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Effects of auxins on *in vitro* reserve compounds of *Phalaenopsis amabilis* (Orchidaceae)

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The effects of auxin and the endogenous levels of reserve compounds of *Phalaenopsis amabilis* (L.) Blume (Orchidaceae) were analyzed *in vitro*. Rootless plants were inoculated in modified MS media supplemented with IBA or NAA (0.0, 0.2, 1.0 and 5.0 mg L^{-1}) and with 2,4-D (0.000, 0.032, 0.160 and 0.800 mg L^{-1}). The biochemical parameters of endogenous levels of soluble carbohydrates and starch and of total soluble protein in roots, leaves and shoots were analyzed after 30 and 120 days. Carbohydrate levels in leaves showed similar patterns for all treatments. At 30 days, there was an increase in the endogenous carbohydrate level along with an increase in the concentration of auxins. At 120 days, the endogenous carbohydrate level in leaves had decreased, while the auxin concentration had continued to increase, demonstrating the mobilization of the carbohydrates. The leaf carbohydrate levels decreased from day 30 to 120; for both IBA and 2,4-D treatments, there was starch accumulation in roots as a function of the collection date. The 2,4-D concentration of 0.0032 mg L^{-1} decreased the level of total soluble protein in roots. The *in vitro* plants exhibit different growth patterns depending on the classes and concentrations of growth regulators. Biochemical analyses exhibited that metabolic activity and the degradation and accumulation of substances occurs in leaves, roots and shoots, demonstrating that roots contribute to the maintenance of plant metabolism and also act as reserve organs, even in epiphytic plants.

Key words: Storage compounds, soluble carbohydrates, starch, soluble proteins.

INTRODUCTION

The genus *Phalaenopsis* has sixty described species and thousands of hybrids derived from crosses between *Phalaenopsis amabilis* (L.) Blume and *Phalaenopsis stuartiana* (Rchb. f) (Harper, 2004). Originally from Northern Australia, Southeast Asia, the Himalayas, Indonesia and the Philippines (Arditti, 2007), these ornamental plants have high commercial value on the international market as one of the few orchids to bloom every six months with flowers of varied colors, ranging from white to bright red, and with long flower durability of up to 45 days. The species has epiphytic monopodial growth, with leaves arranged alternately from the buds, and its commercial propagation is performed by germinating seeds under aseptic conditions, as side-by-side shoots (Arditti, 2007). The *in vitro* culture techniques used for this species have a high propagation rate com-

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pared to conventional methods, with larger quantities of seedlings, better health, high ornamental value and production during a short time (Bosa et al., 2003).

The roots of epiphytic orchids have a complex structure that acts as a storage system for water and nutrients, is responsible for more than 70% of the plant's photosynthesis (Winter and Holtum, 2002) and also functions as protective tissue (Arditti, 2007). Auxins are the only growth regulators that increase consistently with root growth and are responsible for the formation of root primordia, at least in tissues that naturally have a predisposition to root (De Klerk, 2002). Auxins also influence shoot development, cell proliferation and elongation, and high concentrations are lethal with herbicidal activity (George, 1996).

The main compounds of plant reserves, including those in roots, are carbohydrates (starch and water-soluble carbohydrates), acting as an energy source for growth and as carbon skeletons for young tissue, as well as maintaining the osmotic potential of the cell (Itai and Birnbaum, 1996). Proteins correspond to approximately 30% of the total dry mass of a typical plant (Taiz and Zeiger, 2003), and they become part of the cytoskeleton (microtubules and microfilaments), protein reserves in seeds (globulins and prolamins), enzymes, and smaller amounts of peptides and amino acids. In a heterotrophic culture in which the main carbon source (sucrose), organic and inorganic salts are provided by the semi-synthetic substrate on which the plant depends throughout its growth and capacity to absorb these nutrients, which is activated by the differentiation of organs (buds and roots as large users of nutrients) and coordinated by a specific balance of auxin and cytokinin that also permits vegetative propagation.

Meristems of plants are nutrient sinks due to their high division rate, which is related to auxin induction of cell proliferation (Hartig and Beck, 2006), and multiple studies demonstrate that there are several functional types of auxin (Simon and Petrásek, 2011). The increase in soluble carbohydrate flux upon correct auxin action correlates with the C:N ratios, osmotic potentials, and formation of new organs (buds and adventitious roots), and auxins also function as signaling molecules (Gibson, 2005; Börner, 2011).

Carbohydrates and auxins function as signaling molecules and drivers of growth and developmental processes. Auxin metabolism is also regulated by the availability of free sugars, and the regulation of the biosynthesis and degradation of the main auxin, indole-3-acetic acid (IAA) by sugars, requires changes in the expression of multiple genes and metabolites linked to several IAA biosynthetic pathways (Sairanen et al., 2012).

The present study aimed to correlate the effects of different concentrations of the auxins IBA (indole-butyric acid), NAA (α-naphthalene-acetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) with the endogenous levels of soluble carbohydrates, starch and proteins from plants of *P. amabilis* hybrids cultivated *in vitro*, to obtain plants with greater levels of reserve compounds that would thus exhibit better growth.

MATERIALS AND METHODS

Culture of plants

Plantlets of *P. amabilis* were obtained from *in vitro* seed germination on MS medium (Murashige and Skoog, 1962) modified with 10% macronutrient, 30 g L^{-1} sucrose, agar (0.8%), pH 5.8 and autoclaved (15 min at 121°C, 1.2 atm). The plantlets were cultivated in 550 ml glass bottles with polypropylene caps filled with hydrophobic cotton and maintained in a culture room (temperature of 27 ± 2°C, photoperiod of 12 h and 25.0 µmol m^{-2} s^{-1} of photosynthetic active radiation (PAR). After 360 days, plants were selected based on the following criteria: 5.0 cm high, 2.0 cm wide and 2 leaves. Their roots were removed aseptically and transferred to auxin treatments (Sigma-Aldrich reagents): NAA (0.0, 0.2, 1.0 and 5.0 mg L^{-1}) or IBA (0.0, 0.2, 1.0 and 5.0 mg L^{-1}) and 2,4-D (0.0, 0.032, 0.160 and 0.800 mg L^{-1}) added to liquid of modified MS medium (same composition and process as described above). The treatments consisted of 10 glass bottles (as described above) with five plants each, in which 60 ml of culture medium was renewed every 30 days for each auxin and concentration, and the plants were maintained under the same culture conditions.

Two treatment bottles were randomly collected at 30 and 120 days of cultivation, totaling 10 plants analyzed per treatment. Plants were separated (leaves, roots and shoots), weighed for fresh mass, lyophilized and weighed for dry mass, ground in a knife micro-mill (0.2 mm mesh sieve) and divided into three samples of 100 mg for biochemical analysis.

Biochemical analysis

Extraction and determination of soluble carbohydrates

For each treatment and organ sample ground and lyophilized (100 mg), three extractions with 5 ml of 80% ethanol (homogenized for 10 min in each extraction) were made. These extracts was then centrifuged (1,000 *g* for 10 min at room temperature) resulting in the combined ethanolic supernatant extract, and its volume was measured.

The soluble carbohydrates were determined in triplicate using the phenol-sulfuric acid method (Dubois et al., 1956) and read on a spectrophotometer at 490 nm. The values were expressed as soluble carbohydrates (mg g^{-1} dry weight of soluble carbohydrate) with D-glucose as the standard.

Extraction and determination of total soluble protein

After extracting the ethanolic fraction, 5 ml 0.2 M phosphate buffer (pH 6.7) was added for each residue, homogenized for 10 min and centrifuged (1,000 *g* at room temperature), with triplicate of extraction, resulting in the soluble protein supernatant. The supernatants were collected and combined with the corresponding fraction, and the final volume was measured. Protein in the supernatant was determined by a dye-binding assay (Bradford, 1976) in triplicate, using bovine albumin as the standard. Biochemical measurements were performed on a spectrophotometer at 595 nm, and the content of total soluble protein was expressed as mg g^{-1} dry weight of soluble protein.

Extraction and determination of starch

For starch extraction, the previous residue was used with the addition of 5.0 ml perchloric acid (52% v v^{-1}), homogenized at 4°C for 15 min with periodic shaking, and centrifuged at 1,000 *g* at room temperature for 10 min, with two extractions. The supernatants were collected and their volumes were measured, yielding the starch extract, and the residue was discarded. The concentration of starch was calculated using the phenol-sulfuric acid method, with D-glucose as the standard (McCready et al., 1950) and multiplied by the correction factor 0.9 (estimated for plant starch), and the content was expressed as mg g^{-1} dry weight of starch, using the same standard curve of D-glucose as before.

Statistical analysis

Comparisons of the mean levels of soluble carbohydrates and starch and total soluble protein from leaves, roots and shoots at 30 and 120 days of growth were performed by analysis of variance (ANOVA) and the Turkey test. Measurements were conducted on three samples per experiment, and assays were performed in triplicate. Differences were considered significant at $p \leq 0.05$.

RESULTS

The level of soluble carbohydrates in roots (Figures 1B, 2B and 3B) in control samples of *P. amabilis* increased from day 30 to day 120, but it decreased in leaves (Figures 1A, 2A and 3A). The addition of the auxins IBA and 2,4-D reduced the carbohydrate levels in roots from day 30 to day 120 (Figures 1B and 2B), but treatment with NAA had no effect on root carbohydrate levels (Figure 3B).

However, with 5.0 mgL^{-1} NAA, a lethal herbicide effect was observed. Carbohydrate concentrations in leaves were reduced (Figures 1A, 2A and 3A) in all assays. Levels of soluble carbohydrates in shoots induced with 0.160 mg L^{-1} 2,4-D (Figure 2C) was high (approximately 250 mg g^{-1} dry mass) at day 30 and reduced by 80% at day 120 of the same 2,4-D treatment. Levels of soluble carbohydrates in plants treated with 5.0 mgL^{-1} NAA (Figure 3C) for day 30 was similar to those in plants treated with 2,4-D, but NAA was lethal for plants by 120 days.

Starch levels in control roots of *P. amabilis* (Figures 1E, 2E and 3E) were higher at day 120 than at day 30. Starch concentrations in roots increased at day 30 in treatments with 2,4-D (Figure 2E) and IBA (Figure 1E), except for the 5.0 mg L^{-1} IBA treatment. In NAA treatments (Figure 3E), starch levels were constant for 0.2 and 1.0 mgL^{-1} NAA at 30 and 120 days; the lowest concentration was observed for 5.0 mg L^{-1} NAA at 120 days, and there was no root induction with 5.0 mg L^{-1} NAA at 30 days. Leaf starch levels in plants treated with IBA (Figure 1D), NAA (Figure 3D) and 2,4-D (Figure 2D) were higher at day 30 than at day 120.

Shoots induced with 0.160 mgL^{-1} 2,4-D (Figure 2C, 2F and 2I) showed high levels of soluble carbohydrates, starch (both with 200 mgg^{-1} dry weight) and soluble proteins at 30 days, and a reduction was observed (75%) at 120 days, possibly due to the deleterious effects of auxin action in these tissues, not allowing their normal growth. The reserve carbohydrates (starch and soluble) were quantified at approximately 425 mgg^{-1} dry weight of leaves in the treatment with 0.160 mgL^{-1} 2,4-D at 30 days (Figure 4D). Higher amounts of reserve carbohydrate (starch and soluble) were observed in roots, at approximately 480mgg^{-1} dry weight for the 0.2 mgL^{-1} IBA treatment at 30 days (Figure 4B).

Total soluble protein levels varied between 5.0 and 8.0 mgg^{-1} dry weight in leaves of *P. amabilis* (Figures 1G, 2G and 3G), roots (Figures 2H and 3H) and shoots in treatments with 2,4-D and NAA (Figures 2I and 3I). Levels of total soluble protein over 10 mgg^{-1} dry weight were observed in roots upon treatment with 0.032 mgL^{-1} 2,4-D for 30 days, possibly related to the initial phase of organ tissues in multiplication and differentiation, and in shoots upon treatment with 0.160 mgL^{-1} 2,4-D for 120 days (Figure 2I), showing an 88% reduction in soluble protein levels. All other measurements of total soluble protein from leaves and roots showed constant levels (Figures 1G, 1H, 2G, 2H, 3G and 3H).

DISCUSSION

Research into orchids and their growth and biochemical compounds are scarce when compared with studies of *Solanum tuberosum*, a tuberous dicot rich in starch (60-75% dry weight). Both the leaves and roots of *P. amabilis* are rich in carbohydrates (soluble and starch) that may allow their re-mobilization under certain culture and propagation conditions.

Stancato et al. (2002), using pseudobulbs of *Cattleya forbessii* Lindl. × *Laelia tenebrosa* Rolf. described the translocation of reserve compounds from mature shoots to young as developing tissues, showing a direct relationship between source (mature photosynthetic tissues) and drain. Vaz et al. (2004) observed that long days also increase the concentration of starch and soluble carbohydrates in *Psygmorchis pusilla* Dodson and Dressler, an epiphytic orchid. Suzuki (2005) compared the amounts of soluble carbohydrates in the shoots and sub apical regions of *C. fimbriatum* and observed an inverse relationship between the two samples analyzed: the shoot contained approximately 25 mgg^{-1} fresh mass of soluble carbohydrates, with a lower concentration in the sub apical tissue, at approximately 5.0 mgg^{-1} fresh mass of soluble carbohydrates. Increased sucrose concentration in *Dendrobium* leads to a high level of total carbohydrates and starch, and the high level of soluble sugars allows for shoot elongation and growth (metabolic sink) (Ferreira et al., 2011).

Leaves of *P. amabilis* showed higher levels of reserve carbohydrates, mainly soluble carbohydrates, upon treatment with IBA (0.2 mgL^{-1}), NAA (1.0 mgL^{-1}) and 2,4-D (0.160 mgL^{-1}), corresponding to approximately 180 mgg^{-1}

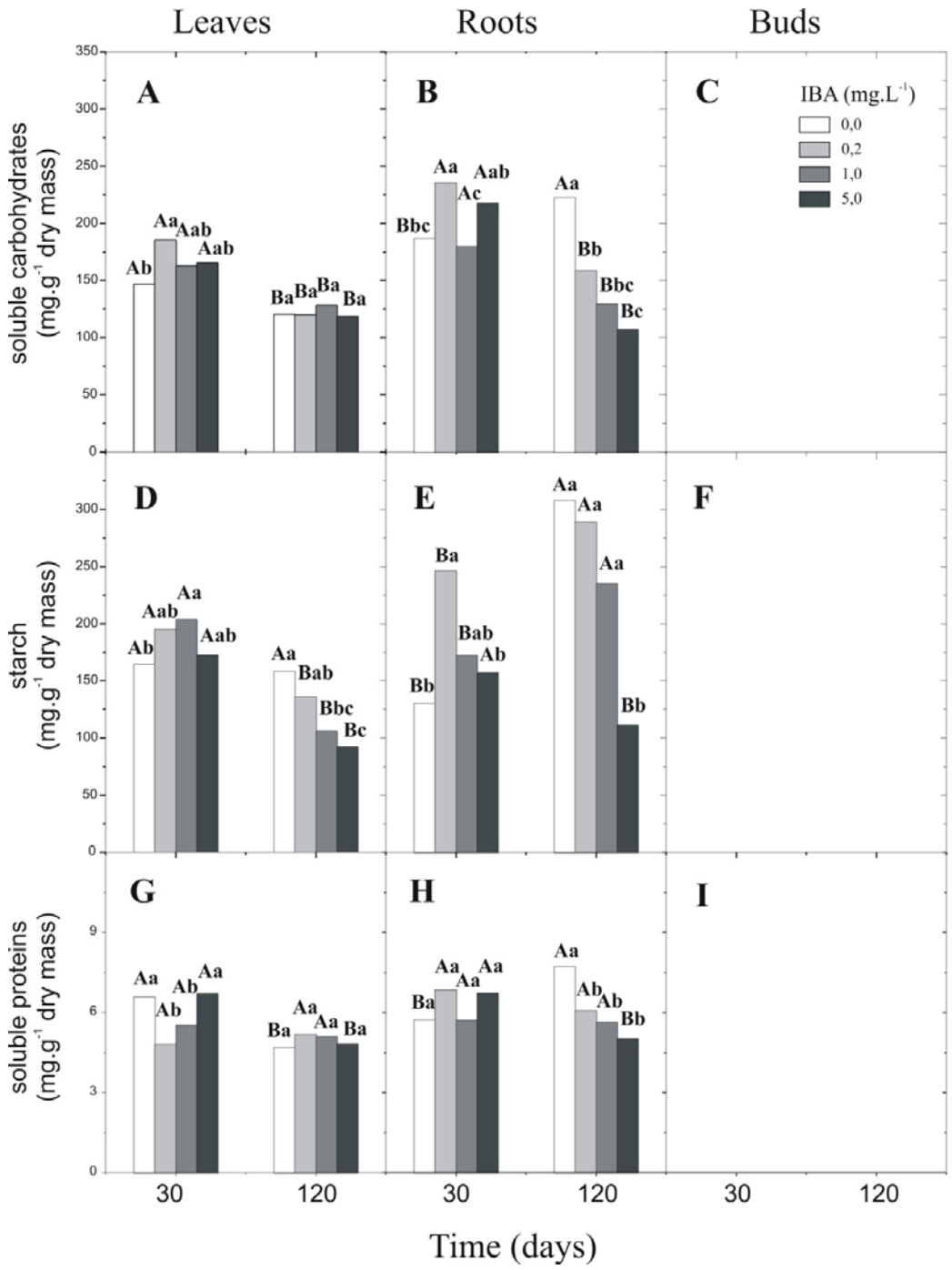

Figure 1. *In vitro* soluble carbohydrates, starch and soluble proteins in leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with IBA at concentrations of 0.0, 0.2, 1.0 and 5.0 mg L^{-1}, (*) there was no induction of shoots in this treatment; means followed by different capital letters indicate significant differences ($P \leq 0.05$) between the treatments and the lowercase letters indicate significant differences ($P \leq 0.05$) between the concentrations of growth regulators.

dry weight at 30 days. *P. amabilis* also has a higher accumulation of starch in leaves (200 and 250 mg g^{-1} dry weight) upon treatment with IBA (0.2 and 1.0 mgL^{-1}), NAA (0.2 and 1.0 mgL^{-1}) and 2,4-D (0.032 and 0.160 mgL^{-1}), resulting in five times more reserves than shown in other studies. This accumulation of carbohydrates may be related to the initial growth cycle in culture, which is followed by a decline upon growth arrest, with cell division having employed all available carbon molecules. This would preclude the existence of sink organs as

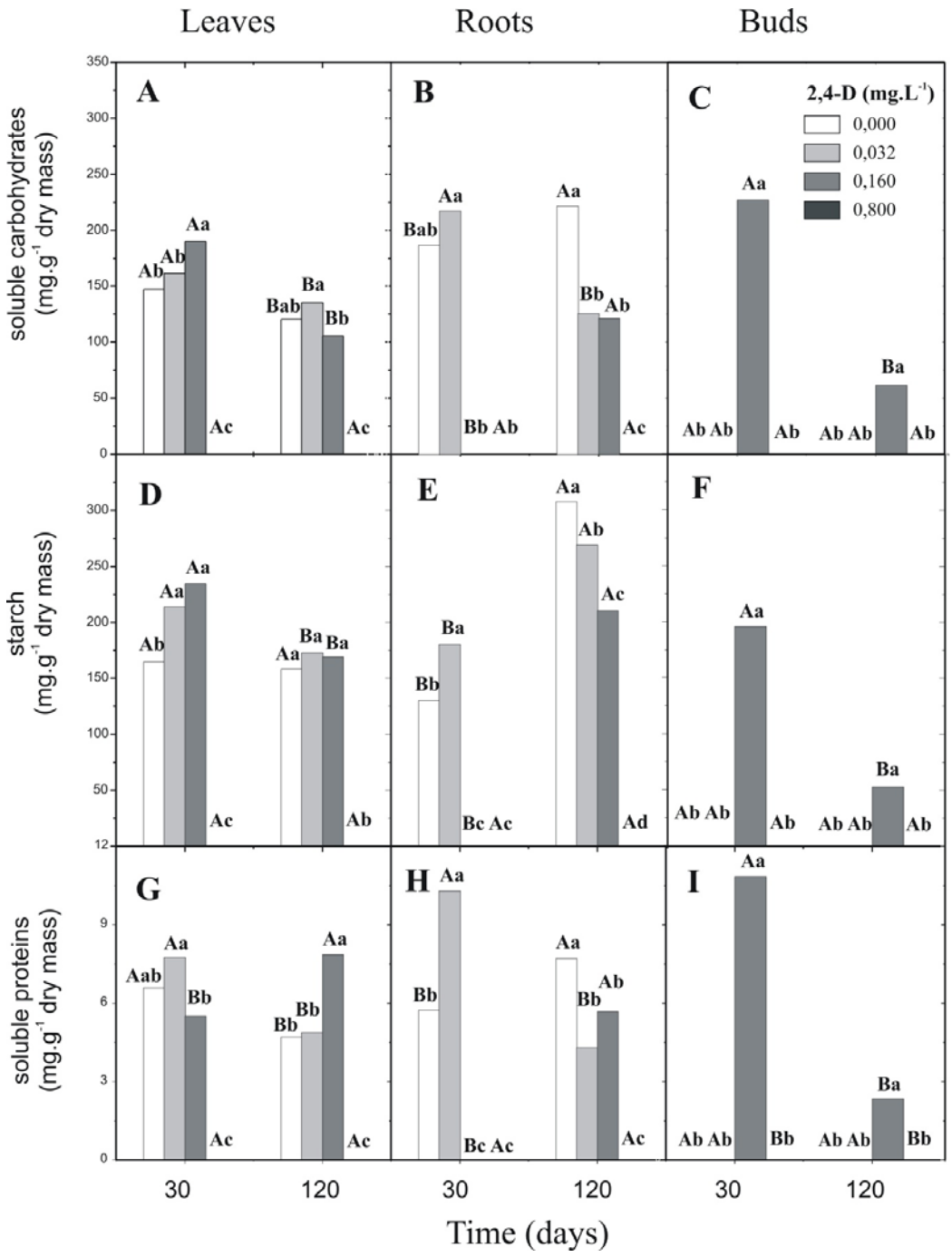

Figure 2. *In vitro* soluble carbohydrates, starch and soluble proteins in leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with 2,4-D at concentrations of 0.000, 0.032, 0.160 and 0.800 mg L^{-1}; means followed by different capital letters indicate significant differences ($P \leq 0.05$) between the treatments and the lowercase letters indicate significant differences ($P \leq 0.05$) between the concentrations of growth regulators.

reported for the life-cycle of sugarcane suspension cells (Goldner et al., 1991). In the callus cells of tomato cotyledons, starch accumulation seems to be a prerequisite for the *in vitro* development of shoots during the first days in culture, irrespective of the future development of the explants, and therefore the culture regime does not influence amylogenesis or changes in the protein pattern at a relatively late stage, when cell differentiation is visible (Thorpe et al., 1986; Stamp, 1987; Branca et al., 1994). Starch can act as a storage reserve

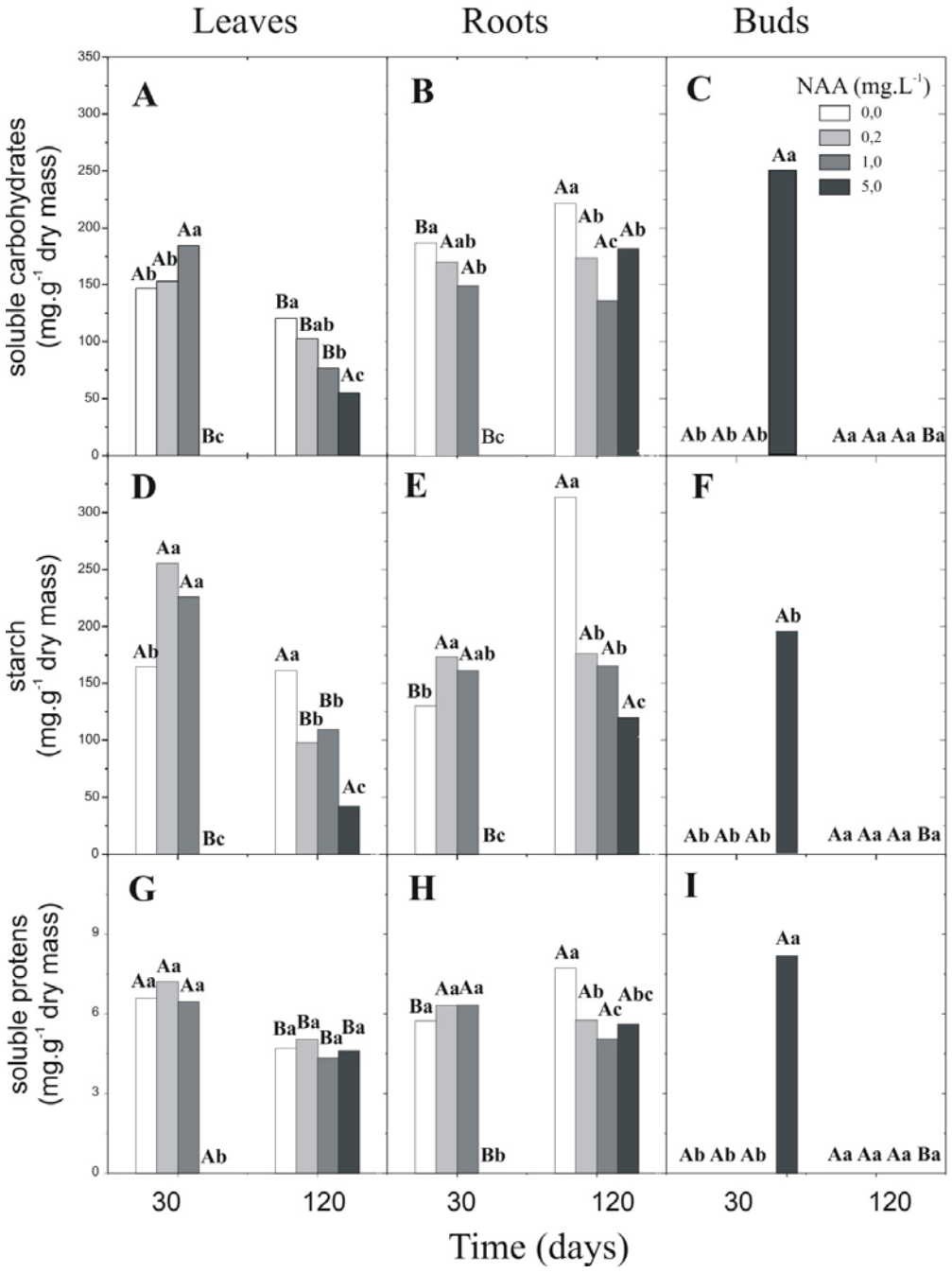

Figure 3. *In vitro* soluble carbohydrates, starch and soluble proteins in leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with NAA at concentrations of 0.0, 0.2, 1.0 and 5.0 mg L^{-1}; means followed by different capital letters indicate significant differences ($P \leq 0.05$) between the treatments and the lowercase letters indicate significant differences ($P \leq 0.05$) between the concentrations of growth regulators.

to support plant respiration and growth through the night, and several projects have focused on the synthesis and turnover of starches (Börner, 2011) and their mobilization in the acclimatization phase as important compounds during root and stem development.

Catasetum fimbriatum (Morren) Lindl. upon 90 days of *in vitro* culture, showed carbohydrate levels of 190 mgg^{-1} dry weight in mature roots, but lower levels were observed upon treatment with IBA treatment (0.061 mgL^{-1}) for 10 days, with approximately 240 to 150 mgg^{-1} dry weight at 30 days of culture. Similar effects were observed for the concentration of starch (approximately 50 mgg^{-1} dry mass for each compound in reserve), with the tips of roots and shoots showing approximately 300

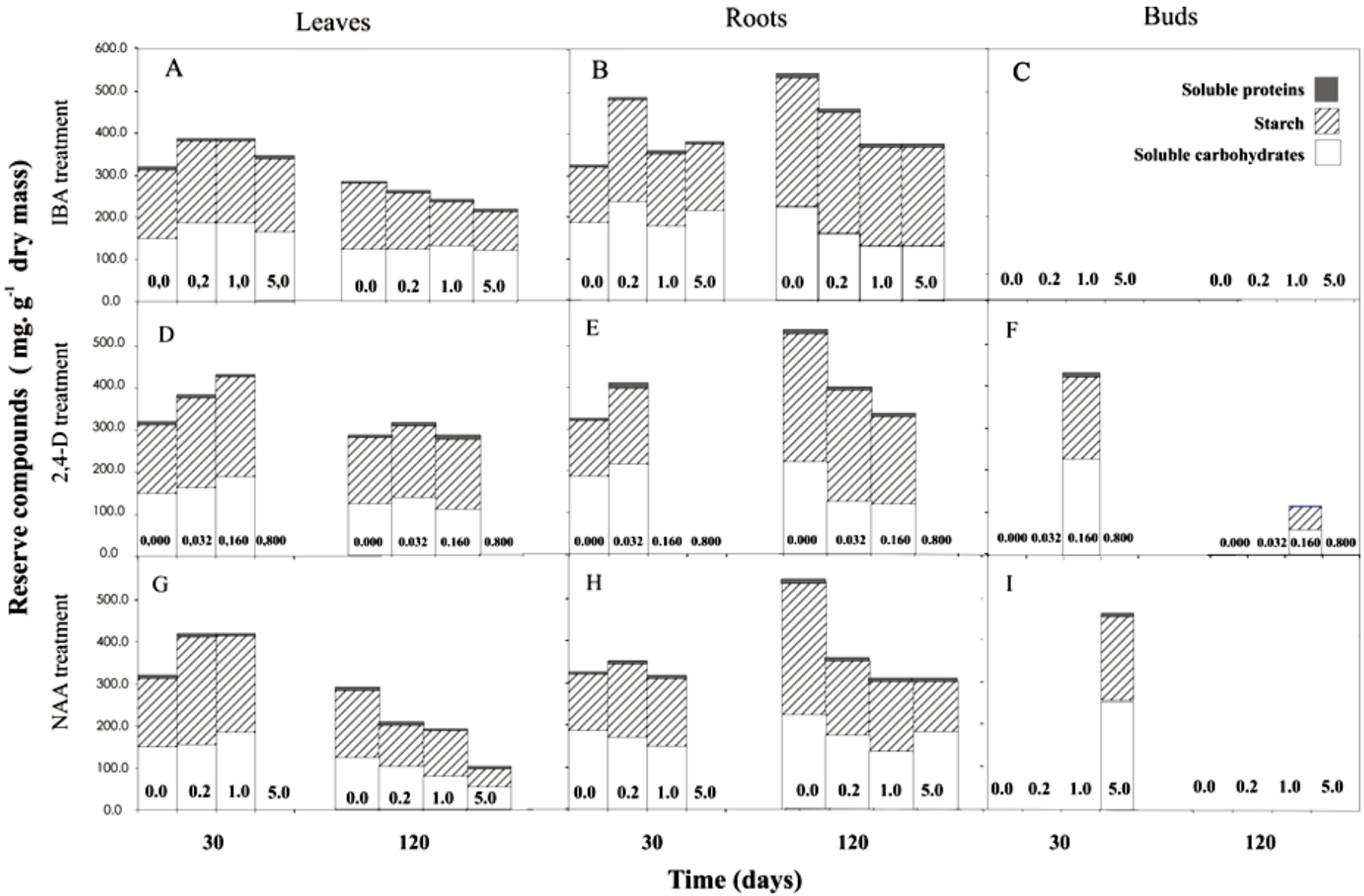

Figure 4. *In vitro* total reserve compounds (soluble carbohydrate, starch and soluble protein) of leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with auxins (IBA, 2,4-D and NAA).

mgg^{-1} and 405 mgg^{-1} dry weight of carbohydrate, respectively, and about 65 mgg^{-1} and 250 mgg^{-1} dry weight of starch, suggesting high carbon and energy costs for the development of new organs (Vaz et al., 1998).

In addition to controlling the assimilate supply to sorghum grains, the capacity to synthesize starch is also under hormonal regulation. Whereas IAA increases starch accumulation by facilitating the transport of sugars into grains and their transformation to this polysaccharide, IAA also increases the activity of the sucrose-hydrolyzing enzymes and decreases the activity of sucrose-phosphate synthase, contributing to a decrease in the proportion of sucrose in grain sugars (Bhatia and Singh, 2002).

Roots of cultivated potato showed the amount of total carbohydrates of 50 mgg^{-1} dry weight at 20 days (Schittenhelm et al., 2004), but *P. amabilis* roots showed 240 mgg^{-1} dry matter when treated with IBA (0.2 mgL^{-1}) for 30 days and approximately 100 mgg^{-1} dry matter when treated with IBA (5.0 mgL^{-1}) or 2,4-D (0.032 and 0.160 mgL^{-1}) for 120 days, both at concentrations four times higher than in potato. Starch levels in roots ranged from 100 mgg^{-1} dry weight after treatment with 5.0 mg L^{-1} IBA at 120 days to almost 280 mgg^{-1} dry matter (0.2 mg L^{-1} IBA at 120 days). Li et al. (2003) observed that *in vitro* explants of *Cymbidium sinense* (Andr.) Willd. had higher concentrations of starch grains in mature roots than in young roots. The same was observed in *C. fimbriatum* (Suzuki, 2005), in which there were an antagonism between the occurrence of sub apical shoots and the higher concentration of starch in shoots at mature stages (approximately 23 mgg^{-1} fresh weight) and lower in the sub apical development phase, with approximately 2.0 mgg^{-1} fresh weight.

Phalaenopsis has a CAM (Crassulatian acid metabolism), which requires an elevated energy level and large carbohydrate stocks. There is a competition between carbon storage for CAM to maintain a high capacity to fix CO_2 and exporting as much carbon as possible for growth (Wild et al., 2010). Four basic carbon partitioning strategies may occur in CAM species: ME (malic enzyme) starch formers, ME extra chloroplastic carbohydrate formers, PEPCK (PEP carboxylase) starch formers, and PEPCK extra chloroplastic carbohydrate formers. ME species can also combine both starch and extra chloroplastic carbohydrate storage, as *Phalaenopsis* does in our study.

The present results show that the total soluble proteins in roots and the photosynthetic aerial parts of *P. amabilis* are most likely not storage proteins, as are those found in potato tubers and soybean seeds. These proteins may be related to enzymes and their synthesis, coenzymes, nucleic acids, chlorophyll and to primary plant metabolism. According to Debergh and Maeno (1981), roots developed *in vitro* are not functional, but the biochemical analysis of roots of *P. amabilis* cultured *in vitro* shows accumulation of organic compounds that could be re-mobilized and translocated to newly formed tissue. Reduced levels of total soluble protein, from 350 to 125 mgg^{-1} dry weight, were also observed in pho-tosynthetic leaves of wild and transgenic potato grown *ex vitro* for 120 days (Schittenhelm et al., 2004), and this phenol-menon was related to the end of the growth cycle with remobilization of compounds to the storage organ. Silveira et al. (2004) observed that intracellular protein levels increased during the growth phase cells of *Pinus taeda* L. in suspension culture of embryos when 2,4-D (0.44 mg L^{-1}) was added to the medium and the starch levels were simultaneously reduced. Auxin may influence substrate distribution by determining the course and orientation of vascular strands through a mechanism by which auxin formation increases the advantage of dominant roots or shoots. The faster a root develops, the more carbohydrates and phloem-transported auxin it will receive, thus denying these to weaker roots, and the auxin in turn would enhance root branching, further increasing local carbohydrate consumption (Sachs, 2005).

The formation of new functional leaves and roots for the establishment of the *ex vitro* plant depends on the *in vitro* induction phase or on the acclimatization phase, with the mobilization of all of the existing reserves in plant tissues. Sucrose and starch (sources of carbon skeleton and energy), peptides and re-assimilated proteins (via proteases and degradation as a source of amino acids for the synthesis of new enzymes) can increase the rate of cell division and growth of the initial establishment until an autotrophic organism is produced. Therefore, increased ability to remobilize organic compounds (soluble carbohydrates, starch and total soluble protein) to perform specific tasks and coordinate developmental programs based on the availability of these crucial nutrients may increase the survival (Gibson, 2005) and growth of these explants when they are transferred from the *in vitro* stage to *ex vitro*, as observed in *Oxalis tuberosa* Mol (Conner et al., 1993) and *Rosa* (Capellades et al., 1991). Schittenhelm et al. (2004) observed that the starch from leaves of wild potato (*Solanum tuberosum* L.) and transgenic potato grown in a greenhouse were reduced from 20 to 120 days, showing nearly complete translocation of these reserves from the tubers during the growth cycle. To comprehend source-sink regulation in relation to plant development and to be able to manipulate these complex interactions for agricultural purposes (Wardlaw, 1990; Williams et al., 2000; Lemoine et al., 2013), it is vital that the underlying induction factors determining a sink organ for sugar (competition/priority system among growing tissues and storage) be correlated with growth regulators, sugar-sensing mechanisms, and the physiological process of sugar transport, and their cellular and temporal expression patterns must be defined.

Our results demonstrate the presence of high metabolic

activity with absorption of nutrients from the medium (macro- and micronutrients including sucrose, vitamins, and other organic compounds), the translocation of nutrients, and the accumulation and remobilization of carbohydrates and starch in leaves, stems and roots of *P. amabilis*. Furthermore, these results indicate the importance of the root system to epiphytic orchids that do not have specialized structural reserves, such as pseudobulbs, for use during growth, development and propagation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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