Evaluation of Seed Viability of *Juniperus Excelsa* (M. Bieb.) found in Balochistan by Tetrazolium Assay

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Abstract

The present study was carried out to determine the seed viability of seeds of Juniperus excelsa by Tetrazolium (TZ) assay. The cones/berries of J. excelsa were collected from three locations in Balochistan. The preparation of the seeds for the biochemical assay was performed as recommended by the International Seed Testing Agency (ISTA) rules 2007. The seeds of J. excelsa were tested for viability in aqueous solution of 2,3,5-triphenyl tetrazolium chloride of three different concentrations. The staining pattern of the seeds differed with respect to the strength of the indicator. Only (1%) seeds from Ziarat, Zarghoon Ghar and Harboi provenances were found to develop a partial and light pink staining of the tissues in (0.5%) aqueous solution of TZ. The percentages of seeds that completely stained in (1%) aqueous solution of TZ were found (5%), (4%) and (5%) for Ziarat, Zarghoon Ghar and Harboi provenances respectively. The percentages of partially stained seeds in (1%) aqueous solution of TZ were found (6%), (5%), and (6%) for Ziarat, Zarghoon Ghar and Harboi provenances respectively. Finally the unstained seeds percentages for Ziarat, Zarghoon Ghar and Harboi provenances were found (89%), (91%) and (89%) respectively.

Key words: Cupressaceae; Juniperus excelsa; seed viability; Tetrazolium chloride.

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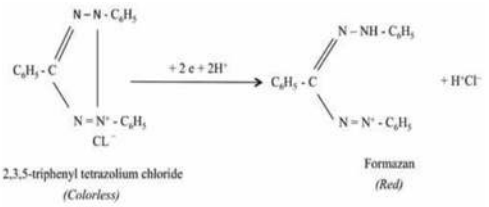
INTRODUCTION

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, whereas this duration is even prolonged for *Juniperus spp.* Furthermore germination test of conifer seeds are also inadequate and do not express their true viability. 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.

The seed viability is considered by the seed technologists as the capacity of a seed to germinate under favourable environmental conditions (Bradbeer, 1988). Seed scientists prefer to perform rapid biochemical assays involving (vital staining) to determine the viaility of seeds as an alternative of time consuming germination assays which require many weeks to complete (Moore, 1976). The seeds of *J. excelsa* are difficult to germinate because of deep physiological dormancy and the presence of hard impermeable seed coats (Cantos et al. 1999). Esmaeelnia et al. (2006) have also reported poor seed germination because of low viability of seeds of *J. excelsa*. The most widely used rapid biochemical assay is probably the tetrazolium or TZ test which is employed to measure the viability of seeds and reproducible standard procedures for different plant species have been developed (Grabe, 1970). The pattern and intensity of staining of the embryo indicates the viability of seeds (Copeland and McDonald, 1985). Given the germination difficulties of the seeds of *J. excelsa* the purpose of present study was to evaluate and estimate the viability of seeds of *J. excelsa* by rapid TZ assay and to compare the quality of seeds collected from *Ziarat*, *Zarghoon Ghar* and *Harboi* provenances.

The tetrazolium or TZ test is a biochemical assay which is employed to differentiate between live seeds from dead ones based on the activity of dehydrogenase respiratory enzymes present in seeds. When the seeds are hydrated and become fully imbibed the dehydrogenase enzyme activity is enhanced which is resulted in the release of hydrogen ions, which in turn results in the reduction of colorless tetrazolium salt solution (2,3,5-triphenyl tetrazolium chloride) into an insoluble reddish chemical compound termed triphenylformazan. Formazan stains the living cells which are respiring

with a red color while the non-respiring dead cells remain color less. On the basis of staining pattern of seed tissues, the amount of area stained and the intensity of staining, the viability of seeds is interpreted (Vankus, 1997). The principal advantages of the tetrazolium test over standard germination are the speed with which seed viability results can be obtained and the ability to determine the viability of even most dormant seeds.



In Balochistan, *J. excelsa* has natural stands distributed between 20°9'N and 30°37'N and between 67°1'E (Rafi, 1965). There are three distinct tracts of Juniper forests in Balochistan, growing at *Ziarat*, *Zarghoon Ghar* and *Harboi* (in districts *Ziarat*, Quetta, and Kalat respectively), forming open and multistoried forests between elevations of 2000 to 3000 m (Sheikh, 1985). The seed germination in *Junipersus spp*. in general is erratic and requires prolonged incubation time; besides the dormancy breaking treatments in the species have not yielded desired results, hence the tetrazolium test may prove a helpful tool for assessing seed viability of the species.

MATERIALS AND METHODS

To estimate the seed viability topographic tetrazolium testing method was used. This technique defines live and dead areas of the embryo and endosperm by differential, topographic staining. The chemical basis for the tetrazolium method is the reaction which is mentioned below:

Tetarzolium chloride + H^+ (from dehydrogenase activity) ⟶ Formazon (red) ↓

In the tetrazolium test method colourless solution of tetrazolium chloride is reduced in the living tissue by dehydrogenase group of enzymes to form a stable, non-diffusible and distinctive red dye (2,3,5-triphenyl formazon). In the absence of active enzymes the dead tissues remain unstained. The distribution and the extent of stained red areas in the treated seeds are examined when the viability of the seeds is assessed. To assess the seed viability following procedure as described by ISTA (Anon., 2007) was employed.

Preparation of Solution

The concentration of solution used for the assessment of viability was 1.0 %. To prepare 1.0% tetrazolium solution 1g of the salt was dissolved in 100 ml of distilled water. To check the influence of TZ concentration and duration on viability assessment, two separate concentrations of 0.1% and 0.5% were also prepared. To avoid reduction by light, tetrazolium solution was stored in flask wrapped with aluminium foil in refrigerator at 5 °C.

Soaking

The seeds were soaked at 25 °C for 24h to hydrate. Hydration is essential for initiating the dehydrogenase enzyme activity. Hydration also softens the seed coat and makes the cutting easier. The seeds were soaked in 9cm petri dish lined with two layers of filter paper and moistened with 8 ml of distilled water.

Cutting

In order to hasten the staining the embryos of the imbibed seeds were exposed to stain by cutting a small portion of seed coat. The seeds were cut longitudinally, off-center through seed coat and nutritive tissues to expose the outline of the intact embryo. To cut the seed coat, seed was held between thumb and forefinger, and starting at one end and orienting the blade edge parallel to the long axis, a small thin layer of megagametophyte was also sliced along with seed coat.

Incubation

Cut seeds were placed in 9cm petri dishes filled with 15 ml of tetrazolium solution. Seed were incubated in 0.1%, 0.5%, and 1.0% solution for 72 h, 48 h, and 24 h respectively at 25 °C in dark.

Evaluation

The seeds incubated in tetrazolium sol. were later evaluated on the basis of staining of different areas of embryo and megagametophyte. For a gymnosperm seeds to be viable, the radicle and cotyledon ends must clearly stained, while megagametophyte should be stained more than two third of its length.

RESULTS AND DISCUSSIONS

The results of biochemical assay tetrazolium (TZ) test of *J. excelsa* seeds with different staining evaluation categories along with their respective percentages are presented in Table 1. The seeds were classified into three evaluation categories on the basis of the intensity of staining and the proportion of the living tissues that developed

staining (as per criterion mentioned in evaluation section of materials and methods). These classes were grouped into the completely stained, partially stained and unstained seed categories. The staining behavior of the seeds of *J. excelsa* differed with respect to (0.1%), (0.5%) and (1%) strength of TZ solution.

The seeds did not stain when soaked in the (0.1%) concentration of TZ solution. However, partial staining was observed when seed lot collected from *Ziarat*, *Zarghoon Ghar* and *Harboi* provenances in were soaked in 0.5% solution of TZ. This partial staining was observed in less than 2% seeds, while no seed was found completely stained (red) from respective provenance. The seeds from *Ziarat* provenance when soaked in (1%) concentration of TZ solution showed that there were 6% completely stained seeds, 9% partially stained seed and 85% unstained seeds. The seeds of *J. excelsa* from *Zarghoon Ghar* when soaked in (1%) concentration of TZ solution showed that 5% seeds were completely stained, 7% were partially stained while 88% seeds were completely unstained. The seed viability results of the seed lot from *Harboi* provenance of *J. excelsa* were practically similar, where the seeds soaked in (1%) concentration of TZ solution showed that 5% were completely stained, 5% were partially stained while 89 % seeds were completely unstained. (Table 1). The TZ staining pattern of *J. excelsa* seeds has been depicted in (Figure 1) which includes both completely stained (red) and partially stained (light pink) embryos. The completely stained embryos which have been exposed to TZ solution by applying a longitudinal cut on the seed coat are shown in (Figure 2). The seeds that were not stained as a result of Tetrazolium assay were observed for further classification into three categories, namely empty, dead, and infested seeds. It was observed that the seed from *Ziarat* provenance consisted of 29% empty seeds, 55% had embryo and megagametophyte however they were dead, whereas 16% seeds were either infested or consumed by larvae. These observations were almost comparable for *Zarghoon Ghar* and *Harboi*, the empty, dead and infested seeds were 14%, 58% and 28% respectively in seeds from *Zarghoon Ghar*, while the corresponding value of empty, dead and infested seeds were 27%, 59% and 14%, respectively for *Harboi* provenance. (Table 2).

Table 1: Percentage of **completely** stained, partially stained and unstained seeds of *Juniperus excelsa* as a result of Tetrazolium assay.

Solution Strength	Evaluation Categories of seeds	Populations with seed staining (%)		
		Ziarat	*Zarghoon Ghar*	*Harboi*
0.1 %	Completely stained	0	0	0
	Partially stained	0	0	0
	unstained	100	100	100
0.5 %	Completely stained	0	0	0
	Partially stained	1	1	1
	unstained	99	99	99
1%	Completely stained	5	4	5
	Partially stained	6	5	6
	unstained	89	91	89

Table 2: Percentage with seed profile of unstained seeds of *Juniperus excelsa*.

Seed profile	Populations with unstained seed profile (%)		
	Ziarat	*Zarghoon Ghar*	*Harboi*
Empty seeds	29	14	27
Dead seeds	55	58	59
Infested seeds	16	28	14

Figure 1: Tetrazolium assay of *Juniperus excelsa* seeds with complete stained (red) and partial stained (light pink) embryos.

Figure 2: Completely TZ stained embryos of *Juniperus excelsa* with longitudinally scarified seed coats.

CONCLUSION

The pattern of staining of the seeds of *J. excelsa* as a result of tetrazolium (TZ) assay was found to differ with respect to the strength of the aqueous solution of tetrazolium (TZ). At 0.1% concentration of the solution the seeds from all the three provenances were not stained at all although incubation time was increased. The optimum concentration of the aqueous solution of TZ was found to be as (1%) which is also mentioned by ISTA (Anon., 2007) the seeds were found partially stained in 0.5% solution. The staining process of seeds was found to complete in 1% solution as some seeds were found completely stained (red) while others were partially stained (light pink) or remained unstained till the termination of the experiment. It was found that (4-5%) seeds were completely stained red while (5-6%) seeds were partially stained and (89-91%) seeds from *Ziarat*, *Zarghoon Ghar* and *Harboi* localities were not stained. The low level of seed viability in the genus *Juniperus* of family Cupressaceae has been reported in the literature by many authors. Wesche *et al*. (2005) have reported that only 2.5 percent of embryos from seeds of *Juniperus sabina* L were viable while the rest of the seeds contained dead or not fully developed embryos. Ahani *et al*. (2013) have also reported that the percentage of empty seeds in *Juniperus polycarpos* was as much as (92.5%). As we found that the majority of seeds (89-91%) which were not stained during TZ assay was consisting of empty, dead, immature embryos or infested seeds by the insects. The percentages of empty seeds, dead seeds and infested seeds among the *Ziarat*, *Zarghoon Ghar* and *Harboi* provenances varied (Table 2) but there were no substantial differences in the quality of seeds with respect to seed viability as determined by staining pattern and percentages of completely and partially stained seed categories. There are some limitations of tetrazolium test, for example it does not distinguish between dormant and nondormant seeds, and it requires proficiency and extensive training in testing and there exist subjectivity in interpreting the results. Due to these limitations it is not accepted as an official viability test, yet it is invaluable test assessing seed viability of deeply dormant seeds of a species like *Junipersus*. The germination in *Junipersus spp*. in general is unpredictable, erratic and requires prolonged incubation time; besides the dormancy breaking treatments in the species have not yielded desired results. Therefore the tetrazolium test may prove a helpful tool for assessing viability, provided an emphasis is paid on consistency, repeatability and reliability while performing the test.

REFERENCES

- Adams RP, Demeke T and Abulfatih HA. (1993). RAPD DNA fingerprints and terpenoids: Clues to past migrations of *Juniperus* in Arabia and East Africa. *Theoretical and Applied Genetics.* 87:22-26.
- Ahani H, Jalilvand H, Nasr HMS, Kouhbanani SH, Ghazi RM and Mohammadzadeh H. (2013). Reproduction of Juniper (*Juniperus polycarpos*) in Khorasan Razavi, Iran. *Forest Science Practice.* 15(3):231-237.
- Anonymous. (2007). The germination test. In: International Rules for Seed Testing. International Seed Testing Association, Bassersdorf, Switzerland.
- Bradbeer JW. (1988). Seed dormancy and Germination. Chapman & Hall, New York.
- Cantos M, Cueva J, Zarate R and Troncoso A. (1998). Embryo rescue and development of Juniperus oxycedrus subsp. oxycedrus and macrocarpa. *Seed Science and Technology.* 26:193-198.
- Copeland LO and McDonald MB. (1985) Principles of Seed Science and Technology. 2nd edition. Burgess Publication Co. Minneapolis, MN.
- Donald WW. (1994). A method of validating the Tetrazolium assay for testing viability of dormant

jointed Goatgrass (*Aegilops cylindrica*) seed. *Weed Science* 42:502-508.

- Esmaeelnia M, Jalali SG, Tabari M and Hosseini SM. (2006). Influence of plant growth regulator IBA on vegetative propagation of *Juniperus excelsa*. *Iranian Journal of Forest and Poplar Research*. 14: 221-227.

- Farjon A. (1998). World checklist and bibliography of conifers, Royal Botanic Gardens, Kew.

- Grabe D. (1970). Tetrazolium Testing Handbook for Agricultural Seeds. Assoc. Official Seed Analysts.

- Khajjak MH, Raza AM, Shawani MN, Fayyaz A, Ghazala S and Saeed M. (2012). Comparative analysis of essential oil contents of *Juniperus excelsa* (M. Beib.) found in Balochistan, Pakistan. *African Journal of Biotechnology.* 11(32):8154-8159.

- Moore RP. (1976). Tetrazolium seed testing developments in North America. *J. Seed Technol.* 1:17-30.

- Rafi M. (1965). Vegetation types of Balochistan Province. Pakistan Govt. Printing Press, Lahore, Pakistan.

- Sheikh IS. (1985). Afforestation in Juniper forests of Balochistan. Pakistan Forest Institute, Peshawar, Pakistan.

- Vankus V. (1997). The Tetrazolium Estimated Viability Test for Seeds of Native Plants. U. S. Department of Agriculture, Forest Service, Pacific Northwest Research Station.

- Wesche K, Ronnenberg K and Hensen I. (2005). Lack of sexual reproduction within mountain steppe populations of the clonal shrub *Juniperus Sabina* L. in semi-arid southern Mongolia. *Journal of Arid Environment.* 63:390-405.