Allelopathic effects of *Hyptis suaveolens* L. on growth and metabolism of pea seedlings

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## A B S T R A C T

The present study deals with the allelopathic stress caused by allelochemicals present in leachate of *Hyptis suaveolens* L. on growth and metabolism of pea seedlings. Seeds were soaked in distilled water for 3 hours. Five seeds of *Pisum sativum* were sown in each pot filled with acid washed sand. Pots were moistened exogenously with different concentrations of leachate viz. 25, 50, 75 and 100% respectively. Twenty days old seedlings were taken for biophysical and biochemical parameters and calculated. Root length and shoot length, fresh weight and dry weight of pea seedlings decreased in dose dependent manner. Pigment content, protein, sugar and activities of nitrate reductase were decline. Activities of enzymes viz. superoxide dismutase, catalase and peroxidase increased under allelopathic stress caused by the allelochemicals present in the leachate of donor plant. Increases in lipid peroxidation were observed in terms of malondialdehyde (MDA) content. Allelopathic stress influence several metabolic activities of plant which lead to inhibition in growth and metabolism of recipient plant.

**Key words:** Allelopathy, Antioxidants, Leachate, *Hyptis suaveolens*, Lipid peroxidation, Nitrate reductase

## Introduction

Biochemical interaction with beneficial and harmful effects among plants caused by secondary metabolites have both on plants as allelopathy (Inderjit, 1996, Rice, 1984). Allelopathy is a natural phenomenon having bio-chemical interaction among plants (Rice, 1984). A variety of chemical compound are known as allelochemicals are released from plants into the environment via., leaching, root exudation, mulching, volatilization and decomposition (Inderjit and Kneating, 1999). Allelochemicals released from plants by different ways may affect the growth of plant of the vicinity is also known as abiotic stress or allelochemical stress (Cruze-ortega et al., 2002; Romero-Romero et al., 2002). Secondary metabolites produced by one plant species that affect another plant species of neighborhood have non- nutritional values (Minorsky, 2002; Calaway and Ridenour, 2004). Rain facilitates leaching of allelochemicals present in the plant. Realease of secondary metabolites from plant to plant, plant to soil and surrounding areas affect the growth of neighboring plants in natural or agro ecosystem or agricultural filled (Inderjit and Duke, 2003).

*Hyptis suaveolens* which belongs to family Lamiaceae usually known as wilayati tulsi is an effective medicinal aromatic plant. Medicinal properties of this plant is due to presence of several chemical compounds like carbohydrate, phenol, tannin, saponin, alkaloids, steroids and flavonoids etc. (Wealth of India, 1964). Native place of *Hyptis suaveolens* is tropical America it is naturalized in India. The plant is sometimes regarded as pantropical weed because now it is widespread throughout the whole world from tropical to subtropical regions (Afolayan, 1993; Sarmiento, 1984).

*Hyptis suaveolens* is a soft suffrutescent and ruderal aromatic weed commonly rigid annual herb with aggressive nature grows adjacent to the road and the moist boundaries of ponds (Mudgal et al., 1997). *Hyptis suaveolens* is one of the aromatic weeds which are most noxious. It is a world’s exotic persistent species invading the natural ecosystems (Padalia et al., 2013). Damage to the surrounding localities is inferior in a large amount caused by the its utilization for the pharmacological and industrial purpose (Raizada, 2006). The growth and establishment of other plant species are quite restricted near their clumps (Raizada, 2006), but the exact reasons which lead to the dominance of *H. suaveolens* is still remain unclear. Phytotoxicity of this plant could be one of the believable reasons for such interference. Some previous studies related to phytotoxicity of *Hyptis suaveolens* on the germination and growth of different test plant species considered as exception but there is no latest data has been reported to deal with the phytotoxic substances of *H. suaveolens*. (Kapoor, 2011; Chatiyannon et al., 2012; Rodrigues et
al., 2012). Germination and seedling growth of several weed and crop species affected by the phytotoxic activities of aqueous methanol extract of H. suaveolens (Islam et al., 2013).

Allelopathic potential of Hyptis is still unknown but it might be give a stong composition to the other aromatic plants which belong to family lamiaceae due to presence of a strong fragrance or odour and volatile oil present in it. (Heisey and Delwiche, 1985). The plants belonging to family Lamiaceae have essential oils, volatile oils, exudates and leachates etc. responsible for allelopathic potential (Qasem and Foy 2001). Hyptis suaveolens is an annual savanna herb of neotropical origin having broad-leaf with tropical and subtropical distribution (Monasterio and Sarmiento 1976; Sarmiento 1984; Afolayan 1993). In savannas where human impact is low it is quantitatively insignificant plant. However, the species has become widespread and very difficult and expensive to control growth in areas where mechanized agriculture and severe cattle raising are skillful, (Holmes 1969; Harrison 1973). Chemical and mechanical control procedures are limited because the overlapping growing season of weed and crops. It inhibits seed germination and seedling growth of some crops and weed species (Kapoor, 2011). In previous studies aqueous extract of Hyptis suaveolense L. aerial part is used to verify the germination of weed and crop plants.

Materials And Methods

Plant of Hyptis suaveolense is collected from the surrounding areas of the Department of Botany, University of Allahabad (24°47’ and 50° 47’N latitude; 81°91’ and 82° 21’E longitude; 78 m above sea level).

Leachate preparation
Whole plants of Hyptis suaveolens L. which is collected from nearest areas were chopped into small pieces and soaked in distilled water at 1:4(w/v), separately and kept in refrigerator for 3 days at 8°C. After 3 days plant parts leachate was filtered. The stock leachate of plant considered as 100% concentration was further diluted with distilled water to prepare different concentration viz. 25, 50 and 75% of stock leachate.

Sand culture
Seeds of pea (Pisum sativum L. var. AP3) were purchased from certified seed agency of Allahabad. The seeds were surface sterilized with 0.1% HgCl₂ (m/w) solution for 3 minutes and soaked in distilled water for 3 hours. Five seeds of Pisum sativum were sown in each pot filled with acid washed sand. Pots were moistened exogenously with different concentrations of leachate viz. 25, 50, 75 and 100% respectively experiment were conducted in the culture room. The experiment was done in triplicate. Biochemical and biochemical parameters were also recorded. First fully expended leaves were selected from biochemical analysis.

Determination of pigment and protein content
Chlorophyll of experimental plant was extracted with 80% acetone. The amount of photosynthetic pigments was determined as per Lichtenthaler (1987). Fresh leaf (10mg) was homogenized in 10mL 80% acetone and centrifuged. Supernatant was taken and optical density was measured at 663, 645 and 470 nm. Protein content was determined as per the method of Lowry et al. (1951). The amount of protein was calculated with reference to standard curve obtained from bovine serum albumin.

Sugar content
Sugar content was estimated following Hedge and Hofreiter (1962). About 0.25 g of the sample was homogenized in 2.5mL of 95% ethanol. After centrifugation, the sugar content was determined in the supernatant. The supernatant (1mL) was mixed with 4 mL of anthrone reagent and heated on boiling water bath for 8 min. Absorbance was taken at 620 nm after rapid cooling. Standard curve was prepared from glucose.

Lipid peroxidation
Lipid peroxidation was measured in terms of malondialdehyde content following the method of Heath and Packer (1968). Leaves (200 mg) of the test plant were homogenized in 5 mL of trichloroacetic acid (0.1% w/v) and centrifuged at 10000 g for 10 min. Malondialdehyde level was used as index of lipid peroxidation and was expressed as nmol g⁻¹ fresh weight. One mL supernatant was added to 4mL 0.5 thiobarbituric acid prepared in 20% trichloroacetic acid. The mixture was incubated at 95°C for 30 min followed by rapid cooling and centrifuged at 10000 g for 10 min. The absorbance of supernatant was recorded at 532 nm and corrected for non specific absorbance at 600 nm. Malondialdehyde content was determined using the extinction coefficient of 155 mM cm⁻¹.
Nitrate reductase

Nitrate reductase activity was assayed by modified procedure of Jaworski (1971) based on incubation of fresh tissue (0.25 g) in 4.5 mL medium containing 100 mM phosphate buffer (pH 7.5), 3% KNO3 and 5% propanol. About 0.4 mL aliquot was treated with 0.3 mL 3% sulphanilamide in 3 N HCl and 0.3 mL 0.02% N-1-naphthyl ethylene diamine dihydrochloride (NEDD). The absorbance was measured at 540 nm. NR activity was calculated with a standard curve prepared from NaNO2 and expressed as μ mol NO2 g-1 FW h⁻¹.

Antioxidant enzyme assay

Enzyme extract was prepared by homogenizing 500 mg of leaves tissue in 10 mL of 0.1 M sodium phosphate buffer (pH=7.0). The homogenate was filtered and centrifuged at 15000 g at for 30 min and temperature was maintained at 40°C. The supernatant was collected and used for analysis of superoxide dismutase, catalase and peroxidase.

Superoxide dismutase (SOD) activity was determined by the nitroblue tetrazolium (NBT) photochemical assay method described by Beyer and Fridovich (1987). The reaction mixture (4 mL) contained 63 μM NBT, 13 mM methionine, 0.1 mM ethylene diaminetetra acetic acid (EDTA), 13 μM riboflavin, 0.5 M sodium carbonate and 0.5 mL clear supernatant. The test tubes were placed under fluorescent lamps for 30 min and absorbance was recorded at 560 nm. One unit of enzyme was defined as the amount of enzyme which caused 50% inhibition of NBT reduction.

Catalase (CAT) activity was assayed following the method of Cakmak and Marschner (1992). The reaction mixture (2mL) contained 25 mM sodium phosphate buffer (pH = 7.0), 10 mM H2O2 and 0.2 mL enzyme extract. The activity was determined by measuring the rate of disappearance of H2O2 for 1 min at 240 nm and estimated using extinction coefficient of 39.4 mM⁻¹.cm⁻¹ and expressed as enzyme unit/g fresh weight. One unit of CAT was defined as the amount of enzyme required to oxidize 1μM H2O2/min. Peroxidase (POX) activity was assayed following the method of Mc Cune and Galston (1959). Reaction mixture contained 2 mL enzyme extract, 2 mL sodium phosphate buffer, 1 mL 0.1-N pyrogallol and 0.2 mL 0.02% H2O2 and determined spectrophotometrically at 430 nm. One unit of enzyme activity was defined as the amount which produced an increase of 0.1 optical density per minute.

Statistical analysis

Standard errors of means were calculated in triplicates. In addition, analysis of variance was carried out for all the data generated from this experiment, employing one way ANOVA test using GPlS software 3.0 (GRAPHPAD California USA).

Results And Discussion

The effect of different concentrations of Hyptis suaveolens leachate on shoot length, root length, fresh weight and dry weight of pea seedlings is significantly decreased with 32.09, 36.63, 26.93 and 62.88% respectively (Table 1). In the present study demonstrates the phytotoxic influence of allelochemical present in Hyptis suaveolens leachate on growth and metabolism of pea seedlings. Plant leachate has potential secondary metabolites or allelochemicals. The leachate have shown toxic effects on the seedlings of test plant. Production of allelochemicals in plants is induced by environmental biotic and abiotic stresses. Our results revealed reduction in seedling height under allelochemical stress. Reduction in plant growth was earlier reported under allelopathic stress (Batish et al., 2006; Singh et al., 2009, Singh et al., 2008). Previous findings support our result in which growth of tomato, radish cucumber and barnyardgrass inhibited via water extract of Lantana camara (Liu et al., 2002) and inhibition in root initiation, number of roots and root length of hypocotyls of mung bean and pea was observed treated with leaf extract of Eucalyptus urophylla (Huang et al., 1997). Five weed sp. and five crop plants were in use for the study of germination of taken plants named Alternanthera sessile, Echinochola colomn L., Tridex procumbens L., Parthenium hysterophorous L., Cyprus tubers and crop plant were used are black gram, Cluster bean, Cotton, ladies finger and rice respectively. The finding of this study is that the aqueous extract inhibits the germination of weed whereas seeds of crop plants were unaffected (Rao et al., 1987). The aqueous methanol extracts and the crude extract and or the residue of Hyptis suaveolens L. have strong allelopathic potential and suggested to apply as bio-herbicide (Islam et al., 2013). Herbicidal activities of Hyptis suaveolens L. is due to chemicals present in leaf. Suaveolic acid, a phytotoxic substance isolated from the aqueous methanol extract of Hyptis suaveolens L. show phytotoxicity effect on growth of shoot and root length of garden Cress, Italian rye grass, barnyard grass and lettuce shoots (Mominul et al., 2014). Increased amount of leachate concentrations considerably decreased seedlings growth. The growth of the test plant adversely affected by the plant leachate of Hypties suaveolens. Allelopathic nature of Hyptis suaveolens leachates affects morphological parameters of plants. It was due to revelation of impaired metabolic activities of plant which leads to decrease in FW, DW, SL and RL of seedlings (Singh et al., 2008).

Photosynthetic pigments were adversely affected by the allelochemical stress. A significant decrease was observed in total chlorophyll and carotenoids content is maximum in 35.58 and 90.31% respectively higher concentration (Table 2). Due to influence of allelopathy of allelochemicals present in the leachate of donor plant reduction in photosynthetic pigment
observed. The gradual decreased in chlorophyll content under all treatments may be due to inhibition in enzyme synthesis, cofactors required for chlorophyll synthesis and protein (Kohli, 1992). Under allelochemical stress extreme degradation of chlorophyll also arise due to influence of allelochemicals (Thaper and Singh, 2006). Allelochemicals reduce the accumulation of chlorophyll content (Hejl et al., 1993; Singh et al., 2010). Similar results were also reported in case of sorghum and radish (Venkateshwara et al., 2001; Bagavathy and Xavier 2007).

Protein and sugars contents gradually decreased in dose dependent manner. Maximum decrease is occurred higher concentration is 29.41% and 23.26%, respectively (Table 2). Seedlings of control group contain maximum protein and sugar contents. Allelochemicals affect plant physiological processes such as cell wall expansion, protein synthesis, antioxidant enzymatic activities (Rice, 1984; Baziramkenga et al., 1997). There is a gradual decrease in sugar content all given treatments under allelochemical stress in comparison to control. Decreased rate of protein synthesis is result of integration of certain amino acids into protein inhibited by phenolic acids (Baziramkenga et al., 1997). The allelochemicals are known to increases the amount of free amino acids resulted protein degradation (Singh and Thapar, 2003).

Nitrate reductase (NR) activity is gradually decreased when concentration of leachate is increased. Allelopathic stress adversely affected the nitrate reductase activity in treated plants in dose dependent manner. Maximum 28.79% decrease was observed in the higher concentration (Table 3). Under stress condition NR activity decreased in seedlings. Process of photosynthesis provided energy rich e-donors and carbon skeleton to regulate NR activity in plants (Kaiser et al., 1993, Singh et al., 2010). In case of sorghum Decreased NR activity was reported by Bagavathy and Xavier (2007). Similar results were also reported in V. radiata (Tripathi et al., 2000).

Lipid peroxidation is measured in terms of malonaldehyde (MDA) content. The increased malaonaldehyde content corresponded to the concentrations of leachate. Maximum increase was observed is 47.37% in higher concentration (Table 3). Malonaldehyde content increased under stress caused oxidative stress and membrane damage (Lin et al., 2000). Increased concentration of leachate increased MDA content. The increased amount of malaonaldehyde content in response to different concentration of leachate is in agreement with previous studies (Smirnov 1993; Baziramakenga et al. 1995, Singh et al., 2012).

In plants, under allelopathic stress, the activity of antioxidant enzymes increased. The increased activity of antioxidant enzyme is extensive or common when plant is suffering from any type of stress. Antioxidant enzymes viz. SOD, CAT and POX increased in oxidative damage caused by allelopathic stress. Activity of SOD increased drastically in response to allelopathic stress. CAT activity is stimulated in dose dependent manner. Maximum stimulation was observed in higher concentration of leachate of donor plant. Increased POX activity is also considered in plant under allelochemical stress which causes oxidative damage in test crop plant. The increased activities of antioxidant enzymes in pea seedlings mitigate the oxidative damage caused by allelopathic stress for the survival of test crop. Maximum increase in antioxidant enzyme viz. SOD, CAT, POX was 43.94, 58.34 and 48.70% respectively was recorded in higher concentration when compared to control (Table 3). ROS caused Oxidative damage, could be responsible for detained biosynthesis of pigment or degradation via impaired metabolic processes (Singh et al., 2010). The membrane damage is the common mark of allelopathic stress (Singh et al., 2006). The membrane damage of test plant increased by the leachate of donor plant. Increased production of antioxidative enzymes indicates stress condition. Plants produce antioxidant enzymes in response of oxidative stress to protect them from oxidative damage caused by ROS (Foyer and Noctor, 2003; Singh et al., 2009, Singh et al., 2010). SOD act as first line of defense to facilitate the Plant to overcome the effects of stress (Gomez et al., 2004). Antioxidant enzymes like SOD, CAT and POX activities enhanced by allelochemicals (Doblinski et al., 2003; Curtze-Ortega, 2002). Present investigation show that the activities of enzyme enhanced as with increased concentration of the leachate. The production of Free radicals increased antioxidant activities. Higher concentration of leachate exhibited inhibitory effect on seedlings (Singh et al., 2008). The allelopathic potential of Hyptis suaveolens reduced in growth. It causes fluctuation in metabolic processes negatively affected the different physiological parameters of recipient plants.

Table 1. Allelopathic effect of Hypties suaveolens on fresh weight, dry weight, root and shoot length of pea seedlings

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fresh weight (gm/2 plants)</th>
<th>Dry weight (gm/2 plants)</th>
<th>Root length (cm)</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.52±0.066a</td>
<td>0.873±0.103a</td>
<td>12.5±0.606a</td>
<td>20.25±0.433a</td>
</tr>
<tr>
<td>H₁</td>
<td>1.96±0.012b</td>
<td>0.752±0.066b</td>
<td>10.6±0.548b</td>
<td>19.0±0.577ab</td>
</tr>
<tr>
<td>H₂</td>
<td>1.78±0.032c</td>
<td>0.581±0.079c</td>
<td>9.6±0.866b</td>
<td>17.5±0.866bc</td>
</tr>
<tr>
<td>H₃</td>
<td>1.30±0.058d</td>
<td>0.445±0.058d</td>
<td>9.1±0.375bc</td>
<td>16.5±0.535c</td>
</tr>
<tr>
<td>H₄</td>
<td>1.84±0.026e</td>
<td>0.324±0.056e</td>
<td>7.9±0.490c</td>
<td>13.7±0.143d</td>
</tr>
</tbody>
</table>

Mean±SEM values followed by same letters within each column are not significantly different at 0.05 (ANOVA and Duncan’s multiple range test), n=3. H₁=25, H₂=50 and H₃=75 and H₄=100% concentrations of Hyptis suaveolens plant leachate, respectively.
Table 2. Allelopathic effect of Hyptis suaveolens on pigment, sugar and protein contents of pea seedlings

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Chlorophyll a (mg/g FW)</th>
<th>Chlorophyll b (mg/g FW)</th>
<th>Total chlorophyll (mg/g FW)</th>
<th>Carotenoids (mg/g FW)</th>
<th>Protein (mg/g FW)</th>
<th>Sugar (mg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3.81±0.048a</td>
<td>0.179±0.104a</td>
<td>3.99±0.058a</td>
<td>2.24±0.021a</td>
<td>21.84±0.573a</td>
<td>15.06±0.0146a</td>
</tr>
<tr>
<td>H1</td>
<td>2.55±0.054b</td>
<td>0.171±0.120ab</td>
<td>2.69±0.044b</td>
<td>1.197±0.087bc</td>
<td>20.22±0.445b</td>
<td>13.19±0.053b</td>
</tr>
<tr>
<td>H2</td>
<td>2.41±0.061b</td>
<td>0.166±0.137abc</td>
<td>2.41±0.165bc</td>
<td>1.169±0.062d</td>
<td>19.27±0.432c</td>
<td>12.27±0.017c</td>
</tr>
<tr>
<td>H3</td>
<td>2.24±0.164bc</td>
<td>0.153±0.147bc</td>
<td>2.24±0.179c</td>
<td>1.185±0.074d</td>
<td>18.19±0.299d</td>
<td>11.85±0.073c</td>
</tr>
<tr>
<td>H4</td>
<td>2.08±0.184c</td>
<td>0.139±0.199c</td>
<td>2.57±0.012bc</td>
<td>0.217±0.095ab</td>
<td>16.76±0.159e</td>
<td>10.63±0.087d</td>
</tr>
</tbody>
</table>

Mean±SEM values followed by the same letters within each column are not significantly different at 0.05 (ANOVA) and Duncan’s multiple range test, n=3. H4=25, H3=50 and H2=75 and H1=100% concentrations of Hyptis suaveolens plant leachate, respectively.

Table 3. Allelopathic effect of Hyptis suaveolens Antioxidant enzymes, lipid peroxidase and nitrate reductase activity of Pea seedlings

<table>
<thead>
<tr>
<th>Treatments</th>
<th>NR (µmol NO2 g-1 FW h-1)</th>
<th>LP (nmol g-1 FW h-1)</th>
<th>SOD (EU/g FW)</th>
<th>CAT (EU/g FW)</th>
<th>POX (EU/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>36.67±0.054a</td>
<td>10.64±0.310e</td>
<td>33.03±0.153e</td>
<td>21.01±0.192e</td>
<td>24.52±0.158e</td>
</tr>
<tr>
<td>H1</td>
<td>34.64±0.072b</td>
<td>13.82±0.281d</td>
<td>36.94±0.148d</td>
<td>30.72±0.038d</td>
<td>31.25±0.143d</td>
</tr>
<tr>
<td>H2</td>
<td>32.83±0.036c</td>
<td>16.44±0.276c</td>
<td>46.38±0.361c</td>
<td>39.06±0.023c</td>
<td>37.62±0.043c</td>
</tr>
<tr>
<td>H3</td>
<td>28.20±0.036d</td>
<td>18.98±0.296c</td>
<td>50.34±0.396c</td>
<td>42.39±0.019b</td>
<td>39.27±0.034b</td>
</tr>
<tr>
<td>H4</td>
<td>26.11±0.018e</td>
<td>20.22±0.303a</td>
<td>58.92±0.233a</td>
<td>50.44±0.012a</td>
<td>47.80±0.057a</td>
</tr>
</tbody>
</table>

Mean±SEM values followed by the same letters within each column are not significantly different at 0.05 (ANOVA and Duncan’s multiple range test), n=3. H4=25, H3=50 and H2=75 and H1=100% concentrations of Hyptis suaveolens plant leachate, respectively.

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