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Diversity of Epiphytic Lactic Acid Bacteria (LAB) on Insect Oviposition Sites

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A B S T R A C T

Alternative strategies are needed in pest management to protect crops from pests. Occurrence of epiphytic lactic acid bacteria (LAB) on crop plants, especially on sites that are selected by pests, may be associated with host selection by insects. We carried out laboratory investigations as well as screen house and field experiments to understand whether epiphytic LAB occur at oviposition sites of agricultural and horticultural crops, probably modulating pest abundance by attraction or repulsion. The diversity of such epiphytic LAB associated with insect oviposition sites is discussed.

Introduction

With pesticides leading to environmental pollution, alternative strategies need to be explored in integrated pest management. Lactic acid bacteria (LAB) such as *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997; Makarova *et al.*, 2006) which produce a variety of antimicrobial compounds and other substances, confer a range of health benefits not only in higher animals but also in insects, e.g. honeybee (Vásquez *et al.*, 2012; Mathialagan, 2014). They are generally recognized as safe (GRAS) food grade microorganisms exploited as probiotics (Salminen, 1998) and in biopreservation (Carr *et al.*, 2002; Dalie *et al.*, 2010). They occur not only in food-related habitats such as milk but also in soil, water, manure, sewage, silage and plants (Harzallah and Belhadj, 2013). The potential of epiphytic LAB as biocontrol agents against phytopathogenic bacteria and fungi has earlier been documented (Trias *et al.*, 2008). While bacterial species such as *Staphylococcus* sp.

and *Bacillus* sp. associated with oviposition resources serve to regulate subsequent insect attraction and colonization (Zheng *et al.*, 2013), LAB may also help regulate pest populations on plants. The objective of this investigation was to understand the diversity of epiphytic LAB that occur on sites selected by lepidopteran insects for oviposition so that they can be exploited to modulate pest populations in IPM.

Materials and Methods

Laboratory, screenhouse and field experiments were conducted at the Department of Agricultural Entomology, Anbil Dharmalingam Agricultural College and Research Institute, Trichy, Tamil Nadu during 2015-16.

Laboratory studies

Crop samples (ca. 1" long, 1 cm wide) that serve as probable oviposition sites were collected between 6.00 and 8.00 am. The impression method was adopted to isolate the epiphytic LAB before enumerating their numbers and morphology in the laboratory (temp. 34 ± 2^0 C, 75 ± 5% RH, 12 ± 1 hr photoperiod) (Table 1). The samples were pressed on Lactobacillus MRS Agar (de Mann Rogosa Sharpe) medium (Himedia Laboratories), a specific medium for LAB growth. Cycloheximide (0.1 %) was added before plating in order to prevent fungal growth and other contamination. Calcium carbonate ($CaCo_3$) was added (0.8 g/100ml) to induce better LAB growth (Wright and Klaenhammer, 1981; Aween, 2012). As the LAB growth differed with the samples, it was observed 12 hours after getting the impression. The colony forming units (CFUs) were counted manually and expressed as CFUs/sample on 1^{st} and 2^{nd} day after plating. Then single colonies from the main cultures were streaked on MRS medium before gram staining for microscopic examination to record the morphology of the culture at 10 x 100 oil magnification in an image analyzer (CETI). The cultures thus obtained were preserved in the form of slants for further identification and storage.

Screen house and field experiments

In a screen house experiment, the response of yellow stem borer *Scirpophaga incertulas* (Wlk.) was evaluated after spraying an LAB culture isolated from rice leaf, where *S. incertulas* / leaffolder (*Cnaphalocrocis medinalis*) moths lay eggs, and releasing neonate *S. incertulas* larvae emerging from field collected egg masses. After inoculating the above LAB isolate in MRS broth, the culture was left for seven days to multiply before mixing 50 ml water-based soft insecticidal soap, 50 ml water and 25 ml of the LAB culture. From this stock solution, 3.75 ml was added to 250 ml water as the spray fluid and sprayed using a 250 ml capacity hand atomizer. Spraying was done at weekly interval after assessing insect damage and LAB numbers. The treatments included i) LAB spray and ii) untreated control. A set of five plants in tube pots (15 high x 12 cm in diameter) served as a treatment. After spraying the LAB culture, 3-5 field collected *S. incertulas* egg masses were placed on the experimental plants in both the treatment cages (90 x 60 cm) before counting the dead hearts two weeks later.

Two field experiments in rice, one with TRY 1 and the other with TRY 3 varieties, were also conducted with the above two treatments in eight one-cent plots (4 for each treatment) to understand the influence of LAB on insects after spraying the above LAB culture. Spraying was carried out at fortnightly interval and counts on LAB by leaf impression method and injury due to *S. incertulas* and *C. medinalis* was recorded from 5 to 10 hills /

plot. The experimental data were subjected to paired t-test analysis with log transformation for LAB counts and angular transformation for insect damage.

Results and Discussion

Diversity of epiphytic LAB

The results indicated that the LAB were present in all the plant samples examined, greatly diversified in their morphological characters (Table 1). Their population ranged from 7.33 ± 1.45/sample in jasmine flower bud where the budworm, *Hendecasis duplifascialis* Hmpsn. lays eggs to 271.33 ± 39.50/sample where the diamond-back moth *Plutella xylostella* (L.) oviposits. Morphologically, the cells were cocci or rods, in singles or doubles, in chains short or long. In the screenhouse experiment, though no significant difference could be observed in LAB population between the treated and untreated control plants (16.00 ± 1.0 - 16.75 ± 2.60 CFU/leaf sample)**,** the dead heart injury due to *S. incertulas* was significantly higher ($P < 0.05$) in untreated control plants (8.63 ± 1.69 %) than that in treated plants (6.79 ± 1.43 %) after spraying the LAB isolated from rice leaf and introducing *S. incertulas* egg masses (Fig. 1). When the data from both the field experiments were pooled and analysed for both LAB population and injury due to insect pests following treatments with the LAB culture isolated from the leaf site where *S. incertulas* and *C. medinalis* lay eggs, no significant difference in LAB population density could be observed between the treated and untreated control plots (32.61 ± 2.52 - 42.15 ± 5.27 CFU/leaf sample) and in white ear (14.06 ± 1.80 - 16.22 ± 1.90 %) (Table 2). However, *C. medinalis* damage was significantly higher ($P < 0.05$) in untreated control plots (16.73 ± 2.83 %) than that in treated plots (13.35 ± 2.47 %). Similarly, the dead heart injury was also significantly higher ($P < 0.05$) in untreated control plots (3.04 ± 0.81 %) than in treated plots (1.91 ± 0.57 %). In sustainable agriculture, lactic acid bacteria are exploited as one of the microbes (Mostafiz *et al.,* 2012) as they are commonly found on fresh fruits and vegetables (Trias *et al.,* 2008). In this study too, LAB were isolated from different plant parts that are preferred by insect pests for egg laying. It is probable that these epiphytic LAB are associated with the health of these host plants, nutritionally, or in its defence against pests and diseases, or both. Strains such as *Lactobacillus paracasei* subsp. *tolerans* and *Lactobacillus paracasei* subsp. *paracasei* have been reported as plant growth promoting bacteria (PGPB) (Murthy *et al.,* 2012). As one of the 'effective microorganisms (EM)' they are antagonistic to plant pathogenic fungi (Higa and Kinjo, 1991). As seed treatment in chilli, *Lactobacillus* sp. inhibited not only *Xanthomonas campestris* but also promoted plant growth (Kannan *et al.,* 2014). This study explored whether they can help rice plants protect themselves from selected pests, probably by influencing host selection through the volatiles they produce. For instance, the LAB present on human skin produce volatiles which may attract or repel *Anopheles gambiae* (Okumu *et al.,* 2010) as the bacterium, *Staphylococcus* sp. converts branched-chain amino acids to highly odorous short–chain volatile fatty acids (James *et al.,* 2004) that play an important role in the host-seeking behaviour of *A. gambiae* (Smallegange *et al.,* 2009; Knols *et al.,* 1997). Choi *et al.,* (2016) reported that the volatile odour diacetyl produced by LAB as an oxidized by-product of fermentation in the presence of citrate in rotting citrus fruit, attracted the bacteriovorous nematode, *Caenorhabditis elegans*, mediated by the diacetyl odour receptor, ODR-10.

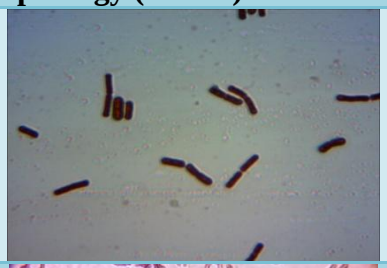
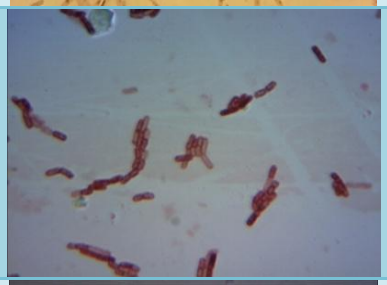
Lactic acid bacteria often occur in abundance in cereals and their fermented products. LAB associated with rice include *Lactobacillus*

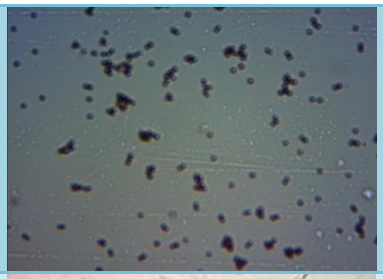
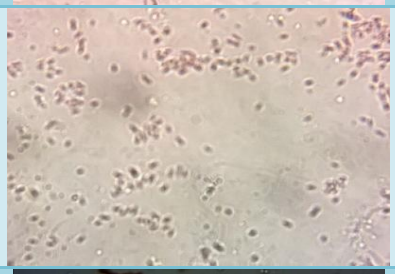
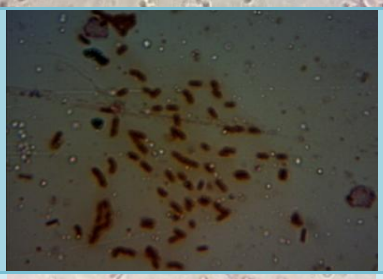
johnsonii (Doi *et al.,* 2013), *Lb. plantarum* (Olympia *et al.,* 1995), *Lactobacillus delbrueckii* and *Sporolactobacillus inulinus* (Fukushima *et al.,* 2004), probably co-evolved over the years similar to those in grapes and sugarcane (Sobrun *et al.,* 2012; Aplevicz *et al.,* 2014). *Lactobacillus fermentum*, *Lb. plantarum* and *Lb. paracacei* have earlier been reported to develop in the natural fermentation products of rice straw (Gao *et al.,* 2008). Similarly, *Pediococcus pentosaceus* is the most abundant LAB species in paddy rice silage (Ni *et al.,* 2015). Many bacteria produce cell-surface polysaccharides involved in a wide variety of biological functions including protection from environmental stresses, adherence to surfaces, pathogenesis and symbiosis (Jolly *et al.,* 2002). They are associated with virulence and cell protection against desiccation, osmotic stress, antibiotics, toxic compounds, and bacteriophage or protozoa attack (Sanchez *et al.,* 2006). Exopolysaccharides which are loosely attached or excreted into the environment (Boels *et al.,* 2001), either homopolysaccharides or hetero polysaccharides (De Vuyst and Degeest, 1999), may help the LAB survive on plants. As most LAB isolated in this study produce exopolysaccharides in the laboratory, these biopolymers may also influence the behaviour of pests and their populations, especially at night when the pests will be more active in the presence of dew coupled with low atmospheric temperature. In addition to the exopolysaccharides, LAB produces several antimicrobial metabolites such as oxygen metabolites (hydrogen peroxide and free radicals) and catabolism end-products (Vandenbergh, 1993; Rattanachaikunsopon and Phumkhachorn, 2010). Many strains of LAB produce specific compounds with antimicrobial activity, called bacteriocins (Piard and Desmazeaud, 1991). Bacteriocins are proteins or protein complexes which have inhibition activity against gram-positive and negative bacteria (Tagg *et al.,* 1976). Since bacteriocins allow the LAB to compete even in non-fermentative ecosystems (Lindgren and Dobrogosz, 1990), they are also likely to produce these compounds on crop surface as well, influencing insect behaviour too. Since LAB have the ability to break down organic matter, thereby releasing amino acids, sugars, alcohols, hormones and similar organic compounds that are absorbed by plants (Alagukannan and Ashokkumar, 2015), these substances may also help modify insect behaviour.

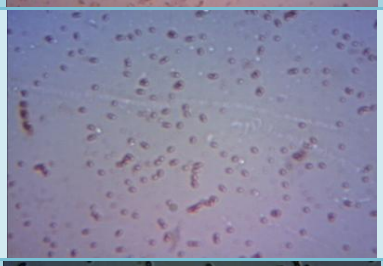
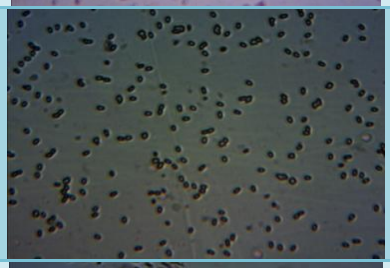
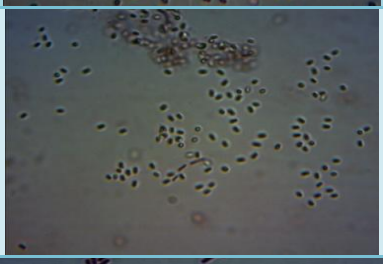
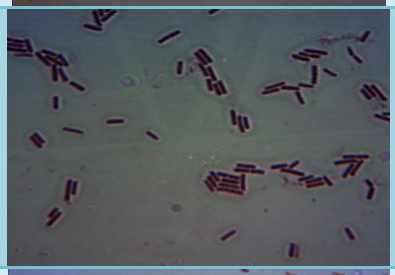
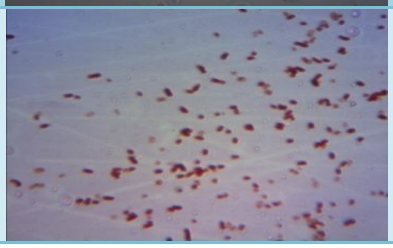
LAB and oviposition

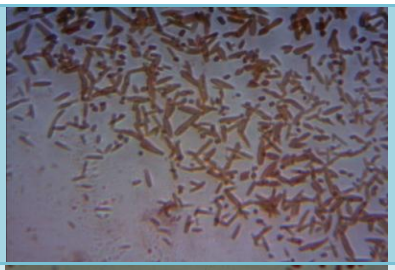
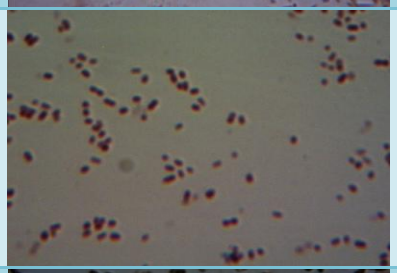
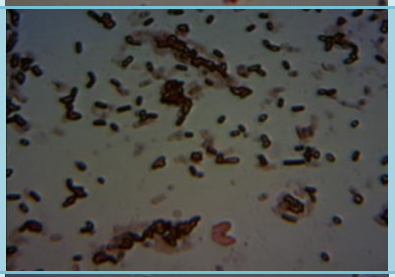
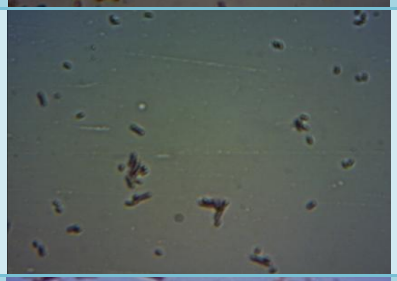
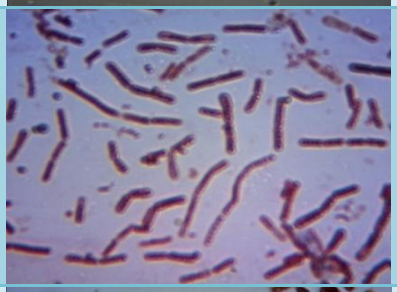
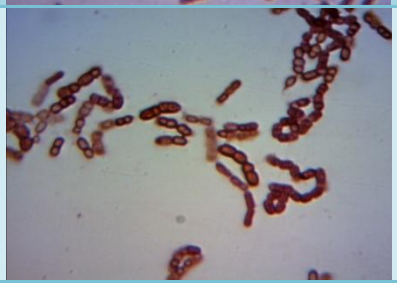
Abiotic and biotic environmental factors often influence the production or release of behaviour-modifying chemicals by a plant, and therefore affect oviposition preferences (Renwick, 1989). In black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), different bacterial species mediate oviposition (Zheng *et al.,* 2013). Gravid house flies *Musca domestica* L. (Diptera: Muscidae) evaluate volatiles produced by microbes on conspecific eggs to ensure synchronous larval development which allows for aggregative feeding and reduced likelihood of cannibalism (Lam *et al.,* 2007). As most crops have LAB on their surface as documented in this study, they may also serve as a mechanism regulating attraction, colonization and succession of insect species. For example, in sugarcane, three different LAB strains were isolated from the sites where three different borer moths {*Chilo infuscatellus* Snell., *Chilo sacchariphagus indicus* (Kapur) and *Scirpophaga excerptalis* Wlk.} lay eggs, all cocci in singles or doubles or short chains (41.66 ± 13.12 to 111.33 ± 39.63 CFU/leaf sample). This shows that the adults may select the right place of egg laying owing to the presence of specific epiphytic LAB even though plant volatiles do attract.

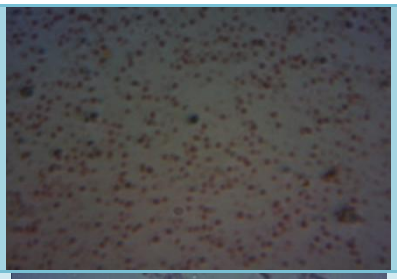
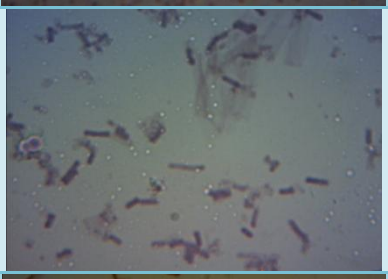
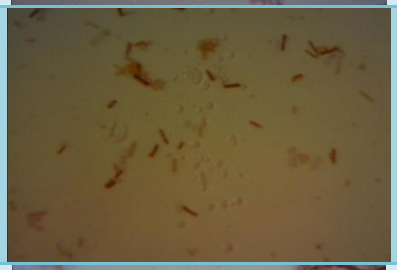
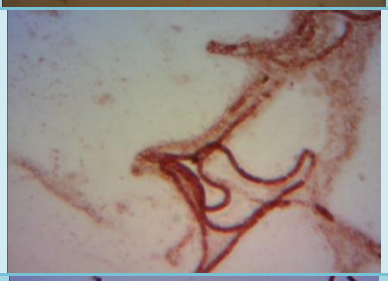
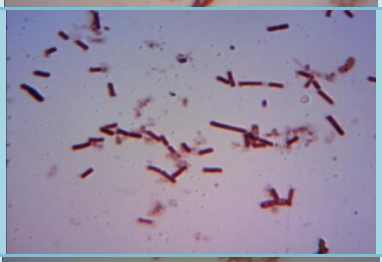
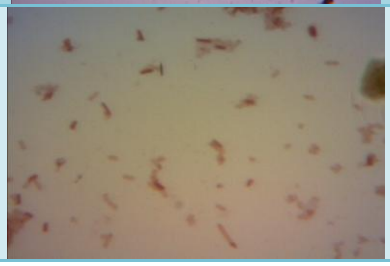
Table.1 The diversity of epiphytic LAB isolated from different oviposition sites on crops

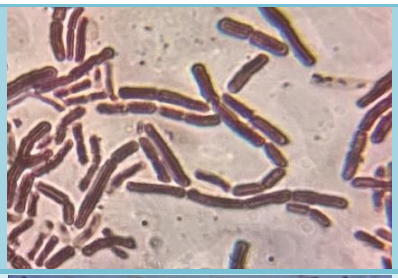
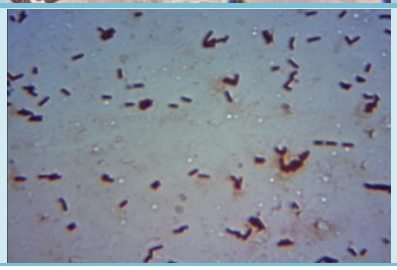
Oviposition site	Pest Name	Lactic Acid Bacteria	
		CFU / sample	Cell morphology (1000 x)
Rice leaf tip	Yellow stem borer, *Scirpophaga incertulas* (Wlk.)	47.33 ± 10.90	Rods in singles or doubles 
Rice leaf sheath	Pink stem borer, *Sesamia inferens* Wlk.	112.33 ± 14.81	Cocci in singles or doubles or chains 
Rice leaf auricle	Gall midge, *Orseolia oryzae* (Wood-Mason) Mani	59.33 ± 10.27	Cocci in singles or small chains 
Rice leaf	Leaffolder, *Cnaphalocrosis medinalis* (Guen.), *Marasmia* sp.	47.33 ± 13.38	Rods in singles or doubles or chains 
Paddy straw	Not preferred by pests	347.66 ± 43.05	Cocci in doubles or short chains 

Ragi leaf sheath	Pink stem borer, *Sesamia inferens* Wlk.	65.00 ± 11.13	Cocci in singles or doubles	
Sugarcane leaf sheath	Early shoot borer, *Chilo infuscatellus* Snell.	111.33 ± 39.63	Cocci in short chains	
Sugarcane leaf sheath midrib	Internode borer, *Chilo sacchariphagus indicus* (Kapur)	105.33 ± 36.97	Cocci in short chains	
Sugarcane top leaves	Top shoot borer, *Scirpophaga excerptalis* Wlk.	41.66 ± 13.12	Cocci in singles or doubles	
Sorghum leaf near midrib	Stem borer, *Chilo partellus* (Swinhoe)	184.66 ± 33.84	Cocci in doubles or short chains	
Cotton flower bud	Boll worms, *Earias* spp., *Helicoverpa armigera* (H.)	34.33 ± 2.66	Rods in singles or doubles or chains	

Gingelly tender leaf	Shoot webber, *Antigastra catalaunalis* D.	33.00 ± 4.16	Irregular rods	
Redgram tender pod	Pod fly, *Melanogromyza obtusa* (Malloch)	47.00 ± 1.00	Cocci in singles or doubles	
Brinjal flower calyx	Shoot and fruit borer, *Leucinodes orbonalis* Guenee	37.66 ± 3.38	Cocci in doubles	
Brinjal abaxial leaf surface	Spotted leaf beetle, *Epilachna vigintioctopunctata* (F.)	30.66 ± 4.37	Cocci in singles or doubles	
Tomato top canopy leaf	Fruit borer, *Helicoverpa armigera* (H.)	30.33 ± 4.91	Rods in singles	
Moringa shoots	Hairy caterpillar, *Eupterote mollifera* Wlk.	10.00 ± 1.73	Cocci in singles or doubles	

Moringa Leaf	Leaf webber, *Noorda blitealis* W.	31.66 ± 6.17	Irregular rods	
Bhendi tender fruit	Shoot and fruit borer, *Earias* spp.	111.66 ± 9.02	Cocci in singles or doubles	
Cucurbit flower ovary	Fruit fly, *Bactrocera* spp.	29.00 ± 3.60	Cocci in singles or doubles	
Snake gourd fruit surface	Fruit fly, *Bactrocera* sp.	262.66 ± 69.21	Cocci in singles or doubles	
Cabbage outer whorl leaves	Diamond- back moth *Plutella xylostella* (L.)	271.33 ± 39.50	Cocci in singles or short chains	
Guava flower ovary	Fruit borer, *Deudorix isocrates* (F.)	154.66 ± 18.52	Cocci in chains	

Banana pseudostem	Pseudostem borer, *Odoiporus longicollis* Olivier	17.66 ± 4.09	Rods in singles	
Citrus tender leaf midrib	Leaf miner, *Phyllocnistis citrella* Stainton	30.66 ± 2.66	Rods in singles	
Citrus tender leaf lamina	Butterfly, *Papilio demoleus* L.	37.33 ± 9.56	Rods in singles	
Sapota tender leaf	Shoot webber, *Nephoteryx eugraphella* Ragonot	44.66 ± 5.48	Cocci in long chains	
Mango marble-sized fruit	Nut weevil, *Sternochetus mangiferae* (F.)	17.66 ± 4.63	Rods in singles	
Grapevine bark on girdled vines	Girdler, *Sthenias grisator* (F.)	25.00 ± 4.93	Rods in singles	

Sides of jasmine flower bud	Budworm, *Hendecasis duplifascialis* Hmpsn.	7.33 ± 1.45	Rods in doubles or chains	
Tip of unopened Jasmine bud	**Blossom midge, *Contarinia maculipennis* Felt**	**13.00 ± 7.50**	**Rods in singles**	

(CFU, colony forming unit; mean of 3 replicates ± SE)

Table.2 Mean LAB population and damage due to pests in rice after spraying LAB culture field experiments

Treatments	LAB population (CFU / leaf sample)	*C. medinalis* injury (%)	% damage due to *S. incertulas*	
			Dead hearts	White ear
LAB culture spray	32.61 ± 2.52 (1.46 ± 0.03)	13.35 ± 2.47 (16.92 ± 2.13)	0.91 ± 0.57 (5.38 ± 1.03)	**14.06 ± 1.80 (20.10 ± 1.70)**
Untreated control	42.15 ± 5.27 (1.52 ± 0.05)	16.73 ± 2.83 (6.53 ± 0.81)	3.04 ± 0.81 (7.10 ± 1.26)	**16.22 ± 1.90 (21.75 ± 1.80)**
Observations (n)	47	50	35	**35**
t - value	**NS**	**6.14 (P < 0.05)**	**2.13 (P < 0.05)**	**NS**

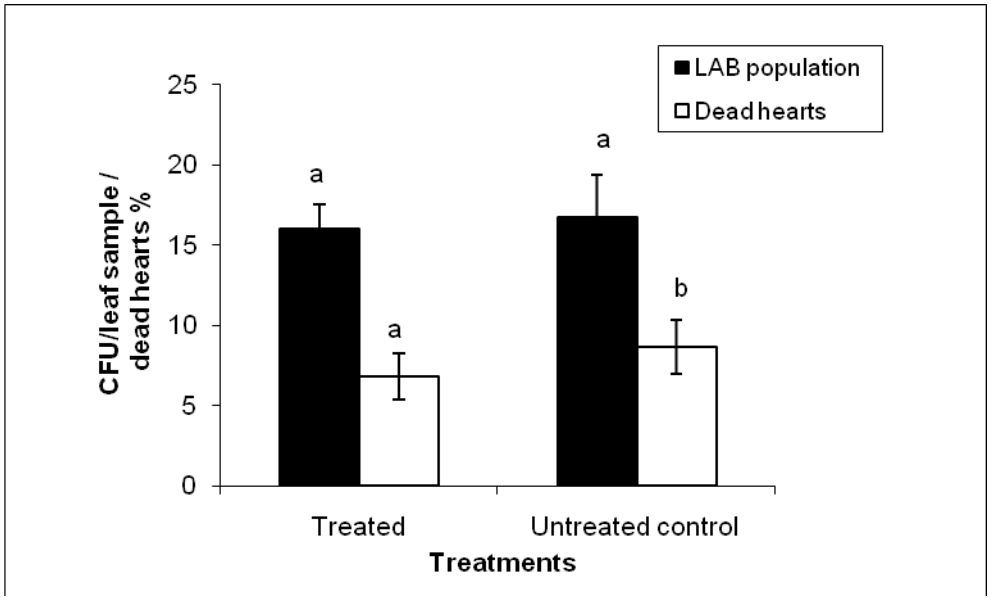
(CFU, colony forming unit; Figures in parenthesis are transformed values; NS, not significant)

Table.3 Diversity of LAB isolated from healthy and mite infested leaves of Rutaceae showing their morphology and population.

Leaves	LAB population (CFU/ leaf sample)	Cell morphology
Healthy citrus leaf	33.33 ± 4.91	Rods in singles
Mite-infested citrus leaf	40.33 ± 8.87	Cocci in singles or chains
Healthy curry leaf	9.0 ± 2.0	Cocci in short chains
Mite-infested curry leaf	164.66 ± 23.1	Cocci in singles or short chains

(CFU, colony forming unit; mean of 3 replicates ± SE)

Figure.1 LAB population (n = 8) and dead hearts damage (n = 60) in rice following LAB spray and *S. incertulas* egg mass introduction in the screenhouse experiment. CFU, colony forming unit. Columns with the same letter are not significantly different according to paired *t* test (P < 0.05). Vertical bars indicate the standard error



Similarly, the LAB differed morphologically on moringa shoots and on leaves where *Eupterote mollifera* Wlk. and *Noorda blitealis* W. lay eggs, respectively. On the other hand, the LAB on young citrus leaves seem to attract both the leaf miner, *Phyllocnistis citrella* Stainton and the butterfly *Papilio demoleus* L. as both the pests select the same site for oviposition. Thus the diversity in the morphology of epiphytic LAB species may be associated with pest susceptibility which needs to be investigated further. Moreover, LAB density may also differ with plant age or part/site. For instance, LAB were more numerous both on mite-infested older citrus and curry leaves (40.33 ± 8.87 - 164.66 ± 23.10 CFU/leaf sample) than on mite-free young citrus and curry leaves (9.0 ± 2.0 - 33.33 ± 4.91 CFU/leaf sample) (Table 3). Therefore pest populations and their behaviour can be manipulated by LAB strains isolated from different crop sites or age or source.

LAB culture spray and rice pests

Screenhouse and field experiments also demonstrated the presence of LAB at different crop growth stages in rice. Along with the native LAB, they appeared to have significantly influenced *S. incertulas* both in screenhouse and field, probably repelling adult moths thereby injury to leaves, though not to tillers. In natural farming, LAB-rich rice rinse water is used to protect crops (Ikeda *et al.*, 2013). With no pest infestation noticed in harvested paddy straw, a cattle feed, LAB on paddy straw would probably deter insects as they occur in large populations on them (347.66 ± 43.05 CFU/leaf sample) (Table 1). Consequently, cow dung is generally considered as a vehicle for the distribution of LAB onto field-grown crops and vegetables (Henning *et al.*, 2015). It has already been established that the behaviour and biology of brown planthopper, *Nilaparvata lugens* (Stal) are significantly influenced by rice plant volatiles extracted as steam distillates (Saxena

and Okech, 1985). It is worth probing the influence of LAB-produced volatiles on BPH too in future as bacterial microbes representing the phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes are found in *N. lugens* (Tang *et al.*, 2010). Rice plant LAB may also be associated with its chemical ecology. Thus LAB on oviposition sites of crops are also likely to play a key role in pest management too as they are as diverse as crops or pests. This, in conclusion, opens up a new research area in agricultural entomology hitherto unexplored: could epiphytic LAB influence insect behaviour and abundance?

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