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Optimization of Cultural Conditions for *Cochliobolus heterostrophus* Isolates from Infected Maize Plants from Different Agricultural Zones of Pakistan

Iffat Naz[1,2*], Shama Sehar[1], Irum Perveen[1], Abdul Rehman[1], Amber Hameed[1], Yasmin Ahmad[2] and Safia Ahmed[1]

[1]Department of Microbiology, Quaid-i-Azam University, Islamabad 45320, Pakistan.
[2]National Agricultural Research Centre, Islamabad 45320, Pakistan.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Southern corn leaf blight caused by *Cochliobolus heterostrophus* is a major foliar disease of maize crop in Pakistan. The disease affects leaves, leaf sheaths, ears and maize grains. Suitable physiological conditions which include different nutrient media, temperature, pH, carbon and nitrogen sources were determined for growth and reproduction of the pathogen. Among all media used viz, the best supporting medium was found to be Richard's agar for the growth of pathogen after 7 days of incubation. Different temperatures (20, 25, 30 and 35ºC) were selected for mycelia growth of the pathogen, among which maximum growth was found to be at 30ºC (80 mm colony size) and minimum at 35ºC (35 mm). Maximum radial colony growth of the pathogen was observed at neutral pH (80 mm). Sucrose and potassium nitrate (KNO_3) were found to be the most appropriate sources of carbon and nitrogen respectively.

Keywords: Cochliobolus heterostrophus; southern corn leaf blight; pathogenicity test; maize.

**Corresponding author: Email: iffatkhattak@yahoo.com;*

1. INTRODUCTION

Maize (*Zea mays* L.) of family Poacea is considered as a leading cereal of the world due to total production (695 million tonnes) and per unit area yield (4815 kg ha) [1]. Maize is of significant importance for the developing countries, where rapidly increasing population has already out stripped the available food supplies. In Pakistan, it is the third major cereal after wheat and rice and plays a considerable role in the overall progress of the national economy [2].This crop is cultivated on 0.95 million hectares with a yield of 1.6 million metric tons and share 4.82% in cereal production in Pakistan [3]. Being a short duration crop, this cereal has tendency to solve the food shortage by growing two crops per year and vacating the land in time for wheat cultivation in the prevailing cropping system.

Despite its high yield potential, one of the major limiting factors to maize grain yield is its sensitivity to numerous diseases [4]. It is mostly grown in low and mid-elevation zones (plains) of Pakistan in months when the climatic conditions are most suitable for the growth and development of different diseases. Approximately 65 pathogens infect maize [5]. Most fungal pathogens of maize are seed borne and cause rottening and discoloration of seeds [6]. Drastic decrease in maize yield is caused by Southern leaf blight (SCLB), northern leaf blight (NLB), gray leaf spots (GLS) and various types of rust. SLB or Maydis leaf blight (MLB) is one of the most important disease of maize caused by fungus *Cochliobolus heterostophus* (*Bipolaris maydis, Helminthosporium maydis*) and constitutes a major threat to maize production throughout the world where maize is grown under warm, humid conditions [7,8]. Three races of *C. heterostrophus* known as O, T and C are known so far [9, 10]. Race O is considered as the most common race in most areas and is controlled by nuclear genes. Race T, the cause of 1970's epidemic in North America is specific to maize containing Texas male-sterile cytoplasm (cms-T) and is controlled mainly by cytoplasmic factors. The most prominent difference between race O and T is that race O only attacks leaves while race T attacks leaves, stalks, leaf sheaths, ear husks, ears and cobs. Race C is a cms-C cytoplasm-specific race reported only in China. SCLB or MLB accounts for 20% or sometimes even more yield losses to maize crop in Pakistan [11].

Most efficient, economical and sustainable way of controlling SCLB is breeding of the resistant varieties to the pathogen *C. heterostrophus* [12]. However, seed borne fungi can be controlled by treatment with fungicides [13,14]. Thus the present research study was planned to investigate the physiological conditions most suitable for the growth of *C. heterostrophus* isolated from samples collected from different agro-climatic zones of Pakistan (Part 1). Then optimized growth conditions of the pathogen would be used as a baseline for *in-vitro* and *in-vivo* evaluation of the efficacy of different fungicides against SCLB (Part 11).

2. MATERIALS AND METHODS

2.1 Isolation of *C. heterostrophus*

The fungus *C. heterostrophus* was isolated by the technique of Ricker and Ricker [15]. The leaves of maize, bearing typical spots (race O) of the disease SCLB were collected from the fields of northern areas, Khyberpukhtoon Khawa (KPK) and Punjab (northern zone), were examined in fungal pathology laboratory, Crop Disease Research Program (CDRP), National Agriculture Research Centre (NARC) and Microbiology Research Laboratory (MRL), Quaid-i-Azam University, Islamabad. The infected portions of leaves were cut into small pieces;

surface was sterilized with 1% aqueous solution of chlorex for one minute. The sterilized pieces were rinsed with sterilized distilled water, blotted and plated on PDA (three pieces per 90 mm petri-plate), agar water medium (three pieces per petri plate) and also on sterilized autoclaved moist filter paper lined in petri plates. The growth of pathogen was visualized under stereo-microscope after incubation at 29ºC for 24 hrs alternate light and dark periods. Pure culture of the pathogen was prepared by single spore technique by picking a single conidium transferred on PDA. Maximum growth of pathogen *C. heterostrophus* was attained after one weak. Then these cultures ware used for further experiments.

2.2 Pathogenicity Test

Pathogenicity of the fungal strain was checked on susceptible maize variety in earthen pots (30 cm in diameter) containing autoclaved sterilized soil. Twelve pots were used, three seeds of maize were sown in each pot, six pots were inoculated at 3-4 leaf stages with the inoculum ($2x10^4$ spores/ ml) of the *C. heterostrophus* (conidial suspension), and six were sprayed with sterilized distilled water (control or un-inoculated). These pots were placed in growth room by maintaining temperature at 29ºC for incubation. After one week of inoculation, the plants were inspected daily for the initiation of SCLB symptoms. Re-isolation of fungus was made from diseased leaves on PDA and was subjected to pathogenicity test for confirmation of Koch's postulates. The disease SLB on the plants was also rated with a 0-5 scale based on leaf area i.e. 0 for no lesion and 5 for heavily blighted leaves. An arbitrary gradation of 10 class scale (i.e. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0) was also used to measure more accurately the disease severity.

2.3 Effect of Culture Conditions on Radial Colony Growth of *C. heterostrophus*

The effects of culture conditions including media, incubation temperature and pH were observed on radial colony growth of pathogen *C. heterostrophus*. For this purpose, different natural and synthetic media (Potato dextrose agar medium, Water agar medium, Basal medium, Richard's agar, Corn meal agar and Czapek`s dox), temperature (20, 25, 30, and 35ºC) and pH (3,5,7,9 and 11) were studied. All the experiments were conducted in triplicate. The radial growth of the pathogen was measured at 3rd, 5th and 7th days of incubation period. The data for radial mycelial growth was subjected to one-way analysis of variances (ANOVA). Means at various treatments were separated using Fisher's protected least significant difference (LSD) at $P = .05$ to visualize the difference between treatments.

2.3.1 Effect of different temperature on radial colony growth of *C. heterostrophus*

To determine the most suitable optimum temperature for the growth of *C. heterostrophus*, temperature i.e. 20, 25, 30, and 35ºC were studied on Richard's agar medium in the petri-plates. The experiment was conducted in triplicates. The colony diameter of the pathogen at each temperature was recorded at 3rd, 5th and 7th days during incubation period. The data for mycelial growth were analyzed statistically to observe the difference between treatments.

2.3.2 Effect of different carbon sources on radial colony growth of *C. heterostrophus*

Five different carbon sources viz., glucose, maltose, fructose, dextrose and sucrose were studied individually as substitute of sucrose in standard Richard's agar medium. These sources were used to give equivalent amount of carbon. The experiment was conducted in triplicate. The data for radial colony growth of the pathogen were taken at 3rd, 5th and 7th

days of incubation period and analyzed statistically to observe the difference between treatments.

2.3.3 Effect of different nitrogen sources on radial colony growth of *C. heterostrophus*

Four nitrogen sources viz., peptone, potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$) ammonium sulphate $(NH_4)_2SO_4$, were used to study their effects on the growth of the fungus. The quantities used were: Ammonium nitrate, 5 g, Ammonium sulphate, 6.6 g, Sodium nitrate 8.5 g, Potassium nitrate and 8.5 g/1000 ml of medium respectively. Calcium carbonate 0.75% was added in each case to maintain the neutral pH of medium. The experiment was conducted in triplicate and incubated at 29ºC. Radial growth of the fungus was taken at 3rd, 5th and 7th days during incubation period. The data for mycelial growth was analyzed statistically to check the difference between the treatments.

2.3.4 Effect of different initial pH levels on radial colony growth of *C. heterostrophus*

The different levels of pH on the growth of *C. heterostrophus* was studied on Richards's agar medium for the growth of the pathogen with pH levels 3, 5, 7, 9 and 11 by addition of appropriate volume of N\2 HCL and normal NaOH solution. The medium was autoclaved, adjusted for respective pH levels before pouring in petri-plates. The experiment was conducted in triplicate and the plates were incubated at 29ºC. Radial growth of the pathogen was recorded at 3rd, 5th and 7th days during incubation period. The data for radial mycelial growth was analyzed statistically to check the difference between the treatments.

3. RESULTS AND DISCUSSION

3.1 Isolation of *C. heterostrophus* and Its Morphology

In the present investigation, foliar maize disease SCLB was examined thoroughly under laboratory and field conditions at NARC and MRL Islamabad. The isolated pathogen was found to be similar in character to that of *C. heterostrophus* on the basis of microscopic and morphological characteristics. Conidia were slightly curved having 3-10 septa, widest in the middle and tapering towards the ends, measuring 30-121µm x 15-22µm in size. Peripheral walls of conidia were thin showing the germination by the production of two polar germ tubes as reported by Drechsler [16] who also described detailed morphology of the pathogen *C. heterostrophus* in pure cultures, measuring 30-115µm x 10-17µm in size. However, there were some differences in size and septation which may be due to environmental variation or due to nutritional factors.

3.2 Pathogenicity Test

Pathogenicity of the fungus was studied on maize plants (susceptible to SCLB) grown in earthen pots. Data on un-inoculated (control i.e. sprayed with distilled water) and inoculated plants were taken as the symptoms of disease appeared on the leaves. Percent plant infection and disease severity on un-inoculated plants was 23.3 and 1.2%, while, on inoculated plants, it was 76.7 and 2.7% respectively. There was 228.6% increase in percent plant infection and 125% increase in disease severity over control.

3.3 Effect of Different Culture Media on Radial Colony Growth of *C. heterostrophus*

In culture media investigation, various growth media were used for evaluating nutrient availability to *C. heterostrophus*. Gao and Liu [17] also signifies mass production (maxi. mycelia and spore yields) is directly related to the nutritional availability from culture media. In the present investigation, growth of the pathogen *C. heterostrophus* was significantly different (*P* = .05) in culture media used. In all media the growth increased with an increase in incubation period. The most effective supporting medium for the growth of the fungus was the Richard's agar which showed 84.7 mm diameter colony growth of the pathogen after an incubation period of 7 days followed by PDA (78 mm), Basal medium (70 mm), Corn meal agar (70 mm) and Czapek`s medium (70 mm), while, Water agar was found to be the least effective (50 mm) (Fig. 1). Two media i.e., PDA and Richard's agar proved excellent for growth and sporulation of *C. heterostrophus* with the highest colony diameter of 78.6 and 66.0 mm, respectively in studies conducted by Kumar et al. [18]. On the other hand, different results were obtained by Earle et al. [19] who studied the pathogen *C. heterostrophus* thoroughly which grew and sporulated well on a variety of natural agar media as well as plant tissues and also found that the pathogen grew well but sporulated sparingly in Richard`s and Czapek`s media. However, Amin et al. [20] used PDA for isolation and for further in vitro screening of fungal endophytes isolate against pathogen of SCLB, *H. maydis* (*C. heterostrophus*). Moreover, maximum mycelial growth and sporulation of genus Helminthosporium (sub- genera *Drechslera bicolor*) was obtained on PDA followed by Malt extract and Richard's media [21].

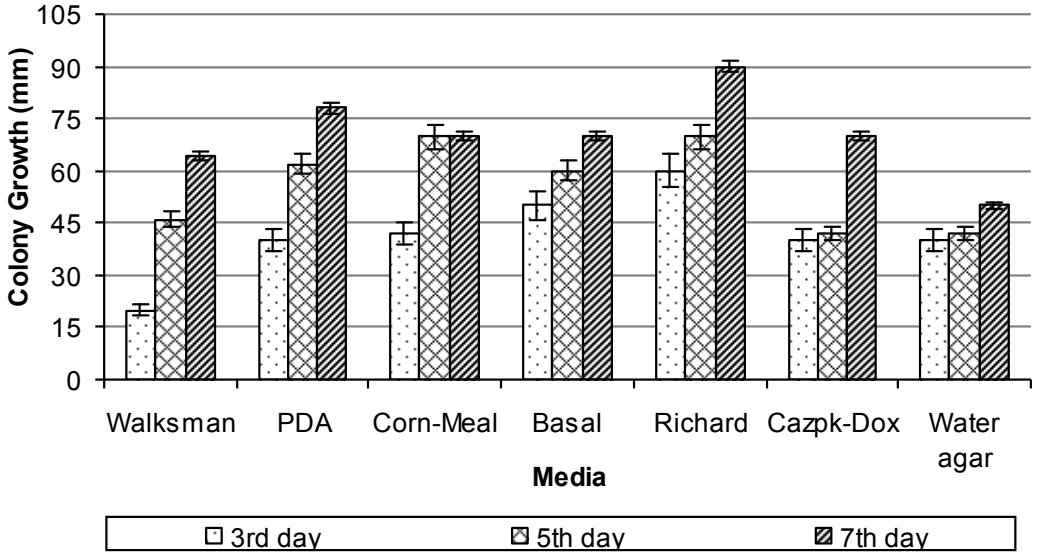

Fig. 1. Radial colony growth of *C. heterostrophus* by using different culture media

3.3.1 Effect of different incubation temperature on radial colony growth of *C. heterostrophus*

The pathogen *C. heterostrophus* from pure culture was grown on Richard's agar at four different temperature levels viz. 20, 25, 30 and 35ºC. The pathogen showed significant

difference at *P* = .05 in colony growth at different temperature levels. The most suitable temperature for mycelial growth of the pathogen was 30ºC as the diameter at this temperature was 80 mm after 7 days of incubation period. Whereas, other temperature levels (25 and 35ºC) showed reduction in the colony growth (45 and 35 mm respectively) while, minimum growth was observed at 20ºC showing 28 mm colony diameter (Fig. 2). Almost similar results were reported by Wallin and Loonan [22]. While Almaguer et al. [23] reported optimum temperature range of 10–40ºC for mycelial growth and germination of air born *Bipolaris* species.

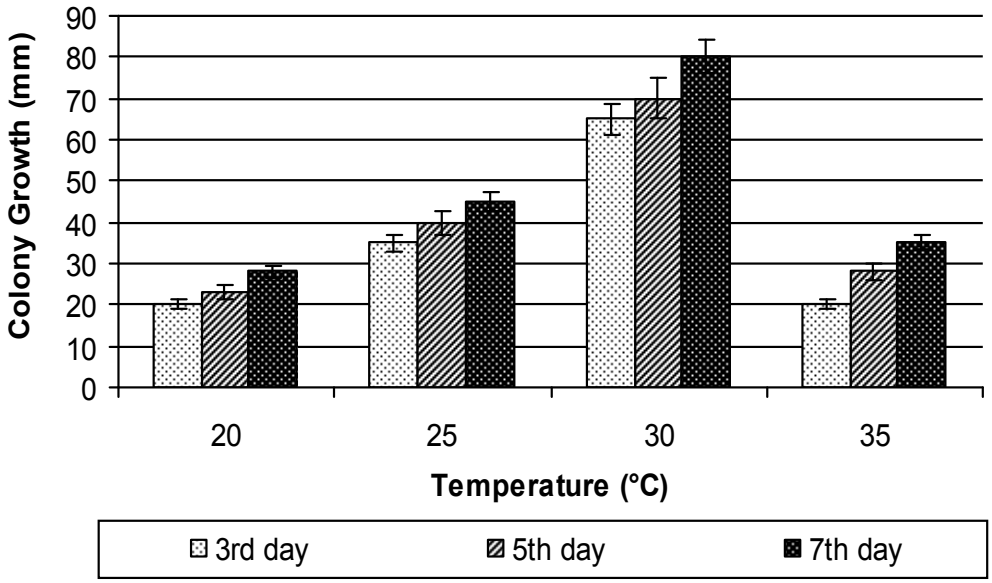

Fig. 2. Radial colony growth of *C. heterostrophus* at different incubation temperature

3.3.2 Effect of different carbon sources on radial colony growth of *C. heterostrophus*

After selection of Richard's agar as best medium at 30ºC incubation temperature, carbon component was replaced with glucose, dextrose, maltose, sucrose and fructose. The results indicated that all the carbon sources vary significantly at *P* = .04 in their effect on the radial colony growth of the pathogen *C. heterostrophus*. However, sucrose was found to be the best carbon sources and showed maximum colony growth (84 mm) followed by glucose (82 mm), dextrose (80 mm), maltose (70 mm) and fructose (68 mm) (Fig. 3). However, Bennett et al. [24] used corn oil as sole carbon source for growth of *C. heterostrophus* Race T (NRRL 5128) in shaken flasks at 28ºC. On the other hand, monosaccharides i.e., glucose and fructose were reported to be the most suitable carbon supplement for colony growth, biomass production and sporulation of *C. heterostrophus* [18].

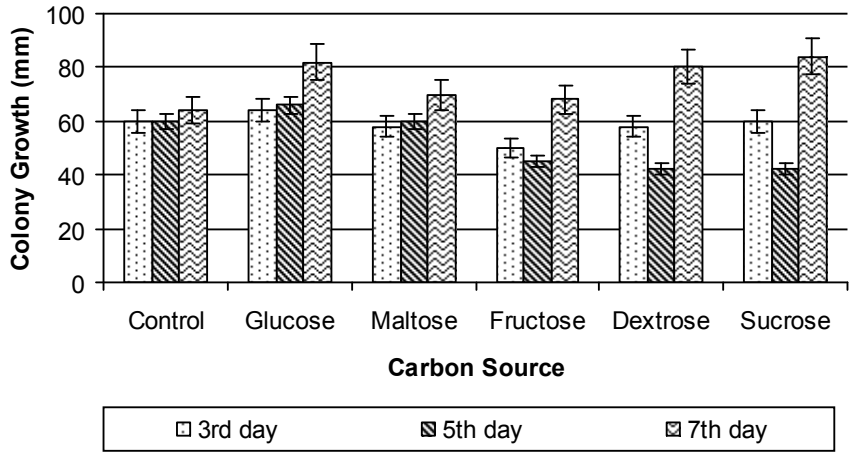

Fig. 3. Radial colony growth of *C. heterostrophus* by using different carbon source

3.3.3 Effect of different nitrogen sources on radial colony growth of *C. heterostrophus*

Four different nitrogen sources viz., potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$), peptone, ammonium sulphate $(NH_4)_2SO_4$ were emended in Richard's media with sucrose as best carbon source. The Nitrogen sources showed significant effect (P = .02) on radial colony growth of the pathogen *C. heterostrophus.* Maximum growth of the pathogen (90 mm in diameter) was obtained with KNO_3 used as nitrogen source in the media. This was inorganic nitrogen responsible for vigorous mycelial growth of *C. heterostrophus*. Evans et al. [25] found organic nitrogen sources generally result in greater dry weight than inorganic ones studied the effect of 23 organic and 3 inorganic nitrogen sources on growth, sporulation and polyphenoloxidase activity in *Bipolaris maydis* race T. Second nitrogen source was $NaNO_3$ which showed 84 mm diameter colony growth after 7 days of incubation period (Fig. 4). Whereas, $(NH_4)_2SO_4$ gave minimum growth (47 mm in diameter) after seven days of incubation. Combined effect of C: N is very important for the growth of *C. heterostrophus*. Various researchers have studied the effect of C:N on fungal growth [26,27,28].

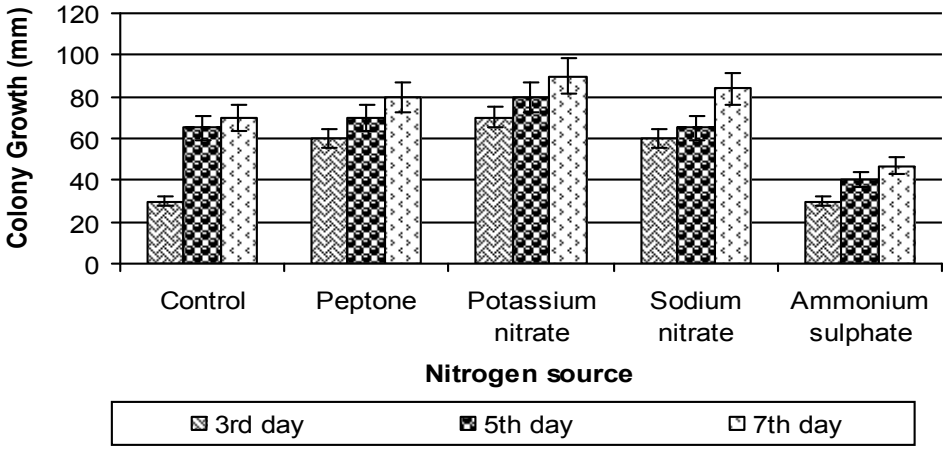

Fig. 4. Radial colony growth of *C. heterostrophus* by using different nitrogen source

3.3.4 Effect of different initial pH levels on radial colony growth of *C. heterostrophus*

Growth of the pathogen was obtained at different initial pH levels (3, 5, 7, 9 and 11) tested but it was maximum (88.0 mm in diameter) at pH 7 after 7 days of incubation. Observation revealed that pH 5 and pH 9 were favorable for colony growth (83.3 and 84.0 mm in diameter) (Fig. 5). Singh and Singh [29] also observed *H. maydis* grew best at pH 5-7. A slightly acidic initial pH of 6.5 in PDA was found to be favorable for growth and reproduction of pathogen *Drechslera bicolor* of genus *Helminthosporium* [21].

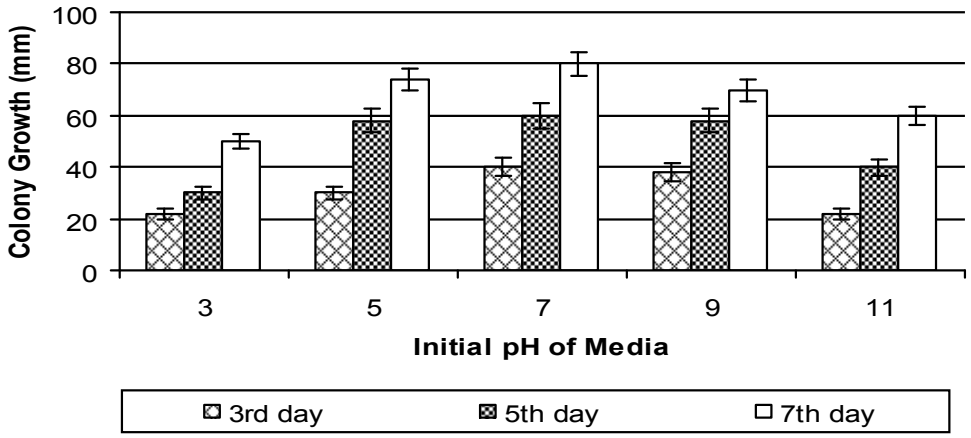

Fig. 5. Radial colony growth of *C. heterostrophus* by using different initial pH levels in growth media

4. CONCLUSION

In the present investigation, the pathogen isolated from infected samples of Maize was *C. heterostrophus* and Richard`s medium was found to be highly suitable for its growth. This pathogen grew well at neutral pH at 30ºC. Best carbon and nitrogen sources include sucrose and KNO_3 respectively. This study will provide a baseline for future *in-vitro* and *in-vivo* experimentation on control of SCLB.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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