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Plant biological warfare: thorns inject pathogenic bacteria into herbivores

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Summary

Thorns, spines and prickles are among the rich arsenal of antiherbivore defence mechanisms that plants have evolved. Many of these thorns are aposematic, that is, marked by various types of warning coloration. This coloration was recently proposed to deter large herbivores. Yet, the mechanical defence provided by thorns against large herbivores might be only the tip of the iceberg in a much more complicated story. Here we present evidence that thorns harbour an array of pathogenic bacteria that are much more dangerous to herbivores than the painful mechanical wounding by the thorns. Pathogenic bacteria like *Clostridium perfringens*, the causative agent of the life-threatening gas gangrene, and others, were isolated and identified from date palm (with green-yellow-black aposematic spines) and common hawthorn (with red aposematic thorns). These thorn-inhabiting bacteria have a considerable potential role in antiherbivory, and may have uniquely contributed to the common evolution of aposematism (warning coloration) in thorny plants.

Introduction

Plants suffer from ceaseless pressure of herbivory by large vertebrates, a consequence of their being sessile and the main constituent of the terrestrial biomass. Thorns, spines and prickles, here collectively termed thorns, are a common antiherbivory defence in thousands of plant species, especially in arid regions (Grubb, 1992). The role of thorns in defence against large herbivores is well established (Cooper and Owen-Smith, 1986; Janzen, 1986; Grubb, 1992; Gowda, 1996). For instance, thorny plants cover many areas in the Mediterranean region that have suffered long and heavy grazing (Zohary, 1983). Many of these thorny plants are aposematic, that is, the unpalatability of these plants is associated with various types of conspicuous coloration; recently it has been proposed that this coloration deters large herbivores (Lev-Yadun, 2001; 2003a,b; 2006; Lev-Yadun and Ne'eman, 2004; Rubino and McCarthy, 2004; Ruxton *et al*., 2004; Speed and Ruxton, 2005). The special character of the possible aposematic function of colourful spines is that unlike poisonous organisms, the spines directly advertise their defensive quality (Ruxton *et al*., 2004; Speed and Ruxton, 2005). An important question is whether the relevant herbivorous animals can see such colour signals. This issue was in debate until recent reviews provided enough data to conclude that these animals are able to see colours (Jacobs, 1993; Kelber *et al*., 2003). It is very likely that large mammalian herbivores do not have the ability to see colours the way trichromatic humans see them. Still, even with herbivores' dichromatic vision, colourful spines or other plant parts may look different from regular green tissues because of their hue, saturation or brightness (see Kelber *et al*., 2003). Moreover, in many plant species, spines and thorns are associated with white coloration, which increases their conspicuousness even for colour-blind animals (Lev-Yadun, 2001; 2003a; 2006).

Aposematic coloration and mimicry of aposematic animals is described and discussed in thousands of publications (e.g. Cott, 1940; Wickler, 1968; Edmunds, 1974; Ruxton *et al*., 2004). Interestingly, animal thorns are also commonly colourful and aposematic (Ruxton *et al*., 2004; Inbar and Lev-Yadun, 2005; Speed and Ruxton, 2005). Thorny plants such as cacti, *Agave*, *Euphorbia* and *Aloa* have colourful spines with red, orange, yellow, black or brown colours that may deter herbivores (Lev-Yadun, 2001). These colourful thorns, like other aposematic types, confer a selective advantage as predators learn to associate conspicuous coloration with unpleasant qualities (Cott, 1940; Gittleman and Harvey, 1980; Harvey and Paxton, 1981; Wiklund and Järvi, 1982; Ruxton *et al*., 2004; Speed and Ruxton, 2005).

We hypothesized that the mechanical protection provided by thorns against large herbivores might not be the whole story in their defensive strategy. Thorns by

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wounding may inject bacteria into herbivores and cause them severe infections that may be much more dangerous and painful than the mechanical wounding itself. To test our hypothesis we first reviewed the published literature and found indications from medical case reports that injuries from the plant thorns can result in septic inflammation (Sugarman *et al*., 1977; Cahill and King, 1984; Vincent and Szabo, 1988; Hodes and Teferedegne, 1990; Freiberg *et al*., 1993; De Champs *et al*., 2000; Ergonul *et al*., 2003; Kratz *et al*., 2003; Pascual *et al*., 2003). None of the published data that we found discussed ecological or evolutionary issues, or aposematism; they were published for medical practice. However, that information showed that plant thorns may regularly harbour various toxic or pathogenic bacteria. *Clostridium tetani* (a Gram-positive, spore-forming anaerobic bacterium), for instance, is the aetiological agent of tetanus, a serious disease in humans and animals that when untreated can be fatal. The national tetanus surveillance in the United States reported that 26 (31%) cases of tetanus in 1998–2000 were caused by injuries sustained by the patient while farming or gardening. Rose bush prickles were one of the causes of puncture wounds that resulted in tetanus (Pascual *et al*., 2003). In Ethiopia, thorn injuries were the known cause of five out of 55 cases of tetanus (Hodes and Teferedegne, 1990). Thorn injury was also reported to be the cause of tetanus in Turkey (Ergonul *et al*., 2003). Other inflammatory states of the musculoskeletal system have similarly been associated with specific pathogens introduced into animal tissues by various thorn injuries, such as *Pantoea agglomerans*, *Staphylococcus aureus*, *S. albus*, *Streptococcus hemolyticus* and *Nocardia pyarthrosis* (Sugarman *et al*., 1977; Cahill and King, 1984; Vincent and Szabo, 1988; Freiberg *et al*., 1993; De Champs *et al*., 2000; Kratz *et al*., 2003).

The aim of this study was to examine the hypothesis that mechanical wounding is not the only role that plant thorns play in antiherbivory defence. Here we show that thorns from date palm (*Phoenix dactylifera*) (with green-yellow-black aposematic spines) and common hawthorn (*Crataegus aronia*) (with red aposematic thorns) harbour an array of pathogenic bacteria. These bacteria potentially pose a greater risk to herbivores than that caused by the thorn injury *per se*. The bacteria that inhabit thorns are most likely involved in defending plants, hence in the avoidance of plants with colourful thorns by large herbivores; therefore, they may have uniquely contributed to the common evolution of aposematic coloration in thorny plants.

Results

We studied the presence of bacteria known to be pathogenic for humans or animals on thorns of two tree species: *P. dactylifera* (date palm) and *C. aronia* (common hawthorn). Every typical mature individual of both trees carries thousands of colourful aposematic thorns (Fig. 1A and B). Although we sampled and grew microbial cultures from only a small number of the thousands found on each tree, we were able to isolate and identify an interesting array of pathogenic bacteria from these thorns. Fifty-eight bacterial isolates from thorns of both tree species were randomly selected and identified by means of 16S rRNA gene analysis in both aerobic and anaerobic growth conditions. After the bacterial isolates were identified, they then were classified as pathogens or non-pathogens according to the scientific literature records. The isolates were found to belong to 22 different bacterial species, 13 of which are known to be pathogenic for animals or humans (Tables 1 and 2).

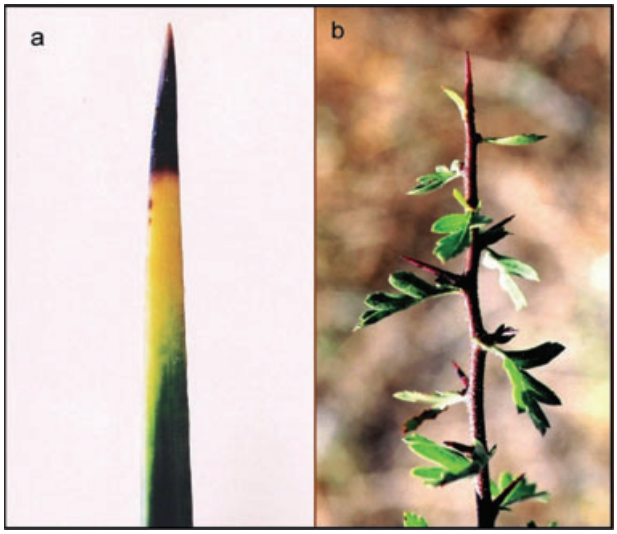

Fig. 1. Thorns of the tree species *Phoenix dactylifera* (date palm) and *Crataegus aronia* (common hawthorn).
A. Date palm spines are sharp leaflets that develop along the proximal parts of the large leaves. Their tips are usually conspicuously yellow and black.
B. Common hawthorn thorns are hard branches that end in a sharp thorn and are found all over the tree. They are red when young and turn grey with time.

Seven isolates from thorns of date palm and common hawthorn were identified as *Clostridium perfringens*, a Gram-positive, spore-forming obligate anaerobe bacterium that is the causative agent of the life-threatening gas gangrene (Tables 1 and 2, Fig. 2). Two isolates of *Clostridium sordellii* and *C. sardiniense* were found on date palm thorns (Table 1) and on common hawthorn thorns (Table 2) respectively. Another isolate (U523A5), which was found only on common hawthorn thorns, showed less than 97% similarity to known bacteria and is therefore probably a new species of *Clostridium* (Table 2).

Table 1. Bacterial species that were isolated from thorns of *Phoenix dactylifera* (date palm).

Isolate	Accession No.	Closest relative in GenBank database (accession No.)	Similarity (%)
Pathogens and opportunistic pathogens			
DF4B	DQ298076	*Clostridium perfringens* (BA000016)	98.6
DF4B1	DQ298077	*Clostridium perfringens* (BA000016)	99.2
DF4B6	DQ298078	*Clostridium perfringens* (BA000016)	99.0
DF4B3	DQ298079	*Clostridium perfringens* (BA000016)	99.9
DF2C21	DQ298111	*Clostridium sordellii* (AB075771)	97.9
DF2C1	DQ298112	*Clostridium sordellii* (AB075771)	98.2
DF2C22	DQ298113	*Clostridium sordellii* (AB075771)	98.4
AD2	DQ298080	*Bacillus cereus* (DQ207729)	99.9
		Bacillus anthracis strain ATCC 4229 (AY920253)	
AD1A	DQ298081	*Bacillus cereus* (DQ207729)	100
		Bacillus anthracis strain ATCC 4229 (AY920253)	
DF2B	DQ298082	*Bacillus cereus* (DQ207729)	99.9
		Bacillus anthracis strain ATCC 4229 (AY920253)	
D1041	DQ298083	*Bacillus cereus* (DQ207729)	100
		Bacillus anthracis strain ATCC 4229 (AY920253)	
AD3B	DQ298084	*Bacillus cereus* (DQ207729)	100
		Bacillus anthracis strain ATCC 4229 (AY920253)	
DF2C3	DQ298085	*Bacillus cereus* strain Delaporte (AF155958)	99.8
ADP5	DQ298086	*Bacillus anthracis* strain JH14 (DQ232742)	99.9
		Bacillus thuringiensis strain HAMBI2389 (AF501348)	
AD4B	DQ298087	*Bacillus licheniformis* strain ACO1 (DQ228696)	100
AD1B	DQ298088	*Bacillus megaterium* strain GSP10 (AY505510)	99.9
Non-pathogenic species			
AD5A	DQ298114	*Bacillus benzoevorans* (AY043085)	99.9
ADP41	DQ298115	*Bacillus benzoevorans* (AY043085)	100
AD5B	DQ298116	*Bacillus endophyticus* (AF295302)	99.8
AD3A	DQ298117	*Bacillus subtilis* strain CICC10147 (AY971364)	100
AD4AI	DQ298118	*Paenibacillus polymyxa* strain WY110 (AY302439)	99.5
DF5A2	DQ298119	*Propionibacterium granulosum* DSM 20700 (AJ003057)	99.6
ADP3	DQ298120	*Erwinia cypripedii* (AJ233413)	97.9

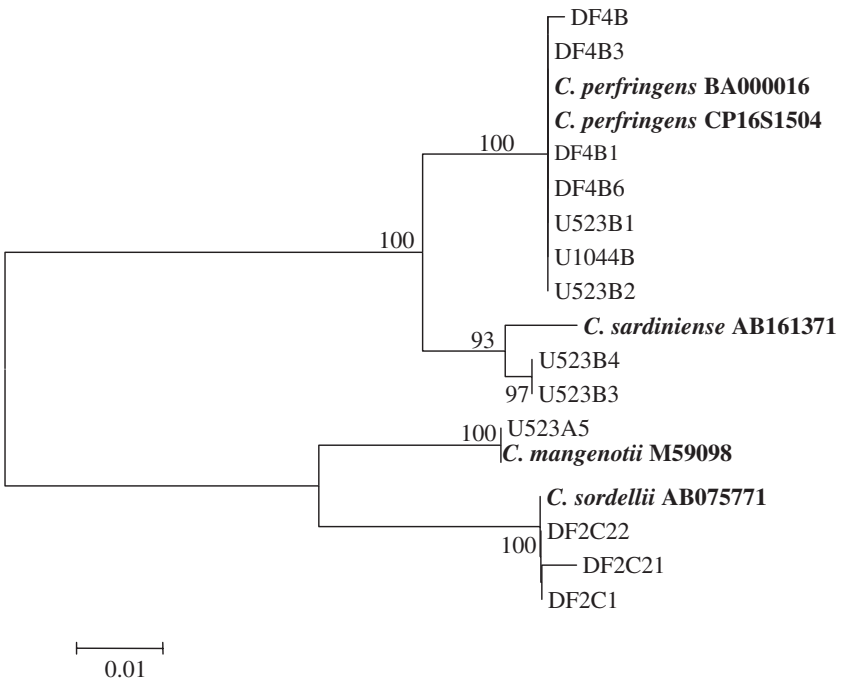

Fig. 2. A phylogenetic tree of *Clostridium* isolates from date palm (name of isolates starting with D or AD) and common hawthorn (name of isolates starting with U or AU). The tree shows the relationship based on partial sequences of the 16S ribosomal RNA gene of selected isolates. The sequence alignment was performed by use of the CLUSTAL W program and the tree was generated by the neighbour-joining method with Kimura 2 parameter distances in MEGA 3 software. Bootstrap values (from 1000 replicates) greater than 50% are shown at the branch points. The bar indicates 1% sequence divergence.

Table 2. Bacterial species that were isolated from thorns of *Crataegus aronia* (common hawthorn).[a]

Isolate	Accession No.	Closest relative in GenBank database (accession No.)	Similarity (%)
Pathogens and opportunistic pathogens			
U1044B	DQ298089	*Clostridium perfringens* (BA000016)	99.8
U523B1	DQ298090	*Clostridium perfringens* (CP16S1504)	99.6
U523B2	DQ298091	*Clostridium perfringens* (BA000016)	99.9
U523B4	DQ298122	*Clostridium sardiniense* strain DSM 600 AB161371	98.2
U523B3	DQ298123	*Clostridium sardiniense* strain DSM 600 AB161371	98.3
U511A1	DQ298092	*Bacillus cereus* (DQ207729) *Bacillus anthracis* strain ATCC 4229 (AY920253)	100
AU511A	DQ298093	*Bacillus cereus* (DQ207729) *Bacillus anthracis* strain ATCC 4229 (AY920253)	100
AU511B	DQ298094	*Bacillus cereus* strain F 528/94 (BCE577291)	99.7
AU523A1	DQ298095	*Bacillus cereus* (DQ207729) *Bacillus anthracis* strain ATCC 4229 (AY920253)	99.9
AU513A2	DQ298096	*Bacillus cereus* strain G9667 (AY138273) *Bacillus anthracis* strain Ames (AE017024)	99.6
U1042A	DQ298097	*Bacillus anthracis* strain JH14 (DQ232742) *Bacillus cereus* strain ATCC 25621 (AY795568)	99.7
U1042A1	DQ298098	*Bacillus anthracis* strain JH14 (DQ232742) *Bacillus cereus* strain ATCC 25621 (AY795568)	99.2
AU2	DQ298099	*Bacillus anthracis* strain JH14 (DQ232742) *Bacillus cereus* strain ATCC 25621 (AY795568)	99.6
U513A6	DQ298100	*Bacillus licheniformis* strain ATCC 14580 (CP000002)	99.6
U513A5	DQ298101	*Bacillus licheniformis* strain ATCC 14580 (CP000002)	99.6
AU513A1	DQ298102	*Bacillus licheniformis* strain R-13585 (AJ586341)	99.4
AU512A2	DQ298103	*Bacillus licheniformis* strain CICC 10084 (AY871103)	99.5
AU522B2	DQ298104	*Bacillus licheniformis* strain CICC 10084 (AY871103)	99.7
AU5513B	DQ298105	*Bacillus licheniformis* strain ATCC 14580 (CP000002)	99.9
U3	DQ298106	*Enterococcus faecalis* strain D3 (DQ239694)	100
AU5A	DQ298107	*Enterococcus faecium* strain RJ16 (AJ874342)	99.8
AU522B1	DQ298108	*Rahnella aquatilis* (X79937)	99.7
AU522A2	DQ298109	*Shigella boydii* strain 5216-70 (AY696668)	100
AU523A2	DQ298110	*Pantoea agglomerans* strain 732 (AY092079)	98.2
Non-pathogenic species			
U523A5	DQ298121	*Clostridium mangenotii* (M59098)	96.2
AU5B	DQ298124	*Bacillus benzoevorans* (AY043085)	100
U4A	DQ298125	*Bacillus benzoevorans* (AY043085)	99.9
U1044C	DQ298126	*Paenibacillus polymyxa* strain WY110 (AY302439)	99.4
U4D	DQ298127	*Paenibacillus polymyxa* strain WY110 (AY302439)	99.8
AU4D	DQ298128	*Paenibacillus polymyxa* strain WY110 (AY302439)	99.6
AU523B3	DQ298129	*Staphylococcus pasteuri* strain ZA-b3 (AF532917)	99.9
U523B6	DQ298130	*Staphylococcus pasteuri* strain ZA-b3 (AF532917)	99.8
U1042B	DQ298131	*Streptococcus sanguinis* ATCC 29667 (AY281085)	98
AU5521A	DQ298132	*Brenneria quercina* ex *Erwninia quercina* (AJ223469)	99.2

a. All isolates of *Crataegus aronia* with a name beginning with U5 or AU5 were sampled and isolated in May 2005.

A total of 16 isolates from the thorns of both trees were identified as members of the *Bacillus cereus sensu lato* group (Tables 1 and 2, and Fig. 3). Twelve isolates showed identical similarity to *B. cereus* and *B. anthracis*, and one isolate showed identical similarity to *B. anthracis* and *B. thuringiensis*, when their partial 16S rRNA sequences were checked against the GenBank database. This indicates that the thorns are a habitat for *B. anthracis* as well as for *B. cereus* and *B. thuringiensis* (Fig. 3). All the isolates that were identified as *B. anthracis* or *B. cereus* showed haemolytic activity on blood agar plates, indicating that their identity is most likely *B. cereus*. Other members of the genus *Bacillus*, namely *B. licheniformis* and *B. megaterium*, were also isolated from date palm tree thorns (Table 1). *Bacillus licheniformis* was also found on common hawthorn (Table 2, Fig. 3).

The pathogenic bacteria *Enterococcus faecalis*, *E. faecium*, *Rahnella aquatilis*, *Shigella boydii*, and *Pantoea agglomerans* were isolated only from common hawthorn (Table 2).

Additional species of bacteria (*Bacillus benzoevorans* and *Paenibacillus polymyxa*) that are not considered pathogenic to mammals were also isolated from thorns of both tree species. *Bacillus subtilis*, *B. endophyticis*, *Propionibacterium granulosum* and *Erwinia cypripedii* were found only on date palm thorns (Table 1), and *Staphylococcus pasteuri*, *Streptococcus sanguinis* and *Brenneria quercina* were isolated only from common hawthorn (Table 2).

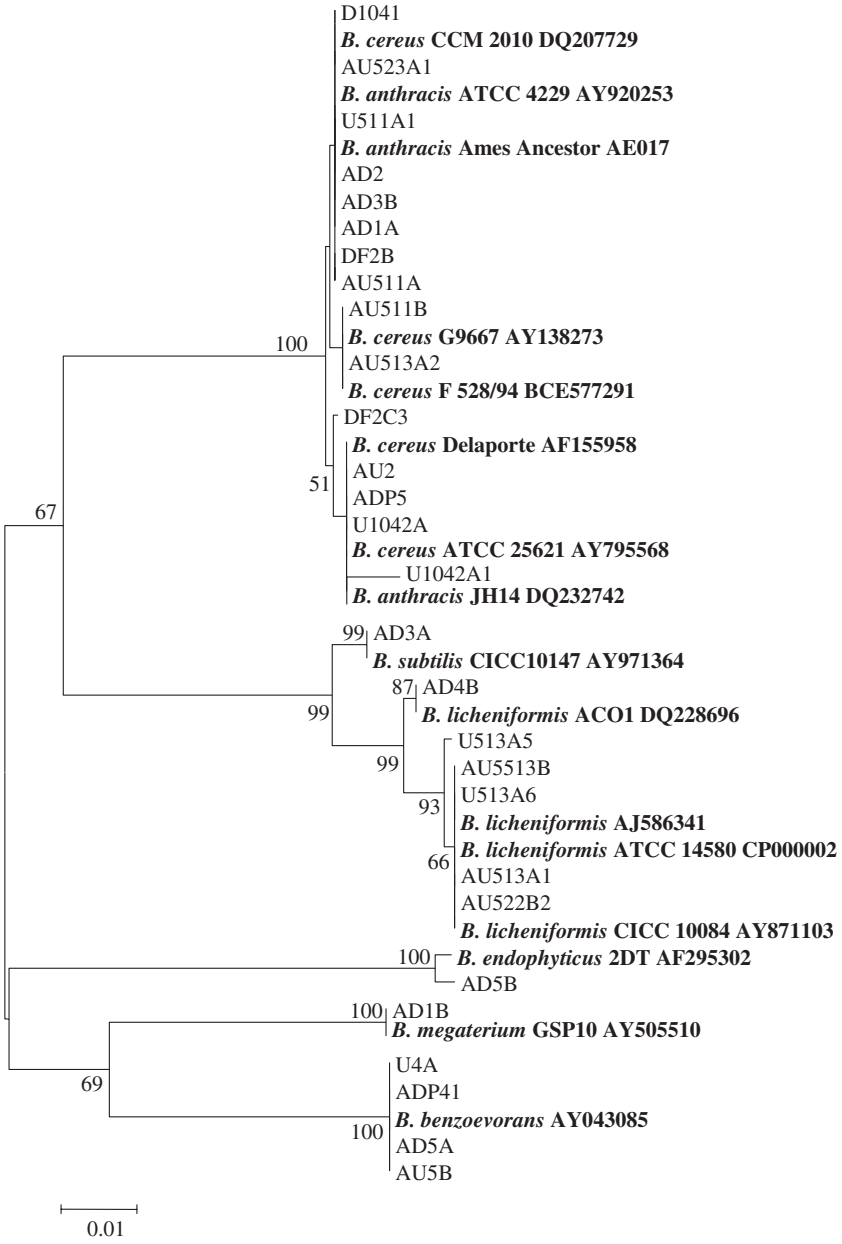

Fig. 3. A phylogenetic tree of *Bacillus* isolates from date palm (name of isolates starting with D or AD) and common hawthorn (name of isolates starting with U or AU). The tree shows the relationship based on partial sequences of the 16S ribosomal RNA gene of selected isolates. The sequence alignment was performed by use of the CLUSTAL W program and the tree was generated by the neighbour-joining method with Kimura 2 parameter distances in MEGA 3 software. Bootstrap values (from 1000 replicates) greater then 50% are shown at the branch points. The bar indicates 1% sequence divergence.

Discussion

Here we provide evidence that bacteria known to be pathogenic for humans or animals inhabit thorns of two tree species: date palm and common hawthorn (Tables 1 and 2, Figs 2 and 3). Thorns, a common antiherbivory defence in plant species, are frequently also aposematically coloured (Lev-Yadun, 2001; 2003a,b; 2006; Rubino and McCarthy, 2004; Ruxton *et al*., 2004; Speed and Ruxton, 2005). Various case studies in the medical literature indicate that pathogenic bacteria can be introduced through the skin of humans or animals by thorn injuries and cause serious infections (Sugarman *et al*., 1977; Cahill and King, 1984; Vincent and Szabo, 1988; Hodes and Teferedegne, 1990; Freiberg *et al*., 1993; De Champs *et al*., 2000; Ergonul *et al*., 2003; Kratz *et al*., 2003; Pascual *et al*., 2003). We discuss here the potential defensive effects of bacteria known to be pathogenic to mammals or humans that we isolated and identified from the thorns of date palm and common hawthorn. We conclude that thorns may regularly act as an antiherbivory defence in plants by delivering mechanical injuries but also by means of the pathogenic bacteria they introduce into the herbivores.

Microorganisms seem to occupy virtually every living and non-living niche on earth. In the past few decades,

plant scientists have begun to realize that all the aerial plant surfaces, including leaves, stems and flowers, are inhabited by diverse assemblages of microorganisms, including filamentous fungi, yeasts, bacteria and bacteriophages (Lindow and Leveau, 2002; Lindow and Brandl, 2003). Most probably, the bacteria species that we isolated from the thorns are inhabitants that multiply on the thorns (hence they are present in large numbers) and not just visitors that landed on the thorns accidentally. Thorns, unlike other plants organs, possess the unique capability of introducing the bacteria by piercing the herbivore's defensive skin. This novel hypothesis is relevant to thousands of plant species in many types of habitats.

Another theoretical aspect is the delay between the thorn's contact and the bacterial action. While the pain from contacting thorns is immediate, bacterial action is delayed. Yet, the same is true for the delayed action of poisons. Nevertheless, there is a general agreement that poisonous and colourful organisms are aposematic (e.g. Cott, 1940; Edmunds, 1974; Gittleman and Harvey, 1980; Harvey and Paxton, 1981; Ruxton *et al*., 2004). Therefore, there is no reason to look at bacterial contamination differently.

Clostridium perfringens, which was isolated in this study from thorns of both tree species, is a Gram-positive, spore-forming obligate anaerobic bacterium (Tables 1 and 2, Fig. 2). It is responsible for several serious diseases in humans and domestic animals. *Clostridium perfringens* is known to be a flesh-eater and the causative agent of life-threatening gas gangrene and mild enterotoxaemia in humans (Shimizu *et al*., 2002). It also causes severe enteritis/enterotoxaemia and diseases belonging to the gas oedema complex in humans (Lin and Labbe, 2003), in Belgian Blue calves (Manteca *et al*., 2001) and in other species including foals and piglets (Garmory *et al*., 2000; Dray, 2004). Microorganisms can grow on plant surfaces in biofilms (assemblages of bacterial cells attached to a surface and enclosed in adhesive polysaccharides excreted by the cells). The biofilm environment exhibits remarkable heterogeneity. For example, within biofilms, highly aerated zones can border anaerobic zones separated by distances of only tens of microns (Hall-Stoodley and Stoodley, 2005). Indeed, in addition to *C. perfringens* six additional isolates belonging to the obligate anaerobic genus *Clostridium* were identified (*C. sordellii*, *C. sardiniense* and *Clostridium* sp., Tables 1 and 2). This implies that members of the genus *Clostridium*, even though they are obligate anaerobes, are common viable inhabitants of thorns of date palm and common hawthorn. Both *C. sordellii* and *C. sardiniense* (a synonym of *C. absonum*; Wang *et al*., 2005) are considered *C. perfringens*-like strains and were isolated from infected tissues in cases of gas gangrene (Masaki *et al*., 1988; el Sanousi and Musa, 1989).

The *B. cereus sensu lato* group comprises three Gram-positive, spore-forming species: *B. anthracis*, *B. thuringiensis* and *B. cereus sensu stricto* (Jensen *et al*., 2003). Recent molecular data suggest that all three are members of a single species, *B. cereus sensu lato* (Daffonchio *et al*., 2000) (Tables 1 and 2, Fig. 3). *Bacillus cereus* is an opportunistic pathogen causing food poisoning manifested by diarrhoeal or emetic symptoms (Ivanova *et al*., 2003), and *B. thuringiensis* is a well-known insect pathogen (Aronson and Shai, 2001). *Bacillus anthracis* is the aetiological agent of anthrax, a notorious acute fatal disease in animals (domesticated and wild, particularly herbivorous) and humans (Jensen *et al*., 2003). The cutaneous form of the disease is usually acquired through injured skin or mucous membranes, a typical thorn injury. Leendertz and colleagues (2004) reported sudden deaths of wild chimpanzees caused by *B. anthracis* in the tropical rainforest, Tay National Park, Ivory Coast. The source of the chimpanzee's infection was not clarified and remained a mystery. It is not impossible that plant thorns were the cause of that incident. Toxigenic strains of *B. licheniformis* caused food poisoning incidents (Salkinoja-Salonen *et al*., 1999). *Bacillus licheniformis* and *B. megatherium* were found to produce a heat-stable toxin, which showed varying levels of toxicity and similar physical characteristics to the *B. cereus* emetic toxin, cereulide (Salkinoja-Salonen *et al*., 1999; Taylor *et al*., 2005).

Enterococcus faecalis and *E. faecium* were isolated from common hawthorn thorns (Table 2). Enterococci are Gram-positive, aerotolerant anaerobes that can cause various complicated infections (abdominal, skin, urinary tract, and of the blood) (Ruoff *et al*., 1990). *Pantoea agglomerans*, *R. aquatilis* and *S. boydii* are all members of Enterobacteriaceae and were isolated from common hawthorn thorns in this study. *Pantoea agglomerans*, a common colonizer of plant surfaces (Lindow and Leveau, 2002), has been reported in the medical literature as the cause of septic arthritis after palm thorn injury (Kratz *et al*., 2003) as well as the cause of osteomyelitis after rose prickle injury (Vincent and Szabo, 1988). *Rahnella aquatilis* is an uncommon human pathogen. Most of its human infections have involved episodes of bacteraemia, urinary tract infections, post-surgical wound infections and endocarditis. The sources for most *Rahnella*-related human illnesses remain unknown (Oh and Tay, 1995; Janda, 2002). *Shigella* is a common cause of diarrhoeal illness that can cause food-borne outbreaks. Recently, *S. boydii* was implicated in an outbreak of food-borne illness in the USA (Chan and Blaschek, 2005) and as a cause of necrotizing enterocolitis (a serious gastrointestinal disease in neonates) (Sawardekar, 2005).

The phenomenon of biological defence by spines that harbour pathogenic bacteria is not confined to plants.

Various bacteria defend animals from predation, and this defence is sometimes associated with spines. For example, *Vibrio vulnificus* causes severe and often fatal infections in humans through contamination of wounds commonly in patients injured while handling pond-cultivated fish (Bisharat *et al*., 1999). Bacteria producing tetrodotoxin (a strong neurotoxin) were isolated from the spine apparatus of the Caribbean sea urchin, *Meoma ventricosa* (Ritchie *et al*., 2000). We argue that pathogenic bacteria-harbouring animal spines are also associated with the evolution of aposematism in spiny animals (e.g. Ruxton *et al*., 2004; Inbar and Lev-Yadun, 2005; Speed and Ruxton, 2005).

In sum, this study suggests for the first time that thorns, by wounding, insert pathogenic bacteria into the body of the herbivores as a sort of natural injection. The injury enables the bacteria to pass the animal's first line of defences (the skin) and to cause a disease. In Israel, for instance, the severity and frequency of infections following date palm thorn wounding of orchard workers has necessitated the costly practice of removal of all the millions of thorns from many of the orchards in Israel by mechanical saws. Indeed, the phenomenon we describe here is natural biological warfare by thorny plants against their herbivores. The microbe–thorn combination seems to be an important factor in the common evolution of the aposematic coloration of thorny plants (Lev-Yadun, 2001; 2003a; 2006; Lev-Yadun and Ne'eman, 2004; Rubino and McCarthy, 2004; Ruxton *et al*., 2004). Hence, bacteria that harbour thorns or spines in plants and animals alike seem to have enhanced the common, convergent evolution of aposematism in these organisms.

Experimental procedures

Thorn sampling

Thorns were sampled in northern Israel from two tree species: *P. dactylifera* (date palm) and *C. aronia* (common hawthorn). Date palm trees were sampled in October 2004 in an orchard 15 km south of the Sea of Galilee, Israel. Date palm spines are sharp leaflets that develop along the proximal parts of the large leaves. The spines are usually yellow and black (Fig. 1A). Five thorns (one from each tree) were randomly sampled at a height of 1–1.5 m (corresponding to the common height of grazing by large mammals), and were further treated as described below. Common hawthorn trees were sampled twice: in October 2004 first on a grazing plot on the northern slope of Mount Carmel, Israel, about 2 km south-east of our laboratory, and again in May 2005, this time both from the earlier sampled population and also from a second one on the mountain top. Hawthorn thorns consist of hard branches that end in sharp thorns; they are found all over the tree. They are red when young (Fig. 1B) and turn grey with time. The hawthorn thorns sampled in October 2004 were all old hard grey branches, and in May 2005 most thorns were young and red. Five thorns (one from each tree) were randomly sampled on each sampling date, again at a height of 1–1.5 m.

Isolation and enumeration of bacteria from thorns

Thorns were inoculated in aseptic conditions, in the field, immediately after sampling into tubes containing Thioglycolate Medium (Difco). The tubes were taken to the laboratory and were incubated at 30°C for 48 h. The cultures were then streaked from each tube onto LB agar plates to obtain single colonies. The plates were incubated in aerobic and anaerobic conditions (Anaerobic Jar) at 30°C for 48 h. Individual colonies from the LB agar were randomly picked and streaked again on LB agar to obtain single colonies. Isolated colonies were subcultured at least four times before examination of cell shape, Gram staining and motility. Bacterial isolates were kept in LB with 30% glycerol (–80°C).

Identification of isolates using 16S rRNA gene

Universal bacterial primers 8f [5′-CAC GGA TCC AGA CTT TGA T(C/T)(A/C) TGG CTC AG-3′] and 1512r [5′-GTG AAG CTT ACG G(C/T)T AGC TTG TTA CGA CTT-3′], based on *Escherichia coli* positions, were used to amplify internal fragments of 16S rRNA genes (Felske *et al*., 1997). Eight microlitres of a bacterial suspension was transferred to a sterile thin-walled PCR tube. One microlitre of each primer (20 pmol μl^{-1}) and 10 μl of PCR master mixture (ReddyMix, ABgene, UK) were added to the tube to make up a final reaction volume of 20 μl. Initial DNA denaturation was performed at 94°C for 4 min followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 50 s, and elongation at 72°C for 2 min and then a final elongation step at 72°C for 10 min. To confirm amplicon production (approximately 1500 bp), the mixture was analysed by electrophoresis on 1.5% agarose gel, followed by staining with ethidium bromide and visualization under ultraviolet light. The amplified PCR products were purified with the Wizard PCR product purification kit (Promega, Madison, WI). Automated sequencing with 3100 Genetic analyser from Applied Biosystems was performed at the sequencing centre of the Technion Medical School, Haifa, Israel. Sequencing was by means of 8f and 1515r primers. For identification of closest relatives, newly determined sequences were compared with those available in the GenBank (http://www.ncbi.nlm.nih.gov) databases with the use of the standard nucleotide-nucleotide BLAST program (BLASTN; http://www.ncbi.nlm.nih.gov) to ascertain their closest relatives. A phylogenetic tree was generated by the neighbour-joining method with NJPlot (MEGA 3) based on alignments from CLUSTAL W (Kumar *et al*., 2004). The bootstrap values obtained were from 1000 iterations.

Haemolytic activity

Haemolytic activity on blood agar plates (Hy Laboratories, Rehovot, Israel), was used to differentiate isolates that were identified either as *B. anthracis* or as *B. cereus* by their 16S rRNA gene sequences. *Bacillus anthracis* lacks the haemolytic activity.

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