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The Use of Tetrazolium as a Measure of the Salt Tolerance of Alfalfa

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THE USE OF TETRAZOLIUM AS A MEASURE OF THE SALT TOLERANCE OF ALFALFA

by

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INTRODUCTION

The task of obtaining and selecting plants which may not only survive under salty conditions, but grow and produce satisfactory yields is varied and complex. It is becoming necessary to select and breed crops for salt tolerance. Plants can be grown in artificially constructed salt basins to test their individual salt tolerance, but this takes time, at least one year. It would be desirable to develop a rapid test to determine the salt tolerance of a given plant. The use of a dye in conjunction with a series of salt solutions has been suggested for determining the salt tolerance of plants.

The objective of this study was to see if a tetrazolium dye could be used to determine the relative salt tolerance of 37 alfalfa varieties. The compound 2,3,5-triphenyltetrazolium chloride was used in this and similar studies. Most of the literature refers to this compound as TTC, which reference will also be used in this manuscript.

REVIEW OF LITERATURE

Evaluating salt tolerance of crop plants

Hayward and Bernstein (1958) reported that salt tolerance can be appraised in at least three ways:

1. The ability of a plant to survive on saline soils. This criterion of survival has limited application in irrigation agriculture but has been widely used by plant ecologists.

2. The absolute yield of a plant on saline soils. This criterion has the greatest agronomic significance.

3. The relative yield on saline soil compared to yield on similar nonsaline soil. This criterion is useful for comparing dissimilar crops whose absolute yields cannot be compared directly. The U. S. Salinity Laboratory (1954), in compiling its plant lists according to salt tolerance, used the third criterion.

Repp, McAllister, and Wiebe (1959) found that protoplasmic salt resistance can be used as a test for predicting the salt tolerance of agricultural plants. This test consists of placing freehand tangential sections containing the epidermis and several subjacent cell layers of the lower portions of the stem in sodium chloride solutions of various molal concentrations. After 24 hours these sections were transferred to a slightly hypertonic solution of glucose for from 3 to 4 hours and then examined under the microscope for plasmolysis. Plasmolyzed cells were considered uninjured while cells which failed to plasmolyze were considered injured or dead. Good correlation was found between the

salt resistance of the protoplasm and salt tolerance of a number of plants.

Monk (1960) developed the technique further in studying the salt tolerance of ornamental plants. His study consisted of two parts:

1. Plasmolytic method of determining salt tolerance.
2. Tetrazolium method of determining salt tolerance.

The plasmolytic method consisted of immersion of plant tissue sections in a graded series of sodium chloride solutions for 24 hours, followed by immersion of the sections in a hypertonic sucrose solution for 4 hours. The tissue sections were then examined microscopically for plasmolysis.

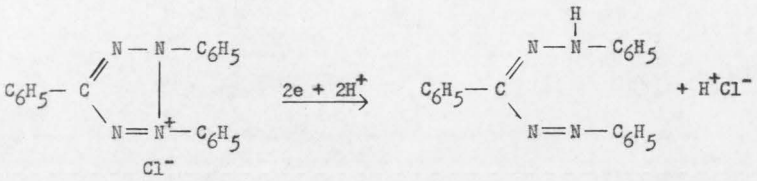
The tetrazolium method consisted of immersing the tissue sections into a graded series of sodium chloride solutions for 24 hours, followed by immersion in a 0.1 percent or 0.05 percent solution of 2,3,5-triphenyltetrazolium chloride for 24 hours. The sections were then examined for pink or red formazan color which appeared in living or slightly injured cells.

Monk (1960) found good correlation between salt tolerance in field or solution cultures and in the plasmolytic and tetrazolium tests for salt hardiness.

Chemical and physical properties of TTC

TTC belongs to a group of chemical compounds known as the tetrazoles. Benson (1947) reviewed the chemistry of the monosubstituted and disubstituted derivatives but did not discuss the trisubstituted members.

TTC occurs as a white to pale yellow crystalline powder that darkens on exposure to light. It has a high solubility in water, and is colorless in solution forming carmine red formazans upon reduction, making it one of the comparatively few organic compounds which is colored in the reduced state. Tetrazolium differs from the majority of redox indicators since, in the reduced state, it forms an insoluble formazan and the reaction is therefore, irreversible (Roberts, 1950).



2,3,5-triphenyltetrazolium chloride triphenylformazan

Formazan is easily visible in minute quantities and, consequently, the reaction is very sensitive. The formazan is insoluble and neither diffuses from the cell in which it is formed, nor oxidizes back to a colorless state on standing. The sensitivity of tetrazolium to reducing agents, in general, is indicated by the observation that a water solution of TTC will color a stainless steel spatula an intense red, due to the slight reaction of the metal with water which liberates hydrogen (Roberts, 1950). He also found that in some cases a faint reaction appeared over the entire cut surface of tissues. Such results were found to be due to the presence of microorganisms and were easily detected since the TTC solution itself was stained red. To eliminate the external effect of the microorganisms on his results, he stained and recorded his results in 24 hours.

Jambor (1954) stated that conditions must be carefully controlled when TTC is used for quantitative experiments. Polarographic study has shown that, in media of pH lower than 6, the reduction of TTC yields chiefly a colorless product. In more alkaline solutions the reduction product was mainly the red formazan. This behavior was due to the change of redox-potentials in the reduction of nitrogen atoms 1 and 2, and atoms 2 and 3, respectively. This explains why reddening can be achieved only in solutions of pH 11 to 14. The redox-potential of formazan formation cannot serve as a basis for predicting whether some reducing agent of known redox-potential will produce formazan or not. This is due to the insolubility of the reduced form of TTC. No redox-equilibrium can be established, and the actual potential of the substance will therefore be more positive than its normal potential. This shows why TTC can be reduced by succino-dehydrogenase which has a more positive normal redox-potential.

Jerchel and Mohle (Smith, 1951) reported the apparent redox-potential of the TTC-formazan equilibrium to be approximately -0.08 volts. Since triphenylformazan is an insoluble compound it interferes with the determination of the redox-potential of a TTC-triphenyl-formazan system and makes a determination difficult to obtain.

Light brings about the reduction of TTC, so most investigations must be carried out in reduced light. The photoreducibility of TTC was found to be optimal at a wave-length of approximately 4500° (Atkinson, et al., 1950).

The use of TTC in biological research

The use of TTC as a measure of living tissues or cells began in Germany around 1940. The topographical selenite method of determining the viability of seeds developed by Lakon (Grabe and Delouche, 1959) stressed the examination of individual parts of the embryo and gave accurate germination percentages. This method, however, had the serious disadvantage that a poisonous gas was slowly liberated which endangered the technicians. As a means of overcoming this obstacle, the Heidelberg chemist, Kuhn, pointed out to Lakon that the tetrazolium salts were equally well adapted to his topographic method as well as having the highly desirable characteristic of being non-poisonous. Lakon then began developing the tetrazolium method for testing agricultural, horticultural, and forest seeds. The use of tetrazolium salts in plant science research thus had its beginning.

Lakon's early tetrazolium work was done during the war years and was not known in this country until 1945. A team of investigators of the Joint Intelligence Objectives Agency, interrogating German scientists concerning their research activities, discovered the work of Lakon and reported it in this country (Grabe and Delouche, 1959).

TTC was introduced into the United States by Dutcher (1945) and by Mattson, et al. (1947). Porter, Durrell, and Romm (1947) used the TTC method and found a close agreement between the percentage of stained embryos and the percentage of normal sprouts obtained in standard germination tests with corn, wheat, rice, buckwheat, popcorn, soybeans, and Bahia grass. Cottrell (1947) also found good agreement between results obtained with the tetrazolium salt test and those

obtained by the standard germination tests. Bennett and Loomis (1949) found that freezing injury in seed corn could be determined with fair accuracy by staining with a 0.05 percent TTC solution, provided viability was fairly high and the corn had been stored for a period after freezing.

Small seeds were found to be difficult to examine and interpret (Cottrell, 1947). Flemion and Poole (Smith, 1951) found great difficulty in interpreting the staining in testing the seed viability of many species, but their trials did not include cereals.

Grabe and Delouche (1959) observed in preliminary experiments that very dilute solutions of TTC did not kill cottonseed, yet stained the seeds satisfactorily for interpretation. Their experiments indicated a method for accurately developing criteria for the interpretation of tetrazolium tests. The seeds could be stained, the various staining patterns observed, and these observations directly compared with subsequent seedling development.

Waugh (1948) observed that sections of living trees and shrubs became stained in the cambium layer, whereas dead sections were unaffected. He concluded that TTC could serve as a valuable aid in studies of living tissue. Gall (1948) used tetrazolium salt to estimate the reducing activity of bean tissue cultured in 2,4-dichlorophenoxyacetic acid. Pratt, Dufrenoy, and Pickering (1948) in studies of cellular physiology found TTC a valuable reagent, which could also be used in penicillin assays with Staphylococcus. In another paper, Dufrenoy and Pratt (1948) discussed its use in testing the reducing activity of sugar-cane stalks.

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Penetration of TTC into living cells appears not to be a limiting factor in its use in biological research. Few cases can be found in the literature suggesting that penetration was a limiting factor. Stein and Gerarde (1950) could not obtain formazan formation in whole, rapidly growing embryonal cells of chick heart fibroplasts. They concluded that the negative results were due to the failure of TTC to penetrate the living cellular membranes.

No particular concentration of TTC can be recommended for testing all tissues. The kind of tissue or organ under investigation influences the concentration to be used. Van der Zweep (1954) reported that with tissue studies of the viability of lower organisms the concentration has to be fairly low, near 0.0025 percent, whereas with tissue studies of higher organisms concentrations of 0.0025 percent to 2 percent were commonly used. Concentrations above 2.5 percent were toxic to living cells. Monk (1960) found that a 0.1 percent solution produced the best results for tissues from various woody species of plants, and that an 0.05 solution proved best for tissues from herbaceous plants.

Agencies responsible for reduction of TTC within the living cell

Gunz (1949) indicated that enzyme systems are the agencies responsible for the reduction of TTC in living tissue. He reported on its rapid reduction by fresh brewers' yeast, and also that he obtained reduction with a cell-free extract. Upon heating the yeast or extract to 60° C. the reaction was inhibited. His experiments showed that tetrazolium salts were reduced by non-viable cells and by cell-free extracts, and that reduction in yeast was independent of fermentation.

The reduction of TTC in living cells takes place at a much lower pH than that required for the formation of triphenylformazan *in vitro*. Van der Zweep (1954) pointed out that in very basic solutions of pH 13 to 14 the formation of triphenylformazan takes place immediately. Roberts (1951) reported the approximate optimum pH to be from 6.5 to 7.5 for tetrazolium reduction in normal tissues. *In vitro* and in the absence of enzymes, TTC never becomes reduced at such low pH values. However, preparations of enzyme solutions have optimal reduction values at lower pH values. Succinic dehydrogenase brings about the reduction at a pH of 7.4 in transferring hydrogen from succinic acid to TTC (Kun and Abood, 1949). The optimum pH for aerobic reduction of TTC by diaphorase was reported by Brodie and Gots (1951) to be in the range of 7.5 to 8.3.

Researchers have different ideas as to which enzymes or groups of enzymes are responsible for the reduction of TTC in living cells. Many of the original ideas were concerned mainly with the dehydrogenases. Mattson, *et al.* (1947), Kun and Abood (1949), and Jensen, *et al.* (1951) showed the presence of dehydrogenated DPN or TPN to be necessary for the reduction to take place. Brodie and Gots (1951) and Shelton and Schneider (1952) questioned their results. Brodie and Gots (1951) were able to reduce DPN by solutions of carefully purified lactic dehydrogenase, glycerol dehydrogenase and phosphoglyceraldehyde dehydrogenase, but TTC could not be reduced by them. Tetrazolium violet, a compound related to TTC, could not be reduced by lactic dehydrogenase or by alcohol dehydrogenase (Shelton and Schneider, 1952). They found that succinic dehydrogenase was able to perform the reduction, but that further purification of the enzyme was

necessary. They questioned the statement that succinic dehydrogenase can reduce TTC (Kun and Abood, 1949).

Belig, Kausche, and Haardick (Brodie and Gots, 1951), through an analysis of the pH dependence and temperature sensitivity of TTC reduction by bacterial systems, suggested the role of a flavoprotein, diaphorase, as the immediate site of the reduction. Kuhn (Brodie and Gots, 1951) also reported that TTC was reduced via the oxidation of reduced coenzyme by diaphorase. The reduction of TTC by a glucose dehydrogenase-coenzyme I system was reported by Mattson, et al. (1947); however, they did not mention whether flavin enzymes were involved.

Brodie and Gots (1951) have shown, as applied to isolated systems, that diaphorase reduces TTC both aerobically and anaerobically. The aerobic system was much less effective than the anaerobic. Xanthine oxidase and DPN-cytochrome C-reductase can also reduce TTC (Shelton and Schneider, 1952).

The research to date suggests more strongly the necessity for the presence of flavoproteins for the reduction of TTC in living cells than the presence of dehydrogenases. It was suggested by Roberts (1951) that the reduction of TTC was due to the presence of a general specific redox-potential level and not due to the action of any specific enzyme system. His idea was based on the lack of a specific influence of enzyme poisons on the reduction of TTC by living tissues.

MATERIALS AND METHODS

On May 31, 1957, seedlings of 38 varieties of alfalfa (table 1) were transplanted into the salt basins on the Greenville Experimental Farm at North Logan, Utah. The artificially salinized field plot technique (United States Salinity Laboratory, 1954) was employed in constructing the 14 x 14 foot basins. The basins were planted in a randomized block design with paired plants of each variety replicated four times in each basin. Salinization treatments were applied beginning July 3, 1957. These consisted of equal parts of sodium chloride and calcium chloride added in prescribed amounts to the irrigation water; basin A1 receiving 0 PPM added salt, basin A2, 8,000 PPM added salt, and basin A3, 16,000 PPM added salt (Olsen, 1958).

In 1957, Olsen (1958) measured the green weight forage yields of each variety and ranked them according to yield. The study was continued through 1958 and 1959 using the same procedure with the exception that each basin received irrigation water containing 16,000 PPM added salt. This level of salt was terminated August 25, 1960, when the plants were clipped. The basins then received irrigation water containing the original salt concentrations.

Testing of the plant tissue began on basin A1 on August 30, 1960. Basin A2 was sampled at a later date. The method of collecting stem sections was similar to the method used by Monk (1960). The stem was split longitudinally in half from bottom to top and cut into sections 1/2 to 1 inch long. Four of these sections from each variety were

Table 1. Alfalfa varieties used in the tetrazolium studies

Number	Variety
1.	A-224 Syn. 1
2.	A-225 Northern Syn.
3.	A-250 (Utah Syn. Y)
4.	A-251 (Utah Syn. Z)
5.	A-252 (Utah Syn. A)
6.	A-253 (Utah Syn. B)
7.	African A 4-35 (Arizona Common)
8.	Arizona Chilean
9.	Atlantic
10.	"Bam"
11.	B. Y. Strain
12.	Buffalo
13.	Caliverde
14.	Cossack
15.	Delta Common
16.	DuPuits
17.	Hairy Peruvian
18.	Kansas Common
19.	Ladak
20.	Lahontan
21.	Narragansett
22.	Nemastan
23.	Nev. Syn. E. 1956 (O.F.S.)
24.	Nev. Syn. K. (O.F.S.)
25.	Nomad N B 21
26.	Rambler
27.	Ranger
28.	Rhizoma
29.	Sevelra
30.	So. African (W3275)
31.	Stafford
32.	Swift Current 3484
33.	Swift Current M A 501
34.	Talent
35.	Terra Verde N. K.
36.	Uruguay Clone 10
37.	Vernal
38.	Williamsburg

placed in each level of salt used and four were placed directly in a TTC solution.

The salt solutions contained C. P. sodium chloride and consisted of a graded series of concentrations of 0, 0.25, 0.50, 0.65, 0.75, 1.00, 1.25, and 1.50 M.

The stem sections were placed in the salt solutions in the field and then brought to the laboratory. After a period of experimentation, 6 hours was selected as the optimum length of time to leave the stem sections in the salt solutions. At the end of this time interval the sections were removed, rinsed off, and the containers rinsed out. The stem sections were then placed back into their containers and a solution of 0.05 percent 2,3,5-triphenyl-2H-tetrazolium chloride was added. The stem sections in each container were examined 24 hours later and given a numerical value for the intensity of red coloration. These assigned values ranged from 4 to 0; 0 representing an absence of formazan formation within the cells of the stem sections.

Ladak and Rhizoma were both sampled in basin A2, which received 8,000 PPM added salt, to determine if border effects affected the results. Samples from both were collected from the center of the basin where maximum effect of the quantity of salt present was exhibited and from the margins of the basin where border effects were noticeable.

Variety 22, Nemastan, was omitted as it was missing in basin A1.

RESULTS AND DISCUSSION

The alfalfa began to grow after the basins were clipped and received their individual prescribed levels of added salt in the irrigation water. The growth of alfalfa in basin A1 exhibited a light green color and was more uniform than that in basins A2 and A3. The growth of alfalfa became noticeably darker green and less uniform as the salt level increased. Border effects were increasingly noticeable as the salt level increased.

Leaves proved unsatisfactory for testing as their green color masked the presence of formazan. Further testing indicated that all portions of the stem cut longitudinally could be used, as the red coloration of the tissue, if present, could be easily observed.

The use of Hoagland's solution No. 2 diluted to 0.1 strength to make the 0.05 percent TTC solution gave results similar to those obtained using Logan tap water. Logan tap water was used in the 0.05 percent TTC solution and in all of the various salt levels. All samples were checked against the container containing the TTC solution in which stem sections were placed directly. If no red coloration was found, all samples were discarded and a new group of stem sections taken.

Determination of the correct length of time to leave the stem sections in the salt solutions proved quite difficult. At first, six varieties were sampled and placed in the salt solutions for a period of 24 hours as Monk (1960) did on herbaceous plants in his studies.

After placement in the 0.05 percent TTC solution for 24 hours, the stem sections exhibited little or only sporadic staining. The stem sections placed in the TTC solution in the field stained a deep red. The stagnant odor of the salt solutions indicated that the time interval was too long and had resulted in the death of the stem sections.

The time interval was reduced to 7 hours. The degree of staining was greater but quite sporadic.

Kansas Common was sampled six times to determine the proper length of time to leave the stem sections in the salt solutions. The time intervals used ranged from 1 to 5 hours. One sample was removed from the salt levels after 1 hour, two samples after 2 hours, one after 3 hours, and two after 5 hours. All samples were placed immediately in the 0.05 percent TTC solution following removal from the salt solutions. The data are presented in table 2.

Table 2. Data obtained using various time intervals of stem sections of Kansas Common in salt solutions

Time (hours)	Salt concentration (M)						
	0	0.25	0.50	0.75	1.00	1.25	1.50
1	4	4	2	0	1	1	1
2	4	4	2	1	0	0	1
2	4	4	3	0	0	0	0
3	4	3	2	1	0	0	0
5	4	4	3	2	0	0	0
5	4	4	3	1	1	0	0

Observation of the six samples tested showed that 1 hour was too short a time interval in the salt solutions. Five hours appeared to be too long. The best time interval appeared to be between 2 and 3 hours. Two hours was selected as the length of time to leave the stem sections in the salt solutions. The data of table 3 were collected on this time interval.

At this point the 0.65 M NaCl level was inserted into the study, as most of the varieties stained at the 0.50 and not at the 0.75 M NaCl level. This gave a more accurate point at which some varieties failed to stain.

A time interval of 2 hours gave good results which could be analyzed, until some condition caused the results to change suddenly, as shown in table 4. The period of time the stem sections were left in the salt solutions was then increased to 4 hours. Still a high degree of red coloration appeared in the stem sections at the 1.50 M NaCl level.

Increasing the period of time in the salt solutions to 6 hours gave satisfactory results. The remainder of the study was carried out using a time interval of 6 hours. Tables 5 to 11, inclusive, report the results of TTC tests on alfalfa varieties.

The pictures of the alfalfa stem sections (figures 1 and 2) exhibiting varying degrees of red coloration developed darker than the colors actually observed. The color assigned the numerical value of 4 was a deep brilliant red. The color assigned the numerical value of 0 was entirely green, having no red coloration.

F tests indicated that there were no significant differences between determinations on varieties from basin A1 and from basin A2,

Table 3. Color values assigned alfalfa stem sections from basin Al after placement in various molar concentrations of NaCl for 2 hours followed by placement in a 0.05 percent TTC solution for 24 hours[a]

Variety	Salt concentration (M)							Variety total
	0	0.25	0.50	0.75	1.00	1.25	1.50	
1.	4	3	0	0	0	0	0	7
2.	4	1	1	0	1	0	0	7
3.	4	4	3	1	0	0	0	12
4.	4	3	1	0	0	0	0	8
5.	4	4	3	1	0	0	0	12
6.	4	4	2	1	0	0	0	11
7.	4	4	3	1	1	0	1	14
8.	4	4	3	2	0	0	1	14
9	4	4	0	0	0	1	0	9
10.	4	4	2	2	0	0	0	12
11.	4	4	2	0	0	0	0	10
12.	4	4	1	0	0	0	0	9
13.	4	4	4	2	0	0	0	14
14.	4	4	3	2	2	1	1	17
15.	4	4	2	0	1	0	0	11
16.	4	4	3	1	1	0	0	13
17.	4	4	4	2	0	2	1	17
18.	4	4	1	0	1	0	0	10
19.	4	4	4	1	1	0	0	14
20.	4	2	2	1	1	0	0	10
21.	4	4	3	1	1	0	0	13
23.	4	4	3	1	0	0	0	12
24.	4	4	1	0	0	0	0	9
25.	4	3	3	1	1	0	0	12
26.	4	4	4	2	0	0	0	14
27.	4	4	3	0	0	0	0	11
28.	4	4	3	1	0	1	1	14
29.	4	4	4	2	3	2	1	20
30.	4	4	1	1	2	2	2	16
31.	2	3	3	1	1	0	0	10
32.	4	3	1	1	1	1	0	11
33.	4	4	0	0	0	0	0	8
34.	4	2	0	0	0	0	0	6
35.	4	4	2	2	1	1	1	15
36.	4	4	4	2	0	1	0	15
37.	4	4	2	1	1	1	0	13
38.	4	4	1	0	0	1	1	11

a The 0.65 M NaCl level was not included in this set of determinations. This table was not included in the analysis of variance.

Table 4. Color values assigned alfalfa stem sections from basin A1 after placement in various molar concentrations of NaCl for 2 hours and for 4 hours followed by placement in a 0.05 percent TTC solution for 24 hours

Variety	Salt concentration (M)								Length of time in salt solutions (hours)
	0	0.25	0.50	0.65	0.75	1.00	1.25	1.50	
1.	4	4	2	0	0	0	0	0	2
2.	4	4	3	0	1	0	0	0	2
3.	4	4	4	4	0	0	1	0	2
4.	4	4	2	0	0	0	1	1	2
5.	4	4	1	3	0	2	1	0	2
6.	4	4	1	1	1	1	0	1	2
7.	4	4	4	4	1	2	0	0	2
8.	4	4	3	4	1	1	2	1	2
9.	4	4	3	4	2	2	2	0	2
10.	4	4	3	1	2	2	2	2	2
11.	4	4	4	3	3	3	2	2	2
12.	4	4	4	4	1	1	1	2	2
13.	4	4	4	4	4	1	1	2	2
14.	4	4	4	2	3	3	3	2	2
15.	4	4	4	4	2	1	2	2	2
16.	4	4	3	1	1	1	1	1	4
17.	4	3	4	3	1	1	1	1	4
18.	4	4	4	2	2	0	0	0	4
19.	4	4	4	3	3	1	1	1	4
20.	4	4	4	2	1	3	1	2	4
21.	4	4	3	3	1	3	2	1	4
23.	4	4	4	4	3	0	2	3	4
24.	4	4	3	3	1	2	3	2	4
25.	4	4	4	4	1	2	2	0	4
26.	4	4	4	2	2	3	1	3	4
27.	4	4	4	4	0	2	2	2	4
28.	4	4	4	3	3	3	2	3	4

Table 5. Color values assigned alfalfa stem sections from basin A1 after placement in various molar concentrations of NaCl for 6 hours followed by placement in a 0.05 percent TTC solution for 24 hours

Variety	Salt concentration (M)								Variety total
	0	0.25	0.50	0.65	0.75	1.00	1.25	1.50	
1.	4	4	4	3	0	0	0	1	16
2.	4	4	4	4	0	0	1	0	17
3.	4	4	4	3	3	3	2	2	25
4.	4	4	4	0	2	2	3	2	21
5.	4	4	4	1	2	2	2	2	21
6.	4	4	4	0	1	1	1	1	16
7.	4	4	4	3	0	0	0	0	15
8.	4	4	4	4	3	2	2	2	25
9.	4	4	4	0	1	0	1	1	15
10.	4	4	1	2	0	0	0	1	12
11.	4	2	2	1	0	0	0	0	9
12.	4	4	4	3	3	2	2	2	24
13.	4	4	4	3	0	0	1	1	17
14.	4	3	1	2	0	0	0	0	10
15.	4	4	4	3	3	1	1	1	21
16.	4	4	4	4	4	1	0	0	21
17.	4	4	4	4	2	0	0	0	18
18.	4	4	3	3	0	0	0	0	14
19.	4	4	4	3	2	2	2	1	22
20.	4	4	2	2	1	0	0	2	15
21.	4	4	4	1	1	0	0	1	15
23.	4	4	4	4	4	4	2	1	27
24.	4	4	2	3	3	2	1	1	20
25.	4	4	4	4	4	2	1	3	26
26.	4	4	4	4	4	2	2	3	27
27.	4	4	2	3	2	1	1	2	19
28.	4	3	3	3	2	2	1	0	18
29.	4	4	4	3	2	2	2	1	22
30.	4	4	4	3	1	0	1	1	18
31.	4	4	4	4	0	1	1	1	19
32.	4	4	4	4	3	3	0	0	22
33.	4	4	4	2	2	1	3	3	23
34.	4	4	4	4	2	1	3	3	25
35.	4	2	2	2	1	1	1	0	13
36.	4	4	4	3	3	3	3	2	26
37.	4	4	2	2	1	0	0	0	13
38.	4	4	4	3	2	2	2	1	22

Table 6. A second determination of color values assigned alfalfa stem sections from basin Al after placement in various molar concentrations of NaCl for 6 hours followed by placement in a 0.05 percent TTC solution for 24 hours

Variety	Salt concentration (M)								Variety total
	0	0.25	0.50	0.65	0.75	1.00	1.25	1.50	
1.	4	4	4	4	1	1	2	1	21
2.	4	4	4	3	3	2	0	1	21
3.	4	4	2	0	0	0	0	0	10
4.	4	4	4	3	3	2	2	2	24
5.	4	4	3	4	3	3	3	2	26
6.	4	4	4	1	1	1	1	2	18
7.	4	4	4	1	2	1	0	0	16
8.	4	4	4	2	3	2	2	2	23
9.	4	4	4	4	4	3	1	0	24
10.	4	4	2	0	2	1	0	0	13
11.	4	4	1	1	0	0	0	0	10
12.	4	4	4	2	4	2	1	2	23
13.	4	4	4	0	1	1	2	2	18
14.	4	4	4	0	2	2	2	1	19
15.	4	4	4	3	1	0	1	0	17
16.	4	4	3	4	3	3	2	1	24
17.	4	4	1	0	0	0	0	1	10
18.	4	4	4	0	1	1	0	1	15
19.	4	4	4	4	1	2	2	0	21
20.	4	4	4	4	2	0	2	0	20
21.	4	4	2	0	0	0	0	0	10
23.	4	4	4	3	3	3	2	2	25
24.	4	4	3	0	2	2	2	2	19
25.	4	4	3	0	2	2	2	1	18
26.	4	4	4	3	3	0	0	1	19
27.	4	4	3	1	2	1	2	2	19
28.	4	4	1	2	0	0	0	0	11
29.	4	4	4	0	1	0	0	0	13
30.	4	4	4	2	3	2	2	3	24
31.	4	4	0	2	0	0	0	0	10
32.	4	4	3	4	1	0	0	0	16
33.	4	4	4	2	1	1	0	1	17
34.	4	4	4	2	2	1	1	1	19
35.	4	4	4	2	2	1	0	0	17
36.	4	4	4	3	3	2	2	2	24
37.	4	4	4	3	3	2	2	2	24
38.	4	4	3	2	1	0	0	0	14

Table 7. Color values assigned alfalfa stem sections from basin A2 after placement in various molar concentrations of NaCl for 6 hours followed by placement in a 0.05 percent TTC solution for 24 hours

Variety	Salt concentration (M)								Variety total
	0	0.25	0.50	0.65	0.75	1.00	1.25	0.50	
1.	4	3	1	2	1	1	0	0	12
2.	4	4	3	2	3	2	1	0	19
3.	4	4	4	4	2	0	0	0	18
4.	4	4	3	3	1	0	0	0	15
5.	4	4	4	4	4	2	0	0	22
6.	4	4	4	3	1	1	1	1	19
7.	4	4	4	4	3	2	2	2	25
8.	4	4	4	4	4	2	1	2	25
9.	4	4	4	4	4	2	2	1	25
10.	4	4	3	4	2	2	2	2	23
11.	4	4	4	2	2	1	2	1	20
12.	4	4	4	4	3	1	0	0	20
13.	4	3	3	1	2	0	0	0	13
14.	4	3	3	1	2	1	1	0	15
15.	4	4	4	4	3	2	0	1	22
16.	4	4	4	4	4	3	3	1	27
17.	4	4	4	1	1	0	0	0	14
18.	4	4	4	4	2	0	0	0	18
19.	4	4	4	4	3	3	3	1	26
20.	4	4	3	4	0	0	0	0	15
21.	4	4	4	3	2	1	0	0	18
23.	4	4	4	2	2	1	0	0	17
24.	4	4	4	4	3	2	1	1	23
25.	4	4	2	3	3	1	0	0	17
26.	4	4	4	3	1	0	1	1	18
27.	4	4	4	4	1	0	1	0	18
28.	4	4	4	4	2	0	0	1	19
29.	4	4	4	2	2	2	2	1	21
30.	4	4	4	4	4	3	2	1	26
31.	4	4	4	3	3	2	2	1	23
32.	4	4	2	0	1	1	1	2	15
33.	4	4	4	4	1	0	1	0	18
34.	4	4	4	3	3	1	1	1	21
35.	4	4	4	2	2	2	1	1	20
36.	4	4	4	3	2	0	0	0	17
37.	4	4	4	2	2	1	1	1	19
38.	4	4	3	3	2	1	1	1	19

Table 8. A second determination of color values assigned alfalfa stem sections from basin A2 after placement in various molar concentrations of NaCl for 6 hours followed by placement in a 0.05 percent TTC solution for 24 hours

Variety	Salt concentration (M)								Variety total
	0	0.25	0.50	0.65	0.75	1.00	1.25	1.50	
1.	4	3	2	1	1	0	0	0	11
2.	4	4	4	3	2	1	0	0	18
3.	4	4	4	1	1	1	1	0	16
4.	4	4	4	3	2	0	0	0	17
5.	4	4	4	4	4	1	0	0	21
6.	4	4	4	3	0	0	1	1	17
7.	4	4	4	4	1	1	1	1	20
8.	4	4	4	2	4	1	1	0	20
9.	4	4	4	4	0	1	0	1	18
10.	4	4	4	4	2	0	2	1	21
11.	4	4	4	0	2	1	0	0	15
12.	4	4	4	4	2	2	0	0	20
13.	4	3	4	2	0	0	1	1	15
14.	4	3	3	4	3	1	0	0	18
15.	4	4	4	2	2	1	0	0	17
16.	4	4	4	3	3	3	2	2	25
17.	4	4	1	4	1	0	0	0	14
18.	4	4	4	4	3	2	2	1	24
19.	4	4	4	4	4	1	0	0	21
20.	4	4	4	4	4	1	0	0	21
21.	4	4	3	3	1	0	0	0	15
22.	4	4	4	4	2	1	1	1	21
24.	4	4	4	3	3	2	1	1	22
25.	4	4	4	3	1	1	0	0	17
26.	4	4	4	4	3	0	0	0	19
27.	4	4	4	3	3	2	1	1	22
28.	4	4	4	4	2	0	0	1	19
29.	4	4	4	1	2	1	0	0	16
30.	4	4	4	4	3	1	0	1	21
31.	4	3	4	4	4	2	1	1	23
32.	4	4	4	3	3	2	2	2	24
33.	4	4	4	2	2	1	0	0	17
34.	4	4	4	3	3	1	1	1	21
35.	4	4	0	1	2	1	1	0	13
36.	4	4	4	2	2	1	1	0	18
37.	4	4	4	4	3	0	0	0	19
38.	4	4	4	3	1	0	0	0	16

Table 9. Color values assigned, after TTC treatment, to Ladak and Rhizoma alfalfa sampled where border effects were noticeable and in the central part of basin A2, containing 8,000 PPM added salt

Variety	Salt concentration (M)								Variety total	Part of basin
	0	0.25	0.50	0.65	0.75	1.00	1.25	1.50		
Ladak	4	4	4	4	3	2	1	1	23	center
Ladak	4	4	4	3	4	2	0	0	21	border
Rhizoma	4	4	3	2	2	1	0	0	16	center
Rhizoma	4	4	4	0	1	1	1	1	16	border

Table 10. Totals of TTC color values per basin for alfalfa varieties

Variety	Basin A1	Basin A1	Basin A2	Basin A2	Basin A1 sum	Basin A2 sum	Means	Variety sum
1.	16	21	12	11	37	23	15.00	60
2.	17	21	19	18	38	37	18.75	75
3.	25	10	18	16	35	34	17.25	69
4.	21	24	15	17	45	32	19.25	77
5.	21	26	22	21	47	43	22.50	90
6.	16	18	19	17	34	36	17.50	70
7.	15	16	25	20	31	45	19.00	76
8.	25	23	25	20	48	45	23.25	93
9.	15	24	25	18	39	43	20.50	82
10.	12	13	23	21	25	44	17.25	69
11.	9	10	20	15	19	35	13.50	54
12.	24	23	20	20	47	40	21.75	87
13.	17	18	13	15	35	28	15.75	63
14.	10	19	15	18	29	33	15.50	62
15.	21	17	22	17	38	39	19.25	77
16.	21	24	27	25	45	52	24.25	97
17.	18	10	14	14	28	28	14.00	56
18.	14	15	18	24	29	42	17.75	71
19.	22	21	26	21	43	47	22.50	90
20.	15	20	15	21	35	36	17.75	71
21.	15	10	18	15	25	33	14.50	58
23.	27	25	17	21	52	38	22.50	90
24.	20	19	23	22	39	45	21.00	84
25.	26	18	17	17	44	34	19.50	78
26.	27	19	18	19	46	37	20.75	83
27.	19	19	18	22	38	40	19.50	78
28.	18	11	19	19	29	38	16.75	67
29.	22	13	21	16	35	37	18.00	72
30.	18	24	26	21	42	47	22.25	89
31.	19	10	23	23	29	46	18.75	75
32.	22	16	15	24	38	39	19.25	77
33.	23	17	18	17	40	35	18.75	75
34.	25	19	21	21	44	42	21.50	86
35.	13	17	20	13	30	33	15.75	63
36.	26	24	17	18	50	35	21.25	85
37.	13	24	19	19	37	38	18.75	75
38.	22	14	19	16	36	35	17.75	71

Table 11. Ranked means of TTC color values for alfalfa varieties from all four determinations, the higher values being more salt tolerant

	Variety	Means	Least significant ranges at the 5 percent level* (Duncan's Multiple Range test)
16.	DuPuits	24.25	
8.	Arizona Chilean	23.25	
5.	A-252 (Utah Syn. A)	22.50	
19.	Ladak	22.50	
23.	Nev. Syn. E. 1956 (O.F.S.)	22.50	
30.	So. African (W3275)	22.25	
12.	Buffalo	21.75	
34.	Talent	21.50	
36.	Uruguay Clone 10	21.25	
24.	Nev. Syn. K. (O.F.S.)	21.00	
26.	Rambler	20.75	
9.	Atlantic	20.50	
25.	Nomad N B 21	19.50	
27.	Ranger	19.50	
4.	A-251 (Utah Syn. Z)	19.25	
32.	Swift Current 3484	19.25	
15.	Delta Common	19.25	
7.	African A 4-35 (Arizona Common)	19.00	
2.	A-225 Northern Syn.	18.75	
31.	Stafford	18.75	
33.	Swift Current M A 501	18.75	
37.	Vernal	18.75	
29.	Sevelra	18.00	
18.	Kansas Common	17.75	
20.	Lahontan	17.75	
38.	Williamsburg	17.75	
6.	A-253 (Utah Syn. B)	17.50	
3.	A-250 (Utah Syn. Y)	17.25	
10.	"Ban"	17.25	
28.	Rhizoma	16.75	
13.	Caliverde	15.75	
35.	Terra Verde N. K.	15.75	
14.	Cossack	15.50	
1.	A-224 Syn. 1	15.00	
21.	Narragansett	14.50	
17.	Hairy Peruvian	14.00	
11.	B. Y. Strain	13.50	

* A significant difference exists between any two means which are not found within the same range. There is no significant difference between any two means within the same range.

Figure 1. Paired alfalfa stem sections after removal from 0.05 percent TTC solution having color values of 4, 3, 2, 1, and 0, respectively.

Figure 2. Paired alfalfa stem sections after removal from 0.05 percent TTC solution. Previous treatment of the stem sections consisted of TTC solution, 0, 0.25, 0.50, 0.65, 0.75, 1.00, 1.25, and 1.50 M NaCl, respectively. The color values are 4, 4, 4, 4, 3, 1, 0, and 1, respectively.

nor were there any significant differences between all four determinations on each variety. The data indicated that similar results could be obtained regardless of the salt level in the growing medium. This was verified by the data of table 9 which were obtained by sampling Ladak and Rhizoma to see if border effects in basin A2 varied the results.

Significance was observed in salt tolerance between varieties of alfalfa (table 11). However, the salt tolerance cannot be attributed to the point of origin of the varieties. The significance obtained may be due to a confounding effect and should be used only as a guide for future development of technique.

A difficulty, encountered several times in this study, was the sudden changes in the results obtained. The 2-hour time interval proved satisfactory in the initial stages of the study. However, using the same technique, the results changed and high color values were obtained at the 1.50 M NaCl level. There seems to be no valid explanation for these variations. The 6-hour time interval in the salt solutions may prove unsatisfactory as did the 2-hour time interval. Further experimentation should be undertaken to see if the results of this study can be duplicated.

SUMMARY AND CONCLUSIONS

1. The objective of this study was to determine if a tetrazolium dye could be used on the stem tissue of 37 alfalfa varieties to determine their relative salt tolerance.

2. Determination of the optimum length of time to leave the stem sections in the salt solutions proved difficult. Six hours was selected as the optimum period of time to leave the stem sections in the salt solutions.

3. F tests indicated that there were no significant differences between determinations on varieties from basin A1 and from basin A2, nor were there significant differences between all four determinations on each variety.

4. Significance was observed in salt tolerance between varieties of alfalfa. However, salt tolerance could not be attributed to the point of origin of the varieties. The significance obtained may be due to a confounding effect and should be used only as a guide for future development of technique.

5. A difficulty, encountered several times in this study, was the sudden change in the results obtained, using the same technique. There seems to be no valid explanation for these variations. The 6-hour time interval in the salt solutions may prove unsatisfactory as did the 2-hour time interval in the initial stages of the study.

6. Since the salt tolerance ratings obtained could not be attributed to the point of origin of the varieties and the results

from the different time intervals varied, the significance between varieties obtained in this study only indicate that with further refinement of technique, a reliable rapid method of determining salt tolerance could result. This technique may be applied to other salt tolerant crop plants.

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APPENDIX