Phytochemistry, Vol. 24, No. 6, pp. 1193–1195, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

ACCUMULATION OF PHYTUBERIN AND PHYTUBEROL IN TOBACCO CALLUS INOCULATED WITH *PSEUDOMONAS SOLANACEARUM* OR *PSEUDOMONAS SYRINGAE* PV. *TABACI*

HIROSHI TANAKA and TAKANE FUJIMORI*

The Utsunomiya Tobacco Experiment Station, The Japan Tobacco & Salt Public Corporation, Oyama, Tochigi 323 Japan; *Central Research Institute, The Japan Tobacco & Salt Public Corporation, 6-2 Umegaoka, Midori-ku, Yokohama 227, Japan

(*Revised received* 6 *September* 1984)

Key Word Index—*Nicotiana tabacum*; Solanaceae; callus; stress compounds; phytuberin; phytuberol; *Pseudomonas solanacearum*; *Pseudomonas syringae* pv. *tabaci*.

Abstract—Tobacco callus tissues inoculated with *Pseudomonas solanacearum* or *Pseudomonas syringae* pv. *tabaci* accumulated phytuberin and phytuberol. Accumulation of the two sesquiterpenoids was dependent upon: the source (cultivar) of the explant; the number of transfers; the period which had elapsed after transfer; the bacterial species, and the time after inoculation.

INTRODUCTION

Fifteen sesquiterpenoid stress compounds have been isolated from diseased and stressed tobacco leaves: capsidiol, glutinosone, 3-hydroxysolavetivone, lubimin, occidenol, occidentalol, occidol, occidol isomer-1, occidol isomer-2, occidol acetate, phytuberin, phytuberol, rishitin, solanascone and solavetivone [1–3]. Four of these are established as stress compounds in the callus tissues of *Nicotiana tabacum*. Thus capsidiol and rishitin are produced on infection with *Phytophthora parasitica* var. *nicotianae* [4, 5], and phytuberin and phytuberol on infection with *Pseudomonas solanacearum* [6].

In this paper, we report the accumulation of phytuberin and phytuberol in tobacco callus after inoculation with *Pseudomonas solanacearum* or *Pseudomonas syringae* pv. *tabaci*, the pathogens of bacterial wilt and wild fire of tobacco, respectively.

RESULTS AND DISCUSSION

Phytuberin was detected in callus tissues from both air-cured cultivars [Burley 21 (B21) and Mito 3 (M3)] and flue-cured cultivars [Bright Yellow 4 (BY4) and Coker 319 (C319)] after inoculation with *P. solanacearum* U-7 (U-7). The amounts of phytuberin in callus tissues from air-cured cultivars were larger than those from flue-cured cultivars. Phytuberol was detected only in callus from B21 and M3 and the ratios of phytuberin to phytuberol were almost the same (10.0 and 11.7) (Table 1).

Production of phytuberin was affected by the number of times the callus tissue had been subcultured. Thus callus tissues from B21 subcultured more than 10 times, produced 6.7 times more phytuberin on inoculation with *P. solanacearum* M23R (M23R) than the callus subcultured twice prior to induction of phytoalexin accumulation by inoculation with M23R. More than three times more phytuberin was also detected in callus tissues from BY4 subcultured more than 10 times than those subcultured twice by inoculation with M23R.

Production of phytuberin and phytuberol was also affected by the growth periods of the callus tissues. The maximum amount of phytuberin was detected in 5-week-old callus tissues inoculated with U-7. The amount of phytuberol was almost the same at every stage of growth except for 6-week-old tissue (Table 2).

Table 1. Production of phytuberin and phytuberol in tobacco callus from four tobacco cultivars

Cultivar of mother plant*	Stress compounds (μg/g dry wt)†		
	Phytuberin (A)	Phytuberol (B)	(A)/(B)
Bright Yellow 4 (BY4)	7.1	—	
Burley 21 (B21)	38.3	3.83	10.0
Coker 319 (C319)	6.0	—	
Mito 3 (M3)	13.4	1.15	11.7

*Transplanted 7 times after callus induction.

†Measured 2 days after inoculation with *P. solanacearum* U-7 (10^7 cells/ml, 1 ml/flask), —, not detected.

Table 2. Production of phytuberin and phytuberol in tobacco callus from Burley 21 at varying time of incubation after transplant by inoculation with *P. solanacearum* U-7

Time after transplant* (weeks)	Stress compound (μg/g dry wt)†		
	Phytuberin (A)	Phytuberol (B)	(A)/(B)
3	4.3	1.6	2.3
4	8.7	1.8	4.8
5	10.5	1.6	6.4
6	3.5	+	

*The 2nd from callus induction. cv.: B21.
†Measured 4 days after inoculation. +, trace.

The accumulation of phytuberin and phytuberol in tobacco callus (B21), incubated four weeks and subcultured > 10, was measured every half day after inoculation with *P. solanacearum* U-7. Production of phytuberin was already detectable 12 hours after inoculation without any visible symptoms in the callus tissues. Two days after inoculation, when the browning of callus tissues began to be observed, the amount of phytuberin was increased (58 μg/g dry callus). Three days after, the maximum accumulation of phytuberin (188 μg/g dry callus) was almost reached. Four days after inoculation, the production of phytuberin began to decrease with complete browning of the callus tissues (Fig. 1).

The progress of browning agreed with a previous report on inoculation of tobacco callus with *P. solanacearum* [7]. Unlike the study on tobacco leaves inoculated with *Pseudomonas syringae* pv. *lachrymans*, a non-pathogen of tobacco, where the maximum accumulation of sesquiterpenoids was observed within 24 hours after inoculation [1], the maximum accumulation of phytuberin in this study occurred two and a half days after inoculation and phytuberol four days after.

Inoculation with *P. solanacearum* U-7 (virulent isolate [8]) induced almost the same amount of phytuberin as that with *P. solanacearum* M23R (an avirulent mutant form of U-7 [8]) (Table 3). Unlike the inoculation with *Phytophthora parasitica* var. *nicotianae* [9], there was no visible difference in the callus tissues following inoculation with *P. solanacearum* U-7 or M23R. The amounts of phytuberin and phytuberol were almost the same after inoculation with either U-7 or M23R. Although the

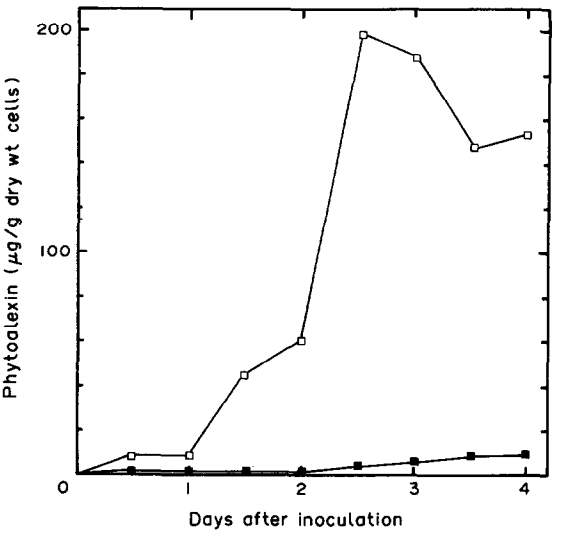

Fig. 1. Accumulation of phytuberin and phytuberol in tobacco callus inoculated with *P. solanacearum* U-7. □, phytuberin; ■, phytuberol.

accumulation of sesquiterpenoids in tobacco leaves may have some relation to the resistant response of the host [1], the results in this study indicate that the accumulation of phytuberin and phytuberol is not the result of resistant response of callus tissues inoculated with *P. solanacearum*.

Callus tissues inoculated with *P. syringae* pv. *tabaci* 6602 (not pathogenic to B21) or 7246 (pathogenic to B21 [10]) produced less than one hundredth of the amount of phytuberin than when inoculated with *P. solanacearum*, but the ratio of phytuberin/phytuberol was lower (Table 3). The amounts of phytuberin and phytuberol were larger in the callus inoculated with 7246 than the callus inoculated with 6602. The ratio of phytuberin to phytuberol in the callus with 7246 was lower than that with 6602. Further investigation will be necessary to establish the pattern of production of phytuberin or phytuberol in respect of wild fire resistance of tobacco callus or plants.

These results indicate that the accumulation of phytuberin and phytuberol in tobacco callus tissues by inoculation with pathogenic bacteria was dependent upon: (1) the source (cultivar) of the explant, (2) the number of

Table 3. Production of stress compounds in tobacco callus inoculated with *P. solanacearum* or *P. syringae* pv. *tabaci*

Inoculum	Stress compounds (μg/g dry wt callus)		
	Phytuberin (A)	Phytuberol (B)	(A)/(B)
P. solanacearum M23R	141	6.7	21
P. solanacearum U-7	159	6.8	23
P. s. pv. *tabaci* 6602	0.49	0.24	2.0
P. s. pv. *tabaci* 7246	1.3	0.41	0.32

Each bacterial suspension was inoculated onto tobacco callus (from cv. B21 transplanted > 10) and the amounts of stress compounds measured 4 days after inoculation.

transfers of the callus after induction, (3) the period of incubation after transfer to new medium, (4) time after inoculation, and (5) the species of the inoculating bacterium.

EXPERIMENTAL

Callus tissues were induced from the pith of tobacco plants of each cultivar as reported previously [6]. They were subcultured by transferring a tiny piece of callus tissues (*ca* 0.5 mm in diameter) to new medium every 4 weeks unless specified.

Pseudomonas solanacearum U-7 and M23R were grown on Kelman's TZC medium [11] and *Pseudomonas syringae* pv. *tabaci* 6602 and 7246 on Kelman's CPG medium [11] at 27° for 2 days. Inocula were prepared by suspending the bacteria in sterilized H_2O (10^7 cells/ml).

Unless specified, 10 flasks of 4-week-old callus tissues were inoculated by dropping the bacterial suspension (1 ml/flask) and incubated at 27° in the dark. At the appropriate times after inoculation, the callus tissues were harvested and freeze-dried. The dried material was extracted with CH_2Cl_2 and the extract analysed by capillary GC and GC-MS. The presence of phytuberin and phytuberol was established by comparison with authentic samples as reported previously [6]. The amounts of phytuberin and phytuberol were measured by comparison with a standard soln of authentic samples by GC (glass capillary column of OV-101 50 m × 0.27 mm, 150–240°, 2°/min.)

Acknowledgement—We are grateful to Dr. K. Ono, The Okayama Tob. Exp. Sta., for kindly providing the isolates of *Pseudomonas syringae* pv. *tabaci*.

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