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ELICITATION OF CAPSIDIOL ACCUMULATION IN SUSPENDED CALLUS CULTURES OF *CAPSICUM ANNUUM**

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Key Word Index—*Capsicum annuum*; Solanaceae; elicitation; phytoalexin; callus cultures; sesquiterpenoid; capsidiol.

Abstract—Capsidiol was elicited in suspended callus cultures of *Capsicum annuum* in response to commercial cellulase (*ex Trichoderma viride*), or pectinase (*ex Aspergillus niger*), or a sterile extract from *Gliocladium deliquescens*. Amounts of capsidiol up to 2.9 mg per 100 ml of culture were accumulated in response to the *G. deliquescens* extract. Capsidiol was the preponderant phytoalexin produced in the cultures: minor congeners were present at levels below 0.1 % of the amounts of capsidiol.

INTRODUCTION

The utility of tissue cultures in the study of phytoalexin formation is beginning to be exploited on a wider scale. Their uses in this respect and in the study of other problems in host–pathogen interactions have been recently reviewed [1]. Extensive work carried out on the formation of flavonoid stress metabolites in cultures of *Petroselinum hortense* [2, 3], and of isoflavonoid phytoalexins in cultures of *Phaseolus vulgaris* [4, 5] and *Glycine max* [6] has provided evidence of the changes in enzyme activities and mRNA levels associated with the induction of phytoalexin biosynthesis. Cultures of *Trifolium repens* [7] and *Medicago sativa* [8] have also been found to produce isoflavonoid phytoalexins in response to fungal inoculation.

No detailed studies have been carried out on the enzymology of sesquiterpenoid phytoalexin production. Among tissue cultures that have been found to produce sesquiterpenoid phytoalexins are the following: callus cultures of *Ipomoea batatus*, which produce furanoterpenes spontaneously on transfer to suspension culture [9]; *Gossypium hirsutum* suspension cultures, which produce hemigossypol in response to spores of *Verticillium dahliae* [10]; *Nicotiana tabacum* callus cultures, which form phytuberin and phytuberol in response to *Pseudomonas* bacteria [11, 12] and rishitin, epirishitin, capsidiol and a vicinal diol (presumed to be debneyol) in response to *Phytophthora parasitica* [13, 14]; *Nicotiana tabacum* suspension cultures, which produce phytuberin, phytuberol, capsidiol and debneyol in response to cellulase (*ex Trichoderma viride*) [15]; and suspension cultures of *Solanum tuberosum*, which produce rishitin, lubimin and solavetivone in response to *Phytophthora infestans* [16, 17]. In addition to the elicitors already cited a number of other agents have been used to induce stress metabolite/phytoalexin formation in tissue cultures. These include ultraviolet light [2, 18], fungal extracts [4–6, 19–23] and SH reagents [24]. Recently, detailed investigations have been made of the induction of 6-methoxymellein formation in carrot cultures using as elicitors either pectolytic enzymes or pectolytic digests of the culture cell walls [25–27]. A recent review covers certain aspects of the use of tissue cultures in phytoalexin studies as well as more general details of the phytoalexin response [28].

We now report the elicitation of capsidiol formation in cultures of *Capsicum annuum* in response to a number of elicitors which we previously used in a study of the whole fruits [29].

RESULTS AND DISCUSSION

Capsidiol was not detected in extracts of the combined tissue and medium from untreated cultures by a procedure that would have detected 5 μg/100 ml of culture. The results are expressed in terms of mg of capsidiol per 100 ml of culture since the suspended callus cultures did not follow a uniform growth curve; 2-week-old cultures were used in elicitation experiments. Suspended callus cultures were found to produce 3–4 times more capsidiol than was formed in cell suspension cultures. This was presumably because of the greater degree of cell–cell contact within the larger aggregates present in the suspended callus cultures. Aggregation in cell cultures has been shown to promote secondary product formation [30].

Treatment of the cultures with crude cellulase (*ex T. viride*) (Figs 1 and 2) caused capsidiol accumulation after a lag of *ca* 8 hr; capsidiol accumulated most rapidly between 12 and 36 hr. The higher concentration of capsidiol in the medium than in the tissue, shown here, was consistently observed. The capsidiol concentration in the cultures reached a plateau and showed little sign of declining during prolonged incubation: capsidiol added to untreated *Capsicum* cultures (5 mg per flask) was not

*Part 3 in the series "Elicitation of Terpenoid Stress Metabolites". For Part 2 see ref. [15].

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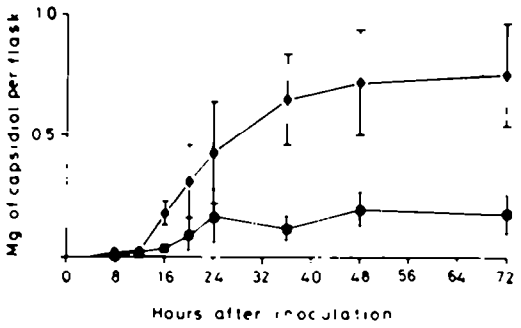

Fig. 1. Time course of capsidiol accumulation in *Capsicum* cultures in response to crude cellulase *ex Trichoderma viride*. ●, Capsidiol concentration in culture medium; ■, capsidiol concentration in tissue. Data points and standard deviations are based on extracts from five separate flasks of 2-week-old culture (each 100 ml) treated with cellulase at 10 μg/ml.

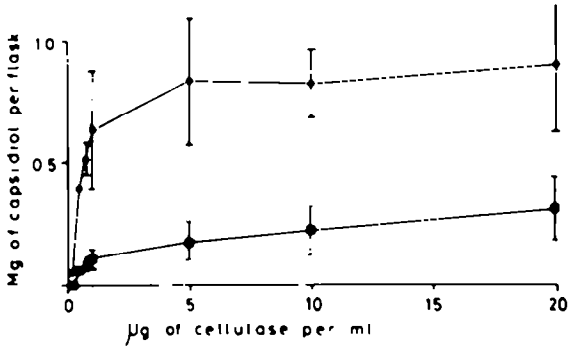

Fig. 2. Dose response of *Capsicum* cultures to crude cellulase *ex Trichoderma viride*. ●, Capsidiol concentration in culture medium; ■, capsidiol concentration in tissue. Data points and standard deviations are based on extracts from three sets of five flasks of 2-week-old culture (each 100 ml) treated with cellulase for 48 hr.

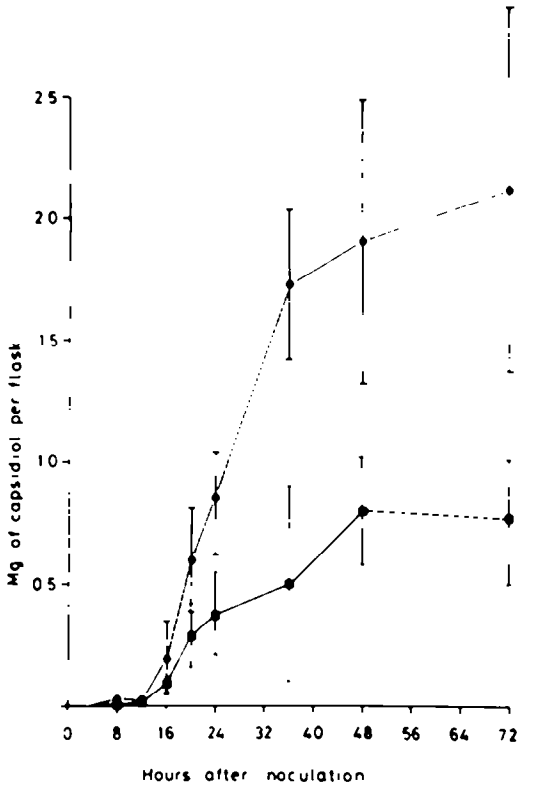

Fig. 3. Time course of capsidiol accumulation in response to a sterile extract from *Gliocladium deliquescens*. ●, Capsidiol concentration in culture medium; ■, capsidiol concentration in tissue. Data points and standard deviations are based on extracts from five separate flasks of 2-week-old culture (each 100 ml) treated with *G. deliquescens* extract at 100 μg/ml.

appreciably metabolized in the course of 5 days, and the presence of capsidiol caused no apparent harm to the cultures during this period. This was in contrast to the findings in *Solanum tuberosum* [Threlfall, D. R. and Brindle, P. A., personal communication] and *Dianthus caryophyllus* [19] cell cultures where the levels of phytoalexins declined markedly with prolonged incubation. Capsicum cultures were responsive to cellulase concentrations as low as 0.5 μg/ml; and high levels of cellulase (300 μg/ml), which lysed a large number of cells in the culture, did not greatly lessen the amounts of capsidiol accumulated. The elicitor activity of crude cellulase *ex T. viride* could be only partly destroyed by boiling or by treatment with trifluoroacetic acid. Commercial cellulases from *Aspergillus niger* and *Penicillium funiculosum* did not have elicitor activity, so the nature of the active components in the *T. viride* preparation merits further examination.

Treatment of *C. annuum* cultures with a sterile extract from *Gliocladium deliquescens* caused capsidiol accumulation after a lag phase of *ca* 8 hr: as indicated in Figs 3 and 4, the rate of accumulation was greatest between 16 and 36 hr. After 36 hr, both the rate and the extent of capsidiol production (up to 2.9 mg per 100 ml of culture) were higher than in the cellulase-treated cultures, possibly reflecting a higher degree of cell lysis in the latter system. The cultures responded to *G. deliquescens* extract at concentrations as low as 10 μg/ml. The apparent two maxima in the dose–response curve (Fig. 4) represent data from only three replicate experiments, and need verification. A similar pattern of response has been reported for the induction, by fungal extracts, of phenylalanine ammonia-lyase activity in *Phaseolus vulgaris*, although this was not reflected in the amounts of isoflavonoids that were accumulated [4, 23].

The *Capsicum* cultures responded more weakly to pectinase (*ex A. niger*) as an elicitor (Fig. 5). The amounts of capsidiol accumulated were no more than 0.3 mg per 100 ml of culture. At pectinase concentrations above 40 μg/ml, the tissue was rapidly macerated, while only a little capsidiol was produced; and below 15 μg/ml, neither tissue damage nor capsidiol formation was observed. There is accordingly a narrow range of concentrations at which elicitation by pectinase predominates over cell destruction. Related observations have been made in capsicum fruits: pectinase was a powerful elicitor of capsidiol in unripe fruits [29], but in ripe fruits it led to rapid maceration of tissue, and to much lower yields of

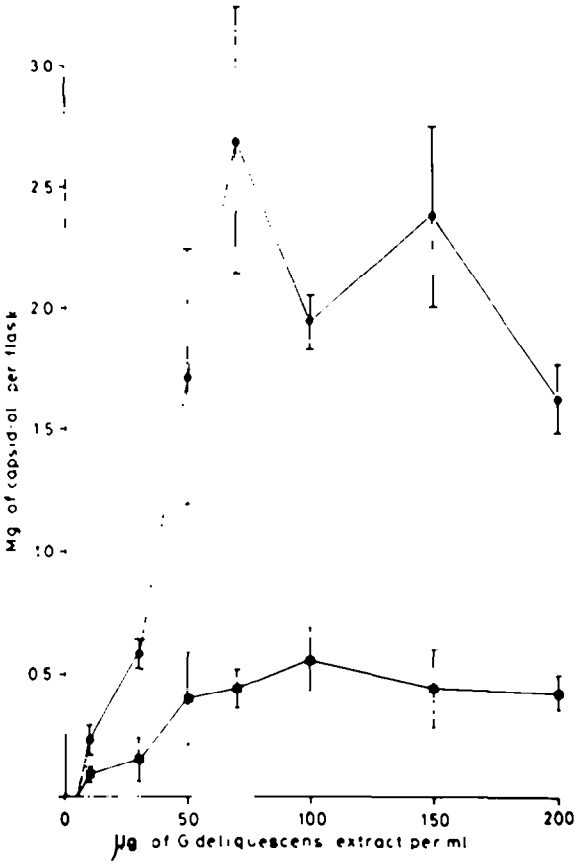

Fig. 4. Dose response of *Capsicum* cultures to a sterile extract from *Gliocladium deliquescens*. ●, Capsidiol concentration in culture medium; ■, capsidiol concentration in tissue. Data points and standard deviations are based on extracts from three sets of five flasks of 2-week-old culture (each 100 ml) treated with *G. deliquescens* extract for 48 hr.

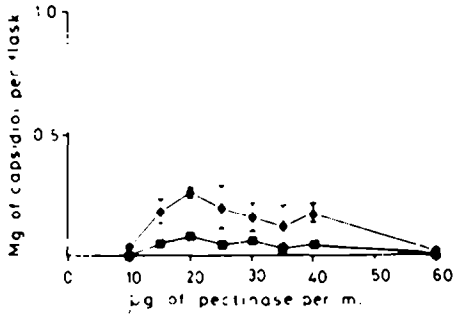

Fig. 5. Dose response of *Capsicum* cultures to crude pectinase *ex Aspergillus niger*. ●, Capsidiol concentration in culture medium; ■, capsidiol concentration in tissue. Data points and standard deviations are based on extracts from three sets of five flasks of 2-week-old culture (each 100 ml) treated with pectinase for 48 hr.

capsidiol. Kurosaki *et al.* [25-27] have studied the elicitation of 6-methoxymellein in carrot cultures by pectinase from *Aspergillus japonicus*. The pectinase was found to release elicitor activity from the cell walls of the cultured cells: such activity was ascribed to pectic fragments by analogy with other systems [12, 31]. The degree of methylation of the pectic fragments appeared to be important in relation to elicitor activity.

Acid hydrolysates of the cell wall polysaccharides from *Capsicum* fruits and cultures were found to have weak elicitor activity in the capsicum cultures. A more consistent release of this type of elicitor material was obtained by hydrolysis of citrus pectin. It has been previously shown that a dodecagalacturonic acid obtained from citrus pectin was an elicitor of phytoalexin accumulation in soybean [32]. The nature of the constituent of hydrolysed citrus pectin that elicits capsidiol accumulation is being further investigated.

The *de novo* synthesis of capsidiol in *Capsicum* cultures was established by addition of sodium [2-^{14}C]acetate to the cultures 12 hr after treatment with *G. deliquescens* extracts. About 10% of the added radioactivity was recoverable in organic extracts of the combined tissue and culture medium. Analysis by GC-RC indicated a maximal incorporation into capsidiol of *ca* 6%; 95% of the labelled capsidiol was extractable from the culture medium. GC-RC and TLC-RC analysis indicated that capsidiol was the only significantly labelled component present in extracts from the medium; small amounts of other labelled materials were present in the extract from the tissue.

The minor congeners of capsidiol which are present in extracts from elicitor-treated fruits [33–35] were present at levels < 0.1% of the amount of capsidiol extractable from the media and tissue cultures. In the fruits, five principal minor congeners occurred in amounts up to 8% of the capsidiol extracted from the flesh and diffusates [35]. Analysis of the minor congeners from the tissue cultures by GLC and GC-MS revealed at least eleven sesquiterpenoids with R_I values in the range 1650–1750 (CP Sil 5CB, 125°). The predominant molecular ions were 216, 218 and 222; in the whole fruits, the two principal minor components are the monohydroxy compounds 1-deoxycapsidiol and capsidesmol, which have molecular ions at 220 [35]. The differences in response between the fruits and tissue cultures may be in part accounted for by the differences in the nature of the tissue subjected to elicitor action.

EXPERIMENTAL

Plant tissue cultures. Capsicum annuum L. cultures were initiated from slices of the fruits, and had been in culture for two years before commencement of this study. The cultures were maintained on Murashige and Skoog's (M/S) medium [36], with kinetin (0.1 mg/l.) and IAA (1 mg/l.) as growth hormones. Suspended callus cultures were produced by transferring pieces of callus (*ca* 1 g) to agitated liquid medium and allowing them to grow for two weeks before treatment with elicitors was initiated. Elicitor materials were added in soln (1 ml) via a 0.2 μm Microflow 25 filter (Flow Laboratories, Irvine). After appropriate periods of incubation, growth medium and tissue were extracted separately with EtOAc, and the extracts were dried over Na_2SO_4 and concd to 3 ml for analysis by GLC.

Cell suspension cultures were established by transfer of callus pieces to M/S medium containing 2,4-dichlorophenoxyacetic acid (0.5 mg/l.) as the only growth hormone. The cell suspension cultures were sub-cultured every three weeks by transfer of inocula (20 ml) to fresh media.

Gliocladium deliquescens IMI101515 was grown and extracted as described earlier [29].

Materials. Solvents were AnalaR Cellulase (0.02 units/mg *ex Trichoderma viride*) was from BDH, pectinase (8 units/mg, *ex Aspergillus niger*) from Sigma, citrus pectin from Sigma. Sodium [2-^{14}C]acetate (714 μCi/mg) was from Amersham International Plc, Lipidex 5000 from Packard Instruments Ltd.

Methods of analysis. Extracts from cultures were analysed without purification using packed-column GC as described earlier [29]. The identity of capsidiol was confirmed by capillary GC, using a 25 m × 0.32 mm i.d. fused silica WCOT SE-54 column (FID), He carrier gas 3 ml/min: capsidiol di-TMSi ether, R_f 1915 (135°); di-TBDMS ether, R_f 2401 (170°); derivatives were made as described earlier [15]. GC/MS of capsidiol and its minor congeners was carried out using an LKB 9000 instrument as described previously [15]. GC-RC and TLC-RC were also carried out as before [15]. Total radioactivity in extracts was measured by scintillation counting; the fluor contained 4 g PPO and 0.2 g dimethyl POPOP per litre of toluene.

Column liquid chromatography. A Lipidex 5000 column (15 × 2.5 cm) was used to effect a group separation of the minor congeners of capsidiol present in a combined extract from the tissue culture medium and tissue after elicitation. The column was eluted with cyclohexane–EtOAc (19:5) and 15 ml fractions were collected: the minor congeners, found in fractions 4 and 5, were analysed by GC/MS, using a DB-1 column programmed from 90° at 2°/min.

Release of elicitor activity from citrus pectin. Portions (0.5 g) of citrus pectin were suspended in 50 ml of TFA and heated at 85° for 5.5 hr. The insoluble residue was filtered off, and the TFA removed by rotary evaporation under red. pres.; the resulting paste was resuspended in MeOH several times, the MeOH being evaporated under red. pres. after each addition. The residue was dissolved in 0.2 M K-Pi buffer (pH 7.0: 2.0 ml for each hydrolysate from 0.5 g of pectin) and the soln was filtered through a Whatman GF/F filter paper. 1 ml portions of the filtrate were added to 20 ml aliquots from 2-week-old suspended callus cultures. The culture media were extracted after 48 hr and analysed for capsidiol.

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