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EFFECTS OF *CAPSICUM* LEACHATES ON GERMINATION, SEEDLING GROWTH AND CHLOROPHYLL ACCUMULATION IN *VIGNA RADIATA* (L.) WILCZEK SEEDLINGS

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Abstract

The effects of three concentrations of *Capsicum* leachates on germination, seedling growth, chlorophyll and chlorophyll supply-orientation (precursors for chlorophyll biosynthesis) in *Vigna radiata* var. NM98 were investigated. *Capsicum* leachates inhibited the germination of *V. radiata* seeds and also negatively affected root and shoot growths at 50 or 75%. Two-week old etiolated seedlings of *V. radiata* were cultured in growth chamber in one-tenth Hoagland culture solution with or without 25, 50 or 75% leachates. Leaves were harvested at 0, 6, 12, 24 and 48h time interval after treatment. The concentrations of chlorophyll and its three biosynthetic porphyrin precursors i.e. proto porphyrin IX (Proto), Mg-proto porphyrin IX (Mg-Proto), and proto-chlorophyllide (Pchlide) were determined and the mole percent of total porphyrins was calculated. Accumulation of chlorophyll and porphyrin contents was inhibited as the leachate concentrations increased. The mole percent of Mg-Proto affected by the leachate exhibited the same pattern as that of Pchlide and Proto. The data strongly suggests that enzymes responsible for the conversion of Proto to Mg-Proto or Mg-Proto to Pchlide may be the major targets of the leachates causing the significant decrease in chlorophyll contents.

Introduction

Capsaicin is a main leaching compound of *Capsicum* spp such as chilli pepper, red pepper and cayenne pepper exudates from the root and fruit of *Capsicum* plant. It is an amide derivative of vanilylamine and 8-methylnon-*trans*-6-enolic acids (Ochoa-Alejo & Salgado-Garacilia, 1992) with number of pharmacological and physiological effects on mammals (Surh & Lee, 1995; Abdel Salam *et al*., 1997). Several reports have shown the potential allelopathic effects of *Capsicum* leachates on germination and plant growth (Cho *et al*., 1992; González *et al*., 1997; Kato-Nogouchi & Ino, 2001; Bennett & Habeck, 1991; Li & Norland, 2001; Morton, 1978; Herro & Callaway, 2003). However, the physiological mechanism behind the *Capsicum* allelopathy needs further elucidation.

Allelopathy through phenolic compounds has been well documented for many years (Rice, 1984) and multiple physiological effects like reduced leaf water potential, stomatal diffusive conductance, plasma membrane perturbation and decreased photosynthetic rate are also reported in different plant species (Patterson, 1981; Einhellig *et al*., 1985; Gerald *et al*., 1992; Einhellig *et al*., 1970; Chou & Lin, 1976; Einhellig & Kuan, 1971; Einhellig *et al*., 1985; Einhellig, 1995; Patterson, 1981; Einhellig, 1986; Mersie & Singh, 1993; González *et al*., 1997; Materska *et al*., 2003).

Even though a reduction of photosynthesis has been widely observed in allelochemical-targeted plants, the component of photosynthesis which is directly or indirectly affected by the allelochemical is still unknown. Chlorophylls are complex

molecules exquisitely suited to the light absorption, energy transfer and electron transfer function that they carry out in photosynthesis. Like all other biomolecules, chlorophylls are made by a biosynthetic pathway in which simple molecules are used as building blocks to assemble more complex molecules. It is presumed that fate regarding photosynthesis reduction due to allelochemicals is related to chlorophyll metabolism. Therefore, it is hypothesized that the leaching compound from chilli pepper and red pepper like capsaicin may behave as allelochemical because of its phenolic property which 1) may partially block the biosynthetic pathway of chlorophyll (i.e. an inhibition of supply-orientation), or 2) stimulate the degradative pathway of chlorophyll (i.e. a stimulation of consumption-orientation) or both, leading to a reduction of chlorophyll accumulation which in turn cause a reduction of photosynthesis and finally results in diminished total plant growth. To test the hypothesis, the present study was aimed at gauging the inhibitory effects of three different concentrations of leachate of *Capsicum* plants on the chlorophyll biosynthetic pathway to provide a physiological explanation.

Materials and Methods

Collection of *Capsicum* leachates: About 200 plants of *Capsicum annuum* were planted in plastic pots and irrigated with tap water at 4 days interval and leachate were collected through draining every week up to the late fruiting stage when senescence was obvious. These leachates were used to study this effect on germination, seedling growth (experiment 1) and chlorophyll metabolism (experiment 2).

Experiment 1: Seeds of *Vigna radiata* (L.) Wilczek var. NM98, were collected from Nuclear Institutes of Agriculture and Biology Faisalabad (NIAB) and surface sterilized in 70% ethanol for 10 min., then in 2% Sodium hypochlorite for 5 min., and finally washed several times with distilled water. The sterilized seeds were soaked in distilled water (control) and respective leachate solutions of *Capsicum* (25, 50 and 75%) for 10min. After that 20 healthy seeds of each treatment and control were transferred in 9cm diameter Petri plates. The whole set-up was kept in Hotpack germinator under controlled conditions (30 ± 2°C during 13hrs light periods from 7 A.M to 4 P.M of about 12 h, 300 µmol. m^{-2} s^{-1} white florescence light and 20.5 ± 2°C during 11hrs darkness). A 5ml of test solution and distilled water were added on alternate days. Final germination percentage and root and shoot lengths were recorded after 15-days. Each treatment and control was replicated four times. The data sets were subjected to factorial analysis of variance in a completely randomized manner using SPSS 11.0 (Anon., 2005).

Experiment 2: Seeds of *Vigna radiata* (L.) Wilczek cultivar NM98 was sterilized in the same manner as described in experiment 1. The sterilized seeds were soaked and incubated in water in the dark at 30ºC for 2 h and transferred in three 40 cm diameter pots (triplicate) kept in dark at 30ºC in each pot more than 200 seedlings were grown. A total 30 etiolated seedlings were transferred in fresh one-tenth Hoagland culture solution containing 25, 50 or 75% of leachates respectively while 0% served as control. The photoperiod, light intensity and relative humidity were 13 h, 300 µmol/m^{-2} s and 70%, respectively. The sample leaves of 25 seedlings from each pot were harvested randomly at 0, 6, 12, 24 and 48 h after treatment to perform the following biochemical examination. All the treatments and control were replicated four times. The data sets were subjected to factorial analysis of variance using SPSS 11.0 (Anon., 2005).

Table 1. Effect of *Capsicum* leachate on germination and seedling growth of *Vigna radiata* (L.) Wilczek.

Leachate Concentration (%)	Germination (%)	Total root Length (cm)	Total shoot Length (cm)	Total seedling Length (cm)
0	95.5 ± 3.5a	2.50 ± 0.88a	7.6 ± 0.45a	10.1 ± 0.88a
25	75.25 ± 2.24b	2.25 ± 0.25a	5.25 ± 0.98b	7.50 ± 0.58b
50	58.25 ± 2.25c	1.50 ± 0.24b	3.70 ± 0.77c	5.20 ± 0.58c
75	45.50 ± 3.5d	0.88 ± 0.045c	1.88 ± 0.66d	2.76 ± 0.33d

Note: For each concentration, values having the same letters are not significantly different at p= 0.05, Bonferroni test (SPSS, 2005)

Chlorophyll determination: Following extraction of liquid-nitrogen frozen leaf with 80% acetone, the concentration of chlorophyll was determined according to the spectrophotometer method of Porra *et al*., (1989). Absorbance was measured with a Hitachi U1680 UV-visible spectrophotometer.

Porphyrins determination: For three porphyrin precursors of chlorophyll biosynthesis i.e., protoporphyrin IX (Proto) magnesium-protoporphyrin (Mg-Proto) and protochlorophyllide (Pchlide), samples of leaves were extracted in ice cold 20mL acetone and 0.1N NaOH (9:1v/v) solvent mixtures. The extracted material was centrifuged at 3000rpm for 15min. The pellets were then extracted twice with 10mL acetone and NH_4OH mixtures in a ratio of 8:2v/v for the maximum recovery. The pooled supernatant and washing were extracted with 30mL of petroleum ether to extract the protochlorphyllide whereas 1.2mL of saturated NaCl and 0.3mL of 0.5M KH_2 PO_4 were added in remaining acetone extract solution for Mg-Proto. Likewise, proto porphyrin IX was extracted in 10mL of ethyl acetate and acetic acid solvents in 3:1v/v ratio. The contents of three porphyrin precursors were then estimated by the method of Kahn *et al*., (1976). The mole percent of porphyrin was calculated in the following manner: (%) = [Proto (or Mg- Proto or Pchlide) /(Proto+Mg-Proto+Pchlide)] × 100%.

Statistical analysis: Analysis of variance (ANOVA) was used to test the significance of the main effects. When significant difference was found among means, a Bonferroni post-hoc test was used to determine whether significant ($p<0.05$) differences occurred between individual treatments (Anon., 2005).

Results and Discussion

Capsicum leachate inhibited the germination of *V. radiata* seeds and also negatively affected root and shoot growth at 50 or 75% leachates (Table 1). Especially the affect on root growth was more evident with the increasing concentrations. Similarly the shoot growth also decreased as the concentration increased. This negative impact on the root and shoot growth also caused a decrease in the overall length of the seedling.

All three tested leachate concentrations also exhibited inhibitory effects on the chlorophyll accumulation of *V. radiata* seedlings and demonstrated similar inhibitory patterns (Fig. 1). Of the three concentrations of leachate, 25% caused the least inhibitory effect. Inhibition could be detected within 6 h of treatment with each of the concentration, and it was extended by increasing either the concentration or the treatment duration. In addition to affecting chlorophyll, leachate also inhibited the accumulation of three porphyrins (Porphyrin, Mg-Proto, and Pchlide) with 75% of *Capsicum* leachate causing

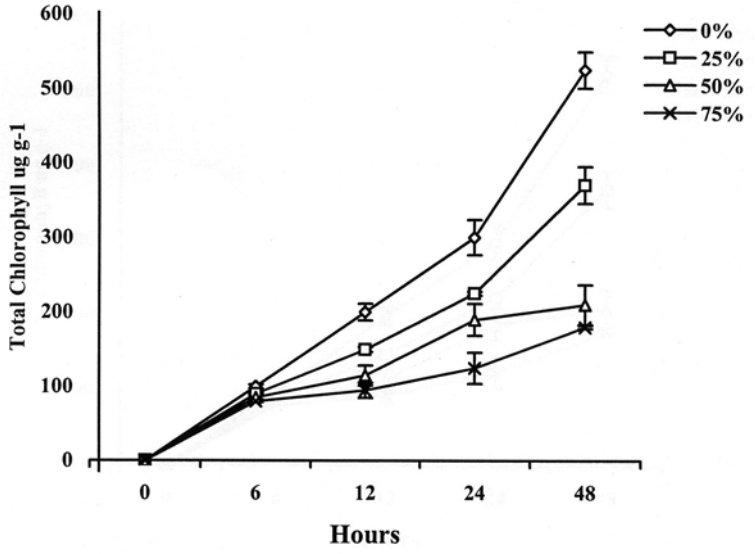

Fig 1. Effect of *Capsicum* leachates on total chlorophyll contents of *Vigna radiata* (L.) Wilczek

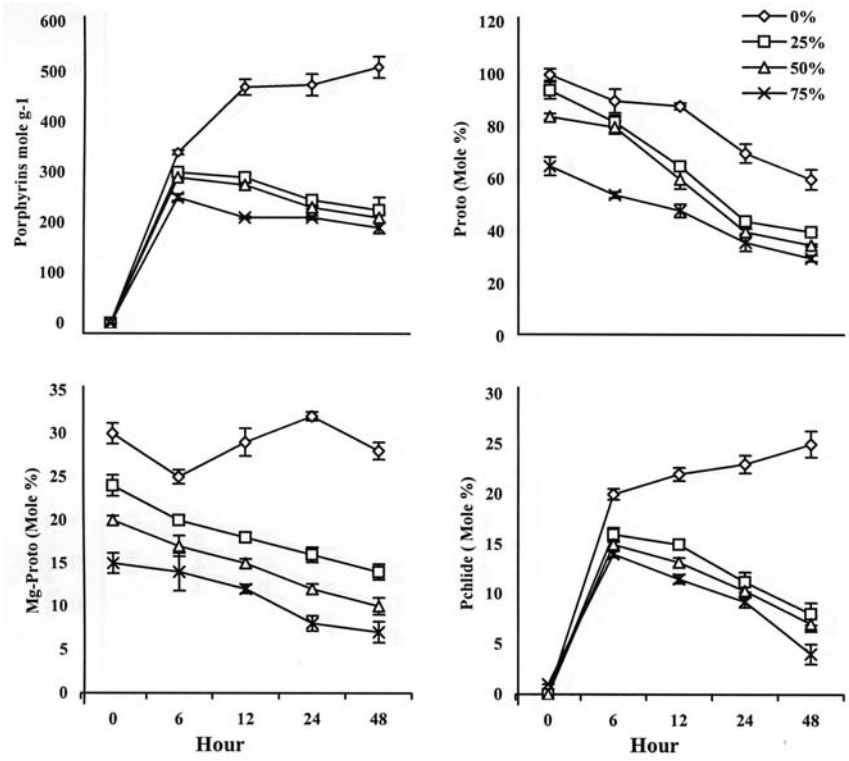

Fig 2. Effect of *Capsicum* leachates on porphyrin, proto, Mg-proto and pchlide contents of *Vigna radiata* (L.) Wilczek

more inhibition than 25 and 50% leachate. With the increase in concentration and treatment duration, the mole percentage declined however, the mole percentages of individual porphyrins were extremely different. Apparently, two distinct phases were observed in the mole percentage of the total porphyrins: the first was a fast phase, occurring within 6 h, and the second was a slow one occurring thereafter. Under controlled conditions, the porphyrin accumulation increased (Fig. 2), the mole percent of Proto plunged from about 75% to 61% within 48h. During the 48-hour period, the mole percent of Mg-Proto gradually decreased (Fig. 2). Apparently, the alteration of mole percentage of Mg-Proto like that of Proto is a monophasic reaction. For the mole percent of Mg-Proto, the three concentrations exhibited significant effects during the whole treatment period. Mole percent of Pchlide followed the same inhibitory pattern caused by leachate (Fig. 2). Under normal conditions, the mole percent of Pchlide increased from about 0% to 20% within 6 h then gradually reached up to 25%. Among the three concentrations, 75% leachate exhibited the most severe inhibitory effect than 50 and 25%. During treatment, 25, 50 and 75% leachate increased the mole percent of Pchlide from 0% to only 15% and then gradually declined goes down to 5%.

The present investigation revealed that *Capsicum* plants leachate inhibited the germination of *V. radiata* seeds and also negatively affected root and shoot growth at concentrations of 50 or 75%. Occurrence of capsaicin in *Capsicum* (Leete & Louden, 1968; Iwai *et al*., 1979) and its effectiveness on germination and growth suggests that capsaicin may act as an allelochemical after being released into soil after decomposition of the pepper or by exudation from their roots. The presence of these exudates then goes on negatively affecting the neighboring or successional plants. It has been reported that *Biden alba* and *Rivina humilis* were negatively affected in terms of germination and biomass accumulation by the irrigation of aqueous extract of Brazilian pepper leaves in field laboratory experiments (Morgan & Overholt (2005). Similar allelopathic impact were also reported by Bennett & Habeck (1991), Li & Norland (2001), Morton (1978) and Herro & Callaway, (2003).

Several studies have reported that many secondary metabolites are released into the environment, either as exudation from living plant tissues or by decomposition of plant material under certain conditions (Rice, 1984; Putnam, 1988, Einhelling, 1995). These chemicals like phenolics, terpenoids and alkaloids and their derivatives are potential inhibitors of germination, seedling growth (Macias *et al*., 1992; Siddiqui, *et al*., 1999; Siddiqui & Zaman, 2004), enzymes activity (Borua & Das, 2000; Cremer & Eichner, 2000)

Accumulation of chlorophyll and porphyrin contents were inhibited as the leachate concentrations increased. The mole percent of Mg-Proto affected by the leachate exhibited the same pattern as that of Proto while Pchlide showed slightly different pattern. Chlorophyll molecules are the core component of pigment-protein complexes embedded in the photosynthetic membranes and play a major role in photosynthesis. It was hypothesized that a chlorophyll reduction might be the cause in a decrease of photosynthesis. Leaching compound like capsaicin and its related compound present in various *Capsicum* spp., may reduce chlorophyll accumulation in three ways: the inhibition of chlorophyll biosynthesis, the stimulation of chlorophyll degradation, or both (Morgan & Overholt, 2005). The present study strongly indicated that chlorophyll biosynthesis of *Vigna* seedlings is inhibited by the three exogenous concentrations of *Capsicum* leachate. The leachate exhibited apparently different degrees of inhibition on

the reactions between Proto and Pchlide in first 6h, and especially on the reaction between Mg-Proto and pchlide. Since the three concentrations are affected on the mole percent of Pchlide, it seems that *Capsicum* leachate inhibited the Mg-Proto methylesterase, Mg-protoporphyrin IX monomethylester oxidative cyclase (Mg-proto ME cyclase), or Mg-2, 4-divinyl-pheoporphyrin at reductase (Mg-DVP reductase). Possibly, enzymes responsible for the conversion of Proto to Mg-Proto or Mg-Proto to pchlide may be the major targets of the leachate. Similar results were obtained by Yang *et al*., (2002) after treatment of rice plant with three allelopathic phenolics. Further studies are needed to clarify the possible physiological mechanism related allelopathic effect on plants.

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