STM JOURNALS

Research & Reviews: Journal of
Botany

ISSN: 2278-2222
Volume 13, Issue 1, 2024
January-April
DOI (Journal): 10.37591/RRJoB

http://sciencejournals.stmjournals.in/index.php/RRJoB/index

Research RRJoB

Optimization of Seed Viability Tests for Humulus lupulus

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Abstract

The Humulus lupulus member of the Hemp family has been extensively explored for their industrial application in diverse sectors. During the cultivation of this high-cost plant, one of the major concerns associated is low seed germination due to physical dormancy. The role of rigid seed coats in augmenting an impermeable barrier for water and nutrient availability to the seed has been well analyzed in previous studies. However, the associated impact on biological dormancy and viability of germplasm has not been explored in detail. Furthermore, in the absence of any well-defined rapid and cost-effective solution, the analysis becomes even more difficult. Thus, there is an urgent need to develop an accurate and reliable, method to check the physiological qualities of the seeds. The present study focuses on the comparative assessment of different staining methods for assessing the viability of Humulus lupulus seeds. The tetrazolium method has been widely used to assess the vigor and viability of various leguminous and horticultural crops. To the best of our literature survey no such methods have been checked to assess the viability of Humulus lupulus seeds. The present study focuses on the qualitative assessment of triphenyl tetrazolium chloride (TTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dyes to assess the seed viability. The result revealed that both these dyes can be used to assess the viability of Humulus lupulus seeds however, range of 0.75-1% can be used for evaluating the viability of seeds within an incubation time of 48 hrs.

Keywords: *Humulus lupulus*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Seed viability, Surface Morphology, Triphenyl Tetrazolium Chloride (TTC)

INTRODUCTION

The *Humulus lupulus* (Hops), plant is dioecious belonging to the family Canabenace [1]. It is native to Europe, Asia, and the Western hemisphere [2]. The dioecious nature of the plant and further commercial use of the plant, obtrude female plants more important as the cones are widely used in beer making [3, 4]. The female plant is reported to exhibit a plethora of bioactivities, including neuroprotective activities, antimicrobial, antiviral, antitumor, antioxidant, estrogenic, and metabolic disorder treatment [2, 5]. The propagation of the plant via seeds has been a major challenge due to the dormant nature of the seeds leading to a lower germination rate [6, 7]. However, the production of good quality seeds and their utilization are important key elements in determining plant productivity [8]. Thus, there is a need to determine an accurate, reliable, and cost-effective method to check the physiological qualities of the seeds. The tetrazolium test has gained much attention due to its ability to determine the vigor of the seeds with some of the plant systems. The method has been widely used for checking the viability of leguminous seeds and other horticultural crops with specifically developed methodologies [8]. To the best of our literature review, no prior study is available on the determination of seed viability for *Humulus lupulus* seeds. This research focused on studying the seed

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Received Date: March 11, 2024
Accepted Date: April 09, 2024
Published Date: April 19, 2024

Citation: T. Singh, A. Mathur. Optimization of Seed Viability Tests for Humulus lupulus. Research & Reviews: Journal of Botany. 2024; 13(1): 1–6p.

morphology and its viability assessment using triphenyl tetrazolium chloride (TTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The study focuses on developing and validating a rapid procedure for assessing the viability of hop seeds.

MATERIAL AND METHODS

The *Humulus lupulus* seeds were procured from Semisauvage Permaculture (France, Lot No-8284). The seed packets were kept in the cold room before assessing the viability of the seeds. Varying concentrations of triphenyl tetrazolium chloride (TTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (w/v) were prepared in 100 ml of autoclaved distilled water. The reagent prepared was stored in an amber bottle due to the light sensitivity of the dye. The scarification solution consisted of sodium hypochlorite (20 ml) and Triton X-100 (100 µl) in 100 ml of autoclaved water [9]. Before testing, the seeds were pre-soaked in water for 48 hrs and kept in the cold chamber. Around 30 seeds were taken for the study each with a set (n=10) in triplicates. The seeds were soaked in scarification solution for 2 hrs (due to the impermeable nature of seeds) and kept on a shaker (100 rpm and at RT). The seeds were further washed 3-4 times using distilled water followed by removing excessive water by keeping them in between tissue paper. After removing the excessive water, the seeds were incubated with TTC and MTT (0.25, 0.5, 0.75, and 1%) solutions respectively at around 30 ℃ for 24, 48, and 72 hrs. The seeds were analyzed under a stereomicroscope (Olympus SZ2, Japan) and the observations were recorded. The seeds were not able to take the dye when kept for 24 hrs in both the dyes used (Data not shown). The heat-killed seeds were taken as a negative control for the assay. Around 10 seeds from each germplasm were kept at 100 ℃ in the water bath for 30 minutes to heat kill the seeds.

RESULTS AND DISCUSSIONS

The metabolic inactivity of seeds is referred to as dormancy which affects embryo growth and development thus affecting seed germination [10]. Recent researchers have assigned two classes for seed dormancy. The first one, called “Inherent” includes the inherited property naturally built among the organisms while the other one, called “Imposed” is controlled by the surrounding environment [11, 10]. A low germination percentage, of around 3-5% is reported for the hop seeds [12, 6]. The dormant nature of seeds accounts for a low germination rate caused by the presence of resins and impermeability to seed tegument thus restricting the intake of water and oxygen via the embryo [6]. The seeds of the plant are small in size and their hardy nature makes it difficult to handle. The seed coat removal and further microscopic analysis reveal that the embryo is encapsulated with a hard shell (Figure 1(a-d)). Seeds that are not able to imbibe water are associated with physical dormancy [13]. Often physical dormancy is accompanied by a layer of palisade cells (macrosclereids) which are impervious to water in nature [14]. While dissecting the hop seeds similar macrosclereids were observed as depicted in Figure 1 (b). After removing the palisade cells layer a hard shell-like structure was observed as shown in Figure 1 (c). This hard shell may act as a protection layer for the embryo and does not allow the intake of dye and water.

The tetrazolium test is based on the principle that a viable seed will exhibit dehydrogenase activity during the respiration process. The presence of this dehydrogenase enzyme catalyzes the 2,3,5 triphenyl tetrazolium chloride, a colorless solution into a red color dye formazan thus confirming the viability nature of the seeds. However, the MTT test is based on the reduction of the yellow tetrazolium salt to a purple-colored complex when a cell is metabolically active. To the best of our literature review seed viability of hops has never been assessed using the tetrazolium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dyes. The efficiency of this test depends upon factors such as the appropriate method development for different species, the nature of the seeds, the concentration of the tetrazolium solution, temperature, and priming time for the seeds [15]. The present research shows that the viable seed was stained fully red (Figure. 2 (d) & (e)) while the non-viable seed didn’t take the red color and are partially viable with low vigor (Figure. 2 (a) &(b)).

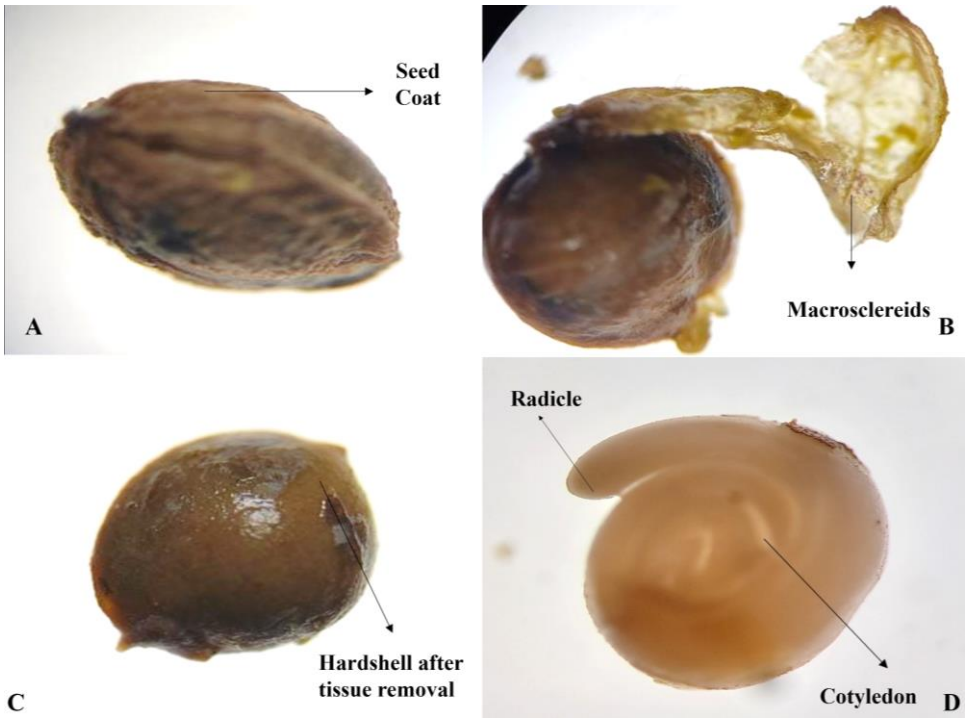

Figure 1. Microscopic analysis (A) Seed (B) Macrosclereids (C) Hardshell (D) Embryo.

Figure 2. Seed viability observed using tetrazolium dye after 48 hrs of incubation (a) 0.25% (b) 0.5% (c) Non-viable (d) 0.75% (e) 1% (f) Negative-control.

Similar results were obtained in the case of staining using MTT dye. The seeds were partially purple stained at lower concentrations of dye making them partially viable Figure 3 (a) & (b) while an intense color of dye was emitted at higher concentrations making the seeds viable.

Previous studies have shown the incubation time of seeds with TTC to be more than 20 hrs [16]. After 24 hrs of incubation, it was recorded that none of the seeds were able to take up the dye (Data not shown). The incubation period in the current investigation was thus further extended to 48 and 72 hrs, seeking the nature of the seeds and their impermeability to water. However, the studies also show that at low concentrations of dye like 0.25 and 0.5%, the seeds were not able to take the tests as seeds have shown partial staining as shown in Figures 2 and 4 when incubated both at 48 and 72 hrs. The seeds

taken as negative control which were heat-killed were colorless despite adding tetrazolium (Figures 2 (c), and 3(f), and MTT dye Figures (3 and 5 (f)). However, the results have revealed that the dye within the range of 0.75–1% has shown a similar color revealing intense red and purple color in both the cases. Thus, it was recorded that 0.75% of TTC when incubated for 48 hrs gave an intense staining pattern of the viable seeds. Similar results were obtained in the case of staining done using MTT dye. No correlation could be established between the concentration of the dye and incubation time. However, genetic and physiological factors play a vital role in affecting seed viability and vigor. The role of factors such as storage conditions, temperature, geographical location, moisture, and seed type cannot be ignored [17]. In the current study, the results showed robust application of the use of tetrazolium dye and MTT dyes for the detection of the viability of the hop seeds.

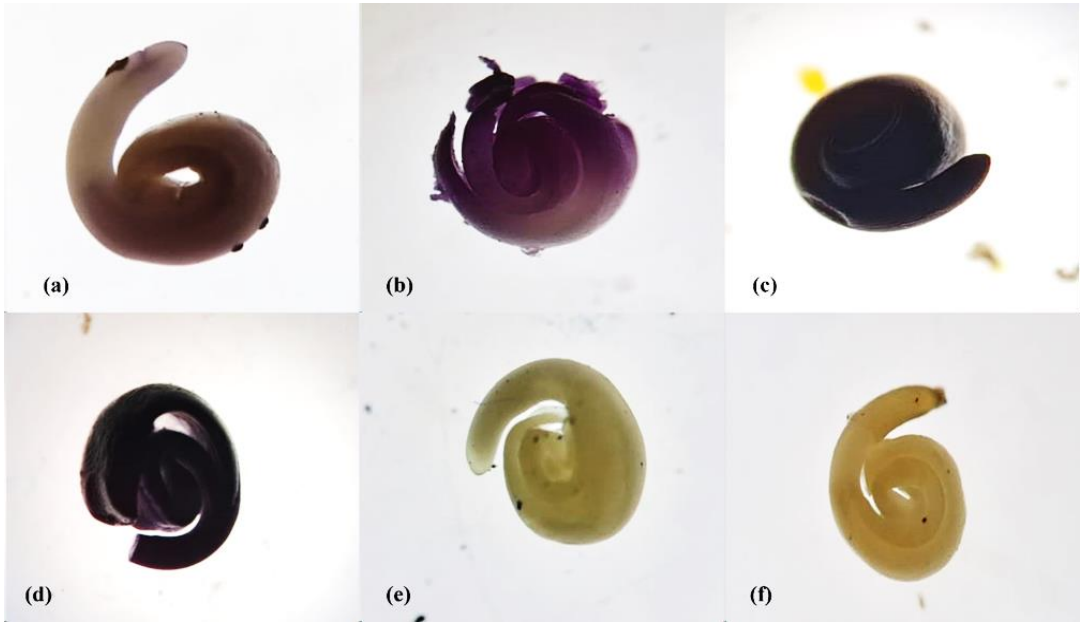

Figure 3. Seed viability observed using MTT dye after 48 hrs of incubation (a) 0.25% (b) 0.5% (c) 0.75% (d) 1% (e) Non-viable (f) Negative- control.

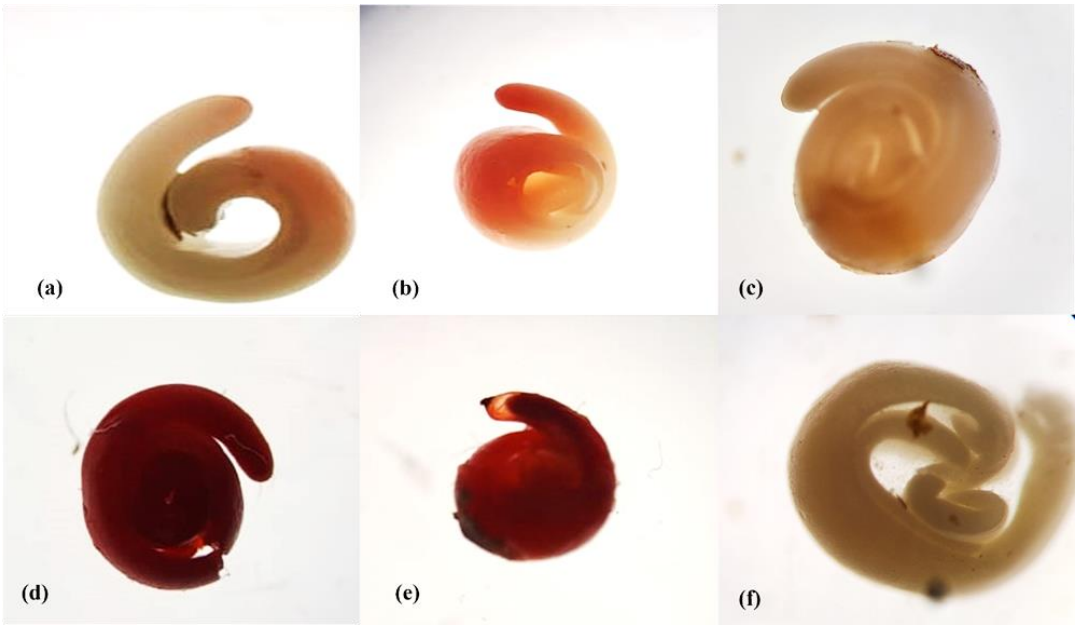

Figure 4. Seed viability observed using tetrazolium dye after 72 hrs of incubation (a) 0.25% (b) 0.5% (c) Non-viable (d) 0.75% (e) 1% (f) Negative-control.

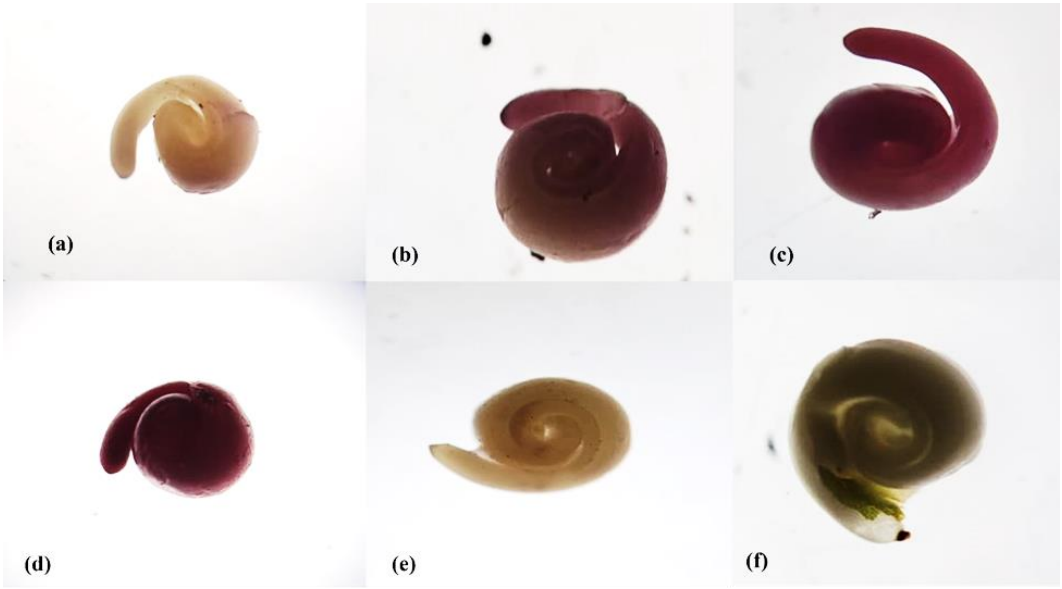

Figure 5. Seed viability observed using MTT dye after 72 hrs of incubation (a) 0.25% (b) 0.5% (c) 0.75% (d) 1% (e) Non-viable (f) Negative-control.

CONCLUSION

The *Humulus lupulus* seeds viability can be evaluated using both the Tetrazolium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dyes. The findings revealed that dye within the range of 0.75-1% can be used for evaluating the viability of seeds within an incubation time of 48 hrs. The study further evaluated that increasing the incubation time does not lead to any change in the color intensity of the seeds. Various factors such as germplasm, the collection date of the seeds, and their storage conditions play a vital role in affecting the viability of the seed species. However, the size and nature of the seeds make it difficult for the researchers to carry out this test. This paper opens an avenue for further studies to be carried out thus optimizing the above parameters.

Acknowledgment

The authors acknowledge the Department of Biotechnology, Jaypee Institute of Information Technology for providing all the necessary support required for carrying out this research.

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