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Cytoplasmic Acidification and Secondary Metabolite Production in Different Plant Cell Suspensions[1]

A Comparative Study

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In this study, a correlation is described between low cytoplasmic pH, measured with the fluorescent probes 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (acetoxymethyl ester) and bis-[3-propyl-5-oxoisoxazol-4-yl]pentamethine oxonol, and the production of secondary metabolites for several plant cell-suspension systems. Anthraquinone production in *Morinda citrifolia* suspensions is negligible in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D), whereas with naphthalene acetic acid (NAA) a significant accumulation is realized. NAA-grown cells showed a lower cytoplasmic pH than did 2,4-D-grown cells. Addition of 2,4-D or parachlorophenoxy acetic acid to NAA-grown cells resulted in an inhibition of anthraquinone production and an increase of the cytoplasmic pH, whereas addition of parachlorophenyl acetic acid had no effect on either parameter. Lignin production in *Petunia hybrida* cells could be induced by subculturing them in a medium without iron. These cells showed a lower cytoplasmic pH than control cells. Addition of Fe^{3+} led to a decreased lignin content and an increased cytoplasmic pH. Two cell lines of *Linum flavum* showed a different level of coniferin and lignin concentration in their cells. Cells that accumulated coniferin and lignin had a lower cytoplasmic pH than cells that did not accumulate these secondary metabolites. Apparently, in different species and after different kinds of treatment there is a correlation between acidification of the cytoplasm and the production of different secondary metabolites. The possible role of this acidification in secondary metabolite production is discussed.

Some plant tissues are able to produce substantial amounts of secondary metabolites. However, the production of these compounds in plant cell and tissue cultures is generally low. A number of treatments have been described that lead to an increased production; these treatments often stimulate the defense response in the cell (Scheel and Parker, 1990).

In a previous paper (Hagendoorn et al., 1991a), we analyzed the effect of elicitors on the plasma membrane of *Petunia hybrida* cell suspensions. It appeared that changes in the activity of the plasma membrane ATPase, and subsequently of ΔpH, were sufficient to increase PAL activity and product formation in these cells. This conclusion was based on two different experimental approaches. First, we tested a number of plasma membrane ATPase inhibitors and ionophores that specifically influence ΔpH, ΔΨ, or both for their ability to activate secondary metabolism. Second, we directly showed changes in cytoplasmic pH upon activation of a secondary metabolic pathway with the aid of the fluorescent probe oxonol VI.

In this paper we show that this relation between low cytoplasmic pH and secondary metabolite production is not limited to *P. hybrida* and the products of the PPP but can also be observed in different model systems. We investigated the production of secondary metabolites by cell suspensions derived from three plant species belonging to three different families: *Morinda citrifolia*, a member of the Rubiaceae, *P. hybrida*, a solanaceaeus species, and *Linum flavum*, belonging to the Linaceae. In *Petunia* and *Linum* the PPP yielding lignin (in both species) and coniferin (in *Linum*) was studied (Hagendoorn et al., 1990, 1991b; Oostdam and van der Plas, 1992). The production of anthraquinones, a secondary metabolite derived from the shikimate pathway, could be switched on in *Morinda* cell suspensions (Zenk et al., 1975; Hagendoorn et al., 1994).

The cytoplasmic pH was measured using the fluorescent probes BCECF and oxonol VI. A correlation between secondary metabolite production and a low cytoplasmic pH was observed for all three species and for both secondary meta-

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Abbreviations: BCECF(-AM), 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (-acetoxymethyl ester); ΔpH, pH gradient; ΔΨ, membrane potential; LFH, *Linum flavum* cell culture initiated from hypocotyl; LFL, *Linum flavum* cell culture initiated from leaf; NAA, naphthalene acetic acid; oxonol VI, bis-[3-propyl-5-oxoisoxazol-4-yl] pentamethine oxonol; PAL, phenylalanine ammonia lyase; pCP, parachlorophenyl acetic acid; pCPO, parachlorophenoxy acetic acid; PPP, phenyl propanoid pathway; RMF, Rosy Morn fertile.

bolic pathways. The significance of these changes for secondary metabolite production will be discussed.

MATERIALS AND METHODS

Chemicals

Oxonol VI was synthesized and kindly donated by Dr. W. Hanstein (Ruhr-Universität Bochum, Germany). BCECF-AM was purchased from Molecular Probes (Eugene, OR). The auxins NAA, 2,4-D, and pCP were purchased from Fluka, and pCPO was from Merck. Stock solutions of oxonol VI (1 mM) and BCECF-AM (1 mg/mL) were prepared in ethanol and dry DMSO, respectively. The auxins were dissolved in water (100 mg/mL) and adjusted to pH 5 to 5.5.

Plant Material and 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 Suc (40 g/L), kinetin (0.2 mg/L), and auxin (1 mg/L 2,4-D or NAA). The cells were subcultured every 14 d (10 mL of culture transferred to 50 mL of fresh medium). The *Petunia hybrida* cells were maintained in 250-mL Erlenmeyer flasks containing 60 mL of Murashige-Skoog medium (Murashige and Skoog, 1962), supplemented with 2 mg/L NAA and 30 g/L Glc, in the absence or presence of 0.1 mM FeNaEDTA. The cells were subcultured every 10 d (10 mL of culture transferred to 50 mL of fresh medium). The *Linum flavum* batch cultures were maintained in 250-mL Erlenmeyer flasks containing 75 mL of medium (Murashige-Skoog salts) supplemented with B5 vitamins (Gamborg et al., 1968), 30 g/L Suc, and 3 mg/L NAA. The cells were subcultured every 14 d (25 mL of culture transferred to 50 mL of fresh medium). Continuous cultures of *L. flavum* were cultivated as described by Oostdam and van der Plas (1992). All batch 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 the cells were harvested on a paper filter on a Büchner funnel. For determination of dry weight the cells were dried at 60°C for 24 h.

Determination of PAL Activity and Lignin Content

Determination of the lignin content and the PAL activity of the *P. hybrida* and *L. flavum* cells was as described earlier (Hagendoorn et al., 1990).

Anthraquinone Determination

M. citrifolia cells (0.02–0.2 g fresh weight) were boiled in 5 mL of 80% aqueous ethanol for 10 min. After the sample was centrifuged (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).

Coniferin Determination

Coniferin was extracted according to the method of van Uden et al. (1990) and determined according to the method of Oostdam and van der Plas (1992).

Fluorescence Assays for Cytoplasmic pH

Fluorescence assays were carried out at 25°C with undiluted cultured cells. To prevent pH changes in the very weakly buffered suspensions, fluorescence assays were carried out immediately after taking the suspension cultures from the incubator. Anaerobiosis was avoided by shaking the cell suspensions at 100 rpm (before the measurements) and by stirring the solution (during the measurements).

Oxonol VI fluorescence intensities at set wavelengths were followed with an Oriel 3090 front face fluorimeter using a stirred thermostated multipurpose cuvette (Kraayenhof et al., 1982). This fluorimeter has the advantage that excitation and emission light have a minimal optical pathlength because excitation and emission take place at the surface of the sample. Therefore, it is useful for measurements in dense cell suspensions or suspensions containing large aggregates. Immediately before measurement oxonol VI was added to a final concentration of 0.5 μM to the cell suspension in the cuvette.

Fluorescence spectra (both BCECF and oxonol VI) were recorded on an SLM-Aminco SPF-500 spectrofluorimeter. BCECF-AM was added to the cell culture at a concentration of 1 μM. This probe shows fluorescence only after uptake into the cells and transformation into BCECF (Haugland, 1992). Loading of the probe and transformation to BCECF was performed on a gyratory shaker (100 rpm) at 25°C and was monitored by taking fluorescence spectra of a sample of the cell suspension every 5 to 10 min until fluorescence no longer increased (after about 1 h). BCECF fluorescence data are presented as excitation spectra and as the ratio of fluorescence excitation at 490 and 439 nm. In the latter case, a number of variables that perturb measurements, such as nonuniform dye concentration, dye bleaching or leakage, and differences in cell density (Haugland, 1992), have been eliminated. In some cases, 450 nm was used instead of 439 nm to determine the ratio, leading to similar results. Fluorescence micrographs were made with a Nikon Labophot microscope, ex. 470–490/DM 510/BA 515 EF.

RESULTS

Localization of Fluorescence Probes as Indicators for Cytoplasmic pH in Cell Suspensions

BCECF-AM is a membrane-permeable derivative of BCECF and has no fluorescence when excited at 490 nm. This nonfluorescent derivative must be hydrolyzed by intracellular esterases yielding impermeable BCECF, which is the fluorescent pH indicator. We have determined the localization of BCECF in *P. hybrida* RMF cell suspensions with a fluorescence microscope (Fig. 1). Only the cytoplasm showed clear fluo-

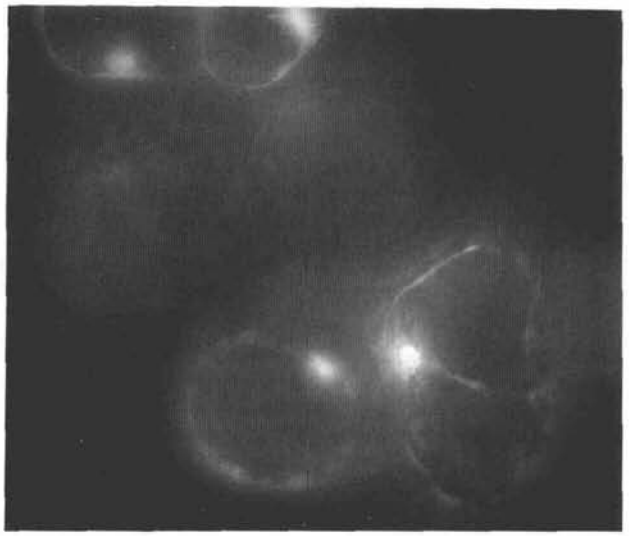

Figure 1. Fluorescence micrograph of *P. hybrida* RMF cells. The cells were loaded with BCECF-AM as described in "Materials and Methods" and observed under a fluorescence microscope.

rescence upon blue light excitation. The same observation was made for *M. citrifolia* cells grown in the presence of 2,4-D (not shown). Experiments indicating that fluorescence changes of oxonol VI represent changes in cytoplasmic pH of plant cells were described by Hagendoorn et al. (1991a).

Anthraquinone Production in *M. citrifolia*

Anthraquinone production by *M. citrifolia* cell suspensions is largely dependent on the type of auxin present in the medium: production in the presence of 2,4-D is negligible, whereas a significant anthraquinone accumulation can be reached with 1 mg/L NAA as the sole auxin present (Fig. 2). Addition of 1 mg/L 2,4-D to cells growing in the presence of NAA led to inhibition of anthraquinone production (Fig. 2).

We studied the cytoplasmic pH in *M. citrifolia* cells, growing in the presence of NAA and/or 2,4-D with two fluorescent pH probes: oxonol VI and BCECF. The BCECF excitation spectrum of 2,4-D-grown cells (Fig. 3A) showed a marked difference from the spectrum of NAA-grown cells. The fluorescence of 2,4-D-grown cells was significantly higher than that of NAA-grown cells, thus indicating a more alkaline cytoplasmic pH. This is confirmed by the ratio of BCECF fluorescence excitation (490/450 nm), which is on average 3.61 ± 0.12 and 1.83 ± 0.19 ($n = 6$) in the case of 2,4-D- and NAA-grown cells, respectively. The oxonol VI emission spectra of both types of cells showed similar differences, thus confirming the BCECF results (Fig. 3B).

To be sure that the changes in fluorescence are indeed the consequence of pH changes and not caused by chemical changes of the probes, we tested whether the fluorescence changes could be influenced by addition of acid or base. Figure 4 shows that the BCECF fluorescence in *Morinda* cells grown in the presence of NAA is not significantly changed

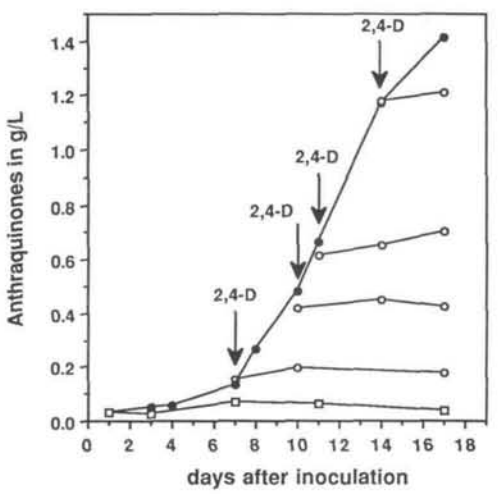

Figure 2. Time course of anthraquinone accumulation of *M. citrifolia* cell suspensions grown in the presence of 1 mg/L NAA (closed circles) and after addition of 2,4-D (final concentration 1 mg/L, open circles). Open squares represent the anthraquinone content of cell suspensions continuously grown in the presence of 2,4-D. Means of duplicate measurements are depicted. Anthraquinone concentration is expressed in g/L cell culture.

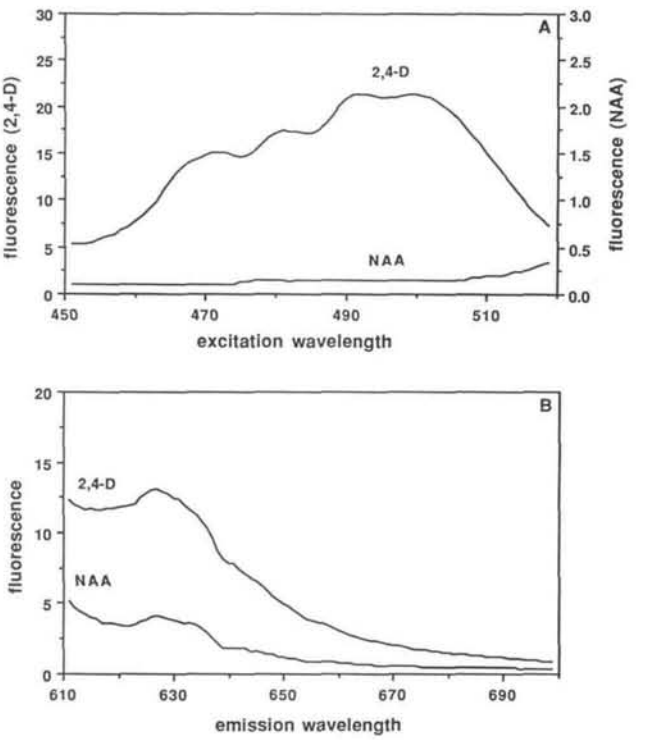

Figure 3. A, Fluorescence excitation spectra of BCECF loaded into *M. citrifolia* cells grown in the presence of NAA or 2,4-D. The emission wavelength was set at 535 nm. Fluorescence was expressed in arbitrary units. Cells were used 3 d after inoculation. B, Fluorescence emission spectra of oxonol VI added to *M. citrifolia* cells grown in the presence of NAA or 2,4-D. The excitation wavelength was set at 594 nm. Fluorescence was expressed in arbitrary units. Cells were used 4 d after inoculation.

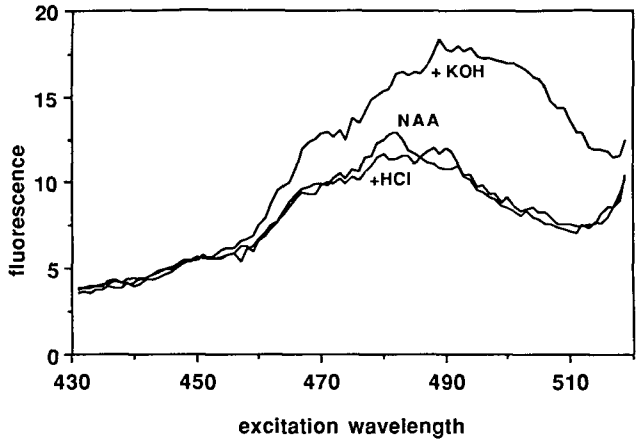

Figure 4. Effect of HCl and KOH addition on the fluorescence excitation spectra of BCECF loaded into *M. citrifolia* cells grown in the presence of NAA (see Fig. 3 for conditions). First, a BCECF-loaded cell suspension without acid or base addition was used (NAA); thereafter, 0.4 mM HCl was added (+HCl) and subsequently 1.6 mM KOH was added (+KOH). HCl and KOH were added immediately before the respective measurements.

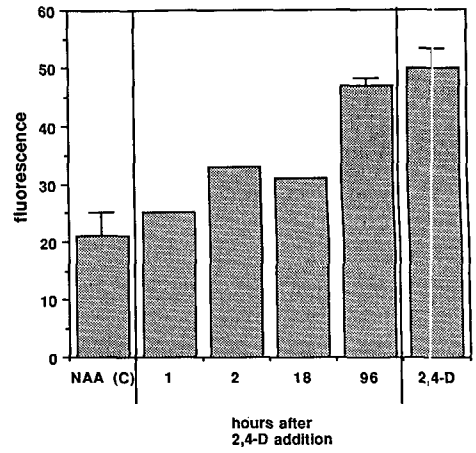

Figure 5. The fluorescence level of oxonol VI (arbitrary units) added to *M. citrifolia* cells. Cells were grown in the presence of NAA or 2,4-D; also NAA cells were used at different times after addition of 2,4-D (2.5 mg/L). The excitation and emission wavelengths were set at 556 and 654 nm, respectively. Means of three to four independent experiments are shown. 2,4-D was added to NAA cells 3 d after inoculation. NAA and 2,4-D cells were measured at different times between 3 and 7 d after inoculation.

by HCl addition, indicating an already very low internal pH. Subsequent addition of KOH substantially increased the fluorescence to the level of cells grown with 2,4-D. On the other hand, HCl addition to cells grown in the presence of 2,4-D decreased the BCECF fluorescence, indicating a relatively high initial cytoplasmic pH (not shown). Fluorescence spectra of oxonol VI in plant cells upon addition of acid and base were given by Hagendoorn et al. (1991a).

Figure 5 shows the oxonol VI fluorescence of *M. citrifolia* cells at a fixed excitation and emission wavelength; the fluorescence of 2,4-D-grown cells is approximately 2.5 times higher than that of NAA-grown cells. After addition of 2,4-D to NAA-grown cells, the initially low fluorescence increased during the first hours, and within 4 d the fluorescence had reached the level of 2,4-D-grown cells.

In *M. citrifolia* two other auxins were tested for their ability to sustain or inhibit anthraquinone production. In Table I it is shown that addition of pCP to NAA-grown cells does not lead to a large reduction in anthraquinone accumulation. The low oxonol VI fluorescence under these conditions indicates a low cytoplasmic pH. The same conclusion can be drawn based on the low BCECF fluorescence (Fig. 6) and the low 490/439 nm excitation ratio (Table I). Addition of pCPO to NAA cells has a similar effect as 2,4-D addition, i.e. low anthraquinone production, high oxonol VI, and BCECF fluorescence (Table I; Fig. 6).

Lignin Production in *P. hybrida*

When *P. hybrida* RMF cells were transferred to medium without Fe^{3+} (−Fe cells) the growth of the cells was not significantly changed in comparison with the growth of control cells (+Fe cells). A subsequent second subculturing into medium without iron, however, resulted in a decrease in growth rate (Fig. 7A). After a third consecutive transfer to such a medium, growth almost stopped completely. Transfer of −Fe cells to Fe^{3+}-containing medium (−Fe/+Fe, Fig. 7A) resulted in a rapid return to normal growth rates.

The first transfer of +Fe cells to medium without iron led to an immediate induction of lignin synthesis, and, within a week, a significant lignin accumulation was observed (Fig. 7B). The lignin production continued after the next subculturing. After transfer to Fe^{3+}-containing medium, the lignin content decreased again (Fig. 7B).

Using oxonol VI, we compared the cytoplasmic pH of −Fe cells during lignin production with that of control cells (+Fe). Figure 8 shows emission spectra from −Fe cells, control (+Fe) cells, and −Fe cells 1 d after Fe^{3+} addition (−Fe/+Fe). −Fe cells showed a lower fluorescence than control cells and −Fe cells after Fe^{3+} readdition. BCECF fluorescence spectra could not be determined because of the presence of large aggregates in the −Fe cells.

Table II summarizes some data for *Petunia* cells growing in

Table I. *The effect of different auxins on anthraquinone production, oxonol VI fluorescence emission, and corrected BCECF excitation of M. citrifolia cells grown in the presence of NAA*

The extra auxins were added 4 d before the measurements in a concentration of 2.5 mg/L to NAA cells 3 d after inoculation. Means of four measurements are shown. See Figure 4 for original BCECF excitation spectra. Oxonol VI (excitation, 556 nm; emission, 654 nm), BCECF (excitation, 439 and 490 nm; emission, 535 nm).

Added Auxin	Anthraquinone Production	Oxonol VI Fluorescence	BCECF Ratio (490/439 nm)
	mg/g dry wt	*arbitrary units*	
	52.7 ± 2.4	33 ± 3	2.33
pCP	46.0 ± 4.9	23 ± 4	2.17
pCPO	10.1 ± 0.49	46 ± 5	2.79
2,4-D	10.1 ± 0.24	47 ± 1	3.00

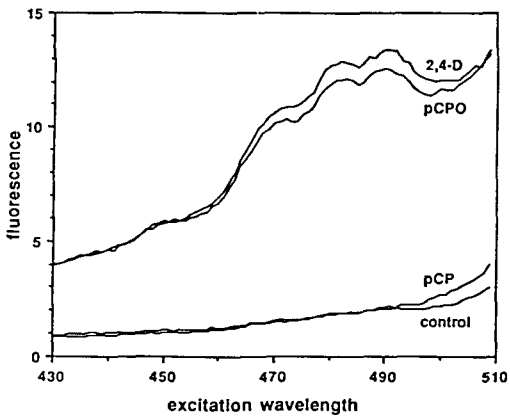

Figure 6. Fluorescence excitation spectra of BCECF, loaded into *M. citrifolia* cells grown in the presence of NAA, 4 d after addition of the auxins 2,4-D, pCPO, and pCP (all 2.5 mg/L). The emission wavelength was set at 535 nm. Fluorescence was expressed in arbitrary units. Before additions NAA cells were 3 d old.

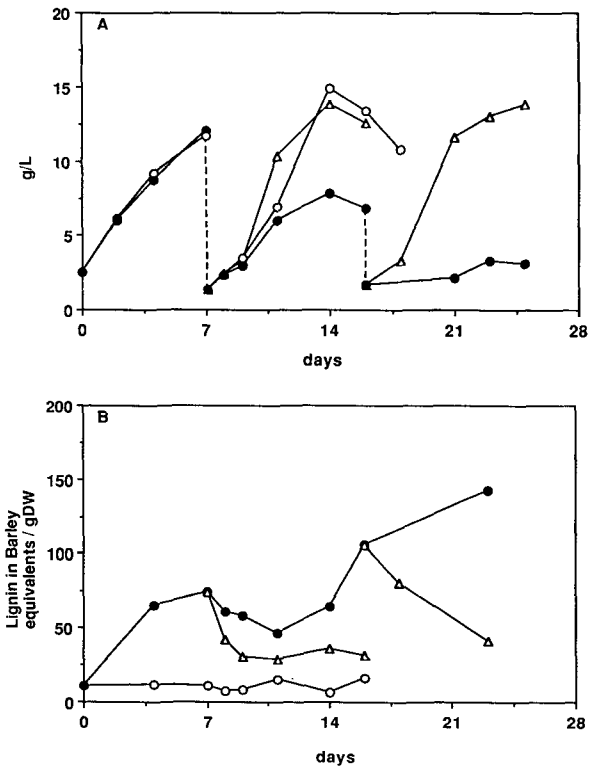

Figure 7. A, Time course of growth of *P. hybrida* cell cultures in the presence of Fe^{3+} (0.1 mM, open circles), subcultured without Fe^{3+} (closed circles), and after readdition of Fe^{3+} (0.1 mM, open triangles). Growth is expressed in g dry weight (DW)/L cell culture. Points represent means of two batch cultures. B, Time course of lignin production of *P. hybrida* cell cultures in the presence of Fe^{3+} (0.1 mM, open circles), subcultured without Fe^{3+} (closed circles), and subsequently subcultured with Fe^{3+} (0.1 mM, open triangles). Points represent means of two batch cultures. Lignin is expressed in g barley equivalents/g dry weight (DW). First inoculation in medium without iron was on d 0. Iron readdition/subculturing occurred on d 7 and 16.

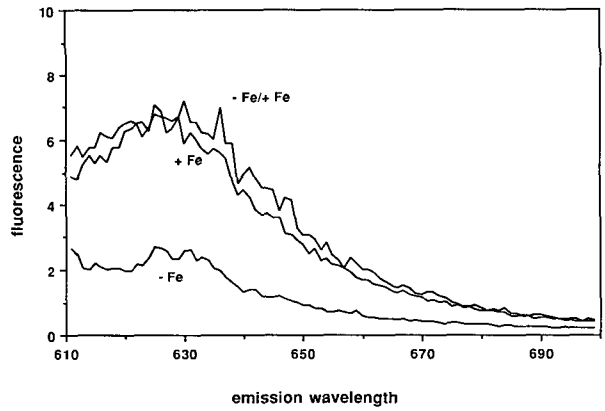

Figure 8. Fluorescence emission spectra of oxonol VI added to *P. hybrida* cell cultures in the presence of Fe^{3+} (+Fe), subcultured without Fe^{3+} (−Fe), and after readdition of Fe^{3+} (−Fe/+Fe). The excitation wavelength was set at 594 nm. Fluorescence was expressed in arbitrary units. Cells 8 d after the start of the experiment (Fig. 7) were used.

the presence and absence of iron. Both the lignin content and the activity of PAL, the first enzyme of the PPP leading to lignin synthesis, were significantly increased in −Fe cells, whereas both decreased again after the readdition of Fe^{3+} to these cells. The oxonol VI fluorescence of −Fe cells was significantly lower than the fluorescence of control cells and −Fe/+Fe cells but could be regenerated upon addition of KOH (not shown).

Coniferin and Lignin Production in *L. flavum*

In Table III, two different *L. flavum* cell lines are compared with respect to their PAL activity, the production of coniferin and lignin, and the oxonol VI fluorescence. The cell line LFL showed an enhanced PAL activity and an increased content of lignin and coniferin both during batch and during continuous culture when compared to the cell line LFH. LFL cells showed a lower level of oxonol VI fluorescence, indicating a lower cytoplasmic pH. The oxonol VI spectra of all three cultures showed that the emission maximum of the LFH culture was approximately twice as high as the maximum of both LFL cultures (results not shown). No BCECF fluores-

Table II. *Effect of the presence of Fe^{3+} during culturing of P. hybrida cells on PAL activity, lignin production, and oxonol VI fluorescence emission, measured 8 d after the start of the experiment (see Fig. 7)*

Means of four measurements are shown. For oxonol VI excitation is 556 nm and emission is 654 nm.

	PAL Activity	Lignin	Oxonol VI
	μKat/kg protein	*mg BLE[a]/g dry wt*	*arbitrary units*
+Fe	5.2 ± 2.5	6.8 ± 3.4	63 ± 1
−Fe	38 ± 1.4	60 ± 2.5	24 ± 4
−Fe/+Fe	19 ± 3.1	43 ± 1.4	73 ± 8

[a] Barley lignin equivalents.

Table III. *PAL activity, coniferin and lignin production, and oxonol VI fluorescence emission in LFL and LFH*

Cells from batch cultures were 5 d old. Two representative experiments performed in duplicate are shown. For Oxonol VI, excitation is 556 nm and emission is 654 nm.

Cell Line	PAL Activity	Coniferin	Lignin	Oxonol VI
	μKat/kg protein	*mg/g dry wt*	*mg BLE[a]/g dry wt*	*arbitrary units*
LFL (batch)	49	38	121	37
LFH (batch)	2	3.3	7	79
LFL (continuous)	20	99	263	21

[a] Barley lignin equivalents.

cence spectra could be obtained because of the presence of large aggregates in the LFH culture.

DISCUSSION

Measurement of Cytoplasmic pH

Since any conclusion from this study critically depends on the method used for measuring cytoplasmic pH, it is necessary to discuss these methods in more detail first. Oxonol VI was originally developed as a $\Delta\Psi$ probe (Haugland, 1992). However, in a previous paper (Hagendoorn et al., 1991a) we showed that oxonol VI also can be used as a pH probe because its fluorescence level is highly dependent on the pH with a critical range below pH 6 (Smith et al., 1976). At lower pH values, oxonol VI shifts from the fluorescent anionic form to the poorly soluble neutral form. As a consequence, a decrease in pH results in a decreased fluorescence. Hagendoorn et al. (1991a) compared oxonol VI with nonpermeating fluorescent pH indicators and described the effect of the ionophore nigericin on oxonol VI fluorescence. Based on these experiments it was concluded that oxonol VI responds to intracellular events and, therefore, can be used for the measurement of changes in cytoplasmic pH in plant cells. Oxonol VI in combination with the Oriel front face fluorimeter can be used for samples containing large aggregates.

For comparison we also used BCECF, a widely used pH probe of approximately pH 7 (Haugland, 1992; Sakano et al., 1992). This probe shows fluorescence only after hydrolysis of the AM bond by intracellular esterases. As a consequence its fluorescence reflects changes in the intracellular environment. A disadvantage is that loading of the probe in the AM form takes about 1 h. The efficiency of loading is not the same for various types of plant cells. To correct for the resulting differences in concentration of cytoplasmic BCECF, the ratio between the excitation maximum (here 490 nm) and the isosbestic point (439 nm) was calculated (Haugland, 1992). Sakano et al. (1992) extensively studied the use of BCECF-AM in *Catharanthus roseus* cells; from their paper it appeared that BCECF specifically accumulates in the cytoplasm (90% of total BCECF). This was confirmed by our own observations (Fig. 1). As with oxonol VI, any BCECF present in the medium or the vacuole does not fluoresce at low pH values (Sakano et al., 1992). BCECF fluorescence cannot be measured in cell cultures containing large aggregates because of scattering. However, for *M. citrifolia*, in which both BCECF and oxonol VI could be used, both probes show a similar change in cytoplasmic pH. Both probes are very sensitive in that the differences in fluorescence are large and both probes react rapidly. Calibration of the probes with nigericin to obtain actual pH values is not readily possible in plant cells (Sakano et al., 1992). In our experiments, therefore, we have focused on qualitative changes in cytoplasmic pH rather than the actual pH value of the cytoplasm.

Cytoplasmic Acidification and Secondary Metabolite Production

Cytoplasmic acidification upon activation of a defense response by a glucan elicitor has also been observed in *Phaseolus vulgaris* cells (Ojalvo et al., 1987) and in parsley cells by a *Phytophthora* elicitor (Kneusel et al., 1989), whereas pH decreases in the total cell sap of tobacco cells were detected after treatment with *Pseudomonas* sp. (Atkinson et al., 1985). In contrast, Horn et al. (1992) could not observe cytoplasmic pH changes (larger than the scattering, approximately 0.1 pH) within 30 min after polygalacturonic acid was added as an elicitor to soybean cells. Most studies have been performed with ^{31}P-NMR. With ^{31}P-NMR actual pH values in the plant cell compartments can be obtained. A disadvantage, however, is that the method is relatively slow and not very sensitive.

The suggestion (Horn et al., 1992) that cytoplasmic acidification is a side effect of elicitor treatment itself and not related to the production of secondary metabolites does not seem very likely because the cytoplasmic pH remains low as long as production occurs for all of the systems investigated by us and increases again when production stops. The same applies to the phaseollin-producing *P. vulgaris* cells of Ojalvo et al. (1987). In *L. flavum* cells, in which production depends solely on the cell line under observation and no external stimulus is needed, a lower cytoplasmic pH was also observed for the producing cells (Table III). The importance of a low cytoplasmic pH for the production of secondary metabolites also appeared from experiments in a previous paper (Hagendoorn et al., 1991a), in which we showed that addition of the ionophore nigericin resulted in both cytoplasmic acidification and direct activation of the secondary metabolism (lignin production in *Petunia* cells).

Whether the observed decrease of cytoplasmic pH is localized, i.e. limited to certain regions at the periphery of the cytoplasm near to the plasma membrane (cf. Roos, 1992)

where oxonol VI is thought to accumulate, or occurs uniformly throughout the cytoplasm cannot be concluded from our measurements. As is the case for ^{31}P-NMR measurements the fluorescence methodology gives an "average" value of the cytoplasmic pH.

Auxins, Cytoplasmic Acidification, and Anthraquinone Production in *M. citrifolia*

This study shows that the effect of auxins very much depends on the type of auxin used: the production of anthraquinones in *M. citrifolia* is induced by NAA and inhibited by 2,4-D. Growth in the presence of NAA decreases cytoplasmic pH in *Morinda* cells, whereas 2,4-D addition leads to an increase in cytoplasmic pH. In the case of *Morinda*, Zenk et al. (1975) have shown that the effect of NAA is concentration dependent; high concentrations hamper production. In our experiments with high NAA concentrations, a concomitant alkalinization of the cytoplasm could be observed (H.S. van Walraven and M.J.M. Hagendoorn, unpublished results). In line with these results, a low concentration of NAA in the medium at the time of elicitation enhanced lignin production of *P. hybrida* Violet 30 cell suspensions (Hagendoorn et al., 1991b). This coincides with a low cytoplasmic pH. Increased NAA concentration led to a decrease in product formation and an increase in cytoplasmic pH (H.S. van Walraven and M.J.M. Hagendoorn, unpublished results).

Iron Deprivation, Cytoplasmic Acidification, and Lignin Production

The production of lignin in −Fe cells was not expected because peroxidases involved in lignin synthesis need iron because they possess an iron-containing heme group. Lignin production was observed during the first subculturing without Fe^{3+} chelate when there was still iron enough left in the inoculum for unaffected growth. Apparently, there is no direct relation between the lack of iron and the activation of lignin synthesis. A number of stress treatments proved to be able to induce lignin synthesis, e.g. short treatment with orthovanadate and (extreme) dilution (Hagendoorn et al., 1991a, 1991b). Also, with these stress treatments lignin production appeared to be possible without a decline in growth rate.

A number of plants possess an iron-efficient uptake system, a so-called "turbo" system (Bienfait, 1988), that reduces Fe^{3+} chelates to provide Fe^{2+} that is taken up in the cell. At the same time protons are excreted into the medium. For soybean suspension cells it has been suggested that this turbo system is always active (Cornett and Johnson, 1991), a situation that also might occur in the *P. hybrida* cells. When iron was omitted from the medium, alkalinization of the culture medium was observed (H.S. van Walraven and A.M. Wagner, unpublished results). This can be explained by the fact that Fe^{3+} chelate is no longer reduced at the plasma membrane, thereby reducing proton extrusion and creating a more alkaline medium pH.

Physiological Role of Cytoplasmic Acidification

Our results show for three plant species and for two different secondary metabolic pathways that there is a correlation between production of secondary metabolites and low cytoplasmic pH. This indicates a relation between low pH in the cytoplasm and active synthesis of secondary metabolites, but a correlation gives no clues with regard to the physiological significance of such an acidification. Previously, we suggested a role for protons (i.e. low cytoplasmic pH) in the signal transduction chain leading from the elicitor treatment to the production of secondary metabolites (Hagendoorn et al., 1991a). Our present results and those of others (Kneusel et al., 1989) suggest that cytoplasmic acidification is probably not serving as a "second messenger." First, decrease in cytoplasmic pH is not a transient effect as would be expected for a second messenger, but the pH remains low as long as production occurs. Second, cells that do not need an external stimulus for production, such as the *Linum* LFL culture (Table III), also show a lower cytoplasmic pH than nonproducing *Linum* cultures. There is no direct correlation with the changes in medium pH upon the various treatments: sometimes internal acidification is accompanied by external alkalinization (e.g. *Linum, Petunia*), whereas in other cases (e.g. *Morinda*) both the cytoplasmic and the medium pH decrease (H.S. van Walraven and M.J.M. Hagendoorn, unpublished results).

Therefore, we suggest that cytoplasmic acidification is not necessarily a prerequisite for the induction of secondary metabolite production but might be part of a metabolic shift that accompanies this production. As long as a significant production persists, the cytoplasmic pH is low, whereas decreases in the production rate are accompanied by an increase in pH, independent of the plant species and the nature of the secondary product. One of the processes that might be connected with this low cytoplasmic pH is the active transport (using H^+-antiport systems) of secondary metabolites from their site of synthesis to another cell compartment, e.g. from the cytoplasm to the vacuole (Blom et al., 1991, and refs. therein).

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