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Hao, Z. P., Wang, Q., Christie, P., & Li, X. L. (2007). Allelopathic potential of watermelon tissues and root exudates. *Scientia Horticulturae*, 112(3), 315-320. <https://doi.org/10.1016/j.scienta.2006.12.030>

Published in:
Scientia Horticulturae

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Allelopathic potential of watermelon tissues and root exudates

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Received 4 September 2006; received in revised form 23 November 2006; accepted 7 December 2006

Abstract

Allelopathic potential of watermelon in hydroponic culture was investigated using the continuous root exudates trapping system (CRETS). Laboratory bioassays and high-performance liquid chromatography (HPLC) were used for quantification of phytotoxins in different plant tissues and root exudates collected using XAD-4 resin. Seedling growth of both watermelon and lettuce was significantly inhibited by watermelon root exudates and the degree of inhibition increased with increasing concentration of exudates. Root exudates had inhibitory effects throughout the experiment and these were most pronounced during the period 41–50 days after transplanting. Inhibitory effects of various plant tissues were demonstrated using the bioassay tests. Frulic acid was the dominant component found using HPLC and the amounts of allelochemicals found in the plant extracts were correlated with the bioassay results using the Petri dish test. The results indicate that the allelopathic potential of watermelon may play an important role in the phenomenon referred to as ‘soil sickness’.

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Keywords: Allelopathy; Watermelon; Autotoxicity; Soil sickness; Phenolic compounds

1. Introduction

Watermelon (*Citrullus lanatus* (Thunb.) Matsum and Nakai) is commonly cultivated in continuous cropping systems in monoculture and this can lead to serious problems including poor plant growth and declining production over time (Zhou and Everts, 2004). Some studies have shown that cucurbit crops are vulnerable to autotoxicity which leads to nutrient uptake disorders (Yu and Matsui, 1994) and promotion of soil-borne disease development (Ye et al., 2004). Watermelon is an important cucurbit crop species whose allelopathic potential has recently attracted widespread interest.

Allelochemicals are low molecular weight compounds excreted from plants during the processes of secondary metabolism (Rice, 1979) and they can accumulate in plants, soils and other organisms. These compounds vary in chemical composition, concentration and localization in plant tissues and

from plant to plant with changes in both biotic and abiotic conditions as discussed by Inderjit and Duke (2003).

Previous studies have shown that cucurbit crops can inhibit other plant species by production of allelopathic toxins and possess allelopathic potential in root extracts and exudates (Yu and Matsui, 1993; Yu et al., 2000). Allelopathic substances were isolated and identified from the exudates of germinating watermelon seeds by Kushima et al. (1998) and a number of phenolic acids have been isolated from their root tissues and root exudates as autotoxins (Yu, 2001). It is not clear, however, whether differences in the allelopathic potential of root exudates are influenced by plant maturity. Previous studies have focused on the allelochemicals from the underground parts of the plants (Hao et al., 2006) and the shoot tissues may also have potential allelopathic effects on the growth of bioassay test species. In the present study a re-circulating hydroponic culture system was used to evaluate changes in the allelopathic effects of exudates with plant age and to assess the allelopathic potential of the shoot tissues. High-performance liquid chromatography (HPLC) was used to identify and quantify the allelochemicals present.

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2. Materials and methods

2.1. Plant materials

Seeds of watermelon (*Citrullus lanatus* L. cv. JF) were obtained from the Vegetable Research Institute, Beijing, China and commercially available lettuce seeds (*Lactuca sativa* L., cv. Triton; Harris Moran Co., USA) were used.

2.2. Continuous root exudates cultivation and trapping system

The watermelon seeds were surface sterilized with NaOCl for 20 min, soaked at 80 °C for 10 min and 55 °C for 30 min, and then germinated for 2 days on moist filter paper at 28 °C in the dark (Demir and van de Venter, 1999).

Seedlings were grown for 10 days in Perlite in a growth chamber and then transplanted into the continuous root exudates trapping system (CRETS) (Fig. 1). The allelochemicals released from root exudates into the liquid culture system were trapped in a column (diameter 15 mm) containing 20 ml XAD-4 resin (Sigma, USA) (Tang and Young, 1982). Two seedlings were placed in a 12-L plastic vessel containing Enshi hydroponic culture solution (Yu and Matsui, 1994). The hydroponic nutrient solution was prepared using deionized water and was changed every 10 days. The pH of the nutrient solution was maintained at 6.0 and electrical conductivity at 2.2 mS cm⁻¹ by daily measurement to ensure that no substantial changes occurred in the nutrient solution throughout the experiment.

The trapping columns were replaced every 10 days by washing with 400 ml of distilled water followed by elution with 200 ml of spectral-grade methanol (Sigma, American) and evaporation on a rotary evaporator (Yarong Model RE-52A, Shanghai, China) at 40 °C to a volume of 3 ml as a stock for use in the bioassay and analysis by HPLC. The control was a recirculating loop with no plants.

Fig. 1. CRETS (continuous root exudates tapping system) culture setup used for watermelon cultivation. Twelve liters of Enshi nutrient solution were continuously circulated through the vessel containing the root systems and extracellular organics selectively adsorbed by the XAD-4 resin column were eluted.

Plants were grown for 100 days in a glasshouse at the Department of Plant Nutrition, China Agricultural University, Beijing. The plants were grown without supplementary illumination with a daytime temperature range of 25–30 °C and a night temperature range of 15–20 °C.

Plant sampling and preparation of extracts were performed following the methods of Chung et al. (2000). At harvest the fresh plants were separated into leaves, stems and roots. Fresh tissue (100 g) from each plant part was soaked in ethanol (400 ml) for 12 h at 25 °C in a lighted room to give a concentration of 250 g wet weight tissue l⁻¹ as a stock solution. The stock solutions were filtered through four layers of cheesecloth and filter paper (Whatman No. 1) to remove the fibrous debris and were evaporated off on a rotary evaporator to a volume of 10 ml for evaluation their allelopathic effects using seedling bioassay tests.

2.3. Bioassay

The activity of suspected biologically active materials was evaluated in germination tests using watermelon and lettuce seeds. The root exudates test solution (0.0, 0.1, 0.2, 0.4, or 0.8 ml plus distilled methanol to 1 ml) was added to filter paper (Whatman No. 1) in sterilized 9-cm glass Petri dishes, evaporated to dryness with ethanol for 24 h at 24 °C and moistened with 10 ml distilled water (Yu et al., 2000). Eight watermelon seeds were surface sterilized in a solution of 10% (v/v) H₂O₂ for 10 min, washed thoroughly in distilled water, and evenly placed on filter paper in a Petri dish and the dishes were placed in a dark chamber at 30 °C. Seedling growth was recorded 72 h later. A similar bioassay was performed using lettuce seeds (10 per dish) incubated at 24 °C and tested after 24 h.

Each tissue stock extract from the various plant parts was diluted appropriately with sterile methanol to give final concentrations of 2.5, 5, 10, 20 and 40 g l⁻¹ with 0, 10, 20, 40, 80 and 160 µl added to each Petri dish. The growth conditions of the lettuce seeds were the same as described above.

2.4. Identification and quantification of allelopathic compounds

The stock solutions were freeze-dried at 40 °C *in vacuo* and re-dissolved in 30 ml distilled water to produce aqueous extracts for further isolation and identification. The isolation methods were modified from Yu and Matsui (1993). The basic extracts were partitioned three times with an equal volume of ethyl acetate by first adjusting the aqueous fraction to pH 11 with 1 M NaOH and then acidifying the remaining residue to pH 7 and pH 2 with 1 mol l⁻¹ HCl to obtain the neutral and acidic fractions. The active residues were finally purified with XAD-4 resin exchange columns in order to obtain the water-soluble fraction. Both fractions were concentrated to a final volume of 10 ml.

The standard phenolic compounds used for HPLC analysis were *p*-hydrobenzoic, vanillic, syringic, *p*-coumaric and frulic

acids (Sigma Co., USA). All chemicals were purchased as high-purity standards and the solvents used were HPLC spectral grade. Allelopathic compounds were identified using an HPLC system (Shimadzu Model LC-10A, Tokyo, Japan). The column was ODS-C18 (6.0 × 150 mm) and the mobile phase consisted of water and methanol with a flow rate of 1 ml min⁻¹. The UV detector wavelength was set at 280 nm. Standard compounds were chromatographed alone and in mixtures. Retention times for the standard compounds and the major peaks in the extracts were recorded. Phenolic compounds from each fraction were identified by their retention times or addition of standards and they were quantified by comparing the areas of their peaks with those of the standards (Banwart et al., 1985).

2.5. Statistical analysis

Bioassays were conducted in triplicate. Analysis of variance was carried out using the SAS software package Version 6.12 (SAS Institute, Inc., Cary, NC). Means were separated by calculation of least significant difference (LSD) at the 5% level.

3. Results

Allelopathic potential was evaluated using watermelon and lettuce seeds as the test plants at four different concentrations of exudates collected during the period of seedling development of 11–20 days in the culture system (Fig. 2). Both plant species showed significant inhibition, which increased with increasing concentration of the root exudates. The radicle length of

Fig. 2. Effects of adding different amounts of watermelon exudates on radicle elongation of watermelon and lettuce. The exudates were collected during the 11–20 day period of watermelon growth. Watermelon seedling growth was determined after 72 h and lettuce 24 h after seedlings were treated on filter paper moistened with the extracts. Data are the means of three replicates with standards errors shown by vertical bars. The separate bar represents least significant difference (LSD) at the 5% level for comparison of pairs of mean values.

Fig. 3. Effects of watermelon root exudates at different growth stages on the elongation of lettuce radicles. Lettuce seedling growth was determined 24 h after seedlings were treated on filter paper moistened with 20 μl of extract. Data are the means of three replicates with standards errors shown by vertical bars. The separate bar represents least significant difference (LSD) at the 5% level for comparison between pairs of mean values.

watermelon decreased significantly to 65% of the control value with 100 μl concentrated solution, but there was no further significant decrease with increasing quantity of added solution until 800 μl were added. Compared with watermelon seeds, lettuce growth was more sensitive to the exudates with inhibition as little as 10 μl and increasing with increasing addition. Thus, although watermelon showed some sensitivity to the autotoxic chemicals, lettuce was a more suitable test plant in this study as the seedlings were easy to grow, were more sensitive in response to the allelopathic chemicals and gave more reliable data.

Allelopathic potential of root exudates collected every 10 days over the whole experimental growth period of watermelon (90 days) was tested by its effect on elongation of lettuce radicles compared with nutrient solution as control (Fig. 3). Significant inhibition was observed over almost the whole growth period with the most pronounced effects at the period of anthesis (41–50 days after transplanting). Before anthesis the degree of inhibition increased as plant growth proceeded but after anthesis the inhibitory effect gradually decreased.

Extracts from fresh watermelon plant roots, leaves and stems also showed inhibitory effects on seedling growth (Fig. 4). At the lowest addition (10 μl), the methanol extracts significantly reduced seedling growth compared with distilled water controls (Fig. 4). The degree of inhibition increased with increasing

Fig. 4. Effects of methanol extracts of plant parts from watermelon on the elongation of lettuce radicles. Lettuce seedling growth was determined 24 h after seedlings were treated on filter paper. Data are the means of three replicates with standards errors shown by vertical bars. The separate bar represents least significant difference (LSD) at the 5% level for comparison between pairs of mean values.

Fig. 5. Effects of different components separated from methanol extracts of plant parts and root exudates from watermelon on the elongation of lettuce radicles. Lettuce seedling growth was determined 24 h after seedlings were treated on filter paper. Data are the means of three replicates with standard errors shown by vertical bars. The separate bar represents least significant difference (LSD) at the 5% level for comparison between pairs of mean values.

concentration of extract. Similar trends were observed in the changes in concentration of allelopathic compounds. There were no significant differences among the different plant parts.

Results from the lettuce seed bioassays indicate that inhibitory substances were present in all the acidic, neutral, basic and water-soluble fractions (Fig. 5). Radicle growth decreased as the concentrations of extracts increased. Of the various test fractions investigated, the neutral fraction showed the least inhibition. The greatest inhibitory effects of extracts of all plant parts occurred at the highest concentrations.

The five major phenolic compounds analyzed in different plant parts and root exudates were all detected. Frulic acid was the dominant component followed by vanillic acid (Table 1).

Taken together, the allelopathic compounds in leaf and stem samples were present in higher quantities compared with roots and root exudates. The neutral fractions contained the lowest amounts of phenolic compounds, and this was in agreement with the trends found in the bioassays using the Petri dish test.

4. Discussion

Our results indicate that watermelon plants can contain and release growth inhibitors that are capable of reducing the growth of watermelon and lettuce. Allelopathic potential can vary with plant part, plant growth stage, amount of extract present and fractions extracted. The identification of the

Table 1
Quantitative determination by HPLC analysis of some phenolic compounds in plant parts and root exudates of watermelon ($\mu\text{g g}^{-1}$)

Plant part	Fraction	ρ -Hydrobenzoic acid	Vanillic acid	Syringic acid	ρ -Coumaric acid	Frulic acid	Total
Leaf	Basic	ND	2.43	0.27	5.72	16.75	25.17
	Neutral	1.80	ND	ND	ND	ND	1.80
	Acidic	ND	ND	ND	ND	ND	ND
	Water-soluble	ND	2.63	ND	0.79	ND	3.42
	Total	1.80	5.06	0.27	6.51	16.75	30.39
Stem	Basic	1.03	ND	0.02	0.46	1.07	2.58
	Neutral	ND	ND	ND	ND	0.10	0.10
	Acidic	0.14	3.85	1.67	2.44	3.23	11.33
	Water-soluble	2.27	1.84	ND	0.81	1.65	6.57
	Total	3.44	5.69	1.69	3.71	6.05	20.57
Root	Basic	ND	0.38	ND	ND	0.04	0.42
	Neutral	ND	0.34	ND	ND	ND	0.34
	Acidic	0.10	ND	0.90	0.66	2.45	4.11
	Water-soluble	0.02	0.23	0.20	0.70	0.13	1.28
	Total	0.12	0.95	1.10	1.36	2.62	6.15
Root exudate	Basic	1.49	1.10	0.32	ND	0.58	3.49
	Neutral	ND	0.04	0.07	ND	0.48	0.59
	Acidic	ND	ND	ND	0.28	0.71	0.99
	Water-soluble	ND	ND	ND	ND	0.20	0.20
	Total	1.49	1.14	0.39	0.28	1.97	5.27

ND: not detected, no phenolic compounds observed on chromatograms.

phytotoxins may contribute to an understanding of the mechanisms of allelopathy.

Combined with the use of XAD-4 resin for selective adsorption to hydrophobic or partially hydrophobic compounds, the CERTS method has been used previously in studies on *Tagetes patula* (Tang et al., 1987), *Rorippa indica* (Yamane et al., 1992), *Cucumis sativus* (Yu and Matsui, 1993), and *Oryza sativa* (Kong et al., 2004). In the present study, allelopathic substances from watermelon trapped by XAD-4 resin greatly inhibited radicle elongation in watermelon and lettuce, presumably due to the allelopathic effects of watermelon tissues or root exudates.

Lettuce radicle length was found to be more sensitive to the allelochemicals than watermelon root growth. The germination of watermelon seeds and seedling growth are influenced by many factors such as the nature of the seed coat and the degree of seed maturity, which lead to high variation in the results (Nerson, 2002). In the present study we therefore included lettuce as a test plant because it is recognized as a sensitive and reliable species that is commonly used for bioassays (Shafer and Garrison, 1986; Lin et al., 2004). However, the inclusion of watermelon as a test plant has also confirmed the existence of potential autotoxicity in this species (Fig. 2). The allelopathic intensity increased as the concentration of extracts or root exudates increased, and the inhibitory effect was observed at the lowest concentration tested (Figs. 2–4).

The concentrations and activities of allelochemicals may be influenced by plant maturity. Our results indicate that allelopathic effects of watermelon existed over the whole experimental period, increasing during vegetative growth up to a peak at anthesis and followed by a decline at the reproductive stage (Fig. 3). Similar trends have been observed in other cucurbit crops (Yu and Matsui, 1993) and are affected by the amount of root exudation, the rate of photosynthesis, and plant growth.

Different plant tissues such as leaves, stems, roots and seeds, and even flowers, can release certain amounts of allelochemicals into the surrounding environment (Miller, 1983; Chung et al., 2000). Ben-Hammouda et al. (2002) studied barley autotoxicity in extracts from the roots, stems and leaves of barley, and showed that the leaves were the most important source of allelopathic substances and the roots were the least important. Our results (Fig. 4) indicate that the various plant parts of watermelon, although having different physiological functions, showed no significant differences in the bioassay tests. Mixtures and fractions according to different pH can vary in their chemical and physical properties. Chaves et al. (2001) detected 11 allelochemicals in *Cistus ladanifer* exudates, tested them separately and in combination in bioassays, and discovered that the mixtures produced a greater negative effect than the individual chemicals acting alone. In the present study, the allelopathic potential of single fractions of the different tissues decreased in comparison with the mixture at the lower concentrations (Fig. 5). Tang and Young (1982) demonstrated that the basic fraction was the most inhibitory, while our results indicate that the most potentially inhibitory fraction was not the same

among all the parts but the neutral fraction was least inhibitory of all the tissues examined.

The phenolic compounds analyzed by HPLC present in methanol extracts have been confirmed to act as allelochemicals in numerous studies as reviewed by Blum (1996) and Inderjit (1996). Of these, frulic acid was detected as the largest component in all of our fractions (Table 1). As found by Chon and Boo (2005), the phenolic compounds were more abundant in stem samples, but our results for root exudates were different. Quantitative and qualitative differences in allelochemicals would be likely to lead to differential allelopathic effects. Our results show that the amounts of allelochemicals found in plant extracts were correlated with the bioassay results using the Petri dish test.

In conclusion, the use of the bioassay and the analysis of the phenolic compounds in different plant tissues and root exudates may contribute to our understanding of the allelopathic potential of watermelon and may provide more information about the allelopathic activities related to the phenomenon of 'soil sickness'. It is extremely important to minimize induced contamination from the culture system and the risk of possible effects of micro-organisms should be further minimized in future studies. Results obtained using hydroponic solution culture also need to be further evaluated using pot and field experiments to better understand the autotoxicity potential of watermelon under field conditions.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (Project 30230250) and the British Council with the UK Department for International Development through their Development Partnerships in Higher Education (DelPHE 64) program.

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