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Colonization Dynamics Difference of *Acidovorax.citrulli* Subgroup I Pslb3 Strains And Subgroup II Pslbtw43 Strains in Melon Plants

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This paper researched the colonization dynamics difference of the causal agent of bacterial fruit blotch of watermelon *Acidovorax.citrulli* subgroup1 pslb3 strains and subgroup 2 strains pslbtw43 in melon plants. Under sunlit greenhouse condition, inoculated melon seeds with subgroup I pslb3 strains and subgroup II pslbtw43 strains marked green fluorescent protein(GFP),collection hypocotya, root, cotyledon and blade periodically, separate pathogenic bacterium and count it. The result showed that both subgroup I pslb3 and subgroup II pslbtw43 can colonization on all organs of melon plant, and it was the strongest in cotyledon. The maximum of bacteria recycled of group I pslb3 strain in cotyledon was 5.26×10^8 CFU/g, it was 2.56×10^7 CFU/g of group II pslbtw43 strain. The bacteria recycled in cotyledons which inoculated subgroup I pslb3 strains or subgroup II pslbtw43 strains were both 0 after 30 days inoculated. The colonized ability of subgroup I pslb3 strains in melon organization was stronger than subgroup II pslbtw43 strains, the colonization ability of both them was weakest in blade.

1. Introduction

Bacterial fruit blotch(BFB) is a an important bacteria disease of cucurbits, it is caused by *Acidovorax citrulli* (formerly *Pseudomonas pseudoalcaligenes* subsp.*citrull*i (Schaad et al.)) (Melo et al. 2014, Dutta et al. 2012, Walcott et al. 2003). It is a gram-negative bacterium (Dutta et al. 2012, Ha et al. 2009), it is seed-born disease that has high potential damage. Under appropriate conditions, the disease spread rapidly in greenhouse and field, lead to seedling blight, late fruit rot (Bahar et al. 2009).
The disease was first reported in 1965 (Feng et al. 2009), and in 1989 it was first reported in commercial watermelon production field in Florida (Lessl et al. 2007), and after that the disease was successively reported in many watermelon production area. The pathogenic bacteria can cause other cucurbitaceae crops disease, such as melon,watermelon, cantaloupe, citron, and pumpkin and so on (Walcott et al. 2000). The disease seriously restrict the production of some crops such as melon, watermelon and so on, and influence the development of seed industry According to reports, cucumber can also infect *Aac* and spread the bacteria by seeds after artificial inoculation (Kubota et al. 2011, Bahar et al. 2009). Walcott and others divided the *Aac* strains from the united states and other countries two subgroups due to the PFGE and rep PCR technology. Subgroup I strains were isolated from melon and pumpkin, and subgroup Ⅱ strains were isolated from watermelon (Yan et al. 2013). Under greenhouse conditions, Walcott, Lessl et. studied the amount of colonization of subgroup Ⅱ strains in watermelon plants (Walcott et al. 2003, Lessl et al. 2007) and the amount of colonization of subgroup I strains in melon plants (Neto et al. 2006). But the adaptability differences such as colonization dynamics and so on of different subgroup strains on the same host has not been reported. This paper studies mainly the colonization dynamics differences of subgroup I strains pslb3 and subgroup Ⅱ strains pslbtw43 in melon plants, it provide theoretical basis for controlling bacterial fruit more effectively.

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2. Materials and methods

2.1 Materials

2.1.1 Strains and plasmids

Subgroup I pslb3 strains and subgroup Ⅱpslbtw43 strains of *Aac* were preserved by the Chinese academy of agricultural sciences institute of plant protection vegetable diseases laboratory; pBGM-kn plasmid vector that carried GFPuv gene with kanamycin(final concentration of 50 μg·mL^{-1}) resistance screening markers was so kind to give by Ph.D. Professor li-qun zhang of china agricultural university. Assistant bacteria DH5α (pRK600) was purchased from Beijing zhong bao ke nong biological technology co., LTD; Melon seeds (Jingxiang8) were provides by the Chinese academy of agricultural sciences institute of plant protection vegetable disease lab.

2.1.2 Media

Assistant and donor bacteria were cultured used LB culture medium (tryptone 10 g, yeast extract 5 g, NaCl10g, constant volume to 1000 mL with water, to pH7.0, 121℃ sterilization 20 min; solid medium AGAR concentration was 1.5%.) Cultivated watermelon acidophilic bacteria used KB medium ($MgSO_4$1.5 g, K_2HPO_4 1.5 g, glycerol 15 mL, peptone 20g, constant volume to 1000 mL with water, to pH 7.0, 121℃ sterilization 20 min, solid medium AGAR concentration was 1.5%, containing 25% sucrose KB medium).

2.2 Test methods

2.2.1 GFP marked watermelon acidophilic bacteria

Subgroup I pslb3 strains and Subgroup II pslbtw43 strains which both existing ampicillin (Amp) resistance were marked with GFP by three close hybrid method. Finally cultivated the KB tablets in 28℃ thermostat training for 2-3 days. Inversion the KB tablets which grew bacteria (containing ka and Amp) on vivo fluorescence microscope and tested positive for cloning, that had the bright green fluorescence was the watermelon acidophilic bacteria strains which had the green fluorescent markers and resistanted Amp. Cultured the marked strains for tested selected getdetransgenerationally 20 generations, to detect the fluorescent stability on fluorescence microscopy.

2.2.2 Preparation the bacteria suspension strains

The marked strains were inoculated in the triangle bottle with 250 mL liquid KB (including Ka and Amp) and culture it oscillating in 28ºC table concentrator for 24 ~ 36 h, adjusted the density of bacteria suspension use sterile water to 3.4×10^7 CFU/mL, spectrophotometer 570 nm, absorbance was 0.36.

2.2.3 The research of colonization ability differences of subgroup I pslb3 strains and subgroup II pslbtw43 strains to melon plants

2.2.3.1 Inoculation method

First washed the melon seeds (Jingxiang8) use tap water for 10 ~ 20min, naturally air drying at room temperature (25 ± 2ºC), immersed in the 3.4×10^7 CFU/mL bacteria suspension for 16 h. Put the seeds in air at room temperature for 16 h, planting the seeds in the plastic pot which the upper aperture diameter was 10 cm, the bottom diameter was 7cm, the height was 10cm, and cultivate it in the constant temperature incubator for 15h day time, 12 h night time alternately, 60% light and 90% or over humidity. Test the seed after vaccination 2 h (0 d); detected the hypocotyl after 6d; detected the root after 9 d ; detected the cotyledons after 12 d; started to test the leaf from 15th d, stop the above test until can't detect the bacteria consecutive two times . The experiment repeated 2 times, each time set up 4 repetitions(Alves at el.2010).

2.2.3.2 Sample retrieve processing

Take the sample randomly every time, rinsed clean the sample use sterile water, weigh them after blot up surface water use sterilization filter papper, 75% alcohol disinfect for 30 s, sodium hypochlorite disinfect for 5 min, use sterile water wash three times, grinding to mud use outer hole mortar, add 1mL sterile water blending, dilution to10^{-1}, 10^{-2}, 10^{-3} concentration respectively, absorb 200 uL to coat KB tablet containing Ka and Amp, cultivate inverted in 28ºC cultivation box for 48 h, statistic fluorescent colony number inverted on the fluorescence microscope, converted to CFU/g, the conversion formula:

Amount of watermelon acidophilic bacteria colonization (CFU/g) = (colony number×5×10×diluted multiples)/sample weight (g)

3. The results

3.1 GFP tag watermelon acidophilic bacteria fluorescence detection

The KB table marked GFP by three close hybrid grew many small colonies after they were cultivated 2 days in 28℃ temperature box, detected these colonies on stereo fluorescence microscope, they all gave out strong green fluorescence (FIG. 1-A,B).The bacterium solution that cultivated used liquid KB for 1d was putted under

fluorescence microscope to detect, visible the short rod-shaped bacteria with bright green fluorescence (FIG. 1-C). It show that the green fluorescent protein granule had been successfully imported in watermelon acidophilic bacteria bacteria cells in this study, these green fluorescent bacteria respectively named pslb3$^{Amp, GFP}$, pslbtw43^{Amp,GFP}. It can still give out strong green fluorescence when detected on fluorescence microscope after successive transfer culture 20 generations.

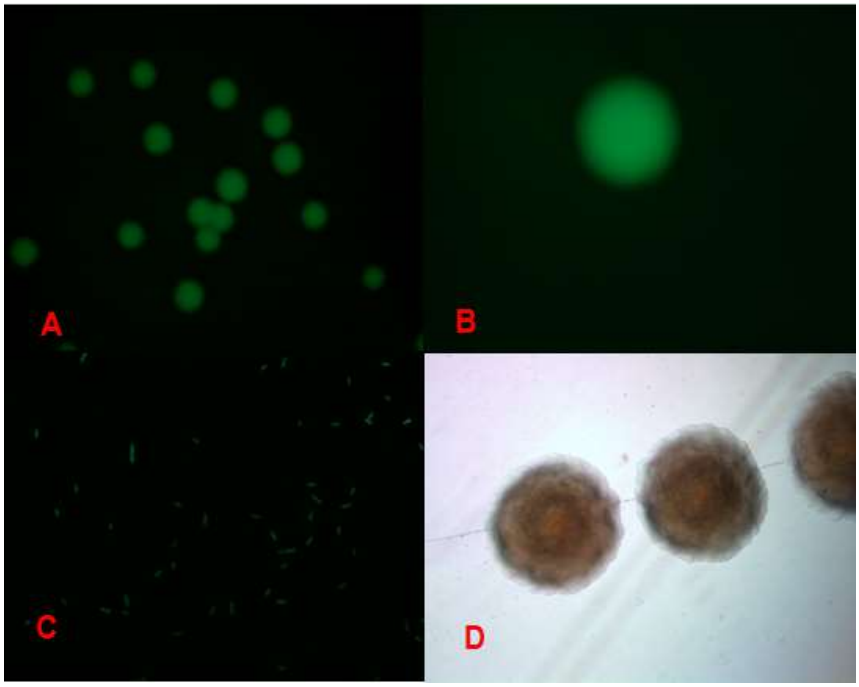

Figure 1: Watermelon acidophilic bacteria marked GFP strains form A multiple colonies B single colony C liquid KB bacterium solution D unlabelled strains

3.2 Colonization of pslb3$^{Amp, GFP}$, pslbtw43$^{Amp, GFP}$ on melon plant

Began to recycle melon hypocotyl after inoculation 6 days, the bacterial recycle of melon hypocotyl inoculated subgroup I and subgroup II strains both all reduced down at first, and then the trend was increased and then decreased again, bacteria quantity of melon hypocotyl that inoculated subgroup I pslb3 strains was zero at 27th day, bacteria quantity of melon hypocotyl that inoculated subgroup II pslbtw43 strains was zero at 21th day(figure 2).

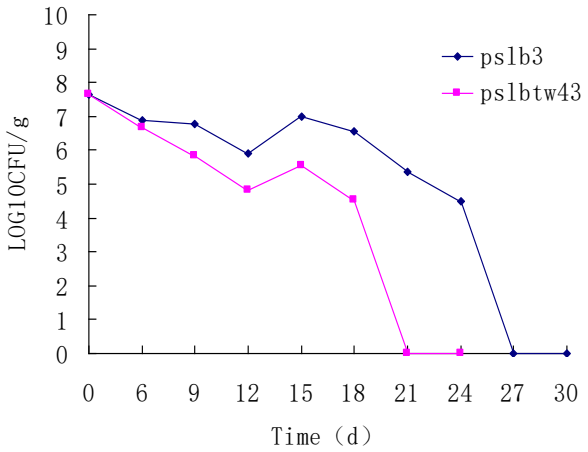

Figure 2: Colonization of pslb3$^{Amp, GFP}$, pslbtw43$^{Amp, GFP}$ in melon hypocotyl

Bacteria quantity of melon root which inoculated subgroup II pslbtw43 bacteria strain showed continuous decreasing trend, it began to 0 at 18th day; Bacteria quantity of melon root which inoculated subgroup Ipslb3 strains reduced to 0 at 30th day(figure 3).

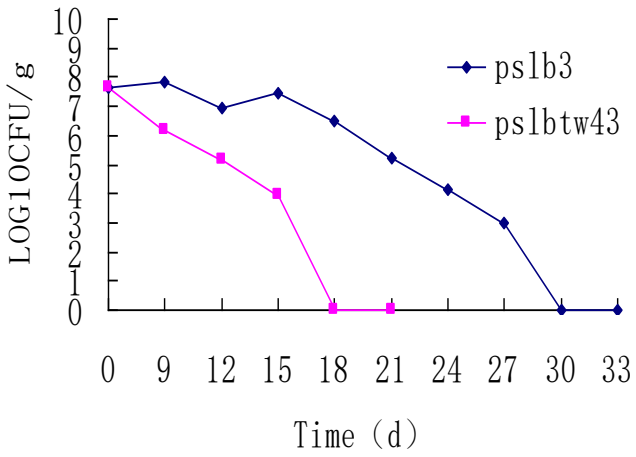

Figure 3: Colonization of pslb3$^{Amp, GFP}$, pslbtw43$^{Amp, GFP}$ in melon root

Started to recycle bacteria in cotyledon from 12th day ,subgroup Ipslb3 strains reported the highest in 12th day, to 5.26×10^8 CFU/g, the recycle bacteria of subgroup II pslbtw43 strains in 12th day was 2.56×10^7 CFU/g. (figure 4)

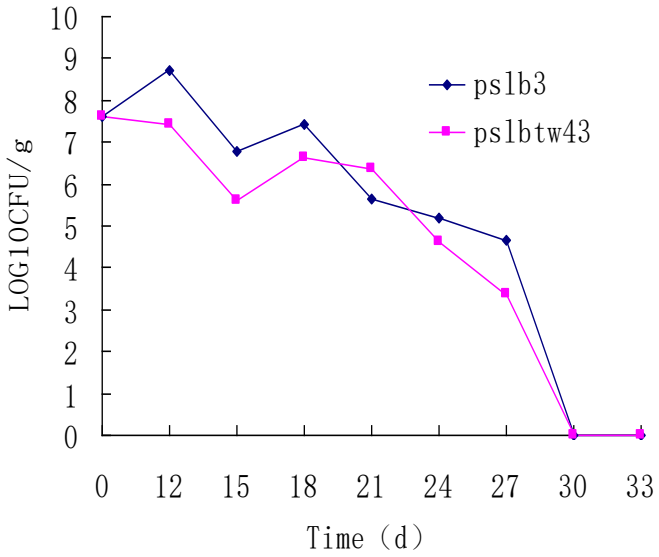

Figure 4: Colonization of pslb3$^{Amp, GFP}$, pslbtw43$^{Amp, GFP}$ in melon cotyledon

In the process of recycling blade, the bacteria recycled of subgroup I and subgroup II are all declining, and the bacteria recycled of subgroup I reduced to zero in 27th day, and the one of group II reduced to 0 in 21th day (figure5).

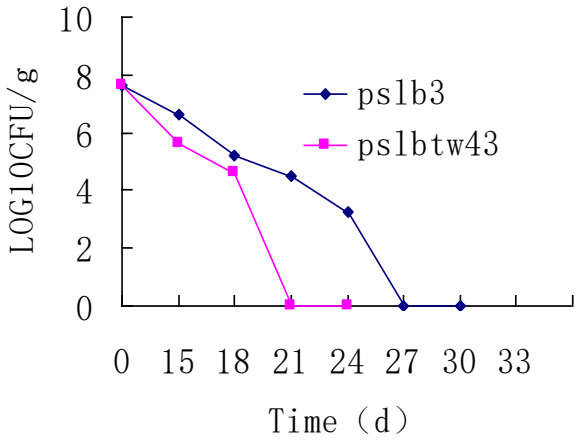

Figure 5: Colonization of pslb3$^{Amp, GFP}$, pslbtw43$^{Amp, GFP}$ in melon blade

4. Discussions

Green Fluorescent Protein (GFP) is the report gene that was found in the glowing jellyfish (*Aequorea victoria*) in 1962 and was applied (Alabouvette and Couteaudier 1992). Because in itself is a kind of light-emitting proteins that have stable, intuitive and easy to operate fluorescent properties and does not need to add the exogenous substrates can be directly tested in living cells and observing, overcome the disadvantages of traditional report gene in molecular biology research (Feng et al. 2009). GFP as a ideal marker that monitoring complete cells and gene expression and protein localization in tissues is widely applied in a variety of bacteria, fungi, plants and mammalian cell research (Chalfie et al.1994), the main areas including transgenic animals, fusion marker, gene therapy, protein function orientation and migration change with in living cells and molecular process of pathogenic bacteria intruding into living cells, etc. (Ni et al. 2001). Because of the small effect on strains, easy to operate, and can be directly observed and evaluate the effect of colonization, GFP marker is receiving much more attention of the researchers at home and abroad (Yang et al. 2013).

Regular recycling hypocotyl, root, cotyledon and leaf after sowing, separated pathogenic bacteria and counting. The results showed that the colonized ability of subgroup Ipslb3 strains to melon plant was stronger than the subgroup II pslbtw43 strains, the colonized ability of both them to the cotyledon was most powerful, Chalupowicz L et al. researched and thought that the cotyledon was the main colonization part of watermelon acidophilic bacteria to melon plant (Chalupowicz 2015), the bacteria recycle of subgroup I pslb3 strains in cotyledon reported the highest at 12th day, to 5.26 $\times 10^8$ CFU/g, the maximum of bacteria recycle of group II pslbtw43 strains in cotyledon was 2.56 $\times 10^7$ CFU/g, in the determination of recycle at the 21th day, there was a phenomenon that the bacteria recycle of subgroup Ipslb3 strains was below subgroup II pslbtw43 strains, this may be linked to a sharp change of temperature and humidity changes in greenhouse. The colonization ability of subgroup Ipslb3 strains and subgroup II pslbtw43 strains on the blades was weakest, as you can see in figure 5 where the bacteria recycle of both them were all declining, subgroup I pslb3 strains in 27th day recycled to 0, and subgroup II pslbtw43 strains recycled to 0 at 21th day. The colonization period of subgroup II pslbtw43 strains in melon root was relatively short, it can not recycling bacteria at 18th day, the bacteria reduced to 1.43 $\times 10^6$ CFU/g at 9th day. Through experiments, watermelon acidophilic bacteria can be colonization in different parts of melon plant as hypocotyl, root, cotyledon and leaf, the colonization ability in the cotyledons was strongest, and was the weakest on the leaf, it provide important theoretical basis for the science cultivation ,prevention and control to BFB. The mechanism of lead to the differences above needs to further research.

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