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Estimation of Induced Secondary Metabolites in Carrot Tissues in Response to Elicitor Preparations from Seaweeds

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Abstract. Secondary metabolites were induced in carrot tissues on treatment with high molecular weight crude elicitor "polysaccharides" preparations (HMWCEPs) from red algae, *Hypnea musciformis*, *Acanthophora delili* and *Botryocladia leptopoda* collected from the coast of Karachi, Pakistan. A simple microtechnique based on UV-visible spectrophotometry is described for the quantification of induced secondary metabolites as a function of time and doses of elicitor preparations.

Keywords: induced secondary metabolites, phytoalexins, elicitors, carrot tissues, seaweeds, elicitors from seaweeds

Introduction

Phytoalexins, which are induced secondary metabolites, are low molecular weight compounds produced by plant tissues in response to microbial invasion as a defence response, or by treatment of various biotic and abiotic elicitors (Darvill and Albersheim, 1984; Keen, 1975). These compounds prevent the growth of most fungi and some bacteria, but they are also toxic to the producing plant cells (Nicholson and Wood, 2001). The determination of induced secondary metabolites (ISMs) in plant tissues is often taken as a measure of the biochemical responsiveness towards agents eliciting defense mechanisms (Bi and Iqbal, 2000). The technique which is generally followed is based on collection of diffusates from treated tissues and the measurement of UV-absorbance after a single organic extraction (Cruickshank and Perrin, 1971).

Carrot phytoalexin, a dihydroisocoumarin, known as 6-methoxy mellein (6MM), is accumulated in carrot roots infected by various fungi, and in cell suspension cultures treated with pronase, pectinolytic enzymes, oligosaccharides from carrot cell walls (endogenous elicitors), and several chemical agents (Marinelli *et al.,* 1991; 1990). Present study was undertaken to estimate the secondary metabolites produced in carrot tissues in response to elicitor preparations obtained from three red algal species, *Hypnea musciformis, Acanthophora delili* and *Botryocladia leptopoda*. A simple and quick microtechnique was used to measure the concentration of induced secondary metabolites by UV-spectrophotometry.

Materials and Methods

Seaweed collection, extraction and isolation of the high molecular weight crude elicitor "polysaccharides" preparation (HMWCEPs) from algal species have been described in a previous communication (Bi and Iqbal, 1999a).

Elicitor treatment. A general method of elicitor application was employed (Whitehead *et al.,* 1982). 250 g fresh carrots (small size), procured from the local market, were washed with tap water, sterilized with 1% Na-hypochlorite solution, washed extensively with distilled water, and finally rinsed with sterile water. Each carrot was cut into thin slices of equal sizes. About 6-8 pieces were placed in petri-dishes over moist filter paper. In the dose response studies, the carrot samples were treated with 75 µl of 10, 40, 70 and 100 µg glucose eq/ml concentration of HMWCEPs of the three algal seaweeds, *Hypnea musciformis, Acanthophora delili* and *Botryocladia leptopoda*. Control samples were prepared by treating the carrot samples with sterile water and by simply wounding the tissues. All the samples were incubated at 25 °C for 24 h in darkness. In another set of experiment, related with time course studies, the treated and control samples were prepared by application of 75 µl test solution of 100 µg concentration. Sterile water, and only wounding, followed by incubation for 6, 12, 24 and 48 h at 25 °C in darkness served as the two controls.

Extraction and estimation of induced secondary metabolites. After the specified period of incubation, the control and treated samples were dipped in about 50 ml redistilled ethanol (95%) and left overnight for complete extraction. Illumination was avoided, as it may oxidize the components. The extracts were filtered through Whatman filter paper No. 1 and concentrated on a rotary evaporator at 45 °C. The weight of residual carrot slices was taken after drying. Stock solutions were prepared by using extracts equivalent to 1 g dry weight of the treated and control tissues of carrot slices in 2 ml redistilled ethanol. 100 µl

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of this solution was further diluted with 100 ml of distilled ethanol (95%) in a volumetric flask for recording the UV-absorbance.

Instrumentation. UV-spectra were recorded on a UV-visible spectrophotometer (Specord 200) of variable wavelengths. The results were recorded in terms of absorption intensity of various alcoholic extracts scanned at the wavelength 190-350 nm using ethanol as the blank.

Results and Discussion

In previous studies, the elicitor activity of the heavy molecular weight crude elicitor polysaccharides (HMWCEPs) obtained from *Hypnea musciformis, Acanthophora delili* and *Botryocladia leptopoda* was determined in the treated tissues of chickpea (Bi and Iqbal, 1999b; 1998). On the basis of preliminary screenings, the potentially active elicitors, the cold aqueous extracts of *H. musciformis* and *A. delili*, and the cold aqueous extract of *B. leptopoda* with low profile of activity, were employed to determine the dose and time dependent activity, in terms of induced secondary metabolites (ISMs) in carrot tissues. It is reported in literature that intracellular production of 6-methoxymellein, a phytoalexin of carrot, was induced by the addition of a wide variety of substances, such as pectinases and proteases secreted by the invading fungi (Kurosaki, 2001; 2000).

In the present study, the alcoholic extracts of treated and control tissues of carrots were analyzed for changes in the concentrations of the ISMs. The metabolite concentration was measured by using the UV-visible spectrophotometer. The high extinction coefficient of many phytoalexins in the ultraviolet region of spectra allows quantification of ISMs (Eva and Earnesto, 1993). Results given in Fig. 1 were recorded in terms of absorption intensity of various alcoholic extracts in the UV-range, scanned from 190-350 nm. Absorption at 266 nm of peak-1 assumed to be proportional to the amount of ISMs, was calculated per g fresh weight of carrot tissues.

In various plant-pathogen/elicitor interactions, the dose response has been determined to optimise the concentration of elicitor molecules with the possible physiological significance for subsequent production of the host resistance response (Mackenbrock *et al.,* 1993). Fig. 1 showing the absorbance peak at 266 nm clearly indicates that secondary metabolites were induced more in the treated carrot samples as compared to both the controls. The effect of various elicitors and their dilutions on the production of ISMs is summarized in Table 1. Elicitor preparations from *H. musciformis* and *A. delili* had followed a similar pattern. Low levels of ISMs were induced at 10 µg elicitor concentration. Significant increases were observed at 40 µg, while

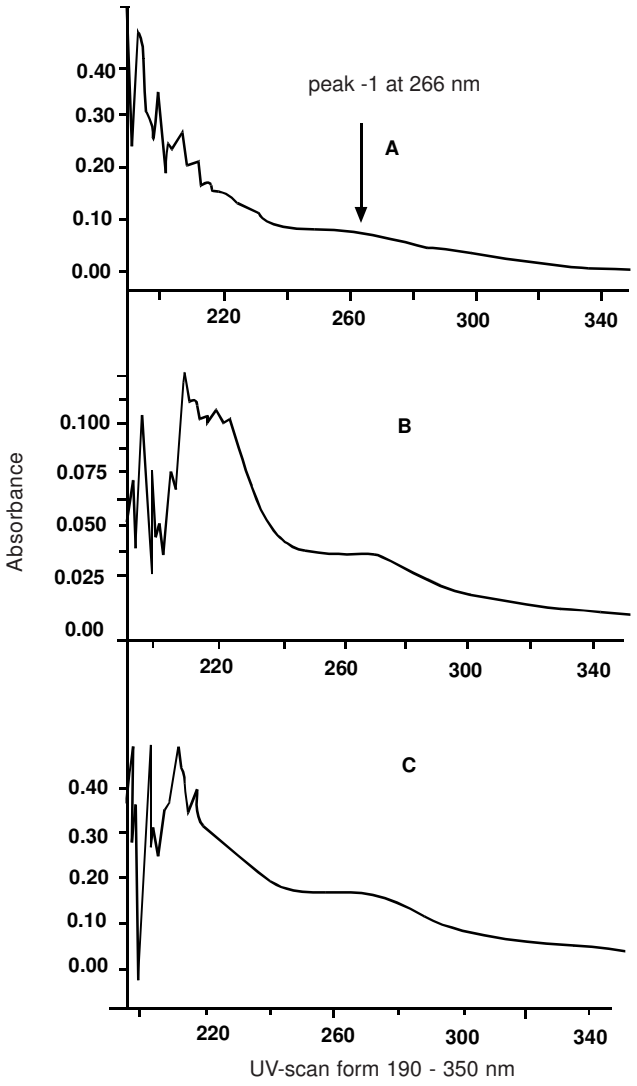

Fig. 1. UV-scanning of ethanolic extracts of elicited carrot tissues for the estimation of induced secondary metabolites (ISMs) during trial with different concentrations of the elicitor; A= wounded tissue (control), B= sterile water treatment (control), and C = *Hypnea musciformis* (75 µl solution; conc 40 µg glucose eq/ml; 24 h incubation period).

values at 70 µg dropped, but increased again at 100 µg glucose eq/ml elicitor concentration. Elicitor preparations from *B. leptopoda* behaved differently; high level of ISMs was induced at 10 µg, while the induction of ISMs was low at 40 µg, which remained almost the same at 70 µg, and a slight increment in the elicitor concentration was observed at 100 µg.

Elicitor activity as a function of time indicated that after the elicitor treatment, ISMs accumulation at 6 h of incubation was

Table 1. UV-absorption of induced secondary metabolites (ISMs) of carrot tissues treated with 75 µl solution of various doses of HMWCEPs of seaweeds, recorded after 24 h of incubation at λ = 266 nm

Algal species	Elicitor conc (µg glc eq/ml)*				
	zero	10	40	70	100
Hyprea musciformis		0.152	0.185	0.155	0.195
Acanthophora delili		0.149	0.177	0.147	0.158
Botryocladia leptopoda		0.162	0.149	0.151	0.156
Tissue wounding (control)	0.10				
Sterile water (control)	0.045				

*glucose equivalent/ml; HMWCEPs = high molecular weight elicitor polysouhorides preparation

Table 2. UV-absorption of induced secondary metabolities (ISMs) of carrot tissues treated with 75 µl solution of 100 µg glc eq/ml* of HMWCEPs of seaweeds incubated for different periods of time at λ = 266 nm

Algal species	Incubation period (h)			
	6	12	24	48
Hypnea musciformis	0.211	0.452	0.491	0.512
Acanthophora delili	0.221	0.376	0.366	0.422
Botryocladia leptopoda	0.201	0.289	0.278	0.375
Tissue wounding (control)	0.224	0.186	0.307	0.351
Sterile water (control)	0.137	0.213	0.320	0.291

*glucose equivalent/ml; HMWCEPs = high molecular weight elicitor polysouhorides preparation

low, almost equal to control (Table 2). Sharp increases were observed after 12 h incubation with the extract preparations from all the three algal species under investigation. A regular increase was observed in the carrot tissues treated with the elicitor isolated from *H. musciformis* at 24 h, whereas in the case of *B. leptopoda* and *A. delili* the values of ISMs were slightly decreased. The induction of ISMs was significantly increased at 48 h of incubation. It is documented that both the rapidity and magnitude, with which the antifungal compounds are produced in the treated tissues, are important in disease resistance, rather than the magnitude alone (Anderson *et al.,* 1991).

The diversity in elicitor activity suggests that either the elicitor active principles of particular preparations were structurally different and their degree of activity maximized at certain dilutions and periods, or some suppressor principles co-existed, which masked the activity at certain dilutions, leaving the elicitor active principles less accessible to the plant. On the basis of present observations it is concluded that carrot tissues responded differentially and positively to various elicitor preparations of the red algae studied. The UV-spectrophotometric method was successfully used for the estimation of induced secondary metabolites. The amplitude and timing of the responses were particularly examined using this method.

References

Anderson, A.J., Rogers, K., Tepper, C., Blee, K., Gardon, J. 1991. Timing of molecular events following elicitor treatment of plant cells. *Physiol. Mol. Plant Pathol.* **38:** 1-13.

Cruickshank, A.M., Perrin, D.R. 1971. Studies on phytoalexins. XI. The induction, antimicrobial spectrum and chemical assay of phaseollin. *Phytopathol. Z.* **70:** 209-229.

Darvill, A.G., Albersheim, P. 1984. Phytoalexins and their elicitors. A defense against microbial infections in plants. *Ann. Rev. Plant Physiol.* **35:** 243-275.

Eva, S., Ernesto, G. 1993. Microassay to measure phytoalexin response in protoplasts of bean leaves. *Phytochem. Analysis* **4:** 82-85.

Bi, F., Iqbal, S. 2000. Estimation of induced secondary metabolites in chickpea tissues in response to elicitor preparations of seaweeds. *Pak. J. Sci. Ind. Res.* **43:** 123-126.

Bi, F., Iqbal, S. 1999a. Studies on aqueous extracts of three green algae as elicitors of plant defence mechanism. *Pak. J. Bot.* **31:** 193-198.

Bi, F., Iqbal, S. 1999b. Chemical investigation and elicitor activity of polysaccharides of red algae *Hypnea musciformis* and *Botryocladia leptopoda. Pak. J. Sci. Ind. Res.* **42:** 223-226.

Bi, F., Iqbal, S. 1998. Chemical investigation and elicitor activity of polysaccharides of red algae, *Dictyota haukiana* and *Acanthophora delili*. *Bangladesh J. Sci. Ind. Res.* **33:** 35-40.

Keen, N.T. 1975. The isolation of phytoalexins from germinating seeds of *Cicer arietinum, Vigna sinensis, Arachis hypogea* and other plants. *Phytopathology* **65:** 91-92.

Kurosaki, F. 2001. Induction and regulation of biosynthetic activity of phytoalexin in carrot cells. *Studies Nat. Prod. Chem.* **25:** 483-512.

Kurosaki, F. 2000. Induction and regulation of carrot phytoalexin biosynthesis. *Recent Res. Develop. Phytochem*. **4:** 173-185.

Mackenbrock, U., Gunia, W., Barz, W. 1993. Accumulation and metabolism of medicarpin and maackiain malonyl glucosides in elicited chickpea (*Cicer arietinum* L) cell suspension cultures. *J. Plant Physiol*. **142:** 385-91.

Marinelli, F., Di, G.S., Nuti, R.V. 1991. Phytoalexin production and cell death in elicited carrot cell suspension cultures. *Plant Sci.* **77:** 261-266.

Marinelli, F., Nuti, R.V., Pini, D., Salvadari, P. 1990. Induction of 6-methoxymellein and 6-hydroxymellein production in carrot cells. *Phytochemistry* **29:** 849-851.

Nicholson, R.L., Wood, K.V. 2001. Phytoalexin and secondary products, where are they and how can we measure them. *Physiol. Mol. Plant Pathol.* **59:** 63-69.

Whitehead, T.M., Dey, P.M., Dixon, R.A. 1982. Differential patterns of phytoalexin accumulation and enzyme induction in wounded and elicitor treated tissues of *Phaseolus vulgaris*. *Planta* **154:** 156-164.