Localizing Starch Reserves in *Mandevilla sanderi* (Hemsl.) Woodson Using a Combined Histochemical and Biochemical Approach

Wahiba Boutebtoub

UMR SAGAH A 462, INRA - Agrocampus Ouest, Université d'Angers, 42 rue Georges Morel, B.P. 57, 49071 Beaucouzé, France

Michel Chevalier and Jean-Claude Maugé

UMR GENHORT A 1259, INRA - Agrocampus Ouest, Université d'Angers, 42 rue Georges Morel, B.P. 57, 49071 Beaucouzé, France

Monique Sigogne, Philippe Morel, and Gilles Galopin¹

UMR SAGAH A 462, Sciences Agronomiques Appliquées à l'Horticulture, INRA - Agrocampus Ouest, Université d'Angers, 42 rue Georges Morel, B.P. 57, 49071 Beaucouzé, France

Additional index words. Apocynaceae, *Mandevilla*, histology, ornamental plant, starch, tuberous roots

Abstract. *Mandevilla sanderi* is a plant of tropical origin of great horticultural interest because of its abundant flowering and its persistent foliage. Vegetative propagation requires the removal of leafy branches on the mother plant to produce cuttings. This loss of biomass must be compensated for by the growth of new branches thanks to the mobilization of reserves within the plant. Lack of knowledge about the physiology of this species therefore makes it necessary to characterize its different organs both at the level of their anatomic organization as well as at the level of their ability to store starch. After histological characterization of the different organs (leaves, stems, and roots), starch reserves were localized by histochemical analysis and quantified by biochemical analysis. Starch grains are mainly found in the parenchymatous cortex, the parenchymatous pith and xylem parenchyma cells, in tuberous roots and stems, and in the palisade and spongy mesophyll of leaves. In 22-week-old plants, the greatest quantity of starch is found in the leaves, whereas the tuberous roots have the highest concentration. The histological description of the different organs of *Mandevilla sanderi* and the localization of starch reserves allow us to assess the potential role of the different organs in plant growth and development. In the particular case of mother plant management, it is hoped that this knowledge will make it possible to optimize conditions for removing leafy branches.

Mandevilla sanderi (Hemsl.) Woodson (Woodson, 1933) is a plant native to Brazil, increasingly used in horticulture for its ornamental aspect, abundant and extended flowering, persistent and glossy foliage, tolerance to limited water availability, and resistance to many plant pests. The genus *Mandevilla* belongs to the family Apocynaceae, subfamily Apocynoideae, tribe Mesechiteae, and includes more than 170 species, most of which are native to the tropical forests of South and Central America (Morales, 1998, 2005; Simões et al., 2007). *Mandevilla sanderi* is characterized by its woody, voluble stems, persistent foliage with decussate phyl-

lotaxy, axillary racemose-type inflorescences, and pink, infundibuliform corollas. Whereas several species of *Mandevilla* have been extensively studied for their pharmacological properties (Biondo et al., 2004), the physiological characteristics of this species have not yet received adequate attention.

This plant is vegetatively propagated by taking cuttings from mother plants. This operation involves the removal of leafy branches, depriving the plant of part of its biomass and, as a result, its glucidic compounds. For *Platanus acerifolia*, this loss of biomass has an impact on the plant's ability to continue its growth (Haddad et al., 1995). Its subsequent development thus requires the mobilization of reserves present in its different organs. The lack of knowledge about the physiology of this species therefore makes it necessary to characterize at the level of their anatomic organization as well as at the level of their ability to store starch.

The purpose of this study was to localize and to quantify starch reserves in the various

plant organs. To do this, we adopted an approach that consisted, first, of carrying out anatomical characterization of these organs and then localizing the starch using histochemical analysis. Finally, biochemical analyses allowed us to quantify these reserves. To know this quantity and the localization of these reserves in the plant tissue allows to assess the ability of the plant to remobilize these resources after pruning, particularly in the case of mother plant management (Latt et al., 2000), and to ensure good rooting of cuttings (Eliasson, 1978).

Materials and Methods

Plant material and culture conditions.

The plant material used consisted of young plants of *M. sanderi*, cv. Rosea foncé, 22 weeks old, grown from cuttings on mother plants cultivated in vitro. They were planted in 11-cm diameter plastic pots in a substrate consisting of a mixture of blond peat and perlite (in a 1:1 volume ratio). After a week of acclimatization in a glass greenhouse, the plants were placed in a growth chamber with an area of 10 m² (4 × 2.50 m). A 4.5-m² (1.5 m × 3 m) subirrigation table was located in the center of the growth room equipped with a 200-L tank for recycling nutrient solution (in mEq·L⁻¹: 7.77 NO₃⁻; 0.85 H₂PO₄⁻; 1.76 NH₄⁺; 3.93 K⁺; 3.30 Ca⁺⁺; 1.40 Mg⁺⁺; pH 5.8; electrical conductivity 1.6 mS·cm⁻²). The lighting system consisted of 24 metallic halide lamps, Model HQI®.BT 400 W (OSRAM®, Berlin, Germany), providing average light intensity of 496 μmol·m⁻²·s⁻¹. The photoperiod was 16 h light (from 1800 HR in the evening to 1000 HR the next morning). Room temperature was regulated by a climate computer with a ventilation set point of 24 °C during the light period and 22 °C during the dark period. Plants were watered every other day with a nutrient solution.

Anatomical and histochemical analysis.

Three homogeneous plants were selected for this study. Samples were cut from roots, tuberous roots, stems, and leaves. Samples were immediately transferred after sampling to a fixation solution containing 4% glutaraldehyde in 0.2 M phosphate buffer at pH 7.2 for 2 h at 4 °C under vacuum. The samples were rinsed in three changes of buffer, washed three times in distilled water, and then dehydrated in a graded ethanol series (70%, 80%, 95%, 100%). They were infiltrated and embedded in Technovit® 7100 resin (2-hydroxyethyl methacrylate) (Heraeus Kulzer, Wehrheim, Germany) according to the procedure used by Kroes et al. (1998). Specimens were stored at 37 °C. Three-micron thick sections were cut with a Reichert-Jung 1140 microtome (Reichert-Jung, Nussloch, Germany) and mounted on clean glass slides.

The following two staining methods were used: 1) periodic acid/Schiff: sections were treated in periodic acid for 20 min, stained with Schiff's reagent for 30 min, differentiated with sulphuric water at 1%, rinsed with distilled water, and mounted in a synthetic resin. Schiff's reagent stained carbohydrate compounds red. It made it possible to visualize cell

Received for publication 5 May 2009. Accepted for publication 31 Aug. 2009.

We thank Lannes et Fils, SA, Marie Tellier, and Catherine Bernard (UMR-GenHort) for their contribution to this study and Gail Wagman for the English translation of the text.

¹To whom reprint requests should be addressed; e-mail gilles.galopin@agrocampus-ouest.fr.

Figs. 1–5. Morphologic structure of *Mandevilla sanderi*. Fig. 1. Aerial apparatus. Fig. 2. Voluble stem. Fig. 3. Young leaves. Fig. 4. Mature leaf. Fig. 5. Adventitious root system. (A) Tuberous roots; (B) fine roots.

Figs. 6–7. Anatomical structure of young roots of *Mandevilla sanderi*. Figs. 6–7. Transverse sections stained with Schiff's reagent. Fig. 6. Transverse section of a young root. Fig. 7. Cortical tissue. (A) Exodermis; (B) parenchyma cells; (C) phloem; (D) xylem; (E) pericycle; (F) endodermis; (G) exodermis; (H) laticifer; arrow (►): starch; Bar (—): 100 µm.

walls and starch grains; 2) iodine potassium iodide: sections were placed in the solution for 2 h and then rinsed with distilled water and mounted in synthetic resin. Starch grains were specifically stained purplish blue.

Samples were observed using a BH-RFC Olympus® microscope (Olympus, Tokyo, Japan) combined with a Sony 3CCD camera (Sony, Tokyo, Japan).

Biochemical analysis. Nine plants were separated into different compartments: roots, tuberous roots, stems, leaves, and inflorescences. These compartments were quickly immersed in liquid nitrogen and then freeze-dried for 48 h (Leybold Heraeus-GT-2; Leybold Didactic GmbH, Hürth, Germany). The dry weight obtained after freeze-drying corresponded to the weight of the apparent dry matter (ADM) (Gomez et al., 2003). A fine powder was obtained by placing the samples in a ball grinder (Retsch® MM301; Retsch, Haan, Germany), maintaining a low temperature, and then bringing them back to room temperature in a dessicator before samples were weighed for assaying. Aliquots of samples of 300 mg of dry powder were prepared. After extraction of soluble sugars with 80% ethanol at 70 °C, the residue was recovered and dried in a ventilated drying oven at 80 °C. Dispersion and hydrolysis of starch using α -amylase enzymes at 100 °C and amyloglucosidase at 50 °C (Megazyme International Ireland Ltd., Bray, UK) freed the glucose,

Figs. 8–12. Anatomical structure of tuberous root of *Mandevilla sanderi*. Figs. 8–10. Transverse sections stained with Schiff's reagent. Fig. 8. transverse section of a tuberous root. Fig. 9. Cortical tissue. Fig. 10. Vascular cylinder. Figs. 11–12. Transverse sections stained with iodine potassium iodide. Fig. 11. Starch localization. Fig. 12. Cortical tissue. (A) Cork layer; (B) parenchyma cells; (C) secondary phloem; (D) secondary xylem; (E) laticifers; (F) ray parenchyma cells; arrow (►): starch; Bar (—): 100 µm.

which was then quantified using the colorimetric method with anthrone in sulphuric medium.

Results

Morphological description of the plant. *Mandevilla sanderi*'s aerial system consists of indefinite and continuously growing stems with axillary inflorescences (Fig. 1). The basal part of the stems is lignified and has persistent and leathery leaves whose upper side is glossy with a thick epidermis (Fig. 2). These leaves are considered to be mature. The distal part of the stems has a growing apex and young anthocyanated leaves in their initial state of development (Fig. 3). The middle part is in the process of lignification. The stem may display voluble growth, which is characterized by long, thin internodes, leaves with small blades, and rarely with inflorescences (Fig. 4). The adventitious root system consists of fine, branched roots and tuberous roots (Fig. 5). To study the localization of starch reserves, this morphological description led to the differentiation of three compartments for the aerial system (stems, leaves, and inflorescences) and two for the root system (fine roots and tuberous roots).

After 22 weeks of growth, the total apparent dry biomass of the plants was 37.5 ± 9.7 g. In relation to the total biomass, the stems represented $26.5\% \pm 5.1\%$ and the leaves $51.1\% \pm 6.5\%$, of which $23.2\% \pm 6.1\%$ was attributed to mature leaves. Then, in decreasing order, the inflorescences represented $12.1\% \pm 3.9\%$, the tuberous roots $6.3\% \pm 2.1\%$, and the fine roots only $3.9\% \pm 0.8\%$ (Fig. 21).

Histological and histochemical study. Young roots have a small diameter; the parenchymatous cortex is limited to five or six concentric cambia and secondary formations are not yet apparent. The primary xylem is tetrarch or pentarch (Figs. 6 and 7). The small number of laticifers is dispersed in the form of isolated cells and with no order in the parenchymatous cortex. Starch is localized in the cells of the parenchymatous cortex, the endodermis, and the pericycle in the form of small grains (Fig. 6).

The parenchymatous cortex and the secondary vascular tissue (phloem and xylem) are the most highly developed in the tuberous roots (Figs. 8 and 11). The phellogen gives rise to a thin cork layer and a phelloderm where the laticifers are concentrated (Fig. 9). Several laticifers can be observed at the edge of the parenchymatous cortex in the form of isolated or grouped cells. The tuberous root is particularly rich in starch (Figs. 11 and 12). Starch grains are present throughout the parenchymatous and cortex with a higher concentration at the edge of the phloem. The starch grains in the xylem ray parenchyma cells are very big (Fig. 10).

The stem is limited by a periderm (Figs. 13 and 14). The vascular system (Fig. 13) consists of vascular bundles that form a continuous cylinder. The secondary xylem is

considerably increased. Xylem vessels are laid out in radial rays separated by the parenchyma (Fig. 15). Laticifers are found in the form of isolated cells in the parenchymatous cortex and the parenchymatous pith and at the edge of these two types of phloem. They are also found in the form of grouped cells in the parenchymatous cortex. Laticifers are also laid out in an almost continuous layer in the phelloderm (made up of a single small monocellular layer). The parenchymatous cortex and the parenchymatous pith of the stem of *M. sanderi* are rich in starch grains, especially at the edge of the parenchymatous cortex (Figs. 16 and 17). As is the case with the tuberous root, very large starch grains are found in the xylem ray parenchyma cells.

The adaxial epidermis is lined with a hypodermis made up of big cells. Latex cells are isolated and numerous in the chlorophyllous palisade mesophyll. They are absent in the epidermis and the hypodermis (Fig. 18). Chlorophyllous palisade mesophyll cells contain more and larger starch grains than

chlorophyllous spongy mesophyll cells (Fig. 19). There is no starch in the epidermis (except at the level of the stoma guard cells) or in the hypodermis.

Observation of the different organs appears to reveal the absence of starch grains in the laticiferous cells.

Biochemical analysis. Of all the organs of *M. sanderi*, the tuberous roots reveal the highest starch concentration (113.1 ± 25.8 mg·g⁻¹ ADM) (Fig. 20). In the other organs, the starch concentration varies according to the following decreasing order: leaves (51.2 ± 15.8 mg·g⁻¹ ADM), stems (36.5 ± 14.3 mg·g⁻¹ ADM), and fine roots (31.8 ± 8.6 mg·g⁻¹ ADM). Inflorescences have the lowest concentration (19.2 ± 7.1 mg·g⁻¹ ADM).

After 22 weeks of growth, the average quantity of starch per plant is 1745.1 ± 549.2 mg. In relation to the total quantity, leaves represent $56.5\% \pm 12.6\%$, stems $20.4\% \pm 7.4\%$, tuberous roots $15.6\% \pm 6.7\%$, inflorescences $5.0\% \pm 2.9\%$, and fine roots $2.7\% \pm 1.0\%$ (Fig. 21).

Figs. 13–17. Anatomical structure of woody stem of *Mandevilla sanderi*. Figs. 13–15. Transverse sections stained with Schiff's reagent. Fig. 13. Transverse section of a woody stem. Fig. 14. Cork and cortical tissue. Fig. 15. Secondary xylem. Figs. 16–17. Transverse sections stained with iodine potassium iodide. Fig. 16. Starch localization. Fig. 17. Pith. (A) Cork; (B) parenchyma cells; (C) secondary phloem; (D) secondary xylem; (E) laticifers; (F) ray parenchyma cells; (G) pith; (H) phellogen; (I) sclerenchyma; arrow (►): starch; Bar (—): 100 µm.

Discussion

The anatomical study of the different tissues of *M. sanderi* confirm that the plant belongs to the family Apocynaceae, particularly because of the presence of the intraxylary phloem at the level of the stem according to observations by Raynal-Roques (1994). The plant is thus characterized by the presence of laticifers (Hamel, 1989). In the case of *M. sanderi*, laticifers are present in all of the plant's organs whether they are below or above ground. They are localized in the parenchymatous cortex of fine and tuberous roots and of stem, in the parenchymatous pith of stems, and in the spongy and palisade mesophyll of leaves. The phelloderm in this species is made up of a majority of laticiferous cells. Laticifer distribution in *M. sanderi* is identical to that of *M. illustris* and *M. pohlinia* (Apezatto-Da-Gloria and Estelita, 1997). In contrast, laticifers are located near the phloem in the two latter species.

Apezatto-Da-Gloria and Estelita (1997) showed that the latex of *M. illustris* and *M. pohlinia* is poor in starch grains, confirming our observations on *M. sanderi*.

Histochemical analysis allowed us to precisely localize starch reserves at the tissue level. In *M. sanderi*, starch grains are mainly found in the parenchymatous cortex, the parenchymatous pith and the xylem parenchyma cells, tuberous roots and stems, and in the spongy and palisade mesophyll of leaves.

Biochemical analysis reveals that the tuberous roots have a high starch storage capacity. These reserves confer commonly on the plant a large capacity for adaptation to difficult conditions (Figueiredo-Ribeiro et al., 1986). Nevertheless, for plants older than 22 weeks, the quantity of starch stored in these organs is low as a result of their reduced biomass. Inversely, because the foliar biomass is considerable, the quantity of starch stored in the leaves is very high despite a concentration that is twice as low as that of the tuberous roots. Stems can also be considered to be non-negligible storage organs with a biomass twice as low as that of leaves but with a similar starch concentration. Using differentiated analysis of the central cylinder and the cortex of the tuberous root, we verified that the starch present in the xylem parenchyma cells was extracted and quantified by the biochemical analysis method used.

The use of biochemistry and histochemistry to study starch reserves combines two complementary methods, one that allows the quantification at the organ scale and the other that enables the localization of starch grains in the different tissues of each organ.

Within the framework of mother plant management to produce cuttings, the productivity and the sustainability of the plant are highly dependent on starch reserves. These carbohydrate reserves and the nitrogen content have also an effect on rooting capacity of cuttings (Zerche and Druege, 2009). In *Gliricidia sepium*, frequent cutting progressively decreased concentrations of starch, probably by hydrolysis of starch reserves to

Figs. 18–19. Morphological structure of *Mandevilla sanderi* mature leaf and starch localization. Fig. 18. Transverse section stained with Schiff's reagent. Fig. 19. Transverse section stained with iodine potassium iodide. (A) adaxial epidermis; (B) hypodermis; (C) palisade mesophyll; (D) spongy mesophyll; (E) laticifer; (F) stoma; (G) abaxial epidermis; arrow (►): starch; Bar (—): 100 µm.

Fig. 20. Starch concentration ($\text{mg} \cdot \text{g}^{-1}$ apparent dry matter) of different organs of *Mandevilla sanderi*.

Fig. 21. Starch quantity (mg) and apparent dry matter weight (g) of different organs of *Mandevilla sanderi*.

maintain the growth (Latt et al., 2000). In our particular case, the plant is pruned on a regular basis, resulting in the regular export of young growing organs (young leaves and stems) and the conservation of perennial organs (mature leaves and woody stems, fine and tuberous roots). These perennial organs make up the main part of the starch reserves.

In 22-week-old plants, it is primarily the mature leaves and the woody stems that ensure this role. For older plants, we can assume that the quantity of tuberous roots constitutes a complementary starch reserve essential to the mother plant. However, it is not absolutely certain that all of these reserves are easily mobilized for the growth of

the mother plant. The highest density of starch in the parenchymatous cortex cells, located at the edge of the phloem, implies that this starch is more easily mobilized than the reserve observed in the xylem parenchyma cells. This hypothesis could be verified by a dynamic monitoring of reserved starch in different tissues at each cut throughout the life of the mother plant. To know the quantity and the localization of starch reserves would allow optimizing the mother plant management (Galopin et al., 1996). The morphology of cuttings and the frequency of taking off must preserve the synthesis and the storage of starch in the stem and leaves kept on the mother plant (Latt et al., 2000).

Literature Cited

- Appezato-Da-Gloria, B. and M.M.E. Estelita. 1997. Laticifer systems in *Mandevilla illustris* and *M. velutina* (Apocynaceae). Acta Societatis Botanicorum Poloniae 66:301–306.
- Biondo, R., A.M. Soares, and B.W. Bertoni. 2004. Direct organogenesis of *Mandevilla illustris* (Vell) Woodson and effects of its aqueous extract on the enzymatic and toxic activities of *Crotalus durissus terrificus* snake venom. Plant Cell 22:549–552.
- Eliasson, L. 1978. Effect of nutrient and light on growth and root formation in *Pisum sativum* cuttings. Physiol. Plant. 43:13–18.
- Figueiredo-Ribeiro, R.C.L., S.M.C. Dietrich, E.P. Chu, M.A.M. Carvalho, C.C.J. Vieira, and T.T. Graziano. 1986. Reserve carbohydrates in underground organs of native Brazilian plants. Revista Brasileira de Botânica 9:159–166.
- Galopin, G., F. Beaujard, and M. Gendraud. 1996. Intensive production of juvenile cuttings by mother microplant culture in *Hydrangea macrophylla* 'Leuchtfleur'. Can. J. Bot. 74:561–567.
- Gomez, L., M.O. Jordan, S. Adamowicz, H. Leiser and L. Pagès. 2003. Du prélèvement au dosage: Réflexions sur les problèmes posés par la mesure des glucides non structuraux chez les végétaux ligneux. Cahiers d'études et de recherches francophones/Agricultures 12:369–386.
- Haddad, Y., D. Clair-Maczulajtys, and G. Bory. 1995. Effects of curtain-like pruning on distribution and seasonal patterns of carbohydrate reserves in plane (*Platanus acerifolia* Wild) trees. Tree Physiol. 15:135–140.
- Hamel, M.-C. 1989. Contribution bibliographique à la connaissance botanique des Apocynacées. IV. Relations avec les familles affines dans l'ordre des Gentianales. Revue de Cytologie et de Biologie Végétale. Le Botaniste 12:139–158.
- Kroes, G., R.P. Baayen, and W. Lange. 1998. Histology of root rot of flax seedlings (*Linum usitatissimum*) infected by *Fusarium oxysporum* f. sp. Lini. Eur. J. Plant Pathol. 104:725–736.
- Latt, C.R., P.K.R. Nair, and B.T. Kang. 2000. Interactions among cuttings frequency, reserve carbohydrates, and post-cuttings biomass production in *Gliricidia sepium* and *Leucaena leucocephala*. Agrofor. Syst. 50:27–46.
- Morales, J.F. 1998. A synopsis of the genus *Mandevilla* (Apocynaceae) in Mexico and Central America. Brittonia 50:214–232.
- Morales, J.F. 2005. Estudios en las Apocynaceae Neotropicales XII: Tres nuevas especies de *Mandevilla* Lindl. (Apocynoideae, Mesechiteae) para Colombia. Candollea 60:51–58.
- Raynal-Roques, A. 1994. Organisation des Angiospermes. In: Belin-INRA Editions (ed.). La botanique redécouverte. Belin-INRA, Paris, France. p. 191–250.
- Simões, A.O., L.S. Kinoshita and M.E. Endress. 2007. New combinations in *Mandevilla* Lindley (Apocynaceae). Novon: A Journal for Botanical Nomenclature 17:87–90.
- Woodson, R.E. 1933. Studies in the Apocynaceae. IV. The American genera of Echioideae. Ann. Mo. Bot. Gard. 20:605–790.
- Zerche, S. and U. Druège. 2009. Nitrogen content determines adventitious rooting in *Euphorbia pulcherrima* under adequate light independently of pre-rooting carbohydrate depletion of cuttings. Sci. Hort. 121:340–347.