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RAPID VIABILITY TEST—A TOOL TO AID CONSERVATION EFFORTS IN *ZANTHOXYLUM RHETSA*

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Zanthoxylum rhetsa is a dioecious forest tree species with significant commercial value, due to its diverse culinary and pharmaceutical uses. The fruits and seeds are part of cuisine in many countries of South Eastern Asia, and it has global relevance as a component of diverse pharmaceutical products. It occurs in wild forest habitat and is facing the threat of getting endangered, due to commercial exploitation and loss of habitat. The situation warrants intervention through extensive conservation efforts for sustaining and enhancing its population. The successful establishment of *Z. rhetsa* tree population depends on the availability of high quality seeds, with superior growth potential. Low seed viability and vigour are pertinent issues in this species because the bulk harvest of the fruits is preferentially carried out prior to harvest maturity, for commercial use. A pre-sowing assessment of seed viability and vigour would facilitate assured plant establishment by screening out the large number of immature or deteriorated seeds. Standard seed viability assessment protocols require more than four weeks, hence, a quick viability testing protocol will be a highly beneficial tool for conservation efforts of *Z rhetsa*. In this study, a rapid testing protocol viz., treatment of 1% TTZ at 35 °C with a soaking duration of 24 hours, was identified as the standard protocol for quick viability testing of *Z. rhetsa* seeds.

Keywords: Germination test, seed viability, germination percentage

INTRODUCTION

Zanthoxylum rhetsa is a pan-tropical dioecious tree species belonging to the family Rutaceae. It is found in the natural forest habitats of South-Eastern Asia including India, Sri Lanka, Myanmar, Thailand, South Vietnam, Malay Peninsula, Java, Philippines, Moluccas and South Papua (Thu et al. 2010, Rao et al. 2016). It is an integral part of Thai and Japanese cuisine, where it is commonly known as Chinese prickly ash pepper or Sichuan pepper. In India, it occurs in the wild habitat, across Western Ghats, North eastern states and in Andaman & Nicobar Islands. It has high commercial value due to the diverse culinary and pharmaceutical uses of the fruits, seeds, leaves and bark. In India, fresh fruits are an item of commerce in Konkan region, whereas dried fruits and leaves are sold in markets of North eastern states as spice and vegetable, respectively (Joseph et al. 1999). The fruits of *Z. rhetsa* are borne on panicles and each fruit is botanically identified as a follicle. Its round blackish seeds are enclosed in a thick pericarp which splits open on maturation. Both the seedcoat and pericarp are rich in mono-terpene based essential oil which possess significant antioxidant attributes and therapeutic properties, and has also been proven to be an effective mosquito repellant (Rabha et al. 2012, Bhalla et al. 2013, Muppalla & Chawla 2018, Theeramunkong & Utsintong 2018, Antony et al. 2019). The stem and root barks have abundance of alkaloids and phenolic compounds belonging to the lignin and coumarin subgroup. These phyto-constituents, prevalent in the various plant parts, render high ethno-medical qualities, thus, it is highly sought after as anti-inflammatory, analgesic, antibacterial and anti-diarrheal components of various traditional medicine systems across the world (Supabphol & Tangjitjareonkun 2015, Okagu et al. 2021, Aziz et al. 2022).

The diverse use of this tree species is leading to over-harvesting from forest habitats and there is significant reduction in its population in the native countries. It has been listed by IUCN in its red list category, indicating increasing risk of extinction (Barstow 2019). Ground survey studies have reported it to be critically endangered in several regions of the Western and Eastern Ghats of India and is red listed as a medicinal plant of conservation concern in India (Jonnakuti 2014,

Patil et al. 2014, Rajasekharan & Wani 2020). This species is also red listed in Malaysia and is a priority species for genetic conservation in Indonesia (Chua et al. 2010, Country Report 2011).

Conservation efforts for re-establishment of this species in its natural habitat involve mass multiplication of saplings and introduction into the forest plantation, for facilitating in situ conservation. The most cost-effective approach for this endeavor is establishment of sapling nurseries from seeds. However, this procedure is hindered by the low seed viability characteristic of the species. The low germination capacity of seeds can be primarily attributed to the high level of immature seeds in the panicles, since the seeds are bulk harvested when the seed coat is still green, which is the ideal stage for use as spice. Seed sterility due to low pollination caused by high temperature and humidity during flowering, and lack of proximity between male and female trees are also other major factors. Storage of the harvested oil-rich seeds in ambient tropical conditions, where it is produced, further accelerates deterioration and loss of seed viability. When such seed lots with low viability are used in nurseries, it delays the plantation re-establishment efforts by a whole year since seedling emergence results are evident only after almost two months of sowing, and if found unsuitable, re-sowing cannot be carried out due to low quality of stored seeds. The successful establishment of the emerged seedling also depends on its ability to survive under various biotic and abiotic stress factors that prevail in the forest habitat, which in turn is determined by the vigour status of the seedling. Hence, there is a need to develop a suitable seed testing protocol that can ascertain the germination capacity and vigour status of *Z. rhetsa* seeds. The standard seed germination test for estimation of normal seedlings is not having practical utility in this regard, due to the lengthy duration involved. The lack of *ex situ* conservation efforts for *Z. rhetsa* seeds also adds to the lacunae in seed availability. As per available records, there are no conserved seeds of this species in any genebanks. Accessions are being maintained in either field genebanks or in botanical gardens (Joseph et al. 1999, Yulistyarini & Hadiah 2021).

In case of such species, the International Seed Testing Association (ISTA) recommends the quick viability test using tetrazolium reagent, viz., 2, 3, 5 triphenyl tetrazolium chloride (TTZ test), as the best option for viability assessment (ISTA 2019). The TTZ test is based on the reduction reaction that occurs only in the presence of dehydrogenase enzyme, thus restricting the reaction to the living cells of the seed. The water-insoluble product called formazan causes all living cells to be stained red. The intensity of the stain is determined by the level of enzyme activity, and thereby, is directly proportional to seed vigour. The staining pattern of the seed, and the extent of staining of seed parts that give rise to essential seedling structures, are used for assessing their viability and vigour status. This has been successfully demonstrated in *Eucalyptus* spp., *Genipa americana*, *Ailanthus altissima*, *Acer pictum, A. rubrum*, *Juglans mandshurica, Fraxinus mandshurica*, *Juglans nigra* and *Eremanthus elaeagnus* (Velten & Queila 2005, Gang et al. 2015, Ma et al. 2016, Wickert et al. 2017, Virgens et al. 2019, Afroze et al. 2021). Earlier reports clearly indicate the need to standardise TTZ testing protocol for individual species based on seed morphology, which has not been carried out for *Z. rhetsa*. Such a protocol will be a handy tool for conservation scientists and other stakeholders for ensuring better establishment of *Zanthoxylum* seeds in its natural habitat. In the current study, an easy and reproducible protocol was developed for TTZ staining test in *Z. rhetsa* seed lots.

MATERIALS AND METHODS

In this experiment we used freshly harvested mature seeds that were collected from a wild tree in the premises of ICAR-National Bureau of Plant Genetic Resources, Regional Station Thrissur, Kerala, India. The collected seeds were subjected to germination test, using 400 seeds in four replicates of 100 seeds each, using Between Paper method. The samples were maintained at 25 °C. The germination percentage was calculated based on the number of normal seedlings counted on the 28th day of the test, as recommended by the International Seed Testing Association (ISTA 2019).

Standardisation of 2, 3, 5 triphenyl tetrazolium chloride (TTZ) test

After ascertaining the initial viability percentage, the seeds were preconditioned through overnight soaking in distilled water. This step is essential

for pre-moistening of tissues and uniform TTZ reaction. Subsequently, the seed coat was cracked, without damaging the embryo and the seeds were dipped in TTZ solution.

For the standardisation of the protocol, three levels of incubation temperatures (30, 35 and 40 °C), three concentrations of TTZ (0.1, 0.5 and 1.0%, with pH 7.0, prepared in distilled water) and three different durations of soaking in TTZ (14, 24 and 36 hrs) were attempted, and each treatment was replicated thrice.

The preconditioned seeds were soaked in TTZ for the proposed durations and incubated at the indicated temperature levels, under dark condition. After the soaking period, the seeds were removed from TTZ and thoroughly rinsed in distilled water. The seeds were then scrutinised for their topographical staining pattern, using hand lens. Seeds were considered viable if they qualified any of the following five criterias: (1) seeds contained fully stained embryos, (2) seeds possessed less than 50% of area having lighter red stain colour, (3) one third or less part of radicle was unstained, (4) half of distal end of cotyledons or less was superficially unstained and (5) one third of distal end of cotyledons or less had pervading unstained areas. The TTZ viability percentage, as obtained in each of the treatment, was subject to arcsine transformation, and the data was analysed using SPSS software. The TTZ treatment that gave potential viability percentage values which best correlated with the actual germination percentage was identified as the standard TTZ protocol.

RESULTS

Initial germination test

The test conducted on the fresh seed lot showed a mean germination value of 96%. There were no abnormal seedlings present in the evaluated sample.

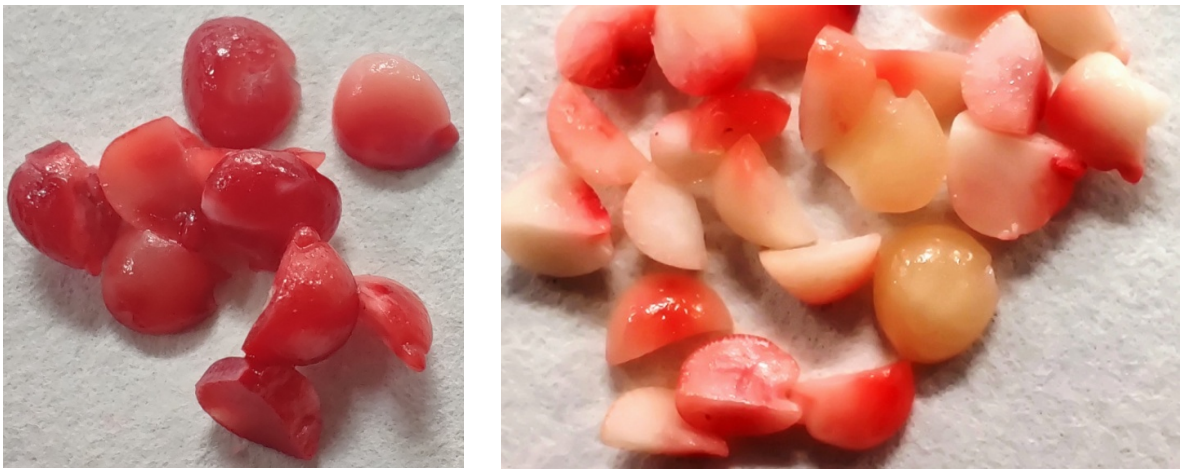
Standardisation of TTZ test

When the seeds, after removal of seed coat, were exposed to the 3 soaking durations (14, 24 and 36 hrs), it was observed that 24 hrs was required for effective staining of seed embryo, irrespective of the temperature. Below 24 hours, the staining was restricted to the cotyledons and the radicle. The *Z. rhetsa* seeds have characteristic thick cotyledons which require sufficient duration of exposure to TTZ, and it was observed that effective soaking was attained in 24 hours (Figure 1).

Hence, all further treatment combinations were carried out by including a single soaking duration of 24 hours. The effect of temperature and concentration of tetrazolium on the staining profile (and hence, on viability percentage) is indicated in Table 1.

DISCUSSION

The analysis revealed significant effect of incubation temperature and concentration of TTZ on the staining pattern. Within the duration of 24 hours soaking, exposure to



(a) (b)

Figure 1 2, 3, 5 triphenyl tetrazolium chloride (TTZ) stained seeds of *Zanthoxylum rhetsa* depicting (a) viable (stained as per criteria mentioned in 2.1) and (b) non-viable (unstained) seeds

Table 1 Effect of incubation temperature and concentration of 2, 3, 5 triphenyl tetrazolium chloride (TTZ) solution on the staining pattern of *Zanthoxylum rhetsa*

Concentration of TTZ (%)	Temperature (°C)		
	30	35	40
0.1	64 (53.13)	90 (71.57)	82 (64.89)
0.5	74 (59.34)	94 (75.82)	84 (66.42)
1	82 (64.89)	100 (90)	88 (69.73)

Critical difference at 0.05% = 3.56; figures in parenthesis are arcsine transformed values

30 °C temperature could not generate the staining pattern equivalent to actual germination percentage, at any of the TTZ concentrations. At 40 °C, TTZ concentrations resulted in over staining, due to which the true topographical staining pattern could not be analysed, thereby resulting in false viability values. The treatment of 1% TTZ at 35 °C had the best value of TTZ viability percentage that correlated with the actual germination percentage. Hence, this treatment, with a soaking duration of 24 hours, was identified as the standard protocol for quick viability testing of *Z. rhetsa* seeds using TTZ.

CONCLUSION

The experiment could identify a standard protocol for quick viability testing in *Z. rhetsa* seeds, which will serve as an easy and effective technique for assessing the viability status in commercial seed lots. This protocol will be a handy tool for monitoring vigour and viability of seed lots prior to nursery establishment during reforestation and conservation programmes. This quick testing protocol will enable assured seedling establishment/regeneration, thereby facilitating improved population of this threatened species in its natural forest habitat.

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