Reserve mobilisation following germination of italian ryegrass caryopses

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Abstract

The utilisation of food reserves following germination of Italian ryegrass (*Lolium multiflorum* Lam.) caryopses has been investigated. Concurrent time-course determinations of caryopsis dry weight changes, starch content and the activity of the starch degrading enzyme α-amylase have been performed. Enzyme activity was first detected two days after the initiation of germination coinciding with the onset of starch depletion. Peak α-amylase activity was observed on the sixth day of the time-course while starch levels were virtually exhausted by day eight. Ryegrass α-amylase was shown to exist as multiple isoenzymes which appear to be under differential developmental control. The spectrum of ryegrass α-amylase isoenzymes was also distinctive from that of germinated wheat and phalaris caryopses.

Additional key words: *pasture grass, α-amylase,* Lolium multiflorum, *seedling establishment.*

Introduction

The attractiveness of ryegrass species, e.g., Italian ryegrass (*Lolium multiflorum* Lam.), for pasture is in part due to the speed with which the seed (strictly a caryopsis) germinates and the seedling subsequently grows. Such attributes give *Lolium* species a competitive advantage when sown as mixtures with slower growing species such as tall fescue (*Festuca arundinacea*) (Hill *et al.*, 1985a,b; Brock *et al.*, 1982). The intrinsic factors responsible for these species differences remain largely unknown especially at a biochemical/molecular level.

Following germination a growing seedling is dependent on the food reserves stored in the seed until such time as it becomes autotrophic (Bewley and Black, 1985). The ability of the plant to mobilise and use these reserves is therefore, likely to influence seedling establishment, particularly under circumstances such as shading, when the onset of autotrophic growth may be delayed.

Our understanding of the mechanisms of reserve mobilisation in pasture grass seed lags far behind that of the cultivated cereals which have been the subject of intensive study (see Fincher, 1989). Results reported here represent the initial findings of an ongoing study to investigate seed reserve mobilisation in grass seed with a view to identifying factors responsible for the differences that exists in early seedling growth among pasture grass species. Attention has initially focused on reserve mobilisation in germinating Italian ryegrass (*Lolium multiflorum* Lam.) seeds as an example of a species with rapid seedling growth.

Materials and Methods

Germination conditions

Untreated seeds of Italian ryegrass (*Lolium multiflorum* Lam. cv. Paroa) and phalaris (*Phalaris aquatica* L.) were obtained from Hodder and Tolley Seeds, Palmerston North and DSIR Grasslands (now AgResearch Grasslands), Palmerston North, respectively. Seeds were surface sterilised with sodium hypochlorite solution (1% available chlorine) containing a drop of Tween 20, for 10 min after which they were washed with copious amounts of sterile distilled water. Surface sterilised seeds were germinated on moist blotting paper in darkness at 20°C for up to 11 d.

At each point of the time-course replicate samples, each of 25 seeds, were harvested. Observations were made to determine the time of emergence of the radicle, coleoptile, first true leaf plus lateral and adventitious roots. Root and shoot lengths were determined using a graduated ruler. Material used for biochemical analysis was rapidly frozen using liquid air or a -80°C glycol bath

after the shoot and root tissue had been removed from the seed. Frozen material was stored at -20°C until required.

Seed extracts

Extracts of seed material were prepared as follows. Replicate batches of 25 seeds were powdered in liquid nitrogen using a pestle and mortar. The resultant powder was transferred to a 1.5 ml micro-centrifuge tube and 1 ml 50 mM Tris-maleate buffer pH 6.2 containing 10 mM $CaCl_2$ added. Extraction was for 5 min at room temperature with vigourous shaking. The mixture was then centrifuged at 10,000g for 3 min to remove debris and the supernatant assayed for α-amylase activity and protein content as described below.

α-Amylase activity

α-Amylase activity of extracts was assayed using Phadebas blue starch (Pharmacia Diagnostics, Sweden) as substrate, according to Cornford *et al.* (1987). The reaction mixture contained 0.5 ml of extract or an appropriate dilution, plus fresh buffer to a volume of 4 ml. One ml of substrate (10 mg/ml), made up in the same buffer, was added to start the reaction. The mixture was incubated for 20 min at 37°C after which the reaction was stopped by the addition of 1 ml 0.5 M NaOH. Undigested substrate was removed by centrifugation at 2000g for 5 min and the absorbance of the supernatant fluid read at 620nm. Absorbance readings were converted to enzyme units using a standard curve prepared with barley malt α-amylase. One enzyme unit is defined as that amount of enzyme which will hydrolyse 1.0 mg of maltose from starch at pH 6.9 at 37°C.

α-Amylase isoenzymes were detected following isoelectric focusing according to the method described by Garcia-Maya *et al.* (1990). Isoelectric focusing was done using an LKB Multiphor flatbed electrophoresis unit. Aliquots (13 µl) of extract were placed on filter paper segments applied on the cathode side, to the surface of precast Ampholine polyacrylamide gel plates pH range 3.5 to 9.5 (Pharmacia LKB Biotechnology, Uppsala, Sweden). Running conditions for isoelectric focusing were as recommended by the manufacturer. After separation, the α-amylase isoenzymes were visualised by overlaying the gel with one containing β-limit dextrin as substrate. After an appropriate incubation period the gel overlay was stained with a potassium iodide solution and α-amylase activity visualised as white bands against a dark staining background of undigested substrate. The isoelectric point of individual isoenzymes was estimated by comparison with a set of defined pI marker proteins.

Starch

Starch levels were determined by quantifying glucose levels following enzymatic digestion of ethanol insoluble material using a method modified from that described by Holligan *et al.* (1974). Seed material was ground in 1 ml of 80% ethanol and boiled for 5 min. The mixture was centrifuged at 2000g for 10 min at room temperature and the supernatant fluid discarded. The pellet was washed twice with 80% ethanol after which it was resuspended in 2 ml of 50 mM citrate buffer pH 4.5 containing amyloglucosidase (200 µg/ml). The mixture was incubated for 2h at 45°C after which the reaction was stopped by heating to 100°C for 5 min. The solution was centrifuged as described above and the supernatant retained. The pellet was washed with 2 ml of water and the washings added to the supernatant. The glucose content of the resulting solution was determined using the glucose oxidase method (Holligan *et al.*, 1974).

The reaction mixture for glucose determinations consisted of 1 ml of an appropriate dilution of extract to be assayed plus 4 ml of glucose oxidase reagent (0.011% glucose oxidase, 0.002% peroxidase and 0.007% o-dianiside in 0.1 M sodium phosphate buffer pH 7.0). The mixture was incubated at room temperature for 15 min after which the reaction was stopped by the addition of 0.1 ml 4 M HCl and the absorbance measured at 420nm. Results are compared with a standard curve prepared using glucose and expressed as µmoles glucose.

Protein

Soluble protein levels were determined using the Coomassie Brilliant Blue G250 dye binding assay described by Bradford (1976).

Results

Germination and subsequent development of Italian ryegrass seedlings at 20°C was rapid as shown in Figure 1a. Radicle emergence was first observed on the second day of incubation following the initiation of germination with coleoptiles appearing a day later. Lateral seminal roots and the first true leaf were first observed on the fifth day of the time-course while adventitious roots were not seen until days 6-7. By the 8th day of the time-course a substantial seedling had formed as shown in Figure 1b.

The transfer of dry weight from the ryegrass seed to the growing seedling following germination is illustrated in Figure 2. Seed dry weight showed a marked decline between day 3 and 6 of the time-course after which little change was detected. Mean seed dry weight at the start of the time-course was 3.52 mg/seed. This fell to 1.84

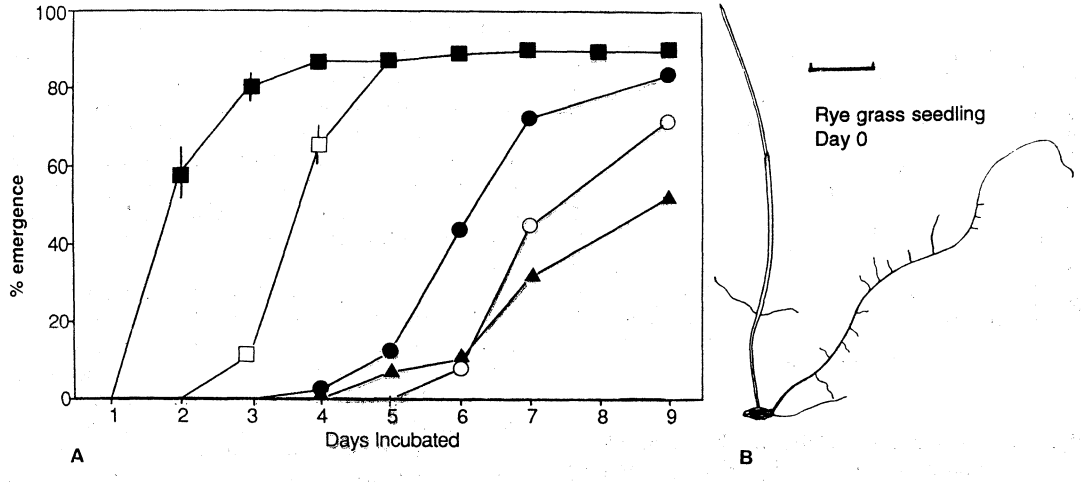

Figure 1. a) A developmental time-course for germinating Italian ryegrass (*Lolium multiflorum* Lam. cv. Paroa) indicating the % emergence of different seedling components. Each point represents the mean of 4 replicates each consisting of 25 seeds. The bars represent standard errors. Seeds were germinated in darkness at 20°C. (■), radicle; (□), coleoptile; (●), first true leaf; (▵), lateral seminal roots and (○), adventitious roots.
b) The appearance of a typical seedling 8 d after the initiation of germination. The scale bar represents 1 cm.

mg/seed by day 8 which represented a 40% reduction over the period covered. It is presumed that the weight loss was due to the depletion of endosperm reserves with the residual seed weight being due to seed coat and glume material. Seedling dry weight by contrast, rose rapidly from day 3 reaching 1.12 mg/seedling by day 8.

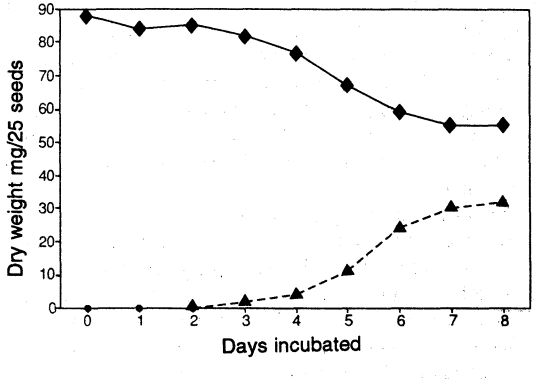

Figure 2. Time-course of seed (♦) and seedling (▲) dry weight changes for germinating Italian ryegrass.

Figure 3 shows the affect of germination on the starch content and α-amylase activity of the seed. The starch content of ryegrass seeds fell rapidly after the second day of incubation and was effectively depleted by the 8-10th day of the time-course. In contrast, activity of the starch degrading enzyme, α-amylase was absent from ungerminated seeds. It was however, detected on the second day of incubation coinciding with radicle emergence and the onset of starch loss from the seed. Enzyme activity rose rapidly, reaching a maximum (0.6 units per seed) by the sixth day of the time-course after which enzyme activity began to fall. This pattern of enzyme activity was observed irrespective of whether it was expressed as total activity (Fig. 3); specific activity (units per mg soluble protein) or on a dry weight basis (data not shown).

Isoelectric focusing revealed that α-amylase from germinating ryegrass seeds consisted of multiple isoenzymes (the senior author holds photographs of the

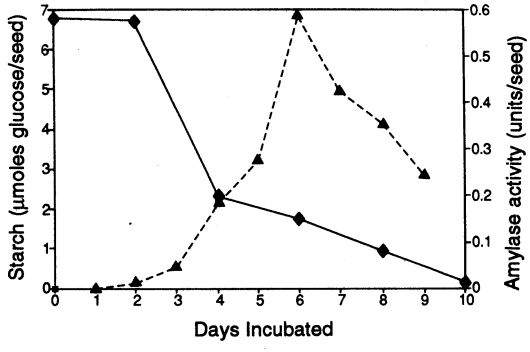

Figure 3. Time-courses of the starch content (♦) and α-amylase activity (▲) of germinating Italian ryegrass seed. Starch levels are expressed as µmoles glucose following enzymatic digestion of ethanol insoluble glucans extracted from the seed.

gel plates - eds.).

Consistent with the quantitative experiment described above, no ryegrass α-amylase isoenzymes were detected until day 2 of the time-course. By the fourth day, up to 6 different isoenzymes were observed. Of these, the strongest staining band represented an isoenzyme with a pI of approximately 5.5. Two changes in the isoenzyme pattern occurred between days 3 and 6 of the time-course. Firstly, a strongly staining isoenzyme (pI 5.0) appeared on day 4, while the weakly staining isoenzymes with the highest pI (approximately 6.0) disappeared. The pattern observed on day 6 remained unchanged for the remainder of the time-course. All ryegrass isoenzymes were of relatively low pI values compared with the major isoenzyme group found in germinating wheat grains.

The α-amylase isoenzyme pattern of germinating phalaris seeds was quite distinct from that of ryegrass and wheat. Phalaris seeds contained just two low pI isoenzymes. Of interest is the change in the isoenzyme profile that occurs as the germination time-course proceeds with the pI 4.5 isoenzyme replacing the pI 5.0 isoenzyme by day 10.

Discussion

In this study Italian ryegrass seeds were germinated under favourable temperature and water conditions but in complete darkness. The growth of the seedling was therefore, dependent upon the food reserves stored within the seed. In Gramineaceous species such as this the major food reserves are stored in the endosperm (Bewley and Black, 1985). Results showed that for Italian ryegrass, the seed food reserves were effectively depleted by 8-10 d after imbibition, as shown by dry weight and starch content changes. The seed reserves were quite sufficient, however, to support the growth of a substantial seedling.

A number of studies have demonstrated a close correlation between seedling growth rate and seed weight (Hill *et al.*, 1985 a,b; Scott and Hampton, 1985). However, in a comparative study of tall fescue, phalaris and annual ryegrass (Hill *et al.*, 1985a) seedlings of tall fescue grew more slowly than those of phalaris although the seeds of the former were heavier. This suggests that factors other than simply the size of the seed are involved. Hill *et al.* (1985b) were able to obtain particulary close correlations between growth rate and seed weight when perenniality (associated with slower growth) and ploidy were taken into account. The mechanism(s) responsible for the influence of these two characteristics were not, however, discussed.

Previous studies have shown endosperm utilisation rates, particularly during the first six days after the initiation of germination to be considerably higher in perennial ryegrass (cv. S24) than tall fescue (cv. S170) (Brock *et al.*, 1982). The importance of this observation with respect to seedling establishment is not yet clear since seed food reserves are often considered to be in excess of the plants' requirements. Under favourable light conditions for example, it has been demonstrated that positive net photosynthesis, i.e., a photoautrophic state, is achieved in growing pasture grass seedlings after only 5 days growth. This is well before the seed food reserves are exhausted (McWilliam, *et al.*, 1970 and this study). Under sub-optimal conditions however, a seedling is likely to be dependent upon mobilised stored food reserves for much longer and thus any species differences in reserve mobilisation are likely to be magnified.

Germinating ryegrass seeds rapidly produced the starch degrading enzyme α-amylase. Increasing enzyme levels were associated with a sharp decline in the amount of starch present in the seed. The source of α-amylase in the seed has not yet been identified nor has the influence of plant hormones such as gibberellins and abscisic acid. These studies are currently underway.

A major international research effort has focused on the hormonal control of reserve mobilisation in germinating cereals. From such studies, particularly

those based on the Himalaya cultivar of barley, it has become widely believed that gibberellins are released from the embryo and trigger enzyme production by the aleurone cells surrounding the endosperm (Bewley and Black, 1985). It is becoming increasingly clear however, that the situation in germinating cereals is rather more complex than this and that differences exist both within and between species in their sensitivity to hormones and the relative importance of the aleurone and scutellum tissue for producing hydrolytic enzymes following germination (Fincher, 1989). Future work will consider whether such factors are implicated in the differences in seedling growth that exist between different pasture species.

Ryegrass α-amylase has been shown to consist of multiple isoenzymes which differ markedly from those extracted from germinated wheat and phalaris seeds. In both wheat and barley two groups of isoenzymes which are encoded by two separate gene families have been identified. They are known as the high or low pI group respectively, on the basis of their isoelectric points and each group consists of multiple isoforms (Ainsworth *et al.*, 1985; MacGregor *et al.*, 1988). Ryegrass α-amylase, by comparison, consisted of only one group of low to intermediate pI isoenzymes. Wheat and barley may be relatively unusual in possessing two groups of α-amylase isoenzymes since other cereals such as maize, oats and sorghum possess only one group of low or intermediate pI isoenzymes (Lecommandeur and Daussant, 1989; MacGregor *et al.*, 1988).

Within the single group of ryegrass amylases, however, changes to the isoenzyme profile were observed as the time-course proceeded. Of particular note was the appearance of a band of activity on the fourth day coinciding with the period in which enzyme activities were rising sharply. Whether these isoenzyme changes reflect activation of preformed enzyme or *de novo* expression of developmentally controlled genes is not known. Nor is it yet known what, if any, role is played by an inhibitor of α-amylase which has been identified in extracts of non-germinated ryegrass seeds (Cornford and Dixon, 1994).

In contrast with both ryegrass and wheat α-amylases which exist as multiple isoenzyme forms only two isoenzymes were detected in extracts of germinated phalaris seeds. Nevertheless, once again a striking change in isoenzyme pattern occurs as the germination time-course proceeded suggesting that individual isoenzymes are under differential control.

The results reported here are those of preliminary studies aimed at characterising aspects of reserve mobilisation in a rapidly germinating and establishing pasture species namely *Lolium multiflorum*. Nevertheless, the differences between ryegrass, phalaris and wheat, with respect to α-amylase production, clearly merit further investigation.

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