APPLICATION OF VOLATILE FRACTIONS FROM AGERATUM HOUSTONIANUM AND TAGETES ERECTA AS SAFE MANAGEMENT OF SOME ROOT PHYTOPATOGENIC FUNGI

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ABSTRACT

Ageratum houstonianum Mill and Tagetes erecta L. (Asteraceae) were subjected to hydrodistillation as well as the stepwise extraction with organic solvents. Crude extracts and the hydrodistilled essential oils (E.O) were bioevaluated against two phytopathogenic fungi Rhizoctonia solani and Phytophthora megasperma in vitro. The volatile fraction from both plants showed a good antifungal activity towards the tested fungi. EC₅₀ were 91 and 1369 ppm for R. solani and 84 and 1571 ppm for P. megasperma with Ageratum essential oil (E.O) and Tagetes E.O, respectively. But generally; Ageratum E.O was found to be more effective in reducing mycelium growth of R. solani and P. megasperma (EC₅₀ - 91 and 84 ppm respectively) than Tagetes E.O. Chemical composition of Tagetes E.O and Ageratum E.O; fractions F1 (solid) and F2 (liquid) fractions were investigated by GC-MS analysis. Interestingly, heterocyclic benzopyran compound (Precocene II) was only component which has been detected in Ageratum E.O fraction F1. Precocene II seems to be the fungitoxic active components in Ageratum E.O and its fractions. However, monoterpenic hydrocarbons were correlated with the fungitoxic effect of Tagetes E.O. In the green house experiments, Tagetes E.O and Ageratum E.O fraction F1 showed a clear selectivity towards tested pathogens; Rhizoctonia solani and Phytophthora megasperma. Ageratum E.O; fractions F1 were found to be much more fungitoxic activity than Tagetes E.O. Tagetes E.O and Ageratum E.O fractions F1 were controlled the 90.91% of Root-Rot disease in bean caused by Phytophthora megasperma, while the percentage of disease control was only 36.36% for Rhizoctonia solani.

INTRODUCTION

Natural products are organic chemical compounds that produced from the secondary metabolic pathways in organs of higher plants, fungi, bacteria and marine organisms (González et al 1991 and Kim et al 2004). Since most of the synthetic chemicals used in plant protection have side effects and cause pesticidal pollution to the ecosystem by their persistence, there is a demand for the development of new safe, biodegradable alternatives which have a maximum efficacy with minimal environmental impact and danger for the consumer (Mishra and Duby 1990).

Ageratum spp is an annual herbaceous plant and it has a history of use in traditional medicine (Ming 1999), bioactivity of plant extract has been reported against bacteria and Fungi (Okunada 2002). Also, Tagetes spp essential oil has been found to effective, nonphytotoxic and easily biodegradable, the essential oil has exhibited very promising antifungal efficacy against many tested organisms (Gary and Dengre 1988; and Bruce et al 2002). Antifungal activity of Tagetes extracts and essential oil have been investigated by several researchers.

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MATERIALS AND METHODS

Plant materials

Two plant species from family Asteraceae have been chosen for this study. Mist flower (Bor-goman) Ageratum houstonianum Mill, and Mari-gold Tagetes erecta L. the aerial parts of the flow-ering plants were collected in the period between 2002 and 2003 from the Botanical Research Farm, Faculty of Agriculture, Shobra El-Khema. Fresh plant samples were cleaned and washed by tap water then dried in attempt room temp in the shade. After dryness, the samples were grinded and kept into tightly sealed plastic pages and stored in closed brown glass bottles.

Preliminary chemical analysis and Volatile oil hydrodistillation

Moisture content and total ash content were determined in collected plant samples according to AOAC (1990). Volatile content was steam distilled according to British Pharmacopoeia (1980). Essential oils of both A. houstonianum and T. erecta were hydrodistilled in Clevenger’s apparatus on a productive scale from the fresh aerial parts according to Clevenger (1928).

Plant extracts preparation

The air dried powder plant samples (20g) were exhaustively extracted in a continuous extraction apparatus with n-hexane, chloroform and then methyl alcohol (99%). The powder after each extraction was freed from the solvent before the next trip. The extract was filtered and the solvent was distilled off. The percentage of the crude extract has been calculated according to Khalil (1982). The preparative extracts were obtained by soaking the sample onto a cold solvent for excessive period of time as described by Iqbal et al (2004).

Fractionation of A. houstonianum essential oil

A dehydrated essential oil from A. houstonia-num was noticed to solidify at low temp Dixit et al (1995). This observation was noticed also in our present research, this phenomenon has been de-veloped to standardize the fractionation of the Ageratum E.O. The dehydrated E.O was dissolved in dry diethyl ether with the ratio of 2:1(w/v) and cooled at -5 °C for 7 days. The white amorphous crystals were identified as F1 and the liquid phase was identified as F2 fraction.

GC-MS analysis

Sample of 1 µl solution (1mg/ml) in n-hexane was injected in HP-5970 GC-MSD instrument, MS detector unit with E1 and CI modes. Mass range is 50-800 m/z, DB-5 capillary column, 30m length x 0.25m I.D. 0.25µm film. The temp program was set to get the best separation.

Fungal isolates

Pathogenic isolates of Phytophthora megasperma and, Rhizoctonia solani originally isolated from diseased snap bean plants were used for all experiments. Phytophthora megasperma isolate was maintained on clarified V8 media while Rhizoctonia solani isolate was maintained on PDA media.

Solid agar bioassay

To test fungitoxic activity in vitro of T. erecta crude extracts and E.Os as well as the A. housto-nianum E.O fractions F1 and F2 against Rhizoctonia solani, and Phytophthora megasperma, each E.Os and fractions were diluted w/v in agar diluted methods according to Hammer et al (1999). V8 agar plates were used for P.
megasperma and PDA plates were used for R. solani. Culture media in different final concentrations of each E.Os or fractions were inoculated by each pathogenic fungus separately. Each experiment was replicated fourth, fungal growth diameters were measured daily up to day 8 after inoculations, incubation temperature was 22°C. EC₅₀ was calculated by using regression equation between log concentrations and probit of percentage growth inhibition of fungi according to Abd El-Naeem et al (2004).

Seeding bioassay

The in-vivo practical applicability of the T. erecta E.O and A. houstonianum E.O fraction F1 was tested in pot experiment by the modification the technique used by Kishore and Dwivedi (1991). In one set, the uninoculated control, 6 pots were filled with normal sterilized soil. In another set, which served as the inoculated control, each of twelve pots contained equal amount of soil infected with mycelia of either R. solani or P. megasperma isolates. In inoculated and uninoculated controls snap bean seeds (Pronco vr.) were soaked only in natural gum solution. The treatment sets, snap bean seeds (Pronco vr.) soaked separately in 3000ppm and 400ppm for T. erecta E.O and A. houstonianum E.O fraction F1 respectively prepared in natural gum solution. Snap bean seeds (Pronco vr.) were sown after one hour soaking time at rate of 3 seeds/ pots, three replicates were considered for each treatment. Records were made on pre-emergence after 7 days of planting as well as post-emergence damping-off after 15 days. The percentage of disease control were determined after 15 days of sowing, also height, dry weight and fresh weight of survival seedling were recorded.

RESULTS

1. Preliminary chemical analysis and Volatile oil hydrodistillation

The data in Table (1) presents the percent of moisture content, total ash and volatile compounds in the air dried T. erecta and A. houstonianum samples. Percentage of moisture content was almost the same for the two samples as well as the percentage of total ash, while the percentage of volatile content was higher in A. houstonianum plant (0.61%) than the T. erecta (0.12%). The percentage of chloroform extract was the same in both samples (1.29%) while the percentage of n- hexane and methanol extract were higher in A. houstonianum compared with T. erecta.

Table 1. Preliminary chemical analysis and percentage of the crude extracts with organic solvents of the air dried A. houstonianum and T. erecta (Asteraceae) plants

<table>
<thead>
<tr>
<th>Chemical &amp; crude extracts properties</th>
<th>T. erecta</th>
<th>A. houstonianum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content (%)</td>
<td>11.56</td>
<td>11.50</td>
</tr>
<tr>
<td>Total Ash (%)</td>
<td>20.19</td>
<td>17.71</td>
</tr>
<tr>
<td>Volatile compounds(%)</td>
<td>00.12</td>
<td>00.61</td>
</tr>
<tr>
<td>Hexane Extr. (%)</td>
<td>02.85</td>
<td>05.07</td>
</tr>
<tr>
<td>Chloroform Extr. (%)</td>
<td>01.29</td>
<td>01.29</td>
</tr>
<tr>
<td>Methanol Extr. (%)</td>
<td>06.10</td>
<td>10.25</td>
</tr>
</tbody>
</table>

2. GC-MS analysis

Data in Table (2) presented the major constituents of both T. erecta E.O and A. houstonianum E.O fractions F1 and F2 in T. erecta E.O there were five major components had been identified against the authentic samples. Monoterpenoid α-pinene was found to be the major component (42.86%) followed by monoterpenoides piperitone (32.22%) and D- Limonene (12.16%) while sesquiterpene β- Caryophyllene was encountered as 3.34% of total constituents of T. erecta E.O.

Table 2. GC-MS analysis of T. erecta E.O and A. houstonianum E.O fractions (F1 and F2)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Volatile concentration in dry samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T. erecta</td>
</tr>
<tr>
<td>α-Pinene*</td>
<td>42.86</td>
</tr>
<tr>
<td>D-Limonene*</td>
<td>12.16</td>
</tr>
<tr>
<td>Piperitone*</td>
<td>32.22</td>
</tr>
<tr>
<td>β-Caryophyllene**</td>
<td>03.34</td>
</tr>
<tr>
<td>Caryophyllene Oxide**</td>
<td>-</td>
</tr>
<tr>
<td>γ-Cadeline**</td>
<td>-</td>
</tr>
<tr>
<td>Precocene II***</td>
<td>-</td>
</tr>
<tr>
<td>Unknown</td>
<td>03.04</td>
</tr>
</tbody>
</table>

* Monoterpenoides compound
** Sesquiterpene compound
*** Heterocyclic benzopyrene compound

Interestingly, precocene II was the only major component which has been detected in fraction F1 (Table 2) by GC-MS. The heterocyclic benzopyran compound precocene II was also detected in F2 fraction; it was formed 48.82% of total components. Sesquiterpenoids β-Caryophyllene, Caryophyllene oxide and γ-Cadenine were also detected in F2 fraction. The percent of the corresponding compounds were 20.80, 11.78 and 9.43% respectively.

3. Solid agar bioassay

The fungitoxic activity of A. houstonianum E.O fractions F1, F2 and T. erecta E.O were tested against, Phytophthora megasperma and Rhizoctonia solani. Data in Table (3) presented the EC50 of all treatments. It was noticed that EC50 of A. houstonianum E.O F2 was much higher than the other two E.Os fractions. However the EC50 for T. erecta E.O against R. solani and P. megasperma were 1571 and 1369 p.p.m respectively. EC50 of fraction F1 was found to be much lower than the EC50 of T. erecta E.O for two tested phytopathogens. The EC50 was 84 and 91 p.p.m for both P. megasperma and R. solanii respectively.

4. Seedling bioassay

Data in Table (4) and Figure (1) showed the effect of treatment with T. erecta E.O or A. houstonianum E.O fraction F1 on the controlling the root rot disease in snap bean seeds (Pronco vr.) caused by either P. megasperma or R. solani in vivo. Better control results was recorded with P. megasperma.

Seed inoculation 90.9% percentage of disease control (% of plant survival) was for both treatments while it was 36.36% for R. solani. Percentage of germination, seedling height and fresh weight were in corresponding with the percentage of disease control, they were lower in seedlings infected with R. solani than those infected with P. megasperma compared with uninoculated control.

DISCUSSION

The recovery of the volatile fraction from leaves of A. houstonianum was ranged from 0.11-0.6 % Dixit et al (1995), and Wandji et al (1996). The steam distillation of T. erecta leaves yielded 0.2% of essential oil Machado et al (1994), meanwhile the percent of volatile fraction was found to be a species dependent in Tagetes spp plant Héthelyi et al (1986).

Stepwise extraction has been carried out to catch the different compounds depending on the gradient differences in their polarity. Aquino et al (2002). Héthelyi et al (1987) were identified Limonene, β-Caryophyllene, Piperitone and γ-Terpinolone in T. erecta E.O by using GC-MS analysis. That was supported the obtained results. In another investigation Krishna et al (2002) were found that the hydrodistilled E.O from the shoots of Tagetes spp contain Limonene, (cis)-β-Ocimene, Terpinoline, Piperitone and β-Caryophyllene. Also Singh et al (2003) reported that the β-Ocimene was the major constituents in Indian Tagetes erecta E.O. In A. houstonianum E.O fractions F1 and F2, the heterocyclic benzopyran compound precocene II was detected as a major constituents , those findings were in agreement with several authors; Kasali et al (2002); Sundufu and Shoushan (2004). Chromone compounds, Sesquiterpenoids and monoterpenoids were contained in Ageratum spp E.O at 71.05, 13.95 and 5.17 % respectively. The main constituents of the hydroidistilled A. houstonianum E.O were precocene I, precocene II and beta- caryophyllene at levels 23.3, 43.99 and 9.18 respectively Suresh et al (1996).

Success of essential oils as biodegradable and environmentally safe fungi toxicants have shown the possibilities for their exploitation as natural fungicides Dixhit et al (1983); Dubey et al (1983); Asthana et al (1986). It was found that the A. houstonianum E.O fraction F1 was much higher than fraction F2 as fungitoxicant against the tested pathogens concluding that the active fungitoxicant (s) may contain in fraction F1. The essential oil of A. conyzoides exhibited a broad range of activity inhibiting 22 out of 35 fungi tested Dixit et al (1995). Similarly, T. erecta E.O showed a strong antifungal activity against 20 tested phytopathogens at 2000 p.p.m Kishore and Dwivedi (1991).

A. houstonianum E.O fraction F1 and T. erecta E.O have shown a strong fungitoxic activity towards the tested phytopathogens in-vitro. It was found that the Phytophthora spp were much more susceptible than Rhizoctonia spp for the treatment with fraction F2. From the obtained results it could be noticed also that the fungitoxic activity of F1 fraction was higher than T. erecta E.O. the obtained results were found to be in agreement with several re-
Table 3. Antifungal activity of *T. erecta* E.O and *A. houstonianum* E.O fraction F1 and F2 against two phytopathogenic fungi (*Rhizoctonia solani* and *Phytophthora megasperma*) in-vitro

<table>
<thead>
<tr>
<th>Plant EOs extracts</th>
<th><em>R. solani</em></th>
<th><em>P. megasperma</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ ppm</td>
<td>Reg. Equation</td>
</tr>
<tr>
<td><em>T. erecta</em> E.O</td>
<td>1369</td>
<td>Log Y=0.521X+3.365</td>
</tr>
<tr>
<td><em>A. houstonianum</em> E.O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction F1</td>
<td>0091</td>
<td>Log Y=2.129X+0.832</td>
</tr>
<tr>
<td>Fraction F2</td>
<td>7949</td>
<td>Log Y=0.584X+2.723</td>
</tr>
</tbody>
</table>

Reg. Equation = Regression equation between log concentration (log y) and propit of % fungus growth inhibition (x)
(R²) = Correlation coefficient of Y and X
EC₅₀ = the effective concentration at 50 % growth inhibition in ppm.

Figure 1. Effect of treatment with *T. erecta* E.O or *A. houstonianum* E.O fraction F1 on the Root-Rot disease in snap bean infected with *P. megasperma* (A) or *R. solani* (B)

Table 4. Effect of treatment with *Tagetes* E.O and *Ageratum* E.O fraction F1 on root-rot diseases caused by *P. megasperma or R. solani* on snap beans

<table>
<thead>
<tr>
<th>Pathogen inoculated</th>
<th>EOs Treatment</th>
<th>Pre-emergence %</th>
<th>Efficacy %*</th>
<th>Height (g)/seedling</th>
<th>Fresh weight (g)/seedling</th>
<th>Dry weight (g)/seedling</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. solani</em></td>
<td><em>Tagetes</em> E.O</td>
<td>22.2</td>
<td>36.36</td>
<td>6.6±5.4</td>
<td>1.75</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td><em>Ageratum</em> F1</td>
<td>22.2</td>
<td>36.36</td>
<td>3.7±0.8</td>
<td>1.50</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>27.78</td>
<td>45.45</td>
<td>8.8±2.3</td>
<td>1.40</td>
<td>0.33</td>
</tr>
<tr>
<td><em>P. megasperma</em></td>
<td><em>Tagetes</em> E.O</td>
<td>55.56</td>
<td>90.91</td>
<td>18.7±2.3</td>
<td>2.90</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td><em>Ageratum</em> F1</td>
<td>55.56</td>
<td>90.91</td>
<td>14.8±5.81</td>
<td>2.80</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>44.4</td>
<td>72.72</td>
<td>16.7±5.95</td>
<td>2.63</td>
<td>0.33</td>
</tr>
<tr>
<td>Non-infected</td>
<td>Untreated</td>
<td>61.11</td>
<td>-</td>
<td>19.2±6.17</td>
<td>2.18</td>
<td>0.26</td>
</tr>
</tbody>
</table>

* Efficacy of treatment = (control – treatment) / control %

In-vivo experiment showed that the treatment with either T. erecta E.O or A. houstonianum E.O fraction F1 was effective in management of Root-Rot disease caused by Phytophthora megasperma much higher than Rhizoctonia solani. Even though the treatments were affected both Phytophthora spp and Rhizoctonia spp in vitro, the obtained results cleared a high selectivity towards the Phytophthora spp. The A. houstonianum E.O fraction F1 was achieved the same effect of T. erecta E.O at Conc Level of 400 p.p.m compared with 3000 p.p.m of T. erecta E.O. It could be concluded that the A. houstonianum E.O fraction F1 was higher fungitoxic than T. erecta E.O. The potential of T. erecta E.O and A. houstonianum E.O fraction F1 as a promising fungitoxicant indicate the possibility of their exploitation as a novel fungitoxicants for the management of Root-Rot disease in snap bean. Kishore and Dwivedi (1991) reported that the possibility of control the Damping-off disease in tomato by using 2000-3000 p.p.m of Tagetes E.O. Also Dixit et al (1995) reported that the whole Ageratum E.O was successful in control blue mold –rot in mandarins.

Structure – fungitoxic relationship of F1 fraction and T. erecta E.O have been investigated. Monoterpenic hydrocarbons were 87.24% of total components in T. erecta E.O. There was a positive correlation between the monoterpenic content and the fungitoxic activity. Several reports have been pointed out to such correlation between the monoterpenoids and the antifungal activity [Kim et al (1995), Caccioni et al (1998), Arras and Usai (2001)]. The mechanism of action was suggested that the monoterpenoids may interact with the fungal cell membrane and disrupting it Thompson (1996). On the other hand, monoterpenoids were detected as minor constituents in A. houstonianum E.O and its fractions; instead, the heterocyclic benzopryrene compound precocene II was detected as a major component. More over, fraction F1 was found to contain the precocenelI mainly and this resulted in increasing the fungitoxicity of F1 fraction. The antifungal effect was decreased dramatically in F2 fraction as a result of decreasing the chromene content. From those observations it could be concluded that the precocene II, a member of chromene compounds, could be correlated with the antifungal activity of A. houstonianum E.O. Chromene compounds showed an antifungal activity against Colletotrichum gloeosporioides (Bandara et al 1992) Phytophthora spp (Widmer and Laurent 2006).

Further separation and isolation guided by antifungal evaluation are required to prove weather or not the chromene compound and / or any other constituents in F1 fraction are responsible for the observed antifungal activity. Despite the work that still needs to be done, this study provides the basis for an alternative method to synthetic chemical application that can be developed quickly for farmer use.

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