13. Körner C., *Alpine Treelines: Functional Ecology of the Global High Elevation Tree Limits*, Springer, Basel, 2012.
14. Shrestha, K. B., Hofgaard, A. and Vandvik, V., *J. Plant. Ecol*., 2015, **8**, 347–358.
15. Liang, E. *et al.*, *Proc. Natl. Acad. Sci. USA*, 2016, **113**, 4380–4385; doi: 10.1073/pnas.1520582113.
16. Dubey, B., Yadav, R. R., Singh, J. and Chaturvedi, R., *Curr. Sci*., 2003, **85**, 1135–1136.
17. Yadava, A. K. *et al.*, *Quat. Int*., 2017, **444**, 44–52.
18. Telwala, Y., Brook, B. W., Manish, K. and Pandit, M. K., *PLoS One*, 2013, **8**, 1–8.
19. Miehe, G., Miehe, S., Vogel, J., Co, S. and Duo, L., *Mt. Res. Dev*., 2007, **27**, 169–173.
20. Esper, J., *Holocene*, 2000, **10**, 253–260.
21. Stokes, M. A. and Smiley, T. L., *An Introduction to Tree-Ring Dating*, University of Chicago, Press, Chicago, 1968.
22. Fritts, H. C., *Tree-Rings and Climate*, Academic Press, London, 1976, p. 567.
23. Holmes, R. L., *Tree-Ring Bull*., 1983, **43**, 69–78.
24. Rinn, F., TSAP-Win time series analysis and presentation for dendrochronology and related applications, version 0.53 for Microsoft Windows. Rinn Tech, Heidelberg, Germany, 1996, p. 110.
25. Cook, E. R., Ph D thesis, University of Arizona, Tucson, AZ, 1985, p. 171.
26. Cook, E. R. and Peters, K., *Holocene*, 1997, **7**, 361–370.
27. Wigley, T. M. L., Briffa, K. R. and Jones, P. D., *J. Clim. Appl. Meteorol*., 1984, **23**, 201–213.
28. Biondi, F. and Waikul, K., *Comput. Geosci.*, 2004, **30**, 303–311.

ACKNOWLEDGEMENTS. KGM, VS, AKY and SM acknowledge the Director, Birbal Sahni Institute of Palaeosciences, Lucknow, for permission (BSIP/RDCC/78/2018-19) and providing all the necessary facilities and support. We also thank the Department of Forest, Government of Himachal Pradesh, India for all necessary help and logistic support during the collection of tree-ring samples. KGM and VS thank the Department of Science and Technology, New Delhi, for providing financial support (SB/DGH-76/2013). RRY acknowledges the support of the Council of Scientific and Industrial Research, New Delhi under the Emeritus Scientist scheme (No. 21(1010)/15/EMR-II).

Received 28 March 2019; accepted 12 October 2019

KRISHNA G. MISRA[1,*]
VIKRAM SINGH[1]
AKHILESH K. YADAVA[1]
SANDHYA MISRA[1]
RAM R. YADAV[2]

[1]*Birbal Sahni Institute of Palaeosciences, 53 University Road, Lucknow 226 007, India*
[2]*Wadia Institute of Himalayan Geology, 33 GMS Road, Dehradun 248 001, India*
**For correspondence.*
e-mail: kg_misra@bsip.res.in

In vitro rearing and gallery tunnelling pattern of Island pinhole borer, *Xyleborus perforans* (Wollaston), a scolytid associated with pomegranate wilt complex

Wilt, a devastating disease in pomegranate (*Punica granatum* L.) plantations causes complete death of young and old plants alike. This disease has become a threat to crop cultivation across the major pomegranate-growing countries like India, China, Iran and Greece, posing a potential crisis for farmers. Wilt-affected plants exhibit gradual yellowing, drying of leaves in a particular branch that spreads to others, leading to dieback and finally the infected plant dies within the next few weeks[1,2]. Pioneering studies have revealed that this disease shows symptoms caused by many contributing biotic and abiotic factors. Several biotic factors like fungal pathogens (viz. *Ceratocystis fimbriata*, *Fusarium* spp., *Macrophomina phaseolina*, *Phytophthora* spp., *Rhizoctonia bataticola*, *Rosellenia necatrix*, *Verticillium dahliae*), insects (scolytid beetle, *Xyleborus perforans* (Wollaston)) and nematodes (root-knot nematode, *Meloidogyne incognita*) were found to play a crucial role in disease progression[3].

The role of Island pinhole borer, *Xyleborus perforans* (Wollaston) (Coleoptera: Scolytidae) popularly known as pomegranate shothole borer (SHB), in causing pomegranate wilt is well established[4–7]. Besides their direct role in the mechanical transmission of wilt pathogens, these tiny beetles breed in the woody tissues of pomegranate plant by excavating galleries, particularly in the collar regions thereby damaging the plant vascular tissues. These scolytid beetles exclusively live in nutritional symbiosis with ambrosia fungi[8,9]. Therefore, the adult females (=foundresses) cultivate the ambrosia fungi in these galleries, as their exclusive source of nutrition for adults and young ones alike[6]. Studying the behaviour of these scolytid beetles, the nature of brood establishment, fungal cultivation and dispersal mechanism is extremely difficult in the field due to their invisible galleries hidden underneath the bark within the collar region of the plant. To overcome such difficulties associated with scolytids in general, which attack several tree species, many researchers have tried developing an artificial medium for rearing them in the laboratory[7–11]. A 'phloem sandwich' technique was explored, where a piece of phloem was sandwiched between acrylic or glass sheets sealed with parafilm to rear scolytid beetles within (that infest pine trees)[12].

In this study, the semi-synthetic medium established previously for other ambrosia beetles was customized to suit the nutrition requirements of *X. perforans* by addition of host wood sawdust (pomegranate), as the earlier medium did not support establishment of the beetles. The modified medium successfully supported the *in vitro* rearing of scolytid beetles that are associated with pomegranate wilt to facilitate the studies on their biology and etiology using *X. perforans* as a model species. The present study not only improves our understanding of tunnelling behaviour and biology of this scolytid beetle, but also demonstrates the feasibility of its *in vitro* rearing.

The study was conducted in the Division of Entomology and Nematology, Indian Institute of Horticultural Research (IIHR), Bengaluru, India. Pomegranate plants (cv. Bhagwa) infested with SHB were collected through extensive surveys conducted in Chitradurga district (14.1823°N, 76.5488°E), Karnataka, India during March 2015. The plants showing symptoms of wilt, viz. leaf yellowing, leaf shedding, branch drying were selected for the study (Figure 1). The infected plants were visually graded for the above symptoms on a 0.00% (when symptoms of leaf yellowing, shedding and branch drying were absent) to 100.00% scale (when plants were completely dried). After grading, plants with wide range of wilt symptoms were carefully uprooted from the base along with the root/primary stem region. The collar regions of such infected plants ($n = 10$) were trimmed using metal shears, placed in plastic bags, labelled and brought to the IIHR laboratory. These collar regions (45–60 cm length) were further used to explore the scolytid galleries and insect collection.

The collar regions of scolytid-infested pomegranate plants were cleansed for external soil deposition using a paint brush (Camlin, 0.25 inch, flat), and the length of each collar region along with its primary stem was measured. Further, the number of exit holes on each collar region was counted. The collar regions were chipped carefully for bark removal using stainless-steel scalpel. The horizontal cross-sections of the collar region were done using a handheld power cutter (Saber saw – GSA 1300 PCE model, Bosch, India) with utmost care using a thin blade (1 mm) without disturbing the insect gallery (Figure 2). Each cross-section was manually stripped using secateurs, needles and small forceps to find live insects inside the galleries. The gallery phenology below the bark was recorded for gallery length, exit-hole diameter and interconnectivity structures between the galleries. The beetles collected from these galleries were stored in sterile disposable petri plates (90 × 15 mm, Himedia, India) for further inoculation into artificial medium. The collected beetles were photographed under a stereomicroscope (Lieca M205A, Lieca Microsystems, Switzerland) for their morphological measurements using the in-built LAS program.

The media were prepared according to Biederman *et al.*[13], with minor modification to suit the pomegranate SHB nutritional requirements and aid its adaptation.

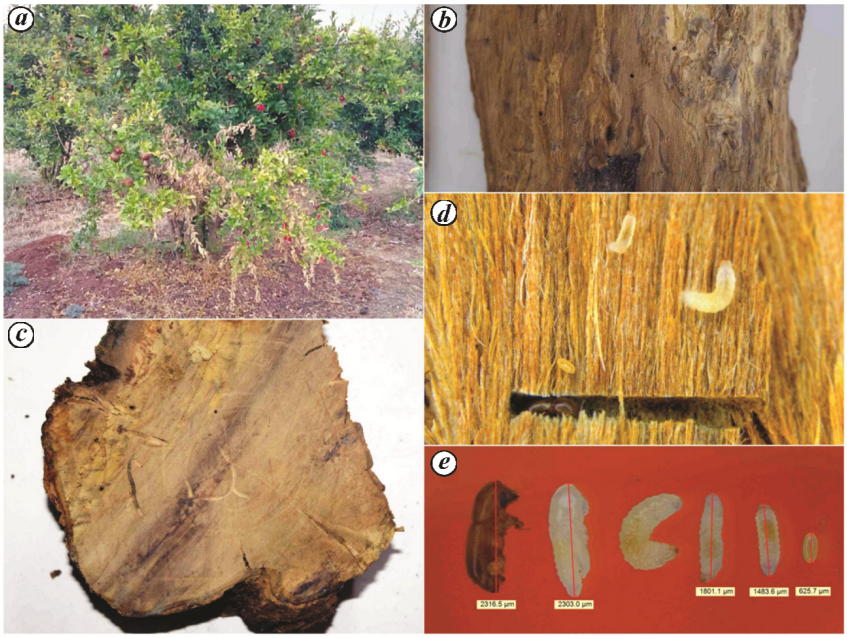

Figure 1. ***a***, Wilt-affected pomegranate plant with typical dieback symptoms. ***b***, Excavated stem with shothole borer infestation with exit holes (pointed arrows). ***c***, Horizontally cut collar region exhibiting gallery structures. ***d***, Gallery structure cut-open tunnel showing eggs, grubs and adult. ***e***, Complete life stages of beetle collected from the gallery shown in (***d***) with measurements (***c***).

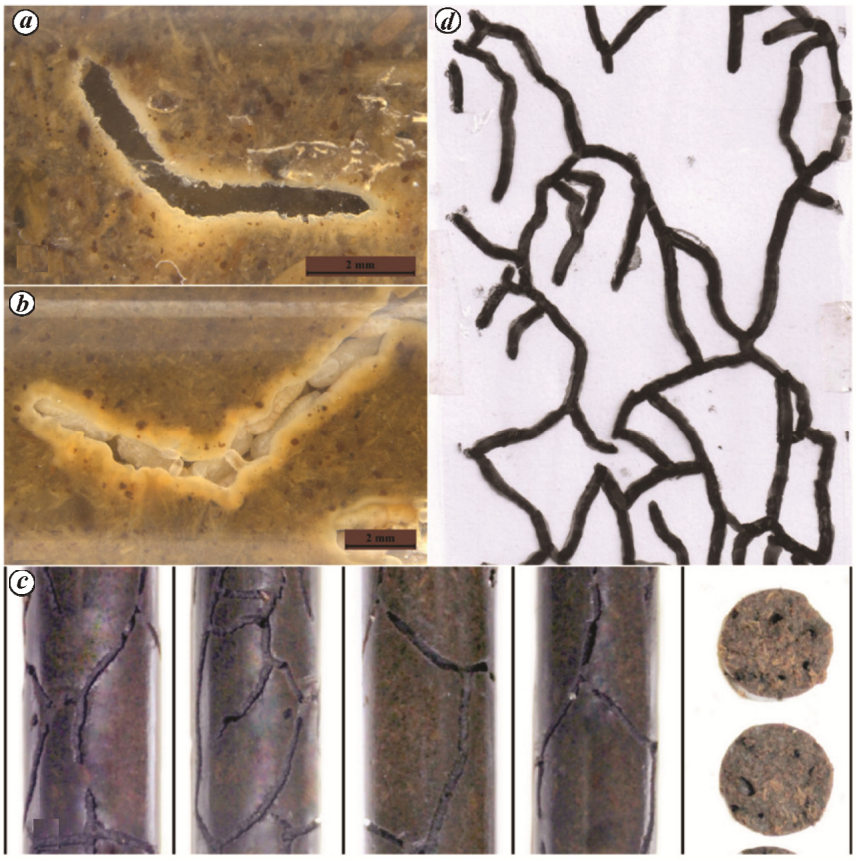

Figure 2. Shothole borer beetle *in vitro* culture and gallery structures. ***a***, The media excavated by foundress. ***b***, Larvae and pupae in the gallery. ***c***, The gallery structure in media tubes. ***d***, Traced gallery structure.

The ingredients, viz. yeast (5 g), casein (10 g), pomegranate plant sawdust (100 g), sucrose (5 g), agar (10 g), Wesson's salt mixture (1.25 g) were mixed thoroughly with sterile distilled water (580 ml) and autoclaved for 15 min at 120°C. To this autoclaved mixture, ethanol (95%; 5 ml) and streptomycin (350 mg) were added, stirred thoroughly and then aliquotes (20 ml per tube) were made aseptically into sterile glass tubes (18 × 150 mm). The aliquoted medium was allowed to set at room temperature and stored in the refrigerator (4°C) until further use. The pomegranate sawdust was prepared by scrapping the hard wood of primary stem/collar region (cv. Bhagwa) with Sabre saw machine.

The collected adult females were allowed to initiate a brood of their own on artificial medium. The individual adult females were surface-sterilized with absolute alcohol for less than 1 min and transferred into media tubes @ 1 foundress per tube using camel-hair brush under sterile conditions. The cultures were incubated at room temperature (27° + 2°C) under dark conditions for a period of 45–60 days. Periodic observations were recorded for gallery length, developmental period of different beetle stages and interconnectivity of galleries established by SHB for each culture. At the end of the incubation period and complete exit of insects from the gallery, each tube was wrapped with a transparent sheet. The outer visible gallery path was traced using a marker pen. These sheets with the tracings were scanned (HP Scanner) along with calibrated scales and total length of the gallery (cm) was measured using Image J software (1.47v, NIMH, USA). The data on gallery length and life period for each stage of beetles were analysed for descriptive statistics using Graphpad Prism.

The SHB activities were observed in the wilt-infected pomegranate plants with sympotoms of branch drying and leaf shedding ranging from 15.00% to 100.00% (Figure 1 *a*). The start or end of the gallery had a typical borehole (=exit/entry hole) externally that was often merged with the dried bark, or had its exit underneath loosened barks of the stem. The bore measured ~1 mm in diameter, which is equivalent to the body width of adult SHB (Figure 1 *b* and *c*). Most of the galleries were found just below the bark and superficially in the hardwood region. Active galleries were scouted in wood through the presence of dust powder expelled near the exit holes to the ground. The average length of 1.8′ stem hosted around 27.7 ± 6.77 (Mean ± SE), boreholes spread around the wood in the collar region, with no preference to above or below soil zone. Apart from superficial ones, galleries were also excavated within the hardwood (20–30 mm depth) away from the bark region. These galleries were simple, narrow tunnels without any special nuptial or egg chambers. The gallery paths were interconnected with each other facilitating to and fro activities of movable brood members, viz. grubs and adults. The immobile stages, viz. eggs, pupae were found at the extreme end of gallery branches. All phenological stages of SHB could be found within the gallery (Figure 1 *d*) and categorized into egg, grubs (I, II, III instars), pupae and adult (Figure 1 *e*). The gallery of SHB inside the pomegranate stem collar region ranged from 34 to 116 mm in length ($n = 10$, 60.95 mm ± 8.48 SE), with simple branching vertically or horizontally across the stem.

The modified medium was accepted SHB with evidence of extensive gallery excavation and brood establishment (Figure 2). The galleries in the medium ran horizontally and branched vertically with interconnected paths for easy brood movements (Figure 2 *c*). The maximum length of the gallery was observed to be 87.29 mm for the duration of 60 days with 27 adults emerging out of the culture. In general, the length of the gallery ranged from 32.97 to 87.29 mm with 10–27 adult beetles from the respective galleries. Thus the length of the gallery is directly proportional to the number of brood members. The complexity of the gallery increased with increased members of the brood due to activity of newly emerged females. The foundress of the gallery kept herself busy with excavation and fungal cultivation in the tunnel for self and brood members. The grubs and pupae were found at the end of gallery branches, as observed in cut stems (Figure 2 *b*).

The beetles cultivated in the semi-synthetic diet were similar in morphology and behaviour to their field-collected counterparts. The cost of medium preparation was INR 650/L of medium, from which a total of 1350 adult SHBs (costing about INR 0.48 per beetle) could be reared. The medium was mainly based on host-plant (pomegranate) stem powder with minimal extra sources of carbohydrates (as sucrose), proteins (from casein and yeast extract) and antibiotics (to prevent microbial degradation).

The newly introduced foundress was found tunnelling in the medium within an hour and fungal cultivation was observed two days after inoculation as a slimy layer on the tunnel surface. Post ten days of tunnelling initiation, the foundress started laying eggs randomly in tunnels. The egg emergence period was nearly 6.7 ± 0.3 days, the larval stage (three instars) completed with an average of 10.6 ± 0.5 days, and pupal emergence took an average of 7.3 ± 0.33 days. Adults lived longer with an average of 18.10 ± 0.53 days. Distinct sexual dimorphism was observed among adult beetles, and usually females were more in number than males with a sex ratio of 13♀ : 1♂ per tube (Figure 1 *e*).

The gallery movements were largely dominated by grubs. In every situation of tunnel activity, the grubs were always given preference to make their way out of the path by other brood members. Their movement intervention was usually found absorbed by the immovable stages, viz. pupae, eggs under no-choice circumstances. Females held onto fungal cultivation while the emerged grubs kept grazing on the grown fungus. Nevertheless, adults kept away (blocking their way) grubs from exit holes, as observed particularly in cut-stem galleries. Adult females took steps to move the unnecessary materials by balling them with hind legs and pushing backwards out of the gallery in reverse motion.

Understanding the role of the pomegranate SHB in wilt disease will help us envisage the progression of this dreadful disease and its management. The information paucity on SHB biology and its tunnelling behaviour demands the need to establish laboratory-rearing methodology. In this study we standardized a semi-synthetic diet for *in vitro* culturing of SHB to understand the basic biological information with respect to its establishment, gallery formation, breeding and dispersing nature.

Several artificial diets have been developed for laboratory rearing of different SHB species with various combinations of diet components, depending upon the individual species requirement as well as their host plant[12–18]. In the present study, the customized semi-natural diet for *in vitro* culture establishment of SHB

involves host plant (pomegranate) stem powder for providing natural texture to the medium along with other carbohydrate and protein sources. The medium developed could support the beetles up to 45–60 days without any replacement, thereby helping us understand its biology and behaviour *in vitro*.

The present study reveals the atypical nature of these beetles in establishing galleries at the collar region. Later, these galleries expand upward (above the soil) with exterior exit/entry holes for adult female dispersal/entry that would allow opportunistic phytopathogens to gain access into the wood system causing wilt or enhancing wilt status of the plant[19]. Nevertheless, how these ambrosia beetles are able to resist the entry of pathogens into their gallery that can affect the brood as well as the pure culture of a symbiotic fungus remains unexplored. This microbial complexity involving both positive and negative microbial interactions with respect to the plant as well as the scolytid beetle is worth exploring to understand the intricate trophic interactions involved within the gallery[20]. This information would support the development of safe pest management strategies for wilt control.

In the SHB tunnelling systems, the first egg appears when the tunnel length ranges between 3 and 8 cm (ref. 21), and the emergent grubs develop and pupate within the gallery system. Each species has its own blueprint of gallery design based on the need. In the present study, the authors did not find special chambers or designs in gallery formation. For example, in *Scolytus multistitus* there are larval-built nuptial chambers for pupation in the gallery architectures[22]. Whereas in this species there are no such special chambers to accommodate eggs; instead, the eggs were laid in the main path of the gallery. The eggs and pupae were found constantly groomed either by adults or grubs, passing through the path. As a consequence, they are carried randomly in different directions of gallery branches and boundaries. Whether this is a random process, or there is a purpose in shifting the eggs/pupae from place to place needs further more analysis. The gallery paths are interconnected from the main path which the foundress initiates, and the gallery length increases with increasing female members in the brood[23].

The present study clearly established the possibility of laboratory rearing of island pinhole borer, *X. perforans* on semi-synthetic diet, and a single foundress could successfully establish a colony and rear grubs on media. Further studies on isolation and identification of symbiotic fungi and the role of scolytid in causing wilt are being carried out to exploit behavioural management strategies for this notorious pest of pomegrante.

1. Somasekhara, Y. M., *Plant Dis.*, 1999, **83**, 400.
2. Alam, M. W., Gleason, M. L., Mehboob, S., Riaz, K. and Rehman, A., *Plant Dis.*, 2016, **101**(1), 255.
3. Darekar, K. S., Shelke, S. S. and Mhase, N. L., *Int. Nematol. Netw. Newsl.*, 1990, **7**, 11–12.
4. Sharma, K. K., Sharma, J. and Jadhav, V. T., In *Fruit Veg. Cereal Sci. Biotechnol.*, 2010 (Spl. Issue 2), 96–101.
5. Somasekhara, Y. M. and Wali, S. Y., *Orissa J. Hortic.*, 2000, **28**, 84–89.
6. Carrillo, D., Duncan, R. E., Ploetz, J. N., Campbell, A. F., Ploetz, R. C. and Peña, J. E., *Plant Pathol.*, 2014, **63**, 54–62.
7. Lira-Noriega, A., Soberón, J. and Equihua, J., *Sci. Rep.*, 2018, **8**, 10179.
8. Baker, J. M. and Norris, D. M., *J. Invertebr. Pathol.*, 1968, **11**, 246–250.
9. De Fine Licht, H. H. and Biedermann, P. H. W., *Front. Zool.*, 2012, **9**, 13.
10. Choe, J. C. and Crespi, B. J., *The Evolution of Social Behaviour in Insects and Arachnids*, Cambridge University Press, 1997.
11. Castrillo, L. A., Griggs, M. H. and Vandenberg, J. D., *Environ. Entomol.*, 2012, **41**, 822–827.
12. French, J. R. J. and Roeper, R. A., *Ann. Entomol. Soc. Am.*, 1972, **65**, 719–721.
13. Biedermann, P. H. W., Klepzig, K. D., and Taborsky, M., *Environ. Entomol.*, 2009, **38**, 1096–1105.
14. Mizuno, T. and Kajimura, H., *J. Appl. Entomol.*, 2002, **126**, 455–462.
15. Sivapalan, V. S., *Entomol. Exp. Appl.*, 1977, **21**, 1–8.
16. Biedermann, P. H. W., Klepzig, K. D. and Taborsky, M., *Environ. Entomol.*, 2009, **38**, 1096–1105.
17. Aflitto, N. C., Hofstetter, R. W., McGuire, R., Dunn, D. D. and Potter, K. A., *J. Vis. Exp.*, 2014, e50793–e50793.
18. Maner, M. L., Hanula, J. and Braman, S. K., *Fla. Entomol.*, 2014, **96**(3), 1042–1051.
19. Kirisits, T., In *Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis* (eds Lieutier, F. *et al.*), Springer, Dordrecht, The Netherlands, 2004, pp. 181–236.
20. Six, D. L. and Klepzig, K. D., *Symbiosis*, 2004, **37**, 207–232.
21. Kalshoven, L. G. E., *Beaufortia*, 1964, **11**, 131–142.
22. Smith, S. M. and Hulcr, J., In *Bark Beetles* (eds Vega, F. E. and Hofstetter, R. W.), Academic Press, San Diego, USA, 2015, pp. 495–531.
23. Peer, K. and Taborsky, M., *Behav. Ecol. Sociobiol.*, 2007, **61**, 729–739.

ACKNOWLEDGEMENTS. We thank the farmers of Chitradurga, Karnataka and Mr T. S. Rajanna and Mr J. Sagar for support during field collection of plant samples and gallery excavation. Funding for this study was provided under the project Consortium Research Platform for Borers, by the Indian Council of Agriculture Research, New Delhi. We thank Dr Meenal Vyas for editing the revised manuscript.

Received 22 April 2019; revised accepted 12 December 2019

P. D. KAMALA JAYANTHI*
T. RAGHAVA
T. NAGARAJA
K. SREEDEVI

ICAR-Indian Institute of Horticultural Research,
Hesseraghatta Lake PO,
Bengaluru 560 089, India
**For correspondence.*
e-mail: jaiinsect@gmail.com