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Superoxide release is necessary for phytoalexin accumulation in *Nicotiana tabacum* cells during the expression of cultivar-race and non-host resistance towards *Phytophthora* spp.

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Abstract

The relationship between superoxide radical generation, the accumulation of the phytoalexin capsidiol and hypersensitive cell death has been examined in *Nicotiana tabacum* following challenges by compatible and incompatible races of *Phytophthora nicotianae*, and the non-host species *Phytophthora palmivora*. Challenging suspension cell cultures of *N. tabacum* with zoospores of incompatible isolates of *P. nicotianae* elicits a biphasic burst of superoxide release. The maximum rate of capsidiol accumulation between 9 and 12 h after challenge coincides with the second oxidative burst and the onset of hypersensitive cell death. Addition of superoxide dismutase or Mn (III) desferal, which scavenge superoxide anions and quench the superoxide burst, suppresses both phytoalexin accumulation and hypersensitive cell death. Mevastatin, an inhibitor of the sesquiterpenoid biosynthesis enzyme HMG-CoA reductase, has no effect on the oxidative burst or hypersensitive cell death, but abolishes capsidiol accumulation. Zoospores of the non-host pathogen *P. palmivora* also elicit superoxide release, but in a single, broad burst between 3 and 12 h after challenge. Capsidiol accumulates to levels similar to those seen in incompatible host reactions, although the onset of capsidiol accumulation is more rapid in the non-host interaction. As in the incompatible interaction, phytoalexin accumulation and hypersensitive cell death are both inhibited by superoxide scavengers, although scavenging does not render host cells susceptible to infection by non-host zoospores. Our findings indicate that phytoalexin accumulation and hypersensitive cell death in both incompatible and non-host interactions are regulated by pathways that diverge downstream of superoxide release. While hypersensitive cell death and phytoalexin accumulation appear to be necessary for gene-for-gene resistance, other factors cause non-host infection to fail.

Keywords: *Nicotiana tabacum*; *Phytophthora nicotianae*; *Phytophthora palmivora*; Capsidiol; Resistance; Hypersensitive cell death

1. Introduction

Disease resistance in plants results from passive and active factors that disrupt the disease cycle. Once a potential pathogen has breached preformed defence barriers, reactive oxygen species are released in an 'oxidative burst' in resistant genotypes of many host species [1,29,46,50]. Neighbouring cells also synthesise and accumulate phytoalexins and oxidised phenolics with antibiotic activity, and deposit cell wall reinforcements [44]. A feature of disease resistance in many plants is the rapid collapse and death of challenged cells in the hypersensitive response, a form of programmed cell death in plants [8,19,23,33]. Simultaneously, signals are released and translocated throughout the plant, activating systemic responses that serve to increase the resistance of the plant to subsequent pathogen attack [20,23,38,42,44]. It is likely that during the coevolution of plants and pathogens multiple recognition and response pathways have evolved, and in a number of plant–pathogen interactions it has been shown that once elicited, the resulting downstream components of the response appear to be independent of each other [9,11,23,32,36,48]. One consequence of this is that elicitors fractionated from pathogens may elicit only part of the response elicited by intact pathogen propagules [16,45].

One feature common to the responses of plant, animal and microbial cells to pathogens is the presence of an oxidative burst early in the response cascade [26,29]. We have shown that release of the superoxide radical ($O_2^{\cdot-}$),

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and its protonated form the perhydroxyl radical (HO_2), is a consistent feature of gene-for-gene resistance in *Nicotiana tabacum* L. plants and cell cultures challenged by zoospores of *Phytophthora nicotianae* Breda de Haan. Superoxide release is first observed between one and 2 h following challenge by incompatible pathogen races. A second, major extracellular burst of superoxide coincides with hypersensitive cell death [1,35], and several experimental approaches indicate a link between superoxide release and hypersensitive cell death. Exogenous scavengers of superoxide radicals inhibit the hypersensitive response [1]. Temporary hypoxia around the time of the major oxidative burst suppresses both superoxide release and hypersensitive cell death, and induces susceptibility in *N. tabacum* and *Glycine max* cells challenged by normally incompatible pathogen isolates [35]. Furthermore, *lsd* and *acd* mutants of *Arabidopsis thaliana*, which are deficient in superoxide dismutase (SOD) and as a result are unable to remove even basal levels of superoxide, develop hypersensitive-like lesions in the absence of pathogen challenge [28,49]. The SOD-mediated dismutation of superoxide appears to be the major source of hydrogen peroxide in *P. nicotianae*-challenged *N. tabacum* suspension cells [2,3] Superoxide also interacts with another reactive oxygen species, nitric oxide (NO), and although the precise interaction remains unclear, it appears that NO may have a dual role as either an antagonist or agonist, depending on the relative timing and intensity of superoxide and NO release [10,31].

In addition to its role in hypersensitive cell death, Doke has shown that superoxide elicits phytoalexin accumulation in potato tuber slices [12,13,14]. The major phytoalexin produced by *N. tabacum* in response to challenge by *P. nicotianae* and other pathogens is the sesquiterpene capsidiol [6,25,37]. We have previously shown that a competitive inhibitor of the biosynthetic enzyme HMG-CoA reductase, mevinolin (6α methyl compactin), abolishes capsidiol accumulation and disease resistance in *N. tabacum* stems, suggesting that this phytoalexin is important in restricting infection by incompatible races of *P. nicotianae* [37]. However, as HMG Co-A reductase is the key regulatory enzyme in terpenoid biosynthesis, the possibility that it affects other, unidentified, terpenoid components of resistance cannot be excluded. Other approaches using host and pathogen mutants have been no more successful in precisely defining the role of phytoalexins in disease resistance, and although a substantial body of evidence correlates rapid phytoalexin accumulation with disease resistance a causative role is yet to be unequivocally established [22].

Non-host resistance, the observed outcome for the vast majority of plant-microbe interactions, renders plants outside the host range of the pathogen unsuitable as hosts. Non-host resistance may arise from a failure of the recognition and pathogenesis mechanisms of the pathogen, or from developmental, morphological or physiological factors already present in the plant at the time of microbial encounter. However, there are many instances where pre-existing factors are supplemented by an active response by the plant, indicating some early recognition process has taken place [4,24]. The degree to which non-host responses mirror those observed in host-cultivar resistance is a matter of some debate. Recently, the responses of resistant tobacco cells towards incompatible races of the pathogen *P. nicotianae* and the responses of the same host genotypes to zoospores of the non-host pathogens *P. palmivora* and *P. cryptogea* were compared [3]. These results indicated that an oxidative burst and hypersensitive cell death still occur, although the timing and degree of these responses to non-host challenge were different.

In this paper we evaluate the significance of phytoalexin accumulation in the cultivar-race responses of tobacco cells to *P. nicotianae* and the relationship of capsidiol production to two previously-studied components of these responses, the oxidative burst and hypersensitive cell death. The generation of phytoalexins during a non-host response induced by challenge with *P. palmivora* has also been examined. In both categories of resistance expression, phytoalexin production and hypersensitive cell death appear to be dependent upon the upstream generation of superoxide ions in signalling pathways.

2. Materials and methods

2.1. Suspension cells

Cultures of the near-isogenic *N. tabacum* cultivars Hicks and NC2326 were established and maintained by weekly transfer into fresh Murashige and Skoog medium, as previously described [1,40]. Four days after transfer, cells to be inoculated were washed twice in 1·0% sucrose in 10 mM phosphate buffer (pH 7·5), and resuspended in the same sucrose/phosphate buffer. One ml aliquots, each containing approximately 0·1 g (fresh weight) cells, were dispensed into 2 ml wells in 24-well tissue culture plates (Greiner #662160), and left for 2 h at 24°C, with shaking at 100 rpm. Viable cells were those able to plasmolyse and concentrate neutral red dye when placed in a hypertonic solution [39]. Cytoplasmic streaming was used to indicate cell viability in the experiment involving mevastatin [35].

2.2. Phytophthora isolates and inoculation

P. nicotianae isolates 4974 (race 0) and 9201 (race 1) were isolated from black shank lesions on field-grown tobacco in Australia. A *P. palmivora* isolate from cocoa was provided from the University of Melbourne collection. Sporangial production was initiated by stripping the mycelium off one week-old oatmeal agar cultures, placing it into a shallow layer of sterile distilled water in a 9 cm Petri dish, and incubating under diffuse light for a further 2–3 weeks. Draining the mycelium then rewetting it with

chilled water triggered zoospore release. Zoospores were collected 30 min later, and then adjusted to $2{\cdot}5 \times 10^3$ ml^{-1}. Each 1 ml aliquot of *N. tabacum* cells was incubated with 1 ml of zoospore suspension and the progress of zoospore motility, encystment, germination and development was monitored by microscopic observation of approximately 200 cells in each replicate [1]. Sterile distilled water was used for sham-inoculated controls.

2.3. Superoxide assays

To visualise the location of superoxide release relative to the point of infection, 200 μl of a 0·5% stock solution of nitroblue tetrazolium chloride (Sigma N-6876) in 10 mM phosphate buffer (pH 7·5) was added to each well, at the appropriate times after zoospore addition. Two 20 μl samples of cells from each well were transferred to microscope slides, and scored for the formation of insoluble NBT formazan between 5 and 30 min following NBT addition. Extracellular superoxide release in the experiment involving mevastatin was monitored using XTT [1].

The superoxide scavengers SOD (SOD; Sigma S-2515) and Mn (III) desferal were included in some experiments by adding 10 μl of stock solutions, freshly prepared in sucrose/phosphate buffer, to final concentrations of 50 SOD equivalent units ml^{-1} [41], to each well at the start of the appropriate experiment. The activities of SOD and Mn (III) desferal were calibrated using a xanthine/xanthine oxidase assay [18].

2.4. Phytoalexin accumulation

At appropriate times after inoculation, 1 ml aliquots of cells and medium were dried overnight at 110°C, weighed, and then extracted twice with 1 ml of ethyl acetate. Filtered ethyl acetate extracts were pooled and evaporated to dryness (Jouan Model RC 10·10 Speed-Vac). The residue was dissolved in 20 μl methanol containing 0·1 mg ml^{-1} methyl myristate as an internal standard for flame ionisation detection on a Hewlett Packard 5890A gas chromatograph using a BP1 capillary column (SGE Aust. P/L, Ringwood; [25]). Concentrations of capsidiol, the major phytoalexin, are given as μg g^{-1} fresh weight. A capsidiol standard was prepared following the ethyl acetate extraction of red capsicums (*Capsicum annuum*) inoculated with *Monilinia fructicola* conidia [30].

Mevastatin (compactin; Sigma M2537), an inhibitor of the key regulatory enzyme in sesquiterpenoid biosynthesis, HMG-CoA reductase, was added to each well to a final concentration of 10 μM 2 h before zoospore addition. At appropriate times after infection, cells and supernatants were processed and assayed for capsidiol accumulation. In addition, 200 cells in each treatment were monitored for cell viability and superoxide release (XTT assay).

3. Results

3.1. Cultivar-race resistance

3.1.1. Capsidiol accumulation

P. nicotianae zoospores swim briefly after being added to *N. tabacum* cells before encysting near clumps of cells. Germ tube growth was strongly directional and appressoria formed on the surface of host cells approximately 4 h after inoculation. In the incompatible interaction between NC2326 and *Pn*4974 zoospores, capsidiol accumulated rapidly between 9 and 12 h after challenge to 88 μg g^{-1} fresh weight and continued to increase to 114 μg g^{-1} fresh weight 24 h after challenge (Fig. 1 and Table 1). During three compatible interactions (Hicks/*Pn*4974, Hicks/*Pn*9201 and NC2326/*Pn*9201) capsidiol concentrations remained below 20 μg g^{-1} fresh weight, and in uninoculated cells of either cultivar capsidiol concentrations remained less than 10 μg g^{-1} fresh weight (Fig. 1).

3.1.2. Effect of superoxide scavengers on capsidiol accumulation

Superoxide dismutase added at the time of zoospore challenge approximately halved subsequent capsidiol accumulation in the incompatible interaction between NC2326 and *Pn*4974 (Fig. 2(a)). Mn (III) desferal had an even more profound effect, reducing capsidiol levels by over 80%, to levels similar to those found in compatible interactions. Eighteen hours after challenge capsidiol levels in the incompatible challenge peaked at 114 μg g^{-1} fresh weight. By contrast, in the incompatible interaction capsidiol levels after 18 h in the presence of SOD were 61 μg g^{-1} fresh weight, and 21 μg g^{-1} fresh weight in the presence of Mn (III) desferal. Neither superoxide scavenger had any effect on the very low levels of capsidiol produced by uninoculated cells or compatible interactions (data not shown).

3.1.3. ROS release and the HR

In the incompatible interaction, two peaks of NBT staining were observed, a minor peak 3 h after challenge (20% of cells), and a major peak 12 h after challenge, by which time 47%, or nearly all remaining viable cells, exhibited formazan formation (Fig. 3(b)). The percentage of cells staining with NBT remained low (less than 3%) in all compatible interactions (Fig. 3(a)), and was not affected by the presence of SOD or Mn (III) desferal (Table 1).

The viability of Hicks cells challenged by zoospores of the compatible isolate *Pn*4974 declined steadily so that 50% of the cells remained viable 19 h after zoospore challenge, and 25% were still viable 24 h after challenge (Fig. 3(a)). Similar trends were observed in the other compatible interactions (Hicks/*Pn*9201 or NC2326/*Pn*9201; data not shown). The viability of incompatibly-challenged cells declined rapidly between 9 and 12 h after zoospore

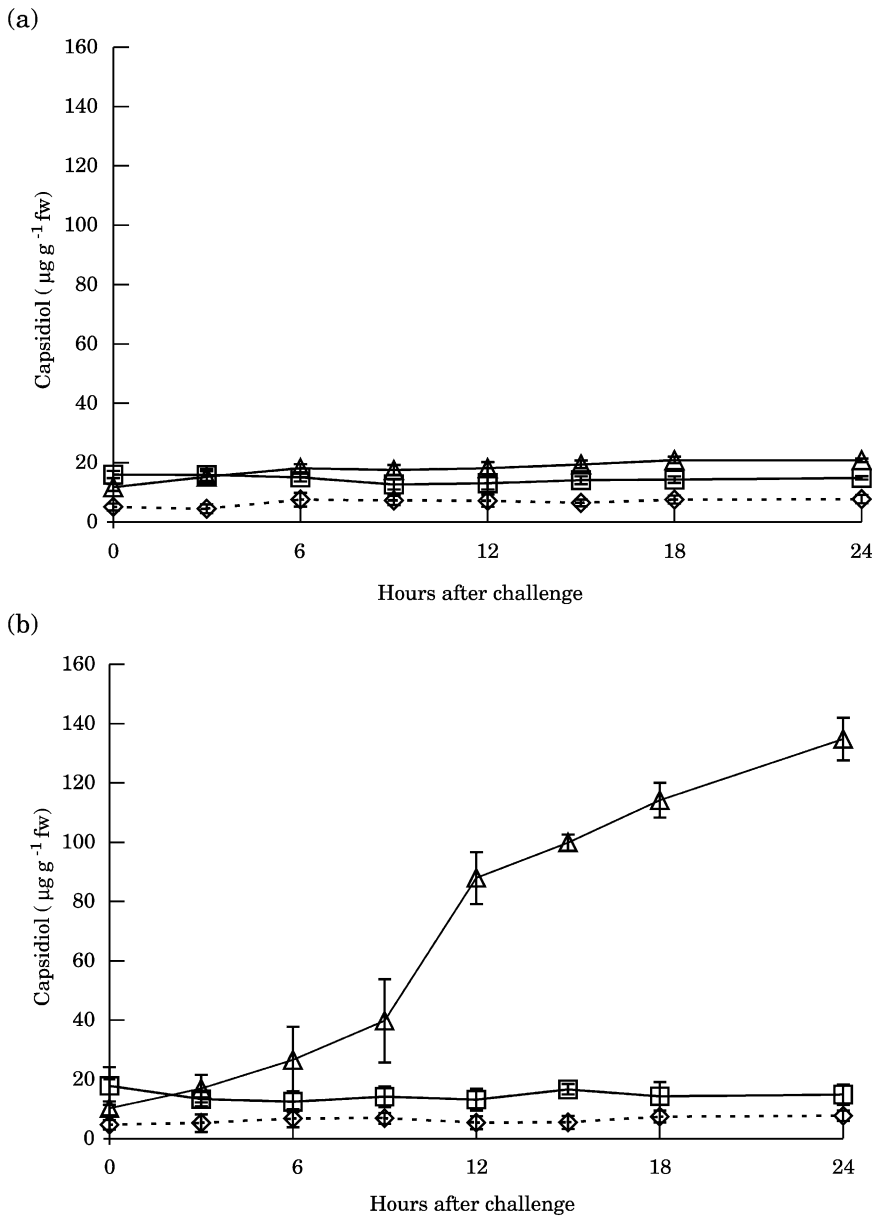

Fig. 1. Capsidiol accumulation in (a) Hicks and (b) NC2326 cells challenged with zoospores of *P. nicotianae* 4974 (-△-), *P. nicotianae* 9201 (-□-), or in uninoculated cells (-◇-). Means and standard deviations were calculated from four replicates.

challenge. Fifty percent of NC2326 cells were dead 12 h after challenge with *Pn*4974 zoospores, and 100% were dead within 24 h (Fig. 3(b)).

3.1.4. Inhibition of capsidiol production

Mevastatin abolished capsidiol accumulation in all treatments, but had no effect on superoxide production or on loss of cell viability in any treatment (Table 1). However, the percentage of NC2326 cells penetrated by the normally incompatible race 0 isolate (*Pn*4974) increased from 8 to 24% in the presence of mevastatin, and these cells became heavily colonised.

3.2. Non-host resistance

Challenge of NC2326 cells (and Hicks cells; data not shown) by zoospores of *P. palmivora* elicited similar levels of capsidiol to those in the NC2326/*Pn*4974 interaction 24 h after zoospore addition (Fig. 3(c)). However, the early pattern of production appears to be subtly different, with both the NC2326 and Hicks non-host reactions to *P. palmivora* eliciting a faster accumulation of capsidiol in the 8 h immediately following challenge, compared to the incompatible NC2326/*P. nicotianae* interaction. The superoxide scavenger Mn (III) desferal almost completely inhibited, while SOD partly inhibited, capsidiol accumulation in the non-host interactions (Fig. 2(b)).

Table 1
Effect of mevastatin on cell viability, superoxide release and capsidiol accumulation in uninoculated Hicks (compatible) and NC2326 (incompatible) suspension cells, and in cells inoculated with zoospores of *P. nicotianae*. Data from four independent experiments was pooled. Means and standard deviations were calculated from observations of 200 cells (viability) or three replicates of 1 ml aliquots of cells (XTT absorbance and capsidiol quantification) for each treatment

	Cultivar	Mevastatin treatment	Viability (% at 10 h)	XTT absorbance (A_{470} at 12 h)	Capsidiol ($\mu g\ g^{-1}$ at 24 h)
Uninoculated	Hicks	Untreated	87 ± 4	0·16 ± 0·02	nd
		Mevastatin	80 ± 4	0·14 ± 0·01	nd
	NC2326	Untreated	76 ± 4	0·19 ± 0·02	nd
		Mevastatin	81 ± 3	0·19 ± 0·02	nd
Inoculated	Hicks	Untreated	81 ± 4	0·15 ± 0·02	29 ± 3
		Mevastatin	73 ± 4	0·13 ± 0·01	nd
	NC2326	Untreated	16 ± 1	0·57 ± 0·03	141 ± 5
		Mevastatin	18 ± 2	0·68 ± 0·04	nd

nd, not detected ($<10\ \mu g\ g^{-1}$).

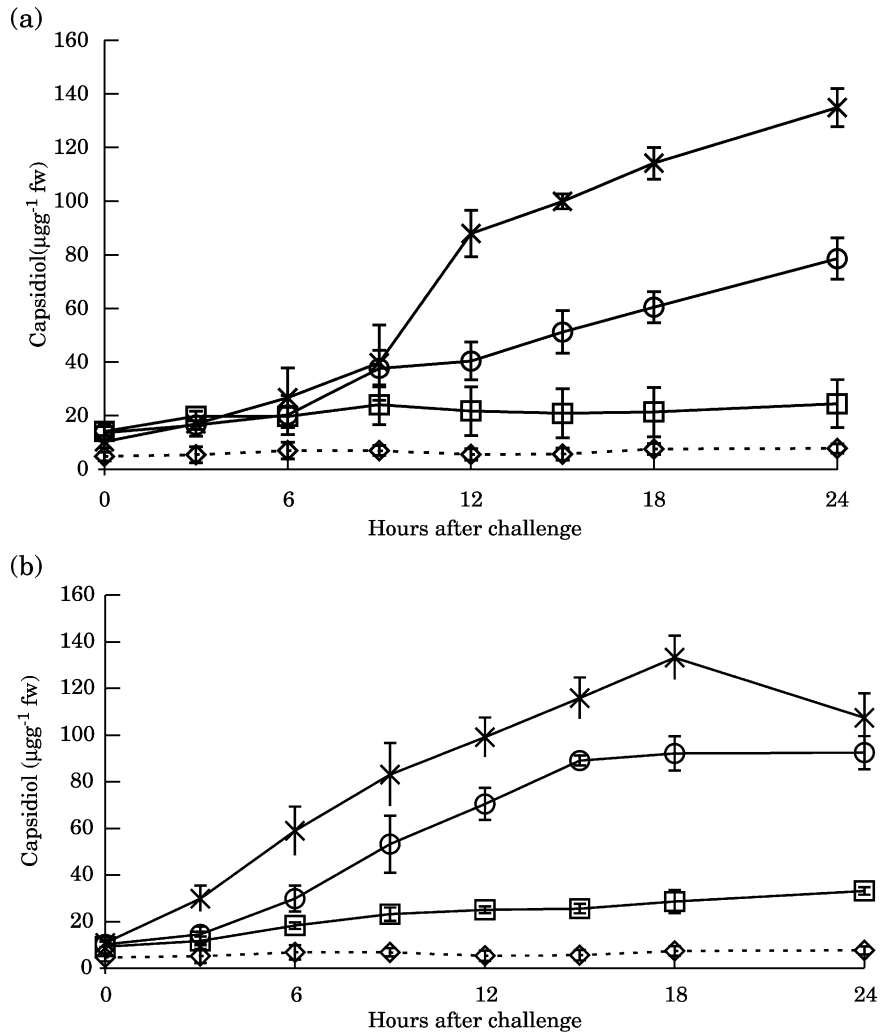

Fig. 2. Effect of superoxide scavengers on capsidiol accumulation in *N. tabacum* cv. NC2326 cells challenged with zoospores of (a) incompatible *Phytophthora nicotianae* 4974 or (b) *P. palmivora* (see Section 2). (-◇-) uninoculated; (- × -) zoospores; (-○-) zoospores + SOD; (-□-) zoospores + manganese (III) desferal. Means and standard deviations were calculated from four replicates.

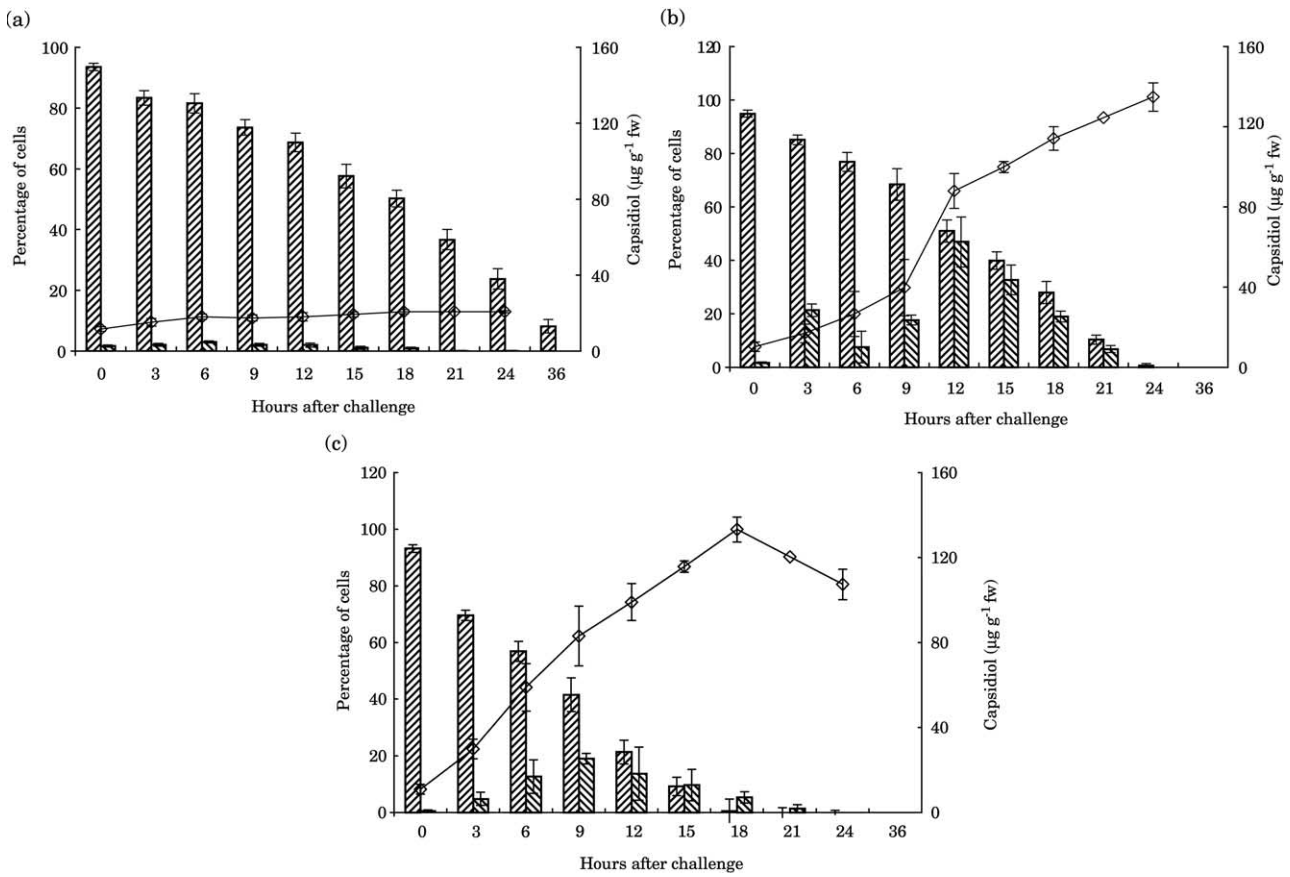

Fig. 3. Cell viability (▨), NBT staining (▧), and capsidiol production (-◇-)in *N. tabacum* cells challenged with zoospores of *Phytophthora* spp. (a) Hicks/*Pn 4974;* (b) NC 2326/*Pn 4974;*(c) NC 2326/*P. palmivora*. The means and standard deviation for cell viability were calculated from 9 replicates, for NBT staining from six replicates and capsidiol accumulation from four replicates (see Section 2).

Although these scavengers inhibited both superoxide release and capsidiol accumulation, *P. palmivora* zoospores still failed to colonise treated tobacco cells. Of 200 *P. palmivora* cysts monitored, 60% failed to germinate, and those that did produce short, randomly-orientated germ tubes that disintegrated within a few hours. This behaviour was unaffected by the superoxide scavengers.

By comparison to the incompatible NC2326/*P. nicotianae* interaction, viability measurements using the neutral red assay indicated a more rapid onset of hypersensitive cell death in the non-host NC2326/*P. palmivora* reaction (Fig. 3(c)). The percentage of cells stained by the NBT test for superoxide production was lower in this non-host interaction and there was no evidence of a biphasic rate of production. The single broad peak of NBT staining occurred earlier than the second major burst of radical production observed in the incompatible interaction. The patterns and extent of NBT staining and cell death observed in Hicks cells challenged with *P. palmivora* reflected those seen in the NC2326/*P. palmivora* interaction.

4. Discussion

Previous studies have reported that tobacco cells in suspension culture produce the sesquiterpenoid phytoalexin capsidiol when immersed in pathogen extracts or bacterial cell suspensions [6,43]. However, the implications of these studies for our understanding of the interaction between pathogens and intact plant tissues is limited because pathogen fractions may selectively activate independent pathways. In addition the synchronous elicitation of suspension cells does not accurately model cells in intact plant tissues where initially only one, or at most a few, individual cells are in direct contact with the pathogen. Surrounding and remote cells and tissues become involved asynchronously, presumably in response to signals emitted by the cell(s) under direct challenge [7,15,21,23]. While the directly challenged cell may enter an irreversible cell death program, neighbouring cells simultaneously activate a range of defence-related responses that include novel gene expression and biosynthesis. Inundation experiments are thus unable to expose the interplay between challenged cells and their neighbours that regulates localised and systemic responses in intact tissues.

In recognition of this fundamental limitation we developed a model system that exploits the tactic attraction of zoospores to clumps of suspended host cells in culture [1,2,35,40]. In our model system, zoospores of the black shank pathogen, *P. nicotianae*, are attracted to suspended clumps of 15–20 cells of *N. tabacum*. Once one zoospore attaches

to a clump of cells, the clump becomes less attractive to further zoospores [40]. In challenges involving gene-for-gene incompatibility, the host cells under attack undergo a rapid oxidative burst of superoxide production, which is necessary for resistance expression [1,2,3,35]. Incompatibility is finally expressed as the hypersensitive death of the entire clump of cells in a temporal and spatial sequence closely resembling observations in intact plants [40]. Although non-host zoospores are not attracted to, and rarely come into direct contact with, clumps of *N. tabacum* cells, they elicit an oxidative burst and phytoalexin accumulation.

Our work places the production of the phytoalexin capsidiol in the time course of previously studied events. As is the case for superoxide production and hypersensitive cell death [1,2], significant levels of capsidiol accumulate only in incompatible and non-host interactions. Capsidiol accumulation is detected after the first burst and rises rapidly both during and after the second burst of superoxide release, coinciding with the onset of rapid cell death in the incompatible plant–pathogen response.

In a series of experiments with aged potato tuber slices, Doke showed that exogenous superoxide elicits phytoalexin accumulation [12,13,14], inferring a link between the two responses, possibly involving the oxidation of membrane lipids via dismutation of superoxide into hydrogen peroxide [5]. Ellis et al. [17] showed that SOD inhibits the arachidonic acid-induced oxidative burst in potato tuber slices, and also reduces phytoalexin accumulation. Our results have now confirmed this link between the oxidative burst and phytoalexin accumulation in incompatible *N. tabacum*/*P. nicotianae* interactions. Mn (III) desferal is a more potent inhibitor of superoxide release in our system than SOD [1], and this correlates well with the relatively greater inhibition of capsidiol accumulation and suppression of hypersensitive cell death [1].

Few researchers have investigated superoxide release, with most restricting their observations to its more stable dismutation product, hydrogen peroxide. While hydrogen peroxide has been proposed to act as a second messenger in plant–pathogen interactions [11,26,34], conclusive proof that this species acts as a primary signal *in vivo* is lacking [16]. We have recently shown that temporary hypoxia prevents both superoxide release and hypersensitive cell death, and induces susceptibility in incompatibly-challenged *N. tabacum* cells [35]. We have now shown that experimental quenching of superoxide blocks phytoalexin elicitation, and conclude that superoxide release is essential to the pathogen-induced elicitation of phytoalexins in the tobacco/*P. nicotianae* interaction. Furthermore, blocking sesquiterpenoid biosynthesis in incompatibly-challenged tobacco stems also induces susceptibilty [37]. While these data support a decisive role for capsidiol in determining the outcome of this host-pathogen interaction, we cannot exclude the possibility that inhibiting a key enzyme such as HMG-CoA reductase may additionally suppress other components of resistance.

This cell culture system has also allowed us to investigate the non-host reactions of tobacco towards *Phytophthora* species unable to infect its tissues. Able et al. [3] recently compared the non-host responses of tobacco cell cultures towards both *P. palmivora* and *P. cryptogea* with the gene-for-gene incompatible response towards a race 0 isolate (4974) of *P. nicotianae*. While the patterns of oxidative burst and hypersensitive cell death were similar to those observed following gene-for-gene incompatibility, superoxide generation was lower in non-host interactions. Our observation that fewer cells deposit NBT formazan in the non-host compared to the incompatible reaction is consistent with this reported lower overall rate of superoxide production [3]. Localised superoxide release at the point of host-pathogen contact was detected earlier using NBT than when using XTT, which detects superoxide released into the medium [3,47].

We also noted fundamental differences in zoospore behaviour in host and non-host interactions. Any direct contact between *N. tabacum* cells and *P. palmivora* was apparently random, and there was no evidence of attempted penetration. Unlike avirulent zoospores of *P. nicotianae, P. palmivora* zoospores remained unable to colonise tobacco cells treated with superoxide scavengers.

In contrast to the lower rate of superoxide release in the non-host interaction, we have observed that capsidiol accumulation not only reaches similar levels to those seen in the incompatible interaction, but it does so earlier. This more rapid accumulation of capsidiol in tobacco cells responding to *P. palmivora* challenge parallels the earlier (and single) peak in superoxide release in this non-host response. Although we observed no evidence for a bimodal oxidative burst in the non-host interaction, it is possible that an early burst occurred, but was missed because our first sampling was 3 h after inoculation. As in the incompatible gene-for-gene response, the production of a burst of superoxide production is a necessary condition for phytoalexin production in the non-host response, indicated by the inhibition of capsidiol production in the presence of a superoxide scavenger.

These observations define a significant difference between incompatible and non-host interactions in this system. We hypothesise that this difference results because *P. nicotianae* zoospores recognise *N. tabacum* cells and only elicit race-specific resistance upon appressorial development, attachment and attempted penetration, while *P. palmivora* zoospores do not recognise *N. tabacum* cells and release non-specific elicitor-active molecules during zoospore motility, encystment or germination. Appressoria fail to develop. This also implies that defence responses elicited by non-host zoospores in cell suspension cultures are redundant. Similarly, although *P. palmivora* zoospores elicit hypersensitive cell death and capsidiol accumulation on wounded *N. tabacum* leaves (Perrone et al., unpublished), these host responses may not be necessary to stop the development of infection structures and parasitism.

While similar response pathways may be initiated by apparent gene-for gene interactions in both non-host and specific resistance [24], the defining recognition events that condition the expression of these responses appear independent. This is indicated by the ability of Hicks cells to express a strong non-host response to *P. palmivora* whereas these same cells are unable to express resistance towards Race 0 of *P. nicotianae* [3]. In non-host resistance in barley, the presence of the barley *mlo5* mutation has no effect on penetration by *Blumeria graminis* f. spp. *tritici*, which susbsequently triggers hypersensitive cell death typical of the normal non-host interaction. However, in barley challenged with isolates of *B. graminis* f. spp. *hordei* virulent on the wild type, the presence of the *mlo5* mutation induces complete penetration resistance and no hypersensitive response is observed [27].

From this and other recent studies on tobacco cells we conclude that superoxide release is necessary, but is not on its own sufficient, for the expression of resistance to incompatible pathogen races. Superoxide release triggers a cascade of events leading independently to hypersensitive cell death and phytoalexin accumulation [1,11,23]. Thus we propose that the primary role of superoxide release is the regulation and signalling, rather than the direct execution, of disease resistance mechanisms in tobacco.

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