

## Zoophthora radicans affecting Zyginidia pullula

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### Abstract

Mortality of the maize leafhopper *Zyginidia pullula* (Boheman) (Hemiptera Cicadellidae) had been observed on maize plants cultivated in northwest Italy since the '80s of last century. In past research several fungi from the dead leafhoppers having a saprophytic or pathogenic activity were isolated, but only lately *Zoophthora radicans* (Brefeld) Batko (Zygomycota Entomophthorales) was recognized as the actual pathogen. A research was conducted in 2003 and 2004 to find out how these fungi infected the leafhopper by weekly collecting 30 adult leafhoppers on maize plants and rearing them individually in the laboratory, repeating an unpublished experiment carried out in 1988. *Z. radicans* was confirmed to be by far the most frequent entomopathogenic agent (8.2% of adult mortality in July-October). It appeared in summer and its presence increased throughout the season, reaching a peak in August (even around 50% of adult mortality). The infestation decreased gradually until the beginning of frosts in November. The unusual drought accompanied by high temperatures during the whole summer in 2003 delayed the peak, that occurred at the beginning of October. Furthermore leafhoppers were infected in the laboratory to check the development of *Z. radicans*, that could complete its life history within four days and a half at 23 °C.

**Key words:** Northwest Italy, natural enemy, mortality.

### Introduction

*Zyginidia pullula* (Boheman) is a leafhopper (Hemiptera Cicadellidae) widespread in the Po Plain (North Italy) in all environments where there are grasses, from the sea level to a little over 1,000 m a.s.l., thus penetrating the Alpine and Apenninic valleys. This leafhopper has several vicariant species: *Zyginidia ribauti* Dworakowska in the Italian peninsula and Sardinia, and *Zyginidia serpentina* (Matsumura) in Sicily (Vidano, 1982).

*Z. pullula* was lately discovered to host a feminizing bacterium, *Wolbachia pipientis*, that infects 30% of females in the field (Negri *et al.*, 2008; 2009).

In the Po Plain *Z. pullula* accomplishes up to four generations a year, overwinters as a fecundated female, and starts laying eggs after the vegetative onset of the host plants (Vidano and Arzone, 1985). The reproductive potentiality of this species is remarkable, as a single female may lay up to 350 eggs (Mazzoglio and Arzone, 1988).

This leafhopper migrates to cereal crops where it multiplies massively, and is sometimes recorded noxious to maize (Naibo *et al.*, 1991), but generally it has no economic consequence. It rather acts as a reservoir for the multiplication of egg-parasitoids, insect and spider predators, and entomopathogenic fungi (Vidano and Arzone, 1988; Ozino and Zeppa, 1988; Mazzoglio, 1989).

*Zea mays* L. is the preferred plant for this leafhopper, whereas the usual wild host plants belong generally to the genus *Bromus*, in particular *B. arvensis* L., *B. inermis* Leyser, and *B. sterilis* L., followed by *Hordeum murinum* L., *Echinochloa crus-galli* (L.) Beauv., *Alopecurus pratensis* L., and *Cynodon dactylon* (L.) Pers., in a decreasing order of preference. This leafhopper is seldom found on *Agrostis*, *Festuca*, *Lolium*, and *Phleum* (Verzè and Mazzoglio, 1994).

A remarkable mortality of *Z. pullula* was observed on maize plants cultivated in northwest Italy since the '80s of last century. Several fungi from the dead leafhoppers

having a saprophytic or pathogenic activity were isolated, such as *Aspergillus versicolor* (Vuill.) Tiraboschi, *Mucor hiemalis* Wehmer, *Verticillium fusisporum* Gams, *V. lecanii* (Zimm.) Viégas, *Drechslera ravenelii* (Curt.) Subram et Jain, *Alternaria alternata* (Fr.) Keissler, *Fusarium oxysporum* Schlecht emend. Snyder et Hansen, *Cladosporium cladosporioides* (Fres.) de Vries, *Penicillium oxalicum* Currie et Thom, *Zoophthora radicans* (Brefeld) Batko (Zygomycota Entomophthorales) (Ozino Marletto, 1982; Ozino Marletto and Maggiora, 1983; Ozino and Zeppa, 1988; 1989; Vidano and Arzone, 1988).

*Z. radicans* was found in 1986 as one of the natural enemies of the leafhopper adults (Ozino and Zeppa, 1988), but only lately it was recognized to be the most effective pathogen (Mazzoglio *et al.*, 2004). In order to evaluate the importance of the above mentioned fungus, a research was carried out in 2003 and 2004, repeating an unpublished experiment carried out in 1988 on field leafhopper mortality caused by fungi and a particular attention was focussed on the mechanism of infection.

*Z. radicans* is an entomopathogenic fungus with a broad host range. It attacks insects in the families Aphidae, Cercopidae, Cicadellidae, Delphacidae, and Psyllidae (Hemiptera Homoptera) (Humber, 1992) besides Curculionidae (Coleoptera), Anthomyiidae (Diptera), Diprionidae (Hymenoptera), Geometridae, Noctuidae, Pieridae, Plutellidae, Pyralidae, Tortricidae (Lepidoptera), and Miridae (Hemiptera Heteroptera) (Ben-Ze'ev *et al.*, 1988; Humber, 1992; Keller, 1991).

*Z. radicans* has potential use as a biological control agent for several economically important insect species (Furlong and Pell, 1997; Poprawski and Wraight, 1998). This fungus includes many strains exhibiting high degrees of host specificity. In general, isolates of *Z. radicans* infect insect species which are closely related to the original host species than other insects (Shah and Pell, 2003).

Among Hemiptera Homoptera different studies of biological control by *Z. radicans* have been carried out on

the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) (Sanchez-Peña, 2000), on the leafhoppers *Empoasca fabae* (Harris) (Sanchez-Peña, 2000; Hodge *et al.*, 1995) and *Empoasca kraemeri* Ross and Moore (Wraight *et al.*, 2003), and on the spotted alfalfa aphid *Therioaphis trifolii* (Monell) (Milner, 1997).

Environmental conditions may affect the success of biological control by *Z. radicans*; in particular the relative humidity on the viability of primary conidia (Griggs *et al.*, 1999) and the influence of solar radiation (Furlong and Pell, 1997). Dried-mycelium formulations (Wraight *et al.*, 2003) and the recent millet-based technology (Hua and Feng, 2005) may improve the mass production of *Z. radicans*, culturable but nutritionally fastidious like all Entomophthorales entomopathogens.

The infection mechanism occurs with the conidia falling onto an insect and, under the appropriate environmental conditions, they germinate and penetrate the host's cuticle. The fungus grows in the host, eventually killing it, after having produced adhesive rhyzoids, so to block the insect on the lower part of the leaf, and then produces conidiophores on the dead insect's integument, that project conidia in the surrounding environment. Some isolates produce resistant spores (zygospores) for long term survival (Sanchez-Peña, 2000), but not all isolates of *Z. radicans* do so (Pell *et al.*, 1993; Yeo *et al.*, 2001).

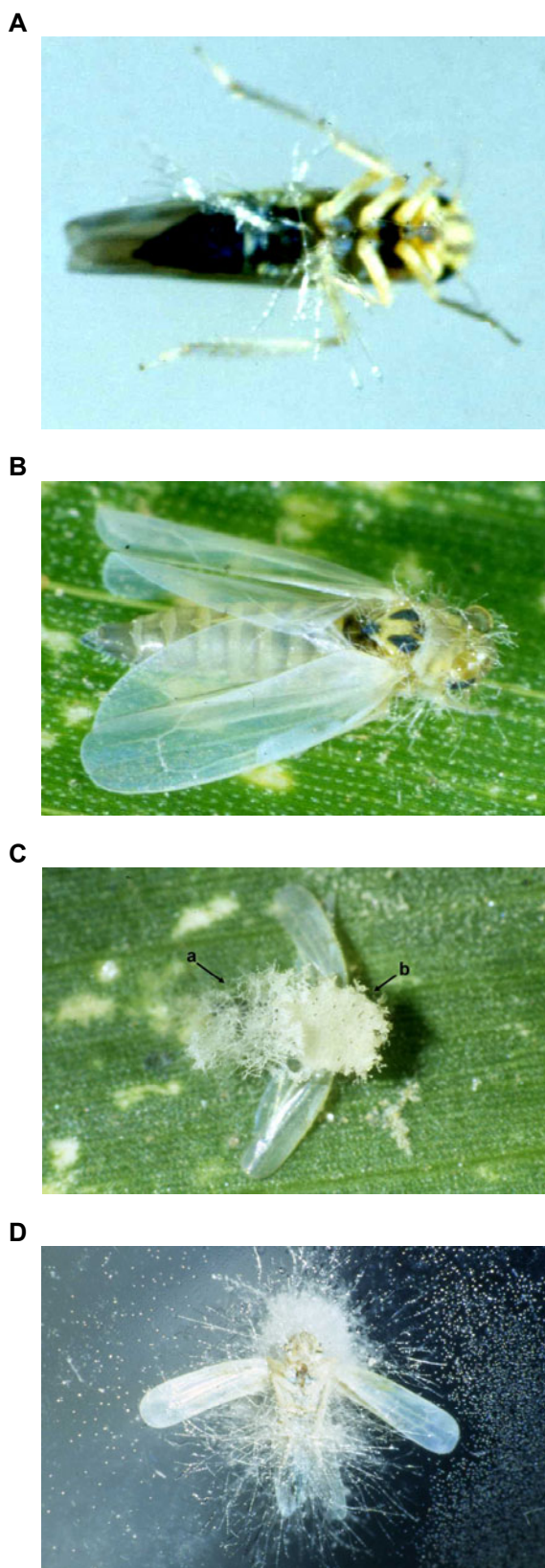
Samplings, rearings and laboratory infection tests were also carried out to determine the life history of this fungus on *Z. pullula* in maize fields in Piedmont.

## Materials and methods

Samplings were carried out in Piedmont, Northwest Italy: during 1988 in Chieri, hamlet of Pessione (44°57'56"N 7°50'05"E); and during 2003-2004 in Carmagnola (44°53'06"N 7°41'02"E), in the experimental field centre of the Faculty of Agriculture of the University of Turin. Both sites have similar environmental and climatic conditions. Weekly collections of at least 30 *Z. pullula* adults were made from mid June to October using a sweep-net in the maize fields. In the laboratory the collected individuals were isolated in sterilized glass tubes including a piece of maize leaf grown in the laboratory, the tube was tapped with a dampened cotton plug. The leafhoppers were reared in darkness at 23 ( $\pm 1$ ) °C and were checked daily so to observe any mortality and determine the immediate cause of it, or just to dampen the cotton plug and replace the maize leaf portion if necessary.

The dead individuals were placed in a sterile humid chamber at the same temperature to favour the development of the mycelium. A part of the leafhopper was examined microscopically for fungus identification.

Considering the external aspect of the fungus growing on the leafhopper, different growth phases were distinguished: rhyzoidal development, rhyzoidal branching (figure 1A); development of trichoid hyphae (figure 1B), development of reticular mycelium (figure 1Ca), development of conidiophores (figure 1Cb), production and spreading of conidia (figure 1D).



**Figure 1.** *Z. radicans*: A) beginning of rhyzoid branching (ventral view of the leafhopper on a glass slide); B) aspect of trichoid hyphae on head and thorax of the leafhopper; C) reticular mycelium (a) and conidiophores (b), with an halo of conidia on the leaf around the leafhopper; D) halo of conidia around the leafhopper (ventral view on a glass slide). (In colour at [www.bulletinofinsectology.org](http://www.bulletinofinsectology.org))

**Table 1.** Meteorological data recorded during the three year period of the experiment. Measure units: Temperature (T): °C; Relative humidity (RH): %; Total Rainfall: mm; Leaf wetness (LW): hours; NR: not recorded.

Period	T max	T mean	T min	RH mean	RH min	Rainfall	LW max	LW mean	LW min
1988									
July	28.9	23.2	17.5	69.0	NR	3.1	NR	NR	NR
August	28.2	22.3	16.3	73.0	NR	3.5	NR	NR	NR
September	24.2	24.2	10.9	72.0	NR	0.1	NR	NR	NR
October	17.9	13.9	10.2	81.3	NR	11.7	NR	NR	NR
2003									
July	31.2	23.9	16.5	68.6	31.0	40.4	303.0	36.0	188.3
August	33.9	24.8	16.5	68.1	25.0	16.8	187.8	71.5	139.0
September	25.3	17.2	9.8	72.7	28.4	129.4	696.1	196.7	463.0
October	16.7	10.2	5.0	83.3	36.0	112.5	1001.9	589.4	781.7
2004									
July	29.3	21.7	13.4	72.5	43.8	33.4	331.3	85.3	182.8
August	28.0	21.0	14.3	72.9	45.0	27.6	188.8	58.5	138.4
September	26.4	18.1	11.3	67.3	36.0	72.0	625.5	290.5	454.8
October	22.0	14.3	8.2	73.8	43.3	20.2	906.5	608.5	749.9

Field data were analyzed via simple linear regression to determine the influence of temperature, relative humidity, rainfall, and leaf wetness on the mortality of *Z. pullula* due to *Z. radicans*. Meteorological data were taken from a nearby meteorological station, and mean values for each year are shown in table 1. Maize leaf wetness data came from measurements made in the Agricultural Technical Institute of Lombriasco (44°50'35"N 7°38'06"E) (1988 data are missing). The comparison of seasonal mortality in different years (i.e. the percentage of dead individuals during August, September, and October in the three years of sampling) was performed with a  $\chi^2$  test, calculating a  $3 \times 3$  contingency table. Different mortality rates in different years were also compared using a  $\chi^2$  test for independence. All data were analyzed with SPSS® 17.0 statistical package.

Infection trials were made to document the development of *Z. radicans*, by using a leafhopper that had just been killed by the fungus and was projecting conidia. The dead leafhopper was introduced into a sterilized glass test-tube (120 × 24 mm) on a 1 cm<sup>2</sup> maize leaf portion on which 10 adult laboratory-reared individuals had to feed for 1 h. The humidity was kept at the highest degree by soaking the cotton plug. Afterwards the leafhoppers exposed to the conidia were isolated individually on a maize leaf in a test-tube and checked periodically until their death and the growth of the fungal mycelium, noting the seven phases derived by the external aspect of the fungus growing on the leafhopper.

Mean, minimum, and maximum value, standard deviation, and confidence limits (95%) were calculated for the different developmental phases of *Z. radicans* on the leafhoppers infected in the laboratory.

## Results

*Z. radicans* appeared in summer and its presence increased throughout the season, reaching a peak in August in 1988 and 2004, whereas in 2003 the abnormal drought accompanied by high temperatures during the whole summer delayed the peak, that occurred until the beginning of October (figure 2). The infection de-

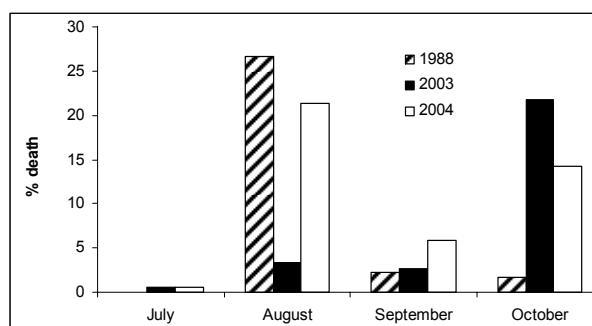
creased gradually until the beginning of November when the maize plants got frosted and the leafhoppers had all moved to grasses where the collecting was not carried on.

Descriptive statistics of time in hours of the different phases of the fungal development are shown in table 2 and figure 3.

The relationship between the duration of leaf wetness and field mortality was significant, although with low R<sup>2</sup> values in 2003, but not in 2004; mortality was negatively associated with the temperature only during 2003, whereas the relative humidity seldom showed some influence. No significant relationship was observed between mortality and rainfall (table 3).

No significant differences were recorded between leafhopper mortalities in different years (table 4). However, the maximum number of dead individuals was recorded in different months in different years (figure 2): significant differences of seasonal distributions of dead individuals between years were always detected (overall:  $\chi^2 = 106.00$ , df = 10, P < 0.001; 1988 vs. 2003:  $\chi^2 = 84.67$ , df = 6, P < 0.001; 1988 vs. 2004:  $\chi^2 = 63.54$ , df = 6, P < 0.001; 2003 vs. 2004:  $\chi^2 = 63.56$ , df = 7, P < 0.001).

Zygosporos of *Z. radicans* (figure 4) were observed sporadically in individuals that did not develop an external mycelium and died without an apparent cause, just falling onto the ground. The microscopic preparation



**Figure 2.** Monthly mortality of *Z. pullula* due to *Z. radicans* in the different sampling years.

**Table 2.** Descriptive statistics of the different development phases of *Z. radicans* in *Z. pullula* under laboratory conditions. Time is given in hours since infection for death, and in hours since death for the other phases. Size refers to the number of insects from which each phenological phase was calculated.

Development phase*	Size	Mean	SD	SE	Range	Max	Min	Confidence	x - d	x + d
1	9	90.1	43.2	14.4	133.5	203.5	70.0	33.23	56.86	123.32
2	3	-0.1	1.0	0.6	1.8	0.5	-1.3	2.58	-2.68	2.48
3	5	1.2	1.0	0.4	2.6	2.0	-0.6	1.24	-0.05	2.43
4	6	5.3	1.5	0.6	4.3	8.0	3.7	1.54	3.74	6.82
5	5	5.6	1.6	0.7	4.0	8.0	4.0	1.93	3.67	7.53
6	3	5.8	1.9	1.1	3.5	7.2	3.7	4.66	1.19	10.51
7	6	14.7	6.6	2.7	16.0	24.0	8.0	6.95	7.75	21.65
8	7	35.2	19.8	7.5	45	59	14	18.3	16.9	53.5
9	2	54.5	21.9	15.5	31.0	70.0	39.0	196.95	142.45	251.45

SD: standard deviation; SE: standard error; x ± d: lower and upper confidence limit.

\* 1: death of the leafhopper; 2: beginning of rhyzoidal development; 3: beginning of rhyzoidal branching; 4: beginning of trichoid hyphal development; 5: wing divarication; 6: beginning of reticular myceliar development; 7: beginning of conidiophoral development; 8: beginning of conidial production; 9: beginning of conidiophoral degeneration.

of these individuals showed the presence of zygospores inside their body (n = 4 in 2003, n = 1 in 2004).

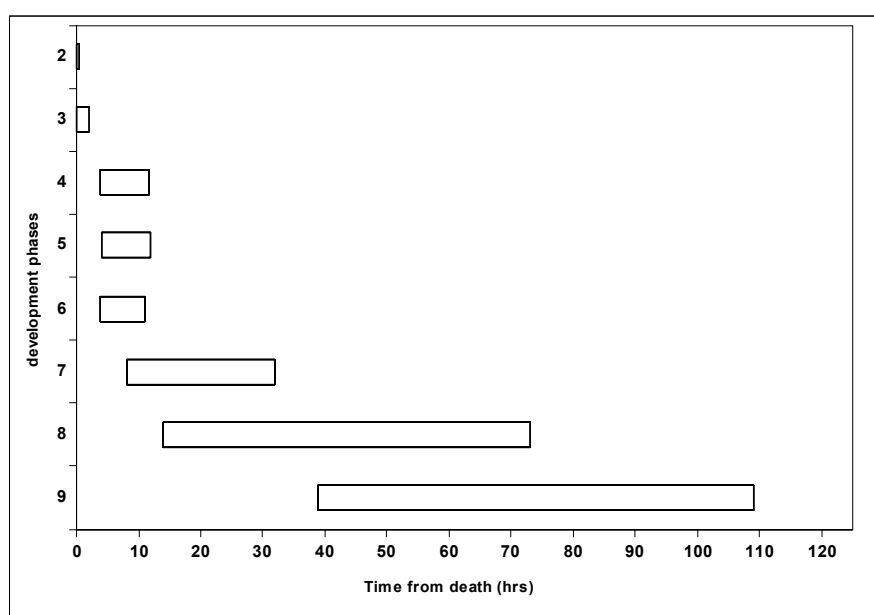
On the other hand, the individuals producing conidia always adhered to the leaves because of the development of fungal rhyzoids that, emerging from the intersegmental membranes of the urosternites while the leafhopper was still alive, took contact with the leaf surface and ramified on it, sticking the leafhopper to it until its death and permitting the fungus to complete its growth and spray conidia on the area beneath.

During the three year investigation, in the period July-October, only 5 individuals of the 1,576 leafhoppers collected in the field died killed by *V. lecanii*, 3 by *Beauveria bassiana* (Balsamo) Vuillemin, 2 by *M. hiemalis*, and 1 by *P. oxalicum*, representing 0.7% of adult mortality, compared with 8.2% of *Z. radicans*.

## Discussion

The results confirm that the most important entomopathogenic agent of *Z. pullula* in Northwest Italy in the period from July to October is *Z. radicans*. This fungal pathogen proved to be efficient above all by infecting the adults of the leafhopper during summer, in particular in August, after the very high temperatures of July, that do not seem to be appropriate for development of the fungus (further studies are necessary to determine the temperature range of the fungal development and its speed). Mortality peaks reached over 53% in a sampling made on 19<sup>th</sup> August 1988 and almost 47% in a sampling made on 8<sup>th</sup> August 2004 (data not shown).

Its peculiar propagation mechanism is particularly efficient in maize fields, where the tall and not too dense plants host the infected leafhoppers on the lower leaf



**Figure 3.** Range of the beginning of the different development phases of *Z. radicans*. Developmental phase number as in table 2.

**Table 3.** Linear regression between meteorological factors (independent variables) and field mortality of *Z. pullula* caused by *Z. radicans* (dependent variable). T: temperature; RH: relative humidity; LW: duration of leaf wetness; min: minimum; max: maximum; field mortality is expressed as number of dead leafhoppers out of 100 individuals.

Year	Independent variable	Intercept	Slope ± S.E.	R <sup>2</sup>	DF	F	P
1988	Tmax	-5.47	1.04 ± 0.80	0.11	1.13	1.69	0.22
"	Tmin	-0.05	0.07 ± 0.01	0.14	1.13	1.14	0.17
"	Tmean	-11.82	1.08 ± 0.76	0.14	1.13	2.03	0.18
"	RHmean	20.97	-0.17 ± 0.77	0.00	1.13	0.05	0.82
"	Rainfall	9.21	-0.67 ± 1.40	0.02	1.13	0.24	0.63
2003	Tmax	17.54	-0.44 ± 0.19	0.22	1.19	5.34	0.03
"	Tmin	12.19	-0.54 ± 0.28	0.17	1.19	3.82	0.06
"	Tmean	15.17	-0.51 ± 0.22	0.21	1.19	5.19	0.03
"	RHmin	-1.00	0.26 ± 0.17	0.11	1.19	2.44	0.14
"	RHmean	-23.90	0.41 ± 0.17	0.23	1.19	5.76	0.03
"	Rainfall	7.79	-0.06 ± 0.11	0.01	1.19	0.26	0.62
"	LWmax	0.25	0.01 ± 0.00	0.31	1.19	8.76	0.01
"	LWmin	-0.43	0.01 ± 0.00	0.33	1.19	9.43	0.01
"	LWmean	2.61	0.01 ± 0.01	0.28	1.19	7.41	0.01
2004	Tmax	17.27	-0.35 ± 0.87	0.01	1.16	0.16	0.69
"	Tmin	-0.67	0.72 ± 0.58	0.09	1.16	1.54	0.23
"	Tmean	0.79	0.37 ± 0.71	0.02	1.16	0.28	0.61
"	RHmin	-5.68	0.32 ± 0.22	0.12	1.16	2.14	0.16
"	RHmean	-22.29	0.42 ± 0.39	0.07	1.16	1.16	0.30
"	Rainfall	6.48	0.16 ± 0.27	0.03	1.16	0.43	0.52
"	LWmax	6.98	0.12 ± 0.50	0.00	1.16	0.05	0.82
"	LWmin	8.35	-0.11 ± 0.71	0.00	1.16	0.02	0.88
"	LWmean	6.82	0.18 ± 0.61	0.00	1.16	0.09	0.77

**Table 4.** Percentage of dead leafhoppers recorded in the different years. Same letters indicate no significant differences between mortalities ( $\chi^2 = 0.25$ ,  $df = 2$ ,  $P = 0.88$ ).

Year	Total leafhoppers	Dead leafhoppers	%
1988	450	36	8.0 a
2003	578	42	7.2 a
2004	548	51	9.3 a



**Figure 4.** *Z. radicans*: zygospores inside and outside a leg of a leafhopper (microscope slide). (In colour at [www.bulletinofinsectology.org](http://www.bulletinofinsectology.org))

surface, so that during the spreading of the conidia, the projection cone is very wide and the probability of hitting leafhoppers flying or standing on the upper side of the leaves beneath is higher.

The mortality of leafhoppers has been observed also in

sequence on a leaf, i.e. leafhoppers feeding close to an infected individual are hit by the conidia and die a few millimetres or centimetres far, thus showing how quick is the infection and the development of the fungus.

In the field, some nymphs were also found to be killed by *Z. radicans*. Their low number is probably due to their poor dispersion and their position always on the leaf underside, thus protected from the projection cone of the conidia. The greater mobility of adults would help to explain their higher infection rate (>84%, data not shown).

The minimum time for the fungus to complete its life cycle is 108 hours and the mean is 143 hours at 23 °C, whereas, once the leafhopper is adhered on the leaf underside, the fungus is present only for 39-54.5 hours at 23 °C. After the decomposition of *Z. radicans*'s mycelium, no trace remains in the leafhopper adhered to the plant except for the position with spread wings and the fact that it adheres to the leaf.

The difficulty to identify *Z. radicans* as the entomopathogenic agent in the past was due to its short life cycle and rapid decay. Material collected in the field and analyzed in the laboratory usually included leafhoppers adhered on the lower leaf side, but that had died at least 2 days before the moment of microbiological analysis, therefore no trace of *Z. radicans* remained and fungal saprophytes grew when the specimen was placed in a humid chamber. The data in Vidano and Arzone (1988) refer exclusively to *Z. radicans*.

The optimal temperatures for the infection and growth of *Z. radicans* in the leafhopper are within 20-25 °C, although laboratory infection tests proved a fungal en-



tomopathogenic activity within a broad range between 15 and 30 °C (unpublished data), that are similar to the data on this fungus cultivated *in vitro* by Ben-Ze'ev and Kenneth (1981), indicating an upper limit of sporulation at 27 °C and a growth range between 8 and 33 °C. This may explain the reason of the peak shift towards October 2003; drought could also be an influence, as our results seem to prove that leaf wetness is significant for conidial production.

On the other hand, zygospores permit the fungus to resist unfavourable climatic conditions (e.g. frost, drought, excessively high temperatures) after which conidiophores should develop and infection starts again.

In conclusion, *Z. radicans*, by infecting a remarkable percentage (up to 50%) of the leafhopper's adult population in summer, in addition to 79.2% of eggs parasitized by the oophagous Mymarid wasp *Anagrus atomus* (L.), represents an important control agent of this leafhopper that otherwise would be a serious pest of maize (Vidano and Arzone, 1988).

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