*Original Research Article*

# Standardization of Quick Seed Viability Protocol for *Pinus roxburghii* Sarg. using Tetrazolium Assay ~~in Himachal Pradesh of India~~


**ABSTRACT**

Chirpine (*Pinus roxburghii*) is one of the important tree species amongfive indigenous pine species of the Himalayan region which is widely distributed throughout the Himalaya. It is used as a commercial timber, fuel wood and pulpwood and also provide resin for various utilities. For the ecological, silvicultural and economical significance, the regeneration of the species is required for restoration and conservation of the species. Before seed sowing in nursery, there is need to know the actual seed vigour and quality of seeds. Therefore, the present experiment was conducted at Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh of India to standardized a quick seed viability testing protocol using tetrazolium (TZ) ~~test~~ and confirmed with laboratory seedgerminationtesting.The tetrazolium test and germination test were conducted with 100 seeds in 4 replications in CRD design as per the procedure devised by ISTA and analyzed with OPSTAT statistical software. After pre-moistened, chir pine seeds were cut longitudinally into two parts away from the radicle end to expose the embryo. The seeds of the species stained with 0.5, 1.0, 1.5 and 2.0 % solution of TZ and incubated at 25, 30 and 35°C temperature in the darkness for 12, 24 and 36 hours.The result of TZ test showed that the seeds of chir pine soaked in 0.5 % tetrazolium solution at 25 °Ctemperature for 24 hours found to be thebest one forthe seed vigour and seed quality testing. For predicting the accurate seed viability, RMS(root mean square)method was used and recognized six staining seed categories distinguished 1 to 6. Seed category 1 denoted as viable seeds and category 6 as non-viable seeds.Result showed that the topographical staining categories 1, 2 and 3 provided least root mean squarevalue and recommendedtheseas viable seed categories for TZ seed viability test. Furthermore, it is also compared with laboratory seed germination test to standardize the seed viability protocol and result in line with the conformity. Overall, the results of the present study fulfilled all the gaps and criteria of quick seed viability testing in chir pine species and standardized protocol could be used for future seed vigour testing within a short period of time for the forest restorationand conservation purpose.



***Keywords:****Chir Pine, Himalaya, Seed Viability, Tetrazolium Test, Seed Quality, Root Mean Square, Germination Test, Seed Vigour*

## INTRODUCTION

Chirpine (*Pinus roxburghii*Sarg.) is one of the important tree species of five indigenous pine speciesof the Himalaya,which is widely distributed an altitudinal range between 450-2300 m elevation inAfghanistan, Pakistan, India, Nepal and Bhutan between latitude range 26°N-36°N andlongitude range 71°E-93°E [1].It is of great ecological, silvicultural and economical significance tree species in the Himalayan region. It provides commercial timber, resin, fuel wood and pulpwood for muciferous end uses. Resin obtained from the trees by tapingmethod is usedin soap, pharmaceutical,paper and paintindustries in India [2]. It is a fire resistant, strong light demander tree species and successful pioneer on exposed sites which can be planted on bare lands where it can withstand frost, drought, snow and free from serious pests and diseases. It has a wide variation in germination, growth, stem forming, resin yield and timber yield with grain angle throughout its natural forests in relation to altitude and site [3]. The artificial and natural regeneration of the species takes place through seeds. The pollination takes places in the spring season by air in this monoecious species and fertilization occurs in the next coming spring season and mature cones disperse their seeds in next or third spring season after pollination. Thus, all most the time taken between pollination and seed maturity is 2 years [4].Good seed year of chir pine is about 4-5 years, so seed can be collected only that year for the regeneration purpose. The seed germination and its attributes are not only irregular but also takes prolonged germination period which resulteduneven seedling growth and poor-qualityseedlings [5]. The precise determination of seed viability prior to nursery germination and field sowing is of great importance for the regeneration of the species in the forest. The accurate quantity of seeds to be sown in nursery is mainly dependent upon the quality or viability of the seeds.

Seed viability testing in tree species is very important to know the quality of seeds [6] which has a great effect on the quality of planting stock. Therefore,it is imperative to test the viability of the seeds before commencing seed sowing in nursery or field. Seed germination test for most of the forest tree species takes places from 30 to 60 days after seed sowing [7]. Moreover,the germination test of pines speciesis also inadequate and unknow their true seed viability [8].There is a need to know the non-viable seeds from viable seeds which is sometimes visually undifferentiated from dead seeds.Various tests have been used to estimate the seed viability, but they are laborious enough in workability [9]. One of the most accepted and reliable method of seed viability is the tetrazolium test (TZ) which is also referred as “quick test” [10]. A topographical method of seed viability assessment was developed first forcereals [10] and later on used for seed viability test in coniferous species [11].The tetrazolium test isalso one most rapid and useful methods to evaluate seed qualityof many species [12]. The historical aspects of the tetrazolium test from its development [13]to the applicability of 2,3,5-Triphenyl Tetrazolium Chloride (TTC) to thequick viability test in conifers species [14]for the restoration [15] and conservation ecology [16] as well as in endangered pine species [17].Seed dormancy is common in most of the forest tree species [18,19]. Tetrazolium test (TZ) also provides a quicker knowledge to obtain seed viability even when a seed in a state of dormancy [20]. Savonen (1999) developed an improved method of topographic tetrazolium testing in Scots pine to overcome the patchily stains in seeds [21] due to the effect of storage time, temperature and container on the viability of the seed of some pine species [22]. Daws et al. (2006) also tried pressure with time dependency of vacuum degassing in17 Pinus species to rapid the method for viability assessment using tetrazolium chloride [23]. Keeping in mind all the knowledge, the present experiment is conducted to standardize a quick seed viability testing protocol for the chir pine species in Himachal Pradesh of India.

## EXPERIMENTAL DETAILS

### Seed collection and extraction

Cones of chir pine tree were collected from the five different tree seed stands in the Hamirpur Forest Division, Himachal Pradesh, India during the year of 2023as geolocation presented in figure 1. After the collection of cones, they were sun dried for 2-3 days and followed the extraction, cleaning and processing of the seeds. Further, chir pine seeds were mixed thoroughly to maintain homogeneousness. Thesefive seed lots were designated from A to Eand stored in well labelled air tight plastic containers at room temperature (25°C±2).

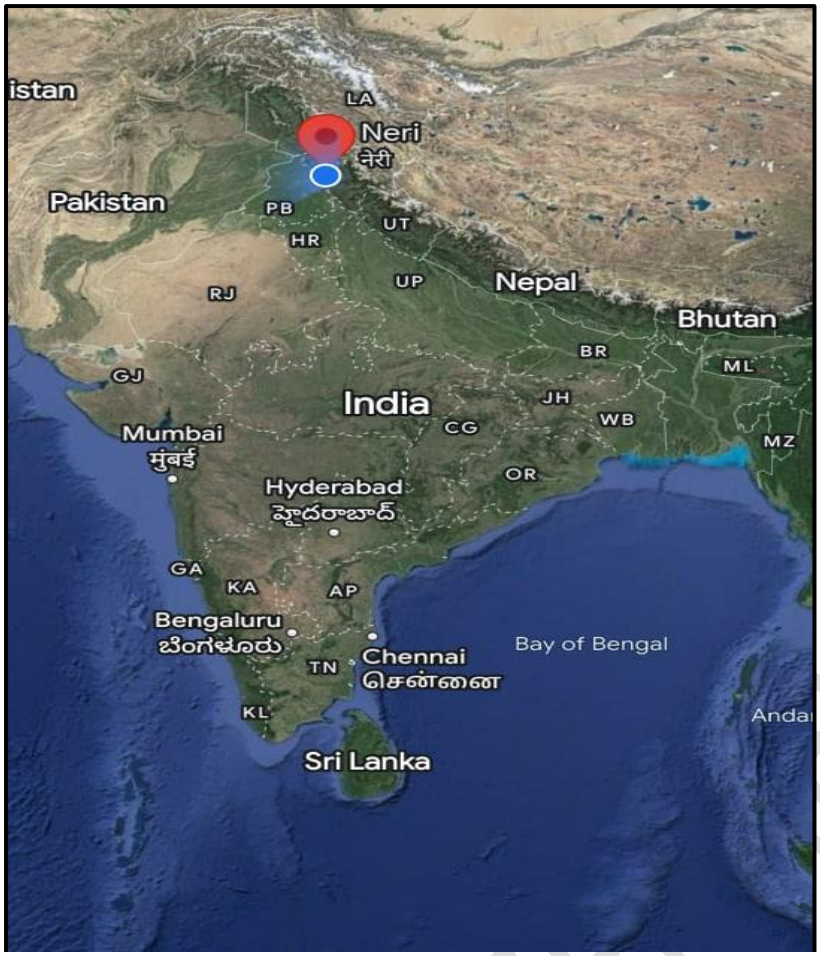

Figure 1. Geolocation of cone collection of *Pinus roxburghii* during the course of study

**TetrazoliumSeed Viability Testing**

The procedure of the tetrazolium test (TZ) was followed as per devised by ISTA [13]. Four replications of 100 seeds were taken from the random samples after mixing somequantity of seeds from each seed lotof *Pinus roxburghii*and employed for each treatments given below to standardize optimum concentration of TTC solution, incubation temperature and time. These samples were soaked in distilled water for 24 hours at ambient room temperature before TZ staining to allow complete moistened of all the seed tissues. After pre-moistened, chir pine seeds were cut longitudinally into two parts away from the radicle end to expose the embryo. Seeds of the species were then stained with 0.5, 1.0, 1.5 and 2.0% solution of TZ prepared in the double distilled water. The chir pine seeds were placed in the Petridisheson the moistened double sheets of Watman filter paper No.1 to incubate with TZ solution at 25, 30 and 35°C temperature in the darkness for 12, 24 and 36 hours. The 2,3,5-Triphenyl Tetrazolium Chloride (TTC) salt solution enters into the living tissue of the seed which produce a reddish or dark purple colour due to water insoluble compound called formazon by the activity of dehydrogenase enzyme [24]. After competition of incubation period, the TZ solution was drained out and chir pine seeds were washed in distilled water.Then, red coloured seeds were counted as viable or germinable seeds and non-coloured seeds as nonviable or non-germinable seeds. Then, the concentration of TZ solution, incubation temperature and time standardized on the basis of optimum seed viability results presented in table 1. After that, the optimised concentration of TTC solution, incubation temperature and time was employed for all the seed lots of chir pine seed separately to standardize the stain topography of viable and non-viable seeds. Seed viability was evaluated according to the topographical staining pattern of the presence, location, extent, intensity of the colouration and nature of tissue staining in all the seed structures with the help of magnifying glass [8, 13]. The staining pattern of each seed structure was recorded under 6 staining categories depicted in figure 2 and details presented in table 2.

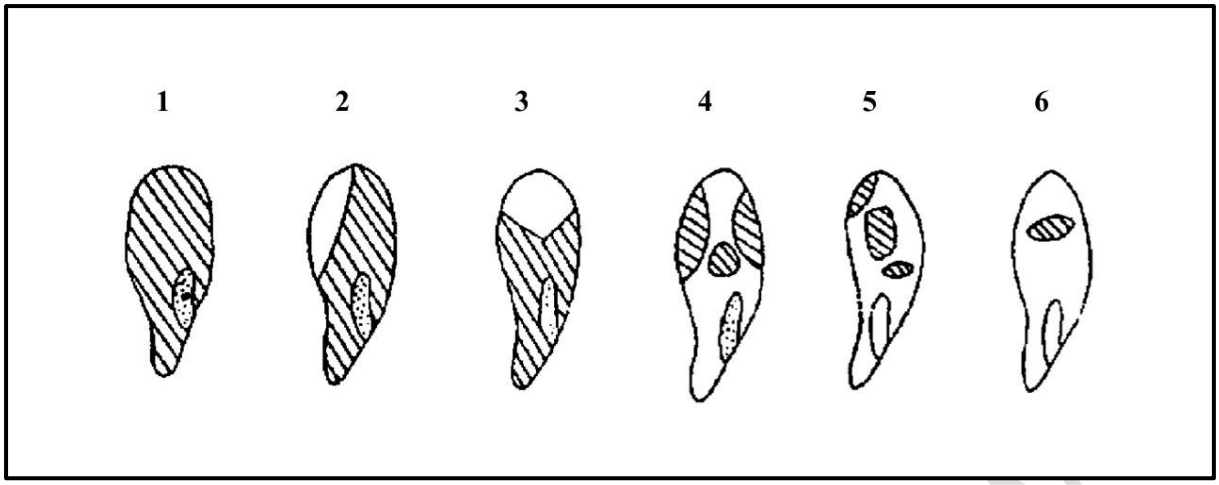

Figure 2. Staining topography category (1-6) for tetrazolium viability test in *Pinus roxburghii*

**Seed Germination Testing**

Seed germination test was conducted in laboratory condition with 100 seeds in four replications taken from each seed lot toknow the viability of seeds by performing the seed germination test. These chir pine seeds wereplaced in the Petri dishes on the moistened double sheets of Watman filter paper No.1 as per divested by ISTA [13]. Before to sowing the seeds, they were soaked in distilled water for 7 days to overcome the seed physiological dormancy which is mainlyfound in conifers [25]. The germination data were recorded to calculatethe germination percentage of chir pine seeds to test the viability or quality of seeds. The emergence of radicle i.e., newly developed root portion of the seedling reached upto the equal length of the seed was taken as the criterion for seed germination [26].

**Statistical Analysis**

The experimental data were subjected to OPSTAT statistical software package [27] for the statistical analysis and appropriate interpretation. ANOVA (Analysis of Variance) was derived using completely randomized design (CRD) with four replications in the laboratory condition. All the treatments were considered to be random and ~~treatments was~~ compared at 5 % level of significance.For predicting the accurate seed viability, the RMS(root mean square)method was usedas described byKuo *et al.* (1996) [28]. This method hadbeen used for predicting the seed viability in *Pinuswallichiana*seeds [6] and recognized six staining categoriesdistinguished 1 to 6.Seed category 1 denoted as viable seeds and category 6 as non-viable seeds. Then, total 15 combinations possibilities were found within the remaining 4categories i.e., 2, 3, 4, 5for the evaluation criteria.Root mean square (RMS) differences between actualgermination percentageas determined by laboratory germination test (G) and potential viability percentage as determined by Tetrazolium test (P) for each evaluationas per~~to~~ the following equation:

$$RMS = \sqrt{\frac{1}{n}\sum_{1}^{n}(G_i - P_i)^2}$$

Where, $G_i$ = Actual germination percentage of seed lots from 1 to n.$P_i$ = Potential viability percentage as determined byTZ staining of seed lots from 1 to n.n = Total number of seed lots in the study.The evaluation criterion used in the study that created least root mean square values was recommended for the Tetrazolium test of the species.

## RESULTS AND DISCUSSION

**Standardization of optimum conditions for TZ staining**

The experimental data is presented in table 1 with respect to know the optimum concentration of tetrazolium solution, soaking temperature and soaking time needed for maximum staining. All the treatments significantly (*P*< .05) affected the staining pattern in *Pinusroxburghii* seeds. The ~~chid~~chirpine seed ~~stained~~staining patterndecreased when the concentration of tetrazolium increased from ~~0.05~~0.5% to 2.0 %, however seed less stained when incubated at high temperature i.e. 35°C and for a longer period (36 hours). The light colour staining pattern is very difficult to differentiate viable

and non-viable seeds.The seeds of chir pine soaked in a 2.0 and 1.5 % TZsolution and incubated at 35°Ctemperature for 36 hours showed only 30 and 30.50 % seed viability, respectively.~~In respect to the highest viability,when~~Thechir pine seed soaked at 25°C for 24 hours in 0.5 % TZ solution indicatedsignificantly higher viability of 64.75 % which is followed by 57.50 %in seeds incubated at 25°C in 0.5 % TZ solutionfor 36 hours.There was a big difference between the seed viability percentage by TZ staining pattern (mean= 40.70 %) andlaboratory germination (mean= 71.25 %) with four replications of 100 seeds. On the other hand, chir pine seed soaked at 25°C for 24 hours in 0.5% TZ solution showed 64.75 % seed viability which is nearby the laboratory germination (71.25 %). Thus, the above concentration of TZ solution, incubation temperature and time could beconsidered as anoptimum for TZ staining in *Pinusroxburghii*seed and standardized for the future use for the seed viability testing in this species.The results obtained from the present study are configured with the tetrazolium test in *Pongamia pinnata*[29]; in *Moringa oleifera*[30]; in *Acer caesium* [26];*Ulmus wallichiana*[26] and in *Pinus wallichiana* [8].This is also corroborated with the ISTA rules of tetrazolium test [13].Masullo et al. (2017) optimized ~~of~~thetetrazolium tests to assess the viability and quality of *Platymiscium floribundum*, *Lonchocarpusmuehlbergianus* and *Acacia polyphylla* seeds for different concentration of tetrazolium solution, incubation time and temperature[31].Thus, TZ solution of 0.5 %, incubation at 25°Ctemperature for 24 hours standardized as an optimum condition for TZ staining in *Pinusroxburghii*seeds for the seed viability and quality testing.

Table 1. Effect of incubation in concentration of TZ solution, temperature and soaking time on seed staining pattern in *Pinus roxburghii*

| Sr. No. | Treatments | | | Seed viability percentage (%) | |
|---|---|---|---|---|---|
| | TZ conc. (%) | Soaking temp. (°C) | Soaking time (hours) | Mean | ±S.E. |
| 1 | 0.5 | 25 | 12 | 50.50 | 0.65 |
| 2 | 1.0 | 25 | 12 | 43.75 | 0.85 |
| 3 | 1.5 | 25 | 12 | 40.50 | 0.96 |
| 4 | 2.0 | 25 | 12 | 34.50 | 0.65 |
| 5 | 0.5 | 30 | 12 | 39.50 | 1.04 |
| 6 | 1.0 | 30 | 12 | 35.00 | 0.41 |
| 7 | 1.5 | 30 | 12 | 33.50 | 0.65 |
| 8 | 2.0 | 30 | 12 | 30.00 | 0.71 |
| 9 | 0.5 | 35 | 12 | 27.50 | 0.29 |
| 10 | 1.0 | 35 | 12 | 26.00 | 0.41 |
| 11 | 1.5 | 35 | 12 | 24.00 | 0.41 |
| 12 | 2.0 | 35 | 12 | 23.00 | 0.41 |
| **13** | **0.5** | **25** | **24** | **64.75** | **1.25** |
| 14 | 1.0 | 25 | 24 | 57.00 | 1.29 |
| 15 | 1.5 | 25 | 24 | 53.50 | 1.19 |
| 16 | 2.0 | 25 | 24 | 47.75 | 0.85 |
| 17 | 0.5 | 30 | 24 | 53.00 | 1.29 |
| 18 | 1.0 | 30 | 24 | 48.50 | 0.65 |
| 19 | 1.5 | 30 | 24 | 46.25 | 0.85 |
| 20 | 2.0 | 30 | 24 | 43.00 | 1.08 |
| 21 | 0.5 | 35 | 24 | 40.50 | 0.65 |
| 22 | 1.0 | 35 | 24 | 39.50 | 0.65 |
| 23 | 1.5 | 35 | 24 | 38.25 | 0.48 |
| 24 | 2.0 | 35 | 24 | 37.25 | 0.48 |
| 25 | 0.5 | 25 | 36 | 57.50 | 0.65 |
| 26 | 1.0 | 25 | 36 | 50.75 | 0.85 |
| 27 | 1.5 | 25 | 36 | 47.00 | 0.91 |
| 28 | 2.0 | 25 | 36 | 41.50 | 0.65 |
| 29 | 0.5 | 30 | 36 | 46.50 | 1.04 |
| 30 | 1.0 | 30 | 36 | 41.75 | 0.48 |
| 31 | 1.5 | 30 | 36 | 39.75 | 0.85 |
| 32 | 2.0 | 30 | 36 | 36.50 | 0.87 |
| 33 | 0.5 | 35 | 36 | 33.75 | 0.48 |
| 34 | 1.0 | 35 | 36 | 32.75 | 0.48 |
| 35 | 1.5 | 35 | 36 | 30.50 | 0.65 |
| 36 | 2.0 | 35 | 36 | 30.00 | 0.41 |
| **Overall Mean of TZ test (%)** | | | | **40.70** | |

| | | | |
|---|---|---|---|
| | | C.D. | 2.20 |
| | | ±SE(m) | 0.78 |
| | | C.V. | 3.85 |
| **Overall Mean of Germinationtest (%)** | | | **71.25** |

**Standardisation of staining topography of viable and non-viable seed**

Tetrazolium (TZ) solution is a colourless before staining of seeds and when seeds aresoaked in ~~it~~TZ, then it imbibed by the seed tissues of chir pine to interferes in the reductionprogression of living cells and accept hydrogen ions fromdehydrogenase which resulting in the production of red stable colour and non-diffusible substance i.e., Triphenyl formazon in theliving tissues.Then, it is possible to distinguish the redcoloured living tissues of the seed from the colourlessdead tissues. Based on this type of phenomenon, the topographical staining pattern of the presence, location, extent, intensity of the colouration and nature of tissue staining, the tetrazolium (TZ) stained seeds were classified into six staining categories asshown in the figure 2 and detailed in table 2. Data presented in table 2 showed that the seed viability ranging from 1.25 to 62.25 % in the five saining topographical categories (1-5) on the basis of the stained area of radicle axesand cotyledons. The comparison of staining category1 alone (mean= 55.15 %) with laboratory germination(73.35 %) among all the seed lots showed that there was big differencein viability test. The similar results also observed by Aslam et al. (2010) in *Pinus wallichiana* seed viability test [8].

Table 2. Staining topography of TZ test results and laboratory germination percentage for five seed lots of *Pinus roxburghii*

| Staining topography category | Details of staining category | TZ viability percentage in five seed lots (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | Mean |
| **1** | Embryo and cotyledon fully stained. | 48.50 | 54.25 | 62.25 | 52.50 | 58.25 | **55.15** |
| **2** | Embryo fully stained and minor unstained areas (i.e. less than 3/4) on cotyledon. | 10.50 | 8.25 | 5.25 | 10.25 | 9.25 | **8.70** |
| **3** | Embryo fully stained and less than 1/2 portion of the cotyledon unstained. | 8.25 | 7.25 | 4.50 | 7.50 | 8.25 | **7.15** |
| **4** | Embryo stained and stained patches on cotyledon. | 5.25 | 3.25 | 2.25 | 4.75 | 2.75 | **3.65** |
| **5** | Embryo unstained and stained patches on cotyledon. | 4.25 | 2.50 | 1.25 | 3.50 | 2.25 | **2.75** |
| **6** | Embryo and cotyledon unstained or stained in very small patches. | 23.25 | 24.50 | 24.50 | 21.50 | 19.25 | **22.60** |
| **Laboratory germination (%)** | | **70.25** | **73.50** | **76.25** | **72.25** | **74.50** | **73.35** |

However, this is an under estimate and to overcome it with the procedure of RMS (Root Mean Square) determination [26]. Therewere15 possible combinations(table 3) for RMS values of staining categories2 to 5 and together with the fully viable staining category1. Staining category 6 was clearly showed non-viable seeds as the radicle axesand cotyledons were fullycolourless or unstained. The experimental results showed that staining categories from 1 to 3 included as viable seedsbecause of the category2 showed a fully stained embryo and minor unstained areas on cotyledon,and staining category3 showeda fully stained embryo and less than 1/2 portion of the cotyledon unstained. After pooling together, the RMS values ofstaining categories 1,2 and 3 provide least root mean squarevalue (18.89) and therefore, it is recommended to include staining categories 2 and3 in the viable categories. However, the lowest RMS value of 13.47, 13.76, 4.43 and 14.60 showed when staining category 1 pooled with either on one of the categories 2 or 3 or 4 or 5. Staining category 4 stained only embryo and stained some patches on cotyledon. However, staining category 5 not stained embryo and stained only some patches on cotyledon.In the other combinations, the mean root square values were much higher as compared to the actualgermination percentage which leading to faulty determinationof seed viability.Anatomically, staining categories 2 and 3 also represent normal cotyledons and TZ staining as described earlier by several authors [8,26]. Aslam et al. (2010) categories six different staining ~~categories~~patternsto distinguish the difference between colouration,

radicle germination and surface area of staining of chir pine seeds for potential or real seed viability testing. They also reported that thepooling together the root mean square (RMS) values ofstaining categories1, 2 and 3 contributed the least RMS value which is justified to include staining categories 2 and3 as in viable categories.These staining categories are also anatomically visible [8]. The staining category4 and 5 included in non-viable seeds due to they are anatomically non-visible. Phartyal et al. (2003)also categories six staining topography in *Acer caesium* seed viability test and results showed that staining categories1 to 3considered as viable due to least RMS values in this combination [26].Santos et al. (2014) also standardized the preparation and staining procedure and seed viability classes in *Pinus taeda* seeds using tetrazolium test [32]. Thus, topographical staining categories 1, 2 and 3 provided least root mean squarevalue and recommendedtheseas viable seed categories for TZ seed viability testing in *Pinus roxburghii*.

Table 3. RMS (Root Mean Square) determination for depiction of viable seed categories (1-5) in *Pinus roxburghii* seeds after tetrazolium staining

| Sr. No. | Staining categories considered as viable | Root mean square |
|---|---|---|
| 1 | 1,2,3,4,5 | 27.41 |
| 2 | 1,2,3,4 | 23.49 |
| 3 | 1,2,3,5 | 23.60 |
| 4 | 1,2,4,5 | 24.00 |
| 5 | 1,3,4,5 | 24.16 |
| **6** | **1,2,3** | **18.89** |
| 7 | 1,2,4 | 19.39 |
| 8 | 1,2,5 | 19.52 |
| 9 | 1,3,4 | 19.60 |
| 10 | 1,3,5 | 19.72 |
| 11 | 1,4,5 | 20.20 |
| 12 | 1,2 | 13.47 |
| 13 | 1,3 | 13.76 |
| 14 | 1,4 | 14.43 |
| 15 | 1,5 | 14.60 |

Whenthe seed viability percentage of first three categories (1,2 & 3) was pooled together, the potential seed viability wascomparable to the actual laboratory germination in all five seed lots (table 4).The relationship between potential seed viability and actual viability for all the fiveseed lots showed that potential viability was positively comparable with laboratory germination for *Pinus roxburghii* seeds. This is also confirmed the standardised protocol for quick seed viability test in *Pinus roxburghii* in the stipulated time period. Aslam et al. (2010) reported that the seed viability percentage in tetrazolium test (79.82%)~~was~~did notvary significantly than~~that~~ the germination percentage (82.69 %)in seed germination test in *Pinus wallichiana* [8]. Gagliardi et al. (2011) advocated that the tetrazolium test (TZ) proved to be a definite test for seed viability than germination viability test which takes longer time in days to complete it, hence tetrazolium test is efficient for evaluating the physiological potential seed viability in short time of period [33]. Thus, the standardized protocol was confirmed with the laboratory seed germination test in *Pinus roxburghii*.

Table 4. Relationship between laboratory germination and potential viability as determined by TZ test for five seed lots of *Pinus roxburghii* seeds

| Sr. No. | Seed lots | Laboratory germination (%) | Potential viability (%) |
|---|---|---|---|
| **1** | A | 70.25 | 67.25 |
| **2** | B | 73.50 | 69.75 |
| **3** | C | 76.25 | 72.00 |
| **4** | D | 72.25 | 70.25 |
| **5** | E | 74.50 | 75.75 |
| **Mean** | | **73.35** | **71.00** |

When seed vigor test was compared with germination test both in the laboratory and in nursery beds for loblolly and slash pines then the correlation was found between tetrazolium staining and leachate conductivity with seed germination [34].The basic objective of seed vigor or quality testing is to provide a precise or accurate physiological potential among different seed lots before seed sowing as similar to germination percentage mostly to identify good seeds lots [35] for higher germinabilityas well as seed storage. The results of the present study fulfilled all the gaps and criteria of quick seed

viability testing in *Pinus roxburghii* and the standardized protocolcould be used for future seed vigour and quality testing within a stipulated time period.

## CONCLUSION

From the present experiment, it can be concluded that the seeds of *Pinus roxburghii* should be soaked in 0.5 % tetrazolium solution at 25 °Ctemperature for 24 hours to test the ~~seed vigour~~viability and quality of seeds before ~~seed~~ sowing with the counting of seed staining categories 1, 2 and 3 within a shortperiod of time for the forest restorationand conservation.

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