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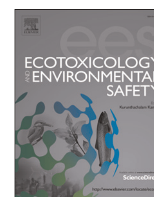

Phytotoxic mechanisms of bur cucumber seed extracts on lettuce with special reference to analysis of chloroplast proteins, phytohormones, and nutritional elements

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ABSTRACT

Bioherbicides from plant extracts are an effective and environmentally friendly method to prevent weed growth. The present investigation was aimed at determining the inhibitory effect of bur cucumber seed extracts (BSE) on lettuce plant growth. Bur cucumber seeds were ground with water, and two different concentrations of seed extracts (10% and 20%) were prepared and applied to lettuce plants. Decreased plant height, number of leaves, leaf length, leaf width, an Prod. Type: FTPd leaf area were found in lettuce exposed to BSE as compared with controls. A significant reduction in lettuce biomass was observed in 20% BSE-treated plants due to the presence of higher amounts of phenolic content in the extracts. Moreover, a significant inhibitory chemical, 2-linoleoyl glycerol, was identified in BSE extracts. The mechanism of plant growth inhibition was assayed in lettuce proteins by 2-dimensional electrophoresis (2-DE) and the LC-MS/MS method. In total, 57 protein spots were detected in plants treated with 20% BSE and control plants. Among these, 39 proteins were down-regulated and 18 proteins were up-regulated in plants exposed to 20% BSE as compared with controls. The presence of low levels of chlorophyll a/b binding protein and oxygen-evolving enhancer protein 1 in BSE-exposed plants reduced photosynthetic pigment synthesis and might be a reason for stunted plant growth. Indeed, the plant-growth stimulating hormone gibberellin was inhibited, and synthesis of stress hormones such as abscisic acid, jasmonic acid, and salicylic acid were triggered in lettuce by the effects of BSE. Uptake of essential nutrients, Ca, Fe, Mg, K, S, and Mo, was deficient and accumulation of the toxic ions Cu, Zn, and Na was higher in BSE-treated plants. The results of this study suggest that extracts of bur cucumber seeds can be an effective eco-friendly bioherbicide for weed control that work by inhibiting mechanisms of photosynthesis and regulating phytohormones and nutritional elements.

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1. Introduction

Because the growth of weeds can decrease crop productivity, synthetic herbicides are widely used to prevent weed growth. The increasing use of synthetic herbicides to control weeds can leave deposits in soil and pollute the environment, and prolonged application of herbicides often increases herbicide-resistant weed biotypes (Batish et al., 2007; Aktar et al., 2009). Therefore, the use of environmentally friendly methods of weed management that avoid chemical hazards is an emerging research area. Plant growth-inhibiting bacteria, fungi, and plant products that can control weed growth have been investigated to find suitable bioherbicides (Bethlenfalvay et al., 1996; Dayan et al., 2000; Boyette and Hoagland, 2015). Water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes, ketones, lactones, long-chain fatty acids, polyacetylenes, quinines, phenolics, cinnamic acid, coumarins, flavonoids, tannins, steroids, and terpenoids are secreted into the environment from roots, leaves, stems, flowers, fruits, and seeds and affect germination and growth of neighboring plants (Soltys et al., 2013). The release of allelochemicals from plants or their decomposed residues into the soil inhibits weed growth (Dayan et al., 2000).

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Moreover, herbicides from natural sources may prevent weed growth without environmental damage (Haig et al., 2009). Various allelochemicals containing extracts have been tested on seeds and seedlings and their detrimental effects can inhibit seed germination and cause abnormalities in roots and shoot growth (Tigre et al., 2012). However, little information is known about the allelopathic effects of plant extracts on metabolism of the affected plants. Recently, Omezzine et al. (2014) demonstrated that an aqueous extract of fenugreek degraded cellular membranes in lettuce by increasing lipid peroxidation and electrolyte leakage while reducing mitochondrial respiration and pigment synthesis. They suggested that understanding the different mechanisms of action of allelochemicals derived from plants may help develop efficient natural plant growth regulators and pesticides for sustainable agriculture.

Bur cucumber (*Sicyos angulatus* L.) is an annual plant belonging to the family Cucurbitaceae. Although bur cucumber has been reported as an invasive species in Korea (Moon et al., 2008), it is able to control fusarium disease in plants (Kwon et al., 2005), and biochar derived from bur cucumber is effective in reducing the mobility of veterinary antibiotics in remediation of antibiotic-polluted soils (Rajapaksha et al., 2014). In this study, we made the first attempt to elucidate the bioherbicide effect of bur cucumber. Aqueous extracts of plants may act as a weapon against weeds. Several studies have been conducted to determine the allelopathic activity of a number of plants based on germination and growth impact studies, but in these methanol, acetone, and ethanol extracts were used more often than water extracts (Hill et al., 2007). In addition, water is the only solvent available in the natural environment and all organisms use it, thus water extracts of plants are a practical, possible way to control weeds (Harun et al., 2014). Moreover, the presence of phenolic substances in organic extracts is higher than in water extracts, and their allelopathic effects may not be similar in the plant–weed ecosystem. Reports on physiological changes in plants exposed to environmental allelochemicals are rare. No previous studies have been carried out on the mode of action of aqueous plant-herbicide-extract-induced disruption of interactions among gibberellins, abscisic acid, salicylic acid, and protein metabolism. The aim of this study was to determine the bioherbicide effects of bur cucumber and its mode of action in phytohormonal, nutritional, and protein regulation in a model plant, lettuce.

2. Materials and methods

2.1. Bur cucumber extract preparation and seed germination

Dried bur cucumber pods were collected from Gumi, Republic of Korea, in January 2014, and the seeds were carefully removed and freeze dried. To prepare seed extracts, freeze-dried seeds were ground in a grinder. The various concentrations of bur cucumber seed extracts (BSE: 0.5%, 1.0%, 2.0%, 3.0%, 5.0% 10%, and 20%) were prepared by adding sterile distilled water and agitating them in a shaker at 140 rpm at 30 °C for 12 h. The filtrates were collected after passing them through a muslin sieve and filtered again through syringe filters (0.45 μm and 0.22 μm). The different concentrations of clear filtrates were separately applied to petri plates containing weed (*Digitaria ciliaris* Retz.) and lettuce seeds. The percentage of seed germination was observed for 7 days.

2.2. Bur cucumber extract and lettuce plant growth

Lettuce seeds were sown in pots containing an autoclaved horticulture soil mixture (peat moss (13–18%), perlite (11%), cocopeat (63–68%), and zeolite (6–8%) with NH_4^+ ~0.09 mg g^{-1}; NO_3^- ~0.205 mg g^{-1}; P_2O_5 ~0.35 mg g^{-1}; and K_2O ~0.1 mg g^{-1}) and were kept in a greenhouse (30 ± 2 °C) and periodically irrigated. To determine the bioherbicide effects of bur cucumber, 10% and 20% extracts were sprayed on six-leaf-stage lettuce plants once a week for 3 weeks. Each plant received 30 ml of BSE. The plant growth experiments were conducted in a completely randomized block design. Plant height, leaf numbers, leaf length, leaf width, leaf area, fresh weight, and chlorophyll content were measured at 7 days after treatment. The harvested samples were ground with liquid nitrogen to identify proteins and freeze-dried samples were used for studies of plant metabolites and nutritional elements.

2.3. Isolation and identification of proteins

2.3.1. Protein sample preparation

Plants treated with 20% BSE and non-treated lettuce plants were homogenized by mortar and pestle (Power Gen125, Fisher Scientific) with a lysis solution composed of 7 M urea and 2 M thiourea containing 4% 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), 2% pharmalyte, and 1 mM benzamidine. Plant tissues were vortexed at room temperature for 1 h, and the crude extract was centrifuged at 15,000 rpm and held at 15 °C for 1 h. The insoluble material was discarded and the soluble fraction was used for 2-dimensional gel electrophoresis.

2.3.2. 2-Dimensional gel electrophoresis

IPG dry strips (4-10 NL IPG, 24 cm, Genomine, Korea) were equilibrated for 16 h by using 7 M urea and 2 M thiourea containing 2% CHAPS, 1% DTT, and 1% pharmalyte, and a protein sample (200 μg) was added to the IPG strips. Isoelectric focusing (IEF) was performed at 20 °C in a Multiphor II electrophoresis unit with EPS 3500 XL power supply (Amersham Biosciences). For IEF, the voltage was linearly increased from 150 to 3500 V before sample entry, and a constant 3500 V was maintained to the completion of the experiment. Prior to 2D SDS-PAGE electrophoresis, the strips were incubated for 10 min in an equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS, and 30% glycerol) with 1% DTT and followed by 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 × 24 cm, 10–16%). SDS-PAGE was performed using the Hoefer DALT 2D system (Amersham Biosciences). 2D gels were run at 20 °C for 1700 volt-hours. The protein spots on the gels were visualized using Coomassie G250 stain as described by Anderson et al. (1991).

2.3.3. Image and data analysis

Quantitative analysis of digitized images was carried out using PD Quest (version 7.0, BioRad) software according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. Protein spots that showed significant expression variation between BSE-treated and control plant samples were selected.

2.3.4. In-gel protein digestion

The selected protein spots were excised and digested in-gel with sequencing grade modified trypsin (Promega, Madison, WI) as previously described (Bahk et al., 2004). In brief, each protein spot was excised from the gel, placed in an Eppendorf tube, and washed 4–5 times with 150 μl acetonitrile and 25 mM ammonium bicarbonate (1:1), pH 7.8. The gel slices were dried in a Speedvac concentrator and then rehydrated in 30 μl of 25 mM ammonium bicarbonate, pH 7.8, containing 20 ng trypsin. After incubation at 37 °C for 20 h, the liquid was transferred to a new tube. Tryptic peptides remaining in the gel matrix were extracted for 40 min at 30 °C with 20 μl 50% aqueous acetonitrile containing 0.1% formic

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