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Chitinolytic Endophytic Bacteria as Biocontrol Agents for Phytopathogenic Fungi and Nematode Pests: A Review

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

Cultivation of crops and other plants is crucial and always at a risk due to the attack by phytopathogens. Among these pathogens, fungi are considered to be the predominant pathogens responsible for a range of diseases in plants and a drastic decrease in crop yields. Currently, there is an increasing public concern on the continuous use of agrochemicals to control the pathogens and pests causing diseases in plants. Several kinds of research have been conducted to find less hazardous options for controlling plant pathogens among which the biological control using the microorganisms has been demonstrated to be a feasible alternative. Endophytic microorganisms, especially chitinolytic bacteria can act successfully as biocontrol agents and improve plant health and yield by controlling plant pathogens and pests and suppressing plant diseases. In the context of fungal disease management, chitinases can enhance the plant defense system by acting on chitin, the major component of fungal cell walls and render them inactive without causing negative impacts on plants.

Keywords: Chitinases; endophytes; bacteria; biological control; phytopathogens.

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1. INTRODUCTION

Plant diseases need to be prevented or controlled to maintain the quality and yield of food and feed produced. Currently, different approaches are using to prevent or control plant diseases by growers around the world. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical control methods of plant diseases which in return cause hazardous effects to the environment and the human and animal health. Various efforts have been made to find less hazardous options for controlling plant diseases in sustainable agriculture and among which the biological control using effective microorganisms has been demonstrated to be a feasible mechanism [1]. Use of biocontrol agents is an effective, safe and promising alternative to the extensive use of synthetic fungicides and pesticides.

Endophytes are microorganisms (bacteria or fungi or actinomycetes) that inhabit different tissues in a wide range of plants without causing any apparent harm to the host [2,3]. They form different relationships with plants providing several advantages to the plants. Endophytic bacteria are the bacteria that remain colonized the internal tissues of plants without causing any harmful effects to the host. Indirectly, endophytic bacteria can act as biocontrol agents and improve plant health by controlling disease causing pests and pathogens and suppressing plant diseases. Some endophytic chitinolytic bacteria have shown potentiality as biological control agents against various phytopathogenic fungi [4,5,6,7]. In this review, literature concerning the experimental studies of the effectiveness and mechanisms of some specific chitinolytic endophytic bacteria isolated from various plants as potential biocontrol agents for phytopathogenic fungi and nematodes is discussed.

2. WHAT IS CHITIN?

Chitin [$(C_8H_{13}O_5N)_n$] is a linear polymer of β-1,4-*N*-acetylglucosamine (GlcNAc) or NAG monomers [8], which are 2-acetamido-2-deoxy-β-D-glucose units attached to each other via β(1→4) linkages. Chitin is the second most abundant polysaccharide in nature after cellulose. Chitin is a major constituent of the outer skeleton of insects, cell wall of fungi as well as in the internal structures of most of the other invertebrates [9]. Chitin exists in nature in three different forms: α-chitin, β-chitin, and γ- chitin. α-Chitin is the most abundant and more compact form and chitin chains are arranged in an antiparallel fashion. β-Chitin chains are loosely packed and are arranged in a parallel fashion with weaker intermolecular forces. γ -chitin is a mixture of both α- and β-chitin chains [10]. Chitin does not accumulate in the environment in the presence of chitinase (EC 3.2.2.14), the enzyme which catalyzes the hydrolysis of glycosidic bonds in chitin by either endo or exo type of cleavage, producing disaccharides and longer oligosaccharides.

3. WHAT ARE CHITINASES?

Chitinases belong to the Family glycosyl hydrolase (GH) are widely distributed across diverse biological systems. Several organisms including bacteria, fungi, insects, plants, and animals produce chitinases [11]. Chitinases which catalyze the degradation of β-1→4-linkages in chitin are divided into two groups: endochitinases (EC 3.2.1.14) and exo-chitinases (EC 3.2.1.52) based on their cleavage and hydrolysis mechanisms [12]. Endochitinases cleave the polymer of chitin randomly at internal sites generating low molecular mass multimers of glucosamine residues such as chitotriose, chitobiose, and diacetylchitobiose. Exochitinases have been classified into two categories, namely, chitobiosidases (EC 3.2.1.29) which catalyze progressive release of diacetylchitobiose from terminal non-reducing end of the chitin polymer and *N*-acetylglucosaminidases (EC 3.2.1.30) which cleave the products obtained by endochitinases into monomers of *N*-acetyl glucosamine (GlcNAc) [13].

Further, chitinases are divided into families 18, 19 and 20 of glycosyl hydrolases based on the similarity of their amino acid sequences [14]. Chitinases of GH19 and GH18 do not share sequence similarities and they have completely different 3-dimentional structures and molecular mechanisms [15]. Family 18 comprises chitinases from viruses, bacteria, fungi, animals and certain plants. Family 19 comprises some plant chitinases and chitinases from some of the Gram-positive bacteria like *Streptomyces*. Family 20 includes *N*-acetylglucosaminidases from bacteria, certain fungi, and humans [14]. Additionally, chitinases have been categorized into five classes according to their sequence, structure, and phylogenetic relationships [16,17,18,19,20]: classes I, II, and IV are in GH19 and classes III and V together form GH18. Chitinases have received an increased attention in biological control of fungal pathogens due to their inducible nature and *in vitro* antifungal activities.

4. BACTERIAL CHITINASES

Most of the bacterial chitinases, which have been isolated and sequenced so far, are included in GH18 of the glycosyl hydrolases with the exception of chitinases isolated from several Actinomycetes, particularly species of *Streptomyces,* which contain chitinases of both GH18 and GH19 families. These chitinases show different chitinolytic efficiencies on different substrates, but only the GH19 chitinases have antifungal properties [21]. For example, chitinases isolated from *Streptomyces griseus* HUT 6037 belongs to the family 19 of the glycosyl hydrolases [22]. Chitinolytic bacteria generally produce multiple chitinases derived from different genes. The presence of multiple chitinase producing enzymes has been described in various bacteria such as *Aeromonas* sp. No. 10S-24, [23] *Pseudomonas aeruginosa* K-187, [24] and *Bacillus circulans* WL-12 [25]. One of the best studied chitinolytic bacteria, *Serratia marcescens*, has been reported three chitinase genes ChiA, ChiB and ChiC, producing three types of extracellular multiple chitinases, chitinase A, B and C [26]. Bacterial chitinases generally have a molecular weight range of 20-60 kDa and are smaller than insect chitinases (40-85 kDa) [26]. They are active over a wide range of pH and temperatures, depending on the source of the bacteria from which they have been isolated. For example, endochitinase from *Streptomyces violaceusniger* [5] and thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520 [27] have an optimum temperature of 28°C and 80°C respectively.

5. BACTERIA AS PLANT ENDOPHYTES

The word 'endophyte' originated from two Greek words; 'endon' means 'within' and 'phyton' means 'plant'. Endophytes were mentioned for the first time by [28] in 1866 and have been defined in many ways. Further, [29] defined endophytes as the fungi that live internally and remain as asymptomatic for at least part of their life cycle. But in addition to fungi, a wide range of bacterial endophytes growing and surviving in different plant tissues have been identified. According to [30], endophytes are plant-associated prokaryotes that form associations with their host plants by colonizing the internal tissues, which has made them important in agriculture as a tool in improving crop production. Many recent reviews showed that the term endophyte should refer to 'habitat only and not function' and should include all organisms which colonize the internal tissues of a plant during their complete life cycle or part of their lifetime [31]. Similar to the definition given by [2], an amended definition was given by [31] as 'endophytes are microbes which occur within plant tissues at least part of their life cycle without causing disease under any circumstance'.

Endophytic bacteria have been isolated and characterized from diverse type of plant hosts including agronomic crops, prairie plants, plants growing in extreme environments and wild and perennial plants [32]. Further, bacterial endophytes have been isolated from different plant tissues that are above and below ground, such as seeds, tubers, roots, stems, leaves, and fruits, where roots of many plants generally have the greatest number of bacterial endophytes as compared to above ground tissues [33]. They are commonly present in intercellular spaces of living tissues because these areas have an abundance of carbohydrates, amino acids, and inorganic nutrients [34] and in addition in xylem vessels [35]. The presence and survival of both endophytic and rhizospheric microbes are regulated by biotic and abiotic conditions. But endophytic bacteria are more protected from biotic and abiotic stresses than rhizospheric bacteria due to their residence in internal tissues of plants [33]. It is thought that bacterial endophytes originate from the bacterial communities of the rhizosphere and phylloplane, as well as from endophyte-infested seeds or any other planting material. They enter the plant tissues through natural openings or wounds [2]. During last decades, many researchers described the presence of endophytes in seeds of several plant species including the plants belong to family Poaceae, such as rice and maize, where Proteobacteria, Actinomycetes and Firmicutes were particularly dominant [36,37]. *Bacillus* and *Pseudomonas* were the most frequently found bacterial genera in plant seeds, but *Paenibacillus*, *Micrococcus, Staphylococcus, Pantoea* and *Acinetobacter* were also reported [38].

Many studies on biocontrol of plant pathogens have tended to focus on some commonly isolated bacterial endophytes belonging to the genera of *Pseudomonas, Streptomyces, Bacillus, Enterobacter* and *Agrobacterium* [2,39]. They colonize ecological niches similar to that of phytopathogens, which makes them suitable as potential biocontrol agents [40]. Indeed, various research work and the findings have revealed that the endophytic microorganisms have the capacity to control phytopathogenic fungi [4,5,7], insects [30] and nematodes [41,42,43]. Bacterial endophytes have been shown to reduce or prevent disease development in plants through endophyte-mediated de novo synthesis of novel compounds and antifungal metabolites including antibiotics, volatile organic compounds, antifungal, antiviral and insecticidal agents. As effective biological control agents, endophytic bacteria producing mycolytic enzymes, especially chitinases and glucanases have the potential to lyse the cell walls of fungal pathogens and prevent or inhibit their growth in plants.

6. FUNGI AS PHYTOPATHOGENS

Plant pathogenic fungi cause dangerous diseases in many crop plants resulting in growth reduction and yield losses. Although the majority of fungi is saprophytic, some of them are parasites and attack living organisms especially animals and plants causing diseases. Among the plant pathogenic fungi, the majority belongs to the Ascomycetes and Basidiomycetes [44]. Plant pathogenic Ascomycete fungi are in various classes such as Dothideomycetes (eg. *Cladosporium* spp.), Sordariomycetes (eg. *Magnaporthe* spp.) and Leotiomycetes (eg. *Botrytis s*pp.). Basidiomycetes are represented by two large orders Urediniales (rusts) and Ustilaginales (smuts). According to the type of parasitism and infection strategy, fungi are classified as necrotrophic (kill the hosts and feed on dead material) and biotrophic (colonize the living tissues) [45]. In addition to these two groups, hemibiotrophic pathogens start as biotrophs and then becomes nectrophs causing necrosis and eventually the death of the infected plant. For successful invasion, fungi have to overcome the physical and chemical defense barriers in plants by employing several strategies.

7. BIOCONTROL POTENTIAL OF CHITINOLYTIC ENDOPHYTIC BACTERIA

Microbial antagonists are widely used for the biological control of many plant diseases caused by pathogens and pests. Among them, endophytic bacteria have received a considerable attention for their potential as positive biocontrol agents particularly for fungal phytopathogens. Biological control may operate via different modes of action including parasitism and lysis, antibiosis, competition and induced resistance of host plants [46,47,48]. Induced resistance may result in the protection of plants against the attack by a wide range of pathogens and it can be divided broadly into two categories; systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR develops locally or systemically in response to an infection by pathogens or treatment with certain chemicals. This process is mediated by a salicylic acid (SA)-dependent process [49]. In contrast, ISR develops as a result of colonization of plant roots by plant-growth-promoting rhizobacteria (PGPR) and fungi in the rhizosphere and is mediated by a jasmonate or ethylene-sensitive pathway [50]. SAR is characterized by the activation and expression of pathogenesis related (PR) proteins such as chitinases and β-1,3-glucanases and in contrast, ISR is generally mediated by a SA-independent pathway and typically functions without activation of PR proteins [50,51,52]. Among the PR proteins evaluated, chitinases have been studied largely because these enzymes are produced by a variety of endophytic micro-organisms including bacteria [53]. Among bacteria, various species of *Streptomyces* [5,7], *Pseudomonas* [6] and *Bacillus* [4] have been shown to secrete chitinases, inhibiting the growth of several phytopathogenic fungi as well as pests like nematodes [42]. Chitinase enzyme has an important role in nematode control because the enzyme is capable of degrading the middle layer of nematode eggs [54,42]. On the other hand, chitinase could inhibit the nematode egg hatching and control the population [41,43].

7.1 Endophytic and Chitinolytic *Bacillus* Species as Biological Control Agents

*Bacillus s*pecies are the most studied antagonists among the bacteria employed as biological control agents against phytopathogens. *Bacillus* strains can secrete various secondary metabolites, which inhibit or prevent the growth of pathogenic fungi. It is also known that they are able to produce various hydrolytic enzymes, including chitosanases, chitinases, *N*-acetyl-β-hexoaminidases, proteases and laminarinases in order to inhibit fungal growth by degrading their cell walls [55].

The work by [4] demonstrated that *B. cereus* strain 65, isolated as an endophyte from mustard

(*Sinapis*) plant and inoculated into cotton seedlings, was able to reduce the root rot disease in cotton caused by *Rhizoctonia solani*. The chitinolytic activity was induced by growing the bacterium in a synthetic medium containing 0·2% (w/v) colloidal chitin. The extracellular proteins produced by *B. cereus* strain 65 showed a decreased rate of spore germination of *R. solani* compared to the control for which water was added instead of the crude protein preparation (64.1% and 85·3%, respectively). Further, the application of this bacterial strain to soil in which cotton seeds had been planted showed a protective effect against *R. solani*. When the strain was applied directly to the soil, the percentage of seedlings with root rot symptoms was approximately 30%, compared to 58% in the non-treated control soil. The results of the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing showed only one chitinolytic enzyme had been produced by *B. cereus* strain 65. Further studies showed that the strain had an ability to produce a chitinolytic enzyme characterized as a chitobiosidase.

Black pepper cultivation in Vietnam has faced many problems due to a disease caused by the root-knot nematode, *Meloidogyne* species. Chemical nematicides have been used for a long time, however, this treatment caused environmental problems and retaining the residues in agricultural products [56]. Hence, researchers were investigating some cost-effective and environmentally friendly ways for controlling the root-knot nematode in Black pepper cultivation in Vietnam. In a study carried out by [57], bacteria living in healthy black pepper plant roots were isolated and identified based on 16S rRNA gene sequencing. Out of thirty-four endophytic bacteria isolated, seventeen strains displayed the potential antinematode activity with the mortality higher than 85.56%. Of these, five strains demonstrated the most effective antinematode activity with the mortality of 100%. The active strains were identified as *Bacillus flexus* DS5, *Bacillus* sp. DS8, *Bacillus megaterium* DS9, *Bacillus* sp. DR10, and *Bacillus* sp. DR2. These five strains were used for greenhouse experiments to evaluate their effect on nematode inhibition in soil and in roots, as well as their interaction with pepper plants. *B. megaterium* DS9 demonstrated active antinematode activity both *in vitro* and in the greenhouse experiments. Thus, this strain was used to test for chitinases responsible for the anti-nematode activity. A chitinase assay was performed using the culture supernatant of the bacterium grown in a medium containing chitin. The detected chitinase activity (2.72 IU/ml) showed inhibition of both nematode growth (52.22%) and their egg hatching ability (71.11%). The results suggested that the *B. megaterium* DS9 could be a good biocontrol agent for the nematode *Meloidogyne*.

Cocoa (*Theobroma cacao* L.) is an economically significant crop, as the seeds are processed into variety of edible cocoa products. According to [58], nearly 30% of the cacao crop is lost globally due to different diseases every year. To determine whether natural endophytes in and on cacao leaves can act as potential antagonists of cacao pathogens, a study was carried out by [59] in 2011 and were able to isolate sixty-nine endospore-forming bacterial endophytes consisting of fifteen different species of five genera from leaves, pods, branches, and flower cushions of *T. cacao*. The researchers found that forty-seven isolates were members of either the *Bacillus* a or *Bacillus* c clades while *B. pumilus* and *B. subtilis* were common inhabitants of internal cacao tissues. *B. cereus* group comprised 29.0% of isolates and less common species were *B. flexus*, *B. firmus, B. megaterium, Solibacillus silvestris*, and *Brevibacillus* species. Chitinase assay results revealed that fourteen of the 69 isolates (20.3%) were chitinolytic *in vitro* and 56.3% of these isolates were belong to *B. cereus* group. *In vitro* antagonism assay was conducted against the cacao pathogens *Phytophthora capsici*, *Moniliophthora roreri* and *M. perniciosa* and the results revealed that 80% of *B. subtilis* isolates inhibited the growth of all three pathogens. Only one *B. cereus* isolate SPEC 541 1.1.1 inhibited the growth of all pathogens tested and this was the only *B. cereus* strain which inhibited the growth of *M. perniciosa*.

Another chitinolytic bacterium *B. cereus* 28-9, isolated from lily plant in Taiwan exhibited biocontrol potential on *Botrytis* leaf and flower blight of lily which causes severe economic loss of cut-flower production in Taiwan [60]. Detached leaf disc assay and dual culture assay were used to test the antagonistic effect of the bacterium against the *Botrytis elliptica* B061, the target fungus. Fluorometric assay was used to determine the chitinase activity using 4-methylumbelliferyl-N,N',N''-chitotriose as a substrate. Sodium dodecyl sulfate polyacrylamide gel electrophoresis results showed that at least two chitinases (ChiCW and ChiCH) excreted by *B. cereus* which were antifungal in nature. An *in vitro* assay results showed that the purified ChiCW had an inhibitory activity on conidial germination of the fungus *B. elliptica*.

7.2 Endophytic and Chitinolytic *Streptomyces* Species as Biological Control Agents

Actinomycetes have been largely exploited mainly because of their capability to produce bioactive compounds, such as antibiotics and lytic enzymes [61]. Endophytic Actinomycetes have been tested as potential biocontrol agents, either acting directly on fungal pathogens or initiating increased plant responses against disease development [62]. Their mode of action includes the release of antifungal compounds, siderophores, hydrogen cyanide (HCN) and hydrolytic enzymes such as β-1.3-glucanase and chitinase [63,64]. In nature, *Streptomyces* species have a quite widespread distribution and are found in soils of different structure and texture, in surface waters and in plants as rhizosphere colonizers or true endophytes. As endophytic microorganisms, they colonize the internal parts of plants, mainly the root system and the xylem tissues of the stem, causing no apparent change to the morphology and physiology of their host.

Chitinase produced by *S. hygroscopicus*, an endophytic actinomycete isolated from peanut plants and maintained on solid starch medium [65] was tested for its antifungal activities against few phytopathogenic fungi such as *R. solani, Fusarium oxysporum, Alternaria alternate, Aspergillus niger, A. flavus, Sclerotinia scleotiorum, Phytophthora parasitica* and *B. cinerea* [7]. The potential biocontrol activity of *S. hygroscopicus* was tested on agar plates using dual culture assay. Inhibition of fungal growth by purified enzyme extract was tested using paper discs coated with the purified chitinase enzyme. According to the observations, larger growth inhibition zones were obtained for *R. solani, S. scleotiorum, B. cinerea* and *F. oxysporum*. In addition, purified chitinases of *S. hygroscopicus* exhibited a degraded appearance of the fungal hyphae after the treatment.

Another study performed by [5], using the chitinolytic endophyte *S. violaceusniger* XL-2, isolated from the bark of trees of Dehradun in India against various wood-rotting fungi such as *Phanerochaete chrysosporium, Postia placenta, Coriolus versicolor* and *Gloeophyllum trabeum*. When the supernatant of the bacterial culture was tested against the fungi, there was no inhibition. But surprisingly, when the bacterium was cultured in a solid medium with the fungus, growth inhibition of *P. chrysosporium* was observed. When the supernatant fraction of the bacterial culture was tested against the fungi, a strong antagonism inhibiting the normal growth of all four fungi was observed. These results indicated that the biocontrol agent produced by the bacterium was inducible and it was found as a 28. 259 kDa endochitinase.

Crown and root rot disease caused by *F.oxysporum* f. sp. *radicis lycopersici*, is a common disease in tomato, which reduces the yield both in field and greenhouse grown plants. Some endophytic actinobacteria isolated from root samples of few native plants were evaluated by [66] for their antagonistic potential against three phytopathogenic fungi including *F. oxysporum* f. sp. *radicis lycopersici*. Among the sixteen endophytic actinobacteria isolated, three strong antagonistic isolates were selected and characterized. These three isolates were tested for their biocontrol traits such as production of hydrogen cyanide, indole-3-acetic acid and chitinase and β-1,3-glucanase activities. Among them, strain *Streptomyces* sp. SNL2 revealed promising features exhibiting the highest protective activity including the production of chitinases. The isolate SNL2 showed strong antagonistic activity against the phytopathogenic fungi tested, especially *F. oxysporum* f. sp. *radicis lycopersici*.

7.3 Endophytic and Chitinolytic *Serratia* Species as Biological Control Agents

Serratia marcescens which has been described to be an important rice endophyte [67] as well as a bacterium producing multiple chitinases [26] has been isolated from many other plants or plant parts such as flowers of summer squash [68], healthy tissue of edible cactus plants [69] and from the medicinal plant *Centella asiatica* [70].

It was investigated that the endophytic bacterium isolated from peanut hulls and identified and designated as *S. marcescens* strain JPPI was a chitinase producer and it significantly reduced the growth of *Aspergillus parasiticus* [71]. The antagonistic effect was determined by visual agar plate assay and tip culture method and the strain JPP1 exhibited remarkable inhibitory effect on mycelial growth (antifungal ratio >95%) of the fungus. These findings suggested that *S. marcescens* JPP1 could potentially be utilized for the biological control of *A. parasiticus* in areas in China where peanut is growing as one of the main crops.

Pepper (*Piper nigrum* L.) is one of the oldest crops growing in Indonesia but, the production is affected to some extent due to the infections caused by *F. oxysporum*, one of the most important and ubiquitous phytopathogenic fungus and the nematode *M. incognita.* Since the use of fungicides and nematicides against these two infections were not successful, as a solution, sixteen endophytic bacteria isolated from root tissues of pepper plant were tested and successfully used to control both *F. oxysporum* and *M. incognita* [43]. The results of the experiments carried out showed that all sixteen endophytic bacteria were able to suppress the growth of *F. oxysporum* in different intensities and seven of them were able to produce chitinases.

Banana is among the most important and popular fruit crops globally. Fusarium wilt, also known as Panama disease is one of the most destructive diseases in banana [72]. An investigation was carried out to test the effectiveness of a bacterium isolated from rubber tree and identified as a strain of *S. marcescens* as a biocontrol agent for the Fusarium wilt disease in Banana [73]. The antifungal activity of the bacterial strain against the wilt causing pathogen *F. oxysporum* f. sp. *cubense* race 4 was tested using visual agar plate assay. The inhibition effect of the bacterium became significant on the agar plate at day 3. At day 7 of incubation, the mycelial tips that grew closer to the bacterial growth were detected as partially decomposed and the conidial formation of the fungus was also affected.

8. CONCLUSION

Since the use of various chemicals in the control of plant diseases is raising concerns, finding environmentally friendly means of control became a necessity. Chitinolytic microorganisms are a potential alternative to these chemicals because they are already part of the soil and endophytic microbiome. Endophytic bacteria provide important benefits to plants such as promoting plant growth through nutrient acquisition and suppression of phytopathogens respectively. Among the endophytic bacteria, some are able to produce chitinases which degrade the fungal cell walls containing chitin, and hence they could be used as potential biocontrol agents especially for fungal phytopathogens. Chitinases could be used directly or indirectly, as a purified protein or through gene manipulation respectively in biological control of most disastrous fungal pathogens. It is expected that control of plant diseases caused by fungal pathogens using chitinolytic endophytic bacteria will be a promising approach in the situations where environmental pollution and detrimental effects on human and animal health are in concern.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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