Osmotic priming and carbohydrate metabolism in *Pinus radiata* seeds

Kusmintardjo, Ian G. Andrew and David W. Fountain

Institute of Molecular Biosciences, Massey University, Palmerston North.

Abstract

The performance of seeds in the field can be enhanced by various pre-sowing treatments, of which osmotic priming has been much used in recent years. During osmotic priming, metabolic changes are initiated which mirror those occurring before radicle emergence in water-imbibed seeds. In pine seeds, these changes include ATP synthesis, degradation of lipid and protein stores, nucleic acid and protein synthesis, and carbohydrate metabolism. In *Pinus radiata*, there are no starch reserves, but starch is formed in both embryo and megagametophyte during germination and during priming. The significance of this starch metabolism is discussed, and unanswered questions concerning the process are raised.

Additional key words: *conifer, germination, Pinaceae, pine, seed performance, starch*

Introduction

Commercial forestry is one of New Zealand's principal export earners (Ministry of Forestry, 1995), and is dominated by one species, *Pinus radiata* (Radiata pine). As quantitative and qualitative improvement of the product is the ultimate goal in crop production of agriculture and forestry, it is essential to begin with good seed. Although clonal forestry is widely used, with cuttings used to raise plants for field transplantation, seeds remain the most economic and most efficient vehicle for large-scale propagation.

The importance of starting with high quality and vigorous seeds has been highlighted by many authors in a recent book on "Seed Quality" (Basra, 1995). Quality attributes include genetic quality, high physical purity, uniformity in seed size, high germination capacity, high vigour and freedom from seedborne diseases. Such attributes ensure a rapid, uniform and complete germination when seeds are planted. Osmotic priming (Pill, 1995) is a promising method for performance enhancement and is considered in this review.

The metabolism of germinating seeds is a very broad field, and this review will concentrate primarily on carbohydrate metabolism and the effects of osmotic priming. The emphasis is on *Pinus radiata*, but other species, particularly of the genus *Pinus*, are discussed to place matters in context and to complement the studies with *P. radiata*.

Performance Enhancement in Seeds

Osmotic priming is one of several methods developed for increasing seed vigour. This is defined by the Association of the Official Seed Analysts (AOSA) as: "Seed vigor comprises those seed properties which determine the potential for rapid, uniform emergence, and development of normal seedlings under a wide range of field conditions" (AOSA, 1983). A similar definition is used by the International Seed Testing Association (ISTA) (Perry, 1978). Various aspects of seed performance in these definitions include: (i) biochemical processes and reactions such as enzyme activity and respiration, (ii) rate and uniformity of seed germination and seedling growth in the field, and (iii) emergence ability of seedlings under unfavourable environmental conditions.

A range of approaches have been used to improve the performance of seeds and seedlings (Heydecker and Coolbear, 1977; Bergsten, 1987). These include:

i Breeding of genetically improved seeds.
ii Elimination of pests and diseases.
iii Conditioning, e.g., with nutrients.
iv Mechanical treatment, e.g., irradiation.
v Selection of better performing seeds by sizing, flotation, etc.
vi Dormancy breaking, e.g., by scarification (abrasion), stratification (moist chilling) or hormone treatment.

vii Advancement of germination, e.g., by a controlled increase in water content, in which the water potential is raised to a limited extent (as in osmotic priming) or for a limited period and the seeds then redried.

We shall briefly consider these different approaches before turning in more detail to osmotic priming.

With *Pinus radiata*, most of the seed used is genetically improved in some way and this will not be discussed further in this review. Treatment with pesticides, fungicides, nutrients, etc., and irradiation treatments discussed by Heydecker and Coolbear (1977) likewise do not concern us further.

Selection processes such as sizing or flotation produce marginal improvements with fresh high quality seed. Sizing can significantly improve performance in the nursery, in that larger seeds have higher germination rate and seedling dry weight (Griffin, 1972; Kandya and Ogino, 1986; Kusmintardjo *et al.*, 1994). Flotation is sometimes used to separate bad seed out of a seed lot. In the IDS process (imbibing-drying-separation), bad seed is separated out by flotation in water. With fresh *Pinus radiata* seed of registered genetically improved lots, there is very little bad seed and the main improvement in performance is due to the advancement of germination incurred during imbibing (Kusmintardjo, unpublished).

Stratification is widely used to break dormancy in pines (e.g., Carpita *et al.*,1983), as are treatments with gibberellins and/or cytokinins (e.g., Pitel and Wang, 1985), but neither of these treatments have led to significant improvement in performance of *Pinus radiata* in New Zealand tests (Kusmintardjo, unpublished).

On the other hand, advancement of germination by a controlled increase in water content can have profound effects. In these pre-sowing treatments, the initial metabolic processes of germination are allowed to proceed, but radicle protrusion is prevented because of a controlled water imbibition. The IDS treatment has already been referred to, but the improvement it gave to performance in *Pinus radiata* was small compared with that produced by priming (Kusmintardjo, unpublished). In some conifers, including *Pinus banksiana* (Downie *et al.*, 1993), another moisture equilibration technique (membrane tube invigoration) has been shown to advance germination more than osmotic priming. This has not yet been tested for *Pinus radiata*.

Osmotic Priming

In the last decade, osmotic priming has been used to increase the rate and uniformity of emergence in many vegetable and flower species. Basically, this treatment involves imbibing seeds in an aqueous polyethylene glycol (PEG) or salt solution of a given negative water potential. Through control of solute concentration, temperature and duration of soaking, the seeds are permitted to undertake initial processes of germination such as the mobilisation of food reserves, nucleic acid metabolism (Coolbear *et al.*, 1980) and the formation of enzyme systems, while the emergence of the radicle is prevented. If we assume these processes are essential and irreversible, then more rapid germination should occur when seeds are primed before sowing in the field. The osmotic priming treatment is relatively inexpensive and simple to apply, but it is not yet used on a wide scale for tree seeds.

Priming with PEG has been shown to advance germination in a number of pine species (Zou and Huang, 1987; Hallgren, 1989; Bourgeois and Malek, 1991), but Kusmintardjo (1992) reported that, for *Pinus radiata*, priming with salt solution gave better performance than a PEG solution of the same water potential. Priming was performed at 20 °C for a 10-day period, and this treatment shortened the time for field emergence of seedlings by 30 to 50 %, which could amount to more than two weeks with early sowing. This quick start in the cooler early part of the growing season was maintained, so that, after ten months in the field, seedlings showed 20 % weight gain over those from non-primed seeds (Kusmintardjo, unpublished).

Osmotically primed seeds are normally dried before sowing. Their shelf life is limited, however, by an accelerated ageing process and both in *Pinus sylvestris* (Huang and Zou, 1989) and in *Pinus radiata* (Kusmintardjo, 1992), the improved performance of primed seeds was lost after prolonged drying.

The technology and physiological mechanism of priming have been reviewed by Pill (1995). During osmotic priming, water uptake is osmotically restricted by a low (negative) water potential, Ψ, and germination is prevented, but metabolic changes still occur in the embryo, presumably reflecting early steps in the germination process. Obroucheva (1995) discussed the sequence of metabolic events involved in germination in relation to water imbibition. The earliest effects, at low water content, were respiration (detected at Ψ = -15 MPa in some seeds) (Bradford and Haigh, 1994) and amino acid metabolism, followed at higher water potential by mobilisation of storage reserves, then activation of the plasma membrane H^+ ATPase, and finally elongation of the embryo axis and radicle emergence. It was postulated that certain threshold water potentials were required to trigger successive stages.

Bradford and Haigh (1994) discussed the advancement of germination in terms of a hydrothermal time model. Using PEG-treated tomato seeds, they noted a threshold Ψ of -2.50 MPa was needed for germination advancement, well below the -0.71 MPa required for radicle emergence. In between these levels, the degree of germination advancement was proportional to the accumulated hydrothermal time - a product of water potential, temperature and time. Most workers use a water potential in the range of -0.8 to -1.5 MPa for osmotic priming.

As noted, PEG and salt priming had different efficiencies with *Pinus radiata* seed. They also may involve different mechanisms. Dell'Aquila and Spada (1991) showed that PEG priming is accompanied by synthesis of the same set of proteins as in normal pre-emergence imbibing of water, but that salt induced a set of 'S' proteins which disappeared on transfer to water.

Metabolism of Germinating Pine Seed

Pine seeds consist primarily of three integument layers, the remnant of the nucellus, the megagametophyte, and an embryo (Stone and Gifford, 1997). The two outer integument layers form a hard, thick seed coat or testa, sometimes covered by a wax layer, but it does not prevent water absorption. An air chamber is usually formed between the seed coat and the megagametophyte where, in the course of imbibition, the water absorbed by the coat gradually fills out the chamber. The innermost integument layer is a papery layer covering the entire megagametophyte.

The megagametophyte (female gametophyte or endosperm) is a haploid homogeneous storage tissue. Apart from providing nutrition to the embryo during germination and post-germination growth, the endosperm also protects the embryo from bacterial and fungal attacks to which the naked embryo is very susceptible.

The embryo or new sporophyte is diploid and occupies the central part of the seed. It is small compared with the megagametophyte, making up about five percent of the seed weight (Mirov, 1967; Kusmintardjo, unpublished). The pine embryo usually has about 7 to 9 cotyledons, surrounding a small epicotyl. The rest of the embryo consists mainly of a hypocotyl as a shoot axis and the radicle, with its rootcap oriented toward the micropyle at the narrower end of the seed.

Cells of both megagametophyte and embryo are packed with storage reserves of lipid and protein, and in *Pinus radiata* these are the principal stores. In some other pine species, carbohydrate reserves may also be important. The lipid is almost entirely triacylglycerol, comprising 31 % of the seed in *Pinus radiata*, with 90 % or more of its fatty acids unsaturated. The predominant ones in *Pinus radiata* are oleate 19 %, linoleate 44 % and pinolenate 22 % (Wolff *et al.*, 1998). Some pines, such as *Pinus sylvestris* (Nyman, 1969), *Pinus banksiana* (Durzan *et al.*, 1971) and *Pinus edulis* (Murphy and Hammer 1994) also have small starch reserves, but in *Pinus radiata* starch is not detected until imbibition (Riding and Gifford, 1973). Starch is a major reserve in some other gymnosperms, such as *Araucaria araucana* (Cardemil and Varner, 1984).

Sugars in resting pine seeds are mostly sucrose, raffinose and stachyose, and the totals can be relatively high in some species, e.g., 4 % of the megagametophyte and 10 % of the embryo dry weight in *Pinus banksiana* (Durzan and Chalupa, 1967). In sugar pine, *Pinus lambertiana*, the resting embryo may contain 20 % sugar, mostly as stachyose (Murphy and Hammer, 1988). In *Pinus radiata*, the levels are about 3 % (I.G. Andrew, unpublished).

Pine seeds have a relatively high ash content, about 2 to 8 %, including phosphate mostly in the form of phytate (inositol *hexakis* phosphate) (West and Lott, 1993). The latter may also act as a carbohydrate store, as inositol is used in the synthesis of cell wall polysaccharides (Loewus and Loewus, 1983).

During imbibition of water, respiration is accompanied by a large increase in ATP (Ching and Ching, 1970; Bourgeois and Malek, 1991), and this is a requirement for subsequent metabolism. The major obvious chemical changes quantitatively are the disappearance of lipid and protein reserves, in both megagametophyte and embryo, and also changes in starch content, and the discussion below centers on these aspects. For radicle emergence, a softening of the seed coat also occurs, which in some conifers, as well as angiosperms, has been reported to involve the action of a b-mannanase on the cell walls in the micropylar region (Downie *et al.*, 1997). Cold stratification in *Picea glauca* was shown to result in an elevation of b-mannanase activity, which may assist in the breaking of dormancy in this and other conifer species. This and other enzymes have been proposed to have a role in regulating germination of a range of seeds (mostly angiosperms) and the possible roles of these enzymes have been reviewed by Welbaum *et al.* (1998).

Mobilisation of protein reserves in pine seeds has been documented by several workers (Stone and Gifford, 1997). A number of proteinases have been implicated in mobilisation of the storage protein in *Pinus sylvestris* (Salmia, 1981). Similar proteinases have been observed in *Pinus radiata* seeds (Kusmintardjo, unpublished). The proteinases in *P. sylvestris* are accompanied, in both

megagametophyte and embryo, by proteinase inhibitors. These inhibitors act specifically on the pine seed proteinases in vitro, but it seems unlikely that they are involved specifically in regulating the degradation of storage protein and a protective role toward other essential proteins has been suggested (Salmia, 1980). Studies by Gifford *et al.* (1989) have suggested that aminopeptidase is important in mobilisation of reserve protein of *Pinus taeda* seeds.

Mobilisation of the lipid involves lipolysis, b-oxidation, the glyoxylate pathway and gluconeogenesis (Ching, 1970; Young and Anderson, 1984; Hammer and Murphy, 1994). Isocitrate lyase is a key enzyme of the glyoxylate pathway and it has been demonstrated in the megagametophyte of a number of pine species, being induced during imbibition and rising to a peak after about 10 to 12 days in *Pinus radiata* (Young and Anderson, 1984). It is also induced in both megagametophyte and embryo during stratification of *Pinus lambertiana* (Noland and Murphy, 1984) and its production in the megagametophyte of *Pinus ponderosa* has been reported to be under control of the embryo (Bilderback, 1974). In *Pinus taeda* another glyoxylate cycle enzyme, malate synthase, increases mainly after radicle emergence (Mullen and Gifford, 1995). Several glyoxysomal enzymes have also been demonstrated in *Pinus ponderosa* (Ching, 1970). The main ultimate product of lipid mobilisation is carbohydrate, and this may be the major source of carbohydrate in many pine seeds.

In *Pinus banksiana*, the starch reserves are used up during the first 3-4 days of imbibition, but starch reappears in the chloroplasts after germination (Durzan *et al.*, 1971; Cecich and Horner, 1977). The disappearance of storage starch in *Pinus sylvestris* is related to the appearance of an a-amylase (Nyman, 1971). In *Pinus radiata*, starch reserves are not detected in the resting seed, but appear shortly after imbibition (see below).

Transient Starch in Germination

In seeds of *Pinus radiata*, starch first appears during water imbibition. Amyloplasts are localised primarily around the shoot apex (Riding and Gifford, 1973). Murphy and Hammer (1994) showed that starch levels in *Pinus edulis* embryos increased during germination, but that the major increase occurred after radicle emergence, resulting in accumulation of starch to about 46 % of the dry weight in the hypocotyl and to 65 % in the cotyledons, with some also in the root cap. In this species, sucrose produced in the megagametophyte is transported to the embryo where it is converted in part to starch. Murphy and Hammer (1994) showed that storage material from the megagametophyte of *Pinus edulis* is transported to the embryo as sugar, and there the excess is converted to starch. Starch was reported to accumulate transiently to relatively high levels in dark-grown seedlings of *Pinus radiata* (Andrew and Little, 1997).

Further studies of *Pinus radiata* (Kusmintardjo *et al.*, in prep.) showed that transient starch appears in both megagametophyte and embryo during imbibition, but the levels are much lower than those reported for *Pinus edulis*. In the megagametophyte, starch rose to a peak of about 0.03 % by weight at radicle emergence, declining thereafter. Levels in the embryo rose more slowly, but were more sustained. Since starch is an insoluble storage carbohydrate and cannot be transported, the starch stored in the megagametophyte must be degraded for transport before it can be reassembled to starch in the embryo (Fig. 1).

The role of the starch formed during imbibition, in particular that in the megagametophyte, needs to be considered. Starch in plants has been viewed as a buffer for sucrose metabolism (Stitt, 1984). In photosynthetic tissue, starch synthesis and degradation are controlled by the levels of inorganic phosphate (P_i) and phosphoglycerate (3PGA) (Heldt *et al.*, 1977). When the rate of photosynthesis exceeds the rate of sucrose synthesis, P_i falls and an increasing proportion of fixed carbon is retained in the chloroplast as starch. Phosphorolysis is stimulated by increased P_i but inhibited by 3PGA. In non-photosynthetic tissues, starch synthesis is controlled by 3PGA acting on starch synthase and ADP-glucose pyrophosphorylase (Preiss and Levi, 1980).

In cucumber, an oilseed analogous to pineseed but with the main reserves in the cotyledons, Chapman and Galleschi (1985) reported a transient accumulation of

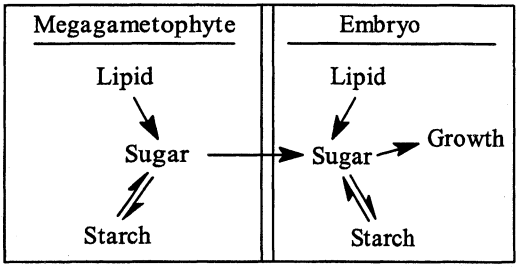

Figure 1. Carbohydrate metabolism of pine seeds during imbibition.

starch in the cotyledons similar to that observed in the megagametophyte of *Pinus radiata*. In excised cotyledons, however, with the embryonic axis removed, starch accumulated to much higher levels. Starch accumulation correlated with the granule-bound starch synthase activity. These authors propose that starch synthesis may reflect the need to produce an internal metabolic sink for soluble sugars in the cotyledons. This is only temporary in the intact system. During incubation of excised cotyledons, or in the intact system during PEG imbibition (Davies and Chapman, 1979), lipid breakdown is reduced and sugar levels increased. Starch may then represent the formation of a secondary pool of carbon and could prevent the increase of sugar concentration to inhibitory levels (Davies and Slack, 1981). Isolated embryos of *Pinus radiata* did not expand significantly when imbibing water, but inclusion of sucrose (0.1 M) led to expansion and spreading of the cotyledons (Kusmintardjo, unpublished). It is likely that sucrose normally supplied to the embryo by the megagametophyte would instead be stored as starch in the isolated megagametophyte.

In germinating pine seed, although the cotyledons may be green they are non-photosynthetic until activated by light (Ou and Adamson, 1995). As in cucumber seed, starch accumulates in amyloplasts rather than in chloroplasts, and it could well have a similar role as a buffer for sucrose metabolism. Storage of carbohydrate as starch would have the effect of restricting its movement, as the starch is confined to plastids until mobilised enzymically, whereas sucrose, although it cannot cross the plastid membrane, is freely transported across plasma membranes by carrier proteins. Thus, in *Pinus radiata*, if the rate of carbohydrate synthesis in the megagametophyte exceeds that of sucrose transport, or if it exceeds the rate at which it can be used by the embryo, the excess could be stored transiently as starch (Fig. 1). The mechanism for this would involve phosphate intermediates (Smith *et al.*, 1997). In mammalian liver, the storage of excess glucose as glycogen serves to maintain the osmotic potential in the cells (Elliott and Elliott, 1997), but such a role for starch formation in plant cells may be less important because the cell walls should be strong enough to withstand the excess turgor pressure of sucrose.

Kusmintardjo (unpublished) also showed that application of exogenous sucrose to excised embryos of *Pinus radiata* led to starch accumulation throughout the embryo. This concurs with observations in photosynthetic tissue, where high sucrose concentrations have been shown to inhibit starch mobilisation (Gordon *et al.*, 1980).

Metabolism of Osmotically Primed Pine Seeds

Most published work on metabolism of seeds during priming relates to angiosperms. Metabolic events are initiated during priming which are part of the normal germination process. These include respiration and ATP synthesis, RNA and protein synthesis and turnover of storage materials (protein, lipid, carbohydrate). The same set of proteins are expressed during priming as in the pre-germination phase of water imbibition (Dell'Aquila and Spada, 1992). Many of the same enzyme activities are also induced under the two conditions (Smith and Cobb, 1991). Most studies on primed seeds have been carried out with PEG, but the limited studies with salt solutions suggest a similar situation (see, for example, Dell'Aquila and Spada, 1992).

Like osmotic priming, moist chilling (stratification) also involves a restricted water uptake and induces some of the changes associated with germination. For example, Noland and Murphy (1994) demonstrated a gradual increase in ATP and isocitrate lyase during stratification of sugar pine seeds. Osmotic priming at around -1.0 MPa usually enables further development than moist chilling and advances germination accordingly (see, for example, Khan and Karssen, 1981).

Embryo enlargement occurs during the limited water uptake occurring in osmotic priming. In pine seeds, the embryo swells to fill the embryo cavity between it and the surrounding megagametophyte, but the radicle is prevented from emerging (Kusmintardjo, unpublished). In lettuce seeds priming was suggested "to lead to the irreversible initiation of cell elongation" (Cantliffe *et al.*, 1984).

As in angiosperms, ATP synthesis is an early metabolic event during priming of jack pine seeds (Bourgeois and Malek, 1991). The same authors also reported that protein hydrolysis occurred in primed pine seeds, but there was no concomitant increase in pepstatin-sensitive proteinase activity.

Microscope studies (Kusmintardjo, unpublished) have revealed that, during priming, depletion of protein stores and accumulation of starch were early events, as when imbibed in water. The starch in primed seeds was principally located in the radicle, where starch grains appeared to occupy up to 5 % of some cells, but quantitative assays of whole embryo (Kusmintardjo *et al.*, in prep.) showed much lower levels, comparable to those observed during water imbibition. Starch development was slower in both megagametophyte and embryo than during water imbibition. The concentration in the mega-

gametophyte was lower than in the embryo but, with its much greater bulk, stores in the megagametophyte could possibly provide a substantial reserve. When primed seeds were subsequently transferred to water, the starch metabolism was advanced over that in untreated control seeds. When primed seeds were surface dried and then treated with water, a decline was observed in the starch content of the megagametophyte, while that in the embryo peaked sooner than in the unprimed seeds.

Conclusion

Germination in pine seeds involves mobilisation of lipid and protein reserves in both megagametophyte and embryo tissues, and transport of nutrient as sucrose from megagametophyte to embryo. Excess carbohydrate can be stored as starch in either tissue, but that in the megagametophyte disappears quite rapidly.

The role of the megagametophyte in pine seed germination has been little studied in terms of carbohydrate metabolism. We expect that a study of the isolated megagametophyte will reveal a large increase in starch during imbibition. Starch is quantitatively minor in *Pinus radiata* seed, with the main increase seen in the hypocotyl and cotyledons after germination, but some *Pinus* species have substantial starch reserves in the dry seed. Observations in *Pinus radiata* will not necessarily relate to those in pine species with larger starch reserves either in the dry seed or during imbibition.

During priming, the same changes are observed as during water imbibition, but the rate or extent are limited by water stress, which also prevents radicle emergence. The changes observed during priming lead to an advancement of both carbohydrate and other metabolism as well as overall germination. The sum of the metabolic changes occurring during priming presumably bear directly on the improved germination performance of primed seeds.

One major disadvantage of the priming process is the short term of the gains in performance. The accelerated ageing observed with primed seeds is of vital interest. Sun and Leopold (1995) showed that loss of seed viability during accelerated ageing of soybeans, induced by salt stress, correlates with formation of Maillard reaction products. It was suggested that this resulted from the gradual increase of reducing sugars occurring through hydrolysis of oligosaccharides. Since priming in *Pinus radiata* seeds involves marked changes in carbohydrate metabolism, it will be of some interest to see whether a similar phenomenon might be responsible for the loss of viability on storage of primed pine seeds.

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