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RECENT ADVANCES IN SEED PRESERVATION TECHNIQUES

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ABSTRACT

Although seed longevity and storage have been of interest to humankinsd since the beginning of agriculture, the systematic collection and long-term preservation of seed germplasm is only a relatively recent practice of the 20th century. Seed longevity claims of thousands of years have not been verified by the scientific community However, seed viabilities in the range of a few hundred years have been well documented. Even some vegetable seeds, which have been thought to survive only a few decades, have recently been shown to germinate after more than 50 years of storage. The search for physiological, biochemical, and/or biophysical mechanisms of seed deterioration has begun to shed new light on how seeds lose their vigor and germinability. Some studies of particular interest have focused on the integrity of cell and organelle membranes, the fragility of DNA and chromosomes, and more recently the role of water in controling longevity at various temperatures. In the case of membrane integrity, we have learned that membranes undergo phase transitions between the gel and liquid crystalline configurations, depending upon the moisture content; and, that this can be controlled by temperature. The genetic integrity of a seed or a population of seeds can be altered considerably during seed aging and deterioration, thus resulting in loss of variability or genetic shifts in populations. A better understanding of the interrelationships between seed moisture content and storage temperature has just recently shed new light on the potential longevity of orthodox seeds. This understanding of deterioration may lead to the rescue of aged seeds and the retrieval of valuable genes once thoughtlost. In addition to the factors of moisture content and temperature, the seed developmental stage, is now known to be vitally important in the preservationof recalcitrant seeds. In

this day, modern genebanks cannot limit themselves to orthodox seed preservation alone.

INTRODUCTION

I am indeed proud and honored to have been invited to participate in the dedication of the new National Plant Genetic Resources Center here in Taichung. This new genebank is certainly one of the most modern and beautiful buildings in the world dedicated to the preservation of plant germplasm. The Council of Agriculture was wise in choosing to locate this Center here on the campus of the Taiwan Agricultural Research Institute as it provides the leadership and research atmosphere to insure a strong and fruitful program in acquiring and preserving your valuable plant genetic resources. I congratulate you all and wish you the greatest success in the future.

In discussing recent advances in plant germplasm preservation, I would like to review current information on seed longevity, deteriouation, cryopreservation, storage protocols, image analysis techniques, seed rescue and include a brief discussion on preservation of non-seed germplasm. Much of this work is being done at the National Seed Storage Laboratory in Fort Collins, Colorado and i hope this presentation will provide stimulus for scientific exchange and cooperation between our laboratory and this new genebank in Taiwan.

SEED LONGEVITY

We can only imagine the great excitement generated by the *Gardener's Chronicle* article which appeared November 11, 1843, describing the germination of 3000 year old wheat seeds, presumably taken from a tomb in Egypt (Anonymous, 1843; Priestley, 1986). Such claims of extreme longevity have appeared frequently in the popular press without any scientific proof of authenticity (see review by Roos, 1986, 1989). The longest claimed longevity was for seeds of *Lupinus arcticus* (Table 1) which were said to have still germinated after 10,000+years buried in the frozen tundra of Alaska (Porsild et al., 1967). However, as with the 'mummy wheat' seeds, no compelling scientific supporting evidence was presented to document the claim. The subject of seed longevity is a fascinating one and it becomes more intriguing when results of actual experiments are examined, such as the reports of the Beal buried seed experiment (Kivilaan

and Bandurski, 1981) in which weed seeds buried in the soil were dug up after 100 years and found to still have some viability.

Longevity experiments: I have been fortunate to have become involved with two long-term storage experiments. The first involves a continuation of a vegetable seed longevity study first reported on by James et al. (1964). Seeds of 15 vegetable seeds were germinated after storage for 15 to 30 years in an office in Cheyenne, Wyoming. Retesting some of these same lots 29 years later showed that some viability was still present after 50 to 60 years (Roos and Davidson, 1992). the greatest reported longevity for most of these species (Table 2).

The second experiment, initiated by Went and Munz (1949). concerend storage of 98 species and varieties of California desert plants. Recently I was able to obtain the actual residual seeds of this experiment from the Rancho Santa Ana Botanical Garden in San Diego, California where they have been stored unnoticed for several years. These seeds, vacuum sealed in glass vials, have been transferred to the National Seed Storage Laboratory and will be tested in the coming year. Of some significance is that, to my knowledge, they have never been stored under sub-freezing conditions.

The advent of modern seed genebanks, such as the one here in Taiwan, present the interesting challenge to preserve our seed genetic resources indefinitely, under optimal conditions as a safeguard for the future of all peoples of the world. The knowledge of the past will be enhanced and transferred to future generations.

SEED DETERIORATION RESEARCH

Several theories have been advanced to explain why seeds deteriorate, even under relatively good storage conditions. These have been discussed previously (Roos, 1989) and are summarized here.

Depletion of Food Reserves: This theory suggests that over time essential substrates become depleted. The major food reserves (storage carbohydrates, proteins, and lipids) may show minor losses over time as a result of respiration. However, examination of non-viable seeds shows that plenty of reserve food remains. Also, we now know that seeds stored at low moisture contents(less than 8%) have essentially no mitochondrail activity. Thus, depletion of food reserves by mitochondrial path ways is likely minimal. Loss of enzyme cofactors (vitamins and minerals) has not been linked with loss of viability.

Alteration of Chemical Composition: Although food reserves may not be depleted, they may be altered chemically so that they are not in a usable form. For example, Crocker and Groves (1915) suggested that seed death resulted from a "coagulation of protein." Also it is well known that lipids undergo oxidation, and an increase in fat acidity has been associated with deterioration. Proteins also underge changes as seen by decreased solubility, partial breakdown, and decreased digestibility. Many other compounds may change quantitatively or qualitatively during storage.

Membrane Alteration: The theory that membrane alteration causes deterioration has its basis in the observation that aged seeds have a great propensity to lose cell constituents upon imbibition. This "leakiness" is attributed to a loss of membrane integrity. Although the exact mechanism causing the membrane alteration is not known, speculation surrounds the role of peroxidation of unsaturated fatty acids. In essence this theory could be considered a corollary to the previous theory. Much of the current research on seed deterioration is concentrated on examining membrane alteration.

Enzyme Alteration: Duvel (1902), in his Ph.D. dissertation, and later White (1909) proposed that respiration and enzyme activity were important in the preservation of vitality. Many enzymes have been studied in an attempt to correlate seed viability with the amount of enzyme activity. The basic problem with regard to loss of enzyme activity seems to be that of cause and effect. Is loss of enzyme activity the cause of lost viability or is there another cause which also affects loss of enzyme activity?

Genetic Damage: Alteration of the cell's DNA, such that it is unable to duplicate and divide, could occur from either chemical mutagenesis or from ionizing radiation. It is a well-established phenomenon that deteriorating seeds produce an abundance of chromosomal aberrations as seen in root tips of germinating seeds. Seed viability has been inversely correlated with the frequency of these aberrations. Again, the mechanism for the production of the aberrations is not known. However, evidence has been advanced that the increase in chromosomal aberrations with aging, may be a result of the breakdown in the cell's natural DNA repair mechanism(s).

Research Approaches: Current research has focussed on the last three theories and I would like to present some additional thoughts on each of these, beginning with genetic changes and their potential importance for germplasm preservation. Basically there are two topics of concern. The

first involves potential damage to the DNA itself and is reflected in mutations, chromosomal aberrations and failure of replication, and/or transcription and translation of the genetic code. Most of the recent research has concentrated on the phenomenon of chromosomal aberrations, as seen in the root tips of germinating seeds. Aged seeds have long been known to produce these aberrations (Barton, 1961; Roos, 1982); however, newer research (Murata et al., 1984) has shown that these abnormal cells do not pass on the aberrations to the next generation of seeds (Fig. 1). Thus, it appears that germplasm accessions are not subject to an accumulation of these changes over successive generations.

The other genetic concern involves the potential for genetic shifts to occur in heterogeneous germplasm populations, typical for many of our landraces and wild populations. Some of my research (Roos, 1984a,b) on mixed bean populations (Fig. 2) demonstrated how rapidly such shifts can occur as a result of differential survival of various components during storage or during regeneration, and the effect of sample size. In the case of genetic shifts, it is absolutely imperative that genebank managers be aware of this and take the necessary precautions to adequately represent the diversity within and accession during regeneration. Base collections should be preserved at sub-zero temperatures and at periodic (every 3 cycles) intervals be used for regeneration of working collections.

Loss of enzyme activity in aged seeds has been studied by many researchers and is the basis for one of the most commonly used seed viability and vigor tests, the topographical tetrazolium test, which measures non-specific dehydrogenase activity. The problem with most enzyme assays is that, although good statistical correlations with seed viability may be obtained, they do not reveal the mechanism for deterioration, nor do they discriminate between the cause and effect of deterioration. The TZ test itself is very subjective and relies on the ability of the analyst to interpret the degree and pattern of staining of a tissue as it relates to seed viability.

Studies on the enzyme cytochrome *c* oxidase (CcO) may offer a new approach to the study of seed deterioration. The catalytic function of this key bioenergetic enzyme is the reduction of molecular oxygen to water, the terminal step in the mitochondrial electron transport chain of all organisms. During seed germination mitochondria and CcO activity increase dramatically, and germination cannot occur in the absence of this key enzyme. We have examined the results of various inhibitor molecules, such as carbon monoxide, nitrous oxide and deuterium oxide, on the activity of CcO and their effects on seed germination and growth of roots

of beans (Sowa et al., 1993). Perturbing the active site of the enzyme affects seedling respiration, CcO activity and growth of the root. This has led us to propose that seed aging might be caused by a decrease in the efficiency of the enzyme. Partial denaturation of the enzyme could result in release of partially reduced oxygen species. These oxyradicals might be a source of lipid peroxidation and membrane alteration (Sowa et al., 1993), which has also been advanced as a prominent theory of seed deterioration.

Recent work has shown that membrane lipids undergo reversible phase transitions between the gel and liquid crystalline configurations, depending upon the moisture content (mc); this can be controlled by temperature. These configurations are important in determining the degree of permeability for the membrane. Alterations to the lipid moiety of the membrane, via peroxidation could lead to the production of free organic acids, an increase in free fatty acids, a decrease in unsaturated fatty acids, and a general production of toxic compounds. All of these can result in overall membrane dysfunction and deterioration of other macromolecules (proteins and DNA). Once again, this theory of seed aging as a result of membrane deterioration has led to a common viability/vigor test, the seed leachate conductivity test. Initially, this test consisted of soaking 50 or 100 seeds in distilled water and measuring the conductivity of the water after 24 hours (Matthews and Bradnock, 1967; 1968). The development of an instrument to measure 100 individual seed leachates has enabled us to look at a population of seeds. Forming the data into a frequency distribution can allow us to analyze the shape of the distribution, and how this might change as seeds deteriorate (Moore et al., 1988).

NEW PRESERVATION TECHNIQUES

New seed preservation techniques have concentrated on two factors, the temperature of storage and the optimum seed moisture content for storage. Recent research has examined the use of cryopreservation, and more recently, the lowering of seed moisture content to very low levels. Am important new approach has been taken at NSSL to develop a theoretical basis for seed storage protocols.

Cryopreservation: Cryopreservation refers to preservation at cryogenic temperatures. Cryogenic, is defined, although somewhat vaguely, as "of or relating to the production of very low temperatures." One view holds cryogenic refers to subfreezing (<0 ℃) temperatures; however, water may not freeze at this temperature and can supercool to as low as -40 ℃ ,

which is the homogeneous nucleation temperature of water. In this report cryopreservation will be restricted to storage in or over liquid nitrogen (LN, -196°C).

Survival of seeds exposed to temperatures at or near that of LN has been known for nearly 100 years. In experiments of Brown and Escombe (1897), seeds of 12 species, air dried to a mc of 10 to 12%, were slowly cooled in thin glass tubes, immersed in liquid air (-183 to -192°C) and held for 110 hours. After seeds were slowly warmed (over a 50-hour period) there was no apparent effect on germination. Research conducted at the NSSL has emphasized development of seed cryopreservation technology. Questions which are important in assessing this technology include: (a) Can seeds of a given plant species withstand being subjected to LN without loss of viability? (b) Does seed mc at the time of exposure to LN affect seed response? (c) How long will seeds remain viable in LN? (d) How do costs of LN storage compare with costs of conventional refrigerated storage? (Stanwood and Bass, 1987). To these questions the following may be added: (e) Can all orthodox seeds be cryopreserved? (f) Can recalcitrant seeds be cryopreserved? (g) Does cryopreservation have any genetic effects on the seed? Some of these questions have been answered or partially answered, but others may never be answered.

A large number of orthodox seed species have been shown to survive LN exposure (Stanwood and Roos, 1979; Stanwood, 1980; Stanwood and Bass, 1981; Stanwood, 1985). Physical damage (splitting, seed coat cracking) to seeds of some species (garden beans, flax, radish, soybean and alfalfa) has been reported (Stanwood, 1985); however, careful control of cooling rate, seed mc and rewarming rate may overcome this damage. For example, bean seeds exposed only to LN vapor (ca. -150°C) showed no breakage. One of the most important factors controlling seed response to LN exposure is mc. Fortunately, for most orthodox seeds, this factor is easily controlled.

One of the most commonly asked questions concerning the adoption of cryopreservation technology for seed germplasm involves the economics of LN vs conventional storage. Stanwood and Bass (1981) attempted to analyze costs for storing onion seeds in LN vs -18°C by making certain assumptions regarding equipment and facilities, yearly operations, and seed regeneration costs. Over a 100 year period, annual costs for maintaining one accession were $1.65 and $0.42 for conventional (-18°C) vs LN storage, respectively. Thus, yearly operational costs (mainly viability monitoring) and seed regeneration costs (resulting from seed deterioration)

were much less under LN storage.

In addition to seeds, many other forms of germplasm, including pollen, dormant buds, and apical meristems are amenable to cryopreservation. Indeed, cryopreservation may be most valuable for these forms and for storage of recalcitrant seeds, for which there is presently essentially no other alternative for long-term preservation.

Theoretical Basis for Seed Storage Protocols: Genebank managers desire to know the optimum conditions for storage to maintain seed germplasm for the future. It has been known for many years that dry, cold conditions will extend longevity: however, the optimum conditions and the theoretical basis for this are poorly understood because research on long-term storage necessarily takes many years to complete. Scientists have relied on experiments done under less optimal conditions and then extrapolated their results to predict longevity under optimal conditions via mathematical equations (Roberts, 1973; Ellis and Roberts, 1980, 1981). The assumption of these equations has been that the effect of water content on the rate of seed deterioration is a logarithmic relationship and thus, the drier the tissue is, the longer it will maintain viability (Ellis and Roberts, 1980; Ellis et al., 1986; Ellis, 1988).

One way to circumvent the problems that arise from making such assumptions is to analyze the physical state of water in seeds. Such analyses indicate, on a theoretical basis, the rates of deteriorative reactions in seeds with different mc and at different temperatures. Both of these parameters affect the state of water in seeds. It is relatively easy to demonstrate that the state of water binding in seeds is dependent upon temperature (Vertucci and Leopold, 1987; Vertucci, 1990). Recently we have also demonstrated that at very low seed moisture contents the seed deterioration rate begins to increase (Fig. 3) and, thus, there is an optimum moisture content for storage (Vertucci and Roos, 1990). This has recently been addressed from a thermodynamic perspective and it is now possible to predict the optimum moisture level as a function of the temperature of drying and the eventual storage temperature (Vertucci and Roos, 1993). This has important implications for genebanks desiring very long storage for base collections, of for storage of active collections at less than optimum temperatures.

The recent suggestion of using "ultradry" storage in areas where refrigeration is not reliable (Cheng et al., 1990; FAO/IBPGR, 1993; IBPGR, 1992) is based on substituting the benefit of low temperature for that obtained by reducing moisture content further. The problem is that using

moisture content per seed as the desired parameter is somewhat meaningless because it does not indicate the activity of water (the critical parameter in deteriorative reactions). RH in the air with which the seeds are equilibrated more accurately reflects water activity. Since seeds equilibrate to different moisture contents, depending upon their chemical composition, RH might be better used to specify optimal conditions for storage (Vertucci and Roos, 1990). The problem becomes then to define what is the optimum RH and what is the effect of temperature on this value. The most significant finding of this work is that the optimum seed moisture content for a given species increases as storage temperature decreases (Fig. 4).

OTHER TECHNOLOGIES

Before concluding this presentation I would like to briefly mention a few other technologies that are emerging and should have important implications for genebank managers. These include the dramatic increase in computer imaging techniques, non-destructive testing of seed viability, the potential for rescue of deteriorated seeds, and finally the storage of high moisture tissues, such as recalcitrant seeds, dormant buds, shoot tip meristems and pollen.

Video Imaging Technologies: High speed computers are an integral part of any genebank program as they contain all the data about accessions in a form that is readily retrieved. The advent of increased data storage capacity and the software to analyze video images has opened up the prospect of storing data in an entirely new format, namely a combined picture (image) plus the usual data associated with an accession. The analysis of the image can provide such data as seedling growth rate (Howarth and Stanwood, 1993) and seed/plant characteristics (Howarth and Stanwood, 1992). Combining such data about a seed accession on a CD ROM can give the user (breeder) an objective assessment of the germplasm of interst and should assist in the collection and evaluation of new data about the genetic variability within a species.

Non-destructive Testing for Seed Viability: We are currently investigating several approaches to determine seed viability non-destructively. These include: use of seed X-ray analysis; calorimetric analyses; examining leachate conductivity of partially imbibed seeds, followed by drying back to the original mc; using infra-red (FTIR) analysis of minimally imbibed seed. Of these, the FTIR technique appears to be the

most promising and has been used to assess viability of cell suspensions and pollen (Sowa and Towill, 1991; Sowa et al., 1991), and bean seeds (*Phaseolus vulgaris*) (Sowa and Roos, 1992).

Rescue of Deteriorated Seed: Unfortunately, genebanks occasionally receive seed in very poor condition, with only minimal germination. In many cases this represents valuable germplasm in danger of being lost. Research is underway to rescue this material. The approach usually taken is to provide optimum conditions for germination, including control of microorganism growth, seed priming, and providing various nutrients and/or hormones. Alternatively, embryos may be excised and grown in culture to produce plants which can then be transferred to the greenhouse of field, as appropriate. Regeneration of plants from callus and protoplasts is also a possibility and new reports on special techniques appear almost daily in the literature.

Other Germplasm Preservation: Although this presentation has mainly concerned preservation of orthodox seeds, it should be recognized that important breakthroughs have recently been made on the storage of recalcitrant seeds (Vertucci et al., 1991; Wesley-Smith et al., 1992). It should soon be possible to store these materials, probably in liquid nitrogen, on a routine basis. Similarly, long-term storage of non-seed germplasm, particularly of clonally propagated species, is now possible (Towill, 1989; 1990; Towill and Jarret, 1992).

CONCLUSION

Germplasm preservation is fundamental to future crop improvement. The new information now emerging should answer many questions about seed longevity, deterioration, and preservation. Modern genebanks, such as the one we are dedicating this week, can not limit themselves to orthodox seed storage. We must be capable of preserving germplasm in several forms. The facility here in Taiwan should now take its place in the world community to preserve germplasm for the benefit of all Humankind. Finally, I would like to once more thank the organizers of this symposium for extending an invitation to me to participate in this joyous event.

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Table 1. Seed longevities of 100 or more year. (data from Roos, 1989)

	Species	Longevity	Comments	Reference
1.	*Lupinus arcticus* S. Wats	10,000 +	^{14}C dating of assoc. rodent bones	Porsild et al., 1967
2.	'Mummy' wheat and barley	2000-3000 +	Egyptian tombs	Barton, 1961; Bewley & Black, 1982; Justice & Bass, 1978
3.	*Nelumbo nucifera* Gaertn.	3075	^{14}C dating of assoc. wood canoe	Libby, 1954; Wester, 1973
4.	*Chenopodium album* L.	1700	Archeological dated soil samples	Odum, 1965
	Spergula arvenis L.	1700		
5.	*Nelumbo nucifera*	120-1080	^{14}C dating, geologic, and historical records	Godwin & Willis, 1964; Libby, 1951; Ohga, 1923; Ohga, 1927
6.	Various weed species (36 different species)	110-600 +	Archeological dated soil samples	Odum, 1965
7.	*Canna compacta* Rosc.	530-620	^{14}C dating of assoc. materials	Lerman & Cigliano, 1971; Sivori et al., 1968
8.	*Nelumbo nucifera*	237	Museum specimen	Anonymous, 1942
9.	*Medicago polymorpha* L.	200	From adobe bricks	Spira & Wagner, 1983
	Hordeum leporinum Link	200	Positive TZ reaction	
	Malva parviflora L.	183-200	Positive TZ reaction	
	Trifolium sp.	193	Positive TZ reaction	
	Chenopodium murale L.	183	Positive TZ reaction	
	Chenopodium album	143	Positive TZ reaction	

Table 1. (Continued). Seed longevities of 100 or more year. (data from Roos, 1989)

	Species	Longevity	Comments	Reference
10.	*Cassia multijuga* A. Rich.	158	Museum specimen	Becquerel, 1934
11.	*Albizzia julibrissin* Durazz.	147	Museum specimen	Anonymous, 1942
12.	Barley and oats	123	Building cornerstone	Aufhammer & Simon, 1957
13.	*Hyoscyamus niger* L.	116	Museum specimen	Odum, 1965
14.	*Cassia bicapsularis* L.	115	Museum specimen	Becquerel, 1934
15.	*Goodia lotifolia* Salisb.	105	Museum specimen	Ewart, 1908
	Hovea linearis (Sm.) R. Br.	105	Museum specimen	
16.	*Malva rotundifolia*	100	Buried seed	Kivilaan & Bandurski, 1981
	Verbascum blattaria L.	100	Buried seed	
	Verbascum thapsus L.	100	Buried seed	
17.	*Trifolium pratense* L.	100	Museum specimen	Youngman, 1951
	Lotus uliginosus (L. *pedunculatus* Cav.)	100	Museum specimen	

Table 2. Longevity of 15 vegetable seed species. (Roos and Davidson, 1992)

Species (common name)	Age (yr)	Germ. (%)
Abelmoschus esculentus (okra)	50	34
Allium cepa (onion)	52	20
Beta vulgaris (beet)	59	8
Beta vulgaris (Swiss chard)	48	70
Capsicum annuum (pepper)	51	28
Citrulus lanatus (watermelon)	58	32
Cucumis melo (muskmelon)	58	42
Cucumis sativus (cucumber)	58	28
Daucus carota (carrot)	46	24
Lycopersicon esculentum (tomato)	60	82
Phaseolus vulgaris (bean)	48	24
Pisum sativum (pea)	51	94
Solanum melongena	56	4
Spinacia oleracea (spinach)	45	60
Zea mays (corn)	50	56

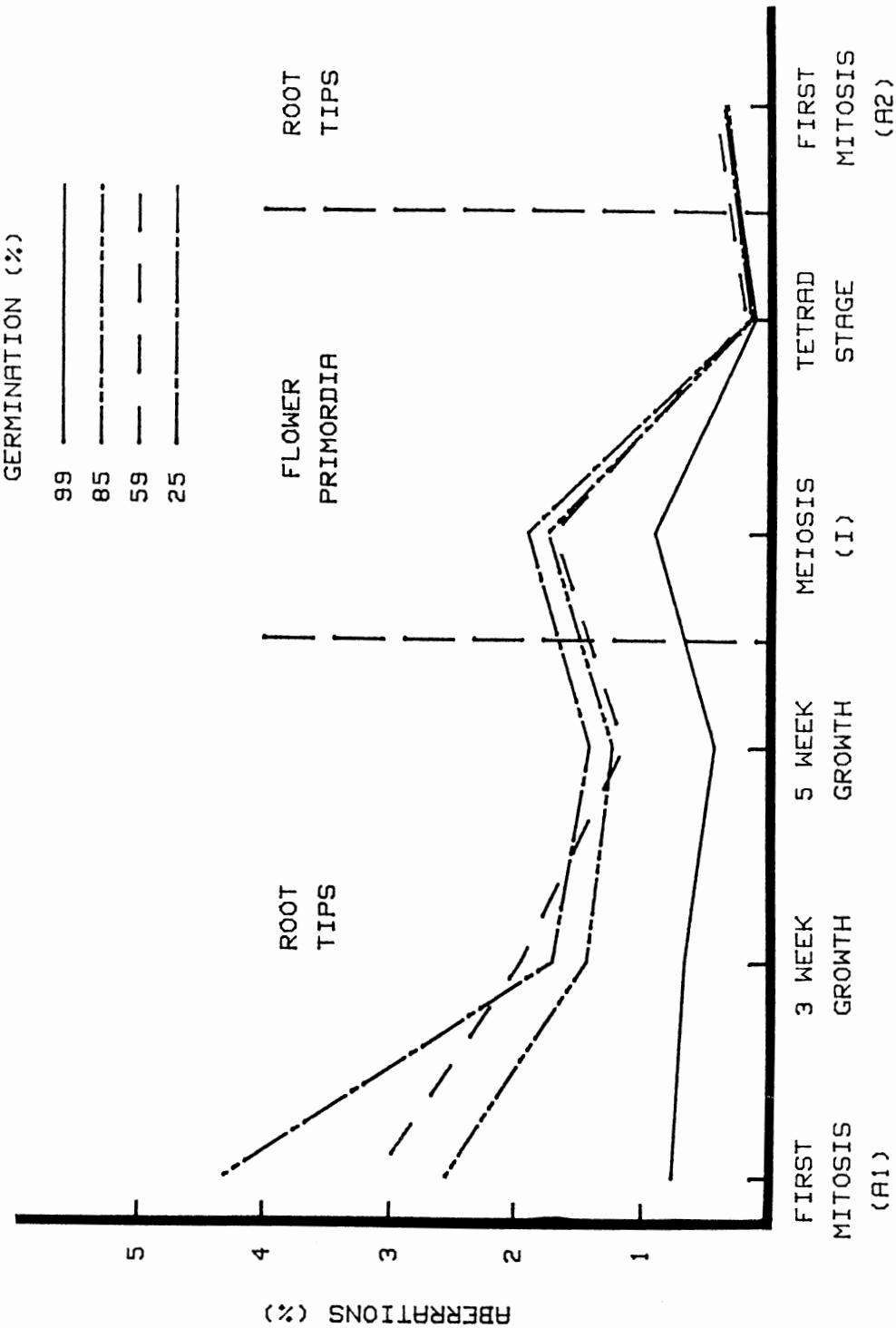


Figure 1. Effects of plant growth on elimination of aging-induced chromosomal aberrations in barley seeds. (data from Roos, 1982)

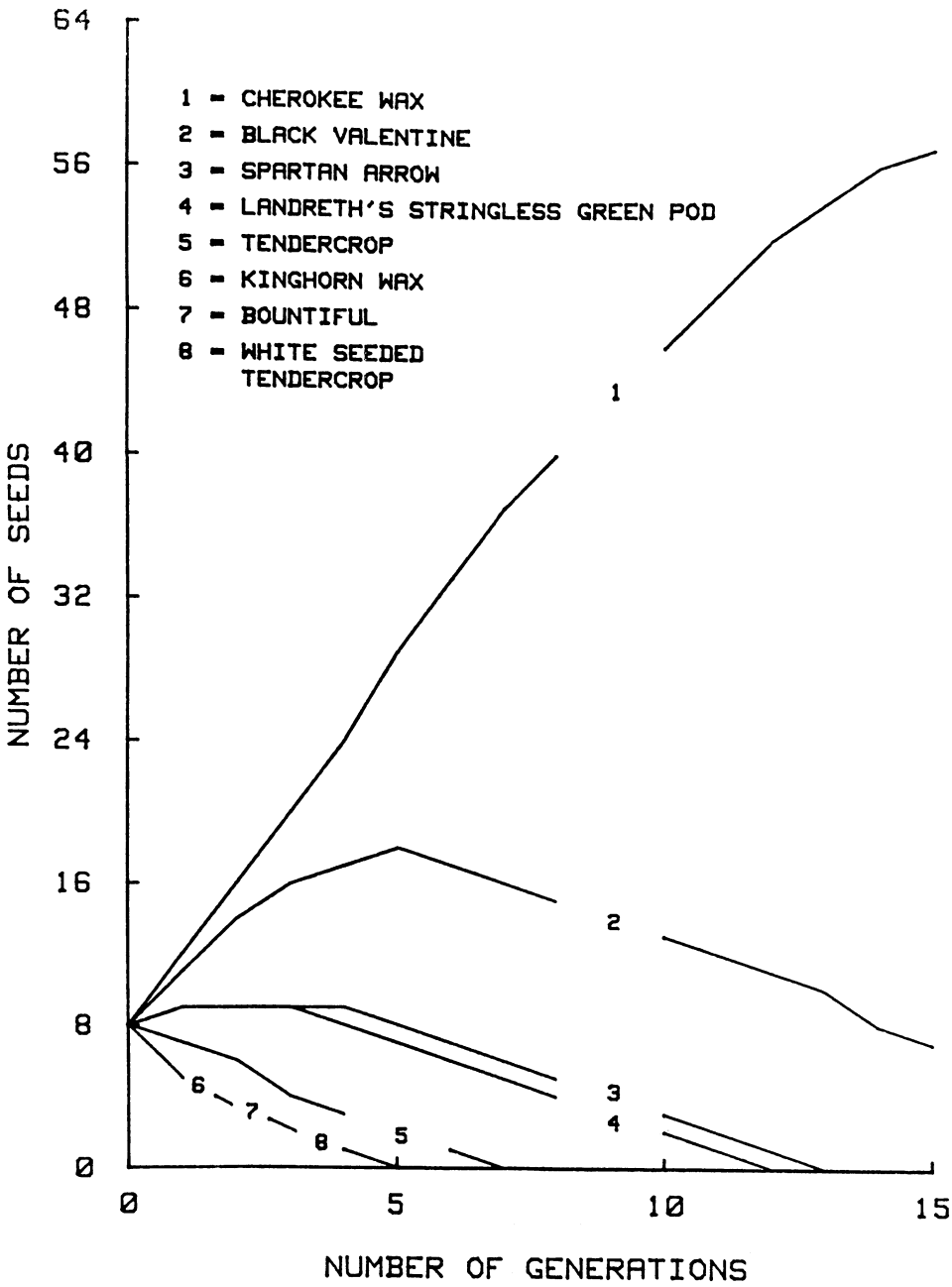


Figure 2. Computer simulation of genetic shift in composition of a mixed snap bean population following several generations of seed aging (to 50% population germination) and seed increase. The initial population consisted of 8 seeds of 8 cultivars (population size = 64 seeds). After 15 generations only two cultivars remain in the population. (data from Roos, 1984b)

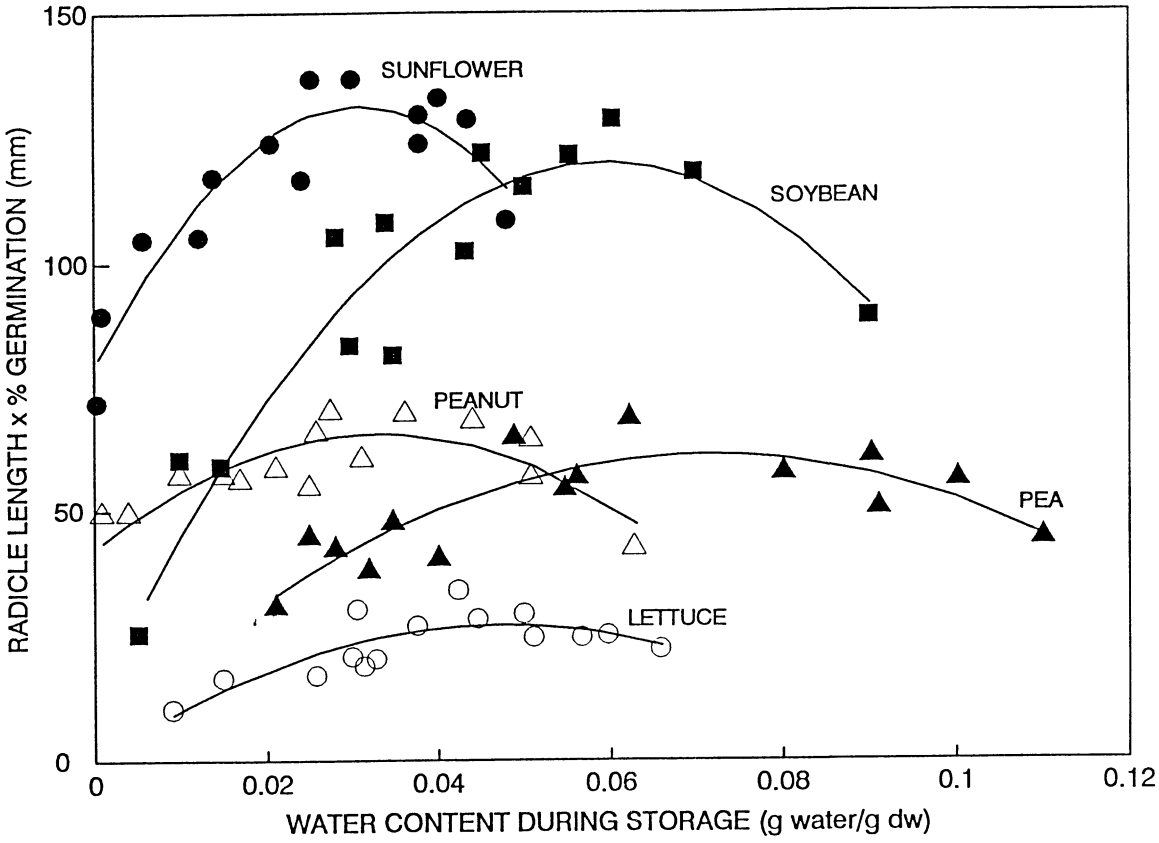


Figure 3. Efect of moisture content during storage at 35℃ on seed vigor. Data are presented for soybean and sunflower stored for 2 months and peanut, pea, and lettuce stored for 4 months. Vigor is expressed as the germination index: the mean radicle length × the percentage germination. The curves are drawn from fitted second order polynomial equations. (data from Vertucci and Roos, 1990)

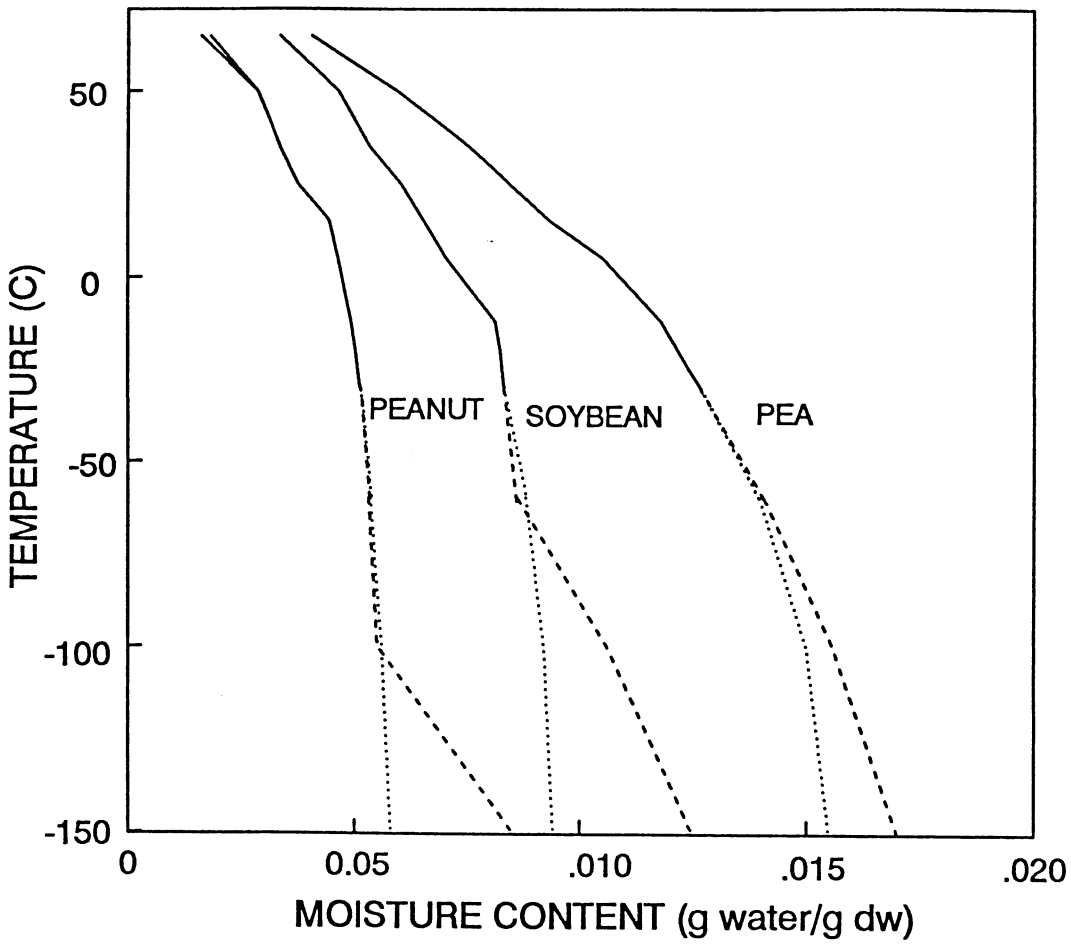


Figure 4. The optimum moisture content for storage of pea, soybean and peanut seeds at storage temperatures between-150 ℃ and 65 ℃. Dashed and dotted lines represent determinations based on isotherms calculated from heat capacity measurements and van't Hoff analyses, respectively. (data from Vertucci and Roos, 1993)