that reduced seed viability and germination. Firstly, there was steady fall in moisture level during storage at RT and secondly visual analysis

Table 2. Mean seed viability (1% TTC) and *in vitro* germination percentage of *H. rhamnoides* on optimized medium kept under different storage conditions with initial 29.49% moisture content

Storage condition	Moisture Content %	Viability %	Germination %
7 days RT	29.49±0.41 ^a	89.3±2.3 ^{ad}	85.33±5.8 ^a
6 month (4°C)	9.64±0.77 ^b	77.0±2.0 ^{bd}	63.63±3.2 ^{bc}
6 month (RT)	3.72±0.26 ^c	0.0±0.0 ^c	0.0±0.0 ^d
1 year RT* (Laddakh) September 2010– September 2011	5.38±0.57 ^c	83.6±2.6 ^d	76.66±1.2 ^c

Values are mean ± SEM of three experiments with fifty replicates/experiment; Values followed by same letter are not significantly different according to ANOVA (P =0.05) and Tukey's test. RT (Room temperature Guwahati (27±7°C); RT* (Room Temperature Laddakh (-30 to +30°C).



Fig. 2. A. Polyphenol secretion in 6 month old RT stored seeds of *H. rhamnoides* at Guwahati; B. Non-viable seeds of *H. rhamnoides* as revealed by 1% TTC test after 6 month storage at RT in Guwahati; C. Intact seeds of *H. rhamnoides* without polyphenol secretion after 1 year RT storage at Laddakh; D. Viable seeds of *H. rhamnoides* as revealed by 1% TTC test after 1 year RT storage at Laddakh

revealed excessive secretion of polyphenols from the seed cotyledons due to increased temperature leading to browning and death of the embryo.

In vivo seed germination in glass house

As compared to other substrates, fresh seeds of *H. rhamnoides* showed maximum germination in soilrite under *in vivo* glass house condition at $22 \pm 2^\circ\text{C}$. A maximum of 73% seed germination was recorded in soilrite within 15 days with 100% survivability which is significantly higher as compared to other substrates (Table 3; Fig 3. A, B). On the other hand, a

Table 3. Mean seed germination percentage of *H. rhamnoides* under *in vivo* glass house condition ($22 \pm 2^\circ\text{C}$) on various substratums

Soil substratum	Seed Germination (%)	Seedling Mortality (%)	Seedling Establishment (%)
Soilrite	70.66 ± 2.9^a	0.0	100 ± 0.0^a
Sand: Soil: Vermicompost (1:1:2)	48.0 ± 4.6^c	29.43 ± 2.4^a	70.57 ± 2.4^c
Sand: Soil: Vermicompost (1:1:1)	57.33 ± 1.7^b	11.79 ± 3.4^b	88.21 ± 3.4^{bc}
Sand: Soil: Vermicompost (2:1:1)	39.33 ± 3.5^d	6.61 ± 1.05^b	93.39 ± 1.0^{ab}

Values are mean \pm SEM of three experiments with fifty replicates/experiment.

Values followed by same letter are not significantly different according to ANOVA.

($P = 0.05$) and Tukey's test.

maximum of only 57.33% seed germination was recorded in readily available low cost mixture of sand, soil and vermicompost mixture at equal ratios and could be an alternative for soilrite for reducing cost.

Acclimatization and seedling establishment

The growth and the development of seedlings was better in almost all the substratum viz. humus enriched surface black soil, bottom porous red soil, bottom porous red soil mixed with vermicompost and soilrite (Table 4). More than 90% seedlings established well within one month on these entire substratum which is statistically non-significant. However the survivability of seedlings was significantly lower in rocky sandy soil with nearly 37% mortality. Seedlings attained a maximum height of 18 cm in bottom porous red soil and vermicompost mixture at 5:1 ratio which is significantly higher than other substratum.

Ex situ reintroduction in North East India

Survivability of seedlings of *H. rhamnoides* was greatly reduced during their acclimatization to the climatic condition of Guwahati and Shillong of North East India. Only 20% seedlings survived in hilly areas of Laban, Shillong whereas only 45% seedlings survived at Amingaon, Guwahati within two months of their transfer. The plants suffered initial loss during their acclimatization in newly introduced locations due to biotic and as abiotic factors. Severe wilting of plants was recorded immediately after their introduction under warm ($30\text{--}36^\circ\text{C}$) climatic condition of July



Fig. 3. A. *In vivo* seed germination of *H. rhamnoides* in soilrite after 15 days, B. *In vivo* seed germination of *H. rhamnoides* in sand: soil: vermicompost (2:1:1) after 15 days

Table 4. Establishment and growth of *H. rhamnoides* seedling in different substratum under glass house condition (22±2°C)

Composition of Substratum	Seedling Establishment %	Mortality %	Shoot length cm
Soilrite (pH : 6.5)	98.66±0.66 ^a	1.34±0.66 ^a	9.02±0.84 ^a
Surface black soil rich in humus (pH : 6.5)	94.0±1.2 ^a	4.0±1.2 ^a	15.6±1.4 ^b
Bottom porous red soil (pH : 6.4)	94.0±1.2 ^a	4.0±1.2 ^a	13.8±3.2 ^{ab}
Bottom porous red soil + vermicompost (5:1) (pH:6.8)	99.3±0.66 ^a	0.7±0.66 ^a	17.5±1.3 ^{cb}
Surface black soil rich in humus + vermicompost (5:1) (pH : 6.6)	83.3±1.7 ^b	16.7±1.7 ^b	11.6±0.80 ^a
Rocky sandy Soil (pH : 6.0)	63.3±1.7 ^c	36.7±1.7 ^c	6.6±1.05

Values are mean ± SEM of three experiments with fifty replicates/experiment; Values followed by same letter are not significantly different according to ANOVA; (P =0.05) and Tukey's test.

– August at Guwahati, India. Pest like the cut worms was also responsible for reducing the numbers of the seedlings at Guwahati. Although temperature at Shillong (17–23°C) was optimum during July–August, roots of the seedlings rotted severely due to excessive rainfall resulting into reduced survivability. Sprouting of new buds from the newly acclimatized seedlings was observed during October–November. Appropriate time for acclimatization is late winter (March–April) as observed from the healthy growth of seedlings during these seasons. Further trials must be carried out at other parts of North East India for successful massive introduction of this plant.

Discussions

Breeders need genes of plant resources and their combinations for development of new varieties. Therefore, at present more than 80% of plant genetic resources in the world are conserved as seeds in genebanks (Luan 2001). The conservation of seeds in gene banks allows ready availability of seeds for future utilization. The seeds maintained in genebanks should keep their germination capacity and genetic integrity. Therefore, the present study on seed characteristics of *H. rhamnoides* becomes very vital for its conservation and sustainable breeding. Study of seed germination in this species is very crucial since it shows epigeal germination that is considered to be evolutionarily more primitive than hypogeal germination. Thus, seed germination of this species could be enhanced only under optimized nutrient condition.

Although there are some reports of seed dormancy in *H. rhamnoides*, freshly collected seeds from Ladakh, India show vigorous germination under *in vitro* and *in vivo* conditions. However, low moisture content and secretion of polyphenols from the seeds reduced germination efficiency in short-term stored seeds. Numerous polyphenolic compounds have been reported in the berries of *H. rhamnoides*. Salicylic acid is the predominant phenolic acid besides protocatechuic, coumaric, cinnamic and ferulic acids which are also present at higher quantities in seeds (Zadernowski et al. 2005). Inhibition by ferulic acid and coumarin on lettuce seed germination were reported earlier (Williams and Hoagland 1982; Li et al. 1993; Yamamoto and Fujii 1997). Similarly, many workers have reported inhibitory effect of ferulic acid on cucumber seed germination and seedling growth (Blum and Rebbeck 1989; Booker et al. 1992; Lehman et al. 1994). These compounds act by disrupting the microtubule formation thereby preventing cell division and elongation. However, in the present study, the germination was significantly enhanced under *in vitro* condition containing optimum concentration of MS salts supplemented with activated charcoal. The presence of ammonium nitrate in MS medium may explain the high germination rate because NH₄⁺ is readily assimilated during the initial stages of development and greatly influences growth and differentiation (Raghavan and Torrey 1964; Kramer and Kozłowski 1979). The high germination and strong further development in MS medium could be attributed to the fact that MS medium is also especially rich in both macro and micro nutrients. Germination was progressively suppressed at higher MS strength signifying the inhibitory action of the major salts at high concentration for this species (Atia et al. 2006; Bairu et al. 2009). This effect could partly be attributed to the role minerals play as osmotica. Since any germination process is preceded by imbibitions, anything that affects this process is likely to affect germination. The effect of ions like chloride, sodium, and magnesium on seed germination and plant growth is well established (Hardegree and Emmerich 1990). For instance, sodium chloride caused a significant delay and inhibition (at higher concentration) on *ex vitro* germination of *Pteroceltis tatarinowii* seeds by regulating water potential and reducing the availability of water to the germinating seed (Fang et al. 2006). According to Yam et al. (1989), the nutritional requirements of germinating seeds vary due to their physiological state and this may be species specific. MS salts at reduced concentration (1/2 and 1/4 strength) have also been reported to promote better seed germination in orchids such as *Dendrobium tosaense* and *Anoectochilus formosanus* (Shiau et al. 2002; Lo et al. 2004) and medicinal herb *Aconitum heterophyllum* (Srivastava et al. 2011). It was noted that excessive secretion of poly-

phenols in medium lacking activated charcoal drastically withheld seed germination. However, addition of activated charcoal in the MS medium had profound effect in adsorbing the polyphenols secreted out from the seeds that favoured enhancement of *in vitro* seed germination. Positive effect of activated charcoal on seed germination and morphogenesis may be due to one side surface imbibitions of inhibitor compounds in medium which minimizes toxic metabolism and exudation of phenolic compounds (Wang and Huang 1976; Thomas 2008). Similarly, positive effects of activated charcoal on seed and embryo germination of *Fraxinus excelsior* and *Taxus baccata* (Mojarabi et al. 2011; Tafreshi et al. 2011) as well as several orchid species (Moraes et al. 2005; Pacek-Bieniek 2010) has been recently reported. Under natural *in vivo* conditions, propagation becomes difficult as a result of various germination obstacles in *Hippophae* sp. seeds (Li and Schroeder 2003; Busing and Slabaugh 2008; Airi et al. 2009; Frochot et al. 2009). These obstacles are mainly because of hard seed coat or embryo dormancy that could be overcome by various chemical and mechanical treatments (Landis et al. 1996). In the present *in vivo* germination study, it was found that fresh seeds germinate vigorously in soilrite at $22 \pm 2^\circ\text{C}$ under glass house condition. This is in accordance with the seed germination of *H. rhamnoides* from Poland wherein nearly 100% seed germination was recorded in sand and peat (1:1 pH 5.5–6.5) substrate at higher temperature $20\text{--}30^\circ\text{C}$ (Tylkowski 2010). Unlike other substratum, soilrite (a mixture of perlite, Irish peat moss and vermiculite) constitutes optimum balanced source of macro and micro nutrients with better porosity and water sieving capability to maintain optimum moisture level that permits profuse seed germination. The porosity of soilrite also perhaps helps in sinking the excessive polyphenols secreted out during seed germination of *H. rhamnoides*. This is not permissible in other substratum including semi-solid agar medium where accumulation of polyphenols nearby the seeds prevents the emergence of plumule. Unlike other substratum, higher porosity also allows the better penetration of roots in soilrite promoting 100% seedling establishment in this study. Low seed germination on other substratum which consists mainly of sand, soil and vermicompost mixtures might be due to high nutrient composition and high water retention resulting into decay of seeds before germination. Recently, it has been reported that vermicompost preparations exhibits inhibitory effect on seed germination and early seedling development in certain vegetable crops. This could be due to the inhibitory effects of both humic and phenolic (gallic acid and chlorogenic acid) substances present in vermicompost (Ivinskaya 2011). Similarly, low seed germination was reported earlier when trials were conducted directly in nursery soil for *H. rhamnoides*

which recommends sowing of seeds in the glass house (or plastic tunnel) on sand and peat substrate (Tylkowski 2010). It was found that the efficacy of germination falls gradually with prolonged storage of seeds at warmer climatic condition. Present findings suggest using of fresh seeds instead of stored ones to obtain better results while working with *H. rhamnoides* outside its natural habitat.

Conclusion

This is a first report on *in vitro* and *in vivo* seed germination of *H. rhamnoides* under controlled temperature and humidity conditions of laboratory and glass house. From the present study it is clear that freshly harvested seeds of *H. rhamnoides* can be efficiently germinated under optimized *in vitro* conditions. However, seeds lose its viability on storage at warmer room temperature conditions and must be kept under low temperature for retaining higher viability for prolonged period. Under natural climatic condition of Ladakh where temperature recedes below 0°C , seeds remain viable for prolonged period. However, room temperature storage at other place is highly detrimental and must be avoided completely. The study has been conducted outside its natural climatic condition and becomes highly imperative for disseminating information on the use of *H. rhamnoides* seeds for research and breeding programmes as the condition of its storage has been well established. As literature reveals the growth of *Hippophae* species could occur even at diverse temperature conditions, the present investigation to introduce seedlings at cold region of Shillong, Meghalaya and also at warmer condition of Guwahati, Assam, India becomes very useful in understanding the actual acclimatization behaviour of seedlings at these two extreme climatic conditions. Reintroduction of the seedlings at the present locations is not so appealing due to several factors. However, it is felt that trials at other colder parts of North East India such as Arunachal Pradesh and Sikkim would reveal appreciable results in future. Although introduction through cuttings is faster as compared to seedling establishment, seed derived plantlets would allow dissemination of diverse germplasm at the introduced site and is more exigent for conservation and breeders concern.

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