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Modification of Disease Resistance of Tobacco Callus Tissues by Cytokinins[1]

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ABSTRACT

The effects of differing cytokinin and auxin concentrations on resistance of tobacco (*Nicotiana tabacum* L.) tissue cultures to race 0 of *Phytophthora parasitica* var. *nicotianae* were examined. With 1 micromolar kinetin and either 11.5 micromolar indoleacetic acid or 1 micromolar 2,4-dichlorophenoxyacetic acid, tissues from resistant cultivars exhibited a "hypersensitive" reaction to zoospores of the fungus and subsequently were colonized only slightly. With susceptible cultivars or with tissues from resistant cultivars supplied with higher cytokinin levels (*e.g.* 10 micromolar kinetin), this hypersensitive reaction did not occur and tissues were heavily colonized. Benzylaminopurine and kinetin were particularly effective in eliminating both the hypersensitive reaction and disease resistance. Zeatin and 6-(3-methyl-2-butenylamino)purine were less effective. Increases in indoleacetic acid levels reversed the effects of high cytokinin concentrations. The balance of phytohormones apparently controls the host response to the fungus; thus, in this system, resistance or susceptibility can be studied without changing either host or fungal genotype.

Previously, we reported (5) that a single, dominant gene for disease resistance of tobacco (*Nicotiana tabacum* L.) is expressed in tissue cultures. Rooted cuttings and pith callus tissues from 185 plants of parental, F_1, F_2, and F_3 progeny were compared directly for their resistance to race 0 of *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker, the causal agent of the black shank disease of tobacco. In each case, resistant plants yielded only resistant callus and susceptible plants yielded only susceptible callus. These results indicated that the tissue culture system can be used for the study of physiological and biochemical events associated with the expression of disease resistance in plants.

During development of this model system, we noted that the tissue morphology, incubation temperature, and phytohormone concentrations in the medium quantitatively affected the amount of tissue colonization (6). In particular, increasing the cytokinin concentration appeared to increase the rate and the extent of colonization of the resistant genotype by the fungus. It seemed possible that resistance of the resistant genotype might be controlled experimentally by the hormonal regime. If so, then both resistance and susceptibility could be studied without changing the genotype of either host or pathogen. A preliminary account of this work has been presented (4).

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MATERIALS AND METHODS

Two plants, one homozygous resistant (designated line 46–8) and the other homozygous susceptible (designated line 49–10), were used as parental materials in earlier studies (5, 6) and have since been maintained clonally. The susceptible parent (49–10) served as the source of susceptible callus tissues for this study. A heterozygous resistant plant (designated line 1–4) derived from a cross between the two parents, was used as the source of resistant callus. This line was used because it maintained a "tight" morphology after one passage on 0.1 μM kinetin (as did 49–10). The isolate of race 0 of Ppn[3] (designated 1156) was used exclusively for these experiments (6).

Pith tissues were isolated from the source plants and cultured on Linsmaier and Skoog medium (8) containing 11.5 μM IAA and 1 μM kinetin. Stock tissues were maintained on this medium by repeated transfers at 5-week intervals. Prior to experiments, tissues were subcultured for 4 weeks on the experimental hormonal regimes.

The procedures for maintaining fungal cultures, obtaining zoospores, and inoculating tissues were as described previously (5, 6). Callus tissue colonization was rated by a numerical system (6). Values assigned were: 0 = no visible fungus on tissue or medium surrounding the callus piece; 1 = fungus on the medium surrounding the piece, but no visible aerial mycelium growing on the piece; 2 = aerial mycelium growing on the piece, but not completely covering the piece; 3 = aerial mycelium completely covering the outside of the piece (see Fig. 2 of ref. 5 for the appearance of the tissues). Typically, five or six Petri plates, each containing six pieces of callus, were used for each treatment. Numerical ratings usually were made at 2- or 3-day intervals from 3 to 14 days after inoculation. Each piece was rated separately and the reported values are the averages for each treatment (±SE).

RESULTS

Comparison of Stock Callus and Newly Isolated Pith Callus Tissues. In our comparisons of intact plant and tissue culture responses to Ppn, we used pith tissue isolated from stems, cultured for 28 days, and then inoculated. For the experiments reported here, we used uniform stock tissues that had gone through one passage on the experimental hormonal regimes before inoculation. The responses of stock and newly derived callus tissues of a given genotype were identical, even though the stock callus had been in culture for over 2 years (Fig. 1). Typically, tissues from the resistant plant showed little or no fungal colonization and susceptible tissues were heavily colonized when grown on our standard regime of 1 μM kinetin and 11.5 μM IAA.

Effect of Kinetin Concentration on Resistance. When the ki-

[3] Abbreviations: Ppn: *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker; HR: hypersensitive reaction; 2ip: 6-(3-methyl-2-butenylamino)purine.

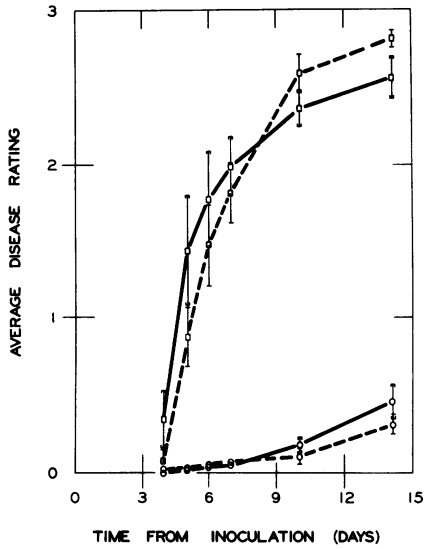

FIG. 1. Disease ratings followed inoculation with race 0 of *P. parasitica* var. *nicotianae* of stock tobacco callus (——) and newly derived pith callus (– – –) tissues grown on Linsmaier and Skoog medium containing 1.0 μM kinetin and 11.5 μM IAA. Genotypes are susceptible (□) and resistant (○). Each datum represents an average of at least three experiments.

netin concentration was increased from 1 to 10 μM (and the auxin concentration kept at 11.5 μM IAA), the difference in colonization of callus from the two genotypes was reduced greatly (Fig. 2). For example, the average 10-day rating of the resistant genotype increased from a value of 0.2 ± 0.2 to 2.4 ± 0.3. This increase represented a change from an average of one piece out of five showing some fungal mycelium on the medium surrounding the piece to aerial mycelium growing on each piece.

The appearance of tissues grown at the two cytokinin concentrations differed strikingly soon after inoculation. By the 1st day after inoculation, necrotic tissue appeared in the inoculated area of resistant tissues supplied with 1 μM kinetin and 11.5 μM IAA (Fig. 3A). We interpreted this change to be a hypersensitive reaction. In contrast, inoculated tissues supplied with high cytokinin levels or tissue from the susceptible genotype showed general water-soaking and a gold-brown coloring (Fig. 3A). After 7 days, the extent of fungal colonization was the differentiating feature;

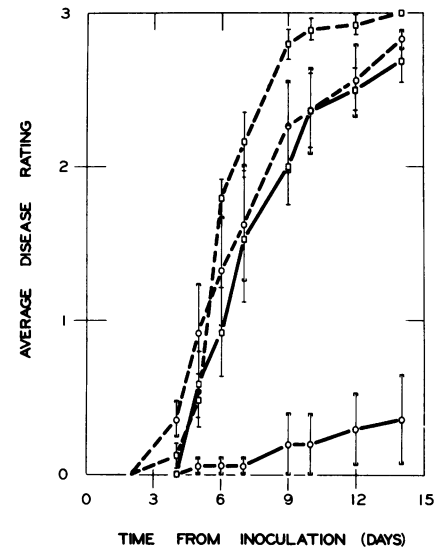

FIG. 2. Disease ratings following inoculation with race 0 of *P. parasitica* var. *nicotianae* of callus tissue from resistant (○) and susceptible (□) genotypes grown on Linsmaier and Skoog medium containing 11.5 μM IAA and either 1.0 μM (——) or 10.0 μM (– – –) kinetin.

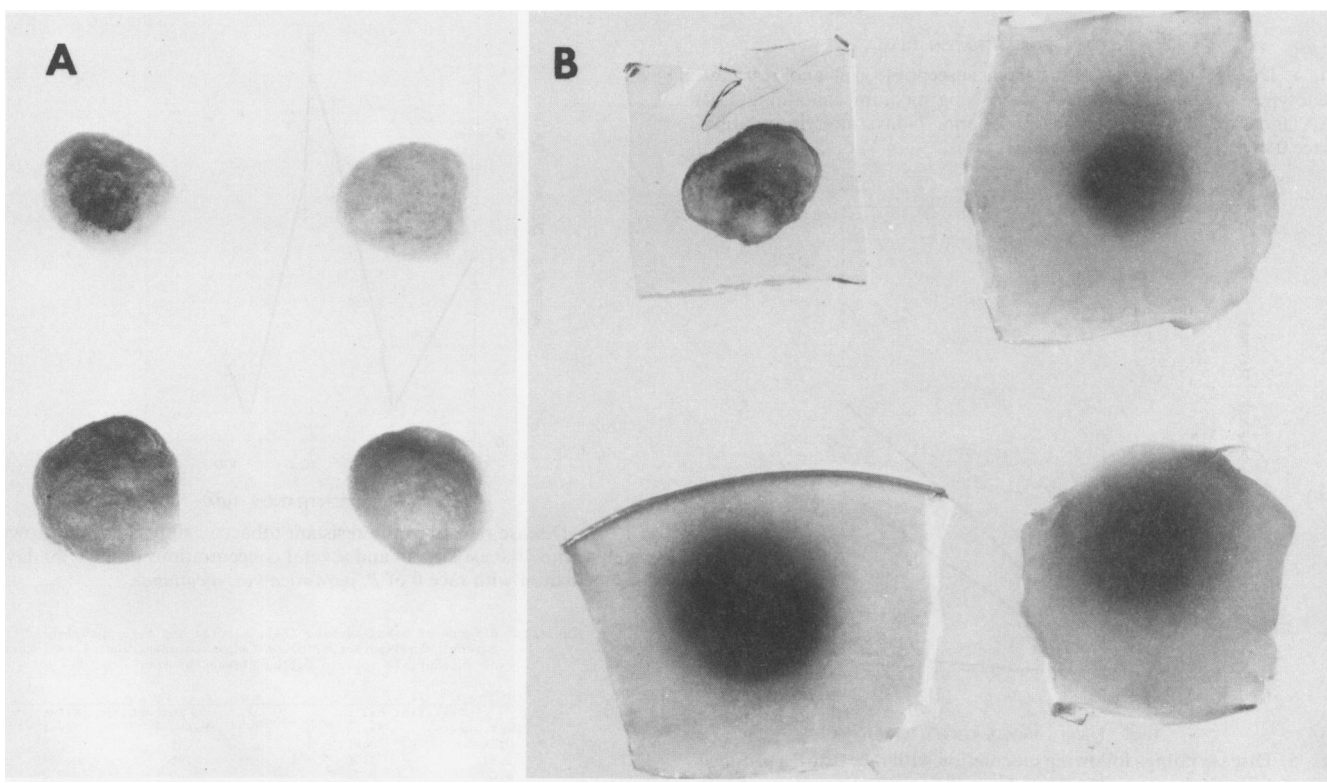

FIG. 3. Appearance of tissues 18 hr (A) or 7 days (B) after inoculation with race 0 of *P. parasitica* var. *nicotianae*. In each figure the tissues are (clockwise from top left): resistant tissues on 1 μM kinetin, resistant tissues on 10 μM kinetin, susceptible tissues on 10 μM kinetin, and susceptible tissues on 1 μM kinetin. All media contained 11.5 μM IAA.

only those tissues which did not show the intense HR were heavily colonized (Fig. 3B).

When the kinetin concentration was varied from 0.125 to 10 μM and the IAA concentration was kept at 11.5 μM, the results shown in Figure 4 were obtained. Tissues from the resistant genotype remained resistant on kinetin concentrations from 0.125 to 2 μM. Then, loss of resistance became more pronounced as kinetin concentrations were increased. Tissues from the susceptible cultivar remained susceptible on all kinetin regimes.

Effect of Variation in Auxin Concentration. Although an increase in tissue colonization was obtained with 11.5 μM IAA and 5 μM kinetin, when IAA concentrations were increased to 17.55 or 23 μM, the effect of 5 μM kinetin was reduced greatly (Fig. 5). These results suggested that the balance of cytokinin and auxin was an important factor in maintaining or eliminating resistance of the tissues. This point was examined in detail with tissues grown with 10 μM kinetin and various IAA concentrations from 0.115 to 115 μM (Fig. 6). As expected, colonization of tissues was very extensive with 10 μM kinetin and 11.5 μM IAA. Tissue colonization decreased with auxin concentrations above 23 μM. In addition, a substantial reduction in tissue colonization was observed when auxin concentrations were decreased below 5.75 μM. Also, the vigor of the tissues grown at low IAA regimes was substantially reduced.

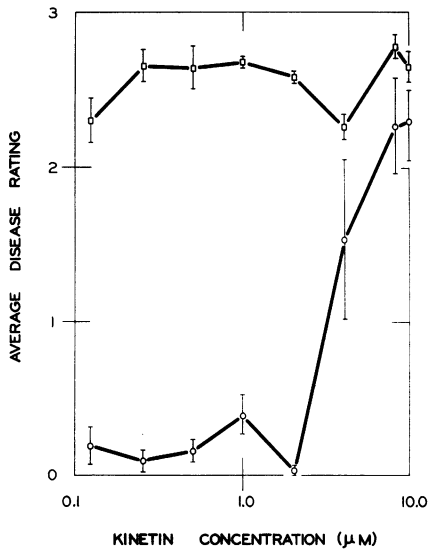

FIG. 4. Disease ratings of callus from susceptible (□) and resistant (○) genotypes grown on Linsmaier and Skoog medium containing 11.5 μM IAA and several concentrations of kinetin, 7 days after inoculation with race 0 of *P. parasitica* var. *nicotianae*.

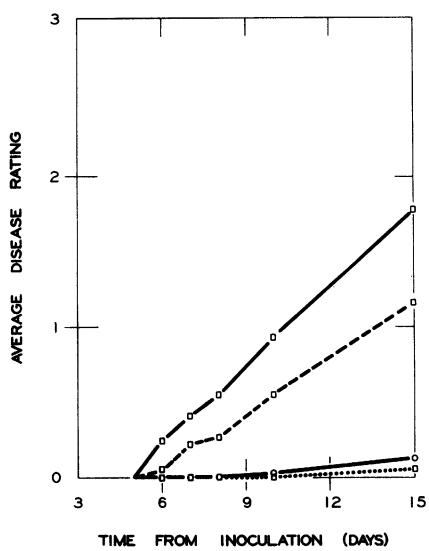

FIG. 5. Disease ratings following inoculation with race 0 of *P. parasitica* var. *nicotianae* of tobacco callus tissue from the resistant genotype grown on 11.5 μM IAA (——) and 1 μM (○) or 5 μM (□) kinetin. (– – –), and (· · · ·): ratings from tissues grown on media containing 5 μM kinetin and either 17.5 or 23 μM IAA, respectively.

Effect of Substituting 2,4-D for IAA. The experiments reported above were also done with 1 μM 2,4-D. In each case, the results of varying concentrations of cytokinins with constant 1 μM 2,4-D were similar to those obtained with constant 11.5 μM IAA (Table I).

However, when 2,4-D concentrations were increased, the effects of higher cytokinin levels were not reversed (data not shown). In fact, tissues of the resistant genotype supplied with 10 μM kinetin and as high as 10.0 μM 2,4-D remained susceptible. These results were not unexpected because of the observation by Witham (12) that high levels of 2,4-D but not of IAA supported growth of tissues in the absence of exogenously supplied cytokinin.

Effects of Other Cytokinins. To test whether resistance could be modified by cytokinins other than kinetin, 6-(3-methyl-2-butenylamino)purine (2ip) and BA were used in comparative trials (Table I). BA at 10 μM was equally as effective as kinetin in eliminating resistance. However, 2ip did not alter tissue colonization. In another experiment, even 25 μM 2ip did not alter resistance. In other experiments, zeatin at 10 μM appeared to be intermediate between kinetin and 2ip in its ability to modify colonization rates.

Tests with Additional Callus Lines. High concentrations of kinetin (with 1 μM 2,4-D) also eliminated resistance of callus from the homozygous resistant parent. For example, in five experiments the average numerical ratings after 10 days were 0.32 ± 0.12 and 1.82 ± 0.49 with 1 μM and 10 μM kinetin, respectively. Unlike the

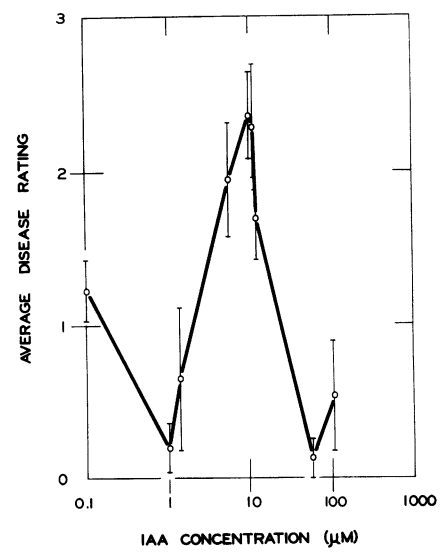

FIG. 6. Disease ratings of the resistant tobacco callus genotype grown on media with 10.0 μM kinetin and several concentrations of IAA, 10 days after inoculation with race 0 of *P. parasitica* var. *nicotianae*.

Table I. Effects of benzyladenine (BA), kinetin and 6-(3-methyl-2-butenylamino)-purine (2ip) on fungal colonization of resistant or susceptible tobacco callus tissues by Ppn.

Cytokinin		Resistant callus		Susceptible callus	
		Expt. 1[1]	Expt. 2[2]	Expt. 1	Expt. 2
BA	1 μM	0.16	0.60	2.91	2.80
	10 μM	1.91	3.00	2.96	2.79
Kinetin	1 μM	0.00	0.76	2.65	2.26
	10 μM	2.18	2.76	2.98	2.83
2ip	1 μM	0.31	0.00	1.94	1.76
	10 μM	0.31	0.00	1.86	2.00

[1]Average numerical ratings of callus pieces 9 days after inoculation
[2]Average numerical ratings 10 days after inoculation

49–10 and 1–4 lines used above, the 46–8 tissue became friable after only one cycle at cytokinin concentrations below 1 μM. As shown previously (6), this morphological change resulted in heavy colonization of this tissue.

To test whether the cytokinin modification of resistance was peculiar to the three lines tested above, we examined several of the F_1, F_2, F_3 and backcross plant lines developed previously (5) and since maintained clonally. In each case, a kinetin concentration of 10 μM substantially increased colonization of tissues from the resistant genotype.

DISCUSSION

We showed previously (5, 6) that tobacco tissue carrying the gene for resistance to race 0 of Ppn were not colonized extensively by race 0, but tissues without this gene were. In addition, we have shown that race 1 of the fungus, against which the resistance gene is ineffective in intact plants, can colonize both genotypes of callus tissues. Thus, resistant or susceptible reactions could be obtained by the appropriate selection of host genotype or fungal race. However, some change of either fungal race or host genotype was required in order to vary the disease reaction. The findings presented here provide a new dimension to study resistance in this model system. By controlling the cytokinin concentration, we can obtain either resistant or susceptible reactions with the same host genotype and fungal race combination.

When roots of resistant tobacco plants are penetrated by Ppn, they rapidly undergo the HR in response to the invading pathogen (3). Further growth of the fungal hyphae is restricted. In contrast, susceptible cultivars do not exhibit the HR when challenged by the fungus, and hyphal growth proceeds rapidly. We suggest that the rapid necrosis appearing below the point of inoculation of resistant tissue cultures (Fig. 3A) is a reaction similar to the HR reported in intact roots.

Dropkin *et al.* (1) noted that resistant tomato roots supplied with exogenous cytokinins no longer underwent the HR in response to the root knot nematode, *Meloidogne incognita.* Similarly, Novacky (9) reported that the HR of tobacco leaves to incompatible bacteria could be eliminated by addition of kinetin. Our results indicate that the HR also can be prevented in callus tissues supplied with high cytokinin levels. Coincident with the prevention of the HR is a marked loss of resistance (Fig. 3B).

There are several structurally related cytokinins that differ in their relative activity in various bioassays. In the promotion of cell division, zeatin and 2ip are clearly more active than either kinetin or BA (7, 10). However, in the prevention of senescence, BA and kinetin are considerably more active than zeatin or 2ip (2, 7, 11). The relative effectiveness of various cytokinins in eliminating resistance in tissue cultures appears to parallel their effectiveness in preventing senescence.

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