AN ABSTRACT OF THE THESIS OF

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Title: THE EFFECT OF OSMOTIC MOISTURE STRESS DURING GERMINATION ON DOUGLAS-FIR (PSEUDOTSUGA MENZIESII)

Signature redacted for privacy.

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Commercially collected Douglas-fir seed from a moist coastal ecotype (LaPush, Washington) and a dry inland ecotype (Kaibab National Forest, Arizona) were subjected to osmotic stresses ranging from 0 to -8 atmospheres using Carbowax polyethylene glycol 6000 during imbibition and germination. To determine the effect of osmotic stress on the initiation and progression of germination, daily germination was recorded during a 25 day period. Results were compared on the basis of total germination, number of days to the first germinant, and a combined index, germination value.

To determine the effect of osmotic stress on growth and moisture uptake during germination, whole seed fresh weight, dry weight, and moisture content were obtained. To determine the effect on respiration and the respiratory quotient, measurements were made during germination using the direct method of Warburg. The total soluble protein content as estimated by Lowry's method and

isocitratase activity as estimated by a method proposed by Jacks and Alldridge were determined from the endosperm of four-day old germinants. In addition, seedling fresh and dry weight and radicle length were measured. To determine the effect of osmotic stress on soluble nucleotide and total RNA and DNA contents, samples were obtained from the embryos during the first 12 days of germination using a modified Schmidt-Thannhauser method. The results of all determinations were compared using factorial analyses of variance.

The results indicated that: 1) the start of germination was delayed and total germination reduced by osmotic stress; 2) rates of water uptake, oxygen uptake, and carbon dioxide evolution were reduced while the respiratory quotient was unaffected by osmotic stress; 3) endosperm total soluble protein content of four-day old germinants increased initially in the inland seed source and decreased in the coastal seed source with increasing osmotic stress; 4) enzyme synthesis in the endosperm as measured by isocitratase activity was reduced by osmotic stress; 5) nucleotide and nucleic acid contents of the embryo were reduced by osmotic stress; 6) growth as measured by radicle length of four-day old seedlings was reduced by osmotic stress; and 7) the differences induced by osmotic stress tended to increase with time.

The inland source generally had larger relative values than the coastal source for all factors except specific activity of isocitratase which decreased an average of 29 percent in the former and 54 percent

in the latter between 0 and -4 atmospheres. This difference in response was felt to be partially due to a combination of a larger initial seed and embryo size, a higher imbibitional water content under stress, and a greater ability to maintain the specific activity of key enzymes. The changes induced by osmotic stress in both sources were felt to be the result of either reduced reactivation of protein synthesis and/or respiration. These changes were induced by a decreased level of cell hydration after imbibition and carried over from the reactivation phase to the de novo synthesis phase of germination. The basis of resistance to moisture stress during germination appeared to be the ability to maintain the specific activity of key enzymes either by synthesis or by maintenance of the active conformation of the enzyme.

The Effect of Osmotic Moisture Stress During Germination on Douglas-fir (Pseudotsuga menziesii)

by

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INTRODUCTION

Undoubtedly one of the most critical stages in the life cycle of a plant occurs during germination of the seed and initial establishment of the seedling. Environmental selection is most intense during this relatively short period of time and unadapted genotypes are rapidly eliminated. Successful establishment depends on the combination of a seed with a desirable genetic composition by chance occurring in a suitable microenvironment.

Environmental factors acting during this stage are very similar to those affecting the long-term growth and development of the plant. These include biotic factors and the physical factors of light, atmosphere, nutrients, temperature, and water which act as trigger mechanisms during stages of plant development by timely induction or suppression of physiological and biochemical processes. The effect and mode of action of an environmental stimulus depends on the genetically controlled program and stage of development of the individual plant, the presence or absence of preconditioning effects, the intensity of other stimuli, and the duration and intensity of the particular stimulus of interest. An adapted genome will progress through an orderly series of developmental sequences induced by the

proper timing and intensity of environmental stimuli.

The nature of the stimulus is only beginning to be understood for some of the environmental factors, and then most commonly for the seedling stages of the life cycle. Germination of Douglas-fir (Pseudotsuga menziesii (Mirb) Franco) is also influenced by these environmental factors. However, the process of germination is most strongly affected by light, temperature, and moisture supply. Germination of moist seed has been observed in Douglas-fir during stratification in the dark at temperatures near 4°C which may indicate that moisture supply is a major control factor in the initiation of germination. Moisture deficits early in the growing season are not uncommon throughout the natural range of Douglas-fir and may retard or prevent germination and establishment.

Water is required by the plant to maintain turgor, to act as a chemical reactant, to provide a medium where myriad cellular functions are performed separately but maintain a unified goal, and to serve in the distribution and balance of ions in the plants by diffusion, mass flow and active uptake. Moisture deficits in the soil may trigger alterations in physiological processes in the germinating seed by acting at one or more levels of control. These levels include: 1) phase change involving termination or initiation of developmental stages by cell division, 2) transcription involving changes in DNA repression and derepression which sends specific information by

means of a messenger RNA from the nucleus to the cytoplasm, 3) translation involving the decoding of m-RNA with the assistance of s-RNA, ribosomes, various cofactors, and substrates necessary for polypeptide formation occurring in the cytoplasm, and 4) assembly involving the organization of the polypeptides into functional conformations occurring in the cytoplasm and cellular organelles.

Little information is available regarding the level or levels at which moisture stress exerts its effect in disrupting the normal genetically programmed development and growth pattern. In fact the general physiological and biochemical responses to moisture stress are not yet fully defined in tree species. Therefore this study is designed to determine these general responses in Douglas-fir during germination. In addition, by comparing these responses between seed from one moist environment ecotype and one dry environment ecotype of Douglas-fir, the mechanism of drought resistance might be elucidated.

REVIEW OF LITERATURE

Normal Germination

A discussion of the germination process under ideal conditions is necessary to obtain an understanding of the modifications resulting from moisture stress. The review of literature on the germination process presented here is by no means complete, but represents a discussion of pertinent aspects for seed in general and for Douglas-fir in particular where available.

Definition and Stages of Germination

Germination is essentially the resumption of growth by a dormant embryo, with rupture of the seed coat, and subsequent development of the embryo into an independent seedling (70). The general physiological processes involved are: 1) absorption of water largely by imbibition, 2) beginning of cell enlargement and cell division, 3) increase in enzymes and enzyme activity leading to digestion of stored food, 4) translocation of metabolites to growing regions, 5) increase in respiration and assimilation, 6) increase in cell division and cell elongation, and 7) differentiation of cells into various tissues and organs characteristic of the seedling (50, 70, 134, 140).

The exact order of the various steps is not clear (70) nor is it possible to specify the order in which metabolic reactions are initiated during imbibition (50, 140). Further, it is not certain whether metabolic events observed are the result or the cause of germination (65). It is not presently possible to specify the limiting reaction which is affected by dormancy-breaking agents to initiate germination (140).

Imbibition

> Imbibition of water softens hard seed coats, and the swelling of the embryo as it imbibes water bursts the seed coat, permitting emergence of the radicle (70, p. 410).

Shull (115) disproved early assumptions that seed coats were permeable to most water-soluble substances and that cell walls and membranes of seed coats do not modify stimuli acting through them because they are dead by demonstrating selective permeability of the seed coats of several species. In later work, Shull (114) measured the internal imbibitional forces of seeds and concluded that water enters the seed in response to large internal forces. These forces owe their existence and magnitude to the fact that the organic material in the seed is colloidal in nature and therefore exhibits a surface force.

Goo (41) described three phases of water absorption for tree seeds: 1) rapid initial imbibition, 2) a period of slow uptake, and

3) a rapid uptake again as germination begins. This pattern has been confirmed for Jeffrey pine (129), bean (96), and sugar pine (122). By examination of the moisture uptake curves presented by Ching (24), this also may be demonstrated for Douglas-fir. Stanley (123) felt that for sugar pine, the level of endosperm water content attained at the beginning of the slow uptake period provided the proper intracellular environment essential to the functioning of the particulate enzyme units. Oota (96) found that the initial rapid rise probably represented uptake by diffusion, that the slow uptake or transient suspension phase was shortened with increasing temperature, and that the final elevation was of an aerobic nature.

Owen (99) demonstrated that water uptake in living and dead seeds was similar until germination effects are apparent. He concluded that water uptake in the early stages was probably due to physical rather than physiological processes and that germination causes an exponential increase in the rate of water uptake, perhaps due to starch hydrolysis. Oota (95) felt that cell division and probably nitrogen change are not involved in water uptake.

Shull (116) found that the velocity of water uptake at any given moment was approximately an inverse exponential function of the amount of water previously absorbed. This result was confirmed for peas (1, 116), corn (117, 125), cotton (125), and wheat (99).

The increased metabolic activity in seeds is a function of

protoplasmic hydration so that germination rates are frequently correlated with rate of water uptake (68). The rapid initial uptake facilitates the mobilization of reserve material and the utilization of this material for axis growth (50, 58, 70).

More water is required for germination than is taken up in initial imbibition (70, 129). Further, the degree of hydration necessary for germination varies among species (68, 125).

Cell Division and Elongation

It is not known whether cell elongation or cell division are primarily affected by seed dormancy-breaking factors (65). Cell elongation may procede or follow cell division or both may occur together (50, 65). Nevertheless, the first visible step of germination is cell elongation, and the synthesis of enzymes for the utilization of main seed reserves apparently is not essential to this step but closely follows it (134).

Germination is a growth process of the embryo of Douglas-fir and is accompanied by large increases in fresh and dry weight (24). This pattern is common to germination of most species and is aptly shown for corn by Ingle, Beevers, and Hageman (50). The deoxyribonucleic acid (DNA) content of embryo tissues increases during germination while that of the storage tissues decreases or remains nearly constant (21, 24, 50, 51). The change in DNA content of the

embryo parallels dry weight changes suggesting cell division is involved.

Catabolism and Translocation of Reserves

The metabolism of the cotyledons and endosperm of germinating seeds is dominated by the degradation and mobilization of reserve materials (140). Activity of the enzymes responsible for the breakdown of food reserves, whether proteinases, lipases, or amylases, increases during germination and then decreases as the level of reserves decreases (10, 26, 65, 86, 91, 140). Transport of the end products of this metabolism to the growing embryo has been demonstrated for many species (24, 50, 65, 86, 140).

Douglas-fir seed has a very small reserve of starch in the embryo which increases during germination apparently at the expense of reserve lipids (23, 24). Oligosaccharides probably are one type of reserve available for rapid utilization during stratification and seedling emergence (23). The lipids are the true reserves which are utilized during germination of Douglas-fir while glycerides are probably the major component providing energy from oxidative degradation and carbon fragments for synthesis of cellular material (23). In common with other species, there is a decrease in acid chain length and an increase in the proportion of saturated fatty acids in the fat but there is no accumulation of free fatty acids in Douglas-fir

as opposed to other species (10, 25). The trends in carbohydrate metabolism of Douglas-fir were shown to be consistent with the pattern of germinating fatty angiosperm seed (23).

Respiration

Some useful respiratory capacity exists in the dry seed but how it is maintained and then reactivated during or following imbibition is largely unknown (140). However, the functional integrity of the mitochondria in germinating pea cotyledons requires the presence of axis tissue (147). It is generally agreed that all of the enzymes necessary for respiration are present in the seed at the time of activation (86, 123, 140). Once the preformed respiratory capacity is fully activated, further increases presumably parallel cell division and embryo growth and are not unique to the germination process (140).

Positive correlations between the respiration rate of corn during imbibition and later stages of germination and seedling growth have been observed (145). Germination is an energy requiring process (65) and both oxygen uptake and carbon dioxide output increase during germination but not at the same rate, so that the respiratory quotient changes (22, 65, 86). In Douglas-fir (22) and castor bean (86, 93), the respiratory quotient decreases during germination.

In seeds, glycolysis, the tricarboxylic acid (TCA) cycle, and

the pentose phosphate pathway all seem to be operating but do not appear to be equally important at all stages (65). Changes in oxidative pathways and in terminal oxidases might be a distinctive feature of germination (65). Changes in the level of terminal oxidases and in the ability to utilize various substrates during germination have been observed (21, 123, 124).

The gas exchange pattern usually comprises three distinct stages: an initial increase characteristic of the hydration of protein; a plateau during which respiration remains more or less constant; and a subsequent rise, probably due to the respiration of the developing seedling (65, 61, 126). In eastern white pine (69) and Douglas-fir (22), the period of relatively constant respiration ends with rupture of the seed coat.

The pattern of respiration parallels that for moisture content (96, 97, 122). Opik and Simon (97) and Markowski and Korlakowska (85) found respiration to increase during imbibition of bean and winter wheat. Ragai and Loomis (107) demonstrated that respiration increased in an exponential manner with increasing moisture for corn.

Nucleic Acids and Protein Synthesis

In a review article on seed germination, Koller, et al. (65) conclude that nucleic acids are rapidly metabolized during germination and that increases in ribonucleic acid (RNA) and DNA are common.

Increases in embryo content of nucleic acids are well documented for corn (50), barley (73), and Douglas-fir (24). Nucleic acids, particularly RNA, tend to rise in storage tissue during the early stages of germination and then decline as shown for corn (50), peanut (21), pea (5), barley (73), and Douglas-fir (24).

Ching (24) noted that the sigmoid curve of RNA increase in the developing Douglas-fir seedling closely paralleled fresh and dry weight and DNA increases. This suggested the accumulation of ribosomal RNA and to a lesser extent messenger and soluble RNA.

The level of nucleic acids and nucleotides is frequently very low in all portions of the seed (50). Since nucleic acid reserve is often very small or non-existent, increases observed during germination are probably the result of de novo synthesis (50). Reductions in nucleotide or nucleic acid content in the storage tissues of barley (73), peanut (20), and Douglas-fir (24) may represent translocation to the growing axes of the embryo or may serve a metabolic function (83). The pattern of RNA metabolism in storage tissue is apparently related to the depletion of storage reserves with smaller seed showing an earlier decline (20).

Decreases in RNA content are frequently accompanied by increases in ribonuclease (RNase) activity (4, 21, 65, 73, 140). The nature of the RNase may change during germination and may lead to the formation of different products in the various tissues (4, 94).

Increases in enzyme activity during germination result from a combination of reactivation and de novo synthesis (10, 81, 86, 91, 141, 148). The protein synthesis system is present in the dry seed (81, 84, 86, 141, 142) and reconstruction of the ribosomal system may be an absolute requirement for the progress of germination (86). The state of seed hydration is a major factor in the inactivation of the ribosomal system during maturation and its resynthesis during germination (86). Increased activity of the ribosome system after imbibition is associated with the formation of polysomes (84, 86), and polysomes are the active components of amino acid incorporation into protein (84).

From studies of polysome formation, Marcus and Feeley (84) found that three explanations of the changes occurring during imbibition were consistent with the data: 1) hydration allows the combination of the messenger RNA and the ribosome to form a polysome, both being functional in the dry seed but spatially separated; 2) the messenger RNA is made available to the ribosome by synthesis or liberation of an inhibitor; or 3) the ribosome is made available to messenger RNA.

The activation or formation of messenger RNA during imbibition may be a general phenomenon in seed germination (81). A stable messenger RNA has been found in dry seeds of cotton (31, 142), hazel (144), and wheat (19). Imbibition may trigger the activation of

this conserved messenger RNA and thus induce enzyme synthesis permitting germination to proceed at a stage when the genome is not yet active (19, 31, 142).

In some instances, the synthesis of particular species of messenger RNA having DNA-like characteristics is required for embryo growth (52, 63). Auxin and gibberellic acid may cause the formation of specific messenger RNA species and thereby influence the synthesis of particular enzymes (87, 141). While axis control of enzyme formation has been demonstrated, it is not a univeral phenomenon in seed germination (82).

Fat Metabolism and Isocitratase

It has been shown that the major reserve material in Douglas-fir seed are lipids (23, 24) and the first step in conversion is the hydrolysis of triglycerides to free fatty acids and glycerol (140). This is accomplished by an increased neutral lipase activity (140) which increases fourfold during Douglas-fir germination in conjunction with a sevenfold increase of acid lipase (26). There is no accumulation of free fatty acids during germination indicating that they are rapidly converted to acetyl-coenzyme A via β-oxidation (23, 140).

The acetyl CoA is metabolized by the glyoxylate cycle which was first described by Kornberg and Krebs (67). The cycle accounts for

the net synthesis of four carbon dicarboxylic acids from acetate and provides oxaloacetate for operation of the TCA cycle (67). By operation of the glyoxylate cycle and reversal of glycolysis, all cellular constituents may be derived from two carbon precursors (55). Sinha and Cossins (118) demonstrated that acetate was utilized for the biosynthesis of carbohydrates in castor bean endosperm and could be used for amino acid biosynthesis in sunflower, pumpkin, linseed, and watermelon.

The reactions converting acetyl CoA to succinate via the glyoxylate cycle are localized in the glyoxysomes (12, 13) while conversion of succinate to malate probably occurs in the mitochondria (13).

The two key enzymes of the glyoxylate cycle are malate synthetase and isocitratase (isocitrate lyase). These two enzymes are particularly interesting for study of enzyme activation during germination. Both are either absent or present in extremely small amounts in dry seeds (140), and most evidence indicates that they arise by de novo synthesis at the time of the appearance of their enzymatic activities (40, 75, 86). In peanut, the presence of axis tissue is apparently required for the synthesis of isocitratase (40), but the mechanism for triggering synthesis is generally unknown (140).

Isocitratase is confined to tissues in which fats are actively being metabolized (7, 16, 118) and usually cannot be detected in

adjacent parts of the seedling proper (7). The enzyme activity rises during the early stages of germination and reaches a peak at about the time of most rapid fat breakdown and sucrose synthesis (7).

The enzyme catalyzes an aldol reaction which does not involve water nor thio-ester cleavage (119). The reaction catalyzed is:

$$\text{D(L) - Isocitrate} \xrightleftharpoons{Mg^{++},\ \text{R-SH}} \text{Glyoxylate + Succinate}$$

with a Michaelis constant (the substrate concentration required to yield half the maximum reaction velocity) of 4.5×10^{-4} M for microorganisms (119). Enzyme activity occurs over a wide pH range but is maximum at a pH of 8.0 to 8.5 (119). The nature of the carbon source utilized for growth influences isocitratase formation in microorganisms (66, 109). Firenznoli, et al. (35) found no correlation between enzyme activity in several conifers and the level of a particular fatty acid. They did observe that linoleic acid had a higher concentration than other fatty acids in all species studied. Ching (25) found a preferential utilization of linoleic acid in glycerides and an increase in linoleic and palmitic acids in phospholipids of Douglas-fir, but no attempt was made to relate this to enzyme activity.

The Effect of Moisture Stress

Studies on the effect of moisture stress during germination are not available for Douglas-fir. Further, some desirable information

for other species is unavailable. Thus, this review will be concerned with the observed effects on other species and, where information is lacking, will deal with stages other than germination.

Germination

Ayers (3) stated that seeds must overcome surface-force action of soil particles and osmotic-force action due to dissolved salts to obtain water from the soil. He observed that soil moisture stresses should be reflected in an increased emergence time, a decreased total germination, or both. Stresses less than one atmosphere probably do not influence imbibition and germination (60) but seeds become more sensitive to drought from imbibition onwards (45).

Progressive delay and reduction in total germination with increasing moisture stress have been shown for alfalfa (29, 110, 137, 149), barley (39), corn (2, 101, 102, 103), clover (39), fourwing saltbush (120), grain sorghum (32), medusahead (146), navy beans (103), peas (79, 103), radish (133), various range grass species (64, 77, 121), various vegetable crop species (28), and wheat (43, 98, 103). This effect has also been observed in the angiosperms eastern cottonwood (33), pin oak (8), and sweetgum (9) and the gymnosperms Pinus rigida (88), P. ponderosa (72), P. palustris (6), P. elliottii (6), P. densiflora (111), P. Thunbergii (111), Cryptomeria japonica (111), and Chamaecyparis obtusa (111). The results

observed may be more complex than moisture stress because most of the chemicals used to produce osmotic solutions may be taken up by the seed (80).

Species and variety differences in the ability to germinate under moisture stress are easily demonstrated (28, 29, 39, 43, 64, 77, 103, 120, 149) indicating that drought response is heritable. Seed coat effects are involved in alfalfa (110, 149) and differences in embryo soundness in corn (102). Owen (98) found that seeds exhibit reduced germinating capacity under conditions where germination is post-poned perhaps as a result of increased susceptibility to infection.

Imbibition

Manohar (79) showed that two varieties of peas germinated only when their embryos had absorbed a certain absolute amount of water which remained more or less constant per unit embryo length at all osmotic potentials. Satoo and Goo (111) found that the ability to germinate under increasing moisture stress paralleled the suction force of the seed.

Increasing moisture stress reduces the rate of water absorption and water content (6, 144). Delayed emergence further reduces the ability to absorb additional water as the osmotic value of the cells continuously drops as a result of pre-emergence respiration (39).

Cell Division and Elongation

Moisture stress usually reduces embryo growth (33, 43, 72, 101, 135, 136). Jansson (54) found a high positive correlation between seedling growth rate and moisture content in barley. Shoot growth of corn seedlings is affected to a greater extent than primary root growth as moisture stress increases (101). Root penetration, root dry weight, and cotyledon length of ponderosa pine seedlings decreased with increasing osmotic stress (72). Seedlings that germinated at low osmotic potentials grew poorly even when watered with solutions of high osmotic potential. Seedling growth of winter wheat varied inversely with moisture stress but not necessarily in a linear manner (43).

DNA synthesis may be reduced or stopped under moisture stress (14, 37, 61, 62) suggesting a cessation of cell division.

Metabolism

Stocker (127) proposed that plants responded to drought by an over-compensation of metabolism. Hydrolytic reactions dominate over synthetic reactions during dehydration (44, 90, 128). Drought-resistant plants may maintain synthetic reactions at a higher level than nonresistant plants (44). Synthesis of compounds of low molecular weight rather than high is intensified (132).

Polysaccharides may be hydrolyzed to more simple sugars, proteins broken down to amino acids, and starch may disappear without sugar accumulation (44, 48, 57). The conversion of dry matter from corn endosperm to embryo decreased with increasing soil moisture tension (136).

Increasing moisture stress usually reduces oxygen uptake and carbon dioxide evolution of seeds and seedlings (17, 48, 59, 132, 135, 136). In corn, growth tends to decrease faster than respiration (135, 136). Low water potentials did not directly inhibit respiratory pathways in Chlorella (46) but in many plants the contribution of the pentose phosphate pathway increases while the glycolytic pathway decreases (104). The ratio of the two depends on the degree of tissue hydration and the increase of dehydrogenase activity may be a process of enzyme synthesis induced by drought (104). Dehydration may inhibit the accumulation and transformation of energy in plants by increasing protoplasmic viscosity (44).

Dehydration in seedlings and older tissues may lead to changes in amino acid content. Proline frequently accumulates in wilting leaves (71, 100, 112) and this may be accompanied by an increase in phenylalanine (100), or valine and arginine (112). In some instances, alanine may increase (132). Savitskaya (112) feels that plants respond to unfavorable conditions by forming substances that detoxify ammonia such as amides and organic acids.

Nucleic Acids and Enzyme Synthesis

Moisture stress may markedly affect the content and nature of RNA in germinating seeds and older plants. Changes in the ratio of base pairs as a result of dehydration vary by species and age of plant. In germinating corn seedlings, moisture stress increased RNA, depressed adenosine triphosphate (ATP) synthesis, promoted production of guanosine and uridine triphosphates, and resulted in a high ratio of guanosine plus uridine to adenine plus cytosine in the RNA (143). In sugar beet leaves, the RNA had a purine to pyrimidine base ratio greater than one, however the ratio changed with age (113). During moisture stress the soluble RNA increased while the other fractions, particularly ribosomal RNA, decreased (113). In drought tolerant olive, the proportion of guanosine and cytosine in the RNA of leaves increased under moisture stress (62). Net accumulation of RNA ceases in leaves of tomato seedlings as shown by Gates and Bonner (37) and Kessler (61). Presowing treatment of sunflower seeds with aluminum and cobalt nitrate increased both RNA and DNA contents while lowering RNase activity under stress (11).

Observations of changes in RNA base composition have led West (143) and Shah and Loomis (113) to propose that changes in growth caused by moisture stress may be related to changes in RNA and protein metabolism. Gates and Bonner (37) felt that the block to

RNA synthesis was a result of increased destruction of RNA. In support of this, Dove (30) and Kessler (61) found increased RNase activity in stressed tomato leaves.

The synthesis of DNA ceased in Vicia faba seed (14), tomato leaves (37, 61), and sunflower leaves (61) suggesting impaired cell division. In drought-tolerant olive leaves, on the other hand, DNA synthesis was initiated in non-dividing cells which was paralleled by an accumulation of RNA (62). With optimum provision of water, wheat, wheat-wheatgrass hybrids, and sugar beets accumulate more DNA, and the stages of cell division and differentiation are prolonged (106).

Protein content tends to decrease under moisture stress (38, 56, 61, 131, 132, 143). This was attributed to reduced proteolysis of aleurone grain protein in barley thus reducing the amino acid pool (56), to an impaired nucleic acid system in tomato and flower (61), and to replacement of normal RNA by an altered one in corn (143).

The integrity of the ribosome system depends, among others, on the ionic concentration of the cytoplasm. Soluble RNA and ribosomal RNA do not associate in high ionic strength magnesium solutions (42). Marre (86) proposed that water shortage would primarily affect the ability of the cells to form active polysomes which was affirmed by Genkel', Satarova, and Tvorus (38) for corn and bean

plants. They attributed the absence of polysomes to increased destruction of messenger RNA by ribonuclease. More recently, Sturani, Cocucci, and Marre (130) suggested that some factor affecting messenger RNA or its interaction with the ribosomes, rather than modified ribosomal structure is responsible for cessation of protein synthesis in germinating castor bean endosperm.

Inactivation and destruction of long-lived, stable messenger RNA during moisture stress has been shown for wheat embryos during germination (18). This definitive study by Chen, Sarid, and Katchalski demonstrated that only trace amounts of complementary RNA were transcribed during a 24-hour rehydration period of embryos presoaked for 72-hours and dehydrated for 48-hours. This RNA represented a false message which could not be translated *in vivo*. The DNA of these embryos breaks down upon dehydration which might explain the slow transcription rate *in vivo* and the formation of false messages. They conclude that dehydration of the wheat embryo causes damage at the programming level but does not affect ribosome activity *per se*.

METHODS

To provide for an orderly development of the study, the areas of interest were divided into logical work units termed phases. The order of progression for each phase was planned to provide direction for subsequent phases through assessment of the results obtained and reappraisal of objectives in terms of those results.

Seed Sources

Previous studies concerning drought resistance in Douglas-fir indicated that seedlings grown from interior sources were generally more drought hardy than those from maritime sources (34, 105, 150). For this study, an inland source from Kaibab National Forest, Arizona collected at 8500 feet elevation and a coastal source from LaPush, Washington collected at 0 to 250 feet elevation were obtained from commercial seed dealers.

Climatological data for the station nearest each collection zone are summarized in Table 1. The data for Arizona indicate a continental climate with cold winters and warm summers, while that for Washington indicate a maritime climate with moderate seasonal temperature fluctuations. There is an average of 153 days between the last Spring and first Fall minimum of 32°F and 214 days between minimums of 16°F or below in Arizona. The Washington station

Table 1. Mean Monthly Temperature and Precipitation from Representative Stations in the Seed Collection Zones (139).

Station	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Mean Annual
Williams, Arizona[a]													
Temperature (°F)	31.0	33.2	38.5	46.9	54.8	63.6	68.7	66.6	61.4	51.0	40.4	34.1	49.2
Precipitation (in.)	1.89	2.15	1.85	1.38	0.66	0.54	2.59	3.73	1.85	1.32	1.06	2.23	21.25
Clallam Bay, Wash.[b]													
Temperature (°F)	38.3	39.7	42.0	46.4	50.5	54.0	56.3	56.5	54.5	49.7	43.4	40.7	47.7
Precipitation (in.)	13.55	9.81	8.43	5.25	3.15	2.44	1.65	1.52	3.15	7.85	11.16	14.29	82.25

[a]Williams, Coconine County, Arizona. Latitude 35°15'N, Longitude 112°11'W. Elevation 6750 feet. Temperature means based on 60 years of record; precipitation based on 59 years of record.

[b]Clallam Bay 1NNE, Clallam County, Washington. Latitude 48°16'N, Longitude 124°15'W. Elevation 30 feet. Temperature and precipitation means based on 36 years of record.

averages 191 days between minimums of 32°F and has never recorded temperatures of 28°F or below.

Seed Preparation

Seed preparation for all phases of the study was conducted in an identical manner varying only in the amount of seed required and the specific osmotic moisture stresses used. The required amount of seed from the inland and coastal sources was weighed or counted out and surface sterilized for five minutes in a one percent sodium hypochlorite solution followed by five rinses in distilled water.

Manohar, Bhan, and Prasad (80) found that most osmotic substrates may enter the seeds and therefore their effects are more complex than producing drought. True osmotic stress may be produced by using polyethylene glycol solutions (80). Various molecular weights of polyethylene glycol have been found to be suitable osmotic substrates for simulating moisture stress (72, 78, 80, 102, 133, 146). Manohar (78) did not find toxic effects of polyethylene glycol solutions of nominal molecular weights of 4000 or higher on peas.

After rinsing, the seed was presoaked for 24 hours in solutions of Carbowax polyethylene glycol 6000, a polymer of ethylene oxide with the generalized formula $HOCH_2(CH_2OCH_2)_nCH_2OH$ where n is the average number of oxyethylene groups (102), obtained from the Union Carbide Corporation. The average molecular weight of the material

is 6000. Aqueous solutions of 0 -1/2, -2, -4, and -8 atmospheres were obtained by dissolving 0, 60, 129, 190, and 260 grams of Carbowax 6000 in one liter of distilled water. The amounts used were determined from a calibration curve obtained by Zur (151).

After presoaking, the liquid was drained off, and a paper towel saturated with the appropriate osmotic solution was placed in the jar. The seeds were then subjected to naked stratification at 3°C in the dark for 14 days. Germination was conducted in covered plastic germination dishes on Sponge Rok at 25°C day and 15°C night temperatures with a 16 hour photoperiod. The Sponge Rok was saturated with the desired osmotic solution to maintain the same moisture stress conditions used in presoaking. One hundred to 150 ml of solution were normally required to saturate the Sponge Rok without causing it to float. Distilled water was added during the course of germination when required to maintain saturation.

Phase I

Germination

To determine the effect of osmotic moisture stress at 0, -1/2, -2, -4, and -8 atmospheres on the initiation and progression of germination, four 100 seed samples of each source were prepared as described above. The four replicates of each treatment (two

sources x five stresses) were germinated for 25 days in a randomized block design. Seeds were considered germinated when the radicle protruded a minimum of 2 mm.

Daily total and increase in germination were recorded and the germinated seed removed as they were counted. Germination value (G. V.), an index of speed and completeness of germination was used to compare germination response among the treatments (27). The index weights germinative energy more than total germination (27) and was calculated from:

$$G.\ V. = (PV)(MDG)$$

where PV is the maximum number obtained when daily percent germination is divided by the number of days to that level of germination and MDG is the average number of seeds germinating per day of the actual test period. Larsen and Schubert (72) found germination value to be a better index of the effect of moisture stress on germination than either total germination or germinative energy.

Moisture Content

To determine differences in whole seed moisture content during the progression of germination, 560 seeds were prepared for each source at 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress. Four replications from each treatment consisting of 20 seeds each were removed in conjunction with respiration determinations after 24

hours of presoaking, and 0 (end of stratification), 3, and 5 days of germination. An additional sample of 20 seeds was obtained for each treatment using one- to two-day old germinants. Due to delayed germination, this final sample was obtained at varying times after sowing depending on the level of osmotic stress.

After rinsing in distilled water to remove adhering Carbowax and surface drying with a paper towel, the 20 seed sample was placed in a weighing bottle and the fresh weight determined to the nearest 0.1 mg. Dry weight was determined after oven drying for 48 hours at 75°C. Moisture contents were then calculated on a dry weight basis.

To determine differences in equilibrium moisture contents, rates of water uptake, and water distribution within the seed between seed sources, 150 seeds from each source were prepared at 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress. Groups of three treatments were taken in a randomly staggered sequence at one-half hour intervals with respect to the beginning of presoaking. Three replications consisting of 10 seeds each were obtained for each source and stress after 0, 24, and 48 hours of presoaking, and after 24 hours of presoaking plus 24 hours moist at room temperature, according to the random sequence used for the start of presoaking. The seeds were immediately rinsed in distilled water, surface dried with a paper towel, and after separation, the seed coats and endosperm plus

embryo were placed in separate weighing bottles for fresh weight determination. After determining dry weight as described above, the moisture contents were calculated on a dry weight basis.

The rates of water uptake were calculated for the various time periods from the general formula:

Rate of uptake for specified time interval =

$$[\text{M. C. (\%) at time } t_2 - \text{M. C. (\%) at time } t_1 / t_2 - t_1][0.01]$$

= grams water uptake/gram dry weight/hour

Changes in distribution of water between the seed coat and the endosperm plus embryo were determined on the basis of differences in moisture contents between the two tissues over time.

Phase II

Respiration/Respiratory Quotient

To determine the effect of osmotic stress on respiration and the respiratory quotient (RQ), 500 seeds from each source were prepared at 0, -1/2, -2, -4, and -8 atmospheres of stress. The starting times for presoaking were randomly assigned for groups of five treatments at one hour and 45 minute intervals. Four 20 seed samples of each treatment were removed after 24 hours of presoaking, after 0, 3, and 5 days of germination testing, and then terminated with one- to two-day old germinants occurring at varying times

depending on the treatment.

After surface drying and weighing, the four samples of 20 seeds per treatment were placed in separate Warburg flasks of approximately 15 ml capacity. A paper wick and 0.2 ml of ten percent potassium hydroxide were added to two of the flasks for determination of oxygen uptake. The other paired flasks were used for determining carbon dioxide evolution by the direct method of Warburg as described by Umbreit, Burris, and Stauffer (138). This provided two replications each for oxygen uptake, carbon dioxide evolution, and RQ. Twelve treatment flasks plus a thermal barometer were run at one time.

The flasks were equilibrated for 10 minutes in the temperature bath at 30°C with shaking of 130 strokes per minute. The system was then closed to record pressure change to the nearest 0.5 mm at 15 minute intervals for one hour. The seed was then removed and oven dried at 75°C for 48 hours in the same weighing bottles as used for fresh weight for determination of dry weight.

To calculate the flask constants, it was necessary to determine the seed volume. Four replications of 20 seeds each were obtained for each treatment after presoaking to determine the fresh weight and volume by water displacement. The inverse of specific gravity, 1/S. G., was calculated from:

1/S. G. = volume of seed/fresh weight of seed

= ml g^{-1}.

A three factor analysis of variance for source, stress, and replication indicated that there was a significant difference in the inverse of specific gravity only between sources and it was significant at the 99 percent level. Seed volume was then calculated from:

Seed volume (ml) = [fresh weight (g)] [1/S. G. (ml g^{-1})]

where 1/S. G. was 0.913 ml g^{-1} for the coastal source and 1.000 ml g^{-1} for the inland source.

The oxygen uptake (Q_{O_2}) and carbon dioxide evolution (Q_{CO_2}) were expressed as μl of gas (mg dry weight)$^{-1}$ hour^{-1}. No corrections were applied to the measured carbon dioxide evolution to account for carbon dioxide fixation.

The respiratory quotient was calculated from:

RQ = μl CO_2 evolved/μl O_2 uptake

= Q_{CO_2}/Q_{O_2} .

Isocitratase Activity

To determine the specific activity of isocitratase in the two sources under the influence of osmotic moisture stress, approximately 300 seeds of each source were prepared at 0, -2, and -4 atmospheres of stress. These stresses were chosen on the basis of germination performance to provide a representative range of response. Starting

times for presoaking were staggered in order to obtain about 100 four-day old germinants for each treatment on the same day.

Three replications of each source and stress containing 20 endosperm of the four-day old germinants were used for the enzyme isolation. The procedure used for the isolation and the determination of enzyme activity was developed by Dr. Te May Ching[1].

All procedures were conducted at 0° to 4°C and glass distilled water was used for all reagents. The fresh and dry weights of the germinants and the length of radicle extrusion of ten germinants were determined.

The 20 endosperm were then homogenated in a Servall Omni mixer at 60 volts for five minutes in 10 ml of cold 0.1 M Tris buffer plus 0.002 M EDTA (pH 7.5 at 5°C). The homogenate was filtered through four layers of washed cheese cloth saturated with the grinding medium. The filtrate was then centrifuged at 0°C for 10 minutes at 18,000 x g. The clear internatant was drawn with a syringe into a graduated cylinder to avoid the top fat layer. The volume of internatant was determined and 20 mg of acid washed charcoal was added per 10 ml of volume. The material was then recentrifuged to remove nucleotides and protein bodies. The clear internatant was drawn with

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[1]Ching, T. M., Associate Professor of Farm Crops. Laboratory manual for crop seed physiology. Personal communication. Oregon State University Farm Crops Department, Corvallis, Oregon. July, 1969.

a syringe into a graduated cylinder and the volume determined. Finally, the enzyme preparation was placed in a freezer in a capped test tube for storage until use.

The method employed for the enzyme assay was a slight modification of the procedure described by Jacks and Alldridge (53) using mercuric acetate to remove cysteine interference. All of the reagent volumes proposed by Jacks and Alldridge were halved to obtain a final volume of 5 ml. Two 0.7 ml aliquots of enzyme preparation with either 0.3 ml of plus or minus substrate solution were incubated for 5 minutes at 30°C with shaking in a pyrex test tube. After adding the mercuric acetate, 2,4-dinitrophenylhydrazine, ethanol, and sodium hydroxide solutions, the mixture was centrifuged for five minutes at 1085 x g at room temperature.

The supernatant was immediately decanted into a test tube and the optical density of the plus substrate was read at 540 nm against the minus substrate in a double beam spectrophotometer. The glyoxylate content was estimated from a standard curve where:

$$\text{nmoles of glyoxylate} = \Delta OD_{540}/0.00075.$$

The protein content of a 0.35 ml aliquot of enzyme preparation was estimated by Lowry's method (65). The content was then doubled to obtain the protein concentration of the 0.7 ml aliquot used for the enzyme assay. The specific activity of the enzyme was then expressed as nmoles of glyoxylate produced (mg protein)$^{-1}$ minute^{-1}.

The total protein content of the enzyme preparation was estimated by expanding the content of 0.35 ml to the total volume of the extract. This value provided a measure of the total soluble protein content of the endosperm under the particular osmotic stress. The total activity was then estimated by multiplying the total protein content by the specific activity. The total activity, which corrects for differences in protein content between treatments, was expressed as nmoles of glyoxylate produced minute^{-1}.

Phase III

To determine the difference in soluble nucleotides, RNA, and DNA contents of embryos from the two sources during the early stages of germination under conditions of osmotic moisture stress, 300 seeds from each source were prepared at 0, -2, and -4 atmospheres of osmotic stress. Three replications of 20 seeds each were removed from each treatment after 24 hours of presoaking, and 0, 4, 8, and 12 days after sowing. The embryos or seedlings were removed and placed in chilled weighing bottles.

The sample was immediately brought into a coldroom and the nucleotides and nucleic acids extracted using a Schmidt and Thannhauser method modified from those proposed by Ingle (49) and Munro and Fleck (92) which was developed by Dr. T. M. Ching[1] as described below.

All procedures were at 0° to 4°C unless otherwise specified, and centrifugation was at 0°C for 5 minutes at 10,000 x g. Embryos were ground in a mortar with 5 ml of cold 0.25 N perchloric acid, the material transferred to a 50 ml polyethylene centrifuge tube, the mortar washed with an additional 5 ml of 0.25 N perchloric acid, and the rinse combined in the centrifuge tube. Germinants were cut to small pieces in a watch glass, the material transferred to the centrifuge tube, the watch glass rinsed once with 5 ml of perchloric acid, and the rinse combined. The seedling material was then homogenized in a Servall Omni mixer for 10 minutes at 60 volts.

Free nucleotides, nucleotide coenzymes, sugars, inorganic phosphate, and low molecular weight phosphorus compounds were extracted by standing the homogenate for 10 minutes and then centrifuging. The pellet was washed twice with centrifuging in 5 ml of 0.25 N perchloric acid and the supernatants combined in a 30 ml Corex centrifuge tube. The supernatant was neutralized to a pH of 6.9 in an automatic titrator with 2 N potassium hydroxide to precipitate out potassium perchlorate. The neutralized extract was then centrifuged and the supernatant decanted to a flask.

When necessary, phenolic compounds were removed from the neutralized extract by adding 1.5 g of acid washed Polyclar and centrifuging at 10,000 x g for 5 minutes. The volume of the extract and the optical density at 260 nm were determined, and then a

100 O.D. unit aliquot was purified using ion exchange chromatography (49, 50) with 10 g of 50 to 100 mesh Dowex 1-X8 resin in a 1.5 x 50 cm glass column. The column was eluted with 75 ml of 4 N formic acid-0.8 N ammonium formate. The optical density of the eluate was read at 260 nm against the eluting buffer in a double beam spectrophotometer.

The soluble nucleotide content was then estimated from:

$$\text{Nucleotide content } (\mu g) = \left[\frac{\text{extract volume}}{\text{eluted volume}}\right]\left[\frac{OD_{260} \cdot \text{eluted volume}}{14}\right]\left[450\ \mu g\ \mu mole^{-1}\right]$$

where 14 is an arbitrary 260 nm millimolar extinction coefficient (50) and 450 $\mu g\ \mu mole^{-1}$ is the average molecular weight of the nucleotides.

To extract the RNA, the remaining pellet was washed three times each with 10 ml of an ether-ethanol solution (1 : 1 by volume) at room temperature to remove lipids and pigments. The extraction was simplified by centrifuging at 10,000 x g for 5 minutes each time. The washed pellet was then hydrolyzed with 1 ml of 0.3 N potassium hydroxide for one hour at 37°C and then 9 ml of distilled water was added. The hydrolysate was cooled to 0°C on ice in the cold room and then 0.6 ml of 1.1 N perchloric acid was added. After standing for 10 minutes, the hydrolysate was centrifuged, and the pellet washed twice with 5 ml of 0.2 N perchloric acid. The combined washings containing the hydrolysate of RNA were neutralized to a pH

of 6.9 in an automatic titrator using 2 N potassium hydroxide, and the precipitate removed by decanting the supernatant into a flask after centrifuging.

The total RNA content of a 2 ml aliquot of neutralized hydrolysate was estimated by a cupric ion catalyzed orcinol reaction (74) with a microgram absorption coefficient of 0.036 ml $\mu g^{-1} cm^{-1}$. The chromogenic centers of adenosine and guanosine, and to a lesser extent, uridine are responsible for the color reactions (74). Total RNA content in μg was then obtained by expanding the content of the 2 ml aliquot to the total extracted volume.

The DNA was extracted from the residual pellet by hydrolyzing in 5 ml of 0.5 N perchloric acid twice at 70°C for 20 minutes, centrifuging, and then combining the hydrolysate. The deoxyribose content was then estimated in a 1 ml aliquot by Burton's modification of the diphenylamine reaction (15). Only the purine-bound deoxyribose reacts in the diphenylamine reaction, however Burton's modification using acetaldehyde is believed to be more specific for DNA in many cases (92). The optical density at 600 nm was converted to μg of DNA from a standard curve prepared from highly polymerized calf thymus. The total DNA content was then estimated by expanding the 1 ml sample content to the total extracted volume.

The μg contents of soluble nucleotides, RNA, and DNA were expressed on a per seedling basis to provide a uniform comparison.

Presentation of Results

Data from all phases of the study were subjected to three factor analyses of variance for seed source, time, and moisture stress using a standard computer program. For purposes of making inferences from the data, observed differences had to occur by chance no more than five times in 100 observations (95 percent level) to be considered significant. Trends in the data were more important than absolute differences for synthesizing the data into a consistent view of moisture stress response. Therefore, tests of individual differences were not conducted. Further, without preconceived hypotheses to test, such tests are unwarranted.

Because of the length of the tables required and the difficulty in assimilating such data presentations, the results were presented graphically. The graphs were drawn to show all data points. Curves were drawn through the mean values for each sample space to show trends.

Regression models for germination value over moisture stress and respiration over moisture content were designed through use of stepwise multiple linear regression and nonlinear regression computer program routines.

RESULTS

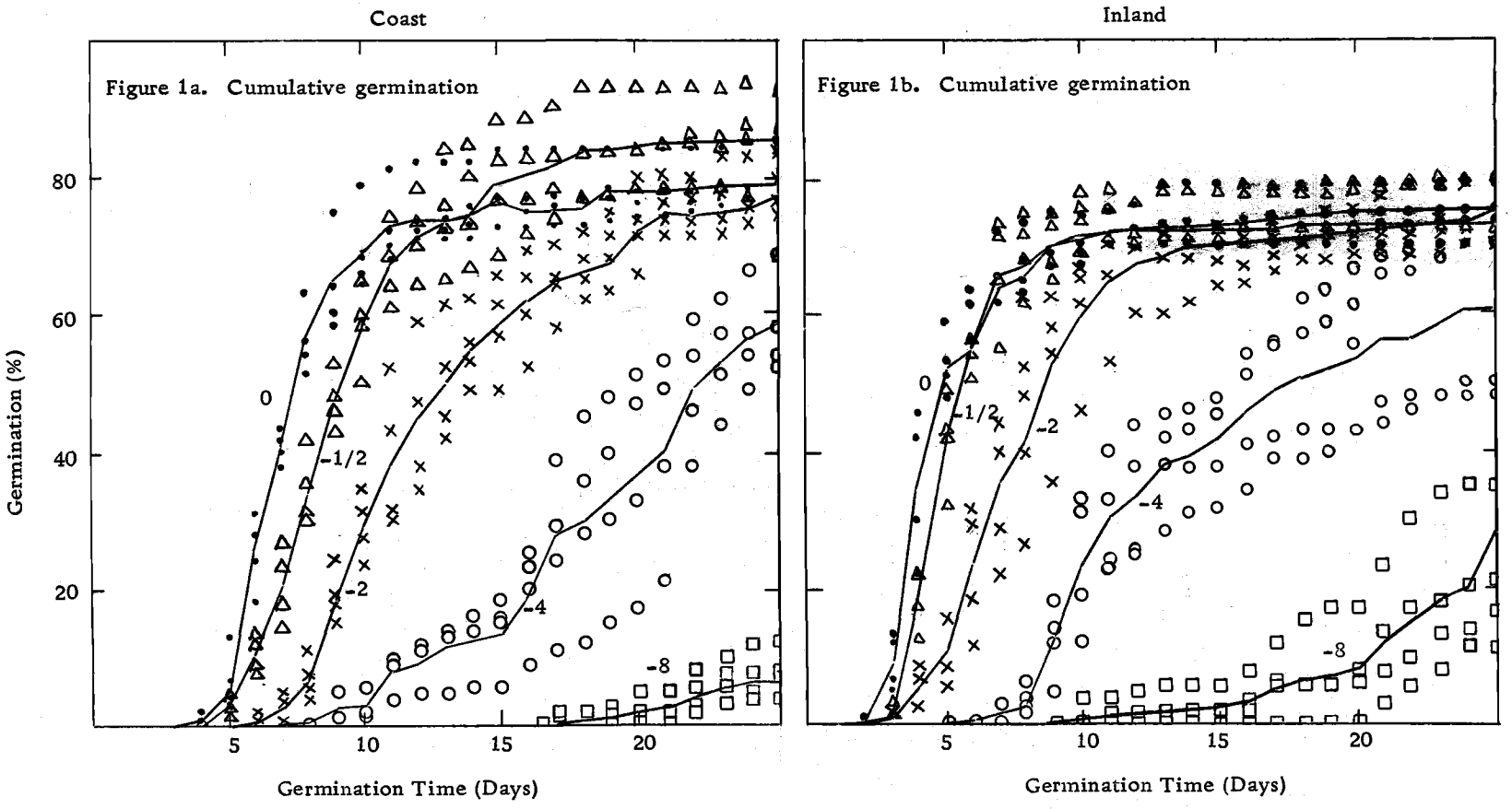
For purposes of discussion, no distinction has been made between differences significant at the 95 and 99 percent levels. However, the actual level of significance was indicated in tabular form using a single asterisk (*) to denote an F ratio significant at the 95 percent level and a double asterisk (**) to denote significance at the 99 percent level. The term "source" has been used in the discussion to indicate seed from the two commercial seed collections.

Germination

The daily progress of germination for the two seed sources under the influence of moisture stress is shown in Figures 1a and 1b. The three factor analyses of variance of source, stress, and blocks for total germination and delay of germination (number of days to the first germinant) are summarized in Table 2.

Table 2. Summary of Analysis of Variance for Delay of Germination and Total Germination Under the Influence of Osmotic Stress for Two Seed Sources

Source	d.f.	Delay of Germination F Ratio	Total Germination F Ratio
Source	1	125.859**	<1
Stress	4	162.154**	82.648**
Blocks	3	<1	<1
Source x Stress	4	15.603**	2.041
Source x Blocks	3	<1	<1
Stress x Blocks	12	<1	<1
Error	12		



Figures 1a and 1b. Cumulative germination for a coastal and inland Douglas-fir seed source under the influence of 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress.

Total germination was equally reduced by osmotic stress for both Douglas-fir seed sources. However, the delay in initiation of germination induced by moisture stress was greater in the coastal source than in the inland source. The two sources were delayed to a different extent by an increase in stress. This progressive delay and reduction in germination was consistent with the response shown for a wide variety of species. Among gymnosperms, similar effects have been shown for Pinus species (6, 72, 88, 111), Cryptomeria japonica (111), and Chamaecyparis obtusa (111).

It was observed that the incidence of fungal infection tended to increase as germination was delayed. This undoubtedly reduces the capacity to germinate at a later date as demonstrated by Owen (98) for wheat.

By combining speed and completeness of germination into a single index, germination value provided an overall measure of the effect of osmotic stress on the progress of germination. The regression of germination value over moisture stress is shown in Figure 2 for both sources. The regression for the coastal source, $Y = 23.377 - 5.962X + 0.382X^2$ where Y is the germination value and X is the osmotic stress in atmospheres, had a correlation coefficient (r^2) of 0.936. The regression for the inland source, $Y = 32.285 - 7.924X + 0.500X^2$, had a correlation coefficient of 0.951. An F test of the two regressions indicated that the parameters were

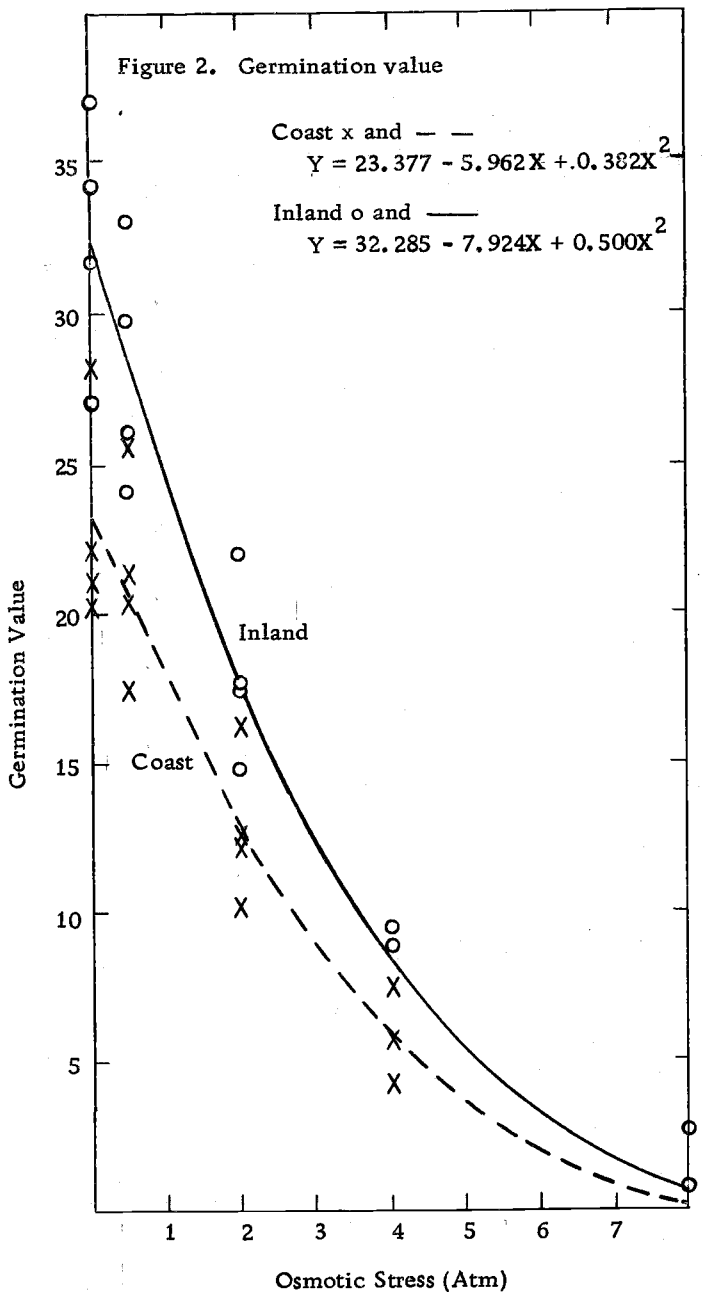


Figure 2. The relationship of germination value for a coastal and an inland Douglas-fir seed source to osmotic stress between 0 and -8 atmospheres.

different for the two sources at the 95 percent level.

Because both sources are affected to the same degree in relation to total germination, the difference in germination value response must be due to differences in delay of germination caused by moisture stress. The inland source has a higher germination value than the coastal source at identical moisture stresses.

The general conclusion is that osmotically induced moisture stress delays the phase shift from the dormant to the active embryo. Further, it is likely that at some stress slightly greater than -8 atmospheres, the phase shift would be completely inhibited. The inland source is affected by moisture stress to a lesser extent and therefore may possess adaptations to drought during germination.

Moisture Content

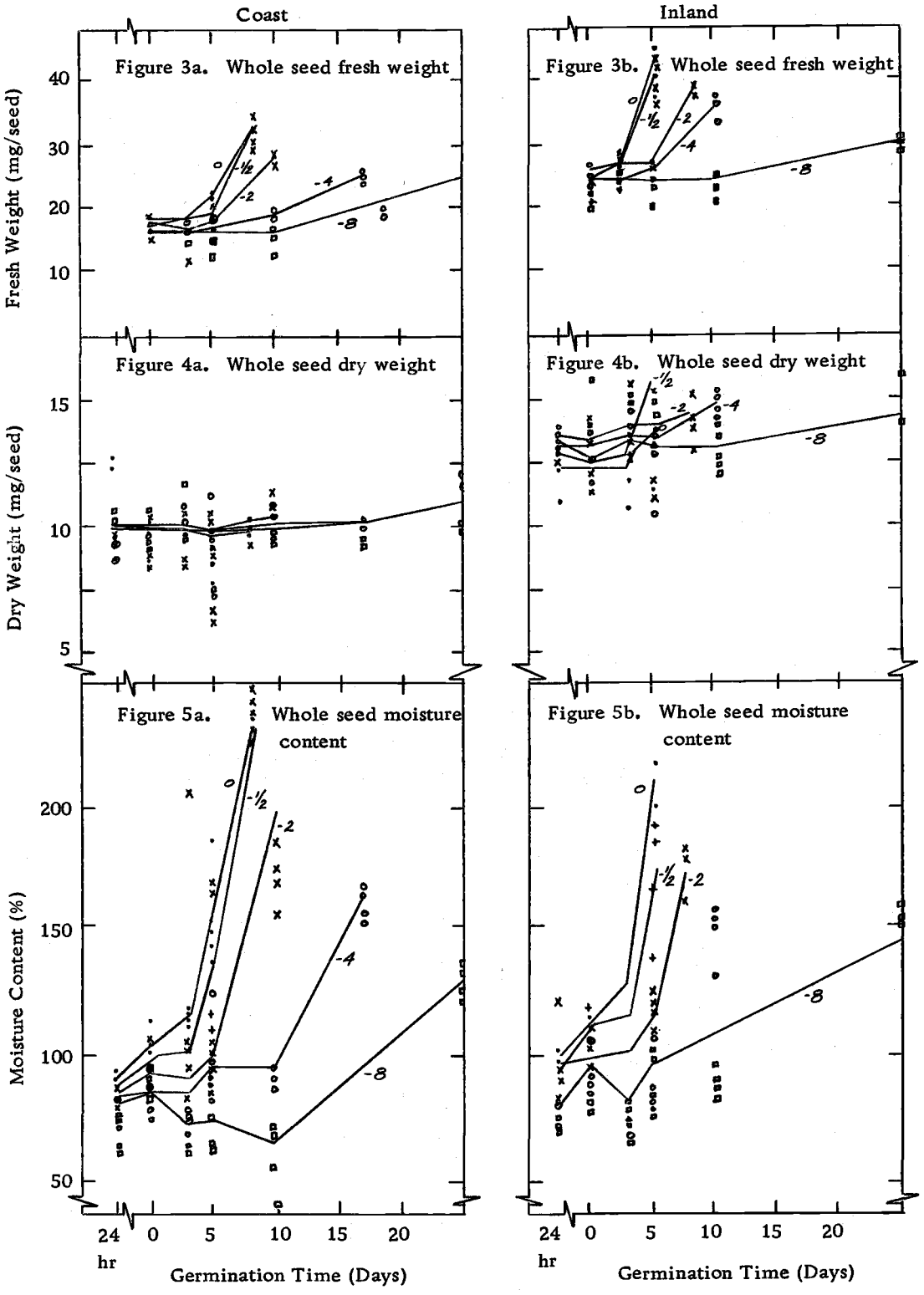
The changes in whole seed fresh weight, dry weight, and moisture content during germination are shown in Figures 3a, 4a, and 5a for the coastal source and Figures 3b, 4b, and 5b for the inland source.

Because the moisture content determinations were terminated at different times, there were an unequal number of levels of the time factor. For purposes of analysis, three physiologically definable time periods were selected. These were: 1) after 24 hours of imbibition, 2) 0 days of germination (end of stratification), and 3) one- to two-day

Figures 3a and 3b. Whole seed fresh weight for a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress.

Figures 4a and 4b. Whole seed dry weight for a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress.

Figures 5a and 5b. Whole seed moisture content for a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress.


Coast
Inland
Figure 3a. Whole seed fresh weight
Figure 3b. Whole seed fresh weight
Fresh Weight (mg/seed)
40
30
20
10
0
-½
-2
-4
-8
Figure 4a. Whole seed dry weight
Figure 4b. Whole seed dry weight
Dry Weight (mg/seed)
15
10
5
Figure 5a. Whole seed moisture content
Figure 5b. Whole seed moisture content
Moisture Content (%)
200
150
100
50
24 hr
0
5
10
15
20
Germination Time (Days)

old germinants. Observation within these time periods should be comparable. Three factor analyses of variance of source, time, and stress for fresh weight, dry weight, and moisture content are summarized in Table 3.

Table 3. Summary of Analysis of Variance for Fresh Weight, Dry Weight, and Moisture Content for Two Seed Sources and Three Time Periods Under Five Levels of Osmotic Stress

Source	d.f.	Fresh Weight F Ratio	Dry Weight F Ratio	M.C. % F Ratio
Source	1	760.121**	470.585**	2.517
Time	2	854.763**	19.541**	788.484**
Stress	4	18.871**	4.805**	70.423**
Source x Time	2	4.405*	5.058**	30.539**
Source x Stress	4	2.465	<1	3.888**
Time x Stress	8	13.882**	<1	17.934**
Source x Time x Stress	8	1.743	1.862	4.156**
Error	90			

The mean fresh and dry weights of the inland source were higher than those of the coastal source. As is common in germination, seed fresh weight increased during germination. Moisture stress reduced the rate of increase in fresh weight, and the rate of increase averaged over stress was different for the two sources. Whole seed dry weight remained constant or increased slightly during the period of observation which is contrary to normal germination behavior. This difference probably resulted from a large variability in seed size and subsequent sampling error. At the time periods examined, stress

tended to reduce dry weight but the results were inconsistent. The fact that the source x stress interactions were not significant for either fresh or dry weight would indicate that the higher weights of the inland source may not be related to a greater response to stress but rather may be a function of initial seed size and moisture uptake.

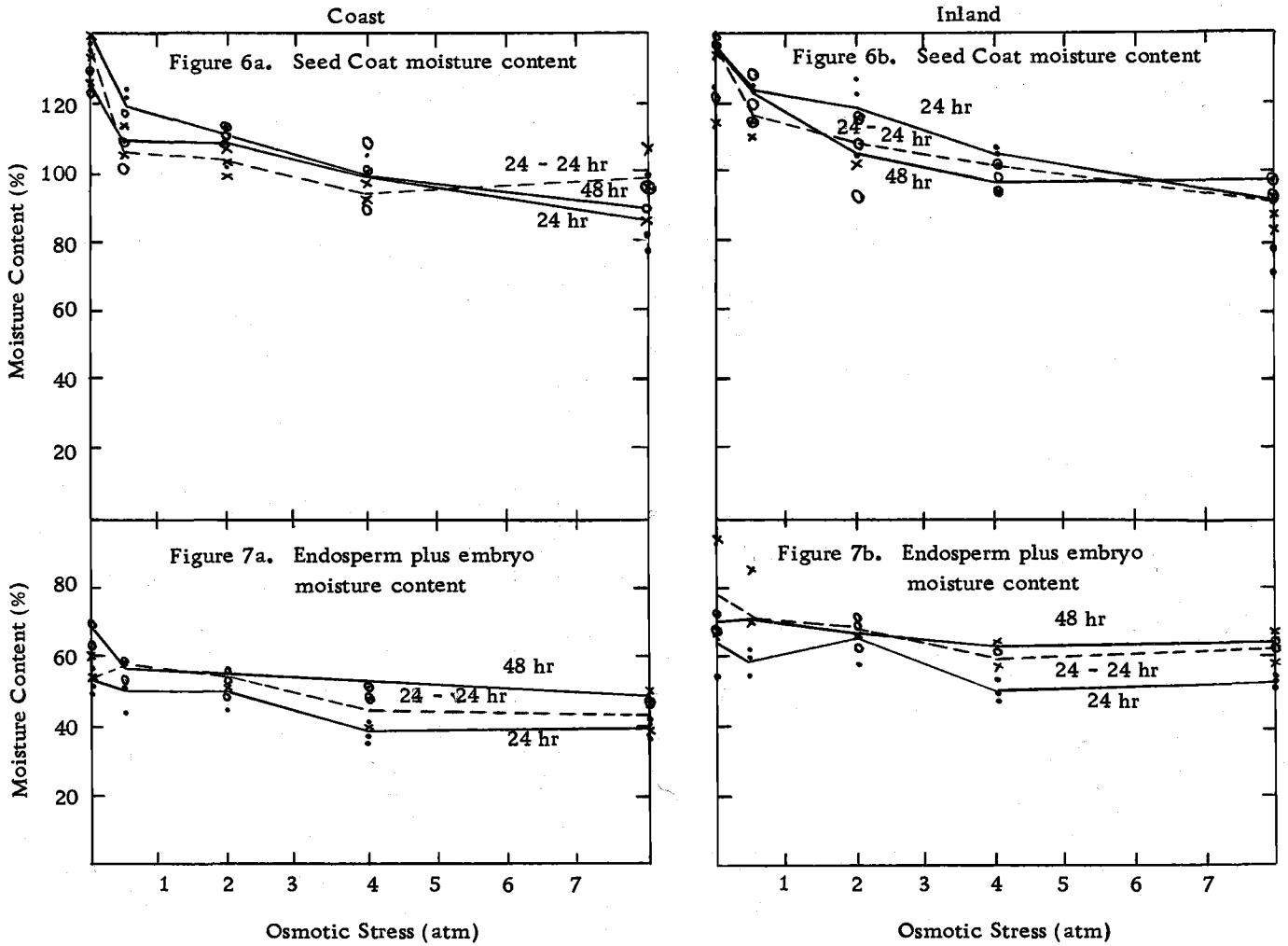
The mean whole seed moisture content increased during germination, but the rate of increase was attenuated by moisture stress. Similar reductions in water content by moisture stress have been demonstrated for other species (6, 137). The general pattern of moisture uptake was consistent with the three phases proposed by Goo (41). The final abrupt increase in moisture content corresponded well with the rupture of the seed coat and extrusion of the radicle.

The two sources had different uptake characteristics averaged over stress and responded differently to moisture stress. This probably was a result of a higher moisture content in the inland source from imbibition up to rupture of the seed coat, but a higher moisture content in the coastal source at germination. The difference in moisture content between stresses increased with time and was a function of seed source.

The change in moisture content of the seed coat and the endosperm plus embryo after 24 hours of imbibition, 48 hours of imbibition, and 24 hours of imbibition plus 24 hours moist at room temperature is presented in Figures 6a and 7a for the coastal source and 6b

Figures 6a and 6b. Seed coat moisture contents of a coastal and an inland seed source as a function of osmotic stress between 0 and -8 atmospheres during 24 hours, 48 hours, and 24 hours plus 24 hours moist imbibitional periods.

Figures 7a and 7b. Endosperm plus embryo moisture contents of a coastal and an inland seed source as a function of osmotic stress between 0 and -8 atmospheres during 24 hours, 48 hours, and 24 hours plus 24 hours moist imbibitional periods.


Coast
Figure 6a. Seed Coat moisture content
Moisture Content (%)
120
100
80
60
40
20
24 - 24 hr
48 hr
24 hr
Figure 7a. Endosperm plus embryo moisture content
Moisture Content (%)
80
60
40
20
48 hr
24 - 24 hr
24 hr
1
2
3
4
5
6
7
Osmotic Stress (atm)
Inland
Figure 6b. Seed Coat moisture content
24 hr
24 - 24 hr
48 hr
Figure 7b. Endosperm plus embryo moisture content
48 hr
24 - 24 hr
24 hr
1
2
3
4
5
6
7
Osmotic Stress (atm)

and 7b for the inland source. The mean rates of water uptake during these time periods are shown in Table 4. Because the 24 hour imbibition period was used for all treatments in this study, two factor analyses of variance of source and stress for water uptake of seed coat and endosperm plus embryo were conducted for this time period only. The results of these analyses are presented in Table 5.

Comparison of the rate of imbibition during the first 24 hours and the second 24 hours indicated that moisture uptake was initially rapid in both the seed coat and endosperm plus embryo but decreased with time. This would indicate that moisture uptake followed an inverse exponential function during imbibition as proposed by Shull (116). The rate of uptake initially was much higher in the seed coats, however sometime during the second 24 hours moisture was lost faster than it was gained. This moisture loss in the seed coats occurred even when the seed was surrounded by water and presumably represented transfer of water to the endosperm and embryo. There was some indication that moisture stress prolonged the period of net water uptake in the seed coats.

Stiles (125) found that the seed coats of cotton and corn seed serve as transporting agents for water from the external water supply to the internal parts of the seed. Ching (24) felt that the seed coat aided water uptake during the early stages of Douglas-fir germination. Stiles (125) indicated that seed coats absorb water passively by

Table 4. Mean Rates of Water Uptake Under Various Conditions of Osmotic Stress for 24 hr. Imbibition, 48 hrs. Imbibition, and 24 hr. Imbibition-24 hr. Moist.

		Mean Uptake Rate [g water (g dry weight)$^{-1}$ hr^{-1}]							
		Seed Coat				Endosperm + Embryo			
Source	Stress	1st 24 hrs	2nd 24 hrs of 48 hr	2nd 24 hrs moist	48 hrs	1st 24 hrs	2nd 24 hrs of 48 hr	2nd 24 hrs moist	48 hrs
Coast	0	0.0540	-0.0062	-0.0036	0.0239	0.0188	0.0047	0.0013	0.0117
	0.5	0.0441	-0.0034	-0.0046	0.0203	0.0175	0.0025	0.0035	0.0100
	2	0.0403	0.0004	-0.0023	0.0204	0.0174	0.0016	0.0013	0.0095
	4	0.0358	0.0006	-0.0020	0.0182	0.0129	0.0051	0.0028	0.0090
	8	0.0308	0.0010	-0.0034	0.0159	0.0135	0.0032	0.0020	0.0084
Inland	0	0.0493	-0.0004	-0.0006	0.0244	0.0230	0.0027	0.0057	0.0129
	0.5	0.0446	-0.0005	-0.0033	0.0221	0.0211	0.0054	0.0062	0.0133
	2	0.0431	-0.0048	-0.0040	0.0191	0.0238	0.0007	0.0010	0.0123
	4	0.0376	-0.0026	-0.0015	0.0175	0.0180	0.0049	0.0033	0.0115
	8	0.0319	-0.0017	-0.0005	0.0169	0.0186	0.0050	0.0047	0.0118

hygroscopic and imbibitional processes and that seed coats of different seeds have different absorption capacities throughout germination. These capacities are due to differences in seed coat morphology and to differences in the activity of internal seed parts in rate and percentage of water absorption.

Table 5. Summary of Analysis of Variance for Rate of Moisture Uptake of Seed Coat and Endosperm Plus Embryo During the First 24 Hours of Imbibition for Two Seed Sources and Five Osmotic Stresses

Source	d.f.	Seed Coat F Ratio	Endosperm plus Embryo F Ratio
Source	1	<1	56.979**
Stress	4	28.870**	12.272**
Source x Stress	4	1.024	<1
Error	20		

Examination of the curves for moisture content over osmotic stress indicated that moisture content, thus rate of water uptake, was reduced by stress for both the seed coats and the endosperm plus embryo. This was confirmed for the first 24 hours of imbibition by the analysis of variance. The rate of water uptake by the seed coats was the same for both sources, while uptake was higher in the endosperm plus embryo of the inland source. The rate of uptake in both was reduced by moisture stress but to the same extent for both sources. The results implied that the seed coat characteristics are nearly the same in both sources in terms of water uptake. The higher

rate of uptake in the endosperm plus embryo of the inland source would perhaps indicate a higher imbibitional force due to a higher osmotic or matrix potential or a combination of these. This would be the case if imbibition is strictly a physical force as some evidence indicates (95, 96, 99). Imbibitional water uptake also depends on the chemical composition of the seed, however, due to the hydration characteristics of various substances such as starch, fat, and protein. Thus the differences in uptake also may be related to different proportions of various materials in the two seed sources.

Respiration - Respiratory Quotient

The change in oxygen uptake, carbon dioxide evolution, and respiratory quotient are shown in Figures 8a, 9a, and 10a for the coast source and 8b, 9b, and 10b for the inland source.

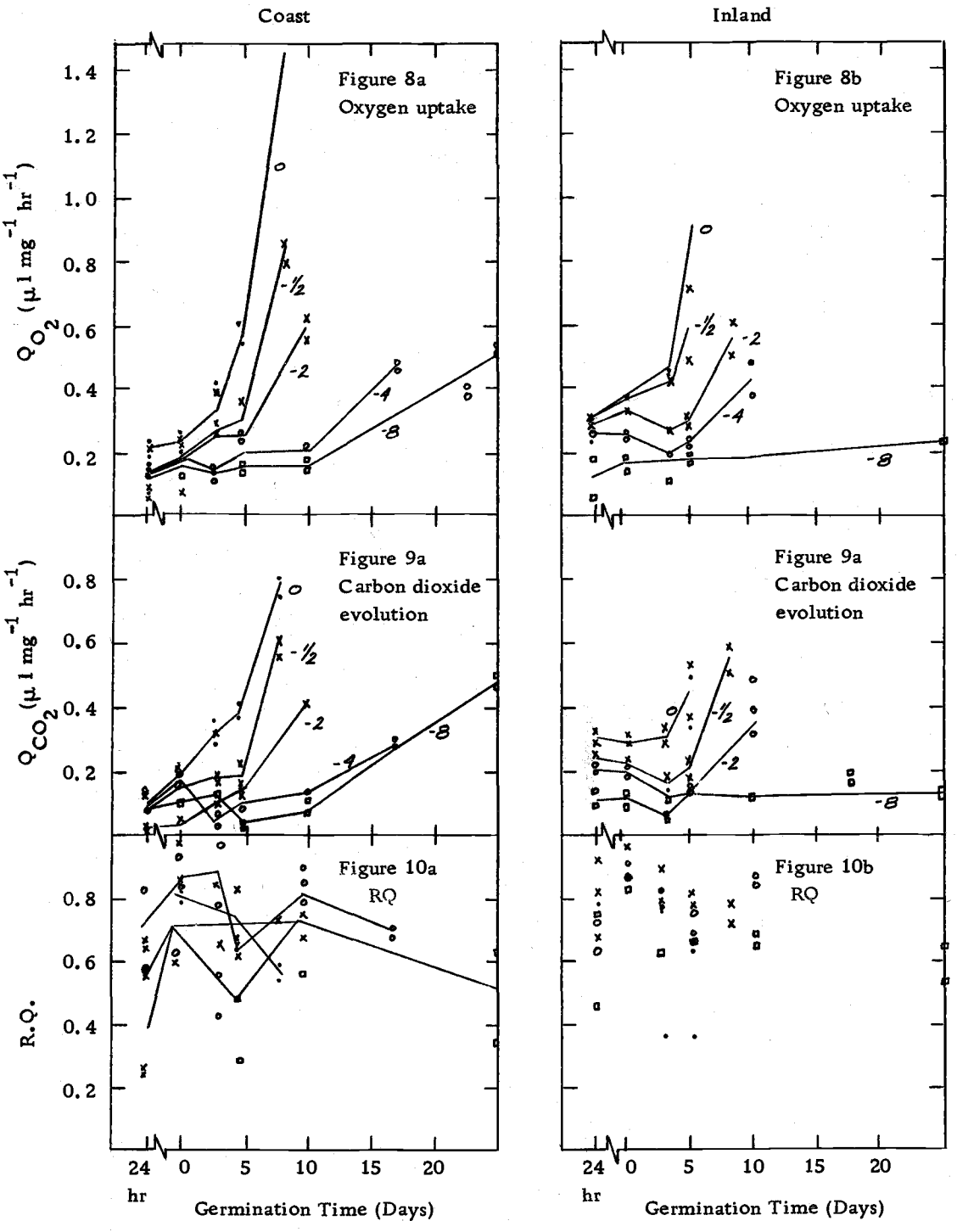
As discussed previously, there were an unequal number of samples through time for respiration and respiratory quotient. Therefore three time periods were chosen for analysis. These were: 1) after 24 hours of imbibition, 2) 0 days of germination, and 3) one- to two-day old germinants. The three factor analyses of variance of source, time, and stress for oxygen uptake, carbon dioxide evolution, and respiratory quotient are summarized in Table 6.

Both oxygen uptake and carbon dioxide evolution increased from imbibition onward which compared with previous studies (22, 65, 86).

Figures 8a and 8b. Rate of oxygen uptake of a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress.

Figures 9a and 9b. Rate of carbon dioxide evolution of a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress.

Figures 10a and 10b. Respiratory quotient of a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -1/2, -2, -4, and -8 atmospheres of osmotic stress.


Coast
Inland
Figure 8a
Oxygen uptake
Figure 8b
Oxygen uptake
Q_{O_2} (μl mg^{-1} hr^{-1})
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-½
-2
-4
-8
Figure 9a
Carbon dioxide
evolution
Figure 9a
Carbon dioxide
evolution
Q_{CO_2} (μl mg^{-1} hr^{-1})
0.8
0.6
0.4
0.2
Figure 10a
RQ
Figure 10b
RQ
R.Q.
0.8
0.6
0.4
0.2
24
hr
0
5
10
15
20
Germination Time (Days)
24
hr
0
5
10
15
20
Germination Time (Days)

The pattern of increase included the three stages reported by Koller, et al. (65), Kozlowski and Gentile (69), and Stiles and Leach (126) if the rapid initial rise from dry seed to imbibed seed was included. The final distinct rise in respiration occurred at the time of seed coat rupture and radicle extrusion consistent with observations on Douglas-fir (22) and eastern white pine (69).

Table 6. Summary of Analysis of Variance for Respiration and Respiratory Quotient for Two Seed Sources Under Five Levels of Osmotic Stress

Source	d.f.	Q_{O_2} F Ratio	Q_{CO_2} F Ratio	RQ F Ratio
Source	1	2.008	<1	7.677**
Time	2	377.630**	55.668**	17.649**
Stress	4	68.425**	16.011**	2.147
Source x Time	2	43.065**	7.715**	<1
Source x Stress	4	7.591**	3.170*	<1
Time x Stress	8	32.553**	2.750*	1.054
Source x Time x Stress	8	4.924**	1.541	<1
Error	30			

Mean oxygen and carbon dioxide exchange were not significantly different for the two sources, however the rate of increase was attenuated to a different extent in seed from the two sources by increasing moisture stress. This reduction due to stress was expected on the basis of earlier studies on seeds and seedlings of other species (17, 48, 59, 132, 135, 136). The respiration rate of the inland source appeared to be higher than that of the coastal source at all except the lowest and the highest moisture stress until rupture

of the seed coat. Respiration rates were higher in the coastal seed source once germination began. The difference in oxygen uptake induced by osmotic stress increased with time and was a function of seed source.

As reported for other species (22, 65, 86), the rates of increase for the two respiratory gases were not the same so that the respiratory quotient changed during germination. The mean quotients were different for the two sources but were not altered by osmotic moisture stress. This would indicate that the basic metabolism of respiration is not altered under stress as shown for Chlorella (46). The respiratory quotient increased during imbibition and stratification and then decreased during germination. This decrease has been reported for Douglas-fir (22) and castor bean (86, 93) and may represent a combination of respiratory combustion of stored fats with lower RQ, respiratory combustion of sugars produced from fat breakdown with a higher RQ, incomplete respiration as intermediates of metabolism are diverted into synthesis of cellular constituents, and an increase in the contribution of the glyoxylate cycle in relation to the TCA cycle.

Comparison of the respiration and whole seed moisture content curves indicated their similarity in shape. Such a relationship has been demonstrated for other species (96, 97, 122) and Ragai and Loomis (107) proposed a relationship of respiration to moisture

content of the form $y = ae^{bx}$ for maize grain between 14 and 24 percent moisture contents. The parameters of the exponential model were not evaluated and the form of the relationship presented implied that respiration did not become zero until moisture content dropped to zero.

As described previously, moisture content determinations during germination were made in conjunction with respiration measurements using the same samples. Plotting the data as shown in Figure 11 indicated that the same relationship should hold for both seed sources and that observed differences in respiration rates between them might be due to moisture content differences. Further, the data indicated that an asymptotic exponential relationship similar to that proposed by Ragai and Loomis (107) might hold. However, respiration would apparently fall to zero at some moisture content greater than 30 percent.

It was therefore necessary to include a constant in the model to account for the fact that the relationship should not pass through the origin. The model, $Y = B_1 + B_2e^{B_3X}$ where Y was the respiration rate in $\mu l\ O_2$ (mg dry weight)$^{-1}$ hr^{-1} and X was the moisture content in percent, was evaluated using a nonlinear least squares computer program. The evaluation of the parameters was based on 96 independent observations from the combined data of both sources.

The best estimate of the parameters provided a relationship of

$Y = 6.2747 - 6.4411\, e^{-0.00069X}$ with Y and X defined as above. The usual tests appropriate in the linear model case are, in general, not appropriate when the model is nonlinear. The unexplained variation for the model was 0.5598 based on approximately 93 degrees of freedom, however this would not provide an unbiased estimate of σ^2 even if the model was correct.

Examination of the function indicated that respiration became zero when moisture content dropped to 40 percent which was reasonable, and examination of the residuals did not indicate any obvious lack of fit. Further, the form of the model was asymptotic, however evaluation of the first derivative implied that the maximum respiration rate of $6.2747 \mu l\, mg^{-1}\, hr^{-1}$ was attained only as X approached infinity. This was a consequence of the small value of B_3 in the exponent. Thus the relationship within the range of moisture contents observed and under the study conditions deviated only slightly from linearity. In fact, the major advantage of the exponential form, that respiration should reach a maximum at some finite moisture content, was negated.

For this reason, a linear model to evaluate the form of the relationship was conducted using a stepwise multiple regression computer program. A linear and a logarithmic form were evaluated simultaneously using the general model $Y = B_0 + B_1X + B_2(\ln X)$ where Y and X were defined as above. The results again indicated

that the data did not differ greatly from linearity. The logarithmic parameter was insignificant, and the final form of the relationship was $Y = -0.1414 + 0.0040 X$. The residual sum of squares was 0.5597 with 94 degrees of freedom and the r^2 was 0.8235. The relationship implied that respiration was zero when the moisture content dropped to 32.8. The residual sum of squares and the moisture content where respiration became zero were very similar for both the exponential and the linear regressions. Neither demonstrated a functional relationship, but for predictive purposes, the linear model was preferred because of its simplicity. Both regressions are presented in Figure 11.

The failure of the data to conform closely to the exponential model proposed by Ragai and Loomis (107) may be related to the nature of the data and the period of time over which data was collected as described below.

Lack of observations at the extremes of moisture content made it difficult to evaluate the function more exactly. Further, it should be noted that the majority of the data was clustered between moisture contents of 60 and 120 percent. Also, the variance of the regression line increased at the higher moisture contents. The most probable explanation for this is that the higher moisture contents occurred at a later period in the course of germination. Consistent with the observations in this study, Opik and Simon (97) found that the

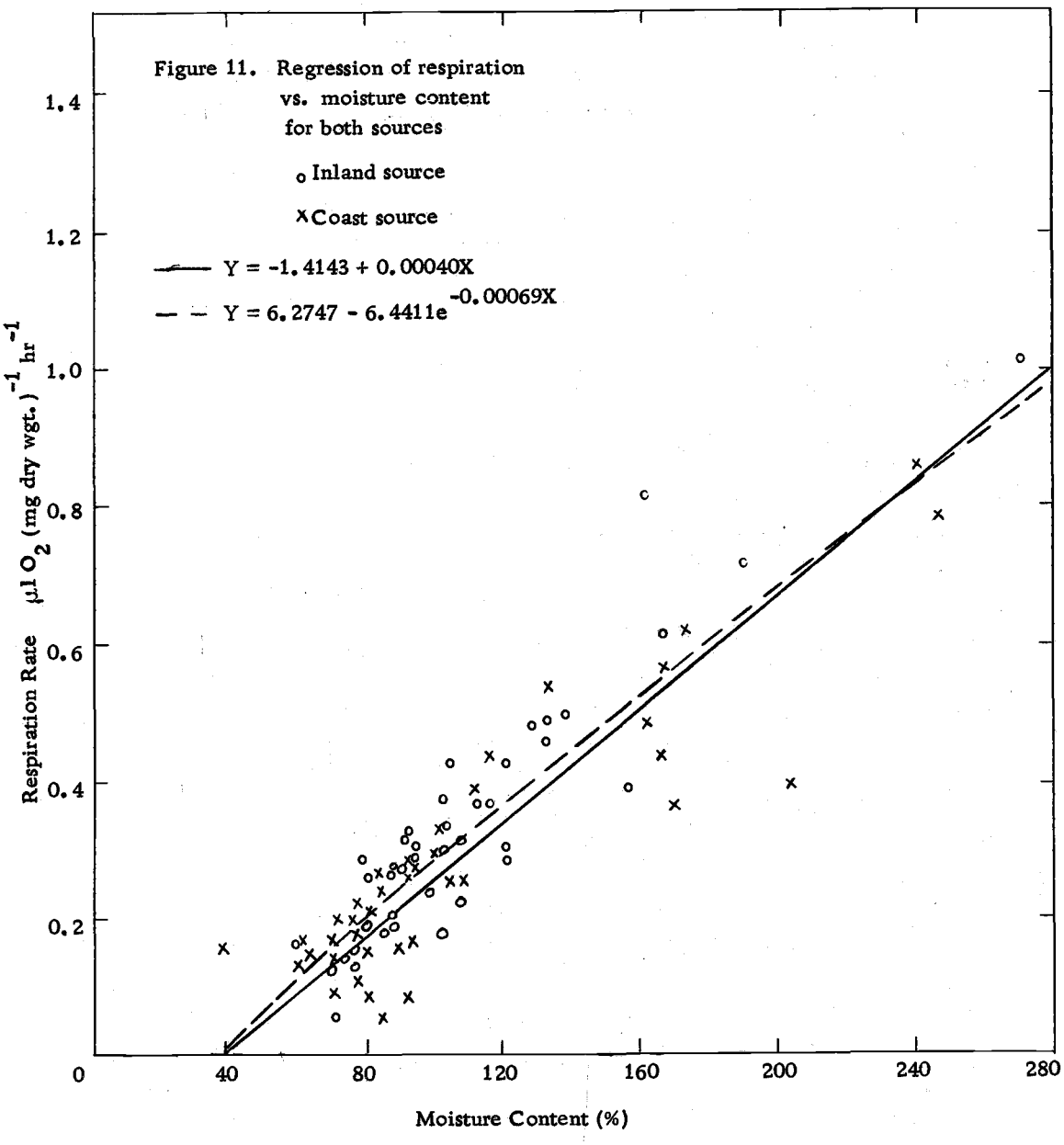


Figure 11. Relationship of rate of oxygen uptake to moisture content for seed from the coastal and the inland sources using an exponential and a linear model.

respiration rate of bean cotyledons was closely dependent on water content only during imbibition. They suggest that this apparent control was related to the effect of water content on the proportion of fully hydrated cells, on the rate of enzymatic reactions, on the opening of disulphide linkages to form free sulphydryl groups, or to the diffusion rates of reactants. These all involved reactivation of a preformed respiratory capacity through increasing hydration. The observations of Ragai and Loomis (107) were probably concerned only with this imbibitional phase.

At higher moisture contents which occur at later stages, the potential capacity for respiration increases due to enzyme synthesis. During this phase, a certain water content is required to fulfill the respiratory capacity (97), however the respiration rate is less dependent on water content than previously.

During the period of observation in this study, much of the respiratory capacity depended on de novo synthesis of the respiratory enzymes and associated particulate systems. If osmotic stress reduces enzyme synthesis, then the relationship of moisture content to respiration would be indirect through the effect of hydration on respiratory enzyme synthesis rather than directly through the functioning of the respiratory system. Thus the relationship as evaluated would not be absolute.

Further variation in the data was expected as a result of the

effect of increasing moisture stress on the depression of moisture content. This often resulted in observations of respiration at similar moisture contents but representing a considerable range in time depending on the level of osmotic stress imposed. It was therefore surprising that the simple linear model accounted for 82 percent of the variation observed in the data.

Examination of the moisture content and respiration curves and the relationship of respiration to moisture content leads to an interesting question. Does respiration decrease under stress due to decreased moisture content or does moisture content decrease due to decreased respiration? For both, the differences imposed by moisture stress are initially very small but tend to increase as germination proceeds. The answer cannot be determined on the basis of this study, however some conjecture is in order. Respiration during the early stages of imbibition and stratification does not depend on the synthesis of enzymes, but largely represents activation of a preformed respiratory capacity (86, 123, 140, 141). If the respiration rate during these early stages is highly dependent on the hydration state of proteins, the availability of water for hydrolytic reactions, or the diffusion rate of gases as suggested by some researchers (65, 97), then the small differences in moisture content during these stages would directly influence differences in respiration.

Further increases in respiration during germination are dependent on growth and the synthesis of enzymes. It is proposed that during this stage moisture content acts indirectly by affecting protein synthesis and thereby the level of respiratory enzymes. While imbibition is largely a physical process, subsequent moisture uptake apparently is an aerobic process requiring metabolic energy (95, 96, 99). Thus reduced water uptake in later stages under osmotic stress could be a result of reduced respiration rates and reduction of the osmotic value of the seed as a consequence of pre-emergence respiration (39).

Isocitratase Activity

The relationship of endosperm total soluble protein content to moisture stress for seed from the two sources is shown in Figure 12 and the relationship of specific activity and total activity of isocitratase in the endosperm to moisture stress is shown in Figure 13. Two factor analyses of variance of source and stress for total soluble protein content and specific and total activity of isocitratase are summarized in Table 7.

The results of the analyses indicated that the inland source has a higher average total soluble protein content than the coastal source. Averaged over both sources, moisture stress did not affect soluble protein content, but seed of the two sources did respond differently

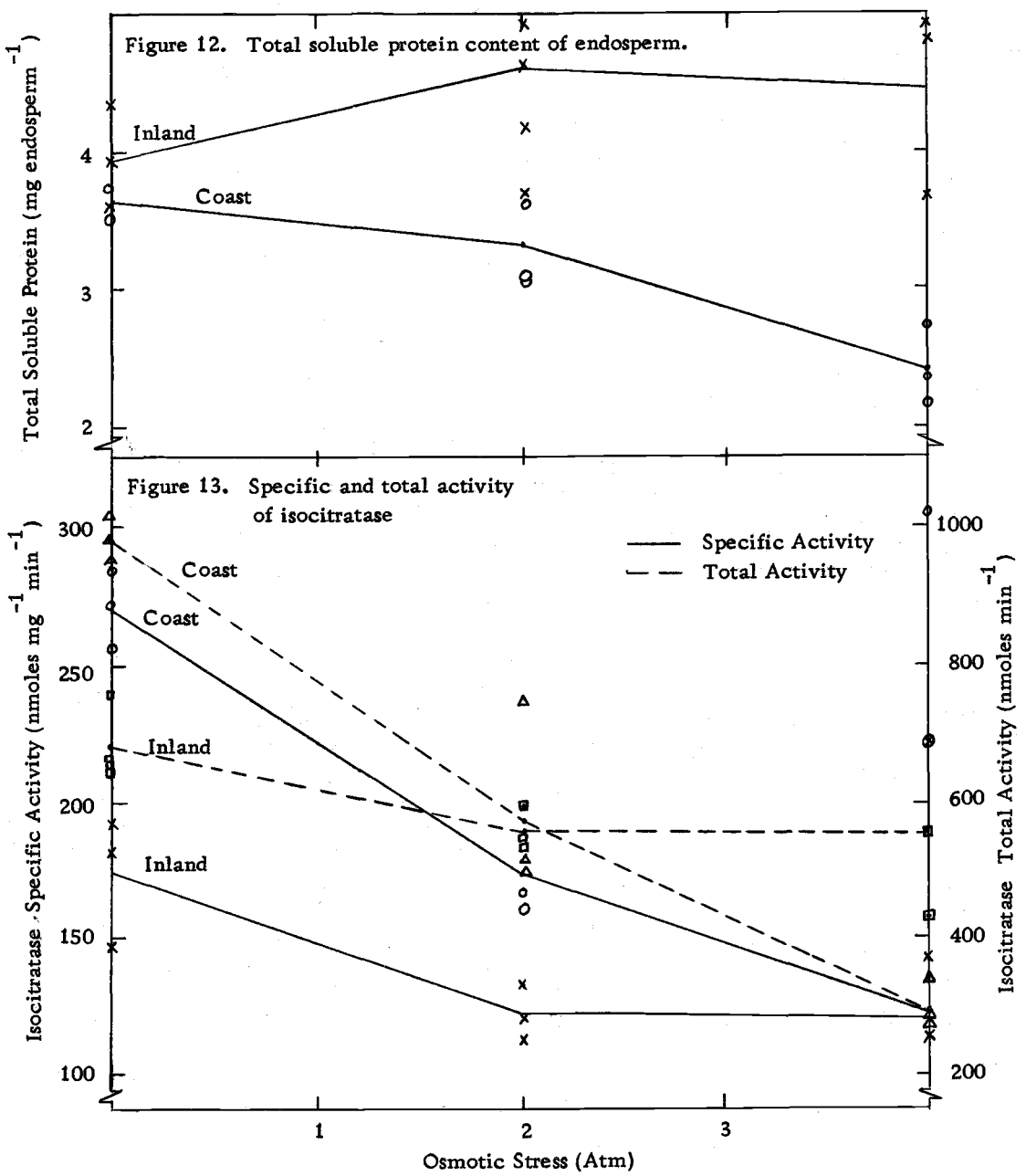


Figure 12. Total soluble protein content of the endosperm of four-day old germinants of a coastal and an inland seed source as a function of osmotic stress between 0 and -4 atmospheres.

Figure 13. Specific and total activity of isocitratase from the endosperm of four-day old germinants of a coastal and an inland seed source as a function of osmotic stress between 0 and -4 atmospheres.

to stress. The indication was that soluble protein content of the coastal source decreased with stress while that of the inland source increased. Decreases in soluble protein content with increasing moisture stress are common in plants (38, 56, 61, 131, 132, 143). The increase in soluble protein content observed in the inland source might be explained by its genetic capability for synthesizing protein under stress. The higher initial content in the inland source is undoubtedly related to the larger seed size.

Table 7. Summary of Analysis of Variance for Total Soluble Protein, Specific Activity, and Total Activity of the Endosperm of Four-Day Old Germinants of Two Sources Under Three Levels of Osmotic Stress

Source	d.f.	Total Soluble Protein F Ratio	Specific Activity F Ratio	Total Activity F Ratio
Source	1	41.391**	50.045**	<1
Stress	2	2.538	74.694**	41.948**
Source x Stress	2	7.152**	16.211**	19.141**
Error	12			

The specific activity of isocitratase was different for the two sources averaging 189.14 nmoles mg^{-1} min^{-1} for the coastal source and 140.38 nmoles mg^{-1} min^{-1} for the inland source. Because of the higher total soluble protein content in the inland source, the total activity was statistically the same for both sources. Both specific activity and total activity declined with increasing stress with the

coastal source having a greater rate of decrease. A study of enzyme activity in wheat leaves during moisture stress demonstrated an increase in total peroxidase activity per unit protein with water stress (131). Increases in hydrolytic reactions under moisture stress are known (44, 90, 128), and it may be expected that various enzyme systems respond differently to stress.

Isocitratase is a key functional enzyme in the endosperm involved in the degradation of fats to sugars before translocation to the embryo for growth. It is synthesized during germination (40, 75, 86, 140) and therefore is related to the growth potential of the seed and should be extremely sensitive to environmental conditions during germination. A decreased specific activity means that the proteins synthesized have less functional enzyme as isocitratase or that this enzyme is less efficient due to, for example, loss of active sites under a less hydrated condition. The specific activity of isocitratase decreases with osmotic stress in both seed sources. However, the mean decrease is 54 percent between 0 and -4 atmospheres in the coastal source and only 29 percent in the inland source. This ability in maintaining the specific activity of a beneficial enzyme might be the key difference between drought resistance and drought sensitivity during germination.

Nucleotides and Nucleic Acids

The change in soluble nucleotide, RNA, and DNA contents of the embryo during germination are shown in Figures 14a, 15a, and 16a for the coastal source and 14b, 15b, and 16b for the inland source. The three factor analyses of variance for source, time, and stress for nucleotides and nucleic acids are summarized in Table 8.

Table 8. Summary of Analysis of Variance for Soluble Nucleotide, RNA, and DNA Content of Embryo from Two Seed Sources Under Three Levels of Osmotic Stress

Source	d.f.	Nucleotide F Ratio	RNA F Ratio	DNA F Ratio
Source	1	11.638**	68.809**	5.097*
Time	4	125.514**	171.200**	84.343**
Stress	2	22.820**	70.068**	11.783**
Source x Time	4	1.431	7.012**	2.946*
Source x Stress	2	<1	<1	1.042
Time x Stress	8	2.854**	16.449**	4.279**
Source x Time x Stress	8	1.368	2.693*	<1
Error	60			

The soluble nucleotide, RNA, and DNA contents on the average were different for the two sources. In all cases, the mean contents of the inland source were higher which probably reflected the larger seed size. This is indicated by the lack of a significant source x stress interaction combined with the higher values at 0 atmospheres of stress. Both nucleotides and nucleic acids increased with time and the mean contents were reduced by stress. Averaged over

Figures 14a and 14b. Embryo soluble nucleotide content of a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -2, and -4 atmospheres of osmotic stress.

Figures 15a and 15b. Embryo RNA content of a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -2, and -4 atmospheres of osmotic stress.

Figures 16a and 16b. Embryo DNA content of a coastal and an inland seed source after 24 hours of imbibition and during germination under 0, -2, and -4 atmospheres of osmotic stress.

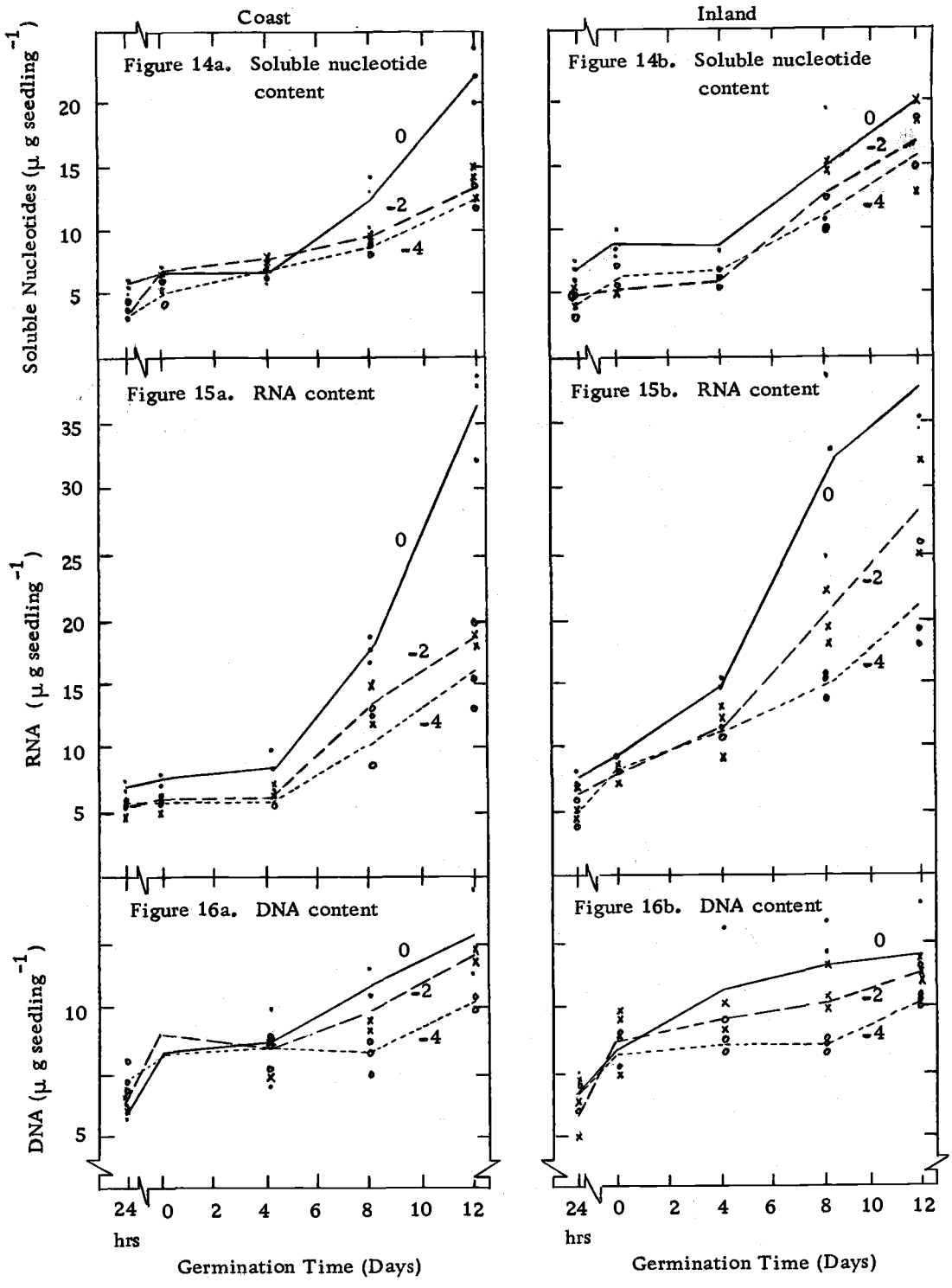


Figure 14a. Soluble nucleotide content

Figure 14b. Soluble nucleotide content

Figure 15a. RNA content

Figure 15b. RNA content

Figure 16a. DNA content

Figure 16b. DNA content

stress, RNA and DNA contents were different for the two sources through time. Averaged over seed source, nucleotides, RNA, and DNA contents responded differently to stress through time. This apparently is a result of an increasing difference in contents between stresses with increasing time.

Decrease in RNA and DNA content under stress have been shown for other species (14, 37, 61), but changes in the soluble nucleotide content have not generally been observed. The attenuation of the rate of DNA increase induced by moisture stress coupled with the observed delayed emergence and reduced growth of the seedlings appeared to indicate a reduced mitotic index.

Whether the reduced RNA content is due to increased RNase activity as proposed by Gates and Bonner (37) for tomato or to partial inhibition at the level of RNA synthesis (transcription level) as proposed by Sturani, Cocucci, and Marre (130) and Chen, Sarid, and Katchalski (18) cannot be ascertained from this study. Increased RNase activity should be reflected in slightly increased soluble nucleotide content. However RNase acts only on messenger RNA which comprises only a small fraction of the total RNA content. Some other factor must be found to explain the relatively large reduction in total RNA content through time.

It is difficult to determine cause and effect relationships in this instance, however the parallel effect of moisture stress on soluble

nucleotides, RNA, and DNA suggests that there may be some relation between them. For example, the size of the nucleotide pool, that is the soluble nucleotide content, undoubtedly affects the rate and amount of nucleic acid polymerase activity. The reduced nucleotide content would therefore directly account for reduced RNA and DNA levels. If reduced nucleotides are the cause of decreased nucleic acids, then the cause of reduction in nucleotide content must be determined. The reduced rate of respiration observed could conceivably indicate a reduced rate of synthesis of nucleotides along with other compounds utilizing carbon skeletons produced via the glycolytic, pentose phosphate, and TCA cycle pathways. This further would indicate a reduction in the energy supply from ATP produced by phosphorylation.

On the other hand, reduction in protein synthesis induced by moisture stress could reduce the level of nucleic acid polymerases and enzymes participating in nucleotide synthesis. Reduced enzyme synthesis could lead to a simultaneous reduction in the amount of nucleotides synthesized and the amount of nucleic acids polymerized into RNA and DNA.

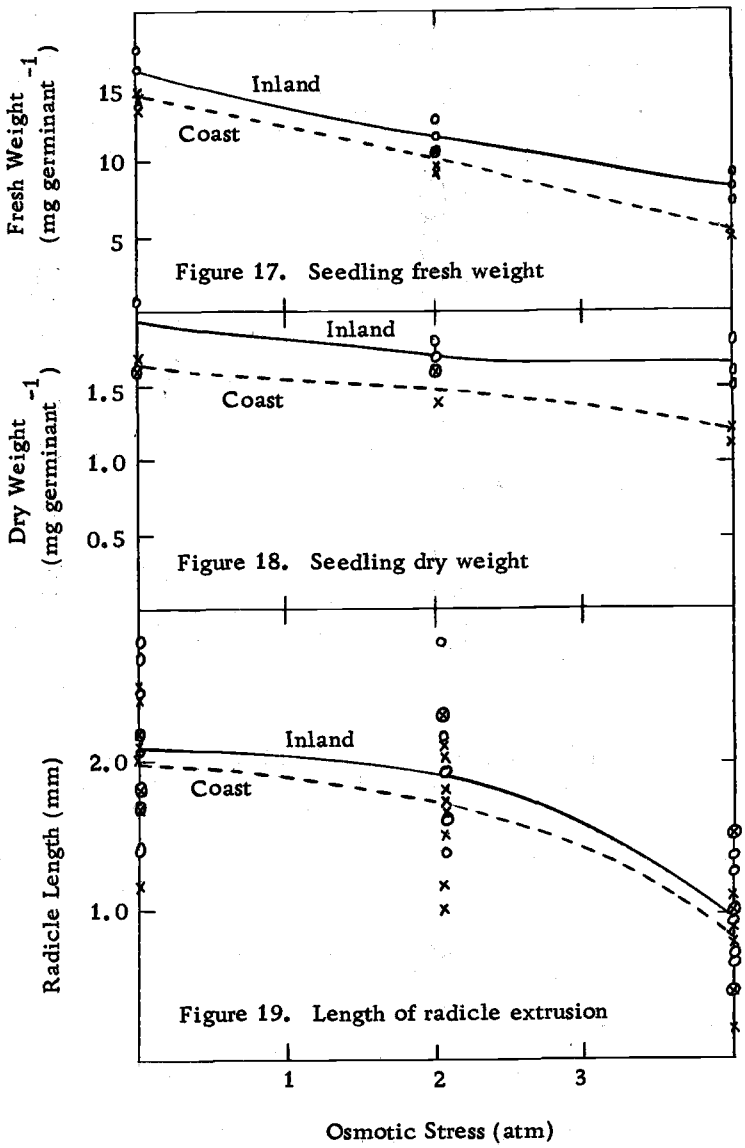
Growth

Fresh and dry weights and length of radicle extrusion of four-day old germinant seedlings expressed as a function of osmotic stress are shown in Figures 17, 18, and 19 for both sources. Two factor

Figure 17. Seedling fresh weight of four-day old germinants as a function of osmotic moisture stress between 0 and -4 atmospheres for a coastal and an inland seed source.

Figure 18. Seedling dry weight of four-day old germinants as a function of osmotic moisture stress between 0 and -4 atmospheres for a coastal and an inland seed source.

Figure 19. Length of radicle extrusion from the seed coats of a coastal and an inland seed source as a function of osmotic moisture stress between 0 and -4 atmospheres.


Fresh Weight -1 (mg germinant)
15
10
5
Inland
Coast
Figure 17. Seedling fresh weight
Dry Weight -1 (mg germinant)
1.5
1.0
0.5
Inland
Coast
Figure 18. Seedling dry weight
Radicle Length (mm)
2.0
1.0
Inland
Coast
Figure 19. Length of radicle extrusion
1
2
3
Osmotic Stress (atm)

analyses of variance are summarized in Table 9 for fresh and dry weight and radicle length.

Table 9. Summary of Analysis of Variance for Fresh and Dry Weights and Radicle Length of Four-Day Old Germinant Seedlings of Two Seed Sources Under Three Levels of Osmotic Stress

Source	d.f.	Fresh Weight F Ratio	Dry Weight F Ratio	d.f.	Length F Ratio
Source	1	19.431**	20.577**	1	1.882
Stress	2	99.902**	8.690**	2	41.362**
Source x Stress	2	<1	1.571	2	<1
Error	12			54	

The results indicated that the mean fresh and dry weights are higher for the inland source. Lack of a significant source x stress interaction indicates that this difference is not due to a difference in response to stress, but is probably related to a larger initial embryo size based on the higher seed weights of the inland sources demonstrated previously. Both fresh and dry weight were depressed by increasing moisture stress, but the response was parallel in both sources. Apparently decreased fresh and dry weights with increasing stress observed in earlier stages persisted at least until four days after radicle extrusion.

Radicle length was decreased by increasing osmotic stress but both sources responded the same and had statistically identical mean lengths.

Reductions in seedling fresh weight, dry weight, and growth

with moisture stress have been demonstrated for other species (33, 43, 72, 101, 135, 136). The most pertinent study using ponderosa pine (72) showed a growth response consistent with that found in this study for Douglas-fir. Fresh weight, dry weight, and length vary inversely in a nonlinear manner with increasing stress which confirms results with winter wheat seedling growth (43) and ponderosa pine root penetration (72). The rate of decrease in radicle length increased with each increment of stress in the range of 0 to -4 atmospheres.

Reduced growth may arise from reduced elongation, reduced cell division, or a combination of these. The reduced dry weight with increasing stress coupled with observed decreases in DNA synthesis indicated that reduced cell division was at least partially involved.

DISCUSSION AND CONCLUSIONS

An environmental stimulus triggers the unfolding of a genetically programmed sequence of events leading to a change in the physiologic state of the organism. This stimulus may act through changes in a hormonal balance, in enzyme substrate and product relations, or in DNA repression. In any case, the effect will be on processes at the phase change, transcription, translation, or assembly level of control and will be manifest in changes in enzyme activities.

The process of germination is most strongly affected by the relative and absolute levels of temperature, light, and water and deficiencies of one frequently may be compensated in part by a proper balance of the other two. With temperature and light constant, osmotic moisture stress during imbibition and germination of Douglas-fir affected a variety of processes examined in this study. Any hypotheses advanced to explain the effects of osmotic moisture stress under the study conditions must be consistent with the following observations: 1) the shift from a dormant embryo to an active seedling is delayed and at some stress greater than -8 atmospheres probably is completely inhibited; 2) rates of water uptake, oxygen uptake, and carbon dioxide are reduced with increasing osmotic stress and the observed differences due to stress tend to increase

with time; 3) enzyme synthesis in the endosperm as measured by isocitratase activity is reduced with increasing osmotic stress; 4) endosperm total soluble protein content in four-day old germinants increases with osmotic stress initially in the drier site ecotype and decreases in the moister site ecotype; 5) nucleotide and nucleic acid levels are reduced with increasing osmotic stress; 6) growth as measured by radicle length of four-day old seedlings is reduced with increasing osmotic stress; and 7) there are observed differences in these responses between moist and dry site seed sources.

One of two general explanations is possible: either osmotic stress directly affects one or two processes which ultimately indirectly affect the others or it acts independently in a direct manner at each level. The first involves determination of perhaps a single effect which would produce all of the others while the second requires determination of a complex set of effects to explain each of the observations. The concept that moisture stress affects each level of control independently is difficult to reconcile due to the interactions and interdependency of the processes at each level, and consequently the plausibility of an effect at one level producing the observed results at the others. Further, a hypothesis of this nature is more complex and assumes a greater degree of independence of cellular processes than actually exists. It is therefore believed that the first general explanation is more satisfactory and provides the

basis for further discussion.

Two general stages during germination should be recognized. The first is characterized by the predominance of reactivation and functioning of preformed systems, and the second by increased capacity of these systems and establishment of additional ones by de novo synthesis. The fact that moisture content, dry weight, respiration, and nucleotides and nucleic acids change very little during stratification of Douglas-fir as shown by this study and that of Ching (24) indicates that the second stage does not dominate until after the first few days of germination conditions and coincides with the initiation of cell division. It is proposed that during the initial phase, osmotic stress reduces the amount of water physically imbibed and the resulting reduced degree of hydration directly affects the reactivation process. During the second phase, osmotic stress acts indirectly through changes in one or more vital processes induced during the first phase.

At least two major changes induced by moisture stress during the reactivation phase are likely and either is sufficient to explain the majority of effects observed during the synthesis phase. The first is concerned with the reactivation of the protein synthesis system and the second with reactivation of respiration.

Reconstruction of the ribosomal system may be an absolute requirement for the progress of germination (86). Requisite to this

is the activation or formation of messenger RNA during imbibition (81). The most recent evidence suggests that moisture stress affects protein synthesis during germination probably through this messenger RNA (18, 38, 130). Genkel', Satarova, and Tvorus (38) with corn and beans, Sturani, Cocucci, and Marre (130) with castor bean, and Chen, Sarid, and Katchalski (18) with wheat conclude that moisture stress does not affect the ribosome system; rather it causes inactivation or destruction of messenger RNA.

Based on observations from other species (19, 31, 142, 144), it is reasonable to assume that early protein synthesis during imbibition and perhaps stratification is under the direction of a conserved messenger RNA. This stable RNA is undoubtedly synthesized during the late stages of seed formation and may be activated by imbibition (19, 31, 142). It is also reasonable to assume that the proteins coded on this RNA are essential to the normal progress of germination. Interruption of the decoding process by inactivation or destruction of messenger RNA could conceivably prevent subsequent activation of the genome essential for continued development. The degree of inactivation, destruction, or both may depend on the relative hydration either directly or indirectly if a hormonal balance is involved. Thus osmotic moisture stress would influence the effectiveness of this conserved messenger RNA through its influence on the degree of hydration attained.

Acceptance of a stable messenger RNA component in Douglas-fir seed is not essential to the hypothesis that moisture stress affects protein synthesis. If germination of Douglas-fir seed involves the synthesis of new messenger RNA as a prerequisite, then reduced hydration may lead to either a partial or total repression of the DNA or a shift in the kind and amount of protein coded through changes in repression. The first would result in a reduction or absence of messenger RNA and the second in either false coding or a change in the programmed sequence of events leading to germination. Results of this study and others concerned with different species (18, 19, 31, 38, 130, 142, 144) do not provide a basis for determining which effect might be operating in Douglas-fir seed.

If the assumption that protein synthesis is reduced is correct, other factors besides DNA and messenger RNA may be involved. For example, reduced cell hydration may result in a higher ionic concentration and thereby prevent the formation of polysomes which requires a specific concentration of magnesium, for instance. However, present evidence from wheat (18) and castor bean (130) seeds indicates that polysomes are formed and phenylalanine is incorporated under moisture stress in the presence of an exogenous supply of RNA, poly U. This suggests that the ribosomes remain functional and the necessary cofactors are present but polysomes cannot form due to the absence of messenger RNA.

Acceptance of a primary effect of moisture stress during germination on protein synthesis could account for effects observed during the synthetic phase. Moisture stress may initially affect reactivation of preformed systems by reducing cell hydration. Differences during this phase would tend to be small and would most likely be related to the effect of increasing stress on the number of cells reaching a hydration level sufficient for functioning. During the synthesis phase, the continued reduction in protein synthesis would be manifested in a reduced activity of catabolic and synthetic pathways with a progressive delay in the unfolding of the genetically programmed germination sequence. This would lead to increasing differences in the response of control and stressed treatments as the observed capacities become more and more dependent on de novo synthesis and the initiation of functions are delayed. Change or curtailment of the sequence would ultimately lead to a delay or inhibition of the germination process. This postulated effect is diagrammed schematically in Figure 20 in relation to the results observed in this study.

This hypothesis is consistent with the observed changes in respiration, nucleotides, nucleic acids, and isocitratase activity. However, it does not explain the increase in total soluble protein content observed in the four-day old germinant endosperm of the inland seed source. The nature and content of this protein fraction

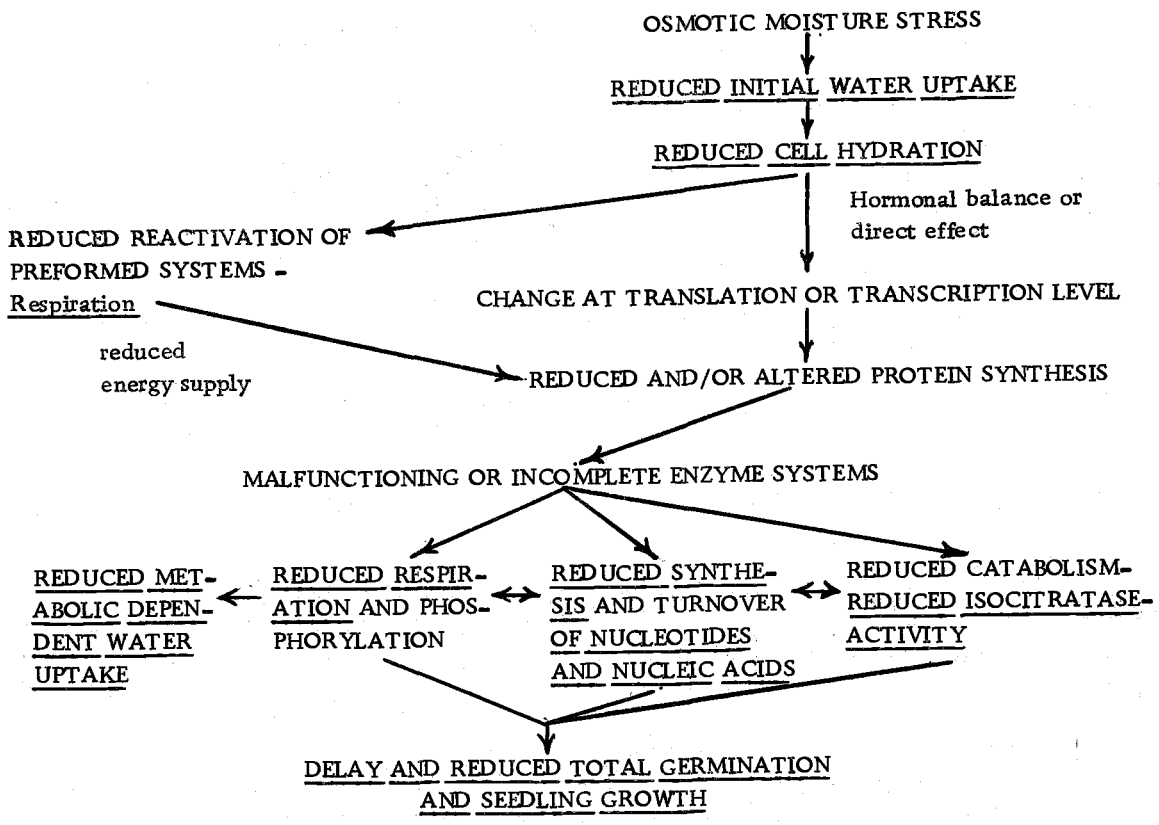


Figure 20. Diagram of proposed effect of osmotic moisture stress during Douglas-fir germination acting through reduced protein synthesis. (Underlining indicates observed effects)

is unknown and therefore it is difficult to determine the cause for the observed increase. In fact, some of the free amino acids might react with the Folin reagent and result in an overestimation of the soluble protein content. If the observed increase arises from an initial accelerated rate of stored protein breakdown, it does not necessarily negate the hypothesis. If, however, it results from protein synthesis, it casts some doubt on curtailment of protein synthesis by moisture stress in at least the inland seed source. In this case, the protein may be "nonsense" polypeptides formed by false coding of messenger RNA as shown in drought sensitive wheat seed (18) or it may possess a composition different from that of the control as a result of a change in DNA repression. Replacement of normal RNA with an altered one may lead to decreased growth in response to water stress as indicated for corn seedlings (143).

The second major change induced by osmotic moisture stress is the reduction of respiration rate. As observed in this study, the basic metabolism probably is not altered as the respiratory quotient is not influenced by stress. Thus the relative proportions of the TCA and glyoxylate cycles are not altered. This does not negate the possibility of a change in the relative contribution of the pentose phosphate and glycolytic pathways as shown for corn and horse bean plants (104), however. Further, the effect of reduced hydration probably is manifested during imbibition by decreasing the level of reactivation

of the respiratory system. Water uptake during imbibition probably contributes toward a proper intracellular environment necessary for the functioning of the particulate enzyme units (123) and toward protoplasmic hydration (68). Reduced hydration may then have a direct effect on the respiratory capacity during the reactivation phase of germination.

Continued depression of respiration may indirectly reduce the level of other activities during the synthesis phase of germination. Enzyme synthesis requires the presence of ribosomes, various co-factors, messenger RNA, energy, and an activated amino acid pool (47). It is through these last two that dehydration may readily act. Dehydration may inhibit the accumulation and transformation of energy directly by increasing protoplasmic viscosity (44) or indirectly by reducing respiration. Depressed ATP synthesis has been shown in germinating corn seedlings subjected to moisture stress (143) and may be a factor in Douglas-fir. Each major synthetic route for an amino acid branches off from an intermediate of either the glycolytic or TCA cycle pathways (36). However the majority of amino acids arise from degradation of protein bodies during this stage of germination. Reduced respiration may then directly lead to a reduced amino acid pool in the long run, and by reducing the energy level, a decrease in the amount of amino acids activated in both the short and long run. Thus increases in activity dependent on de novo

synthesis of enzymes may be reduced through a reduced respiratory dependent energy and amino acid turnover.

Other synthetic pathways requiring metabolic energy or intermediates from the respiratory pathways may similarly be reduced. If the primary role of osmotic moisture stress during germination is to reduce respiration, then the effects observed in this study might arise as diagrammed in Figure 21.

No direct evidence for the protein synthesis or respiratory capacity hypothesis or some combination of these is available from this study. However, it is believed that the many observations are, in the main, in agreement with either of them in at least the coastal seed source.

Thus far, no attempt has been made to explain the difference in response between the two seed sources. The fact that the ultimate response is more prompt germination for the drier site inland source than the moister site coastal source under identical stresses would indicate that some adaptation to moisture stress during germination exists in this source. The trends in response observed were similar for all factors studied except total soluble protein content of the endosperm and specific and total activity of isocitratase. The higher mean values for the inland source may be a partial result of a larger initial seed or embryo size where the measured response is higher for this source at 0 atmospheres stress and a significant source x

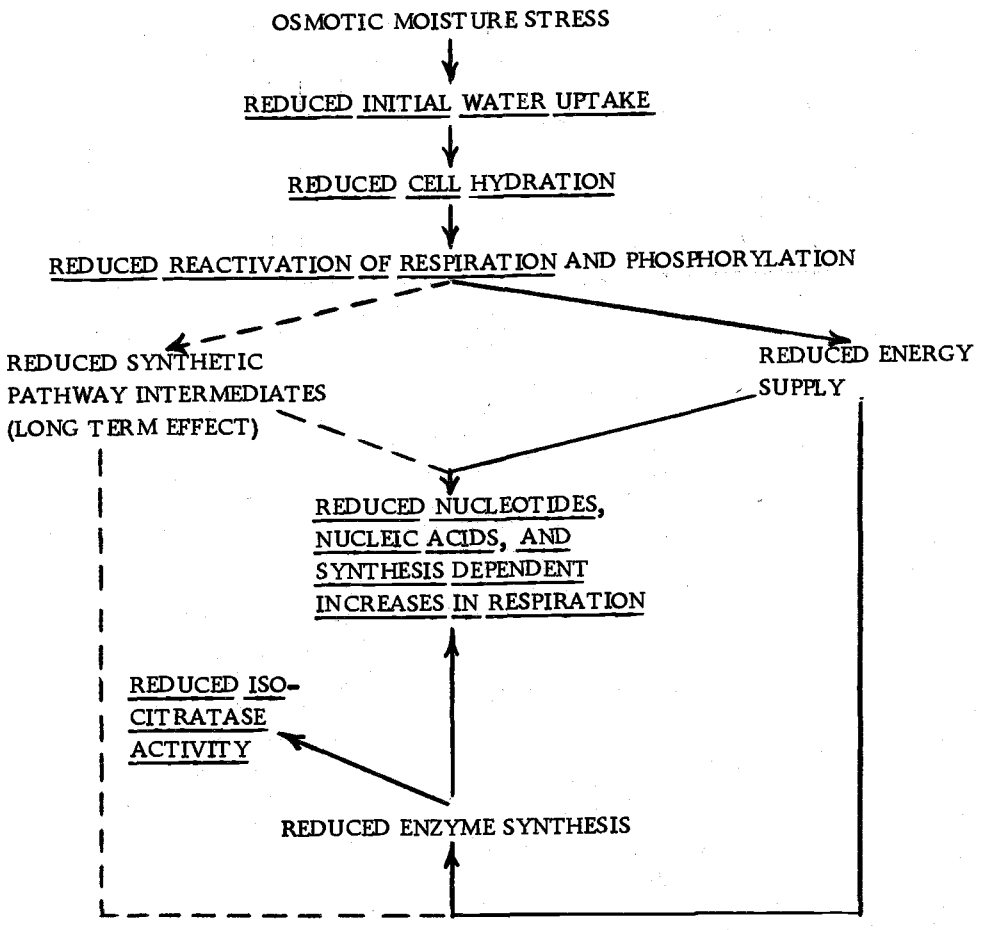


Figure 21. Diagram of proposed effect of osmotic moisture stress during Douglas-fir germination acting through reduced respiration. (Underlining indicates observed effects)

stress interaction is absent. However, at a more fundamental level, the inherently higher level of hydration under stress in the inland source probably allows a greater degree of reactivation of respiration and protein synthesis than in the coastal source seed. This effect would carry over into the synthetic phase providing for a higher rate of protein synthesis under stress.

At identical moisture contents (not stresses) and stages of germination, the inland seed source may respond identically to the coastal seed source. For example, this is indicated by the similarity between the respiration rate versus moisture content relationship shown in Figure 11. However, some adaptation in moisture uptake provides a higher degree of hydration in the inland source at identical osmotic stresses. Thus the existence of a significant source x stress interaction may reflect a difference in hydration response rather than an inherently different capacity to respond to reduced hydration. Ideally, the comparison should be between identical hydration levels for a more valid test of true drought resistance. This, of course, assumes that the relative degree of hydration required to attain a sufficient intracellular environment is similar for both seed sources.

Maximov (89) maintained that xerophytic plants tend to have higher osmotic values. If a higher imbibitional water uptake is characteristic of the inland source, it may be directly related to a

higher osmotic value of the cells of the endosperm or embryo. Apparently seed coat characteristics are less important in Douglas-fir than in other species such as alfalfa (110, 149) except for transitory water storage (24). Rate of water transfer may be more rapid through the seed coats of the inland source, but this may be a function of a larger diffusion pressure gradient across the diffusion pathway.

The major objection to the two hypotheses advanced for the effect of moisture stress and the hypothesis concerning the nature of seed source differences is that they do not explain the opposite responses in total soluble protein content of the endosperm in both seed sources. The hypotheses in general are consistent with a decrease in total soluble protein content as shown for the coastal seed source. However, the inland source shows an increase between 0 and -2 atmospheres followed by a slight decline. As mentioned previously, this protein increase may represent accumulation of amino acids as a result of decreased protein synthesis or accelerated protein synthesis. The response to moisture stress was similar in both seed sources for all of the other factors examined. Thus the major differences in endosperm total soluble protein content and isocitratase activity may provide the key to resistance to moisture stress during germination.

Fat and protein bodies occupy about 85 percent of the volume

and 90 percent of the weight of endosperm tissue of Douglas-fir (24). Solvation of protein, degradation of storage protein, and active transport and synthesis of new protein occur in the endosperm during the early stages of germination under normal conditions (24). The higher endosperm moisture content of the inland source under stress may provide a higher level of reactivation of protein body degrading enzymes. At the same time, the reduced cellular hydration may interfere with transport of the soluble material to the embryo and utilization of the material locally in enzyme synthesis. This would lead to an accumulation of amino acids until increasing stress reduced hydration sufficiently to adversely affect the reactivation of the degrading enzymes. At this point, amino acid and soluble protein content would decrease as it does in the inland source above -2 atmospheres.

Rather than an accumulation of amino acids, the higher soluble protein content in the inland source may indicate a higher level of enzyme synthesis, particularly of those enzymes involved in the degradation of storage materials. The response of isocitratase activity to osmotic stress is indicative of this possibility. While the specific activity of this important enzyme in the glyoxylate cycle is reduced by stress in both seed sources, the mean decrease is 54 percent in the coastal source and only 29 percent in the inland source between 0 and -4 atmospheres. This smaller rate of decrease in

activity in seed from the inland source may be the result of an ability to synthesize this enzyme at a relatively higher rate under stress or an ability to maintain the efficiency of the enzyme under reduced hydration. After germination, the respiration rate is lower in the inland source than in the coastal source. Thus, if enzyme synthesis is involved in the inland source, it occurs with a greater efficiency in energy utilization. While the RNA content is decreasing in a similar manner in both seed sources, synthesis of isocitratase is declining more rapidly in seed from the coastal source, perhaps indicating a difference in RNA coding or utilization between the sources. In any case, this ability to maintain the specific activity of key enzymes, either by synthesis or by maintenance of enzyme efficiency (for example, by maintaining the conformation of active sites), may be the difference between resistance and sensitivity to moisture stress during germination.

Of course the results of this study cannot be directly extrapolated to field conditions as the study used a highly artificial environment. It does seem reasonable to assume that delays in germination and reduced seedling growth imposed by moisture stress will reduce the probability of field establishment. In a dry environment, adaptation to moisture stress should be a positive selection force and the amount of variation in response to drought should be relatively uniform. In a moist environment, adaptation to drought

has a neutral selection value. Therefore, such adaptations are neither selected for or against and normal survival does not depend on their existence. In this situation, adaptation is not rigidly enforced and variation in response to drought is likely to be large.

Osmotic moisture stress during germination of Douglas-fir resulted in reduced moisture uptake, reduced respiration, reduced nucleotide and nucleic acid content, reduced seedling growth, and reduced isocitratase activity in both a coastal and an inland seed source. The observed level of a factor was generally higher in the inland source and it is proposed that this was a result partially of a larger initial seed size, a greater ability to imbibe water under osmotic stress, and a greater ability to maintain the specific activity of key enzymes either by synthesis or maintenance of active conformation. Further, the observations are consistent with moisture stress acting through reduced protein synthesis, reduced respiration, or a combination of these. This effect is established during imbibition by preventing complete reactivation of the enzyme and respiratory systems.

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