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INFECTION OF RED PALM WEEVIL, *RHYNCHOPHORUS FERRUGINEUS*, BY A YEAST*

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Internal habitat of red palm weevil (*Rhynchophorus ferrugineus* F., Coleoptera: Curculionidae) makes the chemical and mechanical control methods ineffective. Therefore, self perpetuating entomopathogens are expected to be suitable for suppression of the pest. Although a cytoplasmic polyhedrosis virus (Gopinadhan *et al.* 1990) and a predacious earwig, *Chelisoches moris* (Abraham and Kurian 1973) were recorded as biocontrol agents of the pest, their efficiency could not be confirmed. Therefore, the present study was envisaged to detect and exploit different natural pathogens of the pest. During this study, a yeast was isolated from the haemolymph of the natural pest population. The entomogenous yeasts are generally symbiotic or commensalistic and occasionally pathogenic organisms but rarely maintain transitional (between symbiotic/commensalistic and pathogenic) relation with the hosts (Krieg 1971; Van der Walt and Yarrow 1984). Entomopathogenic yeasts are rare and no such pathogen of the red palm weevil has been recorded so far. Therefore, the significance and potency of the yeast as a biocontrol agent were assessed.

The yeast cells were pelleted (15000g, 20 min, 3°C) from the haemolymph (2-3 ml) drawn from the immobilized (in ice-bath) and surface-sterilized (0.1% mercuric chloride, 2 min and 90% ethanol, 5 min) larvae, pre-pupae and pupae of red palm weevil. The cells were thoroughly washed, suspended in 0.25 ml sterile distilled water, counted under a phase-contrast microscope (x40) and inoculated separately in each of 3 ml of two insect tissue culture media (0.22 µm membrane filter-sterilized) containing either horse serum or haemolymph of the red palm weevil pupae (Grace 1962; Lery and Fediere, 1990), and nutrient, brain-heart infusion, PPLO, potato dextrose, malt extract and corn meal broths (autoclave-sterilized). Adaptability of the yeast growing in tissue culture media was checked and subsequently

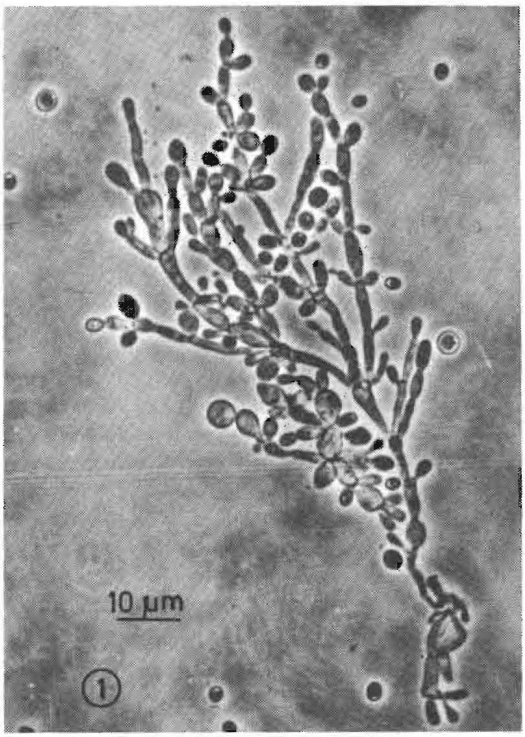

Fig. 1. Phase contrast photomicrograph of the yeast (water mount) grown in potato dextrose broth.

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Table 1. ***Occurrence and population of yeast cells in the haemolymph of field-collected smaller (LI) and larger (L2) larvae, pre-pupae (PP) and pupae (P) of red palm weevil***

Insect	Insect with yeast* (%)	Yeast population (No./ml haemolymph)
LI	48.81±5.23	12.11±2.30
L2	46.32±8.21	4.21±1.96
PP	44.88±6.11	3.29±2.02
P	45.87±7.25	3.33±2.40

Results are means of 5 observations from 42 insects ±SE.

LI=Smaller larvae, 1.88±0.21 cm in length and 1.90±0.67 g live wt. L2=Larger larvae, 4.25±1.11cm in length and 4.26±1.43g live wt. PP=Pre-pupae, 5.31±1.26cm in length and 5.56 ±1.42g live wt. P=Pupae, 4.45±1.36 cm in length and 4.78±1.11g live wt.

dilution-plated in the above mentioned microbiological media, and colony and vegetative cell characters, and reproductive structures were recorded. The cultures were maintained on nutrient agar slopes.

The layer of yeast growth (12h) was scraped off from the nutrient agar plates, washed and suspended in sterile distilled water, cell concentration was adjusted to 10^{10} cells/ml and serially diluted. Test-insects were made yeast-free (haemolymph was checked periodically up to 30 days under a phase-contrast microscope) by injecting 250 µg cycloheximide/g insect (MIC for the yeast was 100µg/ml) for 3 successive days and maintained aseptically feeding on surface-sterilized (1.6% sodium hypochlorite, 5 min) coconut petioles. Batches (25 each) of immobilized (in ice-bath) and surface-sterilized (90% ethanol, 2 min) larvae (small and large), pre-pupae and pupae (Table I) were injected and force-fed (except the pupae) individually with 10-10^{10} yeasts/insect. Equal number of reference insects were inoculated with 0.1ml sterile distilled water/insect. Treated insects were maintained aseptically, and mortality, and behavioural and morphological changes were recorded up to 30 days, and LD_{50} and LT_{50} were estimated (Litchfield 1949; Finney 1971). Yeast population in the haemolymph of moribund and dead insects were checked under a phase-contrast microscope (x40).

The yeast was free-living in the haemolymph and no mycetome-like structure was detectable in the haemocoel of the insect. It is reminiscent of the symbiotic or commensal yeasts of different insects including the Coleoptera (Krieg, 1971; Noda and Omura, 1992). Natural insect population expressed no external symptoms although

Table 2. ***Susceptibility of smaller (LI) and larger (L2) larvae, pre-pupae (PP) and pupae(P) of red palm weevil to intra-haemocoelic inoculation of the yeast and resultant yeast population within the haemolymph of moribund insects.***

Insect	Dose of yeast (nx10^6 cells/insect)	Death of insect(%)	Yeast population	LD_{50} (nx10^6) (nx10^8cells/ ml haemolymph	LT_{50} (Days) cells/insect)
LI	8.08	51.25±1.60	3.62±0.83	7.94±0.90	4.21±1.61
L2	8.62	40.06±2.16	0.94±0.19	NA	NA
PP	8.43	19.81±1.22	0.11±0.03	NA	NA
P	8.39	36.29±1.42	0.44±0.08	NA	NA

Results are means of 5 replications ±SE. For each group, 125 insects (5 batches) were tested. Details of the insects are given in Table I. All control insects remained healthy. NA=Could not be assessed. n=Data mentioned in respective columns.

about 50% of them harboured 3-12 yeasts/ml haemolymph (Table I). Probably meagre yeast population in the haemocoel could not impart lethal effect to the pest because the lethal dose of the pathogenic yeasts varied from about 10^4-10^7 cells/insect (Martignoni *et al.*, 1969; Van der Walt and Yarrow, 1984). From the haemolymph, the yeast could establish only in the tissue culture media within 3-6 days (Table I) but subsequently it could grow in the microbiological media also. The observations depicted that the yeast would neither be a true symbiont/commensal organism nor an obligate pathogen but would be at a transitional position in between the two status which is intriguing but has great evolutionary significance (Krieg, 1971). The organism produced creamy-white, raised, glistening, slightly irregular and non-pigmented colonies of 2-3 mm dia. on agar plates; the cells sedimented and pellicle was formed in liquid microbiological media and fermented the tissue culture media. *In vivo*, the yeast cells were spindle shaped (1.46±0.16x0.96µm) or spherical (1.23± 0.10µm dia). In tissue culture media, the cells were capsular (5.36±0.94x2.85 ±0.18µm) and spherical (7.80±0.10µm dia) with single or multiple buds but in nutrient broth and PDB, the cells were slightly thinner and elongated (3.48±0.41x0.30±0.10µm and 5.56±0.32x 2.08±0.01µm). Profusely branched pseudofilamentous growth was also noticed in PDB (Fig. 1). Although unconfirmed, the yeast occasionally formed spore-like structures in the moribund insects but not in culture. Therefore, at present the organism could not be identified. Intra-haemocoelic inoculation of about $8x10^6$ yeasts/insect could kill all groups of the insect but force-feeding was ineffective (Table II) and higher doses resulted non-specific death of the insects within 12h. Although ineffective, force-feeding increased the yeast population to about 12-26 cells/ml haemolymph (Table II). The results indicate that the yeast could invade into the haemocoel but failed to multiply significantly which implies that it would be a facultative pathogen (Martignoni *et al.*, 1969; Bang 1975; Codreanu and Codreanu-Bolcescu, 1981). Intrahaemocoelic inoculation depicted the LD_{50} and LT_{50} of the smaller larvae to be about $8x10^6$ yeasts/insect and 4 days respectively but the same dose of the yeast could kill only about 19-40% of other categories of the insect (Table II). The observations suggest that the smaller larvae are more susceptible to the yeast which supported the opinion of Bang (1975). Furthermore, the LD_{50} of the yeast against the smaller larvae was comparable to that of the pathogenic yeasts (about 10^7 cells/insect) of other insects (Martignoni *et al.*, 1969).

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