Comparison of the effect of different fungal elicitors on *Rubia tinctorum* L. suspension culture

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Abstract

The effect of two elicitor types prepared from three different fungi on the alizarin content and the ultrastructure of *Rubia tinctorum* cells was studied. The *de novo* alizarin synthesis took place predominantly during the first day of treatment then it was followed by a constant release of alizarin into the medium. The alkalinization of the medium was similar in every treatment. The number of the living cells did not changed during 24-h elicitor treatments, but it decreased significantly after 96 h. The appearance of vacuolar bodies, and the change of the plasmalemmasome structure from vesiculo-reticular to reticulo-lamellar were the most typical morphological syndromes due to elicitation. *Phytium* elicitor proved to be the least effective, and *Botrytis* elicitor seemed to be the most effective.

Additional key words: alizarin, defence response, fluorescence, madder, ultrastructure.

Introduction

Madder, *Rubia tinctorum* L. (*Rubiaceae*), is a perennial herb with bright red fibrous roots containing alizarin. The phytopathogenous fungi *Verticillium dahliae* Klebahn, *Botrytis cinerea* Persoon and *Pythium debaryanum* Hesse are non-host-specific pathogens colonising several plant species which cause wilting, "damping off", and wet rot of the invaded plants (Hudson 1986). Both the crude homogenate of liquid cultures and the purified cell wall material of these fungi are able to elicit plant defence responses (Nádaská *et al.* 1994, Suvarnalatha *et al.* 1994, Brady and Fry 1997) including increased production of reactive oxygen species (Kauss and Jeblick 1995, Bestwick *et al.* 1997), changes in enzyme activities (Małolepsza and Urbanek 1994, Bohland *et al.* 1997, Chen *et al.* 1997), cell wall reinforcement (Brady and Fry 1997), elevated secondary metabolite content (Dörnenburg and Knorr 1994), gene expression (Doares *et al.* 1995, Niki *et al.* 1998) and hypersensitive response (Bennett *et al.* 1996). These processes are strictly

regulated and the spectrum and the intensity of single events and the timing of their appearance is characteristic (Dixon and Paiva 1995). Beside the huge number of available physiological and biochemical data on elicited plant materials a limited number of papers concentrate on the structural changes appearing in elicited cell cultures (Eilert and Constabel 1985, Eilert *et al.* 1987).

Some aspects of the defence response to non-host-specific elicitors like oligogalacturonides, fungal polysaccharides, oligoglucosides or chitosan (DiCosmo and Misawa 1985, Dörnenburg and Knorr 1994, Doares *et al.* 1995, Hammond-Kossack and Jones 1996) can be studied in plant cell cultures. The induced synthesis and storage of phytoalexins around the infection site as well as in other organs via long term signalling is a part of the defence mechanism.

The alizarin is produced both in intact plants (Kączkowski 1985) and their tissue cultures (Kurosaki *et al.* 1992). The production was expected to increase by

elicitors as in the case of many other secondary metabolites (DiCosmo and Misawa 1985, Furze et al. 1991, Kurosaki et al. 1992, Suvarnalatha et al. 1994, Nádaská et al. 1994, Moreno et al. 1994, Dixon and Paiva 1995). Alizarin is believed to be stored in the vacuoles but the release of secondary metabolites into the solid or liquid medium was also shown (DiIorio et al. 1993). The differentiating cells of suspension cultures show enhanced capability for defence responses and are optimal objects for initiation of changes in the secondary metabolism caused by elicitation (Komamine et al. 1989,

Dixon and Lamb 1990, Hammond-Kossack and John 1996).

In the experiments reported here, R. tinctorum suspension culture was used as a model system, in which the synthesis of alizarin stimulated by fungal elicitors (crude culture homogenate and purified cell wall material) was monitored. The reproducible induction and the easy detection of this plant response allowed us to make a comparison between the effects of the different elicitors as well as to study their influence on the physiological state and the structural characteristics of the cultured R. tinctorum cells.

Materials and methods

Plant and fungal cultures: The intact madder (*Rubia tinctorum* L.) plants were supplied by the Botanical Garden of the Research Institute of Medical Plants (Hungary). The callus tissues were initiated from roots on MS medium solidified with 0.6 % agar, the cell lines were selected on the base of their growth and alizarin content. The suspension cultures used in our experiments originated from the RF1-413 strain and were grown on MS liquid medium at room temperature and at natural lighting. The suspension cultures were shaken on a rotary shaker (125 rpm) and were subcultured every 14 d. In the preliminary experiments we found that the cell division reached its maximum between the 7th and the 14th day, than this period was followed by a stationary phase (Fig. 1). During this phase the differentiation became predominant. The beginning of this stationary phase has been proved to be optimal for elicitor treatments (Komamine et al. 1989).
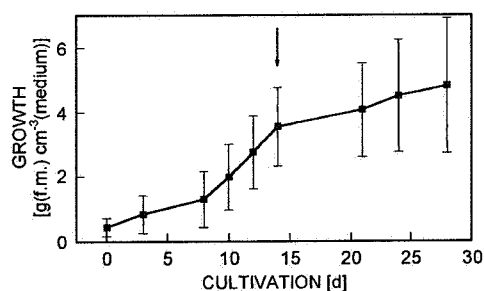

Fig. 1. Growth of RF1-413 strain of *Rubia tinctorum* in cell suspension culture (means ± SE, $n = 3$). *Arrow* indicates the beginning of the stationary phase when the elicitor was added.

The phytopathogenous fungi used were purchased from the Department of Cell and Molecular Biology of Drugs, Comenius University, Bratislava, Slovakia (*B. cinerea*, *V. dahliae*) and the Institute of Plant Protection, Hungarian Academy of Science, Budapest, Hungary (*P. debaryanum*). The fungi were stored on 3 % malt medium solidified with 2 % agar. For elicitor preparation, all fungi were grown on 2,4 D free B5 liquid medium (Gamborg et al. 1968) on a gyratory shaker (125 rpm) at room temperature and subcultured weekly and grown.

Elicitors preparation: The 7-d-old fungal cultures were autoclaved at 121 °C for 20 min. After cooling, the mycelium was homogenised in culture medium in a blender for 10 min. The obtained crude homogenates was refrigerated (-20 °C) until further use.

The fungal oligosaccharide elicitors were prepared in the similar way. The harvested mycelium was filtered through *Miracloth* and stored at -20 °C until further processing. The frozen mycelium was suspended in distilled water (DW), then it was autoclaved for 10 min on slow exhaust. The mycelium collected on *Miracloth* was blended for 30 s in 0.5 M NaCl solution. The filtered homogenate was washed with DW then it was treated with 3 M Tris buffer (pH 7.5, 30 min), DW (5 min), chloroform-methanol 1:1 mixture, hexane and finally with acetone (each 30 min). The fungal material was air dried overnight, suspended in DW, and dialysed against 0.5 M acetic acid at first for 2 h, then against fresh acetic acid overnight at 4 °C. The dialysis was carried on against DW for 2 h and then against fresh DW overnight at 4 °C. The lyophilised mycelium was placed into a glass bottle (*Kimax*) treated with 2 M trifluoroacetic acid (TFA) shaken at 90 °C for 2.5 h in a water bath. The volume of filtrate separated from the fungal wall debris was reduced by evaporation at 45 °C. Then it was dissolved in DW and rotoevaporated again to syrup state. This material was diluted with 10 cm^3 DW to every gram mycelium hydrolysed. The solution gained was neutralised with 10 M KOH and adjusted to 20 $cm^3 g^{-1}$ mycelium with DW. The reducing sugar content of the elicitor was determined by 2 % anthrone dissolved in concentrated sulfuric acid. The glucose equivalent was calculated on the base of A_{625} measurement. For calibration glucose solutions were used and the glucose equivalents of the elicitors were set to the range 12 - 14 $mg\ cm^{-3}$ glucose.

Determination of molecular mass and the amount of oligosaccharides: The oligosaccharide composition of the elicitors obtained by the TFA hydrolysis of the mycelium was screened by *Sephadex G-200* (*Pharmacia*,

Uppsala, Sweden) column (25 × 1 cm) chromatography. The molecular mass of the fractions was calculated according to Determann (1967). The oligosaccharide concentration of the separated fractions was checked by two methods. First it was measured spectroscopically at 220 nm, and then estimated by anthrone test as described above.

Elicitor treatments: 100 cm³ of the 2-week-old suspension cultures was treated with 3.75 and 7.5 cm³ crude fungal homogenates (*Pythium* - PC, *Verticillium* - VC and *Botrytis* - BC) for 24 or 96 h. The same experiment was carried out with hydrolysed fungal cell wall elicitors (*Pythium* - PH, *Verticillium* - VH and *Botrytis* - BH) in concentrations 1.25 and 2.5 cm³ elicitor per 100 cm³ culture. After elicitation, the pH of the medium, the dry mass of the cells and the alizarin content of the cell fraction and the media was measured. The changes in the fluorescence and the ultrastructure were also checked. Each experiment was performed in triplicate and all the treatments were done three times.

Measurement of the alizarin content: The amount of alizarin was measured both in the cell fractions and the supernatants of the suspension cultures. According to the method of Zenk *et al.* (1975), the filtered plant material was lyophilised, and 0.1 g was suspended in 10 cm³ 80 % ethanol, sonicated for 10 min and centrifuged at 1 500 *g* for 10 min. The alizarin concentration of the clear supernatant was calculated on the basis of the A_{434} values.

Morphological investigations: Light microscopical studies were carried out with an *Olympus BH-2* microscope (Tokyo, Japan), and fluorescence microscopical observations (with fluorescein diacetate (FDA) and aniline blue stainings) were made using *Olympus BH2-RFCA* epifluorescence equipment.

For transmission electron microscopy (TEM), the samples were fixed for 2 h in 2 % glutarealdehyde dissolved in 0.1 M phosphate buffer (pH 7.2), and postfixed in 1 % OsO_4. After fixative steps, the samples were rinsed in the same phosphate buffer. The dehydration with ethanol was followed by embedding in *Durcupan* resin. The sectioning was carried out by a diamond knife using *Reichert Jung UltracutE* ultramicrotome (Vienna, Austria). The sections were stained with 5 % uranyl acetate for 4 min and lead citrate for 6 min (Reynolds 1963). The ultrathin sections were studied by *Hitachi 7100* TEM (Tokyo, Japan).

Results

All the three purified elicitors showed similar characteristics in their composition. The calculated molecular size range of the oligosaccharide fractions separated by column chromatography was 0.56 to 736.4 kDa. The higher sugar concentration appeared in the 9th - 17th fractions (1.5 - 67.3 kDa) in all the three hydrolysed elicitors. The highest sugar concentration was always measured in the 13th fraction (9.9 kDa). There was a good accord with the sugar quantity values obtained by anthron reaction and the spectroscopic measurement (*cf.* Fig. 2 for *Pythium*; very similar data of *Verticillium*
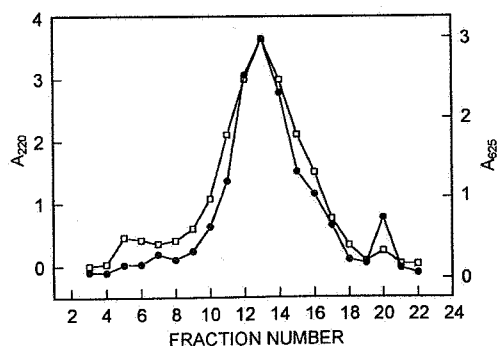

Fig. 2. Sugar content of the purified *Pythium debaryanum* fungal cell wall elicitor obtained by TFA hydrolysis followed by column chromatography (*squares* - A_{220} measured in the effluent, *circles* - A_{625} measured after anthrone reaction.

and *Botrytis* elicitors are not shown).

During a 24-h-treatment all the elicitor types applied caused the alkalinisation of the medium. There were no significant differences between the effects of the elicitors, apart from the PH type in both concentrations applied (Fig. 3*A*).

After the elicitor treatments, the alizarine content of the cells was raised to 109 - 142 % of that in the control cultures. This type of response was very similar in all the elicitor treatments (Fig. 3*B*). Comparing the increase of the alizarine content detected in the cells and in the medium, the later showed a higher increase, about 10 times that of the control (Fig. 3*C*). The dry mass of the plant material has not changed significantly as a result of the treatment (data not shown).

The light microscopic observation of the cells treated with different elicitors at various concentrations showed that the number of the viable cells did not change significantly during the first 24 h. However, during the same period there were detectable differences in FDA fluorescence (Table 1). In the long term experiments the number of the living cells decreased after 96 h of elicitation, particularly in cultures exposed to *B. cinerea* elicitors. Besides the FDA staining, there were changes detected in vacuolar fluorescence. In function of the effectiveness of the elicitation, the central vacuole showed brownish-red fluorescence (at blue light

Table 1. Assessment of the fluorescence characteristics of cells of the control and elicited *R. tinctorum* suspension cultures. The viability of the cells was tested by FDA staining. P - *Pythium debaryanum*, V - *Verticillium dahliae*, B - *Botrytis cinerea*, H - hydrolysed fungal cell wall material, C - crude homogenate of fungal culture, 1.25, 2.5, 3.75, 7.5 - volume of the elicitor in cm^3 added into 100 cm^3 plant suspension culture, +++ - numerous/strong, ++ - common/medium, + - few/weak, d - detected, n - not detected.

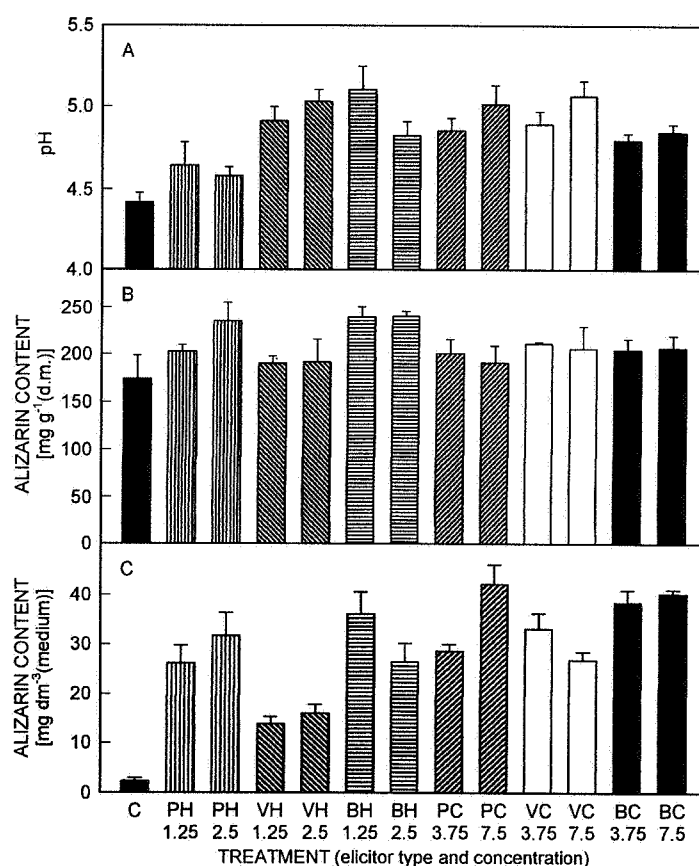| | Fluorescence after 24 h | | | | Fluorescence after 96 h | | | |
| | FDA fluorescence | | Fluorescence of vacuole | | FDA fluorescence | | Fluorescence of vacuole | |
	Number of living cells	Intensity of staining	Diffuse	Bright red particles	Number of living cells	Intensity of staining	Diffuse	Bright red particles
Control	+++	+++	n	n	+++	+++	n	n
PH 1.25	+++	+++	n	n	++	++	d	n
PH 2.5	+++	+++	d	n	++	++	d	d
VH 1.25	+++	++	n	n	++	++	d	d
VH 2.5	+++	+	d	d	+	+	d	d
BH 1.25	+++	+	d	d	+	+	d	d
BH 2.5	+++	+	d	d	+	+	d	d
PC 3.75	+++	++	n	n	+	+	d	d
PC 7.5	+++	++	d	n	+	+	d	d
VC 3.75	+++	++	n	d	+	+	d	d
VC 7.5	+++	+	d	d	+	+	d	d
BC 3.75	+++	+	d	d	+	+	d	d
BC 7.5	+++	+	d	d	+	+	d	d

Fig. 3. Medium alkalisation and changes in the alizarin content of *Rubia tinctorum* cell suspension culture treated with different elicitors for 24 h. *A* - the pH of the culture medium after elicitor treatments, *B* - the amount of alizarin in cells collected from elicitor-treated *Rubia tinctorum* cell suspension cultures, *C* - alizarin content in the media, P - *Pythium debaryanum*, V - *Verticillium dahliae*, B - *Botrytis cinerea*, H - hydrolysed fungal cell wall material, C - crude homogenate of fungal culture, 1.25, 2.5, 3.75, 7.5 - volume of the elicitor in cm^3 added into 100 cm^3 plant cell suspension culture, C - control. Means ± SE, $n = 9$.
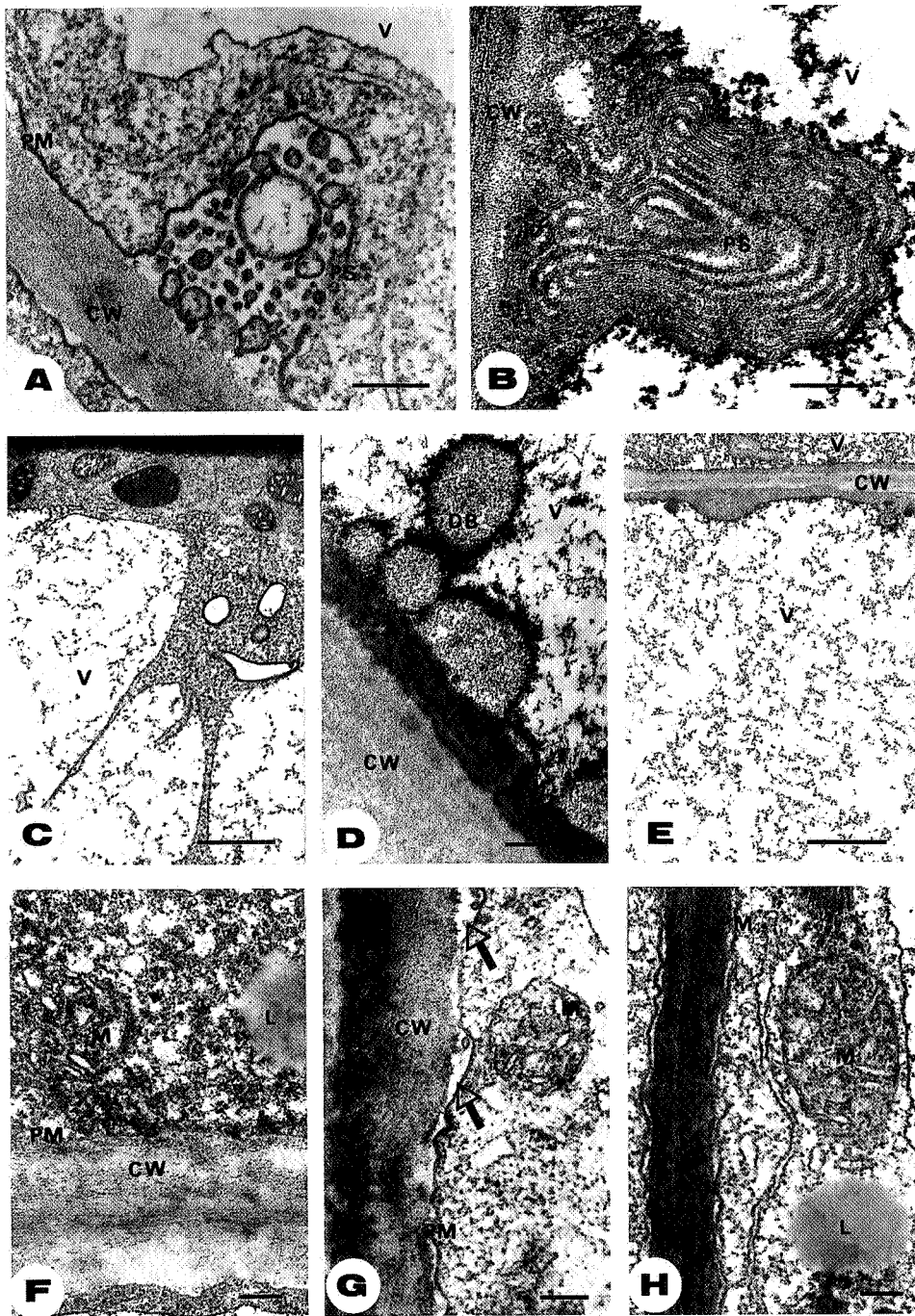
Fig. 4. Ultrastructural characteristics of elicited cells: *A* - vesiculo-reticular plasmalemmasome in a control *R. tinctorum* cell (*bar* 0.2 μm); *B* - reticulo-lamellar plasmalemmasome in a cell treated by BH 1.25 (*bar* 0.2 μm); *C* - control cell suspension culture cell devoid of dense vacuolar particles (*bar* 1 μm); *D* - electron-dense vacuolar bodies joined to tonoplast in elicited (BH 1.25) *R. tinctorum* cells (*bar* 0.5 μm); *E* - appearance of a cell treated with crude homogenate of BC 7.5, vacuolar precipitate content is higher then in the control cells but is only slightly aggregated (*bar* 1 μm); *F* - electronmicrograph of the area near the plasmalemma of a control cell, the plasmalemma is slightly wavy, electron-transparent deposition at the cell wall is undetectable (*bar* 0.2 μm); *G* - deposition of electron-transparent material (marked by *arrows*) in BH 1.25 treated cell suspension culture cell (*bar* 0.2 μm); *H* - the region as shown on parts *F* and *G* but photographed in BC 7.5 treated cells, the wavy plasmalemma appears without considerable deposition of electron-transparent material (*bar* 0.2 μm). CW - cell wall, DB - electron-dense body, L - lipid droplet, M - mitochondrium, PM - plasmalemma, PS - plasmalemmasome, V - vacuolum. Other symbols as on Fig. 3.

Table 2. Assessment of the ultrastructural characteristics of control and elicited *R. tinctorum* cell suspension culture cells after 24 h. For symbols see Table 1.

	Number of plasmalemmasomes	Structure of plasmalemmasomes	Vacuolar precipitate	Plasmalemma waving	Electron-transparent layer	Tonoplast breaking
Control	++	Vesiculo-reticular	Fine	n	n	n
PH 1.25	++	Vesiculo-reticular	Fine	n	n	n
PH 2.5	++	Vesiculo-reticular	Fine	n	n	n
VH 1.25	++	Vesiculo-reticular	Aggregating	n	n	n
VH 2.5	+++	Reticulo-lamellar	Aggregating	d	n	d
BH 1.25	+++	Vesiculo-reticular	Aggregating	d	d	d
BH 2.5	+++	Reticulo-lamellar	Aggregating	d	d	d
PC 7.5	++	Vesiculo-reticular	Fine	n	n	n
VC 7.5	+++	Vesiculo-reticular	Fine	d	d	d
BC 3.75	+++	Reticulo-lamellar	Fine	d	d	d
BC 7.5	+++	Reticulo-lamellar	Aggregating	d	d	d

excitation) in correlation with the weakening of FDA staining (Table 1). The intensity of fluorescence depended on the type and the concentration of the elicitor, as well as the timetable of events (Table 1). It was shown both in the aggregates and the single cells. By aniline blue staining just a weak fluorescence appeared in patches (data not shown).

The study of the fine structure of the treated cells revealed some alterations in correlation with the elicitor types and the concentration applied. As a general symptom it was seen that the number of plasma-lemmasomes present also in untreated cells, increased in the treated cells, and their structure and size changed too. In the elicitor affected cells, plasmalemmasomes were more extended and rather lamellar-reticular in contrast to the reticulo-vesicular type of the unaffected cells (Fig. 4 *A,B*). The other peculiarity evoked by fungal elicitors is the change in the vacuolar content. The purified *Verticillium* and *Botrytis* elicitors caused a massive accumulation of polyphenolic material in the compact electron-dense bodies, often joined with the tonoplast. The BH 1.25 treatment resulted in the appearance of electron-dense units in the central vacuole of the cells. A crude elicitor (BC 7.5) also increased the amount of a disperse precipitate, uniformly distributed in the vacuolar space (Fig. 4 *C-E*), or slightly aggregated. The plasmalemma at VH, BH, VC and BC treatments were often wavy. The electron-transparent layer between the cell wall and the plasmalemma was present in that group, however, not as a continuous deposition but as an irregular, thin, often disrupted coating (Fig. 4 *F-H*; Table 2).

During the 96-h long treatments the sequence of events following the administration of the BH elicitor was observed. There was a considerable change in the pH of the treated cultures. After 24 h, a significant rise was shown, followed by a plateau after 48 h. On the 4th day of the treatment, a new alkalisation step was ensued

resulting a rise of 1.0 pH during these 24 h. The pH of the control culture medium changed from 4.85 to 5.2 during the experiment (Fig. 5*A*).
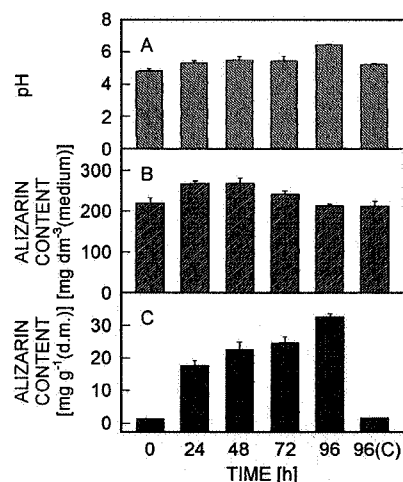

Fig. 5. 96-h elicitor (BH 1.25) treatment of *R. tinctorum* cell suspension culture. *A* - alkalisation of the medium; *B* - alizarin content of the elicited cells; *C* - changes in the alizarin content of the media of treated cultures. C - control, 96(C) - 96-h-old control culture.

While the dry mass of the plant material in the control and the treated cultures has not diverged significantly (data not shown), the alizarine content of the cells and the medium have differed considerably. The alizarin content of the cells showed a maximum after 48 h and decreased to the control level after 96 h (Fig. 5*B*). At the same time, the alizarine content of the medium increased permanently during the whole experiment, while the medium of the control culture showed only a slightly higher alizarine content than at the beginning (Fig. 5*C*). The fluorescence characteristics of the elicited cells obviously altered after 96 h, and the viability, indicated by FDA staining, dramatically changed excluding the PH 1.25 treatment, which otherwise caused the weakest symptoms (Table 1).

Discussion

To investigate the changes after the exposure of the cells to elicitors, several authors use different types of fungal elicitors, prepared by different methods and of diverse origin. The crude homogenates are easy to prepare but their composition is unknown and uncertain (Godoy-Hernandez and Loyola-Vargas 1991). For more exact elicitation and reproducibility purified fungal cell wall extracts can be used (Rokem et al. 1984, Dörnenburg and Knorr 1994). However, the comparison of the efficiency of the different fungal elicitors is almost missing.

In the present paper we report data gained at different concentrations of the two elicitor types (the total culture homogenates and the purified hydrolysates from fungal cell wall) originated from Oomycetes (P. debaryanum) and Deuteromycetes (V. dahliae and B. cinerea), which all are non-host-selective phytopathogenes. The hypha wall hydrolysates contained oligosaccharides with molecular masses ranging between 1.5 to 67.3 kD eluted as a single peak. All the three fungal wall hydrolysates gave a similar curve in the molecular mass composition and the the reproductivity of the hydrolysis was demonstrated (Fig. 1).

Some authors prefer to use intact plants emphasising the importance of the unity of the organism, while others give preference to in vitro cultures. In this work, we voted for the use of suspension cultures to avoid the differences in genetic background, which could interfere with the elicitation outcome. An additional advantage is the simple and homogenous administration of the elicitors directly to the cells without extra influences such as wounding, infiltration or pH changes. However, the main disadvantage of shaken suspension cultures is the permanent weak mechanical stress produced by the circulation of the medium (Scragg 1990).

On the basis of the responses induced, the differentiating cells seem to be better targets for elicitation than younger or older cultures (DiCosmo and Misawa 1985). The constancy of fresh/dry mass ratios measured both in control and treated suspensions showed that the elicitation did not affect cell division capability, even during the long term experiments.

A change has been observed in the pH and the conductivity of the medium (Marinelli et al. 1991) as a consequence of the alterations in the physiological and metabolic state of cells mainly attributed to membrane changes (ion channels, H^+ pumps, permeability; Anderson et al. 1991, Messiaen et al. 1993, Bennett et al. 1996, Dangl et al. 1996, Naton et al. 1996). The present data are in a good accordance with some of the earlier results. The elicitors used in the present work all evolved an alkalisation of the medium in the first 24 h. The degree of pH change was irrespective of the type and origin of the elicitor. However, PH 1.25 and 2.5 treatments resulted in a weak effect on the pH of the medium (Fig. 3A). During the 96-h treatments, after a slow

changing rate up to 72 h, a second, stronger rise was shown during the last 24 h (Fig. 5A).

The alizarin production of the elicited cultures was enhanced soon after the treatments, so the total amount of the pigment was typically higher compared to the control, as a result of any type of elicitation. However, the distribution of the pigment between the cells and the mediums was different. In the medium, the increase was almost ten times higher after 24 h of treatment, while in the cells the alizarin content ranged between 109 - 142 % of that in the control (Fig. 3B,C). It was shown that in the first day the de novo alizarin synthesis took place, and a significant amount of the pigment was released into the medium. The increased secondary metabolite production of Rubia continued during the following days, but its rate was quite different. After 96 h, alizarin content of cells returned to the control level while its concentration became twenty times higher in the medium (Fig. 5B,C). This means that the proportion of the synthesis and the release changed during the examined period. This accelerating secretion may be due to the similar membrane changes as were described in senescent leaf cells (Dhindra et al. 1981).

After short elicitor treatments, the appearance of living cells did not change under light microscope considerably, while at long term treatments the number of collapsed and reddish cells increased significantly. Under the fluorescence microscope there was a symptomatic alteration in the habit of cells. The number of living cells did not change significantly after 24 h in consequence of the exposition to elicitors irrespective of their type, origin and concentration. It does not mean that the viability of cells was not influenced by elicitors. The results of FDA tests showed that the intensity of FDA fluorescence was considerably weaker at higher elicitor concentrations (especially at VH, BH and BC treatments), often recognisable only at the area of the nucleus. In contrast to this, in those cells the reddish fluorescence of the vacuolar space became typical, showing again their altered metabolic status. After 96 h, the number of the living cells strongly decreased as a result of the elicitation, while in the control culture the viability did not changed. In the died cells the reddish-brown patches were seen without any other remarkable fluorescence. The changes in the autofluorescence of pathogen challenged cells were described earlier. It is thought to be due to the elevated level of secondary products, mainly flavonoids and phenoloids (Bennett et al. 1996). In intact plants, and cultured plant cells infected with pathogenous fungi, local callose deposition was described (Naton et al. 1996). Apart from a weak fluorescence often appearing in patches in some cells after anilin blue staining, we could not detect any considerable amount of callose in the elicitor treated Rubia cells.

Our ultrastructural observations are in agreement with

the few statements on elicited suspension cultures. Eilert and Constabel (1985) found that there were no dramatic fine structural changes in poppy (*Papaver somniferum*) cultures, in spite of their strong sanguinarine accumulation. Eilert *et al.* (1987) observed enlarged lipid droplets in the cytoplasm of cells of periwinkle (*Catharanthus roseus*) cultures. Between these two works there was a contradicting point: the vacuolar content. When the *B. cinerea* homogenate was added to the poppy cells, a dense vacuolar precipitate appeared, often joined to the tonoplast, while in the periwinkle cells, after *P. debaryanum* homogenate treatment, the vacuolar space remained devoid of precipitate. Both effects are consistent with our results. In *R. tinctorum* suspension cultures, *B. cinerea* proved to be a potent elicitor leading to the formation of electron-dense vacuolar precipitates. On the other hand, the *P. debaryanum* treatment applied in both forms resulted in an elevated alizarin level without inducing vacuolar body creation. In *V. dahliae* treated cells, a smaller number of less dense vacuolar particles was detected. It is believed that they are composed of polyphenols.

The polyphenol content of this precipitate was directly proved by gold-complexed fungal laccase labelling (Benhamou *et al.* 1996). Similar vacuolar precipitates were reported in cell cultures and in intact plants affected by elicitors or different stress factors (Eilert and Constabel 1985, Eilert *et al.* 1987, Keresztes *et al.* 1989, Cline *et al.* 1990, Bezaková *et al.* 1996, Benhamou *et al.* 1996). On the other hand, Robinson *et al.* (1998) reported similar dense vacuolar bodies appearing during the process of starvation-induced autophagy and senescence. According to them, these bodies originate from autolytic vesicles.

Beside the appearance of vacuolar bodies, another characteristic feature was detectable: the change in the plasmalemmasome structure. In the cells of the control culture, they are present in small number and their structure is vesiculo-reticular (Fig. 4A). As a consequence of the elicitor effect their texture became reticulo-lamellar (Fig. 4B) and they appeared most frequently in the elicited cells. As a result of stress, elicitation and infection, the transport in the cells is enhanced. Also the cytoplasmic membrane structures show alterations, e.g., ER swelling, plasma membrane waving, mitochondrial internal membrane system alterations (Eilert and Constabel 1985, Lopez-Carbonell *et al.* 1994, Naton *et al.* 1996). Extended and multiplied plasmalemmasomes were described in differentiating trachea elements by Verbelen (1977). The author proposed a possible degradative role of these plasmolemmosomes during the late developmental stage.

Under the electron microscope we could not detect any expanded electron-transparent regions in the cell wall near the plasmalemma, as it was seen in the infected cells of the plants near the infection site (Benhamou *et al.* 1996). In some places there were light, thin areas between the cell membrane and the wall, but their occurrence was also detectable in the control (Fig. 4F-H) possibly due to the permanent shaking stress present in the suspensions.

The above results strongly suggest that the different fungal elicitors used in these experiments evoked similar effects, and the applied concentrations caused qualitatively comparable data on the alizarin production in the *R. tinctorum* cell suspension cultures. The *de novo* synthesis of alizarin, the intense release of it into the medium, as well as the medium alkalisation and the fluorescence microscopical and electron microscopical observations allow us to make clear distinctions between the effectiveness of the different fungal elicitors. Not only the concentration, but the type and the origin of the elicitor seems to be important. In our work *Pythium* turned out to be the weakest as elicitor, while *Botrytis* caused the clearest symptoms.

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