Bio-ethology of *Anisandrus dispar* F. and Its Possible Involvement in Dieback (Moria) Diseases of Hazelnut (*Corylus avellana* L.) Plants in Central Italy

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Abstract

Hazelnut is one of the most important orchards in central Italy (Viterbo province). More than eighty phytophagous insect pests adversely affect hazelnut orchards, but only a few of these, such as *Anisandrus dispar* F. (Coleoptera, Scolytidae), induce severe damage. A bacterial disease (called moria) constitutes one of the main phytopathological problems of hazelnut plants in central Italy. Two years ago, the Lazio Regional Government and local hazelnut cooperatives supported a research into the bio-ethology of *A. dispar* and its possible association with moria disease on hazelnut plants in Viterbo. In 2003 and 2004 two experimental hazelnut areas were selected in the Capranica and Caprarola districts (Viterbo), where eighteen chemio-chromotrophic traps were installed to study the dynamic population of *A. dispar* and to catch live Scolytidae females. Representative samples of live *A. dispar* females were used to isolate and identify the bacterial populations present both outside and inside the insects. After two years 5,726 *A. dispar* females had been caught. Of more than 1,400 live *A. dispar* females, 10% were submitted to microbiological analyses by morphological, physiological, biochemical and molecular techniques. The populations of the main bacteria (by outside and inside) associated with the phytophagous were identified as *Erwinia billingae*, *Brenneria quercina*, *Pantoea cedenensis* and *Pseudomonas* spp. Studies are currently in progress to: *i)* clarify the biological cycle of *A. dispar*; *ii)* identify the role (direct and/or indirect) of the insect respect to the epidemiology of moria disease; *iii)* carry on pathogenicity tests on bacterial isolates to prove their involvement in bacteriosis; *iv)* develop specific primers to identify the presence of these bacteria when associated with the insect and with asymptomatic hazelnut plants; *v)* verify the influence of environmental parameters on the biology of both the insect and the disease.

INTRODUCTION

Hazelnut (*Corylus avellana* L.) orchards are the most common orchards in the Cimini Hills (Viterbo province). They are important not only for the local, but also for the national economy. In the Lazio region, of a total of more than 23,300 ha dedicated to the production of stone fruits, more than 16,400 ha produce hazelnut (Carbone et al., 2004).

More than 80 phytophagous can infest hazelnut plants (Pollini, 1998) but only a few of them, such as Coleoptera Scolytidae *Anisandrus dispar* F. are able to cause severe damage and loss (Viggiani, 1984), as they prefer to attack twigs and young hazelnut stems (∅ 1-3 cm) (Pollini, 1998).

A. dispar is present in the paleoartic region and infests many fruit plants (apple, pear, apricot, plum, peach, walnut, and hazelnut) and broad-leaved wood plants such as chestnut, oak, beech, elm, poplar, etc. This ambrosia beetle establishes a complex symbiosis with a fungus (*Ambrosiella hartigii* B.) that allows larvae to develop in wood tissues, which are notoriously poor in nutritious substances (Pollini, 1998).

In the adult stage, the females, which are the only ones able to fly, make small

cortical perforations that sometimes provoke rugosity and bark deformations. They subsequently bore new prolification galleries (horizontal semicircular galleries from which they vertically depart) (Natali et al., 1994). The females then lay about fifty eggs, in piles, at the end of the vertical galleries. Larvae are born after a few days and exclusively nourish on the mycelium of the symbiont fungus, which is transported by the females and grows inside the galleries. The last larvae instar is reached within a period of 30-40 days, they turn into a chrysalis. This stage lasts an average of 15 days. The new adults stay inside the galleries until the following spring (Pollini, 1998).

Scolytidae can cause direct damage to branches and sometimes even the death of young hazelnut plants. It also seems to be involved in spreading a serious bacterial disease called "moria" (Balestra et al., 2003).

Hazelnut plants attacked by this disease initially show a light green/yellow colouring. Within a few days, one or more whole branches show evident signs of dieback. The yellow leaves then remain attached to twigs and branches for the whole vegetative season and then finally the hazelnut plants die.

Since 2002, a Lazio Regional project on different aspects related to this disease, was financed in collaboration with the hazelnut cooperatives of Viterbo province (Fig. 1). It has examined a possible link between *A. dispar* populations and the spread and infection processes associated with moria disease in the hazelnut orchards of central Italy. The aims of the present research include trying to clarify the bio-ethology of this Scolytidae and studying which bacterial populations are carried (internally and externally) by the *A. dispar* that are normally present on hazelnut plants and in neighbouring forests.

MATERIALS AND METHODS

During 2003 and 2004 samples of Scolytidae *A. dispar* were collected from two hazelnut orchards ("**a**" and "**b**") located close to Lake Vico in the Caprarola district (Viterbo, Italy) whose plants showed typical symptoms of moria (Fig. 1). The sampling process involved the use of "Rebell® Red" chemio-chromotropic traps. These were specifically selected for the capture of *A. dispar* females and characteristically contained ethylic alcohol with toluol 1% diluted in water at 50% and no soluble glue (Temocid® by Kollant).

The traps were installed 12th and 19th March 2003. They were monitored on a weekly basis as part of a process that is still ongoing. The traps were taken into the lab once a week, where the insects caught were counted and collected.

During the 2004 hazelnut season, samples were carried taken at two hazelnut experimental areas: "**a**" near Lake Vico and "**c**", in Caprarola and the Capranica (VT) district, respectively (Fig. 1). In that specific season, Mastrap L® (ISAGRO) traps were modified and used. These traps are normally used to catch the insect *Traumatocampa pityocampa* (Den. & Schiff) which attacks pine trees. The traps were installed not only in healthy and diseased hazelnut areas ("**a**", "**c**") but, were also - considering the polyphagy of the insect - set up in the "**forest**" (characterized by oaks, walnut, chestnut, beech, maple, elm and ash trees) very close to hazelnut area "**a**" (Fig. 1). Samples were also taken on a weekly basis in 2004.

During 2003 the manual capture of insects was carried out in stressed hazelnut plants within hazelnut area "**a**", coinciding with the different biological phases (larvae, pupae and female adults) of the pest. From May 1st until 30th June, numerous isolations of the bacterial cells present, were collected from these insects (both externally and internally). Sampling for the 2004 hazelnut season started on March 15th and was still in progress when this article was written. In the current season, given the large number of *A. dispar* collected, groups of 10 insects were worked together each time and these were selected from the different areas ("**a**", "**c**", and "**forest**").

In both years, each insect was washed (in 1 ml of sterile distilled water, SDW) for 2 hrs at 28°C, using an orbital shaker at 100 rpm. Later, 0.1 ml of each suspension was plated on Petri dishes containing nutrient agar supplemented by 5% of sucrose (NAS). The Petri dishes were subsequently placed in an incubator at 28±1°C for 48-72 hrs and

then the developed bacterial colonies were observed by stereomicroscope, described, and collected on nutrient agar supplemented by 2% of glycerol (NAG) at 4°C. To isolate them from the internal part of the Scolytidae, they were externally sterilized (NaClO 3%) for 3 min. Each *A. dispar* was later homogenized in 1ml of SDW, under sterile conditions. 5 plate dilutions (0.1ml each) were made from each insect (or group). These were then plated onto NAS Petri dishes, incubated at 28±1°C for 48-72 h, and then observed under a stereomicroscope. Bacterial colonies obtained from internal isolations were also morphologically characterized and then collected on NAG at 4°C (Natali et al., 1994). They were subsequently submitted to morphological, physiological, nutritional, biochemical and molecular tests to identify all of the bacterial strains obtained.

Bacterial cells grown on NSA agar for 24 h, were resuspended in 10 µl of SDW using a sterile toothpick and boiled for 10 min at 99.9°C. The cells were centrifuged for 2 min at maximum speed and the supernatant was transferred to a PCR tube containing 40 µl of PCR reaction mixture, consisting of 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 mM of each ribosomial primer (6F, 1510R) and 1 unit of Taq DNA polymerase (Pharmacia Biotech, Sweden). PCR was carried out on a 2400 Thermocycler (Perkin Elmer-Applied Biosystems, Warrington, UK) according to the following conditions: an initial denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.30 min and a final elongation step at 72°C for 10 min (Fig. 2). The amplified PCR products were purified using Microcon-PCR filter units as described in the manufacturer's instructions (Millipore S.p.A.) and sequenced using an ABI PRISM 310 automatic sequencer (Applied Biosystems Inc., Foster City, California). The sequences were analyzed with the FastA program from the EMBL database, to give sequence alignment and percent identity.

To verify the pathogenicity of the bacterial strains isolated from *A. dispar*, artificial inoculations in greenhouse and in a growth chamber were carried out on one-year-old hazelnut plants cv. Tonda Gentile Romana in spring 2004.

RESULTS

A total of 5726 *A. dispar* F females were captured using chemio-chromotropic traps during the two-year period 2003-2004. In 2003 the capture dynamics showed a typcial Gaussian trend, with respective peaks of 288 and 208 females/trap (average value) on 30th April in hazelnut orchards "**a**" and "**b**". On April 9th 2003, heavy rainfall (33.6 mm) and an abrupt fall in temperature (to -3.9°C) were associated with a sharp fall in the number of the captures in both hazelnut orchards. Even considering the 28th May sample, a slight decrease in captures was observed (Fig. 3).

Insect captures in hazelnut grove "**a**" continued until 25th June and until 2nd July in hazelnut grove "**b**".

In 2004, the first captures of *A. dispar* females in the experimental hazelnut orchards ("**a**", "**c**", "**forest**") were on 15th March.

By 3rd May, 553 and 101 females/trap had been respectively captured from healthy and diseased hazelnut plants in area "**a**" using Mastrap L traps; 38 and 46 females/trap from healthy and diseased hazelnut plants in area "**c**"; and 326 females/trap from the forest close to area "**a**".

Using Mastrap L traps, the peak number of captures was registered on April 26th. 124 and 23 females/trap (mean value) were respectively captured from healthy and diseased plants in area "**a**". Using Rebell Red traps, the peak number of captures for the same area was 114 females/trap (mean value). Using Mastrap L traps in area "**c**" the peak number of captures (mean value) were 10 females/trap for healthy and 9 for diseased hazelnut plants. In the forest close to area "**a**", the peak number of captures using Mastrap L traps was 65 females/trap (mean value). In samples taken on 13th and 19th April 2004, no captures were registered.

Different bacterial populations were isolated from inside and outside the Scolytidae by applying phytobacteriological procedures. The forty most representative bacterial populations most commonly associated with *A. dispar* insect were isolated and

selected. These bacteria were identified and mainly associated to the *Enterobacteriaceae* and *Pseudomonadaceae* families. These bacterial strains identified were particularly associated with the *Erwinia*, *Pantoea*, *Brenneria* and *Pseudomonas* genera. *Brenneria, Erwinia* and *Pantoea* spp. seemed to be the bacterial populations most closely associated with forest samples while, *Brenneria*, *Erwinia*, *Pantoea*, and *Pseuodomonas* spp. were mainly associated with hazelnut samples. Most of these bacteria were saprophytic micro-organism populations but ongoing pathogenicity tests suggest that some (*Erwinia* spp., *B. quercina*, *Pseudomonas* spp.) may require further studies to clarify their taxonomical position.

DISCUSSION

After two years of sampling *A. dispar* in hazelnut producing areas of central Italy, the flights of this Scolytidae showed it to be particularly susceptible to variations in temperature and rainfall. The reduction in *A. dispar* flight dynamic in hazelnut orchards "**a**" and "**b**" on April 30th 2003 seemed to be strictly associated with the temperature (minimum temperature -3.9°C) and rainfall intensity (33.6 mm) registered during the week of capture (2nd-9th April). Even the sample taken on 28th May showed a slight decrease in numbers captured, which was also due to the inclement weather conditions.

Comparing captures from hazelnut orchard "**a**" made with Rebell Red traps for the same period (March/May) in the two years (2003-2004), it is evident that of a total capture of 980 females/trap, 81% of captures occurred in the first year of sampling, and only 19% in the second. An analysis of weather data pointed to the fact that the rainfall total for 2003 was 76.5 mm, while it was 169.2 mm for the same period in 2004, and that this had had a great influence upon the flight of *A. dispar*. In the period in question, average temperatures (minimum and maximum) were also higher in 2003 (7.1°C and 16.2°C) than in 2004 (5.9°C and 14.3°C).

No captures were registered in samples taken on 13th April and 19th April 2004 in hazelnut groves "**a**" and "**c**" and in the "**forest**". This absence of captures is largely explained by an analysis of weather data and, in particular, the maximum temperatures registered over the two weeks capturing period (6th-19th April). These were rather low, with maximums of 15°C in orchard "**a**" and 11.7°C in "**c**". Minimum temperatures were particularly low, with absolute minimums of -1.6°C and 2°C for orchards "**c**" and "**a**", respectively, on 10th April and 19th April

On the evidence provided so far, *A. dispar* populations seem to have played an important role in the development of bacterial populations. In particular, isolates of *B. quercina*, *Erwinia* spp. and *Pseudomonas* spp. were obtained from inside and outside the Scolytidae. *B. quercina* have been included in group III of *Enterobactericeae* (Hauben et al., 1998) and in the "true erwiniae" group (Schroth and Hildebrand, 1980), and also with a species of *Pseudomonadaceae.* These are already considered as causal agents of the moria disease in hazelnut orchards in central Italy (Varvaro et al., 1990; Scortichini et al., 1994). Considering that a clarification is required with respect to taxonomical status of the *Erwiniae* spp. so far isolated, it will be necessary to improve on present studies and to indicate whether different bacterial pathogens are involved in the disease and establish the role of *A. dispar* in the processes associated with the infection and the spread of bacteriosis in hazelnut orchards.

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Figures

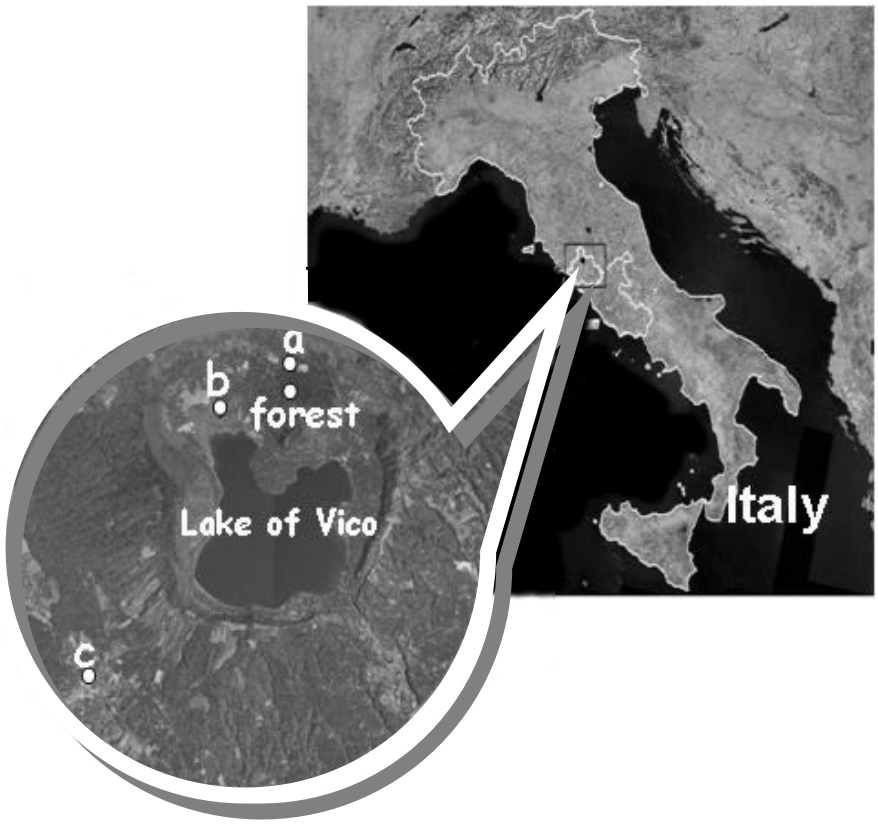

Fig. 1. Satellite images of experimental hazelnut areas in the province of Viterbo.

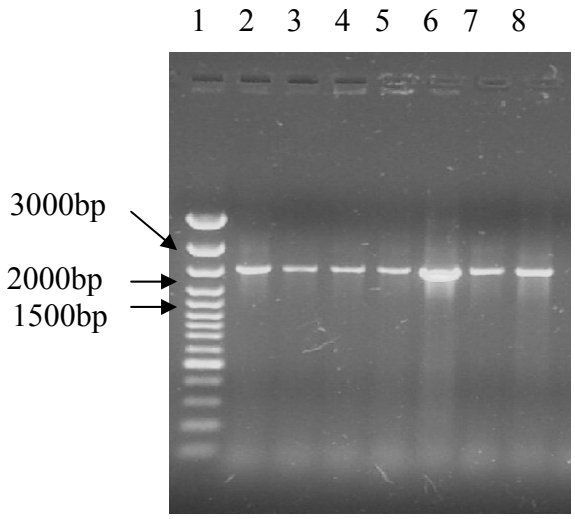

Fig. 2. Lanes 2-8, PCR product, formed with primers rRNA16S from bacterial cells isolated from the internal organs of the insect. Lane 1, 100bp DNA ladder plus (Fermentas).

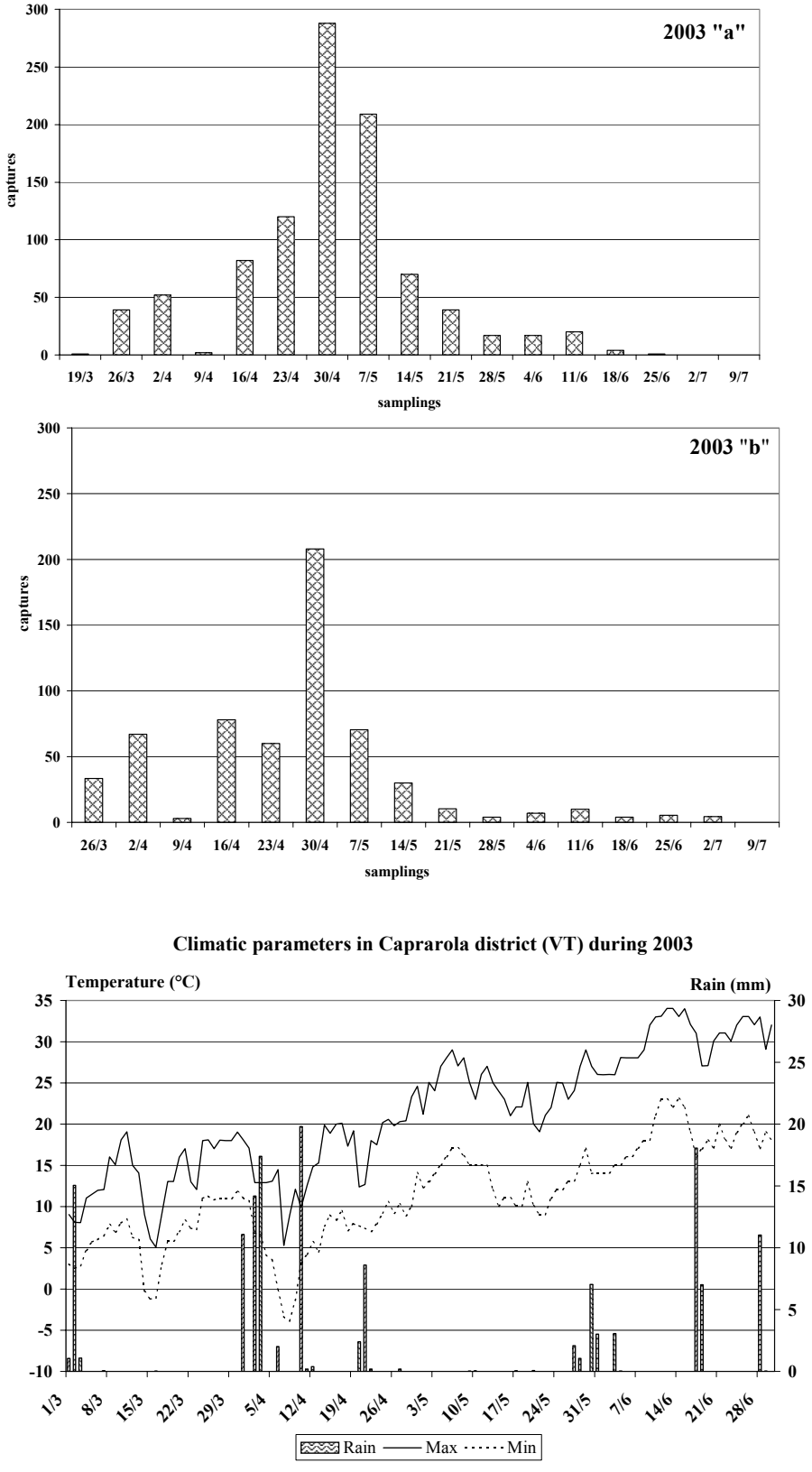

Fig. 3. Females of *A. dispar* captured (mean value) during the 2003 hazelnut season, by Rebell®. Red traps in experimental areas "**a**" and "**b**" and related climatic parameters.

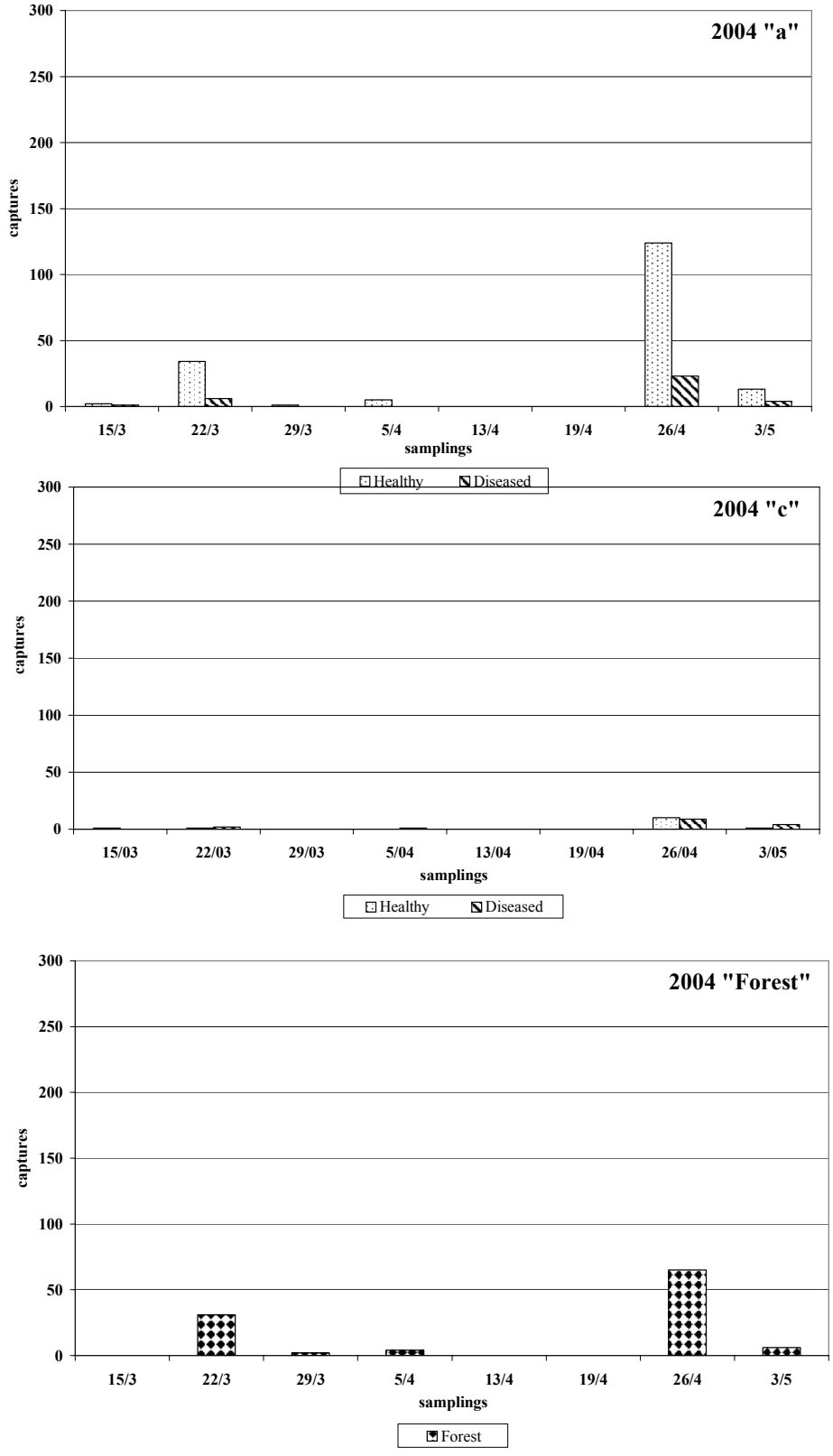

Fig. 4. Females of *A. dispar* captured (mean value) during the 2004 hazelnut season, by Mastrap®. L traps in experimental areas "**a**", "**b**", "**forest**".

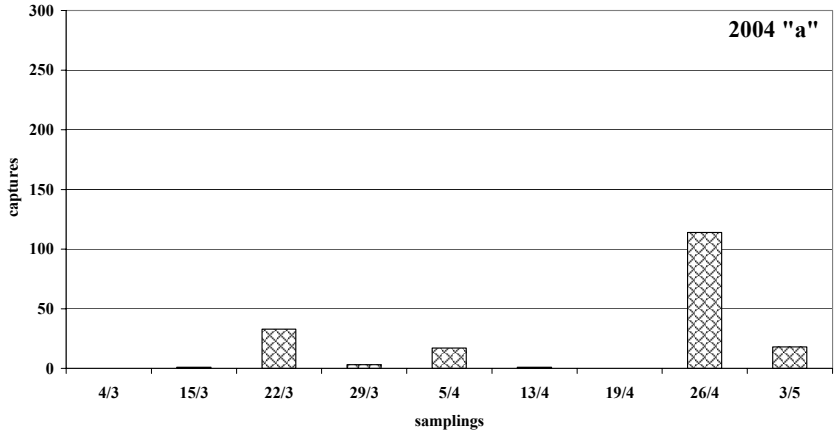

Fig. 5. Average of *A. dispar* females captured during the 2004 hazelnut season by Rebell®. Red traps in area "**a**".

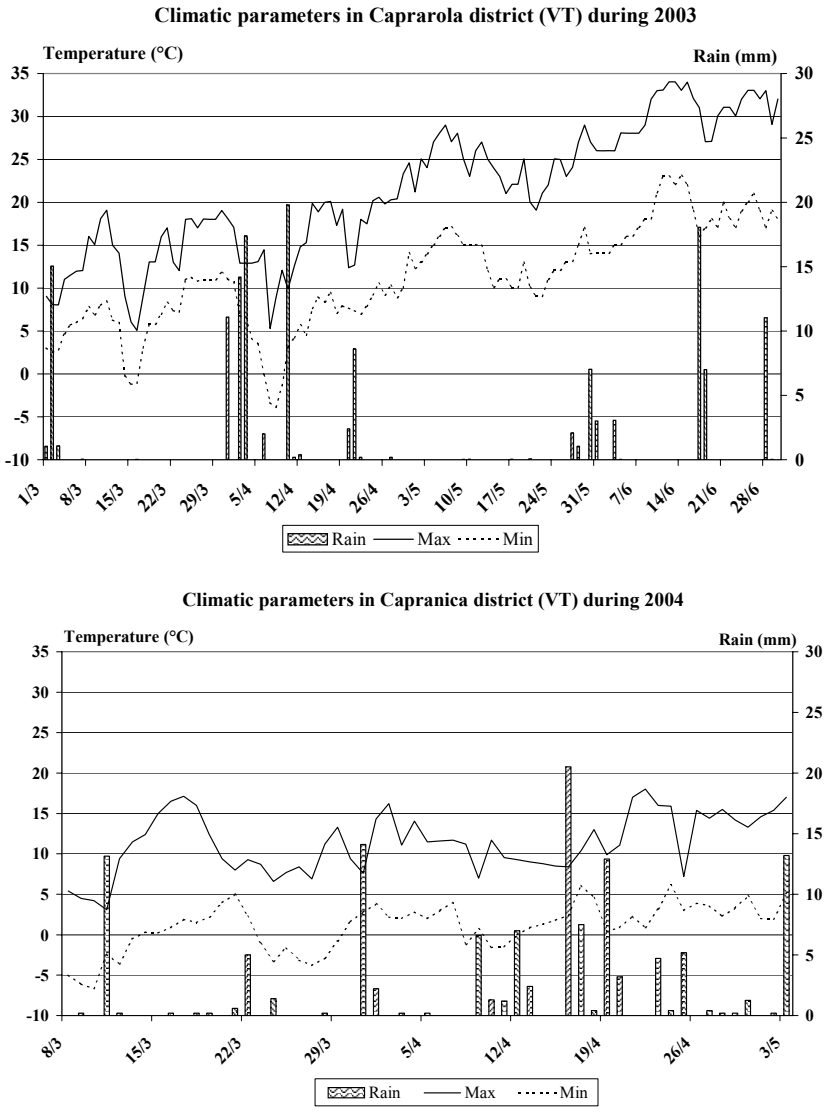

Fig. 6. Climatic parameters in the Caprarola (VT) and Capranica (VT) districts during the 2004 season.