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A comparative study on dehydrogenase activity using the TTC test

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Abstract
The dehydrogenases include several oxidoreductases functioning in different parts of plants. The TTC (2,3,5-triphenyl tetrazolium chloride) test is a biochemical test used all over the world to determine the viability of seeds. The test is based on the conversion of TTC, a water-soluble colourless compound, to an insoluble red/ pink coloured compound formazan by dehydrogenases present in the tissues. In the present investigation, dehydrogenase activity using the TTC test has been compared in the germinating seeds of three pulses, namely *Cicer arietinum* (chickpea; black and Kabuli) and *Vigna radiata* (green gram), and sweet corn *(Zea mays)* grains. Dimethyl sulfoxide (DMSO) was used as the solvent for extracting the formazan formed, and the technique of colorimetry was used for relative quantification of formazan based on absorbance values. All the four materials studied were TTC test positive and, therefore, contained dehydrogenases. The absorbance values showed that the activity of dehydrogenases was maximum in *V. radiata* and minimum in *C. arietinum* (black).

Keywords: Dehydrogenases, TTC, pulses, sweet corn

Introduction

Dehydrogenases are oxidoreductases commonly present in plants. The enzymes function in different organs as well as at different developmental stages of the organs (Moin *et al*., 2024; Subramanyan & Bahri, 2013; Subramanyan *et al.*, 2025 a, b) [1, 2, 3, 4]. Cells contain different dehydrogenases, to name a few glyceraldehyde-3-phosphate dehydrogenase involved in glycolysis and dark reaction of photosynthesis, pyruvate dehydrogenase that links citric acid cycle to glycolysis, succinate dehydrogenase and malate dehydrogenase in citric acid cycle, and alcohol dehydrogenase and lactate dehydrogenase in fermentation (Lehninger *et al.,* 1993; Taiz & Zeiger, 1998) [5, 6]. During the anaerobic phase of seed germination of cowpea *(Vigna unguiculata)* seeds, the increased dehydrogenase activity had a role in the breakdown of reserve food materials; lactate dehydrogenase, alcohol dehydrogenase, and succinate dehydrogenase were present, respectively, in a 60>31>9 percent ratio (Oaikhena *et al.,* 2013) [7].

The colourless water-soluble compound 2,3,5-triphenyl tetrazolium chloride (TTC) is a redox indicator which is used to detect dehydrogenase activity. On being absorbed by the hydrated respiring seed, TTC gets reduced to 1, 3, 5-triphenyl formazan (formazan in short) by dehydrogenases. The TTC (also called TZ) test is recognized all over the world as a method to determine viability of seeds of a variety of cultivated plants [8]. For seeds the test is performed using 0.5 to 1.0% TTC solution of pH 6-8 (aqueous or phosphate buffer is used), and at 30-35ºC in darkness because TTC is sensitive to light and heat [9]. Formazan is red/ pink and insoluble and is retained by the tissues where the reaction occurred. The colour development by an experimental material indicates that the dehydrogenases are active; thereby allowing to determine whether the material is viable or not. The TTC test was developed by Lakon to assess seed viability and with the results available in 1-2 days compared to the commonly used seed germination test which may take several weeks (Lakon, 1949) [10]. The test is a simple and quick biochemical method to determine seed viability and seed quality, and can be conducted on dormant seeds as well (Bajracharya, 1999) [11]. The TTC test is also used widely as a test for pollen viability (Shivanna, 2003; Shivanna & Rangaswamy, 1992) [12, 13]. The TTC-dehydrogenase activity method has been used as a reliable and quick method for analysing bioactivity of sludge and treating tomato paste wastewater (Sun *et al.,* 2012) [14].

The intensity of red/ pink colour of formazan can be simply visualized or the formazan formed can be extracted by dissolving in a suitable solvent and quantified by colorimetry. In maize following the TTC test, formazan was extracted using different solvents, namely trichloroacetic acid (TCA)/ acetone or ethanol or acetone (Zhao *et al*., 2010) [15]. The absorption spectra of the extracts were similar and maximum absorbance was at 485 nm; however, the extraction efficacy of TCA/ acetone was better than that of acetone and ethanol (Zhao *et al*., 2010) [15]. Using barley, the highest recovery of formazan was obtained when 10% (w/v) TCA/methanol was used for extraction compared to the acetone- and methanol-only based methods; and in the extraction method developed the formazan recovered and response of seeds to temperature, and seed viability of artificially aged seed lots showed a linear statistically significant relationship (Del Egido *et al.,* 2017) [16]. A rapid and quantitative method to determine seed viability was developed for wheat combining the optimal germination stage (24 hour-germination), allowing the TTC reaction for 1 hour at 25ºC in dark, followed by extracting the formazan formed in dimethyl sulfoxide (DMSO) at 55ºC for 1 hour, measuring the absorbance of the extract at 483 nm, and calculating the seed viability using a calibration curve (Wang *et al.,* 2023) [17]. In the present study the dehydrogenase activity using the TTC test has been compared in four materials, namely the germinating seeds of three pulses and sweet corn (maize) grains, using DMSO as the extraction solvent for formazan and colorimetry as the technique for relative quantification of formazan based on absorbance values.

Materials and Methods

The experimental materials and the parts used in the study are given in Table 1.

Table 1: The experimental materials used.

S. No.	Common name	Scientific name	Family	Part studied
1	Chickpea (black)	*Cicer arietinum* L.	Fabaceae	Cotyledons*
2	Chickpea (Kabuli/ white)	*Cicer arietinum* L.	Fabaceae	Cotyledons*
3	Green gram/mung bean	*Vigna radiata* (L.) R. Wilczek	Fabaceae	Cotyledons*
4	Sweet corn	*Zea mays* L.	Poaceae	Fresh grains

*The cotyledons were obtained from seeds soaked in water for 36 hours.

Seeds of chickpea (black, Kabuli) and green gram were rinsed thoroughly and then soaked in water in separate beakers. The soaked seeds were periodically rinsed with fresh water. Once the radicle started emerging, the amount of water added just covered the seeds. Germinating seeds 36 hours from soaking were used in the study. Fresh grains of sweet corn were bought from the local market, rinsed and used without soaking.

Detection of dehydrogenase activity by the TTC test

Three large Petri dishes were taken and the base Petri plate rims were labelled as (i) TTC (ii) water and (iii) TTC (boiled and cooled materials). A filter paper disc was placed on the inner surface of the base Petri plate. TTC (1 %, aqueous) was added to plates (i) and (iii) using a dropper. Distilled water was added to plate (ii). The seed coats of the germinating seeds of chickpea and green gram were removed, and each cotyledon of a seed was sliced parallel to the long axis into two. Sweet corn grains were cut into two halves by slicing parallel to the broad surface of the grain. One gram of each experimental material was placed on the base Petri plates (i) and (ii) with the cut surface in contact with the filter paper. One gram of each of the sliced material was taken in separate boiling tubes containing sufficient distilled water and boiled. Thereafter, the tubes were held under a running tap and cooled. Each boiled and cooled material was taken out using a spatula, blotted and placed on the base Petri plate (iii) with the cut surface of the material in contact with the filter paper. The three Petri dishes were incubated in dark for three hours at room temperature (around 35^{o}C), following which the sliced plant materials were observed for red/ pink colour development.

Quantification of formazan

Dimethyl sulfoxide (DMSO, 5 mL) was taken in four separate labelled test tubes. Each of the four experimental materials in Petri dish (i) following the TTC test, was blotted and transferred to the labelled test tube containing DMSO. The test tubes were shaken and allowed to stand for 1 hour at room temperature for the formazan to dissolve in DMSO: the formazan had almost completely dissolved within 1 hour. The test tubes were shaken before taking the sample in the cuvette. The absorbance of the four extracts containing dissolved formazan was recorded at 580 nm using a colorimeter. The colorimeter had been set for zero absorbance using DMSO. The experiment was repeated two more times and the average absorbance of the three replicates was taken for interpreting the results.

Results

The sliced plant materials turned pinkish red only in the first Petri dish (Fig 1 A) but not in the other two dishes (Fig 1 B, C). There was no formazan formation in the controls, namely where the materials were placed on filter paper moistened with water (Fig 1 B), and where the materials had been boiled and cooled and placed on filter paper moistened with TTC (Fig 1 C). All the four materials studied showed formazan formation when the fresh sliced material was placed on TTC solution (Fig 1 A, D). In case of green gram and chickpea (black and Kabuli) the entire cotyledon slice turned pinkish red whereas in sweet corn grains only the embryo turned deep pinkish red and the surrounding endosperm was either pale red or colourless. The aleurone layer was red when observed under a compound microscope because the cells of the aleurone are live and show dehydrogenase activity. Formazan formation was maximum in green gram, and least in chickpea (black) (Fig 1 D, E, Table 2, Fig 2). Interestingly, the average absorbance of the formazan extracted from green gram was nearly double the absorbance of the extract from sweet corn.

Discussion

The sliced plant materials could reduce TTC to pinkish red formazan only in Petri dish (i). Petri dishes (ii) and (iii) did not show colour development (Fig 1 A-C). This was because Petri dish (ii) did not contain TTC to be acted upon, and Petri dish (iii) contained boiled and cooled plant material. Boiling killed the tissues and the enzymes got denatured. Denatured dehydrogenases cannot act on TTC and hence there was no formazan formation in Petri dish (iii). Dehydrogenase activity was detected in all the materials studied, i.e., cotyledons of chickpea (black), chickpea (Kabuli) and green gram; and grains of sweet corn.

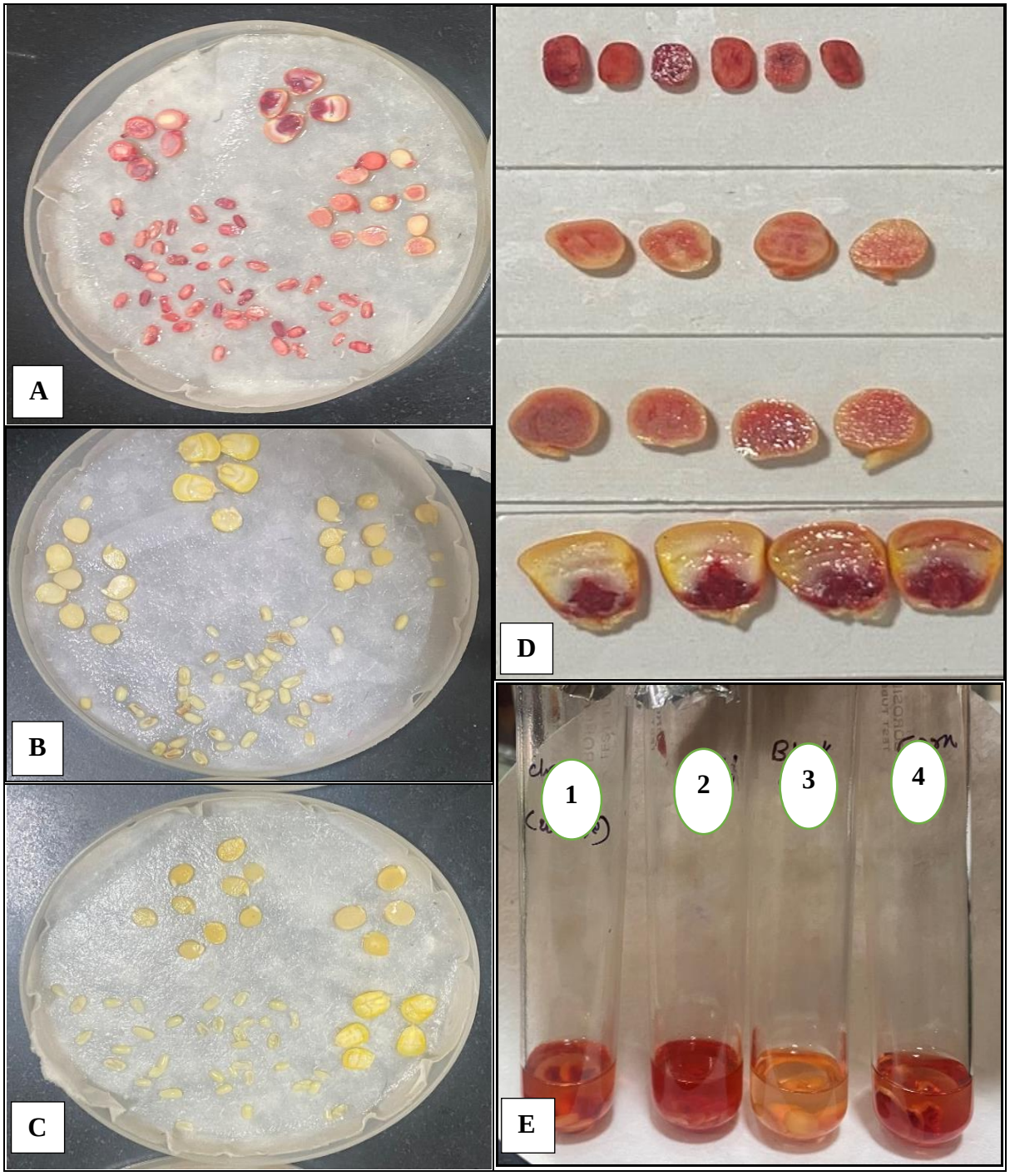

Fig 1: Representative Petri dishes three hours from incubation in dark with the sliced plant materials. Fresh plant materials: A. on TTC; B. On distilled water. C. Boiled and cooled slices on TTC. In A, clockwise the sliced materials are sweet corn, chickpea (black), green gram and chickpea (Kabuli). D. The sliced plant materials from Petri dish A following the TTC test; from top to bottom: slices of the cotyledons of seeds of green gram, chickpea (black) and chickpea (Kabuli); and half-grains of sweet corn. E. the DMSO extracts containing formazan, one hour from transfer of the plant material to DMSO following the TTC test: 1. Chickpea (Kabuli), 2. Green gram, 3. Chickpea (black), 4. Sweet corn.

Table 2: Absorbance of the DMSO samples with the dissolved formazan in the four experimental materials**.**

Plant material	Absorbance (580 nm)			
	Replicate 1	Replicate 2	Replicate 3	Average
C. arietinum (black)	0.14	0.15	0.20	0.16
C. arietinum (Kabuli)	0.16	0.25	0.29	0.23
Vigna radiata	0.64	0.64	0.77	0.68
Zea mays	0.37	0.35	0.32	0.35

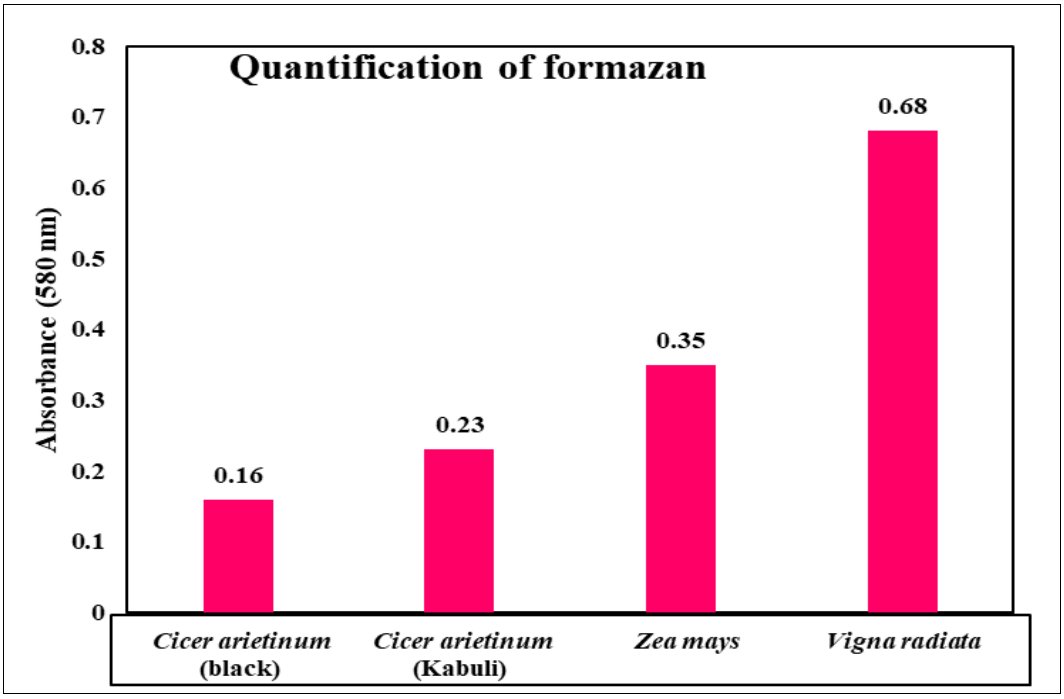

Fig 2: Absorbance of the DMSO solutions containing 1 g of the experimental material following the TTC test

In case of the pulses studied the cotyledons completely turned pinkish red which showed that the cotyledons were made up of live cells (1 D). In sweet corn the starchy endosperm is made of living cells when the grain is young; but as the grain grows and matures the cells start dying. Dead cells do not show any enzyme activity; hence in the mature endosperm of sweet corn grains there are no dehydrogenases to reduce TTC to formazan. Only the embryo and aleurone layer of the endosperm contain living cells and, therefore, turn pinkish red (1 D). Among the four materials studied green gram had the maximum average absorbance, i.e., 0.68; which showed that it had the highest dehydrogenase activity. Chickpea (black) showed the minimum average absorbance, i.e., 0.16; and, therefore, the minimum dehydrogenase activity of all the four plant materials studied (Table 2, Fig 2).

Increased dehydrogenase activity involved in mobilizing stored food materials has been reported in germinating cowpea seeds (Oaikhena *et al*., 2013) [7]. Both total and succinate dehydrogenases activity have been reported to increase during germination of barley grains and green gram seeds (Subramanyan *et al.*, 2025) [4]. An analysis of the proteome of mitochondria in *Arabidopsis* seeds within the first 0–24 hours of germination showed that certain dehydrogenases, namely glutamate dehydrogenase 1 or 3, glyceraldehyde-3-phosphate dehydrogenase, and succinate-semialdehyde dehydrogenase, were upregulated; however, the flavoprotein subunit of succinate dehydrogenase remained at a constant level (Farooq *et al.*, 2021) [18]. Four plastidal and two cytosolic isoforms of glucose-6-phosphate dehydrogenase (G6PDH) have been reported in *Arabidopsis* and many other crops, and the cytosolic G6PDH controls seed germination by modulating reactive oxygen species (ROS) homeostasis and hormonal signalling (Jiang *et al*., 2022) [19]. The mitochondrion is an organelle where ROS are produced (Farooq *et al.*, 2021) [18]. It is known that metabolic activity resumes soon after water enters the cells of the imbibing seeds using the enzymes already present in the seed (Bewley *et al*., 2000) [20]. In our work seeds soaked in water for 36 hours and fresh sweet corn grains have been used for the TTC test. In the hydrated state, repair of pre-existing mitochondria and biogenesis of new mitochondria occurs. Consequently, the seeds shift to aerobic respiration, and the citric acid cycle and electron transport chain start operating rapidly. Pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase in citric acid cycle; and NADH dehydrogenase complex in mitochondrial electron transport chain will contribute to the variety of dehydrogenases in the hydrated seed (Lehninger *et al.*, 1993; Taiz & Zeiger, 1998) [5, 6].

Conclusion

The soaked seeds of *C. arietinum* (black and Kabuli) and *V. radiata*, and fresh grains of *Z. mays* (sweet corn) gave a positive TTC test which showed that they all contained dehydrogenases. Colorimetry showed that the reduction of TTC to insoluble pinkish red formazan was maximum in *V. radiata* and minimum in *C. arietinum* (black); therefore, *V. radiata* had the maximum dehydrogenase activity.

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