

## **Antagonistic and synergistic effects of main phenolic compounds present in continuously cropped sick cotton soil on its own seeds germination, seedling growth and antioxidant enzyme activities**

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### **ABSTRACT**

Effects of 3-main phenolic compounds [*p*-hydroxybenzoic acid (*p*-HA, 22.5 mg/L), ferulic acid (FA, 6.6 mg/L) and vanillin (VA, 8.4 mg/L)], present in continuously cropped sick cotton soil and their combinations were studied on cotton seed germination and seedlings growth, root activity and antioxidant enzyme activities. Results showed that *p*-HA, VA, FA, *p*-HA+FA and VA+FA treatments stimulated the germination of cotton seeds. While the treatments VA, *p*-HA+VA, VA+FA and *p*-HA+VA+FA inhibited the seedlings growth. All phenolic compounds combinations inhibited the root activity of cotton seedlings. The VA, FA, *p*-HA+FA and VA+FA treatments reduced the malondialdehyde content. The superoxide dismutase activity was inhibited by *p*-HA, VA, VA+FA, *p*-HA+VA+FA, catalase activity was inhibited by VA, FA, VA+FA, *p*-HA+VA+FA and the peroxidase activity was stimulated by FA and *p*-HA+VA+FA. There were antagonistic effects between *p*-HA and VA on the cotton seeds germination. The antagonistic effects between *p*-HA and VA. However, there were significant synergistic effects between VA and FA.

**Key words:** Antagonism, antioxidant enzyme, cotton, interaction, phenolic compounds, seeds germination, seedling growth, soil sickness, synergism

### **INTRODUCTION**

In Xinjiang Uyghur Autonomous Region, cotton (*Gossypium hirsutum* L., Malvaceae) is most important cash crop. The allelochemicals enter the soil through decomposition of residues and their root exudates and affects the growth of crops (5,14,15,18,17) This phenomenon is called allelopathy (11,33). Some phenolic compounds [syringic acid (35), vanillic acid (32), *p*-hydroxybenzoic acids (16) etc.], are allelochemicals with strong allelopathy activity (9,32). Syringic acid inhibits the growth of seedlings and altered the rhizosphere microbial communities (34). Besides it has direct phytotoxicity. While the *p*-coumaric acid inhibited the growth of seedlings through negative plant-soil microbial interactions (18,34). Sun (24) detected phenolic compounds (*p*-hydroxybenzoic acid, vanillin, ferulic acid) in the cotton growing soil and found that these phenolic compounds at high concentration inhibited the growth of cotton seedlings.

Phenolic compounds had variable effects on different organs of same plant (24). The allelopathic effects of phenolic compounds are concentration dependent (21). The low

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concentrations of phenolic compounds did not affect the plant growth or even promoted the plant growth (24,31), but their high concentrations inhibits the plant growth (24,25). Plants can produce one or more allelochemicals. The allelopathic effects of allelochemicals on plant development depends on their interactions (8,18,23). Einhellig (11) suggested that allelopathic potential of most plants is due to the interactions of two or more compounds. As the number of phenolic compounds added to soil increased, the individual phenolic compounds concentration required for growth inhibition becomes lower (6,7). Although the effects of single exogenous phenolic compounds on cotton seed germination and seedling growth had been investigated, but the interactions between many phenolic compounds on cotton growth remains poorly understood. Thus, it is necessary to find the effects of concentrations of phenolic compounds accumulated in soils on cotton growth.

This study aimed to, (i). investigate the effects of different combinations of 3-test phenolic compounds [*p*-hydroxybenzoic acid (*p*-HA), ferulic acid (FA) and vanillin (VA)] on seeds germination and seedling growth of cotton and (ii). explore the interactions between these phenolic compounds.

## MATERIALS AND METHODS

The seeds of Cotton (*Gossypium hirsutum* L., Malvaceae) variety (Xinluzao 42) was obtained from Shihezi University, Shihezi city (45°19' N, 86°03' E). Mean height above sea level: 443 m, Continental climate, average annual precipitation: 208 mm (Rainy season: from June to August), Mean annual evaporation: 1,660 mm. The mean maximum temperature: 26.7 °C and the mean minimum temperature: -17.8 °C (Frost-free period: 160 d). The test phenolic compounds were *p*-hydroxybenzoic acid (*p*-HA), ferulic acid (FA) and vanillin (VA).

### 1. Experimental details

The experimental treatments consisted of two factors (i). Phenolic compounds combinations: 8 (CK, *p*-HA, VA, FA, *p*-HA+VA, *p*-HA+FA, VA+FA, *p*-HA+VA, *p*-HA+VA, *p*-HA+VA, *p*-HA+VA+FA) and (ii). their concentrations: *p*-HA (22.5 mg/L), VA (8.4 mg/L), FA (6.6 mg/L). The treatments were replicated thrice in randomized block design.

The distilled water was used as control. The concentrations of *p*-hydroxybenzoic acid, ferulic acid and vanillin were determined from the 30-years continuous cotton cropping fields. Experimental Station Agriculture College, Shihezi University. The concentrations of *p*-hydroxybenzoic acid, ferulic acid and vanillin were 1.91, 0.56 and 0.71 µg/g, respectively (24). The concentration of phenolic compounds in aqueous solution was calculated as under (14)

$$\omega \text{ (mg/L)} = (S \times H \times W_s \times P \times 10^4) / I \text{ (1);}$$

Where,  $\omega$ : Concentration of phenolic compounds in aqueous solution (mg/L), S: Unit area of land (ha), is 1, H: Depth of soil layer 0.2m (20 cms),  $W_s$ : Soil specific gravity (g/cm<sup>3</sup>), is 2.65, P: Phenolic compounds content in soil (µg/g), I: Volume of water per

irrigation by drip irrigation (m<sup>3</sup>/ha) per pot was 450 mL. The phenolic compounds concentrations in aqueous solution of *p*-HA, FA and VA were 22.5 mg/L, 6.6 mg/L and 8.4 mg/L, respectively. The combinations of 3- phenolic compounds in aqueous solution are given in Table 1.

Table 1. The 3-Phenolic compounds concentrations used in different treatments

Treatments	<i>p</i> -HA(mg/L)	VA(mg/L)	FA(mg/L)
CK	-	-	-
<i>p</i> -HA	22.5	-	-
VA	-	8.4	-
FA	-	-	6.6
<i>p</i> -HA+VA	22.5	8.4	-
<i>p</i> -HA+FA	22.5	-	6.6
VA+FA	-	8.4	6.6
<i>p</i> -HA+VA+FA	22.5	8.4	6.6

**2. Bioassay**

**Seed germination**

Cotton seeds were selected and disinfected with 20 % (v/v) H<sub>2</sub>O<sub>2</sub> for 20 min and then sown in germination boxes (19 cm×13 cm×12 cm) with 200 cm<sup>3</sup> (500 g vermiculite) sterilized (at 126 °C for 25 min). Vermiculite was used instead of field soil, because it is a clay mineral that does not contain phenolic acids and other allelochemicals. If field soil was used, the presence of phenolic acids or other allelochemicals in the soil may have unknown effects on the germination of cotton seeds or the growth of seedlings. One hundred mL solution was added to each germination box as per treatment. Fifty seeds were sown in each pot. The treatments were replicated thrice in each design. Germination boxes were placed in artificial climate chamber (28°C, 50 % relative humidity in dark). The seeds were sown on July 3, 2018, and the germination data was recorded from July 4 to July 10, 2018.

**Germination parameters**

The number of germinated cotton seeds were counted daily for 7 days (from July 4 to July 10). The germination potential (GP), germination rate (GR) and germination index (GI) of the seeds were measured and calculated on the 7th day as under:

$$GP = \text{Number of total germinated seeds in 3 days} / \text{Number of total seeds} \times 100\% \quad (2);$$

$$GR = \text{Number of total germinated seeds in 7 days} / \text{Number of total seeds} \times 100\% \quad (3);$$

$$GI = N_1/1 + N_2/2 + N_3/3 \dots\dots N_7/7 \quad (4);$$

Where, N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>,.....N<sub>7</sub>, number of seeds which germinated on day 1, 2, 3.....7.

**Seedling growth**

On July 13, 2018, cotton seeds were disinfected with 20 % (v/v) H<sub>2</sub>O<sub>2</sub> for 20 min. One seed was placed per 1 cm<sup>2</sup> in the germination box (19 cm×13 cm×12 cm). Two layers of gauze (19 cm ×13 cm) were moistened with 50 ml distilled water. These seeds were covered with two layers of moist gauze, then these pots were placed in

growth chamber (28°C, 10 h light). On July 15, 15 identical germinated cotton seeds were selected and transplanted in plastic pots (10 cm dia, 20 cm depth, 10 holes in bottom) containing 2250 g vermiculite per pot. In each pot 400 mL treatment solution (different combinations of phenolic compounds) was added. The treatments were replicated thrice in randomized block design. Then these pots were placed in growth chamber (28°C, 10 h light). Twelve days after transplanting, seedlings were randomly selected to determine the morphological and physiological indicators.

#### **Morphological indicators**

Twelve days after transplanting (July 27), 8 seedlings were selected to measure (i). Seedling height, (ii). Main root length, (iii). Root mass and (iv). Seedling mass (Root + Shoot). We used the seedlings shoot to measure its height.

#### **Physiological indicators**

Twelve days after the transplanting (July 27), 9- seedlings were selected to measure (i). Roots activity (RA), (ii). Malondialdehyde content (MDA) and (iii). Antioxidant enzyme activity (in groups of three, repeated thrice).

**I. Root activity (RA):** It was measured as per Luo (19). The samples of root tissue (1.5 g) were immersed in 30 mL TTC solution in phosphoric acid buffer (66.67 mmol/L, pH = 7.0). Blanks were also prepared simultaneously by adding 2 mL vitriol (H<sub>2</sub>SO<sub>4</sub>, 1M) to root tissue. The solutions were allowed to set for 1 h at 37 °C; then 2 mL vitriol (1M) was added to each sample to stop the reaction. The roots were removed and ground in ethyl acetate (3 mL). The solutions were put into tubes and ethyl acetate was added to make the volume to 10 mL. Absorbance was measured at 485 nm. The degree of tetrazolium reduction was calculated using a standard curve. Root activity was expressed in micrograms (µg) of tetrazolium reduction per g of roots per hour.

**II. MDA content:** It was measured as per He (13) with method of thiobarbituric acid (TBA), and calculated in micromole of malondialdehyde per g of aboveground seedlings based on absorption at 450, 532 and 600 nm wavelength respectively.

**III. Superoxide dismutase (SOD):** It was measured as per Gao (12) with method of nitrogen blue tetrazolium (NBT), and expressed as superoxide dismutase activity unit per g of leaves.

**IV. Peroxidase (POD):** It was measured as per Gao (12) with method of guaiacol. H<sub>2</sub>O<sub>2</sub> oxidizes guaiacol to a dark brown substance under the catalysis of peroxidase. This substance has maximum light absorption at 470 nm. Peroxidase activity was expressed in unit per g of leaves per min.

**V. Catalase (CAT):** It was measured as described by Gao (12) and expressed in milligrams (mg) of decomposed H<sub>2</sub>O<sub>2</sub> per g of leaves per min.

#### **3. Inhibition rate (IR)**

Inhibition rate = (control-treatment)/control ×100%.

When IR >0, it represents inhibition effect; when IR < 0, it represents promotion effect. The absolute value of IR stands for strength.

#### 4. Synthesis effect

The synthesis effect (SE) is comprehensive evaluation index of receptor growth index and expressed as the arithmetic mean of several Data of IR.

#### 5. Statistical analysis of data

Data were analyzed by analysis of variance (ANOVA) and mean comparison between treatments was performed based on Duncan's multiple range method at 5 % level.

## RESULTS AND DISCUSSION

#### Seed germination

The VA+FA, VA, FA, *p*-HA+FA, *p*-HA stimulated the germination of cotton seeds (Table 2, Figure 1-a). The germination potential and germination rate of cotton seeds treated with VA+FA, VA, FA, *p*-HA+FA and *p*-HA were significantly increased ( $P < 0.05$ ). The germination index of cotton seeds treated with VA+FA was significantly increased ( $P < 0.05$ ). The different combinations of phenolic compounds stimulated the germination of cotton seeds and the order of stimulation intensity was: VA+FA > VA > *p*-HA+FA > FA > *p*-HA > *p*-HA+VA+FA > *p*-HA+VA. Among them, VA+FA had the strongest stimulatory effect, and *p*-HA+VA had the weakest stimulating effect.

Table 2. Effects of different combinations of phenolic compounds on germination indicators ( $\bar{x} \pm s$ )

Treatments	Seed germination potential		Seed germination rate		Seed germination index	
	Value (%)	IR	Value (%)	IR	Value (%)	IR
CK	68.0±2.0 c	-	70.0±5.3 c	-	17.7±4.4 b	-
<i>p</i> -HA	81.3±7.0 ab	-0.20	84.7±8.1 ab	-0.21	21.9±2.8 ab	-0.23
VA	83.3±7.0 ab	-0.23	88.0±3.5 ab	-0.26	21.4±3.0 ab	-0.21
FA	82.0±4.0 ab	-0.21	87.3±5.0 ab	-0.25	21.3±4.1 ab	-0.20
<i>p</i> -HA+VA	72.7±4.2 bc	-0.07	78.0±7.2 bc	-0.11	19.7±3.9 ab	-0.11
<i>p</i> -HA+FA	82.0±10.4 ab	-0.21	88.7±11.0 ab	-0.27	21.6±1.9 ab	-0.22
VA+FA	85.3±6.4 a	-0.25	92.0±2.0 a	-0.31	25.1±3.1 a	-0.42
<i>p</i> -HA+VA+FA	75.3±6.1 abc	-0.11	78.7±3.1 bc	-0.12	21.1±0.5 ab	-0.19

Different letters in the same column mean significant difference at 0.05 probability level.

Phenolic compounds had obvious effects on seed germination and were concentration dependent (4,10,30). The concentration thresholds of *p*-HA, FA and VA for inhibiting cotton seed germination were 200, 200 and 50 mg/L (28), respectively. In this study, VA+FA, VA, FA, *p*-HA+FA and *p*-HA stimulated the germination of cotton seeds, because the concentrations used were far below the concentration thresholds of the three phenolic compounds.

#### Seedlings growth

The main root length and seedling height of cotton seedlings were significantly inhibited by *p*-HA+VA+FA, *p*-HA+VA, VA and *p*-HA+VA, VA+FA, VA, respectively ( $P < 0.05$ ). The root mass and seedling mass were significantly stimulated by all treatments

except *p*-HA+VA and *p*-HA+VA+FA, FA, respectively ( $P < 0.05$ ). It could be seen from the synthesis effect of inhibition. In the inhibition of cotton seedling main root length and seedling height, the inhibition intensity was in the order VA > *p*-HA+VA > *p*-HA+VA+FA > VA+FA > *p*-HA > FA > *p*-HA+FA. In the stimulation of root mass and seedling mass of cotton seedlings, the stimulation intensity was in the order FA > *p*-HA+VA+FA > VA > VA+FA > *p*-HA+FA > *p*-HA > *p*-HA+VA (Table 3, Figure 1-b).

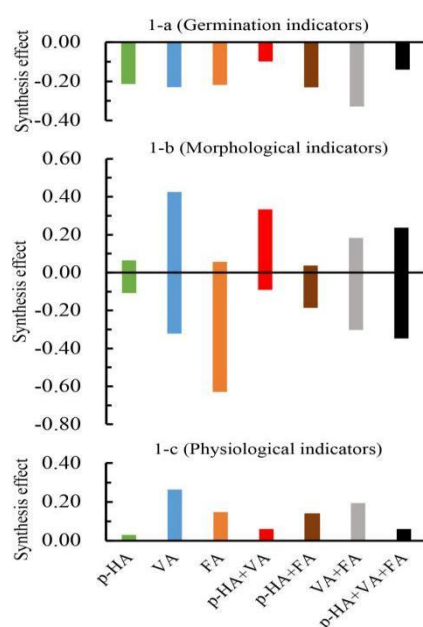


Figure 1. Effects of different combinations of phenolic compounds on the synthesis effect (SE) on cotton seeds germination, seeding growth and physiological activities. Synthesis effect was calculated using the IR arithmetic mean of several test items Date. In Figure 1-b, the upper part of X-axis: Synthesis effect of main root length and seedling height, and the lower part of the X-axis: Synthesis effect of root mass and seedling mass.

Table 3. Effects of different combinations of phenolic compounds on morphological indicators of cotton seedlings ( $\bar{x} \pm s$ )

Treatments	Main root length		Root mass		Seedling height		Seedling mass	
	(cm)	IR	(mg)	IR	(cm)	IR	(mg)	IR
CK	15.8±0.2 a	-	27±5 e	-	10.4±0.1 a	-	525±6 b	-
<i>p</i> -HA	14.1±2.5 a	0.11	34±1 d	-0.27	10.2±0.2 a	0.02	498±40 b	0.05
VA	7.2±0.4 b	0.54	44±3 b	-0.67	7.2±0.2 d	0.31	513±59 b	0.02
FA	15.3±0.3 a	0.03	0.055±5 a	-1.07	9.5±0.1 abc	0.08	627±48 a	-0.19
<i>p</i> -HA+VA	8.1±1.7 b	0.49	30±2 de	-0.13	8.5±1.6 bc	0.18	555±81 ab	-0.06
<i>p</i> -HA+FA	15.6±0.6 a	0.01	36±6 cd	-0.37	9.7±0.4 ab	0.07	526±45 b	0.00
VA+FA	13.2±3.3 a	0.16	45±1 b	-0.69	8.3±0.3 cd	0.20	483±33 b	0.08
<i>p</i> -HA+VA+FA	9.2±1.5 b	0.42	40±2 bc	-0.51	9.8±1.1 ab	0.06	620±6 a	-0.18

Roots are the earliest organs of crops that sense environmental signals and directly contact the allelochemicals in the soil, affecting the development of the above-ground parts of plants (3). Previous studies suggested that the elongation of plant tissues mainly depends on cell division in the meristematic zone, but allelochemicals could reduce the mitotic index (31). In this study, some combinations of phenolic compounds inhibited the main root length and seedling height of cotton seedlings, which might be related to phenolic compounds inhibiting the mitosis of meristem region in the root tip and stem growth point. The roots of cotton seedlings were more sensitive to the phenolic compounds than above-ground, which was consistent with the researches of Wang and Tomar (25,28).

#### Physiological activity of cotton seedlings

All combinations of phenolic compounds significantly ( $P < 0.05$ ) inhibited the root activity of seedlings; The content of malondialdehyde was significantly inhibited by VA+FA, *p*-HA+FA, FA and VA, respectively ( $P < 0.05$ ). Different combinations of phenolic compounds affected the antioxidant enzyme activity of cotton leaves. The SOD and CAT activities were significantly inhibited by *p*-HA, *p*-HA+VA+FA, VA, VA+FA and VA+FA, FA, *p*-HA+VA+FA, VA, respectively ( $P < 0.05$ ). The POD activity was significantly stimulated by *p*-HA+VA+FA and FA ( $P < 0.05$ ) (Table 4). The different combinations of phenolic compounds inhibited the physiological activity of cotton seedlings and the order of inhibition intensity was: VA > VA+FA > FA > *p*-HA+FA > *p*-HA+VA > *p*-HA+VA+FA > *p*-HA. Among them, VA had the largest inhibitory effect (Figure 1-c).

Plants have developed complete antioxidant system during their long-term evolution,

Table 4. Effects of different combinations of phenolic compounds on physiological indicators of cotton seedlings ( $\bar{x} \pm s$ )

Treatments	RA		MDA		SOD		POD		CAT	
	Activity/ [ $\mu\text{g}/(\text{g}\cdot\text{h})$ ]	IR	Content / ( $\mu\text{mol}/\text{g}$ )	IR	Activity/ (U/g)	IR	Activity/ [ $\text{mg}/(\text{g}\cdot\text{min})$ ]	IR	Activity/ [ $\text{mg}/(\text{g}\cdot\text{min})$ ]	IR
CK	84.2±6.2 a	-	10.07±0.27 a	-	421.7±3.1 a	-	614.3±150.2 b	-	1502.9±54.5 a	-
<i>p</i> -HA	76.8±4.0 b	0.09	9.49±0.30 a	0.06	397.5±3.7 b	0.06	717.5±285.3 ab	-0.17	1325.9±37.1 ab	0.12
VA	49.2±1.5 d	0.42	2.96±0.16 d	0.71	356.0±10.1 c	0.16	831.3±187.2 ab	-0.35	912.0±94.3 c	0.39
FA	38.4±0.7 f	0.54	4.91±0.21 c	0.51	416.8±8.2 a	0.01	985.1±59.8 a	-0.60	1090.1±88.0 c	0.27
<i>p</i> -HA+VA	60.2±5.5 c	0.28	8.88±1.31 a	0.12	419.3±5.9 a	0.01	710.6±77.8 ab	-0.16	1426.1±69.0 a	0.05
<i>p</i> -HA+FA	39.8±0.6 ef	0.53	5.42±0.67 c	0.46	410.9±2.3 a	0.03	854.2±68.3 ab	-0.39	1398.4±125.3 a	0.07
VA+FA	37.7±0.7 f	0.55	7.26±0.55 b	0.28	281.5±11.9 d	0.33	889.8±217.5 ab	-0.45	1121.1±219.2 bc	0.25
<i>p</i> -HA+VA+FA	44.5±0.7 de	0.47	9.79±1.77 a	0.03	396.0±6.2 b	0.06	967.9±171.1 a	-0.58	1025.1±164.7 c	0.32

including enzyme and non-enzyme constituents (2,20). The non-enzymatic antioxidants are generally small molecules (ascorbate, glutathione and carotenoids) (1,12). In the enzymatic pathways, when the activity of one antioxidant enzyme inhibited, plants will compensate for each other's deficiencies by increasing the activity of other antioxidant enzymes (22,29). In this study, the non-enzymatic system in seedlings reduced the oxidative damage of cell membranes, may be due to the reduced MDA content. The inhibition of SOD and CAT activity may be due to the inhibition of enzyme activity after the removal of reactive oxygen species, but POD was stimulated to compensate for the

loss of SOD and CAT. Actually, low concentration of some phenolic compounds stimulated the growth of seedlings, which were determined by the concentration-dependent properties of phenolic compounds (24,25,31). It is possible that some phenolic compounds are adsorbed in the soil or chelated with other chemical substances (27), and thus decreasing the detectable phenolic compounds concentration in the cotton mono-cropping management systems.

### **Synergistic and antagonistic effects on seed germination**

The synthesis effects intensity of *p*-HA+VA was 0.1, which was significantly higher than that of *p*-HA and VA, proving that there was an antagonistic effect between *p*-HA and VA. The synthesis effect intensity of VA+FA was 0.33, which was significantly higher than that of VA and FA, proving that there was a synergistic effect between VA and FA. In *p*-HA+VA+FA, the antagonistic effect between *p*-HA and VA and the synergistic effect between VA and FA appeared together. The synthesis effect intensity of *p*-HA+VA+FA was 0.14, which was lower than that of *p*-HA, VA and FA. Therefore, the antagonistic effect between *p*-HA and VA was greater than the synergistic effect between VA and FA (Figure 1-a).

Phenolic compounds are secreted during the growth of cotton, and these phenolic compounds may be similar in chemical structure or may be different than test phenolic compounds concentration. When different phenolic compounds have the same action site on the cell membrane, there may be an antagonistic effect between the two. Antagonism between phenolic compounds is determined by the concentration level of each phenolic compounds (23). In this study, the interaction relations between phenolic compounds and their interaction intensity were investigated. In terms of the effect of phenolic compounds on the cotton seed germination, there was a significant antagonistic effect between *p*-hydroxybenzoic acid and vanillin, and a significant synergistic effect between vanillin and ferulic acid. The antagonistic effects between *p*-hydroxybenzoic acid and vanillin were stronger than the synergistic effects between vanillin and ferulic acid.

## **CONCLUSIONS**

The concentrations of *p*-hydroxybenzoic acid, ferulic acid and vanillin used in this study were kept similar to these phenolics concentration accumulated in 30-years continuous cotton growing fields. Some combinations of phenolic compounds stimulated the cotton seeds germination and the seedlings growth, reduced the stress on seedlings, and made cotton seedlings shorter and thicker; while other combinations, did not affect the cotton seed germination and seedling growth. In addition, we found that the antagonistic effects between the *p*-HA and VA were stronger than the synergistic effects between VA and FA.

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