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COMBINED EFFECTS OF SOIL AMENDENTS AND EFFECTIVE MICROBIAL AGENTS ON CONTAINER SEEDLING GROWTH AND INCIDENCE OF CITRUS CANKER

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Abstract

To investigate the combined effects of soil amendments and effective microbial agents for the cultivation of container-planted citrus seedlings, *Citrus reticulata* Ehime 28 was used as the test material. Bentonite clay and attapulgite were mixed with natural soil, and effective microbial agents were then added. The combination comprising attapulgite, bentonite, and effective microbial agents could reduce the soil bulk density and increase the total porosity, pH and EC values, and contents of Olsen-P and Olsen-K in the soil. The combination contains 60% natural soil, 10% attapulgite, 30% bentonite, and 300 times the amount of EM microbial agents significantly promoted the growth of citrus leaves and the formation of chlorophyll; was beneficial to the biomass accumulation of the plants; and promoted the uptake of total nitrogen, phosphorus, and potassium. The incidence of cankers on plant leaves was significantly reduced. This combination has the potential to improve the efficiency of nurseries in producing healthy citrus container seedlings.

Introduction

The cultivation of citrus plants is a significant contributor to the agricultural economy in numerous mountainous regions of southern China (Guo *et al.* 2019). As a result, the promotion of citrus container seedling cultivation has become an important development direction for the future of citrus production. The frequency of continuous planting of citrus varieties is increasing, as is the demand for citrus container seedlings from growers on a daily basis (Deng *et al.* 2022). Nevertheless, a considerable number of nursery bases in China continue to utilise open-air nurseries. While the open-air nursery method enhances space utilisation, it also results in a decline in soil fertility, an imbalance in soil nutrients, and a high incidence of pests and diseases due to the continuous cultivation of the planting soil. The presence of harmful microorganisms in the soil can have a detrimental impact on crop growth. Continuous cropping can disrupt the physicochemical properties and nutrient balance of the soil, thereby impeding crop growth (Lei *et al.* 2019, Wang *et al.* 2023).

Previous studies utilising diverse soil amendments have demonstrated their capacity to regulate soil nutrient imbalances, enhance soil physicochemical attributes, and modify microbial communities (Lv *et al.* 2018). Earlier studies have demonstrated that attapulgite is an effective agent for enhancing the physicochemical properties of soil. Bentonite possesses a series of excellent physicochemical properties, which have a beneficial impact on soil improvement and restoration. These properties include strong swelling, high water absorption, and effective adsorption and adhesion. Effective microorganism (EM) is a composite bacterial flora comprising a variety of microorganisms. It can regulate soil pH and reduce soil salinity. The application of EM has been observed to have effects on phosphorus, potassium solubilisation, and nitrogen fixation. It has also been demonstrated to inhibit microbial metabolism during the synthesis of

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secondary metabolites, which can improve the inhibition of harmful pathogens (Kong *et al.* 2013). However, there is a paucity of research examining the impact of inorganic soil conditioners with EM microbial agents on soil quality in citrus container seedling cultivation. This study explored the effects of soil amendments and effective microbial agents on the growth of container seedlings and the incidence of citrus canker, and provided improvement measures for producing healthy citrus container seedlings.

Materials and Methods

The experiment was conducted in a greenhouse of Zunyi Normal University, China under an average room temperature of 25°C and a humidity level of 70%. The citrus variety tested in the experiment was the two-year-old *Citrus reticulata* Ehime 28. The plants were cultivated in containers with an approximate diameter of 28 cm, a height of 34 cm, and a total capacity of 18 L. The soil used in the experiment was collected from a citrus plantation. The bentonite, attapulgite and EM microbial agents used in the experiment were provided by Chengdu Hengyi Chemical Products Co. and Shanghai Sansheng Biotechnology Co., respectively.

A pot experiment was designed by mixing different proportions of bentonite, attapulgite, and natural soil, and irrigating the roots with different concentrations of EM bacterial solutions. The control group (CK) used natural soil, and all treatments are presented in the Table 1. All management measures, such as fertilisation, irrigation, pest control, and weed control, during cultivation were consistent across the control and treatment groups. The relevant indicators were measured after 50 days.

Table 1. Treatments designed.

Ttreatments	Natural soil proportion (%)	Attapulgite proportion (%)	Bentonite proportion (%)	EM microbial agents proportion (Times)
T1	70	20	10	300
T2	60	30	10	300
T3	70	10	20	300
T4	60	10	30	300
T5	70	20	10	200
T6	60	30	10	200
T7	70	10	20	200
T8	60	10	30	200

In this study, a ring cutter of a specified volume was employed to excise an unstirred soil sample in its natural state, and the excised soil was subsequently weighed to calculate the ratio of the weight of a unit volume of dried soil (105°C) to the weight of the same volume of water. The soil weight ratio was determined by placing a known weight of the soil in a liquid, draining the air, and measuring the volume of the liquid displaced by the soil. This volume was then divided by the weight of the soil in its dried at 105°C to obtain the soil weight. The total soil porosity was calculated. The soil pH was determined using a standard protocol. The soil electrical conductivity (EC) was determined using a conductivity meter (BEC-520, Bell Analytical Instruments Ltd.). Soil Olsen-P was determined using the $NaHCO_3$ extraction molybdenum antimony colorimetric method. Soil Olsen-K was determined via the NH_4OAC extraction flame photometry method. Soil total nitrogen was determined using the Kjeldahl nitrogen determination method, and soil organic matter was determined using the potassium dichromate volumetric method with dilution calorimetry.

The SPAD value of the leaves was determined by wiping the samples with a moist and measuring them using a portable chlorophyll meter (Konica Minolta, Japan, SPAD-502plus). The leaf area was calculated. The main root length was measured, using a straight edge to determine the distance from the root tip of the plant's main root to the point where the root separated from the stem. The dry weight of the entire plant was determined using an electronic scale. The H_2SO_4-H_2O_2 method was used for digestion, and the semi-trace Kjeldahl nitrogen determination method was used to determine the total nitrogen content in the sample. The molybdenum antimony colorimetric method was used to determine the total phosphorus content, and flame photometry was used to determine the total potassium content.

The total number of leaves and diseased leaves at each level were investigated. The grading standard for diseased leaves was: level 0: no lesion; level 1: 1-5 lesions per leaf; level 3: 6-10 lesions per leaf; level 5: 11-15 lesions per leaf; level 7: 16-20 lesions per leaf; and level 9: more than 21 lesions on each leaf. The formula for calculating the disease prevention effect is as follows:

$$\text{Disease index} = \frac{\sum \text{(number of diseased leaves at each level} \times \text{relative level value)}}{\text{(total number of surveyed leaves} \times 9)} \times 100$$

Three biological replicates were used for each treatment. The data were subjected to ANOVA using the SPSS software, and significance of differences was analysed via multiple comparisons ($p < 0.05$).

Results and Discussion

The physical and chemical indicators of the soil samples subjected to different treatments are presented in Table 2. The bulk density of the diverse soil samples ranged from 1.29 to 1.43 g/cm^3, the total porosity from 42.0 to 56.2%, and the EC value from 297.0 to 468.3 μS/cm, indicating that the soil samples subjected to different treatments exhibited disparate physicochemical properties. The different treatments had varying effects on the soil unit weight, total soil porosity, pH, and EC. The T1, T2, and T3 treatments resulted in a notable reduction in soil unit weight, with the T1 treatment exhibiting a 9.8% decrease. Conversely, all treatments demonstrated an increase in total soil porosity. The T4 treatment exhibited the most pronounced effect, with an increase of 33.8%. All treatments also demonstrated a significant elevation in soil pH relative to the control (CK), with the T6 and T7 treatments displaying the most pronounced effect, showing an increase of 33.8%. Compared to the control treatment, all other treatments resulted in a significant increase in soil pH. The most pronounced increase was observed in the T6 and T7 treatments, which exhibited an increase of 7.9 and 8.5% in pH, respectively. Additionally, all treatments, with the exception of T2, led to an elevation in soil EC. The greatest increase was observed in the T6 and T7 treatments, which demonstrated an increase of 50% in EC. These results demonstrated that the combination of bentonite, attapulgite, and EM microbial agents resulted in a reduction in soil unit weight but an increase in total porosity, as well as pH and EC values.

Table 3 illustrates the impact of different treatments on soil active ingredients. The soil organic matter content ranged from 25.67 to 29.93 g/kg, while the total nitrogen content ranged from 1.02 to 1.16 g/kg. The Olsen-P content was found to range from 25.00 to 72.73 mg/kg, and the Olsen-K content ranged from 25.23 to 43.27 mg/kg. No notable alterations in soil organic matter content were discerned following the implementation of the treatments. However, in all treatments except for T4 and T7, there were discernible changes in the soil total nitrogen content. However, all treatments resulted in a significant increase in the soil Olsen-P content, with the T8 treatment demonstrating the most pronounced effect, followed by the T1, T2, and T4 treatments.

Concomitantly, all treatments demonstrated a significant increase in the soil Olsen-K content, with T4 and T5 exhibiting the most pronounced effect, showing an increase of 71.5 and 62.0%, respectively. These results demonstrated that the formulation comprising bentonite, attapulgite, and EM microbial agents was unable to enhance soil organic matter and total nitrogen content. However, it was effective in augmenting the levels of Olsen-P and Olsen-K in the soil. The T4 treatment exhibited the most pronounced impact when all indicators were considered in a holistic manner.

Table 2. Soil physical and chemical indicators under different treatments.

Treatments	Soil unit weight (g/cm^{-3})	Total porosity (%)	pH value	EC value (μS/cm)
CK	1.43 ± 0.05a*	42.0 ± 1.80e	6.32 ± 0.11c	297.0 ± 13.0e
T1	1.29 ± 0.06c	54.2 ± 1.95ab	6.64 ± 0.06b	356.0 ± 14.2bc
T2	1.34 ± 0.02bc	52.7 ± 1.06bc	6.62 ± 0.11b	317.0 ± 16.8de
T3	1.36 ± 0.04b	53.2 ± 2.58abc	6.66 ± 0.09b	346.0 ± 15.9c
T4	1.40 ± 0.01ab	56.2 ± 2.07a*	6.63 ± 0.13b	329.7 ± 9.6cd
T5	1.38 ± 0.05ab	50.5 ± 0.91c	6.64 ± 0.18b	354.0 ± 13.5bc
T6	1.39 ± 0.01ab	45.2 ± 1.50d	6.82 ± 0.07a*	468.3 ± 17.0a*
T7	1.41 ± 0.02ab	46.0 ± 1.99d	6.86 ± 0.07a*	455.0 ± 15.1a*
T8	1.38 ± 0.03ab	45.9 ± 1.16d	6.60 ± 0.14b	377.7 ± 17.8b

*Lower-case letters represent 0.05 level.

Table 3. Soil active ingredients under different treatments.

Treatments	Organic matter content (g/kg)	Total nitrogen content (g/kg)	Olsen-P content (mg/kg)	Olsen-K content (mg/kg)
CK	28.33 ± 0.92ab	1.12 ± 0.07ab	25.00 ± 4.36e	25.23 ± 1.50e
T1	29.96 ± 2.69a*	1.18 ± 0.06a*	56.33 ± 7.09b	28.60 ± 0.82d
T2	29.93 ± 0.32a*	1.03 ± 0.08ab	58.37 ± 1.42b	34.07 ± 1.38c
T3	29.51 ± 1.85a*	1.16 ± 0.06a*	39.00 ± 7.01cd	37.24 ± 1.39b
T4	25.67 ± 0.78b	1.02 ± 0.04b	61.67 ± 1.53b	43.27 ± 2.15a*
T5	28.66 ± 0.60ab	1.08 ± 0.11ab	44.70 ± 1.99c	40.86 ± 1.18a*
T6	29.43 ± 2.38a*	1.08 ± 0.07ab	39.67 ± 1.53cd	30.45 ± 1.00d
T7	27.08 ± 2.71ab	1.00 ± 0.05b	34.30 ± 1.49d	33.28 ± 1.15c
T8	29.85 ± 0.33a*	1.11 ± 0.10ab	72.73 ± 8.51a*	30.25 ± 2.31d

*Lower-case letters represent 0.05 level.

This study used a combination of inorganic soil amendments and EM microbial agents and found that it had significant effects on the soil unit weight, pH value, and EC value. The effective components, such as organic matter, protein, total nitrogen, available phosphorus, and available potassium, in the soil were also significantly improved. These results are similar to the results reported in previous studies regarding the effects of inorganic amendments on soil improvement in the cultivation of crops such as garlic and tea (Zhou *et al.* 2013).

In this study, the treatments significantly increased the plant leaf area and SPAD value; promoted plant biomass accumulation; and increased the nitrogen, phosphorus, and potassium contents. Previous studies have also shown that the application of conditioning agents can effectively induce an increase in citrus leaf weight and SPAD value, as well as promote the absorption and utilisation of nitrogen, phosphorus, and potassium in leaves (Sun *et al.* 2021).

Table 4 illustrates the impact of different treatments on the growth of citrus plants. As shown in the table, the primary root length of the citrus plants exhibited minimal variation under the various treatments. The T1, T2, and T4 treatments notably enhanced the leaf area of the plants, with a 27.7% increase observed for the T4 treatment. Additionally, the SPAD value of the leaves was significantly elevated by 13.7% under this treatment. All treatments had an impact on the biomass of the citrus plants, with the T4 treatment demonstrating the most significant increase in whole-plant dry weight (21.3%), followed by T3 and T8. The formulation comprising 60% natural soil, 10% attapulgite, 30% bentonite, and 300 times EM bacterial solution was found to significantly promote the growth of leaves and chlorophyll formation, as well as favouring the accumulation of plant biomass.

Table 4. Effects of different treatments on the growth of citrus plants.

Treatments	Length of main root (cm)	Leaf area (cm^2)	SPAD value	Dry weight of plant (g)
CK	30.03 ± 2.16ab	2349.16 ± 116.56bc	27.27 ± 2.66bc	340.13 ± 8.03cd
T1	26.47 ± 1.91ab	3193.17 ± 157.61a*	30.58 ± 1.41ab	353.63 ± 18.30cd
T2	26.40 ± 4.43ab	3177.47 ± 265.04a*	30.41 ± 2.19ab	344.20 ± 17.71cd
T3	27.10 ± 1.25ab	2529.58 ± 83.91bc	29.87 ± 1.70abc	382.27 ± 9.32b
T4	30.97 ± 2.76a*	3000.75 ± 187.40a*	31.01 ± 3.06a*	412.53 ± 17.81a*
T5	27.13 ± 1.05ab	2557.43 ± 165.59bc	27.91 ± 1.90abc	350.87 ± 8.40cd
T6	26.67 ± 1.99ab	2575.13 ± 144.71b	26.99 ± 1.63bc	331.43 ± 9.32d
T7	26.00 ± 3.21b	2262.79 ± 114.50c	26.41 ± 1.14c	359.73 ± 16.42bc
T8	26.73 ± 1.46ab	2487.92 ± 153.80bc	28.13 ± 1.77abc	380.23 ± 19.61b

*Lower-case letters represent 0.05 level.

Table 5. Total N, P and K content of citrus plants under different treatments.

Treatments	Total N content (g/kg)	Total P content (mg/kg)	Total K content (mg/kg)
CK	1.66 ± 0.03d	44.20 ± 2.89c	59.37 ± 0.95d
T1	1.25 ± 0.04e	51.78 ± 5.96bc	56.90 ± 1.30de
T2	1.34 ± 0.11e	44.35 ± 3.19c	56.50 ± 0.72de
T3	2.13 ± 0.03b	54.38 ± 3.20b	74.03 ± 1.91c
T4	2.38 ± 0.04a*	61.74 ± 2.19a*	73.87 ± 2.37c
T5	1.83 ± 0.02c	51.07 ± 3.90bc	71.73 ± 2.27c
T6	1.60 ± 0.11d	52.04 ± 2.00bc	88.97 ± 3.88a*
T7	1.88 ± 0.07c	55.70 ± 8.75b	83.63 ± 3.23b
T8	1.57 ± 0.17d	52.02 ± 2.06bc	53.27 ± 1.31e

*Lower-case letters represent 0.05 level.

The total nitrogen content of the citrus plants across all treatments ranged from 1.25 to 2.38 g/kg, the total phosphorus content ranged from 44.20 to 61.74 mg/kg, and the total potassium content ranged from 53.27 to 88.97 mg/kg (Table 5). The total nitrogen content of the citrus plants was significantly increased by the treatments, with the exception of T1, T2, and T8, which showed no significant change. The T4 treatment demonstrated the greatest increase in total nitrogen content, at 43.4%. Similarly, the T4 treatment showed the greatest increase in total phosphorus content, followed by T3 and T7. Furthermore, the T3, T4, T5, T6, and T7 treatments all demonstrated a significant increase in total potassium content. The aforementioned results illustrated that the mixture of 60% natural soil, 10% attapulgite, 30% bentonite, and a 300-fold

EM bacterial solution with natural soil can facilitate the growth of citrus plants and the accumulation of total nitrogen, total phosphorus, and total potassium.

The exchangeable cations and reactive -OH groups on the surface of a concave/convex rod can increase soil pH and promote the conversion rate of soil organic phosphorus. $Ca^{2+}$and Mg^{2+} in concave/convex rod materials can also reduce the content of soil acidic substances and enhance the buffering capacity of soil through ion exchange (Yang *et al.* 2020). This is consistent with the findings of this study, which found that mixing attapulgite and bentonite with natural soil and adding EM microbial agents can effectively increase the soil pH and EC values. Researchers have demonstrated in field experiments over two consecutive years that the application of attapulgite increased the pH value of sandy farmland soil by 3.7%, reduced the soil bulk density by 3.6%, and increased the soil total nitrogen content by 6.22% as well as the soil organic matter content to a certain extent (Niu *et al.* 2023). Another field experiment showed that the application of attapulgite increased the bulk density of sandy soil, and promoted the aboveground biomass of corn and the nutrient retention capacity of the 0-40 cm soil layer, while reducing the amount of chemical fertilisers applied (Liu *et al.* 2023). In this study, the potting test was employed, and it is evident that the combination of bentonite clay with natural soil, along with the simultaneous addition of an EM fungicide, could effectively reduce the soil bulk weight and increase the soil porosity. The soil organic matter and total nitrogen content remained unaltered; however, the soil quick-acting phosphorus and potassium contents exhibited a discernible increase. Another pot experiment on winter wheat (*Tritium aestivum* L.) showed that the application of attapulgite soil not only inhibited the absorption of cadmium, a heavy metal, by roots, but it also had a strong nutrient adsorption effect, which could reduce nutrient loss and improve fertiliser utilisation efficiency (Liang *et al.* 2019). As a nutrient carrier in soil, attapulgite clay minerals can effectively adsorb and retain nutrients in the soil. This adsorption and ion-exchange ability allows attapulgite to act as a stable nutrient reservoir in soil, reducing nutrient loss and leaching, which is beneficial for plants to absorb and utilise nutrients. At present, research on the mechanism of increasing the yield of palygorskite clay is mainly based on the fact that palygorskite clay contains many elements required by crops and has an impact on soil structure. However, most research studies still remain at the level of individual application or combined fertiliser application.

This study found that combining an EM bacterial solution with bentonite and attapulgite mixed into natural soil not only regulated soil physical and chemical properties, but also increased the availability and effective absorption of nutrients, as well as improved soil quality. After eight years of continuous application of bentonite in the field, the total nitrogen content of the soil was significantly higher than that of the untreated soil. One study found that using 12 kg/m^{-2} of bentonite significantly increased the organic matter content in the soil (Czaban *et al.* 2013). Researchers have reported that applying bentonite and zeolite to wheat, alone or in combination, promoted plant growth and increased underground yield and chemical composition (Youssef *et al.* 2013). An Australian study used cationic bentonite and demonstrated its ability to improve soil exchange properties and plant growth (Noble *et al.* 2001). Multiple studies have confirmed that bentonite can be widely used as a soil amendment in many humid or arid regions around the world to improve crop growth and yield. This is consistent with the results of this study, which used a pot experiment to visually assess that mixing attapulgite and bentonite with natural soil, and adding EM microbial agents, can improve soil physicochemical properties; increase plant total nitrogen, total phosphorus, and total potassium contents; increase leaf number, leaf area, and leaf SPAD value; and promote biomass accumulation as demonstrated in the increase in whole-plant dry weight. Among the different treatments, T4 (60% natural soil + 10% attapulgite + 30% bentonite + 300 times EM bacterial solution) exhibited the most significant effects.

All treatments demonstrated a superior preventive efficacy against canker disease compared to the control. The total number of leaves examined across all treatments exceeded that of the control, with T4 exhibiting the highest number of leaves. Additionally, the proportion of diseased leaves in T7 and T8 was significantly lower than that observed in other treatments. The incidence of canker disease was markedly lower in all treatment groups compared to the control, with six treatments-T3, T4, T5, T6, T7, and T8 exhibiting the lowest disease indices, followed by the T1 and T2 treatments. When evaluated using a combined disease index, the T4 treatment (60% natural soil, 10% attapulgite, 30% bentonite, and a 300-fold dilution of the EM bacterial solution) demonstrated the greatest impact on the leaf number and the prevention of citrus canker disease.

Table 6. Effects of different treatments on incidence of citrus canker.

Treatments	No. of leaves	No. of infected leaves	Infection rates (%)	Disease index
CK	110 ± 7.02d	21 ± 3.06a*	18.78 ± 1.56a*	1.12 ± 0.05a*
T1	124 ± 4.00bc	15 ± 2.31ab	11.87 ± 2.17b	0.53 ± 0.08b
T2	131 ± 6.11abc	17 ± 3.51ab	13.19 ± 2.63b	0.56 ± 0.07b
T3	126 ± 8.14bc	13 ± 2.52b	10.03 ± 1.49b	0.33 ± 0.07c
T4	140 ± 5.00a*	16 ± 2.08ab	11.17 ± 1.21b	0.31 ± 0.07c
T5	127 ± 5.69bc	15 ± 2.65ab	11.17 ± 1.89b	0.40 ± 0.04c
T6	134 ± 5.13ab	18 ± 4.51ab	13.60 ± 2.96b	0.40 ± 0.07c
T7	130 ± 7.64abc	14 ± 4.04b	10.93 ± 2.57b	0.29 ± 0.07c
T8	122 ± 3.46c	14 ± 3.21b	11.26 ± 2.91b	0.33 ± 0.06c

*Lower-case letters represent 0.05 level.

It has been reported that *Bacillus subtilis* can achieve the prevention and control of citrus canker disease by inhibiting growth *in vitro* or by lysing bacterial cells (Jorge *et al.* 2021). In this study, the treatment of 70% natural soil + 10% attapulgite + 20% bentonite + 300 times EM bacterial solution significantly reduced the incidence rate of plant leaf cankers and control the disease index. The application of soil amendments and microbial agents has a synergistic effect on improving the physical properties and ecological environment of the soil plow layer, while also effectively promoting the growth of citrus plants. These findings provide a theoretical and practical basis for soil improvement in citrus container seedling cultivation.

EM is an effective microbial community composed of various microorganisms such as photosynthetic bacteria, lactic acid bacteria, yeast, and actinomycetes. EM microbial agents have been proven to be an effective soil activator that can improve soil properties and nutrient cycling, increase crop yield and quality, reduce the adverse effects of continuous cropping, increase beneficial soil microorganisms, and control pathogens through competitive exclusion (Javaid *et al.* 2011, Ndona *et al.* 2011, Ismail *et al.* 2013). This is consistent with the findings of this study, which used a pot experiment to visually assess the effects. The combined application of EM microbial agents and two inorganic amendments improved soil physicochemical properties, promoted plant nutrient absorption and transport, promoted biomass accumulation, and effectively reduced the occurrence of citrus canker disease. Among the treatments, the T4 treatment (60% natural soil + 10% attapulgite + 30% bentonite + 300 times EM bacterial solution) had the greatest effect on the prevention and control of citrus canker disease. This study combined beneficial microorganisms and two inorganic soil amendments to improve the soil for citrus container seedling cultivation. It is of great significance to improve soil structure and physicochemical properties, enhance soil fertility, and prevent diseases in citrus plant cultivation.

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