Plant Cell, Tissue and Organ Culture **43**: 111–116, 1995.

Relation between primary and secondary metabolism in plant cell suspensions

Competition between secondary metabolite production and growth in a model system (Morinda citrifolia)

Linus H.W. van der Plas, Conny Eijkelboom & Marc J.M. Hagendoorn
Department of Plant Physiology, Agricultural University Wageningen, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Key words: anthraquinones, auxins, growth, *Morinda citrifolia*, secondary metabolite production

Abstract

Cell suspensions of *Morinda citrifolia* are able to produce large amounts of anthraquinones (AQ) when they are cultivated on a B5-medium containing 1 mg l^{-1} naphtyl acetic acid (NAA); this production is inhibited by addition of 2,4-dichloro-phenoxyacetic acid (2,4-D). Also during cultivation on 1 mg l^{-1} 2,4-D AQ-production is absent.

It appeared that in the presence of NAA a kind of 'AQ-production' program is switched on: cell division rate is low as well as metabolic activity, while endogenous sugar levels are high. The same properties develop in the presence of auxins like indolyl-acetic acid and p-chloro-phenylacetic acid. With 2,4-D and related auxins (like p-chloro-phenoxyacetic acid) AQ production is absent and emphasis is laid on a developmental program characterized by high cell division rates, high metabolic activity and low endogenous sugar contents. Independent of the type of auxin applied, the cells grow as a suspension consisting of finely dispersed cells. The 'AQ-producing differentiation program' cannot be maintained during a consecutive series of subculturings: with increasing AQ-contents the viability of the cells and the cell division rate decrease.

The possible mechanisms of regulation of AQ-production by auxins are discussed as well as the advantages of the use of the *Morinda* model system in the study of the relation between growth, primary and secondary metabolism.

Abbreviations: AQ – anthraquinones; 2,4-D – 2,4-dichloro-phenoxylacetic acid; DW – dry weight; EFW – extractive free weight; FW – fresh weight; IAA – indolyl acetic acid; NAA – naphtyl acetic acid; pCP – p-chloro-phenyl-acetic acid; pCPO – p-chloro-phenoxy-acetic acid.

Introduction

In each plant two conditions have to be met before a significant production of secondary metabolites can occur: the various enzymes directly involved in secondary metabolism have to be induced and also a sufficient supply of precursors from primary metabolism is necessary before product accumulation can be observed. Primary metabolism not only supplies the secondary metabolism with precursors, but many of the same precursors are also important for the synthesis of cell constituents. For that reason there is often a competition for these precursors between growth processes and secondary metabolism. As a consequence, synthesis of secondary metabolites occurs especially when growth is slow or absent, often after the completion of a differentiation process.

In many plant tissue culture systems, the quantitative significance of the synthesis of secondary metabolites is low. As a consequence, the impact of the synthesis of these products on cell metabolism is difficult to study. However, a few types of cell suspen-

sion are able to perform such biosynthetic reactions at high rates, resulting in concentrations of more than 10% on a dry weight basis. Such a system is formed by cell suspensions of *Morinda citrifolia* producing anthraquinones, first described by Zenk *et al.* (1975). Together with berberine production by Coptis cells (Sato & Yamada 1984) and rosmarinic acid synthesis in *Coleus blumei* and *Anchusa officinalis* (Ulbrich *et al.* 1985; De-Eknamkul & Ellis 1985), *Morinda* cell suspensions belong to the 'top ten' of producing cell suspensions. Most of these AQs are stored in the vacuole as glycosides (i.e. with a glucose-xylose-tail, Inoue *et al.* 1981).

The *Morinda* cell suspension system has a number of other properties, which makes it very suitable as a model system for the study of the regulation of secondary metabolite production and especially of the competition between growth and this production. In the first place, it is very easy to switch the production on and off in this system. Zenk *et al.* (1975) already described that the AQ-production is differentially affected by the type of auxin present in the medium; with 1 mg l^{-1} 2,4-D no sign of production of AQ is found while accumulation of AQ is observed very clearly in the presence of 1 mg l^{-1} NAA. In the second place, production of AQ in *Morinda*-cells is not limited to non-growing cells in the stationary phase. The AQ-production already occurs during the growth phase of NAA-cells and continues also in the stationary phase (Hagendoorn *et al.* 1994a).

However, in this paper, we show that cell metabolism and cell division rate are differentially affected by NAA- and 2,4-D-types of auxin, suggesting that in *Morinda* cells two different developmental programs can be switched on: a program without secondary metabolite production, focusing on fast cell division and a program in which production prevails and cell division is inhibited. A rapid switch from producing to non-producing state can be realized by the addition of 2,4-D-type of auxins: the study of the physiological changes during this switch may shed new light on the regulation of the relation between primary and secondary metabolism.

Materials and methods

Tissue culture

The *Morinda citrifolia* cultures (Zenk *et al.* 1975) were maintained in 250-ml Erlenmeyer flasks containing 60 ml of culture medium (B5, Gamborg *et al.* 1968) supplemented with sucrose (40 g l^{-1}), kinetin (0.2 mg l^{-1}) and auxin (1 mg l^{-1} 2,4-D or NAA). The cells were subcultured every 14 days (10 ml of culture transferred to 50 ml of fresh medium). The cultures were grown in the dark at 25 °C on a gyratory shaker at 100 rpm.

Fresh weight/dry weight

Fresh weight was determined after harvesting the cells on a paper filter on a Büchner funnel. For determination of dry weight the cells were dried at 60 °C for 24 h.

Anthraquinone determination

Morinda citrifolia cells (0.02–0.2 g FW) were boiled in 5 ml of 80% aqueous ethanol for 10 min. After centrifugation (1500 *g*, 5 min) the supernatant was collected and the pellet was again boiled in 5 ml of 80% aqueous ethanol. The supernatants were pooled and made up to 10 ml. More than 99% of the anthraquinones was extracted from the cells by this method (data not shown). The absorption at 434 nm was determined and the anthraquinone content was estimated using a millimolar extinction coefficient of 5.5 (Zenk *et al.* 1975).

Determination of carbohydrates

For determination of the cellular carbohydrate content, circa 10 mg DW was boiled for 15 min in 75% aqueous methanol. As an internal standard raffinose was added. Subsequently the methanol was evaporated under vacuum at 20 °C. The dried pellet was taken up in water and the suspension was centrifuged in an Eppendorf centrifuge (5 min 14000 rpm). The supernatant was directly injected in a Dionex HPLC system (Dionex corporation, Sunnyvale, USA) equipped with a Carbopac PA 100 column and a pulse amperometric detection system. Samples were eluted isocratically with 0.1 N NaOH. Carbohydrates were determined by comparing retention times with sugar standards.

Sugar content was also determined with the anthrone method (Mokrasch 1954), using saccharose as a standard.

Extractive free weight

Cells (5 g fresh weight) were washed with boiling water and filtered through a Büchner funnel. The residue was extracted with 96% ethanol and the extractive free

weight (EFW) obtained by this method was dried (60 min, 90 °C). This EFW consists mainly of cell wall material (Hagendoorn *et al.* 1990).

Protein content

The proteins were extracted from the cells by grinding about 0.1 g FW in liquid nitrogen with a mortar and pestle. Two ml of 1 N NaOH was added and after centrifugation (10 min, 3000 rpm) the protein content of the supernatant could be determined according to Bradford (1976). Bovine serum albumin was used as a standard.

Determination of cell number

After an appropriate dilution two samples of cells in the suspension culture were counted in a haemocytometer (Fuchs-Rosenthal). The cells in 16 squares were counted and the mean was determined.

Determination of lipid content

Lipid content of circa 0.2 g FW was determined according to Hoekstra *et al.* (1989). After extraction (Hoekstra *et al.* 1989) the samples were injected in a gas chromatograph (GC-8A, Shimadzu) applied with a J & W Megabore column (DB 225, 30 m 210 °C) and a flame ionisation detector. The total amount of lipids was determined.

Results and Discussion

Analysis of growth and production in Morinda cells

To analyse the competition between growth and secondary metabolite production, the properties of cells growing in the presence of 2,4-D (producing no secondary metabolites) and of AQ-producing cells growing with NAA, were compared. When growth is expressed on a dry weight basis, it is clear that both auxins lead to growth with almost the same kinetics. Only the total increase in dry weight of NAA-cells is somewhat higher than that of 2,4-D cells (Hagendoorn *et al.* 1994a). This suggests that there seems to be no competition between growth and production in *Morinda* cells and that the growth of the producing (NAA)-cells is even 'better' than that of the non-producing 2,4-D-cells. However, this is not necessarily a correct

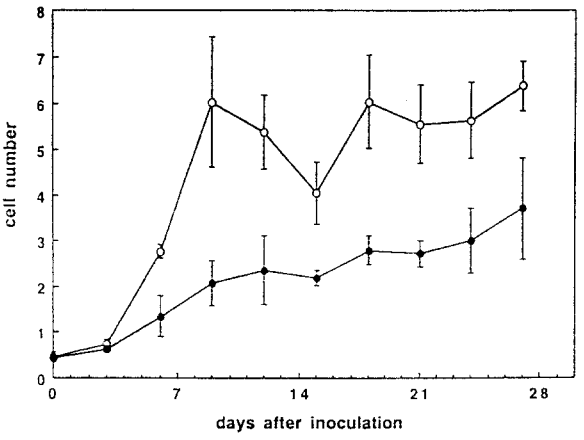

Fig. 1. Time course of growth of *Morinda citrifolia* cells cultivated in the presence of 1 mg l^{-1} NAA (closed circles) and 1 mg l^{-1} 2,4-D (open circles). Growth is expressed as cell number (1 unit is 10^9 cells l^{-1}). Means of 4 experimental series are depicted. NAA-cells were twice subcultured in medium containing 1 mg l^{-1} NAA.

interpretation of these growth data, as is shown by Fig. 1. When growth is depicted as increase in cell number, there is a large difference in growth kinetics for these two types of auxin, a difference that does not correspond with the dry weight data. The cell number in the 2,4-D-culture rapidly increases between 5 and 10 days after inoculation and stays rather stable afterwards in the 'stationary phase'. With NAA-cells a completely different picture is seen: there is no rapid growth phase but only a slow increase in cell number that goes on for at least four weeks; even then cell number is only half that in 2,4-D-cells.

These data show that cell division is inhibited in the producing *Morinda* cells. Apparently AQ-producing cultures contain less cells, that have a higher dry weight per cell when compared with non-producing cultures. This also indicates that one has to be careful with the use of data on dry weight increase as a measure for growth.

Sugar metabolism, metabolic activity, growth and production

As the *Morinda* cells are cultivated heterotrophically, growth and secondary metabolite production is completely dependent on the presence of sugars in the nutrient medium. The fate of these medium sugars was studied by Hagendoorn *et al.* (1994a). Medium sucrose appeared to be converted rapidly in glucose and fructose, of which glucose was preferentially taken up by the cells. The total rate of sugar uptake appeared to be

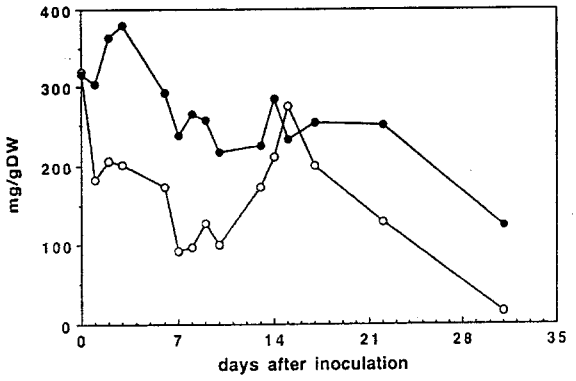

Fig. 2. Time course of changes in total sugar contents of *Morinda citrifolia* cells grown in the presence of 1 mg l^{-1} NAA (closed circles) and 1 mg l^{-1} 2,4-D (open circles). Total sugar was calculated by adding the amounts of sucrose, glucose and fructose at each measuring point, expressed as mg g^{-1} DW. Duplicate measurements of two independent experimental series are depicted.

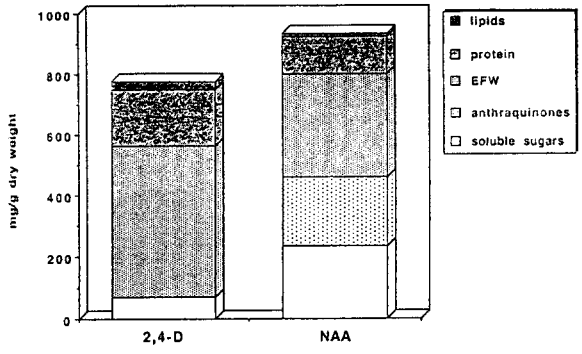

Fig. 3. Dry weight composition of *Morinda citrifolia* cells grown in the presence of 1 mg l^{-1} NAA and 1 mg l^{-1} 2,4-D. Cells that had grown for 18–27 days after inoculation were used for the determination of the various compounds. Non-determined compounds (e.g. ions, organic acids etc.) made up about 200 (2,4-D-cells) and 50 (NAA-cells) mg g DW^{-1}, respectively. Means of 4 experimental series are depicted.

very much the same for non-producing 2,4-D-cells and producing NAA-cells.

When the sugars inside the cells were analyzed, a number of differences were observed that might be connected with the differences in production between these two treatments (Hagendoorn *et al.* 1994a). The total amount of sugars is especially high in the producing, NAA-cells and remained at a rather stable level (at about 25% of the dry weight) for at least 3 weeks (Fig. 2). In 2,4-D-cells there is a drop in internal sugar during the first two weeks; at the end of the growth phase, total sugar level increases again from about 10 to almost 30%. At the end of the cultivation period, the exhaustion of sugar from the nutrient medium leads to starvation phenomena and decrease of internal sugar under both growth conditions.

The drop in sugar level in the 2,4-D-cells in the growth phase, when the cells need energy for cell division and growth, corresponds with a respiratory increase in these cells (up to 3–4 times the values found for NAA-cells; Hagendoorn *et al.* 1994a). The lack of large respiratory changes in the producing NAA-cells corresponds to the gradual, slow (but continuous) increase in cell number for these cells (Fig. 1). Not only is the cell division rate of the 2,4-D-cells higher, but these cells also need more energy for growth and maintenance because of differences in cell composition (Fig. 3) They contain more 'expensive' cell constituents (such as lipids and especially proteins) and less 'cheap' cell components like sugars, which prevail in NAA-cells; The anthraquinones, a main constituent of the latter cells are stored as glycosides making these compounds also rather 'cheap'.

Simultaneous growth and production?

Summarizing, one might argue that in the presence of NAA (or better in the absence of 2,4-D-like auxins) a kind of differentiation occurs, which transforms the cells in slowly dividing AQ-producing cells, although the cell suspension still consists of finely dispersed, separate cells.

This suggests that in *Morinda* cells two developmental programs can be switched on:

- with 2,4-D-type auxins, emphasis is laid on growth, with high cell division rate and high metabolic activity and no AQ-production;
- with NAA-type auxins, emphasis is laid on production, lower cell division and metabolic activity rates and high endogenous sugar levels.

This differentiation program cannot be maintained during a consecutive series of subculturings: generally the viability of the cells decreases with increasing AQ-contents leading to further decreases in cell division rate and finally cell death (Fig. 4). Anthraquinones in concentrations higher than 15% (on a dry weight basis) seem to be poisonous.

Apparently the original suggestion that in *Morinda* cell suspensions, comparable growth rates are possible with and without production, resulting in a very attractive model system (two nearly identical growth situations only differring in secondary metabolite production) is not true: In the presence of NAA-type aux-

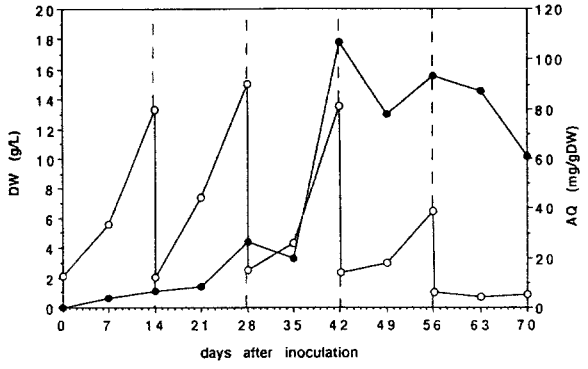

Fig. 4. Changes in dry weight production and anthraquinone content during cultivation of *Morinda citrifolia* cells for 5 consecutive subculturings in the presence of 1 mg l^{-1} NAA. Dry weight (open circles) is expressed as g l^{-1}, anthraquinone content (closed circles) as mg g DW^{-1}. The cells were transferred to fresh medium every two weeks (at day 14, 28... etc.). At t = 0 cells were inoculated from a 2,4-D-preculture.

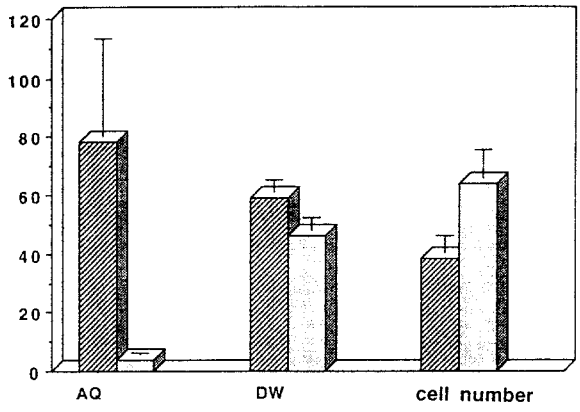

Fig. 5. Comparison of anthraquinone content, dry weight production and cell number of producing and non-producing cell cultures of *Morinda citrifolia*. Producing cell cultures were cultivated in the presence of 1 mg l^{-1} of NAA, IAA or pCP (hatched bars); non-producing cell cultures grew in the presence of 1 mg l^{-1} 2,4-D or pCPO (dotted bars). For both types of cell cultures, the values for the various parameters at the end of the growth period (after 15 days) were averaged. The cells were twice subcultured in the various medium types. Anthraquinone production (AQ) is expressed as mg g DW^{-1} (1 unit = 1 mg). Dry weight production is expressed as g DW l^{-1} (1 unit = 0.3 g). Cell number is expressed as U l^{-1} (1 unit = 10^8 cells).

ins a kind of developmental change occurs which leads to impaired growth in combination with differentiation into an AQ-producing cell type. Analysis of the growth of *Morinda citrifolia* cell suspensions in the presence of some other auxins, also indicates the existence of such a 'syndrome' of mutually related properties: The use of IAA and p-chlorophenyl acetic acid not only leads to AQ-production (just like the use of NAA) but also to lower cell numbers, and a relatively high dry weight production, while p-chlorophenoxy acetic acid prevented production, at the same time leading to high cell numbers and a relatively low dry weight production just like 2,4-D (Fig. 5).

Interaction of auxins that permit and inhibit AQ-production

The switch to anthraquinone production as part of the switch to the 'production state' occurs as a response to the inoculation in nutrient media containing NAA as an auxin instead of 2,4-D. The role of this NAA might be one of two sorts: in the first place the NAA-type auxin might have an active role and might be necessary for the induction of the AQ-synthesis pathway, e.g. by activating transcription. In the second place NAA might just have a 'permissive' role, allowing production (i.e. not inhibiting it) without having an active, activating role. The last situation seems to occur in the *Morinda* cell suspension system, as inoculation of cells in a nutrient medium without hormones appeared to induce AQ production (results not shown). Of course, such cells cannot be kept growing and viable for a series of subculturings, because the cells need an auxin for continuous growth.

Starting with non-producing 2,4-D-cells, both after inoculation in a medium with a 'permissive auxin' as well as after inoculation in medium without auxin, at least one subculturing is necessary before production becomes visible (Fig. 4). This inhibition is caused by the carry-over of 2,4-D present in the inoculum. A point that still has to be elucidated, is whether there are really 'permissive' and 'inhibiting' auxin types or whether it is just a question of concentration: In other words will low 2,4-D concentrations also permit production of AQ in *Morinda* cells? Zenk *et al.* (1975) stated that at none of the concentrations he tested, 2,4-D permitted accumulation of AQ.

Instead of the comparison of growth and production by cells growing in NAA- and in 2,4-D-medium, the study of the changes following the addition of production-inhibiting auxins (like 2,4-D and pCPO) to producing cells might be a better approach. When 2,4-D is added to producing (NAA-) cells, an almost instantaneous inhibition of the production is seen without a direct effect on the dry weight increase (Hagendoorn *et al.* 1994a, b). Further analysis of this inhibition showed that a significant difference in AQ-content between treated and non-treated cells develops between 10 and

24 h after addition. Already low concentrations of 2,4-D (as low as one tenth of the NAA-concentration) are able to exert this inhibitory effect. Soon after the secondary metabolism is shut off by this 2,4-D-addition, the cells regain all the characteristics of actively growing, 2,4-D-type cells (data not shown).

With regard to the question on the primary target of the inhibiting action of 2,4-D, there are two possible answers:

- a direct inhibition by 2,4-D-type auxins of one of the steps in the secondary metabolism, leading to the observed changes in the primary metabolism of the cells, or
- a stimulation of growth and cell division which affect the precursor supply for secondary metabolite production, leading indirectly to its switching off.

The contrast might be not as large as suggested here: a coordinated effect of 2,4-D on steps of both the primary and secondary metabolism might occur, resulting in a continuously controlled adjustment of these two aspects of plant cell metabolism.

In conclusion, study of the *Morinda* system shows that a close relation exists between primary and secondary metabolism. For a better understanding of the regulation of the secondary metabolite production in plant cells, further research into this relation is crucial.

References

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72: 248–254

De-Eknamkul W & Ellis BE (1985) Effects of auxins and cytokinins on growth and rosmarinic acid formation in cell suspension cultures of *Anchusa officinalis*. Plant Cell Rep. 4: 50–53

Gamborg OL, Miller RA & Ojima V (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158

Hagendoorn MJM, Traas TP, Boon JJ & Van der Plas LHW (1990) Orthovanadate induced lignin production, in batch and continuous cultures of *Petunia hybrida*. J. Plant Physiol. 137: 72–80

Hagendoorn MJM, Van der Plas LHW & Segers GJ (1994a) Accumulation of anthraquinones in *Morinda citrifolia* cell suspensions. Plant Cell Tissue Org. Cult. 38: 227–234

Hagendoorn MJM, Wagner AM, Segers G, Van der Plas LHW, Oostdam A & Van Walraven HS (1994b) Cytoplasmic acidification and secondary metabolite production in different plant cell suspensions. A comparative study. Plant Physiol. 106: 723–730

Hoekstra FA, Crowe LM & Crowe JH (1989) Differential desiccation sensitivity of corn and *Pennisetum* pollen linked to their sucrose content. Plant Cell Environ. 12: 83–91

Inoue K, Nayeshiro H, Inouye H & Zenk M (1981) Anthraquinones in cell suspension cultures of *Morinda citrifolia*. Phytochemistry 20: 1693–1700

Mokrasch LC (1954) Analysis of hexose phosphate and sugar mixtures with the anthrone reagent. J. Biol. Chem. 208: 55–59

Sato F & Yamada Y (1984) High berberine-producing cultures of *Coptis japonica* cells. Phytochemistry 23: 281–285

Ulbrich B, Wiesner W & Arens H (1985) Large-scale production of rosmarinic acid from plant cell cultures of *Coleus blumei* Benth. In: Neumann K-H, Barz W & Reinhard E (Eds) Primary and Secondary Metabolism of Plant Cell Cultures (pp 293–303). Springer-Verlag, Berlin, Heidelberg

Zenk MH, El-Shagi H & Schulte U (1975) Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. Planta Med. Suppl. 79–101