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Molecular diagnosis and induced systemic protection against rhizome rot disease of ginger caused by *Pythium aphanidermatum*

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Polyclonal antibodies and antigens of host and pathogen were used for early diagnosis of rhizome rot disease of ginger. *Pythium aphanidermatum*, a causal organism was detected in ginger rhizome after eight weeks of inoculation by agar gel double diffusion and immuoelectrophoretic tests, but only one week after inoculation by indirect ELISA (enzyme linked immunosorbent assay). Systemic protection against *P. aphanidermatum* was induced in ginger (Cv. Suprabha) by soaking rhizome seeds separately in selected synthetic chemicals or specific herbal extracts for 1 h prior to sowing. Among 12 plant defence activators tested, jasmonic acid (JA, 5 mM) and 10% leaf extract of *Acalypha indica* (ALE) reduced disease significantly, with concomitant increase of defence-related proteins (DRPs). Analysis of protein profiles of leaves of JA-treated and inoculated plants by SDS–PAGE and Image Master VDS-ID Gel Analysis Version 3.0 revealed 18 protein bands, including four DRPs having molecular masses 32, 24, 18 and 14 kDa respectively. At least four DRPs were detected in leaves of ALE-treated inoculated plants. Growth response of pathogen to both JA and ALE was evaluated *in vitro*. ALE stimulated growth, while JA inhibited growth at high concentration (0.5 mM) and slightly stimulated growth at low dose (0.005 mM). Results suggest their host-mediated role in induced systemic protection against disease.

RHIZOME rot is a serious disease of ginger (*Zingiber officinale* Rosc.), causing considerable economic loss to growers in different countries. To ensure effective and sustainable disease management, early detection and authentic identification of pathogen are considered as prerequisites. On the contrary, exploitation of host defence is one of the most reliable non-conventional strategies of plant disease management. Several synthetic chemicals and natural products could induce or activate host resistance by producing or accumulating defence-related proteins (DRPs) or other antimicrobial compounds, as reported earlier by several workers[1–4]. Foliar spray with potassium phosphate (0.1 M) induced systemic protection against *Sphaerotheca fuliginea* (powdery mildew) in cucumber[5]. Similarly, potato leaves sprayed with jasmonic acid (JA) or jasmonate methyl ester (JME) also induced systemic protection against *Phytophthora infestans* and enhanced levels of two proteinase inhibitors[6]. In this communica-

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tion, however, we report the results of immunological tests for early and accurate identification of pathogen of rhizome rot disease of ginger, a few tested potent plant-defence activators, including a promising herbal extract for disease management and some newly detected DRPs in ginger leaves after induction of resistance.

Rhizome seeds of two ginger (*Z. officinale*) cvs. Varada and Mahima and the culture of *Pythium aphanidermatum* Str. SR_2 were obtained from the Indian Institute of Spices Research, Calicut. Cv. Suprabha was collected from Amtala Seed Stores, South 24-Parganas. The cultures of *P. aphanidermatum* Str. IR and *Colletotrichum capsici* were procured from the Indian Agricultural Research Institute, New Delhi and maintained in PDA medium.

Antigens from healthy and infected ginger rhizomes and fungal mycelia were prepared following the method described by Purkayastha and Banerjee[7]. Fungal antigens were extracted from twelve-day-old mycelia grown in sterilized glucose-asparagine medium. Normal (pre-immunized) sera were collected from two male rabbits and antisera raised separately against antigens of virulent pathogen, *P. aphanidermatum* Str. SR_2 and healthy ginger rhizome (Cv. Varada). Each rabbit received intramascular injections of antigens (2 mg ml^{-1} of protein of either host or pathogen) emulsified with an equal volume of Freund's complete adjuvant (Difco) followed by Freund's incomplete adjuvant (Difco) at seven days interval for four consecutive weeks. Immunized blood was collected by ear-vein puncture on the 36th day after first injection. Serum was clarified from blood clot by centrifugation and stored at –15°C with 0.1% NaN_3 until use.

Titre values of antigens and antisera of *P. aphanidermatum* Str. SR_2 and ginger (Cv. Varada) were determined as described by Ouchterlony[8] and Clausen[9]. The titres of antigens of both *P. aphanidermatum* and Cv. Varada against their homologous antisera were 32, while titres of antisera of *P. aphanidermatum* and Cv. Varada against their homologous antigens were 4 and 8 respectively.

Indirect ELISA technique of Alba and DeVay[10] was adopted with modifications. Plant and fungal antigens were diluted to known concentrations (1, 10 and 100 µg ml^{-1} protein) with carbonate buffer (0.05 M, pH 9.6) and were placed on microtitre plates (200 µl/well). Antisera were diluted to 1:2000 with phosphate buffer saline (pH 7.6) containing 0.1% BSA. Goat anti-rabbit horseradish peroxidase (HRPO) conjugate (1:10,000 dilution) and tetramethyl benzidine with hydrogen peroxide were used as enzyme-conjugated secondary antibody and enzyme substrate respectively. Absorbance at 450 nm was determined by ELISA reader (Multiskan Excut Off V.2.1-0).

Rhizome seeds (5 cm long) of ginger (Cv. Suprabha) were disinfected with 0.1% $HgCl_2$, washed thoroughly with sterile distilled water and soaked separately in a desired concentration of synthetic chemical or leaf extract prior to sowing. Control seeds were also soaked in distilled water for a similar period. Leaf extracts were prepared by homogenizing leaves in an electric blender with distilled water (0.5 g ml^{-1}), the homogenate strained through two layers of muslin and the filtrate centrifuged at 5000 *g* for 15 min. Subsequently, the clear supernatant was diluted with distilled water (1:5) to obtain 10% leaf extract.

Both treated and untreated rhizome seeds were sown in disinfected earthenware pots (one rhizome seed/pot, 20 cm dia, 4400 cm^3) containing sandy soil (1:3, 3 kg/pot) and arranged in a completely randomized design. After four weeks of sowing, 50% of the pots were inoculated with *P. aphanidermatum* Str. SR_2. The inoculum was prepared by growing the pathogen in sand–maize meal medium (9:1) for 10 days. Usually, 50 g of inoculum was mixed with the top soil in each pot, except when otherwise stated. The plants were kept under daylight temperature (28–32°C) and moisture content (77–85%) of the experimental garden and supply of water (approx. 200 ml/pot) once a day was maintained, except during rainy days. The disease severity was usually assessed after four weeks of inoculation. Among the symptoms, yellowing index/plant was calculated as follows: the total number of leaves showing yellowing symptom divided by the total number of plants/treatment. The percentage loss in fresh weight of rhizomes in relation to control was also noted after four weeks of inoculation.

Proteins were extracted from ginger leaves (eight-week-old plant) using 0.1 M Tris-HCl buffer (pH 7.5, 1 ml g^{-1} fresh wt), strained through muslin, centrifuged at 10,000 rpm for 20 min at 4°C. The protein concentration in the supernatant was adjusted to 3 mg ml^{-1} following the method of Lowry *et al.*[11], taking bovine serum albumin (BSA) as standard. Proteins were extracted from the leaves and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 13% (w/v) acrylamide gels. The gels were stained by Coomasie brilliant blue R250 and further analysis of proteins was carried out using Image Master VDS-ID Gel Analysis Version 3.0.

Growth response of *P. aphanidermatum* to plant defence activators was tested *in vitro* by growing the fungus in glucose–asparagine medium (glucose 30 g, asparagine 1 g; KH_2PO_4 1.5 g, $MgSO_4.7H_2O$ 0.5 g, distilled water 1 l) supplemented with appropriate quantity of either JA or *Acalypha indica* leaf extract (ALE). The final concentrations of JA in the medium were 0.5, 0.05 and 0.005 mM and that of ALE were 5, 0.5 and 0.05%. Flasks containing the medium (50 ml/250 ml flask) were inoculated with agar block (3 mm dia.) containing three-day-old mycelia of *P. aphanidermatum* and incubated for 12 days at 30 ± 1°C. After incubation, mycelia were collected, dried at 60°C for 96 h, cooled and weighed.

For early detection and authentic identification of pathogen causing rhizome rot of ginger, immunological techniques were followed, as stated earlier. Initially, immunodiffusion tests were carried out using polyclonal antibodies

and antigens of different ginger cvs, virulent (SR_2) and avirulent strains (IR) of *P. aphanidermatum* and a non-pathogen of ginger, *Colletotrichum capsici* and the results are given in Table 1. Cross-reactive antigens (CRAs) were not detected between antigens of infected (four-week-old infection) rhizome or non-pathogen (*C. capsici*) and antiserum of the virulent strain of *P. aphanidermatum* SR_2 (Figure 1 *a*), but CRA was easily detected when antigens of heavily infected (eight-week-old infection) ginger (cv. Mahima) were cross-reacted with antiserum of the pathogen (Figure 1 *b*), which was confirmed by immunoelectrophoretic and cross-immuoelectrophoretic tests. Results showed four and three precipitin lines between antiserum of host cv. and antigens of infected and healthy rhizomes respectively (Figure 1 *c*). The above-mentioned tests are reliable and confirmatory, but not suitable for early detection of pathogen in ginger. Hence, indirect ELISA technique was followed for the purpose. Antigens were prepared from infected (one-, two- and four-week-old infection) rhizomes of ginger (cv. Suprabha) and different concentrations (1, 10 and 100 µg ml^{-1}) of these antigens were cross-reacted separately with antisera of *P. aphanidermatum* Str. SR_2 (1 : 2000) and healthy ginger rhizome (1 : 2000). The results are summarized in Table 2. Earliest detection of pathogen in rhizome was possible after one week of inoculation by ELISA. Results of ELISA suggest that the period of incubation, concentration of antigens of infected rhizome and appropriate dilution of pathogen antiserum are important factors that may affect early detection of pathogen.

Rhizome seeds of ginger were soaked separately in six synthetic chemicals, viz. JA, acetyl salicylic acid, 5,6-dichloronicotinic acid, 2,2-dichloro-1-methyl cyclopropane carboxylic acid (5 mM in each case); K_2HPO_4 and potassium oxalate (50 mM in both cases) and in 10% leaf extracts of six plant species, viz. *A. indica* L., *Catharanthus roseus* (L) Don., *Andrographis paniculata* Nees, *Spinacea oleracea* L., *Curcuma longa* L. and *Centella asiatica* Urb. for 1 h prior to sowing. The rest of the procedure was as described earlier and the results are given in Table 3. Among the chemical compounds, JA and K_2HPO_4 are more or less equally effective in reducing the disease. More than 70% reduction in disease was noted in both cases in relation to control. Leaf extracts of all the six plant species reduced the disease, but markedly by *A. indica*.

Protein profiles of ginger leaves were analysed after induction of resistance. Level of DRPs increased in the

Table 1. Agar gel double-diffusion tests using different cvs of ginger, strains of *P. aphanidermatum* and a non-pathogen of ginger

Host/pathogen/non-pathogen with code	Combination Antiserum × antigen (A) (a)	Reaction positive/negative
Cv. Varada (V)	VA × Va	+
Cv. Suprabha (S)	VA × Sa	+
Cv. Mahima (M)	VA × Ma	+
P. aphanidermatum Str. SR_2 (P_2)	$P_2A \times P_2a$	+
P. aphanidermatum Str. IR (P_i)	$P_2A \times P_ia$	+
C. capsici (C)	$P_2A \times Ca$	–
Cv. Mahima Healthy (M H)	$P_2A \times MHa$	–
Cv. Mahima Infected (M I)	$P_2A \times MIa$	+

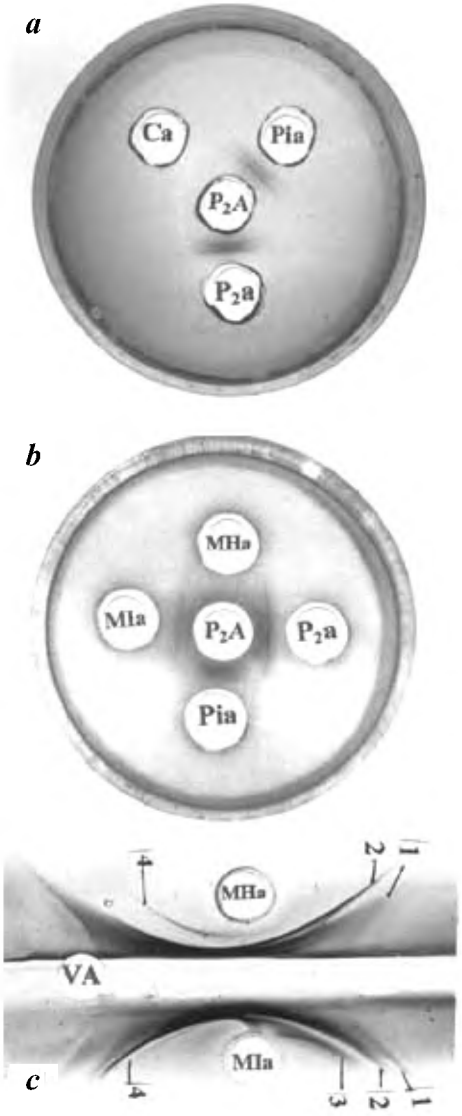

Figure 1. Agar gel double-diffusion and immunoelectrophoretic tests using antigens and antisera of host and pathogen. ***a***, P_2a, Antigen of *P. aphanidermatum* Str. SR_2; P_ia, Antigen of *P. aphanidermatum* Str. IR; Ca, Antigen of *C. capsici*; P_2A, Antiserum of *P. aphanidermatum* Str. SR_2. ***b***, P_2a, Antigen of *P. aphanidermatum* Str. SR_2; P_ia, Antigen of *P. aphanidermatum* Str. IR; MHa, Antigen of healthy ginger rhizome (cv. Mahima); MIa, Antigen of infected ginger rhizome (cv. Mahima); P_2A, Antiserum of *P. aphanidermatum* Str. SR_2. ***c***, MHa, Antigen of healthy ginger rhizome (cv. Mahima); MIa, Antigen of infected ginger rhizome (cv. Mahima); VA, Antiserum of ginger cultivar (cv. Varada). 1–4, Precipitin lines.

Table 2. Detection of pathogen (*P. aphanidermatum*) in infected ginger rhizomes by indirect ELISA

Antigen from infected rhizome of ginger (cv. Suprabha) weeks after inoculation	Concentration of antigen (μg ml^{-1} protein)	Absorbance at 450 nm	
		Antiserum of *P. aphanidermatum* Str. SR_2 (1 : 2000 dilution)	Antiserum of ginger cv. Varada (1 : 2000 dilution)
1	1	0.529 ± 0.015*	0.900 ± 0.031
	10	0.727 ± 0.014	1.468 ± 0.014
	100	1.164 ± 0.015	2.916 ± 0.032
2	1	0.653 ± 0.014	0.916 ± 0.032
	10	0.879 ± 0.150	1.370 ± 0.017
	100	2.107 ± 0.007	3.057 ± 0.015
3	1	0.831 ± 0.067	1.004 ± 0.080
	10	1.715 ± 0.010	1.761 ± 0.047
	100	2.859 ± 0.025	3.042 ± 0.007
Non-inoculated (control)	1	0.488 ± 0.014	0.786 ± 0.105
	10	0.585 ± 0.015	1.689 ± 0.026
	100	0.665 ± 0.009	3.041 ± 0.030
Antigen without antiserum	100	0.078 ± 0.003	0.067 ± 0.002
Antiserum without antigen	0	0.066 ± 0.001	0.075 ± 0.003
Substrate + conjugate	0	0.062 ± 0.001	0.064 ± 0.002

*Average of three replicates.

Table 3. Effect of synthetic chemicals and herbal extracts on the development of rhizome rot disease of ginger

Treatment (concentration)	Yellowing index/plant (four weeks)	Average fresh wt (g) of rhizomes (4 weeks)**		Percentage loss in wt of rhizome (four weeks)#
	Inoculated*	Non-inoculated	Inoculated	
Untreated (control)	1.64	33.00 ± 1.79	23.00 ± 1.54	30.30
Treated with DCNA (5 mM)	1.25	29.00 ± 1.62	25.50 ± 1.56	12.06
DMCCA (5 mM)	0.87	33.30 ± 1.22	28.32 ± 0.91	14.95
Untreated (control)	1.75	44.00 ± 1.90	32.00 ± 1.36	27.27
Treated with JA (5 mM)	0.80	34.00 ± 0.93	31.25 ± 0.65	8.08
ASA (5 mM)	1.28	33.75 ± 1.51	29.12 ± 2.01	13.71
Untreated (control)	1.83	62.25 ± 3.41	45.00 ± 2.62	27.71
Treated with potassium phosphate dibasic (50 mM)	0.82	50.33 ± 2.05	47.25 ± 4.48	6.11
Potassium oxalate (50 mM)	1.38	43.25 ± 0.95	37.00 ± 1.43	14.45
Untreated (control)	2.65	52.50 ± 1.60	32.40 ± 3.34	38.28
Treated with leaf extract (10%)				
Catharanthus roseus	1.58	53.30 ± 0.94	42.60 ± 2.50	20.07
Acalypha indica	0.54	45.00 ± 2.95	42.91 ± 2.42	4.64
Spinacea oleracea	1.61	40.50 ± 3.43	37.20 ± 1.75	8.14
Andrographis paniculata	1.84	43.00 ± 1.36	33.80 ± 2.27	21.39
Centella asiatica	1.36	45.50 ± 2.08	38.00 ± 0.93	16.48
Curcuma longa	1.55	51.00 ± 1.70	38.60 ± 3.41	24.31

*For leaves of non-inoculated plants yellowing index is zero.
**Average of four replicate rhizome seeds/treatment.
#Four weeks after inoculation; JA, Jasmonic acid; ASA, Acetyl salicylic acid; DCNA, 5,6-Dichloronicotinic acid; DMCCA, 2,2,Dichloro-1-methyl cyclopropane carboxylic acid.

leaves of JA-treated, inoculated plants. Among the 18 protein bands detected, four were DRPs having molecular masses 32, 24, 18 and 14 kDa respectively (Table 4). The DRPs also increased in JA-treated non-inoculated and untreated inoculated plants. There is evidence that JA affects a variety of physiological processes, including plant response to abiotic stresses and pathogens. Exogenous application of JA stimulates the expression of defence compounds in plants. Besides, JA has been implicated in gene regulation and several of these genes are involved in plant defence[12]. The results of the present study also suggest that JA plays a role in inducing systemic protection in ginger against *P. aphanidermatum* and in enhancing DRPs in treated and inoculated plants. Leaves of ALE-

Table 4. Effect of JA on DRPs in ginger leaves of untreated inoculated, JA-treated non-inoculated and inoculated plants. (Results based on analysis of SDS–PAGE by Image Master VDS-ID Gel Analysis Version 3.0)

Untreated inoculated			JA-treated non-inoculated			JA-treated inoculated		
Serial no. of protein band*	Concentration of defence protein**	Molecular mass (kDa)	Serial no. of protein band*	Concentration of defence protein**	Molecular mass (kDa)	Serial no. of protein band*	Concentration of defence protein	Molecular mass (kDa)
4	++	67	4	+++	67	8	+++	32
8	++	32	8	++	32	11	++	24
11	+	24	15	++	18	15	+++	18
18	+	14	18	++	14	18	+++	14

*Total no. of protein bands is 18.
**Only these defence proteins increased in relation to untreated non-inoculated control. (Defence proteins detected only after inoculation or treatment with a plant activator).
+, Low; ++, moderate; +++, high.

Table 5. Effect of different concentrations of JA and ALE on the growth of *P. aphanidermatum*

Treatment	Concentration	Average mycelial dry wt (mg) with SE*
Basal medium (BM)	0	258.00 ± 2.95
BM + ALE	5.0%	289.67 ± 1.52
	0.5%	278.65 ± 2.76
	0.05%	265.33 ± 4.19
BM	0	261.00 ± 2.49
BM + JA	0.5 mM	220.00 ± 2.16
	0.05 mM	242.00 ± 4.32
	0.005 mM	278.50 ± 3.01

Incubation period is 12 days.
*Average of three replicates/treatment.
Temperature is 30 ± 1°C.

treated inoculated plants also exhibited four DRPs (molecular masses 67, 56, 40 and 18 kDa) which could be associated with reduction in disease severity.

The results (Table 5) of growth response experiments reveal that ALE stimulated growth of *P. aphanidermatum in vitro*, while JA inhibited growth (approx 16%) at high concentration (0.5 mM), but stimulated growth slightly at low dose (0.005 mM). These findings also suggest the host-mediated effect of these activators on disease instead of direct effect on the pathogen.

Latent infection or delayed expression of symptoms often creates problems in early diagnosis of disease and accurate identification of pathogen by conventional biological methods, and hence immunological techniques are preferred. In case of rhizome rot of ginger, the pathogen (*P. aphanidermatum*) was detected in the rhizome after one week of inoculation by indirect ELISA, although plants showed no visible symptoms. Immunodiffusion and immunoelectrophoretic tests were positive at the advanced stage of disease only.

Plant defence activators which induced systemic protection against rhizome rot of ginger also activated the synthesis of DRPs. Usually, defence response of plants could be induced specifically or non-specifically by a broad range of biotic and abiotic agencies. In both cases, *de novo* transcription of the plant genes encoding various components of defence response appears to be an early determinative event. Induction of DRPs/pathogenesis related proteins and disease resistance in plants have been discussed in detail by previous researchers[13–16].

Among selected synthetic compounds and herbal extracts used for induction of systemic protection in ginger, JA (5 mM) and ALE (10%) appeared to be most effective in reducing the disease (more than 70%). Vijayan *et al.*[17] demonstrated that a mutant of *Arabidopsis* unable to accumulate jasmonate was susceptible to root rot caused by *Pythium mastophorum*, although the neighbouring wild-type plants were unaffected. But application of exogenous methyl jasmonate substantially protected mutant plants from disease. These findings support the positive role of JA in induced resistance in ginger.

Significant reduction in rhizome rot by ALE is not unusual, since systemic resistance was induced earlier in cucumber against anthracnose (*Colletotrichum lagenarium*) by spraying leaf extracts of spinach or rhubarb. Oxalate was identified as the active component of both extracts, but it did not inhibit germination or mycelial growth of *C. lagenarium in vitro*[18]. *A. indica* contains an alkaloid 'acalyphin' and a cyanogenetic glucoside along with other substances[19,20], but the active component associated with induced resistance in ginger is yet to be identified.

Analysis of protein profiles of ginger leaves of JA- and ALE-treated and inoculated plants showed increasing trend in DRPs suggesting their involvement in induced systemic protection in ginger against *P. aphanidermatum*.

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Coral bleaching 2002 in the Palk Bay, southeast coast of India

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The bleaching of corals and their recovery in chosen study sites of the reefs of Palk Bay, southeast coast of India were monitored. The bleaching phenomenon occurred during the period from April to June 2002. A minimum of at least 50% and a maximum of 60% bleaching were noticed among the six different sites monitored. However, the corals started to recover quickly in August 2002 and as much as 52% recovery could be noticed. The coral-bleaching phenomenon might have occurred due to unusual rise in surface sea-water temperature, which was 32°C in the peak of summer. This, confounded with the failure of the southwest monsoon winds during this period, might have exerted a stress on the corals leading to expulsion of zooxanthellae from their bodies, causing the bleaching phenomenon. Coral bleaching might affect the distribution of coral-associated animals, especially the food fishes which depend on the corals for feeding and breeding. This, in turn, can affect the livelihood of the reef resource users, particularly the fishermen in the Palk Bay region.

CORALS, the marine invertebrate animals which live in association with the algae zooxanthellae, form colonies that appear in attractive and beautiful hues and colours. As they live in and build colonies, they form massive underwater structures similar to, and often much larger than, those built by honey bees or termites. However, they are not as advanced as these insects. Hence they neither have the ability to move away from their living structures – e.g. to run away from danger or disasters such as unusual temperature rise in the ambience – nor do they have the division of labour in their colonies to effectively manage crisis situations. As a result, they succumb to even minor changes in the ambient environmental conditions, resulting in disaster. One such disaster has struck recently, killing a large section of the corals in the Palk Bay, which may have far-reaching consequences on the fisheries of this region.

Massive structures of coral colonies form large reefs in the sea which not only give refuge to a variety of organisms, including food and ornamental fishes, but also act as the feeding and breeding grounds for them[1]. The reef-building corals are normally restricted to shallow coastal waters of tropical climate, where the temperature ranges

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