Low Concentrations of Phytoalexins Correlate with Resistance in Regenerated Plants from Meristem Cultures of *Vicia faba* L.

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Meristem Culture, Regeneration, Resistance, Phytoalexins

In tissue cultures from shoot apex meristems with leaf primordias of *Vicia faba cv.* TP667, addition of low concentration of auxins (0.01 $mg \cdot l^{-1}$) induced regeneration of whole plants at high frequency (100%). The combination of NAA and kinetin or GA_3 also induced a high yield of plant regeneration. Regenerated plants from various cultivars on a medium with 2,4D (0.01 $mg \cdot l^{-1}$) were infected with *Botrytis cinerea, Phytophthora megasperma* and *Rhizoctonia solani.* Accumulation of phytoalexins, ethylene production and the resistance to fungal diseases were studied. In general, production of phytoalexins occurred at a high level in all cultivars infected with *B. cinerea.* Ethylene production varied more in the seven cultivars studied than phytoalexin accumulation. No cultivar was resistant to *B. cinerea.* The highest resistance and the lowest concentration of phytoalexin was found after infection by *R. solani*, and phytoalexin accumulation and resistance were intermediate in plants infected by *P. megasperma.* The data suggest that only low to medium concentrations of phytoalexin in faba beans are correlated with resistance of regenerated plants.

Introduction

An application of tissue culture techniques for disease control is the propagation of a pathogen free plant from a meristem culture. Because of rapid propagation, meristem cultures have opened a new way to achieve inexpensive maintainance breeding. The development of an *in vitro* system to increase resistance has stimulated activity in unconventional breeding [1].

Plants of the genus *Vicia* showed variable behaviour when they were cultivated in *in vitro* [2]. *Vicia faba* (faba bean) is rather recalcitrant to most of the classical *in vitro* manipulation [3]. One barrier for *in vitro* culture of faba bean is the formation of black tissue. *I.e.,* calli with or without roots or shoots growing on agar or in liquid culture become black and die afterwards [4]. In the field, some shoots also often turn black in young seedlings [5]. Because plant regeneration from somatic tissue of this plant is difficult, additional information is essential for a better understanding of the factors controlling regeneration in the faba bean.

Abbreviations: 2,4 D, 2,4-dichlorophenoxyacetic acid; NAA, naphthalene acetate; IAA, Indole-3-acetic acid; BAP, 6-Benzyl-aminopurine; GA_3, Gibberellic acid A_3; K, kinetin; T.F., Tannin Free cultivar.

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Tissues of faba beans produce in response to microbial infection furanoacetylenic phytoalexins: wyerone, wyerol, wyeronic acid, dihydrowyerone, dihydrowyeronic acid, wyerone epoxide and dihydrowyerol [6]. The main phytoalexins in the callus of the faba bean, which turned black without infection by a pathogen were wyerol and wyerone, with some additional wyeronic acid and dihydrowyerone [4].

Regeneration from the meristem culture of pea [7–9], *Lathyrus* [10], soybean, cow pea, peanut, chickpea and bean [11], bean [12] and pea and bean [13] has been reported. However, they have not been used to study resistance aspects of fungal diseases. In this work we used regenerated plants from meristem cultures to study the accumulation of phytoalexins, ethylene production and resistance to fungal infection.

Materials and Methods

Growth of plants and infection

Faba bean seeds were surface sterilized with 30% H_2O_2 for 30 min, followed by thorough rinsing in 3 changes of sterile distilled water and soaked on the shaker for 4 h. Then the seeds were allowed to germinate on NB agar plates at 28 °C in the dark. Shoot apical meristem with adjacent tissues approximately 2–4 mm in length and leaf primordias were obtained from seeds germinated for 3–5 d. Explants were es-

Table I. Effect of phytohormones on meristem cultures of faba beans (B5 medium).

Tested substances concentrations [mg·l^{-1}]		Regeneration whole plant [%]	shoot [%]	Differentiation bud [%]	root [%]	Remarks
Controls		–	–	–	–	No visible growth
2,4D	0.01	100	–	100	100	Multiple buds and large calli
	0.05	17	83	75	17	1–3 buds, calli
	0.1	8	92	58	8	Small buds, no further development of buds
NAA	0.01	100	–	–	100	Short roots
	0.05	100	–	–	100	Short roots
	0.1	58	–	–	58	Short roots
IAA	0.01	100	–	100	100	Small buds, no further development of buds
	0.05	100	–	42	100	Small buds, short roots
	0.1	100	–	–	100	Short roots
K	0.01	100	–	–	100	Long roots
	0.05	17	–	33	100	Long roots, small buds
BAP	0.01	33	–	100	100	Short roots, no further development of buds
	0.05	–	–	–	–	Only calli
GA_3	0.01	100	–	17	100	Long roots, no further development of buds
	0.05	100	–	17	100	Developed buds
2,4D 0.01 + K	0.1–0.5	–	–	–	–	Only calli
BAP	0.1	–	–	50	–	No further development of buds
	0.5	–	–	67	–	Same as above
GA_3	0.1	33	–	–	33	Small roots
	0.5	8	–	8	8	Small buds
NAA 0.01 + K	0.1	100	–	58	100	Long roots, small buds
	0.5	100	–	50	100	Same as above
BAP	0.1–0.5	–	100	100	–	Developed buds
GA_3	0.1–0.5	100	–	100	100	Small buds, long roots
IAA 0.01 + K	0.1	–	33	–	–	Shoots with calli
	0.5	–	–	–	–	Only calli
BAP	0.1	17	83	100	16	Developed buds
	0.5	25	75	100	25	Same as above
GA_3	0.1	41	–	–	50	Long roots
	0.5	17	–	17	17	Small buds

Results after 6 weeks of cultivation.

tablished on a modified B5 medium as previously described by Thynn and Werner (1987) [14] with various phytohormones and incubated at 23 °C with a 16:8 h light and dark regime. For the test of host-fungi interaction, the excised meristematic tissues were explanted on a medium with 0.01 mg·l^{-1} of 2,4D where they regenerated strongly. After 2 weeks of culture the regenerated plants were infected with various fungi. The mycelium of fungi with well developed spores was suspended in sterile distilled water. 200 µl of the spore suspension were placed on the medium of the regenerated plants. The resistant plants were transplanted into pots containing perlite and allowed to grow in a phytotron. After 1 d of infection ethylene production was measured by gas chromatography.

Extraction, identification and quantification of phytoalexins

After 6 d of infection, the plants were harvested and stored at −20 °C before extraction. Plants were homogenized in ethanol and stirred overnight at room temperature. The homogenate was centrifuged at 3000 × *g* for 5 min. The supernatant was evaporated to dryness, then resuspended in an appropriate volume of ethanol and analyzed by HPLC as described by Wolff *et al.* 1988 [4].

Results and Discussion

Effects of phytohormones on plant regeneration in meristem culture

Data summarizing the effect of phytohormones on plant regeneration and somatic differentiations in meristem cultures of faba beans are given in Table I.

Generally, a low concentration of auxins (0.01 mg·l^{-1}) had an effect on whole plant regeneration. The highest frequency of regeneration was obtained with a medium containing 0.01 mg·l^{-1} 2,4D, whereas a higher concentration of this auxin allowed only a low level of regeneration. Buds were differentiated in media with all concentrations of 2,4D but the frequency decreased from the low to the high concentration. With various concentrations of IAA (0.01 to 1.0 mg·l^{-1}), plants regenerated vigorously at a high level. Buds formed in a medium with 0.01 mg·l^{-1} of IAA, but not with high concentrations. NAA induced whole plant regeneration even though it had no effect on additional bud formation.

All concentrations of GA_3 had an effect on whole plant regeneration. In a medium with a concentration of 0.01 mg·l^{-1} BAP whole plant regeneration appeared. However, neither bud nor root differentiation occurred in media with other concentrations of BAP. Whole plant regeneration was observed in kinetin concentrations ranging from 0.01 to 0.1 mg·l^{-1}. In contrast, no regeneration was found with a higher concentration of this cytokinin.

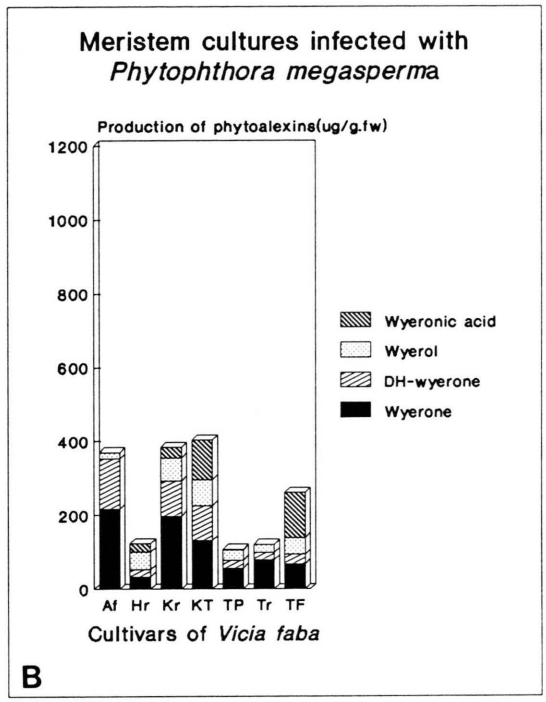
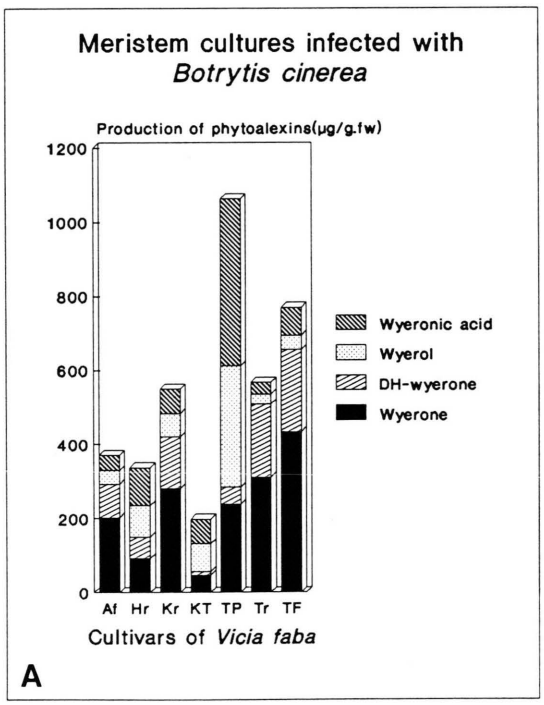
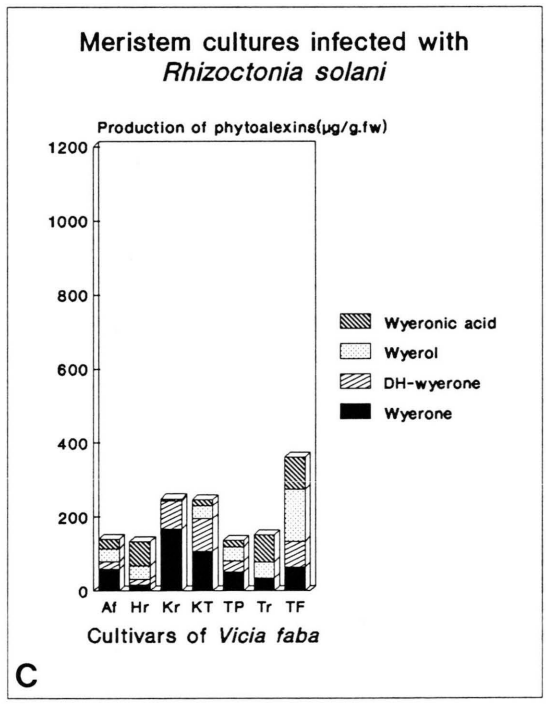


Fig. 1. Phytoalexin production in meristem cultures of faba beans 6d after infection.
A: infected with *B. cinerea;* B: infected with *P. megasperma;* C: infected with *R. solani; Vicia faba* cultivars: Af = Alfred, Hr = Herra, Kr = Kristall, KT = Kleine Thüringer, TP = TP667, Tr = Troy, TF = Tannin Free.





The combination of 0.01 mg·l^{-1} 2,4D and 0.1–0.5 mg·l^{-1} GA_3 induced whole plant regeneration. Regeneration was not found in media with 2,4D and cytokinins. A combination of IAA and kinetin had no detectable effect on plant regeneration, whereas in a medium with IAA and BAP or GA_3 plants did regenerate. The best result of whole plant regeneration was obtained with the combination of NAA and kinetin or GA_3.

Infection of Vicia faba cultivars by fungi

Phytoalexin accumulation after infection by *B. cinerea*

Wyerone was found at the highest level in *cv.* T.F. and was also observed in *cvs.* Troy, TP667 and Kristall. Only cultivar TP667 produced a high concentration of wyeronic acid and wyerol. The highest total concentration of phytoalexins was detected in *cv.* TP667 and *cv.* T.F. (Fig. 1,A). In contrast, low concentrations were observed in *cv.* Kleine Thüringer.

Phytoalexin accumulation after infection by *P. megasperma*

Wyerone was detected in high concentrations in *cv.* Kristall and *cv.* Alfred, up to 200 µg/g·fresh weight (Fig. 1,B). All cultivars produced a low level of wyerol. Wyeronic acid was absent in *cv.* Alfred and only a little was found in *cv.* TP667 and *cv.* Troy. A high accumulation of combined phytoalexins was recorded in *cvs.* Alfred, Kristall and Kleine Thüringer whereas cultivars TP667, Troy and Herra accumulated only low concentrations.

Phytoalexin accumulation after infection by *R. solani*

Cultivar T.F. responded with a high production of phytoalexins whereas a low level was found in *cvs.* Alfred and TP667 (Fig. 1,C). Cultivar Kristall produced a high yield of wyerone. Wyerol was detected at high levels in *cv.* T.F., but was absent in *cv.* Kristall and present only in small amounts in *cvs.* Alfred, TP667 and Kleine Thüringer.

Fungal resistance of the regenerated plants

No cultivar of *Vicia faba* tested in this study was resistant to *B. cinerea.* Six days after infection with *B. cinerea* all regenerated plants gradually turned black and died. The results of the responses of the regenerated plants to all fungi tested are given in Table II. Cultivars Alfred and Herra were less resistant than other cultivars. A high level of resistance was found in *cv.* Kristall infected with *R. solani,* whereas a low level against *P. megasperma* was observed. All cultivars except *cv.* Alfred were resistant to *P. megasperma.*

Table II. Resistance of different cultivars of faba beans regenerated from meristem cultures (% surviving plants).

Cultivars	Infecting fungi		
	B. cinerea	*P. megasperma*	*R. solani*
TP667	0	20	44
Kleine Thüringer	0	11	44
Kristall	0	22	66
Alfred	0	0	25
Herra	0	12	25
Troy	0	44	37
T.F.	0	25	44

Results 6d after infection.

Ethylene production was studied using the regenerated plants from meristem cultures infected with *B. cinerea.* After 6 d of infection with *B. cinerea* all regenerated plants gradually turned black and died. Their ethylene production increased from 5 d to 16 d

Fig. 2. Resistance of regenerated plants from meristem cultures of faba beans 2 weeks after infection.
A: *cv.* Troy plant infected with *P. megasperma.* B: *cv.* Kristall infected with *R. solani.* C: *cv.* TP667 after a 4 week culture on B5 medium with 0.01 mg·l^{-1} of 2,4D (control).

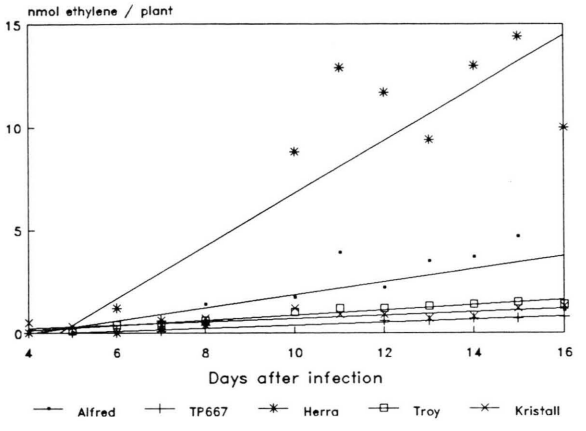


Fig. 3. Ethylene production of *Vicia faba* cultivars regenerated from meristem cultures, infected by *B. cinerea.*

after infection (Fig. 3). The highest response was found in *cv.* Herra.

The total concentration of phytoalexins in meristem cultures of the faba bean infected with fungi varied between 100–1100 μg/g fresh weight (Fig. 1, A). In infected cotyledons the phytoalexin concentration can also reach up to 1200 μg/g fresh weight [15, 16]. In calli of faba beans 10–150 μg/g fresh weight were observed [4]. Cain *et al.* [17] found 386 μg/g fresh weight of wyerone in the cotyledon of the faba bean infected by *B. cinerea,* compared to 420 μg wyerone per g tissue we obtained from the meristem culture of *cv.* TP667 infected by the same fungus. A large difference in wyeronic acid accumulation was observed with 450 μg/g fresh weight after *B. cinerea* infection in *cv.* TP667 and less than 30 μg/g fresh weight in *cv.* Alfred.

Wenzel [1] observed resistance of suspension cultures of soybean calli against *Phytophthora megasperma* and calli of potatoes were resistant to *P. infestans* [19]. Deaton *et al.* [20] found resistance to *P. parasitica* in calli of *Nicotiana longiflora.* In the present study, no resistance against *B. cinerea* was observed. However, most regenerated plants derived from meristem cultures were resistant to *R. solani.*

Generally, regenerated plants, infected with *B. cinerea* produced a high level of phytoalexins and had no resistance. The regenerated plants had the highest resistance and accumulated the smallest amounts of phytoalexins when infected with *R. solani.* Resistance ability and phytoalexin production were in between in *P. megasperma* infected plants. In addition, the responses of the seven cultivars of *Vicia faba* tested show marked differences in the total amount of phytoalexins and also in the ratio of wyeronic acid, wyerol, DH-wyerone and wyerone. A different accumulation of these four components of furanoacetylene phytoalexins is apparently under genetical control of the plant cultivar, since the plants were grown under identical conditions and not affected by other stress factors or microorganisms. The method developed allows the plant breeder to study resistance characters of new plant cultivars with very small number of new seeds and meristem cultures, multiplied during a few weeks.

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