*Pak J Sci Ind Res 2001 **44** (1) 18-21*

Studies on Seaweed Extracted Elicitors and their Analysis for Antimicrobial Activity in Chickpea Cotyledons

Fatima Bi*, Seema Iqbal, Shahnaz Ahmed and Nasim F Usmani

PCSIR Laboratories Complex, Karachi-75280, Pakistan

(Received 11 March 1999; accepted 17 June 2000)

High Molecular Weight Crude Elicitor Preparations HMWCEP obtained from *Hypnea musciformis* (red algae) were used for elicitation of biologically active secondary metabolites of chickpea (*Cicer arietinum*) cotyledons. Elicited tissues were extracted with 95% alcohol, concentrated and stored in the fridge. A yellow ppt was collected and fractional extraction of the supernatant provided total alcoholic Ext.1, pet ether Ext.2, chloroform ethyl acetate (3:7) Ext.3 and the residual aqueous Ext.4. Yellow ppt and various extracts were evaluated for their antifungal activity. Ext.2,3 and 4 showed elevated antifungal activity on treatment against some pathogenic and non pathogenic fungi. HPLC method was developed for the separation of complex mixture of induced secondary metabolites of chickpea tissues by the treatment of seaweed elicitors.

Key words: *Cicer arietinum*, Elicitor, *Hypnea musciformis*, Induced secondary metabolites, Antimicrobial activity.

Introduction

As a result of infection or stress, plants exhibit some natural resistance mechanism (Darvill and Albersheim 1984; Joseph Juc 1995). Disease resistance involves not only static protection but also some inducible defence or hypersensitive responses leading to, i) tissue browning associated with cellular necrosis, ii) leakage of electrolyte from isolated cells, iii) phytoalexin 'induced secondary metabolite' production, iv) increased level of enzymes for induction of phytoalexins, v) ethylene production and accumulation of hydroxyproline rich glycoprotein etc. Phytoalexins are plant antibiotics which have been shown to contribute to disease resistance which has been deduced from their antifungal activity (Arnold and Merlin 1990). Natural or synthetic molecules that are able to elicit resistance response against diseases in plants are grouped together and known as Elicitor (Keen *et al* 1972). Elicitor molecules have been isolated from cell wall culture filtrate and cytoplasm of various pathogenic and non pathogenic fungi (Gunia *et al* 1991; Koga *et al* 1996), they also include host derived endogenous elicitors (Legender *et al* 1993) and abiotic elicitors (Satakopan *et al* 1992).

Elicitor molecules obtained from microbial source are diverse in nature including substances of microbial origin i.e. polysaccharides, proteins and fatty acids they also induce phytoalexin synthesis and accumulation (Clarence and Edward 1991; Vogelsang *et al* 1994; Castoria *et al* 1995). It is reported that in most of the cases elicitor activity was associated with the polysaccharide fraction of various preparation of microbial origin (Kessmann *et al* 1988; Hughes and Dickerson 1991). Seaweeds are generally comprised 40-69% carbohydrate and our major interest of studies are to exploit these polysaccharides as an inducer of hypersensitive response in general. The work presented here includes the treatment of chickpea tissues with the High Molecular Weight Crude Elicitor Preparations (polysaccharide in nature) obtained from *Hypnea musciformis* (red algae). Antifungal activity of total alcoholic extract and other fractional extracts were determined. HPLC method was developed for the separation of complex mixture of induced secondary metabolites.

*Author for correspondence

Materials and Methods

Hypnea musciformis (red algae) collected from Hawksbay of Karachi coast in the month of February 1994, washed and air dried material was extracted with dilute acid (0.1N HCl). HMWCEP were obtained as described earlier (Fatima and Seema 1999).

Elicitor activity. A general method of elicitor application was employed in all expermients as described by Whitehead *et al* (1982). Over night soaked, 2 kg of desi (local) channa, chickpea seeds (*Cicer arietinum*) purchased from local market, were germinated on filter paper, placed on wet cotton bed incubated at 25°C in dark. The excised cotyledons of 2-3 days were surface sterilised by immersion in 1% sodium hypochlorite for 2-3 minutes and then washed with sterile water three times. Sterilised seeds, in four batches (500g of each),

were wounded and pricked randomly and flooded with HMWCEP of *H.musciformis* at a concentration of 100µg glucose equivalent ml^{-1}, spreaded on moist filter paper lined over stainless steel trays. Trays were covered with aluminium foil with holes for aeration and stored at room temperature (25-27°C) for 24 h in dark.

Extraction of induced secondary metabolites. Treated tissues of chickpea were extracted with 95% ethanol and concentrated below 45°C. The concentrated total alcoholic extract (Ext.1) was kept at 4°C (in Fridge) for three to four days. A yellow precipitate was collected and the supernatant was partitioned between pet ether and pooled concentrated pet ether extract designated as (Ext.2). The aqueous phase was re-extracted with chloroform: ethyl acetate (7:3 v v^{-1}) as Ext.3 leaving behind a residual aqueous extract (Ext. 4) each extraction was repeated twice. Various extracts and yellow precipitate (ppt. 5) were evaluated for their antifungal activity.

Antifungal activity. Five comnon plant pathogens i.e *Macrophomina phaseolina, Fusarium moniliforme, Curvularia lunata, Aspergillus niger and Rhizoctonia solani* were procured from Botany Department of Karachi University. Cultures were maintained on sabourauds glucose agar and were sub cultured on sabourauds glucose broth for testing. Inocula were prepared using 72 h old broth culture. The antifungal activity was done by agar cup plate method (Raddish 1950). As extracts 1-4 and ppt 5 were not soluble in water, a 2% stock solution of each extract was prepared in methonal 95%. A working solution of concentration mg ml^{-1} (1:1 v v^{-1}) of each sample in methonal was used to evaluate their antifugal activity. Each cavity received 0.15 µg of the test sample respectively. Test was run in triplicate controls, were run simultaneously using 95% methanol in same quantity. All the plates were incubated at 27-29 °C for 72 h and tabulated. Each experiment was run thrice to confirm the results.

HPLC analysis of induced secondary metabolites. HPLC method was developed for the separation of complex mixture of induced secondary metabolites. LC Shimadzu, LC 6A model, UV-visible wavelength Shimadzu R 64A Chromopac were used for data collection and presentation. A 3.9 x 300mm Nova-Pak C_{18}, 60 A°, 4 µm (Water Millipore Corporation) analytical column was used. A guard column of pellicular C_{18} hydrocarbon chemically bonded to glass beads (37-53 µm) was placed immediately before the analytical column. The gradient elutions empolyed were acetonitrile and water. Both the phases contained 1% acetic acid. The gradient was run 20% to 80% acetonitrile in 25 min. A flow rate of 1ml min^{-1} was used.

Sample preparation for HPLC analysis. 2% stock solutions of total alcoholic and other fractional extracts were prepared in methanol. 10 µl of this stock solution of each extract was further diluted with 3 mL of initial gradient solvent, filtered through 0.2 µm Whatman filters; clear solution was injected via a Rheodyne model (loop volume 250 µl). At the time of dilution the solution of yellow ppt turned milky and later it ruined our column.

Results and Discussion

Elicitor preparation of *H. musciformis,* their chemical analysis and elicitor activity in terms of induced browning was determined and established in our previous communication (Fatima and Seema 1999).

It appeared from the data collected (Table 1), that growth of *M.phaseolina and F.moniliforme* were inhibited by the application of all the extracts 1-4 and ppt 5 under test. The chloroform-ethyl acetate extract exhibited maximum activity and found to be the most active fraction. Low levels of activity were shown by the total alcoholic ext. and ppt 5. Alcoholic extract was active against *F.moniliforme, M.phaseolina* and *A.niger* whereas ppt-5 inhibited growth of *F.moniliforme, M.phaseolina and C.lunata,* although the level of inhibition was low. It is evident from these studies that component induced in chickpea tissues by the treatment of elicitor preparations of *H.musciformis* showed inhibitory effect on the growth of test organism and their inhibitory effect was more pronounced with extracts in the order: chloroform-ethyl acetate ext.3>pet ether ext.2> residual aqueous ext.4 > total alcoholic ext.1> yellow ppt-5. No work has been reported yet about the use of seaweed polysaccharides as inducers of plant defence responses. Recently, antifungal property of proteins (agglutinins) from *H.musciformis* was reported against the fungi *Trichophyton rubrum and Colletotrichum lindemuthianum* with total inhibition of spore germination at a concentration of 500, 1000 and 2000 mg ml^{-1} (Melo *et al* 1997). The work presented here is an indirect approach to the problem, HMW crude preparations of *H.musciformis* were applied to the cut surfaces of chickpea tissues and the induced secondary metabolites were evaluated for their antifungal activity.

Fractions (Table 1) which exhibited substantial amount of antifungal activity and yellow ppt 5 lowest antifungal activity were analysed by HPLC to examine the pattern and the levels of induced component in various extracts. Previously HPLC method which was used to analyse vacuolar extracts from protoplast of chickpea cell cultures showed that malonyl glucosides of isoflavones, isoflavanones and pterocarpons are exclusively located in the vacuole (Mackenbrock *et al* 1992).

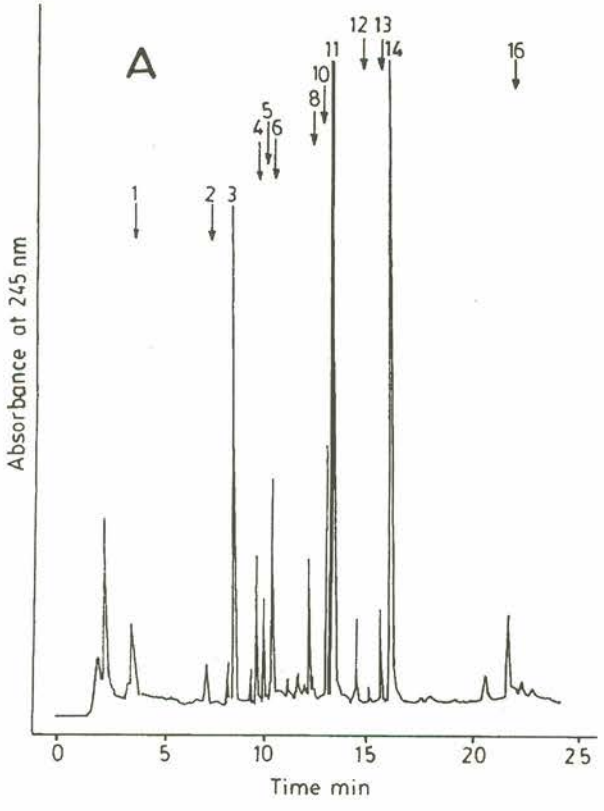
Table 1
Antimicrobial activity of induced secondary metabolite in chickpea tissues by the treatment of seaweed elicitors

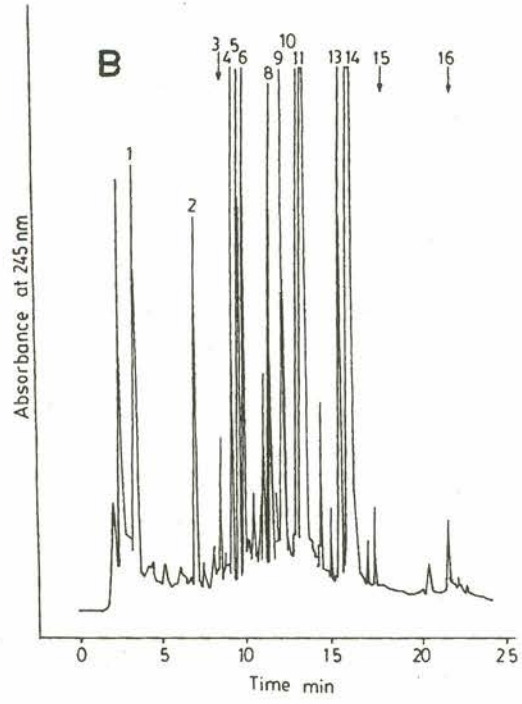
Name of culture	Total alc. ext.	Pet ether ext.	Chloro. ethyl acetate	Res. aqu. ext.	Yellow ppt
Fusarium moniliforme	+ +	+	+ + +	+	+
Macrophomina phaseolina	+	++	+	+	+
Curvularia lunata	-	++	+++	+	++
Aspergillus niger	+	++	++	-	-
Rhizoctonia solani	-	+	-	++	-

Results are average on triplicate samples and findings of two determinations.

Zone of inhibition with average diameter.

+ = 5-9.9mm; ++ = 10-14.9 mm; +++ = 15-20mm; - = no zone of inhibition





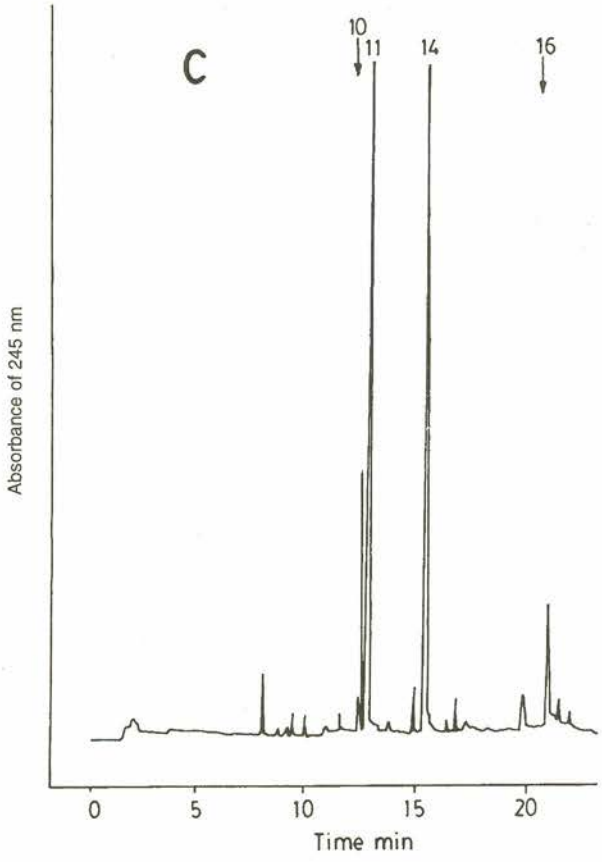


Fig 1. Typical chromatogram for separation of induced secondary metabolites extracted in different solvents from germinated seeds. A) Total alcoholic ext. B) Chloroform ethyl acetate ext. C) Yellow ppt.

In this study an extraction procedure and a successful HPLC method was developed for the separation of complex mixtures of induced secondary metabolites. Separation was achieved on reversed phase column with a gradient elution of acetonitrile and water, monitored by UV-absorption at 254 nm. Typical chromatogram in (Fig 1), illustrates that in a gradient elution, extracts were satisfactorily resolved into individual components, marked by the peak numbers (1-16) in the chromatogram B. Peak Nos.10, 11, 14 and 16 were the common components and found in almost all three extracts in varying proportions. The number of peaks and their concentration in chromatogram B, pertaining to chloroform ethyl acetate ext.3 were far greater as compared to the chromatogram A and C of total alcoholic ext.1 and yellow ppt.5. In turn ext.3 exhibited higher antifungal activity, (Table 1). It is reported that treatment of chickpea cell culture with a polysaccharide elicitor from *Ascochyta r abie* induced five fold higher amounts of the phytoalexin medicarpin and macckian in the resistant cultivar of chickpea (Daniel *et al* 1990).

The HMWCEP of *H.musciformis* were found to be potent elicitor and resistance responses were induced in treated chickpea tissues. Secondary metabolites extracted from elicitor treated tissues possess antifungal activity of various degrees. Extraction procedure and a reliable HPLC method was developed for the separation of induced components in treated and control tissues of chickpea.

References

Arnold A, Merlin L 1990 Lipophilicity-antifungal activity relationship for some isoflavonoid phytoalexin. *J Agric Food Chem* **38** 834-8.

Castoria A R, Maria F M, Anna T A, Marisa C F 1995 Interrelationship between browning and phytoalexins accumulation elicited by arachidonic acid. *J Plant Physiol* **145** 209-14.

Clarence R A, Edward F E 1991 Oligosaccharides signals in plants. *Ann Rev Physiol Mol Biol* **42** 651-74.

Daniel S, Tiemann K, Wittkampf U, Bless W, Hinderer W, Barz W 1990 Elicitor-induced metabolic changes in cell culture of Chickpea (*Cicer arietinum L*) cultivars resistant and susceptible to (*Ascochyta rabie 1*) Investigation of enzyme activities involved in isoflavone and peterocarpon phytoalexins. *Planta* **182** 270-8.

Darvill A G, Albersheim P 1984 Phytoalexins and their elicitors. A defence against microbial infection in plants. *Ann Rev Plant Physiol* **35** 243-75.

Fatima Bi, Seema I 1999 Studies of aqueous extracts of three green alga as an elicitor of plant defence mechanism. *Pak J Botany* **31** 193-198.

Gunia W, Hinderer W, Wittkampf U, Barz W 1991 Elicitor induction of cytochrome P-450, mono oxygenases in cell suspension cultures of Chickpea (*Cicer arietinum L*) and their involvement in pterocarpon phytoalexin biosynthesis. *Z Naturforsch C: Biosci* **46** 58-66.

Hughes R K, Dickerson A G 1991 Modulation of elicitor induced chitinase and B-1, 3-glucanase activity by hormones in *phaseolus vulgaris: Plant cell physiol* **32** (6) 853-61.

Joseph Juc 1995 Phytoalexins metabolism and disease resistance in plants. *Annu Rev Phytopathol* **33** 275-97.

Keen N T, Zaki A, Sams J J 1972 Pathogen-produced elicitor of a chemical defence mechanism in soybeans monogenically resistant to *phytophthora megasperma var sojae. Phytochemistry* **22** 2729-33.

Kessmann H, Daniel S, Barz W 1988 Elicitation of pterocarpon phytoalexins in cell suspension cultures of different Chickpea (*Cicer arietinum L*) cultivars by an elicitor from the fungus *Ascochyta rabie, Z Naturforsch C:BioSci* **43** 529-35.

Koga J, Ogawa N, Yamauchi T, Kikuchi M, Ogasawara N, Shimura M 1996 Functional moeity for the antifungal activity of phytocassane E, a diterpene phytoalexins from rice. *Phytochemistry* **44** 249-253.

Legendre L, Rueter S, Heinstein P F, Low P S 1993 Characterization of the oligogalacturonoid-induced oxidative burst in cultured soybean (glycine max) cells. *Plant physiol* **102** 233-4.

Mackenbrock U, Vogelsang R, Barz W 1992 Isoflavone and pterocarpon malonylglucosides and B-1, 3-glucan and chitin hydrolases are vacuolar constituents in Chickpea *Cicer arietinum L. E.Naturforsch C: Biosci* **47** 815-22.

Melo V M M, Medeiros D A, Rios F J B, Castelar L I M, De F F U, Carvalho A 1997 Antifungal properties of proteins (agglutinins) from the red algae, *Hypnes musciformis. Bot Mar* **40** (4) 281-284.

Raddish G F 1950 *Bacteriologic method of testing disinfectant antiseptics and fungisides drugs, Allied Industries* **36** 18.

Satakopan V N, Baskaran G, Sankar M 1992 Changes in the levels of RNA, proline, phenol and ascorbic acid in aluminium toxicity in Chickpea seedlings. *Indian J Plant Physiol* **35**(3) 272-4.

Vogelsang R, Berger E, Hagedorn T, Muehlenbeck U, Tenhaken R, Barz W 1994 Charactersization of metabolic changes involved in hypersensitive-like browning reactions of Chickpea (*Cicer arietinum L*). Cell cultures following challenge by *Ascochyta rabiei* culture filtrate. *Physiol Mol Plant Pathol* **44** 141-55.

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