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Standardization of Seed Viability Testing Protocols in Dinanath Grass (*Pennisetum pedicellatum*) and Berseem (*Trifolium alexandrinum*)

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ABSTRACT

Experiments conducted to standardize the suitable preconditioning period, seed preparation method, Triphenyl Tetrazolium Chloride salt solution concentrations and Incubation period for the seed viability studies in Dinanath grass and Berseem seeds at AC and RI, Kudumiyanmalai, between 2018 and 2020. For standardization of seed viability testing Dinanath grass seed was exposed to five different preconditioning durations viz., 2h, 4h, 6h, 8h and 12h; preparation methods (removal of glumes); four different concentrations of 2, 3,5 Triphenyl Tetrazolium Chloride (TZ) salt solutions (0.25, 0.5, 1.0 and 1.5%) and four different incubation durations in TZ (2h, 4h, 6h and 8h) @ 40 °C. Berseem seeds exposed to three different preconditioning durations (2h, 3h and 4h); preparation methods (splitting of cotyledons); followed by exposure to four different concentrations of 2, 3,5 Triphenyl Tetrazolium Chloride salt solutions (0.1, 0.2, 0.5 and 1.0 %) and three different incubation durations in TZ (1h, 2h and3h) @ 40 °C. The Tetrazolium test results were correlated with regular germination test. From the studies it could be concluded that Dinanath grass seeds preconditioned for 4h followed by removal of glumes and soaked in 0.5 % TZ solution for 6h @ 40 °C stained perfectly. In Berseem, seeds preconditioned for 2h followed by splitting of cotyledons, and soaking in TZ @ 0.1 % for 1h at 40 °C was the best possible combination leading to proper staining, and the same combinations were positively correlated with the normal germination test.

Key words: *Dinanath grass, Berseem, 2,3,5 triphenyl tetrazolium chloride salt, Preconditioning durations, Seed preparation, Tz concentrations, Incubation durations in Tz and staining pattern, Seed Viability, Seed germination test.*

Introduction

Dinanath is an indigenous grass of Ethiopia belonging to the family Poaceae and used as fodder, mainly for cattle and milk buffalo. It is a herbaceous perennial grass which have high nutritive values and in naturally palatable for livestock. Berseem (Egyptian clover, berseem) an annual forage legume species native to western Asia is an annual clover cultivated mostly in irrigated sub-tropical regions and used as fodder, mainly for cattle and milk buffalo.

Livestock production is the backbone of Indian agriculture and also plays a vital role contributes 7% to national GDP. It is the major source of employment and ultimate livelihood for 70% population in rural areas. The human population in India is expected to reach over 1400 million by 2025. Urbanization has brought a marked shift in the lifestyle of people in feeding habits towards milk products, meat and eggs with resultant increase in demand of livestock products. Due to ever increasing popula-

tion pressure of human, arable land is mainly used for food and cash crops, thus there is little chance of having good quality arable land available for fodder production to feed the livestock. Moreover, there is decline in practice of fodder production in rural areas due to strengthened focus on field crops and animals were allowed to graze naturally grown grasses and shrubs which are of low quality in terms of protein and available energy, they are thus heavily dependent on seasonal variations and this resulted in fluctuation in fodder supply round the year affecting supply of milk, milk products and poultry products.

At present, the country faces a net deficit of 61.1% green fodder, 21.9% dry crop residues and 64% feeds. It could be compensated by increasing productivity by using quality seeds or utilizing untapped feed resources or increasing land area or through imports. Availability of quality seed in forage crops to enhance production and productivity is long felt need, even then the availability of improved varieties are the bottlenecks of forage seeds. Apart from forage grasses, legumes are shy seed producers; maximum are blank seeds with lower seed germination, higher seed dormancy and poor seed standards. Use of low quality seeds leads to delay of emergence of seedlings in the field, which results in smaller growth of the aerial plant parts as well as smaller growth of the root system low and irregular plant stand in the field ; and lower tolerance to drought resulting in lower yield. Therefore, to increase the seed production first we should use high quality seeds.

To check verify the quality status of forage seeds, proper seed testing protocols and standards are not established so far. Germination based seed quality assessments could take several weeks to months, to overcome these problems, tetrazolium (2,3.5 / triphenyl tetrazolium chloride, TTC) test it is a rapid method and allows the assessment of viability and vigour of seed lots in less than 24 hours.

The test relies on the reduction of the colourless and water soluble 2, 3,5triphenyl –tetrazolium chloride (TTC) to an insoluble red compound (Formazon). This reaction occurs as a consequent of hydrogen ions donated to the TTC upon dehydrogenase activity in metabolically active tissues, such as the seed embryo. The tetrazolium test has been successfully used on evaluating vigour of seeds of several plant species, such as: corn, soybean, watermelon, tomato (Santos *et al.,* 2007). There is no proper standardized procedure established to predict the viability status of Dinanath grass and berseem in India. Hence, the present work has been formulated to standardize suitable seed viability test procedure in Dinanath grass and Berseem seeds.

Materials and Methods

Genetically pure seeds of Dinanath (*Pennisetum pedicellatum*) grass and Berseem (*Trifolium alexandrinum*) were oobtained from Indian Grass Land and Fodder Research Institute, ICAR, Jhansi, Uttar Pradesh and used as base material for this study. The standardization and evaluation of seed viability studies were carried out at the Agricultural College and Research Institute, Kudumiyanamalai during 2018-20.

Studies on Seed Germination

Dinanath Grass were selected randomly and 4 sets of 100 seeds were placed in top of paper method. The test conditions of 25 ± 2°C and 95 ± 3% RH were maintained in the germination room. At the end of 7 days the number of normal seedlings were counted and the mean expressed as Percentage (ISTA, 2005).

Berseem seeds were selected randomly and 4 sets of 100 seeds were placed in germination towel to measure the germination at 25 ± 5 °C and final count measured after 7 days (Sanjay Kumar *et al.,* 2018).

Studies on Seed Viability

Twenty five seeds were subjected to seed viability studies in 4 replicates based on Tetrazolium staining as per ISTA (2005).

Preparation of staining Solution

The chemical used for this test is a cream or light yellow coloured water soluble powder called 2,3,5,-triphenyl tetrazolium chloride. To standardize the optimum concentration of 2,3,5 triphenyltetrazolium chloride salt solution, different concentrations of tetrazolium salt solution was prepared and poured on the seeds. The pH of the solution was maintained at 7.0. The experimentation was conducted under dark condition at 40 °C with different incubation periods *viz.,*

Standardization of Preconditioning Duration and preparation of forage seeds for Topographical Tetrazolium Staining Studies

Dinanath grass seeds were subjected to preconditioning by soaking in water for 2h, 4h, 6h, 8h and 12h. After stipulated period the glumes were removed and placed in the various concentrations of 2,3,5 Triphenyl Tetrazolium Chloride salt solutions (0.25, 0.5, 1.0 and 1.5% with a pH of 7.0.). Seeds were soaked in Tz solution (50 no of seeds in 5 ml of solution) with four different incubation durations of 2h, 4h, 6h and 8h @ 40 °C and allowed to staining.

Berseem seeds were subjected to preconditioning durations of 2h, 3h and 4h. After stipulated period, seeds were de-coated and the cotyledons were split to reveal the embryonic axis. The cotyledon half with the radicle and plumule attached was subjected to TZ Staining by exposure to four different concentrations of 2, 3,5 Triphenyl Tetrazolium Chloride salt solutions (0.1, 0.2, 0.5 and 1.0 % with a pH of 7.0). Seeds were soaked in Tz solution (50 no of seeds in 5ml of solution) with three different incubation durations (1h, 2h and 3h) @ 40 °C and allowed to Staining.

Detailed method of preconditioning, seeds preparations, Tz Concentrations and Incubation durations for staining was given in the (Table 1 and 2).

Evaluation and Interpretation of Staining

After stipulated period of Incubation, the tetrazolium chloride solution was decanted and the seeds were thoroughly washed with water for the evaluation. The essential structures such as cotyledons, embryonic axis (radicle and leaf primordial), region of cotyledon attachment with hypocotyls and growing points were observed for staining based on the staining pattern the seeds were classified as Germinable or Non Germinable (Moore, 1962). The germinable category seeds were counted and the mean of stained cum germinable seeds were expressed in Percentage.

Statistical analysis

The data obtained from each experiment were correlated with germination test results and the best method of Tetrazolium test was derived based on highest positive correlation with germination test.

Results and Discussion

In Dinanath grass seeds germination studies, after 7 days of sowing the number of seeds germinated was 16 %. The poor performance of the seed was due to presence of dormancy and blanks seeds (Table 3).

Among the various preconditioning durations *viz.*, 2h, 4h, 6h, 8h and 12h seeds preconditioned for 4h recorded the perfect staining, while raising the period of preconditioning over staining recorded. Among the different incubation periods viz., 2h, 4h, 6h and 8h, seeds incubated in Tz for 6h recorded the

Table 3. Germination % of Dinanath (*Pennisetum pedicellatum*) grass seeds

Number of seeds germinated	**16 %**

Table 1. Preparation of seeds, Duration of Preconditioning, Tz Solution concentration and incubation duration for Dinanath (*Pennisetum pedicellatum*) grass seeds

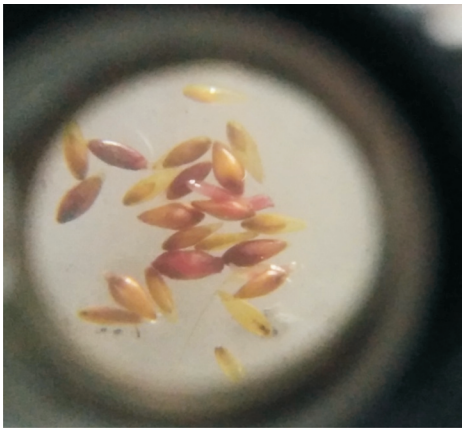
Treatments	Duration of preconditioning	Preparation of seeds	Tz Solution Concentration	Incubation Duration
T1	2h	Removal of Glumes	0.25%	2 h
T2	4h		0.5 %	4 h
T3	6h		1.0%	6 h
T4	8 h		1.5 %	8 h
T5	12 h			

Table 2. Preparation of seeds, Duration of Preconditioning, concentration of Tz Solution and incubation duration for Berseem (*Trifolium alexandrinum*) seeds

Treatments	Duration of preconditioning	Preparation of seeds	Tz Solution Concentration	Incubation Duration
T1	2h	De coating followed by	0.1 %	1 h
T2	3h	splitting with cotyledon	0.2 %	2 h
T3	4h	embedded with	0.5%	3 h
		embryonic axis	1.0 %	4 h

perfect staining and over staining observed in the higher durations of incubations. Among the four different concentrations of TZ solution *viz.*,0.25%, 0.5%, 1.0% and 1.5 %, seeds soaked in 0.5 % recorded the perfect staining than other concentrations (Table 4).

The percent germination of berseem seed lot using standard germination test was 88% (Table 5, Plate 1).



4h Pre conditioning followed by removal of glumes and stained in Tz @ 0.5 % for 6h @ 40°C

Plate 1. Standardization of Preconditioning, Staining concentration and incubation duration for Dinanath (*Pennisetum pedicellatum*) for Tz Staining

Regarding staining pattern among the various preconditioning durations *viz.*, 2h, 3h and 4h seeds preconditioned for 2h recorded the most perfect staining. Among the different incubation periods *viz.*, 1h, 2h, 3h and 4h, seeds incubated in TZ for 1h recorded the perfect staining. Among the four different concentrations of TZ solution viz., 0.1%, 0.2%,

Table 5. Germination % of berseem seeds (*Trifolium alexandrinum*)

Number of seeds germinated	**88 %**

0.5% and 1.0 %, seeds soaked in 0.1 % recorded the perfect staining than other concentrations (Table 6, Plate 2).

2h Pre conditioning followed by decoating and splitting with cotyledon embedded with embryonic axis and stained in Tz @ 0.1 % for 1h @ 40°C

Plate 2. Standardization of Preconditioning, Staining concentration and incubation duration for Berseem (*Trifolium alexandrinum*) for Tz Staining

Irrespective of preconditioning durations, TZ Concentrations and their incubation durations, Over staining observed while raising the period of preconditioning, durations of incubations increasing and increasing the concentration.

From the results, Dinanath grass seeds preconditioned in water for 4h followed by removal of

Table 4. Per cent of seeds stained by different preconditioning methods, concentration of tetrazolium solution and incubation duration for Dinanath (*Pennisetum pedicellatum*) grass seeds

Preconditioning method (P)	Concentration of Tetrazolium/Incubation duration (h)															
	0.25%				0.5%				1%				1.5%			
	2h	4h	6h	8h	2h	4h	6h	8h	2h	4h	6h	8h	2h	4h	6h	8h
2h water soaking	0	0	12	100	0	0	24	100	0	0	44	100	0	0	100	100
4h water soaking	0	0	20	100	0	0	16	100	0	0	60	100	0	0	100	100
6h water soaking	0	0	24	100	0	0	32	100	0	0	68	100	0	0	100	100
8h water soaking	0	0	100	100	0	0	100	100	0	0	100	100	0	0	100	100
12h water soaking	0	0	100	100	0	0	100	100	0	0	100	100	0	0	100	100
	P		C		D		PC		PD		CD		PCD			
SEd	0.217		0.194		0.194		0.433		0.433		0.387		0.866			
CD (P=0.05)	0.428		0.383		0.383		0.856		0.856		0.765		1.711			

glumes and soaking in 0.5 % Tz solution for 6h at 40 °C recorded the perfect staining as prescribed by Moore (1985) and positively correlated with seed germination percentage and the correlation value was (1). Among all Berseem seeds preconditioned in water for 2h and decoated followed by splitting the cotyled on embedded with embryonic axis and soaking in 0.1 % Tz solution for 1h at 40 °C recorded 88 % staining and it was positively correlated with the standard seed germination test and the correlation value was (0.952).

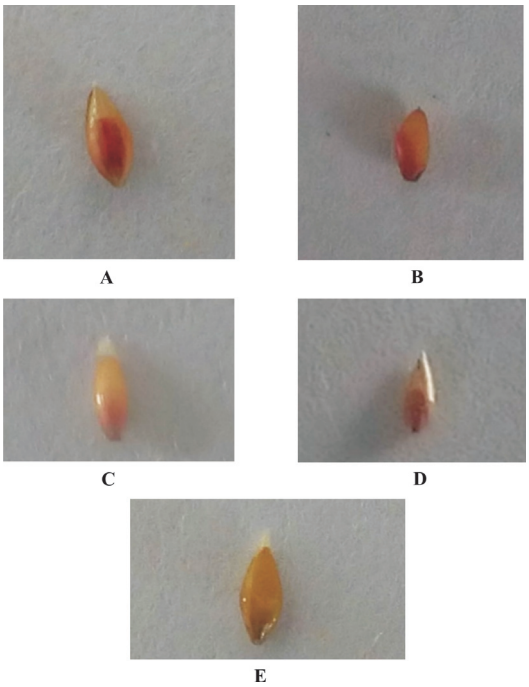
Marco - Filho (2005) developed a procedure for preconditioning. They suggested that seeds that were previously preconditioned or moisturized showed improved enzymatic metabolism which leads adequate staining incontact with Tz salt solution. Over staining was observed at higher preconditioning, incubation duration and at higher concentration. Lakon (1949) they reported that tetrazolium is a better indicator of germinability by correlating the quick viability test with standard germination. Decoated seeds followed by placing one half of split cotyledon (with embryonic axis) in Tz solution found effective. Marcos - Filho (2005) suggested that the preconditioned or pre moisturized seeds showed improved enzymatic metabolism which leads adequate staining in Topograpical Tetrazolium staining.

Seed Viability and Germinability status

As per the classification of (Moore, 1962) based on the staining pattern, There were five different staining patterns were recorded for Dinanath grass among them four for viable category and one for non viable category (Plate 3) as follows

Viable and Germinable category

A. 1/3rd of the Embryo unstained;



A: Embryo Completely stained **B:**1/3rd of the embryo unstained
C: Margins of scutellum unstained **D**: Upper half of embryo unstained
E : Embryo completely unstained

Plate 3. Staining Pattern and classes of viable and nonviable seeds of Dinanath (*Pennisetum pedicellatum*)

B. Embryo Completely Stained;
C. Margins of Scutellum unstained;
D. Upper half of Embryo unstained;

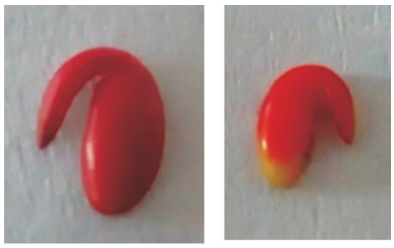
Non-Viable and Non Germinablecategory

E. Embryo Completely unstained.

In the stained seeds 16 % registered under viable and germinable category and 84% shown nonviable

Table 6. Standardization of Preconditioning methods, TZ concentration and incubation duration for Berseem (*Trifolium alexandrinum*) seed viability studies.

Preconditioning method	Concentration of Tetrazolium/Incubation duration (h)															
	0.1%				0.2%				0.5%				1.0%			
	1h	2h	3h	4h	1h	2h	3h	4h	1h	2h	3h	4h	1h	2h	3h	4h
2h water soaking	88	92	100	100	84	92	100	100	100	100	100	100	100	100	100	100
3h water soaking	96	100	100	100	90	92	100	100	100	100	100	100	100	100	100	100
4h water soaking	92	100	100	100	96	96	100	100	100	100	100	100	100	100	100	100
	P		C		D		PC		PD		CD		PCD			
SEd	0.208		0.241		0.241		0.417		0.417		0.481		0.833			
CD (P=0.05)	0.413		0.477		0.477		0.827		0.827		0.955		1.653			



1 2

3

1. Cotyledon and radicle completely stained
2. Radicle completely stained and less than 1/3rd of the cotyledon unstained
3. Cotyledon and radicle stained but less than 1/3rdportion unstained

Plate 4. Staining Pattern and classes of Viable / Germinable seeds of Berseem (*Trifolium alexandrinum)*

and non germinable category (Table 7).

Eight different staining patterns recorded and sorted as viable and non-viable In Berseem seeds . Under Viable and Germinable category three staining patterns recorded and under Non viable and non germinable five staining patterns recorded as follows.

Viable and Germinable category (Plate 5)

1. Cotyledon and radicle completely stained;
2. Radicle completely stained and less than 1/3rd of the cotyledon unstained;
3. Cotyledon and radicle stained but less than 1/3rd portion unstained;

Non-Viable and Non Germinablecategory (Plate 6)

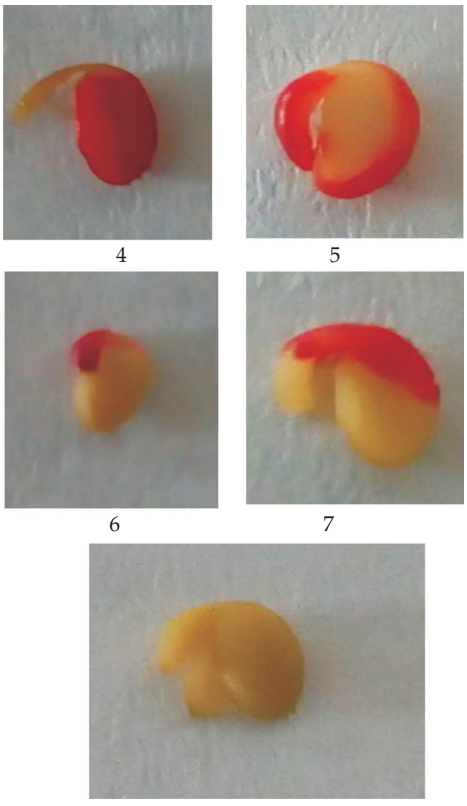
4. Cotyledon stained and radical completely unstained;
5. V. Unstained areas in Cotyledon attached to the Radicle
6. VI. Radicle stained and cotyledon completely unstained;
7. VII. More than 1/3rd of the cotyledon and extreme tip of radicle unstained

Table 7. Staining pattern for Dinanath (*Pennisetum pedicellatum*) grass seeds

Pattern of Staining	Category of seeds	Number of seeds stained (%)
1/3rd of the Embryo unstained (P1)	Viable and germinable	5
Embryo Completely Stained (P2)		7
Margins of Scutellum unstained (P3)		2
Upper half of Embryo Unstained (P4)		2
Embryo Completely Unstained (P5)	Non viable and Germinable	84

Table 8. TZ Staining pattern in Berseem (*Trifolium alexandrinum*) seeds

Pattern of Staining	Portions of Staining	Number of seeds stained (%)
I. Viable and Germinable Category		
1	Cotyledon and radicle completely stained	80
2	Less than 1/3rd of the cotyledon unstained	6
3	Small portions or minor areas of cotyledons unstained	2
	Total Viable and Germinable Seeds	88
II. Non Viable and Non Germinable Category		
4	Cotyledon completely unstained	3
5	Radicle completely unstained	3
6	Unstained areas in Cotyledon attached to the Radicle	1
7	More than 1/3rd of the cotyledon and extreme tip of radicle unstained	1
8	Cotyledons and Radicle completely Unstained	4
	Total Non Viable and Non Germinable Seeds	12



4. Cotyledon stained and radicle completely unstained
5. Cotyledon attached to the radicle unstained
6. Radicle stained and cotyledon completely unstained
7. More than 1/3rd of the cotyledon and extreme tip of radicle unstained
8. Cotyledon and radicle completely unstained

Plate 5. Staining Pattern and classes of Nonviable/Non Germinable seeds of Berseem (*Trifolium alexandrinum*)

8. VIII. Cotyledons and Radicle completely Unstained

In the stained seeds 88 % registered under viable and germinable category and 12% shown nonviable and non germinable category (Table 8).

TZ is a rapid way to assess the seed viability of forage seeds which give the viability status of seed lot with in 3 to 12 hours since the standard germination test has taken 7 to 10 days. TZ test provides significant results for viability than germination test which takes days to complete.Bonner,1974; Moore, 1985; ISTA, 1991; Sivasubramaniam and Vijayalakshmi, 2012 reported that Topographical Tetrazolium Chloride Test is a rapid method to assess the viability and vigour of seed lots in less than 24 hours which allows interpretation of seed viability according to the staining pattern of seed tissues

Conclusion

Dinanath grass seeds preconditioned for 4h followed by removal of glumes and soaked in 0.5 % TZ solution for 6h @ 40 °C stained perfectly and can be recommended for TZ based viability studies in Dinanath grass.

Berseem seeds preconditioned in water for 2h followed by decoating and splitting with cotyledon embedded with embryonic axis and soaking in 0.1 % Tz solution for 1h at 40°C recorded the perfect staining and can be recommended for TZ based viability studies in Berseem.

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