J. Plant Biol. (2011) 54:15–22
DOI 10.1007/s12374-010-9137-4

ORIGINAL RESEARCH

Contrasted Responses to Root Hypoxia in Tomato Fruit at Two Stages of Development

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Received: 25 September 2010 / Revised: 25 October 2010 / Accepted: 5 November 2010 / Published online: 18 November 2010

Abstract The biochemical consequences of root hypoxia have been documented in many sink organs, but not extensively in fruit. Therefore, in the present study, the response to root hypoxia in tomato fruit (*Solanum lycopersicum* L.) was investigated at two developmental stages, during the cell division and the cell expansion phases. Our results showed that in dividing fruit, root hypoxia caused an exhaustion of carbon reserves and proteins. However, ammonium and major amino acids (glutamine, asparagine and γ–aminobutyric acid (GABA)) significantly accumulated. In expanding fruit, root hypoxia had no effect on soluble sugar, protein and glutamine contents, whereas starch content was significantly decreased, and asparagine and GABA contents slightly increased. Metabolite contents were well correlated with activities of the corresponding metabolising enzymes. Contrary to nitrogen metabolising enzymes (glutamine synthetase, asparagine synthetase and glutamate decraboxylase), the activities of enzymes involved in sugar metabolism (invertase, sucrose synthase, sucrose phosphate synthase and ADP glucose pyrophosphorylase) were significantly reduced by root hypoxia, in diving fruit. In expanding fruit, only a slight decrease in ADP glucose pyrophosphorylase and an increase in asparagine synthetase and glutamate decarboxylase activities were observed. Taken together, the present data revealed that the effects of root hypoxia are more pronounced in the youngest fruits as it is probably controlled by the relative sink strength of the fruit and by the global disturbance in plant functioning.

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Keywords *Solanum lycopersicum* · Root hypoxia · Dividing fruit · Expanding fruit · Fruit development · Sink strength

Introduction

Waterlogging, a major environmental stress, is a severe constraint on crop growth and productivity in many regions and situations (Jackson and Colmer 2005; Ahsan et al. 2007). Soil is considered to be waterlogged if there is freestanding water on the soil surface or if the available water fraction of the surface layer is at least 20% higher than the field capacity (Aggarwal et al. 2006). For terrestrial species, such complete submergence imposes stress because gas exchange rates between the shoot and the environment are severely reduced, since the diffusion rates of gases are 10^4 times lower in water than in air (Armstrong 1979). As a result, oxygen deficiency is considered to be the major factor negatively affecting survival and growth of submerged plants (Vartapetian and Jackson 1997; Voesenek et al. 2004), because it leads to a decrease in the ATP/ADP ratio and the adenylate energy charge (Gharbi et al. 2007) due to hampered aerobic metabolism (Crawford and Brändle 1996).

Waterlogging currently has become an important global crop production constraint causing significant yield reduction in several crops that affects about 16% of production areas worldwide (Boyer 1982). Tomato, one of the most widely produced and consumed vegetable in the world, both for the fresh produce market and the processed food industries, is highly sensitive to waterlogging stress (Bray et al. 2001). The decrease in stomatal conductance, due to stomatal closure, is considered a general reaction induced by root hypoxia (Zhang and

Davies 1986; Vartapetian and Jackson 1997; Horchani et al. 2008a). In addition, hypoxia limits the transport of water and minerals, causes leaf epinasty (Pezeshki 2001; Horchani et al. 2009; Horchani et al. 2010) and reduces photosynthetic activity (Irving et al. 2007). It may also affect the synthesis and/or transport of cytokinins that are required for the normal development of the aerial part of the plant (Rahayu et al. 2005).

In addition to the environmental conditions, plant growth and development largely depend upon the partitioning of assimilated carbon between photosynthetic sources such as mature leaves and sink tissues, which include roots and fruits (Devaux et al. 2003). The plant life cycle is accompanied by changes with respect to the sink strength of individual organs and the number of sink organs competing for a common pool of carbohydrates (Ho 1988; Wardlaw 1990). Such a competition based upon sink strength may influence fruit development (Bohner and Bangerth 1988). The early development of tomato fruit can be divided into two distinct phases (Gillaspy et al. 1993). In cherry tomato, the first phase lasts up 8–10 days after fertilisation and fruit set, and is characterised by a very active period of cell division inside the ovary. During the second phase, which proceeds for further 13–16 days, fruit growth is mostly due to cell expansion phenomena, thus leading to a fruit that exhibits its finale size and is able to ripen. The final fruit size depends on the number and the size of cells that originate from the cell division and expansion phenomena, respectively (Gillaspy et al. 1993). Sink activity of developing fruit is comprised of three important physiological features: (1) the phloem unloading of assimilates, (2) post-phloem transport and retrieval by sink cells, and (3) the consumption and storage of imported carbohydrates (Ho 1988). Hence, the fruit sink strength results from its size and its metabolic activity.

The physiological and biochemical responses to water-logging stress have been extensively investigated in different sink organs in many plant models (Pezeshki 2001; Horchani et al. 2010). However, such data are scarce on fruits. In as much as root hypoxia is known to disturb whole plant growth, and as fruit growth depends widely on leaves and roots functioning, the aim of this work was to determine whether, as priority organ for photoassimilates, the fruit is susceptible to root hypoxia or not, and whether the sensitivity to root hypoxia relies on its developmental stage or not. This investigation compares the response to root hypoxia between fruit at the cell division phase and fruit at the cell expansion phase. The effects of root hypoxia on fruit at these two distinct developmental stages are also discussed relative to sink strength and to global disturbance in plant functioning.

Material and Methods

Plant Material and Growth Conditions

Tomato (cv. Micro-Tom) seeds were germinated for 10 days in vermiculite, and then grown hydroponically in a growth chamber (16-h light at 23°C/8-h dark at 18°C with an irradiance of 350 μmol m^{-2} s^{-1}, and 75–80% relative humidity). Each seedling was placed in a 25-mL vermiculite plug on a polystyrene tray floating on the nutrient solution, with six plants per 20-L tank. The culture medium, consisting of Algospeed solution (1 g L^{-1}) was renewed weekly and the pH was checked and readjusted daily close to 5.8. For control plants, the nutrient solution was continuously bubbled with air. Hypoxic treatment was applied at first flower anthesis by stopping air bubbling. These plants are called “hypoxically treated plants” (HT) and are compared to “control plants” (NT). Flowers were tagged when fully opened (anthesis). Six fruits were allowed to develop per plant, usually with three fruits per truss. Secondary stems and additional trusses were eliminated. Fruits were harvested at 5 and 15 days post-anthesis (DPA), referred to the cell division and cell expansion phases, respectively. Fruit dry weight (DW) was obtained by weighing the fruit equatorial pericarp after drying at 80°C until a constant mass was reached. For metabolic analyses, the equatorial pericarp of fruits was hand dissected and immediately frozen in liquid nitrogen, ground to a fine powder and stored at −80°C until use.

Leaf Properties

Total leaf area, leaf chlorophyll content, transpiration rate, stomatal conductance and photosynthesis were measured 15 days after root hypoxia application. Total leaf area and chlorophyll content were measured as described in Horchani et al. (2008a). Transpiration rate, stomatal conductance and photosynthesis were analysed using an infrared CO_2 analyser (LCA3-Analytical Development Corporation, Hoddeson, UK) following the recommendation of the manufacturer.

Sugars, Amino Acids, Proteins and Ammonium Extraction and Assays in Fruit

Sugars and amino acids were extracted from 10–15-mg dried powder using the alcoholic extraction method described by Brouquisse et al. (1991). Glucose, fructose and sucrose were assayed as described in Devaux et al. (2003). Starch was assayed in the residue of ethanolic extraction as described by Moing et al. (1994). Amino acids were analysed by reverse-phase high-performance liquid chromatography according to the AccQ-Tag method of Cohen and De Antonis (1994).

Total soluble proteins were extracted in 500 mM Tris-HCl (pH 7.5) and measured according to Bradford (1976) using γ-globulin as a standard. NH_4^+ was extracted with 0.1 M HCl and assayed according to the phenol-hypochlorite method (King et al. 1990).

Enzyme Assays

Sucrose synthase (SuSy) and invertase (INV) activities were extracted as in Alonso et al. (2005). SuSy and INV activities were measured according to Alonso et al. (2007) by monitoring NAD reduction at 340 nm. Sucrose phosphate synthase (SPS) was extracted and measured in the sucrose synthesis direction as in Sasaki et al. (2001). ADP-glucose pyrophosphorylase (L-AGPase) was extracted and assayed as described in Schaffer and Petreikov (1997). Glutamine synthetase (GS) was extracted and assayed according to Horchani et al. (2010). Asparagine synthetase (AS) and glutamate decarboxylase (GDC) were extracted and assayed as in Brouquisse et al. (1992) and Rolin et al. (2000), respectively.

Statistics

Statistical data analysis was made using the Student's *t* test. The results are given as means with standard errors of at least six replicates per treatment. The significance of differences between the control and the treatment mean values was determined at the significance level of $p<0.05$. Experiments were replicated two to three times.

Results

Plant Responses to Hypoxia

As previously described (Horchani et al. 2008b), root hypoxia was applied at first flower anthesis. In aerated solutions, oxygen concentration remained close to 21%, the oxygen partial pressure in air. In non-aerated solutions, oxygen shortage appeared progressively as the roots consumed the oxygen present in the medium. The oxygen concentration, which was similar for both the control and hypoxic tanks at the time of removing the medium, decreased continuously to reach 3% within 1 day and stabilised at about 2% for the rest of the week. This experimental setup, with a gradual development of hypoxia, allows root tissue acclimation as is the case under natural flooding (Saglio et al. 1988).

Total leaf area, chlorophyll content, stomatal conductance and photosynthetic activity were evaluated 15 days after hypoxic treatment application. No difference was observed for leaf area between control aerated (NT) and hypoxically treated (HT) plants (Table 1). However, chlorophyll content, stomatal conductance and photosynthetic activity were reduced by 35%, 29% and 40%, respectively, in HT relative to NT plants (Table 1).

Changes in Fruit Growth

Tomato fruit at 5 and 15 DPA, referred to as dividing and expanding fruit, were used to analyse the effect of root hypoxia. Table 2 shows two parameters of the developing fruit from NT and HT plants, the fruit growth rate defined as the increase of the diameter and the change in DW/FW ratio. For the dividing fruit, neither fruit diameter, nor DW/FW ratio were affected by root hypoxia. In expanding fruit, root hypoxia resulted to an increase in its diameter reflecting a more rapid growth for fruits of HT plants. However, DW/FW ratio was maintained (Table 2) reflecting probably an equal level of assimilate importation in fruits of NT and HT plants.

Changes in Sugar Contents

Under both normoxic and hypoxic conditions, tomato fruit was characterised by an increase in the soluble sugar (glucose, fructose, and sucrose), and starch contents as expected for sink organs during development (Fig. 1a and b). The accumulation process of starch last up to the end of expansion phase and then slow down, whereas the soluble sugars continued to accumulate up to the maturity (Rolin et al. 2000). Our results showed that, in the dividing fruit, the soluble sugar and starch contents were reduced by approximately 30% in HT compared to NT plants (Fig. 1a). In the expanding fruit, no obvious difference was observed in soluble sugar content, whereas starch content was reduced by only 18% (Fig. 1b).

Changes in Amino Acid Contents

Because asparagine, glutamine and γ–aminobutyric acid (GABA) accounted for 25–50% of the total amino acid content, only these three amino acids were represented in Fig. 2. In dividing fruit, glutamine, asparagine and GABA contents were increased by 45%, 50% and 30%, respectively in HT compared to NT plants (Fig. 2a). In expanding fruit, root hypoxia had no effect on glutamine content, whereas asparagine and GABA contents were increased by only 28% and 15%, respectively (Fig 2b).

Changes in Protein and Ammonium Contents

The analysis of the protein content revealed that it varied according to the fruit developmental stage. Indeed, it was decreased by 37% in dividing fruit of HT plants compared to that of NT plants, whereas no obvious difference was observed in the protein content of the expanding fruit

Table 1 Total leaf area, leaf chlorophyll content, stomatal conductance and photosynthesis of tomato plants with roots in aerated (NT) and hypoxic (HT) nutrient solution for 15 days

Treatment	Total leaf area (cm^2 $plant^{-1}$)	Chlorophyll content (mg g^{-1} FW)	Stomatal conductance (mol m^{-2} s^{-1})	Photosynthesis (pmol CO_2 m^{-2} s^{-1})
NT	162±25	0.82±0.11	25.6±2.7	31.4±4.1
HT	140±16	0.53±0.07[a]	18.2±1.6[a]	18.8±3.6[a]

Results are the mean ± S.D. of six measurements on six NT and HT plants

[a] The significance of differences between the control and the treatment mean values was determined by the Student's *t* test at the significance level of $p<0.05$

(Table 3). The changes in NH_4^+ content was the most spectacular in both fruit stages as it increased by root hypoxia in values to ten- and sevenfold level, in dividing and expanding fruits, respectively (Table 3).

Changes in Enzyme Activities

The activities of enzymes involved in sugar (INV, SuSy, SPS), starch (L-AGPase) and amino acid (GS, AS, GDC) metabolism were analysed in fruit collected from HT plants and compared to fruit from plants grown under control aerated conditions (Table 3). Contrary to INV, SPS and AS, the activities of other enzymes (SuSy, L-AGPase, GS and GDC) were higher in the dividing compared to expanding fruit.

Contrary to GS, AS and GDC, the activities of INV, SuSy, SPS and L-AGPase enzymes were significantly reduced by root hypoxia, in diving fruit. However, only a slight decrease in L-AGPase and increase in AS and GDC activities were observed in expanding fruit.

Discussion

In previous works (Horchani et al. 2008b, 2009), we have investigated the effects of prolonged root hypoxia on tomato fruit development and metabolism at the maturation phase (Mature Green and Red Ripe steps). Since the effects of hypoxia have been demonstrated to depend on the state of the plant development, on the duration of treatment application and on the balance between sources and sink organs (Klieber et al. 1996; Jackson, 2002), we initially investigated, in this study, the biochemical processes occurring in the young developing fruits (5 and 15 DPA). Globally, the plant response to root hypoxia is usually a rapid decrease in stomatal conductance and photosynthesis (Table 1; Pezeshki 2001; Horchani et al. 2008a), leading to a rapid consumption of the carbohydrate reserves and/or an arrest of the processes of carbon storage (Devaux et al.

Table 2 Diameter and dry weight/fresh weight (DW/FW) ratio of 5 and 15 DPA fruits growing on aerated (NT) and hypoxically treated (HT) plants

Fruit stage	Treatment	Diameter (mm)	DW/FW ratio (%)
5 DPA	NT	4.3±0.4	8.5±1.1
	HT	4.7±0.3	7.8±0.6
15 DPA	NT	8.4±0.7	6.7±0.8
	HT	13.2±1.3[a]	7.1±0.6

Each value represents the mean (±SD) of measurements on eight fruits from four plants per condition

[a] The significance of differences between the control and the treatment mean values was determined by the Student's *t* test at the significance level of $p<0.05$

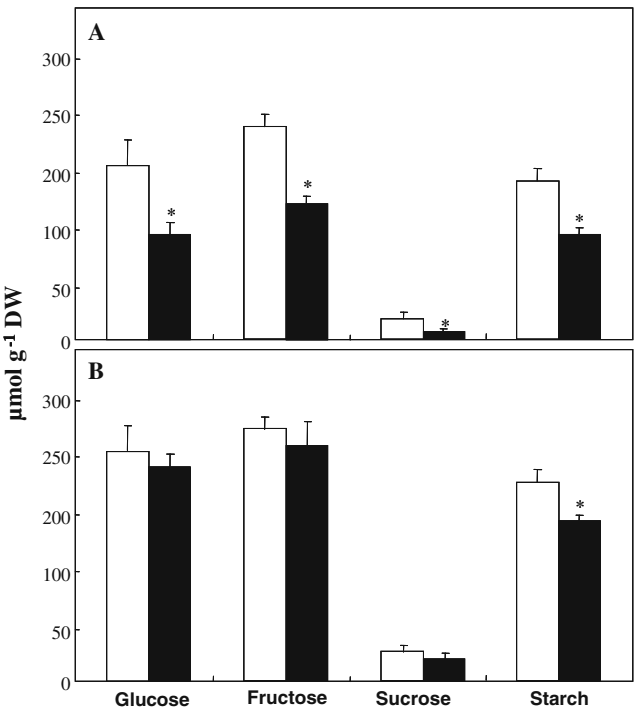

Fig. 1 Soluble sugars (glucose, fructose and sucrose) and starch content in tomato fruits growing on aerated (*white bar*) and hypoxically treated (*black bar*) plants, at 5 (**a**), and 15 (**b**) DPA. Results are the mean (±SD) of at least six replicates. *Single asterisk* (*) The significance of differences between the control and the treatment mean values was determined by the Student's *t* test at the significance level of $p<0.05$

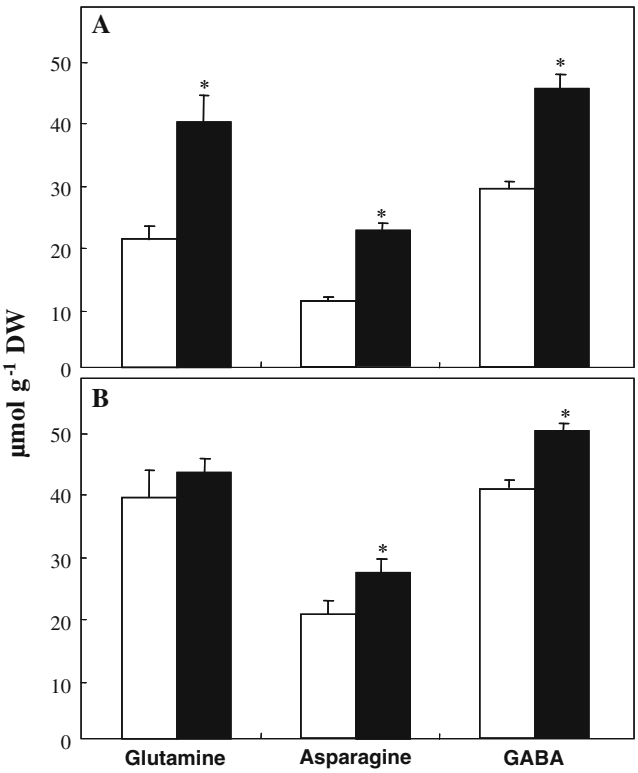

Fig. 2 Glutamine, Asparagine and GABA content in tomato fruits growing on aerated (*white bar*) and hypoxically treated (*black bar*) , at 5 (**a**) and 15 (**b**) DPA. Results are the mean (±SD) of at least six replicates. *Single asterisk* (*) The significance of differences between the control and the treatment mean values was determined by the Student's *t* test at the significance level of $p<0.05$

2003; Ricard et al. 2006). Compared with other sink organs such as root, tomato fruits appear to be less sensitive to the photosynthesis decrease induced by prolonged root hypoxia (Horchani et al. 2009). Two explanations can account for this observation. First, it may be ascribed to the priority rank among sinks in the partitioning of assimilates (Wardlaw 1990). Under root hypoxia, fruit appear to be the priority sink relative to other developing sink organs and thus, the last to undergo assimilate depletion (Ammerlaan et al. 1986; Horchani et al. 2009). A second potential explanation is that among tomato sink organs, those which contain the greatest carbohydrate reserve as a starch pool and sustain the photosynthesis decrease induced by root hypoxia for much longer period. This is much more obvious and accentuated in expanding fruit (Fig. 1). Fruits can be also supplied with assimilates from starch reserves, which are considerable in leaf and stem tissue of tomato plants (Ammerlaan et al. 1986).

Table 3 Protein and ammonium contents in 5 and 15 DPA fruits growing on NT and HT plants

Fruit stage	Treatment	Proteins (mg g^{-1} FW)	NH_4^+ (µmol g^{-1} FW)
5 DPA	NT	4.3±0.4	5.2±1.1
	HT	2.7±0.3[a]	52.4±0.6[a]
15 DPA	NT	5.2±0.2	8.7±0.8
	HT	4.8±0.3	57.1±0.6[a]

Results are the mean (±SD) of at least six replicates

[a] The significance of differences between the control and the treatment mean values was determined by the Student's *t* test at the significance level of $p<0.05$

In plants, many factors including nutritional and hormonal balance, and oxygen availability regulate the activities of carbohydrate-related enzymes (Koch 1996; Mustilli and Bowler 1997). In dark-treated expanding tomato fruit, Grange and Andrews (1994) described a decrease in starch content, and related it to the down-regulation of L-AGPase activity (Guan and Janes 1991). In our case, this behaviour was observed in dividing as well as expanding fruit growing on HT plants (Fig. 1, Table 4). It has been demonstrated that developing fruit undergo a transient accumulation of starch that represents a carbohydrate reservoir contributing to the soluble hexose level in the mature fruit (Dinar and Stevens 1981). In the case of root hypoxia, this starch pool is probably used for the survival of the fruit. The slight decrease in starch content in the expanding compared to the dividing fruit (Fig. 1) may be explained by the adaptation of the fruit, and generally speaking of plants organs, to environmental signals that often induce slow adjustment in order to establish a coarse control system in response to major alterations (Gieger et al. 1993). Thus, key enzymes of the starch synthesis such as SuSy and L-AGPase exhibit their maximum activities during the expansion phase (Schaffer and Petreikov 1997). Consequently, once plants are submitted to root hypoxia, the fruit reserves totally the metabolism from synthesis to degradation of starch. The amounts of starch obtained for expanding fruit, in the present study, were significantly lower than those obtained by Schaffer and Petreikov (1997). This may be related to the difference in the growth type between the two tomato cultivars (determinate for Mico-Tom vs indeterminate for F144). In dividing fruit, the significant decrease in soluble sugar content under hypoxic conditions (Fig. 1) was correlated with a decline in INV, SuSy and SPS enzyme activities (Table 4).

Root hypoxia, while not affecting the growth of dividing fruit (Table 2), decreased significantly its protein content (Table 3), reflecting a protein degradation process. In the expanding fruit, no obvious difference was observed in protein content. Assayed amino acids, except glutamine, decreased but not to the same extent as in dividing fruit (Fig. 2). In the remobilization processes for alternative carbon sources, nitrogen released by protein degradation is differentially distributed into amino acids and ammonium according to the fruit developmental stage (Fig. 2). Aspar-

Table 4 Invertase, sucrose synthase, sucrose phosphate synthase, ADP-glucose pyrophosphorylase, glutamine synthetase, asparagine synthetase and glutamate decarboxylase enzyme activities in 5 and 15 DPA fruits growing on aerated (NT) and hypoxically treated (HT) plants

	5 DPA		15 DPA	
Enzyme	NT	HT	NT	HT
Invertase (μmol glc min^{-1} g^{-1} FW)	5.2±0.3	3.4±0.3[a]	6.5±0.5	5.3±0.9
Sucrose synthase (μmol glc min^{-1} g^{-1} FW)	1.3±0.2	0.5±0.3[a]	0.8±0.1	0.6±0.3
Sucrose phosphate synthase (μmol suc min^{-1} g^{-1} FW)	13±1.2	8.0±1.4[a]	18.7±2.1	16.5±1.8
ADP-glucose pyrophosphorylase (μmol glc min^{-1} g^{-1} FW)	1.4±0.3	0.6±0.2[a]	0.9±0.1	0.4±0.2[a]
Glutamine synthetase (μmol GHM min^{-1} g^{-1} FW)	0.38±0.06	0.52±0.1[a]	0.27±0.05	0.23±0.04
Asparagine synthetase (μmol asparagine min^{-1} mg^{-1} FW)	0.13±0.02	0.18±0.03[a]	0.17±0.04	0.26±0.03[a]
Glutamate decarboxylase (μmol GABA min^{-1} g^{-1} FW)	1.3±0.1	1.9±0.2[a]	0.9±0.2	1.4±0.1[a]

Results are the mean (±SD) of at least six replicates

[a] The significance of differences between the control and the treatment mean values was determined by the Student's *t* test at the significance level of $p < 0.05$

agine is a well-known detoxification compound when high levels of ammonium result from very active proteolysis (Mazelis 1980). When the amount of assimilate produced by photosynthesis decreases (Table 1), asparagine is consumed to supply the tricarboxylic acid cycle with carbon skeletons (Brouquisse et al. 1992) and this results in the increase in NH_4^+ content (Table 3). In dividing tomato fruit, glutamine also appears as transient form of storage for nitrogen in addition to asparagine (Fig. 2). GABA is a non-protein amino acid that accumulated under root hypoxia (Ricard et al. 1994; 2006). In tomato fruit, GABA has been proposed firstly to act as a temporary store for nitrogen, and secondly to play a role in the transport of nitrogen and the regulation of cytoplasmic pH (Rolin et al. 2000). Moreover, the accumulation of GABA has been observed under a variety of environmental stress conditions, thus suggesting that as for asparagine the nitrogen released by protein catabolism in dividing fruit (Table 3) could firstly be transiently stored as GABA and then released as NH_4^+ when GABA is used as a substrate to sustain respiration via the GABA shunt (Satya Narayan and Nair 1990). In expanding fruit, the changes in asparagine and GABA contents (Fig. 2) could not result from proteolysis at this developmental stage because no net protein degradation occurs (Table 4). Hence, we hypothesise that the increase in asparagine and GABA, as well as NH_4^+ content results from the proteolysis that occurred in the division phase or from a net influx of nitrogen-compounds into the fruit from senescing organs (stem, mature leaves, roots) of less sink priority than fruit (Brouquisse et al. 1998). Moreover, as in dividing fruit, the increase in asparagine and GABA content observed in expanding fruit (Fig. 2) was associated with an increase in AS and GDC activities (Table 4). Hence, we cannot exclude that the massive importation of NH_4^+ in expanding fruit is partly detoxified through the AS and GDC activities.

Dividing fruit are much more sensitive to root hypoxia than expanding fruit, as their carbon and nitrogen metabolisms were significantly impaired (Fig. 1 and 2, Tables 3 and 4). This suggests the existence of a critical point related to the physiological capacity of cell proliferation. Thus, in expanding fruit the effects of root hypoxia are less pronounced, as if expanding fruit had overcome this critical point of cell division by being engaged in the process of metabolite storage. The fact that expanding fruits appear as priority organs is in accordance with the "priority rank order" supporting the concept of competition for assimilates between alternative sinks (Wardlaw 1990). Sink size may explain why expanding fruit attract more efficiently at the expense of dividing fruit. According to the definition of sink activity, SuSy, SPS and INV participate in the control of sugar import into tomato fruit (D'Aoust et al. 1999; Delrot et al. 2000). Under hypoxic conditions, the activities of these sink-related enzymes were decreased (Table 4). This could hamper the capacity of dividing fruit to compete for assimilates. However, the strong decrease of SuSy activity alone cannot explain the weak competitiveness for assimilates by dividing fruit. Indeed, D'Aoust et al. (1999) have demonstrated that SuSy does not participate in the control of the sucrose import capacity in dividing tomato fruit to attract assimilates when the photoassimilate supply is limited, as in the case of root hypoxia. The aptitude of tomato fruit to attract assimilates under unfavourable conditions of sugar supply depends also on the mechanisms controlling long-distance transport of reserve assimilates (Farrar 1996). As a consequence, the position of the fruit within a truss, expanding fruit are at proximal settings and thus, in a more favourable position for assimilate supply than the youngest fruit at distal settings (Bertin 1995).Under root hypoxia, dividing fruit experience a shortage in assimilates that could lead to fruit set failure, an accident which occurs in nature under several environmental stresses.

This work shows that tomato fruits are sensitive to root hypoxia. Increasing knowledge of the mechanisms that regulate carbon and nitrogen metabolisms in whole plant organs, as well as unravelling the reasons for the marked response of fruits to root hypoxia, may be useful for tomato breeders in order to improve fruit production and consequently fruit quality.

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