Comparing Different Methods for Isolating *Fusarium* from Soybean Rhizosphere Soil

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Abstract: *Fusarium* root rot of soybean is one of the soil-borne diseases which is difficult to control. In soil, distribution of *Fusarium* is surprisingly diverse, which can efficiently influence the pathogenic *Fusarium* populations and host resistance. Isolation of *Fusarium* is very useful in the study of soil *Fusarium* diversity. Many methods for the isolation of *Fusarium* have been reported, but the efficiency was certainly different. In this paper, some related contents which affect the effeciency of *Fusarium* separation were researched, and dilution plate method and direct soil plating method were compared. The results showed that bacteria could be inhibited excellently when the concentration of antibiotic reached fourfold, but there was no significant impact on the number of fungi. Interestingly, it seems that different combination of antibiotics had effect on *Fusarium* isolation. Between colony forming units (CFU) of fungi and different sample volume, well linear relationship were detected in both sterile water and Water Agar treatment ($R^2 > 0.9$). The isolation rate of *Fusarium* in sterile water treatment was 21.0%, a bit higher than that in Water Agar treatment, which was 10.0%. No significant effects on *Fusarium* isolation between two culture media were observed. Compared with Malachite Green Agar (MGA), Peptone PCNB Agar (PPA) behaved higher isolation rate of *Fusarium* which was 20.93%. The difference between *Fusarium* isolation rates derived from two culture methods were significant. The isolation rate of *Fusarium* derived from direct soil plating method were all above 60.0%, which is significantly higher than that derived from dilution plate method which were all below 6%.

Key words: Soybean; Rhizosphere soil; *Fusarium*; Isolation methods

大豆根际土壤镰孢菌不同分离方法比较

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摘 要: 镰孢菌(*Fusarium*)引起的大豆根腐病是难防治的土传病害之一。土壤中镰孢菌多样性水平很高, 并对其中致病菌种群动态及寄主抗性表现有一定影响。镰孢菌分离在其多样性研究中起重要作用, 报道的镰孢菌分离方法很多, 但分离效果有一定差异。本文对影响分离效果的相关内容进行了研究, 比较了稀释平板法和土粒平板法。结果表明, 当抗生素浓度增加到常量的 4 倍时可很好抑制细菌, 并对真菌数目没有显著影响; 不同抗生素组合对镰孢菌分离有一定影响; 无菌水和水琼脂处理不同样品量与菌落数 (CFU) 之间均呈线性关系($R^2 > 0.9$), 随着样品量的增加菌落数呈递增趋势; 无菌水处理中镰孢菌的分离比率为 21.0%, 略高于水琼脂处理的 10.0%; 供试 2 种培养基对镰孢菌分离比率没有显著影响, 与孔雀绿琼脂培养基(MGA)相比, 蛋白胨-五氯硝基苯琼脂培养基(PPA)对镰孢菌的分离比率较高为 20.93%。培养方法对镰孢菌分离比率影响较大, 土粒平板法分离比率均大于 60.0%, 远高于稀释平板法(分离比率均低于 6%)。

关键词: 大豆; 根际土壤; 镰孢菌; 分离方法

The genus *Fusarium* is a widely-distributed phytopathogen, which host to more than one hundred plant

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species (Kistler,1997). The soybean root rot caused by *Fusarium*, is one of soil-borne diseases which is difficult to control (Kistler et al., 1999; Isabel et al., 2003). So far at least seven species of *Fusarium* isolated from infected soybean root was identified as pathogen (Wang et al.,2004; Gao et al.,1992; Tai,2003). This fungi has a highly diverse variant, including several dozens of pathogenic formae speciales and ubiquitously present nonpathogenic strains (Armstrong and Armstrong,1981). In soil, *Fusarium* diversity level is very high (Alves-Santos et al.,1999; Gordon and Martyn,1997; Postma and Rattink,1992) which has a definite impact on the pathogenic *Fusarium* population dynamics and host resistance (Li et al.,2002). But the diversity of *Fusarium* in soybean rhizosphere of China is not clear.

Fusarium isolation is the primary problem in soil *Fusarium* diversity study, so some selective culture media, such as Peptone PCNB Agar (PPA), Komada's Medium, Selective *Fusarium* Agar (SFA), or Malachite Green Agar (MGA 2.5) have been developed for isolating and enumerating *Fusarium* from natural samples. However, some of them are not very selective because they allow the growth of many other fungal species (Bragulat et al.,2004). PPA used most prevalent in all of these media. It is highly inhibitory to most other fungi and bacteria and allows slow growth of *Fusarium* (John and Brett,2006). Komada's Medium was developed for quantitative isolation of *F. oxysporum* from soil (Komada,1975). SFA was developed for the selective isolation of *Fusarium* species from soil debris (Burgess et al.,1977). In MGA, the PCNB is replaced by 2.5 mg L^{-1} malachite green, this medium probably will become more important as the availability of PCNB declines (Castellá et al.,1997); MGA is also reported to be more inhibitory of common contaminants without reducing the number of colonies of *Fusarium* recovered in PPA (Bragulat et al.,2004).

Although many media can be used, effective separation of *Fusarium* from soybean rhizosphere was still difficult. In *Fusarium* isolation, interference from bacteria and other fungi is a major problem which difficult to solve. To achieve the best *Fusarium* isolation, some researchers improved the medium such as altered antibiotic species or dosages. However, some antibiotics are difficult to buy or have no satisfactory results. Microbial growth may affect by soil texture and pH, the bacteria may also have resistance against some antibiotics. So isolate *Fusarium* from a specifically sample (for example soybean rhizosphere soil) using the same medium above may need some changes. Diluted plate was widely used in *Fusarium* isolation, but it required amount of agar medium and labor. Soil plate was rarely used, but its easy to operate (Helle and Susanne,1999; Liang and Lv,2000). To enable separation category and quantity of Fusarium species, 0.05% or 0.1% Water Agar was used more to soil dilution abroad than sterile water at home.

Above all, some related contents which affecting the effeciency of *Fusarium* separation were researched, and dilution plate method and direct soil plating method were compared, together for isolating *Fusarium* from soybean rhizosphere soil.

1 Materials and methods

1.1 Field sites and Sampling

The infections of root rot could be affected by large differences in soil properties (Gill et al.,2001). To minimize the soil effects, sampling files were at the same locations in soybean long-term experiment spot of Hailun Agroecology Experiment Station, CAS, MN (126°38′N;47°26′W). Soybean rhizosphere soil was collected in four different soybean rotation systems spot on 18 October 2006, in which soybean cultivar Heinong 35 was planted. Four samples were soybean continuous cropping (SSS), Wheat-Corn-Soybean (WCS), Corn-Soybean (CS) and Corn-Soybean-Soybean (CSS). The samples were air-dried and sieved then stored at 4℃ until processed.

1.2 Culture media

Three culture media were used in this study, ⅰ) Peptone PCNB Agar (PPA), which components are 15 g of peptone, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of PCNB, 20 g of agar, in 1 liter of distilled water (John and Brett, 2006). ⅱ) Malachite Green Agar (MGA), which components are 15 g of peptone, 1 g of

KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2.5 mg of malachite green oxalate, 20 g of agar, in 1 liter of distilled water (Castell'a et al., 1997). ⅲ) Nash and Snyder Medium base (NSMB), which components are 15g of peptone, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 20 g of agar, in 1 liter of distilled water (Castell'a et al., 1997). Before poured the plates with medium which had been autoclaved and cooled to about 50℃, different antibiotics were added. In addition, sterile water and 1% Water Agar were used as dilution medium.

1.3 Antibiotics and dilution medium

Antibiotics were necessary when isolation *Fusarium* from soil using selective medium, but some antibiotics were expensive and difficult to acquire or had dissatisfied effect. Considering the convenience and feasibility, streptomycin, penicillin, gentamicin and rifampicin were chose for test. Among them streptomycin was effective against Gram-negative bacteria, and penicillin against Gram-positive bacteria, and they were usually used together in order to acquire satisfactory inhibition effect. Gentamicin and rifampicin both were board spectrum antibacterial.

1.4 Experimental design

Experimental design based on different antibiotics concentration and combination, the relationship between colony forming units (CFU) of fungi or *Fusarium* and different sample volume with different dilution media, and the effect of different culture medium and method on *Fusarium* isolation rate and total CFU of fungi genus.

1.4.1 Antibiotic concentration In order to value the inhibition effects of antibiotic concentration on bacteria, three antibiotics concentration treatments were designed. The initial concentration was 0.067 g L^{-1} streptomycin and 0.11 g L^{-1} Penicillin, which as first treatment. The second was two folds of the two antibiotics and the last one was fourfold. This experimental unit was used the sample WCS and dilution plate method on *Fusarium* selective medium, using sterile water as dilution medium. After cultured, all the CFU of bacteria and fungi were counted.

1.4.2 Antibiotic combinations According to PPA, MGA and some other *Fusarium* selective medium, most of components were same, only one component (such as PCNB) or antibiotic was changed in type or quantity. Four combinations were designed with PCNB and four antibiotics to detect the effects of antibiotic combination on total CFU of fungi and *Fusarium*. The four combinations were all base on NSMB and components as follow: (ⅰ) NSMB + PCNB (1.0 g L^{-1}) + streptomycin; (ⅱ) NSMB + PCNB (1.0 g L^{-1}) + treptomycin + rifampicin; (ⅲ) NSMB + streptomycin + gentamicin + rifampicin; (ⅳ) NSMB + streptomycin + gentamicin + penicillin.

1.4.3 Sample volume and dilution media To ensure the veracity and validity of isolation, we detected the relationship between total colony forming units per plate of fungi and different sample volume, and the CFU difference of Fusarium between two dilution media. The two dilution media were sterile water and Water Agar, four sample volumes added to each plate were 0.125, 0.25, 0.5 and 1.0 mL.

1.4.4 Culture medium compared PPA and MGA as culture media for selectively isolating *Fusarium* from soil were reported. In this test we counted the total CFU of fungi and number of different fungi genus.

1.4.5 Culture method compared Dilution plate method and direct soil plating method were compared by detecting the difference of *Fusarium* isolation rate in four samples.

1.5 Culture method

1.5.1 Dilution plate method Sub sample of soil (10 g) was added into 100 mL soil dilution medium and mixed thoroughly in shaker. Further dilution series were made using 1 mL of the above mentioned solutions into 9 mL soil dilution medium. 0.5 mL of dilution (1 :000, based on preliminary tests) was transferred to three Petri dishes containing *Fusarium* medium and dispersed by a glass "hockey stick" applicator to spread the suspension (Siegrid and Ingrid, 2004). The Petri dishes were incubated at 25℃ in dark. After 3 – 5 days the total colonies were counted and expressed as mean CFU/plate. Then the representative colonies were transferred to potato-dextrose-medium for further identification.

1.5.2 Direct soil plating method Direct poured

0.05 g soil into Petri dishes with *Fusarium* selective medium, following operation was the same as the dilution plate method (Lv et al., 2006).

1.6 Statistical analyses

The data were analyzed using ANOVA. Mean values were compared using Duncan's Law. The analysis was conducted using the SAS System for Windows (version 6.12) and DPS (version 7.55).

2 Results

2.1 Inhibition effects of antibiotic concentration on mean CFU/plate of bacteria and fungi

The inhibition of bacteria results showed that the mean CFU/plate of bacteria decreased when increased the concentration of antibiotics. In this study, inhibiting effect was best at fourfold concentration of antibiotics, and significant differences among them were observed ($P<0.01$). At the same time, the mean CFU/plate of fungi maintain stable, no significant differences among them were observed ($P=0.769$). So the increase of antibiotic concentration could inhibit the growth of bacteria but have no effect on fungi (Fig. 1).

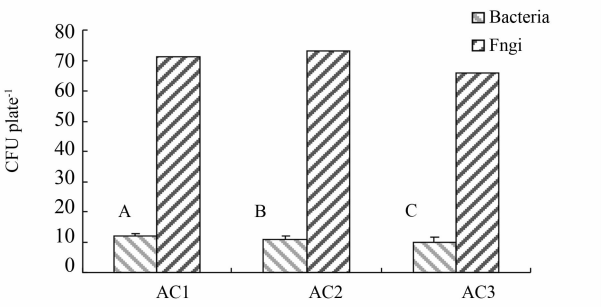

Fig. 1 Inhibition effects of antibiotic concentration on mean CFU/plate of bacteria. AC1, AC2 and AC3 means three antibiotic concentrations: the initial, two fold and fourfold. The letter A, B and C means significantly difference of bacteria between three treatments ($P<0.01$).

2.2 Effect of antibiotic combination on total mean CFU/plate of fungi and Fusarium spp

When using sterile water as medium, the mean CFU/plate of fungi were similar between combination (ⅲ) and (ⅳ), but had significant differences from combination (ⅰ) ($P<0.01$); When using Water Agar as medium, there were no significant differences among different combinations observed. Selected colonies were subcultured in different combinations of antibiotics using Water Agar as media to identify the CFU for *Fusarium spp.* in four combination, (ⅰ) was the highest one, and significant differences among them were observed ($P<0.01$), which suggested that different antibiotic combinations had some effect on *Fusarium* isolation (Fig. 2).

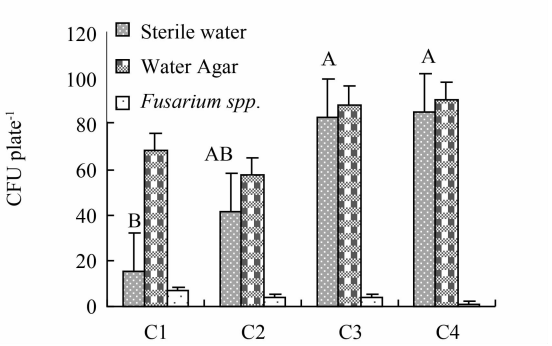

Fig. 2 Effects of antibiotic combination on total mean CFU/plate of fungi and *Fusarium spp.* C1 to C4 meant four antibiotic combinations above (ⅰ, ⅱ, ⅲ and ⅳ). The letter A, AB and B means significant difference in sterile water between four treatments ($P<0.01$).

2.3 Sample volume and dilution media

2.3.1 The relationship between total mean CFU/plate of fungi to different sample volume　Regression analyses with the sample volume as the independent variable, total mean CFU/plate of fungi as the dependent variables were formed. A significant (sterile water: $R^2=0.936$; Water Agar: $R^2=0.985$) linear relationship indicated that CFU of fungi was positively related to sample volume. No significant differences between sterile water and Water Agar were observed ($P=0.4233$) (Fig. 3).

2.3.2 Effect of dilution media on different fungi isolation rate　Representative colonies were subculture from two treatments, and three different fungi genus were identified. The most frequent one was *Penicillium spp.*, following was *Fusarium spp.*. The isolation rate of *Penicillium spp.* was lower in sterile water than in Water Agar. Reversely, the isolation rate of *Fusarium* spp. was higher in sterile water than in Water Agar. No significant differences among them were observed ($P>0.05$) (Fig. 4).

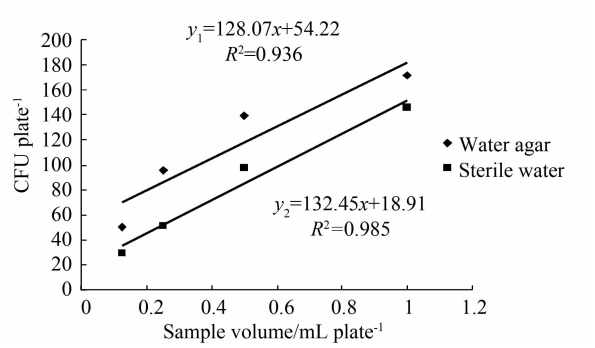

Fig. 3 The relationship between total mean CFU/plate of fungi and sample volume. Formula y_1 and y_2 represented Water Agar and Sterile Water respectively.

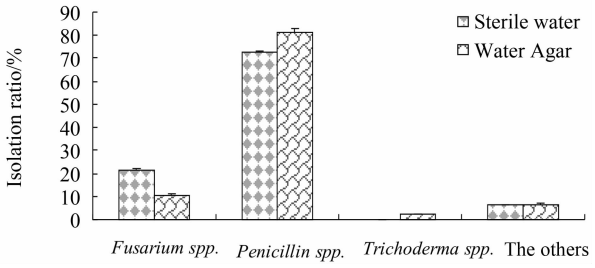

Fig. 4 Effect of two dilution media on *Fusarium* isolation rate

2.4 Effect of culture medium on *Fusarium spp.* isolation rate and total CFU of fungi genus

The number of mean CFU of fungi in PPA was higher than in MGA, and significant differences among them were observed ($P < 0.05$). The isolation rate of *Fusarium spp.* in PPA was 20.93%, about double than that in MGA, but had no significant differences among them ($P > 0.05$). Colony grew faster in PPA than that in MGA, after incubated two days the colonies could be seen clearly in PPA and then had less new colony. By contrast, visible colony appeared in MGA after three days and some colonies might emergence slower. More fungi genus were discovered in PPA rather than in MGA, testify MGA had more inhibition against fungi (Fig. 5).

2.5 The effect of culture method on isolation rate of *Fusarium spp.*

The isolation rate of *Fusarium spp.* of direct soil plate method was usually more than 60%. Although soil particles size and uniformity on selective medium plate were impacted by operation, direct soil plate method still had distinct advantage on purification when the quantity and quality of soil particles were appropriately controlled. Generally, in dilution plate method *Fusarium spp.* isolation rate was lower than 6%. Significant difference between them were observed ($P < 0.01$).

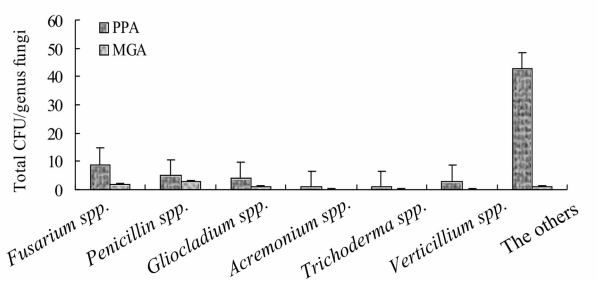

Fig. 5 The total CFU/plate of different fungi genus

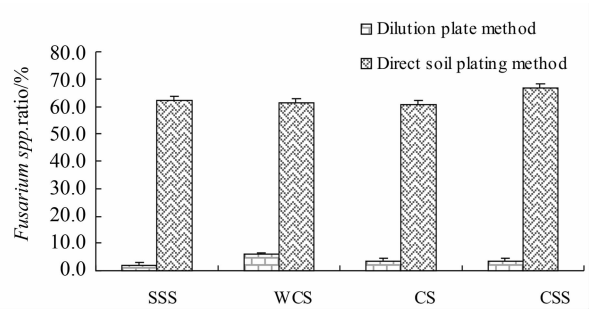

Fig. 6 *Fusarium spp.* isolation rate of different soybean rotation systems compared two culture methods. SSS = Soybean continuous cropping, WCS = Wheat-Corn-Soybean, CS = Corn-Soybean and CSS = Corn-Soybean-Soybean.

3 Discussion

Effective isolation *Fusarium* in soil was affected by variety factors. It is proposed that inter and intra specific variation in the *Fusarium* species community, in particular soil niche, is an outcome of the influence of bioclimatic, multi-soil-edaphic and biotic factors (Qaher, 2006). The interference form other fungi and bacteria to the isolation effect of *Fusarium* species is also inevitable, so selecting medium or improvement by different researchers were needed. Bragulat et al. (2004) used different strains of *Fusarium spp.* and natural samples to compare six selective efficacy culture media, among them no statistical differences were detected in colony counts of the *Fusarium spp.* although the colony diameters in MGA 2.5 were significantly lower than the other five media within strains tested; with natural samples, MGA 2.5 performs as a potent selective medium for

Fusarium spp., whereas the other recommended selective media allows the growth of many different fungal species including Zygomycetes and yeasts (Bragulat et al.,2004;Castellá et al.,1997). In this study, MGA 2.5 is better than PPA in selectivity for *Fusarium* and inhibitory to other fungi. This conclusion is the same as the previous research. But the isolation rate of *Fusarium* in MGA 2.5 is lower than in PPA, which maybe one of the reasons why MGA 2.5 was not popular.

Considering both the concentration and types of the antibiotics are variable to different cases, so we just choose the suitable concentration and types to the specific sample. No significant deference between sterile water and Water Agar on total CFU of *Fusarium spp.* were observed, but Water Agar had been suggested to acquire more *Fusarium* species. At present, most researchers use dilution plate method to isolate *Fusarium* in soil, but this method was time consuming, huge amount of medium and labors were required, which maybe a restrictive factor when handling tremendous samples. Comparison between dilution plate method and direct soil plating method showed that the latter method has several advantages, such as well isolating ratio, easily to purification when the soil sample volume is appropriate and resource-saving. So direct soil plating method is feasible for fungal distribution studying between microhabitats and soil and it could replace the dilution plating method to reduce both the required amount of medium and labor (Helle and Susanne, 1999).

The research was based on soybean rhizosphere of black soil as sample. *Fusarium* diversity in the soil was impacted by tillage, seed treatment, host resistance (Siegrid and Ingrid,2004;Wang et al.,2004) and other factors, so application of this method still have some restriction.

4 Conclusion

The bacteria could be inhibited excellently when antibiotic density reached fourfold, but there was no significant impact on the number of fungi. Interestingly, it seems that different combination of antibiotics had effect on *Fusarium* isolation. Between CFU of fungi and different sample volume, well linear relationship were detected in both sterile water and Water Agar treatment ($R^2 > 0.9$). The isolation rate of *Fusarium* in sterile water treatment was 21.0%, a bit higher than that in Water Agar treatment which was 10.0%. No significant effects on *Fusarium* isolation between two culture media were observed. Compared with MGA, PPA behaved higher isolation rate of *Fusarium* which was 20.93%. The difference between *Fusarium* isolation rates derived from two culture methods were significant. The isolation rate of *Fusarium* derived from direct soil plating method were all above 60.0%, which is significantly higher than that derived from dilution plate method which were all below 6%.

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