Wheat / Faba Bean Intercropping Improves Physiological and Structural Resistance of Faba Bean to Fusaric Acid Stress.

Yu Li

Yunnan Agricultural University

Jiaying Lv

Yunnan Agricultural University

Qian Zhao

Yunnan Agricultural University

Ling Chen

Chongqing

Yan Dong (✉ dongyanyx@163.com)

Yunnan Agricultural University <https://orcid.org/0000-0002-5523-4977>

Kun Dong

Yunnan Agricultural University

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Abstract

Background

The succession cropping obstacle of faba bean are main common and increasingly serious. Soil-borne fusarium wilt is one of the most serious diseases threatening faba bean production. *Fusarium oxysporum* f. sp. *fabae* (FOF) is the causal agent of fusarium wilt. Fusaric acid (FA), produced by FOF, plays an important role in the occurrence of disease, and intercropping is an effective measure for control of disease and for improving host resistance in plants. Intercropping is a traditional farming measure that uses inter-species interaction to control pests and diseases and increase food production. The objective of the current study was to investigate the physiological and biochemical responses, and mechanisms of tissue structure resistance of intercropped faba beans following exposure to FA.

Results

Results demonstrated that increasing concentrations of FA had greater effects on faba beans and intercropping significantly reduced red ink absorption of faba beans (33.2%), increased water content (3.1%) and increased activity of the root antioxidant enzymes peroxidase (POD) and catalase (CAT) (26.3% and 2.2.%, respectively). Furthermore, increased lignin content and callose deposition in plant vessels were observed (12.5% and 42.7%) when subject to the high concentration of FA (200mg.L⁻¹) stress, respectively. Intercropping resulted in more intact root cell morphology, increased occurrence of intracellular vacuoles, cell wall thickness and the number of mitochondria and rough endoplasmic reticulum.

Conclusions

This study contributes to us to understand the wheat / faba bean intercropping system at the physiological, tissue and subcellular levels to effectively alleviate the wilting effect of *Fusarium* toxin on faba bean.

Background

Faba bean fusarium wilt is a soil-borne disease which restricts production of faba bean [1, 2]. *Fusarium* wilt is a common vascular disease caused by *Fusarium oxysporum* which is difficult to control due to its host-specific characteristics, long time of survival and rapid spread in soils [1]. *Fusarium* wilt directly infects the plant or damages the host root, interferes with host defense systems and impacts the vascular bundle of the host [3]. Frequent occurrence of fusarium wilt over recent years has resulted in reduced crop resistance and reduced yields [4].

In recent years, crop soil-borne diseases caused by species of *Fusarium* have received extensive research attention due to the severity of hazards and difficulty in their management. During infection of plants by species of *Fusarium*, many toxins are secreted and contribute to infection and colonization of the host by

pathogenic fungi, ultimately resulting in plant wilt and death [3]. Fusaric acid (FA, 5-n-butyl-2-pyridine carboxylic acid), a well-known host non-specific toxin, is known for its high phytotoxicity [3, 5, 6]. For example, there is a positive correlation between FA content and the pathogenicity of *F. oxysporum* [3, 6, 7]. A role for FA in the development of disease symptoms has been confirmed in a variety of crops, including beans [8], watermelons [9], bananas [10], tomatoes [11], and cucumbers [7]. Invasion of the pathogen triggers a series of changes in the host's resistance response. Evidence suggests that FA reduces production of defense enzymes and respiratory activities of infected plants and induces programmed cell death [12], interferes with host metabolism, causes pathological changes in plant cell tissue structure [9], changes activities of leaf-related enzymes and root cell membrane potential of infected plants [9], increases intracellular electrolyte leakage and reduces intracellular ATP [12]. Overall, FA significantly impacts host resistance response.

Intercropping is a common cultivation practice in agriculture. Rational intercropping can effectively prevent disease, reduce insect and weed pressure while improving utilization of land resources. In addition, intercropping is an effective, economic and environmentally-friendly prevention and treatment method for overcoming soil-borne diseases caused by continuous cropping practices [13]. Intercropping, as a mitigation strategy for soil-borne diseases, has been used in multiple systems such as intercropping soybean with maize to reduce severity of soybean red crown rot [14]; watermelon/wheat companion system to reduce watermelon fusarium wilt [15]; and watermelon intercropping with aerobic rice to suppress fusarium wilt [16]. Characterization of the mechanisms of disease control in intercropping models has focused on interactions between root exudates and pathogens, and soil microorganisms [17, 18], however, limited information is available related to the resistance of intercropping to host. Recent work has demonstrated that intercropping and other diversified cultivation methods can enhance host plant resistance to pathogens, and control occurrence of soil-borne diseases. Mechanisms of host defense responses included; increased expression of defense-related genes in watermelon roots of a wheat/watermelon companion cropping system [19]; increased activity of defense enzymes in roots of tomato in a potato/tomato intercropping system [20]; improved defense enzyme activity in garlic leaves and increased garlic physiological resistance in an eggplant/garlic intercrop system [21]. To our knowledge, it is not known whether intercropping affects host resistance to FA stress, especially in wheat / faba bean intercrop systems, or how the defense response of faba bean changes. The present study aimed to investigate mechanisms of damage of faba beans following exposure to FA in leaves and roots, and to compare resistance to stress of monocropped and intercropped faba bean via physiological and tissue structure endpoints.

Results

Effect of intercropping on faba bean seedlings under FA stress

As presented in Fig. 1A, water content of faba bean seedlings decreased with increasing concentrations of FA regardless of being monocropped or intercropped. When compared with the C0 treatment, C1, C2 and C3, FA significantly reduced water content of monocropped faba bean seedlings by 3.3%, 4.9% and 8.9% respectively, with an average decrease of 5.7%; the water content of intercropped faba bean seedlings were significantly reduced by 3.4% and 6.4% by C2 and C3 concentrations treatments, respectively when compared to control. When compared to water content of monocropped faba bean, the water content of intercropped faba bean were significantly greater by approximately 2.5% and 3.1% when treated with FA treatments C1 and C3, respectively.

As the concentration of FA increased, red ink absorption of faba bean leaves increased significantly, where average absorption increased by 183.5% in monocropped faba bean and 166.37% in intercropped faba bean (Fig. 1B). When compared with monocultured beans, absorption of red ink by intercropped faba bean leaves decreased significantly by 14.3%, 19.1%, 33.2% when exposed to the C1, C2 and C3 treatments respectively, with an average decrease of 22.2%.

Physiological and biochemical response of faba bean seedlings to FA stress

Effect of intercropping on membrane lipid peroxidation

As concentrations of FA increased, MDA content of faba bean roots increased significantly when monocropped or intercropped. Average increase in MDA content was 79.9% in roots of monocropped faba bean and 45.3% in intercropped faba bean (Fig. 2A). When compared with monocropped faba bean, MDA content in roots of intercropped faba bean decreased by 22.3%, 38.3% and 39.6% when treated with FA C1, C2 and C3, respectively.

Effect of intercropping on defense enzyme activity

Catalase activity of monocropped and intercropped faba bean significantly decreased as FA concentration increased (Fig. 2B). When compared with C0, CAT activity of monocropped and intercropped faba bean roots were significantly reduced by 25.3%, 33.3%, or 50.0%, and 18.1%, 34.9%, or 43.9%, with an average reduction of 36.2% and 32.3%, respectively. When compared with monocropped faba bean, CAT activity of intercropped faba bean was significantly greater by 14.1%, 20.0%, and 26.3%, with an average increase of 20.1% for the C0, C1, and C3 FA treatments.

When compared with C0, POD activity of monocropped faba bean root was significantly less by 44.0% under the high FA treatment (C3). When treated with C1, C2 and C3, intercropped faba bean POD activity was significantly reduced by 12.1%, 23.7%, and 49.3%, respectively, with an average reduction of 28.4%. Activity of POD in intercropped faba bean roots was significantly higher when compared to monocropped beans by 57.1%, 30.2%, 33.9% or 2.2% for the C0, C1, C2 or C3 treatments (Fig. 2C).

Tissue structure of faba bean seedlings

Effect of intercropping on lignin in faba bean seedling roots

When compared with C0, treatment with FA resulted in increased accumulation of lignin in monocropped and intercropped faba bean by 136.5% and 122.8% on average, respectively. As concentration of FA increased (C0, C2, or C3) an increasing trend in the lignin content was observed in both culturing systems (10.0% and 12.5%, for intercropped and monocultured). However, when treated with C1, lignin content of intercropped faba bean was lower than that of monocropped beans, but differences were not significant (Fig. 3).

Effect of intercropping on callose deposition of faba bean roots treated with FA

As the concentration of FA increased, monocultured and intercropped faba bean roots had higher callose accumulation and stronger fluorescence intensity when compared to C0 (Fig. 4, 5). Differences were most obvious following treatment with C2 and C3, where callose accumulation in monocultured beans increased 118.7% and 181.7%, and intercropped beans increased 76.0% and 122.2%, respectively (Fig. 5). However, stronger fluorescence intensity was observed in intercropped faba bean root when compared to monocropped faba beans across treatments (Fig. 4). When compared with monocropped faba beans, callose accumulation in intercropped faba beans decreased by 42.5% and 41.7%, respectively, when treated with C2 and C3 (Fig. 5).

Effect of intercropping on ultrastructure of faba bean roots

Effect of treatment with FA on the ultrastructure of intercropped faba bean roots as determined by TEM are presented in Fig. 6. In general, cell walls of monocropped faba bean roots were thinner, cellular morphology was irregular and deformed, nuclei had leaked and looked granulated, the number of rough-surfaced endoplasmic reticulum and mitochondria were lesser, and increasing amounts of vacuolization and plasmolysis were observed with increasing concentration of FA (Fig. 6A, B, C, D). Analysis of the ultrastructure of intercropped faba beans demonstrated lesser cell deformation, normal cell wall thickness and more defined vacuoles in the cytoplasm, however nuclear proliferation was greater with greater FA treatment when compared to monocultured beans (Fig. 6E, F, G, H).

When compared to monocropped faba beans, the organelles mitochondria, rough-surfaced endoplasmic reticulum and vacuole were more easily observed in intercropped faba bean root cells, and were more healthy in the C0 treatment. Defined vacuoles were apparent in intercropped faba bean cells, lesser prevalence of mitochondria and rough-surfaced endoplasmic reticulum were observed when compared with monocropped faba beans while cell walls were thicker and had apparent papillae. Emulsion bumps likely helped to alleviate occurrence of plasmolysis in the C1 treatment. In the C2 treatment, the cell wall of some cells of intercropped faba beans were ruptured, had increased vacuolization and fewer organelles. In addition, nuclei were seriously deformed and swollen, had released their organelles, severe plasmolysis was observed. Intercropped faba beans in the C3 treatment had similar characteristics as the C2 treatment however, intercropped beans had a small number of rough-surfaced endoplasmic reticulum and mitochondria remaining when compared to monocropped.

Discussion

Effect of intercropping on water content and red ink absorption in faba bean leaves

Fusaric acid, the main pathogenic factor of *F. oxysporum*, plays a vital role in infection of plants by pathogens [22]. Dong Xian et al. (2012)'s [23] demonstrated that water loss of *Fusarium*-infected banana plants is caused by interference of FA rather than by the pathogen itself. In addition, earlier evidence suggests that FA is the direct cause of non-stomatal water loss in *Cucumis sativus* L., ultimately resulting in cucumber wilting [24]. As previously reported, and confirmed in this study, treatment of faba bean with FA resulted in decreased water content however, water content was of intercropped beans was greater than monocropped beans (approximately 2%; Fig. 1A), suggesting that wheat / faba bean intercropping alleviated adverse effects of FA. Although FA treatment reduced absorption of water by faba bean, it significantly increased absorption of large particle red ink (Fig. 1B). Similar results were observed in a study of water imbalance after cucumber plants were infected with *Fusarium oxysporum* f. sp., where *Cucumerinum* had reduced water absorption, stem hydraulic conductance and accumulated significant amounts of red ink in leaves [25]. The observed effects might be due to FA damaging the cell membrane of plant leaves, resulting in accumulation of red ink. More importantly, faba bean roots were extensively damaged following addition of FA, resulting in reduced ability for selective absorption of nutrients and greater absorption of red ink by faba bean roots. Interestingly we observed that red ink absorption in intercropped faba bean leaves was 22.2% lower than that in monocultured beans, and might be related to differences in defense responses of faba bean roots.

Effect of intercropping on physiological and biochemical resistance of faba bean roots to FA stress

Intercropping is at the heart of traditional Chinese agriculture [26], can improve ecological stability of farmland by improving nutrient absorption [27], reduce toxicity of allelopathic substances [14], improves microecological environment and control of soil-borne diseases [28]. Intercropping is considered an important model of sustainable agricultural development due its increased production, efficient use of resources and better control of disease [29]. Most studies have demonstrated that treatment with FA inhibits physiological and biochemical responses of plants to disease [30, 22, 31]. In our study, we found that treatment with FA increased MDA content and decreased CAT and POD activities in faba bean roots, which increased with FA concentration (Fig. 2) however limited information is available in regards to host resistance of intercropped systems to FA induced wilting of plants. To gain a greater understanding of host resistance in intercrop systems to FA induced wilting of plants, we determined membrane peroxidation of faba bean after intercropped with wheat. Overall, we observed that MDA content of faba bean roots in the intercropped systems was lower than that in the monoculture system at all tested concentrations, especially at low concentrations (C1 and C2), indicating that intercropping systems reduced FA related damage (Fig. 2A). However, no significant change in MDA content at high concentrations of FA, suggesting that the high concentration of FA produced a strong enough oxidative stress response, which was more pronounced than effects related to lipid peroxidation [32]. The

abundance of oxidative stress from exposure to FA likely exceeded the oxidative reducing capacity of the intercropped system.

When plants are subject to various stress treatments, antioxidant defense systems are used to maintain the redox balance in cells [33, 30]. In the current study we observed that POD and CAT activity in intercropped faba bean roots was greater when compared to monocropped beans. Furthermore, enzyme activities increased at high concentrations of FA stress (Figs. 2B, 2C). These results differed from previous studies in which antioxidant enzyme activity of plants treated with different concentrations of FA temporarily increased then decreased [34]. This might be explained by the fact that high concentrations of FA induced an imbalance of oxidative stress in faba bean root system [32], which initiated a response from the antioxidant defense system as observed by the increase in antioxidant enzyme activity in intercropped faba bean under high concentration FA stress.

Effect of intercropping on tissue structure in response to FA stress.

Deposition of lignin on the host cell wall is a well-known response of plants following infection by pathogens or other external stressors [35, 36]. A more consolidated structure of plants improves the ability to resist pathogen invasion and increases host resistance. A previous study observed a positive correlation between increased lignin content and plant resistance to pathogens [37]. Infection of *Arabidopsis* by *Pseudomonas syringae* pv. tomato and *Xanthomonas campestris* increased the expression of the gene lignin synthetase and its concentration in *Arabidopsis* [38]. In the current study we observed that treatment with FA resulted in increased lignin content of faba bean roots. In addition, lignin content of intercropped faba beans increased significantly by 25% and 15.4% when compared to monocropped beans under C2 and C3 stress (Fig. 3). Similar results were observed in cotton varieties, where increased lignin content was observed in more resistant varieties whereas those more susceptible to infection had lesser amounts when inoculated with *Verticillium dahliae* for 14d [35]. Our results showed that intercropping enhanced the degree of lignification of the cell wall of faba bean roots as induced by treatment with FA, thereby enhancing its physical barrier to the toxic effects of FA.

Furthermore, high callose synthesis in the cell wall of most plants seems to be related to plant defense responses [39]. For example, *Arabidopsis* resistance to *Alternaria brassicicola* infection was due to callus deposition [40]. In addition, we observed that callose deposition increased with increasing concentration of FA, whether monocropped (Fig. 4A, C, E, G) or intercropped (Fig. 4B, D, F, H), our results showed that the increase in callose deposition in faba bean tissue may be part of the defense response as induced by FA, which confirms the above view. However, Cohen et al. [41] suggested that although callose deposition in muskmelon leaf cells inoculated with *Pseudoperonospora cubensis* prevented the growth of fungal hyphae, excessive callose deposition interrupted the flow of nutrients from and into the invaded host cells. In the current experiment, the callose fluorescence intensity of intercropped faba bean in root cells were fainter than that of monocropped faba bean under high concentrations of FA (C2 and C3) (Fig. 4E, F, G, H), and the callose accumulation significantly decreased by 42.5% and 43.7%, respectively (Fig. 5), which indicated that the intercropping system reduced vessel blockage of faba bean root tissue caused

by callose deposition [41, 42], thus alleviated the toxic effect of high concentrations of FA on faba bean root.

At the subcellular level, TEM has been used to observe infiltration of FA and treatment induced tomato leaf cell death [30]. *Fusarium oxysporum* f. sp. *cucumerinum* infection has been observed to destroy the chloroplast structure of cucumber leaf cells, damage the plasma membrane, impair metabolism and nutrient absorption, and aggravated occurrence of diseases [25]. Ultrastructural observation of susceptible cotton treated with the toxin of *Verticillium dahliae*, resulted in obvious plasmolysis and cell membrane and cell wall damage [43]. In addition, we observed that monocropped and intercropped faba bean, had obvious ultrastructural changes following treatment with FA, including enlarged vacuoles and nuclei, displaced and less prevalent mitochondria and rough endoplasm displacement, and disruption of the plasma membrane (Fig. 6). The first noticeable change in intercropped faba bean after exposure to FA was thickening of the cell wall (Fig. 6B, D, F, H) and reduction of plasma wall separation at low concentrations (Fig. 6C and D). Changes in the cell wall is directly related to plant defense responses [44, 45, 46]. Treatment of plants with FA induces their defenses, induces lignin production and callose in the cell, resulted in greater mechanical strength of the cell wall and blockage of passages used by pathogens [37].

In the current study we observed greater restoration of plasmolysis in root cells of intercropped faba bean under C1 stress when compared with monocropped faba bean (Fig. 6C and D). Currently, a thorough understanding of the observed effects is not available however molecular investigations might provide greater insight in the mechanisms of this effect. Some papillae were observed in the C1 treatment (Fig. 6D). Previously it has been reported that formation of papillae is an early defense response in plants, when pathogens attack a host, they form papillae quickly earn time to induce additional defense responses [47]. Similarly, papillae appeared in intercropped faba bean root when treated with low concentrations of FA, which improved their early response. A large number of vacuoles were observed in the intercropped faba bean root cells (Fig. 6B, D, F, H), vacuoles have a lytic function in plant cells [48], thus intercropping improved the ability of faba bean root cells to dissolve toxins. Mitochondrial effects is a common mechanism for pathogens to attack host plants, for example increased oxidative respiration of potatoes following exposure to a pathogen has been observed [49]. In addition, there is evidence that toxins produced by pathogens can disrupt mitochondrial function and mitochondrial ATP synthesis in *Arabidopsis* [50]. In the current study, the intercropping system increased mitochondria number in faba bean root cells when treated with C0, C2 and C3 (Fig. 6B, F, H), provided sufficient energy for cell activity, and decomposed FA toxin by increasing respiration of faba bean root cells. Studies have found that the endoplasmic reticulum plays a vital role in peroxisome biosynthetic processes, acting as the source of peroxisome pro-vesicles, which fuse to form larger vesicles and eventually mature into peroxisomes [51]. Although plasmolysis occurs in root cells of intercropped faba beans under C3 stress, there was still rough endoplasmic reticulum (Fig. 6H), which helps to explain why POD and CAT activity in intercropped faba bean roots were significantly higher than those of monocropped under C3 stress (Figs. 2B and C).

Conclusions

Overall, our study explored the effect of a wheat / faba bean intercropping system on reducing wilting and toxic effects of FA on faba bean by reducing the degree of membrane lipid peroxidation (MDA content) in faba bean root and excessive callose induction following treatment with high concentrations of FA leading to obstruction of plantlets, increased antioxidant enzymes activities (POD and CAT) and lignin content. In addition, the structure of tissue cells changed following treatment with FA. Physiological, biochemical and subcellular responses of faba beans to different cropping systems were investigated and our results provide a good theoretical basis for practical production.

Methods

Plant materials and exogenous additives

Wheat seeds (*Tricum aestivum* L. var. *Yunmai* 53) and faba bean seeds (*Vicia faba* L. var. 87–147) were obtained from the Yunnan Academy of Agricultural Sciences (Yunnan province, China). The fungal toxin FA (fusaric acid) was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Purity: 95% Package: 1 g, Molecular weight: 179.22, molecular formula: $C_{10}H_{13}NO_2$, color: off-white to faint yellow,

form: crystalline powder. Structure:

Analytical-grade FA was diluted to generate a series of concentrations of 0, 50, 100, or $200\text{mg}\cdot\text{L}^{-1}$ with sterilized 1/2 Hoagland nutrient solution with a small amount of 95% ethanol. Plants were fed daily using a modified full-strength Hoagland nutrient solution (50 L) [52]: 33.5 g of $\text{CaCl}_2\cdot 6\text{H}_2\text{O}$; 25.5 g of KNO_3 ; 24.5 g of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$; 7 g of KH_2PO_4 ; 3 g of H_3BO_3 ; 1 g of $\text{MnCl}\cdot 4\text{H}_2\text{O}$; 2.5 g of $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$; 4 g of $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$; 4.5 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$; 100 ml of NaFe-EDTA.

Greenhouse Experiment

Pot experiments were carried out between October 2017 to January 2018 in the greenhouse of Yunnan Agricultural University, China, Kunming, Yunnan province. 150 faba bean seeds and 300 wheat seeds were used for germination, and seeds which germinated were transplanted into flowerpots. The experiment followed a two-factor design with concentrations of fusaric acid C0 ($0\text{ mg}\cdot\text{L}^{-1}$, CK), C1 ($50\text{ mg}\cdot\text{L}^{-1}$), C2 ($100\text{ mg}\cdot\text{L}^{-1}$), C3 ($200\text{ mg}\cdot\text{L}^{-1}$), and two planting patterns (monocropped faba bean [MF, 6 faba bean plants per pot]; intercropped faba bean with wheat [IF, 3 faba bean plants and 9 wheat plants per pot]). The experiment was repeated three times, each with 8 treatments for a total of 24 pots ($3\text{ replicates}\times 2\text{ planting patterns}\times 4\text{ concentrations}$). Overall, 36 faba bean seedlings and 36 wheat seedlings were used for each replicate. The control consisted of Hoagland nutrient solution alone. Wheat were naturally germinated by washing seeds and then placing them on two layers of filter free paper along the bottom of a container at $20\text{--}25\text{ }^\circ\text{C}$. The filter paper was soaked with sterile water, and wheat seeds were evenly spread on the bottom of the box. Faba bean seeds were treated with 10% (v/v) H_2O_2

for 30 min and germinated in a saturated CaSO_4 solution in the dark for 12 h in a porcelain tray in the dark for 48 h. After germination, seeds were sown in sterile quartz sand moistened with Hoagland nutrient solution at 25°C. When emergence of true leaves occurred, plants were transferred to plastic pots (24 cm in diameter, 16 cm in height) containing 2L Hoagland nutrient solution for 5 days, and then replaced with nutrient solution containing different concentrations of FA. During the growth period, plant roots were washed with sterile water, every three days nutrient solutions (with or without FA) were replaced and the position of pots were randomly changed every five days. Seedlings were maintained under natural light and temperature conditions (26/22°C day/night) in a greenhouse and the relative humidity ranged from 70–85%. During hydroponic culture, the nutrient solution were continuously aerated using air pumps with two small air filters. Twenty five days after sowing, faba bean seedlings were sampled when seedlings exhibited symptoms of wilting.

Measurement of plant water content

Plant samples were washed with tap water and then distilled water. Water on the plant surface was blotted off with filter paper, and plant material was weighed to obtain the fresh weight (FW). Water was removed from plant samples in a drying oven at 105°C for 30 min and then at 65°C until a constant weight to obtain dry weight (DW). Plant water content (WC) was calculated as:

$$\text{WC (\%)} = [(\text{FW} - \text{DW}) / \text{FW}] \times 100$$

Where FW is fresh weight and DW is dry weight.

Plant red ink absorption

Faba bean roots were exposed to different concentrations of FA for 25d in plastic pots and then immersed in dilute 5% red ink (Hero 201, Shanghai ink factory, Shanghai, China) solution (diluted 20 times with sterile water) for 24 h as described by Wang et al. (2015)[21]. After red ink absorption, leaves were washed with distilled water and a perforator was used to drill holes (5 mm × 5 mm) in the blade to avoid the aorta. Leaf pieces were transferred to test tubes containing 15 ml distilled water and bathed in boiling water for 30 min. After cooling to room temperature, solutions were filtered and 10 ml aliquots of sample solutions were read at 515 nm with a spectrophotometer (T6, Shanghai Jinghua Technology Instrument Co., Ltd., Shanghai, China). Measurements of absorption of red ink solution was measured in the wavelength range of 400–800 nm using an Ultraviolet–visible (UV–vis) spectrophotometer (SHIMADZU, UV-1601 PC, Japan). Maximum absorptive peak of red ink solution occurred at 515 nm.

Measurement of antioxidant enzymes and membrane lipid peroxidation.

Peroxidase (POD) and catalase (CAT) activities, and malondialdehyde (MDA) content was measured in fresh root samples according to the methods of Li et al. (2000) [53]. Briefly, a 1.0 g sample of plant material 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 and centrifuged at 3000 rpm for 10 min. The

supernatant was transferred to a 25 ml volumetric flask, remaining sediment was extracted twice with 5 ml phosphate buffer and the supernatant was transferred to the volumetric flask. Peroxidase activity was measured using the guaiacol method. The reaction mixture was prepared by adding 2.9 ml $0.05\text{mol}\cdot\text{L}^{-1}$ phosphate buffer, 1.0 ml 2% H_2O_2 , 1.0 ml $0.0\text{mol}\cdot\text{L}^{-1}$ guaiacol, and 0.1 ml enzyme solution. The reaction mixture was placed in a 37°C water bath for 15 min, and then transferred to cooling water. Absorbance was measured at 470 nm at 1 min intervals for 5 min. Results are presented as D470 per minute per gram of fresh root ($\text{U}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$).

One gram of root material was ground into a homogenate with an appropriate amount of phosphate buffer (pH 7.8). The homogenate for enzymatic analysis was centrifuged at 4000 rpm for 15 min and the supernatant was transferred to a volumetric flask. Catalase activity was measured using potassium permanganate titration. The reaction mixture contained 2.5 ml phosphate buffer and $2.5\text{mL } 0.1\text{mol}\cdot\text{L}^{-1}$ H_2O_2 and was held 10 min in a water bath at 30°C , then 2.5 ml 10% H_2SO_4 was added immediately. The solution was then titrated with a $0.1\text{mol}\cdot\text{L}^{-1}$ KMnO_4 standard solution until a pink color began to appear (approximately 30 min). Catalase activity was expressed in milligrams of H_2O_2 per gram of fresh root in 1 min ($\text{mg}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$).

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 measure MDA content using a thiobarbituric acid (TBA) reaction. Two ml of the supernatant was combined with 2 ml 0.6% TBA, and the mixture was heated in boiling water for 15 min and then centrifuged after cooling. Absorbance at a wavelength of 450, 532 and 600 nm was measured. Concentrations of MDA are presented as the amount of substance per gram of fresh root ($\mu\text{mol}\cdot\text{g}^{-1}$).

Extraction and measurement of lignin.

Extraction and determination of lignin was completed using the thioglycolic acid method as described by Liu et al. (2018) [54]. Briefly, root tissues (2 g) were homogenized in 6 ml of 99.5% ethanol, and the crude extract was centrifuged at $10000\times g$ for 30 min. Extracts were precipitated and air-dried overnight. Dried solids were weighed (50 mg) into a centrifuge tube, and 5 ml of 2N HCl and 0.5 ml of thioglycolic acid were added. The tube was heated in a water bath for 8 h. After cooling, the contents were centrifuged at $10000\times g$ for 30 min at 4°C . Precipitate was washed by resuspension in 5 ml of distilled water followed by centrifugation, then resuspended and incubated in 5 ml of 1N NaOH at 25°C for 18 h, under gentle stirring. The supernatant was centrifuged at $10000\times g$ for 30 min at 4°C , and then 1 ml of concentrated HCl was added to the supernatant. After centrifugation at $10000\times g$ for 30 min, the precipitate lignin thioglycolic acid was collected. The final precipitate was dissolved in 3 ml of 1 N NaOH and absorbance was measured at 280 nm wavelength, and absorbance was measured against a NaOH blank at 280 nm.

Staining and observation of callose deposition of faba bean root.

Callose deposition in faba bean root tissues was completed using paraffin sectioning and aniline blue fluorescence staining. Preparation of faba bean paraffin sections was completed as described by Wang et al. (2013) [55] and callose deposition was determined by aniline blue staining. Paraffin sections of faba bean root tissues were immersed in xylene I and xylene II for 20 minutes respectively for dewaxing. Samples were decolorized in anhydrous ethanol I, anhydrous ethanol II and 75% alcohol for 5 min respectively. After bleaching, roots were rinsed 6 to 7 times with tap water then incubated in aniline blue solution (0.01% staining) for 5 min. Rinsed samples were dried in an oven at 60°C, then soaked in xylene for transparent sample treatment, and finally sealed with neutral gum. Callose was observed with an upright optical microscope (Nikon Eclipse E100) at blue excitation light. Quantification of callose by immunofluorescence surface density analysis (areal density) was completed using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) to convert green fluorescent monochrome photos into black and white images. Black areas acted as the unified standard to estimate the positive (white) area of photos and to estimate the positive integrated optical density (IOD) and pixel area of tissues (AREA). All green fluorescent spots were counted and classified as callose deposits per mm². Area density was calculated as:

$$\text{Area density} = \text{IOD}/\text{AREA}$$

Transmission electron microscopy (TEM)

Samples were cut about 5 mm from the root tip, 1-2cm were fixed with 2.5% (w/v) glutaraldehyde and incubated for primary fixation for 4 h at room temperature, and then held overnight at 4°C. Samples were rinsed three times with 0.1 M phosphate buffer solution (PBS, pH 7.0) for 15 min, and then immersed in 1% (w/v) OsO₄ fixed in 0.1 M PBS (pH 7.0) for 5 h. Next, samples were dehydrated through a graded alcohol series and embedded in Epon 812 (Shell Chemical Company, USA). Ultrathin sections were cut with a Leica Microsystem UC7 ultramicrotome, then double stained with 2.5% (w/v) uranyl acetate followed by lead citrate. After drying at room temperature, the ultrastructure of faba bean roots were observed with an transmission electron microscope (TEM) (Wuhan servicebio technology CO., LTD, HT7700, Hubei, China) at 80kv.

Statistical analysis.

Mean and standard errors were calculated for individual measurements with Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA) software. The statistical analysis software package SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) was used for analysis of variance (ANOVA) of experimental data. Significant differences between the treatments were evaluated using a one-factor ANOVA, followed by Duncan's test at the 5% probability level. The callose and ultrastructure cross-sections images of faba bean root cell were obtained using the Case Viewer software 2.0 (Wuhan servicebio technology CO., LTD, Hubei, China).

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

The authors declare agree that the articles published in BMC plant biology.

Availability of data and material

All data generated or analysed 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, YD and KD contributed to the design of the study. YL, QZ 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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Figures

Figure 1

Effect of faba bean intercropping with wheat on water content (A) and red ink absorption (B) under FA stress. Data are mean ($n=3$), and error bars indicate standard deviations. MF represents monocropped faba bean and IF represent intercropped faba bean. Significant differences (Duncan test, $p < 0.05$) among treatments are indicated by different letters.

Figure 2

Effect of intercropping on MDA content (A), activity of CAT (B) and POD (C) in faba bean roots under FA stress. Data are presented as mean ($n=3$), and error bars indicate standard deviation. MF represent monocropped faba bean and IF represent intercropped faba bean. Significant differences (Duncan test, $p < 0.05$) among treatments are indicated by different letters.

Figure 3

Effect of intercropping on lignin content of faba bean roots treated with FA. Data are presented as the mean ($n=3$), and error bars indicate the standard deviation. MF represent monocropped faba bean and IF represent intercropped faba bean. Significant differences (Duncan test, $p < 0.05$) among treatments are indicated by different letters.

Figure 4

Callose deposition of monocropped and intercropped faba bean roots following treatment with different concentrations of FA. Fluorescence microscopy was used to visualize callose deposits in faba bean roots following staining with Aniline Blue, whereby stronger fluorescence intensity indicates greater callose deposition. Scale bars = 100µm. (A and B) Callose deposition of monocropped and intercropped faba bean root in the absence of FA stress, respectively. Callose deposition of monocropped (C) and intercropped (D) faba bean roots in the C1 treatment. The callose deposition of monocropped (E) and intercropped (F) faba bean root in the C2 treatment . Callose deposition of monocropped (G) and intercropped (H) faba bean roots in the C1 treatment.

Figure 5

Quantification of callose in faba bean roots by immunofluorescence areal density analysis. Data are mean ($n=3$), and error bars indicate standard deviation. MF represent monocropped faba bean and IF represent intercropped faba bean. Significant differences (Duncan test, $p < 0.05$) among treatments are indicated by different letters.

Figure 6

Transmission electron micrograph images of faba bean root cells from the intercropping system with applied FA. A and B. Root cells of monocropped and intercropped faba beans without FA treatment. Root cells of monocropped (C) and intercropped (D) faba beans following treatment with C1. Root cells of monocropped (E) and intercropped (F) faba beans following treatment with C2. Root cells of monocropped (G) and intercropped (H) faba beans following treatment with C3. Abbreviations: Nu, cell

nucleus M, mitochondria V, vacuole CW, cell wall P, plasmalemma RER, rough-surfaced endoplasmic reticulum Pa, papillae Scale bar= 5µm (A, C, D, E) Scale bar= 2µm (B, F, G, H)

Figure 7

Schematic diagrams of faba bean monocropping and intercropping with wheat (one repetition).

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