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Research Article

BREAKING THE STONE: OVERCOMING SEED DORMANCY AND SEEDLING EMERGENCE OF THE RARE *ZIZIPHUS BUDHENSIS* IN BHUTAN HIMALAYAS

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ABSTRACT
Ziziphus budhensis, a newly identified *Ziziphus* species belonging to the Rhamnaceae family is a tree of religious, economic and conservation significance. This particular species is poorly distributed in nature and rarity is often linked to poor natural regeneration and very low propagation success. There is also no documentation or scientific studies on the species. In order to provide basic information that will be relevant for its multiplication and conservation, we aimed to understand the seed treatment and germination response of this species using different pre-treatments (sanding, cracking, Acid, hot water and control). The study was carried out in two phases and in a completely randomized block design with three replications at controlled temperature of 25-30 ^{0}C and 12 hours light. The study revealed that seed germination and seedling emergence was highest (90% ± 7.3 SE) when the hard seed coat was cracked and sown, followed by sanding of the seed stone to a thin layer (76.7 % ± 5.3 SE). The duration taken by the seeds to germinate was also faster in these two treatments (≈ 2 - 5 days). Acid treatment for 20 minutes gave a low germination and seedling emergence percentage (10 % ± 1.0 SE) with a longer duration to germinate (≈ 23 days), while treatments that has no effect on the hard seed stone (seeds soaked in hot water and control) did not germinate and emerge at all. Seed storage and viability of the species was significantly reduced after 4 months of storage which is very peculiar compared to other *Ziziphus* species. In the context of this study, it can be concluded that sowing the endosperm by cracking the hard seed stone soon after seed collection is the best method to propagate and conserve *Z. budhensis* in Bhutan Himalayas.

Keywords: *Seed Stone, Pre-treatment, Zizyphus, Bhutan Himalayas*

INTRODUCTION
Ziziphus budhensis is a deciduous shrub-tree attaining a height of 10m and belongs to the Rhamnaceae family. The species is found growing in small pockets of Western Bhutan and Central Nepal and remained unidentified until recently. The tree has been lately described as a new species *Z. budhensis* by Khem Raj Bhattarai and Mitra Lal Pathak from Nepal (Bhattarai & Pathak, 2015) which is published in the Indian Journal of Plant Sciences.
In Bhutan, the species is commonly known as *Chemshing* which literally translates to “rosary bead tree” in English language. The fruits particularly inner stony endocarp from this tree is used to make rosary beads for religious purposes by the Buddhist people since long time. Legend has it the current few populations of the species in Bhutan had been brought by a great Buddhist saint Thangtong Gyalpo (15th Century) from Nepal during his pilgrimage to the present Boudhanath temple. Since then, there are many accounts on use of the stony fruits as the religious beads by the Central Monastic Body in Bhutan even in the 16th century (Wangda, 2008). However, the tree gained more popularity recently due to the high economic value of the beads which were made into a garland of 108 seeds (Bhattarai & Pathak, 2015). Either due to extensive collection of the seeds for making beads or for some natural reasons the tree is known to be very poor in both natural and artificial regeneration which leads to rarity in nature. There are only few trees growing in the Western Bhutan and similarly, the distribution in Nepal is also confined to a small area of Central Nepal (Bhattarai and Phatak, 2015). There are no records of distribution of this species from other countries.

Considering the strong religious sentiments towards this tree and poor natural regeneration coupled with the aging of the existing parent trees in Bhutan, study on understanding the ecology and propagation techniques was initiated in 2007. There are general views that the poor natural regeneration is associated with human interferences in the form of extensive collection of fallen seeds which removes the basic requisite of regeneration.

Propagation and multiplying this species is important in order to conserve the relict plant. Like all the *Zizyphus* species, the seeds of *Z. budhensis* are enclosed in a woody endocarp (stone) which is mostly mistaken as the seed (Maraghni, *et al.*, 2010). It is this hard stone that prevents the germination and seedling emergence of Ziziphus species. Pre-treatment of the hard stone prior to sowing is therefore essential for germination and emergence of seedlings. There are many pre-treatment methods described by various researchers on some related species. Germination is known to be promoted by scarification of the hard stone with concentrated sulphuric acid in *Ziziphus spina-christi* (Aboutalebi *et al.*, 2012) and digestion by cattle in case of Z. mauritiana (Grice,1996) and by spider monkey in case of *Z. cinnamomum* (Zhang and Wang, 1995). Commercially important species such as *Z. mauritiana, Z. mucronata, Z. nummularia and Z. lotus* are well studied and their information along with propagation methods are described in detail by Asenga & Otsyina, 1996, Hassen *et al.*, 2005, Enayatollah *et al.*, 2012 and Maraghni *et al.*, 2010 respectively. However, there has been no documentation on this species except general myths and legends narrated by the elders on the newly described Himalayan species, *Z. budhensis.* Therefore, any single information obtained on the species is important for its conservation and management (Wangda, 2008). Several attempts to propagate the species through artificial propagation such as cuttings, layering, grafting and even tissue culture were tried since 2005 in Bhutan however, the success and survival of the species was very low. Unlike its closely related cousins which germinates easily from seeds (Asenga & Otsyina, 1996), *Z. budhensis'* germination and seedling emergence is very poor, which may be associated with its thick impermeable endocarp. The tree produces good quantity of seeds every year but germination and regeneration is very rare. It appears that the lifespan of seeds within the stony endocarp is also very short lived though there is no literature available on it. Removal or abrasion of the hard endocarp artificially is known to promote germination in many related species such as *Z. mucronata* (Hassen *et al.*, 2005), *Z. mauritiana* and *Z. abyssinica* (Mankar *et al.*, 1997; Murthy and Reddy, 1989). In the absence of proper documentation on the species itself, propagation and conservation of this species is a challenging task. Thus, in order to fill up the information gap, current study was undertaken with the objective to determine the best practical method to break seed dormancy and enhance seedling emergence in the multiplication and conservation efforts of this species within shortest possible time.

MATERIALS AND METHODS

Study Site

Z. budhensis grows at an elevation of 1000 – 2000 m in Nepal (Bhattarai and Phatak, 2015). According to the Bhattarai and Phatak (2015), the stony fruit is 1.5-2.5 cm in diameter and the mesocarp is thin up to 1 cm.

The woody endocarp inside the mesocarp is thickly cartilaginous, 1-3 loculed with 1 or 2 seeds. The tree flowers from March-April and fruiting takes place from May-August. Good rainfall during the flowering and fruiting period results in abundance seed production (Wangda, 2008). The tree sheds its leaves in winter months November-December and remains leafless until late February.

In Bhutan, *Z. budhensis* is located at an altitude of 2250 m. a.s.l. (N 27º22'28.4", E 89º27'17.5") facing south-east along a relatively steep slope with a slope percent of 35-40 % (Figure 1). *Z. budhensis* appeared on a shallow and dry soil with a soil moisture content of 10-15 % at 10 cm soil depth (Wangda, 2008). The species is known for its survival on harsh environmental conditions with the mean monthly temperature varying from a maximum of 20 ºC to a minimum mean monthly temperature of about 2 ºC. Mean annual rainfall at the site varies from 400 mm to 600 mm and a clear dry spell from October to late January (Wangda, 2008).

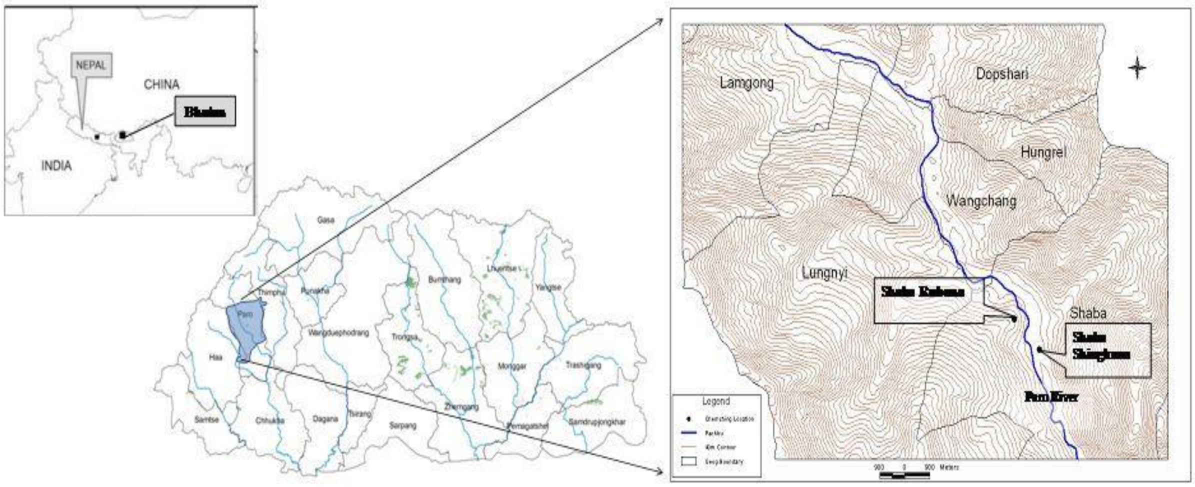

Figure 1: Map of Asia showing the distribution of *Z. budhensis* in Nepal (adapted from Bhattarai & Pathak, 2015) and Bhutan (Top left). Map of Bhutan and study area showing the location of Z. budhensis (Centre and right)

The seeds of *Z. budhensis* were collected in October 2013 and brought to the Research and Development Centre Yusipang, Thimphu where seed treatments and germination experiments were conducted in the laboratory and nursery, in two phases: 1. Experiment established soon after seed collection in November 2013 and 2. Experiment established after 4 months of seed storage at 5 ^{o}C in a refrigerator.

Experimental Design and Data Recording

About 600 seeds of *Z. budhensis* were collected in October 2013. Seeds were collected directly from the tree as fallen seeds may have low viability in late October. Seeds were brought to the laboratory and the outer pulp was removed to the woody endocarp (stone). Five treatments were used on the woody endocarp to break the seed dormancy 1. Scarification of hard endocarp between two pieces of sanding paper until the endocarp is reduced to a very thin layer. 2. Scarification of the endocarp with conc. sulphuric acid (H_2SO4) for 20 minutes and washing in running tap water for 10 minutes. 3. Cracking of the endocarp and sowing the seed directly 4. Hard endocarp soaked in hot water for 20 minutes and 5. Endocarp sown directly was used as control. A completely randomized block design with three replications for each treatment was used for this experiment. The different pre-treated stones and seeds were sown in the germination trays containing sterilized river sand at a depth of 1-1.5 cm and were kept in the germination incubators at controlled temperature of 25-30 degree Celsius and 12 hours light and 12 hours dark. Adequate moisture was provided through a sprayer using distilled water regularly. The germination status was recorded daily and the experiment was kept for a little more two months (65 days).

Statistical Analysis

The germination and emergence percentage was analysed using windows SPSS ver. 16 (SPSS Japan Inc., Tokyo, Japan). One-way analysis of variance (ANOVA) was performed after checking the normal distribution tests in the data set to see any significance difference amongst the treatments. Fisher's LSD post hoc test was deployed to check which treatment means are significantly different from each other.

RESULTS AND DISCUSSION

Results

Germination of Immediately Sown Seeds to Different Pre-Treatments

Germination responses of *Z. budhensis* seeds to different pre-treatments varied greatly under similar light, temperature and moisture conditions. By deploying different pre-treatment methods, dormancy induced by the woody endocarp was prevented to achieve successful germination of seeds and their progress into seedlings and saplings. Different pre-treatments significantly affected the final germination of *Z. budhensis* (Anova, F (4, 8) = 32.349, $p < 0.001$, Table 1).

Table 1: Analysis of variance of the effect of treatments on the germination percent of *Z. Budhensis*

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Dependent Variable: Germination percentage

Source	df	Type III Sum of Squares	Mean Square	F	Sig.
Treatment	4	23506.667	5876.667	32.349	0.000
Block	2	13.333	6.667	0.037	0.964
Error	8	1453.333	181.667		
Corrected Total	14	24973.333			

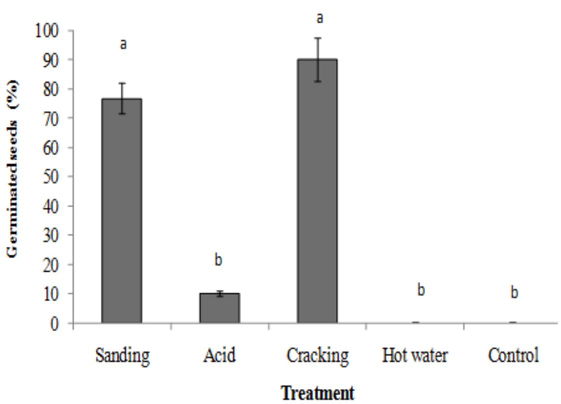

Figure 2: Comparison of the effect of treatments on the germination percentage of Z. budhensis. Different letters indicate significant differences (LSD, P < 0.05)

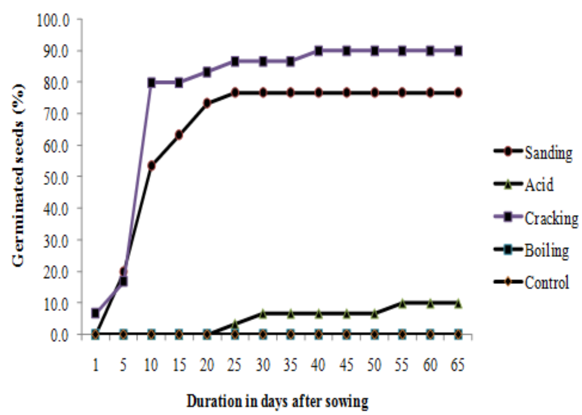

Figure 3: Comparison of the effect of treatments on days/duration taken to germinate after sowing

Figure 2 describes the degree of effectiveness of different treatments on the germination of *Z. budhensis*. Cracking of the hard endocarp and sowing the lone seed gave the highest germination (90 % ± 7.3 SE) followed by sanding of the hard endocarp to a very thin layer (76.7 % ± 5.3 SE) and then by acid treatment of the hard endocarp (10 % ± 1.0 SE). The two other treatments viz. soaking in hot water for 20 minutes and the control (soaking of seeds in cold water for 24 hours) did not germinate at all under the same temperature, light and moisture environments (Figure 2). The results indicate that the germination of *Z. budhensis* is purely controlled by the hard endocarp and any treatments that abrade the hard stone would provide favourable environment for the seed inside to germinate. For the seeds to germinate, adequate moisture and gaseous exchange must reach the embryo, however, in the case of *Z. budhensis*, the hard seed coat acted as an impermeable barrier to moisture and gaseous exchange. Complete removal of seed coat or abrassion of seed coat (sanding and acid treatments) therefore gave good germination compared to treatments that did nothing to the hard seed coat (hot water and control).

Germination Duration of Immediately Sown Seeds

The duration or time taken by seeds to germinate also differed greatly amongst the pre-treatment of seeds (Figure 3). The total duration from the emergence of first seed of any pre-treatments varied from one day to as long as 23 days. Cracking of the hard seed stone treatment took only one day for about 7% of the seeds to germinate while sanding of the hard seed stone treatment took about two days to germinate about 3% of the seeds. After 5 days of trial initiation cracking and sanding treatments have achieved about 17% and 20% germination of the total seeds sown respectively (Figure 3). The cracking and sanding treatment reached their peak germination percent in about 40 days (90%) and 23 days (77 %) respectively. These two treatments were followed by the acid treatment which resulted in the initial germination of about 3% in 23 days and attained a peak germination value of only about 7 % in 29 days. The other two treatments; hot water and control did not germinate at all even 65 days after sowing. The un-germinated seeds were cracked to see the condition of the endosperm under hand lens and found that the embryo had decayed and become empty inside.

Germination Response 4 Months after Storage

The germination and emergence of seeds four months after storage was very poor. The sanding pre-treatment gave only a seed germination of 6.7 % (± 0.8). All other seeds failed to germinate indicating short viability of seeds (Table 2). On further examination by cracking the stored seeds, we found that the seeds have become dark and mostly empty inside.

Table 2: Effect of seed storage (4 months) on the germination and seedling emergence of *Z. budhensis*

Treatments	Seeds sown directly	Seeds sown after storage
Sanding of the stone in between sand papers	76 ± 5.2	6.7 ± 0.8
Acid treatment of the stone for 20 minutes	10 ± 1.0	0
Cracking of the stone and sowing the seed	90 ± 7.3	0
Soaking in Hot water	0	0
Control	0	0

(± SE), values expressed as percentages

Seedling Growth and Survival

Seedling growth and survival did not differ significantly amongst the treatments (data not presented) however, height growth was faster for the cracking of the stone treatment followed by others. The average height of seedlings 1.5 years of pricking varied from 22-25 cm and collar diameter of about 0.2- 0.5 cm. The mortality rate from seedling emergence to planting size varied from 30-40% (Data not presented).

Discussion

Propagation and multiplication of *Z. budhensis* is seen as an important step towards conservation of this small population. Several attempts to propagate the species via artificial means were tried in Bhutan, however with little success. Propagation through seed therefore remains a single most hope in the propagation of the species (Figure 5). But the seeds are enclosed in a very hard woody endocarp andis in a dormant state. Seed dormancy is a condition in which the viable seeds fail to germinate even when they are exposed to favourable environmental conditions such as moisture, temperature and light. There are many types of dormancy but dormancy induced by the woody endocarp or hard seed stone is very common for stony fruits like *Zizyphus* species. By comparing the different dormancy breaking methods on the germination response of *Z. budhensis*, it appears that they could be described as having a form of mechanical dormancy induced by the impermeable endocarp which restricts the gaseous and moisture exchange to the seed. Our results indicated that failure of *Z. budhensis* to germinate may be due combinations of two main dormancy types: 1. the very hard seed tone serves as a mechanical barrier for the projection and enlargement of embryo (Mechanical dormancy) and, 2. The hard seed stone acts as a physical barrier to water and gaseous exchange which is essential for growth of the embryo (Physical barrier). Our study justified this statement as the treatments like cracking, sanding and acid treatments which broke or abraded the hard seed stone gave significantly higher germination percent compared to the other treatments where seeds are sown with intact seed stone. Our result is therefore in agreement with several other studies reporting that seed stone induced dormancy can be gradually overcome by breaking or abrading of the seed stone during pre-treatment of the seeds such as *Z. spina-christi* (Saied *et al.*, 2008), Z. mucronata (Hassen, 2005), *Z. mauritiana* (Grice, 1996) and *Z. nummularia* (Hussain *et al.*, 1993). The woody endocarp also acted as an impermeable barrier to the seeds inside to grow and emerge which is evident from the duration taken by the seeds to germinate. Seeds sown alone after cracking the woody endocarp imbibed moisture quickly and germinated as fast as less than one day and that of sanded seeds (in which the endocarp is removed to a thin layer) took as less as two days. Acid treated woody endocarp took some time for moisture to reach to the seeds and germinated after about 23 days of sowing. The control and hot water treatment was ineffective as they virtually had no impact on the woody endocarp. Hassen *et al.*, 2005 also reported similar results, where *Z. mucronata* (buffalo thorn) seeds gave significantly higher germination and emergence when cracked and soaked in water. In the absence of any action that can abrade the hard endocarp and expose the seed, germination and emergence of *Z. budhensis* is nil. Therefore in nature, any physical conditions or forces that abrades the woody stone should be quicker. In the natural state, the abrading forces tend to occur over a long period of time during which the seed viability inside is already lost. This could be a reason why there is no natural regeneration of the species. Our study supports this because seeds stored under dry and cool conditions (below 5 ºC) even lost their viability significantly within a period of 4 months quite contrary to other Ziziphus species which can be stored up to 1-2 years under similar storage conditions. The seeds became dark and decayed completely upon observation by cracking the hard endocarp. In such a case seed storage may not be recommended but rather seeds are treated and sown soon after collection.

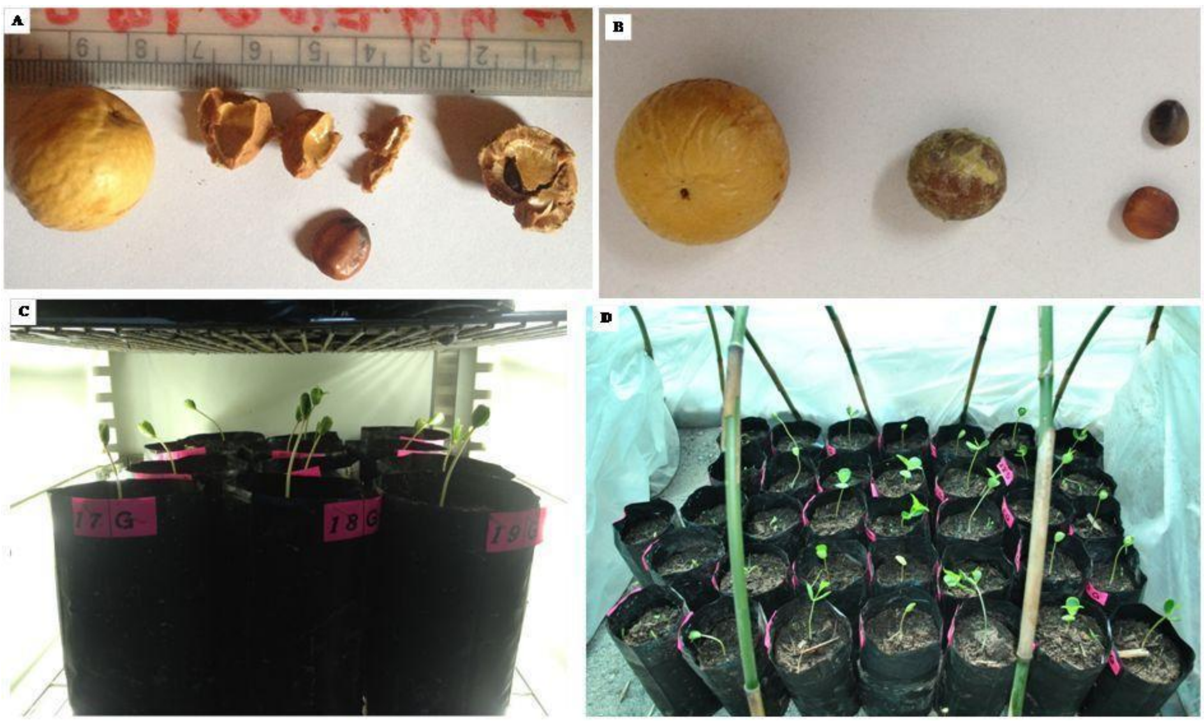

Figure 4: Seed anatomy and successful seedling emergence. A) Cracked seeds revealing the seed inside. B) Fully ripened seed with fleshy mesocarp, mesocarp removed and two seeds which are inside the stony shell when cracked. C) Successfully emerged seedlings after pricking out in to the polybags inside the incubators and D) inside the green house

There is only limited population of *Ziziphus budhensis* which gives seeds in the month of September. It should be noted that even if the tree sheds seeds in abundance, the germination or emergence of seedlings and saplings will be very rare without the physical abrasion of stony endocarp. It is reported in nature that abrasion of the stony endocarp of long lived other *Ziziphus* species happen over time from the action of fire, heating and thawing, wind and water, microbial attack, digestion by animals and cracking by animal hoofs (Hassen *et al.*, 2005; Hanley and Lamont, 2000; Pearson and Ison 1997). Considering the nature of short lived seeds and extreme hardness of the woody endocarp in the case *Z. budhensis*, the chances of cracking the hard stone by animal hoofs, heating and thawing, action of fire or even microbial attack which will lead to abrasion of endocarp is very low as these actions occur over a long period of time by which the viability of seeds inside will be already lost. However, it cannot be ignored and there is enough evidence that acid digestion and excretion of *Ziziphus* seeds by animals can contain viable seeds that can germinate (Aboutalebi *et al.*, 2012; Varela and Bucher, 2006; Grice, 1996; Zhang & Wang, 1995). Thus,

allowing grazing animals such as wild goats, deer or even domestic cattle around the mature tree during the seeding period could be an option to promote germination and encourage natural regeneration.
Further, the tree is well known for its endurance to survive on dry and shallow soils exhibiting xeric vegetation types (Wangda, 2008). Bhutanese people have high respect and love to grow this species due to religious beliefs. Thus, besides conservation goals, multiplication of the species and plantation on marginal, dry and barren lands in Bhutan will generate income opportunities thereby promoting the religious and spiritual wellbeing of Buddhist people.

Conclusion

Many studies have been carried out around the world in an effort to obtain superior cultivars of *Zizyphus* for medicinal, ornamental and horticultural purposes. These properties or purposes have never been explored in case of newly described *Ziziphus budhensis* which is an important religious/economic tree of strong conservation impetus in Himalayan countries like Bhutan and Nepal. The hard seed stone is used to make the rosary beads and are in great demand by the Bhutanese people. A garland of beads has been known to fetch high prices in Nepal (Bhattarai & Pathak, 2015). Our study therefore serves as a basic documentation for choosing a practical method to break seed dormancy and enhance seedling emergence in the multiplication process of the species. We would like to draw the following conclusions:

- In order to propagate this species in future, we recommend that seeds should be collected as soon as they ripe and seed treatment targeting at either removal or abrasion of the hard seed stone is necessary.
- Complete removal by cracking the stone or scarification with sand paper (sanding) of the stone to a thin layer appeared to be the best pre-treatments of seeds. However, sanding of the hard seed stone is a tedious task, labour intensive and is applicable to only for treating small amounts of seeds as also reported by other researchers.
- For optimum results and under the conditions of the current study, controlled temperature of 25-30 ^{0}C is best suited to germinate the pre-treated seeds of the species.
- Massive propagation and plantation of *Z. budhensis* on dry, marginal and barren lands as a part of greening projects will offer income opportunities to local communities. Encouraging domestic and wild animals such as cattle, wild goat and other ungulates to graze around the tree during the seeding period could be used as a management tool to enhance the natural regeneration of *Z. budhensis.*

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