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INDUCTION OF SECONDARY METABOLITES IN CHICKPEA, PEA, CARROT AND POTATO TISSUES IN RESPONSE TO ELICITOR OF *HYPNEA MUSCIFORMIS*

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SUMMARY

Secondary metabolites were induced in chickpea, pea, carrot and potato tissues on treatment with high molecular weight crude elicitor preparations (HMWCEP) of red algal plant, *Hypnea musciformis* of Karachi coast. These metabolites were quantified with time and dose response of elicitor by using UV-visible spectrophotometer. The results indicated that chickpea, carrots and potatoes responded positively to the elicitor while no significant response was observed in pea tissues. The optimum elicitor doses for the maximum induction of induced secondary metabolites (ISMs) were 5 µg for pea tissues and 100 µg for remaining three plants. Similarly 24 and 48 h incubation periods were the optimum timing for the production of ISMs in these plants. High level of metabolites induction suggest that the seaweed polysaccharides, especially from red algal plant *H. musciformis*, can be used as potent plant protectant. The UV spectrophotometric method employed is less time consuming and technique is successfully used for the semiquantitative ISM analysis.

Key words: **Elicitor, *Hypnea musciformis*, induced secondary metabolites, phytoalexins, polysaccharides.**

INTRODUCTION

Phytoalexins, the induced secondary metabolites (ISMs) are low molecular weight compounds induced in plant tissues in response to microbial invasion as a defense response or by treatment of elicitors, the compounds isolated from cell wall, culture filtrate and cytoplasm of various parasitic and non-parasitic plant pathogens (Keen 1975, Heath et al 1997, Darvill and Albersheim 1984). These ISMs 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 ISMs in plant tissues is often taken as a measure of biochemical responsiveness towards agents eliciting defense mechanisms (Fatima and Iqbal 2002).

Recently a relationship between phytoalexin accumulation and defense against pathogenic microorganisms in pea (*Pisum sativum*), alfalfa (*Medicago sativa*), barrel medic (*Medicago truncatula*) and chickpea (*Cicer arietinum*) has been studied (Liu *et al.* 2006). It is reported previously 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). The oligopeptide elicitor Prep-13 and its derivatives induced defense responses and accumulation of jasmonic and salicylic acids in parsley and potato plants (Halim *et al.* 2004).

It is documented that polysaccharides purified from seaweeds as well as derived oligosaccharides have the

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ability to trigger plant defense responses (Potin *et al.* 1999). The aim of the present study was to look at the spectrum of seaweed polysaccharides as an elicitor of disease resistance responses in various cash crops. This research work was carried out to estimate the secondary metabolites induced in chickpea (*Cicer arietinum*), peas (*Pisum sativum*), carrots (*Daucus carota*) and potato (*Solanum tuberosum*) tissues in response to elicitor preparations (polysaccharides) obtained from *Hypnea musciformis*, a red alga. A simple and quick microtechnique established by Cruickshank and Perrin (1971) was employed to measure the concentration of ISMs by UV-spectrophotometry.

MATERIALS AND METHODS

Seaweed collection, hot aqueous extraction and isolation of high molecular weight crude elicitor preparations (HMWCEP) from *H. musciformis* have been described elsewhere (Fatima and Iqbal 1999).

Elicitor treatment: A general method of elicitor application was employed (Whitehead *et al.* 1982). Elicitor activity experiments of chickpea and peas were described earlier (Fatima *et al.* 2006). Fresh carrots and potatoes of small sizes (250 g each), procured from local market were washed with running tap water. The samples were cut into thin slices of equal sizes (about 1-1.5 cm), sterilized with 1% sodium hypochlorite solution and then washed extensively with distilled water and finally rinsed with sterile water. About 8-10 pieces were placed in petri-dishes over moist filter paper. In dose response experiment, sample pieces were treated with 75 µl elicitor solutions of 5, 25, 50, 75 and 100 µg glc eq ml^{-1} concentration and incubated for 24 hours. In time course studies, samples were prepared by the application of 75 µl solution of 100 µg glc eq ml^{1} and incubated for 6, 12, 24 and 48 hours. Control samples were prepared by sterile water treatment (75 µl). All the samples were incubated at 25°C in dark.

Extraction and estimation of ISMs: After specified period of incubation, control and treated samples of the four plants were dipped in distilled ethanol 95% (20-50 ml) and left overnight for complete extraction. Illumination was avoided as much as possible. The extracts were filtered through Whatman filter paper No. 1 in 50 ml volumetric flask and diluted with distilled ethanol up to the mark. A 50 µl of this solution was further diluted with 10 ml of distilled ethanol (95%) for recording the UV-absorbance.

Instrumentation: Spectra were recorded on UV-visible spectrophotometer (Specord-200) of variable wavelengths. The results were recorded in terms of absorption intensity of various alcoholic extracts scanned at wavelength 190 – 550 nm using ethanol as a blank sample. Absorption intensity of the identified peaks was assumed to be proportional to the amount of ISMs and calculated per gram dry weight of the plant tissues.

Statistical analysis: Test for significant difference of mean absorbancies of control and treated plants are made by calculating‘t’ value (Student’s t) and by comparing with critical value of ‘t’ at 95% confidence interval and degree of freedom = 4, critical value of ‘t’is 2.78. Null hypothesis for the difference of two means established that there is no significant difference between two means. Greater value of t-calculated rejects the Null hypothesis which shows that there is significant difference of two mean absorbancies which is indicative of increased ISM concentration by treating with the elicitor (Miller and Miller 1994).

RESULTS AND DISCUSSION

For the last many years scientists have been working for biological alternatives to chemical pesticides. Induced resistance is a term used for protecting plants against diseases (Bostock et al 2001). The present work makes use of seaweed polysaccharides as an elicitor of plant defense mechanism. In the previous elicitor activity experiments, the hot aqueous extract (polysaccharides) of *Hypnea musciformis* showed high elicitor activity in chickpea tissues (Fatima and Iqbal 1999). Earlier reports also showed that algal polysaccharide ‘carrageenans’ can act as an elicitor of plant defense responses (Mercier *et al.* 2001, Patier *et al.* 1995).

In the present study, alcoholic extracts of elicitor-treated and control tissues of chickpea, peas, carrots and potato were analyzed for the estimation of induced secondary metabolites (ISM_S) with respect to various doses of elicitor (5-100 µg glc eq ml^{-1}) and different

incubation periods (6-48 h). The high extinction coefficient of many phytoalexins in the ultraviolet region of spectra allows quantification of ISMs (Eva and Earnesto 1993). Typical UV spectra of control and treated samples represent the absorption intensity of various alcoholic extracts in the UV range, scanned from 190-550 nm (Fig. 1). Absorption values are assumed to be proportional to the amount of ISMs. The spectra showed that ëmax at 254 and 266 nm refer to components of chickpea and carrots, whereas the ëmax at 264 & 308 nm and 287 & 321 nm refer to components of peas and potatoes respectively.

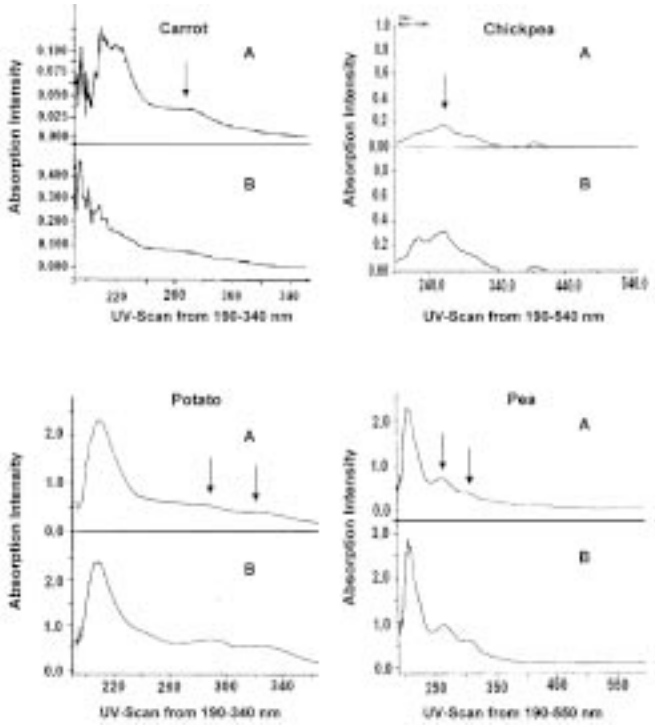

Fig. 1. UV-scanning of ethanolic extracts of various plant tissues for estimation of induced secondary metabolites treated with HMWCEP from *H. musciformis* (red algae), (A) control water (B) Treated samples

Optimization of elicitor concentration and conditions for the subsequent production of host resistance responses were studied previously (Mackenbrock *et al.* 1993). It is clear from table 1 that 100 µg elicitor concentration is enough to induce high level of metabolites in chickpea, carrot and potato tissues, whereas, in peas small increments were observed at 5 and 75 µg. The dose-dependant elicitor activity suggested that molecules were active at a particular dilution or some suppressor molecules coexisted, which masked the activity at certain dilutions, leaving the elicitor active fractions less accessible to the host. Dose-dependent experiments have shown earlier that 100 µg ml^{-1} and 1000 µg ml^{-1} concentrations of algal polysaccharide ë carrageenan were effective dilutions for high induction of florescent compounds and defense-related genes at 168 h and 24 h after elicitation in tobacco leaves (Mercier *et al.* 2001). A novel protein elicitor, 'PaNie' of *Pythium aphanidermatum*, induced multiple defense responses in the cell culture system of carrot, *Arabidopsis* and tobacco leaves (Veit *et al.* 2001). Andreu *et al.* (2001) reported that production of glycoalkaloids and phenolic phytoalexins was low in the susceptible variety of two potato cultivars infected with *Phytophthora infestens*, whereas, the resistant variety had a very high glycoalkaloid content suggesting that these compounds might play a role in plant protection against the fungus.

Effectiveness of various elicitor concentrations was statistically analyzed (Table 1). 't' values for chickpea at each dose were greater than the critical or tabulated value which showed the induction of ISMs at each dose. Peas did not show any significant difference at all, except for the absorbance value at 308 nm at 5 µg elicitor concentration. Carrots showed significant differences at 25, 50 and 100 µg but not at 5 and 75 µg concentration. Potatoes showed a significant difference at 25, 50, 75 and 100 µg dilutions.

The rate of ISM accumulation in these plants at elicitor concentration of 100 µg glc eq.ml^{-1} was examined over a period of 48 hours. Results indicate that ISM accumulation at 6 h incubation was low and in some cases less than in the controls (Table 2). After 6 h of incubation, the induction pattern was quite similar in all the four plants, i.e, the concentration of metabolites was gradually increased with the passage of time and maximized at or after 24 hours. Marked increase (2-5 folds) was observed in treated tissues of chickpea and carrot at late hours of incubation. Effective incubation period at which significant differences were observed at 6 h in chickpea and carrots and at 24 h in potatoes. Peas did not show any significant difference at any interval of time. Cooper *et al*.(2005) reported that during time course studies in peas, application of a new class

Table 1. Estimation of induced secondary metabolites (ISMs) in chickpea, peas, carrot and potato tissues treated with various doses of HMWCEP of *H.musciformis.*

Plants	Wavelength	Control	Elicitor conc. (µg glc eq/ml)*				
tested	(nm)	water	5	25	50	75	100
Chickpea	254	0.1200±0.0351	0.3701±0.0353	0.4517±0.0361	0.6325±0.0355	0.9102±0.0357	1.2137±0.0352
Pea	264	0.5575±0.0354	0.6012±0.0361	0.5227±0.0351	0.4703±0.0359	0.5919±0.0352	0.5602±0.0363
	308	0.2787±0.0359	0.5515±0.0350	0.2730±0.0365	0.2534±0.0343	0.3012±0.0358	0.2712±0.0361
Carrot	266	0.0901±0.0271	0.1632±0.0252	0.1831±0.0241	0.1930±0.0281	0.1681±0.0231	0.2765±0.0291
Potatoe	287	0.3798±0.0291	0.2569±0.0309	0.5840±0.0285	0.6765±0.0251	0.5355±0.0192	0.8679±0.0352
	321	0.2689±0.0321	0.2349±0.0291	0.4347±0.0251	0.5360±0.0351	0.4020±0.0321	0.7620±0.0421

Results were obtained by averaging triplicate samples, *glucose equivalent/ml, HMWCEP = High molecular weight crude elicitor preparations.

Table 2. Estimation of induced secondary metabolites (ISMs) in chickpea, peas, carrot and potato tissues treated with 100 µg glc eq/ml* of HMWCEP of *H.musciformis*, incubated for different periods of time.

Applied plants	Absorption wavelength (nm)	Incubation periods (h)			
		6	12	24	48
Chickpea					
Treated samples	254	0.2651±0.0212	0.3782±0.0253	0.6120±0.0234	1.2011±0.0425
Cont. water		0.1802±0.0191	0.2360±0.0222	0.1250±0.0203	0.3510±0.0352
Pea					
Treated samples	264	0.3762±0.0291	0.4636±0.0351	0.5116±0.0263	0.6292±0.0292
Cont. water		0.3786±0.0254	0.3656±0.0352	0.5436±0.0361	0.5960±0.0212
Treated samples	308	0.1165±0.0312	0.1608±0.0291	0.2735±0.0311	0.3771±0.0256
Cont. water		0.1108±0.0325	0.1309±0.0293	0.2764±0.0321	0.3529±0.0231
Carrot					
Treated samples	266	0.2341±0.0215	0.4390±0.0219	0.5661±0.0312	0.6908±0.0251
Cont. water		0.1265±0.0235	0.2201±0.0191	0.3105±0.0291	0.2514±0.0225
Potato					
Treated samples	287	0.2045±0.0315	0.3588±0.0293	0.4981±0.0312	0.5020±0.0191
Cont. water		0.2646±0.0331	0.3167±0.0305	0.3772±0.0292	0.4185±0.0201
Treated samples	321	0.1306±0.0241	0.2390±0.0282	0.3652±0.0291	0.4452±0.0246
Cont. water		0.1743±0.0232	0.2360±0.0291	0.2664±0.0279	0.3578±0.0251

Results were obtained by averaging triplicate samples, *glucose equivalent/ml, HMWCEP = High molecular weight crude elicitor preparations.

of insect elicitor 'Bruchin B' increased the expression of isoflavone synthase gene within 8 h of elicitor treatment followed by increase in the level of isoflavone phytoalexin 'pisatin' that reached the highest level at 32-64 h after elicitation. In another study, the elicitor from fungus *Ascochyta rabiei* induced a large amount of pterocarpan phytoalexins medicarpin and maackiain in cell suspension cultures of chickpea within 8 h in

resistant varieties, whereas, in susceptible cultivar only small amount of phytoalexins were accumulated 12 h after elicitor application (Kessman *et al.* 1988). Saikia *et al*. (2005) described that the time-course accumulation of pathogenesis-related proteins, chitinase and β-1,3 glucanase, induced in chickpea plants were significantly higher than in the control. Maximum activities of these proteins, observed three days after inoculation, inhibited the growth of various phytopathogenic fungi after purification. Results of the present study show that except peas all the three plants responded well to the seaweed elicitor preparations.

The study indicated that chickpea, carrots and potatoes responded positively to the elicitor under investigation, while pea tissues exhibited no significant response. It is concluded that optimum elicitor doses for the maximum ISM induction were 5 µg for pea tissues and 100 µg for the remaining three plants. Similarly 24 and 48 h incubation periods were the optimum timing for the ISM production in these plants. High level induction of metabolites suggests that the seaweed polysaccharides especially from the red algal plant *(H. musciformis)* can be used as the potent plant protectant.

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