Research Article

Mass Culturing of Mycetophagous Nematode *Aphelenchus avenae* (Nematoda: Aphelenchidae) *in vitro* System by Feeding on Pathogenic Fungus

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Abstract | The production phenomenon of fungal feeding nematode *Aphelenchus avenae* have been thoroughly examined in *vitro* system by ensuring Potato Dextrose Agar (PDA). Two fungal species *Fusarium oxysporum* and *Aspergillus niger* were examined for their aptitude to assist the population rate of the said species. Nematodes were collected from soil around the rhizospheres of sponge gourd (*Luffa cylindrical* L.) field in the vicinity of National Nematological Research Centre, University of Karachi and further processed by Cobb's sieving and Baermann's funnel techniques. Specimens were killed, fixed and identified up to species level under light microscope. Afterward, hundred fresh handpicked nematodes were inoculated on both fully grown fungal species at 25°C. The nematode attraction and mass culture were appeared on *Fusarium oxysporum* only. On the first week, propagation rate was noted approximately $2.5x10^3$ followed by $3.9x10^3$ on the second week and finely after three weeks incubation period the greatest multiplication rate estimated up to $8.9x10^3$; however, the same did not appeared on *Aspergillus niger* and result was found negative. The main purpose of captioned study was to report mass culturing of parasitic *Aphelenchus avenae* and their relationship and further explore these nematodes under field circumstances to control many pathogenic soil-borne fungi because this is the first study conducted on the subject experiment in Pakistan.

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Introduction

The ecosystems consist of various kinds of microscopic organisms including archaea, bacteria, fungi, protozoa, and nematodes. Collectively, these organisms interact with each other and as well as with plants to achieve ecosystem functions. Their interactions ensue through various type of mechanisms likewise mutualism, parasitism, predation or competition (Topalovic and Heuer, 2019). In the terrestrial ecosystem nematodes and fungi are knowing the abundant organisms and generally co-existing around roots of plants as well as crops with substantial effects upon forestry and agriculture. Therefore, the relationships between them whether antagonistic or mutualistic, direct or indirect, attracted significant attention (Zhang *et al*., 2020).

The populace of fungivorous nematodes in soil are basically subordinate than plant parasitic or bacterial feeder nematodes (Freckman and Caswell, 1985) but good fungal hosts being there, the population

densities of these nematodes could rise quickly (Duyck *et al*., 2009; Quénéhervé, 2008; Poornima *et al*., 2007; Arancon *et al*., 2003; Hoffman and S'-Jacob, 1989). However, antagonistic relationship existing between nematodes and fungi are frequent as they varied. Some nematodes likewise *Aphelenchus avenae*, *Aphelenchoides* spp., as well as *Paraphelenchus acontioides* may fed upon fungi (Lamondia and Timper, 2016). Among these, *A. avenae* is ubiquitous fungivores nematode and has been confirmed and verified as biological agent to control soil-borne plant pathogens (Haraguchi and Yoshiga, 2020; Azimi, 2018; Lamondia and Timper, 2016; Jun and Kim, 2004). They are high reproduction rate, limited life cycle, highly colonized capacity and are potential to any disturbances (Bonger, 1990; Kibet *et al*., 2014).

The feeding routine of *A. avenae* is important to recognize their biology and role in ecosystem. A total of 6.1 ng of Nitrogen consumes by *A. avenae* from fungal biomass in 24 hours which rises nitrogen mineralization and release of CO2 (Ingham *et al*., 1985), thus promising to soil fertility (Chen and Ferris, 1999). Synergistic and antagonistic interactions between fungi and nematodes have been reported (Ragozzino and D' Errico, 2011). While, fungivorous nematode parasitizing fungi, feed on their cell contents and subsequently decreasing fungal biomass (Wolfarth *et al*., 2013), they can also co-exist with fungal endophytes in a cultivation-based mutualism, where the nematode established the growth of its favorable fungus (Baynes *et al*., 2012). However, in case of parasitic interaction between fungivorous nematodes and pathogenic fungi, the disease severity is reduced (Karuri *et al*., 2014; Ruess and Dighton, 1996).

Feeding behaviour of fungivorus nematode has dissimilar effect on soil ecology. High grazing on mycorrhizal fungi may limit mycorrhizal growth and nutrient uptake by host plants which is complicatedness to plants. This can direct to lessening in the yield of mycorrhizal host plants (Ruess *et al*., 2000).

Pathogenic *Fusarium oxysporum* is a source of vascular wilt, stunting, chlorosis, die back and death of plant in numbers of crops. They achieve entrance via roots and assault vascular system from where they get access throughout plant. In East Africa Fusarium wilt has reported and as well as in Kenya wherever it effectively minimized the fiber quality and yields (ICAC, 2003). At field level progress of Fusarium wilt is depending upon susceptibility of the crop, fungal virulence, kind of soil and fecundity, environmental circumstances and associations with some other organisms, i.e., nematodes.

Fungivorous nematodes could be cultured on various fungal species, including pathogenic, saprophytic and mycorrhyzal fungi (Freckman and Caswell, 1985; Giannakis and Sanders, 1989; Ruess and Dighton, 1996; Bae and Knudsen, 2001; Tahir *et al*., 2017). *A. avenae* feeds over 52 genera of fungi mostly plant pathogens and is an important biological control agent (Mankau and Mankau, 1963; Giannakis and Sanders, 1989; Okada and Ferris, 2001; Okada, 2006; Karuri *et al*., 2014; Tahir *et al*., 2017). However, our result also agreed with the statements given by researchers (Okada, 2006; Karuri *et al*., 2014; Tahir *et al*., 2017) who said *A. avenae* reproduced on many species of Fusarium. The present objective of this article is to report mass culture of parasitic nematode *A. avenae* and provide their evidence or relationship between them as mentioned in this article, and in future to apply these nematodes under field condition to control various pathogenic soil borne fungi and other plant parasitic nematodes for better agricultural productions and soil fertility. It is basically first systematic study focused on mass culture of fungivorous nematode *A. avenae* under laboratory condition by feeding upon fungus on PDA and is thoroughly described in this article.

Materials and Methods

Isolation of fungus

The pure culture of *Fusarium oxysporum* used in the experiment was obtained from Pakistan Agriculture Research Council (PARC), University of Karachi, Karachi, Pakistan while *Aspergillus niger* were retrieved in laboratory from infected banana leaves. For this purpose, leaves were thoroughly washed and cut into 3mm pieces via sterilized scalpel. Surface sterilized with 70% ethyl alcohol for few seconds followed by 2-3 times washed with sterilized water and transferred aseptically to fresh solidifying PDA plates (Figure 1A and B), supplemented with streptomycin (100 mg/l) and incubated at 25°C for 3-5 days (Aneja, 2003). They were further sub-cultured aseptically on other fresh solidifying PDA plates or slants for maintaining their pure biomass for further work.

Extraction of nematodes

Nematodes were isolated from soil of sponge gourd (*Luffa cylindrical* L.) from field of National Nematological Research Centre, University of Karachi, Karachi, Pakistan. This soil sample was processed by Cobb's sieving and decanting technique (Cobb, 1918). Retrieved nematodes were further purified through modified Baermann's funnel technique (Baermann, 1917).

Mounting of nematodes for confirmation of Aphelenchus species

Fresh and alive *Aphelenchus* were handpicked under stereomicroscope. These *Aphelenchus* were heat killed and conserved in TAF (Tri-ethanol-amine Formaldehyde) including 8% formalin and 2% Tri-ethanol-amine in distilled water (Courtney *et al*., 1955). Permanent mounting was done in a small drop of pure glycerin, protected with 19mm of cover slip and fixed firmly by paraffin wax (Siddiqui, 2000). Measurements were taken by utilizing de Man's formula (1884) with the assistance of an ocular micrometer in a compound microscope up to species level. Photographs were captured via Nikon DS-fi-1 camera fixed with Nikon Eclipse- E-400 compound microscope.

Media preparation

The source of medium used in the experiment was Potato Dextrose Agar (PDA). Peeled potatoes about 200 gm were boiled in 1000 ml distilled water for 45 minutes. Sugar (20gm) and Agar (16gm) were added and autoclaved at 15 psi for 1 hour. Distributed into sterilized petri dishes and test tubes aseptically under laminar air flow.

Inoculation of A. avenae

About 100 nematodes (including 90 females and 10 males) for each culture were added by pipette in petri dishes aseptically to fully grown pure fungal cultures of *Fusarium oxysporum* and *Aspergillus niger* (Figure 1C and D). Subsequently, petri dishes were sealed by parafilm (PM-996) and incubated at 25°C.

Retrieved of nematodes from culture media

After three weeks incubation period, distilled water was added in culture plate, gently shaken and nematodes were collected in large glass petri plate. Average propagation rate was calculated and repeated three times in an open counting chamber in 1ml suspension under binocular microscope.

Data analysis

Collected data were analyzed correlation with respect of growth and time period by using Statistical Analysis System (SAS) program.

Results and Discussion

Identification of nematode species

Aphelenchus avenae Bastian, 1865

Measurements: (Table 1)

Table 1: *Morphometric data of A. avenae* Bastian, 1865. *All measurements are in µm in the form of Mean±SD (range).*

Characters	Female (n=10)	Male (n=5)
L	720 ± 45.1 (666–749)	650 ± 62.0 (600–743)
a	26.6±1.66 (22.8–28.9)	27.1±1.616 (25.1–28.9)
b'	4.22±0.52 (3.4–4.9)	3.8±0.47 (3.4–4)
c	27.3±3.00 (24–33.6)	25.0±2.09 (22.7–27)
c'	1.53±0.14 (1.3–1.8)	1.36±0.12 (1.2–1.4)
V%	74.5±2.17 (69–76.7)	–
Stylet	15.5±0.67 (14-16)	15.2±0.4 (15-16)
Spicule	-	26±1.67 (24-28)
Gubernaculum	-	14.8±0.97 (14-16)

Brief description: Body usually long, cylinder-shaped, ventrally arcuate or straight to some extent. Lateral field with 10-12 incisures. Lip region low, round to flattened, non-offset. Stylet long without basal swelling. Procorpus cylindrical. Median bulb well developed, oval-shaped and conspicuous valve plates located centrally. Dorsal esophageal gland orifice opening into lumen of median bulb. Esophago-intestinal junction located posteriorly to the base of median bulb and measured 70-100 µm from head region. Dorsal oesophageal gland usually well developed, overlapped the intestine on the left subdorsal side. Nerve ring located posterior to metacorpus and excretory pore located posteriorly to nerve ring.

Reproductive system mono-prodelphic. Post uterine sac present and well developed. Vulva protuberant. Female tail broad, cylindrical and bluntly rounded. Male tail short, conoid, narrowing posteriorly to a pointed end. Spicules slender, paired, ventrally arched, cephalated proximally. Gubernaculum linear. Bursa well developed. Four pairs of bursal papillae present.

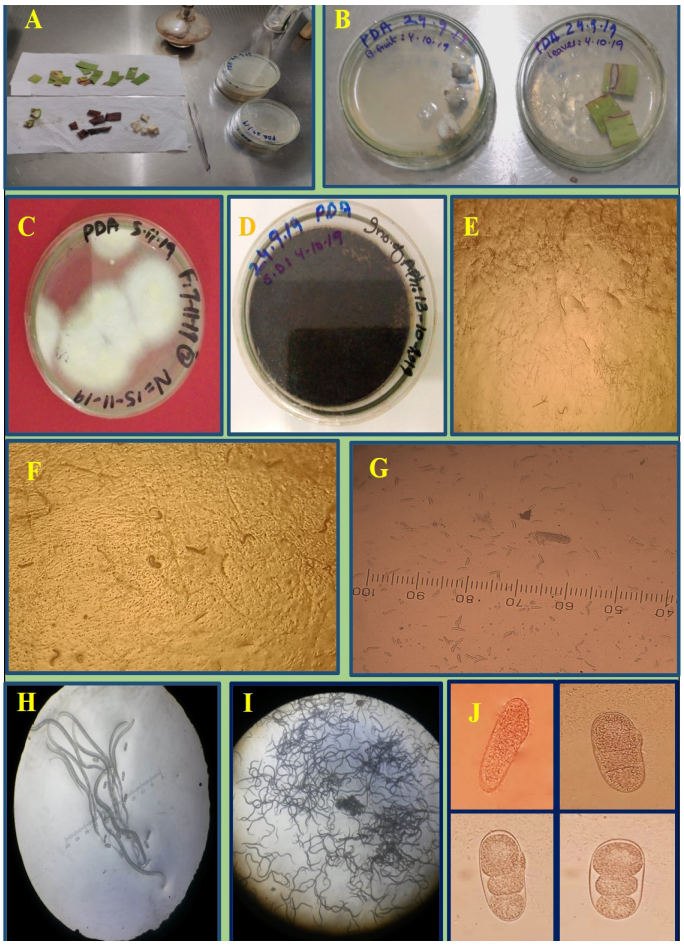

Figure 1: *A and B. Isolation of Aspergillus niger from banana leaves; C. Pure culture of Fusarium oxysporum; D. Pure culture of Aspergillus niger; E and F. Interaction between fungus and nematodes; G. Fungal spores; H. Bunch of nematodes along with eggs; I. Mass culture of A. avenae after grew on F. oxysporum; J. Various embryogenetic stages of A. avenae.*

Eggs: Eggs of *A. avenae* were found in different embryological stages and morphologically same like other nematode species Figure 1J. Temporary slides and measurements were made for morphological studies. The eggs were crystal clear, elongate in shape and about 80–87.5 µm long and 36–37.5 µm wide.

Survival rate and observations highlighted during incubation period

Two fungal species *F. oxysporum* and *Aspergillus niger* were examined for (1) *F. oxysporum* as a good fungal host for nematode, easily mass cultured and pathogenic to plant (2) *Aspergillus niger* as non-fungal host, easily mass cultured and for comparatively result. Fungal feeder nematode *A. avenae* was used for their aptitude to assist the population rate by ensuring PDA medium. After three weeks incubation period, nematode mass culture was appeared on *F. oxysporum* only. On the first week, propagation rate was noted approximately $2.5x10^3$ followed by $3.9x10^3$on the second week and finely after three weeks incubation period the greatest multiplication rate was estimated upto $8.9x10^3$ (Table 2 and Figure 2), while did not observed the attraction of nematode towards the fungus *Aspergillus niger* and resultantly no multiplications were reported after inoculation of initial calculated nematodes (i.e., 100) for each fungus (Figure 3). It has been confirmed the presence of preferable and non-preferable fungal host. Furthermore, it also observed that nematode preferred mycelia rather than conidia. During the course of incubation process, *A. avenae* was observed in close interaction with fungus through contact of their head with the wall of fungal hyphae (Figure 1E and G). This close interaction was exhausted after breaking of fungal hyphae and consuming of entire cell contents by the nematodes. Forming of sunken areas in the colonies was observed because of the damaging by fungivorous nematodes. After one week, a bunch of *Aphelenchus* along with eggs in various embryogenetic stages were surfaced Figure 1H. The numbers of *A. avenae* have been increased many folds and observed that no space existed among them Figure 1I. Moreover, many gravid females in final stage for eviction of eggs from the uterus were also found.

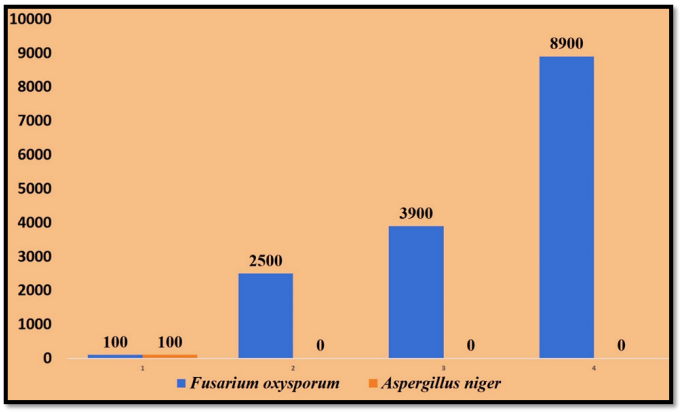

Figure 2: *Increasing in population of A. avenae on F. oxysporum with no growth on Aspergillus niger during three weeks incubation period.*

Table 2: *Reproduction rates of A. avenae on Fusarium oxysporum and Aspergillus niger.*

Species	1st Day	1st Week	2nd Week	3rd Week
Fusarium oxysporum	100	2500	3900	8900
Aspergillus niger	100	0	0	0

The nematodes were also inoculated in a test tube having pure culture of *Fusarium oxysporum* Figure 4A. Nematodes moving tracks were noted on surface of PDA. After few days, gradually the changing was observed in general appearance and lessening in fungal mass (Figure 4B and C). Consequently, the PDA

medium reduced and accordingly moved upward from its primary position inside the tube which indicated that *Aphelenchus* reproduced themselves by consuming fungus as well as medium (Figure 4D and E). After conducted observations, nematodes were retrieved from culture media and inoculated in a pot having sterilized soil for other experimental purpose in future Figure 5.

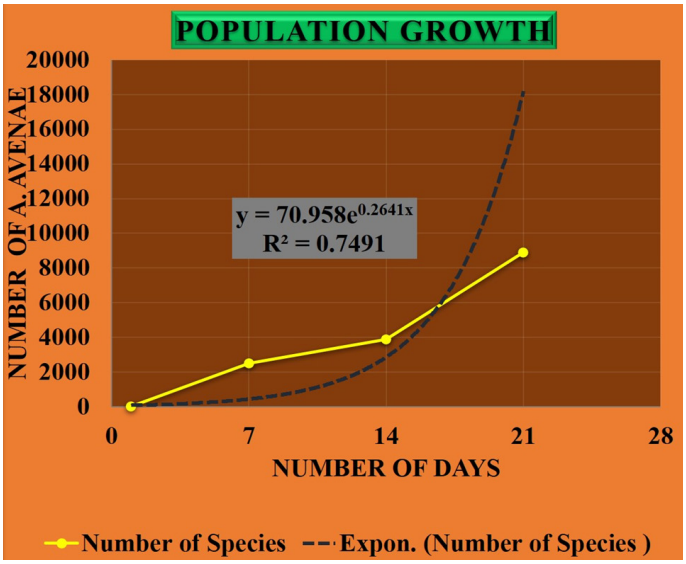

Figure 3: *Exponential growth of A. avenae on F. oxysporum grew on PDA after three weeks incubation period.*

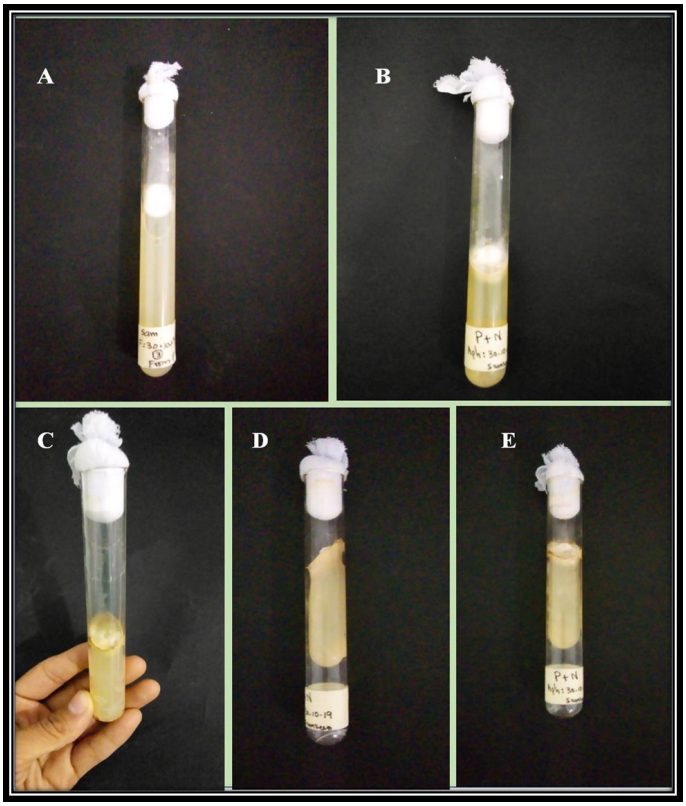

Figure 4: *A. Pure culture of F. oxysporum in test tube; B-C. Change in appearance and lessening of F. oxysporum after inoculation of A. avenae; D-E. Consuming fungus as well PDA medium by A. avenae.*

Figure 5: *Culturing of A. avenae in a pot using brinjal plant.*

The fungal feeder nematode *A. avenae* has great capability to produce itself upon feeding several types of fungi in cultivated and also non-cultivated soil, consequently making it distribute globally (Ishibashi *et al.*, 2005). In the present study *A. avenae* was used as a model predator due to their ubiquitous presence in soils of temperate regions where they cohabit with most of the fungal species and easy reproduced themselves in short time. The current research work represented that propagation of the nematode was substantially dissimilar as a result of different fungal species. In current study *Fusarium oxysporum* was observed the favourable fungal host for support of large multiplication of fungal feeding nematode, *A. avenae*, in short period as compared *Aspergillus niger*. Their mouth is intended with the needle like structure (stylet) and well-developed esophageal part (metacorpus) that facilitate them to penetrate the fungal hyphal cell and consumed entire cell contents. Earlier, Rhoades and Linford, 1959, conducted the first systematic research and observed that the *A. avenae* has ensured for Pythium root rot controlling in maize (*Zea mays* L.) in greenhouse situations. After that, *A. avenae* and some fungivorous species in the genera *Aphelenchoides* has been experienced in laboratory, greenhouse and in field examination to control variety of phytopathogenic fungi in crops (Friberg *et al.*, 2005).

Many fungivorous nematodes could be reared on media having suitable fungus and established strict damages to fungal cultures. In *vitro,* the higher multiplication and number of *A. avenae* on *Fusarium graminearum* (114233) followed by *Fusarium oxysporum* (47013) and *Verticillun dahliae* (1500) after

two weeks incubation period by ensuring solid PDA and Malt extract liquid medium was conducted by Tahir *et al*., 2017. He observed lower reproduction of *A. avenae* and found dead on liquid culture, support their finding with the statement given by (Grewal *et al*., 2005) that *A. avenae* could not propagate and surviving in liquid media. Ishibashi *et al*. (2005) studied that *A. avenae* during feeding in fungal culture, they secrete hydrolytic enzyme which destroyed and hydrolyzed the mycelium of fungus.

In the light of above, few other cases of interrelationships and true parasitism between nematodes and fungi is to be worth mentioning. Ishibashi *et al*. (2000) observed reproductive range of *A. avenae* in semisolid substrate. In another research, *A. avenae* and *Aphelenchoides* species has been experienced to suppress fungal disease of plants and nitrogen mineralization in soil (Okada, 2006). Damping off of radish due to *Pythium* spp was also expressed to be controlled through *A. avenae*. Karuri *et al*. (2014) assess the interrelation between *Fusarium oxysporum* f. sp. *vasinfectum* and *A. avenae* on Bt cotton and its isogenic counterpart in greenhouse situaions. Lagerlof *et al*. (2011) tested the hypothesis through greenhouse experiments about the *Aphelenchoides* spp. and *A. avenae* that could suppress damping-off due to *Rhizoctonia solani* in cauliflower seedlings and improve the disease-suppressive impact of compost. In recent study, Haraguchi and Yoshiga (2020) studied that *A. avenae* may contribute to suppress plant parasitic nematode *Ditylenchus destructor* through fungal control *in vitro* experiments.

The aforesaid experience proved the veracity of fact with respect to interrelation between *A. avenae* and *Fusarium* species (Mankau and Mankau, 1963; Okada, 2006; Karuri *et al*., 2014; Tahir *et al*., 2017). Although the biocontrol potential of *A. avenae* is well recognized, they have not yet been widely used in applied agriculture or horticulture in the formulation of nematode applications in Pakistan even though they are quite easy to proliferate in huge numbers within short time period. In this point of view, their use perhaps economically realistic as well as environmentally welcoming and should be distributed to growers for field applications.

Conclusions and Recommendations

The veracity of Nematodes-Fungus relationships has been successfully established *in vitro* system for the first time in Pakistan. The said experiment providing evidence that the fungivorous nematodes should be proficient biocontrol agent against various phytopathogenic fungi and other plant parasitic nematodes. It is considered that more investigations from various aspects are needed to be determined the biology of fungivorous nematodes and their substantial influences on soil ecology as well as crop production especially in Pakistan.

Novelty Statement

The said research article describes rearing of fungal feeding nematodes on their attractive feeds for agricultural importance for the first time conducted in Pakistan. This research work is significant and meaningful because new valuable information obtained from the said experiment and the same will be assisted/ helpful in future for further culturing of plant-parasitic nematodes. Moreover, the said article being a guideline for researchers in the field of plant nematology.

Author's Contribution

Salma Javed: Supervised the research and critically reviewed the manuscript.
Samreen Khan: Performed the experiment, made photography, analyzed data and drafted the manuscript.

Conflict of interest
The authors declare no conflict of interest.

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