Simultaneous Effects of Ferulic and Vanillic Acids on Peroxidase and Phenylalanine Ammonia-Lyase in Soybean (Glycine max) Roots

By

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Summary


Simultaneous effects of ferulic (FA) and vanillic (VA) acids on peroxidase (POD, EC 1.11.1.7) and phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activities on soybean (Glycine max (L.) Merr.) root growth were analyzed. Three-day-old seedlings were cultivated in nutrient solution containing FA or VA (0.5 mM; 1.0 mM or equimolar mixtures) for 48 h. Acting alone, both compounds (at 0.5 or 1.0 mM) decreased root length (RL), fresh weight (FW), dry weight (DW) and increased soluble POD and cell wall (CW)-bound POD activities. At 1.0 mM, FA increased (but VA decreased) the PAL activity. Acting simultaneously, the effects of the allelochemical interaction were lower than the sum of the effects of each compound tested separately, suggesting antagonism.
Es wurden die Wirkungen von Ferula- (FA) und Vanillin- (VA) Säure auf die Peroxidase (POD, EC 1.11.1.7) und Phenylalanin Ammonia-Lyase (PAL, EC 4.3.1.5) Aktivitäten untersucht. 3 Tage alte Sämlinge von *Glycine max* (L.) MERR. wurden Nährösungen, welche FA oder VA (0.5 mM; 1.0 mM oder equimolare Mischung) enthielten, 48 h lang ausgesetzt. Jede Komponente für sich führte bei 0,5 oder 1,0 mM zu einer Verminderung des Wurzelwachstums (RL), des Frischgewichtes (FW) und des Trockengewichts (DW), bewirkte jedoch einen Anstieg der löslichen sowie der Zellwand (CW) gebundenen POD-Aktivität. Bei 1,0 mM führte die FA zu einem Anstieg der PAL-Aktivität, VA hingegen verminderte sie. Bei gleichzeitigem Einwirken waren die allelochemischen Reaktionen geringer als die Summe der Wirkungen jeder für sich untersuchten Komponente, was auf einen Antagonismus hinweist.

**Introduction**

Different types of chemical compounds identified as allelochemicals, including FA and VA, are commonly found in soils at concentrations between 0.01 and 1 mM, and affect the growth of various plant species at concentrations of up to 1.0 mM. In general, these compounds interfere to some degree with many vital plant processes, including water use, transpiration, shoot and root growth, inhibition of nutrient uptake by roots, photosynthesis reduction and leaf expansion (WINK & LATZ-BRUNING 1995, MACIAS 1995).

It has been reported that mixtures of non-inhibitory concentrations of individual phenolic acids may inhibit plant growth in an additive (equal to the sum of the effects of each allelochemical tested separately), synergistic (greater than the sum of the effects of each allelochemical) or antagonistic (lower than the sum of the effects of each allelochemical) manner (BLUM & al. 1985, LEHMAN & al. 1994). These possibilities have been reported for mixtures of different phenolic acids on cucumber (*Cucumis sativus* L.) radicle growth (BLUM & al. 1984) and leaf expansion (BLUM & al. 1985, 1989, GERIG & BLUM 1991, LEHMAN & al. 1994).

In spite of these reports, no attention has been paid to the simultaneous effects of phenolic acids on the soybean root, which is susceptible to various allelochemicals tested alone (PATTERSON 1981, BAZIRAMAKENG & al. 1995, FERRARESE & al. 2000a, HERRIG & al. 2002). Thus, the purpose of the present work was to investigate the simultaneous effects of FA and VA on POD, which has been implicated in various physiological processes, on PAL which is regarded as the primary enzyme that leads to phenylpropanoids protecting the plants against various biotic and abiotic stresses, and on root growth of soybean seedlings in nutrient solution. FA, a cinnamic acid derivative, and VA, a benzoic acid derivative, were chosen because they have been implicated as potential allelopathic agents (MACIAS 1995) and are the most frequently observed in soil extracts (WHITEHEAD & al. 1982).

**Material and Methods**

*Glycine max* (L.) MERR. (cv. BR-16) seeds, previously sterilized with 2% sodium hypochlorite for two minutes and rinsed extensively with deionized water, were dark-germinated (at 25°C) on two sheets of moist filter paper. Three-day-old seedlings of uniform size were transferred to containers (10 x 16 cm) filled with 200 ml of full-strength Hoagland's solution (FERRARESE & al. 2000a) with or without FA or VA (0.5 or 1.0 mM). Nutrient solution was buffered with 17 mM potassium phosphate buffer, adjusted to pH 6.0 and monitored over time. Each container held 25
uniform seedlings suspended in the solution by floating styrofoam boats. The containers were kept in a growth chamber, at 25°C, under fluorescent light (280 μmol m⁻² s⁻¹) on a 12-h dark/12-h photoperiod. The nutrient solution was aerated continuously by air bubbling, and the roots were exposed to allelochemicals for 48 h. All roots were measured before and at the end of experiments. FW of all roots (25) were determined immediately after 48 h and roots DW were determined after oven drying at 80°C for 24 h. In order to test possible interactive effects, equimolar mixtures containing FA and VA were prepared at the final concentration of 0.5 or 1.0 mM. Phenolic compounds used in this work were purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents used were of the purest grade available or chromatographic grade.

After 48 h, all the roots of treated or untreated seedlings were detached and used for enzyme extraction. For POD, fresh roots (0.25 g) were extracted with 2.5 ml of 67 mM phosphate buffer (pH 7.0) as described by SHANN & BLUM 1987a. The extract was centrifuged at 10,000 xg for 15 min at 4°C and the supernatant was used to determine the activity of soluble POD. To isolate CW-bound POD, the pellet was washed with deionized water (at 4°C) until no activity of soluble POD was detected in the supernatant. The pellet was washed twice with 1 ml of 1 M NaCl. At each step of extraction, the homogenate was centrifuged at 10,000 xg for 15 min. The supernatants of each fraction were pooled and considered to be the CW-(ionically) bound POD. Guaiacol-dependent activities of the soluble and CW-bound POD were determined according to CAKMAK & HORST 1991 with some modifications. The reaction mixture (3 ml) contained 25 mM sodium phosphate buffer (pH 6.8), 2.58 mM guaiacol and 10 mM H₂O₂. The reaction was initiated by the addition of diluted enzyme extract in phosphate buffer. Guaiacol oxidation was followed for 5 min at 470 nm, and the enzyme activity was calculated using the extinction coefficient (25.5 mM⁻¹ cm⁻¹) for tetraguaiacol. POD activities were expressed as μmol min⁻¹ g⁻¹ FW.

PAL was extracted as described by FERRARESE & al. 2000b. Fresh roots (2 g) were ground at 4°C in 0.2 M sodium borate buffer (pH 8.8). The homogenates were centrifuged at 12,000 xg for 15 min and the supernatant was used as enzyme preparation. For the PAL activity assay, the reaction mixture [100 μmoles sodium borate buffer (pH 8.7) and a suitable amount of enzyme extract in a final volume of 1.55 ml] was incubated at 40°C for 5 min, started by the addition of 15 μmoles of L-phenylalanine and stopped after one hour of incubation by the addition of 50 μl of 5 N HCl. Samples were filtered through a 0.45 μm disposable syringe filter (Hamilton® Co., Nevada, USA) and analyzed (20 μl) using a Shimadzu® Liquid Chromatograph (Tokyo, Japan) equipped with a LC-10AD pump, a Rheodine® injector, a SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack GLC-ODS (Shimadzu®, Tokyo, Japan) column (150 x 4.6 mm, 5 μm) was used at room temperature in conjunction with the same type of pre-column (10 x 4.6 mm). The mobile phase was methanol:water (70%:30%) with a flow rate of 0.5 ml min⁻¹ for an isocratic run of 10 min. Absorbance of the samples as well as of the standard were detected at 275 nm, and data collection and integration were performed with the Class-CR10 software (Shimadzu®, Tokyo, Japan). t-Cinnamate, the product of the PAL reaction, was identified by comparison of its retention time with that of the standard's. Parallel controls without L-phenylalanine or with t-cinnamate (added as internal standard in the reaction mixture) were made as described elsewhere (FERRARESE & al. 2000b). PAL activity was expressed as μmol t-cinnamate min⁻¹ g⁻¹ FW.

Statistical tests were performed using the InStat® package, version 1.12 (GraphPAD® Software, San Diego, USA). The statistical significance of the difference between parameters was evaluated by means of Student’s t-test (p<0.05). Data in the tables represents means of four to ten separate experiments ± SE.

Results and Discussion

Table 1 presents all root variables measured after individual treatment with allelochemicals. As may be seen, FA and VA, at 0.5 or 1.0 mM, reduced RL when compared with the control roots. Likewise, FA and VA reduced root FW and root DW. Similar results are described in another plants (EINHELLIG & al. 1982, DEVI & PRASAD 1996, BALERONI & al. 2000, PRAMANIK & al. 2000). The results also show
that the soluble POD and CW-bound POD activities increased under the action of these allelochemicals, for any concentration. However, it was found that the enzymatic activities increased against FA concentration but decreased against VA concentration. The same Table reveals that both compounds, at 0.5 mM, were incapable of stimulating the PAL activity. On the other hand, an expressive increase in the PAL activity after 1.0 mM FA treatment and a significant decrease after 1.0 mM VA treatment were found. The same Table summarizes the effects of equimolar mixtures of the allelochemicals on all root variables evaluated. At equimolar concentration of 0.5 mM, the compounds decreased RL, FW, DW, increased soluble POD (but not CW-bound POD) and reduced PAL activities. Similar behavior was observed at equimolar concentration of 1.0 mM by comparison with the control.

In general, individual treatments with FA and VA increase both soluble and CW-bound POD and correlate with a pronounced decrease in root length (BAZIRAMAKENGA & al. 1995, DEVİ & PRASAD 1996, POLITYCKA 1996). In addition to POD, PAL activity increases in different circumstances but the few studies reported on the effects of exogenous phenolic acids have been controversial. In fact, PAL inhibition by cinnamic acid and derivatives was demonstrated by SATO & al. 1982 in different sources of pure enzyme with the exception to FA, which was ineffective in sweet potato (Ipomea batatas L.). Similarly, SHANN & BLUM 1987b revealed that the PAL activity was unaffected by FA in cucumber roots. Contrarily, POLITYCKA 1998 demonstrated that FA (but not VA) increased PAL activity while decreased the cucumber root growth. Despite these controversies, and based on results reported here, it is likely that FA and VA cause stress on soybean roots. Thus, it is reasonable to suppose that these allelochemicals depolarize the root cell membrane affecting the nutrient uptake and the plant growth (BAZIRAMAKENGA & al. 1995, POLITYCKA 1996, DEVİ & PRASAD 1996). Moreover, the compounds might increase the synthesis of other phenolic acids by the phenylpropanoid pathway (STRACK 1997), their incorporation into lignin (TAN & al. 1992), increase of the cell wall rigidity (SÁNCHEZ & al. 1996) and, therefore, growth reduction.

Table 2 shows percent increase or decrease of the variables obtained with values from Table 1. It is clear that, acting conjointly the combinations tested (0.5 or 1.0 mM) produced less effect on the variables (except PAL activity at 0.5 mM) than the sum of the effects of each allelochemical tested separately. With respect to the effects of phenolic acids mixture on plant growth, it is interesting to emphasize that some studies have been carried out basically with cucumber. In nutrient solution, BLUM & al. 1985 demonstrated that the leaf expansion and dry weight were reduced by single and multiple treatments of FA, VA and p-coumaric acid (p-CA). The effects of the mixture of allelochemicals were additive (to 0.5 mM FA plus 0.5 mM p-CA mixture) and antagonistic (to 0.5 mM FA plus 0.5 mM VA mixture). Using soil systems, BLUM & al. 1989 and GERIG & BLUM 1991 reported that the effects of FA plus VA, FA plus p-hydroxybenzoic acid (p-HB) and p-CA plus p-HB acids mixtures on leaf area expansion were antagonistic. Other combinations among these compounds revealed additive effects. Conducting split-root experiments, LEHMAN & al. 1994 reported that the simultaneous effects of FA and p-CA on leaf expansion were additive. The inhibition of leaf expansion was
Table 1. Changes in the root length (RL), fresh weight (FW), dry weight (DW), soluble peroxidase (POD), (CW)-bound POD and phenylalanine ammonia-lyase (PAL) in roots of soybean seedlings treated with FA, VA and equimolar mixtures.

<table>
<thead>
<tr>
<th>mM</th>
<th>RL (cm)</th>
<th>FW (g)</th>
<th>DW (g)</th>
<th>Soluble POD µmol min⁻¹ g⁻¹ FW</th>
<th>Bound POD µmol min⁻¹ g⁻¹ FW</th>
<th>PAL µmol min⁻¹ g⁻¹ FW (× 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7.0±0.14</td>
<td>4.1±0.17</td>
<td>0.27±0.011</td>
<td>6.6±0.21</td>
<td>2.1±0.29</td>
<td>3.4±0.03</td>
</tr>
<tr>
<td>FA 0.5</td>
<td>5.4±0.24 (-)</td>
<td>3.4±0.15 (-)</td>
<td>0.23±0.008 (-)</td>
<td>7.8±0.38 (+)</td>
<td>3.1±0.08 (+)</td>
<td>3.1±0.12 (ns)</td>
</tr>
<tr>
<td>VA 0.5</td>
<td>3.8±0.14 (-)</td>
<td>3.0±0.08 (-)</td>
<td>0.20±0.005 (-)</td>
<td>8.8±0.79 (+)</td>
<td>2.6±0.07 (+)</td>
<td>3.2±0.15 (ns)</td>
</tr>
<tr>
<td>FA + VA</td>
<td>3.6±0.17 (-)</td>
<td>3.3±0.17 (-)</td>
<td>0.19±0.012 (-)</td>
<td>9.6±0.50 (+)</td>
<td>2.5±0.12 (ns)</td>
<td>2.5±0.02 (-)</td>
</tr>
<tr>
<td>FA 1.0</td>
<td>2.7±0.15 (-)</td>
<td>2.7±0.05 (-)</td>
<td>0.18±0.05 (-)</td>
<td>9.7±0.24 (+)</td>
<td>3.7±0.28 (+)</td>
<td>6.2±0.08 (+)</td>
</tr>
<tr>
<td>VA 1.0</td>
<td>3.2±0.11 (-)</td>
<td>2.8±0.07 (-)</td>
<td>0.22±0.04 (-)</td>
<td>7.7±0.08 (+)</td>
<td>2.4±0.07 (+)</td>
<td>2.3±0.20 (-)</td>
</tr>
<tr>
<td>FA + VA</td>
<td>2.1±0.09 (-)</td>
<td>2.9±0.01 (-)</td>
<td>0.20±0.01 (-)</td>
<td>8.7±0.60 (+)</td>
<td>2.5±0.28 (ns)</td>
<td>2.4±0.07 (-)</td>
</tr>
</tbody>
</table>

Means followed by (-) or (+) are significantly (Student t-test, p≤0.05) different from control. FA + VA = data of equimolar concentrations. ns = not significant.

Table 2. Percent increase or decrease for root length (RL), fresh weight (FW), dry weight (DW), soluble peroxidase (POD), (CW)-bound POD and phenylalanine ammonia-lyase (PAL) of soybean seedlings treated with FA, VA and equimolar mixtures.

<table>
<thead>
<tr>
<th></th>
<th>RL (cm)</th>
<th>FW (g)</th>
<th>DW (g)</th>
<th>Soluble POD µmol min⁻¹ g⁻¹ FW</th>
<th>Bound POD µmol min⁻¹ g⁻¹ FW</th>
<th>PAL µmol min⁻¹ g⁻¹ FW (× 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA 0.5 mM</td>
<td>22.9 (d)</td>
<td>17.1 (d)</td>
<td>14.8 (d)</td>
<td>18.2 (i)</td>
<td>47.6 (i)</td>
<td>0.0</td>
</tr>
<tr>
<td>VA 0.5 mM</td>
<td>45.7 (d)</td>
<td>26.8 (d)</td>
<td>25.9 (d)</td>
<td>33.3 (i)</td>
<td>23.8 (i)</td>
<td>0.0</td>
</tr>
<tr>
<td>Sum</td>
<td>68.6</td>
<td>43.9</td>
<td>40.7</td>
<td>51.5</td>
<td>71.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Mixture</td>
<td>48.6 (d)</td>
<td>19.5 (d)</td>
<td>29.6 (d)</td>
<td>45.5 (i)</td>
<td>0.0</td>
<td>26.5 (d)</td>
</tr>
<tr>
<td>Sum - Mixture</td>
<td>+20.0</td>
<td>+24.4</td>
<td>+11.1</td>
<td>+6.0</td>
<td>+71.4</td>
<td>-26.5</td>
</tr>
<tr>
<td>FA 1.0 mM</td>
<td>61.4 (d)</td>
<td>34.1 (d)</td>
<td>33.3 (d)</td>
<td>47.0 (i)</td>
<td>76.2 (i)</td>
<td>82.3 (i)</td>
</tr>
<tr>
<td>VA 1.0 mM</td>
<td>54.3 (d)</td>
<td>31.7 (d)</td>
<td>18.5 (d)</td>
<td>16.7 (i)</td>
<td>14.3 (i)</td>
<td>32.4 (d)</td>
</tr>
<tr>
<td>Sum</td>
<td>115.7</td>
<td>65.8</td>
<td>51.8</td>
<td>63.7</td>
<td>90.5</td>
<td>114.7</td>
</tr>
<tr>
<td>Mixture</td>
<td>70.0 (d)</td>
<td>29.3 (d)</td>
<td>25.9 (d)</td>
<td>31.8 (i)</td>
<td>0.0</td>
<td>29.4 (d)</td>
</tr>
<tr>
<td>Sum - Mixture</td>
<td>+45.7</td>
<td>+36.5</td>
<td>+25.9</td>
<td>+31.9</td>
<td>+90.5</td>
<td>+85.3</td>
</tr>
</tbody>
</table>

Data followed by (d) or (i) represents % of decrease or % of increase over control (Table 1). Sum = results of FA plus VA tested separately. Mixture = results of equimolar concentrations. Results not significant (from Table 1) are represented as zero.
directly related to the concentrations of the acid(s) and the proportion of roots treated with the acid(s).

Taking into account these reports, the results presented here indicated, at least for soybean, that the effects of the FA plus VA mixture were antagonistic. Although the mechanisms involved are still unknown, the alterations in POD and PAL activities, and on root growth, may be important in explaining allelopathic effects. However, it is necessary to comment that the experiments here were conducted under controlled conditions. Furthermore, an important question about the allelopathy interactions is whether available concentrations of these compounds in soil environment are sufficient to alter seedling growth. In fact, their availability is affected by soil components, and under favorable field conditions, the activity of allelochemicals could be reduced or eliminated through microbial activity (BLUM 1998). Even so, if the plants are continually exposed to allelopathic compounds, the turnover rates of phenolic acid pools in soils will probably be substantial to cause antagonistic effects. Thus, since plant roots encounter the allelochemicals in various combinations and concentration in the soil environment, experiments with other mixtures at different concentrations would yield useful information.

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