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Research Article
In vitro Biocontrol Potential of Agro-waste Compost to Suppress *Fusarium oxysporum*, the Causal Pathogen of Vascular Wilt Disease of Roselle

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Abstract

Background and Objective: Roselle (*Hibiscus sabdariffa* L.) is an important crop used in confectionery, cosmetic and pharmaceutical industries and vascular wilt caused by *Fusarium oxysporum* is the obstacles in roselle production. Compost has been use to control disease infection in various crops through several suppression mechanisms. However, the bio-efficacy of agro-waste compost in suppression of *F. oxysporum* in roselle is still unknown. The aim of this study was to evaluate the bio-efficacy of agro-waste compost that would be potential soil suppressive amendment to control *F. oxysporum* of roselle. **Materials and Methods:** Three types of agro-waste composts: Vermicompost, crop residue and horse manure compost were used in this study. The direct suppression effects of compost and compost extract on the growth of *F. oxysporum* were determined. The total microbial population in compost was also evaluated. **Results:** Generally, the non-sterilized agro-waste compost shown prominent suppressive effects on mycelial growth of *F. oxysporum* over sterilized compost. Similar results were observed when non-sterilized agro-waste compost extracts at 5, 10, 25 and 50% concentrations were used to suppress the growth of *F. oxysporum*. Non-sterilized horse manure compost extracts significantly inhibit *in vitro* mycelial growth of *F. oxysporum* with 70.84%. The total microbial activity in vermicompost was recorded significantly high with 6.46 µg/mL/0.5 h. However, the total microbial activity in vermicompost was not associated with the suppression effect against *F. oxysporum*. **Conclusion:** The suppression activities of compost against *F. oxysporum* was mainly caused by the biotic factor. However, the total microbial activity in agro-waste compost is not sorely contributed to the suppressive activity against *F. oxysporum*. The present of the strong antagonist in the non-sterilized agro-waste compost was the key factor to explain the high suppressiveness of the horse manure compost. Whereas, the abiotic factor involved indirectly influent the compost property and the colonization ability of the microorganisms. All composts used have potential to suppress the growth of *F. oxysporum*, especially the non-sterilized agro-waste composts have higher potential to be developed as soil suppressive amendment against *F. oxysporum*.

Key words: Biocontrol, agro-waste compost, *Fusarium oxysporum*, vascular wilt disease, roselle

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is one of the most important plants of the Malvaceae family which produce a fleshly red calyxes and epicalyxes (sepals)[1]. It has lots of benefits and functions including medicinal and food base purposes. However, due to plants conditions that usually being exposed to an environment either it is from biotic or abiotic stresses likely to inhibit their growth[2]. Most problems that stunted the plant growth are from vascular wilt disease[3]. *Fusarium* spp. were reported as the most common soil borne fungus pathogen in causing rotted root of roselle[4].

Application of chemical pesticide in controlling of vascular wilt disease of vegetable plant is not encouraged due to the hazardous residue. The suppression of soil borne fungal disease by compost application is well known[5], which protect plant directly and indirectly[6]. Various suppression mechanisms involved such as the production of fungitoxic compounds[5], hyperparasitism with the present of the antagonistic microorganism[7], competition for nutrients and evoke antibiosis or induced resistance against plant pathogens[5]. The combination of the biological (biotic) and physico-chemical (abiotic) characteristics of agro-waste compost mainly contributed to the suppressive activity against *Fusarium oxysporum* f. sp., *melonis*[5]. However, little or no literature information exists on exploring the potential of horse manure compost, crop residue compost and vermicompost to be used as potential soil suppressive compost against *F. oxysporum* in vascular wilt disease management of roselle production.

Besides, the recycling of agro-waste as compost amendment as soil conditioners and fertilizers helps to maintain, restore soil productivity[8,9] and improve soil biota through the significant increase of microbial biomass and basal respiration[10] towards sustainability in agriculture production. The disease suppressive phenomenon using agro-waste compost consist of a complex and complicated set of mechanisms, yet the exact mode of action is still unknown. The bio-efficacy of agro-waste compost in suppression of *F. oxysporum* in causing vascular wilt disease of roselle is still unknown. The aim of this study was to evaluate the bio-efficacy of agro-waste compost that would be potential to be used as soil suppressive amendment against *F. oxysporum* of roselle.

MATERIALS AND METHODS

Isolation and purification of the causal organism: The roselle plants with wilting and root rot symptoms were collected from Kuala Terengganu, Terengganu, Malaysia. The infected stem and root were carefully washed with running water to remove the adherence soil particles before surface sterilized with 5% of sodium hypochlorite. The infected vascular tissues were cut into small pieces (3 mm^2) and transferred into Potato Dextrose Agar (PDA). After 5 days of incubation at 28±2°C, the fungal colony growth on the PDA was transferred using hyphal tip isolation technique. The morphological characteristic was identified as described by Burgess *et al.*[11] and Leslie and Summerell[12].

Molecular identification of the causal organism: The obtained fungal culture was first cultured in Potato Dextrose Broth (PDB) in a rotary shaker (180 rpm) for 4 days at 28±2°C. The mycelia were harvested and ground in liquid nitrogen for DNA extraction. The DNA extraction was conducted using Wizard® Genomic DNA purification kit (A1120) supplied by Promega Corporation as according to the protocol provided by the manufacturer.

The DNA primer, Internal Transcribed Spacer (ITS), ITS4 (5′-TCC TCC GCT TT TGA TAT GC-3′) and ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) were used to amplified the DNA template which yield approximately 500 bp[13]. This primer was designed to anneal to the planking 18S to 28 rDNA genes. The PCR was performed by the following protocol: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturing at 90°C for 30 sec, annealing at 52.7°C and extension at 72°C for 1 min. For final extension is at 72°C for 10 min. The PCR product were then purified to obtain the DNA using Wizard® SV gel and PCR clean-up system (A9280) according to the protocol recommended by Promega Corporation. The DNA product were then sent for DNA sequencing.

Direct suppressive effect of compost on *Fusarium oxysporum* growth: A laboratory test was conducted to detect direct suppressive effect of compost[5]. Thirty mililitres of sterilized water was used to dislodge the conidia of *F. oxysporum* from the 7 days old culture. The spore suspension of *F. oxysporum* (0.1 mL) was spread on the fresh PDA plate. After 24 h of incubation at 28±2°C, the sterilized and non-sterilized compost sample (0.5 g) were placed at the centre of each PDA plate. The PDA plates containing compost samples (sterilized or non-sterilized compost) without *F. oxysporum* were served as controls. The plates were incubated at 28±2°C for 10 days and the development of *F. oxysporum* culture was measured. The suppresiveness effects of the compost samples were determined by measuring the diameter of the clear inhibitory zones formed around the compost samples. The pH and Electrical

Conductivity (EC) of the compost were determined with the mixture ration of 1:10. Ten grams of air-dried compost was placed into a conical flash containing 100 mL of distilled water and shook for 30 min in a rotary shaker (150 rpm). The mixture was allowed to precipitate and the supernatant liquid was used to determine the pH and EC value using a pH and EC meters.

Effect of compost extract on suppression of *Fusarium oxysporum*: The vermicompost, horse manure and crop residue composts were extracted following the method of Suarez-Estrella *et al*.[5]. The obtained extracts were used to measure the inhibitory effect of compost extracts on *F. oxysporum* growth. Each of the compost was suspended in sterilized water at ratio 1:2 (v/v) and incubated at 28±2°C for 48 h on a rotary shaker (150 rpm) and filtered through cheesecloth.

Potato Dextrose Agar (PDA) media was prepared and autoclaved with low water content. The compost filtered extract was then added in the respective concentrations (v/v): 0, 5, 10, 25 and 50%. Seven days old mycelia plug of *F. oxysporum* was placed at the centre of PDA plate and incubated for 10 days at 28±2°C before measuring for the colony diameter.

Total microbial activity in composts measuring using fluorescein diacetate hydrolysis (FDA): The total microbial activity in compost was measured by using FDA hydrolysis method[14]. One gram of compost sample were sieved (<2.88 mm) and placed in a conical flask. Then 7.5 mL of potassium phosphate buffer (pH 7.6, 60 mM) was added and shook at orbital shaker at 28±2°C. To start the reaction, 0.1 mL of FDA solution was added, incubated at the shaker for 30 min. The FDA solution was prepared by added 25 mg of FDA powder mixed with 25 mL acetone and kept at -20°C. For blanks that consist of compost and buffer mixture with FDA solution was replaced by 0.1 mL of acetone. After 30 min of incubation, 7.5 mL of chloroform:methanol (2:1) was added and mixed using vortex for 10 sec to stop the reaction. After that, the samples were centrifuged at low speed 2000 rpm for 2 min. The supernatant then was transferred into 1.5 mL micro centrifuge tube and centrifuged at 14000 rpm for 5 min to remove the suspended layer. Lastly the samples were then transferred again into a 1 mL cuvette to measure the absorbance at 490 nm (wavelengths) using a spectrophotometer against the blank.

To construct the calibration curve, fluorescein solution (2000 μg mL^{-1}) was prepared by dissolving fluorescein sodium salt (3' 6'-diacetatyl-fluorescein, Sigma-Aldrich) into potassium phosphate buffer (pH 7.6, 60 mM). A series of concentrations: 0, 50, 100, 150, 200 and 250 μL of this fluorescein solution were then transferred into 100 mL volumetric flasks, respectively and made up to the 100 mL with potassium phosphate buffer to form a fluorescein standard with 0, 1, 2, 3, 4 and 5 (μg mL^{-1}). A 7.5 mL aliquots of each concentration of fluorescein standard solution was pipetted into McCartney bottles and extracted with chloroform:methanol followed by determination the $OD_{490\ nm}$ of the clarified upper phase[14]. The calibration curve was used to calculate the colour intensity of fluorescein produced in each assay and all results expressed as μg fluorescein g^{-1} of dry sample/0.5 h of incubation with sodium salt as substrate.

Statistical analysis: All the *in vitro* experiments were conducted in complete randomized design with 5 replications. The experiment was analyzed using Duncan test with factorial treatment structure and the analysis of treatment effect was performed using SPSS. Means was separated by 95% significant levels. The correlation analysis was also conducted between the total microbial activity in compost and the inhibitory effect of the non-sterilized compost using Person's correlation.

RESULTS AND DISCUSSION

Isolation and identification: The causal pathogen of vascular wilt and root rot of roselle samples collected was identified as *F. oxysporum.* The mycelia of the pure culture were delica, white with purple tinge and the margin slightly lobed or smooth on PDA. Microconidia formed singly, oval to reniform without septa. The PCR amplification of *F. oxysporum* at ITS using primers ITS1 and ITS4 fragments is approximately at 500 bp (Fig. 1). The molecular identification from DNA sequencing results aligned using BLAST network services against NCBI nucleotide database (http://blast.ncbi. nlm.nih. gov/Blast.cgi). GenBank database confirmed that isolate tested was homology with 100% sequence similarity to *Fusarium oxysporum* isolate TVD_Fungal-Culture 71 with accession number of KF494069.1.

Direct suppressive effect of compost on *Fusarium oxysporum* growth: The suppressiveness of compost on plant pathogens was associated to improves plant health and provide direct and indirectly protection[6]. This was in agreement with our findings where all compost used in the experiment demonstrated suppression effects against the growth of *F. oxysporum* with inhibition percentage for more

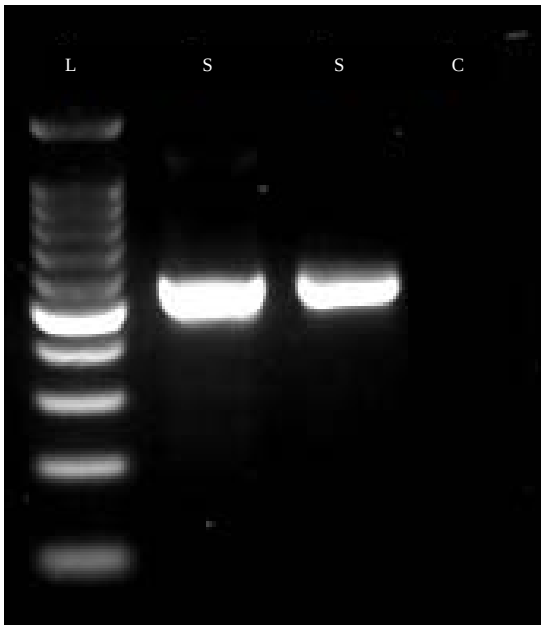

Fig. 1: PCR product of *Fusarium oxysporum* amplified using ITS1 and ITS4. Lane L: Ladder 1 kbp, Lane S: PCR products and Lane C: Control. The single band were observed at approximately 550 bp

Table 1: Direct suppressive effect of non-sterilized composts (inhibitory effect) on *Fusarium oxysporum* growth after 10 days of incubation

Composts	pH	EC (mS cm^{-1})	Mean inhibition (%)
Vermicompost	8.50	4.82	54.09±4.09^{a}
Crop residue	5.14	21.30	52.78±6.41^{a}
Horse manure	8.68	23.90	66.71±7.57^{a}

Means followed by same letters are not significantly ($p \leq 0.05$) different using Duncan test

than 50%. However, the inhibition effect of all composts treated against *F. oxysporum* was not significantly different (Table 1). The highest suppression of *F. oxysporum* was demonstrated by horse manure compost with 66.71% inhibition followed by vermicompost, 54.09% and crop residue compost, 52.78%.

The antagonistic microorganism that present in the compost may play an important role in suppression of the *F. oxysporum* and capable to antagonize the *F. oxysporum* growth. According to Hoitink and Boehm[15] and Ros *et al.*[16] two mechanisms might took part in the inhibition of the *Fusarium* spp., by mycoparasitism which involved direct contact between tested antagonist and phytopathogen in plates which cause further shrinkage in phytopathogen biomass while increase the size of antagonistic microorganisms. On the other hand, microbial antibiosis mechanism may also be involved as a clear inhibition zone between the antagonist

Table 2: Inhibition effect of different concentrations of compost extracts on *Fusarium oxysporum*

	Percentage of inhibition of *Fusarium oxysporum*	
Treatments	Sterilized compost	Non-sterilized compost
Vermicompost (%)		
0	0.00±0^{b,c}	0.00±0^{i}
5	12.58±5.65^{a}	48.96±3.44^{cd}
10	5.15±3.64^{b}	43.13±2.18^{de}
25	1.03±0^{b}	30.83±3.19^{fg}
50	4.91±1.94^{b}	36.25±1.07^{ef}
Crop residue (%)		
0	0.00±0^{c}	0.00±0^{i}
5	0.00±0^{c}	26.84±2.35^{gh}
10	3.74±2.65^{c}	21.00±3.19^{h}
25	1.82±1.82^{c}	38.10±3.90^{ef}
50	2.14±1.39^{c}	55.70±4.82^{bc}
Horse manure (%)		
0	0.00±0^{c}	0.00±0^{i}
5	6.87±1.47^{abc}	54.88±1.03^{bc}
10	3.72±1.81^{c}	58.71±1.93^{b}
25	5.67±0.47^{bc}	70.84±1.03^{a}
50	12.16±2.23^{ab}	52.00±5.08^{bc}

Means within column with same letters are not significantly different by Duncan test at $p \leq 0.05$

from the compost and the *F. oxysporum* culture were observed in the *in vitro* screening.

Effect of compost extract on suppression of *Fusarium oxysporum*: Several researches had already reported the suppression effect of compost was associated to the antagonist microorganism present[7,17]. In this *in vitro* investigation of abiotic factor on the suppressive effect of the compost extracts at various concentrations against *F. oxysporum*, the inhibition of *F. oxysporum* was recorded 10 days after incubation (Table 2). All of non-sterilized compost extracts exhibited higher suppression effects of *F. oxysporum* compared to sterilized compost (Table 2, Fig. 2-4). This was explained by heat eliminates all the potential antagonistic microorganism against *F. oxysporum* and did not show any suppression effect after incubated[18]. Our finding was in line with the results reported by Suarez-Estrella *et al.*[5], where the non-sterilized composts extracts showed stronger inhibitory effect on *F. oxysporum* f. sp., *melonis* over sterilized compost extracts.

The weak inhibitory effect or low antagonist capacity on *F. oxysporum* was detected at higher concentrations when sterilized vermicompost and crop residue compost extracts were applied. The additional nutrients in the medium from the compost extracts might influence the *in vitro* mycelial growth[19]. The micronutrients are essential for microbes, however, elevation of concentrations above threshold cause toxicity to microorganisms[20] including *F. oxysporum*[4].

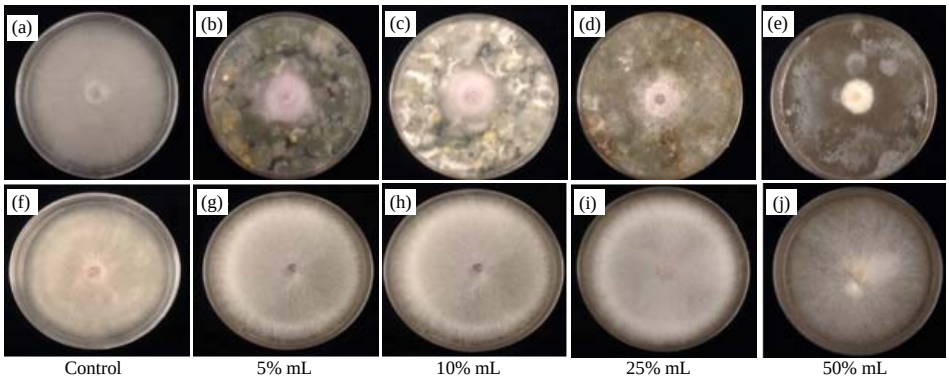

Fig. 2(a-j): Suppression effects of horse manure compost extract at different concentrations on *Fusarium oxysporum* development. (a-e) Non-sterilized horse manure compost extracts and (f-j) Sterilized crop residue compost extracts

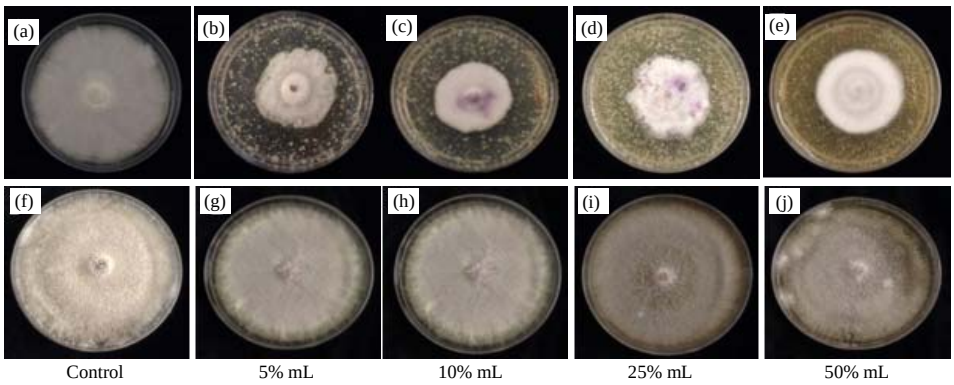

Fig. 3(a-j): Suppression effects of vermicompost extract at different concentrations on *Fusarium oxysporum* development. (a-e) Non-sterilized vermicompost extracts and (f-j) Sterilized vermicompost extracts

Table 3: Total microbial activity present in different compost based on the total enzymatic activity

Types of compost	FDA (μg/mL/0.5 h)
Vermicompost	6.46[a]
Crop residue compost	4.53[b]
Horse manure compost	0.86[c]

Means with the same letters are not significantly different by Duncan test $p \leq 0.05$

The suppression effect of compost towards *F. oxysporum* was not associated to the concentration of the compost extract used. For instance, in non-sterilized horse manure compost extract at 25% exhibited significantly higher inhibitory effect against *F. oxysporum* with 70.84% over other treatments (Table 2, Fig. 2). Vermicompost extract at all concentrations exhibited less than 50% inhibition effect against *F. oxysporum* (Table 2, Fig. 3). Fifty percent concentration of crop residue compost extract and horse manure compost extract at all concentrations exhibited more than 50% suppression of *F. oxysporum* (Table 2, Fig. 3, 4). This study indicated that the absent of microorganism and modification of heat sensitivity biotic and abiotic components after autoclaving caused totally or partially reduced the capacity of compost to suppress the growth of *F. oxysporum*[5]. According to Hoitink and Boehm[15], microbial communities in compost are likely to influence the inhibitory effect towards pathogen. In addition, the present of the strong antagonist in the compost could provoke the important effect on suppressive capacity towards pathogen.

Total microbial activity in composts measuring using fluorescein diacetate hydrolysis (FDA): Fluorescein diacetate (FDA) hydrolysis is a simple method for measuring the total microbial activity in a range of environmental samples, including soils and composts. The distribution of the total microbial activity present in three different types of compost represents the richness of microorganism activity in each types of compost that used in this experiment (Table 3). There were significantly different of total microbial activity between horse manure, crop residue and vermicompost. The highest microbial activity was found in vermicompost with

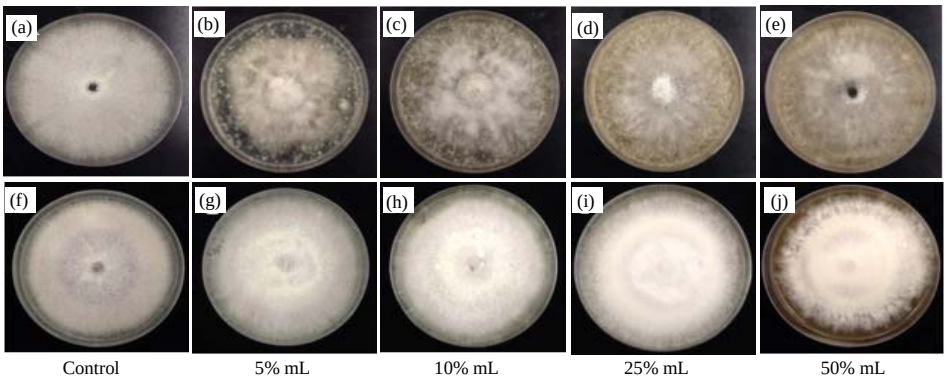

Fig. 4(a-j): Suppression effects of crop residue compost extract at different concentrations on *Fusarium oxysporum* development. (a-e) Non-sterilized crop residue compost extracts and (f-j) Sterilized crop residue compost extracts

6.46 µg/mL/0.5 h, followed by crop residue compost with 4.53 µg/mL/0.5 h and horse manure compost which was 0.83 µg/mL/0.5 h. All of these values were measured by the amount of hydrolysis which the end product fluorescein that absorb at 490 nm wavelengths.

However, the total microbial activity in compost obtained were negative correlated with the inhibitory effect of the non-sterilized composts ($r = -0.9084$, $R^2 = 0.8252$). Therefore, the total microorganism population (based on total microbial activity) may not exactly reflect the compost suppression ability. In this study, high microbial activity in vermicompost did not indicate the presence of the antagonistic microflora that gives suppressive effect to the *F. oxysporum*. This was possibly explained that the strong antagonist that present in horse manure compost play a driving factor to inhibit *F. oxysporum* that might absent in other composts used. Moreover, compost suppressiveness effect was also reported due to the combination of biological (biotic) and physico-chemical (biotic) properties[5,21]. Although, no clear relationships were observed between the inhibitory effects related to pH and EC values, the abiotic factors could be acting indirectly on the establishment of the compost property[5].

CONCLUSION

Here concluded that the agro-waste composts used have *in vitro* suppression effects against *F. oxysporum.* The execution of *in vitro* data obtained is important to imply that the suppression activities of compost against *F. oxysporum* was mainly caused by the biotic factor especially the present of the strong antagonist in the compost. Whereas, the abiotic factor involved indirectly influent the compost property and the colonization ability of the microorganisms. Moreover, screening and identification of the strong antagonist that present in horse manure compost is needed to increase the suppression capability towards sustainability of *F. oxysporum* management in roselle cultivation.

SIGNIFICANT STATEMENTS

- No study has been conducted on the suppression of *F. oxysporum* using agro-waste compost in vascular wilt disease management of roselle
- This study will provide an alternative in vascular wilt disease management of roselle by reducing chemical fungicide application
- Agro-waste compost application as soil suppressive amendment against *F. oxysporum* helps to sustain roselle production and the agriculture ecosystem

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