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Carbohydrate metabolism and tissue differentiation during potato tuber initiation, growth and dormancy induction

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Abstract

The duration of potato tuber dormancy has economic importance for both ware potatoes and seed tubers. The aim of this study was to shed light on the time at which tuber dormancy is induced. Potato tubers were selected at different stages of tuberisation: initial swelling of the stolon tip and early stages of tuber growth (tuber diameter 3, 7 and 14 mm). At each stage of tuberisation, the diameter of the pith and the cortex was measured, the activity of the enzymes β-amylase, glucose-6-phosphate dehydrogenase and succinate dehydrogenase was determined, and starch and RNA levels recorded. It was observed that during tuber initiation the pith and perimedullary zone showed the greatest increase in size, whereas the cortical parenchyma increased mainly when the tuber diameter was 7-14 mm. Moreover, during stolon swelling and initial tuber development (3 mm diameter) total RNA accumulation was observed. Starch accumulation varied with the stages of development. Glucose-6-phosphate dehydrogenase and succinate dehydrogenase exhibited their highest activity during stolon swelling whereas β-amylase activity was highest both during stolon swelling and at the 3 mm diameter stage. From the changes in tuber anatomy/morphology and the assays of enzyme activity, it is clear that dormancy is not induced in all the tissues of the tuber at the time of tuber initiation, but is imposed on the individual tissues as they are formed. Consequently, we may refer to *tuber dormancy* only when the last bud has completed its differentiation.

Keywords: β-amylase; glucose-6-phosphate dehydrogenase; *Solanum tuberosum*, succinate dehydrogenase.
Abbreviations: BSA_bovine serum albumin; DTT_dithiothreitol; EDTA_ethylenediaminetetraacetic acid, GA_3_gibberellic acid; G6PDH_glucose-6-phosphate dehydrogenase; NBT_nitroblue tetrazolium; PVP_polyvinylpyrrolidone; SDH_succinate dehydrogenase.

Introduction

Tuberisation in potato is initiated by the cessation of stolon elongation and swelling of the stolon tip as a result of changes in the direction of cell division (Cutter, 1992; Vreugdenhil et al., 1999). It is also associated with a switch from apoplastic to symplastic phloem unloading in the subapical region of the stolon (Viola et al., 2001) and an alteration in the activity of enzymes involved in carbohydrate metabolism (Geigenberger et al., 2004); for example, a reduction in invertase and an increase in sucrose synthase (Appeldoorn et al., 1997) and adenosine-5-diphosphoglucose pyrophosphorylase (AGPase) activity (Geigenberger, 2011). The changes in carbohydrate metabolism lead to an increase in starch and storage protein content in the swollen part of the stolon (Kloosterman and Bachem, 2014). However, the quality properties of the starch (e.g. granule size, structure) are influenced by the genotype and the environmental conditions (Wasserman et al., 2015). Tuber growth then occurs as a result of an increase in volume of the pith and cortex due to cell division and expansion (Xu et al., 1998), a process that is influenced by environmental factors such as temperature and photoperiod and the levels of endogenous phytohormones (Aksenova et al., 2012; Roumeliotis et al., 2012). Dormancy in potato tubers has been defined as 'the period during which no visible development of buds is observed even under conditions that are favourable for sprouting' (Reust, 1986). Initially, dormancy was thought to start after harvest (Burton, 1989), but Claassens and Vreugdenhil (2000) subsequently expressed the opinion that dormancy begins before harvest and even from the time of tuber initiation. The length of dormancy is affected by the environmental conditions that exist during tuber development on the mother plant and during storage (Suttle, 2007; Muthoni et al., 2014), e.g. temperatures lower than 10 ^{o}C delay dormancy breakage and sprout development (Burton, 1989). The duration of dormancy is also affected by phytohormones (Sonnewald and Sonnewald, 2014). Dormancy breakage may be achieved by the application of gibberellic acid (GA_3) during tuber growth (Alexopoulos et al., 2006), although tuber carbohydrate metabolism may be affected (Alexopoulos et al., 2007a). Dormancy breakage is also promoted by the postharvest application of gibberellin and cytokinin (Alexopoulos et al., 2007b; Hartmann et al., 2011), bromoethane or rindite (Akoumianakis et al., 2000; Akoumianakis et al., 2008).

Carbohydrate metabolism is intimately associated with potato tuber dormancy and the induction of sprouting (Alexopoulos et al., 2008; Rentzsch et al., 2012; Aksenova et al., 2013; Sonnewald and Sonnewald, 2014). For example,

glucose-6-phosphate dehydrogenase (G6PDH) activity was higher in developing tubers than in the stolons (Von Schaewen et al., 1995) and the activity of adenosine-5-diphosphoglucose pyrophosphorylase (AGPase) increased during tuber development (Appeldoorn et al., 1999). According to Sergeeva et al. (2012), the activity of the enzymes AGPase and starch phosphorylase (STP) was higher in developing micro-tubers than in older micro-tubers during storage. Claassens (2002) reported that the activity of α-amylase in micro-tubers was consistently less than that of β-amylase and decreased throughout dormancy. Following dormancy breakage, starch breakdown was accompanied by an increase in the activity of α-amylase and β-amylase only in the apical part of the micro-tubers (Sergeeva et al., 2012).
Most studies of carbohydrate metabolism in potato tubers have concentrated on the role of this process during dormancy and sprouting of mature tubers. However, for a young tuber developing on the mother plant and excised before maturity, a metabolic pathway which permits the induction of dormancy and subsequent dormancy release must either already be present or induced shortly after tuber separation from the plant. In the present paper, we examine some morphological, anatomical and metabolic changes that occur in the potato tuber during initiation and early development and which may shed light on the time at which tuber dormancy is induced.

Results and Discussion

Changes in tuber anatomy and morphology

At the stage of tuber initiation (stage A) the principal anatomical tissues were the periderm, cortical parenchyma, pith and xylem vessels of the vascular ring (Fig. 1A); these tissues were also observed in the subsequent stages of tuber development (Fig. 1B-D). However, at stage A the xylem vessels were relatively sparse, as also observed by Cutter (1992), and the cortical parenchyma consisted of 7-9 layers of cells (Fig. 1E) with the pith and perimedullary zone occupying the largest part of the sectioned swollen stolon tip (i.e. as in a stem), whereas in stages B and C (Fig. 1B, C) a large increase in the pith and perimedullary zone was observed (an increase of 3.5-4 times in comparison with the corresponding tissues at stage A) and to a lesser extent the cortical parenchyma (about 2 times compared with stage A) (Fig. 2). Similarly, Xu et al. (1998) observed a smaller increase in the diameter of the cortical parenchyma of potato tubers developing *in vitro* compared with the pith and perimedullary zone, while Cutter (1992) reported that during the early stages of tuber growth there is only a small increase in the number of cell layers of the cortex. Tuber size increases during the early stages of development due to an increase in cell number as a result of cell divisions in the cortex and pith (Xu et al., 1998) and in the perimedullary zone (Reeve et al., 1973a, b). This observation is supported by Xu et al. (1998) who noted that the small size of potato tubers cultivated *in vitro* stems from the absence of a perimedullary zone in these tubers.

At stage D (14 mm diameter), the diameter of the pith was about 2.3 times more than at stage C (7 mm diameter), whereas the cortical parenchyma was approximately 5 times larger (Fig. 2) due to an increase in both the number of cell layers and cell size (Fig. 1D). This increase in growth of the cortex indicates that after stage C there is a change in the pattern of tuber development. According to Borzenkova and Borovkona (2003), tuber growth from the middle (diameter: 2.5-3 cm) to large (diameter: 6-7.5 cm) size involves cell

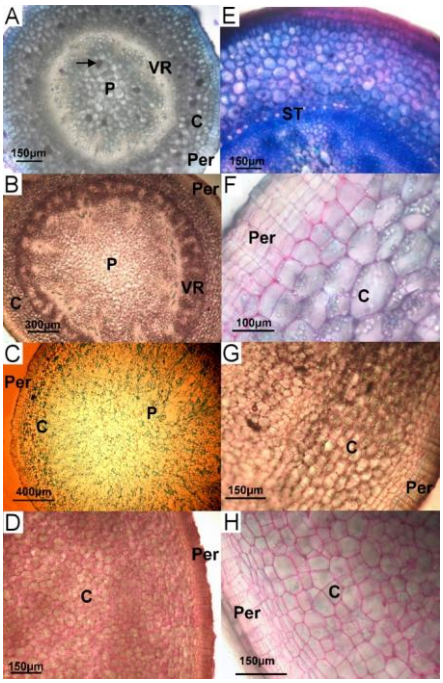

Fig 1. Transverse section of potato tubers at different stages of development. A: during swelling of the stolon tip, where the arrow shows calcium oxalate; B: at 3 mm diameter; C: at 7 mm diameter; D: at 14 mm diameter. Details for the periderm and cortical region of each section are shown in the adjacently placed photographs (E-H). ST: sieve tubes, Per: periderm, C: cortical parenchyma, VR: vascular ring, P: pith. Staining was performed with toluidine blue (A, E) and safranine fast green (B, C, D, F, G, H).

division, especially in the cortex, and cell enlargement in the pith. However, the pith still constitutes the largest part of tubers at stage D (Fig 2), as has been observed in medium-sized and large tubers (Borzenkova and Borovkona, 2003).
Calcium affects tuberisation and it is usually stored as calcium oxalate crystals in plants (Nookaraju et al., 2012). Calcium oxalate crystal, identified at stage A of tuber development (Fig. 1A), may act as a defense mechanism against insects (Webb, 1999) or as a means of removing excess CO_2 (Cabot et al., 2009) from the metabolically active internal cells of the developing tuber. Another significant event during tuber development is the expansion of internodes already present in the apical bud meristem (Cutter, 1992; Wohleb et al., 2014) coupled with the appearance of buds (eyes) at each node (Fig. 3A). As in an aerial stem, the youngest bud is that which forms nearest to the apex of the stolon and since dormancy induction relates to age, this last-formed bud is the last to enter dormancy (Van Es and Hartmans, 1987). By this time, the other buds along the tuber have already completed their development (Fig. 3B) and may be dormant. According to Viola et al. (2007), potato tuber dormancy occurs only in the tuber buds, in which cell division does not occur (Campbell et al., 1996). Nevertheless, dormant tuber buds are metabolically competent, but with low rates of metabolism, probably due to their symplastic isolation (Viola et al., 2007). Thus, dormancy of the potato tuber *as a whole* may be said to occur only once the last-formed bud is dormant.

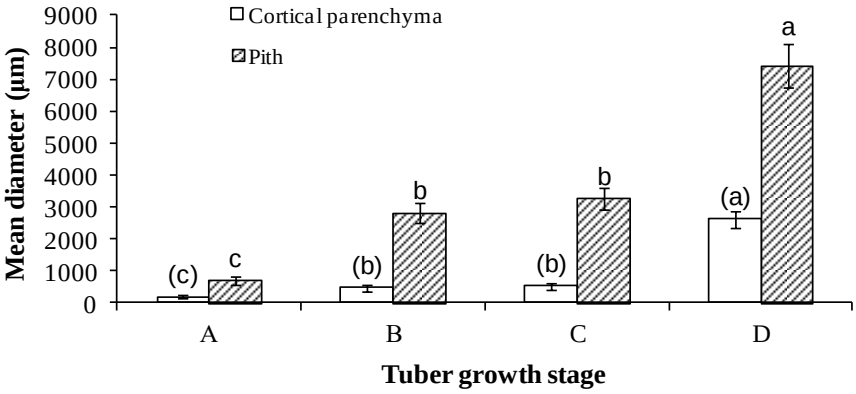

Fig 2. Mean diameter of the pith and cortical parenchyma of potato tubers during development. Different letters at the tops of the columns indicate a significant difference between the stages of tuber growth, in the parenthesis for the cortical parenchyma and without parenthesis for the pith (bars show ± standard deviation).

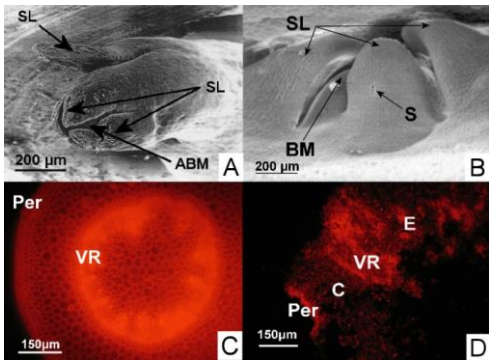

Fig 3. Morphological characteristics of the tuber apical meristem during swelling of the stolon tip-stage A (A) and a bud of the tuber at the 14 mm diameter stage-stage D (B) and RNA localization in transverse sections of potato tubers at growth stage A (C) and growth stage B (D). SL: scale leaf, BM: bud meristem, ABM: apical bud meristem, Per: periderm, C: cortical parenchyma, VR: vascular ring, P: pith. RNA was coloured red with acridine orange.

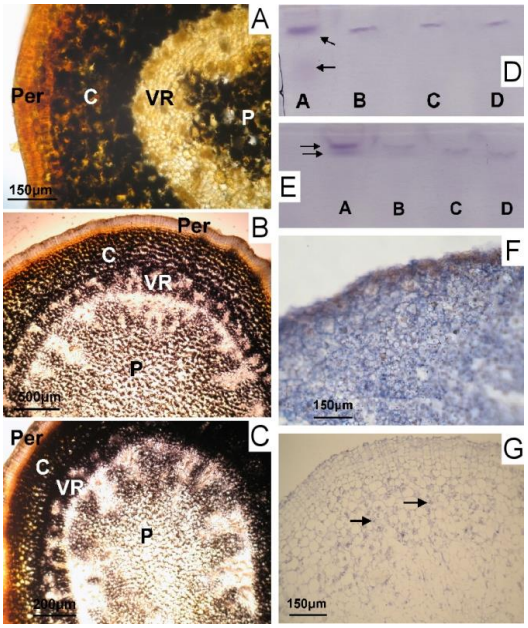

Fig 4. Transverse sections of potato tubers at stages (A) swelling of the stolon tip, (B) at 3 mm diameter, (C) at 7 mm diameter; electrophoretic patterns of (D) glucose-6-phosphate dehydrogenase and (E) succinate dehydrogenase activity at stages A-D of potato tuber development, and (F, G) the immunolocalisation of β-amylase in transverse sections of potato tubers at growth stages B and C, respectively. Per: periderm, C: cortical parenchyma, VR: vascular ring, P: pith. The blue-mauve colour indicates the presence of β-amylase, which is located at the starch grains (arrows).

Starch and total RNA accumulation

Tuber induction and initial enlargement apparently related to the accumulation of RNA at stages A and B, which occurred mainly in the region of the vascular ring, less in the periderm and to a much lower extent in the other tissues of the tuber (Fig. 3C). In the subsequent stages of tuber development RNA accumulation was less, but occurred throughout the entire tuber (Fig. 3D). In dormant buds, meristems are arrested in the G1 state (Campbell et al., 1996) and exhibit reduced levels of RNA (Korableva and Ladyzhenskaya, 1995), whereas at the onset of sprouting in GA_3-treated tubers RNA was detected mainly in the region of the buds (Alexopoulos et al., 2008). It is known that RNA accumulation correlates with protein synthesis and cell division, which are intense during the early stages of tuber development (Reeve et al., 1973b; Hannapel, 2010). The presence of RNA in the proximity of the vascular ring is not surprising since the tissues of this region are active in the transport of nutrients and in the biosynthesis of xylem cell walls and specialized phloem parenchyma cells, which play a role in the transfer of energy to other cells (Peterson et al., 1981; Kragler, 2010; Kloosterman and Bachem, 2014). Similarly, the relatively high concentration of RNA in the periderm may be attributed to intense metabolic activity associated with cell division, growth and differentiation into cork (or phellem) (Kloosterman and Bachem, 2014).

Although starch accumulation and tuber growth are independent processes (Sergeeva et al., 2000), and tubers may form even in the absence of starch (Muller-Rober et al., 1992), the increase in tuber size is affected by starch accumulation (Kloosterman and Bachem, 2014). In addition, imported carbohydrates (e.g. sucrose) not only provide a substrate for starch biosynthesis but also play a role in tuber morphogenesis (Vreugdenhil et al., 1998; Aksenova et al., 2012). Starch was detected from the first stage of swelling and tuber initiation (stage A), as noted by Ross et al. (1994). According to Zeeman et al. (2010), the activity of the enzyme AGPase significantly affects the early steps of starch biosynthesis. Throughout tuber development, more starch was located within the cells of the cortical parenchyma, which has a storage function (Fig. 4A-C), than in the pith, while in the vascular ring the presence of starch is restricted since the sieve elements and accompanying cells contain little or no starch (Cutter, 1992). The presence of starch in the pith at stage A (Fig. 4A) and its subsequent decrease as the tuber enlarged (Fig. 4B, C) indicates that the starch of this region was used in other metabolic processes involved in tuber growth and not replaced. This is in consistent with the observation that starch synthesis and starch degradation occurs during tuber development (Sergeeva et al., 2012). However, the changes in starch metabolism, and carbohydrate metabolism as a whole, appear to be directly associated with tuber dormancy (Aksenova et al., 2013) since during tuber growth and the progressive imposition of dormancy on the buds starch accumulation occurs, except in the cells just below the buds (Davies and Viola, 1988). Liu et al (2015) reported that several proteins involved in sucrose and starch metabolism were found to be up-regulated in tubers after dormancy breakage. Moreover, when dormancy is broken by bromoethane (Alexopoulos et al., 2009) or GA_3 (Alexopoulos et al., 2008) the concentration of reducing sugars in tissues associated with the buds changes, starch is broken down and the activity of enzymes associated with starch metabolism (e.g. α-glucosidase) increases before visible sprouting.

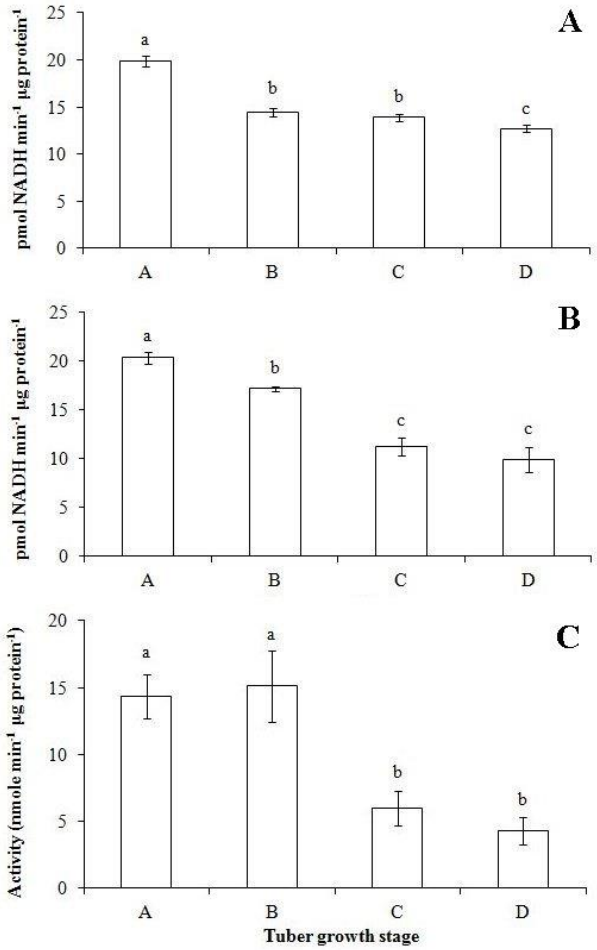

Fig 5. The activity of (A) glucose-6-phosphate dehydrogenase, (B) succinate dehydrogenase and (C) β-amylase at stages A-D (swelling of the stolon tip, tuber diameters 3, 7 and 14 mm, respectively) of potato tuber development. Different letters at the tops of the columns indicate a significant difference between the stages of tuber growth (bars show ± standard deviation).

Changes in the activity of enzymes

The activity of glucose-6-phosphate dehydrogenase (G6PDH) was highest at stage A and fell from stages B to D (Fig. 5A), as may be seen also in the electrophoresis gels (Fig. 4D), indicating that sucrose metabolism and the supply of reducing sugars change during tuber development. This enzyme catalyzes the first step of the oxidative pentose-phosphate pathway and influences the availability of reduced NADPH which sustains reductive biosynthesis, e.g. of fatty acids (Singh et al., 2012). The intense activity of G6PDH at stage A was apparently essential for the requirements of the rapidly growing tissues of the young tuber. Von Schaewen et al. (1995) found a higher expression of the cytosolic isoform of G6PDH in the developing tuber than in the stolon, whereas with the appearance of new buds and the imposition of dormancy the activity of this enzyme fell. Claassens (2002) reported that during dormancy imposition in tubers produced *in vitro* there was a further decline in G6PDH activity, which did not significantly increase even after dormancy breakage. The presence of two electrophoresis bands at stage A (Fig. 4D) suggests the presence of two different isoenzymes or different forms of the same enzyme (e.g. cytosolic and plastidic). In potato plants, two plastidic (P1 and P2) and a

cytosolic isoform of the enzyme have been reported (Hauschild and Von Schaewen, 2003). The cytosolic isoform and the plastidic isoform (P2) of G6PDH are transcribed in potato tubers (Von Schaewen et al., 1995; Wendt et al., 2000).The activity of succinate dehydrogenase (SDH), similar to that of G6PDH, was highest at stage A, followed by a progressive decrease in activity between stages B and D (Fig. 5B), as seen also in the electrophoresis gels (Fig. 4E). Since SDH expresses the respiratory activity of the mitochondria (Araújo et al., 2014), it is clear that the energy requirements at tuber initiation (stage A) are high due to rapid cellular metabolism, but that respiratory activity (hence metabolism) declines as the tuber matures (Burton, 1989). In addition, SDH enhances assimilation in plants (Araújo et al., 2011), contributes to the production of reactive oxygen species (ROS) in plant mitochondria and regulates plant development (Jardim-Messender et al., 2015). Interestingly, SDH at stage A presented two electrophoresis bands (similar to G6PDH), which were not observed during the subsequent stages of tuber development (Fig. 4E). Since SDH activity has been related to dormancy breakage of yam (*Dioscorea esculenta* (Lour.) Burk.) tubers (Panneerselvam et al., 2007), it may be hypothesized that changes in the activity of SDH in developing potato tubers might be related to potato tuber dormancy. Although there is little published work relating to the presence of β-amylase in developing potato tubers, this enzyme was detected in the tubers of the present experiment and verified from cDNA libraries, which show the existence EST clones of β-amylase in developing potato tubers. The activity of β-amylase was high during the initial stages of tuber development (stages A and B) and subsequently decreased (Fig. 5C). It appears that β-amylase activity in developing potato tubers is associated with the provision of reducing sugars to the cells that are active in tuber growth and differentiation (internode and bud formation). According to Sergeeva et al. (2012), the activity of amylases during tuber development is related to starch cycling. The activity of β-amylase decreases with the onset of dormancy and then increases again after the initiation of sprouting (Sergeeva et al., 2012). Moreover, the activity of β-amylase increases in the sub-eye regions of the tuber after the onset of sprouting, but not in the parenchyma (Biemelt et al. 2000). Immunolocalisation indicated that β-amylase was present near the starch grains of cells (Fig. 4F, G) and especially in cells near the vascular bundles (Fig. 4G). This suggests that β-amylase activity relates to the production of sugars that can be readily transported to other metabolically active tissues (e.g. newly-formed internodes). In addition, Sergeeva et al. (2012) hypothesized that sugars produced from starch degradation by amylases in the parenchyma are transported towards the vascular tissue, where starch is resynthesized as a result of starch phosphorylase activity.

In conclusion, the changes in anatomy and morphology in developing tubers may relate to the changes in the activity of the enzymes examined in this work. Since carbohydrate metabolism plays an important role in the regulation of tuber dormancy (Aksenova et al., 2013), the changes in carbohydrate metabolism observed here during tuber development may relate to the induction and duration of tuber dormancy.

Materials and Methods

Plant materials

One hundred and sixty tubers of certified seed potato (*Solanum tuberosum* L. cv. Liseta) were planted singly in 15 cm diameter plastic pots containing perlite and peat (1:1 v/v) in January and placed in an unheated greenhouse of the Vegetable Production Laboratory of the Agricultural University of Athens. Plants were harvested at the following stages of tuberisation: (A) stolon swelling following the hook stage, (B) tuber induction (3 mm diameter), (C) tuber growth (7 mm diameter), and (D) tuber diameter 14 mm. The time of harvest was determined by random sampling of plants during growth following stolon induction, which occurred 22 days after planting. For each stage of tuberisation, four replicates of 10 plants each were harvested.

Microscopy

Immediately after harvest, the stolon tips (stage A) or young tubers (stages B-D) were excised by a sharp blade, placed on moist filter paper in 11 cm Petri dishes and transferred to the laboratory. Transverse sections approximately 1 mm thick were taken from the centre of the small tubers or stolon tips and the diameter of the pith and cortex was measured under an optical microscope (Carl Zeiss, AxioLab, Jena, Germany). For scanning electron microscopy, intact tubers harvested at stages B-D, were placed in 3% glutaraldehydein and 0.1M phosphate buffer (pH 7.2) at 41^{o}C for 4h, dehydrated with acetone, and mounted on stubs with self-adhesive double-sided carbon discs.

Histochemical detection of starch and total RNA in tuber tissues

Starch in sections was localized with a 3% KI+1.3% I solution, whereas for standard anatomy of fixed sections in all tissues except those at stage A saffranin-fast green was used (Jensen, 1962). However, due to tissue hardening and discoloration during xylene infiltration for the safranin-fast green method, fresh sections at stage A were stained with toluidine blue according to O'Brien et al. (1981). RNA was localized with acridine orange (Harris and Oparka, 1994).

Enzyme activities

For enzyme activity determination, tubers were ground in liquid nitrogen and homogenized in 2.5 volumes of a modified extraction medium containing: 50mM Tris pH 7.5, 5% polyvinylpyrrolidone-40 (PVP-40), 20mM sodium sulphite, 1.5 mM dithiothreitol (DTT), 7.0 mM KCl, 1.0 mM $MgCl_2$, 0.5mM ethylenediaminetetraacetic acid (EDTA) and 0.3% BSA (Viola et al., 2001). The homogenates were centrifuged at 15000g for 15min and the supernatants were collected for further measurements. Total proteins were determined according to Bearden (1978). Native protein electrophoresis was performed in discontinuous gels of 7.5%T, 2.7%C for resolving and 3%T, 2.7%C for stacking. Each lane was loaded with 50μg of total proteins from tuber extracts. The in gel activity of G6PDH was localized according to Sergeeva and Vreugdenhil (2002), while SDH activity was localized according to Pette and Tyler (1983). The *in vitro* activity of G6PDH and SDH was determined according to Sergeeva and Vreugdenhil (2002) and Pette and Tyler (1983) respectively with the omission of nitroblue tetrazolium (NBT). For β-amylase activity the Betamyl kit (Megazyme) was used. Immunodetection of β-amylase was determined using polyclonal antibodies directed against the specific enzyme (Biodesign K5941R) according to Tsaniklidis et al. (2015).

Statistical analysis

Results were subjected to analysis of variance (ANOVA) in accordance with a completely randomized experimental design. For each stage of tuber growth, the mean value of the diameter of the pith and cortex was calculated from four replicates of ten tubers each, while the mean value of enzyme activity was calculated from four replicates of eight tubers each. Differences between means were subjected to the least significant different test ($P \leq 0.05$), when the value for F proved significant. Statistical analysis was performed using the statistical package StatGraphics Centurion (Statpoint Technologies, Warrenton, USA).

Conclusions

From the present study, it is clear that from the moment of initiation until the completion of its growth and maturation, the potato tuber presents a series of morphological, anatomical and physiological/biochemical changes. During the first stages of potato tuber growth, in which morphological and anatomical changes occur (increase in size, formation of internodes etc.), a progressive reduction in the activity of enzymes involved in carbohydrate metabolism was observed, but at no stage during this process could the tuber be considered to exist in a dormant state since one or more tissues were continuing to grow or differentiate. For example, β-amylase activity reduced when the tubers had 5-7 visible buds, but finally 8-10 buds per tuber formed in total. In consequence, we submit that potato dormancy does not occur at the onset of tuberisation, as generally accepted in the literature (e.g. Burton, 1989), but is progressively induced within the tuber tissues and is finally imposed only on completion of the differentiation of the youngest (terminal) bud, which if then excised from the tuber has the ability to form a shoot.

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