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Seed Characterization, Viability and Promotion of Seed Germination in Nervine Tonic Plant *Evolvulus alsinoides* Linn.

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ABSTRACT

Evolvulus alsinoides Linn. (Convolvulaceae; Shankhpushpi) is one of the important medicinal plants used as a nervine tonic. The aim of the present study was to characterize seed, to assess viability and to enhance germination of *E. alsinoides* seed. Seed characterization and viability testing showed that about 80% yellowish-green seeds and 45% brown seeds were viable. Black seeds found in the capsules were non-viable. Yellowish-green seeds subjected to different pre-sowing treatments for improved germination included physical scarification using sand paper, acid scarification, presoaking treatment of wet heat (hot water and boiling water), cold water and different types of plant growth regulators including cytokinins, auxins and gibberellic acid (GA_3). Significantly highest (78.7 ± 1.8%) rate of seed germination and higher germination speed (GS), germination value (GV), emergence index (EI) and vigor index (VI) were observed in the seeds soaked in 5.00 mM GA_3 for 36 h. Fresh and aged seeds (0, 6, 12, 18 and 24 months old) were subjected to TTC test and presoaking in 5.00 mM GA_3 for 36 h resulted in lower viability and seed germination percentage, indicating that *E. alsinoides* seeds have a storage life of about 2 years.

Keywords: Convolvulaceae, gibberellic acid, plant growth regulators, scarification, Shankhpushpi, TTC

INTRODUCTION

Evolvulus alsinoides Linn. (Convolvulaceae), commonly known as "Shankhpushpi", is an important multipurpose medicinal herb occurring all over India and subtropical parts of the world (Austin 2008). The plant is used traditionally in East Asia, India, Africa and the Philippines to cure fever, cough cold, venereal diseases, azoospermia, adenitis and amnesia, and has an adaptogenic property (Siripurapu *et al*. 2005). It is a one of the versatile medhya drugs which contributes considerably to the improvement of memory power and intellect. Preclinical research has justified its ancient claim as nervine tonic and useful on several severe brain disorders like insomnia, irritability, epilepsy as well as psychological depression as it has a relaxing effect on the brain, removes nervous weakness and enhances memory power (Pal and Jain 1998; Dash *et al*. 2002; Singh 2008; Sethia *et al*. 2009).

The herbal extracts of Shankhpushpi possess strong anti-inflammatory activity (Lekshmi and Reddy 2011) and could provide an alternative approach to the treatment of arthritis (Ganju *et al*. 2003). Leaf juice is useful in treating internal haemorrhage, given for bowel complaints; root and stem extracts are used to treat dysentery (Pal and Jain 1998). Flowers are good for treating uterine bleeding (Daniel 2006). The plant shows antiphlogistic, antioxidant (Auddy *et al*. 2003) and antimicrobial activity (Omogbai and Eze 2011). Preliminary phytochemical studies were carried out to confirm the presence of active chemical constituents in the plant extract, and the results showed a positive report for the presence of alkaloids, flavonoids and cardiac glycosides (Gupta *et al*. 2007; Singh 2008; Lekshmi and Reddy 2011).

Because of its multifold uses, the plant is being over exploited by the local population as well as Ayurvedic and pharmaceutical companies. It has therefore become imperative to study the seed characterization, propagation and cultivation. The propagation of *E. alsinoides* is affected due to seed dormancy of several months (Baskin and Baskin 1998; Jayasuriya *et al*. 2008). Germination and early seedling development stages are critical periods for the establishment of plant species (Zhou *et al*. 2012). The seed characterization and application of various pretreatments have been proven effective to overcome the dormancy in the seeds of several taxa (Baskin and Baskin 1998).

In vitro propagation of *E. alsinoides* was reported using nodal explants (Tejavathi and Purshothama 2004). However, plant tissue culture is cost intensive and sensitive compared to seed propagation. Therefore, the aim of this study was to characterize the seeds and seed germination by applying physical and chemical treatments and to develop a feasible method for propagation to increase the availability of biomass for Ayurvedic and pharmaceutical preparations.

MATERIALS AND METHODS

Seed source, characterization and authentication

The matured capsules of *Evolvulus alsinoides* Linn. were collected from the plants grown in the Pune district of Maharashtra (India). The collection was made in the month of September and October during (2009 - 2011). The capsules were dried at under shade room temperature and allowed to break naturally. During collection, the number of capsules per plant was noticed. The data on seeds per capsule was recorded on the basis of visual observation and the seeds were categorized on the basis of color as black, brown and yellowish-green. Then the seeds were stored separately in glass bottles with screw cap until use. The weight of 100 shade dried seeds were taken (initial weight) and were kept in oven at 60°C for 48 h and weight of dried seeds were taken (final weight) and moisture content was calculated by using the formula

Moisture content (%): Initial weight - final weight/ final weight × 100

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Authentication of plant material was carried out from Botanical Survey of India (BSI), Western circle, Pune and sample specimen was deposited in the herbarium section of BSI (Ref.: BSI/WRC/Tech/2009/506).

Seed viability

Viability of each lot of collected seeds was tested with the 2,3,5-triphenyltetrazolium chloride (TTC; HiMedia, Mumbai, India) test (Hartman *et al*. 1997). Sodium phosphate buffer (pH 7.0) was used for preparing the 0.5% TTC solution. Randomly selected 40 seeds from each lot were soaked in TTC solution for 24 h in dark. After treatment, the seeds were rinsed three times with sterilized distilled water (SDW). The embryos were dissect out using forceps and needle under simple microscope and observed for change in color to red.

Surface sterilization of seeds and germination conditions

The seeds were surface sterilized using 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) solution for 5 min. Then the seeds were washed with SDW for 5 times to remove the traces of $HgCl_2$. The surface sterilized seeds were used for different treatments and germination trials.

Seeds without treatments (Control) and treated seeds were geminated on germination paper (1 mm thick, Modern paper Ltd., Pune, India) overlying a layer of about 5 mm thick, absorbant cotton in a transparent plastic Petri dishes (90 × 15 mm; Axygen, India). Treated and untreated (control) 50 seeds were sown in each Petri dish. Initially, the germination paper and cotton was moistened with 15 ml SDW and later 4 ml SDW was added at two days interval. The Petri dishes were maintained at 25 ± 2°C under an 8-h photoperiod and light intensity of about 30 µmol m^{-2} s^{-1} provide by cool white florescent tube lights (Champion 40 W, Philips Electronics India Ltd., Kolkata, India).

Pre-sowing treatments

1. Sandpaper scarification

Surface sterilized seeds without any pre-treatment were placed in the Petri dishes were considered as control. The seeds were placed between the flaps of 0-grade sandpaper and scarified by applying little friction force manually for 10 s. During scarification some seeds were damaged therefore, the damaged seeds were observed visually and discarded and the intact, undamaged seeds were selected and placed in the Petri dishes for germination test.

2. Cold water treatment

The seeds were treated with cold water for different time duration. The seeds were separately immersed in a test tube containing cold water (4°C) and placed in refrigerator at 4°C for 12, 24, 36 and 48 h, after which the seeds were placed in the Petri dishes for germination.

3. Wet heat treatment

The seeds were soaked in hot water (60°C) separately in test tubes and kept in a water bath at 60°C for 5, 10 and 15 min. In another set of experiment the seeds were immersed in boiling water (100°C) for 30, 60 and 120 s. After specific temperature treatments, the seeds were transferred to SDW till it reaches at room temperature. Then the seeds were transferred to Petri dishes for germination.

4. Acid scarification

The seeds were presoaked in concentrated H_2SO_4 and HCl (Qualigens, Mumbai, India) for 2.5, 5.0, 7.5 and 10 min. After treatment the seeds were washed 5 times with SDW to remove the traced of acid and transferred to Petri dishes for germination.

5. Treatment of plant growth regulators

The surface sterilized seeds were suspended separately in 00, 0.65, 1.5, 2.5, 5.0 mM sterilized solutions of plant growth regulators such as indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), kinetin (Kin), thidiazuron (TDZ) and gibberellic acid (GA_3) (all from HiMedia) for 12, 24, 36, 48 h. The seeds soaked in SDW for similar duration was served as control. The treated and control seeds were placed in Petri dishes and observed for germination.

Data collection

1. Daily and cumulative germination counts

The cultures were observed daily and the data on daily seed germination was collected until the completion of germination (maximum up to 30 days). The seeds with 0.5 mm or more radical growth occur were counted as germinated seeds. The final germination percentage (FGP) was calculated from the total seeds that germinated on the day of completion, using the formula: No. of seeds germinated on day of completion / No. of seeds sowed × 100.

Daily germination percentages (No. of seeds germinated on a particular day / No. of seeds sowed × 100) were summed up to obtain cumulative germination percentage (CGP) for each treatment (Ahire *et al*. 2009).

2. Germination speed and germination value

The germination value (GV), a composite value that combines both germination speed (GS) and total germination which provides an objective means of evaluating the results of germination values. It is calculated using formula availed by Aldhous (1972) and Djavanshir and Pourbeik (1976):

Germination speed = Final germination percentage / Day of completion of germination.

Germination value (GV) = ($\sum$ DGs/N) GP/10.

where GP = germination percentage at the end of the test, DGs = daily germination speed obtained by dividing the cumulative germination percentage by the number or days since sowing, $\sum$ DGs = Total germination obtained by daily counts, N = The total number of daily counts, starting from the date of first germination, 10 = constant.

3. Emergence index

Emergence index were calculated by using the formula as follows:

Emergence index (EI) = dn/n

where dn = number of seeds germinated on a particular day (emergence) and n= day of emergence (Thakur *et al*. 2004).

4. Seedling vigor

Seedling vigor Index (SVI) was calculated as per the recommendation of ISTA (1976):

SVI = Germination percentage × Shoot length

For seedling vigour normal seedlings from standard germination tests were further classified as strong or weak seedlings by visual observation and expressed as percentage. The erect and sturdy seedlings with well developed seedling parts were kept in the category of strong seedlings (ISTA 1976). Root length and shoot length of the seedlings were recorded and root to shoot ratio was calculated.

Seedling development

Plastic cups of 5 cm in diameter and 10 cm in height were filled out with 1.1 dm^3 of Garden soil, and seedlings of *E. alsinoides*, were sown. The cups were saturated with water by surface irrigation. During plant growth, cups were irrigated daily by spraying water until water drained from the bottom of the cup.

Statistical analysis

The experiments were set up in a completely randomized design with minimum three replicates per treatment. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using Duncan's multiple range test (DMRT) at the 5% probability level. Variability in data has been expressed otherwise as mean ± SE.

RESULTS AND DISCUSSION

Seed collection and characterization

The fruits are 4-valved, loculicidal, dehiscent, rounded, smooth capsules, yellow green in color and become yellowish brown at the time of maturity (Singh *et al*. 2010). In the mid rainy seasons, fruits are not matured with respect to its normal growth and seed setting. The fruits attained their maturity up to the months of September and October. Therefore, the fruits were collected during the months of September and October (2009 – 2011). After collection the fruits were air dried under shade at room temperature. Each matured capsule contains 4 seeds, of which two seeds were yellowish-green in color, and one seed is brown and another one is black in color. Sometimes 3 yellowish-green seeds were also observed. On the basis of color, seeds were distributed in three lots namely yellowish-green, brown and black seeds. Each lot was stored separately in glass bottles with screw cap at room temperature. Seeds with different colors were also differs in their size. Yellowish-green seeds were larger than the brown and black colored seeds (**Table 1**).

In many species, the growth of plant under varying environmental condition or variation in the environment during the time of fruit development may cause changes in seed characteristics such as different sizes, colors and shapes (Baskin and Baskin 1998). The seeds produced in different flower of the same inflorescence, and inflorescence borne at different position on the mother plant may also brought variation in seed characteristics (Baskin and Baskin 1998). The basal seeds in spikeltes of *Aegilops* spp. were heavier than those produced in more distal position (Datta *et al*. 1970; Maranon 1989). In dehiscent fruits of *Fedia* spp., three different shapes of seeds were observed depending on where they are produced in the inflorescence (Mathez and Xena de Enrech 1985). Kedia (2006) recorded light brown, dark brown and black seeds with significant differences in their weight and size in *Eclipta alba*. Thus, the preconditioning environment appears to be the major cause of seed polymorphism (Baskin and Baskin 2005). This might be the reason for appearance of three distinct types of seeds in *E. alsinoides*.

Table 1 Morphological characters of capsule and seeds of *E. alsinoides*.

Character	Observation
Capsule per plant	59.6 ± 4.9
Total no. of seeds per capsule	04.0 ± 0.0
Black seeds per capsules	01.0 ± 0.0
Brown seeds per capsules	01.0 ± 0.0
Yellowish-green seeds per capsules	02.0 ± 0.0
Length of black seeds (mm)	01.4 ± 0.4
Length of brown seeds (mm)	01.8 ± 0.2
Length of yellowish-green seeds (mm)	02.1 ± 0.4
Breadth of black seeds (mm)	00.6 ± 0.1
Breadth of brown seeds (mm)	00.7 ± 0.1
Breadth of yellowish-green seeds (mm)	00.9 ± 0.1

The mean values were obtained from observation of randomly selected 1000 capsules

Seed viability

It has been known that the temperature, seed moisture content and oxygen pressure are most important for viability and longevity of seeds in storage. Seed viability is an indication of capability of the seeds to germinate and produce normal seedlings under suitable germination conditions (Copeland and McDonald 2001). Total germination depends largely on the viability and vigor of the seeds used (Harrington 1972; Ellis *et al*. 1993; Hay and Probert 1995). Therefore, it is necessary to analyze the viability of any seed lot to be used for raising the seedlings (Marrero *et al*. 2007). TTC test provides some indication of propagation success relative to the maximum potential germination (Scianna 2001). In the present study, 50 seeds from three different lots (yellowish-green, brown and black) were randomly selected and soaked separately in 0.5% TTC solution for 24 h dark. Black color seeds showed negative TTC test (data not shown) due to lack of embryo and cotyledons in the seeds. After dissection it was observed that the black seeds were hollow. The yellowish-green and brown seeds stored for different time duration (0, 6, 12, 18 and 24 months) were evaluated for viability testing. Results showed that about 10% decline in viability of yellowish-green as well as brown color seeds (**Fig. 1**). No significant difference in seed viability was observed in freshly collected seeds and 6 months stored seeds. The viability gradually decreases with increase in storage period of the seeds. The seeds stored for more than 24 months showed only about 10% viability in both the seed types (data not shown). The increase in storage time resulted in decline in viability of seeds. Similar results were observed by Kumar *et al*. (2007) in case of *Pongamia pinnata*. Differences in storage behavior are often associated with morphological, physiological, anatomical structure and biochemical composition of the seeds, which affects the longevity of seeds storage (Kumar *et al*. 2007).

Yellowish-green seeds showed about 80% viability and brown seeds showed about 40% viability; whereas black seed did not showed positive TTC test. Therefore, for further germination trials yellowish-green seeds were used.

Effect of treatments on germination behavior

The seeds without any pre-treatment failed to germinate over the period of germination (30 days). The seeds scarified with zero-grade sandpaper and incubated on moist germination paper in Petri dishes were failed to germinate (data not shown). As the seeds are very small in size and non-endospermic, the seeds were damaged during scarification with sandpaper. Remaining seeds were also failed to germinate might have been due to damage caused to embryo. In the present study physical scarification using sandpaper did not help to improve the germination in *Evolvulus alsinoides*. Similarly, Ahire *et al*. (2009) also reported the damage caused by scarification using sandpaper in *Uraria picta* seeds.

Germination is a complex physiological process which depends on several environmental factors such as light, temperature and storage duration (Koornneef *et al*. 2002). Environmental conditions during seed formation and maturation have a remarkable effect on seed germination and dormancy (Perez-Garcia 1997). Cold-moist stratification is a commonly used practice to break dormancy in seeds to attain vigorous, maximum and uniform germination (Roberts and Ellis 1982; Wang and Berjak 2000; Huang *et al*. 2004). In the present investigation, seeds soaked for different time duration in cold water slightly improves the germination percentage as compared with control (**Table 2**). About 28.0 ± 2.3% germination was observed on 24 h cold water pre-soaking treatment. Other germination parameters

Fig. 1 Viability in brown and yellowish-green seeds of *Evolvulus alsinoides* L. using 2,3,5- triphenyltetrazolium chloride (TTC) test. Each value represents mean of three replications and vertical bars indicate SE. The bars with different letters on the same color columns are significantly different at $P < 0.05$.

Table 2 Effect of water temperature and acid scarification on germination of yellowish-green seed of in *E. alsinoides* L.

Treatments	Germination %	GS	GV	VI	EI	Root/shoot ratio
Control	00.0 ± 0.0 j	0.0 ± 0.0 j	00.0 ± 0.0 i	00.0 ± 0.0 j	0.00 ± 0.0 c	0.00 ± 0.00 d
CW 12 h	20.0 ± 1.2 cdef	1.4 ± 0.1 cdef	13.4 ± 1.5 cd	36.7 ± 1.0 cd	0.52 ± 0.1 abc	0.18 ± 0.03 bc
CW 24 h	28.0 ± 2.3 b	2.0 ± 0.0 b	34.0 ± 2.6 a	51.5 ± 1.9 b	0.38 ± 0.1 bc	0.36 ± 0.03 a
CW 36 h	24.7 ± 2.4 bc	1.8 ± 0.2 bc	18.5 ± 0.7 b	38.7 ± 1.2 c	0.83 ± 0.1 ab	0.23 ± 0.02 abc
CW 48 h	15.3 ± 1.3 fgh	1.1 ± 0.1 efg	10.4 ± 1.5 de	21.9 ± 1.4 ef	1.00 ± 0.1 a	0.22 ± 0.04 abc
HW 5 min	19.3 ± 2.4 defg	1.4 ± 0.2 def	09.5 ± 0.1 ef	25.9 ± 2.2 e	0.30 ± 0.1 bc	0.27 ± 0.08 abc
HW 10 min	15.3 ± 0.7 fgh	1.1 ± 0.0 fgh	06.4 ± 0.7 fg	15.1 ± 1.2 gh	0.30 ± 0.1 bc	0.28 ± 0.09 abc
HW 15 min	10.7 ± 1.3 hi	0.8 ± 0.1 ghi	04.7 ± 0.5 gh	11.1 ± 2.7 hi	1.00 ± 0.0 a	0.18 ± 0.04 bc
BW 30 sec	16.7 ± 0.7 efg	1.2 ± 0.0 efg	09.7 ± 0.9 ef	20.1 ± 1.3 f	0.38 ± 0.1 bc	0.31 ± 0.03 abc
BW 60 sec	14.7 ± 1.8 gh	1.0 ± 0.1 fgh	06.7 ± 0.6 fg	14.7 ± 1.0 gh	0.38 ± 0.1 bc	0.26 ± 0.35 abc
BW 120 sec	08.7 ± 0.7 i	0.6 ± 0.0 i	01.9 ± 0.3 hi	09.9 ± 0.5 i	0.55 ± 0.1 ab	0.17 ± 0.03 bc
H_2SO_4 2.5 min	39.3 ± 1.8 a	2.8 ± 0.1 a	17.8 ± 1.4 b	66.5 ± 1.5 a	0.75 ± 0.3 ab	0.15 ± 0.04 c
H_2SO_4 5.0 min	20.7 ± 2.4 cde	1.5 ± 0.2 cde	14.6 ± 1.3 c	33.7 ± 1.6 d	1.00 ± 0.0 a	0.20 ± 0.02 abc
H_2SO_4 7.5 min	14.7 ± 1.8 gh	1.0 ± 0.1 fgh	11.0 ± 1.3 de	25.3 ± 2.2 e	0.55 ± 0.5 ab	0.30 ± 0.01 abc
H_2SO_4 10 min	09.3 ± 1.3 i	0.7 ± 0.1 hi	09.5 ± 0.5 ef	14.1 ± 0.5 ghi	0.63 ± 0.2 ab	0.17 ± 0.02 bc
HCl 2.5 min	22.7 ± 1.3 cd	1.6 ± 0.1 cd	12.0 ± 0.6 cde	25.0 ± 2.0 e	0.58 ± 0.1 ab	0.30 ± 0.03 abc
HCl 5.0 min	16.7 ± 0.7 efg	1.2 ± 0.0 efg	10.3 ± 0.6 de	18.5 ± 0.9 fg	0.97 ± 0.4 a	0.11 ± 0.05 abc
HCl 7.5 min	14.7 ± 1.8 gh	1.0 ± 0.1 fgh	09.4 ± 0.1 ef	13.4 ± 0.7 hi	0.77 ± 0.1 ab	0.32 ± 0.02 ab
HCl 10 min	07.3 ± 0.7 i	0.5 ± 0.0 i	04.8 ± 0.9 gh	09.9 ± 0.4 i	0.67 ± 0.4 ab	0.29 ± 0.11 abc

EI: Emergence index; GS: Germination speed; GV: Germination value; VI: Vigor index; CW: cold water; HW: Hot water (60°C); BW: Boiling water (100°C); Values are mean ±SE of three independent experiments each with three replicates. Mean followed by same letter with in columns are not significantly different at the 5% level (DMRT).

such as GS (2.0 ± 0.0), GV (34.0 ± 2.6), EI (0.38 ± 0.1), VI (51.5 ± 1.9) and root/shoot ratio (0.36 ± 0.03) also remains high (**Table 2**) in the seeds treated with 24 h treatment as compare to other cold water treatment. Okunomo and Bosah (2007) reported about 60% germination in *Acacia senegal* seeds, when subjected to soaking in cold water for 48 h. Hossain *et al*. (2005) also reported the highest germination percentage in *Terminalia chebula* seeds on soaking in cold water for 48 h. Moist chilling treatments have been reported very effective for germination in some species. Bourgoin and Simpson (2004) reported that the soaking of *Acer pensylvanicum* seeds for 48 h, moist chilling (4°C) for 16 weeks, and germinating at 5:15°C produced an average germination of 92%. Seeds stratified at 4°C for 12 weeks germinated to 88% in *Prunus campanulata* (Chen *et al*. 2007).

The seeds treated with hot water for 5, 10 and 15 min and boiling water for 30, 60 and 120 s resulted in decline in germination percentage as well as other germination parameters (**Table 2**). About 20% germination was observed on seeds treated with HW for 5 min. Further increase in the temperature and duration of the treatment resulted in decreased germination percentage (**Table 2**). Wet heat treatment sometimes used to stimulate the germination in the seeds with physical dormancy. Warcup (1980) subjected the soil samples collected from southeastern Australian forests to wet steam at 60-71°C for 30 min and obtained increased germination of species belonging to family Convolvulaceae. But in the present study seeds soaked in hot water or boiling water are not effective for improvement of germination. Hard seed coat can be soften artificially by different treatments such as mechanical or acid scarification, cooling at very low temperature (Pritchard *et al*. 1988) and brief exposure of high temperature (Herranz *et al*. 1998). But the efficiency of such treatments depends on the species, water temperature and immersion time during scarification (Schmidt 2000).

Treatment of concentrated acid resulted in softening of seed coat and destroys the plugged natural openings which helps in entry of water required for germination (Baskin and Baskin 1998). In the present study, treatment of concentrated H_2SO_4 and HCl improves germination percent as compared with non treated seeds (**Table 2**). About 40% germination was observed in the seeds treated with H_2SO_4 for 2.5 min. The GS (2.8 ± 0.1), GV (17.8 ± 1.4), EI (0.75 ± 0.3), VI (66.5 ± 1.5) was also higher in the seeds treated with H_2SO_4 for 2.5 min. Further increase in treatment duration resulted in decreased in germination (**Table 2**). A 30 to 90 min period of acid scarification destroyed the plug-like structure in the bottom of the micropylar depression in seeds and improved the germination of *Convolvulus lanatus*, *C. negevensis* and *C. secundus* (Koller and Cohen 1959). The treatment of concentrated H_2SO_4 hydrolyzes and increases the permeability of seed coat in several plant spe-

Table 3 Effect of presoaking treatments (distilled water and gibberellic acid) on germination of yellowish-green seeds of *E. alsinoides* L.

Treatments	Germination %	GS	GV	VI	EI	Root/Shoot
12 h DW	15.3 ± 1.8 h	1.10 ± 0.13 i	04.6 ± 1.3 k	026.8 ± 2.0 i	1.11 ± 0.1 a	0.24 ± 0.08
12 h 0.65 mM GA_3	16.7 ± 0.7 gh	1.19 ± 0.05 hi	07.0 ± 1.2 ijk	062.8 ± 3.1 gh	0.41 ± 0.2 c	0.39 ± 0.05
12 h 1.50 mM GA_3	23.3 ± 2.4 ef	1.67 ± 0.17 fg	08.3 ± 0.4 hijk	055.5 ± 4.8 h	0.57 ± 0.1 bc	0.39 ± 0.10
12 h 2.50 mM GA_3	26.7 ± 1.3 def	1.90 ± 0.10 efg	10.9 ± 2.4 ghijk	063.1 ± 4.9 gh	0.63 ± 0.2 bc	0.36 ± 0.01
12 h 5.00 mM GA_3	31.3 ± 1.8 cd	2.24 ± 0.13 de	09.0 ± 1.0 hijk	084.1 ± 2.7 de	0.77 ± 0.1 abc	0.19 ± 0.01
24 h DW	21.3 ± 0.7 fg	1.52 ± 0.05 fgh	07.8 ± 1.2 hijk	036.4 ± 3.2 i	0.82 ± 0.1 a	0.30 ± 0.04
24 h 0.65 mM GA_3	24.7 ± 1.8 ef	1.76 ± 0.13 fg	12.1 ± 2.6 fghij	096.1 ± 6.6 d	0.61 ± 0.2 bc	0.60 ± 0.01
24 h 1.50 mM GA_3	27.3 ± 1.8de	1.95 ± 0.13 ef	14.1 ± 2.7 efghi	070.9 ± 4.6 efgh	0.61 ± 0.2 bc	0.54 ± 0.09
24 h 2.50 mM GA_3	31.3 ± 1.8 cd	2.24 ± 0.13 de	34.8 ± 2.6 cd	075.5 ± 7.5 efg	0.77 ± 0.1 abc	0.41 ± 0.03
24 h 5.00 mM GA_3	50.7 ± 1.8 b	3.62 ± 0.13 b	45.7 ± 2.7 ab	134.7 ± 5.1 c	0.44 ± 0.1 c	0.20 ± 0.02
36 h DW	34.7 ± 1.3 c	2.48 ± 0.10 cd	28.7 ± 2.7 d	066.1 ± 6.2 fgh	0.80 ± 0.1 abc	0.22 ± 0.01
36 h 0.65 mM GA_3	36.7 ± 1.8 c	2.62 ± 0.13 c	18.7 ± 2.2 ef	030.6 ± 4.7 c	0.47 ± 0.1 bc	0.54 ± 0.07
36 h 1.50 mM GA_3	31.3 ± 1.8 cd	2.24 ± 0.13 de	20.6 ± 3.8 e	080.1 ± 7.1 ef	0.60 ± 0.1 bc	0.25 ± 0.04
36 h 2.50 mM GA_3	35.3 ± 1.8 c	2.52 ± 0.13 cd	17.9 ± 0.4 efg	095.3 ± 3.8 d	0.47 ± 0.2 bc	0.47 ± 0.04
36 h 5.00 mM GA_3	78.7 ± 1.8 a	5.62 ± 0.13 a	50.3 ± 1.8 a	209.4 ± 5.5 a	0.88 ± 0.1 ab	0.51 ± 0.15
48 h DW	21.3 ± 2.7 fg	1.52 ± 0.19 gh	09.4 ± 2.1 hijk	038.1 ± 6.2 i	0.70 ± 0.4 ab	0.42 ± 0.02
48 h 0.65 mM GA_3	23.3 ± 1.3 ef	1.67 ± 0.10 fg	09.2 ± 2.2 hijk	068.8 ± 3.6 efgh	0.63 ± 0.7 bc	0.78 ± 0.03
48 h 1.50 mM GA_3	24.7 ± 1.8 ef	1.76 ± 0.13 fg	06.5 ± 1.0 jk	063.8 ± 3.6 fgh	0.63 ± 0.7 bc	0.22 ± 0.02
48 h 2.50 mM GA_3	25.3 ± 1.3 ef	1.81 ± 0.10 fg	14.6 ± 2.3 efgh	072.7 ± 7.6 efg	0.45 ± 0.1 bc	0.38 ± 0.06
48 h 5.00 mM GA_3	55.3 ± 1.3 b	3.95 ± 0.10 b	40.3 ± 3.9 bc	102.1 ± 3.1 b	0.88 ± 0.1 ab	0.28 ± 0.05

EI: Emergence index; GS: Germination speed; GV: Germination value; VI: Vigour index. Values are mean ±SE of three independent experiments each with three replicates. Mean followed by same letter with in columns are not significantly different at the 5% level (DMRT).

cies such as *Acacia* species (Kumar and Purkayastha 1972; Gunn 1990) *Astragalus adsurgens* (Kondo and Takeuchi 2004), *Uraria picta* (Ahire *et al*. 2009). A similar trend in germination was observed in the treatment of concentrated HCl but it was less effective than H_2SO_4 (**Table 2**). Similar results were recorded by Ahire *et al*. (2009) in *U. picta*. In the present investigation, 2.5 min treatment of concentrated H_2SO_4 might be sufficient to hydrolyze the seed coat constituents and further improved germination.

Seed germination is influenced by internal factors controlling dormancy, including phytohormones inducing dormancy and seed coat factors. Depending on the plant species and type of dormancy, various growth regulators are used to break dormancy. Pre-soaking treatment of plant growth regulators such as gibberellins and cytokinins has been shown to effective to break dormancy in many species (Dweikat and Lyrene 1988; Karam and Al-Salem 2001). In the present study, on application of different concentrations of cytokinins and auxins, only about 10% germination was observed (data not shown). Among the different plant growth regulators treatments, GA_3 significantly influences the germination in *Evolvulus alsinoides*. Initiation of germination and percentage germination varied with concentration of GA_3 and duration of treatment (**Table 3**). The highest germination percentage (78.7 ± 1.8) was observed in the seeds treated with 5.00 mM GA_3 for 36 h. Further increase in pre-soaking duration for 48 h resulted in decline in germination percentage (55.3 ± 1.3). Maximum daily germination speed was attained on the 7^{th} day after sowing and germination value was found to be highest (50.3 ± 1.8) in the seeds with 5.00 mM GA_3 for 36 h. The emergence index was also found higher (0.88 ± 0.1) in seeds treated with 5.00 mM GA_3 for 36 h. The higher value (209.4 ± 5.5) of vigor index was recorded in the seeds treated with 5.00 mM GA_3 for 36 h followed by 134.7 ± 5.1 in seeds treated with 5.00 mM GA_3 for 24 h. The interest in emergence index and germination speed is based on theory that those seeds germinate rapidly and vigorously under controlled laboratory conditions are capable of producing vigorous seedlings under field conditions (Aldhous 1972; Alamgir and Hossain 2005). The concept of germination value aims to combine an expression of total germination at the end of the test period with an expression of germination energy or speed of germination in a single figure (Djavanshir and Pourbeik 1976; Ahire *et al*. 2009). In the present study, GA_3 enhanced germination more than the control and any other treatment. The role of gibberellins in germination is thought to trigger hydrolysis of storage nutrients in seeds with direct effect on the growth of the embryo (Karssen *et al*. 1989). It was reported that germination can be induced by GA_3 in *Vaccinium corymbosum* (Dweikat and Lyrene 1988), *V. myritilus* (Giba *et al*. 1993), *Fagus sylvatica* (Nicolas *et al*. 1996). Similarly, *Sapindus trifoliatus* seed germination was enhanced to 70-89% on treatment with GA_3 for 10-50 h at concentrations ranging from 250-1500 ppm (Naidu *et al*. 2000). Pretreatment with GA_3 significantly enhanced germination (60-67%) of non-stratified *Morus nigra* L. (black mulberry) seeds (Koyuncu 2005). The seeds of *Stylosanthes scabra* cv. 'Seca' pre-treated with GA_3 (300 ppm) increased germination up to 55% (Bhatt *et al*. 2008). Gibberellic acid is known to break dormancy of several types of seeds including light-promoted seeds, light inhibited seeds, seeds requiring stratification and seeds requiring storage at room temperature in dry conditions (Chen and Chang 1972).

Influence of seeds storage on germination

Maximum seed germination was obtained on seeds treated with 5.00 mM GA_3 for 36 h. Therefore, to find out the influence of seed storage on germination, stored yellowish-green and brown seeds were treated with 5.00 mM GA_3 for 36 h and incubated in Petri dish on moist germination paper under controlled conditions. The results depicted in **Fig. 2** clearly indicate that the storage of seeds influences the germination. Freshly collected seeds and 6 month stored seeds showed almost similar germination percentage in yellowish-green (77%) and brown seeds (37%). Germination percentage declined in the seeds stored for 12, 18 and 24 months (**Fig. 2**). Seeds stored for more than 24 months showed only about 10% germination in yellowish-green and brown colored seeds (data not shown). The seeds of different color viz. black, brown and yellowish-green significantly influenced the germination. The black colored seeds did not germinate as they showed negative TTC test. Yellowish-green seeds showed maximum TTC positive test and same lot of seeds showed maximum germination. Seeds of different color may have different germination requirement. Light colored seeds of *Atriplex inflata* (Beadle 1952) and *A. patula* var. *hastata* (Ungar 1971) were less dormant than dark color ones and brown seeds of *A. rosea* (Kadaman-Zahavi 1955) and *A. hortensis* (Nobs and Hager 1974) were less dormant than black ones. Freshly collected matured brown seeds of *Alysicarpus monilifer* germinated to higher percentage than yellow once (Maurya and Ambasht 1973) and freshly matured brown seeds of *Senna obtusifolia* germinated to higher percentage than green ones (Nann 1992).

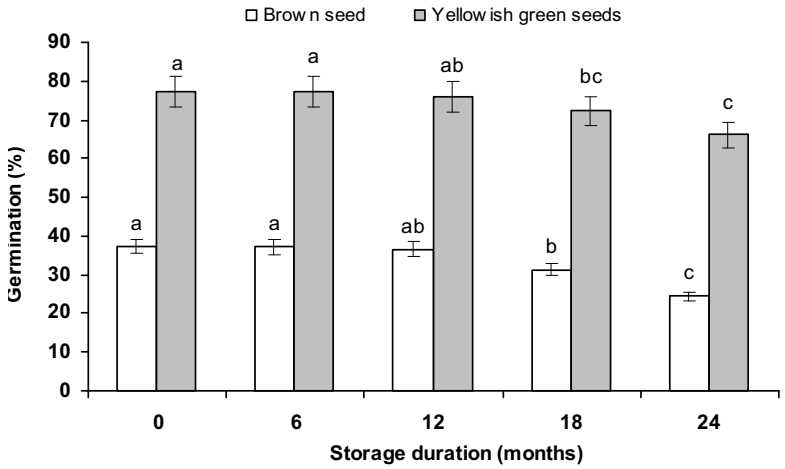

Fig. 2 Effect of storage life of seed on germination in brown and yellowish-green seeds of *Evolvulus alsinoides* L. Each value represents mean of three replications and vertical bars indicate SE. The bars with different letters on the same color columns are significantly different at $P < 0.05$.

CONCLUSION

In conclusion, characterization of seeds in *Evolvulus alsinoides* is an important factor in germination of seeds as black seeds are non-viable and only 40% brown seeds found to be viable. Yellowish-green seeds are the choice to study the germination behavior. Among the various treatments tried, treatment of GA_3 proved to be most effective in enhancing germination. Application of presoaking treatment of 5.00 mM GA_3 for 36 h resulted in 78.7 ± 1.8% germination. Application of wet heat treatment did not promote the germination as compared with the cold water treatment which denotes the temperature plays a role in induction of germination. The stored seed resulted in decline in germination. From the results obtained it can be conclude that the seeds of *Evolvulus alsinoides* had storage life of about 2 years and might have physiological dormancy which could eliminated by using the treatments with GA_3. The protocol describes in the present investigation can be utilized for large-scale cultivation of important memory tonic herb *Evolvulus alsinoides*.

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