Development of an innovative soil disinfestation method based on the medium-chain fatty acid producing *Clostridium* sp.

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Research objective

Soil disinfestation is essential for preventing crop diseases caused by plant pathogens. **A**naerobic **s**oil **d**isinfestation (ASD), has gained increasing attention in recent years as a safe and environmentally friendly alternative to toxic soil fumigants. In ASD, organic matter such as rice bran and molasses is mixed into the soil, irrigated, and the surface is covered and sealed with a plastic film. Oxygen is rapidly consumed by soil microorganisms during ASD, leading to the production of organic acids and metal ions. Additionally, certain anaerobic bacteria dominate, ultimately disinfecting plant pathogens. However, the key disinfestation factor of ASD remains unknown. Moreover, its unstable effectiveness at low temperatures and high costs limit the versatility. Recently, we successfully isolated the strain E801, closely related to *Clostridium kluyveri,* the predominant bacterium after ASD treatment. We found that medium-chain fatty acid (caproate) produced by this bacterium may be a key disinfestation factors[1)]. This study aims to investigate whether caproate can be produced cost-effectively through fermentation with strain E801 and whether it can be applied for soil disinfestation, particularly at low temperatures.

Methods

1. Fermentative production of caproate by strain E801

Strain E801 was cultured anaerobically at 30˚C with varying concentrations of ethanol and acetate as carbon sources. High-performance liquid chromatography (HPLC) was used to measure production and consumption of acetate, butyrate, and caproate using. The effects of yeast extract addition and pH-controlled cultivation were also investigated.

2. Effect of caproate on the growth and viability of *Verticillium dahliae*

Disinfection of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and *Ralstonia solanacearum* using caproate has been reported[1)]. In this study, the effect of caproate on *V. dahlia*, which develops diseases in cool environments, was investigated. Specifically, the effects of caproate on the mycelial elongation, conidiospore growth, and microsclerotic viability were determined.

3. Soil disinfestation by caproate at ambient and low temperatures

Andosol was placed in a glass bottle and inoculated with a spore solution of Fol at 10^5 spores/g soil. Acetate, butyrate or caproate was added to the soil at a final concentration of 10 to 20 mM and incubated at 30˚C or 15˚C for about 2 weeks. Viable Fol was then counted on the agar medium. Similarly, the treated andosol was allowed to dry for an appropriate time, and pregrown tomato seedlings were planted and observed for disease development in a controlled growth chamber.

Results

1. Fermentative production of caproate by strain E801

Strain E801 was grown anaerobically in a liquid medium containing ethanol and acetate at various molar ratios. Strain E801 exhibited good growth at ethanol/acetate molar ratios of 2-5 and produced high concentrations of caproate (approximately 100–120 mM) within 2 weeks (**Fig. 1**). In contrast, when the molar ratio was 0.5 to 1, or when only acetate or ethanol were present, no caproate was produced. The addition of yeast extract to the culture medium did not increase caproate production, and pH-controlled cultivation had no significant effect.

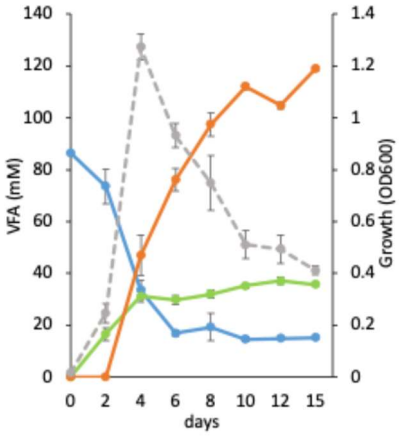

Fig. 1. Acetate (blue), butyrate (green) and caproate (orange) production and consumption by strain E801 cultured with 250 mM ethanol and 100 mM acetate. Gray dots show growth of strain E801.

2. Effect of caproate on the growth and viability of *Verticillium dahliae*

The inhibitory effects of acetate, butyrate, and caproate on the growth of conidiospores of *V. dahliae* was investigated. No significant differences were found between acetate and the control (without organic acids). Butyrate delayed growth in the early stages of incubation, but by day 10 growth was comparable to that of the control. In contrast, caproate clearly inhibited growth at concentrations of 2.5 and 5 mM to less than 1/10

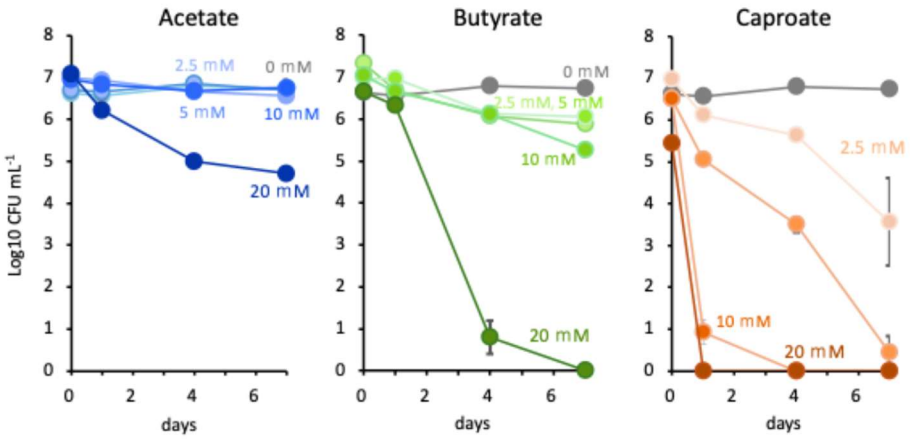

Fig. 2. Effect of organic acids on the viability of microsclerotia of *Verticillium dahliae*.

and 1/1,000 of the control, respectively. In addition, the conidiospores did not grow in the presence of 10 mM caproate. Microsclerotia were then prepared, and the disinfestation effect of the organic acids was investigated (**Fig. 2)**. Acetate showed a weak disinfestation effect at 20 mM; however, it was considered to have a disinfestation effect on the conidiospores included in the microsclerotia preparation. Butyrate had a disinfectant effect at 20 mM but no effect was observed at 2.5 to 10 mM. In contrast, caproate showed a strong disinfection effect at concentrations of 5–20 mM.

3. Soil disinfestation by caproate at ambient and low temperatures

Organic acids (10 mM) were added to andosol inoculated with Fol, and incubated at 30˚C. The strongest disinfestation effect was observed with caproate, although acetate and butyrate also showed some disinfestation effects. When a similar experiment was conducted at 15˚C, caproate showed a strong disinfestation effect against Fol even under these conditions (**Fig. 3**). In contrast, acetate and butyrate exhibit minimal disinfection effects. Tomato seedlings were planted in soil disinfected at 30˚C, but no disease was observed in any soil.

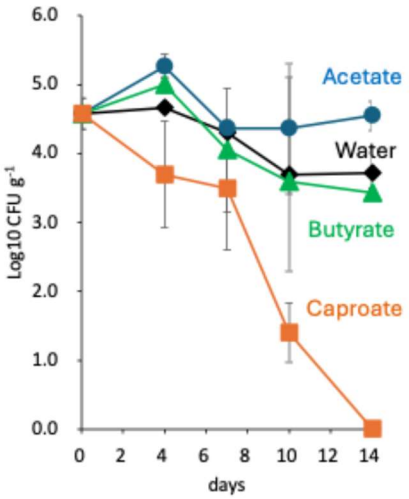

Fig. 3. Effect of organic acids on the viability of Fol inoculated with andosol. The soil was incubated at 15˚C.

Conclusion

Strain E801 produced high concentrations of caproate using inexpensive ethanol and acetate as carbon sources. Caproate was found to have strong disinfestation effects against both conidiospores and microsclerotia of *V. dahliae*. Caproate also completely disinfected soil-inoculated Fol at low temperatures. These results strongly suggest that caproate produced by the fermentation of strain E801 can be used for the disinfestation of plant pathogens at low temperatures and costs. In the future, it will be necessary to plant various crops in the soil after disinfestation to show that these diseases can be controlled and suppressed.

References

1) Shirane, S., Momma, N., Usami, T., Suzuki, C., Hori, T., Aoyagi, T., and Amachi, S. (2023) Fungicidal activity of caproate produced by *Clostridium* sp. strain E801, a bacterium isolated from cocopeat medium subjected to anaerobic soil disinfestation, *Agronomy*, **13:** 747.