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ORIGINAL PAPER

Tomato pollen tube development and carbohydrate fluctuations in the autotrophic phase of growth

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Abstract Ripe pollen has different soluble and insoluble carbohydrates in variable amounts. Pollen germination and pollen tube growth were studied in a tomato cultivar (*Solanum lycopersicum* L. cv. Platense) with atypical pollen among tomatoes due to its very low amount or absence of sucrose. In vitro assays were performed using a culture medium without carbohydrates to explore whether there is an autotrophic phase of pollen tube growth, and if there is, describe it, and to analyze the fluctuations of endogenous carbohydrates (soluble carbohydrates, starch, pectins, and callose). Pollen germination was fast (ca. 10 min) and a definite autotrophic phase was observed. Soluble carbohydrates and pectins showed the most substantial changes during this period, even after 10 min. A small amount of callose was observed in the ripe pollen and pollen tubes. Pectins were the most abundant pollen tube wall component. Pollen can be considered starchless; starch was not involved in the autotrophic phase of growth. Other types of substances must be connected with the carbohydrate metabolism, because the fluctuations of the different substances did not follow balanced stoichiometric relationships. Pollen germination and pollen tube elongation was sustained autotrophically, even though sucrose was absent and starch was negligible in pollen grains. The type of pollen reserves and the fast pollen tube formation could be selective advantages in this cultivar.

Keywords Tomato · Carbohydrate reserves in pollen · Pollen tube growth · Pollen tube wall

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Introduction

Pollen tubes grow through the pistilar tissues on their way to the ovules to achieve fertilization. Fast pollen tube growth requires a high energy supply (Ylstra et al. 1998) and a high level of sugar import because of the fast synthesis of new cell walls (Schlüpmann et al. 1994). Glucose and fructose can be the starting points for glycolysis, whereas glucose is the fundamental molecule of the different polysaccharides of the pollen tube wall. Therefore, sucrose is considered the main nutrient for the developing pollen tubes (Nakamura et al. 1980), which can take sucrose from the surrounding environment (either from the pistilar tissues or an artificial medium) and metabolize it in different ways (e.g., Kessler et al. 1960; Labarca and Loewus 1972, 1973; Nakamura et al. 1980; Schlüpmann et al. 1994; Ylstra et al. 1998). Although the heterotrophic condition of pollen tubes has been recently emphasized (Rounds et al. 2011), pollen tube growth may not be exclusively heterotrophic. The initial period of pollen tube growth is considered autotrophic, when the pollen tube would use the reserves stored in the pollen grain (Shivanna 2003; Stephenson et al. 2003). A range of cytosolic and plastidial carbohydrates are found among pollen reserves, from polysaccharides to small soluble molecules (Pacini 1996). Starch and sucrose are important pollen reserves, whose presence and amount can vary among species (Baker and Baker 1979; Singh et al. 1978; Speranza et al. 1997).

The pollen grains of tomato Platense (*Solanum lycopersicum* L.) can be considered starchless (Carrizo García et al. 2009), as in several other tomato cultivars (Firon et al. 2006; Polowick and Sawhney 1993; Pressman et al. 2002), but their content of soluble carbohydrates is peculiar. In fact, reducing sugars predominate, whereas sucrose can be absent or in a very low amount; the regular presence of maltosaccharides is also interesting (Carrizo García et al. 2009, 2010). Given the importance of carbohydrates in pollen tube development, in particular the role assigned to sucrose, we studied pollen tube growth under autotrophic conditions in this tomato cultivar. The main goal of the present work was to determine if pollen grains can germinate and pollen tubes can develop autotrophically, even if endogenous sucrose is scarce or absent and pollen is starchless, and to analyze different carbohydrates to hypothesize possible relationships between them.

Materials and methods

Plant material

Plants of tomato (*Solanum lycopersicum* L.) cv. Platense (Universidad Nacional de La Plata, Argentina) were grown outdoors in the Botanical Garden of Siena, Italy, in spring–summer. Sampling was conducted in August for 12 days to avoid important changes in soluble carbohydrates among samples (Carrizo García et al. 2009, 2010). On the first day of anthesis, pollen was harvested from flowers randomly selected when the petals were fully reflexed (10-11 am). Pollen viability was tested using the fluorochromatic reaction test (Heslop-Harrison and Heslop-Harrison 1970) to ensure the use of viable pollen (the average percentage was 71.75 ± 4.57).

Analysis of the autotrophic phase of pollen tube growth

In vitro pollen culture was followed for 10, 30, 90, 180, and 240 min, at 25 ± 2 °C, to define the presence and length of the autotrophic phase of pollen tube growth; this phase is considered the period when pollen tubes can lengthen without supply of exogenous nutrients. The culture medium consisted of 2 mM boric acid, 2 mM calcium nitrate, 2 mM magnesium sulfate, and 1 mM potassium nitrate in distilled water; no sucrose was added. A control curve of pollen germination in a culture medium with 5 % sucrose was also plotted. Pollen grains were considered germinated when the pollen tube reached at least the length of the hydrated pollen diameter. The pollen tubes formed were recorded for 900 pollen grains (3 × 300) at each point in time, and the average numbers were compared through ANOVA ($P = 0.05$) between consecutive points along time in each condition (autotrophy and control) and at each point between both conditions.

To assess the possible end of the autotrophic phase, pollen tubes grown in the culture medium without sucrose were measured using the ImageJ software (Rasband 1997–2009) on digital micrographs. Fifty pollen tubes were measured at 30 min, and 150 at 90, 180, and 240 min. A histogram was plotted to compare the frequency of the pollen tube length values recorded; the mean diameter of the hydrated pollen was used to define the intervals. Incipient pollen tubes (length values ranging from one-third to full diameter of the hydrated pollen) were recorded as tip-germination (called tips).

Carbohydrate analyses

Soluble oligosaccharides, starch, pectins, and callose were quantified in pollen grains (0 min) and pollen tubes along with non-germinated pollen at 10, 30 and 90 min. The analyses were focused on the period considered most active (see "Results"); therefore, subsequent stages were excluded. The group of four stages was considered one set of samples; four sets were made. Soluble carbohydrates and pectins were analyzed in all sampling sets. Because starch and callose are in the insoluble residues, two sets were used for quantification of callose and two for starch. The quantities of all carbohydrates were expressed in relation to the initial fresh weight of pollen (accuracy of 0.1 mg). Mean values were calculated at each stage for every substance and a pair-wise comparison was made with ANOVA ($P = 0.05$).

Pollen (0 min) was homogenized with a PRO200 homogenizer in distilled water. Aliquots of pollen were put in a tube containing 100 µL of the culture medium per pollen mg (fresh weight); samples were allowed to incubate at 25 ± 2 °C until each stage was reached, and were homogenized following the same procedure used for pollen grains. After cell rupture, samples were centrifuged at 12,000×*g* and 4 °C for 10 min; the soluble and insoluble fractions were separated and stored at −80 °C. All the standard substances were processed in the same way as the samples.

Soluble oligosaccharides were identified and quantified through HPLC, testing 15 substances, as previously described (Carrizo García et al. 2010). Only one peak had an ambiguous resolution, either fructose-6-phosphate (Fru-6P) or glucose-6-phosphate (Glc-6P) (Carrizo García et al. 2010).

For starch quantification, samples were processed according to Castro and Clément (2007). Starch was quantified based on the absorbance at 600 nm in a spectrophotometer. A standard curve was established using potato starch (J.T. Baker, Germany).

Pectins were estimated by quantifying galacturonic acids following the method used by Aouali et al. (2001). Quantifications were made at 530 nm in a spectrophotometer. Pectin from apple (poly-D-galacturonic acid methyl ester; Sigma-Aldrich Co., USA) was used as standard.

Samples were treated according to Kohle et al. (1985) for callose quantification in a fluorimeter (excitation 393 nm, emission 479 nm: Waldmann et al. 1988). Pachyman (Megazyme International Ireland Ltd., Ireland) was used as 1,3-β-glucan to establish a standard curve. Samples taken at each stage were stained with 0.1 % decolorized aniline blue and observed in a microscope under UV light (365 nm, 40× and 100× magnifications) to reveal the presence of callose.

Results

Pollen germination and pollen tube growth

During the pollen tube growth assay, sporadic pollen/pollen tube bursting was observed from 30 min onwards, except at 240 min, when it was more frequent (but not abundant). The mean diameter of the hydrated pollen was 27.82 ± 2.08 μm; therefore, pollen grains were considered germinated when the pollen tube was at least 27-μm long. Pollen tubes were observed from 30 min in the control (Fig. 1a); the percentage of pollen tubes increased significantly along time until 180 min in this condition (Fig. 1a). In the culture without sucrose, no pollen tube was formed at 10 min, although tip-germination was recorded (Fig. 1a). Different percentages of pollen tubes were observed afterwards (Fig. 1a). The percentage of pollen tubes increased significantly from 30 to 90 min, but after that, germination reached a plateau, showing no significant changes (Fig. 1a). The percentages of pollen tubes were significantly lower than the percentages registered in the control for 90, 180 and 240 min, but not for 30 min. Tip-germination percentage peaked at 30 min (Fig. 1a), which was significantly different from 10 min. Tip-germination then started to decrease, with a significant reduction by 180 min, reaching a final plateau (Fig. 1a).

In the autotrophic culture, all the pollen tubes observed at 30 min fell within the first length interval (27–53.99 μm; Fig. 1b). The distribution of length frequencies was partially overlapped between 90 and 180 min, showing several peaks (Fig. 1b). The main difference between them was the presence of a few longer pollen tubes at 180 min (Fig. 1b). In general, the amount of pollen tubes within each interval increased with increasing length of pollen tubes at 240 min, until a peak was reached around the middle of the range, and then the number of pollen tubes decreased rapidly in the intervals of greater lengths (Fig. 1b). Pollen tubes at 240 min were the most abundant within the latter intervals (Fig. 1b).

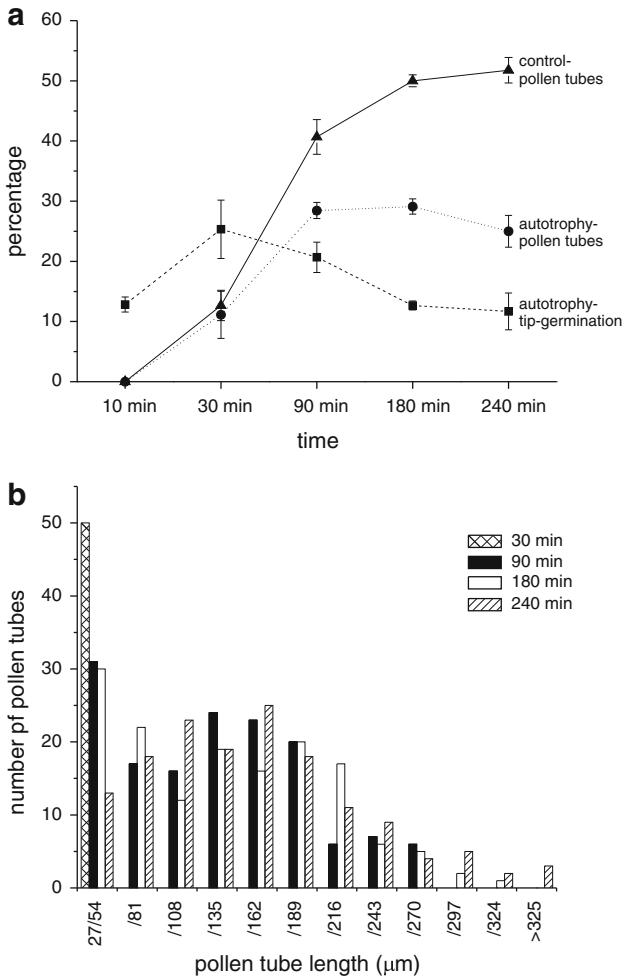

Fig. 1 Characterization of the autotrophic phase of pollen tube growth. **a** Percentages of tip-germination and pollen tubes formed at different periods of time in autotrophic pollen culture, and percentages of pollen tubes formed by pollen cultivated with exogenous sucrose (control). **b** Frequencies of pollen tube lengths at different periods of time in autotrophic conditions. Only the highest value of each interval is shown on the *X* axis, except for the first one

Variations of carbohydrates

Pollen at 0 min contained glucose and fructose as the main soluble sugars, and small quantities of maltopentaose and Fru-6P/Glc-6P (Fig. 2a). No other soluble carbohydrates were found. Glucose and fructose decreased during germination and pollen tube growth (Fig. 2a). The concentration of glucose at 0 min was significantly different from 10 min, with no significant differences between pairs from 10 to 90 min. There were significant differences in fructose

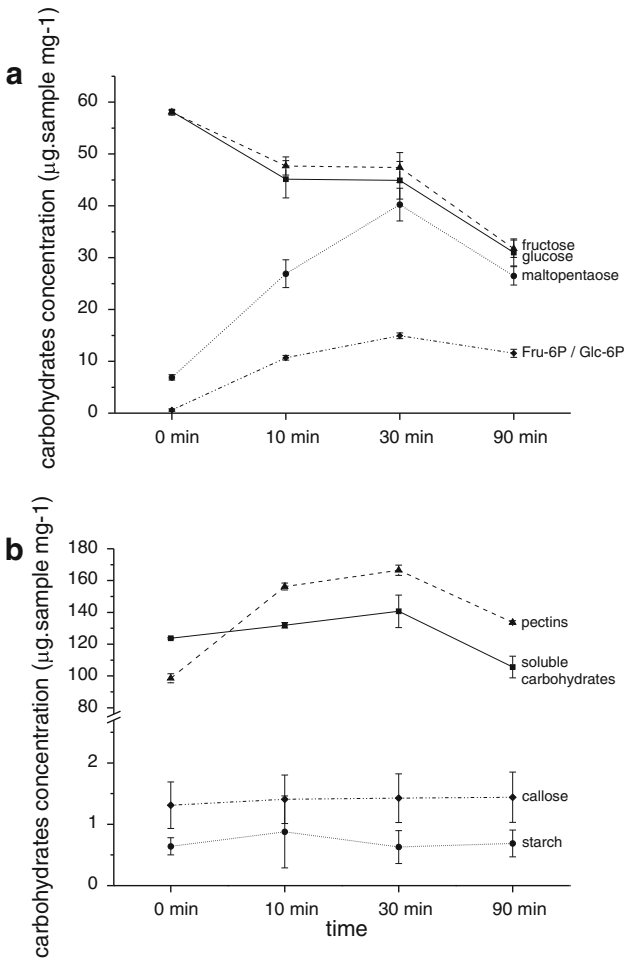

Fig. 2 Carbohydrates in pollen grains and pollen tubes at different periods of time in the autotrophic phase. **a** Soluble carbohydrates. **b** Structural and reserve carbohydrates. Mean values and standard deviations are presented

concentration between pairs of stages, except for 10–30 min. Fru-6P/Glc-6P increased considerably from 0 to 30 min, decreasing at 90 min (Fig. 2a). A similar trend was observed for maltopentaose, although the magnitude of change was bigger (Fig. 2a). In both cases, the initial increase and the final reduction were significant, although concentrations at 90 min were still higher than the levels found at 0 min. Total soluble carbohydrate content increased from 0 to 30 min, decreasing at 90 min (Fig. 2b).

Starch was almost negligible at all stages (Fig. 2b), with no significant differences among them.

Pectins (galacturonic acids) increased from 0 to 30 min, decreasing at 90 min (Fig. 2b). Pectin concentration was significantly different between pairs of stages, except for 10–30 min. Although the final reduction of pectins at 90 min was significant, pectin concentration was still significantly higher than at 0 min (Fig. 2b).

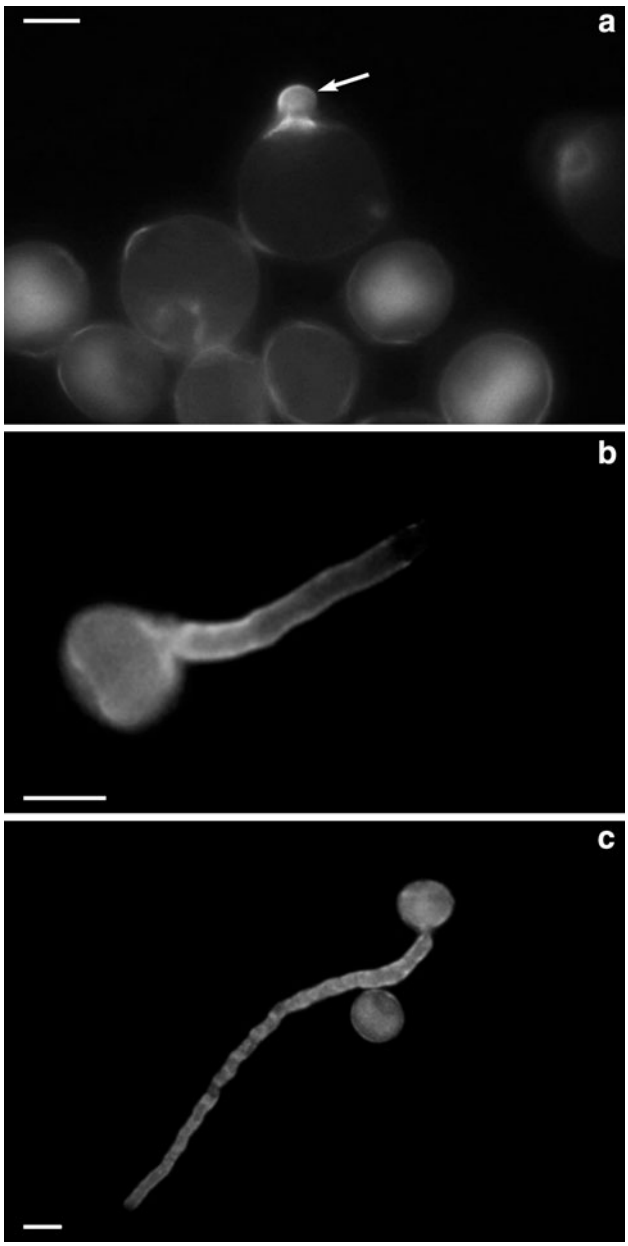

Fig. 3 Callose evidenced under UV light at different periods of time in the autotrophic phase. **a** Pollen grains and emerging pollen tubes at 10 min; the *arrow* points an incipient tip with callose wall. **b, c** Pollen tubes at 30 min **(b)** and 90 min **(c)**, with callose walls (absent in the apical end) and none callose plug. *Bars* = 20 µm (**a**); 15 µm (**b**, **c**)

Callose was always scarce (Fig. 2b); callose amount increased slightly from 0 to 90 min (ca. 10 %; Fig. 2b), but not significantly. Microscope observations did not reveal a callose wall in pollen grains, even though callose was quantifiable. At 10 min, callose could be observed in the pollen grain pores, where the cytoplasm protruded, and in the tips recorded (Fig. 3a). Callose walls were observed in the pollen tubes at 30–90 min (except in the apex), but no callose plug was detected (Fig. 3b, c).

Discussion

Autotrophic phase of pollen tube growth

Pollen tubes developed in tomato cv. Platense when sucrose was provided in the culture medium (control) and also in autotrophic conditions, that is in a medium without carbohydrates. Pollen germination started at the same rate in both conditions, but after 30 min the germination percentages increased in a significant higher degree when exogenous sucrose was supplied. Therefore, the exogenous sucrose would have a positive effect on germination over the time. Nevertheless, a definite autotrophic phase of growth was recorded in the tomato cv. Platense pollen tubes, which elongated in vitro without the exogenous supply of sugars, even though pollen grains lacked sucrose and substantial starch reserves. Pollen hydrated properly and pollen tubes developed under the experimental conditions used, even in the absence of an osmoticant in the medium. Osmotic substances are more critical in the case of recalcitrant or partially hydrated pollen, which actually germinates better on a semi-solid medium (Heslop-Harrison 1979) than on liquid media.

Pollen autotrophic germination was asynchronous and constant, which was evident in the presence of pollen tubes within the interval of shortest length from 30 to 240 min, and by the increasing germination percentage until 90 min. The different peaks observed in the histogram of pollen tube lengths at 90 and 180 min may indicate possible waves of germination and/or pollen tube elongation. A continuous increase in pollen germination until 6–7 h has been recorded in other tomato cultivars, although under different growing conditions (Karapanos et al. 2010; Maisonneuve and Den Nijs 1984). The highest percentage of pollen germination recorded for tomato cv. Platense in autotrophic conditions (90 and 180 min) was similar to, and even higher than, the previous values recorded in other cultivars in a similar period of time and incubation temperature, even though exogenous sucrose was present in those cases (percentage of pollen germination ranging from ca. 5–30 %) (Firon et al. 2006; Karapanos et al. 2010; Maisonneuve and Den Nijs 1984; Pressman et al. 2002). In the control assay of pollen culture with sucrose added, the highest values of pollen germination reached for tomato cv. Platense fall among the highest registered for the species. Records of pollen germination percentages in tomato cultivars above the present results have been sporadic (Abdul-Baki 1992; Karapanos et al. 2010).

The end of the autotrophic phase may have occurred at some point between 90 and 180 min, or 180 and 240 min. The distribution of lengths at 240 min might indicate that late pollen tubes have continued to elongate, reaching the length of earlier pollen tubes that may have slowed down or stopped elongating at that time. Pollen tube elongation in the control condition was not analyzed in detail, but pollen tubes may continue elongating for 12–16 h in this case, even beyond 1 mm (not shown).

Pollen germination started rapidly in tomato Platense, with tips recorded after 10 min and pollen tubes formed after 30 min. It has been suggested that the pollen of tomato Platense may be ready to be activated according to the soluble carbohydrate content (Carrizo García et al. 2010), and the present results support this assumption. Pollen germination in tomato Platense seems to be faster than in other tomatoes (Abdul-Baki 1992; Bellani et al. 1985), although a similar trend has also been described in another cultivar (Karapanos et al. 2010).

Changes of reserve and structural carbohydrates

The start of pollen germination only in 10 min without the supply of exogenous nutrients, suggests that pollen reserves were mobilized. In fact, the concentration of most carbohydrates analyzed changed throughout the period studied, except for starch, even though the degree of change must have been underestimated considering the percentages of non-viable and non-germinated pollen (and possibly also some tips, which will not necessarily develop into pollen tubes).

In other tomatoes, and also in most wild tomatoes, starch stored during microspore development is rapidly hydrolyzed right before flower opening; therefore, pollen grains are mostly starchless (Carrizo García 2007; Polowick and Sawhney 1993; Pressman et al. 2002). This feature was also observed in tomato Platense (Carrizo García et al. 2009); indeed, in this cultivar, the final starch hydrolysis would provide molecules that could be used later, but starch itself is not involved in pollen tube development. In contrast, starch synthesis has been recorded in other species (Dickinson 1968; Hellmers and Machlis 1956; Kessler et al. 1960; Singh et al. 1978), including the wild tomato *S. peruvianum* (Bellani et al. 1985), although in these cases the culture media was supplied with sugars. Because germination began rapidly in the pollen of tomato Platense, there was probably neither time nor reason to synthesize starch, since monosaccharides were immediately used, at least in the absence of exogenous sugars.

The constant decrease of glucose and fructose observed during pollen tube growth can be easily understood, since both substances must have been used as sources of energy and material for the growing pollen tubes. Consumption of soluble carbohydrates during pollen tube elongation has been previously reported, although sucrose showed the most significant changes (Dickinson 1968; Hellmers and Machlis 1956; Nakamura et al. 1980). However, in *Lilium* it was suggested that although glucose and fructose were

also used, sucrose hydrolysis helped to keep levels of both monosaccharides constant (Dickinson 1968). There would be two alternative paths: sucrose consumption and/or use of glucose and fructose. Germination of pollen of tomato Platense and formation of pollen tubes occurred despite the lack of sucrose reserves probably because glucose and fructose are the substances required. Soluble (acid and neutral) and wall-bound invertase activities were detected in the tomato Platense pollen (unpublished data), suggesting that sucrose may be promptly hydrolyzed and its components would be stored. The absence or eventually a low amount of sucrose would not be unfavorable in this case. Actually, the storage material provided by the paternal sporophyte may have determined a selective advantage (Stephenson et al. 2003) in tomato Platense pollen, together with the pathways of nutrient metabolization within pollen during development, since pollen tubes germinated rapidly. In vivo, rapid germination would mean that the nutritional resources of the pistilar-transmitting tissue can be reached faster on the way to the ovules (Stephenson et al. 2003).

Maltopentaose and Fru-6P/Glc-6P showed a major increase when germination started, reaching an absolute maximum at 30 min. No pollen samples presented such high level of both substances (Carrizo García et al. 2010). The increase of maltopentaose does not have an obvious explanation. Since starch is not synthesized, its presence as a possible intermediate in amylogenesis can not be sustained, and because glucose is readily needed, its polymerization in maltosaccharides does not seem logical. Conversely, the increase of Fru-6P/Glc-6P, whether the peak observed is one or the other, can be understood because of their relationship with glycolysis and pollen tube wall formation (Karni and Aloni 2002). Both phosphorilated sugars have been previously detected in developing pollen tubes (Karni and Aloni 2002; Nakamura et al. 1980).

Callose and pectins are the main substances found in the pollen tube wall (Shivanna 2003). Callose was scarce in tomato Platense, unlike in other species in which callose was the dominant polysaccharide in the pollen tube walls (Rae et al. 1985; Schlüpmann et al. 1994), whereas pectins (galacturonic acids) largely predominated in tomato Platense. The accumulation of cell-wall components could be expected as a result of the increasing number and length of pollen tubes (e.g., Ferguson et al. 1998; Lennon and Lord 2000). However, at some point, pectins were significantly reduced in the tomato Platense. This feature is not exceptional. The primary pectic wall became thicker basipetally in *Arabidopsis* (Lennon and Lord 2000), but a loss of part of the primary wall has been observed in the developing pollen tubes of tobacco, lily and petunia (Derksen et al. 1999; Sassen 1974). It may be thought that pectin deposition may not compensate pectin loss, if these opposite pathways were working simultaneously in the pollen tubes of tomato Platense. Such unbalance may be due to the absence of an exogenous supplement of nutrients. The late pectin reduction in tomato Platense was observed in other three samplings made over the flowering season (data not shown). Unlike pectins, callose amount increased between stages, although not significantly. The presence of callose in the pollen grains could be related to the fast pollen tube emission, as suggested for other species with this characteristic (Pacini 1996). Callose wall deposition was fast in the pollen tubes of tomato Platense, observed from 10 min onwards, which is in contrast, for instance, with the 4 h recorded in *Nicotiana tabacum* (Ferguson et al. 1998). However, callose plugs were not formed as the pollen tubes elongated, which is not exceptional, since callose plugs are not always formed in vitro (Shivanna 2003). Callose plugs are deposited during the transition from autotrophic to heterotrophic growth in vivo (Stephenson et al. 2003), thus the trend observed here would be consistent with autotrophic growth.

The fluctuations of the individual carbohydrates analyzed would seem reasonable (oligosaccharides consumed and wall polysaccharides synthesized); however, balanced stoichiometric relationships were not maintained. Other types of reserve substances may be closely linked with the metabolism of carbohydrates, at least in the absence of exogenous nutrients. Following the hypothesis of Baker and Baker (1979), lipids may be an important reserve in these starchless pollen grains, and may also be involved in the nourishment of pollen tubes. In fact, numerous lipid bodies were observed in mature tomato pollen and were considered an important reserve (Polowick and Sawhney 1993). Anyhow, important changes were produced in carbohydrates when the quiescent state of the pollen grains was broken to germinate and pollen tubes elongated. Pollen tube growth could be sustained for some time entirely with the pollen reserves, even though endogenous sucrose were absent and starch were scarce. Furthermore, the type of reserves stored and the fast pollen tube formation could be selective advantages in the tomato Platense pollen.

Author contribution The three authors planned the experiences and analyzed the results in collaboration. CCG did fieldwork and laboratory analysis. All the activities were supervised by EP.

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