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Evaluating the Stability of Post Mushroom Substrate (PMS) and Other Agro-wastes for Mass Production of Entomopathogenic Fungi

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Authors' contributions

This work was carried out in collaboration among all authors. Author PR designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Authors KN, RM and RVK edited the whole draft. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The study was undertaken to evaluate agro-wastes (Post mushroom substrate, Sugarcane bagasse, rice husk and sorghum grains) for mass production of entomopathogenic fungi like, *I. fumosoroseus*:MT997932, *B. bassiana*: MT997933, *L. lecanii*: MT997935 and *H. thompsonii*: MT997936 by solid stat fermentation.
Place and Duration of Work: The study was carried out in the Department of Agricultural Microbiology, GKVK, University of Agricultural Sciences, Bangalore during 2019-20.
Methodology: The substrates were dried, chopped and sieved through 2mm sieve. 100g of all substrates were sterilized and moistened to 60% by adding sterile distilled water fallowed by

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addition of 5ml of spore suspension of fungal isolates. Treatments T_5. T_6, T_7, T_8 and T_{10} were fortified with adding 10ml of molasses. Observation like, growth and spore production were taken at 7, 14 and 21 days after inoculation.

Results: The growth and spore production of entomopathogenic fungal isolates were observed to be increased accordance with the incubation period. Among four substrates maximum mycelial growth and spore production of all the isolates ($\times 10^9$ spores g^{-1}) was observed in sorghum grains (T_4:T_8) fallowed by treatment T_7 (SMS+10% molasses) on 21 DAI. Whereas, sugarcane bagasse and paddy husk substrates were not supported the satisfactory growth and spore production besides the addition of molasses ($\times 10^7$ spores g^{-1}). The addition of molasses has positively influenced the growth and spore production of entomogenus isolates in all treatments.

Conclusion: Based on results, it is evidenced that even though cereal grains are best option for mass production, PMS fortified with molasses will become a better substrate for mass production and reduce the load of using food grains as substrate.

Keywords: Entomopathogenic fungi; agro-wastes; mass production; spent mushroom substrate; B. bassiana; L. lecanii.

1. INTRODUCTION

In modern agriculture, there is a decline in global crop losses due to various pests from 41.1% during 1988-90 to 32.1% during 2001-03 [1] because of extensive use approximately, 2.5 million tonnes of pesticides are annually [2] despite of the alarming problems like, development of resistance and resurgence of sucking pests [3] residual toxic effects to man, insect parasites, predators, animals and also the use will increase the cost of production. In view of these side effects, it is necessary to find an alternative, sustainable and eco-friendly pest management technique is being largely felt in the recent times.

Entomopathogenic fungi are potentially the most diverse and versatile biological control agents, due to their wide host range that often results in natural Epizootics. These fungi have certain advantages in pest control programs over other insect pathogens because they infect all stages of insect, they directly infect pest through cuticle as other agents need ingestion hence these can even infect sucking and piercing pest also (Hajeck and Leger, 1994), high host specificity, negligible effect on non-target organisms and mass production techniques are simpler, easier, cheaper compare to the other microbial agents and their persistence nature.

The growth requirements of most entomopathogenic fungi have been poorly defined despite the fact that this information is essential for mass production. The choice of the nutrients will obviously be directly related to the nutritional requirements of the selected fungus. Entomopathogenic fungi require oxygen, water, an organic source of carbon and energy, a source of inorganic or organic nitrogen and additional elements including minerals and growth factors [4]. Production of adequate quantities of a good quality inoculum is an essential component of the biocontrol programme. A wide variety of organic materials have been evaluated as substrates for the mass production entomopathogenic fungi. Although rice and barley seem to be the major substrates used in the tropics and the Northern Hemisphere respectively [5], there has been considerable effort to identify low-cost agricultural materials, especially byproducts and waste products, as suitable substrates for mass production have taken by numerous researches from different countries (Table 1). There are different methods of mass production like, solid state fermentation [6,7], liquid fermentation, submerged stat fermentation and biphasic culture system [8,9] based on the type substrates, out of all, solid state fermentation has emerged as an appropriate technology.

After mushroom cultivation, the partially degraded paddy or wheat straw and other agricultural wastes, which form as valuable by-products of edible mushroom cultivation have been termed as Spent Mushroom Substrate (SMS). Recently, the term spent compost or spent mushroom substrate has been replaced by a more appropriate term, “post mushroom substrate” (PMS) because it is not ‘spent’ and is ready to be further attacked by a new set of microorganisms. Post mushroom substrate (PMS) normally contains 1.9:0.4:2.4%, N-P-K with a C: N ratio of 9 to 15: 1, pH- 5.8 - 7.7 along with other nutrients like Mg, Ca, Al and Fe [10], hence it can be used as a substrate for

mass production of agriculturally important microorganisms, fungus in particular.

It is estimated that production of 1 kilogram of mushroom generates about 5 kg of PMS. Every year mushroom industry needs to dispose more than 50 million tonnes of PMS. In some countries (e.g., China produces more than 150 thousand tonne SMS per year) the management of spent mushroom substrates poses many difficulties and if not handled properly this may cause various environmental problems, including ground water contamination and nuisance [11]. Based on its nutrient contents it can be used as an alternative substrate for mass production of entomopathogenic fungi through solid stat fermentation (Table 2).

The other agriculture bioproducts or agro-wastes like, sugarcane bagasse produced during sugar production from sugarcane. Average of 140 kg of bagasse are produced for every ton of sugarcane processed thus, this is the most abundant lignocellulosic residue [12]. In general, bagasse composition consists of approximately 30-36% cellulose, 25-28% hemicellulose, and 20-21% lignin [13]. The rice husk, also called rice hull, is the coating on a seed or grain of rice, each kg of milled white rice results in roughly 0.28 kg of rice husk as a by-product of rice production during milling, approximately 120 million tons of rice husk is available each year after it has been removed from the whole rice paddy. Rice husk is composed of 15% carbon, 18% ash, and 67% volatile matter [14].

The success of microbial control of insects/pests depends not only on their pathogenicity, but also on the successful mass production of the microbial control agents. For a successful integrated pest management programme, the agents like the ENPF should be amenable to easy and cheap mass multiplication. The use of agro-industrial wastes in SSF for production of ENPF is of particular interest due to their availability and low cost, besides being an environment friendly alternative for their disposal [15]. Hence the present study was under taken to use this agro-wastes as a substrate for mass production of potential entomopathogenic fungal agents.

2. MATERIALS AND METHODS

2.1 Entomopathogenic Fungal Isolates

Four entomopathogenic fungal isolates, *B. bassiana, L. lecanii, H. thompsonii* and *I. fumoroseus*, were isolated from two Agro-Climatic Zones (Eastern Dry Zone and Southern Dry Zone) in Karnataka, India. The EPF Spore suspension was prepared by adding 10 ml of 0.5% sterile Tween 80 to 10-Day-Old cultures, and the concentration was adjusted to 10^8 Conidia ml^{-1} using an improved Neubauer Haemocytometer.

2.2 Substrate Collection and Preparation

The agricultural waste materials, including sugarcane bagasse (collected from the VC Farm Mandya at the College of Agriculture, University of Agricultural Sciences, GKVK, Bangalore), paddy husk (procured from a paddy mill), and PMS (collected from the Mushroom Lab at the Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bangalore), underwent a preparation process. The materials were first shade dried for 5 days, then chopped into small pieces using a chop cutter. The resulting substrates were ground and sieved through a 2mm sieve. Finally, the processed substrates were packed in airtight polypropylene bags and stored at 25°C.

2.3 Solid State Fermentation

The SSF was performed to in order to select the best alternative substrate other than cereals (sorghum) for mass production of ENF. The study of SSF was carried out in Erlenmeyer flasks (500 ml). Two set of experiments were carried out for all substrates (with and without fortification). The dried substrates (100g) were weighed and transferred to flasks separately and initial moisture percentage was adjusted to 40-50 by adding sterile distilled water. The substrates were inoculated with 5ml of conidial suspension of all four isolates containing 1×10^8 conidia ml^{-1} separately to all flasks contains different substrates. In second set of experiment the substrates were fortified with 10 percent molasses and inoculated with all isolates. The flasks were incubated for 21 days and spore count was checked for every 7 days interval using hemocytometer by diluting 1gm substrate from each treatment in 10 ml water blank [16].

2.4 Statistical Analysis

The conidial production of entomopathogenic fungi from different substrates were subjected to analysis of variance (ANOVA) using SPSS 10.0 for windows software (SPSS, 1999). The means were separated using CRD and differences

between treatments were considered significant at $p = 0.05$.

3. RESULTS AND DISCUSSION

The results of the evaluation of different agro-wastes for mass production of entomopathogenic fungi (EPF) showed that each substrate had a distinct impact on the growth and conidia production of EPF. Out of 10 treatments, the treatment T8 (sorghum grains fortified with 10% molasses) showed the highest spore count ($\times10^{10}$ conidia g-1) at 21 days after inoculation (DAI), followed by t4 (sorghum grains without fortification) and T10 (25% rice husk + 25% bagasse + 25% PMS + 25% sorghum grains + 10% molasses). The evaluation of different agro-wastes for mass production of *B. Bassiana* and *I. Lecanii* revealed that the highest conidial counts (8.43×10^4 and 8.30×10^4 respectively) were recorded in T8 (sorghum grains + 10% molasses) at 7 DAI, followed by T4 (1.33×10^4 and 1.33×10^4) and t10 (5.70×10^4). The lowest conidial count (300 and 700) was observed in treatment T1 (only rice husk) [17].

The spore production increased in all treatments over the incubation period. On the 14th DAI, the yield of conidia by *B. bassiana* significantly increased due to increased growth. The highest spore count (84.6×10^6) was observed in treatment t8 (crushed sorghum grains + 10% molasses), followed by t4 (18.6×10^6). The lowest spore count (6.0×10^4) was recorded in treatments T2 and t3 inoculated with *Beauveria* isolates. At 21 DAI, there was a further increase in the conidial count of entomopathogenic isolates (from 10^6 conidia/gm to 109 conidiospore/gm of the substrate) compared to the results of the 14th DAI. Treatment T8 (crushed sorghum grains + 10% molasses) showed a drastic increase in the spore count ($\times10^9$ conidia/gm of the substrate), followed by treatment T4 (crushed sorghum grains). The lowest spore count was recorded in treatments T1 and T2, with $\times10^6$ conidiospore per gm (Table 3).

Lecanicillium lecanii, Hirsutella thompsonii, and *Isaria fumosoroseus* produced the highest number of conidia per gram of substrate in treatment T8, which consisted of sorghum grains and 10 per cent molasses. The treatment t4 followed the t8 in conidia production, while the lowest conidia count was observed in the treatments T1 and T2. The production of spores was higher in all isolates, with 10^4 conidiospore per gram, compared to *B. Bassiana* isolates, which produced 10^3 conidia per gram, on the seventh day after inoculation. The results of evaluating different agro-wastes for mass-producing the entomopathogenic fungi H. thompsonii and *Isaria fumosoroseus* are presented in Table 4.

The spore density of all isolates significantly increased from 7 days after inoculation (DAI) to 21 DAI. Initially, treatments with sugarcane bagasse, paddy husk, and PMS showed a lower spore count, but it gradually increased compared to treatments that received sorghum grains and 10% molasses. The spore count of *B. bassiana* in T7 and T3 (PMS with and without fortification with 10% molasses) was $1.33\text{x}10^3$, $1.66\text{x}10^6$, $0.30\text{x}10^9$ and $0.30\text{x}10^3$, $0.33\text{x}10^6$, $0.06\text{x}10^9$ at 7th, 14th, and 21 DAI, respectively (Table 3). The spore density of the other isolates (L*. lecanii, H. thompsonii,* and *I. fumosoroseus*) on PMS was also in the same range, but on the 7th day, these isolates produced 10 times more conidia compared to *B. bassiana* on PMS (Table 3).

The post-mushroom substrate is relatively good substrate for the mass production of entomopathogens after cereal grain (sorghum), with a spore count ranging from 10^6 to 10^7 conidia g-1 of substrate. This is due to its properties, such as a low C:N ratio (14:1), pre-decomposed organic substrates, and a 2-4% protein content, which enhance the growth and development of fungi compared to rice husk (104 conidia g^{-1}), which has a C:N ratio of 85:1 and contains complex carbohydrates like lignin, pectin, and hemicellulose [18]. On the other hand, sugarcane bagasse contains approximately 47-52% cellulose, 25-28% hemicellulose, and 20-21% lignin, with a high C:N ratio (70-80:1). All these factors affect the fungus by limiting the availability of nutrients, resulting in poor growth and low spore production [13].

Similarly, Agale et al. [19] used ten different substrates, including chickpea, pigeon pea, black gram, maize, sorghum, soybean, rice, wheat, ground nut, and green gram, as well as two media, PDA and SDA, for the mass production of the entomopathogenic fungus M. anisopliae. The results showed that the highest conidial count (67.6×10^3 conidia/ml) was observed on green gram, followed by sorghum, in 10^3 dilutions, and the highest conidial count (63.7×10^3 conidia/ml) was observed on SDA media, followed by PDA (43.7×10^3 conidia/ml). This was further confirmed by studies conducted many scientists [20-23].

The increased conidia count in treatments T8, T4, and T10 was due to the increased availability of simple carbohydrates and other nutrients; for example, sorghum grains contain 32-57% starch, 8-15% protein, 5-15% sugar, and micronutrients (Fe 41-127; Zn 14-35; Ca 207-447; Mn 10-24; Na 12-54 and Mg 750-1506 @ mg/kg) (15). Since starch is a linear polysaccharide that can be easily utilized compared to complex carbohydrates (lignin, cellulose, hemicellulose, and pectin), the spore count in treatments T7 and T10 was satisfactory ($\times 10^8$) compared to treatments with sugarcane bagasse and rice husk but statistically lower compared to sorghum grains. The use of 10% molasses along with 25% sorghum grains has positively affected the growth and spore production, and there is a possibility of using PMS (presumably "partially molasses substrate") as a substrate for mass production instead of sorghum grains alone. This will reduce cost and the burden of using food grains for mass production. A previous study conducted by Dakshayani and Mallesha (2018) on using SMS as a substrate for the mass production of plant growth-promoting microorganisms revealed that SMS has the potential to be used as a substrate for mass production of biopesticides, including Trichoderma and other beneficial micro-organisms.

Previously, Pal and Prasad [15], were studied on mass production of various ENPF on nine different agriculture andindustrial wastes. The results revealed that maximum yield 278.75×10^6, 171.75×10^6 and 185×10^6 spores per ml of *B. bassiana, M. anisopliae, and V. lecanii* were obtained in FYM respectively, followed by Sabouraud dextrose broth (246.26×10^6, 157.25×10^6 and 180.00×10^6 spores per ml) and lowest yield was obtained in sugarcane bagasse (65.25×10^6, 34.25×10^6 and 39.00×10^6 spores per ml) [15]. similarly, Agale [19], were used ten different substrates (chickpea, pigeon pea, black gram, maize, sorghum, soybean, rice, wheat, ground nut and green gram) and two media like PDA and SDA for mass production of entomopathogenic fungi *M. anisopliae*. The result revealed that significantly highest conidial count (67.6×10^3 spores/ml) was observed on green gram followed by sorghum in 10^3 dilutions and Highest conidial count (63.7×10^3 spores/ml) was observed on SDB media followed by PDB (43.7×10^3 spores/ml) [19]. Similar findings were obtained by many other researchers [20-23].

Among all the treatments, treatments fortified with 10 percent molasses were exhibited higher conidial count in compare with treatment without molasses, this is because molasses composed of roughly 55 percent sucrose and other sugars, 20 percent water, 15 percent organic non-sugars, and 10 percent ash [24]. These easily available sugars and nutrients were promoted the initial growth of fungus in compare with nonfortified treatments where in fungal isolates have to produce enzymes to convert complex carbohydrates in to sugars this process demands and consumes most of the energy generated by the organisms leads to early sporulation with low spore count.

Table 1. Solid substrates evaluated for production of the principal entomopathogenic fungi

Sl. No.	Substrate/s	Organism/s	Reference
1	Green gram, Sorghum	*Metarhizium anisopliae*	[19]
2	Agricultural products	*Beauveria bassiana*	[11]
3	Apple pomace (AP)	*Lecanicillium lecanii*, *Beauveria bassiana, Paecilomyces fumosoroseus*	[25]
4	Broken rice grains, Rice hulls	*B. bassiana namaste Metarhizium anisopliae*	[26]
5	Sorghum, Rice, Wheat, Refuse Potato Chips and Refuse Banana Chips	*Nomuraea rileyi*	[27,28]
6	FYM, Sugar industry Press mud, Sugarcane bagasse, *Corcyra* rearing waste (Maize) and Jawar grain + 1.0 g Dextrose	*Beauveria bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii*	[29]
7	sugar cane, corn, barley, rice, millet and sorghum	*Beauveria bassiana*	[30]

Table 2. Scenario of total production of different agro-wastes in India and world

Sl. no	Substrate	India (MT/annum)	World (MT/annum)	Reference
1	Sugarcane Bagasse	16	300	[31]
2	Molasses	20	**	[31]
3	Post mushroom substrate (PMS)	7.5	50MT	[11]
4	Rice husk	24 mt	120 mt	[32,33]

Table 3. Evaluation of different agro-wastes for mass production of entomopathogenic fungi *Beauveria bassiana* and *L. lecanii*

Treatments	Treatments details	*Beauveria bassiana*			*Lecanicillium lecanii*		
		Spore density (Days after inoculation)			Spore density (Days after inoculation)		
		7 DAI ($\times10^4$)	14 DAI ($\times10^6$)	21 DAI ($\times10^9$)	7 DAI ($\times10^4$)	14 DAI ($\times10^6$)	21 DAI ($\times10^9$)
T_1	Rice husk	0.03 ± 0.02^{ef}	0.06 ± 0.03^{fg}	0.008 ± 0.03^{h}	0.07 ± 0.04^{ef}	0.06 ± 0.02^{g}	0.002 ± 0.10^{h}
T_2	Bagasse	0.06 ± 0.03^{ef}	0.06 ± 0.03^{fg}	0.009 ± 0.18^{gh}	0.07 ± 0.04^{ef}	0.03 ± 0.01^{g}	0.007 ± 0.17^{h}
T_3	Post mushroom substrate (PMS)	0.30 ± 0.05^{cd}	0.33 ± 0.07^{de}	0.06 ± 0.22^{fg}	0.13 ± 0.07^{d}	0.13 ± 0.05^{f}	0.30 ± 0.22^{e}
T_4	Crushed Sorghum grains	1.33 ± 0.07^{b}	18.6 ± 0.47^{b}	1.3 ± 0.35^{b}	5.70 ± 0.09^{b}	18.7 ± 0.36^{b}	6.3 ± 0.27^{a}
T_5	Rice husk + 10 % molasses	0.30 ± 0.44^{cd}	0.63 ± 0.13^{d}	0.07 ± 0.66^{fg}	0.87 ± 0.48^{d}	0.23 ± 0.09^{de}	0.05 ± 0.51^{fg}
T_6	Bagasse + 10 % molasses	0.60 ± 0.49^{c}	0.70 ± 0.36^{d}	0.08 ± 1.12^{fg}	0.93 ± 0.51^{d}	0.67 ± 0.27^{e}	0.06 ± 0.91^{fg}
T_7	Post mushroom substrate + 10 % molasses	0.70 ± 0.53^{c}	1.33 ± 1.00^{cd}	0.50 ± 0.76^{cd}	1.37 ± 0.64^{c}	2.30 ± 0.87^{d}	0.72 ± 1.52^{c}
T_8	Crush Sorghum grains + 10 % molasses	8.43 ± 0.73^{a}	84.6 ± 1.90^{a}	5.6 ± 1.88^{a}	8.30 ± 0.73^{a}	78.7 ± 1.42^{a}	6.6 ± 1.48^{a}
T_9	25 % Rice husk + 25 % Bagasse + 25 % PMS + 25 % Crushed Sorghum grains	0.66 ± 0.04^{e}	1.66 ± 0.42^{c}	0.16 ± 0.48^{e}	0.86 ± 0.04^{d}	0.37 ± 0.32^{de}	0.53 ± 0.45^{cd}
T_{10}	25 % Rice husk + 25 % Bagasse + 25 % PMS + 25 % Crushed Sorghum grains + 10 % molasses	1.33 ± 0.62^{b}	2.66 ± 1.06^{c}	0.60 ± 1.57^{c}	1.53 ± 0.53^{c}	9.63 ± 0.75^{c}	1.60 ± 0.82^{b}

Note: The values represented by same alphabet are statistically on par with each other by DMRT mean of three replications

Table 4. Evaluation of different agro-wastes for mass production of entomopathogenic fungi *H. thompsonii* and *Isaria fumosoroseus*

Treatments	Treatments details	*Hirsutella thompsonii*			*Isaria fumosoroseus*		
		Spore density (Days after inoculation)			Spore density (Days after inoculation)		
		7 ($\times10^3$)	14 ($\times10^6$)	21 ($\times 10^8$)	7 ($\times10^4$)	14 ($\times10^6$)	21 ($\times10^9$)
T_1	Rice husk	0.03±0.02^{ef}	0.06±0.10^{gh}	0.003±0.12^{fg}	0.03±0.02^{de}	0.06±0.12^{ef}	0.002±0.18^{f}
T_2	Bagasse	0.06±0.04^{ef}	0.03±0.12^{gh}	0.006±0.18^{fg}	0.02±0.02^{de}	0.03±0.11^{ef}	0.003±0.21^{f}
T_3	Post mushroom substrate (PMS)	0.13±0.07^{cd}	0.60±0.17^{e}	0.06±0.19^{de}	0.21±0.04^{bc}	0.23±0.20^{d}	0.03±0.25^{de}
T_4	Crushed Sorghum grains	1.66±0.09^{b}	27.3±0.21^{b}	1.30±0.33^{b}	1.33±0.07^{b}	31.3±0.27^{b}	4.20±0.41^{b}
T_5	Rice husk + 10 % molasses	0.26±0.42^{c}	0.70±0.57^{e}	0.03±0.59^{de}	0.66±0.42^{b}	0.27±0.54^{d}	0.07±0.66^{de}
T_6	Bagasse + 10 % molasses	0.31±0.46^{bc}	0.20±0.66^{ef}	0.07±1.00^{de}	0.53±0.51^{b}	0.63±0.77^{d}	0.07±1.09^{de}
T_7	Post mushroom substrate + 10 % molasses	0.66±0.35^{bc}	2.30±0.76^{cd}	0.60±0.83^{c}	0.60±0.33^{bc}	4.30±0.79^{c}	0.40±0.89^{c}
T_8	Crush Sorghum grains + 10 % molasses	4.30±0.78^{a}	84.7±1.00^{a}	9.70±2.10^{a}	5.70±0.84^{a}	79.6±1.09^{a}	7.30±2.27^{a}
T_9	25 % Rice husk + 25 % Bagasse + 25 % PMS + 25 % Crushed Sorghum grains	0.63±0.04^{bc}	0.63±0.18^{e}	0.08±0.53^{de}	0.70±0.04^{b}	0.63±0.27^{d}	0.16±0.64^{cd}
T_{10}	25 % Rice husk + 25 % Bagasse + 25 % PMS + 25 % Crushed Sorghum grains + 10 % molasses	0.98±0.69^{bc}	6.30±0.76^{c}	0.83±1.57^{bc}	0.97±0.64^{b}	4.70±0.81^{c}	0.73±1.71^{c}

Note: The values represented by same alphabet are statistically on par with each other by DMRT mean of three replications

4. CONCLUSION

In conclusion, the spore density and growth of entomopathogenic fungi can be significantly influenced by the type of substrate and medium used for mass production. PMS fortified with 10% molasses was found to be more favorable for the growth and development of entomopathogenic fungi in comparison to other substrates like sugarcane bagasse and paddy husk. The addition of molasses in PMS provided the necessary nutrients and sugars for the initial growth of the fungi, leading to an increase in spore density. Further research is necessary to determine the optimal concentration and type of molasses for mass production of entomopathogenic fungi.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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