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Grafting shaping the Microbial Community Structure to Suppress *Verticillium dahliae* in the Rhizosphere of Eggplants

Yuling Yin[1, *], Shaochun Luo[1], Yunpeng Li[2], Jinsong Zhou[1], Yongping Tang[1], Yuan Liu[3]

[1]Institute of Vegetables and Flowers, Jiangxi Academy of Agricultural Sciences, Nanchang, China

[2]College of Agricultural Resources and Environment, Heilongjiang University, Harbin, China

[3]Liaoning Saline or Alkaline Land Utilization and Research Institute, Liaoning Academy of Agricultural Sciences, Panjin, China

Email address:
yuling_0_2000@163.com (Yuling Yin)

[*]Corresponding author

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Abstract: This study evaluated that grafting as an effective measure on resistance disease in continuous cropping system has a remarkable impact on improving the microbial community population and structure conditions in the rhizosphere of eggplant, which lead to a highly suppressive on the disease incidence. *Verticillium dahliae*, microbial community and soil enzyme activities in the rhizosphere of both grafted and self-rooted eggplants investigations were conducted in different eggplant growth stages in pot trials to determine the effects of grafting on the soil microbiological properties. In addition, an assessment of the bio-control effects of antagonistic microbes isolated from rhizosphere of grafted eggplants on the incidence of *Verticillium* wilt was performed. Grafted eggplants exhibited strong resistance (nearly 90%) with a lower pathological incident index. Amounts of *Verticillium dahliae* in the rhizosphere of grafted eggplants were lower before the fruiting stage compared to non-grafted self-rooting eggplants. Although each general classification of microbes (bacteria, actinomyces and fungi) was reduced over all growth stages by grafting, the ranges of bacterial and actinomycete reduction in the rhizosphere of grafted eggplants were lower compared to self-rooted eggplants. Experimental evidence indicated that grafting increased the relative ratios of Bacteria to Fungi and Actinomycete to Fungi. In terms of functional microbes, amounts of azotobacteria, ammonifying and nitrifying bacteria were significantly higher in grafted eggplants than self-rooted eggplants, especially after floral initiation. Compared to Control, grafting leaded to a significantly positive impacts on invertase, protein enzyme, urease, peroxidase and catalase activities in the rhizosphere of eggplants. Using antagonistic strains have showed strong suppression of *Verticillium dahliae* growth to study the prevention and curative effects on *Verticillium* wilt in pot trials.

Keywords: Grafting Eggplants, *Verticillium* Wilt, *Verticillium dahliae*, Continuous Cropping, Soil Microorganism

1. Introduction

Plants grown in a continuous mono-cropping system often encounter huge problems such as yield and quality reduced sharply, especially in solanum and leguminosae plants [1]. Continuous monoculture on eggplants production is very prevalent in China, and results in serious continuous cropping obstacle of the crop. In the continuous mono-cropping system, eggplant is generally suffered by *Verticillium* wilt. It caused by *Verticillium dahliae*, which is considered to be the most important soil-borne pathogen. *Verticillium dahliae* spreads in plants through the vascular bundles invading from roots of eggplant as the results of leaves turning chloretic, wilt and falling off, which is major obstacle to eggplant yield. *Verticillium dahliae* being able to form microsclerotia can be alive in the lowest temperatures in winter in the Northeast

China, which leads to the long-time accumulation in the soil for its infection to the plants in the coming year. Thus, consecutive cropping of eggplant aggravate the occurrence of *Verticillium* wilt result in drastically yield lower (30%～40%, or even no yield). To the date, only grafting technique is most effective for successful consecutive cultivation of eggplant in same cropland to overcome the destructive disease, which has widespread used in China. Investigation has undertaken that it could remarkably alleviate happen of the disease up to 90% [2]. In terms of mechanism of disease resistance of grafting technique, researchers have studied it from multiple-aspects, such as histological structure, physiology and biochemistry, and resistance induction [3, 4]. A series of physiological response happens on the scion caused by the rootstock, viz, higher levels of endogenous hormones and photosynthesis, compared to these of self-rooted eggplant, and root tissue structure of disease-resistant rootstock effectively protects the rootstock from being infected by pathogens [3, 4]. General views pointed that the plant resistance to soil-borne disease is closely related to its rhizosphere microorganisms [5]. Resistance of plants to soil borne diseases has a tightly linked with the type and amount of microbes in the rhizosphere [6]. On the other hand, benefit microbes in the rhizosphere colonize competitively on the surface of roots, or their metabolites, avoid plants from being invaded by pathogens, which is one of the important ways to control soil-borne diseases [7-9]. Therefore, microorganisms in rhizosphere of grafted eggplants could exit the similar phenomenon mentioned above. However, there are few studies reported about the relationship between microorganisms in the rhizosphere of grafted eggplants and resistance to soil-borne diseases.

This study aimed to 1). Compare the amount of *Verticillium dahliae* between grafted eggplants and self-rooted eggplants. 2) Vary in the population and community structure of microorganisms (bacteria, actinomycetes and fungi) in the rhizosphere of eggplants. 3) Soil enzymes activities difference between grafted eggplants and self-rooted eggplants. These could provide better elucidation for grafted eggplants with highly resistance on soil-borne diseases in terms of microbial community.

2. Materials and Methods

The commonly grown eggplant (*Solanum melongena* L) cultivar Xi'anlu was used as scion. It was grafted with a wild eggplant rootstock (*Solanum torvum*,) as a "Grafting" treatment. Eggplant cultivar Xi'anlu was used as "Control". The grafted and non-grafted eggplants were transplanted in a greenhouse in which eggplants were successively cultivated with normal management. Root washing method was used for taking samples at 45~60-days intervals. The samplings were undertaken at budding stage, florescence, early in fruit and fruiting stage, respectively. Eggplants were slowly uprooted from the pots.

2.1. Soil Sampling

Soil samples were collected around the roots by shaking at different stages from randomly selected five plants. Roots with adhesion of soil were immediately taken to the lab for microorganism isolation. And soil samples were dried by air, then sieved through 100 meshes for measure of soil enzyme activities. There were three replications.

2.2. Disease Investigation in Greenhouse

The resistance level (0-4 scale) of eggplants against *Verticillium* wilt was evaluated 5 times at 12 d intervals, beginning from the first appearance of typical wilt symptoms in non-grafted eggplants after transplanting. Ratings of disease index of *Verticillium* wilt was done as per [10]. Disease index and disease incidence were calculated as under:

Disease index = [∑(Rating number× number of plants with the rating)] ×100%/ (Total number of plants × highest rating)

2.3. Verticillium Dahliae Isolation

The micro sclerotiums of *Verticillium dahliae* in rhizosphere soil of grafted eggplants were separated by water sieve method on improved PDA culture medium every 35 days with 4 replications. The micro sclerotiums of *Verticillium dahliae* were observed under binoculars in the plates after having been incubated at 25°C for 3 weeks. Then amount of micro sclerotiums of *Verticillium dahliae* was counted.

2.4. Microbe Isolation from the Rhizosphere of Eggplants

Bacteria and actinomycetes were isolated from eggplants rhizosphere and incubated on BPA medium and Modified Gause1 medium, respectively. Fungi were isolated from eggplants rhizosphere on Martin agar medium and incubated on PDA culture medium. Soil gradient dilution was used to isolate bacteria, actinomycetes and fungal communities on their selective culture medium in 90-mm dia sterilized plates [11]. Then the plates were incubated at 25°C. Each treatment had 3 replications (3 plates). The amounts of microbes were counted, when the colonies reached 10~100 per plate. Oven drying method was used to determine the weight of rhizosphere soil (per ml of soil suspension). 20 ml diluted soil suspension was put into the evaporative dish for drying up, and dried soil was weighed. Then the soil weight per ml of soil suspension was calculated.

2.5. Functional Diversity of Microbes in Eggplant Rhizosphere

Nitrifying bacteria, ammonifying bacteria and cellulose decomposition bacteria were measured by most probable number (MPN) counting method to the medium of Stephenson, Beef extract-peptone and Hutchinson, respectively. Nitrogen-fixing bacteria were isolated by dilution plate count method to the medium of modified no nitrogen [12]. Each treatment had 3 replications. After the incubation, Microbial growth conditions were observed by the indicator to record the number of tubes where microbes grew in, then record bacteria number approximation was obtained by check the handbook of MPN.

2.6. Soil Enzyme Activities

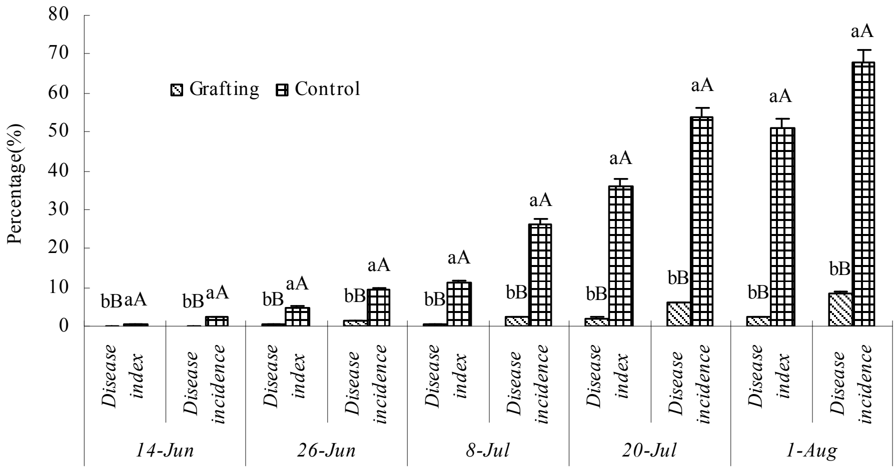
Dehydrogenase activity was determined by TTC (triphenyltetrazolium chloride) colorimetric method, expressed in units of mg of TPF (triphenyl formazan) from enzymatic reaction of 1 g soil after 1 d. Polyphenol oxidase activity was determined by potassium permanganate titration, expressed in units of ml of 0.1mol·L^{-1} potassium permanganate consumption of 1 g soil after 0.5h; peroxidase and catalase activities were determined by pyrogallol colorimetric method, expressed in units of ml of gallic acid of 1 g soil after 3h;

Invertase activity was determined by sodium thiosulfate titration, expressed in units of ml of 0.1mol·L^{-1} sodium thiosulfate consumption of 1 g soil after 1 d; Protease activity was determined by ninhydrin colorimetric method, expressed in units of mg of NH_2-N from enzymatic reaction of 1 g soil after 1 d; Phosphatase activity was determined by colorimetric method of disodium phenyl phosphate, expressed in units of mg of phenolic from enzymatic reaction of 1 g soil after 1 d; Urease activity was determined by colorimetric method of indophenol blue, expressed in units of mg of NH_3-N from enzymatic reaction of 1 g soil after 1 d [12].

3. Results

3.1. Resistance to Verticillium Wilt

Compared to Control, Grafting treatment showed significantly higher disease resistance, with lower disease index and disease incidence each sampling time (Figure 1). The results showed that the resistance of grafted eggplants was mainly due to lower disease infection and disease incidence.



Note: Different capital letters mean P<0.01. Different lowercases mean P<0.05. The same is below.

Figure 1. *Resistance of grafted eggplant to Verticillium wilt.*

3.2. Amount of Verticillium dahliae in the Rhizosphere

The amounts of *Verticillium dahliae* in both rhizosphere of Grafting treatment and Control were increased over the plants growth stages. But compared to Control, grafting treatment showed significantly less amount of *Verticillium dahliae* in the soil every stage (Figure 2). Increment of *Verticillium dahliae* in the soil of Grafting treatment was lower than that of Control. The results indicated that resistance of grafted eggplants was mainly due to reduction of the amount of *Verticillium dahliae* in the soil.

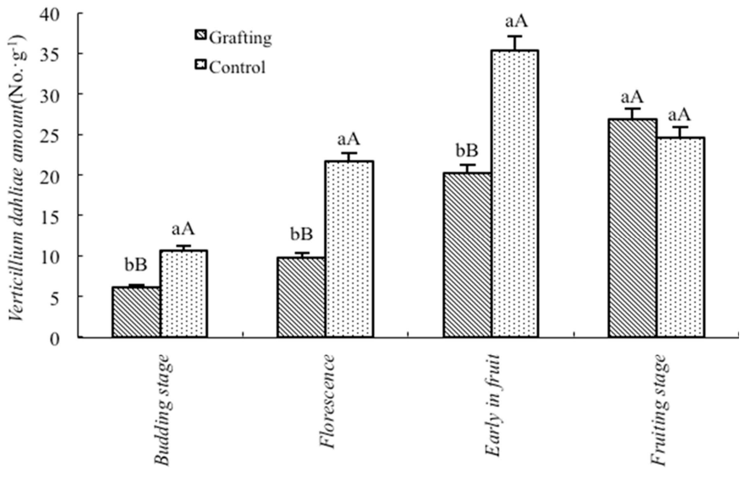


Figure 2. *Effect of grafting on the amount of Verticillium wilt in the rhizosphere of eggplants.*

3.3. Distribution of Three Kinds of Microbes in the Rhizosphere of Eggplants

The ratio of bacteria, actinomyces and fungi in the rhizosphere varied in different stages between Grafting and Control treatments (Figure 3). The ratio of bacteria in the rhizosphere of both treatments decreased from 98.58% to 95.6%, and 99.69% to 89.41%, respectively. The ratio of actinomyces in the rhizosphere of Grafting treatment showed slower increase over the stages than that in Control. The ratio of fungi in rhizophere microorganism of Grafting treatment decreased over the stages, while that in Control increased by a large margin from 0.02% to 3.87%.

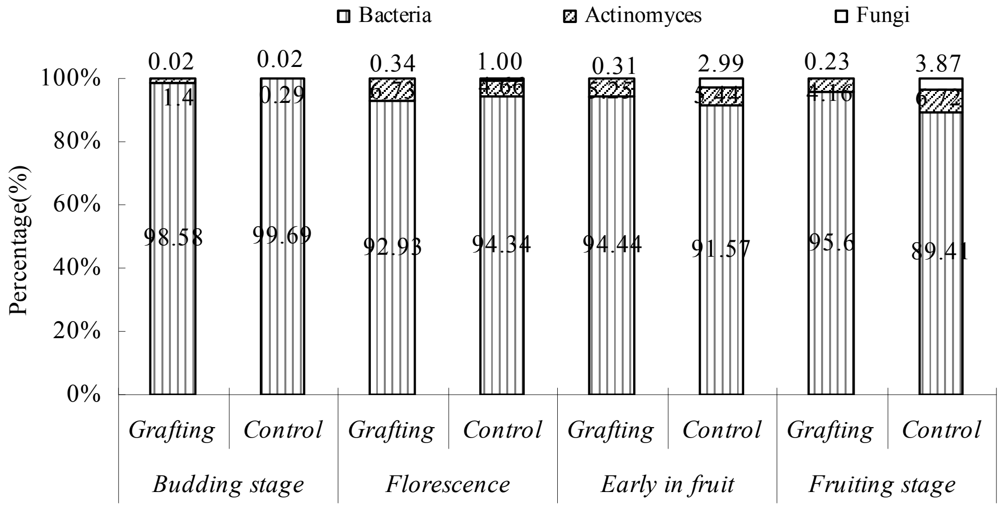


Figure 3. *Distribution of bacteria, actinomyces and fungi in the rhizosphere of eggplants.*

3.4. Rate of Bacteria to Fungi and of Actinomyces to Fungi in the Rhizosphere of Eggplants

In budding stage, both of Grafting and Control treatments appeared the highest value of B/F, and in other stages, they showed lower (Figure 4). Over the stages, value of B/F in self-rooted eggplants gradually decreased. The value of B/F in flowering, early in fruit and fruiting stage were significant larger in Grafting treatment than in Control. Grafting treatment showed the highest value of A/F in the rhizosphere, and significant larger than Control in budding stage. The value of A/F in the rhizosphere of Grafting treatment sharply decreased, while gradually decrease happened in Control over the stages. While the value of A/F in the rhizosphere of Grafting treatment at all the stages were larger than that in Control.

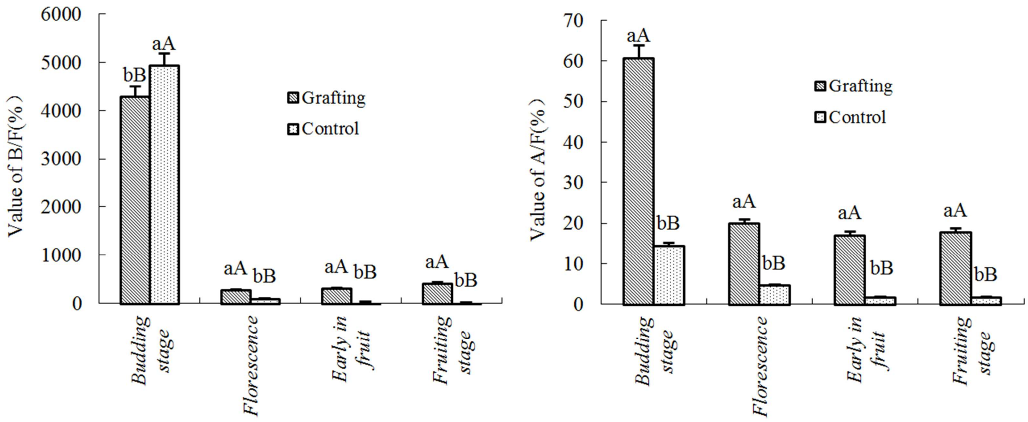


Figure 4. *Value in amounts of bacteria to fungi and bacteria to actinomyces in the rhizosphere of eggplants.*

3.5. Amounts of Functional Microbes in Rhizosphere Soil of Eggplants

At budding stage and florescence, the amounts of azotobacteria, amonifying and nitrifying bacteria were nearly at the same level in the rhizosphere of both Grafting and Control treatments (Figure 5). At early fruit stage, those bacteria amounts all showed higher than these appeared before this stage. Moreover, Grafting treatments appeared significant higher in amounts of all those bacteria than Control. Apart from amonifying bacteria, the amounts of other two bacteria increased over the concentrations. And amounts gaps were enlarged between Grafting treatment and Control at the fruiting stage. For amonifying bacteria, Grafting treatment still showed higher in amount at the fruiting stage than Control.

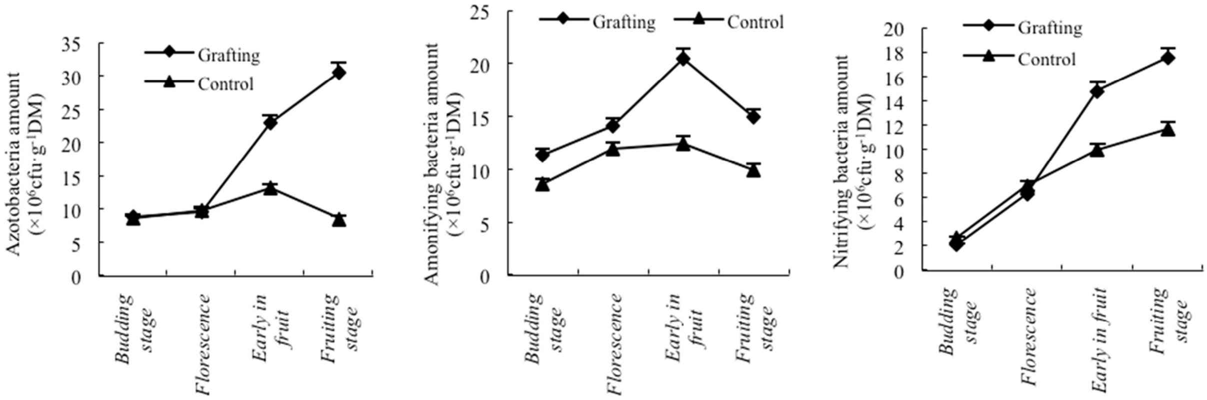


Figure 5. *Effect of grafting on functional bacteria in the rhizosphere soil of eggplants.*

3.6. Hydrolase Activities in Eggplant Rhizosphere

In Figure 6, at budding stage, the values of all soil enzyme activities in the rhizosphere of Grafting treatment have a similar trend with a higher than Control. Of which, invertase and protein enzyme reached a significant level ($p<0.05$); In florescence, the higher values of invertase and urease activities were found in Grafting treatment, but phosphatase activity followed an opposite trend compared with these two enzyme activities. In early fruit stage, apart from invertase and phosphatase activities in the rhizosphere, both values of urease and protein enzyme activities in Grafting treatment were remarkably higher than Control ($p<0.01$). To the fruiting stage, Grafting treatment had significantly higher in invertase and urease activities in the rhizosphere. The other enzyme activities did not vary much across the treatments. By and large, compared to Control, grafting improved soil enzymes activities in the rhizosphere of eggplants.

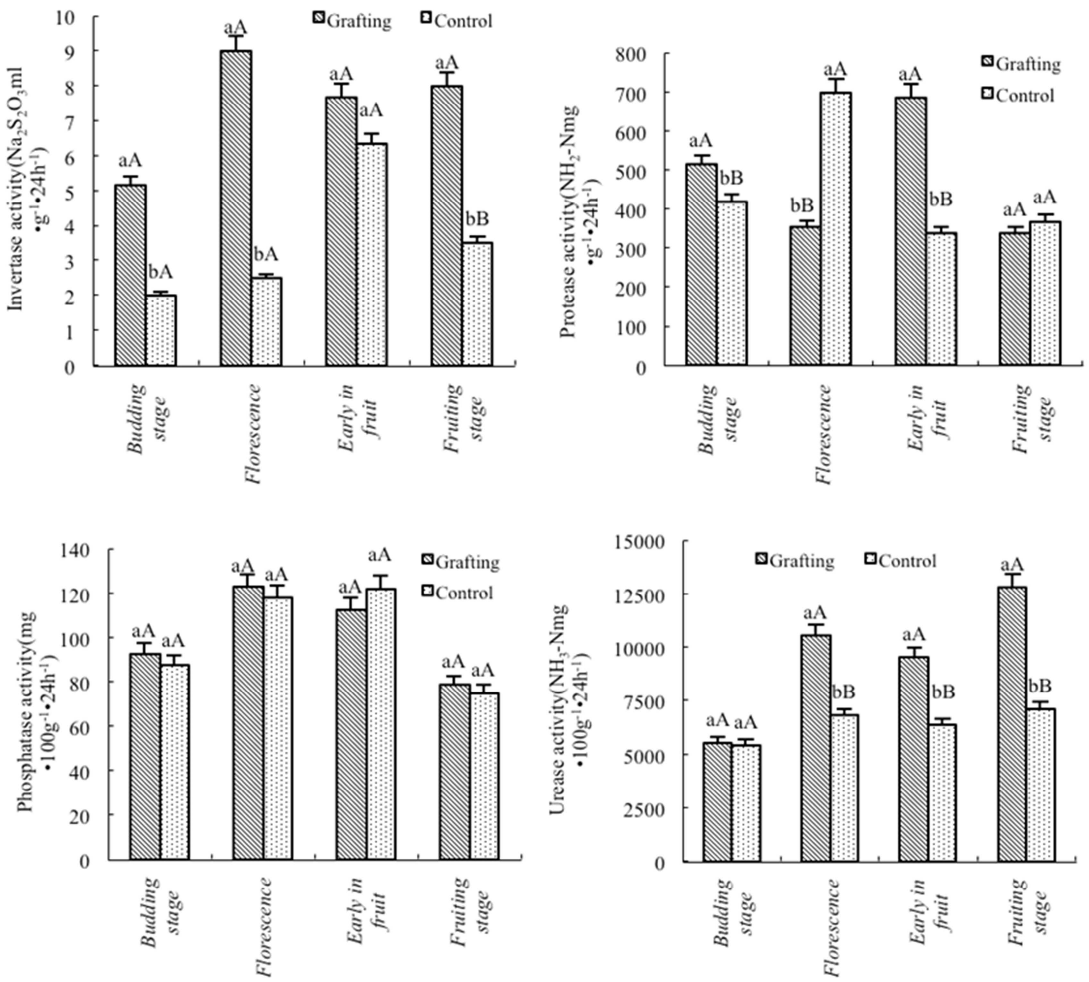


Figure 6. *Effect of grafting on hydrolase activities in the rhizophere soil of eggplants.*

3.7. Oxidase Activities in the Rhizosphere of Eggplants

At budding stage, only value of polyphenol oxidase activity in the rhizosphere of eggplants was remarkable higher than Control (Figure 7). In florescence, the value of peroxidase activity in the rhizosphere of Grafting treatment was significant higher, but catalase activity followed an opposite trend. Apart from catalase, Grafting treatment showed remarkable higher values in the other three soil enzymes activities in their rhizosphere at the early fruit stage. In fruiting stage, the values of peroxidase and catalase activities in Grafting treatment were significant higher, compared to Control. By and large, significant effect of grafting on soil enzyme activities of eggplants mainly focus on peroxidase and catalase activities.

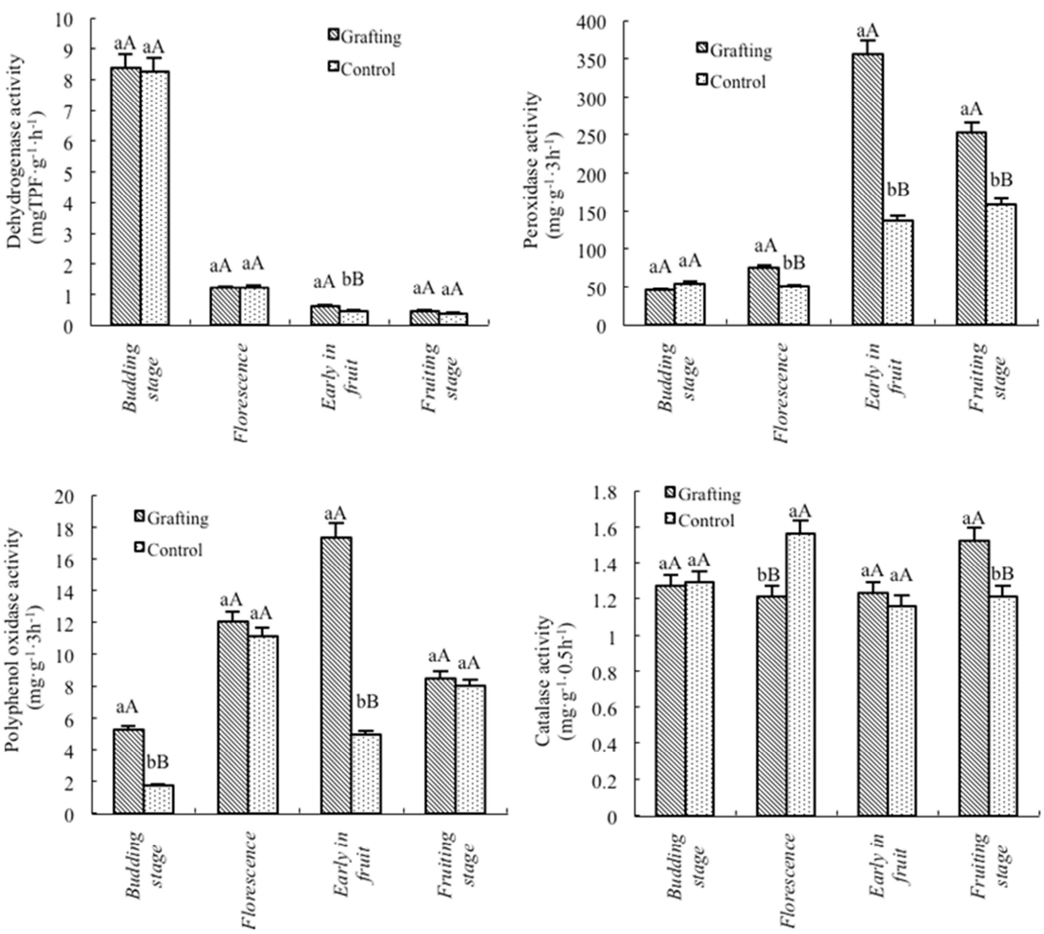


Figure 7. *Variation of oxidase activities in rhizosphere of grafted eggplants.*

4. Discussions

The *Verticillium* wilt incidence and disease index each sampling time of grafting treatment was significantly lower than Control in the study. Compared to Control, Grafting treatment showed significantly less amounts of *Verticillium dahliae* in the soil every stage (Figure 2). Plants grow in close relationship with the microbes that inhabit the soil in which plants live. Soil microbial communities represent the greatest reservoir of biological diversity, which is an approval view worldwide [13-16]. Plants stimulate or inhibit the growth of specific rhizospheric microorganisms through releasing secondary metabolites into the rhizosphere [17]. As a most effective measure alleviating replanting problems, grafted eggplants seem to be a species could impact the soil microbial activities including pathogens and beneficial ones. This summarized from the reports of researches that plant species strongly vary in influence the composition and activity of the rhizosphere microbiota [18-21]. Moreover, plants with varying degree of resistance on soil-borne diseases also have a significant different in microorganism community structure in their rhizosphere. Not only the quantity but also the microbial composition, dominated group and fungistatic action are closely related to their resistance [22, 23]. Soil microbial and enzyme activities play crucial roles in involvement in many ecosystem processes and soil management practices, in which regulate soil quality and function [24, 25]. Compared to monoculture systems, soil microbial biomass and enzyme activities indicated a remarkable increase in multi-cropping systems [26, 27]. Moreover, studies have indicated that vegetables in the continuous cropping system appeared a dramatic decrease in bacterial population in their rhizosphere, but an increase in the fungal and actinomycetes amounts, especially greenhouse cultivation [28]. This study showed that the amounts ratio of bacteria (B) and actinomycetes (A) to the whole microorganism appeared an increase in the rhizosphere of grafted eggplants, and fungi (F) were a decrease over consecutive stage. Meanwhile, grafting increased the ratios of B to F and A to F in the rhizosphere of eggplants. Grafting changed the root system of eggplant and also soil micro-ecology. The fungi dominant soil types changed to bacteria dominant soil, which is considered as an assessment for a healthy soil environment. By and large, grafting also provided an effective measure to enrich the functional bacteria. The amounts of azotobacteria, amonifying and nitrifying bacteria were significant higher than self-rooted eggplants, especially after budding stage. Study showed that the functional activities of microbes at the community level colonizing the soils could serve as useful tool to reduce the level of pathogen infection in the plants [29]. Soil enzyme activities are considered as indications of changes in metabolic capacity and nutrient cycling during agricultural practices [30]. In the study, grafting treatment appeared a positive response on microbial community structure in the rhizosphere of eggplants, and also has similar performance in the soil enzyme activities of rhizosphere. The work concentrated on two groups of soil enzymes, namely hydrolase activities and oxidase activities. Those soil enzymes were identified by their functional in anti-oxidation and nutrient conversion soil. Grafting treatment indicated a remarkable effect on invertase, protein enzyme and urease activities in the rhizosphere of eggplants with significant higher, compared to Control. It also appeared the same trend in oxidase activities, especially in peroxidase and catalase activities.

Rhizobacteria are rhizosphere competent bacteria able to multiply and colonize plant roots at all stages of plant growth, in the presence of a competing microflora [31-33] where they are in contact with other microorganisms. Most soil-borne pathogens need to grow in the rhizosphere to reach their host or to achieve sufficient numbers on their host before they can infect host tissue. The successful infection of a pathogen is influenced by the microbial community of the soil. In turn, Plants are able to recruit protective microorganisms, and enhance microbial activities to suppress pathogens in their rhizosphere. Specific microorganisms are able to protect the plant either directly or indirectly against pathogens [34]. Grafted eggplants have significant disease resistance. Failing infection of *Verticillium dahliae* to the grafted eggplants could be the competition by microbial rival. Studies [35, 36] showed that the amount of antagonistic and nitrogen-fixing bacteria have a higher level in the rhizospheres of resistant cultivars compared to susceptible ones. Moreover, the diversity of antagonistic microbes in the rhizosphere of susceptible plants was lower, and although antagonistic strains were isolated from the rhizosphere of susceptible plants, their antagonistic abilities were more weak compared to these from resistant plants [21]. Sturz and Matheson [37] showed that the population densities of bacterial endophytes with antibiosis to Erwinia soft rot pathogen were more prevalent inside the tubers of resistant than susceptible potato cultivars. Rhizosphere microbiome can be induced by biofertilizer application to suppress banana *Fusarium* wilt [38]. Two antagonistic strains of X631 and Z111 from rhizosphere soil of grafted eggplants were found with inhibitory to *Verticillium dahiae*. In greenhouse study, the disease indexes of eggplants treated with two antagonistic strains were significantly lower than control, perhaps due to systemic resistance induced by the antagonistic strains [39]. This study indicated that grafted eggplants not only reduced disease development, but also changed the microbial community structure in rhizosphere soil and maintained the microorganism environment balance to induce the formation of antagonistic stains against *Verticillium dahliae*, which may be one of the main reasons that grafted eggplants have highly disease resistance and get more yield.

5. Conclusion

Grafting eggplant with stronger resistant to *Verticillium wilt.*. The reasons for this could be summarized that grafting alter microorganism population and structure in the rhizosphere of eggplant. It increased values of ratio of bacteria (B) and actinomycetes (A) to fungi (F), and enriched beneficial microbes, such as azotobacteria, amonifying and nitrifying bacteria, enhanced hydrolase and oxidase activities in the rhizosphere, induced antagonistic stains against *Verticillium dahliae*, all of which finally stimulate inhibitory to growth of pathogen of *Verticillium dahliae*.

Acknowledgements

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