Comparison of pH Changes and Elicitor Induced Production of Glyceollin Isomers in Soybean Cotyledons

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During the incubation of soybean cotyledons with Pmg-elicitor for 22 hours the pH of the diffusion droplets increases from 7.2 to 8.3. This pH-shift is a precondition for the formation of the typical red colour of the diffusion droplet. After inhibiting the pH-shift by the use of 100 mM phosphate or Tris buffer instead of 10 mM buffer as solvent for the elicitor, the red colour is no longer formed with the exeption of 100 mM Ammediol buffer. However, the normal pattern of pterocarpan induction can be measured in the absence of the red colour in the diffusion droplet. Tris and Ammediol buffers exhibited a smaller pterocarpan induction as compared to phosphate buffer as solvent for the Pmg-elicitor.

Introduction

Resistance of plants towards certain pathogens is due in part to the accumulation of fungitoxic compounds called phytoalexins, which seem to be produced as a result of the hypersensitive death of host cells after their contact with the pathogen [1–3]. Phytoalexins are found in the area of localized necrosis, but they are known to be synthesized in neighbouring living cells [4, 5]. Glyceollin can be found during the incompatible host-parasite reaction of soybean tissue (*Glycine max* L. Horosoy 63) and the pathogenic fungus *Phytophthora megasperma* f. sp. *glycinea* [6].

The accumulation of phytoalexins of the glyceollin type (isomers **I, II** and **III**) can also be induced by glucan elicitors which are components of the mycelial cell walls of the pathogen [7, 8]. Recently the structure of the Pmg-elicitor was identified by Sharp *et al.* as hexa(β-D-glucopyranosyl)-D-glucitol [9]. Alternatively, fatty acids as arachidonic acid have been described as elicitors for the induction of stress metabolites in potato tuber slices [10, 11].

At present it is not known how elicitors function as initiators of phytoalexin formation in the host tissue. Hahn *et al.* [12] described the release of endogenous elicitors from pectic cell wall fractions by extraction with hot water or dilute acid. On the other hand lipid peroxidation might also be involved in the induction of stress metabolites such as rishitin and lubimin in *Solanum tuberosum,* infected with *Phytophthora infestans* [13].

Stimulation of phytoalexin accumulation in soybean tissue has been used as a biological assay for elicitor-active compounds. Three different systems have been described: Keen *et al.* [14] used hypocotyl segments while Ebel *et al.* [15] used soybean suspension cultures; the droplet diffusion technique introduced by Frank and Paxton [16] as modified by Ayers *et al.* [8] is also frequently used for testing elicitor active compounds. The advantage of the latter assay is the fact that the diffusion droplet turns red in parallel to the induction of glyceollins, whereby the intensity of the red colour formation is reported to correlate directly with the induced glyceollin concentration.

In this communication, we report on experiments undertaken in order to learn more about the early reactions induced by the Pmg-elicitor in host tissue, *i.e.* whether the intensity of the red colour of the diffusion droplet under all circumstances correlates with induced glyceollin formation and their final concentration in the host tissue.

Experimental procedures

Materials

Soybean seeds (*Glycine max* L. Harosoy 63) were obtained from Prof. J. D. Paxton, University of Illinois, USA.

The Pmg-elicitor was a personal gift from Dr. Ziegler, University of Aachen. Methanol for the

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HPLC analysis was purchased from Promochem D-4230 Wesel.

Methods

Soybean seeds were grown on autoclaved hydroponic stones under continuous light (8000 lx) in a growth chamber at 26 °C and 40% relative humidity.

Glyceollin induction

Six day old cotyledons were sterilized with a hypochlorite solution (0.75%) and sliced with a sterile razor blade from the underside. A 100 µl droplet of elicitor solution (ca. 0.3 µg glucose equivalents) dissolved in Phosphate, Tris or Ammediol buffer (10 mM and 100 mM pH 7.2) was placed on the cut surface and incubated in a growth chamber (26 °C, 98% rel. humidity) for 22 hours. All preparations were carried out under sterile conditions. Twenty cotyledons were used for each experiment. The antibiotic gentamycin sulfate (100 µg/ml) was present in all solutions applied to cotyledons.

Glyceollin detection

The extraction if glyceollin isomers was carried out as described by Ebel *et al.* [15]. Before HPLC analysis, all extracts were subjected to membrane filtration (pore size 0.2 µm). The analysis was carried out using a HPLC gradient system (Beckman, München) in combination with a analytical reversed phase column (ULTRASPHERE-ODS, 5 µm, 4.6 mm × 250 mm). The glyceollin isomers were detected at 280 nm with the aid of glyceollin standards (obtained from Dr. Ebel, University of Freiburg).

Determination of the red colour

For determination of the red colour in the diffusion droplets, diffusion droplets were collected and centrifuged in a bench centrifuge at highest speed for 5 minutes. The absorption of the collected liquid was measured at 525 nm, using a Kontron UVIKON 810 spectrophotometer.

Determination of the pH of the diffusion droplets

After an incubation time of 22 hours in the dark in the growth chamber the diffusion droplets of 20 cotyledons were collected and centrifuged in a bench centrifuge at highest speed for 5 minutes. The pH was measured with a micro pH electrode (INGOLD type: LOT – 406 – M3).

Results

1. Time course of pH increase in the diffusion droplets of cotyledons treated with Pmg-elicitor dissolved in 10 mM phosphate buffer pH 7.2

Fig. 1 shows the development of the pH changes in the diffusion droplets of elicitor treated and untreated cotyledons. The diffusion droplets were measured 0.5, 3, 7, 10, 14, 18 and 22 hours after the start of the experiment. The pH of the buffer control decreased

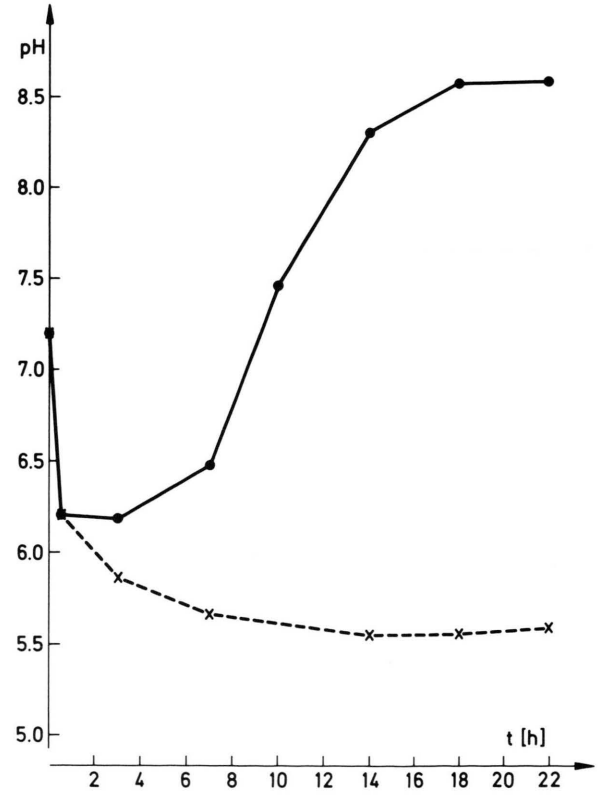

Fig. 1. Elicitor induced pH changes in the diffusion droplet.
●——● Elicitor dissolved in 10 mM phosphate buffer (pH 7.2);
X----X 10 mM phosphate buffer (pH 7.2).
After 0.5, 3, 7, 10, 14, 18 and 22 hours incubation in the dark (26 °C and 98% rel. humidity), the diffusion droplets of 20 cotyledons were collected and the pH was measured with a micro pH electrode. The experiment was repeated twice. The final pH values differ between 8.3 and 8.6 (elicitor dissolved in 10 mM phosphate buffer) and 5.6 to 7.0 (10 mM phosphate buffer).

continuously down to 5.6. The pH of the diffusion droplet containing the Pmg-elicitor increased sigmoidally up to 8.5.

2. Influence of different concentrations of buffers as solvent for the Pmg-elicitor

After an incubation time of 22 hours in the dark only the diffusion droplets in the experiments with 10 mM phosphate buffer, 10 mM Tris buffer and 10 mM Ammediol respectively 100 mM Ammediol buffer were deeply red coloured (Table I). The diffusion droplets of the experiments with 100 mM phosphate and 100 mM Tris buffer showed no red colour. The tissue below the colourless diffusion droplets did not exhibit any red pigmentation. All red coloured diffusion droplets showed final pH values above pH 8.0. The colourless diffusion droplets (100 mM phosphate and 100 mM Tris buffer) exhibited no or only a small pH increase after 22 hours of incubation.

Table I. Influence of different concentrated buffers on the pH increase, the intensity of red colour formation in the diffusion droplet and the pterocarpan induction in soybean cotyledons.

After an incubation time of 22 hours in the dark (26 °C and 98% rel. humidity) the diffusion droplets of each experiment were collected and the pH together with the intensity of the red colour were determined. Following the diffusion droplets together with the whole tissue were extracted for pterocarpans (see Experimental procedures). The experiment was repeated three times with essentially identical results.

Experiments	pH $t = 0$	pH $t = 22$	△ pH	Absorbance (525 nm) cotyledo	THP (μmol/20 cotyledons)	Glyceollin I (μmol/20 cotyledons)	Glyceollin II (μmol/20 cotyledons)	Glyceollin III (μmol/20 cotyledons)	Total (μmol/20 cotyledons)
Elicitor dissolved in 10 mM Phosphate buffer	7.3	8.1	+0.8	0.55	18.7	4.8	0.3	0.8	24.6
Elicitor dissolved in 100 mM Phosphate buffer	7.2	7.2	0.0	0.01	18.4	4.3	0.2	0.9	23.8
10 mM Phosphate buffer	7.3	6.3	−1.0	0.01	0.1	0.0	0.0	0.0	0.1
100 mM Phosphate buffer	7.3	6.7	−0.6	0.00	0.4	0.2	0.0	0.1	0.7
Elicitor dissolved in 10 mM Tris buffer	7.1	8.1	+1.0	0.45	10.8	4.3	0.4	0.9	16.4
Elicitor dissolved in 100 mM Tris buffer	7.2	7.6	+0.4	0.02	7.1	3.2	0.1	0.6	11.0
10 mM Tris buffer	7.2	6.2	−1.0	0.00	0.0	0.0	0.0	0.0	0.0
100 mM Tris buffer	7.3	7.0	−0.3	0.01	0.7	0.4	0.0	0.3	1.4
Elicitor dissolved in 10 mM Ammediol buffer	7.2	8.3	+1.1	0.80	9.8	4.3	0.3	0.9	15.3
Elicitor dissolved in 100 mM Ammediol buffer	7.3	8.0	+0.7	0.21	10.3	4.6	0.3	1.0	16.2
10 mM Ammediol buffer	7.1	5.4	−1.7	0.01	0.0	0.0	0.0	0.0	0.0
100 mM Ammediol buffer	7.3	6.3	−1.0	0.00	0.0	0.1	0.0	0.0	0.1

The pterocarpan induction of soybean cotyledons treated with Tris or Ammediol buffer as solvent for the Pmg-elicitor was reduced as compared to the induction with phosphate buffer. Furthermore 100 mM Tris buffer exhibited a smaller pterocarpan induction than 10 mM Tris buffer. These results could also be measured for the induction of THP and the glyceollin isomers. The ten fold higher concentrated phosphate or Ammediol buffers had no significant effect on the pterocarpan induction of soybean cotyledons compared to the experiments with 10 mM phosphate and 10 mM Ammediol buffer. No red colour formation, no pH increase in the diffusion droplets and only a very small pterocarpan induction could be measured in all the controll experiments.

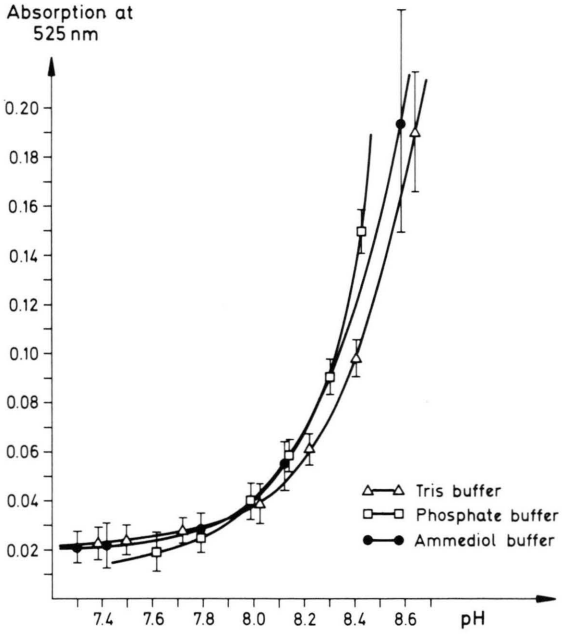

Fig. 2. Influence of different buffers and different pH values on the red colour formation of the colourless diffusion droplet.
The diffusion droplets of twenty cotyledons incubated with Pmg-elicitor dissolved in 100 mM phosphate buffer (pH at the beginning: pH = 7.2) were collected after 22 hours incubation in the dark (26 °C and 98% rel. humidity, pH at the end: pH = 6.2). An aliquot was mixed with 50 mM phosphate, Tris and Ammediol buffer of pH values between 7.2 and 8.7. Colour was measured photometrically at 525 nm for 45 minutes. Red colour could be induced with all three buffers at pH values above 7.8 (data not shown). After ten minutes incubation time (during the linear phase of the different curves) the absorption at 525 nm was determined for each pH values of the tested buffers. Presented values are means of eight replications.

3. Influence of different pH values and buffers on the red colour formation of the colourless diffusion droplets

In fig. 2 the influence of different buffers with different pH values on the start of the red colour formation was investigated. Several colourless diffusion droplets containing the Pmg-elicitor, dissolved in 100 mM phosphate buffer, were collected. An aliquot was mixed with 50 mM phosphate, 50 mM Tris and 50 mM Ammediol buffer of different pH values between 7.2 and 8.7. Red colour formation only developed at pH values above 7.8, whereas the red colour formation with phosphate buffer is significantly stimulated at pH values higher than 8.4 as compared to Tris buffer.

Discussion

The experiments reported here show that a strong pH increase in the diffusion droplet is measured when the Pmg-elicitor is dissolved in 10 mM phosphate, 10 mM Tris and 10 or 100 mM Ammediol buffer. During an incubation time of 22 hours, the pH shifts from 7.2 up to 8.3. The smaller final pH values of all experiments with 100 mM buffer compared to 10 mM buffer is based on the stronger buffer capacity of the ten fold higher concentrated buffers. However, the higher final pH values of the experiments with 100 mM Tris and Ammediol buffer compared to the experiments with 100 mM phosphate buffer may be explained with the smaller buffering capacity of Tris and Ammediol buffer at pH values above 7.0. Only the diffusion droplets with a final pH value above 8.0 were deeply red coloured. No red colour could be measured when the elicitor was dissolved in 100 mM phosphate or 100 mM Tris buffer. Surprisingly the normal pterocarpan pattern could be found also in samples showing no red coloured diffusion droplets. Glyceollin isomers and their precursers can be induced by the elicitor, apparently independent of red colour formation in the diffusion droplets. This result is not in contradiction to Ayers *et al.* [8] who claimed that the intensity of red colour in the diffusion droplets under their conditions correlates whith the elicitor – induced glyceollin concentration. The experiments with Tris and the very similar molecule Ammediol as solvents for the Pmg-elicitor showed a smaller pterocarpan induction than experiments with phosphate buffer. This phenomenon may be ex-

plained by findings of Takahashi *et al.* [17]. They demonstrated that Tris buffer caused cross-linking of thiol groups of chloroplast thylakoids forming disulfide compounds. On the other hand Gustine [18] reported that SH groups are important in regulating the phytoalexin accumulation in *Trifolium repens* callus tissue cultures. Similar results were also described for soybean hypocotyls [19]. One could speculate that similar relations may also be established in soybean tissue during the induction or accumulation of glyceollins.

Fig. 2 demonstrates that the red colour formation is induced at pH values above 7.8. This explaines why no red colour could be measured when the elicitor was dissolved in 100 mM phosphate or 100 mM Tris buffer. This result is in agreement with experiments of Zähringer *et al.* [20] who showed that solutions of THP (3,6a,9-trihydroxypterocarpan) or its derivates turn red after addition of KOH. All three tested buffers exhibit the same properties in forming the red colour, whereas phosphate buffer in addition has a stimulating effect at pH values higher than 8.4.

We speculate that the sigmoidal pH increase in the diffusion droplet (see Fig. 1) may be attributed to membrane bound proton carriers, which may be activated by the Pmg-elicitor. A simultaneous H^+ diffusion from the tissue into the droplet may explain the pH decrease in all treatments without the elicitor. The protons may be derived from compartments such as vacuols after cutting the cotyledons. Furthermore, the proton flux through the cell wall may be responsible for the production of "endogenous" compounds (elicitors) which in turn may induce glyceollin biosynthesis as described by Hahn *et al.* [12].

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