

# Comprehensive analysis of in vitro to ex vitro transition of tissue cultured potato plantlets grown with or without sucrose using metabolic profiling technique

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**Abstract** This study elucidated the effect of exogenous sucrose on growth parameters and metabolic changes during the in vitro rooting (*InVR*) and the ex vitro acclimatization (*ExVA*) stages of potato (*Solanum tuberosum* L.). During *InVR* stage, plantlets were cultured on MS medium with 3 % (S+) or without (S−) sucrose, and were then acclimatized under the same *ExVA* condition. In *InVR* stage, S+ increased photosynthetic capacity ( $A_{max}$ ) and dry matter percentage. Yet, no significant differences in the other growth parameters have been observed. During acclimatization,  $A_{max}$  and respiration were higher in *ExVA* compared to *InVR* plants. Most growth parameters were significantly higher in S+ plants. Principal component analysis and hierarchical cluster analysis of 108 metabolites identified by GC–MS clearly demonstrated that in vitro culture had a profound impact on metabolic profile. In vitro S− and S+ plantlets accumulated large quantities

of amino acids (specially under S+), photorespiration intermediates, putrescine, tocopherol and organic acids, including oxalic and tartaric acid. However, glycolytic and TCA cycle intermediates were found in lower amount. Under *InVR* S+ conditions, proline, gamma-aminobutyric acid, sugars and sugar alcohols accumulated in larger amounts. *InVR* S− plantlets characteristically accumulated large quantity of urea. We suggest that ammonia metabolism was redirected towards urea biosynthesis through urea cycle to sequester nitrogen in condition of low carbon availability. In vitro conditions are causing major disruption in the cellular metabolism, which could produce serious consequences on the capacity of plantlets to adapt to uncontrolled growing conditions and may lead to poor development under these conditions.

**Keywords** Potato · Micropropagation · Stress · Gas chromatography–mass spectrometry · Principal component analysis · Hierarchical cluster analysis · Heat map

## Abbreviations

MS	Murashige and Skoog (1962) medium
$A_{max}$	Light saturated photosynthesis
S−	Culture medium without sugar
S+	Culture medium with sugar
GC–MS	Gas chromatography–mass spectrometry
TAC	Tricarboxylic acid cycle
UC	Urea cycle
GABA	Gamma-aminobutyric acid
PCA	Principal component analysis
3PGA	3 Phosphoglyceric acid
PAs	Polyamines
Put	Putrescine
Spd	Spermidine

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## Introduction

Micropropagation has become a widely used technology for the large-scale and rapid propagation of plants. Over the last three decades, great strides have been made to develop and optimize micropropagation methods and culture media for the large-scale multiplication of many plant species. However, despite the improved propagation efficiency, the method is still plagued with serious problems, which limit in certain cases the profitability of the technique. Indeed, *in vitro* plantlets present many aberrations like, stomatal malfunction, poor epicuticular wax deposition, somaclonal variation, poor rooting, and hyperhydricity. Many species cannot be cost-effectively propagated by tissue culture due to these induced anatomical and physiological modifications (which lead to excessive plant losses during hardening). These developmental problems stem from the particular conditions plants are cultured under. Indeed, the *in vitro* culture environment is typically characterized by low lighting, poor gas exchanges, high relative humidity and high mineral and sugar contents. Yet, even if plantlets are provided with all essential trophic elements, and are cultured under constant temperature and light, they still appear to suffer from many types of stress.

Over the years, sugar has been considered an essential component of the plant *in vitro* culture medium. However, in addition to its nutritional role, sugar regulates many important metabolic processes associated with plant growth and development (signaling functions) (Rolland et al. 2006). At the cellular level, sugars are essential for intermediary and respiratory metabolism and are the substrate for the synthesis of complex carbohydrates such as starch and cellulose. In addition, sugars supply the precursors for amino acid and fatty acid biosynthesis and essentially all other metabolites present in plants. Many plant species benefit from the presence of sugar in the *in vitro* culture medium. For instance, enhanced growth and photosynthesis was observed in sugar beet (Kovtun and Daie 1995), potato (Cournac et al. 1991) and sea oats (Valero-Aracama et al. 2007). In addition, supplementation of sugar to the culture media increases sucrose and starch reserves in micropropagated plantlets, a condition which may favor *ex vitro* acclimatization and accelerate physiological adjustments (Pospisilova et al. 1999). With *Alocasia amazonica* plantlets, Jo et al. (2009) found that increasing sucrose concentration in the culture medium led to increases in foliar starch and sugars content and decreases in water potential in *in vitro* plantlets. During *ex vitro* acclimatization, root length, shoot length, root number and leaf number, were found to be higher when plants were grown with 3 % sucrose. Although the supply of sugar in the medium has many positive effects, it also has negative ones. For instance, exogenous sugar reduces growth,

photosynthesis, expression of photosynthetic genes (Kozai et al. 1995; Hdidier and Desjardins 1994; Ehness et al. 1997; Badr et al. 2011). Hdidier and Desjardins (1994) observed that high sugar concentration in the culture medium was causing feedback inhibition of photosynthesis by reducing the quantity and activation of Rubisco in cultured strawberry and potato plantlets. In addition, Gaspar et al. (2002) reported that, the sucrose added to the medium hindered chlorophyll synthesis, Calvin cycle and photosynthesis, and disturbed the overall carbon metabolism in *in vitro* cultured plantlets. According to these authors, the sugars added in the culture medium were causing an important stress to the plantlets, most likely due to their osmotic effect. Desjardins et al. (2007) suggested that *in vitro* conditions are very stressful for plant growth and that exogenous sugar is the principal cause of plant stress during *in vitro* culture.

The traditional metabolic analysis techniques are restricted to the measurement of a limited number of metabolites. Thus, only a partial interpretation of a physiological phenomenon can be obtained. The ability of metabolomics technology to simultaneously identify and quantify a large number of metabolites allows for a better understanding of plant metabolism. In a seminal work, Roessner et al. (2000) used GC–MS to obtain a comprehensive metabolic profile of a single extracts of soil or *in vitro* grown potato tubers. About 77 metabolites of various biochemical groups were detected and quantified at once. This analytical method proved to be powerful and allowed the simultaneous analysis of a large set of metabolites and revealed major differences between the tubers from different origins. Using the same technique, Jeong et al. (2004) showed that 64 metabolites accumulated differentially during the transition from sink to source of quaking aspen leaves, two-thirds of which showed more than fourfold changes in relative abundance. In this case, the metabolic profiling of three leaf stages yielded distinct biochemical phenotypes.

The purpose of this study is thus to use a metabolomics approach to study the impact of sugar added to the culture media during rooting stage on the profile of metabolites of potato plantlets during *in vitro* culture and to assess how they change after their transfer to *ex vitro* condition (acclimatization).

## Materials and methods

### Plant material and *in vitro* growth conditions

Potato plantlets (*Solanum tuberosum* L., cv Norland) were micropropagated by monthly subculture on 70 mL MS medium (Murashige and Skoog 1962) with 2.5 g/L

phytagel and 3 % sucrose in Magenta polycarbonate vessels (Magenta Corp., Chicago, USA). For the experiments, homogeneous nodal cuttings (120 stem cuttings of comparable size) were transferred to a MS culture medium containing either 0 (S–) or 3 % (S+) sucrose, in culture glass test tubes (diameter, 25 mm; length, 150 mm) closed with polypropylene closure (Magenta Corp., Chicago, USA). The plantlets were grown for 4 weeks under PPF of  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Light was provided by CoolWhite fluorescents, the temperature was maintained at  $23 \pm 2 \text{ }^\circ\text{C}$ , relative humidity was kept at  $50 \pm 5 \%$  and the photoperiod was 16 h. After 4 weeks of the rooting stage (day 0 of acclimatization), 30 uniform plantlets were selected for metabolites analysis (15 plants/treatment), while 8 others were selected for measurement of photosynthetic capacity and growth parameters evaluation (4 plants/treatment).

### Acclimatization

Micropropagated potato plants from the two sugar treatments (80 plants) were transplanted into  $10 \times 7.5$  cm plastic pots with Pro-Mix BX (Premier Peat Moss, Rivière-du-Loup, Canada). The acclimatization phase took place in a greenhouse under natural light supplemented with  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  supplied by 600 W HPS lamp under 16/8 h photoperiod. Temperature inside the greenhouse was kept at  $25 \pm 5 \text{ }^\circ\text{C}$  during the day and  $20 \pm 5 \text{ }^\circ\text{C}$  at the night. The relative humidity was maintained at  $\geq 95 \%$  for the first 2 days by using plastic tunnels, and it was then gradually lowered to about 40 % over a 1-week period. 60 uniform plants were selected for metabolites analysis (15 plant/treatment), while 16 others were selected for measurement of photosynthetic capacity and growth parameters evaluation (four plant/treatment). Determinations were done after 8 days of acclimatization when plantlets adapted to the new condition and after 16 days of ex vitro acclimatization when the first ex vitro leaf started to form.

### Photosynthesis and growth parameters measurements

A portable photosynthesis system (LI-6400, Licor Co., USA) fitted with a leaf chamber fluorometer was used to measure the photosynthesis rates of the fourth fully expanded leaf below the shoot tip. The measurement of photosynthetic capacity was performed at the end of in vitro culture (after 30 days of subculture) and during acclimatization (8 and 16 days post transfer) periods. A light saturation response curve was obtained for each plantlet using light levels of 0, 50, 100, 200, 500, 1000, 2000 and  $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Assimilation chamber temperature was  $22 \text{ }^\circ\text{C}$ ,  $70 \pm 5 \%$  relative humidity and  $400 \mu\text{mol s}^{-1}$  air flows.  $\text{CO}_2$  was maintained at 400 ppm.

To determine photosynthetic parameters, the Photosyn Assistant software (Beharrell and MacFie 1991) was used to establish light response curves and to calculate respiration, apparent quantum efficiency, apparent light saturated maximum, and light compensation point.

Plant fresh and dry weights, percentage of plant dry matter, shoot length and fresh weight, root fresh weight per plant and leaf (surface, number and weight per plant) were determined at the end of in vitro culture period and during ex vitro acclimatization.

### Potato leaf metabolites sample preparation for gas chromatography–mass spectrometry

Plant material preparation for metabolites analysis was as described by Roessner et al. (2000). Briefly, metabolites were measured in 15 plants (15 individual test tubes) for each treatment. The fourth and fifth leaves of each plantlet were collected at the middle of the day and frozen in liquid nitrogen, and they were then stored at  $-80 \text{ }^\circ\text{C}$  until sample preparation. After 8 days in acclimatization, the fourth and fifth leaf was sampled again. After 16 days in the acclimatization stage new leaves, formed ex vitro, were sampled. 100 mg of frozen leaf tissue was ground to a fine powder by mortar and pestle with liquid nitrogen and extracted with 1.4 mL of methanol. 50  $\mu\text{L}$  of ribitol was added as an internal standard (20 mg of ribitol/10 mL  $\text{H}_2\text{O}$ ) to the samples to correct for the loss of analytes during sample preparation or sample injection (ribitol concentration was 0.18 mM just before sample injection). Metabolites were extracted from the sample by incubation for 15 min at  $70 \text{ }^\circ\text{C}$ , one volume of water was added to the mixture which was then centrifuged at 2200g, and dried in a speed-vacuum. The residue was redissolved and 40  $\mu\text{L}$  of a retention time standard mixture was added, 3.7 % (w/v) for heptanoic acid, nonanoic acid, undecanoic acid, and tridecanoic acid; 7.4 % (w/v) for pentadecanoic acid, nonadecanoic acid and tricosanoic acid, 22.2 % (w/v) for heptacosanoic acid; 55.5 % [w/v] for hentriacontanoic acid (dissolved in 50 mg/5 mL<sup>-1</sup> tetrahydrofuran). The derivatization was done for 90 min at  $30 \text{ }^\circ\text{C}$  (in 80  $\mu\text{L}$  of 20 mg mL<sup>-1</sup> methoxyamine hydrochloride in pyridine) followed by a 30-min treatment at  $37 \text{ }^\circ\text{C}$  with 8  $\mu\text{L}$  of MSTFA (*N*-methyl-*N*-[trimethylsilyl]trifluoroacetamide). One  $\mu\text{L}$  volume of sample were injected with a splitless mode.

### GC–MS analysis

Samples were injected with an HP 7683 series automatic sampler separated on a 6890 plus series GC fitted with a split/splitless injector port and identified with HP model 5973 mass selective detector. GC was performed on a 30 m SPB-50 0.25 mm  $\varnothing$  column and 0.25  $\mu\text{m}$  layer thickness (Superlco, Bellfonte, CA). The injector temperature was

250 °C. The carrier gas was helium at a flow-rate 1 mL/min. Oven temperature was maintained for five min at an isothermal temperature of 70 °C, followed by a stepwise 5 °C min<sup>-1</sup> rise temperature until the oven temperature reached 310 °C, after which the temperature was maintained for an additional period of 1 min. The system was then temperature equilibrated for 6 min at 70 °C before the next injection. Mass spectra were recorded at 2.69 scan/sec with the range of 50–600 *m/z*. The chromatographic system was controlled and validated by the ChemStation software (Agilent Technologies). Perfluorotributylamine (PFTBA), with *m/z* of 69, 219, and 502 was used for autotuning.

The mass spectra were deconvoluted, and peaks were assigned identities using the automated mass spectral deconvolution and identification program (AMDIS) and the National Institute of Standards and Technology (NIST library, version 2005). Peaks that were at least 75 (out of 100) match factor were automatically or manually assigned identities compared with the NIST library. We confirmed peaks identification by using authentic standards (100 compounds) and Q\_MSRI\_ID library from Max Planck Institute of Molecular Plant Physiology, Golm, Germany (website: [http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd\\_msri.html](http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html)).

### Statistical analysis of data

All growth parameter results were submitted to ANOVA using SAS software (version 9.1; SAS Institute inc., NC, 2003). Statistical differences between S– and S+ were computed with *T* test on the Least Squares Means. The area of every metabolite peak was divided by peak area of the internal standard, ribitol present in the same chromatogram, to correct for recovery differences. Log<sub>10</sub> transformation was performed on all data for statistical analysis. ANOVA was also performed on individual metabolite data. A protected LSD was used for multiple comparisons among treatment means. Principal component analysis (PCA) was performed on normalized datasets with SAS software. The dataset was further submitted to hierarchical cluster analysis and visual separation through a heat map using Qlucore Omics Explorer 3.0 software (Qlucore, Lund, Sweden). In the hierarchical cluster analysis, squared Euclidean distance and the average linkage method (between groups) were used. In these multivariate analyses, metabolite contents were normalized by Z score.

## Results

### Growth parameters and photosynthesis

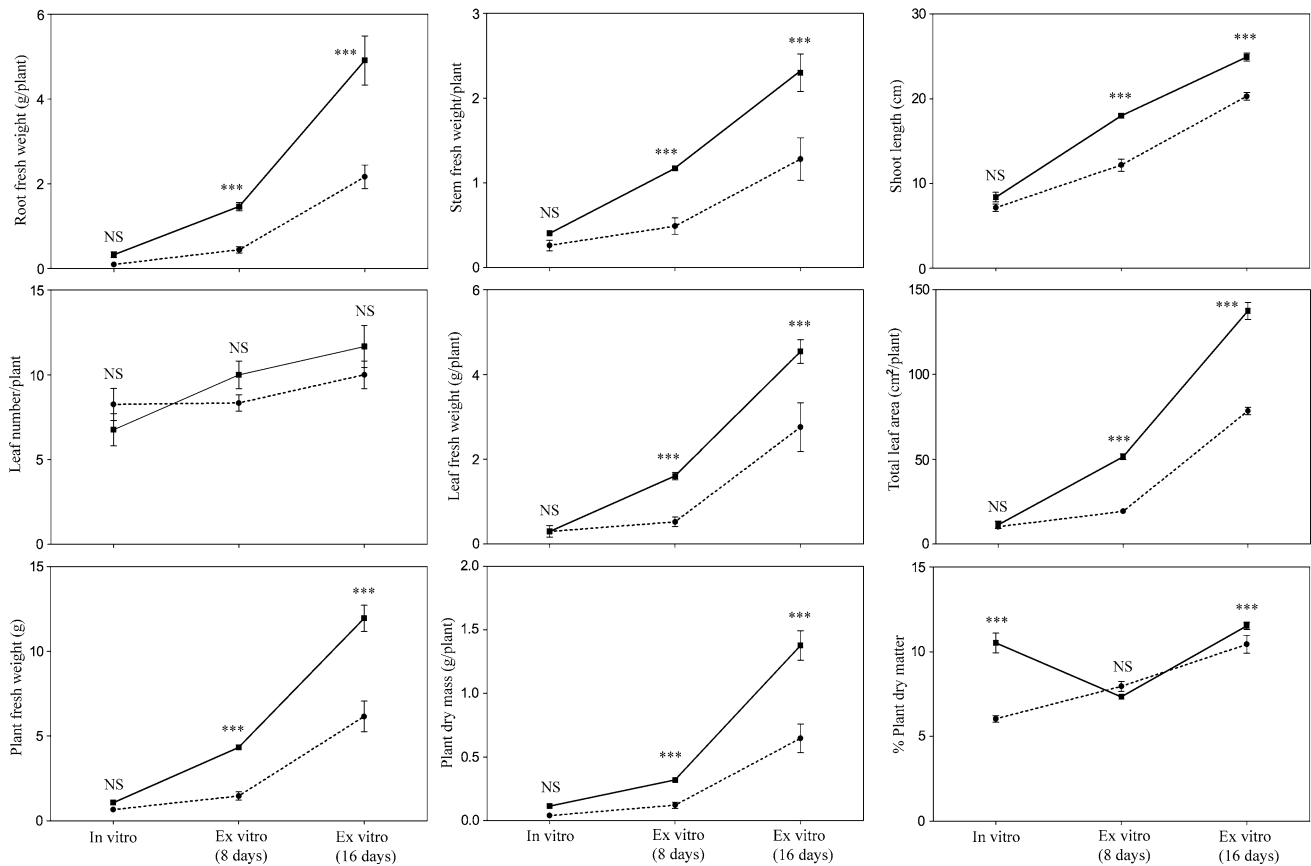
There was no difference between S– and S+ at the end of rooting stage for most growth parameters, i.e. root fresh

weight (g/plant), stem fresh weight/plant, shoot length, leaf number/plant, leaf fresh weight/plant, total leaf area [cm<sup>2</sup>.plant<sup>-1</sup>], plant fresh weight and plant dry mass/plant, except the percentage of plant dry matter that was significantly higher in S+ compared to S– plantlets (Fig. 1). However, S+ plantlets were generally well developed and were darker-green in color with leaves and stem becoming purplish, this color was visually very clear at the end of rooting stage. On the contrary, S– plantlets were very delicate, light-green in color and brittle at the end of the in vitro culture. During the acclimatization stage (ex vitro after 8 and 16 days), there was a statistical difference between S– and S+ plants for all growth parameters except leaf number/plant. Also, the percentage of plant dry matter was slightly higher in S+ than S– after 8 days of acclimatization, yet non-significantly. However, this difference was statistically marked after 16 days of acclimatization.

To determine the effects of sucrose and culture stage on the photosynthetic capacity of potato plants, we measured the photosynthetic parameters such as respiration, photosynthetic quantum efficiency ( $\Phi$ ), light saturated photosynthesis ( $A_{max}$ ) and light compensation point (Table 1). At the end of the in vitro rooting period, respiration was twofold higher in S+ plantlets than in S– plantlets, although the difference was not significant. After 8 days of ex vitro acclimatization respiration increased in both S– and S+ plantlets compared to the same in vitro plantlets, but the differences were still not significant. However, at the end of ex vitro period (16 day) respiration was significantly higher, respectively two- and fivefold in S+ and S–, compared to their corresponding in vitro plantlets.  $A_{max}$  gradually increased during plant transition from in vitro to ex vitro stage under both S– and S+ conditions, and the maximum value was recorded in S+ plants after 16 day of ex vitro acclimatization, while lowest photosynthetic rate was found in in vitro S– plantlets. Light compensation point was significantly higher at the end of the ex vitro acclimatization phase in both S– and S+ plants compared to in vitro plantlets.

### Effect of S– and S+ conditions on the levels of metabolites in potato leaves during in vitro and ex vitro stages

The aim of this experiment was to characterize the biochemical phenotype or metabolic signature of potato leaves produced under different in vitro culture conditions (culture medium with or without sucrose) to evaluate their perturbing influence on overall plantlet metabolism. For this purpose, a metabolomic approach was used to fingerprint potato leaf metabolites using GC–MS. An average of over 108 metabolites (belonging to more than 16 chemical



**Fig. 1** Growth parameters of potato plantlets (*Solanum tuberosum* L., cv Norland) growing on 0 % sucrose (circles) and 3 % sucrose (squares) at the end of rooting stage (in vitro), after 8 days of ex vitro acclimatization and after 16 days of ex vitro acclimatization. Each

value is the mean of 4 replicates ± standard error. Statistical differences between S– and S+ were computed with *T* test on the Least Squares Means computed by ANOVA. NS not significant; \*\*\* = significant at *p* < 0.001

**Table 1** Effect of sucrose on the photosynthetic parameters of the potato plantlets under in vitro (before acclimatization, day 0) and ex vitro (after 8 and 16 days of acclimatization) culture conditions. S–,

plantlets grown on MS medium supplemented with 0 % sucrose and S+, plantlets grown on MS medium supplemented with 3 % sucrose during rooting stage

Treatments	Time (days)	Respiration	QE (Φ)	A <sub>max</sub> (μmol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	Light compensation point
In vitro	S– 0	-0.74b	0.02	8.8c	39.2b
	S+ 0	-1.59b	0.05	16.6b	35.6b
Ex vitro	S– 8	-1.36b	0.04	17.8b	34.3b
	S+ 8	-1.62b	0.05	20.6b	35.3b
	S– 16	-3.78a	0.06	20.9b	69.5a
	S+ 16	-3.69a	0.05	26.2a	83.4a
Pv >  fl		0.0001	0.0829	0.0001	0.0006

a, b, c Different letters within a column is significant according to a protected LSD at 0.05 %

groups) were recovered from leaf methanol extracts and identified between tissue culture conditions (S– and S+) and culture stages (in vitro and ex vitro). These metabolites were plotted in a heat map where metabolites in red are those present in higher concentrations and those in green are those in lower concentrations (Fig. 2).

As shown in Fig. 3, we divided the 107 metabolites (except phosphoric acid) to six groups including amino acids, organic acids, sugars, sugar alcohols, urea and ureides (allantoin) and numerous other miscellaneous compounds. The amount of the majority of the compounds detected changed considerably in both S– and S+ under

in vitro or ex vitro conditions. When taking mean values of metabolite normalized peak area into consideration, we found some interesting trends emerging between the metabolic profiles of plants grown under S<sup>-</sup> versus S<sup>+</sup> and in vitro versus ex vitro conditions. Generally, total metabolite levels in the in vitro S<sup>+</sup> was 3.6- and 1.5-fold higher compared to in vitro S<sup>-</sup> and ex vitro (16 day) S<sup>+</sup> plants, respectively. Moreover, the total metabolites concentration in the in vitro S<sup>-</sup> was only 1/3 of ex vitro S<sup>-</sup> plants after 16 day of acclimatization stage. The quantity of amino acid decreased progressively for both S<sup>-</sup> and S<sup>+</sup> over the in vitro to ex vitro (16 days) transition period (Fig. 3). Under in vitro S<sup>-</sup> condition, amino acids represented 39 % of the total identified metabolites and rapidly declined to represent only 3 % of the metabolites after 16 days of acclimatization. The same trend was been observed under S<sup>+</sup> condition since amino acids represented 34 % of all identified metabolites and only represented 3 % after 16 days of acclimatization. Yet, an inverse relationship was observed for organic acids compared to amino acids in both S<sup>-</sup> and S<sup>+</sup> plants. Organic acids represented 6 and 5 % of total identified metabolites under in vitro conditions in both S<sup>-</sup> and S<sup>+</sup>, respectively, and became prevalent, representing up to 29 and 37 % after 16 day of the acclimatization. The proportion of sugars increased from 36 to 62 % during the transition of in vitro S<sup>-</sup> plantlets to ex vitro (16 day) stage. This proportion also increased in S<sup>+</sup> plantlets during the transition of S<sup>+</sup> plants from in vitro to ex vitro (16 day). The percentage of sugar alcohols was higher under in vitro conditions, 5 and 10 % in S<sup>-</sup> and S<sup>+</sup>, respectively, and then decreased to 4 % in both plant types after 16 days of acclimatization. Interestingly, urea and its derivative ureide (allantoin) were only detected under in vitro S<sup>-</sup> and S<sup>+</sup> conditions, and disappeared under ex vitro condition.

### Response of amino acids to sugar medium concentration and culture conditions

The total quantity of amino acids decreased progressively during in vitro to ex vitro transition. For example, under in vitro S<sup>-</sup> and S<sup>+</sup> conditions, amino acids were 5- and 14-fold higher respectively than under ex vitro conditions. Moreover, for in vitro plantlets, the presence of sugar in the culture medium caused amino acids to accumulate by up to threefold.

Glutamic acid (20 %), glutamine (12 %), serine (19 %) and glycine (20 %) were the most abundant amino acids under in vitro S<sup>-</sup> condition (Fig. 4a). Under S<sup>+</sup> condition, asparagine (33 %), glutamic acid (20 %) and glutamine (16 %) were the most abundant amino acid. Asparagine decreased to very low concentration after 16 day of the acclimatization in both S<sup>-</sup> and S<sup>+</sup> plantlets. Very

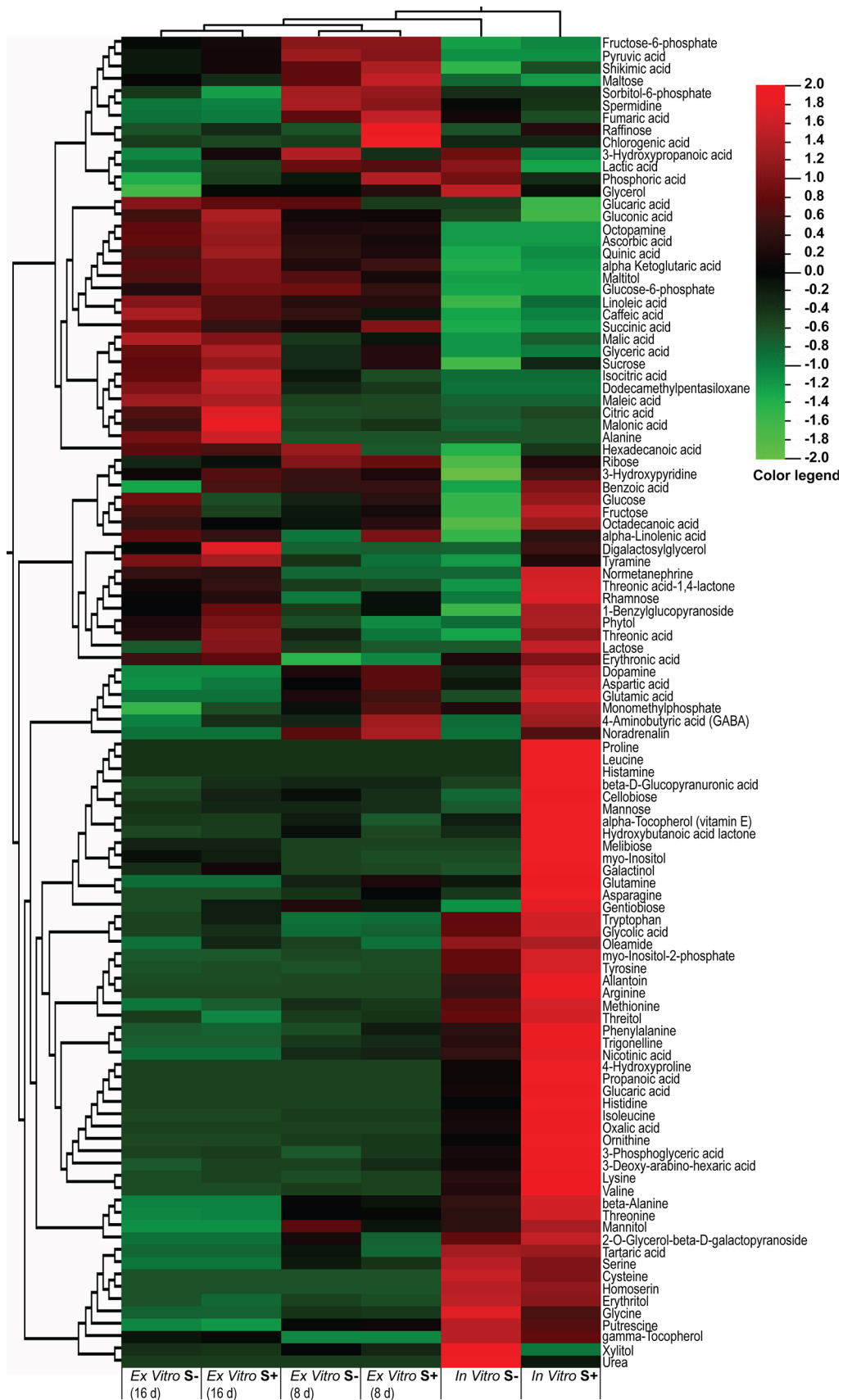
**Fig. 2** Heat map and hierarchical cluster analysis of 108 metabolites recovered in methanolic extracts from leaves of potato (*Solanum tuberosum* L., cv Norland) grown on 3 % sucrose (In vitro S<sup>+</sup>) and 0 % sucrose (In vitro S<sup>-</sup>) at the end of in vitro rooting stage and after 8 days [Ex vitro S<sup>+</sup> (8 day) and Ex vitro S<sup>-</sup> (8 day)] and 16 days [Ex vitro S<sup>+</sup> (16 day) and Ex vitro S<sup>-</sup> (16 day)] of ex vitro acclimatization stage. In the heat map red color indicates high, black intermediate and green low metabolites concentration values. (Color figure online)

interestingly, the most abundant amino acid detected in acclimatized plants was glutamic acid, and its quantity represented 75 and 67 % of the total amino acids detected (25 amino acids) in both types of plantlets.

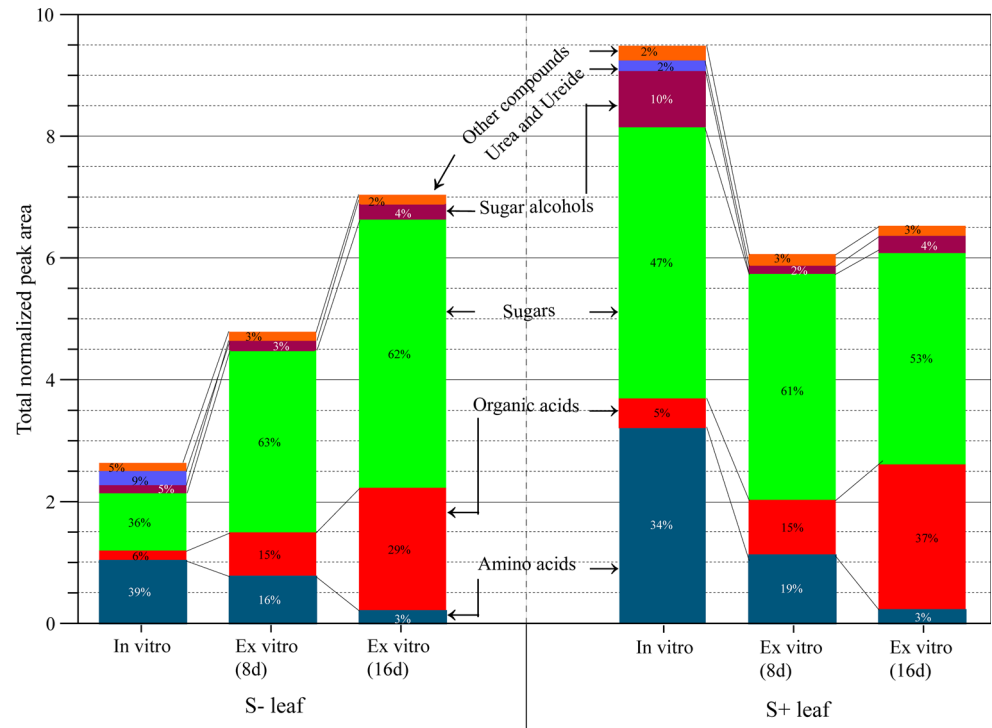
Under in vitro conditions, (See Supplementary data Fig. S1a no. 1-25), the majority of amino acids were found in much higher concentration (2- to 17-fold) in S<sup>+</sup> plantlets compared to S<sup>-</sup> plantlets. In addition, leucine and proline were only detected in S<sup>+</sup> plantlets. Yet, some other amino acids like cysteine, glycine, homoserine and serine were significantly lower under S<sup>+</sup> than under S<sup>-</sup> conditions. When comparing in vitro and ex vitro plantlets, the latter generally had lower amount of amino acid than in vitro plantlets. For example in in vitro S<sup>+</sup> plantlets, isoleucine, glycine, proline and asparagine accumulated by 51-, 79-, 1097-, and 2276-times, respectively, in comparison to ex vitro S<sup>+</sup> plants after 16 day of acclimatization. Also, the quantity of other amino acids like phenylalanine, GABA, tryptophan, glutamic acid, aspartic acid, threonine, methionine, tyrosine, valine, serine and lysine was 2-to 36-fold higher under in vitro S<sup>+</sup> condition compared with 16 day ex vitro condition. Interestingly, some amino acids like arginine, hydroxyproline, histidine, cysteine and homoserine were only detected in in vitro plantlets (in both S<sup>-</sup> and S<sup>+</sup>), while others like alanine was only detected after 16 day of acclimatization in both types of plantlets. As in vitro S<sup>+</sup> plantlets, in vitro S<sup>-</sup> plantlets also accumulated a large quantity of amino acids specially serine, glycine and asparagine which were 42-, 150-, and 254-fold higher respectively compared with S<sup>-</sup> plants after 16 day of ex vitro stage. Other amino acids like isoleucine, tyrosine, valine, threonine, tryptophan and aspartic acid were 3- to 25-fold higher under in vitro compared to ex vitro (16 day) S<sup>-</sup> plants.

### Response of organic acids to medium sugar concentration and culture conditions

We observed that organic acids and total free amino acids content followed an opposite trend during in vitro to ex vitro transition. Through this period, organic acids increased gradually until they reached 5- and 13-fold higher level in S<sup>+</sup> and S<sup>-</sup> plantlets, respectively, after 16 day of acclimatization. Also, during the in vitro rooting stage the



**Fig. 3** Changes in the quantity of all identified metabolites in potato leaves (*Solanum tuberosum* L. cv Norland) through in vitro and ex vitro stages. The percentage of each individual group relates to the total amount of identified metabolites. S–, leaves of plantlet grown on 0 % sucrose during in vitro rooting stage and S+, leaves of plantlet grown on 3 % sucrose during in vitro rooting stage



presence of sugar in the culture medium had a large effect on the accumulation of organic acids as they were threefold higher in S+ compared with S– plantlets. Malic acid was the most abundant organic acid detected in either plant types and under all culture conditions (in vitro and ex vitro) (Fig. 4b). Citric acid was the second most abundant organic acid, but only after 16 day ex vitro notwithstanding plant types. Under in vitro conditions, S+ plantlets had 5- and 14-fold more malic and citric acid respectively, than S– plantlets. Yet, they had 4- and 24-fold less of these respective organic acids, in vitro than after 16 day of acclimatization, while S– plantlets had respectively 26- and 173-fold less of the same organic acids in vitro than after 16 day of acclimatization. Other organic acids like glucuronic acid, propionic acid, oxalic acid, shikimic acid, ketoglutaric acid, malonic acid, 3PGA, quinic acid, threonic acid, nicotinic acid, glucaric acid and succinic acid were between 2- and 4-fold higher in S+ compared to S– plantlets. However,  $\beta$ -lactic acid (3-hydroxypropionic acid), lactic acid (2-hydroxypropionic acid) and fumaric acid were about twofold higher in S– compared to S+ plants. Interestingly, some organic acids were not detected in S– and S+ plantlets such as, isocitric acid, pyruvic acid and ascorbic acid (vitamin C), while some others were only detected in certain treatments, i.e. gluconic acid in S– plantlets and benzoic acid in S+ plantlets. In both types of tissue cultured plantlets, large amounts (between 2- and 13-fold) of certain organic acids accumulated; for example, nicotinic acid, 3PGA, fumaric acid, glycolic acid, lactic

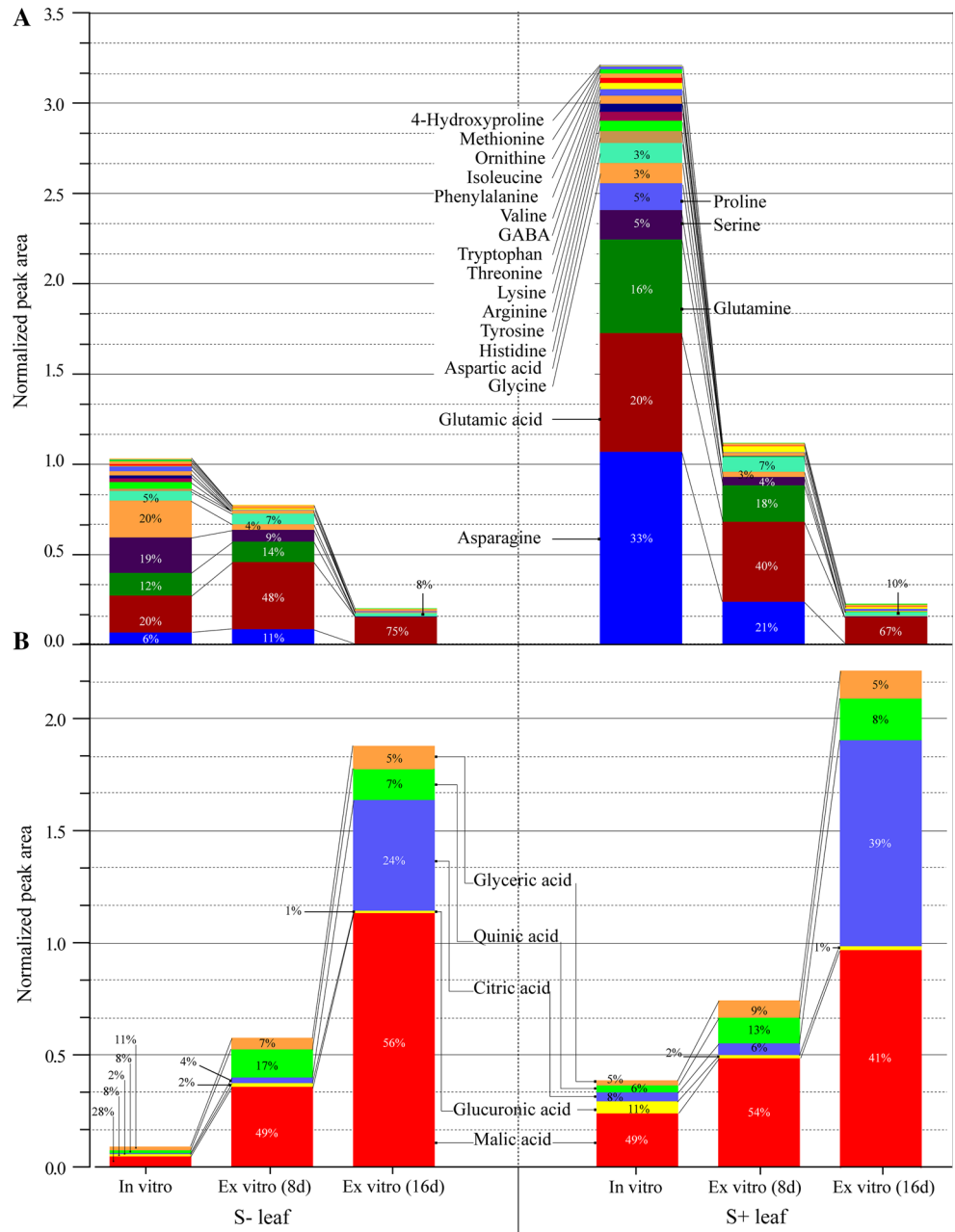
acid (only in S–), 3-hydroxypropionic acid (only in S–) and glucuronic acid (only in S+). In addition, oxalic acid, propionic acid and tartaric acid were only detected in in vitro plantlets (S– and S+), except for the latter, which was also found at low level at the beginning of acclimatization of S– plantlets. A number of other organic acids accumulated to much higher levels (from 2- to 60-fold) in plants acclimatized for 16 day (in both S– and S+) than in in vitro plantlets (i.e. shikimic acid, caffeic acid, succinic acid, glyceric acid, quinic acid, ketoglutaric acid, malonic acid and maleic acid).

### Response of sugars and sugar alcohols to medium's sugar concentration and culture conditions

Throughout the in vitro to ex vitro transition (Fig. 5a), the quantity of all sugars increased progressively for S– plants, while decreasing progressively in S+ plants. Under in vitro S+ condition, the total sugars quantity was 1.3- and fivefold higher compared with ex vitro S+ (16 day) and in vitro S– plantlets, respectively. In vitro S– plantlets were characterized by a lower amount of total sugars; in these plantlets the sugars were fivefold lower compared to their respective 16 day ex vitro acclimatized plantlets. Generally, sucrose, fructose and glucose represented more than 97 % of the identified sugars in plantlets regardless of the culture condition. Interestingly, fructose was the most abundant sugar in in vitro S+ plantlets (45 % of the total detected sugars) and was 2.5- and 12-fold higher compared



**Fig. 4** Changes in amino acids (a) and organic acids (b) in potato leaves (*Solanum tuberosum* L., cv Norland) during the in vitro and the ex vitro acclimatization stages. The percentage of each individual metabolite related to its group is showed on the histograms. S–, leaves of plantlet grown on 0 % sucrose during in vitro rooting stage and S+, leaves of plantlet grown on 3 % sucrose during in vitro rooting stage

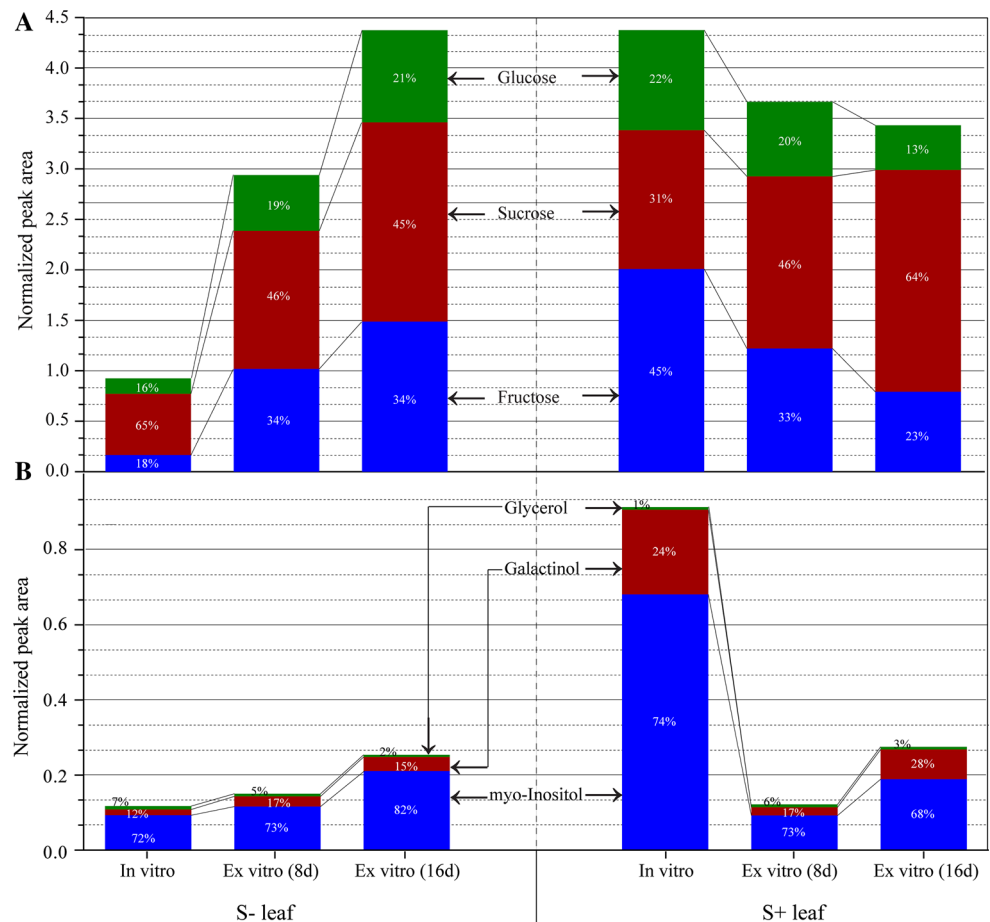


with ex vitro S+ (16 day) plants and in vitro S– plantlets, respectively. Nonetheless, sucrose was the most abundant sugar (representing between 45 and 65 % of the total detected sugars) in all autotrophic plants under in vitro or ex vitro environments. Glucose was two- and sixfold higher in in vitro S+ plantlets compared with ex vitro S+ plants (16 day) and in vitro S– plantlets, respectively. As fructose and glucose, gentiobiose and cellobiose were significantly higher (four- and eightfold) under S+ condition when compared to in vitro S– plantlets. In addition, many other sugars such as mannose, ribose, melibiose, lactose, rhamnose and raffinose were found in in vitro S+ plantlets

and were not detected in in vitro S– plantlets. Only maltose was fourfold higher under in vitro S– compared with in vitro S+ conditions.

Sugar alcohols (Fig. 5b) are another group of metabolites that were strongly affected by the presence of sucrose in the culture medium and the culture stages. The total quantity of detected sugar alcohols was 3- and 7-times higher in in vitro S+ plantlets compared with ex vitro S+ (16 day) plants and in vitro S– plantlets, respectively. The sugar alcohol *myo*-inositol represented at least 68 % of the total identified sugar alcohols in plantlets regardless of the culture condition. It was four- and sevenfold higher under

**Fig. 5** Changes in sugars (a) and sugar alcohols (b) in potato leaves (*Solanum tuberosum* L., cv Norland) during the in vitro and the ex vitro acclimatization stages. The percentage of each individual metabolite related to its group is showed on the histograms. S<sup>-</sup>, leaves of plantlet grown on 0 % sucrose during in vitro rooting stage and S<sup>+</sup>, leaves of plantlet grown on 3 % sucrose during in vitro rooting stage



in vitro S<sup>+</sup> compared with ex vitro S<sup>+</sup> (16 day) and in vitro S<sup>-</sup> conditions respectively. Galactinol was the second most abundant sugar alcohol in all plant tissues. In vitro S<sup>+</sup> plantlets contained the highest concentration of this sugar alcohol compared with all other treatments. It was 3- and 14-fold higher in in vitro S<sup>+</sup> plantlets compared with ex vitro S<sup>+</sup> plants after 16 day of acclimatization and in vitro S<sup>-</sup> plantlets. Under in vitro conditions, xylitol was only detected in S<sup>-</sup> plantlets and was not found in S<sup>+</sup> plantlets. Furthermore, mannitol was only detected under in vitro conditions and maltitol was only found under ex vitro condition (supplementary data Fig. S1b no. 70-71).

#### Response of urea, ureides and other miscellaneous compounds to medium's sugar concentration and culture conditions

In vitro conditions had a large impact on a range of other metabolites including urea, ureides, vitamins, phosphorylated compounds, polyamines, catecholamines, alkaloids, glycerides, fatty acid, fatty acid amide, terpenoids and phenolic compounds (Supplemental data Fig. S1c no. 75-107). For example, urea was only detected in in vitro plantlets (S<sup>-</sup> and S<sup>+</sup>) and rapidly disappeared below the

detection limit after 8 day of ex vitro acclimatization. In vitro S<sup>-</sup> plantlets accumulated a large quantity of urea; eightfold more than in vitro S<sup>+</sup> plantlets. Unlike urea, the ureide allantoin accumulated 2- and 217-fold in vitro S<sup>+</sup> compared with in vitro S<sup>-</sup> plantlets and ex vitro S<sup>+</sup> plants after 16 day of acclimatization, respectively.

The antioxidant tocopherol (vitamin E) levels were altered considerably by the sugar treatments and the culture stages. In general, there were significantly larger amounts of these molecules under tissue culture conditions whether or not sucrose was added to the culture medium. In vitro S<sup>+</sup> plantlets exhibited five- and eightfold higher levels  $\alpha$ -tocopherol in comparison to in vitro S<sup>-</sup> and 16 day ex vitro S<sup>+</sup> plants, respectively. In contrast, the amount of  $\gamma$ -tocopherol was significantly higher in plantlets growing on medium without sucrose compared to those growing mixotrophically in vitro or those S<sup>-</sup> plants ex vitro after 16 day, and was not detectable at the beginning of the acclimatization stage (after 8 day of ex vitro conditions).

Polyamines (PAs) such as putrescine (Put) and spermidine (Spd) were clearly and significantly affected by the presence of sugar in the culture medium and the culture stage. PAs accumulated to high levels under tissue culture conditions particularly in S<sup>-</sup> plantlets (supplementary data

Fig. S1c no. 99,101). During this period, an approximately 18-fold higher Put amount was recorded in S− plantlets as compared to 16 day ex vitro plants, and it was below the detection limit in S+ plants after 16 day of ex vitro acclimatization. Spd increased in S− and S+ plants at the beginning of the acclimatization (after 8 day) compared to in vitro plantlets and then decreased to very low level after 16 day of the acclimatization.

Many other compounds were in significantly higher amounts under in vitro conditions; i.e. oleamide which is an amide of the fatty acid oleic acid. This compound is an endogenous metabolite of mammals. Under in vitro conditions, in both S− and S+ plantlets, oleamide accumulated to levels over twofold higher than ex vitro plants (after 16 day) (supplementary data Fig. S1c no. 97). Trigonelline is also an important bioactive compound of plants. It was observed to accumulate 2.5-fold more in tissue cultured S+ plantlets compared to S− plantlets and it disappeared after 16 day of acclimatization (supplementary data Fig. S1c no. 103).

#### Application of principal component analysis to metabolite data set

Principal component statistical analysis (PCA) compresses large amount of data and provides an overview of class separation and clustering. It reduces hundreds or thousands of variables into a few principal components (Choi et al. 2010). The 107 metabolites present in supplementary Fig. S1 of S− and S+ were analyzed at three different time points (at the end of in vitro stage, after 8 and 16 day of the acclimatization stage). The data obtained were subjected to PCA to discriminate the similarities and differences between S− and S+ at each time-point sampling at the metabolite level. The first two components (PC1 and PC2) explained 70.4 % of the total variance within the dataset (Fig. 6). The PC1, accounting for 46.1 % of total variance, resolved the in vitro and ex vitro conditions. The samples of in vitro plantlets are clearly standing out from the samples of ex vitro plants. The PC2, accounting for 24.3 % of the total variance, resolved the in vitro S− and S+ conditions and ex vitro 8 and 16 days time points. The samples of in vitro S− are separated from the samples of in vitro S+ plantlets. However, during the acclimatization stage, S− and S+ almost formed one cluster at the same time point. The samples of ex vitro 8 days plantlets are differentiated from the samples of ex vitro 16 days plants along the PC2 axis. Principal component analysis was also used to estimate the contribution of individual metabolites to the clustering of the leaf tissues (supplementary data Fig. S3). Metabolites located close to the zero cut off axes contributed in a relatively small manner to the variance, while a number of more distantly distributed metabolites

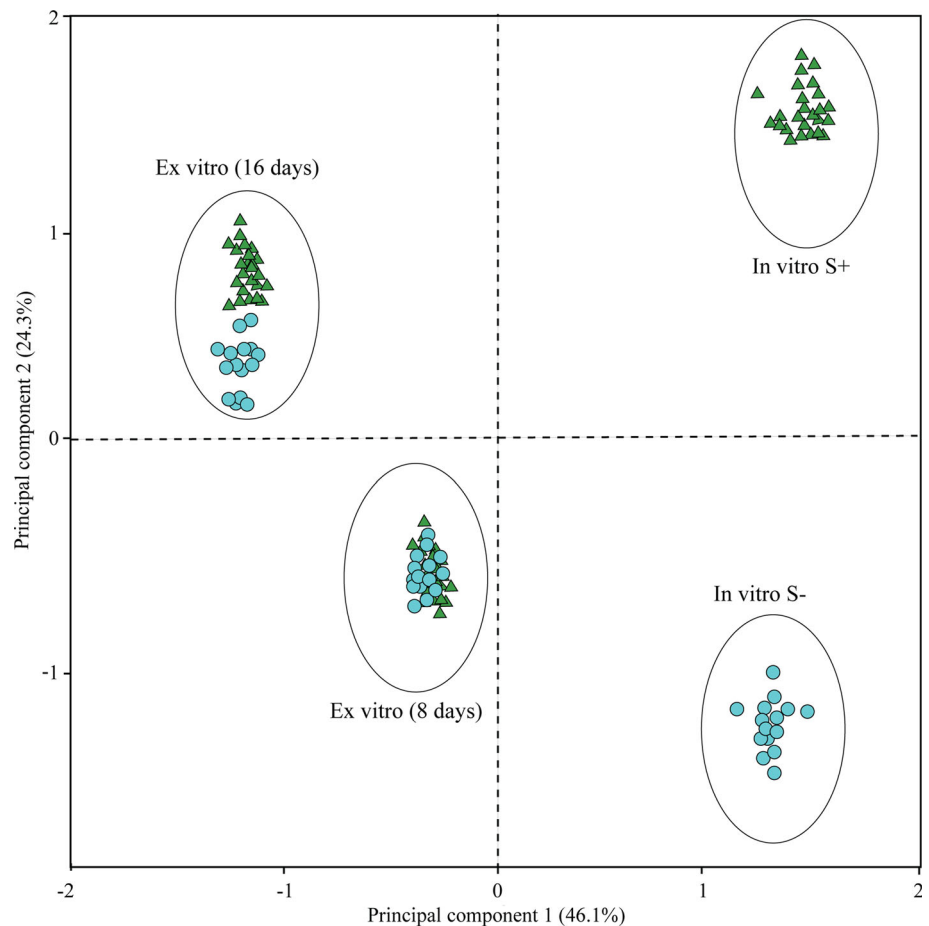
contributed to the separation of S− from S+ leaves clusters during *in* and *ex vitro* conditions.

#### Discussion

Using a metabolomic approach, we were able to comprehensively study the effect of exogenous sucrose on the physiology of potato plantlets during the in vitro rooting stage and recovery of a normal physiology of these plants during acclimatization. We could also track the precise adaptation of the plantlets metabolism in both the presence (S+) and the absence (S−) of exogenous sucrose, and obtain an accurate signature of the metabolite profile of these in vitro plantlets during the acclimatization phase.

The presence of sucrose during the in vitro culture improved by 100 % the capacity of plantlets to fix CO<sub>2</sub> (A<sub>max</sub>) (Table 1; Fig. 1). Yet, it did not modify the light compensation point; under the low light levels provided in vitro (~100 μmol m<sup>-2</sup> s<sup>-1</sup>) photosynthesis was thus restricted and only reached an average of about 1.7 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> (data not shown). The presence of sucrose in the culture medium clearly resulted in improved growth during the first stages of acclimatization (8 day) and later when newly formed leaves were emerging (16 day) (Table 1; Fig. 1). The carbohydrate reserves accumulated during in vitro culture (Fig. 3) may also somewhat contribute to improve growth parameters in the first stage of acclimatization (8 day) and later, when newly formed leaves are emerging (16 day) (Fig. 1). Such a response of exogenous sugar during in vitro rooting stage was previously reported; the exogenous supply of sugar increased plantlets carbohydrate reserves, enabling a smoother transfer to ex vitro conditions, an improved acclimatization success and a faster transition to undisturbed physiological parameters (Van Huylenbroeck and Debergh 1996). In addition, improvements of growth and photosynthesis by exogenous sugars were reported in micropropagated potato plantlets (Cournac et al. 1991). When comparing in vitro to ex vitro plants (in S− and S+), we found that during the in vitro stage A<sub>max</sub> was limited (8.8–16.6 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>); the photosynthetic rate however increases rapidly and significantly as soon as plantlets were transferred to acclimatization (20.9–26.2 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>). In vitro plantlets usually have a low photosynthetic ability, which is probably related to the distinct characteristics of in vitro formed leaves. Indeed, these leaves are anatomically (Sakai et al. 1993) and physiologically (Grout 1988) affected by the culture environment. Photosynthesis reduction during the in vitro stage has been attributed to low activity or quantity of Rubisco (Grout 1988; Premakumar et al. 2001). It is only when new leaves are entirely developed during the acclimatization stage that

**Fig. 6** PCA score plot of potato leaves plantlet (*Solanum tuberosum* L., cv Norland) grown on 0 % (S–, closed cyan circles) or 3 % (S+ , closed green triangles) sucrose in in vitro rooting stage during in vitro and ex vitro conditions. (Color figure online)



higher rates of net photosynthesis can be reached (Van Huylenbroeck and Debergh 1996). Our results confirm those of (Carvalho et al. 2001) who found that grapevine leaves produced during acclimatization stage showed higher photosynthetic capacity than the leaves produced during in vitro stage. Generally, in vitro plants, growing under low light, possess all the characteristics of shade-adapted plants with low  $A_{max}$ , low respiration and lower compensation point. Those of acclimated ex vitro plants behave more like sun leaves and are thus displaying a higher  $A_{max}$ , higher respiration and higher compensation point (Boardman 1977).

Nonetheless, in vitro conditions, with or without sucrose had, on the whole, a negative impact on the  $CO_2$  fixation, with respective rates 20 and 50 % lower compared to those measured after 8 days of acclimatization. Moreover, these conditions dramatically altered the overall metabolism as can be observed from the hierarchical cluster analysis and the PCA (Figs. 2, 6). Indeed, out of the metabolite analysis of the 90 samples submitted to PCA, four distinct clusters could be separated along two principal components explaining close to 75 % of the experimental variation (Fig. 6). The PCA score plots clearly demonstrated that

samples of plants cultured in vitro with sucrose (S+) were much different from those cultured without sucrose (S–). At the beginning of the acclimatization (8 day), many metabolites and in particular sugars declined sharply in S+ plants and increased in S– in response to ex vitro conditions and the metabolic profile converge in many respects. The metabolic profiles of S+ and S– plants became undistinguishable from one another by the end of 16 days of acclimatization (Fig. 6, S1, and S2). In vitro potato plantlets, and particularly those grown under S+ conditions, displayed an entirely different metabolic profile than that of ex vitro plants (Fig. 3, S1 and S2). A closer examination of the profile of these plantlets reveals two clear patterns: 1- they accumulate large quantity of amino acids, depicting high N availability in the in vitro culture medium and 2- they accumulate many stress metabolites, to adapt to the disturbing test-tube conditions.

Characteristically, the presence of sucrose in the medium caused a threefold increase in amino acids content in vitro compared to medium without sugar (S–) (Fig. 4a). The majority of these derived from transamination reactions and the GS-GOGAT pathway with asparagine, glutamate and glutamine representing close to 70 % of all

amino acids accumulated. This fact reflects the abundance of nitrogen in the MS medium (as much as 60 mM, 12 times of that found in soil) and the high availability of  $\text{NH}_4^+$  from either the medium, or from the photorespiratory nitrogen recycling and demonstrates the importance for the plantlets to assimilate this abundant amount of nitrogen into less reactive metabolites. Indeed, asparagine is a central compound involved in nitrogen transport and storage since it is stable and has a high nitrogen-to-carbon ratio (2 N to 4C for asparagine vs. 2 N to 5C for glutamine and 1 N to 5C for glutamate) (Taiz and Zeiger 2010). Assimilation of these amino acids is mediated at the origin through the transamination of 2-oxoglutarate to yield glutamate or oxaloacetate to yield aspartate. Normal plants grown outside in soil dynamically acclimate to the constantly fluctuating levels of C and N supply to optimize the capacity for sustained growth. This adaptation is transient and has mostly been studied following acute changes in either C or N availability (Smith and Stitt 2007). For instance, sucrose initiates a coordinated activation of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  assimilation, and biosynthesis of  $\alpha$ -oxoglutarate leading to the accumulation of amino acids (Morcuende et al. 1998; Stitt et al. 2002). In the present experiment, which is representative of an *in vitro* environment where plantlets are cultured under high C and N regimes, plantlets store available nitrogen into amino acids using sucrose as a source of carbon. *In vitro* plantlets cultured with sucrose (S+), also accumulated ureides such as allantoin, trigonelline and ornithine, as yet another mechanism to detoxify the high amount of inorganic nitrogen added to MS medium. Allantoin, a high nitrogen containing compound (C:N ratio 1:1) derived from purines or urea, is well documented in several plant species (Wang et al. 2007; Castro et al. 2001) to play an essential role in the assimilation, metabolism, transport, and storage of nitrogen in plants (Todd et al. 2006).

Concurrent to the accumulation of amino acids in medium with sucrose (S+), *in vitro* culture caused a remarkable decreased in the content of tri-carboxylic acid (TCA) cycle intermediates, citrate, isocitrate, succinate and malate (Fig. 3, S1 and S2). We presume that the depletion of these intermediates indicates a redirection of the amino acid precursors 2-oxoglutarate and oxoglutarate required for the assimilation of abundant medium nitrogen into amino acids. Such a drain on the TCA cycle, to provide carbon skeleton for the assimilation of the high medium nitrogen has previously been reported by Sima et al. (2001). To ensure that the intra-mitochondrial concentration of TCA intermediates remains constant under unfavourable conditions, plants may resort to metabolic adaptations, like the anaplerotic fixation of  $\text{CO}_2$  by PEPCase. As a matter of fact, in conditions of feedback inhibition of Rubisco due to the presence of sucrose (Hdider

and Desjardins 1994), anaplerotic  $\text{CO}_2$  fixation through PEPCase was stimulated to yield the carbon skeleton used for transamination reactions and the synthesis of amino acid. Sima and Desjardins (2001) found that potato plantlets grown on MS medium with sucrose had the highest PEPCase phosphorylation compared with lower sucrose concentrations. Roessner et al. (2000) have reported such an accumulation of amino acids, which correlated to depletion in TCA cycle intermediates in tissue cultured potato microtubers. Incidentally, we have also observed a low level of pyruvate, in tissue culture plants, which may explain the lack of replenishment of the TCA cycle through glycolysis; this may reflect the poor gas exchange causing reduced  $\text{CO}_2$  fixation and anoxic conditions developing in tissue culture.

In contrast to the stimulation of nitrogen sequestering into amino acids in presence of ample carbohydrate supply observed under S+ conditions, an interesting shift in nitrogen metabolism is revealed in condition of low carbohydrate availability (S–). These *in vitro* plantlets accumulated almost nine times more urea than those grown on medium with sucrose (S+). Since plantlets growing under S– condition are starved for carbon and at the same time have a reduced capacity to fix carbon due to the low light and poor gas exchange in test tubes, they have adopted another strategy to cope with the excessive N supplied by the MS medium, and thus avoid possible ammonia toxicity in their tissues. This response may thus represent an adaptation of the plantlets to assimilate nitrogen while using as little of the plant's carbon reserves as possible. Our data show that, the Krebs–Henseleit ornithine cycle or the urea cycle (UC) may be active to assimilate ammonia. Indeed, urea is very rich in nitrogen (C:N ratio 1:2) requires few carbon skeletons for its biosynthesis; it thus represents a good compound to sequester N under conditions of carbon restriction. In mammalian, the urea cycle involves transformation of toxic ammonia to urea, which is water-soluble and easily excreted from the body; this cycle only operates to eliminate excess nitrogen. Our results show that some of the UC intermediates like arginine and ornithine were found at high concentration in tissue cultured plantlets compared with plants grown *ex vitro* after 16 days. Even if not reported before, there is much evidence that UC is operational in plants under certain specific conditions as it is in mammalian systems. First, all the intermediates and some of the enzymes that cause the inter-conversion of ammonia to urea have been found in higher plants (Reinbothe and Mothes 1962). In addition, urease, which hydrolyses the urea to  $\text{NH}_3^+$  and  $\text{CO}_2$ , was identified in potato tissues (Witte and Medina-Escobar 2001), another evidence to the function of UC in potato.

Another distinct pattern revealed by the close examination of the metabolic profile of *in vitro* and *ex vitro* plantlets is the presence of many abiotic stress metabolites in the formers. Indeed, plants submitted to stress adapt by accumulating osmotically active molecules, by regulating ion transport, by modulating stomatal opening, by storing nitrogenous molecules, by actively maintaining cellular pH, by detoxifying cells, and by scavenging free radicals (Mansour 1998; Noiraud et al. 2001; Hare et al. 1998; Grigore et al. 2011). We have observed that S+ *in vitro* plantlets accumulated proline, asparagine, aspartate, GABA, 4-hydroxyproline, putrescine, trigonelline, myo-inositol, mannitol and many other molecules known to be synthesized in response to abiotic stress (Venkatesan and Chellappan 1998; Roessner-Tunali et al. 2003). More specifically, we observed a high accumulation of proline and 4-hydroxyproline *in vitro* and in particular under S+ conditions. This may be a response to the high osmotic potential of the MS culture medium, which is rich in sucrose, nitrogen and minerals. Several studies have reported that accumulation of proline is a general response to a broad range of stresses (Ashraf and Foolad 2007; Badr et al. 2011; Tekam et al. 2014) and in particular under osmotic stress (Cha-um and Kirdmanee 2008). This molecule is involved in osmotic adjustment, cellular structure stabilization, free radical scavenging and regulation of redox potential under stress conditions. In potato leaves, Levy (1983) observed that water deficit induced the accumulation of a 27 times higher proline accumulation than normal.

Interestingly, GABA also accumulated in large amounts in *in vitro* S+ leaves and remained at the same high level at the beginning of acclimatization (Fig. 4a and S2). After 16 days of acclimatization *ex vitro*, this molecule almost disappeared. A similar trend was also observed in S- plantlets but to a lesser extent. Noteworthy, reduced levels of GABA, observed in both S+ and S- at the end of acclimatization, were accompanied by the appearance of alanine in both cases. This may reflect the fact that GABA is transaminated with pyruvate through the GABA-shunt to yield alanine, which accumulated during acclimatization in both treatments. Apart from playing a role in carbon metabolism, GABA catabolism in mitochondria can supply carbon skeletons to refill carboxylic acids depleted as a result of stress-related metabolism (Kinnersley and Turano 2000), GABA accumulation appears to especially take place in stressed plants (Shelp et al. 1999). Gaspar et al. (2002) suggested that in hyperhydric callus (stressed cells), nitrogen metabolism was reoriented to polyamine biosynthesis and further channeled through the GABA-shunt. This active shunt may have had a role in TCA cycle replenishment in vitrified callus. Numerous reports over the years have confirmed that plants exposed to environmental stresses such as drought, heat, cold, salt stresses, to anoxia,

viral attack and mechanical damage have elevated levels of GABA (Bouché and Fromm 2004; Fait et al. 2008; Obata and Fernie 2012).

Tissue-cultured plantlets accumulate large amounts of ureides and polyamines (PAs) and particularly Put. Spd was also found in higher concentration at the beginning of the acclimatization stage. After 16 days of acclimatization PAs attained a very low level. Interestingly, there is a parallel between the urea cycle and PAs biosynthesis. Many of the UC intermediates are needed to the synthesis of PAs. Put is produced either directly from ornithine by ornithine decarboxylase, or indirectly from arginine by arginine decarboxylase (Martin-Tanguy 2001). Many of UC intermediates were found in higher concentration in tissue-cultured plantlets, probably indicating the activation of PAs biosynthesis pathways under *in vitro* conditions. When higher plants are subjected to environmental stress conditions, for instance when plants are exposed to nutrients shortage, osmotic shock or atmospheric pollutants, there are major modifications in nitrogen and polyamines metabolism (Gill and Tuteja 2010). This class of molecules has been reported to improve stress tolerance due to their poly-cationic nature at physiological pH, so they can bind to negatively charged molecules and stabilize the structure of DNA, RNA, proteins, and membrane lipids and thereby avoid their denaturation (Alcázar et al. 2010; Rodziewicz et al. 2014). Moreover, due to their antioxidant property, they can act as ROS scavengers and improvement ion balance (Liu et al. 2007). Bouchereau et al. (1999) reported that Put plays a role as a protective compound against hypoxia, as there is a relationship between tolerance of some species to oxygen shortage and their capacity to accumulate Put. Since plantlets are cultured in semi-sealed containers where air exchange is limited, they may experience at times hypoxic conditions (George et al. 2007). Activation of Put synthesis might also be the result of a detoxification mechanism in the presence of an excess of nitrogen ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) in MS medium as Put is rich in nitrogen (C:N ratio 2:1). Gaspar et al. (2002) have also showed that PAs accumulate in *in vitro* plants suffering from the vitrification syndrome.

In this experiment, sugars and sugar alcohols were found in significantly higher concentration in *in vitro* S+ compared to other treated plantlets (Fig. 5b). Many plant species accumulate significant amounts of sugars, polyols and other low-molecular-weight compounds in response to a variety of environmental stresses. In fact, metabolic adjustment via the accumulation of compatible solutes is a basic strategy for the protection and survival of plants under abiotic stress (Bohnert and Jensen 1996). The same response appears to take place *in vitro*.

Further confirming the fact that *in vitro* plantlets are subjected to an osmotic stress caused by the presence of

sugar in the culture medium, we observed a much higher concentration of  $\alpha$ -tocopherols (vitamin E) in plantlets with sucrose (S+) than without (S–). Vitamin E is a powerful endogenously produced antioxidant protecting the plants from free radicals generated during abiotic stress (Colakova and DellaPenna 2001). Our results are consistent with the reports of (Shao et al. 2008), who demonstrated that nine out of ten different grass species subjected to water stress had a one to three-fold higher level of tocopherol in their leaves. Stress conditions led to the formation of reactive oxygen species (ROS), which in turn, caused lipid and protein oxidation. To cope with oxidative pressure, plant cells have developed various efficient enzymatic and non-enzymatic defense systems. The non-enzymatic antioxidants are usually small water diffusible molecules, e.g. ascorbate and glutathione, whereas lipophilic antioxidants (tocopherols and carotenoids) are active to protect cell membranes phospholipids (Munné-Bosch and Alegre 2002).  $\alpha$ -tocopherol has been proposed to participate in the detoxification of ROS together with the hydrophilic antioxidants glutathione and ascorbate (Foyer and Noctor 2003). In photosynthetic plant tissues  $\alpha$ -tocopherol levels increase in response to a variety of abiotic stresses (Noctor 2006), such as water stress (Shao and Chu 2005). Interestingly, we noticed that when in vitro plantlets are transferred from tissue culture conditions to greenhouse (acclimatization), they accumulated ascorbic acid instead of tocopherol. This goes in line with the fact that ascorbate concentration in leaves is positively correlated to the intensity of light (Eskling and Åkerlund 1998); this molecule therefore participates in high light acclimation. Muller-Moule et al. (2004) have also shown that both a wild-type and an ascorbate deficient mutants of *Arabidopsis* accumulated more ascorbate when grown under high light levels. Plants have adapted different strategies to cope with sudden increases in light intensity and to protect themselves from excess light. In greenhouse, in vitro plantlets, which have adapted to low light ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) during their in vitro passage, encounter high light intensities, which may generate excessive proton pressures leading to adverse photooxidative damages. Knowing that about 30–40 % of the total cell's ascorbate is found in the chloroplast (Foyer et al. 1983), this molecule thus plays a role of paramount importance in the acclimation of the plants to high light by protecting the photosynthetic apparatus against the adverse effects of excessive light or photooxidative damage.

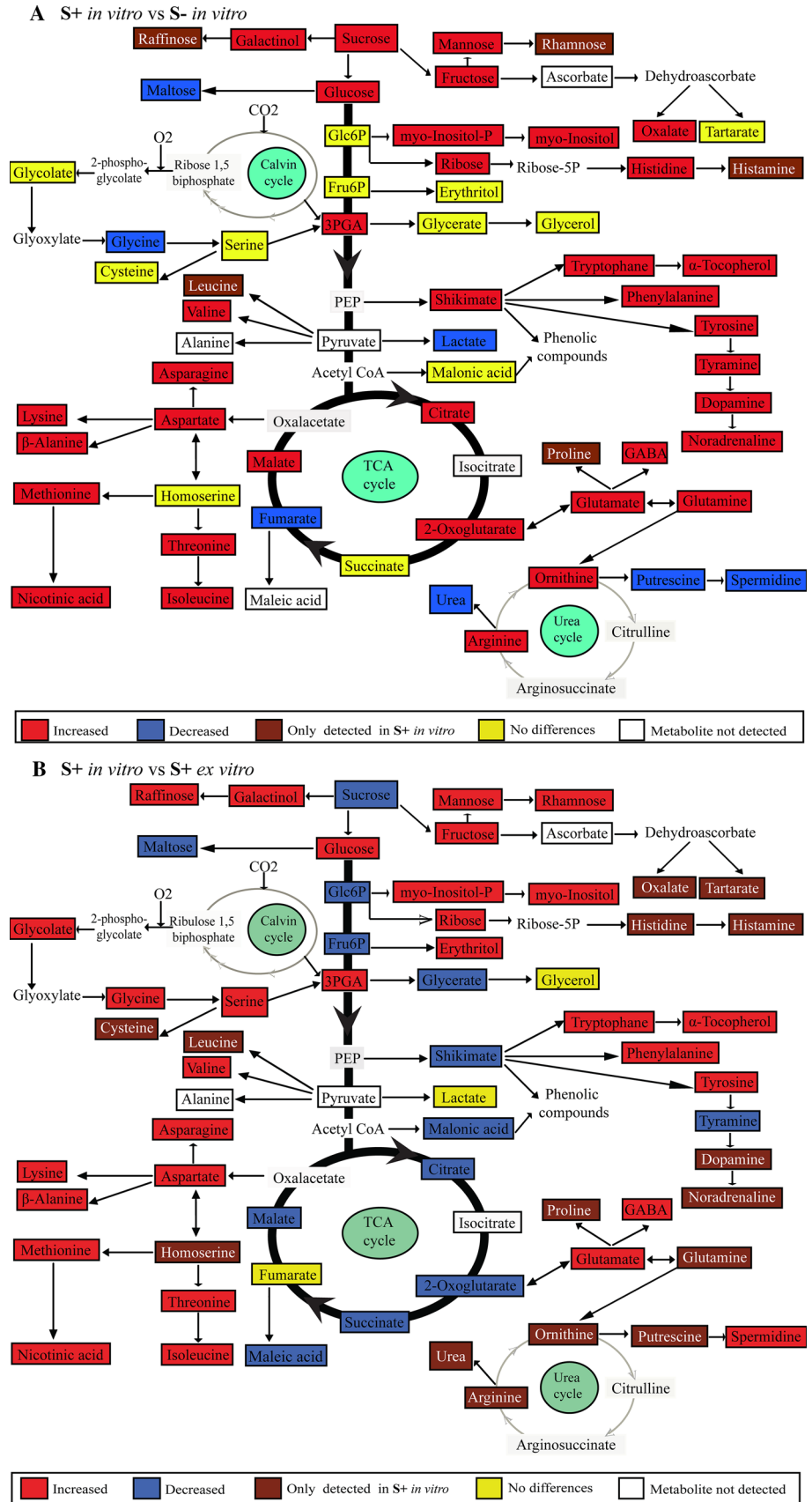
Finally, in vitro cultured plantlets accumulate many other metabolites, which may be involved in mineral nutrient acquisition and metals detoxification. Under in vitro conditions, it is likely that plants increase the production of specific amino and organic acids like histidine, oxalic and tartaric acids to mitigate the effect of high concentrations

of certain mineral nutrients. Stepansky and Leustek (2006) reported that histidine act as chelators and transporters of metal ions such as  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ . Tartaric and oxalic acid (and many others plant organic acids) are directly involved in the plant uptake for P, Al, and Mn. These organic acids are involved in rhizospheric processes, including nutrient acquisition and metal detoxification (Hocking 2001). In a phytoremediation study, tartrate has been used as a chelate to solubilise and enhance metals (Cd, Cu, Pb and Zn) uptake by plants (Doumett et al. 2008). Oxalic acid is a very simple dicarboxylic acid and it is the most oxidized organic metabolite produced in plants (Franceschi and Nakata 2005). It is known to act as a ligand for many metal cations. In addition to its role in nutrients acquisition, oxalate is reported to detoxify hazardous metals such as lead, strontium, cadmium and copper and also regulate  $\text{Ca}^{2+}$  concentration in plant cells (Franceschi and Nakata 2005). Oxalic acid biosynthesis in plants can follow a number possible pathway. It can be formed throughout the oxidation of glycolate and glyoxylate, which can be produced as a by-product of photorespiration. Oxalate can also be formed by the action of isocitrate lyase on isocitrate, and through oxidation of oxaloacetate. Finally, it can form from ascorbic acid. The latter case is believed to be the major source of oxalate in many plants (Franceschi and Nakata 2005).

To summarize, this research, which compares in vitro grown plantlets cultured on medium with (S+) or without (S–), clearly show that the former accumulate large quantity of nitrogenous compounds like amino acids, and osmotically active metabolite like GABA and proline (in red and brown in Fig. 7a). The availability of carbon skeleton enables S+ plantlets to assimilate the high concentration of N provided by the MS medium, which is believed to negatively affect metabolism. These plantlets also accumulated sugars and sugar alcohols, a sign of plant stress adaptation. They also contained high levels of carboxylic acids, like oxalic and tartaric acids, metabolites related to photorespiration, and to oxidation defense network. On the other hand, S– in vitro plantlets did not accumulate amino acids and typically sequestered MS nitrogen into compounds of high C/N ratio like urea and polyamines.

Comparing metabolic profiles of plantlets cultured on sucrose medium in vitro with the same plantlets but after 16 day of acclimatization, it is once again clear that tissue culture grown on MS medium displayed drastic accumulation of amino acids and nitrogen containing compounds that were only detected in vitro (Fig. 7b, red and brown rectangles). After 16 days of acclimatization, coinciding with the emergence of newly developed leaves adapted to ex vitro conditions, plants recovered many metabolites normally encountered under balanced glycolytic and TCA

**Fig. 7** Schematic showing significant variations of the most important metabolites abundance mapped onto the metabolic network. *Colored boxes* indicate changes in particular metabolite in the: **(a)** S+ versus S- plantlets under in vitro conditions, **(b)** S+ in vitro plantlets versus S+ ex vitro plants (after 16 days of acclimatization)





pathways, with reduced amino acids accumulation and an absence of the stress metabolites identified in vitro.

All in all, the results of this comprehensive metabolomic study clearly show that in vitro culture conditions and in particular mixotrophic conditions induce many metabolic aberrations, which may be responsible for the acclimatization problems and poor recovery upon transfer to soil. We demonstrate that the in vitro S+ conditions are triggering many counteracting metabolisms in response to the presence of high quantity of C and N in the medium. Our results confirm the previous suggestions made by Badr et al. (2011), Desjardins et al. (2007), Gaspar et al. (2002) and Joyce et al. (2003) that in vitro culture conditions are particularly stressful to the plantlets. The use of targeted metabolomics approach provides a unique and comprehensive overview of the metabolic adaptation of in vitro plantlets to their culture environment. The technique should be extended to other in vitro growth conditions in order to obtain a better insight of this particular environment and to avoid applying culture conditions that are detrimental to plant development.

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