Seed quality studies of native shrubs
by Gerhard Peter Weber

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Agronomy
Montana State University
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Abstract:
Three forb end eight shrub species native to the Rocky Mountain and Northern Great Plains regions were evaluated for seed viability with 2,3,5-triphenyl-2H-tetrazolium chloride (TZ). Specific staining techniques were developed for each species. Treatments include presoaks, seed coat puncture, or removal, and seed bisection. Species tested and their viability values were Achillea millefolium L., (yarrow) 86%; Linum lewisii (Pursh), (Lewis flax) 93%; RatTbida columnifera (Nutt.) Wooten and Standley, (prairie coneflower) 72%; Amelanchier alnifolia (Nutt.) Nutt., (serviceberry) 84%; Amorpha fruticosa L., (indigobush) 94%; Artemisia tridentata Nutt., (big sage-brush) 66%; Ceretojdes lanata (Pursh) Howell, (winterfat) 91%; Prunus virginiana L., (chokecherry) 98%; Purshia tridentata (Pursh) DC., (antelope bitterbrush) 100%; Rhus trilobata Nutt., (skunkbush sumac) 91%; and Symphoricarpos albus (L.) Blake, (snowberry) 68%.

Skunkbush sumac (Rhus trilobata Nutt.) and serviceberry (Amelanchier alnifolia Nutt.) are native shrubs extensively distributed in the western United States, and have achieved importance for their use in revegetation of disturbed lands. Standard germination tests were performed on each species according to methods outlined in the literature. Seed viability was determined with triphenyl tetrazolium chloride (TZ). Results indicated special techniques would be required to affect the rapid germination which is needed in current seed testing programs. Both species have hard or impermeable seed coats and embryo dormancy normally overcome by cold stratification or fall sowing. Results confirm that skunkbush sumac germination is promoted by 75 minutes acid scarification and that KNO3 or GA produce no additional response. Acid scarification for 30 minutes and a mixture of thiourea (TU) and benzyladenine (BA) as a moistening agent for the media was beneficial to serviceberry germination. Analysis predicted maximum germination to occur at 100 ppm BA and 100 mM TU. An interaction of BA and TU on germination was observed at the lower concentrations tested.

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Date September 2, 1980

SEED QUALITY STUDIES OF NATIVE SHRUBS AND FORBS

by

GERHARD PETER WEBER

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Agronomy

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ABSTRACT

Three forb and eight shrub species native to the Rocky Mountain and Northern Great Plains regions were evaluated for seed viability with 2,3,5-triphenyl-2H-tetrazolium chloride (TZ). Specific staining techniques were developed for each species. Treatments include presoaks, seed coat puncture, or removal, and seed bisection. Species tested and their viability values were Achillea millefolium L., (yarrow) 86%; Linum lewisii (Pursh), (Lewis flax) 93%; Ratibida columnifera (Nutt.) Wooten and Standley, (prairie coneflower) 72%; Amelanchier alnifolia (Nutt.) Nutt., (serviceberry) 84%; Amorpha fruticosa L., (indigobush) 94%; Artemisia tridentata Nutt., (big sagebrush) 66%; Ceretoides lanata (Pursh) Howell, (winterfat) 91%; Prunus virginiana L., (chokecherry) 98%; Purshia tridentata (Pursh) DC., (antelope bitterbrush) 100%; Rhus trilobata Nutt., (skunkbush sumac) 91%; and Symphoricarpos albus (L.) Blake, (snowberry) 68%.

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LITERATURE REVIEW

Seed Viability as Determined by Tetrazolium

Historical

Seed production and marketing depends upon the accurate assessment of seed quality, specifically purity and germination. Whereas, the former may be evaluated in a few minutes, the latter may require a time range of two weeks to several months. Decisions regarding the processing and marketing of a particular seed lot may often be delayed pending the results of germination tests. For this reason rapid methods of assessing seed viability have been studied since the early 1900's (17, 35).

These early investigations concentrated on chemically analyzing ground and pulverized groups of seeds. Unfortunately, the localized defects of individual seeds such as fractures or dead tissues escaped detection with these methods (69). One method was based on the theory that nonviable seeds were more permeable than live seeds and would readily lose electrolytes to a water solution (43). Measurements of the electrical conductivity of the leachate would vary directly with seed viability. This technique has recently been more successfully applied to determinations of seed vigor (35). Another method (16), involved measurements of the relative quantities of heat produced by a seed sample. High heat production was related to extensive microbial

activity, whereas low heat was thought to indicate poor viability and vigor.

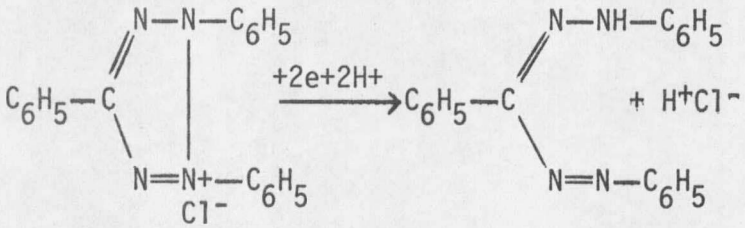
Viability assessment based upon the evaluation of individual seeds was eventually recognized as being superior to group evaluations (69). The techniques used were based on vital staining; that is, the ability of some materials to differentially stain live and dead tissue. Moore (69) credits Dr. Turina of Yugoslavia with the development of vital staining techniques and Dr. Neljubow of Russia for applying the use of indigo carmine, a tontoxic dye, to this technique. He found that this dye would penetrate dead tissue more readily than healthy tissues. Evaluations of viability were based on the relative proportions of colored and uncolored tissues. However, vital staining based on dyes was found to be of limited value.

Hasagawa is credited (69) with the development of a vital staining procedure based on the seed's metabolic processes. With the application of selenium and tellurium salts, enzymatic activity within the seed would affect a color change. As described by others (83, 17, 69, 12) work with these techniques was taken up by the German scientist, Lakon, who developed the topographical viability test. This test stressed the reaction of specific seed tissues and resulted in considerable accuracy in seed viability assessment. The toxic selenium salts were soon abandoned by Lakon in favor of nontoxic tetrazolium salts which functioned in the same manner. He found that when the colorless tetrazolium

solution came in contact with live seed tissue it was reduced to an insoluble red pigment; whereas, nonliving tissue did not stain. Viability was then estimated by evaluation of the extent and location of stained embryo tissue.

Specific Mode of Action

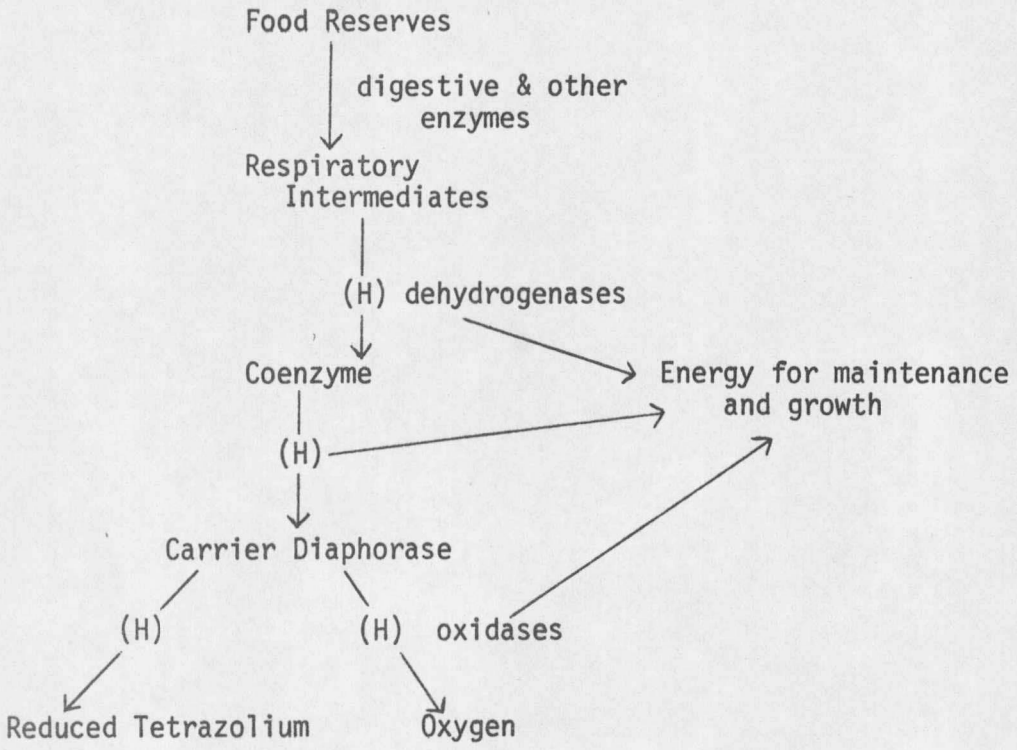
Of the several types of tetrazolium salts, the 2,3,5-triphenyl tetrazolium chloride derivative, now commonly known as TTC or TZ, is best suited for use in the topographical test (17, 35). Tetrazolium chloride occurs as a white to pale yellow crystalline powder, readily water soluble and darkens on exposure to light. In the presence of viable tissue the colorless TZ solutions forms the insoluble red triphenyl formazan according to the following reaction (83):



Colorless Red Color

Detailed studies (51, 83, 84) have indicated that one or more of the dehydrogenase enzyme systems appears to be involved in the reduction reation. Specifically, the reduction of TZ in corn embryo tissues is

catalized by diphosphopyridine nucleotide (DPN)-linked dehydrogenases, particularly the malic and alcohol systems, and is mediated by diaphorase (84). This sequence may be summarized in the following way (85):



The ability to measure dehydrogenase activity is of particular significance. These enzymes are of a delicate nature and are responsible for the maintenance of energy without which the embryo could not remain alive (85). Therefore, the loss of active dehydrogenases probably indicates loss of germinating ability (51). Measurement of

other enzymes have not provided consistent results. For example, the hydrolytic enzymes responsible for food mobilization and other respiratory enzymes such as catalase and peroxidase are of a more stable nature and have been shown to exist in dead seeds (85).

Other advantages of tetrazolium include its nontoxicity and that it is one of the few organic compounds which is colored in the reduced state (83). The insolubility of the reduction product is important. Since the reaction occurs within cells and the pigment is nondiffusable, there is a sharp delineation between viable and nonviable tissue (17). Finally, it has been shown through extensive testing that loss of dehydrogenase enzyme activity tends to parallel loss in seed viability (85). This characteristic coupled with a thorough knowlege of seed structure, makes possible accurate viability assessment in much shorter times than similar results obtained with germination tests.

The Seed

A true seed is a fertilized mature ovule containing the embryonic plant, stored nutrients and the integument(s) differentiated as the protective seed coat or testa (25, 57, 62). The term "seed" is usually applied to the unit of dissemination. Many dispersal units which are often referred to as seeds are not true seeds but single, sometimes two to several seeded fruits. The pericarp remains and may even fuse to the testa, as in cereal grains (57, 8). Physiologically and

biochemically, these dispersal units should be considered as seeds (8, 95).

The seeds of Angiosperms develop as a result of a process called "double fertilization" (62). The embryo is derived from the fertilization of the egg cell by one of the male nuclei from the pollen tube. The "second" fertilization is the fusion of a male pollen nuclei with two mother plant polar nuclei. This fertilization results in the development of the endosperm which may persist as a storage organ, or degenerate and remain rudimentary, possibly fused to the seed or fruit coat (62). The testa or true seed coat comprising the embryo envelope is derived from one or both integuments of the ovule. At times the testa may be derived from tissues other than that of the integuments, e.g., the nucellus, the endosperm or rarely the chalaza (8, 62). The testa is usually a hard coat. Its physiological importance is derived from an often fatty or waxy cuticle, and one or more layers of thickened protective cells (8, 62). These features are responsible for variable degrees of impermeability to water and/or gases and consequently exert some regulatory influence over the respiration of the embryo (8, 62). The features of the testa in some species are apparently lacking, the outer covering being derived from extra-ovular tissue is called a pericarp. Seeds with this structure are actually fruits, for example sunflowers (62).

Germination

Germination, as defined by Bewley and Black (8) consists of those processes which begin with water uptake and successfully terminate with the emergence of the radicle or hypocotyl through the seed coverings. This definition is deceptively simple, as the physiological events leading to the protrusion of some part of the embryo through the seed coat are not well understood. Additionally, where germination ends and growth begins is difficult to delineate. Emergence of the radicle may indicate that germination has occurred but this may already be considered a part of growth (62). Whether this growth is a result of cell expansion, cell division or both is still unresolved (7). Probably the fundamental processes which cause germination are different from those of growth (8, 62, 80).

A seed must be in a favorable environment for germination to occur. Specifically, there must be adequate moisture, suitable temperatures, a correct ambient gas composition, and in certain photoblastic species, a requirement for light (62, 8). A seed which is unable to germinate due to a lack of one or all of these environmental factors is termed quiescent (95, 62, 80).

Dormancy

Dormancy describes the condition in which an otherwise viable seed fails to germinate under environmental conditions suitable for germination (95, 62), and is considered advantageous in adopting the growth

cycles of the plant to variations in the environment. Chances of survival are increased because dormant tissues have a great resistance to adverse environmental conditions (95, 55). Agronomically, seed dormancy is usually a disadvantage. Delayed seedling emergence may result in poor stand establishment or require the use of larger seed quantities than would be required if all seeds germinated equally (61). Vegis (94) defines true dormancy as a state in which normal growth cannot be resumed regardless of the external conditions. Vegis' definition seems overly restrictive if taken literally and has been subject to criticism (95, 70). The application of proper treatments may stimulate the germination of dormant seeds of many species. For example, the removal of the seed coat of antelope bitterbrush (71) or peach (30) allows rapid germination of normally dormant seeds.

The scientific literature on dormancy is vast (95) and the classification schemes too numerous and complex to describe in great detail. This indicates that the nature of seed dormancy itself is a variable and complex condition. Nikolaeva (70) has devised a detailed and complex dormancy classification scheme. He defines dormancy as arising from four major causes, (A) due to properties of the outer coverings, (B) underdeveloped embryos, (C) physiological condition of the embryo and its inner coverings, and (D) types of combined dormancy. The numerous subclassifications of these main categories are of value mainly in providing a means of comparing dormancy mechanisms with

methods used to break dormancy (95).

A simpler and more commonly used classification was published by Crocker in 1916 (15). He describes dormancy as resulting from (A) immaturity of the embryo, (B) impermeability of the seed coats to water, (C) mechanical resistance of the seed coat to embryo growth, (D) low permeability of the seed coats to gases, (E) dormancy resulting from a metabolic block within the embryo itself, (F) a combination of factors A-E, or (G) secondary dormancy. The term secondary dormancy applies to the condition in which quiescent seeds have lost their ability to germinate as a result of being imbibed under conditions unfavorable for germination. Primary dormancy is specified when seeds are dormant at the time of dispersal or harvest (54).

Crocker's seven factors contributing to dormancy remain adequate in summarizing current knowledge of the subject with the exception of embryo dormancy. Endogenous mechanisms are better understood now than in 1916. Identification and characterization of plant growth substances in recent years is largely responsible for this increase in knowledge.

Coat Imposed Dormancy

The various layers of the seed coat provide protection for the embryo from the environment. The seed coat may exert a profound influence by providing a buffer between the embryo and the environment. The morphological features of the seed coat may present a barrier to water uptake and prevent gaseous exchange. The seed coat may also

prevent germination by mechanical restriction and by containing growth inhibitors. An early publication by Crocker (14) reported that seed dormancy of many species was due to seed coat impermeability to water. Intact seeds of Russian pigweed would not germinate until the seed coat was broken to allow water imbibition and subsequently 100% germination. Agronomically, this condition is termed "hard seededness" and is especially prevalent in the legume family. The percentage of hard seeds may vary among varieties and is dependent upon the environment in which the seeds are produced (95, 62). Hard seededness of soybeans is reported to be the result of fat deposits and lignification in the coat palisade cells (3). Hard seeds of white sweet clover can be rendered permeable with vigorous shaking or by the use of moderate heat (37). These procedures remove the strophiolar plug within the strophiolar cleft.

Seed coverings may also prevent gas exchange between the respiring embryo and the environment. Dormancy of the upper seed of cocklebur was shown to be imposed by coat impermeability to oxygen (14). Increasing O_2 tensions or removing the testa would release dormancy. These treatments also improved the germination of wild oats (52). The effect of CO_2 is usually the reverse of O_2. Most seeds fail to germinate if CO_2 tensions are increased (40), but dormancy breaking by CO_2 has been observed in subterranian clover (5) and cocklebur seeds (56).

Dormancy due to mechanical restrictions imposed by the testa are of rare occurrence (62). This type of dormancy is difficult to demonstrate as the release of dormancy by testa removal may be cited as evidence for other germination inhibiting factors. However, through a series of deductive experiments, Ikuma and Thimann (46) propose that light sensitive "Grand Rapids" lettuce seeds are unable to germinate because of the mechanical restrictions imposed by the endosperm layer. Exposure to light stimulates the production of hydrolytic enzymes which help the radicle free itself. Similar results were obtained for lilac (53). More direct evidence is supplied by Esashi and Leopold (23) who measured the thrust developed by germinating cocklebur embryos. Nondormant embryos developed more than twice the thrust of dormant embryos. Additional measurements of the forces required to rupture the testa indicated that these thrust differences of dormant and nondormant embryos were sufficient to account for the prevention of germination (23).

Dormancy imposed by germination inhibitors contained in the seed coat is of ecological significance especially in desert species. Germination of these species is prevented until adequate rainfall leaches the inhibitors from the seed. The amount of moisture necessary for removal of the inhibitor also assures survival of the seedling (80). Seed coat inhibitors are common in members of the rose family. Treating achenes of antelope bitterbrush with thiourea (73) releases

dormancy. Nord (72) postulates that thiourea deactivates a seed coat inhibitor. Attempts to isolate the substance responsible for dormancy have been unsuccessful (20). Similar results were obtained by Jackson and Blundell (48, 49) for field and rugosa rose. Dormancy of these species was believed to be coat imposed by mechanical restrictions of growth. However, when achenes were steeped in various solvents and analyzed chromatographically, fractions were found which prevented the germination of excised embryos. Webb and Wareing (99) also found that inhibitors present in the embryo of sycamore maple are prevented from leaching outward by the testa until the seeds were subjected to moist chilling.

There are many ways in which coat imposed dormancy may be removed. Already mentioned are impaction, moderate heating, leaching with water or other solvents, stratification, and treatment with thiourea. Seed coats can also be rendered permeable by scarifying mechanically in sandpaper lined drums or chemically with concentrated acids. These treatments alter seed coat permeability to water and gases, weaken the seed coat structure, change sensitivity to light, and possibly remove chemical inhibitors (62, 80).

Immaturity of the Embryo

Embryos of seeds of some species especially in the orchis family and buttercup, may not have completed morphological development or achieved maximum size at the time of dispersal (62). Such embryos are

termed immature or rudimentary and are unable to germinate until differentiation and growth are complete. The embryo of seeds of holly are described as a spherical mass of tissue at the time of dispersal (47). Germination in nature is achieved after a period of 8 to 12 months of growth and differentiation. Laboratory germination could only be accomplished by culturing embryos in a 5% glucose medium for 5 months at 25 C. Similarly, rudimentary parsnip embryos will differentiate and increase dry weight by a factor of 25 before germinating. This growth occurs only at temperatures near freezing (89). Work with ash indicates morphologically differentiated embryos must grow in size and assimilate food reserves before normal seedlings are produced (88, 96). Embryos enlarge best when imbibed and kept at 20 C for 2 to 3 months. However, enlarged embryos require an additional cold treatment of 5 C for 2 to 3 months before being returned to an alternating 20-30 C for best germination. Snowberry seeds require a similar treatment for 10 months to produce embroys which will germinate (26). In contrast, embryos of smooth bromegrass are capable of germination 5 days after anthesis (33).

Hormones, Growth Promoting and Inhibiting Substances

Abscisic Acid. The most important naturally occurring plant growth inhibitor is undoubtedly abscisic acid (ABA)(62). ABA is considered to be one of the five classes of plant hormones. When applied exogenously

ABA will prevent seed germination and it has been found to be an endogenous component of many seeds (11, 98). An example of the effectiveness of ABA in controlling germination is demonstrated in cotton fruits (45). During seed development, the ABA content of the ovary wall increases. Isolated embryos will germinate when washed to remove ABA. The cotton embryo germination will be prevented if the ABA or ovary extract is added back to the washed embryos.

Through evidence obtained with ultra violet absorption spectrum analysis, chromatography and plant growth assays, Lipe and Crane (59) were able to show that the germination inhibitor in peach is ABA. Application of either 10 ppm ABA or peach extract to normal seedlings would induce a rosetted growth form, implying that the peach extract contained ABA. Further studies (59) indicated that the ability of seeds to germinate after 6 weeks of moist chilling correlated with the disappearance of the ABA from those seeds.

Gibberellin. Like ABA, gibberellins (GA) are plant hormones, and are important in promoting seed germination. Although more than 50 GA's have been identified, GA_3 is most commonly employed in seed germination studies. GA's stimulate germination in seeds whose dormancy is usually overcome by moist chilling, dry storage after ripening or light (90, 11, 95).

Studies made by Frankland and Wareing (32) in the early 1960's

indicated that dormancy breaking in hazel and beech requires about 12 weeks of moist chilling. During this dormancy breaking period, they detected no changes in seed inhibitor levels. Measurements of GA levels after chilling showed only a slight rise in activity. Several years later Ross and Bradbeer (79) were able to support these results through their own experiments. However, due to improved measuring techniques, GA was shown to be produced in physiologically active quantities. It was also shown that GA synthesis did not take place during the chilling treatment but was initiated once seeds were returned to temperatures suitable for germination.

Work with ash by Villiers and Wareing (97) showed that germination and the production of normal seedlings requires a moist chilling treatment and that soaking unchilled embryos in water resulted in the production of stunted seedlings. Since chilling did not change inhibitor levels within the seed, and inhibitors did not leach from the seed during the soaking treatment, it was postulated that the inhibitor was diluted by imbibition which allowed germination in unchilled embryos. In addition, they concluded that chilling produces a growth promoter, probably GA, which counteracts the effects of growth inhibitors.

A more recent study (2) again with hazel, indicated that the presence of GA synthesis inhibitors, e.g., phosphon D and CCC, prevented GA accumulation and subsequent germination of prechilled seeds. These results were consistent with the theory that GA biosynthesis is

prerequisite for chilled hazel seed germination. In the same study exogenously applied ABA strongly inhibited germination but had little effect on GA accumulation. It was assumed that ABA did not affect GA synthesis but rather its action.

The results of these experiments could be cited in support of the Promoter/Inhibitor Theory of seed germination (1) which postulates that dormancy onset, control and termination is regulated by a balance of growth inhibitors and promoters. Particular emphasis is given gibberellins in the role of promoter, and abscisic acid the inhibitor, although other growth substances such as the cytokinins are not excluded. Accordingly, the termination of seed dormancy and the onset of growth may be accomplished by either decreasing inhibitor content or increasing promoter levels.

Cytokinins and Thiourea. Cytokinin (CK) another of the plant hormones is the generic term for all substances displaying kinetin-like activity; that is, the promotion of cell division or cytokinesis (82). As GA and ABA, the CK's are implicated in seed dormancy and germination. They are most effective in promoting germination when combined with other dormancy breaking agents such as GA. CK's are sometimes more effective than GA in counteracting inhibitor activity (95). CK's are a chemically heterogeneous group (82) and the structural specificity for activity is not very exacting (81). The most commonly utilized synthetic CK is benzyladenine (BA).

Early investigations concerning the effects of CK on seed germination were performed using lettuce seeds (81). BA increased germination to 59% when controls germinated 9% by overcoming the dormancy imposing effects of darkness and warm temperatures. Other investigations with lettuce (66) showed that kinetin, a CK, not only partially overcomes dark imposed dormancy but strongly increases the promotive effect of light on germination. Dormant apple seeds normally requiring 70-80 days of moist prechilling to break dormancy were stimulated to germinate when treated with BA (4). It was postulated that the role of BA was to overcome the effects of endogenous inhibitor. These effects were only partially removed by BA; embryos germinated in this manner produced stunted and abnormal seedlings. CK activity increased progressively during dormancy breaking and decreased once the buds had opened in studies (19) of the dormant buds of deciduous trees. CK was absent in dormant buds and present once dormancy was broken.

Thiourea (TU) while not considered a plant hormone, is important for its effects in stimulating the germination of dormant seeds. TU can stimulate dark germination and substitute for cold moist treatment in some species. Relatively high concentrations of TU must be used. Seeds are commonly soaked in a 0.5 to 3.0% solution of TU then transferred to water. When seeds are to be germinated directly in TU, concentrations of 10^{-3} to 10^{-2} M are usually employed (62).

Thiourea was originally applied to potato tubers to promote sprouting. It was soon being used to stimulate the germination of dormant seeds of sugar and Norway maple, black and red oak (18), lettuce (91), and peach (92). TU will promote the germination of lettuce seeds in the dark, but concentrations slightly above those optimum for germination are inhibitory to growth (91). Peach seedlings produced by the TU stimulation of unprechilled seeds were dwarfed and rosetted, typical for other treatments which artificially stimulate peach germination (92).

Finally, as previously mentioned in the discussion of seed coat inhibitors, TU seems to inactivate this inhibitor in antelope bitterbrush. Application of TU to dormant achenes of this species is as effective in promoting germination as removing embryos from their seed coats (73).

Stratification

Moist chilling has frequently been mentioned as a method for overcoming seed dormancy. Seeds of many species, particularly in the rose family, many deciduous trees, and some conifers will not germinate until exposed to freezing temperatures in the moist condition. Seeds to be preconditioned for germination by this method are frequently layered or stratified in flats containing moist sand or peat. For effectiveness a stratification period of weeks or months may be required. Seeds must be

in the imbibed condition to respond to cool temperature treatments (62).

In review, stratification was reported to be advantageous in removing coat imposed dormancy by changing the properties of the seed coat which prevented germination (99). Stratification is important in promoting growth and morphological development in parsnip (89). Seeds of ash (88, 96) and snowberry (26) require warm followed by cold stratification before embryos are able to germinate. Relative levels of growth promoting and growth inhibiting substances are known to change during the stratification period. ABA disappears in peach after 6 weeks stratification (59).

Stratification stimulates GA production in beech, hazel and ash (32, 79, 97, 2) and this is a prerequisite for germination. It has also been shown that CK's and TU can substitute for the stratification requirement in some species (4, 18, 92).

Many other changes are reported to occur during stratification including increases in acidity, water holding capacity, catalase activity, reducing sugars, respiratory rate, transfer of food reserves to the embryo and changes in enzyme composition (11, 62). Although there is no clear evidence as to any one event responsible for dormancy breaking during stratification (62), a statement by Taylorson and Hendricks (90) summarizes what is generally believed to be the underlying principle:

> Temperature regimes regulate synchrony of processes in seeds by effects on reaction rates and changes in the physical state of cellular components. In seeds, temperature influences (the) integration of partial processes as dormancy continues or is overcome.

If release of dormancy is dependent upon a specific physiological state reached through a complex series of biochemical changes, then synchrony and integration of processes will be the only way in which that physiological condition will be attained.

CHAPTER I: TETRAZOLIUM VIABILITY PROCEDURES FOR NATIVE SHRUBS AND FORBS

INTRODUCTION

The increasing disturbance of large land acreages in recent years especially due to coal surface mining has increased the commercial sale of native species seed for reclamation. Viability testing of this seed has not been practical due to the lack of known testing procedures and the extended time periods necessary for testing. Dormancy in some native species may require long periods of stratification for germination to proceed.

Historically, the need for a rapid method to assess viability led to the introduction and acceptance of staining seed with 2,3,5-triphenyl-2H-tetrazolium chloride (TZ) (69). Seeds difficult to test with conventional germination procedures may successfully be evaluated using the TZ viability method. However, this method does not distinguish between dormant and nondormant seed. Our objective was to develop TZ techniques for three native forb species: Achillea millefolium L. (yarrow); Linum lewisii (Pursh) (Lewis flax), Ratibida columnifera (Nutt.) Wooten and Standley (prairie coneflower); and eight native shrub species: Amelanchier alnifolia (Nutt.) Nutt. (serviceberry), Amorpha fruticosa L. (indigobush), Artemisia tridentata Nutt. (big sagebrush), Ceretoides lanata (Pursh) Howell (winterfat), Prunus virginiana L. (chokecherry), Purshia tridentata (Pursh) DC. (antelope bitterbrush), Rhus trilobata Nutt. (skunkbush sumac), and Symphoricarpos albus (L.) Blake (snowberry). TZ testing procedures for

chokecherry (31, 60), bitterbrush (28, 29), Amelanchier spp., (44), prairie coneflower (13, 87), yarrow (87, 64), and Lewis flax (13) are generally nonspecific and difficult to access. Viability tests using embryo excision have been developed for antelope bitterbrush (71, 42) and skunkbush sumac (42).

Correlations between specific staining patterns and actual germination are obtained after considerable testing experience with a particular species. It is not the objective of this paper to provide this information. TZ viability correlation to germination has been reported for the native shrub Stansbury cliffrose (75). Research by Grabe (34), Delouche (17), and Moore (68) is invaluable as a guide to the interpretation of staining patterns.

The Suggested Procedures section, including Table 2 is presented for quick reference and guide for the seed analyst and researcher. Specific procedures such as soaking times are often not critical to successful staining and are therefore adjusted for convenience in laboratory routine.

Refer to Table 1 for a summarization of specific experimental procedures.

MATERIALS AND METHODS

Seed samples were contributed by the Western Energy Company, Colstrip, MT, from lots currently being used for revegetation of coal strip-mine spoils.

Two replicates of 50 seeds were used for the TZ viability tests. Two replicates of 100 seeds were used for those species which required minimal preparation, i.e., yarrow, winterfat, indigobush, and big sagebrush. Seeds to be preconditioned by moistening were placed in small beakers or between blotter papers moistened with tap water. Preconditioning took place at room temperature. Seeds were bisected and seed coats removed using foreceps, dissecting knives and needles. A stereoscopic microscope with 10 and 20 power magnification and with top and bottom illumination was used to aid preparation and interpretation.

After preparation, seeds were placed in Syracuse watch glasses or small beakers, covered with an ample amount of TZ solution, and covered with a standard watch glass. Seeds were stained in the dark at room temperature. TZ solutions were prepared from 2,3,5-triphenyl-2H-tetrazolium chloride (34): 0.1% being used for seeds with bisected embryos and 1% for intact seeds and embryos. Upon completion of staining, the seed coats of some species were cleared using lactophenol prepared as described in the Tetrazolium Testing Handbook (34). Excess TZ was removed with pipette and blotting paper, before covering seeds

with lactophenol. Clearing was conducted in covered watch glasses at room temperature for a minimum of 1 hour.

RESULTS AND DISCUSSION

Yarrow

Viability of the seed lot tested was 86% (Table 1). Lactophenol induced seed coat clearing, but was not necessary for stain interpretation as the seed has a translucent pericarp (Fig. 1A). Unpunctured seeds for those punctured through the pericarp only, did not stain. This was probably due to an inner seed coat impermeable to TZ. The optimum time for staining was 4 hours. Shorter times were inadequate and longer staining times produced little additional change.

Lewis flax

Viability of the seed lot tested was 93% (Table 1). Lactophenol did not clear the seed coat, and removal was necessary for staining to proceed. Dissection of unimbibed seeds caused extensive embryo damage. Once imbibed, seed coats were removed by applying pressure to the distal end (Fig. 1B) forcing the embryo from the seed coat. This procedure damages cotyledon tips but did not affect stain interpretation (34). Moistening caused the secretion of a gelantinous substance which increased the difficulty of seed manipulation. Optimum staining occurred at 4 hours. Seeds were understained at 2 hours and overstrained at 8.

Table 1. Percentage viability, effect of lactophenol, effect of seed coat preparation on staining, and optimum tetrazolium staining time for eight shrubs and three forbs.

Species	Viability %	Lactophenol effect	Stained with seed coat intact	TZ stain time in hours		
				Understain	Optimum	Overstain
Yarrow	86	+[a]	-	2	4	NC[b]
Lewis flax	93	-[c]	-	2	4	8
Prairie coneflower	72	-	-	2	4	8
Serviceberry	84	-	-	2	4	8
Indigobush	94	+	slow	12	18	NC
Big sagebrush	66	+	+	12	16	NC
Winterfat	91	+	slow	1	4	8
Chokecherry	98	-	-	2	6	12
Antelope bitterbrush	100	-	-	2	4	8
Skunkbush sumac	91	-	-	2	4	12
Snowberry	68	-	-	2	4	NC

[a]Positive (+) effect.

[b]NC = little additional change with increased staining times.

[c]Negative (-) effect.

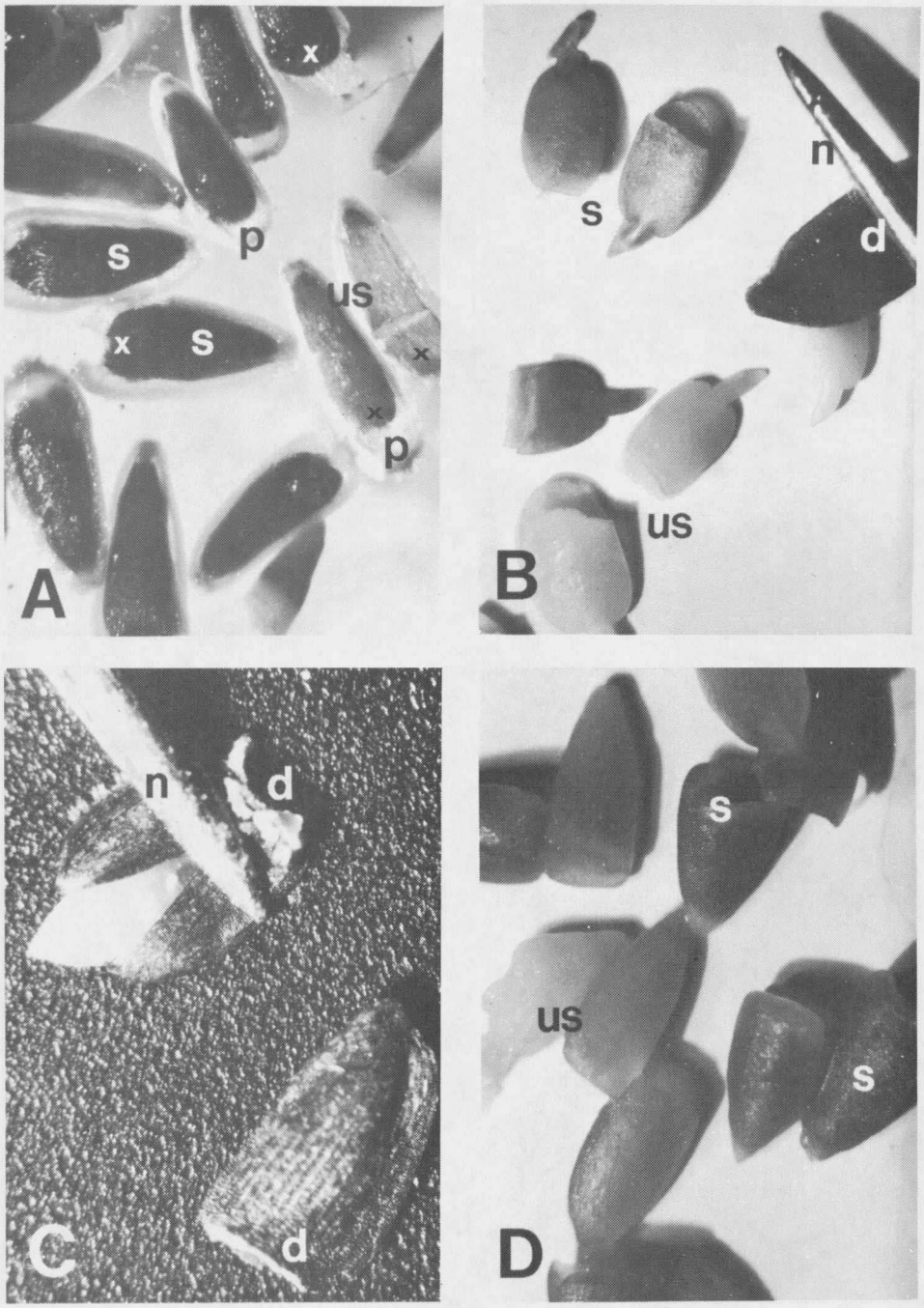


Figure 1. Tetrazolium stained (s) and unstained (us) embryos of: (A) yarrow showing pericarp (p) and puncture point (x), (B) Lewis flax, and (D) prairie coneflower. The method of embryo removal for (B) Lewis flax and (C) prairie coneflower is to apply pressure with a needle (n) to the distal seed end (d).

Prairie coneflower

Viability of the seed lot tested was 72% (Table 1). The seed coat (pericarp) did not respond to the use of lactophenol. Staining did not occur without removing the pericarp and rupturing the impermeable inner membrane (87). Seed coat removal (Fig. 1C) was accomplished in the same manner as for Lewis flax. Seeds were preconditioned using moistened blotter paper. This procedure avoids sampling bias due to the separation of filled and unfilled seeds placed directly in water. Damage to the inner membrane during preparation allowed TZ penetration. Staining was optimum after 4 hours and less than 2 and more than 8 hours yielded inadequate results (Fig. 1D).

Serviceberry

Viability of the seed lot tested was 84% (Table 1). Lactophenol was not effective in clearing the seed coat. Seeds with intact outer seed coats and inner membranes did not stain. Seed coats which were not preconditioned by moistening were difficult to remove. As with Lewis flax, moisture caused the seed coat to secrete a gelatinous material, which made seed manipulation difficult. Embryos were successfully removed by bisection. The seed coat was cut longitudinally along the crease starting at the pore which made it possible to tease the embryo from the seed coat. Staining was optimum after 4 hours. Two hours provided inadequate staining and embryos were overstained

after 8 hours. Stained and unstained embryos are presented in Fig. 2A.

Indigobush

Viability of the seed lot tested was 94% (Table 1). Lactophenol effectively cleared the seed coat permitting stain interpretation without seed coat removal. It was necessary to chip seed coats for staining to occur (Fig. 2B) due to a high occurrence (68%) of hard seeds. Seeds were chipped at the distal end to avoid damage to the embryonic axis. Staining proceeded slowly and was optimum at approximately 18 hours. Extended staining times produced little change. Times shorter than 12 hours were inadequate.

Big sagebrush

Viability of the seed lot tested was 66% (Table 1). Lactophenol effectively cleared the seed coat and facilitated stain evaluation. Sagebrush seeds (achenes) are similar to those of yarrow with a pericarp as an outer covering. Neither the pericarp nor the seed coat required puncture or removal for staining. Seeds placed directly into a 1% TZ solution stained in 16 hours. Staining times less than 12 hours were inadequate. Few changes in staining characteristics were observed with extended times.

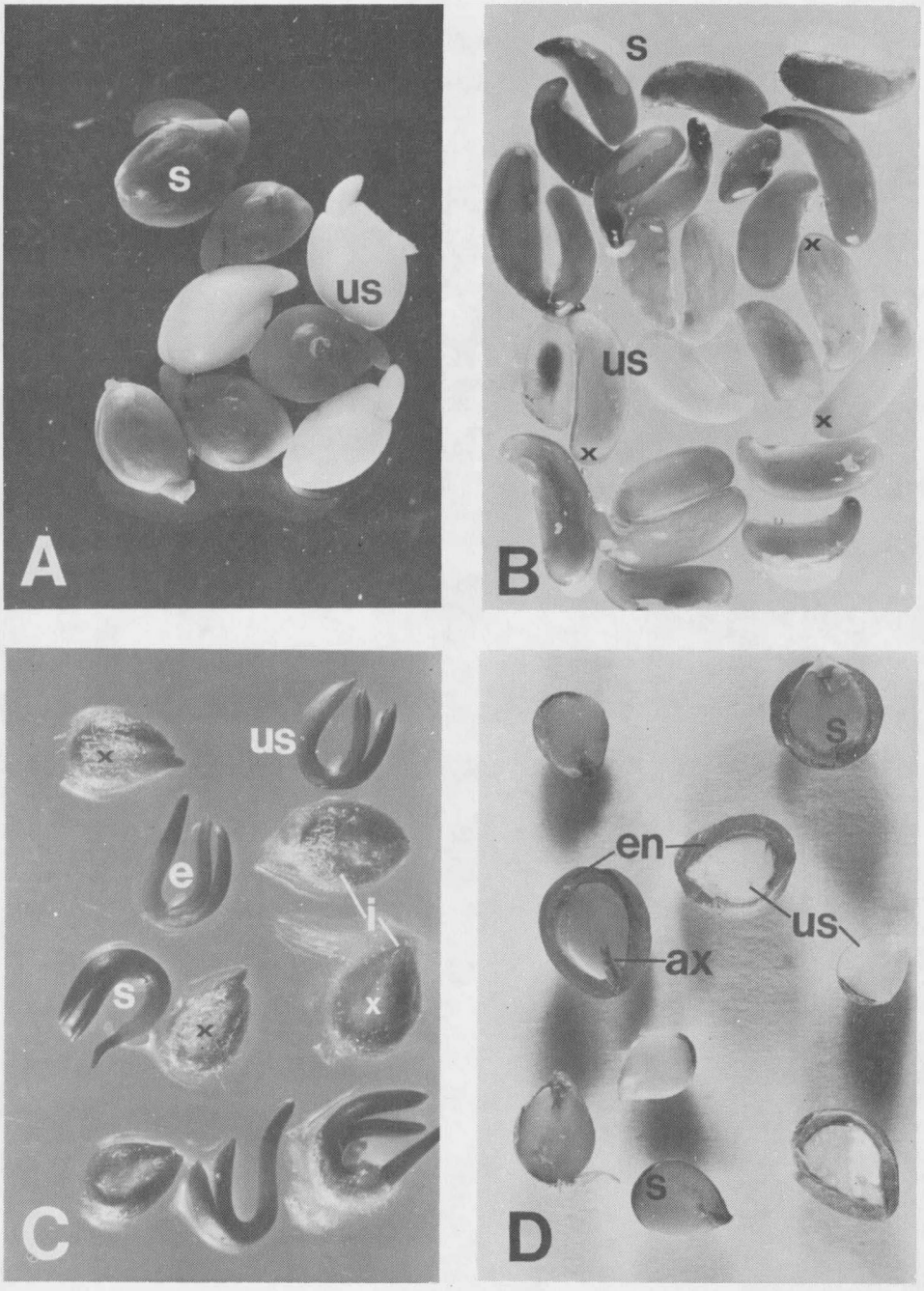


Figure 2. Tetrazolium stained (s) and unstained (us) embryos of: (A) serviceberry, (B) indigobush showing chipping point (x), (C) winterfat showing intact seed (i), seed coat removed (e) and puncture point (x), and (D) chokecherry showing stony endocarp (en) and highly stained embryonic axis (ax).

Winterfat

Viability of the seed lot tested was 91% (Table 1). Lactophenol was effective in clearing the seed coat, but its use was not necessary since the seed coat was removed prior to staining. Seeds placed directly in TZ solution stained slowly and nonuniformly. This was probably due to the non-wetting tendencies of intact seeds. Preconditioning between moistened blotters for several hours overcame this tendency. Seed coats were easily removed once imbibed by puncturing the central area which is surrounded by the embryo (Fig. 2C). Naked embryos were placed directly in TZ and staining proceeded rapidly with 4 hours being optimum. Some staining was evident after 1 hour. Seeds were overstained after 8 hours.

Chokecherry

Viability of the seed lot tested was 98% (Table 1). The thickened stony endocarp does not respond to treatment with lactophenol. Although the endocarp is reported to be permeable to water (36), seeds with an intact endocarp did not stain. This may be due to the presence of an impermeable inner membrane which surrounds the embryo. Many techniques for endocarp removal are possible. Splitting the stone along the prominent midrib or suture was an adequate method (Fig. 2D). This exposed the cotyledons and often left the embryonic axis intact, allowing accurate viability assessment. Seeds preconditioned by

moistening for 24 hours were easier to split than dry seeds. Split seed halves stained in 6 hours. Staining for 2 hours was inadequate while 12 hours produced overstain.

Antelope bitterbrush

Viability of the seed lot tested was 100% (Table 1). Lactophenol was not effective in clearing the seed coat. Embryos with intact seed coats did not stain. Seed coat removal was facilitated by soaking in water for 16 hours. Cutting the seed coat from end to end along the thin lateral edge followed by carefully removing the embryo was an effective method. Embryos stained satisfactorily in 4 hours. Staining for 2 hours was insufficient and 8 hours produced overstaining. Stained and unstained embryos are presented in Fig. 3A.

Skunkbush sumac

Viability of the seed lot tested was 91% (Table 1). Lactophenol was ineffective in clearing the seed coat (endocarp). Seeds with an intact endocarp did not stain. Soaking in water for several hours softened the endocarp and facilitated bisection. Cutting seeds laterally (Fig. 3C) yielded proximal seed halves with intact embryonic axes. Most seeds were extensively damaged during preparation making stain interpretation difficult. However, it was possible to identify dead embryos, unfilled seeds, and parasitism. Viability was based on identifying stained embryonic parts. Staining for 4 hours was optimum,

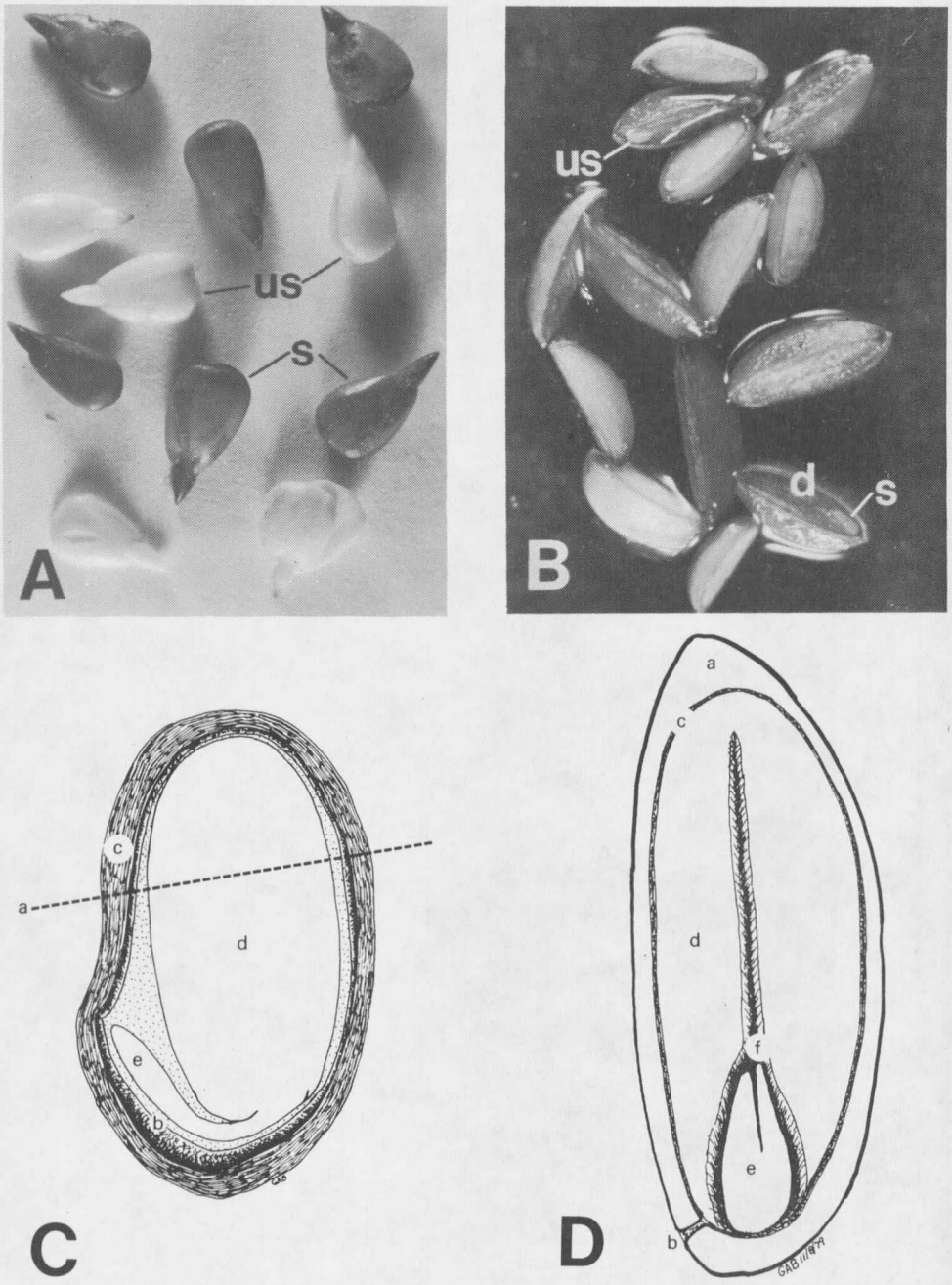


Figure 3. Tetrazolium stained (s) and unstained (us) embryos of: (A) antelope bitterbrush and (B) snowberry; note dark, abnormal staining of endosperm (d). (D) Schematic x-section of snowberry corresponding to B: endocarp (a), micropile (b), seed coat (c), endosperm (d), embryo (e) and central cavity (f). (C) Schematic x-section of skunkbush sumac: bisection line (a), seed coat (b), endocarp (c), cotyledon (d), and radicle (e).

two hours were insufficient, while 12 hours resulted in overstaining.

Snowberry

Viability of the seed lot tested was 68% (Table 1). Lactophenol did not clear the seed coat (endocarp). Seeds with an intact endocarp did not stain. Bisection was facilitated by pre-soaking in water for 24 hours. Longitudinal bisection directly through the micropore (Fig. 3 B & D), resulted in embryo exposure. Embryos are small when compared with endosperm material, and reported to be rudimentary (26).

The endosperm of seeds in the sample evaluated showed abnormal brick-red staining. The cause of this was unknown, but was postulated to be the result of extensive microbial activity. Embryos were stained after 4 hours. Two hours were found to produce inadequate staining. Overstaining was not observed.

SUGGESTED TETRAZOLIUM TEST PROCEDURES

Yarrow

Precondition by placing seed between moistened blotter paper overnight (Table 2). This will soften the seed coat for puncturing with a fine pointed needle. Puncture in the opaque area at the broad, distal end. Puncturing in the translucent margin (pericarp) (Fig. 1A), when viewed under a dissecting scope, will not be sufficient for proper staining. Place punctured seeds in 1% TZ for 4 hours. Interpret as a dicotyledonous seed (34).

Lewis flax

Precondition by soaking seed in water overnight (Table 2). After soaking place on blotter paper and use the side of a curved probe to apply pressure to the rounded, distal end of the seed (Fig. 1B). Press toward the pointed, radicle end to force the embryo from the seed coat. Stain in 1% TZ for 4 hours. Interpret as a dicotyledonous seed (34). The seed secretes a gelatinous substance when moistened, making it somewhat difficult to manipulate.

Prairie coneflower

Precondition seed by placing between moist blotter paper overnight (Table 2). The seed coat may be removed after preconditioning by applying pressure to the broad, distal end of the seed. Squeeze toward

Table 2. Suggested tetrazolium viability procedures for eight shrubs and three forbs.

Species	Precondition	Preparation	TZ conc. %	Stain time (hrs.) @ room temp.	Remarks
Yarrow	Moist blotters overnight	Puncture seedcoat	1	4	
Lewis flax	Soak in water overnight	Remove seedcoat	1	4	Gelatinous seedcoat
Prairie coneflower	Moist blotters overnight	Remove seedcoat	1	4	
Serviceberry	Soak in water overnight	Remove seedcoat	1	4	Gelatinous seedcoat
Indigobush	None	Chip seedcoat	1	16-20	Remove pod clear with lactophenol
Big sagebrush	None	None	1	16	Clear 2 hours with lactophenol
Winterfat	Moist blotters overnight	Puncture	0.1	4	Remove bracts initially
Chokecherry	Soak in water 24 hours	Split endocarp	1	4-6	
Antelope bitterbrush	Soak in water overnight	Remove seedcoat	1	4	
Skunkbush sumac	Soak in water 24 hours	Bisect laterally	0.1	4	
Snowberry	Soak in water 24 hours	Bisect longitudinally	1	4	

the pointed end of the seed which will force the embryo, radicle first, from the seed coat (Fig. 1C). Immediately place the embryo in 1% TZ and stain for 4 hours. Interpret as a dicotyledonous seed (34) (Fig. 1D).

This method of preparation will damage the terminal cotyledon end and allow more rapid TZ uptake through the impermeable membrane surrounding the embryo. The damage to the cotyledon must be ignored when interpreting the TZ staining.

Serviceberry

Precondition by soaking seed in water overnight (Table 2). When bisecting place on moist blotter paper and orient with forceps so the crease faces upward. This may be awkward due to a secreted gelatinous substance which causes the seed to stick to the forceps. Bisect the seed coat longitudinally starting at the pore and cut along the crease. Remove the embryo and place in 1% TZ for 4 hours. A short additional soak, followed by rubbing between fingers will affect removal of the inner membrane if it did not become detached during dissection. Seeds will not stain with the membrane intact. Staining should be interpreted as a dicotyledonous seed (34) (Fig. 2A).

Indigobush

The seed pod must be removed by hand or with a belt thresher before testing (Table 2). This species is a member of the legume

family and may contain a high percentage of hard seed. Precondition by chipping the rounded cotyledon end of the seed coat with a razor or scalpel to permit TZ imbibition (Fig. 2B). Place chipped seeds in 1% TZ overnight or longer if necessary. Stained seeds should be cleared with lactophenol for approximately 1 hour. Clearing will permit interpretation of staining with the aid of magnification, top illumination, and transmitted light.

Big sagebrush

Separate the small, dark colored seed from the usually chaffy material and place directly in a 1% TZ solution with no preconditioning (Table 2). Stain 16 hours or overnight and clear for 2 hours in lactophenol.

Interpret using magnification and strong illumination from below the seed. Stained seeds will show a uniform red color, whereas unstained seeds will remain yellow. Radicle tips may sometimes be unstained, but these seeds were shown to germinate normally.

Winterfat

The outer covering of the utricle (bracts) must be removed by hand or with a thresher before initiating the TZ test (Table 2). Precondition seeds between moist blotter paper overnight. Seeds (nutlets) are hydrophobic and do not imbibe moisture readily if placed directly into water.

The seed is composed of an embryo embedded in a fibrous matrix which is the seed coat and endosperm. After the presoak, it is possible to puncture (Fig. 2C) and partially remove this matrix before placing seeds in a 0.1% TZ solution. Staining proceeds rapidly and should be complete within 4 hours.

Chokecherry

Precondition by soaking in water for 24 hours (Table 2). The seeds possess a stony endocarp which may be split by exerting pressure with a scalpel or razor blade along the midrib or suture which runs longitudinally from the attachment scar to the radicle containing end of the seed. This procedure will separate the cotyledons, exposing the inner surface of each, and the embryonic axis (Fig. 2D). Place one seed half with radicle/epicotyl still attached in 1% TZ and stain 4-6 hours. Some damage will undoubtedly occur during preparation. Assessment of damage caused by preparation may be difficult to separate from natural defects. Care must be taken not to term a seed abnormal due to mechanical injury during preparation.

Antelope bitterbrush

Precondition seed by soaking in water overnight (Table 2). When dissecting, place seed on moist blotter paper, and hold the seed with forceps to keep the thin edge of the seed upright. Bisect the seed coat longitudinally. Carefully remove the embryo from the seed coat

and immediately place in 1% TZ for 4 hours. The seed should stain completely and is easily interpreted as a dicotyledonous seed (34) (Fig. 3A).

Skunkbush sumac

Seeds possess an extremely hard endocarp. Soak in water for 24 hours. Place seed on moist blotter paper, hold with forceps and bisect laterally (Fig. 3C). The embryonic axis which is the broader seed end should be placed in TZ. Care should be taken not to injure this portion when bisecting. Often seed parts will fly or be damaged during bisection. Place the half containing the axis in 0.1% TZ and stain for 4 hours. The damage that occurs during preparation makes interpretation difficult. It is difficult to identify seed abnormalities, however, information on seed fill, parasitism, and viability can be determined.

Snowberry

Precondition by soaking seed in water for 24 hours (Table 2). Remove and place on blotter paper, flattened side down. Bisec longitudinally, directly through the micropore. Place one half of each seed in 1% TZ and stain for 4 hours. Embryonic tissue (relatively small compared to endosperm) is located near the micropore (Fig. 3B & D). Endosperm may show abnormal staining.

CHAPTER II: IMPROVING GERMINATION OF SKUNKBUSH SUMAC AND SERVICEBERRY SEED

INTRODUCTION

Rhus trilobata Nutt. (skunkbush sumac) and Amelanchier alnifolia Nutt. (serviceberry) are native shrubs extensively distributed in the western United States. Skunkbush sumac is a small shrub often forming large thickets. It is valuable as wildlife cover, a characteristic which is important when restoring big game ranges even though palatability is relatively low (77). Skunkbush sumac is unusually persistent, will endure extreme drought, and is well suited for revegetating areas of eroded and depleted soils (93, 77).

Serviceberry grows as a large shrub or a small tree and is often interspersed with other shrubs. Because of its good palatability, wide distribution, and availability, it is recognized as one of the most important browse species for big game and livestock (93, 67, 77).

Both of these species are increasingly being used for range reclamation and restoration. Neither the Association of Official Seed Analysts (12) nor the International Seed Testing Association (60) makes recommendations for laboratory seed germination testing of these species. Seed of both shrubs exhibit varying degrees of dormancy that is normally overcome by lengthy stratification treatments (9, 10). Unfortunately, recommended stratification procedures for these species are extremely time consuming and are not practical for use in seed testing programs (42).

Germination of skunkbush sumac is reported to be inhibited by embryo dormancy and the presence of a hard, impervious seed coat (42, 10). Unscarified seeds do not germinate (13, 78) and seeds stratified for 3 months without seed coat removal also remain dormant (21). Heit (42) achieved maximum germination by scarifying seeds for 90 minutes in concentrated sulphuric acid followed by stratification for 1 month at 3 to 5 C. These seeds achieved 70% germination in 10 days when placed in an alternating 20-30 C temperature regime.

Embryo dormancy of serviceberry seeds is usually overcome by cold stratification (9). Previous research has shown that seeds stratified at 1 to 5 C from 3 to 20 months will germinate from 84 to 100% (78, 76, 41, 63, 65, 38). In other tests (39) seeds have germinated 3% after 3 months stratification. Scarification and stratification were necessary to produce a 4% emergence rate for seeds which were planted in soil (74). Acid scarification was of benefit to the seed germination of A. laevis Wieg. It was postulated that this treatment was responsible for the removal of germination inhibitors (44).

Seed coat scarification increases germination of hard seeds of Fabacae (62). Growth promoters such as the gibberellins, cytokinins, and thiourea substitute for the stratification or light requirement in some species (62). The objective of this study was to facilitate rapid laboratory germination testing by reducing the time required to achieve germination equal to tetrazolium viability for skunkbush sumac and

serviceberry. Various dormancy breaking treatments to eliminate the need for the lengthy stratification requirement were evaluated.

MATERIALS AND METHODS

General Procedure

Seed samples of skunkbush sumac and serviceberry were provided by the Western Energy Company, Colstrip, Montana, from lots being used for land revegetation. Unless otherwise specified, germination of both species was conducted at an alternating 20-30 C[1] (9, 10) with 16 hours of light provided by cool white flourescent tubes (standard conditions). Seeds were placed in plastic boxes on blotter paper moistened initially with various chemicals and/or plant hormones, and subsequently with tap water. Treatments were applied to 3 replicates of 50 seeds per box and germination counts were made weekly for 3 weeks. Stratification procedures were the same as for germination except temperatures were maintained at 1 to 3 C without light, and for durations of 3 and 5 months. Viability was determined by a standard tetrazolium (TZ) test using published procedures (100). Microbial activity was reduced by lightly dusting seeds with tetrachloro-para-benzoquinone (Spurgon)[2]. Seeds

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[1]Temperatures were maintained at 20 C for 16 hours and 30 C for 8 hours. The light treatment was applied continuously for 8 hours of 30 C and 4 hours of 20 C preceeding and subsequent to the 30 C treatment for a total of 16 hours.

[2]Mention of a trademark or proprietory product does not constitute a guarantee or warranty of the product by the Montana Agricultural Experiment Station and does not imply its approval to the exclusion of other products that may also be suitable.

were scarified with 96% H_2SO_4, stirring continuously, then rinsing for 30 minutes with flowing tap water. $Gibberellin_3$ (GA), benzyladenine (BA), thiourea (TU), and potassium nitrate (KNO_3) were applied aqueously to the germination blotters as the initial moistening agent. This required approximately 25 ml per box.

Main effects were tested using factorial treatment combinations in a completely randomized design. Factorial experiments are typically analyzed by analysis of variance techniques which provide tests of treatment effects after separation into main effects and interactions. This kind of analysis was not done because of the nature of the response variable. Rates of germination varied between 0 and 35%, with a very frequent occurrence of 0. Thus, germination counts per replication were frequently 0 and at most 20 seeds. Such counts likely follow a Poisson probability distribution for which the variance is equal to the mean. The requirement of a normally distributed response with homogeneous variance for the usual analysis of variance was clearly not met. A square root transformation of the response followed by the usual analysis of variance procedure would have been acceptable if the zero germination occurrences had been less frequent.

Actual analysis employed two approximately correct techniques. An ordinary Duncan's Multiple Range Test was employed initially on all treatment combination mean counts after completing a one-way analysis of variance utilizing treatment combination cells not containing

exclusively zero counts. The chi-square calculation proposed by Snedecor and Cochran (86) for testing equal expectation for Poisson variables was also used to test various main effects as well as equality of two or more treatment combinations. Such a test can correctly accommodate zero responses. Interactions can be tested by a two-way chi-square contingency table.

Skunkbush sumac

Seeds were scarified for 75 minutes unless otherwise specified. Initially, scarified and nonscarified seeds were germinated with H_2O, 400 ppm GA or 0.2% KNO_3. To clarify responses to GA and KNO_3, scarified seeds were soaked 0, 8, 16, or 24 hours in GA or H_2O and placed in standard conditions. KNO_3 was tested as a moistening agent of scarified and nonscarified seeds at concentrations of 0, 0.1, 0.2, or 0.4%. The effects of scarification for 0, 60, 75, or 90 minutes combined with soaking for 0 or 24 hours in 0.2% KNO_3 or H_2O were evaluated by planting treated and subsequently air dried seeds in flats of soil in the greenhouse. Speed of emergence indices (SE) were determined using the formula

$$SE = X_1 + \frac{X_2}{2} + \frac{X_3}{3} + \ldots + \frac{X_n}{n}$$

where X_n = the number of seeds emerging on day n. Means are the average of 4 replicates totaling 200 seeds per treatment, planted in a randomized block design.

Serviceberry

Seeds were scarified 30 minutes unless otherwise specified. Excised embryos (100) were surface sterilized in 0.3% hydrogen peroxide (HP), aseptically placed in sterile petri dishes, and maintained under standard germination conditions (9). Acid scarification was evaluated by treating seeds for 0, 10, 30, 45, or 60 minutes and germinating with H_2O or a mixture of 5 ppm BA and 50 mM TU. Dormancy breaking effects of GA were tested by germinating scarified seeds with 0, 250, 500, or 1,000 ppm solutions as initial moistening agents for the media. To test for the presence of water soluble inhibitors, scarified seeds were held under cold running tap water for 24 hours before being placed in standard conditions with 0, 200, 400, or 800 ppm GA. The effect of O_2 enrichment was tested using the hydrogen peroxide barley germination method (27). Scarified and unscarified seeds were placed in 125 ml Ehrlenmeyer flasks and covered with 0.3% HP or distilled water (DH_2O). The stoppered flasks were kept in standard conditions and solutions were changed every 24 hours for 3 weeks. Cold soaking with aeration was tested in the manner described by Barnett (6). Scarified seeds placed in 125 ml flasks were covered with 100 ml DH_2O and kept at 1 to 3 C (cold) or in an alternating 20-30 C germinator (warm). Various degrees of aeration were obtained by changing the DH_2O daily or by continuously bubbling air through the solution in the flasks. Seeds were transferred to germination boxes at weekly intervals for 3 weeks and placed

under standard conditions. Various concentrations of BA and TU were tested separately and in combination as initial moistening agents for blotters. Scarified seeds were germinated either with 0, 1, 10, or 100 mM TU or with 0, 10, 50, or 100 ppm BA. A third experiment utilized mixtures of BA and TU in all combinations of the levels previously tested. Finally, mixtures of 0, 100, 200, or 400 mM TU and 0, 100, 200, or 400 ppm BA in all possible combinations were evaluated. Statistical analysis was done for the square root of the count of seeds germinating per replication. Such counts tend to have a Poisson distribution when the proportion germinating is small. Standard references (86) suggest use of the square root transformation to bring about homogeneity of error. This transformation also brought about an improvement in fit and increased simplicity of a polynomial model. The effects of seed size on dormancy were evaluated by using 5/64 x 3/4 inch slotted and 8/64 inch triangular screens to separate scarified seeds into 3 size classes. Four 100 seed samples per size class were germinated with a mixture of 100 mM TU and 100 ppm BA.

RESULTS AND DISCUSSION

Skunkbush sumac

The TZ viability of the seed lot tested was 91%. Preliminary tests supported other reports (42) that acid scarification for approximately 75 minutes is necessary to elicit a significant germination response. It also appeared that combining scarification with GA or KNO_3 could possibly improve germination over that of scarification alone (Table 3), although the maximum response of 25% was far below viability. However, follow-up tests failed to confirm the promotive effects of KNO_3 (Table 4) and GA.

Finally, an emergence study was conducted in the greenhouse. Treatments of 0, 60, 75, or 90 minutes of scarification were combined factorially with soaking treatments of 0 or 24 hours in 0.2% KNO_3 or DH_2O. Speed of emergence (SE) (Table 5) for unscarified seeds was slow as compared to scarified seeds with either soaking treatment. Scarified but unsoaked seeds were intermediate in speed of emergence. Total emergence counts were made after 3 weeks (Table 6). Overall emergence of unscarified seeds was poor. Regardless of soaking treatment, all scarified seeds emerged similarly and better than unscarified seeds. The scarified but unsoaked seeds, although slower to emerge, equalled the total emergence of scarified and soaked treatments.

Table 3. Comparison* of mean percentage germination of skunkbush sumac as affected by acid scarification, GA_3 and KNO_3.

Scarification** (min)	H_2O	GA_3 (400 ppm)	KNO_3 (0.2%)
0	1b	1b	0b
75	15a	22a	25a

*Means followed by the same letter are not statistically different at P = 0.05.

**Main effect of 0 vs 75 minutes scarification is significantly different ($P \leq 0.01$).

Table 4. Comparison* of mean percentage germination of skunkbush sumac as affected by acid scarification and four levels of KNO_3.

Scarification** (min)	Percent KNO_3***			
	0	0.1	0.2	0.4
0	1b	1b	1b	4b
75	31a	29a	31a	35a

*Means followed by the same letter are not statistically different at P = 0.05.

**Main effect of 0 vs 75 minutes scarification is significantly different ($P \leq 0.01$).

***Main effect of KNO_3 levels is not significant ($P \leq 0.01$).

Table 5. Effect of acid scarification for 0, 60, 75, or 90 minutes and 0 or 24 hour soaks in H_2O or 0.2% KNO_3 on speed of emergence* (SE)[a] of skunkbush sumac after 17 days in the greenhouse

	Scarification time in minutes			
Soak time	0	60	75	90
0	.2c	4.7b	6.5b	5.9b
24 hr H_2O	.07c	11.4a	10.0a	9.7a
24 hr KNO_3	.02c	9.7a	10.9a	10.0a

*Means followed by the same letter are not statistically different at P = 0.05.

[a] $SE = X_1 + \frac{X_2}{2} + \frac{X_3}{3} + \ldots + \frac{X_n}{n}$, where X_n is the number of seedlings emerging on day n.

Table 6. Effect of acid scarification for 0, 60, 75, or 90 minutes and 0 or 24 hour soaks in H_2O or 0.2% KNO_3 on total emergence percent* of skunkbush sumac after 3 weeks in the greenhouse.

	Scarification time in minutes			
Soak time	0	60	75	90
0	2c	37b	48ab	46ab
24 hr H_2O	1c	49ab	55a	52ab
24 hr KNO_3	1c	47ab	54a	56a

*Means followed by the same letter are not statistically different at P = 0.05.

These results support the findings of Heit (42) who reported that scarification is beneficial to the germination of skunkbush sumac. Acid scarification of seeds may remove coat-imposed dormancy by changing permeability to water or gases, sensitivity to light, removal of mechanical restriction or possibly by destroying germination inhibitor substances (62). Scarification did not completely relieve dormancy as indicated by the difference between actual germination and TZ viability. GA and KNO_3, substances known to break embryo dormancy in some species by substituting for a stratification requirement (62), were ineffective at the levels tested. Efforts to isolate seed coat effects by embryo excision were unsuccessful due to the extreme hardness of the endocarp. How much of the residual dormancy can be attributed to endogenous mechanisms or to the inefficiencies of the scarification method cannot be assessed from these experiments. Efforts directed at relieving endogenous dormancy by the use of KNO_3 or GA were unsuccessful.

Serviceberry

The TZ viability of the seed lot tested was 84%. Stratification of unscarified dormant seeds for 3 or 5 months produced 34 and 73% germination, respectively. Untreated seeds and excised embryos did not germinate. Preliminary tests showed a slight germination stimulation of dormant seeds due to combining scarification and a

benzyladenine/thiourea (BA/TU) mixture. Scarification times of 10, 30, 45, or 60 minutes, were equally effective (Table 7); however, the longer scarification times were observed to produce physical damage to embryos. As a result, 30 minutes of scarification was used in all subsequent experiments and although beneficial to germination, this treatment reduced the 84% viability of nonscarified seeds to an average of 60% (Table 10).

Gibberellins substitute for the stratification or light requirement in the seeds of some species (62). Levels of 250, 500, or 1,000 ppm GA did not promote the germination of scarified unstratified seeds.

Table 7. Comparison* of mean percentage germination of serviceberry as affected by acid scarification and a benzyladenine/thiourea (BA/TU) mixture.

Moistening agent	Scarification (min)				
	0	10	30	45	60
H_2O	0	0	0	0	1
BA/TU (5 ppm/50 mM)	1b	2ab	5ab	12a	8ab

*Means followed by the same letter are not statistically different at P = 0.05.

Scarified, tap-water leached or unleached seeds were germinated with 200, 400, or 800 ppm GA solutions as moistening agents to test the promoter/inhibitor hypothesis of seed germination (1). Germination was not improved over the controls by leaching or GA (Table 8).

Forty-three percent of the embryos from nonscarified and 65% of the embryos from scarified seeds were released from their seed coats after a continuous 3-week soak in 0.3% HP. No additional growth was observed once seeds were transferred to germination boxes.

Aerated and cold water soaks have been shown to substitute for or reduce the stratification requirement in dormant seeds of southern pine (Pinus palustris Mill.) (6) and sugar maple (Acer saccharum Marsh.) (50). Serviceberry seeds did not germinate during or subsequent to cold and warm water aeration treatments.

Table 8. Comparison* of mean percentage germination of serviceberry as affected by leaching for 24 hours and GA_3.[a]

Leach time hours	GA_3 moisture in ppm			
	0	200	400	800
0	1	4	4	1
24	0	5	7	1

*Means are not statistically different at P = 0.05.

[a]Seeds of all treatments were acid scarified for 30 minutes.

Experiments were conducted to determine the optimum concentrations of BA and TU which would improve germination. Initially, TU and BA were tested separately at concentration ranges found stimulatory to the germination of dormant seeds of other species (62, 24). BA or TU did not individually stimulate germination at the concentrations tested. It had been previously determined that a mixture of 5 ppm BA and 50 mM TU produced minimal germination of scarified seeds (Table 7), therefore two experiments were designed to test the effect of combining various concentrations of BA and TU. Maximum germination responses were achieved when 100 mM TU was combined with either 100, 200, or 400 ppm BA (Figs. 4 and 5). A model using the original count data as the dependent variable showed a BA linear by TU linear form of interaction to be present. When the dependent variable was transformed to the square root of the count, the interaction was accommodated by a simpler model which contained only linear and quadratic terms for each main effect (Fig. 6). Although the regression model is highly significant (Table 9) and accounts for 83% of the variation in germination ($R^2 = 0.83$) there is less confidence in the predictive ability of the model when concentrations of TU exceed 100 mM due to fewer germination data points. A 95% confidence statement for the maximum predicted germination response of 20% is 296 $\pm$ 57 ppm BA and 100 $\pm$ 4 mM TU.

Scarified seeds were germinated after being separated into large (1.359g/100), medium (.9530 g/100), small (.6657 g/100), and unsized

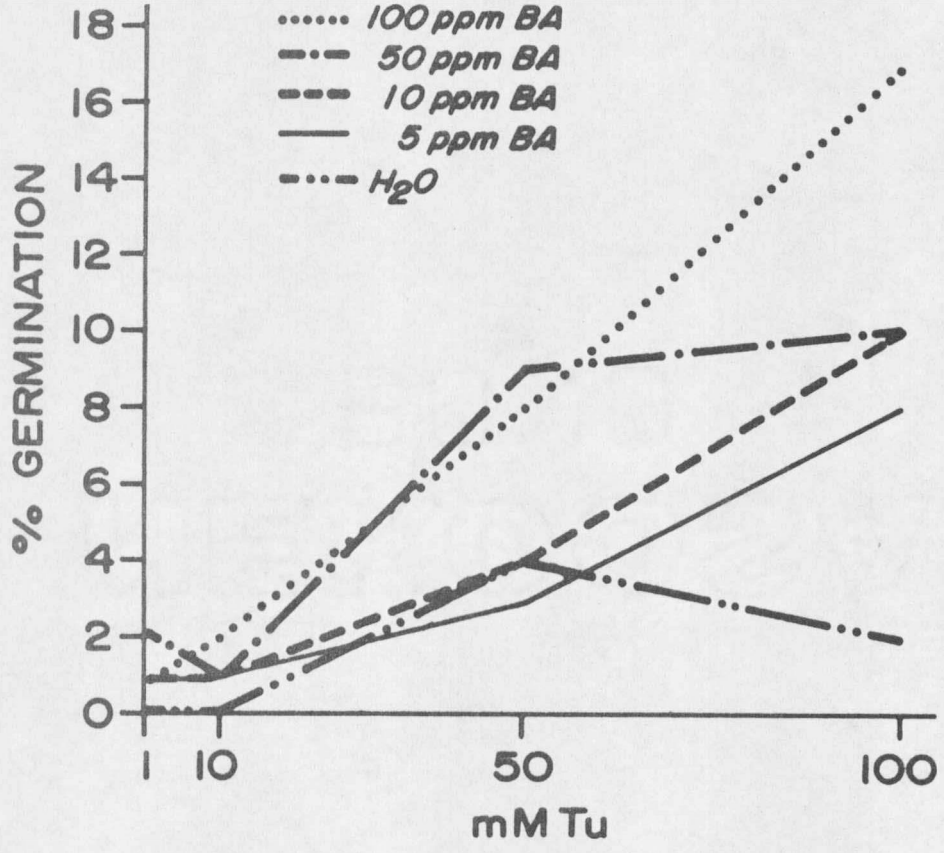

Figure 4. Effect of low concentration BA/TU mixtures on the germination of serviceberry.

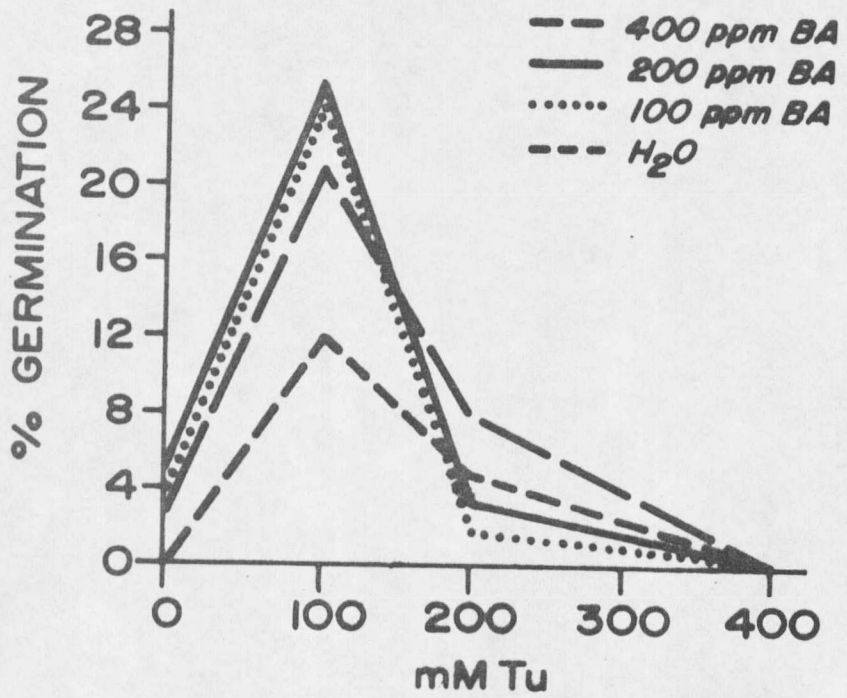

Figure 5. Effect of high concentration BA/TU mixtures on the germination of serviceberry.

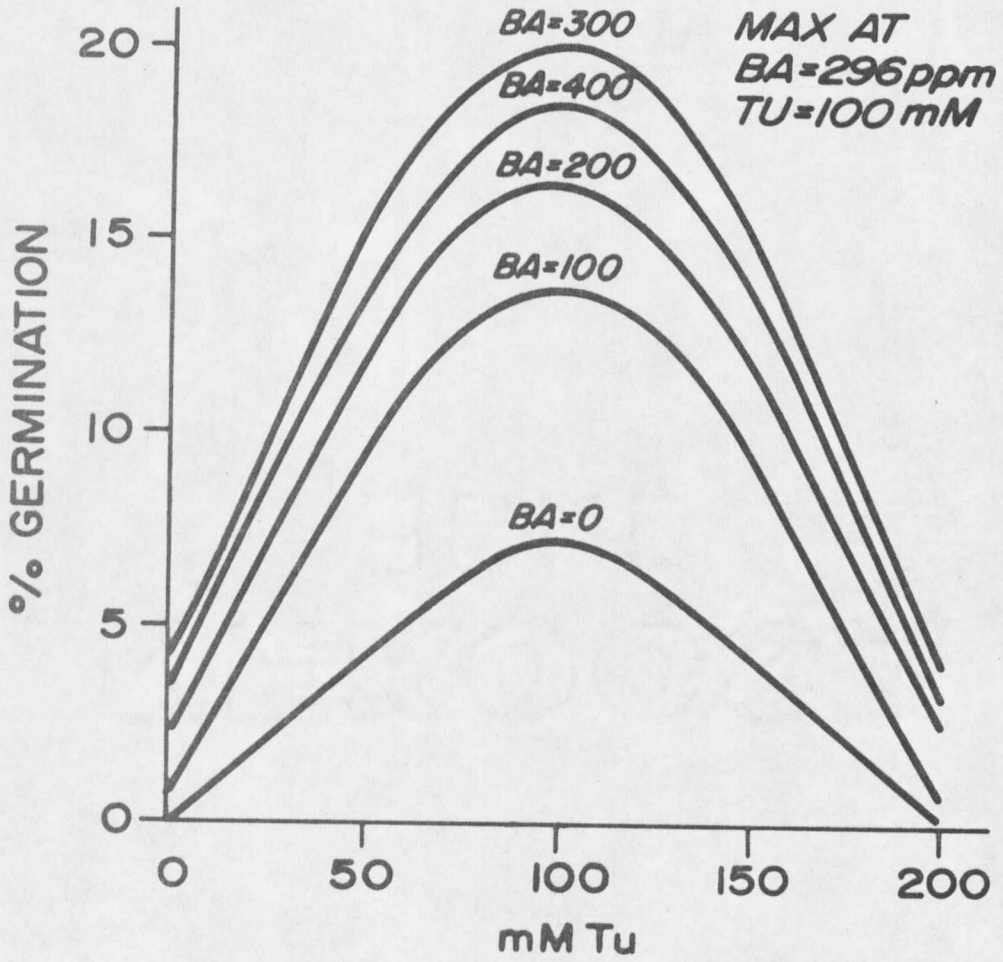

Figure 6. Effect of BA/TU mixtures at various concentrations on the germination of serviceberry as predicted by regression analysis. Refer to Table 7 for regression model.

Table 9. Analysis of variance for the germination response of serviceberry to mixtures of BA and TU.[a]

Source	Degrees of freedom	Mean square	F
Regression model[b]	4	19.73	29.44**
Lack of fit of regression model	32	0.50	0.75 NS
Total for treatments in two experiments	36	2.64	3.93**
Experimental error	74	0.67	-
Total	110	-	-

[a]Data are square root of count of germinated seeds per replicate of 50 seeds.

[b]Percentage germination =

$$2 \times \left[\begin{array}{l} 0.159 + (8.65 \times 10^{-3})BA - (1.46 \times 10^{-5})BA^2 \\ + (3.48 \times 10^{-2})TU - (1.75 \times 10^{-4})TU^2 \end{array} \right]^2$$

(1.043 g/100)size classes to determine whether dormancy could be attributed to some phenological characteristic such as stage of maturity (Table 10). TZ viability was determined for each size class in order to assess the relative dormancy of the class. Viabilities between size classes did not vary greatly; however, the small size class contained a large percentage of unfilled seeds. Germinations were compared using a germination index (GI = germination ÷ viability x 100) which eliminates the nonviable seeds from the comparison. The medium seed GI of

Table 10. Tetrazolium (TZ) viability and germination* of serviceberry as affected by seed size.

Size class	Average weight g/100 seeds	% of sample by weight	TZ viability[a] class**	% empty seeds of size class	% germ[b] of size class**	GI[c] of size class	GI of sample
Large	1.359	40	63a	0	22b	35b	14
Medium	0.953	41	60a	8	38a	63a	26
Small	0.666	19	56a	19	8c	14c	3
Unsized	1.043	100	60[d]	7[d]	26b	43b	43[d]

*Means of a column followed by the same letter are not statistically different using Duncan's MR test at P = 0.05.

**Germination percents and TZ viabilities are significantly different within size classes by chi-square analysis at P = 0.05.

[a]TZ viability determinations were made on seeds scarified for 30 minutes.

[b]Seeds of all treatments were scarified for 30 minutes then germinated with 100 ppm BA/100 mM TU mixture.

[c]GI (germination index) = germination ÷ viability, to yield percent germination of viable seeds.

[d]Calculated data.

63% was significantly greater than the 36% and 14% GI for large and small seeds.

Stratified but unscarified seeds germinated normally, while excised embryos of unstratified seeds do not. Scarification plus the addition of a BA/TU mixture to the germination medium was found to have a pronounced promotive effect on germination. Scarification or the BA/TU mixture applied individually were ineffective. Leaching, soaking, and GA had no effect on promoting germination. Scarification appears to induce increased seed coat permeability to the BA/TU mixture which stimulates embryo growth. TU and BA have been shown to promote the germination of other dormant species (62, 24). Esashi, et al. (24) showed that of the growth and germination promoting substances tested, TU and BA used separately were most effective in stimulating the axes and cotyledonary growth of dormant cocklebur (Xanthium pennsylvanicum Wallr.) embryos. Concentrations of 100 mM TU or approximately 100 ppm BA (the highest tested) were found to be optimum for germination of whole dormant seeds. This correlates with our results for serviceberry.

Erez (22) reported that in the absence of purine cytokinins, concentrations of 0.1 to 1 mM TU were optimum for promoting growth in kinetin requiring callus tissues. Combining TU with zeatin, kinetin or BA produced synergistic (more than additive) effects on the growth response of soybean callus. Concentrations of 0.2 to 1 ppm BA combined

with .01 to 1 mM TU were optimum. These concentrations are of considerably lower magnitude than found to be optimum for serviceberry; however, it has been shown (62) that TU stimulates germination when the internal concentration is relatively low. The growth stimulating effect of TU is postulated by Erez (22) to be a result of an enhancing effect on cell division and not enlargement. Zeatin affected cell division, and the interaction between TU and purine cytokinins is suggested to be due to differences in the modes of action of these two compounds on cell division.

Eashi, et al. (24) report that TU stimulates growth of shoot axes, whereas BA has a greater affect on growth of cotyledons. This cytokinin stimulation of cotyledon expansion is primarily a function of cell enlargement not cell division as in other species (58).

Evidence of this nature leads us to suggest that the interaction of BA and TU on the promotion of germination in dormant serviceberry seeds may function in one or more ways. TU may preferentially promote cell division with BA promoting increases in cell size. Additionally, or alternatively as for Xanthium, TU may stimulate axial growth while BA promotes cotyledon expansion; or, as postulated by Erez (22), interaction may be due to differences in mode of action on cell division.

The occurrence of empty seeds associated with smaller seed size may have practical significance for the seed producer and processor.

Seed quality could easily be improved by adjusting cleaning equipment to discard the small and light seeds. Germination of the entire lot would also be improved since the small seed size class produced significantly lower germination than either large or medium classes. Dormancy may be reduced by selecting for seeds in the medium weight range.

APPENDIX

Appendix Table 1. Common and scientific names of plants referred to in the literature review.

Common Name	Scientific Name
Antelope bitterbrush	*Purshia tridentata* (Pursh) DC.
Apple	*Malus* spp. Mill.
Ash	*Fraxinus* spp. L.
Beech	*Fagus sylvatica* L.
Buttercup	*Ranunculus* spp. L.
Clover, subterranian	*Trifolium subterraneum* L.
Clover, white sweet	*Melilotus albus* Desr.
Cocklebur	*Xanthium pennsylvanicum* Wallr.
Cotton	*Gossypium hirsutum* (Cult.)
Hazel	*Corylus avellana* L.
Holly	*Ilex opaca* Ait.
Legume family	*Fabaceae* Reichenb.
Lettuce	*Lactuca sativa* L.
Lilac	*Syringia* spp. L.
Maple, Norway	*Acer platanoides* L.
Maple, sugar	*Acer saccharum* Marsh.
Maple, sycamore	*Acer pseudoplatanus* L.
Oak, black	*Quercus nigra* L.
Oak, red	*Quercus rubra* L.
Oats, wild	*Avena fatua* L.
Orchid family	*Orchidaceae* Lindl.
Parsnip	*Heracleum sphondylium* L.
Peach	*Prunus persica* Batsch
Potato	*Solanum tuberosum* L.
Rose family	*Rosaceae* Juss.
Rose, field	*Rosa arvensis* Huds.
Rose, rugosa	*Rosa rugosa* Thunb.

Appendix Table 1 (continued)

Common Name	Scientific Name
Russian pigweed	*Axyris amaranthoides* L.
Smooth bromegrass	*Bromus inermis* Leyss.
Snowberry	*Symphoricarpos albus* (L.) Blake
Soybean	*Glycine max* (L.) Merr.
Sunflower	*Helianthus annuus* L.

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