STANDARDIZATION OF SEED VIABILITY PROTOCOL FOR *PINUS WALLICHIANA* A.B.JACKSON IN KASHMIR, INDIA

Mohd. Aslam*[1], Zafar A. Reshi[2], T. O. Siddiqi[3]
[1]State Forest Research Institute, Shiekhbagh Forest complex, Srinagar, Kashmir-190001, Jammu & Kashmir, India.
[2]Department of Botany, University of Kashmir, Srinagar-190006, Jammu & Kashmir, India.
[3]Department of Botany, Jamia Hamdard (Hamdard University), New Delhi-110062, India.
***Email:** maslamdar@yahoo.co.in

ABSTRACT

Pinus wallichiana seeds were subjected to tetrazolium test in order to evaluate their viability characteristics. Seeds were soaked in distilled water for 24 hours followed by soaking in 1% tetrazolium salt and incubated at 30°C ±1 temperature in dark for 36 hours. Six staining patterns were recognized. Root mean square method was applied to determine the viable categories. Three staining patterns represented viable and three non-viable seeds. The tetrazolium staining, an established method of testing seed viability was compared with germination test. Nine seed lots were tested by each method .A close relationship was observed in the results of TTC test and germination test in the present investigation. The tetrazolium staining technique thus potentially provides an immediate and rapid method for determining seed viability.

Keywords: Kail, *Pinus wallichiana*, Tetrazolium test, seed viability protocol, seed germination.

INTRODUCTION

Seed viability testing is important for understanding the quality of seeds, which has great impact on the quality of planting stock. If the quality of the seed is poor at the time of sowing, then no amount of post sowing case will be of any avail. It is therefore imperative to have certainty about the quality and viability of the seed before commencing sowing and planting work. Germination test is commonly used for the assessment of tree seed quality but it is time consuming as it requires a minimum of thirty five days for its completion. Furthermore germination test of conifer seeds are also inadequate and do not express their true viability[1].A method is needed for separating all the non-viable from viable seeds, because live seeds are sometimes visually indistinguishable from dead seeds. Testing for seed viability is therefore required to estimate the number of living seeds in a seed bank.

A viable seed is a seed that has the potential to germinate, emerge through the soil surface and form a normal and healthy seedling under a wide range of field conditions. Viability is the percentage or proportion of viable seeds in a seed lot. In the present study, viability testing was accomplished by testing the seeds with tetrazolium (TZ), which stains respiring tissues red[2, 3, 4] and is generally considered to be the most accurate method for determining seed viability. Viability testing with tetrazolium is always preceded by germination testing to develop a close relationship between the two methods largely used for testing the quality of seeds before starting sowing work[5].

Several methods have been used to estimate the viability of seeds, but all are laborious enough to limit the scope of seed bank studies. One of the most reliable technique is the tetrazolium test[6], often referred to as a "quick test". The tetrazolium staining is an established method of testing seed viability[7], and most commonly practiced biochemical staining method[8, 9].The TZ test remains as one of the seed industry's most rapid and useful methods to assess seed quality. The history of TZ test development is highlighted by[10, 11]. The applicability of TTC (Triphenyl Tetrazolium Chloride) as useful viability indicator has already been reported for the seeds of conifers[12-14], restoration and conservation ecology[15-17] and natural ecosystems, particularly those with frequent fires[18-,20]. Perhaps one of the greatest assets of the test is its applicability to a large number of different species largely recognized by International Seed Testing Association[9]. Tetrazolium testing provides a quicker way to obtain seed viability data, also when in a state of dormancy[21,7]. Several research workers have successfully used it for the determination of tree seed viability[22-27].

Pinus wallichiana A.B.Jackson (Blue pine/Kail), the finest pines of north-western Himalayan region, is well known for its commercial and ecological importance[28]. The species grows naturally along the entire length of temperate Himalayas usually at altitudes ranging from 2000 to 3,500 meter above mean sea level[29]. Of the Indian pines, the wood of the blue pine is considered to be the best and stands next to deodar in value[30,31]. Though exploited mainly as a source of timber, the species is good source of oleoresin also, which is used for the production of turpentine oil, rosin, needle oil and camphor[32,33]. In addition, it is a dominant species of the vibrant but fragile forest ecosystems of the Kashmir Himalaya[34]. Seeds are the principal means of perpetuation of this species but its seed germination attributes are not only erratic but also low and takes more time to complete which results in prolong germination, irregular seedling growth and thus poor quality of the seedlings. Lot of variation has been observed in the tetrazolium staining pattern in our earlier

experiments with the seeds of this species[31]. Thus, it becomes imperative to standardize the efficacy of tetrazolium staining in assessing the viability of the seeds of the species for the production of quality planting material (QPM) in large scale for afforestation and reforestation programmes currently underway in Kashmir Himalaya of Jammu and Kashmir State, India.

MATERIALS AND METHODS

Sources of seed lots

The tetrazolium and germination tests were compared in nine seed lots obtained during September- October, 2007 from the entire distributional range of the species in Kashmir Himalaya of Jammu and Kashmir State, located between 32° 17' to 37° 16' North latitudes and 73° 26' to 80° o6' East longitudes and 81° East Greenwich, falling in the north-western extremity of Himalayan region of the country. After following extraction, cleaning and processing, these seeds were mixed thoroughly into a homogenous lot. The seeds were then air dried and subsequently divided randomly into 9 seed lots (hereafter designated from a to i) and then kept in separate well labeled air tight plastic containers at room temperature. All the seeds prior to evaluation with regard to viability and germination attributes were first visually assessed beneath a dissecting microscope, and the badly deteriorated seeds were removed. After removing the deteriorated seeds, each lot of stored seeds were further divided into two sub-samples, one were tested with tetrazolium staining and another were tested with the germination test under controlled conditions.

Viability Testing

The tetrazolium test was performed according to the procedure devised by International Seed Testing Association[5].200 seeds from each seed lot were used for this test in four replications of 50 seeds each. The seeds were soaked in distilled water for 24 hours before staining to allow complete hydration of all the tissues. This process permits the activation of germination enzymes and makes the seed tissues less fragile. The seeds were then bisected longitudinally to expose the embryo and stained with 1% solution by weight of triphenyl tetrazolium chloride made by dissolving the 2, 3, 5 TTC in double distilled water. The seeds were then placed in 1% TTC solution in petridishes on double sheets of Watman No.1 filter paper moistened with distilled water, which were then covered with aluminum foil and incubated at 30°C ±1 temperature in dark for 36 hours (Table 1).

Table 1: Details of pre-treatment, concentration of solution and staining time for the seeds of *Pinus wallichiana*

Pre-treatment	Concentration of TZ solution (%)	Staining time at 30°C ±1 temperature (hrs.)
Soaking the seeds in distilled water for 24 hours followed by longitudinal cut.	1.0	36.0

It is an established fact that colourless solution of TTC salt on entering the living cells produces a reddish, water insoluble compound called formazon by the activity of dehydrogenase enzyme. After 36 hours, seeds whose embryos had stained red and had a firm flesh were classified as viable This property of TTC salt helps to differentiate living cells from the dead cells i.e. the living cells stained red and are, therefore, considered as viable or germinable while the dead cells of the seed do not take up stain and are colourless, therefore, considered as non-viable or non- germinable. The tissues of the living cells of the seed took up the stain in different patterns during this period. After staining, the solution was drained off and seeds were rinsed with tap water. Viability of each seed was interpreted according to the topographical staining pattern of the embryo and the intensity of the colouration with the help of magnifying glass and the pattern of each individual seed was recorded under six staining categories (Table 3) as per the instructions prescribed by the[35]. The staining pattern of each seed was recorded to 6 categories presented in Fig.2.

Germination Testing

Germination test was also conducted following the procedure devised by[9] 200 seeds from each seed lot were taken for performing the germination tests separately. These were sown in 4 replications of 50 seeds each in sand medium in germination trays (Fig.1) as recommended by[35, 36] and subsequently incubated at 35°C. The experiment was laid in a complete randomized design (CRD).Prior to sowing, the seeds were soaked in water for 7 days, which is recommended to overcome the physiological dormancy mostly encountered in conifers[37,38]. Utmost care was taken to keep the germinating medium moist by adding a small quantity of double distilled water, whenever needed. The first count on germination, recorded as the initial germination percentage, was made on the 7th day of the test and thereafter observations were made daily at 11.00a.m. till the end of the experiment. The final count on germination, recorded as final germination percentage, was made on the 35th day of the test after following[39], when the experiment was terminated. The protrusion of radicle was taken as the criterion for germination[40,41]. Age of the seed and germination percentage at the time of experimentation is given in Table 2.

Table 2: Age of *P. wallichiana* seed and germination percent at the time of TTZ test

Age of the seed (months)	Germination percent (%)
7 months	82.69

Table 4: Comparison of germination test and viability test for estimating seed viability in *P.wallichiana*

Germination percent[1]	Viability percent[2]
82.69	79.82

[1]Actual germination percent under controlled conditions.
[2]Assumed viability percent by tetrazolium staining.

Table 3: Staining results of Tetrazolium test and mean germination percentage of seeds in *P.wallichiana*

Tetrazolium staining category[1]	Tetrazolium staining percent in each seed lot [2]									Mean[3]
	a	b	c	d	e	f	g	h	I	
1.Embryo and cotyledon fully stained.	35.5	45.5	25.10	50.05	35.5	45.5	28.8	35.5	35.1	37.10
2. Embryo fully stained and minor unstained areas (i.e. less than 3/4) on cotyledon.	20.5	24.3	15.8	22.30	25.7	35.4	26.3	30.2	10.5	23.44
3. Embryo fully stained and less than 1/2 portion of the cotyledon unstained.	18.0	15.5	22.5	14.8	20.5	16.5	20.5	20.5	24.8	19.28
Sum of categories 1,2 & 3 =	74.0	85.3	63.4	87.6	81.7	97.4	72.6	86.2	70.4	79.82
4. Embryo stained and stained patches on cotyledon.	2.8	6.3	4.0	6.5	6.8	9.0	14.8	10.5	10.2	7.87
5. Embryo unstained and stained patches on cotyledon.	1.0	0.8	7.0	3.0	1.0	2.5	2.0	2.5	4.5	2.70
Sum of categories 4 & 5 =	3.8	7.1	11.0	9.5	7.8	11.5	16.8	13.0	14.7	10.5
6. Embryo and cotyledon unstained or stained in very small patches.	13.5	2.5	12.0	9.5	3.0	9.5	24.5	6.5	5.5	9.61
Germination (%)[4]	86.0	72.5	84.50	85.50	85.50	90.75	78.50	69.50	88.50	82.69

[1]Tetrazolium staining categories 1-6.
[2]Tetrazolium staining percent in 9 seed lots i.e. from a-i.
[3]Mean values of nine seed lots each with four replications in six tetrazolium staining categories.
[4]Mean values of nine seed lots each with four replications in germination test.
[a-i] Number of seed lots tested for both tetrazolium staining and germination test.

Data Analysis

For predicting the viability, the root mean square (RMS) method as described by[42] was used. The method had earlier been used by[43] for predicting the viability in *Pinus roxburghii* seeds, within 6 staining categories, 1 and 6 were recognized as viable and non- viable respectively. Of the remaining four types, each has two possibilities and together yield 15 combinations of evaluation criteria. Root mean square differences between standard germination test (G) and TZ- predicted viabilities (P) of each evaluation was calculated as:

$$RMS = \frac{\sqrt{\{(G_1-P_1)^2+(G_2-P_2)^2+\text{----------}+(G_n-P_n)^2\}}}{n}$$

Where, G_{1-n} = Germination % of seed lots from 1 to n.

P_{1-n} = TZ staining % of seed lots from 1 to n.

n = Total number of seed lots.

The evaluation criterion that created least RMS value was recommended for the TZ test of the species.

RESULTS AND DISCUSSION

Tetrazolium solution is a colourless one and when imbibed by the seed tissues interferes in the reduction process of living cells and accept hydrogen from dehydrogenase resulting in the production of red stable and non-diffusible substance, Triphenyl formazon in the living cells which makes it possible to distinguish the red coloured living parts of the seeds from the colourless dead ones. Based on this phenomenon, tetrazolium stained seeds were classified into six staining patterns as shown in the Fig.2. Table 3 shows the percentage of seeds stained and classified under different categories for nine seed lots (i.e. from a to i) as well as the germination percentage of each seed lot. A look into the Table 3 and Fig.2 indicates the category 1 has been identified as fully viable as the cotyledons and embryos were fully stained. An average of 37.10% of seeds stained in this category while category 6 was clearly non-viable as the embryos and cotyledons were full unstained or stained in very small patches. An average of 9.61% of the seeds stained in this category. In addition to completely stained viable

seeds (category1) and completely unstained non-viable seeds (category 6), partially stained seeds also occurred (category 2-5). The viability of categories 2-5 was determined by using root mean square method. Table 5 shows the root mean square values of different combinations of categories 2-5 with fully viable category 1.

Figure 1: Seed germination and seedling emergence of *Pinus wallichiana* in germination trays.

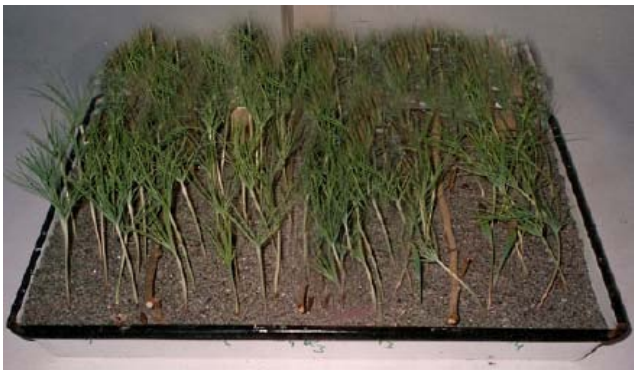
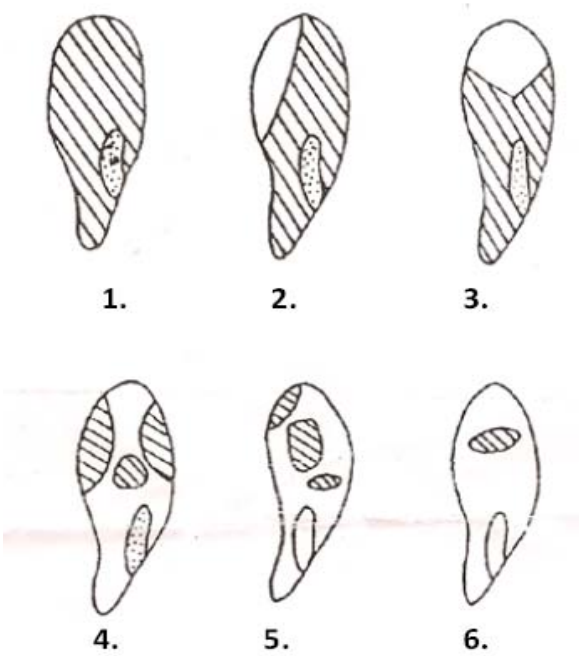


Figure 2: Staining pattern of seeds in *Pinus wallichiana.*



1. Embryo and cotyledons fully stained.
2. Embryo fully stained and less than ¾ portions of cotyledons unstained.
3. Embryo fully stained and less than ½ portions of cotyledons unstained.
4. Embryo stained with cotyledons stained in patches.
5. Embryo unstained and cotyledons stained fully or in patches.
6. Embryo and cotyledons unstained or stained in very small patches.

After pooling together the root mean square values of staining categories, it can be seen that combinations of categories 1, 2 and 3 gave the least root mean square value and hence it is justified to include categories 2 and 3 as viable categories. It can be seen that when any other category is either included or deleted from this combination, the root square value will give a much higher viability percentage as compared to the actual germination percentage leading to faulty determination of viability. Anatomically also, categories 2 and 3 represent normal cotyledons as described earlier by[44]. Category 4 has stained embryo but the cotyledons are unstained (i.e. damaged) at the point of attachment. Also less than 50% tissues are alive, justifying the category to be non- viable. Similarly category 5 has unstained or partially stained embryo with unstained point of attachment with cotyledon which is also partially unstained. Therefore it is proper to include categories 1, 2 and 3 as viable for tetrazolium staining test in the seeds of blue pine. The viability of categories with least RMS value is 79.82%.Analyzing the data on germination studies of 9 seed lots (4 replications of 50 seeds in each seed lot), about 82.69 % of the seeds germinated. This value did not vary significantly than that of the percentage viability (79.82%). Similarly[45] also noted close relationship in the results of germination test and TTC test in *Albizia procera* seed. The results of the present investigation are also in line with[43], who also predicted the viability of *P. roxburghii* through tetrazolium staining. Similar studies have already been conducted by[26], who advocated that tetrazolium test proved to a definite test for viability than germination test which takes days to complete.

Table 5: Root Mean Square (RMS) determination for representation of viable categories 1-5 in *P. wallichiana* seed after tetrazolium staining.

S.No	Categories considered as viable	Root mean square
1.	1,2,3,4,5	15.06
2.	1,2,4,5	18.66
3.	1,3,4,5	18.15
4.	1,4,5	36.48
5.	1,2,3,5	13.62
6.	1,2,3,4	24.02
7.	1,2,5	24.92
8.	1,3,5	43.95
9.	1,2,4	4.69
10.	1,3,4	21.15
11.	1,2,3	20.76
12.	1,5	39.28
13.	1,4	12.86*****
14.	1,2	26.59
15	1,3	27.64

***** The category group with least root mean square value.

CONCLUSION

Efficacy of tetrazolium test in determining the seed viability of *P. wallichiana* was evaluated against the germination test. The percentage viability and germination were 79.82% and 82.69% respectively. Therefore the present study has further proved it a rapid, effective and valuable research technique and confirmed its suitability for prediction of seed viability and determining reasons for poor germination of the seed of this pine species. Hopefully the present studies will also pave the way for successful plantation and multiplication of this species, which is crucial for environmental, ecological and economic-well being of the mankind.

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REFERENCES

1. Mirov NT.Viability of pine seed after prolonged cold storage. Journal of Forestry. 1946; 44:193-195.
2. Harradine AR. Seed longevity and seedling establishment of *Bromus diandrus* Roth. Weed Research.1986; 26:173-180.
3. Leguizamon ES. Seed survival and patterns of seedling emergence in *Sorghum halepense* (L.) Pers.Weed Research. 1986.26:397-403.
4. Gross KL.A comparison of methods for estimating seed numbers in the soil. Journal of Ecology.1990; 78:1079-1093.
5. International Seed Testing Association. International rules for seed testing. Seed Science and Technology.1985; 13:300-520.
6. Lakon GC. Topographicher Nachweis dier kiemfahigkeit der Getriedefruchitee durch Tetrazolium- saize.Berichte- Deutsceh Botanische Gesellshchaft, 1942;60:299-305.
7. Sawma JT, Mohler CL. Evaluating seed viability by an unimbibed seed crush test in comparison with the tetrazolium test. Weed Technology .2002; 16 (4):781-786.
8. Moore RP. History supporting tetrazolium seed testing. Proc. Int. Seed Test. Association. 1969; 34:242.
9. International Seed Testing Association. International rules for seed testing. Proc. Int. Seed Test. Association.1999; 31:1-152.
10. Delouche JC, Still TW,Raspet M,Lienhard M. The tetrazolium test for seed viability. Mississippi State University Mississippi State, MS. Miss.Agri.Exp. Sta.Tech. Bullitin. 1962; 51:1-64.
11. Moore RP. Tetrazolium seed testing developments in North America. Journal of Seed Technology.1976; 1(1):17-30.
12. Parker J. New methods for determination of forest tree seed germinability. Journal of Forestry.1953; 51:34.
13. Yanagisawa, Asakawa S. Woody plant seed viability tests with tetrazolium salts. J.Jap.For. Society.1953; 35(2):40-42.
14. Simak M, Kamra SK. Comparative studies on scots pine seed germinability with tetrazolium and X-ray contrast methods.Proc.Int.Seed Test.Association.1963;28:3-16.
15. Bertiller MB, Aloia DA. Seed bank strategies in Patagoniam semi-arid grasslands in relation to their management and conservation. Biodiversity Conservation. 1997; 6:639-650.
16. Dalling JW, Denslow JS.Soil seed bank composition along a forest chronosequence in seasonally moist tropical forest. J.Veg.Science. 1998; 9:669-678.
17. Fischer M, Matthies D. Experimental demography of the rare *Gentianella germanica*: seed bank formation and microsites effects on seedling establishment. Ecography. 1998; 21:269-278.
18. Ashton PMS, Harris PG, Thadani R. Soil seed bank dynamics in relation to topographic position of a mixed-deciduous forest in Southern New England, USA. For.Ecol.Management.1998; 111:15-22.
19. Morgan JW. Composition and seasonal flux of the soil seed bank of species-rich *Themeda triandra* grasslands in relation to burning history.J.Veg.Science. 1998; 9:145-156.
20. Whittlee CA, Duchesne LC, Needham T. Soil seed bank of a Jack pine (*Pinus banksiana*) ecosystem.Int.J.Wildland Fire.1998; 8:67-71.
21. Gasper S, Nagy J. A new method for tetrazolium analysis of wheat seeds without embryo excision. Seed Science and Technology. 1981; 9:553-556.
22. Gopal M, Thapliyal RC.Topographical tetrazolium test of Indian tree seeds-1. Van Vigan. 1969; 7 (1&2):37-45.
23. Gupta BN, Raturi AS. Tetrazolium staining of seeds for interpretation of viability for six Indian tree species. Indian Forester.1975; 101 (11):659-673.

24. Pasha MK, Das RK.Quick viability test of soybean seeds by using tetrazolium chloride. Seed Science and Technology.1982; 10:651-655.
25. Babeley GS, Kandya AK.Use of TTC for rapid testing of viability of *Lagerstoemia parviflora* Roxb. seeds. Jour. Trop. Forestry. 1986; 2(3):226-231.
26. Sivasubramaniam K, Prabakaran. Topographical tetrazolium test for sekakai (*Acacia concinna*).Van Vigyan.1991; 29(1):5-8.
27. Purohit M, Mishra GP, Jamaluddin. Tetrazolium staining of seeds for testing of viability of some tropical tree species. Jour.Trop. For.1996; 12(3):166-170.
28. Dar AR. Taxonomic studies on gymnosperms in Kashmir. M.Phil. Dissertation, University of Kashmir, Srinagar-190006, J&K, India, 2004.
29. Troup RS. The Silviculture of Indian Trees. Clarendon Press, Oxford.1921; 3: 1013-1066pp.
30. Gamble JS. A manual of Indian Timbers. Sampson Low, Marston and Co.Ltd., London. 1902; 868pp.
31. Aslam M. Studies on selection, growth characteristics and macro-propagation of *Pinus wallichiana* A.B. Jackson in Kashmir, India. Ph.D. Thesis, Jamia Hamdard (Hamdard University), New Delhi-110062, India, 2005.
32. Verma RL. Blessing of pine forests. Van Sandesh, April-June. 1988;9-13pp.
33. Aslam M, Reshi ZA, Siddiqi TO. Genetic divergence in half-sib progenies of *Pinus wallichiana* A.B. Jackson plus trees in the Kashmir Himalaya, India. Tropical Ecology, 2010 (accepted).
34 Aslam M, Salathia HS. Final progress report on ICFRE-WB funded, RGF project no.40-5/97-ICFRE(R).Coordinated Research Project on improvement of *Pinus wallichiana* (Blue pine).Submitted to Indian Council of Forestry Research and Education- Dehradun/ Himalayan Forest Research Institute- Shimala. State Forest Research Institute- J&K, 2001. p71.
35. Association of official Seed Analysts Tetrazolium Testing Handbook for Agricultural Seeds. In: Grabe DF (Ed.), Association of Official Seed Analysts Handbook. (1972); No.29.
36. Kiran DJS, Venkaiah K, Saibabu A. Effect of gibberellic acid (GA_3) on germination of *Givotia rottleriformis* Grief, seeds. Indian Forester. 2001; 127 (8):944-946.
37. Fowler DP, Dwight TW. Provenance differences in the stratification requirements of white pine. Canadian Journal of Botany.1964; 42:669-675.
38. Willan RL. A guide to forest seed handling with special reference to troipics.Danida Forest Seed Center, DK-3050, Humlebaek, Denmark,1985.
39. Gera M, Gera N, Aslam M. Improving germination in *Pinus wallichiana* through pre-sowing seed treatments. Seed Research.2003; 31 (1):110-112.
40. Jann RC, Aman RD. What is germination? The physiology and Biochemistry of Seed Dormancy and Germination.In: Khan AA (Ed).North Holland Publishing Co., Amsterdam.1977; 7-28pp.
41. Copeland LO, Mc Donald MB. Principles of Seed Science and Technology. Mac Millan Publishing Company, New York,1985.
42. Kuo WHJ, Yan AC,Leist N. Tetrazolium tests for the seeds of *Salvia splendens* and *S. farinavea* . Seed Science and Technology.1996; 24:17-21.
43. Gera N,Gera M, Dar FA. Standardization of tetrazolium test for seeds of *Pinus roxburghii.* Seed Research. 2003; 31 (2):190-193.
44. Bekendam J, Grob R. Handbook for Seedling Evaluation. ISTA, Zurich, Switzerland. 1979.
45. Purohit M, Pant NC, Lal RB .Preliminary observation of storage of *Albizia procera* (Roxb). Benth seeds. Australian Forestry.1997; 2(4):9-12.

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