Salicylic acid has allelopathy, but wheat and faba bean intercropping can alleviate faba bean fusarium wilt under salicylic acid stress.

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Research Article

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Abstract

Background

The goals of this study were to evaluate the role of salicylic acid in the continuous cropping obstacle of faba bean (*Vicia faba* L.) and explore how intercropping with wheat alleviates these obstacles. We designed a hydroponic pot experiment to study the effects of exogenous salicylic acid on the occurrence of Fusarium wilt, seedling growth, physiological resistance of faba bean and pathogenicity of *Fusarium oxysporum* f. sp. *fabae* (FOF).

Results

The results showed that salicylic acid significantly increased the incidence and disease index of faba bean, inhibited the growth of seedlings and reduced the physiological resistance of faba bean. An *in vitro* study of FOF found that salicylic acid increased the ability of the organism to produce fusaric acid, cellulase and pectinase, which increased the susceptibility of faba bean to Fusarium wilt. Interestingly, intercropping with wheat significantly reduced the exudation of salicylic acid from the faba bean root system, which directly reduced the deleterious effects of salicylic acid. Alternatively, intercropping also increased the ability of faba bean to defend itself from the aspect of physiological resistance and indirectly reduced the autotoxicity of salicylic acid.

Conclusions

In conclusion, we found that salicylic acid, as an autotoxic substance, deleteriously affected the growth of faba bean, but intercropping with wheat could alleviate its autotoxicity. This finding suggests the existence of an important mechanism in which intercropping alleviates the obstacles in continuous cropping and controls Fusarium wilt.

Introduction

Continuously planting a crop, even under normal cultivation and management conditions, can result in obstacles, such as poor crop growth and development, severe disease and decreased yield and quality [1,2]. A large number of studies conducted over many years have shown that soil nutrient deficiencies or disorders, reduced soil enzyme activities, degraded rhizosphere microflora and a high incidence of soil-borne diseases can result in continuous cropping disorders [3,4]. Recent studies have shown that autotoxicity is an important factor in the obstacles to continuous cropping, and this state is a member of a special category of allelopathy [5]. Recent studies have shown that the accumulation of phenolic acids in continuous cropping soil is an important cause of continuous cropping obstacles in various crops, it has been proven that these phenolic acids are autotoxic substances that cause continuous cropping obstacles in crops, such as Chinese chives (*Cunninghamia lanceolata*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), and tomato (*Solanum lycopersicum*) [6,3].

Faba bean (*Vicia faba* L.) is a widely cultivated leguminous crop that is highly nutritious and adaptable and is most commonly grown in developing countries, primarily Asia and Africa [7]. China is the world's largest producer of faba beans, with an annual planting area of 2×10^6 hectares [8]. Faba bean would have serious continuous cropping obstacles when subjected to continuous cropping, which will cause soil-borne diseases to occur and restrict the production of faba beans. Fusarium wilt is one of the main diseases that occurs during continuous cropping. This disease is caused by *Fusarium oxysporum* f. sp. *fabae* (FOF), which results in severe economic losses worldwide and limits the sustainable development of faba bean [9]. There is evidence that the occurrence of Fusarium wilt is closely related to autotoxicity. This involves the release and accumulation of phenolic acids in root exudates, and the decomposed residues of faba bean is the direct cause of imbalance of soil microflora, stimulation of pathogen growth, the poor growth of faba bean and aggravation of Fusarium wilt [10]. Many methods are used to control faba bean wilt, such as using resistant varieties or chemicals, but these methods are not effective, economical or environmentally friendly.

Planting two or more plants at a close range is called intercropping, and it is the most popular method of planting to increase the diversity of species. Intercropping utilizes plant allelopathic effects and has long been used as a simple and inexpensive alternative to disease control and appears to be a more promising solution than the application of chemicals [11,12]. For example, intercropping between wheat and watermelon can significantly control watermelon wilt [13]; intercropping between soybean and maize can inhibit the occurrence of corn red crown rot [11], and intercropping between garlic and eggplant can protect crops from multiple types of pathogens [14]. However, there is limited data on how intercropping can help crops mitigate autotoxicity.

Root exudates of salicylic acid occur widely in the rhizosphere of various plants. It has been reported that salicylic acid, as a signal molecule, participates in plant disease resistance and defense responses and can directly inhibit pathogens [13,15]. In contrast, a study by Asaduzzaman et al. [16] found that salicylic acid, as an autotoxic substance exuded from faba bean roots, deleteriously affected the growth of faba bean plants. Salicylic acid was also found in our study, but information on how it affects the growth and resistance of broad bean seedlings in the rhizosphere is limited, and its effect on FOF, the pathogen of Fusarium wilt, is also unknown. Therefore, we hypothesized that salicylic acid may have a toxic effect on faba bean and play an important role in the interaction between FOF and faba bean, thus, promoting the occurrence of Fusarium wilt. However, intercropping with wheat can alleviate the autotoxic effects of salicylic acid, increase the resistance of faba bean in physiology and control the occurrence of Fusarium wilt. Therefore, the purposes of this study are: (1) to evaluate the effect of salicylic acid on growth and Fusarium wilt of faba bean, and (2) to explore the effect of intercropping on the alleviation of autotoxicity of salicylic acid from the aspect of physiological resistance.

Results

Analysis of components and contents of phenolic acids in root exudates of faba bean

We determined the components and contents of phenolic acids in the root exudates of faba bean. Seven phenolic acids were identified, including *p*-hydroxybenzoic acid, vanillic acid, syringic acid, ferulic acid, benzoic acid, salicylic acid and cinnamic acid (Fig. 2). Salicylic acid was the most prevalent phenolic acid, comprising 59% of the total. In addition, compared with monocropping, intercropping significantly reduced the exudation of salicylic acid from faba bean roots by 27.16%.

Effect of SA stress on the occurrence of Fusarium wilt of faba bean

When faba bean was subjected to monocropping, compared with the C0 treatment, the C1, C2 and C3 treatments significantly increased the incidence of Fusarium wilt, which infected 16.7%, 33.3% and 50.0% of the plants, respectively. The disease index also increased significantly by 62.5%, 111.9% and 288.6%, respectively (Table 1).

Compared with the monocropping of faba bean, intercropping significantly reduced the incidence of Fusarium wilt under the C1 and C2 conditions by 11.9% and 10.0%, respectively, but had no significant effect on C0 and C3. Compared with monocropping, intercropping significantly reduced the disease index under C0, C1, C2 and C3, which was 25.0%, 31.9%, 28.3% and 11.6%, respectively.

Effects of SA stress on the growth of faba bean seedlings

The growth parameters (leaf number per plant, maximum leaf length, maximum leaf width, height, main root length, shoot dry weight, root dry weight) of faba bean seedlings decreased as the concentration of salicylic acid increased in both monocropping and intercropping (Table 2). Compared with the C0 treatment, the C1, C2 and C3 treatments significantly reduced the dry weight of faba bean roots by 24%, 51.9% and 72.2%, respectively.

Intercropping with wheat could alleviate the inhibition of salicylic acid on growth of faba bean seedlings (Fig. 5). Intercropping significantly increased the max leaf length, shoot dry weight and root dry weight of faba bean seedlings by 13.3%, 31.3% and 41.5%, respectively, under C1 conditions compared with monocropping. Under C2 conditions, intercropping significantly increased the main root length, shoot dry weight and root dry weight by 13%, 32% and 42%, respectively, compared with monocropping. There was no detectable effect between monocropping and intercropping under C3 conditions.

Effects of SA stress on the physiological resistance of faba bean seedling roots

The activities of POD and CAT in faba bean roots gradually decreased as the concentration of SA increased, while the content of MDA gradually increased in both monocropping and intercropping. Compared with monocropping, intercropping significantly increased the activity of POD of faba bean root by 13.0%, 10.0% and 18.8% under C0, C1 and C2 conditions, respectively (Fig. 3A). Compared with monocropping, intercropping significantly increased the activity of CAT under C0 and C1 conditions by 9.8% and 18.4%, respectively, and decreased the content of MDA by 25.5% and 18.3%, respectively (Fig. 3B, C).

Effect of SA stress on disease-related proteins of faba bean seedlings roots

SA treatment inhibited the activities of chitinase and β-1, 3-glucanase in faba bean roots, and the inhibition increased in parallel with the concentration of SA during both monocropping and intercropping. Compared with monocropping, intercropping increased the activity of chitinase by 6.7%, 11.5%, 9.3% and 7.7% under C0, C1, C2 and C3 conditions, respectively (Fig. 4A), while intercropping also significantly increased the activity of β-1,3-glucanase by 11.4% and 8.9% under C1 and C2 stresses, respectively (Fig. 4B).

Effect of SA on pathogenicity of FOF in vitro

The C2 and C3 treatments significantly reduced the colony diameter by 11.0% and 26.2%, respectively, compared with the C0 treatment and significantly reduced the mycelial dry weight by 44.4% and 33.33%, respectively (Fig. 6A, B), figure 6C shows the concentration effect of salicylic acid on mycelial diameter more intuitively. Salicylic acid significantly promoted the production of fusaric acid (FA) in FOF, and the increase was enhanced as the concentration of salicylic acid increased. Compared with the C0 treatment, the content of FA in the C1, C2 and C3 treatments increased by 23.4%, 45.40% and 70.92%, respectively, (Fig. 6D) and the activities of pectinase and cellulase increased by 66.67%, 55.56% and 66.69% and 85.4%, 107% and 119%, respectively (Fig. 6E, F).

Discussion

Autotoxicity of salicylic acid

Autotoxicity is a type of internalization, which can inhibit growth by releasing toxic chemicals to the environment [3]. Allelopathy and autotoxicity are common in agricultural production and are closely related to obstacles in continuous cropping. Ye et al. [17] found that cinnamic acid secreted by cucumber can significantly inhibit the growth of cucumber and promote the occurrence of Fusarium wilt. *p*-Hydroxybenzoic acid secreted by the roots of *Pogostemon cablin* significantly reduced the plant height, root length and total fresh weight of this plant [18]. In our study, seven phenolic acids were detected from the root exudates of faba bean, and salicylic acid was the found in the highest concentration. We added exogenous varying concentrations of salicylic acid and found that the compound significantly increased the rate of incidence and disease index of faba bean wilt and inhibited the growth of faba bean seedlings. This effect increased in parallel with concentration of exogenous salicylic acid. This indicates that salicylic acid, as an autotoxic substance, contributes to the occurrence of Fusarium wilt. However, not all studies have considered salicylic acid to be a type of autotoxic substance. For example, salicylic acid is found in the watermelon/wheat system as a signal molecule and can activate plant defense responses under different biological and abiotic stress conditions and play an active role in controlling watermelon wilt [13]. Similar results were obtained in a watermelon/rice intercropping system [6]. This contradicts our results. We evaluated this phenomenon and found that it may be caused by two reasons: (1) the concentration of salicylic acid exuded from plant roots differs. In watermelon/wheat and watermelon/rice systems, the concentrations of salicylic acid exuded from watermelon were 9.8 and

0.148 μg·g^{-1}, respectively. In our study, the salicylic acid exuded from faba bean roots reached 38.29 μg·g^{-1}. Under such a high concentration of salicylic acid, faba bean roots are likely to suffer acute toxicity. (2) Plant species are different. Owing to this difference, their response to salicylic acid may vary, and the specific reasons merit further study.

Autotoxic substances can affect many physiological and biochemical reactions, such as cell division, interference with water relationship and ion absorption, inhibition of photosynthesis, redox balance and defense reactions [3]. For example, the lipophilicity of benzoic acid and cinnamic acid is closely related to the inhibition of ion absorption and root elongation of cucumber [19]. Cucumber can increase membrane peroxidation, decrease membrane ATPase and increase the incidence of Fusarium wilt when subjected to cinnamic acid stress [3]. In this study, exogenous salicylic acid significantly reduced the activities of POD and CAT in faba bean roots and increased the amount of MDA, and the effect became more pronounced as the concentration increased the effect. POD and CAT can remove H_2O_2 from plants, maintain the balance of plant oxidation and metabolism and eliminate the toxic effects of H_2O_2 to plants. These enzymes are involved in plant physiological processes, including cell wall construction and final lignification, resistance to insects and pathogens and wound healing [20,21,22]. MDA is one of the products of membrane lipid peroxidation, which is related to the degree of membrane lipid peroxidation [14]. This suggests that salicylic acid, as a self-toxic substance, seriously damages the root system of faba bean.

When plants are attacked by pathogens, they will activate their own defense systems and increase the expression of disease-related proteins, such as chitinase and *β*-1,3-glucanase. Chitinase and *β*-1,3-glucanase can hydrolyze the cell wall of pathogens, directly inhibit the growth of pathogens and protect plants from pathogens [15,23]. Exogenous salicylic acid can significantly reduce the activities of chitinase and *β*-1,3-glucanase and damage the defense system of faba bean. This increases the risk of infection and promotes the occurrence of Fusarium wilt.

Wheat intercropping alleviates the autotoxicity of salicylic acid

In this study, intercropping significantly alleviated the autotoxicity of salicylic acid, increased the activities of POD and CAT at the concentrations of C1 and C2, and increased the activities of chitinase and *β*-1,3-glucanase, which is an important reason for the intercropping control of Fusarium wilt. However, there was no significant difference in the activities of POD and CAT, chitinase and *β*-1,3-glucanase under the conditions of C3. This may be because under the double stress of a high concentration of salicylic acid and the presence of a pathogen, faba bean was seriously poisoned, and its root system was critically damaged, resulting in a loss of function. Thus, the intercropping could not manage such a high degree of plant stress. This suggests that the effect of interaction is limited and may not work in an extreme external environment.

We studied the pathogenicity of FOF in more detail and found that exogenous salicylic acid inhibited the colony diameter and mycelial dry weight of FOF but significantly increased the activities of cellulase and

pectinase and promoted the production of fusaric acid by FOF. It is well known that this is an important method for FOF to cause plant disease. Cellulase and pectinase hydrolyze and cleave the polymers, such as cellulose and pectin, that comprise the important components of plant cell wall. This renders the plant more vulnerable, and fungi are more likely to infect it [24, 25]. Fusaric acid is an important virulence factor that causes plants to wilt, which affects plant growth in many ways [26, 27]. Intercropping significantly reduced the amount of salicylic acid and the pathogenicity of FOF, which has strong ramifications for disease control.

Wheat and faba bean intercropping can control Fusarium wilt

Biodiversity is the natural barrier of disease prevalence. It has long been an effective means of disease control instead of chemicals and has become a research hotspot in China and elsewhere in recent years [28, 29, 30]. Intercropping is the most popular planting pattern for sustainability and an increase in species diversity, which has been proven to control disease and increase production in many systems [31, 32]. Our previous study found that under field conditions, wheat/faba bean intercropping significantly controlled the wilt of faba bean and increased the yield. This has strong practical applications. However, in actual production, disease and yield are often the result of multiple factors. For example, different soil types, fertility conditions and climate factors may affect disease control and increases in yield. Yang et al. (2014) [33] showed that the effect of intercropping maize and Capsicum on the control of Capsicum blight was affected by the distance between two crops. The closer the intercropping distance, the more effective was the control of capsicum blight. The effect of intercropping corn and soybean on the control of soybean red crown rot was affected by the levels of phosphorus applied. Increasing the supply of phosphorus could promote the control of soybean red crown rot, and this effect is related to the change in phenolic acid content and components in root exudates [11]. Therefore, additional verification and exploration of the effect of our research in different environments with varying soil types, fertility and climate is merited. Such research should be highly interesting and relevant to practical applications.

Conclusions

In conclusion, we found that salicylic acid, as an autotoxic substance, can significantly inhibit the growth of faba bean seedlings, improve the pathogenicity of FOF, and promote the occurrence of Fusarium wilt. Wheat/faba bean intercropping alleviated the inhibition of salicylic acid on faba bean seedlings, presumably from changes in physiology. Simultaneously, the exudation of salicylic acid from faba bean roots was significantly reduced in the intercropping system, which may be an important mechanism of alleviating the autotoxicity of faba bean by intercropping.

Methods

Materials

Seeds of wheat (*Triticum aestivum* L. var. *Yunmai 53*) and faba bean (*Vicia faba* L. var. *87-147*) were tested as plant materials and were obtained from the Yunnan Academy of Agricultural Sciences (Kunming, China). Analytical grade salicylic acid (SA) was purchased from the China Pharmaceutical Group Shanghai Medical Instrument Co., Ltd. (Shanghai, China), and the standard phenolic acids used for HPLC analysis were *p*-hydroxybenzoate, vanillic acid, syringic acid, ferulic acid, benzoic acid, salicylic acid and cinnamic acid, which were purchased from Sigma-Aldrich (St. Louis, MO, USA). The pectin and cellulose used in this study were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich, respectively. The pathogenic fungus was FOF that was isolated from infected faba bean soil and stored at 4ºC in the Plant-Microbe Laboratory at Yunnan Agricultural University, China. Spore suspensions of FOF were obtained from 14-day-old cultures on potato dextrose agar medium (PDA). The pathogenic mycelia were scraped into sterile water with a sterile L-shaped glass rod and incubated at 28 ℃ for 7 d. The spore suspension was filtered using two layers of gauze and served as the inoculum to infect faba bean.

Experimental design

Greenhouse Experiments

The hydroponic experiment was conducted in a glass greenhouse at Yunnan Agricultural University, China, from September to December 2017. The experiment was designed as a two-factor experiment, in which one factor was different concentrations of salicylic acid, including C0 (0 $mg \cdot L^{-1}$, CK), C1 (50 $mg \cdot L^{-1}$), C2 (100 $mg \cdot L^{-1}$) and C3 (200 $mg \cdot L^{-1}$). The other factor was two planting patterns (monocropping of faba bean [M]; intercropping of faba bean with wheat [I]). Each treatment was conducted four times, resulting in 32 pots (4 replicates × 2 planting patterns × 4 concentrations). The detailed planting pattern is shown in Fig. 1. The experiments were conducted under 24 h pump ventilation.

Faba bean and wheat seeds were soaked at room temperature for 24 h, germinated at 25 ℃ and then sown in sterile quartz sand that had been moistened with Hoagland nutrient solution. After the true leaves emerged, six faba bean plants were transplanted into 2 L containers that contained different concentrations of SA. The faba bean plants of the M and I system were inoculated with an FOF spore suspension of 1×10^6 $cfu \cdot mL^{-1}$ near the roots after two days of transplantation. The seedlings were maintained under natural light and temperature conditions (26/19 ℃ day/night) in a greenhouse, and the relative humidity ranged from 70% to 85%. The nutrient solution was replaced every 2 d.

Laboratory experimental design

A laboratory experiment was conducted to verify the effect of exogenous SA on the pathogenicity of FOF. The concentration of SA was consistent with the design of greenhouse experiment. Various concentrations of SA were treated by high temperature steam sterilization and added to steam-sterilized PDA media to determine their effects on FOF. Four replicates per treatment were conducted.

Analytical methods

Collection and analysis of root exudates

Cleaned roots were placed in containers that contained 300 mL of 0.005 mmol·L^{-1} $CaCl_2$, which was covered by a black membrane to avoid contamination and light. The root exudates were collected under light ventilation for 4 h (09:00 – 12:00) and then placed back in the container. A volume of 50 mL of the collected liquid was added to a centrifuge tube, microbial activity was inhibited by adding a drop of concentrated phosphoric acid. The root exudates were lyophilized, and storedat -20 ℃. Each treatment was conducted in quadruplicate.

The lyophilized powder of root exudates was dissolved in deionized water, diluted to 1 mL and filtered using a 0.45 μm Millipore membrane (Burlington, MA, USA). The contents of phenolic acid in root exudates were analyzed using High Performance Liquid Chromatography (Agilent 1260 Infinity, Agilent Technologies, Carpinteria, CA, USA). The analytical conditions were as follows: Kinetex column, 2.6 μm, 4.6×100 mm; temperature of column: 30 ℃; injection volume: 10 μL; DAD detector wavelength: 280nm; velocity of flow: 0.5 mL·min^{-1}; mobile phase: methanol (A) and 0.1% (v/v) phosphoric acid solution (B), which were used as mobile phases with a gradient elution (B: 80% (0min) →5% (15.0min) →5% (18.0 min) →80% (18.5 min) →0% (20.0 min) → stop (25.0 min)). All solvents were spectral grade HPLC. The types of phenolic acids were determined by the retention time, and the contents of phenolic acids were calculated using external standards.

Determination of faba bean growth indices

The samples were collected 45 days after transplantation. Three faba bean plants were randomly selected from each replicate for study. The leaf number per plant, maximum leaf length, maximum leaf width, height, main root length, shoot dry weight and root dry weight were measured.

Assessment of the incidence of Fusarium wilt

Three faba bean plants were randomly selected from each replicate. The severity of disease on individual plants was rated on a level from 0 to 5: 0 indicates no infection; 1 indicates initial symptoms of Fusarium wilt; 2 indicates that the base of the stem or the root had lesions, although they were not contiguous; 3 indicates that 1/3–1/2 of the stem base or root exhibited lesions, discoloration or wilt, and the lateral roots were significantly reduced; 4 indicates that the base of the stem was surrounded by lesions, or most of the roots were discolored and wilted, and 5 indicates that the plants had died or totally wilted. The incidence of Fusarium wilt on faba bean and the disease index were calculated using the following formulae:

Incidence (%) = (Number of diseased plants / total number of plants studied) ×100

$$\text{Disease index} = \frac{\Sigma(\text{Number of diseased plants at each level} \times \text{level})}{\text{The highest level} \times \text{total number of plants studied}} \times 100$$

Relative control efficacy (%) = [(AM - AI)/AM] × 100 (Where A represents the disease index; M represents the monocropping system, and I represents the intercropping system)

Determination of antioxidant enzymes and membrane lipid peroxidation

Fresh root samples from each treatment and replication were used to determine the activities of peroxidase (POD) and catalase (CAT) and content of malondialdehyde (MDA) as described by Li et al. (2000) [34].

A 1.0 g sample was ground with 2.9 mL of cold extraction buffer (0.05 mol·L^{-1} phosphate buffer, pH 7.8), and the crude extract was transferred to centrifuge tubes, which were centrifuged at 3000 rpm for 10 min. The supernatant was transferred to a 25 mL volumetric flask; the precipitate was extracted twice with 5 mL phosphate buffer, and the supernatant was transferred to the same volumetric flask. The POD activity was measured using guaiacol as a substrate. A reaction mixture was prepared by combining 2.9 ml of 0.05 mol· L^{-1} phosphate buffer, 1.0 ml of 2% H_2O_2, 1.0 ml of 0.05 mol·L^{-1} guaiacol and 0.1 mL of enzyme solution. The reaction mixture was immediately immersed in a 37 ℃ water bath for 15 min and was then rapidly transferred to cooling water. The absorbance at 470 nm was recorded at 1 min intervals for 5 min. The results are shown as A_{470} per minute per gram of fresh roots (U·g^{-1}·min^{-1}).

A total of 1.0 g of root material was ground to a homogenate with phosphate buffer (pH 7.8). The homogenate was centrifuged at 4000 rpm for 15 min, and the supernatant was transferred to a volumetric flask and assayed for enzyme activities. The activity of CAT was determined using a titration of potassium permanganate. The reaction mixture contained 2.5 mL phosphate buffer and 2.5 mL 0.1 mol·L^{-1} H_2O_2. The solution was incubated at 30 ℃ for 10 min, and 2.5 mL 10% H_2SO_4 was immediately added. The solution was titrated with a standard solution of 0.1 mol·L^{-1} $KMnO_4$ until the solution turned pink for at least 30 min. The CAT activity was expressed in milligrams of H_2O_2 degraded per gram of fresh roots in 1 min (mg·g^{-1}·min^{-1}).

A 1.0 g root sample was homogenized in 5 mL of 5% trichloroacetic acid (TCA), and the homogenate was centrifuged at 3000 rpm for 10 min after grinding. The supernatant was used to determine the content of MDA using thiobarbituric acid (TBA). A volume of 2 mL of the supernatant was added to 2 mL 0.6% TBA. The mixture was boiled for 15 min and then centrifuged after cooling. The absorbance values at 450, 532 and 600 nm were measured. The content of MDA is shown as the amount of substance per gram of fresh roots (μmol·g^{-1}).

Determination of the activities of pathogenesis-related proteins

The activities of chitinase and *β*-1,3-glucanase were measured using kits purchased from Sino Best Biological Technology Co, Ltd. (Shanghai, China). One unit of chitinase activity was defined as the amount of chitin that produced 1 mg of N-acetyl-D-(+)-glucosamine per gram of tissue per hour. One unit

of β-1,3-glucan enzyme activity was defined as the amount of enzyme required to produce 1 g of reducing sugar per gram of tissue per hour.

In vitro test of FOF

Determination of FOF growth

A 9-mm agar hole punch taken from a 7-d-old PDA culture was placed in the center of the plate and incubated at 28 ℃ for 7 d with 1 mL of SA at concentrations of C0, C1, C2 and C3. The colony diameter was measured in three different directions on each plate after incubation for 3 and 7 d.

FOF was grown in 30 mL conical flasks consisting of 30 ml potato dextrose broth and inoculated with a 9-mm agar plug from a 7-d-old PDA culture. The cultures were incubated at 28 ℃ in a rotatory shaker (170 rpm) for 7 d. The fungal biomass (dry weight) was determined after filtration and drying at 80 ℃ for 12 h when a constant weight was achieved.

Extraction and quantification of mycotoxin

FOF was inoculated in Richard's medium, which consists of 10 g of KNO_3, 0.02 g of $FeSO_4$, 5 g of KH_2PO_4, 2.5 g of $MgSO_4$, 34 g of glucose, brought to 1 L with distilled water amended with SA. One ml spore suspension of 1×10^6 cfu·mL^{-1} was added to Richard's medium and cultured at 28 ℃ for 7 days. Eight strains were removed with a 9-mm diameter punch, transferred to a 250 mL conical flask containing 125 mL of culture medium and incubated at 28 ℃ on a rotary shaker (180 rev/min) for approximately 15 days. The culture medium was centrifuged at 5000 rpm for 10 min and filtered with a 0.45 μm Millipore membrane (Burlington, MA, USA) to remove mycelia and spores. The supernatant was collected and autoclaved to obtain the crude FOF toxin solution.

The crude toxin solution was mixed with an equal volume of ethyl acetate, shaken for 2 min and incubated at room temperature for 30 min. The organic phase was collected and centrifuged at 4000 rpm for 10 min. The supernatant was dried and condensed at < 40 ℃. The entire dried residue was re-dissolved in 5 mL of ethyl acetate, and the absorbance was measured at 269 nm. The content of FA was expressed as mg·L^{-1}.

Measurement of pathogenesis-related hydrolytic enzyme activities

The enzyme-producing culture medium utilized a synthetic medium formula that contained 1% of pectin and cellulose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.4 g of KH_2PO_4, 0.2 g of KCl, 1 g of NH_4NO_3, 0.01 g of $FeSO_4$, 0.01 g of $ZnSO_4$, and 0.01 g of $MnSO_4$ in a total volume of 1 L of distilled water. A volume of 25 mL of this medium was transferred to 100 mL conical flasks and inoculated with FOF fragments that were 9 mm in diameter. The culture medium was incubated at 28 ℃ and 200 rpm for 7 days. It was then collected and centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a 0.45 μm filter. The filtrate served as the crude enzyme solution, which was stored at 4 ℃ until use.

The activities of pectinase and cellulase were determined using 3,5-dinitrosalicylic acid (DNS). A total of 3.15 g of DNS was added to 500 mL of water while stirring for 5 s. The solution was heated to 45 ℃. A volume of 100 mL of 0.2 g·mL^{-1} sodium hydroxide solution was then gradually added while stirring until the solution became transparent. It is imperative that the solution not exceed 48 ℃ during the addition of the sodium hydroxide. A total of 91.0 g of sodium nitrate potassium tartrate, 2.5 g of phenol and 2.5 g of anhydrous sodium sulfite were added and heated to 45 ℃ in a water bath, while 300 mL of water was added with constant stirring until the material had completely dissolved. Finally, the solution was cooled to room temperature, and distilled water was added to a final volume of 1 L. The solution was stored in the dark at room temperature for 7 days before use. A unit of pectinase was defined as the amount of enzyme required to produce 1 μmol of galacturonic acid, and a unit of cellulase was the amount of enzyme required to produce 1 μmol of glucose per min.

Statistical analysis

All the data were analyzed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA) and SPSS ver. 20.0 (SPSS Inc., Chicago, IL, USA). The least significant difference test was used to determine differences between the treatments at a significance level of $P \leq 0.05$.

Abbreviations

FOF: *Fusarium oxysporum* f. sp. *fabae*

FA: Fusaric acid

POD: peroxidase

CAT: catalase

MDA: Malondialdehyde

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for Publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YL, JL, YG, YD and KD contributed to the design of the study. JL and LC performed the experiments which contributed in the collection of plant samples and all laboratory work, data analysis and elaboration of the work. YD and KD contributed to the supervision of laboratory work and data audit. YL and JL wrote the manuscript and translated the article into the English language and YD contributed to the critical reading of the manuscript. All authors read and approved the final manuscript and approved the submission.

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Statement

This study is not a study that directly or indirectly damages the survival of plants. The plant research methods are in compliance with the relevant provisions of the IUCN policy statement.

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Tables

Table.1 Effects of intercropping with wheat on the incidence and disease index of Fusarium wilt of faba bean under SA stress.

Treatment	Incidence (%)		Disease index		RPCE (%)
	M	I	M	I	
C0	66.67±3.60d	66.67±3.33d	17.78±2.04ef	13.33±1.67f	25.05±0.02
C1	77.78±2.67c	68.56±1.84d	28.89±1.92d	19.56±1.84e	31.85±0.11
C2	88.89±3.47b	79.96±3.44c	37.67±5.13c	26.89±2.27d	28.25±0.05
C3	100.00±0.00a	94.44±6.94ab	69.09±7.67a	61.11±6.31b	11.63±0.07

Note: Incidence and disease index were measured as described in the Materials and Methods. M: monocropped faba bean; I: intercropped faba bean. RPCE: relative control efficacy (%) to Fusarium wilt of faba bean. All the data are the average of four replicates ± SE (standard error). The different lower-case letters after numbers indicate significant differences at $P \leq 0.05$.

Table.2 Effects of intercropping with wheat on faba bean growth parameters under salicylic acid stress.

Growth parameters	C0		C1		C2		C3	
	M	I	M	I	M	I	M	I
Leaf number per plant	21.67±0.58 b	24.00±1.00 a	16.17±0.76 c	14.00±1.00 d	14.77±0.68 cd	15.83±1.04 c	10.67±0.76 e	11.83±0.29 e
Maximum leaf length/(cm)	8.73±0.38 b	9.37±0.12 a	6.33±0.06 d	7.17±0.49 c	5.70±0.26 e	6.10±0.10 de	5.53±0.55 e	6.60±0.53 cd
Maximum leaf width/(cm)	5.43±0.51 a	5.53±0.12 a	3.77±0.12 bc	3.89±0.13 b	3.27±0.17 cd	3.33±0.35 c	2.66±0.20 e	2.79±0.18 de
Height/(cm)	39.23±0.49 a	37.87±1.44 a	32.19±1.16 b	29.83±0.71 c	25.33±1.07 d	22.30±0.61 e	20.98±1.09 e	17.67±1.56 f
Main root length/(cm)	18.33±1.46 b	19.83±0.97 a	17.30±0.56 b	18.28±0.39 b	13.47±0.76 d	15.23±0.38 c	10.60±0.35 e	11.53±0.49 e
Shoot dry weight/(g)	2.23±0.05 b	3.28±0.21 a	1.76±0.04 c	2.31±0.02 b	1.18±0.08 d	1.56±0.1 c	0.76±0.07 e	0.96±0.06 e
Root dry weight/(g)	0.54±0.03 b	0.86±0.06 a	0.41±0.10 c	0.58±0.02 b	0.26±0.0.2 d	0.37±0.02 c	0.15±0.03 e	0.21±0.04 de
Root-shoot ratio/(%)	0.24±0.02 ab	0.26±0.01 a	0.23±0.03 abc	0.25±0.01 ab	0.22±0.02 bc	0.24±0.04 ab	0.20±0.01 c	0.22±0.01 bc

M: monocropped faba bean; I: intercropped faba bean. All the data are the average of four replicates ± SE (standard error). The different lowercase letters after numbers indicate significant differences at $P \leq 0.05$. SA, salicylic acid.

Figures

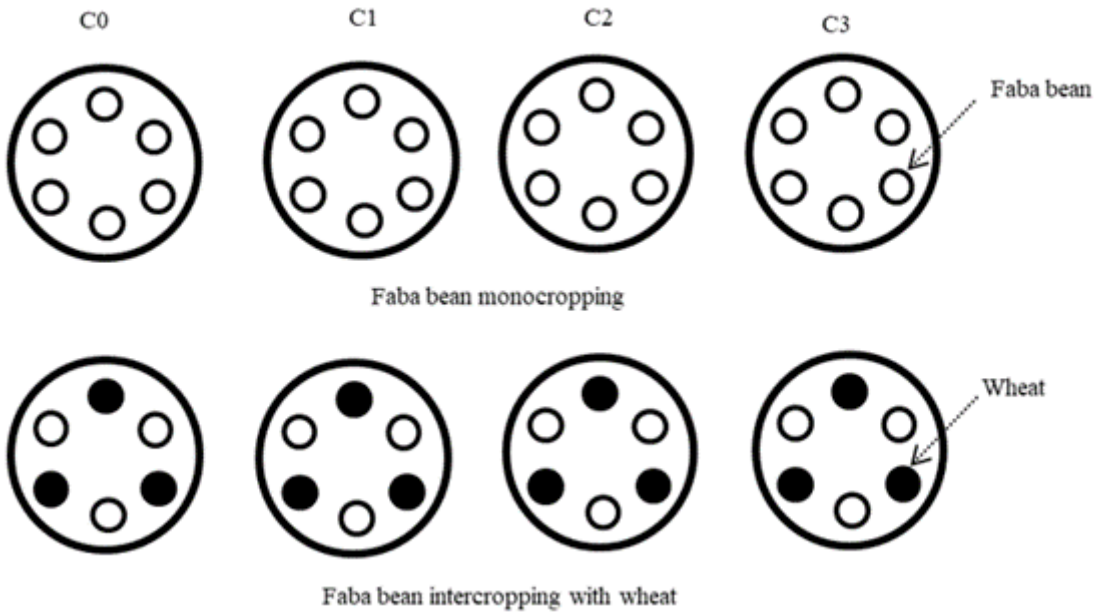

Figure 1

Diagram of the planting patterns.

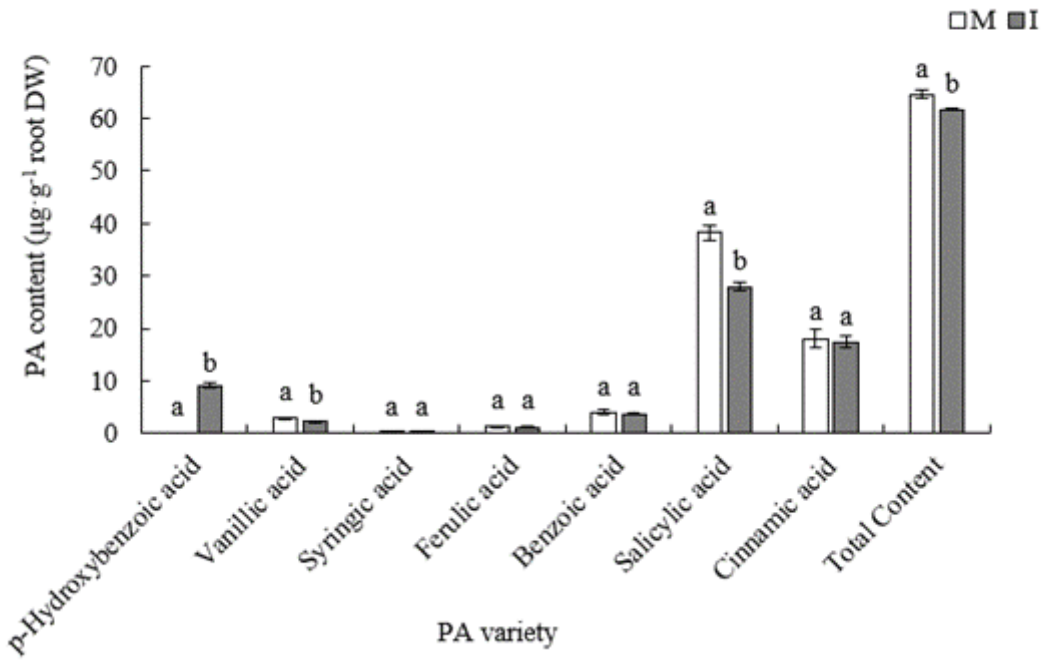

Figure 2

Quantity of phenolic acids in the root exudates of faba bean and total phenolic acids. PA: phenolic acid; M: monocropped faba; I: intercropped faba bean. All the values are presented as the mean ± SE (standard error). The different letters on the mean values of the same phenolic acid indicate significant differences among the treatments ($P \leq 0.05$).

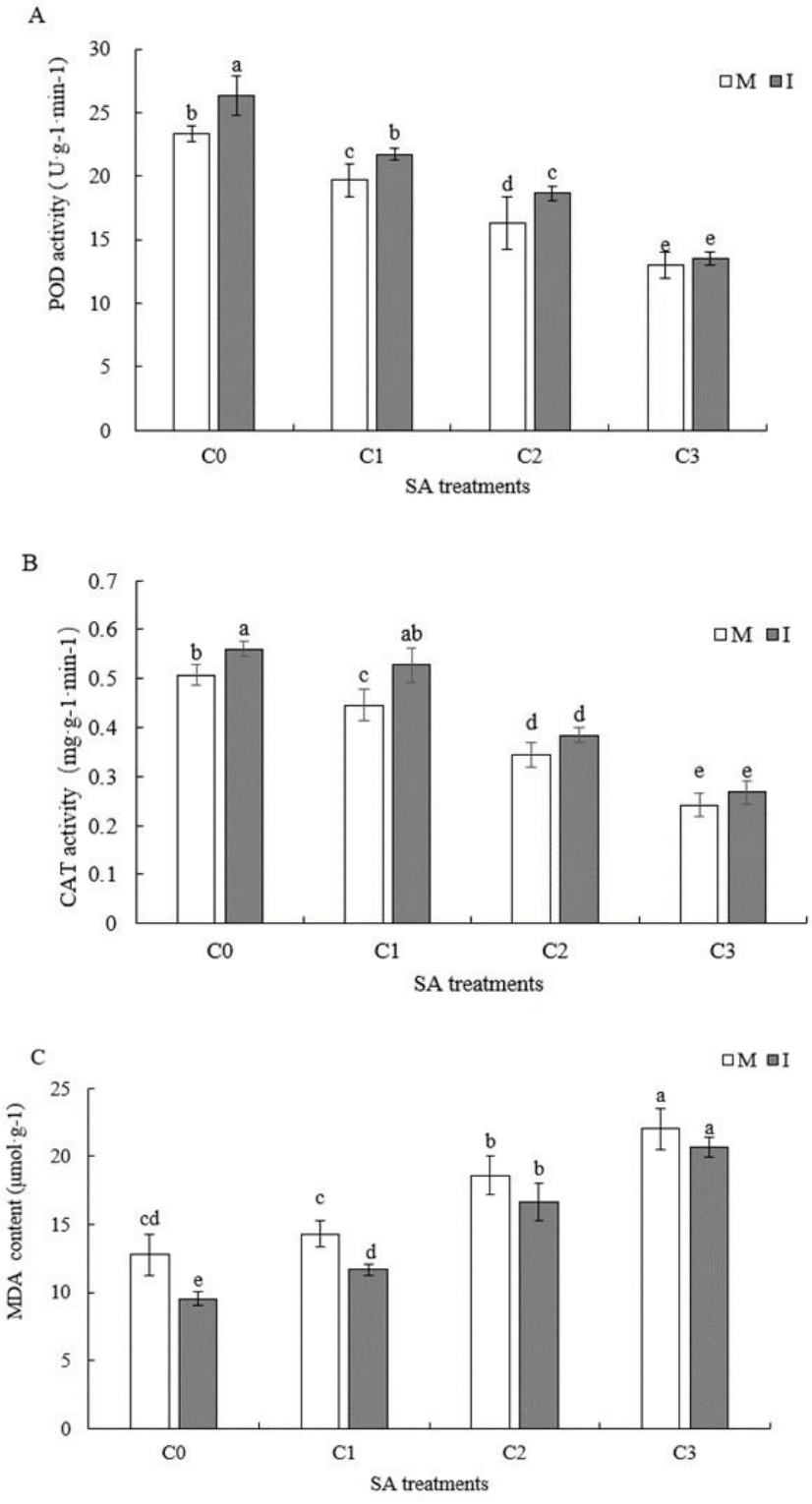

Figure 3

Effects of intercropping with wheat on the activities of POD (A) and CAT (B) and content of MDA (C) in faba bean roots under SA stress. M: monocropped faba bean; I: intercropped faba bean. All the data are the average of four replicates ± SE (standard error). Different letters for each index indicate significant differences at P≤0.05.

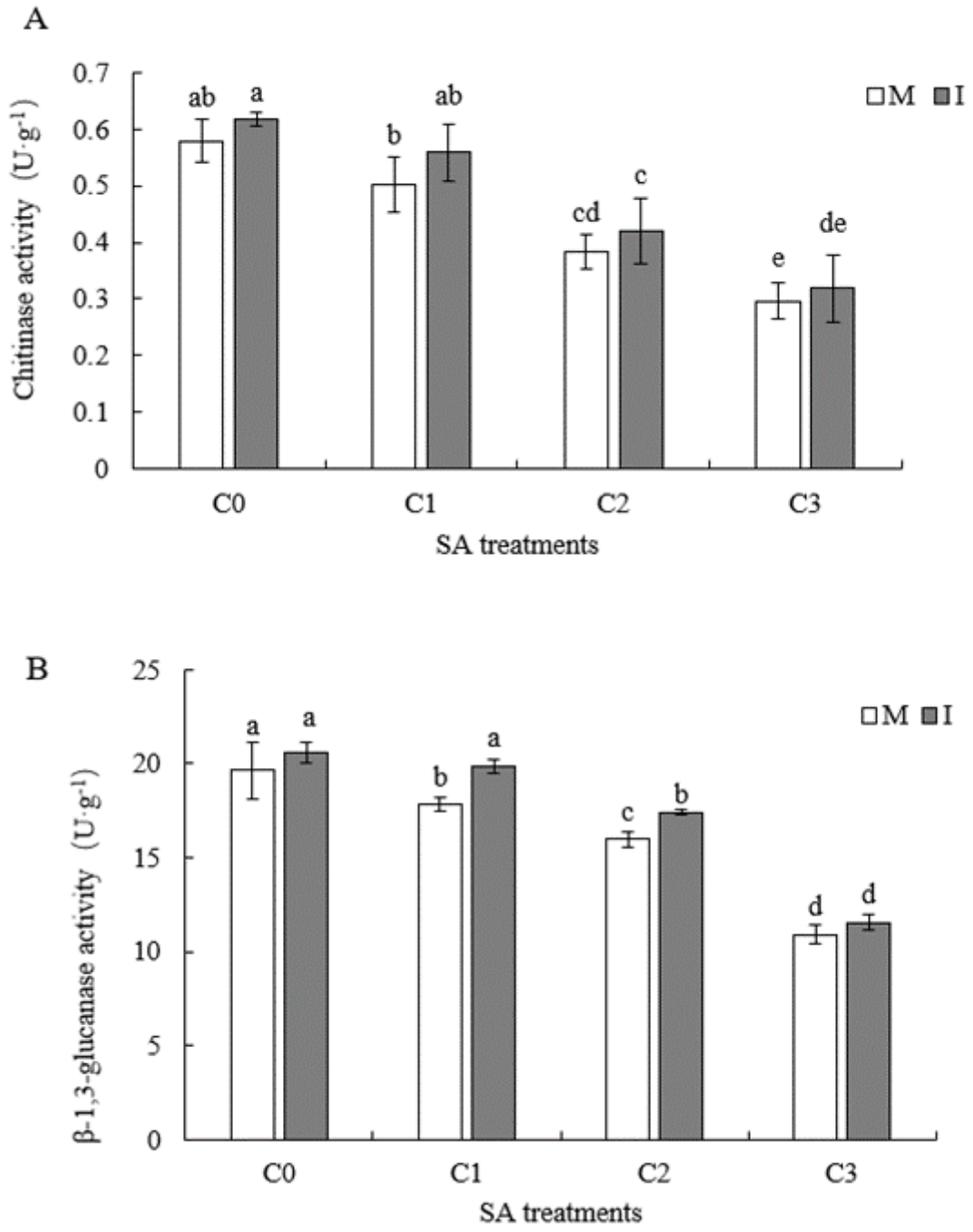

Figure 4

Effects of intercropping with wheat on the activities of chitinase (A) and β-1, 3-glacanase (B) in faba bean roots under SA stress. M: monocropped faba bean; I: intercropped faba bean. All the data are the average of four replicates ± SE (standard error). Different letters for each index indicate significant differences at $P \leq 0.05$.

Figure 5

Effects of intercropping with wheat on the growth of faba bean under SA stress.

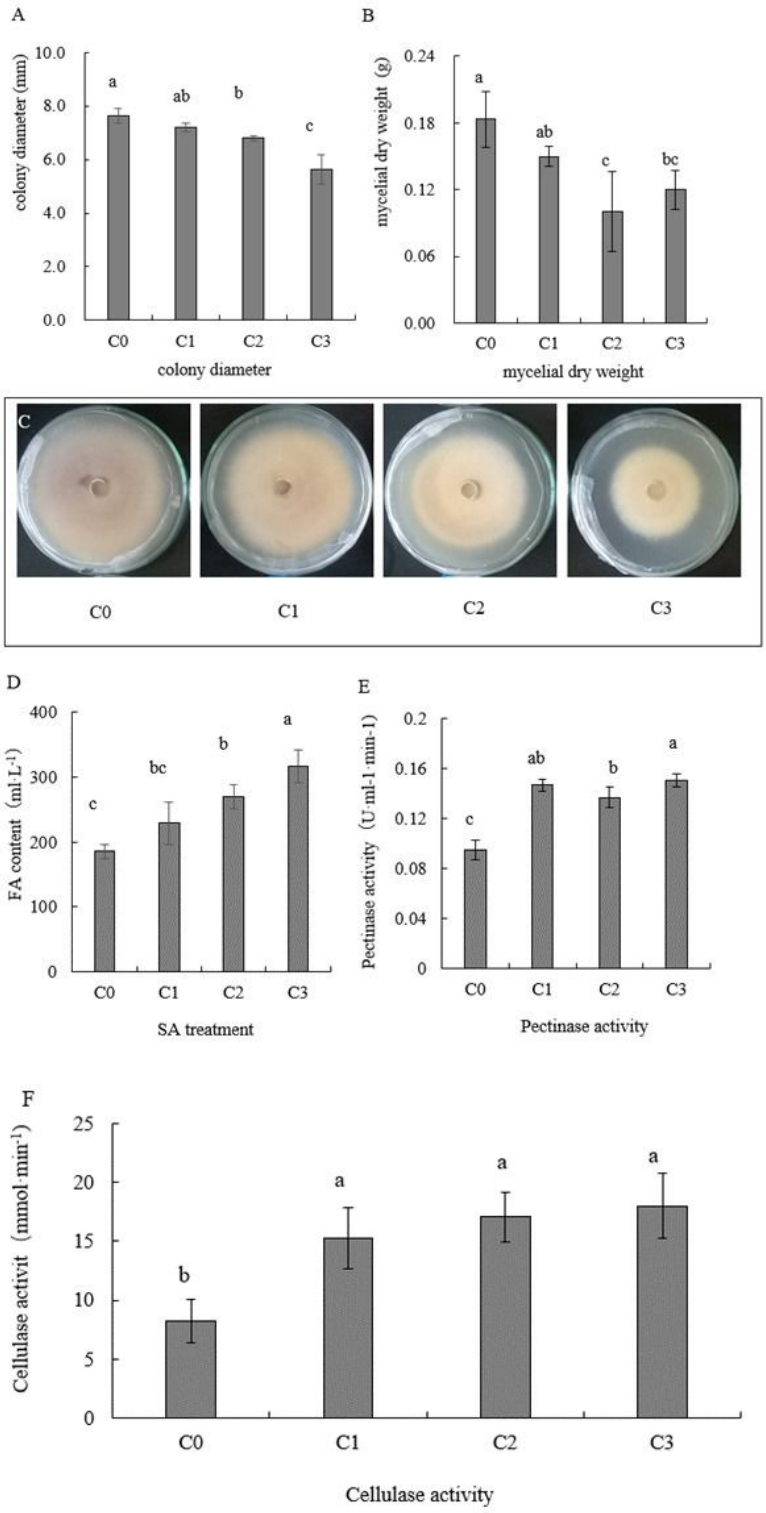

Figure 6

Effects on intercropping with wheat on the colony diameter (A), mycelial dry weight (B), colony growth (C), fusaric acid production of FOF (D) and activities of pectinase (E) and cellulase of FOF (F) under SA stress. All the data are the average of four replicates ± SE (standard error). Different letters for each index indicate significant differences at $P \leq 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx