Determination of the Genotoxic Effects of *Verbascum speciosum* Schrad. Extracts on Corn (*Zea mays* L.) Seeds

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Abstract

The methanolic extracts of *Verbascum speciosum* Schrad. were tested for genotoxic and inhibitor activity against corn *Zea mays* L. seed. The methanol extracts of leaf, stem and root of *Verbascum speciosum* were diluted 10, 5, and 3.3% and applied to corn seed. The germination percentage, mitotic index and random amplified polymorphic DNA (RAPD) results were compared to control. The results showed that especially 10% diluted leaf, stem and root extracts had a strong inhibitory activity. Also, different concentration methanolic extract caused a decrease mitotic index and an increase chromosomal aberration, and changed RAPD profiles. The changes occurred in RAPD profiles of *Verbascum* extract treatment included variation in band intensity, loss of bands and appearance of new bands compared with control. The results suggested that RAPD analysis could be applied as a suitable biomarker assay for the detection of genotoxic effects of plant allelochemicals.

Keywords: Genotoxic; Methanolic Extract; Mitotic Index; RAPD; *Verbascum speciosum*

Introduction

Allelopathy is a phenomenon observed in many plants that release chemicals in to the near environment either from their aerial or underground parts in the form of root exudation leaching by dews and rains and volatilization or decaying plant tissues [1,2]. Different plant parts, including flowers, leaves, leaf litter and leaf mulch, stems, bark, roots, soil and soil leachates and their derived compounds, can have allelopathic activity that varies over a growing seasons.

Allelopathic inhibition is complex and can involve the interaction of different classes of chemicals like phenolic compounds, flavonoids, terpenoids, alkaloids, steroids, carbohydrates, and amino acids [3,4]. There are also stimulatory effects of allelochemicals [5,6]. The mixtures of different compounds sometimes have a greater allelopathic effect than individual compounds alone [7-9]. Allelopathic chemicals can also persist in soil, affecting both neighboring plants as well as those planted in succession [3,10-12].

Commonly cited effects of allelopathy include reduced seed germination [13-17] and seedling growth. Like synthetic herbicides [18,19] there is no common mode of action or physiological target site for all allelochemicals [20] even though some plant extracts are known to have allelopathic effects on cell division, pollen germination [6,17] nutrient uptake, photosynthesis, and specific enzyme function [21-23].

To the point of plant protection view, pesticides and herbicides are not eco-friendly and the use of these chemicals have resulted in the evolution of resistant biotypes and therefore
the new biotypes have been reported to tolerate between 2 and 8 times higher dose of application than susceptible ones [24].

The allelopathic effects of weeds on crop plants have been intensively studied since 1970 [1,25]. In some of the previous works, Verbascum was reported to be one of the plants which possess significant allelopathic activity [25,26]. Some Verbascum species, such as Verbascum thapsus and Verbascum sinuatum, have potent allelopathic activity due to various chemical substances they have. These substances include verbascoside, luteolin, ajugol, sinuatol, aucubin, iridoid glycosides [25-28].

PCR-based random amplified polymorphic DNAs [29] has been widely used to survey ecotoxicological studies because this technique relatively inexpensive and does not need any prior information about target sequence on the genome [30]. Recently, several studies have demonstrated that extracts of Verbascum reduced seed germination in plant. However, extracts of Verbascum speciosum effect genotoxic in plant has not reported up to the present. This study investigated whether methanol extracts of Verbascum speciosum has any genotoxic effect in Zea mays.

Materials and Methods

Plant material
The weed plant used in the present study was collected in August 2007 from different locations of Erzurum vicinity, Turkey. The collected plants at flowering stage were dried in shadow, and the leaves and roots were separated from the stem, and all plant parts ground in a grinder with a 2 mm in diameter mesh.

Preparation of the methanol extracts (MeOH)
Methanol is a common solvent used for allelochemical analyses because this combination exhibits a good ability to extract both polar and non-polar compounds, therefore most potential phytotoxins of the plant can be extracted [2].

The dried and powdered leaves, stems and roots (500 g) were extracted with 1 L of methanol using a Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent. The extracts were filtered using filter paper (Whatman No.1) and then concentrated in vacuo at 40ºC using a Rotary Evaporator. The residues obtained were stored in a freezer at -80ºC until further tests (2).

Treatments of extracts on corn seeds
Corn seeds, selected by uniform size, were surface sterilized with 2.5% (w/v) NaOCl for 3 min, washed with sterile distilled water and paper-plotted. The seeds were soaked in sterile distilled water for 1 h and then 15 seeds were germinated in 15 cm dia Petri dishes on four layers of sterile Whatman No. 1 filter paper. The plant extract were diluted with deionized water to prepare the concentrations of 3.3, 5.0 and 10.0 % and 20 ml extract was added per Petri dish as per treatment. The seeds in Petri dishes were allowed to germinate at 25ºC in the dark. When the extrusion of the radical reached 1.5-3.0 cm, then, seedlings were collected and fixed to investigate chromosome abnormalities and mitotic index. The RAPD assays were performed 8 days after incubation.

Mitotic index
Cytological preparations were carried out using Feulgen’s squash technique. Different slides for each treatment were examined to determine the effects of plant extracts on mitotic
Chromosomal aberrations (CA) were counted in the different mitotic stage (abnormal mitoses) and interphase stage. Mutagenic effect was estimated as the percentage of cells with bridges and fragments. % of aberrant cells (CA) = Total number of mitotic aberrations/ Total number of dividing cells x100.

**DNA extraction**
Genomic DNA was extracted from powdered plant materials using a modified method described by Lin et al. (2001). Approximately 10-15 mg tissue samples from each plant species were snap frozen in liquid nitrogen in 2 ml Eppendorf tubes. 1000 μl DNA extraction buffer [100mM Tris-HCl (pH 8.0); 50mM EDTA (pH 8.0); 500mM NaCl; 2% SDS (w/v); 2% 2-mercaptoethanol (v/v); 1% PVP (w/v)] was added and the whole mixed well. The mixture was incubated at 65°C in a water bath for 40 min with intermittent shaking at 5 min intervals. It was centrifuged at 12 000xg for 15 min at 4°C, the supernatant was transferred into a new 1.5 ml tube and mixed with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), and centrifuged. The supernatant was collected and mixed with 1/10 volume 10% CTAB-0.7M NaCl in a new tube. After centrifugation (12 000xg for 15 min), the supernatant was collected and an equal volume of chloroform:isoamylalcohol (24:1) was added and mixed gently. The DNA was precipitated by the addition of 0.6 volume of cold isopropanol, left at -20°C for 10min. The DNA was pelleted by centrifugation (12 000xg for 15min) and the isopropanol was poured off; the DNA was allowed to air-dry before being dissolved in 100 μl of TE buffer.

**RAPD**
Samples were screened for RAPD variation using standard 20-base primers supplied by Operon. Thirty μl of reaction cocktail was prepared as follows: 10x Buffer 3.0 μl, dNTPs (10mM) 1.2 μl, magnesium chloride (25mM) 1.2 μl, primer (5μM) 2.0 μl, Taq polymerase (5unit) 0.4 μl, water 19.2 μl sample DNA 3.0 μl (100ng/μl). Twenty five oligonucleotide primers were screened on *Vrebasacam speciosum* and among them, 7 primers showed clear polymorphic patterns.

The thermalcycler (Eppendorf Company) was programmed as 2 min at 95°C; 2 cycles of 30 sec at 95°C, 1 min at 37°C, 2 min at 72°C; 2 cycles of 30 sec at 95°C, 1 min at 35°C, 2 min at 72°C; 41 cycles of 30 sec at 94°C, 1 min at 35°C, 2 min at 72°C; followed by a final 5 min extension at 72°C then brought down to 4°C.

**Electrophoresis**
The PCR products (27 μl) were mixed with 6x gel loading buffer (3 μl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5XTBE (Tris-Borate- EDTA) buffer at 70 V for 150 min. The gel was stained in Ethidium bromide solution (2 μl Etbr/100ml 1xTBE buffer) for 40 min and visualized under UV in Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK).

Polymorphism observed in RAPD profiles includes disappearance of normal band and appearance of a new band in comparison to control. The average was then calculated for each experimental group exposed to different methanol extract treatment.
Results and discussion

Effect of methanol extract on germination

Root, leaf and stem extracts obtained from Verbascum speciosum showed significant inhibition effect on seed germination compared with control. In generally the germination (%) of corn seeds dramatically decreased with increasing dilution level of methanol extract. The results obtained in these experiments were showed in Table 1. The percentage of corn germination little effected by 3.3% extracts (80, 80, 85%). When the concentration of extracts 5.0% the percentage of corn seed germination 70% leaf, 70% root, 75% stem was found at 7 days after methanol extract exposure. When the concentration of leaf, root and stem extracts was 10.0 the inhibitory rate of germination was up to 15, 50, and 20% in comparison to control. As a result of the higher concentration of leaf, root and stem extracts the more serious the inhibitory effect on % germination.

Table 1. The effects of different concentration of Verbascum speciosum extracts on corn seed germination (%). Means ± SD from three independent experiments

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>10.0%</th>
<th>5.0%</th>
<th>3.3%</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.speciosum(leaf)</td>
<td>15±3.12</td>
<td>70±8.70</td>
<td>80±11.25</td>
</tr>
<tr>
<td>V.speciosum(root)</td>
<td>50±4.60</td>
<td>70±7.12</td>
<td>80±10.22</td>
</tr>
<tr>
<td>V.speciosum(stem)</td>
<td>20±5.21</td>
<td>75±5.60</td>
<td>85±13.54</td>
</tr>
<tr>
<td>Control</td>
<td>90±5.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effect of methanol extract on mitotic index

Mitotic index was observed that all the applications resulted in decreased mitotic activity compared to control. The lowest mitotic activity was observed in the treatment of 10.0 concentration of the extract of Verbascum speciosum (Table 2).

Table 2. The effects of different concentration of Verbascum speciosum extract on the levels of mitotic index and chromosome abnormalities. Means ± SD from three independent experiments with three assays for each determination are shown

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>3.3%</th>
<th>5.0%</th>
<th>10.0%</th>
<th>3.3%</th>
<th>5.0%</th>
<th>10.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.speciosum(leaves)</td>
<td>4.72±1.31</td>
<td>4.58±1.3</td>
<td>3.78±0.99</td>
<td>23.74±7.20</td>
<td>29.98±10.0</td>
<td>32.30±12.7</td>
</tr>
<tr>
<td>V.speciosum(roots)</td>
<td>2.75±0.75</td>
<td>2.11±0.55</td>
<td>1.16±0.89</td>
<td>26.44±10.8</td>
<td>15.22±5.70</td>
<td>13.10±3.56</td>
</tr>
<tr>
<td>V.speciosum(stems)</td>
<td>4.81±1.52</td>
<td>4.88±1.48</td>
<td>3.49±0.77</td>
<td>11.90±2.70</td>
<td>13.92±4.90</td>
<td>17.67±4.83</td>
</tr>
<tr>
<td>Control</td>
<td>20.64±0.61</td>
<td></td>
<td></td>
<td>5.37±1.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chromosome abnormalities are also another issue that related to mitotic activity. Almost all the applications revealed higher chromosome abnormalities compared to controls. The most injurious effect was observed in all the applications of leaf extracts. The minimum chromosome abnormality was observed in root extracts compared to other extract applications (Table 2). Our results suggest that, an increase in the extract of Verbascum speciosum concentrations caused to decrease the frequencies of cell division and to increase the frequencies of chromosomal aberration.

Effect of methanol extract on RAPD profiles

Of the twenty five decamer oligonucleotide primers tested, only 7 gave specific and stable results (Table 3). The RAPD results showed that leaf, root and stem extract changed the number, size and intensity of amplified DNA fragments. Table 3 indicated that changed bands
observed in RAPD profiles for example appearance, disappearance of bands, increase and decrease in band intensity in comparison to RAPD profiles of the control. The decrease band intensity was particularly obvious for corn exposed to different concentration methanol extracts for primer OLIGO6, OPD08 OLIGO8 and OPB04. On the other hand an increase in band intensity occurred mainly for 3.3 concentration and 5.0 concentration of leaf, root and stem extracts for especially primer OPB07, OPB04, OLIGO6 AND OLIGO 7. Also new RAPD bans appeared with primer OPB07 (5 extra bands for L1, 3 extra bands for L2, 6 extra bands for R1 and 2 extra bands for R2), primer OPB04 (2 extra bans for L1, R1 S1 and S2, 1 extra bands for L2), primer OLIGO7 (1 extra bands for L1, L2, R2, R3 and 5 extra bands for S1), primer OLIGO 6 (4 extra bands for L3, 1 extra bands for R3 and 2 extra bands for S1), primer OPD08 (2 extra bands for R1,S2 S3) (Fig. 1).

Table 3. Changes of total bands in control, and of polymorphic bands and varied bands in different concentration of Verbascum speciosum extract on corn seed

<table>
<thead>
<tr>
<th>Primer</th>
<th>Control</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPB07</td>
<td>0</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
<td>a</td>
</tr>
<tr>
<td>OPB04</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>OLIGO7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>OLIGO6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>OPD08</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>OLIGO8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>OPC05</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

- a: number of new bands, b: similar bands to control, c: increase in band number, d: decrease in band number, L: leaf, R: root, S: stem

Figure 1. RAPD profiles of genomic DNA, obtained from primer OPD08 in corn seed exposed to varying Verbascum speciosum extracts. C: control, L: leaf, R: root, S: stem

Molecular techniques have been increasingly adapted for ecotoxicological studies (Wolf et al. 2004). Most of these studies have thus far focused primarily on individual level effects.
such as DNA damage and gene expression [31,32]. Relatively few studies have used molecular techniques to examine genotoxic effect of plant allelochemicals.

In the present investigation the PCR based RAPD method was adapted to examine genotoxic effect of \textit{V. speciosum} methanolic extract on corn seed. After suitable optimization of the PCR conditions, RAPD used successfully to detect the modifications to genomic DNA induced by genotoxins in plant and animals [33-38]. The presence of the above diverse types of DNA lesions and mutations may also induce important structural changes that are likely to have a substantial effect on kinetics of PCR events. New PCR produced can be amplified because some oligonucleotide priming sites become accessible to oligonucleotide primers after important structural modification or because the same mutations have occurred in genomic DNA sequence. Not only can they result in DNA structural variations, but can also inhibit the polymerization of the DNA or block the Tag polymerase in the PCR reaction, leading to decreased in RAPD band intensity, or alternatively to a disappearance of amplified produced in the case of extensive DNA damage, as reported by Atienzar et al. [39].

The higher the concentrations stem, root, and leaf extracts, the proportions of abnormal c mitosis, chromosomal aberrations increased. Similar results have been noted different plant extract [17]. This result means that different extracts of \textit{V. speciosum} exposed caused damage to the genomic DNA. The low mitotic activity, chromosome abnormalities and changes RAPD band profiles compared to control is due to addition of all DNA alterations induced by allelochemicals such as mutations, rearrangements, structural modifications and these events can effect polymerization of DNA in the PCR reaction [39,40].

Conclusions

The results our study prove that methanolic extracts of \textit{V. speciosum} have genotoxic and antigerminative effect on corn seed and further studies are in progress to identify the active compounds and their modes of action on genotoxic activity of \textit{V. speciosum}.

References