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Investigations into the Role of Sucrose in Potato cv. Estima Microtuber Production *in vitro*

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Sucrose has been the carbohydrate traditionally used for potato microtuber production. Added to nutrient media, sucrose can act solely as a carbon source, or as an osmoticum, or both. Preliminary tests showed that the osmolarity of sucrose solutions was increased by autoclaving, indicating some breakdown of the sugar. This was taken into consideration in experiments which involved supplementing 4% sucrose media with sucrose, maltose, glucose or fructose, while keeping the osmotic potential of the media constant. A medium concentration of about 400 mM with only sucrose was more suitable for microtuber production than media supplemented with maltose, glucose or fructose. However, a better microtuber yield was obtained when hexoses were added than with unsupplemented 4% sucrose media. When glucose was supplied at concentrations which had the same number of carbon atoms as 8% sucrose, the high osmolarity inhibited microtuberisation. Sugar movement in the tubering plantlet was followed using radio-labelled sucrose, glucose and fructose. The sucrose was translocated and used at a faster rate than the other sugars, which tended to remain in the roots of the plantlets. Furthermore, there was no difference in microtuber production on media to which the sucrose was added before or after autoclaving, indicating that levels of breakdown were not severe enough to affect the process. Therefore, it is concluded that sucrose acts primarily as a suitable carbon source for uptake and utilization by the plantlets, but, at 8%, it also provides a favourable osmolarity for the development of microtubers.

Key words: *Solanum tuberosum* (L.), potato, microtuber, media, sugar, sucrose, osmolarity, pH.

INTRODUCTION

Sucrose has been used as the carbohydrate source in most *in vitro* investigations of potato (*Solanum tuberosum* L.), whether at the organ, tissue or cell level. It has been added to culture media at a variety of concentrations, depending on the method and media used to induce microtuber initiation. As early as 1954 it was established that a high sucrose concentration (5% as opposed to 1%) favoured tuberisation (Mes and Menge, 1954). The addition of growth regulators seems to enhance microtuber development only if an adequate supply of sucrose is given (8% rather than 2%) in the culture medium (Harmey, Crowley and Clinch, 1966). There have been several reports comparing the effects on microtuberisation of a range of sucrose concentrations. Wang and Hu (1982) added 1, 3, 6, 8 and 9% sucrose to liquid media containing BAP, and found the highest percentage of tuberising plantlets on the medium with 8% sucrose. Koda and Okazawa (1983) tested media containing zeatin riboside with 2, 4, 6 or 8% sucrose. They also obtained the highest microtuber yield from 8% sucrose, but did not test it at this concentration with other growth regulators. Abbot and Belcher (1986) recorded microtuberisation from plantlets grown on media containing 3, 6, 9 and 12% sucrose. Their best results were obtained from adding 6% sucrose and BAP to the medium. Hussey and Stacey (1984) showed that 6% sucrose was optimal for microtuberisation when BAP and CCC were supplied, while at the International Potato Centre (CIP) the favoured method is the addition of 8% sucrose in combination with BAP and CCC (Tovar *et al.*, 1985). More recently, Garner and Blake (1989) found that, on media free of growth regulators, 8% sucrose coupled with transfer to short days promoted the greatest microtuber formation. The discrepancies between these findings are probably due to the use of different genotypes, different mixtures of nutrient salts, and/or different concentrations of the growth regulators added, together with possible variations in the size of vessel, light intensity and temperature. However, the consensus seems to be that a concentration of about 8% sucrose gives the greatest microtuber yield.

There does not appear to be an explanation in the literature of the function of sucrose in microtuberisation. The question can be posed as to whether it performs an osmotic role or purely a nutritional one. The role of sucrose as an osmotic agent in plants is well established. Along with its component hexoses, it can account for up to 35% of osmotic adjustment in tobacco callus cells subjected to high sodium chloride (NaCl) stress (Gibbs *et al.*, 1989). These sugars were also found to increase dramatically in carrot (Fallon and Phillips, 1989) and tomato (Handa *et al.*, 1983) cells placed under osmotic stress. It is thought that the sucrose dissociates to allow a higher osmotic potential

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within the cells. Thus the role of sucrose in plant tissue culture media as an osmoticum as well as a carbohydrate source has been established.

Microtuber induction may depend on the osmotic shock of a high sucrose solution. However, developing microtubers are a sink for sucrose from the culture medium. All early work on microtuberisation, from the first report in 1953 (Barker, 1953) until recently, has focused on the role of sucrose as an energy source for the developing tuber. In the intervening period, only a few workers have investigated the possible osmotic role which the sugar plays in the microtuberisation media. Fung, Irvine and Barker (1972) cultured etiolated stolon nodes on White's (1943) medium to which sucrose was added at 2, 8 or 12%. The osmotic potential of the media was equated using different concentrations of mannitol. Rapid tuberisation was observed on cultures growing on 8 and 12% sucrose, but none on media to which only mannitol was added. Only a few small tubers were formed after a great delay on low sucrose media, regardless of the presence of mannitol. Thus they concluded that microtuberisation was a response to the high sucrose concentration, and was independent of the osmotic potential of the medium. Garner (1987) attempted a similar experiment, when he used mannitol to raise the osmolarity of MS (Murashige and Skoog, 1962) medium with 4% sucrose to that of MS medium with 8% sucrose. He found that microtuberisation was delayed by 4 weeks on media containing mannitol, and by the end of the experiment (14 weeks), only a few microtubers had developed on that medium, compared to around twice as many on 4% sucrose alone, and around four times as many on 8% sucrose. It was assumed from these results that mannitol inhibited microtuberisation. Oparka and Wright (1988) showed that sucrose uptake and its conversion to starch in potato tuber discs was sensitive to the osmotic potential of the medium and of the cells themselves. They found that starch synthesis was optimal at 300 mM mannitol, but decreased sharply above and below this. The amounts of mannitol needed to equate the osmotic potential of a 2 or 4% sucrose solution to that of 8% are less than 300 mM, and starch synthesis may have been inhibited by mannitol in Garner and Blake's experiments (1989).

There appears to be only one report on the effect of carbohydrates other than sucrose on microtuberisation. In this, sucrose, glucose, fructose, mannose and mannitol were compared at concentrations ranging from 4 to 12% (Chandra, Dodds and Tovar, 1988). It was again found that 8% sucrose was optimal for the development and growth of microtubers, while smaller microtubers were obtained on media containing glucose or fructose, and none on that with mannose or mannitol. Unfortunately, these results were not discussed further, either in terms of osmolarity or carbon supply. Furthermore, the system used would most probably have included the use of certain growth regulators in the medium (cf. Tovar *et al.*, 1985), and could not be extrapolated to other systems.

Thus, the question of whether sucrose is playing an osmotic role instead of, or as well as, a nutritional one still remains, and was investigated in these experiments. To facilitate osmotic measurements, the cultures were grown on liquid media. Liquid media have been used successfully by Tovar *et al.* (1985) and Rosell, De Bertoldi and Tizio (1987) induced heavier microtubers on liquid media than on agar-solidified media. A small preliminary experiment verified that the system as used for these experiments does form microtubers in the normal way.

MATERIALS AND METHODS

Nodal explants of *Solanum tuberosum* cv. Estima were supported on polyurethane foam in 7·5 cm tubes containing 10 ml of liquid media. The base medium was 4·71 g l^{-1} Murashige and Skoog (MS, 1962) medium with 36·7 mg l^{-1} NaFeEDTA, and 40 g l^{-1} sucrose. This was supplemented with different amounts of other sugars as shown below. The control consisted of the base medium (MS) with 80 g l^{-1} sucrose. The plants were grown at 55–62 μE m^{-2} s^{-1} provided by Philips TLD fluorescent lamps with a 16 h photoperiod for 3 weeks before being transferred to an 8 h daylength, all at a temperature of 19 °C.

The cultures were scored by destructive harvesting of five replicates taken at random every 7 d for 12 weeks. The height (cm), node number and total branch number of the plantlets were recorded. The osmotic potential (mmol l^{-1}) and pH of the media were measured. When microtubers began to appear, their individual fresh weight (mg) and number per plantlet were recorded.

Equimolar media

The osmotic potential of 40 g l^{-1} sucrose was increased to that of 80 g l^{-1} sucrose using sugars other than sucrose; in amounts to produce a molarity equal to that of the additional 40 g l^{-1} sucrose after autoclaving. Thus it was assured that the osmolarities of the autoclaved media would be similar. There were a total of five treatments: the base medium supplemented with sucrose, maltose, glucose and fructose, plus an unsupplemented control.

The molecular weight of the disaccharides used was 342 and that of the monosaccharides 180. A 40 g l^{-1} sucrose solution therefore has a molarity of 40/342 = 0·117 M. In a preliminary experiment, it was found that the pH of a sucrose solution does not change significantly upon autoclaving, but the osmotic potential rises, indicating a breakdown of some sucrose molecules. This breakdown was calculated as being 10·95% of the total sucrose present, i.e. from 40 g l^{-1} sucrose, 0·0128 mol break down, and 0·1042 mol remain. Breakdown of the monosaccharides however, was found to be negligible. Assuming the sucrose produces nothing but glucose and fructose, the molarity of a 40 g l^{-1} sucrose solution after autoclaving is therefore 1·1042 + 2(0·0128) = 0·1298 M. From this, it is possible to calculate the amounts of other sugars to be added: 40 g sucrose + 40 g sucrose; 40 g sucrose + (0·1298 M × 342 g = 44·39 g) maltose; 40 g sucrose + (0·1289 M × 180 g = 23·36 g) glucose; 40 g sucrose + 23·36 g fructose; and 40 g sucrose on its own.

The glucose content of the media was measured in the third and ninth weeks of plantlet growth, using an enzyme analysis kit produced by Boehringer.

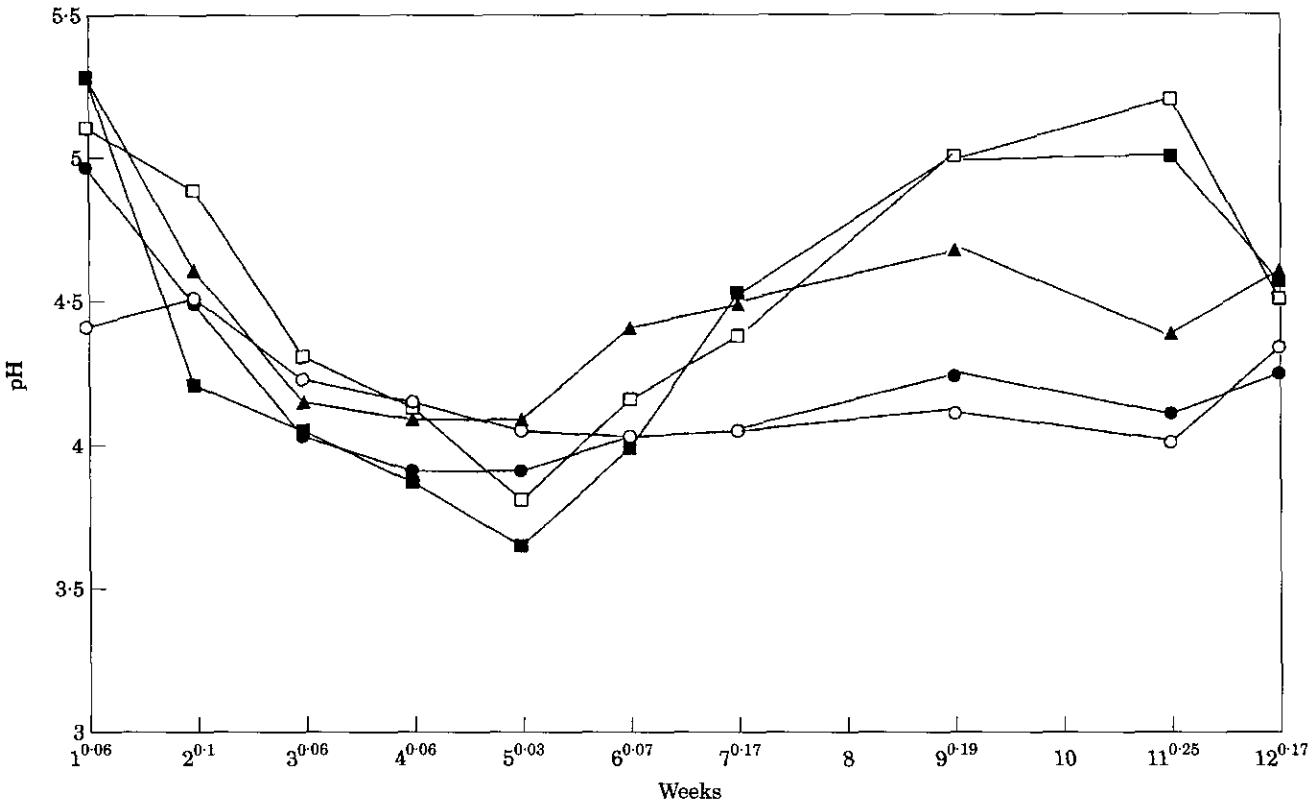

FIG. 1. pH of base medium supplemented with an additional 4% sucrose (-■-), maltose (-□-), glucose (-●-) or fructose (-○-), with an unsupplemented control (4% sucrose) (-▲-), on which *Solanum tuberosum* cv. Estima plantlets were grown for 12 weeks. All the added solutions were equimolar to a 4% sucrose solution after autoclaving. The superscript numbers are the standard errors of the difference between the means of the treatments.

Microtuberisation on glucose

Four per cent sucrose plus 4·71 g l^{-1} MS and 36·7 mg l^{-1} NaFeEDTA was used as the control solution. To this was added 0·234 M glucose (46·7 g l^{-1}) to give a solution equivalent osmotically to 80 g l^{-1} sucrose with 10·95% breakdown. Another solution was prepared such that it had a carbon content equivalent to 80 g l^{-1} sucrose, i.e. 0·519 M glucose (93·42 g l^{-1}).

Pattern of sugar uptake

The uptake and distribution of sugars by the plantlets were studied using radio-labelled sugars. The base medium was supplemented with sucrose, glucose and fructose at the same concentrations as the first experiment, i.e. all the media had the same initial osmotic potential. The media were solidified with 7 g l^{-1} agar to minimize the risk of any radioactive spillages. After autoclaving, known amounts of filter-sterilized labelled sucrose, glucose and fructose solutions were added. An additional treatment was included in which the ^{14}C-sucrose solution was added to the media before autoclaving.

The ensuing cultures were divided into (*a*) the stem, including aerial roots, tubering branches and under-agar stolons, (*b*) the roots, (*c*) the initial explant and (*d*) the microtubers, when they began to form. There were five replicates for each of the four treatments. The first measurements were taken after 3 weeks of growth in a 16 h photoperiod. The cultures were then transferred to an 8 h daylength, and measured every 2 weeks thereafter until week 13. The samples were placed in Universol (ICN Biomedicals Inc.) liquid scintillant in sunlight for 3 weeks to bleach any pigments before being analysed in a scintillation counter (LKB 1211 Rackbeta) for 10 min. The amounts of ^{14}C absorbed were obtained by summing the amounts found in the different plant parts, and the percentage distribution calculated.

RESULTS

Equimolar media

The growth rates of the plantlets on the different media were similar. The pH of the culture medium fell from approximately 5 to 4 in the first 5 weeks, then began to rise (Fig. 1). The media had significantly different pH levels until week 6, but there were no systematic differences between treatments. There was a large variability between replicates in weeks 9 and 11, which meant that the large differences in pH were not significant.

The osmotic potential of all media rose slightly until week 9, but by week 12 they were not different from those at week 1. There were highly significant differences between the

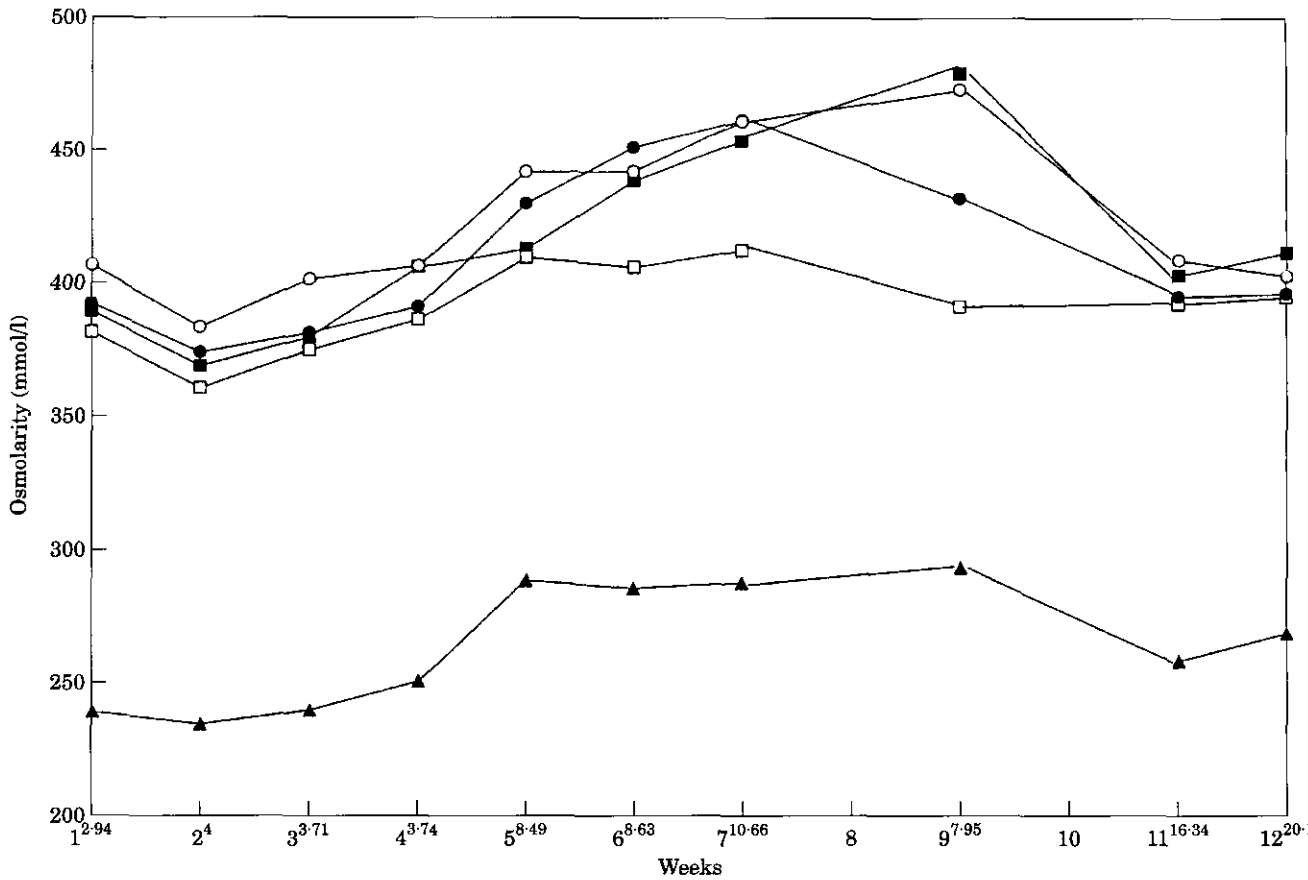

FIG. 2. Osmolarity (mmol l^{-1}) of base medium supplemented with an additional sucrose (–■–) (4%), maltose (–□–), glucose (–●–) or fructose (–○–), with an unsupplemented control (–▲–) (4% sucrose), on which *Solanum tuberosum* cv. Estima plantlets were grown for 12 weeks. All the added solutions were equimolar to a 4% sucrose solution after autoclaving. The superscript numbers are the standard errors of the difference between the means of the treatments.

osmolarities of the different media. These were mainly due to the very low osmolarity of the unsupplemented control, which was around half that of the other media (Fig. 2). A second analysis was carried out without the data for 4% sucrose, and it was seen that media containing fructose maintained a significantly higher osmolarity than other media, whereas that with maltose was the lowest, during the first weeks of the experiment. By week 12, there were no significant differences between the osmotic potentials of the media, except the unsupplemented control.

The results of the glucose analysis show that the glucose concentration of all media increased with time. After 3 weeks of growth, there were still negligible amounts of glucose in all media except that to which glucose was added (Table 1). The amount of glucose in that treatment was lower than the original amount added, showing that glucose had been taken up by the plantlet. At week 9, media supplemented with maltose, fructose or the unsupplemented control had comparable amounts of glucose. In media with fructose or with no additions, this could only have come from the breakdown of the 4% sucrose in the basal medium. The breakdown of maltose yields two glucose molecules, but since the amount found in this medium was not significantly higher than that in the control or in media with fructose, it was concluded that there was negligible breakdown of maltose. The amount of glucose found in media with 8% sucrose at this time was almost twice that found in 4% sucrose (control). As expected, there was a very large amount in media to which glucose had been added. Thus it can be concluded from these results that sucrose was being hydrolysed.

TABLE 1. *Amount (g l^{-1}) of glucose found after 3 and 9 weeks in base medium containing 4% sucrose and supplemented with sucrose, maltose, glucose, fructose or unsupplemented, on which* Solanum tuberosum *cv. Estima plantlets were grown (mean ± s.e.)*

4% sucrose +	Week 3	Week 9
Sucrose	0·76 ± 0·4	27·0 ± 2·9
Maltose	0·68 ± 0·5	18·5 ± 0·9
Glucose	13·92 ± 2·4	44·3 ± 1·0
Fructose	0·48 ± 0·1	17·8 ± 0·6
Unsupplemented	0·19 ± 0·1	17·6 ± 1·4

Microtubers began to form on 8% sucrose medium after week 6, and on 4% sucrose medium supplemented with maltose from week 7. From week 9, the number of microtubers formed on base medium supplemented with sucrose was higher than on that with the other sugars (Fig.

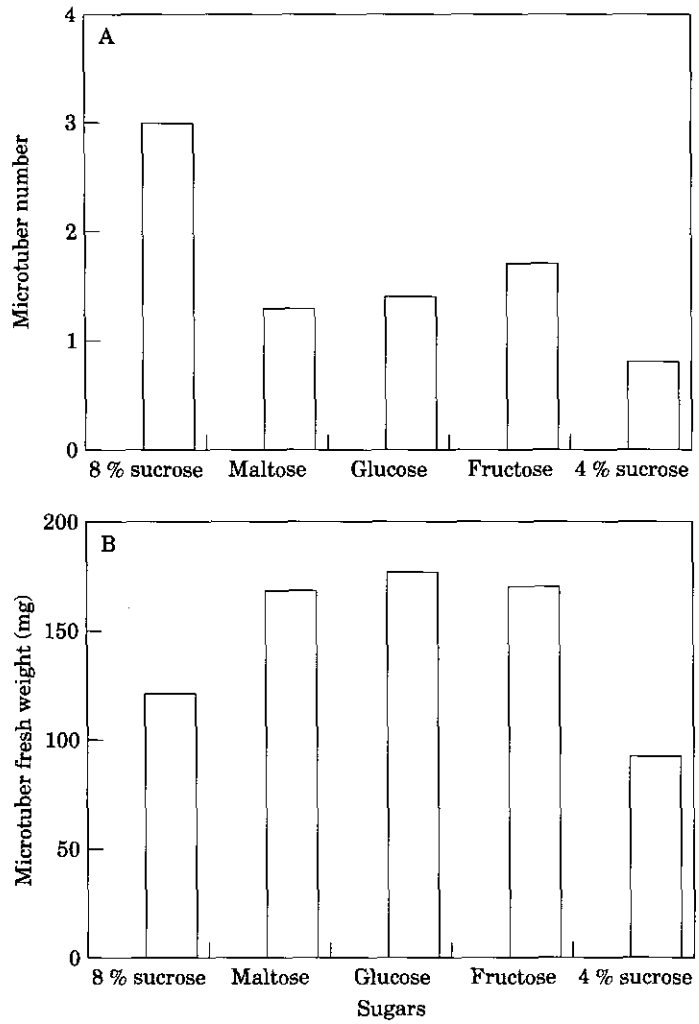

FIG. 3. A, Numbers and; B, fresh weight (mg) of microtubers produced from *Solanum tuberosum* cv. Estima plantlets grown for 12 weeks on base medium supplemented with an additional 4% sucrose, maltose, glucose or fructose, with an unsupplemented control (4% sucrose). All the added solutions were equimolar to a 4% sucrose solution after autoclaving.

3A). The unsupplemented control began to produce microtubers later than all other treatments, and had the lowest number at week 12. The trends in microtuber fresh weight were the inverse of those seen for the number of microtubers. The heavier microtubers were formed on media to which glucose, fructose and maltose were added, and the 4% sucrose control had the lightest microtubers (Fig. 3B).

Microtuberisation on glucose

Growth rates were similar on the 46·71 g l^{-1} glucose to that on media with 8% sucrose, but growth on media containing 93·42 g l^{-1} glucose was significantly less. The plantlets on high glucose medium remained green, but developed many stunted branches which did not grow beyond 1–2 cm in height. The change in pH was similar to that in the previous experiment. It initially fell and then stabilized. The osmotic potential of 8% sucrose and low glucose media was similar throughout the experiment, whereas that of high glucose media was almost twice as high (Fig. 4). This not only affected the growth of the plantlets, but also microtuber development. Microtubers began to form on the 8% sucrose control from the fifth week of culture, and on the other media from week 8. The number and fresh weight of the microtubers formed were similar on the glucose media until week 10, after which they began to develop at a faster rate on low glucose medium (Fig. 5A, B). As expected from previous experiments, fewer microtubers were produced on glucose than on sucrose media.

Pattern of sugar uptake

Figure 6 shows the absorption of sugars by the plantlets. This increased throughout the experiment, but there was a greater uptake of glucose than other sugars. The sugar absorbed through the roots was gradually transferred to the rest of the plantlet, and a decreasing amount remained in the roots. The proportions in the shoots and initial explant remained approximately constant, while the proportion in the microtubers increased, especially in the sucrose treatments. In the first few weeks of the experiment, there were no significant differences between the treatments in the amount of ^{14}C in the roots, but by week 9, more sucrose

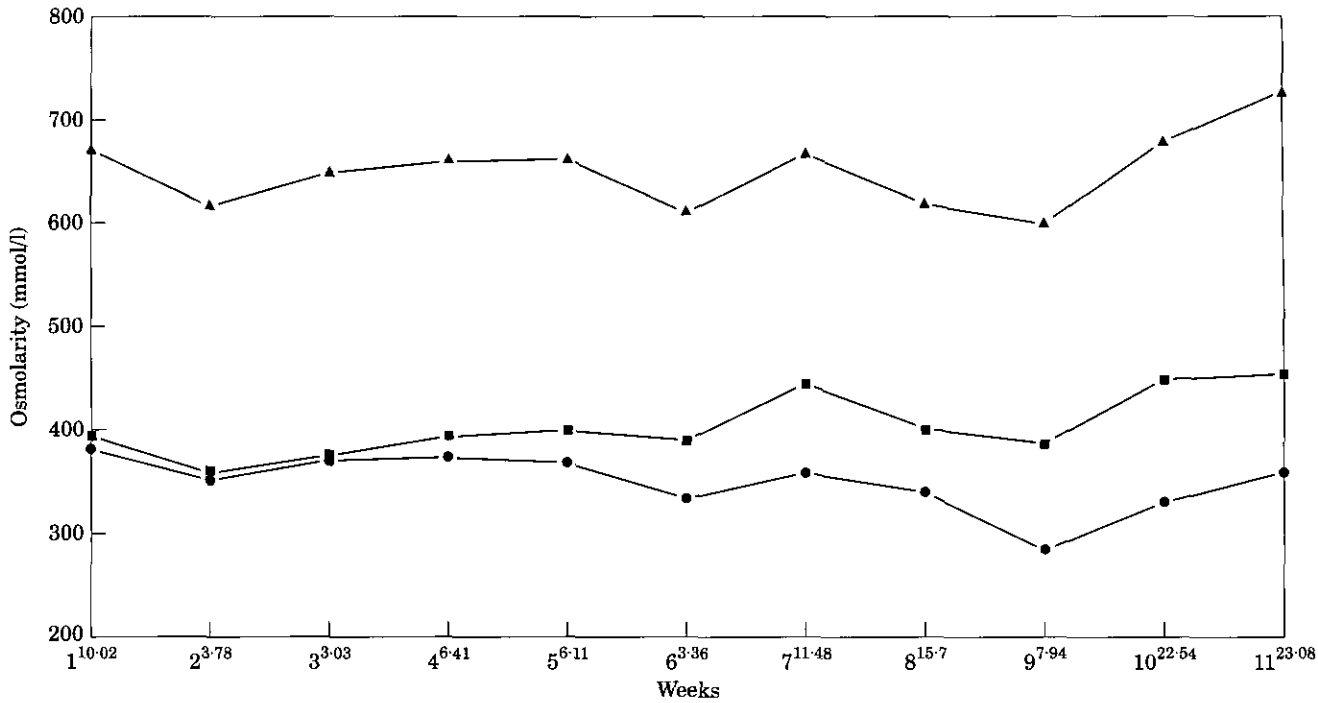

FIG. 4. Osmolarity (mmol l^{-1}) of media on which *Solanum tuberosum* cv. Estima plantlets were grown for 12 weeks and which contained 4% sucrose supplemented with glucose to a molarity equal to 8% sucrose (low glucose, –●–), or at a level that provided an equal number of carbon atoms to 8% sucrose (high glucose, –▲–), with a control base medium containing 8% sucrose (–■–). The superscript numbers are the standard errors of the difference between the means of the treatments.

than hexoses had moved out of the roots (Fig. 7A). This coincided with an increased movement of sugars into the microtubers (Fig. 7B).

As seen previously, fewer microtubers were produced on media containing monosaccharides than on those with sucrose, and microtubers forming on media containing glucose or fructose were much smaller than those forming on sucrose. Plantlets growing on media to which sucrose was added before autoclaving were the first to produce microtubers, but by the end of the experiment, there were no significant differences between media to which sucrose had been added before or after autoclaving.

DISCUSSION

The aim of these experiments was to gain more information about the changes in the media during microtuber development and to shed some light on the role of sucrose in microtuberisation. Media changes included fluctuations in the pH and osmotic potential as well as sugar content. The changes in pH could be explained by the uptake of nutrients by the plantlets, but also by the possible formation of more complex molecules in the media. Similar patterns of pH change were observed by Leifert *et al.* (1992) in *in vitro* cultures of several plant species. The initial decrease in pH is known to be caused by the preferential uptake of ammonia, leaving the more acidic nitrate in the media (Veliky and Rose, 1973; Kirby, Leustek and Lee, 1987). The plantlets later take up the nitrate, so contributing to the subsequent increase in pH. Furthermore, it has been shown that roots growing in solution containing the full complement of nitrogen increase the pH of the solution, possibly by exuding HCO_3^- into it (Moorby, Nye and White, 1985). Another factor to consider is that the plantlets were grown on liquid media. It has been found that pH changes after autoclaving are greater in liquid media than in agar media (Skirvin *et al.*, 1986). It might therefore be that the fluctuations in pH observed were exaggerated by the use of liquid media, and would not be present to such an extent in agar-solidified media.

The initial increase in the osmotic potential of the media could have been caused by the dissociation of the salts in the MS medium into their component ions. Furthermore, it was found that some of the sucrose is hydrolysed during autoclaving, and a large amount of it breaks down during the growth of the plantlets, contributing to the rise in the osmolarity of the media. Kanabus, Bressan and Carpita (1986) measured the daily depletion of sucrose, glucose and fructose from the medium by carrot cell cultures. They found that the sucrose was the first to be depleted, with nearly stoichiometric amounts of fructose and lower amounts of glucose appearing in the medium instead. They attributed this to invertase present on the cell walls or secreted into the medium. The osmolarity in the current experiments stabilized after a few weeks, suggesting an equilibrium between molecules and ions taken up with those exuded by the plantlets, or a balanced uptake of ions and water.

It was interesting to note that media with fructose had the lowest pH, and the highest osmolarity, and that the plantlets did not readily absorb fructose, so that the smallest and lightest microtubers were formed on media containing this

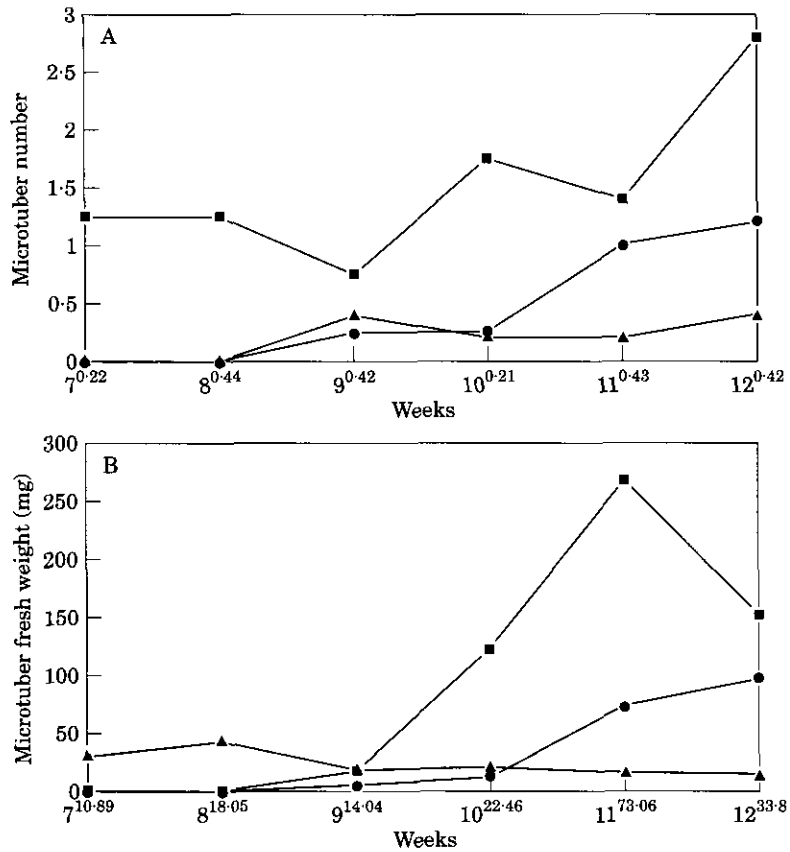

FIG. 5. A, Number and; B, fresh weight (mg) of microtubers produced from *Solanum tuberosum* cv. Estima plantlets grown for 12 weeks on media containing 4% sucrose supplemented with glucose at a molarity equal to an additional 4% sucrose (-●-), or at a level that provided a number of carbon atoms equal to 8% sucrose (-▲-), or on a control base medium containing 8% sucrose (-■-). The superscript numbers are the standard errors of the difference between the means of the treatments.

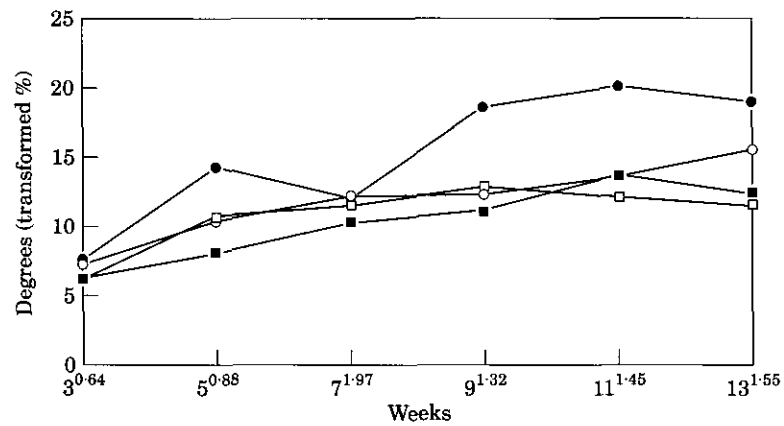

FIG. 6. Absorption of sugars by *Solanum tuberosum* cv. Estima plantlets grown for 13 weeks on base medium supplemented with radiolabelled sucrose after (sucrose:aa, -■-) or before (sucrose:ba, -□-) autoclaving, and with radiolabelled glucose (-●-) or fructose (-○-), both autoclaved. The superscript numbers are the standard errors of the difference between the means of the treatments. The data shown are angular transformations of percentages of sugars absorbed. These range between 0 and 90 and, hence, give a reasonable depiction of the percentages from 0 to 100.

hexose. Guan and Janes (1989) found that the presence of fructose together with sucrose in a medium can inhibit the uptake of sucrose by tomato leaf protoplasts. Furthermore, the fructose molecule is very labile in solution, and its behaviour on autoclaving, particularly in the presence of salts, is unknown. This might explain the low pH in media containing fructose, and also the lack of utilization of this sugar by the plantlets for microtuberisation.

There were significantly fewer microtubers formed on media with 4% sucrose supplemented with monosaccharides or maltose than with sucrose (equimolar media), and even less on unsupplemented medium (with an osmolarity of

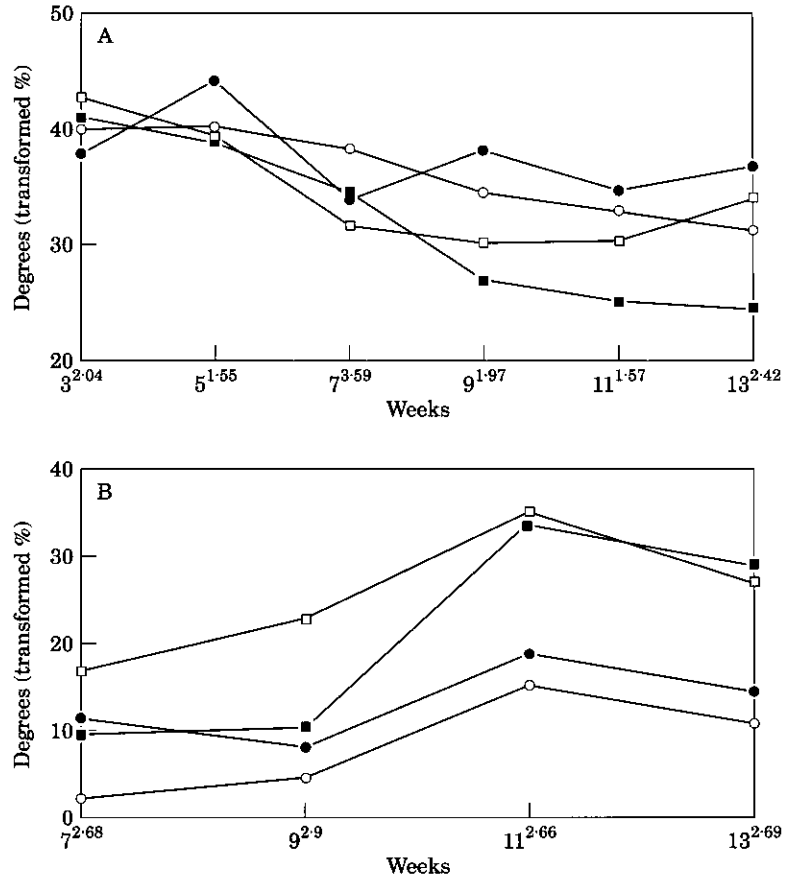

FIG. 7. Accumulation of sugars in the roots (A) and microtubers (B) of *Solanum tuberosum* cv. Estima plantlets grown for 13 weeks on base medium supplemented with radiolabelled sucrose after (sucrose: aa, –■–) or before (sucrose: ba, –□–) autoclaving, and with radiolabelled glucose (–●–) or fructose (–○–), both autoclaved. The superscript numbers are the standard errors of the difference between the means of the treatments. Transformed data, see legend to Fig. 6.

approx. 250 mM). In addition, monosaccharide media containing an equivalent number of carbon atoms to that of 8% sucrose, and therefore with an osmolarity of approx. 600 mM, proved deleterious for both the growth of the plantlets and the development of microtubers. It has been found that starch synthesis is dependent on the osmolarity of the media (Oparka and Wright, 1988) and since this is directly linked to the development of tubers, it becomes clear that there is an optimal osmolarity (approx. 400 mM) for microtuber growth.

In one experiment, glucose was added to 4% sucrose media, but in the other, glucose was added on its own to nutrient salts. Although it is impossible to make a statistically valid comparison between the two sets of results, microtuberisation seems to be better in the presence of the disaccharide and the use of equimolar amounts of monosaccharides was not advantageous. If efficiency of microtuberisation is considered in terms of the number and size of microtubers produced in any time period, then this leads to the conclusion that a disaccharide is needed for the efficient formation of microtubers from plantlets, and that it is not only a matter of the number of carbon atoms present in the culture medium, nor the maintenance of a suitable osmotic potential regardless of the source. This disaccharide could be sucrose or maltose, but there is insufficient information about the function of maltose in potato, and sucrose is the form in which carbon is translocated in potato. Furthermore, because maltose is both more expensive and more difficult to obtain than sucrose, sucrose remains the better choice.

Thus, even though it was not possible to separate unequivocally the effects of carbon source and osmoticum in these experiments, the results implied that the microtuberisation process is facilitated by a high osmolarity, but relies on an adequate supply of sucrose for optimal production. A tentative suggestion would be that the excess sucrose triggers microtuber initiation, and the high osmolarity ensures the continuous production of starch in the microtubers during their growth. In conclusion, therefore, sucrose plays a dual role in microtuber development; primarily that of an easily-assimilated carbon source which is also readily converted to starch for the development of microtubers. Its secondary role is as a non-inhibitory osmoticum, keeping the culture medium at the optimum osmolarity of 400 mM during microtuber development.

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