Wyerol and Wyerone Accumulation in Tissue Cultures of *Vicia faba*

Andreas B. Wolff, Maung Thynn, Elisabeth Görge, and Dietrich Werner

Fachbereich Biologie, Botanisches Institut der Philipps Universität Marburg, Karl von Frisch Straße, D-3550 Marburg, Bundesrepublik Deutschland

Z. Naturforsch. **43c,** 636–640 (1988); received March 7/May 30, 1988

Vicia faba, Callus and Liquid Cultures, Regeneration, Phytoalexins

Callus cultures of various cultivars of *Vicia faba* were studied for regeneration. During callus growth on agar or in liquid medium a black tissue was often observed correlated with an autolysis of some cells. With those tissues callus growth and regeneration of plants was inhibited. In black callus the phytoalexin synthesis is induced without infection by microorganism. The main phytoalexins in the callus tissue are wyerol and wyerone, with some wyeronic acid and dihydrowyerone. Wyerol was found especially in the cultivar Troy at a high concentration. In four cultivars the pooled phytoalexin concentration varied between 10 and 150 $\mu g \cdot g^{-1}$ fresh weight.

Introduction

Regeneration of plants from callus cultures is a method to produce clones from a single plant with special properties, often used in plant breeding. Grain and feed legumes are after cereals the second largest group of crop plants for human and animal nutrition. For several legume plants regeneration from callus cultures was reported [1–5]. Recently a method also for the regeneration of *Vicia faba* from callus cultures was developed [6]. In that work explants from organs were studied, however only epicotyl tissue was regenerated. One of the main barriers in the *in vitro* culture of faba bean was the so-called "blackening", which means that calli growing on agar surfaces or in liquid culture became black because of autolysis of some tissue. Also in the field the "blackening" of shoots occurs in young seedlings and the plants ultimately die [7].

Phytoalexins are antimicrobial compounds with a low molecular weight. They are synthesized and accumulated in plants, exposed to microorganisms or other elicitors such as heavy metals salts, detergents or wounding. In contrast to other legumes which mainly synthesize isoflavonoids as phytoalexins, *Vicia faba* accumulates furanoacetylenes. The main phytoalexins are wyerone, dihydrowyerone, wyerone epoxide, wyeronic acid, dihydrowyeronic acid, wyerol, wyerol epoxide, dihydrowyerol [8]. While in leaves and pods of *Vicia faba* wyeronic acid and dihydrowyeronic acid are the major components, in seeds wyerone and dihydrowyerone are mainly accumulated. Phytoalexin concentration varies from 300 to 1200 $\mu g \cdot g^{-1}$ fresh weight after infection [9]. We established in this contribution the accumulation of phytoalexins in uninfected tissue cultures of faba beans, with large differences in the cultivars studied.

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin layer chromatography; 2,4D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzyl-aminopurine.

Reprint requests to Prof. Dr. Werner.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0900–0636 $ 01.30/0

Materials and Methods

Plant material, culture conditions and media

Seeds of faba bean (*Vicia faba* L.) of the tannin free cultivar "TF" were received from Dr. Nagel, Bundesanstalt für Pflanzenbau, Vienna, Austria and cv. TP 667, cv. Kristall and cv. Troy from Norddeutsche Pflanzenzucht, 2331 Holtsee (F.R.G.). After surface sterilization with 30% H_2O_2 for 20 min they were rinsed 3 times with sterile distilled water and presoaked in water on a shaker for 5 h. The seeds were then allowed to germinate on NB plates (Nutrient Broth 8 $g \cdot l^{-1}$ and agar 16 $g \cdot l^{-1}$) at 28 °C in the dark. Immature pods with seeds (about 7 mm length) were harvested from 2 months old plants grown in a green house and surface sterilized for 1 min in 70% ethanol, followed by 10 min immersion in NaOCl. 3–4 days old hypocotyls, epicotyls and cotyledons from germinated seeds were obtained from the tannin free cultivar. Immature seeds from open pods were removed by carving the funicle. From seeds shorter than 7 mm it was difficult to remove embryos. Immature embryos were separated from the seeds by dissection and were cultured in

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

(2 × 18 cm) test tubes. Pieces of 4–6 mm were excised from immature cotyledons and placed in petridishes containing 20 ml of agar medium.

B5 medium [10] with various phytohormones and 10 amino acids was used for all studies [6]. All cultures were kept in the dark at 28 °C for 2 weeks and then transferred to 23 °C with a 16 h light and 8 h dark regime. All media used were solidified with 0.75% agar and had a pH of 5.5.

For suspension cultures, 6 weeks old calli obtained from epicotyl explants of the tannin free cultivar were cut into small pieces and placed in 100 ml Erlenmeyer flasks with 50 ml liquid medium. Incubation was at 23 °C on a shaker (120 rpm).

Extraction, identification and quantification of phytoalexins

Tissues were stored at −20 °C before extraction. Callus tissue was homogenized in ethanol by a mixer and stirred overnight at room temperature. The extract was centrifuged for 5 min at $3000 \times g$. The supernatant being evaporated to dryness was resuspended in an appropriate volume of ethanol and subjected to HPLC or TLC.

With TLC, wyerone derivatives can be detected as blue fluorescent bands under UV at 366 nm [8]. For further identification we used the R_f-values published by Hargeaves [11] followed by a wavelength scan from 200–400 nm to detect the main absorption bands. Wyerone has main UV absorption bands are at 351, 292 and 224 nm in ethanol, dihydrowyerone at 340 and 235 nm in ethanol, wyerol at 312 nm in methanol and wyeronic acid at 356, 284 and 222 nm in methanol [8].

The identification of phytoalexins after separation by HPLC was carried out by comparison with published R_f-values [9], [11] and by UV absorption bands as described above. Quantitative assays on HPLC were performed by comparing peak areas to an internal standard and by using the extinction coefficient at 351 nm according to Fawcett *et al.* [12].

High performance liquid chromatography (HPLC)

For HPLC we used a two pump system (LKB 2150) from LKB Instrument GmbH (Gräfling, F.R.G.), a controller (LKB 2152) and an UV/Vis variable wavelength monitor (LKB 2151). Injection was performed *via* a Rheodyne injector (Rheodyne Inc., Cotati, C.A., U.S.A.) with a 20 µl loop. Quantitative data were obtained by using a Shimadzu Data Processor Chromatopac C-R3A (Shimadzu Corporation, Kyota, Japan) with *n*-butyl salicylate as a standard.

Reversed phase HPLC was carried out as described by Porter *et al.* [11]. Aliquots of 20 µl of extract with butyl salicylate as internal standard were injected to a Serva Octadecyl = Si100 Polyol 0.005 mm (4.6 × 200 mm) column with a precolumn (4.6 × 75 mm) filled with the same material. Gradient eluation was carried out under room temperature with a flow rate of 1 ml/min and UV detection at 330 nm. Initial solvent conditions were 5% acetic acid – methanol (65:35 vol%). The linear gradient was used for 60 min to a final mixture of 5% acetic acid – methanol – acetonitrile (30:50:20 vol.%). For calibration of the internal standard *n*-butyle salicylate, the peak area after HPLC separation was compared to a known concentration of wyerone and wyeronic acid.

Thin layer chromatography (TLC)

TLC analysis was done as described by Hargreaves *et al.* [13] using Merk silica plates (0.2 mm) with a solvent system of hexane – acetone 2:1 (v/v).

Results and Discussion

Callus cultures in Vicia faba

In *Vicia faba* a difficulty in the *in vitro* culture is the often observed so-called "blackening" of tissue. After placing excised plant tissue for a few hours on different media, some of them became black rapidly. When well formed calli or calli with roots or shoots were transferred to a new medium, some of these also turned black and died afterwards. In cell suspension cultures this problem also occurred when the suspended cell aggregates were transferred to a new medium, especially on agar solidified medium. The phenomenon of "blackening" differs from senescence of calli or dried calli. In the latter cases, colours of calli changed from cream white or green to pale brown. For this change at least one week is needed. In the case of "blackening", calli or plant material immediately changed to a black colour.

Phytoalexins in calli

When well growing calli were compared with black calli by TLC some new bands occurred.

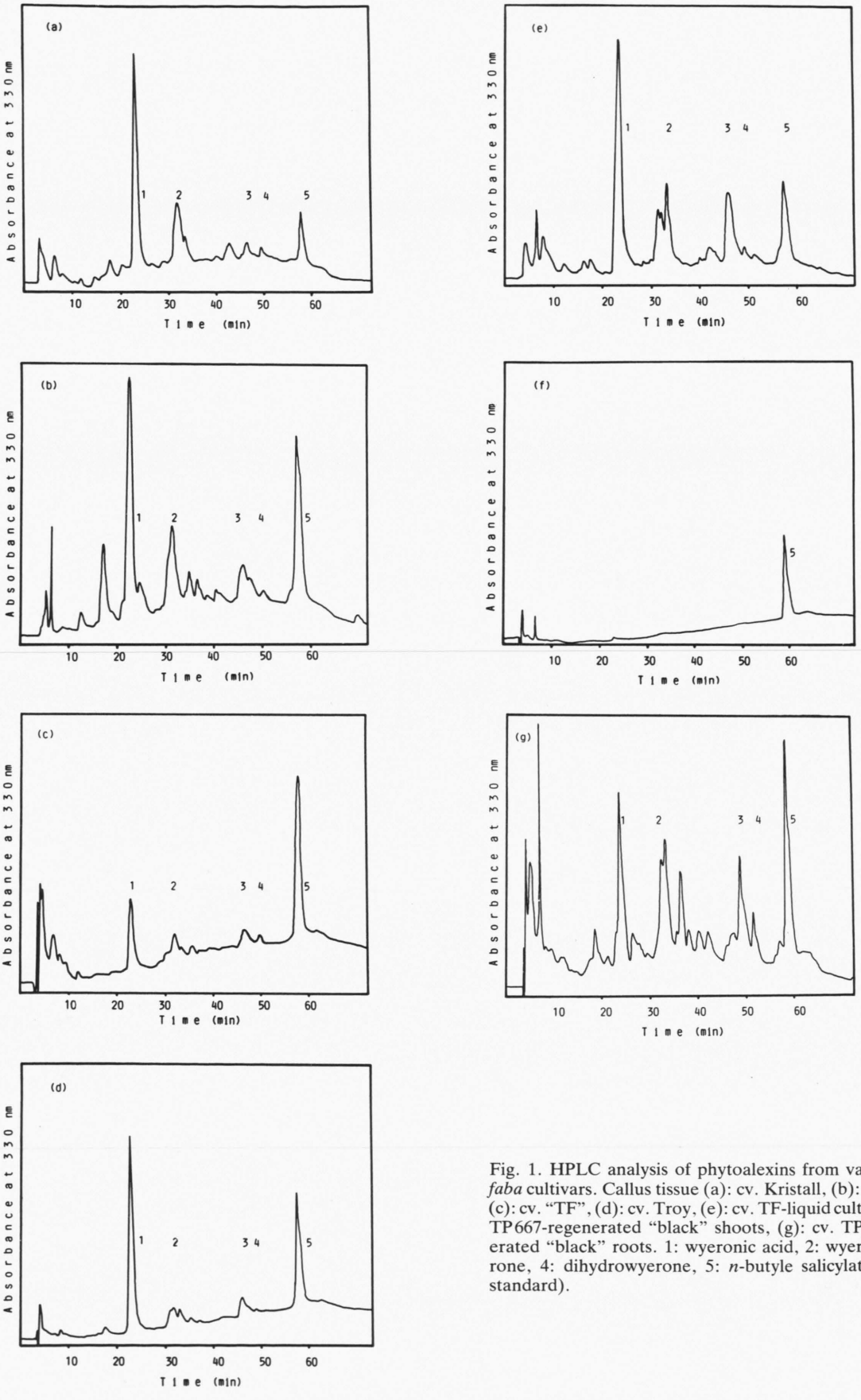

Fig. 1. HPLC analysis of phytoalexins from various *Vicia faba* cultivars. Callus tissue (a): cv. Kristall, (b): cv. TP667, (c): cv. "TF", (d): cv. Troy, (e): cv. TF-liquid culture, (f): cv. TP667-regenerated "black" shoots, (g): cv. TP667-regenerated "black" roots. 1: wyeronic acid, 2: wyerol, 3: wyerone, 4: dihydrowyerone, 5: *n*-butyle salicylate (internal standard).

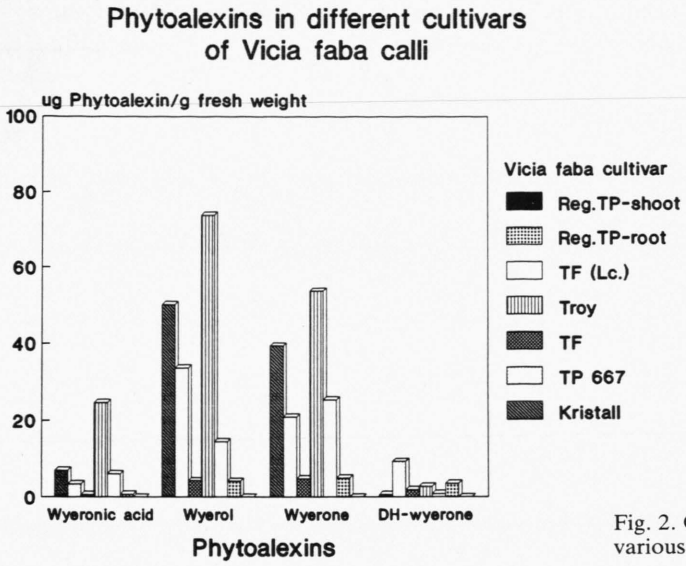

Fig. 2. Concentrations of phytoalexins of "black" calli from various *Vicia faba* cultivars.

Some of these bands could be identified as phytoalexins. Synthesis of phytoalexins is induced by elicitors, components from infecting microorganisms or by other stress factors. Since the calli were not infected by microorganisms other stress factors during or before "blackening" may be involved. Calli of *Vicia faba* are very sensitive to become black. When they were transferred from old medium to new medium with a tweezer and dissected with a knife they were injured and accumulated phytoalexins rapidly. Another factor may be a transfer to a new medium. Calli grew well in a medium with BAP, when the calli were transferred from media with kinetin. But calli rapidly became black, when transferred from media with BAP to media with kinetin.

"Blackening" and the accumulation of phytoalexins are not specific for a single *Vicia faba* cultivar, but occurred in all of our cultivars tested.

Wyerone and dihydrowyerone ($R_f = 0.48$) were found in black calli of all cultivars. Wyerol ($R_f = 0.35$) could be found in every sample except the shoots of the regenerated cultivar TP 667. Wyeronic acid ($R_f = 0.05$) can only be detected with great insecurity because there are other substances with the same R_f-value. The concentration of wyeronic acid was larger in the calli of all cultivars than in black regenerated TP 667 roots or shoots.

The concentrations and quality of phytoalexins accumulated in the tissue of the cultivars differs. While in calli of the cultivar Kristall wyerol and wyerone are dominant, in TP 667 also dihydrowyerone is at a higher level, whereas cultivar Troy also accumulates relatively high amounts of wyeronic acid. The tannin free cultivar has a very low level of all phytoalexins. This cultivar may be less sensitive against the unknown, perhaps endogenous elicitors. It is the only cultivar studied where never some cell aggregates in liquid cultures turned black.

In calli the total content of phytoalexins varies between 10 and 150 $\mu g \cdot g^{-1}$ fresh weight. In infected cotyledons the concentration can reach up to 1200 $\mu g \cdot g^{-1}$ fresh weight [9, 14]. Since the water

Table I. Concentrations of phytoalexins. HPLC analysis of black calli from various *Vicia faba* cultivars. TP667-S: regenerated black shoots from cultivar TP667, TP667-R: regenerated black roots from cultivar TP667, TF-Lc: "TF" in liquid culture.

	Wyeronic acid	Wyerol	Wyerone	Dihydro-wyerone
	[$\mu g \cdot g^{-1}$ fresh weight]			
Kristall	6.9	50.2	39.4	0.6
TP667	3.4	33.7	20.9	9.2
TF	0.6	4.2	4.7	1.8
Troy	24.6	73.8	53.8	2.7
TF-Lc	6.0	14.3	25.3	0.8
TP667-S	0	0	0	0
TP667-R	0.7	4.1	4.9	3.5

content in calli is much higher than in cotyledons, the phytoalexin accumulation per dry matter is more similar. Beside the handling and the transfer of calli to new media as elicitoring factor for phytoalexin synthesis also endogenous elicitors from cell walls of autolyzing cells may be of importance. Sensitivity of *Vicia faba* callus cultures from the cultivars studied towards different kinds of phytopathogens are unknown. It is an interesting question, whether the potential to accumulate phytoalexins in calli of different *Vicia faba* cultivars can be correlated to resistance against phytopathogens under field conditions.

Acknowledgements

The work was supported by the BMFT programme „Wechselwirkungen Pflanzen–Mikroorganismen".

[1] S. Arcioni, M. R. Davey, A. V. P. dos Santos, and E. C. Cooking, Z. Pflanzenphysiol. **106,** 105 (1982).
[2] A. Atanassov and D. C. W. Brown, Plant Cell Tissue Organ Culture **3,** 149 (1984).
[3] U. B. Barwale, H. R. Kern, and J. M. Widholm, Planta **167,** 473 (1986).
[4] P. A. Lazzeri, D. F. Hidelbrand, and G. B. Collin, Plant Mol. Bio. Rep. **3,** 160 (1985).
[5] L. A. Mroginski, K. K. Kartha, and J. P. Schyluk, Can. J. Bot. **59,** 826 (1980).
[6] M. Thynn and D. Werner, Angew. Bot. **61,** 483 (1987).
[7] T. B. Jha and S. K. Roy, Cytologia **47,** 465 (1982).
[8] J. L. Ingham, Phytoalexins from the Leguminosae. In: Phytoalexins (J. A. Bailey and J. W. Mansfield, eds.), Blackie & Son Ltd, Glasgow and London 1982.
[9] J. W. Mansfield, A. E. A. Porter, and R. V. Smallman, Phytochemistry **19,** 1057 (1980).
[10] O. L. Gamborg, R. A. Miller, and K. Ojima, Exp. Cell. Res. **50,** 151–158 (1980).
[11] A. E. A. Porter, R. V. Smallman, and J. W. Mansfield, J. Chromatogr. **172,** 498 (1979).
[12] C. H. Fawcett, D. M. Spencer, R. L. Wain, A. G. Fallis, E. R. H. Jones, M. Le Quan, C. B. Page, V. Thaller, D. C. Shubrook, and P. M. Whitham, J. Chem. Soc. (C) **1968,** 2455.
[13] J. A. Hargreaves, J. W. Mansfield, and S. Rosall, Physiological Plant Pathol. **11,** 227 (1977).
[14] J. W. Mansfield, Y. M. Barlow, and A. E. A. Porter (G. P. Chapman & S. A. Tarawali, eds.), Nijhoff/Tunk 1984.